Comparison of cumulative drip sampling with whole carcass rinses for estimation of *Campylobacter* species and quality indicator organisms associated with processed broiler chickens

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**ABSTRACT** The whole carcass rinse (WCR) procedure is routinely used as a sampling method for determining the presence and number of quality-indicator organisms or pathogens associated with broiler chicken carcasses in processing facilities. Collection of a cumulative drip sample by placing collection vessels under the processing line could potentially capture a more representative sample of bacterial populations associated with an entire flock with less labor than individual bird rinses. The purpose of this study was to evaluate a cumulative drip sampling method for recovery of *Campylobacter* spp. and 3 types of quality indicator organisms from broiler carcasses. Cumulative drip and WCR samples were collected on 14 d from a commercial broiler processing facility over a 3-mo period. No statistically significant difference was demonstrated between the WCR and cumulative drip sampling methods in recovery of *Campylobacter* spp., total aerobes, Enterobacteriaceae, or *Escherichia coli* associated with the postvisceralization samples (*P* > 0.01). Analysis of the pyrosequencing census data demonstrated high interbird variability and indicates cumulative sampling may be required to obtain fully representative sampling of a flock. For most bacterial taxa, the relative abundance in individual WCR was correlated with cumulative drip samples, but some taxa were undercounted or missed entirely by individual WCR. Consequently, individual carcass rinses may not be representative of the flock microbial community. The cumulative drip sampling technique may save labor and provide a more representative summary of process control in poultry processing facilities.

**Key words:** *Campylobacter*, indicator organism, broiler chicken, carcass rinse, sampling methodology

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**INTRODUCTION**

*Campylobacter* is an important human pathogen, responsible for 15% of hospitalizations resulting from foodborne illness in the United States and 400 to 500 million cases of infection each year worldwide (EFSA, 2010; CDC, 2011). There is a strong association between *Campylobacter jejuni* and poultry with more than 80% of broiler chicken flocks positive for the pathogen at time of processing (Herman et al., 2003; EFSA 2010). There is increasing evidence that poultry may be the leading contributor to campylobacteriosis in humans (Hermans et al., 2012). Efforts are ongoing to reduce the presence of campylobacters associated with broilers on the farm and in the processing plant; therefore, reliable methods for isolation and enumeration of pathogens are required. Because isolation and enumeration of pathogens such as *Campylobacter* can be difficult, the presence of other more easily culturable organisms commonly associated with fecal contamination is frequently used as an indicator of process control (Ayres et al., 1980). However, no reliable indicator organisms demonstrating a high correlation with *Campylobacter* spp. contamination have been reported.

The whole carcass rinse (WCR) procedure is routinely used as a sampling method for determining the presence and number of quality-indicator organisms as well as pathogens associated with broiler chicken carcasses in processing facilities (USDA Food Safety Inspection Service, 1998). Briefly, the technique involves using sterile gloves to place a processed broiler carcass (typically postchill) into a sterile plastic bag. A suitable diluent is added and the carcass is vigorously shaken in the bag for approximately 1 min. After the carcass is removed, the rinse is poured into sterile plastic containers and held on ice for transport to a laboratory for microbiological analysis. This process can be labor intensive when numerous birds each weighing up to 3 kg must be sampled. It is also possible that the microbiota recovered from each rinse sample is primarily represen-
tative of only that individual carcass and is not representative of the overall microflora associated with the entire flock being processed. Variability in the numbers of specific bacteria associated with individual carcass rinses is common and interbird variability can be high (Stern and Line, 2009; Line et al., 2011). Additionally, studies have shown that rinsing the same carcass repeatedly up to 4 times results in no statistically significant difference in populations of some microorganisms recovered between samples (Izat et al., 1991).

Collection of a cumulative drip sample by placing collection vessels under selected locations in the processing line for set periods of time could reflect a potentially more representative sample of bacterial populations associated with an entire flock. Also, collection of cumulative drip samples would be less labor intensive than performing individual bird rinses. As each bird passes over the collection vessel, random drops of liquid from the processed broilers are collected. By carefully selecting locations and the specific time periods for collection, bird-to-bird variability would be minimized and a potentially more representative sample would be obtained for assay. The objectives of this study were to evaluate a novel cumulative drip sampling method for recovery of Campylobacter spp. and 3 types of quality indicator organisms (total aerobes, Enterobacteriaceae, and Escherichia coli) for comparison with the more traditional whole broiler carcass rinse sampling method. To measure interbird variability of the entire microbial community and determine how this variability may influence sampling strategies, broad-range pyrosequencing of 16S rRNA genes was used to compare the total microbial community of several individual carcass rinses and cumulative drip samples. Pyrosequencing has emerged as a new tool to quickly and comprehensively take a census of an entire microbial community (Andersson et al., 2008; Rothberg and Leamon, 2008), and we apply it here for the first time in a poultry-processing environment to compare bacterial communities recovered by individual carcass rinses versus cumulative drip sampling of an entire flock.

