Campylobacter jejuni is the greatest causative agent of bacterial food-borne illnesses in the developed world (Wagenaar et al., 2013). The incidence rates of campylobacteriosis in the United States increased 14.3% in 2012 compared with 2006 to 2008 (USDA, Food Safety and Inspection Services, 2013). In the European Union, this disease is the most frequently reported food-borne illness, with a total of 220,209 Campylobacter cases in 2011, 2.2% more than in 2010 (EFSA, 2013). The major source of C. jejuni infection remains the handling and consumption of chicken meat (Uyttendaele et al., 2006; Tam et al., 2009). Campylobacter jejuni is able to efficiently colonize the chicken gut in high numbers without causing disease. Thus, the intestinal tract of chickens provides a reservoir from which C. jejuni may spread via fecal material before or during slaughtering. Most processing operations reduce but cannot eliminate this microorganism; therefore, retail poultry meat is often contaminated with Campylobacter (Suzuki and Yamamoto, 2009). As Campylobacter possesses a low infective dose of about 500 cells, even chicken batches contaminated at a low level may be considered to be a threat to public health. If chicken meat is not handled properly, consumer exposure to Campylobacter is unavoidable either by cross-contamination from raw poultry meat to ready-to-eat food or if the meat is un-

**Rapid identification of Campylobacter jejuni from poultry carcasses and slaughtering environment samples by real-time PCR**

Mirena Ivanova,* Randhir Singh,† Muthu Dharmasena,‡ Chao Gong,‡ Albert Krastanov,* and Xiuping Jiang†

*Department of Biotechnology, University of Food Technologies, 26 Maritza Blvd, 4002 Plovdiv, Bulgaria; †School of Public Health and Zoonoses, Guru Angad Dev Veterinary and Animal Science University, Ludhiana, India PB-141004; and ‡Department of Food, Nutrition, and Packaging Sciences, Clemson University, Clemson, SC 29634

**ABSTRACT** The objective of this study was to develop a real-time PCR assay for rapid identification of Campylobacter jejuni and to apply the method in analyzing samples from poultry processing. A C. jejuni-specific primer set targeting a portion of the C. jejuni hippuricase gene was developed. The specificity of the newly designed primer pair was verified using 5 C. jejuni strains and 20 other bacterial strains. Sensitivity was determined to be as low as 1 genome copy per reaction. A total of 73 samples were collected at different sites along the processing line during 2 visits to a poultry slaughterhouse and were examined by direct plating onto modified charcoal cefoperazone deoxycholate agar or after enrichment in Bolton broth followed by plating on modified charcoal cefoperazone deoxycholate agar. The newly developed real-time PCR assay was used to identify the presumptive colonies as belonging to C. jejuni. A real-time PCR assay targeting 16S ribosomal RNA was also applied to determine Campylobacter spp. prevalence. Results from the real-time PCR analysis indicated considerable variability in Campylobacter contamination, with incidence rates of 72.7 and 27.6% for sampling days A and B, respectively. Campylobacter was isolated from 100% of prescaled and previscerated carcasses on sampling day A. In contrast, on sampling day B, the highest number of Campylobacter-positive carcasses was recovered after evisceration (60%). The chilling process significantly reduced (P < 0.05) Campylobacter population, but the percentage of positive samples on sampling day A increased to 80%. All samples collected from the processing environment, except scalding tank 3 and the prechiller and chiller tanks, were 100% positive on day A, whereas no campylobacters were isolated from machinery on sampling day B. Our results revealed the widespread of C. jejuni in poultry processing and proved that the newly developed real-time PCR assay is a simple, specific, and inexpensive method for rapid C. jejuni identification. The newly developed PCR method can be easily used in laboratories for reliable and unambiguous identification of C. jejuni in poultry samples.

**Key words:** Campylobacter jejuni, real-time PCR, poultry carcass, slaughtering environment, hippuricase gene

©2014 Poultry Science Association Inc. 
Received November 6, 2013. 
Accepted February 8, 2014. 
1 Corresponding author: xiuping@clemson.edu

1587
ndercooked (Humphrey et al., 2001; Kusumaningrum et al., 2003). In broiler processing, Campylobacter numbers found on poultry carcasses usually vary among operations, with a peak at defeathering and sometimes at evisceration steps followed by a rapid reduction at postchilling (Guerin et al., 2010).

Phenotypic methods routinely used for Campylobacter detection often result in unreliable identification, requiring further testing. Therefore, interest has been growing in the development of molecular approaches to allow sensitive, unambiguous, and rapid detection and identification of Campylobacter in retail poultry products and in samples from poultry processing (Melero et al., 2011). Real-time PCR is the most advanced molecular method available for rapid detection of foodborne pathogens in food and food processing. Compared with the conventional phenotyping methods for pathogen identification, real-time PCR is a sensitive, accurate, and rapid method, and does not require post-amplification steps that eliminate the risk of cross-contamination (Barletta et al., 2013). Thus far, numerous studies have been published to estimate the prevalence and concentration of Campylobacter in poultry samples during processing using different approaches (Rosenquist et al., 2006; Debretsion et al., 2007; Mackiw et al., 2008; Hue et al., 2010; Habib et al., 2012; Trimble et al., 2013). As far as we are aware, no previous study has reported on the application of a simple and rapid real-time PCR method to allow sensitive and highly specific identification of C. jejuni at different stages of poultry processing. Therefore, the objectives of the current study were to develop a rapid and inexpensive real-time PCR assay based on FAST SYBR Green (Kapa Biosystems, Boston, MA) and apply the new method for identification of C. jejuni on broiler carcasses during processing and in samples from the poultry slaughtering environment.

