Concurrent Quantitation of Total \textit{Campylobacter} and Total Ciprofloxacin-Resistant \textit{Campylobacter} Loads in Rinses from Retail Raw Chicken Carcasses from 2001 to 2003 by Direct Plating at 42°C

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This is the first report on the use of a normally lethal dose of ciprofloxacin in a \textit{Campylobacter} agar medium to kill all ciprofloxacin-sensitive \textit{Campylobacter} spp. but allow the selective isolation and quantitation of naturally occurring presumptive ciprofloxacin-resistant \textit{Campylobacter} CFU in rinses from retail raw chicken carcasses (RTCC). Thermophilic-group total \textit{Campylobacter} CFU and total ciprofloxacin-resistant \textit{Campylobacter} CFU (irrespective of species) were concurrently quantified in rinses from RTCC by direct plating of centrifuged pellets from 10 or 50 ml out of 400-ml rinse subsamples concurrently on \textit{Campylobacter} agar and ciprofloxacin-containing \textit{Campylobacter} agar at 42°C (detection limit = 0.90 log_{10} CFU/carass). For 2001, 2002, and 2003, countable \textit{Campylobacter} CFU were recovered from 85%, 96%, and 57% of RTCC, while countable ciprofloxacin-resistant \textit{Campylobacter} CFU were recovered from 60%, 59%, and 17.5% of RTCC, respectively. Total \textit{Campylobacter} CFU loads in RTCC rinses ranged from 0.90 to 4.52, 0.90 to 4.58, and 0.90 to 4.48 log_{10} CFU/carass in 2001, 2002, and 2003, respectively. Total ciprofloxacin-resistant \textit{Campylobacter} CFU loads in RTCC rinses ranged from 0.90 to 2.0, 2 to 3, 3 to 4, and 4 to 5 log_{10} CFU/carass, respectively, were recovered from 16%, 32%, 26%, and 5% of RTCC tested over the 2 1/2-year sampling period. For the same period, total ciprofloxacin-resistant \textit{Campylobacter} loads of 0.90 to 2.0, 2 to 3, 3 to 4, and 4 to 5 log_{10} CFU/carass, respectively, were recovered from 24%, 11%, 7%, and 0.2% of RTCC tested. There was a steady decline in total \textit{Campylobacter} and total ciprofloxacin-resistant \textit{Campylobacter} loads in RTCC rinses from 2001/2002 to 2003.

Many recent reports show that the usage of the fluoroquinolone group of antibiotics in poultry apparently creates a reservoir of ciprofloxacin-resistant \textit{Campylobacter jejuni} and other \textit{Campylobacter} spp. in the food chain in the United States (1, 4, 9, 10a, 21, 29, 31, 32). Recent surveillance data by the National Antimicrobial Resistance Monitoring Program (NARMS) clearly illustrate the emerging ciprofloxacin (a fluoroquinolone antibiotic) resistance of \textit{Campylobacter} in humans (2, 5, 13). Molecular subtyping showed an association between ciprofloxacin-resistant \textit{C. jejuni} from chicken products and acquired \textit{Campylobacter} infections among Minnesota residents (29) who had contact with these products. Among \textit{Campylobacter} spp., both \textit{C. jejuni} and \textit{C. coli} are recognized as predominant human-pathogenic species, showing the presence of ciprofloxacin-resistant strains, and these were frequently found in retail raw chicken carcasses (RTCC) (1, 3, 21, 26). While ciprofloxacin resistance in \textit{Campylobacter} can occur following the treatment of humans with the antibiotic, raw poultry is considered a significant source of ciprofloxacin-resistant \textit{Campylobacter} (1, 4, 11, 16, 21, 29, 31, 32). Therefore, there is a need for monitoring their persistence and quantitative reduction of the total ciprofloxacin-resistant \textit{Campylobacter} load in the food chain, particularly from raw chicken products, in efforts to control human campylobacteriosis.

