Research Note

Development of Macrolide-Resistant Campylobacter in Broilers Administered Subtherapeutic or Therapeutic Concentrations of Tylosin†

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ABSTRACT

The use of antimicrobials in food animal production, particularly those commonly used to treat infections in humans, has become a source of debate in recent years. However, limited data are available regarding the development of resistance following the subtherapeutic or therapeutic administration of antimicrobials in animal production. The objective of this study was to evaluate the effect of the administration of therapeutic and subtherapeutic concentrations of tylosin on the erythromycin susceptibility of Campylobacter jejuni and Campylobacter coli isolated from the ceca of treated broilers. In three replicated studies, day-old hatch chicks were exposed to macrolide-susceptible C. jejuni or C. coli. At 2 weeks of age, tylosin was administered at subtherapeutic (22 ppm, continuously in the diet) or therapeutic concentrations (529 ppm, in the drinking water for 5 days). Broilers were sacrificed weekly. Total and erythromycin-resistant Campylobacter spp. were enumerated from individual ceca plus cecal contents. Overall erythromycin resistance was observed at a higher frequency (P < 0.01) among C. coli isolates (70.8%) than among C. jejuni isolates (36.8%) following tylosin administration. Across Campylobacter species, erythromycin resistance was observed at a higher frequency (P < 0.001) when tylosin was administered at subtherapeutic (62.7%) than at therapeutic (11.4%) concentrations. Subtherapeutic administration resulted in the recovery of 83.3 and 56.1% erythromycin-resistant isolates compared with only 33.3 and 7.9% of the isolates expressing erythromycin resistance following the administration of therapeutic concentrations for C. coli and C. jejuni, respectively. Further studies are needed to determine the factors involved in the apparent difference in the acquisition of macrolide resistance in C. coli compared with C. jejuni.

Campylobacter spp. are estimated to account for 1.5 million cases of human gastroenteritis every year in the United States (40). Campylobacter jejuni and Campylobacter coli are the species most frequently isolated from cases of human infection, with C. jejuni accounting for over 90% of infections and C. coli being identified in most of the remaining cases (25, 30). Most cases of gastroenteritis result in a self-limiting diarrheal disease that does not require antimicrobial therapy. However, prolonged duration of illness, or altered immune function in some individuals, may warrant antimicrobial therapy (1, 5).

A number of studies have investigated the epidemiology of Campylobacter in poultry production (18, 21, 22, 37, 44, 49), because poultry products are considered a significant source of Campylobacter infections in humans (10, 11). Prevalence studies in swine (26) and cattle (6, 14, 27, 41, 51) indicate that Campylobacter is a common commensal in other livestock production systems as well. Accordingly, pork, beef, and dairy products can also be a source of human Campylobacter infections (17, 32, 36, 43, 47).

Considerable debate surrounds the use of antimicrobials in food animal production, especially those commonly used to treat human infections. Concerns regarding the emergence of resistant bacterial pathogens resulting from the use of antimicrobials in animals and the potential transfer of resistant strains from food products to humans have led to changes in antimicrobial use in food animal production worldwide (3, 29). In U.S. food animal production, macrolides (erythromycin, tilmicosin, and tylosin) can be used to treat and prevent disease in poultry, swine, and beef cattle (8). In addition, tylosin and oleandomycin are approved for use in poultry and swine diets for increased rate of weight gain and improved feed efficiency (8). Erythromycin also serves as a primary treatment option for campylobacteriosis in humans (33). Even though the four aforementioned compounds have slight structural differences, their mode of action is the same; discrete point mutations in the 23S rRNA gene of Campylobacter confer cross-resistance among all members of the macrolide class of drugs.
(12, 23, 46, 48). Efflux systems have also been shown to provide low-level macrolide resistance in Campylobacter (9, 35). Other mechanisms conferring macrolide resistance, such as mutations in ribosomal proteins, methylation of the drug binding site, and drug inactivation, have not yet been observed in Campylobacter (15, 52).