**MATERIALS AND METHODS**

**Sample Collection**

Samples were collected on 14 separate occasions from a Georgia commercial broiler processing facility during a 3-mo period. On each sampling day, WCR samples were obtained postvisceralion (n = 10). Clean latex gloves were used to place each carcass in a large plastic bag (37 × 52 cm; Cryovac, Duncan, SC). Sterile water (100 mL) was added to each bag, and the carcass was shaken in a prescribed manner for 1 min (Stern et al., 2001). Approximately 50 to 75 mL of rinsate was retrieved from each carcass, poured into individual sterile 150-mL sample cups, and kept on ice after collection. Cumulative drip samples were obtained by placing multiple (replicate) collection vessels (approximately 20 cm in diameter, 2 L volume) at various points beneath the postvisceralion drip line (n = 4) on each collection day. Collection points were carefully selected to obtain drip directly from carcasses while not interfering with the processing line operation or plant personnel.

![Figure 1. Enumerations of total viable aerobes (TVC; A), Enterobacteriaceae (EB; B), Escherichia coli (EC; C), and Campylobacter (D) recovered from 4 sample types. Horizontal bars represent means; letters (a–c) denote significance (P = 0.01) of pairwise t-tests as detailed in Materials and Methods. MPN = most probable number.](https://academic.oup.com/ps/article-abstract/92/1/218/1554368)
tion vessels were left in place during the time required (approximately 1 h) for all the birds in a selected flock (about 15,000) to pass the collection point. Postchill WCR samples (n = 10) and postchill cumulative drip (PCD, n = 4) were similarly collected from the same flocks by sampling at the appropriate times and points as the flocks proceeded through the plant. Samples were transported on ice to the laboratory for microbiological analysis. *Campylobacter* spp. were counted by traditional direct plating of multiple dilutions on Campy-CEFEX agar (Stern et al., 1992). Plates were incubated at 42°C for 24 to 48 h under microaerobic conditions (10% CO2, 5% O2, and 85% N2).

**Automated MPN Determination**

Total viable count, *Enterobacteriaceae* (EB), and *E. coli* (EC) populations were determined using the TEMPO automated MPN procedure (bioMerieux, St. Louis, MO) per the manufacturer’s instructions in a manner similar to Line et al. (2011). Portions of each sample (~30 mL) were aseptically transferred to individual sterile filter bags (bioMerieux) and held on ice. Dehydrated media in vials specific for each test were rehydrated by aseptically adding sterile water. The media contain fluorescent tags indicative of growth of the organisms of interest. Samples were added to vials of prepared dehydrated media, mixed thoroughly, and introduced into the TEMPO cards in an automated vacuum chamber. Microchanneled cards of the instrument automatically create the dilution series necessary for MPN estimation. The cards were then removed from the vacuum chamber and incubated as appropriate. The total viable count cards were incubated at 35°C for 24 h and the EB and EC cards were incubated for 48 h at 35°C. Following incubation, the cards were placed in a reading chamber equipped with a detector capable of determining fluorescence associated with growth of organisms in specific channels on the cards. Computer software (TEMPO Read, bioMerieux) was used to in-

**Figure 2.** Correlations between indicator taxa for cumulative drip samples postvisceral drip line (PED; circles, n = 52), and individual carcass rinse samples postvisceral whole carcass rinse (triangles, n = 130). Dotted lines represent smoothed curves fitted with a LOWESS (locally weighted polynomial regression) function as described in the Materials and Methods. Numbers within each plot represent Pearson correlation coefficients. EB = *Enterobacteriaceae*; TVC = total viable count; EC = *Escherichia coli*. Color version available in the online PDF. MPN = most probable number.
were determined in R.