MATERIALS AND METHODS

Bacterial Strains, Culture Conditions, and Media

Bacterial strains used in this study are listed in Table 1. Campylobacter jejuni strains were obtained from W. Miller (USDA-Agricultural Research Service, Albany, CA), Campylobacter coli #1 and Campylobacter lari #1 were provided by M. T. Musgrove (USDA-Agricultural Research Service, Athens, GA). All other strains belonged to the culture collection at X. Jiang’s food microbiology laboratory (Department of Food, Nutrition, and Packaging Sciences, Clemson University). All strains were stored at −80°C in tryptic soy broth (Becton, Dickinson, and Co., Sparks, MD) containing 20% (vol/vol) glycerol (Fisher Scientific, Fair Lawn, NJ). Campylobacter strains were cultured on modified charcoal cefoperazone deoxycholate agar (mCCDA; Oxoid, Hampshire, UK) and incubated at 42°C for 48 h under microaerobic conditions (5% O2, 10% CO2, and 85% N2) in a GasPak jar (BBL Microbiology Systems, Cockeysville, MD). Helicobacter pylori strains were grown on brain heart infusion agar (Difco Laboratories, Detroit, MI) supplemented with 7% horse serum (Sigma Chemical Co., St. Louis, MO) and incubated at 37°C for 72 h under the same microaerobic conditions. The other bacterial strains were cultured on tryptic soy agar (Becton, Dickinson, and Co.) and plates were incubated aerobically at 37°C for 24 h. Bolton selective enrichment broth (Oxoid) without supplementation was used to prepare carcass rinses and supplemented with 20 mg/L of vancomycin HCl (Sigma Chemical Co.), 20 mg/L of sodium cefoperazone (Toronto Research Chemicals, Ontario, Canada), 20 mg/L of trimethoprim HCl (Sigma Chemical Co.), and 50 mg/L of cycloheximide (Sigma Chemical Co.; Hunt et al., 2001) for selective enrichment of the samples. The mCCDA consisting of selective supplement (Oxoid) was used for direct plating or subculturing of the enriched carcass rinses.

DNA Extraction

The DNA of each Campylobacter isolate was prepared using freshly grown Campylobacter colonies on mCCDA by adding a loopful to 300 μL of sterile distilled water and boiled at 100°C for 10 min. After centrifugation at 6,800 × g for 5 min at room temperature (20°C) to remove cell debris, the supernatant was immediately used for PCR reactions. The DNA concentrations were determined by a NanoDrop 2000 Spectrophotometer (Thermo Scientific, Wilmington, DE).

Primers

Primer sequences used for the real-time PCR assays are listed in Table 2. Primers and conditions described by Linton et al. (1996) were used to confirm isolates as belonging to Campylobacter genus. Two different pairs of primers, one designed in this study and one published (Wang et al., 2002), both targeting C. jejuni hippuricase gene (hipO), were compared for their specificity and sensitivity. The forward and reverse primers designed in the present study were constructed according to C. jejuni ssp. jejuni NCTC 11168 hipO sequence (GenBank accession no. NC_002163) and tested for specificity using the nucleotide BLAST program (http://blast.ncbi.nlm.nih.gov/Blast.cgi). The primer set was designed using online software from PrimerQuest (Integrated DNA Technologies, Coralville, IA). All oligonucleotides were synthesized by Invitrogen (Carlsbad, CA) and reconstituted in nuclease-free water (HyClone Laboratories Inc., Logan, UT) to 1 mM stock solution.

Detection of C. jejuni by Real-Time PCR

The real-time PCR assay was optimized using the Mastercycler ep realplex2 (Eppendorf AG, Hamburg,
Germany) and fast-cycling PCR conditions. Gradient technology in the Eppendorf unit was used to determine the optimal annealing temperature of the designed primer set. The PCR reaction was carried out in a 20-µL total volume reaction mixture consisting of 10 µL of Kapa SYBR FAST qPCR 2x Master Mix Universal (Kapa Biosystems), 0.4 µL of each primer (200 nM final concentration), ~20 ng/µL of template DNA, and nuclease-free water to the volume. The following PCR conditions were used for amplification of the target *hipO* gene: an initial denaturation step at 95°C for 1.5 min followed by 35 cycles of denaturation at 95°C for 15 s, primer annealing at 58°C for 15 s, and extension at 72°C for 15 s. To determine the specificity of PCR reactions, a melting curve analysis was carried out after amplification by slow heating from 55 to 95°C, with fluorescence acquisition at 0.5°C intervals and a 20-s hold at each increment. All PCR experiments were carried out in duplicate and included negative control without target DNA and *C. jejuni* ATCC 43430 as positive control.