The objective of this study was to evaluate the effect of administering therapeutic and subtherapeutic concentrations of tylosin on the erythromycin susceptibility of C. jejuni and C. coli isolated from the ceca of treated broilers.

**MATERIALS AND METHODS**

**Study design.** In each of three replicate studies, 175 day-old hatch chicks were obtained from a commercial broiler hatchery and were allotted to one of seven groups of 25 birds each. Treatment groups were placed in separate isolation rooms on pine shavings and were provided a standard nonmedicated grower diet and water ad libitum. All procedures were administered in accordance to protocols approved by an institutional animal care and use committee.

At placement, three groups of 25 birds each were exposed to C. jejuni by commingling with two seeder chicks that had been challenged by oral gavage with 10^7 CFU of a cocktail of three strains of macrolide-susceptible C. jejuni. In a similar manner, three groups were exposed to three strains of macrolide-susceptible C. coli. Seeder birds were marked at challenge and were not included in sample collection. A control group was not exposed to Campylobacter to determine the Campylobacter colonization status of chicks from the hatchery.

At 2 weeks of age, tylosin phosphate (Tylan 10, Elanco Animal Health, Indianapolis, Ind.) was administered in the diet at a subtherapeutic concentration of 22 ppm (20 g of active ingredient per ton; U.S. Food and Drug Administration–approved concentration for the treatment of chronic respiratory disease in broilers) to one group of birds exposed to C. jejuni and one group exposed to C. coli. Tylosin-medicating feed was provided ad libitum to these two groups for the remainder of the study (4 weeks). At this same time, tylosin tartrate (Tylan Soluble, Elanco Animal Health) was administered at a therapeutic concentration of 529 ppm (0.5291 g/liter; U.S. Food and Drug Administration–approved concentration for the treatment of chronic respiratory disease in broilers) in the drinking water for 5 days to one group of birds exposed to C. jejuni and one group exposed to C. coli. Medications were administered in accordance with the manufacturer’s label directions. Three control groups, one exposed to C. jejuni, one exposed to C. coli, and one unexposed group, were not administered tylosin in either the feed or drinking water.

**Campylobacter strains and inoculum preparation.** All strains used in these studies were obtained from the animal arm of the National Antimicrobial Resistance Monitoring System Campylobacter collection located at the U.S. Department of Agriculture, Agricultural Research Service, Russell Research Center in Athens, Ga. Strains were originally isolated from poultry carcasses. Previous testing had determined that the isolates were susceptible to azithromycin and erythromycin. In addition, they were susceptible to ciprofloxacin, clindamycin, chloramphenicol, gentamicin, nalidixic acid, and tetracycline. Cocktails were prepared that consisted of three strains of C. jejuni (3936, 4820, and 39364) or three strains of C. coli (6647, 97756, and 98544). Challenge cultures were prepared by inoculating frozen stock cultures of each strain onto blood agar (tryptic soy agar with 5% sheep blood; Difco, Becton Dickinson, Sparks, Md.) and incubating at 42°C for 24 h in a sealed bag flushed with a microaerobic gas mixture (5% O2, 10% CO2, and 85% N2). Freshly grown colonies of each of the three strains were suspended as a mixture in phosphate-buffered saline (PBS, 0.9%, pH 7.2) and adjusted to a final concentration of 10^6 CFU/ml with an A540 of 0.45 (Spectronic 20, Spectronics Instruments Inc., Rochester, N.Y.). Inoculum levels were confirmed by spread plating serial dilutions of each inoculum in duplicate.

**Campylobacter recovery and identification.** At 2 weeks of age, prior to medication treatments, and at 3, 4, 5, and 6 weeks of age, five broilers per group were necropsied. Ceca were removed aseptically, placed in Whirl-Pak bags (Nasco, Modesto, Calif.) on ice, and processed within 2 h. Total and resistant Campylobacter spp. were enumerated from individual ceca plus contents as described below.