At the present time, the occurrence of ciprofloxacin-resistant \textit{C. jejuni} or other \textit{Campylobacter} spp. on raw chicken carcasses is determined by enrichment methods which provide only qualitative presence or absence tests per carcass sampled (12, 13, 26, 34). Selective quantitative methods are yet to be developed for the quantitative monitoring of total ciprofloxacin-resistant \textit{C. jejuni} and other \textit{Campylobacter} loads persisting on raw and raw further-processed poultry products. The standard \textit{Campylobacter} selective broth enrichment methods will not provide estimates of the original numbers of \textit{Campylobacter} cells present per carcass. Also, culture isolation by broth enrichment techniques does not permit the total differentiated enumeration of the numbers or diversity of the ciprofloxacin-resistant versus ciprofloxacin-sensitive \textit{Campylobacter} strains present in foods. The faster-growing strains will overgrow other strains. Genetic-based resistance to ciprofloxacin in human and animal isolates of \textit{Campylobacter} has been established (7, 14, 31). A PCR-based TaqMan method was developed for the detection of \textit{C. jejuni} isolates that carry the C→T transition in codon 86 of gyrA (24, 33, 35). However, there appears to be the involvement of multiple genes for resistance to ciprofloxacin (10, 14), and a comprehensive set of PCR probes for these other loci has not yet been developed.

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Confirmative tests based on complete gene sequencing or multiple PCR tests are indispensable for the complete characterization of ciprofloxacin-resistant Campylobacter. However, it is currently impractical by any DNA-based methods to isolate and quantitatively enumerate ciprofloxacin-resistant Campylobacter load from crude carcass rinses without destroying the Campylobacter cells present in those samples. Recently, a direct real-time PCR quantification of Campylobacter jejuni in chicken fecal and cecal samples has been published, but the minimum quantitation limit was 4 log_{10} CFU/g (27). A culture-based direct plating method was recommended for the isolation and enumeration of total Campylobacter spp. from broilers (17, 28), but no such method was published for ciprofloxacin-resistant Campylobacter load. This is the first report of a direct plating method for selectively quantifying the persisting ciprofloxacin-resistant subpopulation of the total Campylobacter load on retail raw chicken carcasses.

**MATERIALS AND METHODS**

*Campylobacter* media and chemicals. Campylobacter agar (CA) was prepared from Food and Drug Administration (FDA)-recommended *Campylobacter* enrichment broth medium (Bolton formula, LAB 135; J&K Microbiologies, Inc., North Ridgeville, OH) (15) by adding 1.5% agar (Becton-Dickinson Microbiology Systems, Sparks, MD). After autoclaving and tempering CA medium, 5% lysed defibrinated horse blood (MT 59074; Quad Five, Ryegate, MT) and FDA- approved Campylobacter selective antibiotic supplement (X132, comprised of four selective antibiotics for Campylobacter isolation from raw poultry products, namely, 20 mg/liter sodium cephaloridine, 20 mg/liter vancomycin, 20 mg/liter trimethoprim, and 25 mg/liter natamycin; J&K Microbiologies, Inc., North Ridgeville, OH) were added and mixed thoroughly prior to pouring CA plates. Ciprofloxacin-containing Campylobacter agar (CCA) containing 8.6 mg/liter ciprofloxacin was prepared by adding 10 mg/liter ciprofloxacin hydrochloride (purity = 86.2% ciprofloxacin; Serological Proteins, Inc., Kankakee, IL) to CA along with 5% horse blood and other selective antibiotic supplement described above before pouring plates.

**Retail raw whole chicken carcass sampling source.** From July 2001 through December 2003, individual commercially packaged refrigerated raw whole chicken carcasses (approximately 3 lb each) were sampled within their printed shelf dates generally at weekly intervals from local retail grocery stores in the Fayetteville, AR, area, at the rate of four carcasses per week. The samples were transported to the laboratory and sampled within 2 h of purchase.