### Sample Collection

Samples were collected in May to June 2012 during 2 visits to a commercial broiler processing plant in South Carolina. Poultry carcasses (n = 40) were randomly chosen and collected by hand (while wearing new latex gloves) for each carcass from 4 sites along the processing line: prescalding, preevisceration, postevisceration, and postchilling. A total of 5 carcasses were collected at each sampling site per visit. Environmental samples were collected during processing operations, including 50 mL of water samples (n = 15) from scalding tanks 1 and 3, prechiller tank 1, and chiller tank 2. In addition, swab samples (n = 18) were taken from an area of defeathering machines 1 and 2 and scalding machines 1 and 2 measuring 5 × 5 cm². As *Campylobacter* was not recovered from the water samples from scalding tank 3 or the prechiller and chiller tanks in the first visit, these sites were not included in the sampling plan for the second plant visit. All carcasses were subjected to a whole carcass rinse in the plant. Feathered carcasses collected

### Table 1. *Campylobacter* species and non-*Campylobacter* microorganisms used in the current study to determine specificity of the designed and published primers

<table>
<thead>
<tr>
<th>Organism</th>
<th>Reference number</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>Positive control</td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Campylobacter jejuni</em></td>
<td>RM 1221</td>
<td>W. Miller USDA-Agricultural Research Service, Albany, California</td>
</tr>
<tr>
<td></td>
<td>ATCC 43430</td>
<td></td>
</tr>
<tr>
<td></td>
<td>ATCC 43431</td>
<td></td>
</tr>
<tr>
<td></td>
<td>D781</td>
<td></td>
</tr>
<tr>
<td></td>
<td>S1–176</td>
<td></td>
</tr>
<tr>
<td>Negative control</td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Campylobacter coli</em></td>
<td>#1</td>
<td>M. T. Musgrove, USDA-Agricultural Research Service, Athens, Georgia</td>
</tr>
<tr>
<td><em>Campylobacter fetus</em></td>
<td>#1</td>
<td></td>
</tr>
<tr>
<td><em>Escherichia coli</em> O157:H7</td>
<td>ATCC 43888</td>
<td></td>
</tr>
<tr>
<td></td>
<td>ATCC 43889</td>
<td></td>
</tr>
<tr>
<td></td>
<td>ATCC 43895</td>
<td></td>
</tr>
<tr>
<td></td>
<td>ATCC 43890</td>
<td></td>
</tr>
<tr>
<td><em>Escherichia coli</em></td>
<td>ATCC 25922</td>
<td></td>
</tr>
<tr>
<td></td>
<td>ATCC 8739</td>
<td></td>
</tr>
<tr>
<td><em>Salmonella Typhimurium</em></td>
<td>32463</td>
<td>S. Zhao, Food and Drug Administration</td>
</tr>
<tr>
<td><em>Salmonella Newport</em></td>
<td>N635</td>
<td>S. Zhao, Food and Drug Administration</td>
</tr>
<tr>
<td><em>Salmonella Enteritidis</em></td>
<td>H2292</td>
<td>M. Doyle, University of Georgia</td>
</tr>
<tr>
<td><em>Salmonella Heidelberg</em></td>
<td>21380</td>
<td>S. Zhao, Food and Drug Administration</td>
</tr>
<tr>
<td><em>Listeria monocytogenes</em></td>
<td>ATCC 43258</td>
<td></td>
</tr>
<tr>
<td></td>
<td>ATCC 51414</td>
<td></td>
</tr>
<tr>
<td><em>Helicobacter pylori</em></td>
<td>ATCC 26995</td>
<td></td>
</tr>
<tr>
<td></td>
<td>D5136</td>
<td></td>
</tr>
<tr>
<td><em>Staphylococcus aureus</em></td>
<td>ATCC 6358</td>
<td>Human isolate; B. Gold, Emory University</td>
</tr>
<tr>
<td></td>
<td>ATCC 29213</td>
<td></td>
</tr>
<tr>
<td><em>Listeria innocua</em></td>
<td>ATCC 33090</td>
<td></td>
</tr>
<tr>
<td><em>Shigella sonnei</em></td>
<td>10304–98</td>
<td>Centers for Disease Control and Prevention</td>
</tr>
<tr>
<td><em>Enterococcus faecalis</em></td>
<td>ATCC 29212</td>
<td></td>
</tr>
</tbody>
</table>