For the enumeration of total and macrolide-resistant Campylobacter, individual ceca were crushed to expose the contents, diluted 1:3 (wt/vol) with sterile PBS, and mixed in a stomacher (Seward Ltd., London, UK) for 30 s. Serial dilutions were prepared and plated onto duplicate Campy-Cefex agar (CCA) (45) and Campy-Cefex agar supplemented with erythromycin (eCCA) at 8 µg/ml. All plates were incubated at 42°C for 36 to 48 h microaerobically. Total and resistant populations of Campylobacter were estimated by plate counts on CCA and eCCA and are reported as log CFU per gram of ceca plus contents.

One presumptive Campylobacter colony was selected from CCA and one from eCCA for each sample for species identification and susceptibility testing. Presumptive identification consisted of observation of cellular morphology and motility by phase-contrast microscopy. Isolates were identified by a commercial multiplex PCR specific for C. jejuni and C. coli (BAX PCR, DuPont Qualicon, Wilmington, Del.), as previously described (13). To determine which Campylobacter strains colonized broilers and which strains developed macrolide resistance, reference sequences of the short variable region (SVR) of the flaA gene of each of the inoculum strains were prepared and compared with flaA SVR sequences of subsets of macrolide susceptible and resistant isolates recovered throughout the study. The SVR was amplified as previously described with flaA-specific primers (31). Sequencing reactions were performed with the BigDye Terminator 1.1 Cycle Sequencing kit (Applied Biosystems, Foster City, Calif.) and an ABI 3100 Genetic Analyzer (Applied Biosystems) according to manufacturer’s directions. Forward and reverse sequence data were assembled and compared by Sequencher version 4.2 (Gene Codes Corporation, Ann Arbor, Mich.).

**Antimicrobial susceptibility testing.** The MICs of erythromycin for all Campylobacter isolates recovered from individual ceca plus contents were determined by the agar dilution method recommended by the Clinical and Laboratory Standards Institute (formerly the NCCLS) (34). Erythromycin is recommended for macrolide susceptibility testing of Campylobacter, as interpretative standards for tylosin susceptibility testing have not been established. Nine doubling concentrations of erythromycin (Sigma, St. Louis, Mo.) were tested (range, 1 to 256 µg/ml) with Mueller-Hinton agar containing 5% defibrinated sheep blood. Isolates were tested on duplicate plates incubated at 42°C for 24 h under microaerobic conditions and were considered resistant to erythromycin if the MIC was ≥8 µg/ml. C. jejuni ATCC 33560 was used as a quality control strain, and its erythromycin MIC remained at 1 µg/ml throughout the study, which falls within the Clinical and Laboratory Standards Institute recommended range (1 to 4 µg/ml) under the growth conditions described (34).
TABLE 1. Campylobacter strain recovery and development of erythromycin (ERY) resistance in each experimental replications (reps)

<table>
<thead>
<tr>
<th>Species and isolate</th>
<th>Strain recovery</th>
<th>ERY-resistant strains recovered</th>
</tr>
</thead>
<tbody>
<tr>
<td>C. jejuni 3936</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>C. jejuni 4820</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>C. jejuni 39364</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>C. coli 6647</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>C. coli 97756</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>C. coli 89544</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>

Statistical analysis. Campylobacter counts were log transformed and analyzed by the general linear model (Statistica, StatSoft, Tulsa, Okla.). Count means were separated by Tukey’s honest significant difference. The χ² test was used for evaluating differences in prevalence and differences in macrolide resistance frequencies between C. jejuni and C. coli.

RESULTS

Throughout the study, no Campylobacter was recovered from broilers that had not been experimentally exposed to Campylobacter. C. jejuni was the only Campylobacter species recovered from birds exposed to C. jejuni, and C. coli was the only species recovered from birds exposed to C. coli, as determined by BAX PCR. The SVR of the flaA gene was sequenced for 46 macrolide-susceptible Campylobacter isolates (28 C. jejuni and 18 C. coli) recovered from broiler ceca at 2 weeks of age, prior to the administration of medication treatments. Sequence comparisons with flaA SVR reference sequences prepared from each of the inoculum strains showed that two of the three strains utilized in each of the challenge cocktails (C. jejuni and C. coli) were recovered from broiler ceca (Table 1). These findings were consistent across replications.

