

## Arsenic Resistance in *Campylobacter* spp. Isolated from Retail Poultry Products

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**Organoarsenicals are commonly used for growth promotion in U.S. poultry production. Susceptibilities to arsenite, arsenate, and the organoarsenical roxarsone were measured in 251 *Campylobacter* isolates from conventional and antimicrobial-free retail poultry products. Isolates from conventional poultry products had significantly higher roxarsone MICs ( $\alpha = 8.22$ ;  $P < 0.0001$ ).**

Over 8 billion broiler chickens are produced annually in the United States (15). For the purposes of promoting growth and improving feed efficiency, broilers are fed nontherapeutic levels of antimicrobials, including arsenic, which is usually in the form of the organoarsenical compound roxarsone (4, 11). Roxarsone is added to poultry feed at concentrations ranging from 22.7 g/ton to 45.4 g/ton (9). Approximately 70% of the U.S. broiler industry utilizes roxarsone (4), and researchers have calculated that  $9 \times 10^5$  kg of roxarsone is excreted in poultry litter each year (5). Once roxarsone is excreted, it degrades into metabolites such as arsenite [As(III)] and arsenate [As(V)] (3). Since these inorganic metabolites are classified as human carcinogens, researchers have begun to investigate the fate of arsenic in poultry meat, poultry litter, soil, and water (3, 5, 6, 8, 13). However, there have been no published studies regarding the effects of roxarsone, As(III), and As(V) on important human bacterial pathogens, such as *Campylobacter* spp., that are prevalent in the poultry production environment.

*Campylobacter* spp. are gram-negative, spiral bacteria that infect most broilers by the time they reach 4 weeks of age, making the consumption of fresh chicken a major pathway of human exposure to *Campylobacter* spp. (2). In a previous study, the presence of DNA inserts similar to arsenic resistance genes was described for *Campylobacter jejuni* 81116, providing the first evidence that *Campylobacter* spp. could possess arsenic resistance determinants (1). However, neither the expression of arsenic resistance nor its association with roxarsone use in poultry production has been examined in *Campylobacter* spp. Thus, the objectives of this study were (i) to investigate whether *Campylobacter* spp. isolated from retail poultry products express resistance to roxarsone, As(III), and As(V) and (ii) to explore whether arsenic resistance in *Campylobacter* spp. is associated with roxarsone use.

*Campylobacter* spp. ( $n = 251$ ), including *C. jejuni*, *C. coli*, and *C. lari*, were isolated from separate retail poultry products, using previously described methods (12). Briefly, fresh poultry products were purchased on a weekly basis from grocery stores

in the Baltimore metropolitan area during the following two periods: (i) 23 February to 13 May 2003 and (ii) 21 January to 7 June 2004. One piece of poultry was sampled from each package, transferred to a stomacher bag containing 200 ml Bolton broth amended with laked horse blood (Oxoid, Ogdensburg, NY; Quad Five, Ryegate, MT), shaken by hand for 2 min, and removed, while the remaining enrichment was incubated for 22 to 26 h at 42°C under microaerophilic conditions. Ten microliters of each enrichment was streaked onto Abeyta-Hunt agar and incubated for 22 to 26 h at 42°C under microaerophilic conditions. A single presumptive *Campylobacter* colony was then streaked and purified on *Campylobacter* blood agar (Fisher Scientific, Hampton, NH). Each presumptive *Campylobacter* isolate was confirmed and identified to the species level, using a PCR and restriction digestion protocol as previously described (12). One hundred sixty-two isolates were from fresh retail poultry produced by the following conventional producers: Tyson Foods (Springdale, AR), Perdue Farms (Salisbury, MD), Wampler Foods (Dallas, TX), Trader Joe's (Los Angeles, CA), Allen Family Foods (Seaford, DE), and Goldkist Farms (Atlanta, GA). Eighty-nine isolates were from fresh retail poultry produced by the following three producers that claim not to use antimicrobials (including roxarsone): Bell & Evans (Fredericksburg, PA), Eberly Poultry (Stevens, PA), and Murray's Chicken (South Fallsburg, NY).