**Carcass rinse collection, subsample rinse concentration, and direct plating on CA and CCA.** After removing from the retail package, each raw whole chicken carcass was placed in a 15-in. by 20-in. sterile poultry rinse bag (Nasco, Fort Atkinson, WI) and Butterfield’s phosphate diluent (400 ml) was added to the bag. One-half of the Butterfield’s phosphate diluent was poured into the interior cavity of the carcass and the other half on the outside of the carcass. To make sure all surface areas of each carcass were sampled, each carcass was rinsed inside and out with a rocking reciprocal motion in an 18- to 24-in. arc for 2 min. Then, the bag was aseptically cut at the lower corner to recover the whole carcass rinse into a sterile 500-ml bottle. The carcass rinse sample collected was mixed by gentle shaking prior to removing large subsamples for assay. Rinse subsamples were tested concurrently for each carcass for the determination of total Campylobacter counts and total ciprofloxacin-resistant *Campylobacter* counts by direct plating. Subsample volumes of 10 ml and 50 ml from the 400-ml rinse per carcass were concentrated by centrifugation at 8,000 × g for 20 min, and the supernatant was carefully decanted without disturbing the pellet. Using all of the small volume (approximately ~250 µl) of rinse left in the tube after discarding the supernatant, the entire pellet was resuspended by pipetting and then direct plated on CA or CCA. Pellets obtained by centrifuging rinse subsamples (one 10 ml and one 50 ml for CA; both 50 ml for CCA) per carcass were directly plated on CA or CCA. CA and CCA plates were incubated under microaerophilic conditions in a Campy gas mixture (5% O2, 10% CO2, 85% N2) at 42°C for 48 h. Typical presumptive *Campylobacter* microcolonies obtained from each rinse subsample concentrate on CA or CCA were enumerated as described below.

**Campylobacter colony presumptive confirmation on CA and CCA, calculation of CFU counts, and statistical analysis of data.** Characteristic total *Campylobacter* colonies on CA or CCA were presumptively identified after 48-h incubation at 42°C based on typical morphological characteristics (17, 18, 20, 25). Typically, two types of *Campylobacter* colonies were found on CA or CCA after 48 h: (a) round, 1- to 2-mm-diameter, small, raised, smooth, shiny, convex, with a defined clear or translucent edge and a dirty brownish opaque center, and (b) flat, large, spreading with an irregular edge, clear, translucent, light cream, or grayish. Only a few non-*Campylobacter* contaminants grew on CA or CCA, and these were easily distinguishable from typical *Campylobacter* by their differences in colony morphologies. Representative characteristic *Campylobacter* colonies were examined by wet mounts for the presence of thin, curved, spiral cells with corkcrew motility. The representative presumptive *Campylobacter* colonies after their isolation on CA and CCA (a total of 16 *Campylobacter* isolates representing four carcasses per week) were randomly selected and preserved as frozen stocks after growth expansion. Genus and species identities for some selected *Campylobacter* isolates from CA and CCA were confirmed by PCR assays (19).

Total *Campylobacter* CFU recovered per carcass were calculated based on the following formula: n2 = n1 × V1/V2, where n2 is the total number of typical *Campylobacter* CFU recovered on CA at 42°C within 48 h per concentrated rinse subsample, V1 is the total volume of chicken carcass rinse (400 ml), and V1 and V2 are the volume of subsample rinse used for concentration, 10 and 50 ml, respectively. Total ciprofloxacin-resistant *Campylobacter* CFU recovered per carcass were calculated based on the following formula: n2 = n1 × V1/V3, where n2 is the total number of typical *Campylobacter* CFU recovered on CCA at 42°C within 48 h per concentrated rinse subsample and V1 and V3 are as described above. All *Campylobacter* count data were transformed to log_{10} CFU/carcass using the Microsoft Excel program (Microsoft Corp., Redmond, Wash.). Mean log_{10} CFU/carcass of total *Campylobacter*, mean log_{10} CFU/carcass of total ciprofloxacin-resistant *Campylobacter* were calculated from standard deviation, standard error of the mean, correlation, and regression analyses were calculated using the Excel program.