A higher prevalence of Campylobacter detection was observed among broilers exposed to C. jejuni than among broilers exposed to C. coli (Table 2). At 2 weeks of age, prior to the administration of medication treatments, 75.6% (68 of 90) of all birds exposed to Campylobacter were detected positive, 100% (45 of 45) of those exposed to C. jejuni were found positive, while only 51.1% (23 of 45) of those exposed to C. coli were found positive. Campylobacter prevalence rates remained at 100% (15 of 15) across sampling dates in nonmedicated broilers exposed to C. jejuni, whereas the prevalence rate in nonmedicated broilers exposed to C. coli was only 20% (3 of 15) at 2 weeks of age and never exceeded 66.7% (10 of 15) throughout the study.

By 3 weeks of age (1 week posttreatment), the prevalence of Campylobacter-positive broilers (Table 2) and the levels of Campylobacter observed in ceca plus contents (Tables 3 and 4) were inversely related to the administration of tylosin. Total Campylobacter counts from CCA and eCCA plates did not differ significantly for broilers administered tylosin (data not shown). Campylobacter counts (presented in Tables 3 and 4) for nonmedicated broilers and medicated broilers prior to medication treatment were from CCA plates. Campylobacter counts for medicated broilers, following the administration of medication treatments (weeks 3 to 6), were from eCCA plates. Among broilers exposed to C. jejuni, Campylobacter prevalence and cecal counts (CFU per gram of ceca plus contents) were significantly reduced (P < 0.05) 1 week after the administration

TABLE 2. Prevalence of C. jejuni- and C. coli-positive birds at 2, 3, 4, 5, and 6 weeks of age in broilers receiving no medication (0), subtherapeutic (22-ppm) concentrations, or therapeutic (529-ppm) concentrations of tylosin

<table>
<thead>
<tr>
<th>Age (wk)</th>
<th>C. jejuni</th>
<th>C. coli</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0</td>
<td>22 ppm</td>
</tr>
<tr>
<td>2</td>
<td>15 (100)</td>
<td>15 (100)</td>
</tr>
<tr>
<td>3</td>
<td>15 (100)</td>
<td>12 (80)</td>
</tr>
<tr>
<td>4</td>
<td>15 (100)</td>
<td>15 (100)</td>
</tr>
<tr>
<td>5</td>
<td>15 (100)</td>
<td>15 (100)</td>
</tr>
<tr>
<td>6</td>
<td>15 (100)</td>
<td>15 (100)</td>
</tr>
</tbody>
</table>

*Data combined from three replications; 5 broilers were sampled weekly in each replication for a total of 25 broilers for each replication.

*Within species and age group, the probability that differences existed in the group was calculated with the chi-square test for independence.

TABLE 3. Mean counts of Campylobacter jejuni in ceca samples at 2, 3, 4, 5, and 6 weeks of age from broilers receiving no medication (0), subtherapeutic (22-ppm) concentration, or therapeutic (529-ppm) concentration of tylosin

<table>
<thead>
<tr>
<th>Age (wk)</th>
<th>Log CFU/g (SE)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0</td>
</tr>
<tr>
<td>2</td>
<td>7.09 A (0.32)</td>
</tr>
<tr>
<td>3</td>
<td>7.02 A (0.18)</td>
</tr>
<tr>
<td>4</td>
<td>7.87 A (0.20)</td>
</tr>
<tr>
<td>5</td>
<td>8.04 A (0.18)</td>
</tr>
<tr>
<td>6</td>
<td>7.70 A (0.11)</td>
</tr>
</tbody>
</table>

*Medication treatments were initiated after sample collection at 2 weeks of age.