### Table 2. Primer sequences used in this study for the identification of *Campylobacter* isolates

<table>
<thead>
<tr>
<th>PCR target gene</th>
<th>Primer</th>
<th>T&lt;sub&gt;α&lt;/sub&gt;&lt;sup&gt;1&lt;/sup&gt; °C</th>
<th>Sequence (5′ to 3′)</th>
<th>Amplicon size, bp</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Campylobacter jejuni</em></td>
<td>CJF</td>
<td>59</td>
<td>ACTTCTTTATTTGCTTGCTGC</td>
<td>332</td>
<td>Wang et al. (2002)</td>
</tr>
<tr>
<td><em>hipO</em></td>
<td>CJR</td>
<td>59</td>
<td>GCCACAACAGTTAAAGAAAGC</td>
<td>131</td>
<td>This study</td>
</tr>
<tr>
<td><em>C. jejuni</em></td>
<td>hipOF</td>
<td>58</td>
<td>GTGGTCATGGAAGTGTCCAGAAA</td>
<td>131</td>
<td>This study</td>
</tr>
<tr>
<td><em>hipO</em></td>
<td>hipOR</td>
<td>58</td>
<td>AGCTCCCATGCTTAAACAATGCTGA</td>
<td>816</td>
<td>Linton et al. (1996)</td>
</tr>
<tr>
<td>Genus <em>Campylobacter</em></td>
<td>C122R</td>
<td>60</td>
<td>GGATGACACTTTTCGGAGC</td>
<td>816</td>
<td>Linton et al. (1996)</td>
</tr>
<tr>
<td><em>16S rRNA</em></td>
<td>C1228R</td>
<td>60</td>
<td>CATTGTAGCAGGCTTGTGC</td>
<td>816</td>
<td>Linton et al. (1996)</td>
</tr>
</tbody>
</table>

<sup>1</sup>T<sub>α</sub> = annealing temperature.
at the prescaling site were shaken with 400 mL of Bolton selective enrichment broth for 60 s, whereas carcasses collected at the preevisceration, postvisceration, and postchilling sites were shaken with 100 mL of Bolton selective enrichment broth for 60 s, as per Bargh and Dickens (2000). The rinses were poured into 500-mL sterile plastic bottles, covered with frozen ice packs in insulated containers and immediately transported to the laboratory. Samples were filtered through sterile cheesecloth to remove larger particles of meat and fat and analyzed within 5 h post-collection.

**Isolation of Campylobacter spp.**

Both direct plating and sample enrichment were used to recover *Campylobacter* spp. from carcass rinses and water samples. For direct plating, 10-fold dilutions of each sample were prepared and 100-μL aliquots were spread-plated in duplicate onto mCCDA supplemented with antibiotics. All plates and tubes were incubated at 42°C for 48 h under a microaerobic atmosphere. Swab samples were submerged in 3 mL of Bolton broth supplemented with antibiotics as described previously. The caps were screwed loosely and the tubes were incubated at 42°C for 24 h, followed by spreading onto mCCDA. For enrichment, 30 mL of carcass rinses or water samples were mixed with 30 mL of Bolton broth supplemented with antibiotics. Samples were preenriched at 37°C for 4 h with agitation and then incubated at 42°C for further 24 to 48 h. If direct plating did not show colonies typical of *Campylobacter*, Bolton broth enrichment cultures were plated on mCCDA and incubated as described previously.

**Identification of Presumptive Campylobacter spp. Isolates**

After incubation, the number of colony-forming units for the direct plating procedure was counted. From each plate resulting from direct plating or after enrichment, up to 5 presumptive *Campylobacter* colonies were streaked onto mCCDA to obtain pure isolates. All isolates were confirmed as members of *Campylobacter* genus by microscopic observation of wet mount for motility, Gram staining for typical *Campylobacter* morphology, absence of growth at 42°C aerobically or at 25°C microaerobically, and real-time PCR reaction based on 16S ribosomal (r) RNA gene as described previously. *Campylobacter* colonies were further identified as thermophilic *C. jejuni* by means of a positive real-time PCR reaction targeting the *hipO* gene. All *Campylobacter* isolates were purified and stored at −80°C in tryptic soy broth containing 20% (vol/vol) glycerol.

**Sequencing Analysis**

Sequencing analysis was performed on 6 of *C. jejuni* strains isolated at different sites. Primers and methods used for PCR amplification, purification of the PCR products, and sequencing were performed according to the method described by Kim et al. (2011). The amplified PCR products were sequenced by the Clemson University Genomic Institute. The closest matches of the partial 16S rRNA sequences were identified through a BLAST search.

**Data Analysis**

All bacterial counts calculated from the rinsate were converted to log_{10} cfu of *C. jejuni* per milliliter and mean ± SD log values were calculated. Differences in the prevalence of *C. jejuni* among sampling sites were determined by the least significant differences test using SigmaPlot 12.3 (Systat Software Inc., San Jose, CA) and were considered to be significant when *P* < 0.05.

**RESULTS**

**Optimization of a FAST Real-Time PCR Method for Detection of *C. jejuni***

Annealing temperature (T_a) of the designed primer set was determined using a Mastercycler realplex² Gradient PCR system in a temperature range of 55 to 70°C. Results showed that temperatures between 55 and 65.8°C produced very similar C_T (threshold cycle) values (10.07 ± 0.23) without amplification of nonspecific products or primer-dimer formation. Based on these results, we chose a T_a of 58°C for further optimization of the real-time PCR assay. To optimize the real-time PCR performance, several primer concentrations (50, 100, 200, 300, and 400 nM) were tested. Of all concentrations, 200 nM showed the lowest C_T value (12.61 ± 0.00) and was considered as the optimal primer concentration. The C_T values obtained at lower concentrations (100 and 50 nM) were significantly higher (14.80 and 19.57, respectively). Using the newly developed FAST real-time PCR protocol, the total time required for the detection of *C. jejuni* was reduced to approximately 1 h (10 min for the DNA extraction and 49 min for the real-time PCR assay) with detection limit of 1 genome DNA copy per PCR and results being available immediately at the end of the PCR cycling.