Ciprofloxacin resistance confirmation of random *Campylobacter* colony picks on CA and CCA. Representative presumptive characteristic *Campylobacter* colonies randomly picked on CA or CCA (total of 16 new *Campylobacter* isolates representing four carcasses weekly) were spotted as individual spots (0.5-cm diameter) to generate fresh spiral cell growth; cell suspension (approximately 10^7 CFU/ml) was prepared by lifting each spot into 1 ml of Campylobacter enrichment broth (Bolton formula), each was then labeled as an individual *Campylobacter* isolate and then spotted (10 µl per spot, approximately 0.5-cm diameter) onto a series of CCA plates containing different basal concentrations of ciprofloxacin (0, 2, 4, 8, 16, 32, 64, or 128 µg/ml), and all plates were incubated at 42°C for 48 h under microaerophilic conditions to determine the ability of *Campylobacter* to grow at each ciprofloxacin concentration. Based on the agar dilution method according to NCCLS recommendations (23), a diverse set of *C. jejuni* and other *Campylobacter* spp. isolated on CA and CCA were tested concurrently for ciprofloxacin MICs on both CCA and Mueller-Hinton agar containing 5% defibrinated sheep blood, with *C. jejuni* ATCC 33560 as the quality control organism. The genetic basis for ciprofloxacin resistance of some selected *Campylobacter* isolates was confirmed by CampyMAMA PCR (36).

**RESULTS AND DISCUSSION**

One of the highest-priority research needs on *Campylobacter* was to develop laboratory methods for quantifying an antibiotic-resistant *Campylobacter* load persisting on raw poultry products to aid in risk assessment, to evaluate intervention strategies, and to develop meaningful baseline data for this pathogen. Currently, there is no published method for estimating loads of ciprofloxacin-resistant *Campylobacter* CFU within the total *Campylobacter* CFU load per chicken carcass. The recently published direct-plating method by U.S. Department of Agriculture (USDA)-Agricultural Research Service (17, 18) permitted the quantitative enumeration of *Campylobacter* CFU but not of antibiotic-resistant *Campylobacter*. Ge et al. (12) recently examined the antimicrobial susceptibilities of 378 *Campylobacter* species isolates obtained by an enrichment method from retail meats, but their method did not permit quantitation of the numbers of such antibiotic-resistant *Campylobacter* present in those meat products. Stern and Robach (30) and Siragusa et al. (28) enumerated the total
Campylobacter spp. on processed broiler carcasses by a direct plating method but did not enumerate ciprofloxacin-resistant subpopulations present on those carcasses. Using the CA- or CCA-based direct-plating method, we determined quantitative trends in the numbers of total Campylobacter CFU (on CA medium) and total ciprofloxacin-resistant Campylobacter CFU (on CCA medium) at the rate of four carcasses per week by sampling a total of 420 carcasses in 105 weeks during the period from July 2001 through December 2003.