Antimicrobial susceptibility testing for roxarsone (4-hydroxy-3-nitrobenzenearsonic acid; Acros Organics, NJ), As(III) (NaAsO<sub>2</sub>; Sigma, St. Louis, MO), and As(V) (Na<sub>2</sub>HAsO<sub>4</sub> · 7H<sub>2</sub>O; Sigma, St. Louis, MO) was performed using the MIC agar dilution method (10). Briefly, each isolate was suspended in 3 ml Mueller-Hinton broth, adjusted to a 0.5 McFarland standard using a Vitek colorimeter (Hach, Loveland, CO), and replicated using a Cathra replicator system (Oxoid Inc., Ogdensburg, NY) onto Mueller-Hinton agar plates with 5% sheep blood that were previously prepared with the appropriate concentrations of arsenicals. Plates were incubated under microaerophilic conditions at 42°C for 24 h. The MIC was recorded as the lowest concentration of arsenical that completely inhibited bacterial growth. Although no quality control ranges exist for arsenical antimicrobials, the quality control strain *C. jejuni* ATCC 33560 was used to examine the precision of the method in determining arsenical MICs. Concentrations of arsenicals tested ranged

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TABLE 1. Roxarsone, arsenite, and arsenate MICs ( $\mu\text{g/ml}$ ) for *Campylobacter* spp. isolated from conventional and antimicrobial-free poultry products

Arsenical	MICs for <i>Campylobacter</i> spp. ( $n = 162$ ) isolated from conventional poultry products			MICs for <i>Campylobacter</i> spp. ( $n = 89$ ) isolated from antimicrobial-free poultry products		
	MIC <sub>50</sub>	MIC <sub>90</sub>	MIC range	MIC <sub>50</sub>	MIC <sub>90</sub>	MIC range
Roxarsone	64	256	8–512	32	64	4–256
As(III)	8	22.4	2–256	8	38.4	1–256
As(V)	256	1,024	32–2,048	256	512	64–1,024

from 0.25  $\mu\text{g/ml}$  to 512  $\mu\text{g/ml}$  ( $9.5 \times 10^{-4}$  mM to 1.95 mM) roxarsone, 0.5  $\mu\text{g/ml}$  to 512  $\mu\text{g/ml}$  ( $3.8 \times 10^{-3}$  mM to 3.94 mM) As(III), and 16  $\mu\text{g/ml}$  to 2048  $\mu\text{g/ml}$  ( $5.1 \times 10^{-2}$  mM to 6.56 mM) As(V).

Two-sample Wilcoxon rank sum tests were used to determine whether patterns of resistance to roxarsone, arsenite, and arsenate were significantly different between *Campylobacter* spp. from conventional poultry products and *Campylobacter* spp. from antimicrobial-free poultry products.  $z$  scores and  $P$  values were calculated for each test, and all analyses were performed using Stata 7.0 (StataCorp, College Station, TX).

All *Campylobacter* isolates from retail poultry products expressed some degree of phenotypic resistance to roxarsone, As(III), and As(V) (Table 1). Statistical analyses indicated that *Campylobacter* spp. isolated from conventional poultry products had significantly higher roxarsone MICs than *Campylobacter* spp. isolated from antimicrobial-free poultry products ( $z = 8.22$ ;  $P < 0.0001$ ) (Fig. 1). These results provide the first evidence that roxarsone use in conventional poultry facilities

could be associated with the development of high-level roxarsone resistance in *Campylobacter* spp. present in poultry. There were no statistically significant differences in As(III) MICs ( $z = 0.26$ ;  $P$  value = 0.80) and As(V) MICs ( $z = 1.14$ ;  $P$  value = 0.25) between isolates from conventional poultry products and isolates from antimicrobial-free poultry products.