**Comparing Sensitivity and Specificity of 2 Primer Sets for Detection of *C. jejuni***

In the current study, a designed and a published primer set, both based on the amplification of the *hipO* gene, was compared for sensitivity and specificity. To choose a published primer set, we performed an extensive search through the published primers based on the amplification of the *hipO* gene and tested them for specificity using the nucleotide BLAST program.
Purified genomic DNA of *C. jejuni* ATCC 43430 was used to test sensitivity of the 2 primer sets. The DNA copy numbers, ranging from $1 \times 10^7$ to $1 \times 10^0$ genome copies as calculated according to the molecular weight of *C. jejuni* genome, were used. The detection limit of the designed primer pair was determined to be 1 genome copy with $R^2 = 0.992$, the efficiency of the PCR reaction as 101% and slope as $-3.29$, whereas the sensitivity of the published primers was $10^3$ genome copies with $R^2 = 0.994$, efficiency of the PCR reaction as 84% and slope as $-3.76$ (Figure 1).

To evaluate the specificity of the designed and published primer sets, 26 bacterial strains were examined, including *Campylobacter*, *Helicobacter*, and strains from other unrelated genera (Table 1). It was demonstrated that the newly designed primer set was highly specific for the detection of *C. jejuni* strains only and produced a mean $C_T$ value of $11.43 \pm 1.24$. All other *Campylobacter* spp., *Helicobacter*, and non-*Campylobacter* organisms were considered negative, as their $C_T$ values were more than 35. Published primer sets had a mean $C_T$ value of $13.31 \pm 3.39$ for all *C. jejuni* strains and did not show any amplification against the other tested microorganisms. Specificity was confirmed by performing a melting curve analysis after the PCR amplification. Reproducible distinct melting points ($T_m$) of 79.6 ± 0.25 or 80.1 ± 0.24°C were observed for the designed and published primers, respectively (Table 3). Negative controls were confirmed to be negative and did not show any peaks at $T_m$ of 79.6 or 80.1°C. Specificity of both primer sets and absence of nonspecific products or primer-dimers were confirmed by analyzing the amplicons in 1.5% agarose gel stained with ethidium bromide (data not shown). Molecular weight of the amplified products was as expected (131 and 323 bp for designed and published primers, respectively) and no other bands were visible. Moreover, database searches carried out through the NCBI using the nucleotide BLAST program showed that the designed primers were predicted to be highly specific only for *C. jejuni* strains and no other bacterial species were predicted. Therefore, based on these results, we chose the designed primer pair as more suitable for further experiments.

### Prevalence and Concentration of *Campylobacter* on Poultry Carcasses and in Samples from the Slaughtering Environment

A total of 73 poultry carcass samples were collected and enumerated for *Campylobacter* spp. Presumptive *Campylobacter* isolates ($n = 40$) were confirmed by real-time PCR assays targeting 16S rRNA and *hipO* genes and further 16S rRNA sequencing. Thirty-six out of 40 *Campylobacter*-positive samples were positive by the direct-plating method, and 4 samples that were

---

**Table 3.** *Campylobacter jejuni* strains used to compare specificity of the designed and published primer sets

<table>
<thead>
<tr>
<th>Strain</th>
<th>Designed primer</th>
<th>Published primer</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$C_T$</td>
<td>Melting point, °C</td>
</tr>
<tr>
<td><em>Campylobacter jejuni</em> RM 1221</td>
<td>12.74</td>
<td>79.8</td>
</tr>
<tr>
<td><em>Campylobacter jejuni</em> ATCC 43430</td>
<td>12.38</td>
<td>79.4</td>
</tr>
<tr>
<td><em>Campylobacter jejuni</em> ATCC 43431</td>
<td>10.00</td>
<td>78.4</td>
</tr>
<tr>
<td><em>Campylobacter jejuni</em> D781</td>
<td>11.78</td>
<td>78.9</td>
</tr>
<tr>
<td><em>Campylobacter jejuni</em> 81–176</td>
<td>10.25</td>
<td>79.4</td>
</tr>
</tbody>
</table>

$^1$Threshold cycle.
negative by the direct-plating method were confirmed as positive after 24 h of enrichment. Among all Campylobacter spp. isolates, 38 (95%) were identified as C. jejuni and only 2 (5%), isolated from samples collected at the prescalding site, belonged to other Campylobacter species. The prevalence of Campylobacter spp. on broiler carcasses and in the processing environment at the commercial poultry plant surveyed was 54.8% with incident rates of 72.7 and 27.6% for sampling days A and B, respectively (Table 4). Mean counts of C. jejuni contamination on poultry carcasses were $2.44 \pm 0.83$ and $0.83 \pm 1.00 \log_{10} \text{cfu/mL}$ for sampling days A and B, respectively (Table 5).