Currently, there is no universally accepted best plating medium for isolating Campylobacter spp. In our direct-plating method, FDA-recommended Bolton formula was used in CA or CCA while Food Safety and Inspection Service and Agricultural Resource Service scientists adopted Campy-Cefex or modified campylobacter charcoal differential agar or Campy-Line agar media (17, 25, 28, 30) for Campylobacter enumeration. We did not use NCCLS-recommended Mueller-Hinton agar supplemented with 5% defibrinated sheep blood because it was not recommended for the isolation of Campylobacter from crude carcass rinses. A normally lethal dose of ciprofloxacin in CCA permitted the direct isolation of primary colonies on CCA from a naturally occurring subpopulation of the ciprofloxacin-resistant Campylobacter cells if such cells are preexisting in crude chicken carcass rinses. The British Society for Antimicrobial Chemotherapy advised a minimum breakpoint of 2 µg/ml for ciprofloxacin resistance in Campylobacter. Conversely, both Danish Veterinary and Food Administration and NARMS in the United States have adopted a breakpoint of 4 µg/ml. In this study, we used a higher concentration of 8.6 µg/ml ciprofloxacin in CCA medium (equivalent to 10 µg/ml ciprofloxacin hydrochloride in CCA with 86% purity) due to the following criteria: (a) the lethal dose of 8.6 µg/ml ciprofloxacin in CCA is greater than the 2× ciprofloxacin breakpoint concentration of ≈4 µg/ml used by NARMS and NCCLS for Campylobacter, which killed all ciprofloxacin-sensitive cells on CCA; (b) this lethal dose in CCA is lower than the minimum threshold limit of frequently found higher levels of ciprofloxacin resistance (MIC, ≧16 µg/ml) in naturally occurring ciprofloxacin-resistant Campylobacter in chicken (12, 22) and therefore allowed their selective recovery on CCA; and (c) this lethal dose in CCA excluded the recovery of intermediate or lower levels of ciprofloxacin-resistant Campylobacter (MIC be-
between Campylobacter ciprofloxacin MICs between ciprofloxacin resistant, but none of the resistant isolates had rofloxacin-resistant Campylobacter harbored within the clinically significant ciprofloxacin-resistant sources (12, 22). For example, in a recent survey by Ge et al. MICs of /H11350 Campylobacter isolates from humans, 288 MICs between breast, and four Campylobacter isolates from ground turkey but resistant isolates from chicken or human or other sources (12, 22). For example, in a recent survey by Ge et al. (12) of 378 Campylobacter isolates from retail meats, 35% were ciprofloxacin resistant, but none of the resistant isolates had ciprofloxacin MICs between >4 and ≤8 µg/ml but all had MICs of ≥16 µg/ml. NARMS tested 297 Campylobacter isolates from humans, 288 Campylobacter isolates from chicken breast, and four Campylobacter isolates from ground turkey but reported that none of the resistant isolates had ciprofloxacin MICs between >4 and ≤8 µg/ml but that all had MICs of ≥16 µg/ml (22).

Our direct-plating method accounted for the thermophilic group of total Campylobacter CFU on CA and total ciprofloxacin-resistant Campylobacter CFU on CCA, irrespective of species, and all recoverable at 42°C under microaerophilic conditions. The species-specific counts of different thermophilic campylobacters that may co-occur in crude carcass rinses, e.g., C. jejuni, C. coli, C. lari, or C. upsaliensis, were not determinable in this method. Using the recommended USDA-Food Safety and Inspection Service carcass rinse sampling procedure (25), this method permitted the detection and enumeration of a lower minimum level of total Campylobacter or total ciprofloxacin-resistant Campylobacter (about 8 CFU/carcass = 0.90 log10 CFU/carcass) due to the direct plating of pellets from centrifuged rinse subsample volumes of up to 50 ml from the total 400-ml rinse (equal to one-eighth of the total rinse volume), compared to the substantially higher minimum levels (about 1,000 to 4,000 CFU/carcass = 3.0 to 3.6 log10 CFU/carcass) obtainable by the direct plating of 0.1-ml subsample rinses of the total 100- or 400-ml rinse (equal to 1/1,000 or 1/4,000 of total rinse volume), as was used for Campylobacter enumeration by other researchers (17, 18, 25, 30). Our direct-plating method, like those of Line et al. (17), Siragusa et al. (28), and Stern and Robach (30), determines only the numbers of Campylobacter CFU released from carcass skin into the rinse. Thus, this method does not reveal what numbers or percentages of total Campylobacter or total ciprofloxacin-resistant Campylobacter still remained firmly attached to carcass surfaces during the rinse sampling.