These findings are the first report of phenotypic arsenic resistance in *Campylobacter* spp. They also provide evidence that nonantibiotic antimicrobials added to poultry feed may contribute to the changing ecology of bacterial pathogens present in poultry environments. Although this study included poultry products collected from grocery stores in a limited geographical area, the results are likely generalizable to other areas because the brands tested in this study are widely distributed in the United States. However, additional questions remain. While *Campylobacter* spp. from conventional poultry products had significantly higher roxarsone MICs, there were no significant differences in As(III) and As(V) MICs between isolates from conventional and antimicrobial-free poultry products. This may indicate that different resistance mechanisms exist for different arsenical compounds. Roxarsone resistance mechanisms could result from the unique selective pressures arising from the use of organoarsenicals within the industrial animal production environment. In contrast, since As(III) and As(V) occur naturally, originating from geochemical sources (14), it is possible that As(III) and As(V) resistance mechanisms in *Campylobacter* spp. could be similar to those mechanisms reported for other gram-negative bacteria (14), which evolved long before the advent of industrial poultry production.

Another important question concerns whether arsenic resistance determinants could be linked to antibiotic resistance

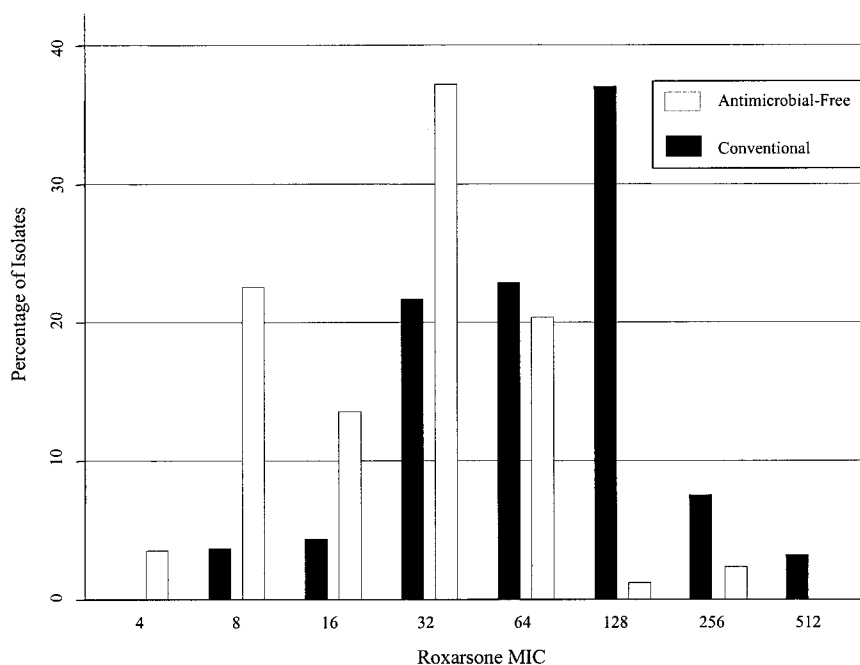


FIG. 1. Distributions of roxarsone MICs among *Campylobacter* spp. isolated from conventional poultry products ( $n = 162$ ) and *Campylobacter* spp. isolated from antimicrobial-free poultry products ( $n = 89$ ). Statistical differences in roxarsone MICs between the two groups were determined by the Wilcoxon rank sum test ( $z = 8.22$ ;  $P < 0.0001$ ).

determinants, similar to the genetic linkages observed between copper, macrolide, and glycopeptide resistance genes in *Enterococcus faecium* (7). If this is true, then the use of roxarsone in conventional poultry production environments could select for antibiotic-resistant *Campylobacter* spp. even in the absence of antibiotic use. In addition, it would be valuable to understand whether arsenic resistance determinants in *Campylobacter* spp. are genetically linked to other genes, including flagellin genes, virulence-associated genes, and genes for other factors that could aid in the invasion and colonization of *Campylobacter* spp. in chickens and humans. In a study by Ahmed et al., where genetic differences were evaluated among *Campylobacter jejuni* strains with various colonization potentials, two arsenic resistance-like inserts were found among a group of 24 inserts present in strain 81116, which proved to be a better colonizer than a strain that lacked the inserts (1). Thus, we echo their suggestion that the role of arsenic resistance genes in the colonization of chickens and humans by *Campylobacter* spp. deserves further examination. These and other questions will require future studies for a full understanding of the potential ecological and public health effects associated with arsenic resistance in *Campylobacter* spp. originating from poultry production environments.

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