As broiler carcasses were processed from prescalding to postchilling, the positive percentage and concentration of C. jejuni changed depending on the sampling day and flock contamination (Figures 2 and 3). On sampling day A, 32 of 44 samples (72.7%) were Campylobacter spp.-positive, and only 8 of 29 (27.6%) were positive on sampling day B. Before scalding and defeathering, 100% of the samples on day A were positive for Campylobacter spp., with a mean population of $3.24 \pm 0.22 \log_{10} \text{cfu/mL}$; only 40% of them ($1.12 \pm 1.53 \log_{10} \text{cfu/mL}$) were positive on sampling day B. Scalding and defeathering steps further increased C. jejuni counts to $4.08 \pm 0.12 \log_{10} \text{cfu/mL}$ before evisceration on sampling day A (Table 5). In contrast, the same operations reduced the number of contaminated carcasses on sampling day B and only 1 sample was C. jejuni-positive after 24 h of enrichment. Evisceration process caused a significant ($P < 0.05$) reduction in C. jejuni counts by $2.55 \log_{10} \text{cfu/mL}$ on sampling day A compared with sampling day B, where the same operation appeared to be a major source of contamination and increased C. jejuni populations on the poultry carcasses by $2.22 \log_{10} \text{cfu/mL}$. Although the percentage of positive samples on sampling day A increased from 60% at postvisceralion to 80% at postchilling, processing significantly reduced ($P < 0.05$) the mean number of C. jejuni on broiler carcasses postchilling to $0.92 \pm 0.89$ and $0 \pm 0.00 \log_{10} \text{cfu/mL}$ for days A and B, respectively.

As shown in Table 4, the rates of contamination with C. jejuni presented in the poultry slaughtering environment varied between 0 and 100% for both processing days. All swab samples taken from defeathering and evisceration machines, as well as water samples from scalding tank 1 were positive on sampling day A, whereas none of the samples from the equipment and only 1 out of 3 water samples (33.3%) from scalding tank 1 were positive for C. jejuni on sampling day B.

To confirm the identity of the isolates as C. jejuni and to prove the accuracy of the results obtained by the newly developed FAST real-time PCR method, 16S rRNA sequencing analysis was performed on 6 of the C. jejuni strains isolated at different stages of the poultry processing. The results confirmed that all sequenced strains belonged to C. jejuni (data not shown).
DISCUSSION

PCR Optimization

Accurate identification of*Campylobacter* spp. using phenotypic methods is difficult because of their low biochemical activity, fastidious nature, and frequent variability in the results obtained (On, 1996). Molecular methods and PCR-based techniques, in particular, are being increasingly applied to detect and quantify food-borne pathogens (Debretsion et al., 2007; Park et al., 2011; Alves et al., 2012; Ryu et al., 2013). As conventional PCR methods have several disadvantages, including cost, time-consuming separation of the PCR products by gel electrophoresis, and inability to analyze

Table 5. Populations of*Campylobacter jejuni* on broiler carcasses and water samples recovered by direct plating

<table>
<thead>
<tr>
<th>Sampling day</th>
<th>Water from scald tank 1</th>
<th>Water from scald tank 2</th>
<th>Water from chill tank 1</th>
<th>Water from chill tank 2</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>3.24 ± 0.22ab</td>
<td>4.08 ± 0.12a</td>
<td>1.53 ± 2.09c</td>
<td>0.92 ± 0.89c</td>
<td>2.5 ± 0.04bc</td>
</tr>
<tr>
<td>B</td>
<td>1.12 ± 1.53</td>
<td>1.33 ± 2.09a</td>
<td>2.22 ± 2.02</td>
<td>0.00 ± 0.00</td>
<td>0.83 ± 1.44</td>
</tr>
</tbody>
</table>

1Cambylobacter jejuni populations expressed as log$_{10}$ cfu/mL ± SD.
2Number of samples positive/number samples examined by direct plating in parentheses.
3NSA = not sampled.

Figure 2. Changes in the populations of*Campylobacter jejuni* on broiler carcasses and in water samples on sampling day A (dark gray) and sampling day B (light gray).

Figure 3. Changes in the prevalence of*Campylobacter jejuni* on broiler carcasses along the processing line and in samples from slaughtering environment. Results for defeathering machine 2, evisceration machine 2, scalding tank 3, prechiller tank 1, and chiller tank 2 represent*Campylobacter* spp. prevalence for sampling day A only. Sampling day A = dark-gray bars; sampling day B = light-gray bars.
large number of samples, the real-time PCR technique provides rapid, highly sensitive, and specific identification. As compared with the fluorescence probe-based assays (Hong et al., 2007; Römer and Lindmark, 2007; Schneider et al., 2010; Toplak et al., 2012), real-time PCR methods using the SYBR Green I technique offer an inexpensive and easy way to perform the analysis and could be adopted for routine laboratory testing of samples during food processing.

In the current study, we developed a simple and inexpensive FAST SYBR Green real-time PCR method for identification of C. jejuni based on the amplification of the hipO gene. The newly developed method is capable of detecting 1 genomic copy per reaction, which is comparable with real-time PCR assays described by other authors. Debretsis et al. (2007) reported a detection limit of 1 genome copy for TaqMan Campylobacter PCR assay, whereas Park et al. (2011) showed a sensitivity of 7.33 × 10^4 copies for pure culture C. jejuni in a uniplex SYBR Green real-time PCR. We optimized various conditions of the amplification process, focusing on the annealing temperature and the concentration of primers to obtain the lowest C_T value during amplification of C. jejuni DNA. Results from the gradient real-time PCR showed constant C_T values over a wide range of temperatures, from 55 to 65°C. Annealing temperature is the most critical component when optimizing a PCR reaction and therefore it should be very carefully selected. At too low a T_a both primers can anneal to sequences other than the true target. This can lead to nonspecific amplification and will, consequently, reduce the yield of the desired product. Conversely, too high a T_a may yield little product, as the likelihood of primer annealing is reduced. Therefore, we chose 58°C as optimal T_a for designed primer set.