- test

bution (Silbernagel and Lindberg, 2002). Means, SD, estimates were converted to log10 counts to more closely match the underlying assumption of a normal distribution. Colony counts or MPN estimates above the detectable range were transformed with the detection limit value. Colony counts or MPN (1.0 cfu/mL) in a manner similar to previous studies (Line et al., 2011). The MPN results above or below the 3.7 log range of detection for the individual TEMPO cards. Nevertheless, some results were above or below the 3.7 log range of detection for the individual TEMPO cards. A second card run at a higher or lower dilution as appropriate for some samples could have been analyzed to capture all possible responses, but this was not done for economic reasons. Results from all enumeration methods were entered into Excel (Microsoft, Redmond, WA) and R (R Development Core Team, 2010) for statistical comparison. For thoroughness we chose to conduct analyses considering all the data points including those above or below the actual detection limit of the analyses which required transforming the raw data. As a conservative rule, results recorded as <X were transformed as 0.1X (i.e., <1.0 cfu/mL was transformed as 0.1 cfu/mL, and those results recorded as <10.0 cfu/mL were transformed as 1.0 cfu/mL) in a manner similar to previous studies (Line et al., 2011). The MPN estimates above the detectable range were transformed with the detection limit value. Colony counts or MPN estimates were converted to log10 counts to more closely match the underlying assumption of a normal distribution (Silbernagel and Lindberg, 2002). Means, SD, t-test P-values, and appropriate correlation coefficients were determined in R.

**Molecular Methods**

Two samples representing cumulative drips and 3 samples from individual carcass rinses were selected for pyrosequencing. The PCR and pyrosequencing were performed at Research and Testing Laboratory (Lubbock, TX: http://www.researchandtesting.com) using tagged amplicon methods similar to those described previously (Dowd et al., 2008). The PCR was performed in 25-μL volumes containing 1 U of Phusion polymerase (NEB, Ipswich, MA), 200 μM dNTP, 2 mM MgCl2, and 0.4 μM of each primer. No-template controls and gDNA extractions from pure cultures served as negative and positive controls, respectively. The PCR products were cleaned with Agencourt AMPure XP beads (Beckman Coulter, Brea, CA). Twenty cycles of PCR (94°C for 30 s, 50°C for 30 s, 72°C for 40 s) were used with a final extension at 72°C for 10 min. We used the broad-range primers 104F (5′-GGGCGVACGCGGTAGTAAA-3′) and 530R (5′-CGCNCNGCNGCTGAC-3′) targeting 16S rRNA genes similar to previous approaches using high-throughput sequencing to perform a census of bacterial communities (Andersson et al., 2008; Dowd et al., 2008; Hamady et al., 2008).

Perl and Bioperl scripts were used to perform initial quality-control and screening of raw sequence data including trimming of tag sequences, screening for presence of both the forward and reverse PCR primers and removal of sequences with any ambiguous base calls. Sequences which passed all the quality-control screens described above were compared with BLASTN to a reference database containing 16S rRNA sequences taken from the Silva project (v104) curated seed database (Pruesse et al., 2007). The highest ranked hit to this database was retained using a significance cutoff of 10−4. To assess phylotype richness and diversity independent of taxonomic assignments as is commonly done in microbial ecology (Schloss and Handelsman, 2005), sequences which passed all the screens described above were grouped into similarity clusters, or sequence types, based on percentage sequence identity using a 95% similarity cutoff (roughly equivalent to a genus level) using the program CD-HIT-EST (Li and Godzik, 2006) with default parameters. Data matrices containing sequence types and the number of sequences belonging to each sequence type for each sample were used to cluster samples using canonical correspondence analysis (Ter Braak, 1986), which essentially provides a multivariate fingerprint of a microbial community in a sample. All graphical and statistical analyses of the pyrosequencing data were conducted in R (R Development Core Team, 2010).

**RESULTS AND DISCUSSION**

**Enumeration**

The MPN data from the Tempo instrument are presented in a strip chart format with means and pairwise
$t$-tests between means shown of log_{10}-transformed values (Figure 1). No differences ($P < 0.01$) were found between the cumulative drip sampling and individual carcass rinse methods in recovery of total aerobes (Figure 1A), EB (Figure 1B), $E.\ coli$ (Figure 1C), or $Campylobacter$ spp. (Figure 1D) associated with the postvisceralation samples. At this point in the processing continuum, the bacterial populations associated with the carcasses are still relatively high because the carcasses have not yet encountered the interventions used to reduce bacterial populations, such as chlorine in the chill tank or other sanitizing solutions.