Total Campylobacter load in rinses from retail raw chicken carcasses from 2001 to 2003. Figure 1A shows the overall distribution of total Campylobacter loads in rinses from 420 RTCC sampled over a 2½-year period. Figure 2A summarizes for 2001 to 2003 the percentage of RTCC yielding different loads of total Campylobacter CFU/carcass. Countable numbers (detection limit = 0.90 log10 CFU/carcass) of Campylobacter were recovered from 85%, 96%, and 57% of carcasses sampled in 2001, 2002, and 2003, respectively. In general, the numbers of total Campylobacter CFU per carcass ranged from 0.90 to 4.52 log10 CFU/carcass (equal to 8 to 34,800 CFU/carcass) in 2001, 0.90 to 4.58 log10 CFU/carcass (equal to 8 to 38,400 CFU/carcass) in 2002, and 0.90 to 4.48 log10 CFU/carcass (equal to 8 to 30,400 CFU/carcass) in 2003. Concerning counts, the Campylobacter loads per carcass did not decline appreciably from 2001 to 2003 but some reductions were seen for carcasses carrying higher Campylobacter loads. For example, about 50%, 30%, and 8% of the carcasses sampled in 2001, 2002, and 2003, respectively, had total Campylobacter loads as high as 3 to 4 log10 CFU/carcass. Overall, 79% of 420 RTCC tested in the 2½-year sampling period from July 2001 to December 2003 had countable numbers of total Campylobacter, out of which 16%, 32%, 26%, and 5% of RTCC yielded total Campylobacter loads of 0.90 to 2.0, 2 to 3, 3 to 4, and 4 to 5 log10 CFU/carcass, respectively. Campylobacter incidence rates of 44% to 91% were frequently reported from retail raw chicken in the United States (6, 26, 34), but to the best of our knowledge, there are only a few reports about Campylobacter counts per carcasses at retail (20).

Total ciprofloxacin-resistant Campylobacter load in rinses from retail raw chicken carcasses from 2001 to 2003. Figure 1B shows the overall distribution of total ciprofloxacin-resistant Campylobacter loads in rinses from 420 RTCC sampled over a 2½-year period. Figure 2B summarizes for 2001 to 2003 the percentages of RTCC yielding different loads of total ciprofloxacin-resistant Campylobacter CFU/carcass. The percentages of carcasses with minimum detectable levels of ciprofloxacin-resistant Campylobacter CFU (log10 0.90 or greater CFU/carcass) ranged from 60%, 59%, and 17.5%, respectively, for 2001, 2002, and 2003. In general, the numbers of total cipro-
In conclusion, our 2 1⁄2-year analysis of RTCC in one geographical area shows continuing persistence of countable numbers of total ciprofloxacin-resistant Campylobacter strains containing relatively higher ciprofloxacin-resistant Campylobacter loads. For example, 11%, 10%, and 0.6% of carcasses, respectively, in 2001, 2002, and 2003 had ciprofloxacin-resistant Campylobacter mean counts as high as 3 to 4 log10 CFU/carcass. Overall, 42% of 420 RTCC tested in carcasses containing relatively higher ciprofloxacin-resistant Campylobacter (equal to 8 to 1,100 CFU/carcass) in 2003. Concerning counts, some reductions were noted in numbers of carcasses containing relatively higher ciprofloxacin-resistant Campylobacter loads. For example, 11%, 10%, and 0.6% of carcasses, respectively, in 2001, 2002, and 2003 had ciprofloxacin-resistant Campylobacter mean counts as high as 3 to 4 log10 CFU/carcass.

In conclusion, our 2 1⁄2-year analysis of RTCC in one geographical area shows continuing persistence of countable numbers of total Campylobacter and total ciprofloxacin-resistant Campylobacter while there were some reductions in their incidence and loads from 2001/2002 to 2003. Random colony picks CA and CCA confirmed the presence of subpopulations of ciprofloxacin-resistant C. jejuni (ciprofloxacin MICs ranging from ≥16 to ≤128 μg/ml in both hippurate-positive and hippurate-negative strains) and of ciprofloxacin-resistant other Campylobacter spp. in RTCC rinses (data not shown), but their differential quantitation in carcass rinses must await further development of selective methods.

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