Campylobacter jejuni-specific primers were designed based on the highly conserved hipO gene in the C. jejuni genome and amplified a 131-bp fragment of this gene. Specificity of the primer pair was confirmed by evaluating several C. jejuni and Campylobacter spp. type strains and 20 strains of other bacterial species (Table 1). The verification of the PCR product in the samples was confirmed by performing a melting curve analysis after each run and comparing their T_m with the T_m of the product from the positive control. The T_m of the PCR amplicons of C. jejuni reference strains was 78.4 to 79.8°C, although some C. jejuni isolates showed a variable peak T_m of 79.0 to 80.3°C in the different assays. Nam et al. (2005) also reported that variations of more than 1°C can occur in the minimum and maximum T_m of PCR amplicons.

**Prevalence of Campylobacter Contamination on Poultry Carcasses During Processing**

A limited number of studies exist that examine Campylobacter prevalence and concentration at multiple points during commercial poultry processing (Guerin et al., 2010), and only a few include the slaughterhouse environment (Klein et al., 2007; Figueroa et al., 2009; Kudirkienė et al., 2011). Findings from recent surveys revealed that Campylobacter occurrence on poultry carcasses varies substantially in different countries (Arsenault et al., 2007; Klein et al., 2007; Son et al., 2007), poultry processing plants (Rasschaert et al., 2006; Barrang et al., 2007; Figueroa et al., 2009), and seasons (Hinton et al., 2004; Reich et al., 2008). In the study of Figueroa et al. (2009), 80 and 41% of the examined poultry carcasses from 2 poultry processing plants were contaminated with Campylobacter spp., whereas Mačkiv et al. (2008), using the traditional method, detected Campylobacter in 74 and 75.4% of the analyzed poultry carcasses in Poland in 2004 and 2005, respectively. Other studies also reported similar results in the prevalence of Campylobacter spp. in fresh broiler carcasses, with incident rates of 56.3 and 87.5% (Hue et al., 2010; Kovalenko et al., 2013). In our survey, a considerable variability in Campylobacter contamination was observed between sampling days A and B. Campylobacter was detected from 72.7% of the analyzed samples on sampling day A and only 27.6% of the samples were positive on day B. A possible reason for this variability could be the variation in Campylobacter presence and numbers by flock, as suggested by Barrang and Dickens (2000). In contrast to day A, the apparently lower prevalence of C. jejuni on poultry carcasses on sampling day B could be due to the recent exposure to contamination of the birds, probably during transport to slaughter (van Gerwe et al., 2009; Berghaus et al., 2013).

Positive percentage and concentrations of Campylobacter changed significantly during different processing steps on both sampling days (Figure 2 and 3). On sampling day A, all pre-scaled and previscerated carcasses were Campylobacter-positive, whereas postviscerated carcasses showed the highest Campylobacter numbers on day B. The major source of Campylobacter contamination on broiler carcasses includes leakage of fecal content from the cloaca and contamination of feathers during transport to the poultry plant (Jacobs-Reitsma et al., 2008). In support of this notion, Stern et al. (2001) reported that Campylobacter populations on the feathers of cooped and transported birds are 10-fold greater than those remaining on the farm. In the same study, the authors reported that many coops were not properly cleaned between flocks, which may contribute to the increased contamination levels observed at the plant.

Defecation is considered as a critical step that potentially spreads microorganisms among carcasses as they pass through the same mechanical picker (Berrang et al., 2001). Wempe et al. (1983) reported that 94.4% of feather-picker drip samples were Campylobacter-positive. In our study, on sampling day A, scalding and defeathering operations led to an increased C. jejuni counts of 0.84 log_10 cfu/mL, whereas the same steps
caused significant ($P < 0.05$) reduction of $1.12 \log_{10}$ cfu/mL on day B. Similar results to ours for sampling day B have been published by Hinton et al. (2004), who reported that the combination of scalding and picking operations decreased the number of Campylobacter recovered from the carcasses by over $3.0 \log_{10}$ cfu/mL.

On sampling day B, the highest isolation rate of C. jejuni (60%) was found after evisceration steps. The reason may be the rupture of internal organs, which resulted in high numbers of Campylobacter on the carcasses, equipment, working surfaces, process water, and air (Jacobs-Reitsma, 2000; Berrang et al., 2001). It should be also noted that, as the skin of poultry carcasses is usually not removed, high levels of Campylobacter may be present on and in it (Lee et al., 1998; Son et al., 2007). Berrang et al. (2004) have reported that even small amounts of intestinal contents can cause a significant increase in the number of C. jejuni on poultry carcasses.