As expected, postchill samples had fewer bacteria associated with them than did postvisceralation samples, which was reflected in significant reductions in all 4 bacterial groups as measured by both sampling methods (Figure 1). For $Campylobacter$, the means of postchill samples were not significantly different ($P = 0.21$) for the 2 sampling methods (Figure 1D). In contrast, for each of the other 3 indicators, means of the cumulative drip samples were significantly lower than the carcass rinse samples (Figure 1A-C). The greater differences observed between the methods for the postchill samples are perhaps due to the long dwell time and exposure of individual bacterial cells in the drip samples containing residual chlorine from the chill tank. Compounds such as fats from carcasses present in the chill tank can bind residual chlorine, making it unavailable and inactive. Physical protection afforded by the carcass to individual bacterial cells is also likely to be important because $Campylobacter$ associated with the carcass can become trapped in crevices and in feather follicles (Chantaranont et al., 2003), which could offer some protection to organisms from sanitizers. The PCD samples could be exposed to higher levels of chlorine or other sanitizing solutions for a longer period of time, which could cause greater stress on the microorganisms, resulting in decreased populations of viable bacteria. It is possible that a neutralizing solution could be developed for inclusion with the PCD samples as they are collected to minimize microbial stress and improve recovery from this sample type.

Figure 4. Relative proportions of sequence types from cumulative drips versus individual bird rinses. Each point in the graph represents a unique taxon at the species level as determined by classification of sequences with the Silva reference database using BLASTN as described in the methods. Panel A shows all taxa for the 2 drip samples combined versus all 3 carcass rinses combined. The 2 solid points are shown in detail in panels B and C for percentages of sequences classified as $Gallibacterium\ anatis$ (B) and $Klebsiella\ pneumoniae$ (C) for each sample type. PED = postvisceralation drip line; PEWCR = postvisceralation whole carcass rinse.
Assessments of Potential Indicator Taxa

We compared the correlations between the human pathogen *Campylobacter* and traditional indicator organisms. Culture-based microbial indicator organisms have been reported to rarely correlate with *Campylobacter* spp. presence (Hellein et al., 2011), and this trend was likewise observed in our study. There were no significant correlations between any of the indicator organisms and *Campylobacter* spp. for any sample type (Figure 2). There were positive correlations observed between the various indicator organisms as expected with the greatest correlation existing between EB and EC for the postevisceration drip line samples (0.93).

Whole-Community Profiling with Pyrosequencing

Clustering of samples based on whole-community similarity profiles showed high interbird variability and suggested cumulative collections may be required to obtain fully representative sampling of a flock. Individual carcass rinse samples (R2, R6, R10) were each separated along both of the principal axes of the canonical correspondence analysis, whereas the 2 samples from the cumulative drip sampling (PED12, PED34) of the same flock were quite similar to one another (Figure 3). This indicates highly similar communities in the 2 cumulative drip samples sequenced. Perhaps not surprisingly, it appears that microbial communities from individual carcass rinses vary among rinses, whereas cumulative drip sampling may effectively integrate this variability by pooling samples from many individual carcasses.

To further compare the individual rinse versus cumulative drip samples, we next compared the relative abundance of each sequence type (classified to the species level) between the 2 methods. Two sequence types in particular, one most closely related to *Klebsiella pneumoniae*, and another most closely related to *Gallibacterium anatis*, were underrepresented in the individual carcass rinse samples (Figure 4A). Queries of the *Gallibacterium* sequence type revealed it was completely absent from one of the individual bird rinses (Figure 4B). Similarly, the *Klebsiella* sequence type was absent from one rinse and only present in the 2 others at a very low percentage (<0.1%, Figure 4C). Based on these results, we concluded that individual carcass rinses can severely underrepresent or even miss important bacterial taxa relative to the cumulative drip samples.

To investigate in more taxonomic detail which genera may be under- (or over-) represented by individual carcass rinses, we next compared the relative abundance of genera by sample type (Figure 5). Several genera were significantly underrepresented in the individual carcass rinses relative to the cumulative drip samples as determined by simple chi-squared tests. These included *Gallibacterium*, *Lactobacillus*, and *Klebsiella* (Figure 5). Only 2 genera were overrepresented in the carcass rinses versus the cumulative drip samples: *Eubacterium* and *Oscillibacter* (Figure 5). All 5 of these genera have been encountered in previous surveys of poultry cecal (Oakley et al., 2012a) and fecal (Oakley et al., 2012b) samples, but to our knowledge, the current study represents the first application of high-throughput sequencing to poultry carcass rinses.
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