Despite the fact that immersion chilling washes off C. jejuni from the surface of the carcasses, this method may also lead to cross-contamination (Wempe et al., 1983; Corry and Atabay, 2001; Guerin et al., 2010). Our survey showed that the average concentration of C. jejuni on broiler carcasses decreased significantly ($P < 0.05$; $0.92 \pm 0.89$ and $0 \pm 0.00 \log_{10}$ cfu/mL for days A and B, respectively), but the number of contaminated carcasses increased to 80% on sampling day A, suggesting that the cross-contamination occurred in the chill tank. The reduction of the mean numbers of C. jejuni on the broiler carcasses is due to the appropriate chlorine concentration and low temperatures used in the chiller tanks. Decreases in C. jejuni counts associated with chilling operation have also been reported in other studies, indicating that it is possible to achieve reductions of up to $2 \log_{10}$ cfu/mL of Campylobacter on the carcasses (Cason et al., 1997; Sánchez et al., 2002; Stern and Robach, 2003; Figueroa et al., 2009).

Prevalence of Campylobacter in Poultry Processing Environment

Equipment and processing water are the main routes for Campylobacter contamination during poultry slaughtering (Kudirkienė et al., 2011). Although a proportion of the bacterial population is killed by the high temperature of scalding, Campylobacter has been periodically recovered from scalding water (Stern et al., 2001; Figueroa et al., 2009). Our study clearly revealed that scalding tank 1 was an important source of contamination and contributed to the increased level of C. jejuni numbers on sampling day A. These findings correlated well with those published by Klein et al. (2007), who reported that even during the slaughtering of Campylobacter-negative flocks the majority of the tested scalding water samples were Campylobacter-positive, though the numbers were lower compared with those collected from Campylobacter-positive flocks. Moreover, all analyzed water samples from scalding tank 1 were C. jejuni-positive on processing day A, whereas no cells were recovered from scalding tank 3 even after 48 h of enrichment on the same day. Similar to our results, Hinton et al. (2004) found significantly fewer Campylobacter cells in the final tank of a multiple-tank counterflow scalding system compared with the first tank and suggested that the reduction in Campylobacter counts could be due to the progressively cleaner water in the tanks and also to the progressive increase in the temperature of water in the tanks. Additionally, all water samples collected from prechiller and chiller tanks were C. jejuni-negative on sampling day A. Our results were in agreement with those published by Figueroa et al. (2009), who isolated thermotolerant campylobacters in only 1 sample of chilling water from a total of 22 samples analyzed. Findings from our survey showed that the low isolation rate from the chilling water is in discrepancy with the percentage of C. jejuni detected on the poultry carcasses postchilling. The possible explanation is the low level of C. jejuni population in the water samples and the limitation of detection sensitivity. Contrary to our results, Wempe et al. (1983) recovered Campylobacter from all chilling water samples and suggested that the chill tank represents a major area where cross-contamination could occur. Trimble et al. (2013) also supported the idea that immersion chilling is considered as a potential site for cross-contamination, as multiple carcasses share the same water bath. Carcasses that enter the chill tank free of bacteria can become contaminated, whereas heavily contaminated carcasses might exit the tank with fewer bacteria.

As machinery cannot accommodate to the natural variation in the size of the carcasses being processed, rupture of the intestines and leak of fecal material on the equipment and carcasses are often observed (Rosenquist et al., 2006). In our study, the percentage of positive samples from defeathering and evisceration machines varied from 0 to 100% depending on the sampling day and flock contamination. Similar results have been reported by Figueroa et al. (2009), who found that 64% of the swab samples from evisceration machines were Campylobacter-positive.

Our survey has some limitations, such as small sampling size, sample collection from a single poultry processing plant, and samples taken within a short period of time. Nevertheless, our main objective was to apply the newly developed FAST real-time PCR method and evaluate if the assay is a rapid and simple method for identification of C. jejuni in poultry samples. Based on our results, the real-time PCR described herein can be used in a future comprehensive survey of poultry processing as a quantitative assay for initial screening of C. jejuni in poultry samples. By using membrane-impermeant dyes, such as propidium monoazide, this method may be used for direct quantification of only viable cells (Josefsen et al., 2010). Furthermore, the newly developed PCR method can be easily adapted in laboratories for reliable and unambiguous identification of C. jejuni in poultry samples.
In summary, our study revealed the widespread of \textit{C. jejuni} on poultry carcasses and in samples from the processing environment and confirmed that the newly developed FAST real-time PCR assay is a simple, rapid, sensitive, and inexpensive system for identification of \textit{C. jejuni}. An advantage of this technique is that it does not require time-consuming postamplification analysis and enables researchers to process more samples compared with the conventional phenotyping methods or standard real-time PCR method. Moreover, the cost of the materials necessary to analyze 1 sample was under $3.00 and the total time required to perform the PCR assay was less than 1 h.

**ACKNOWLEDGMENTS**

This study was partially supported by the Borlaug Fellowship Program (USDA FAS, Washington, DC) and a USDA-National Integrated Food Safety Initiative grant (2007-51110-03812). Mirena Ivanova was awarded a grant by the Borlaug Fellowship Program for Bulgaria.

**REFERENCES**