*Values with different letters within a row are significantly different (P < 0.05) by Tukey’s honest significant difference.
Sequences showed that macrolide resistance was induced in feed or drinking water. Cluster analysis of the treatment groups, respectively. The SVR of the C. coli tylosin (4 weeks of age) and 3 weeks after the administration of medication.

The erythromycin-resistant strains were recovered from broilers that had not been administered tylosin at a subtherapeutic concentration.

Significantly lower than nonmedicated broilers and those receiving tylosin at a subtherapeutic concentration.

Values with different letters within a column are significantly different (P < 0.05) by Tukey’s honest significant difference.

The distribution of erythromycin MICs for C. jejuni and C. coli recovered after the initiation of medication treatments are shown in Table 5. Erythromycin resistance was observed at a higher frequency (P < 0.01) among C. coli isolates (70.8% [17 of 24]) than among C. jejuni isolates (36.8% [35 of 95]). In addition, erythromycin resistance was observed at a significantly higher frequency (P < 0.001) when tylosin was administered at subtherapeutic concentrations (62.7% [47 of 75]) than at therapeutic concentrations (11.4% [5 of 44]). Eighty-three percent (15 of 18) of the C. coli isolates recovered following the administration of tylosin at subtherapeutic concentrations were resistant to erythromycin compared with only 33.3% (2 of 6) following the administration of therapeutic concentrations. The erythromycin MIC50 for C. coli isolates recovered following the subtherapeutic and therapeutic administration of tylosin were >256 and 1 μg/ml, respectively. Similarly, 56.1% (32 of 57) of the C. jejuni isolates recovered following the administration of tylosin at subtherapeutic concentrations were resistant to erythromycin (MIC50: 128 μg/ml) compared with 7.9% (3 of 38) of the C. jejuni isolates recovered following the administration therapeutic concentrations of tylosin (MIC50: 1 μg/ml).

**DISCUSSION**

The C. jejuni strains used in this study were substantially better colonizers of broilers than the C. coli strains. Differences in the colonizing potential of Campylobacter strains in broilers have been previously reported (4, 28, 42), and several genes, including sodB, racR, and cadF (7, 38, 53), have been identified as colonization factors. Genetic variation in the isolates used or host factors may have contributed to the lower prevalence of Campylobacter observed in broilers exposed to C. coli strains in our study.

We observed erythromycin resistance at a higher prevalence among C. coli than among C. jejuni broiler isolates. Antimicrobial resistance surveys from Belgium, Denmark, and Japan have reported analogous findings, with 18 to 35% of C. coli isolates recovered from broilers expressing macrolide resistance compared with only 0 to 6% of C. jejuni isolates (2, 20, 50). However, Sáenz et al. (39) reported no macrolide-resistant C. coli or C. jejuni among broiler isolates in Spain. Prevalence data similar to ours have also been observed among human isolates where 8 to 34% of C. coli and 0 to 3% of C. jejuni were reported to be erythromycin resistant (2, 16, 26, 39). Survey data indicate that the prevalence of macrolide resistance among swine Campylobacter isolates tends to be two- to threefold higher than among strains isolated from other food animals or humans, with resistance among C. coli ranging from 48 to 81% and among C. jejuni from 0 to 33% (2, 20, 39, 50). C. jejuni is most commonly associated with poultry and human campylobacteriosis, while C. coli is primarily associated with swine. This host specificity and difference in each of the strains that colonized broilers; however, the recovery of resistant strains varied across replications (Table 1). Induction of resistance to macrolides occurs as a result of mutation of the 23S rRNA gene. Mutations, as stochastic events, can be expected to vary between trials.

**TABLE 4. Mean counts of Campylobacter coli in ceca samples at 2, 3, 4, 5, and 6 weeks of age from broilers receiving no medication (0), subtherapeutic (22-ppm) concentration, or therapeutic (529-ppm) concentration of tylosin.**

<table>
<thead>
<tr>
<th>Age (wk)</th>
<th>Log CFU/g (SE)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>22 ppm*</td>
</tr>
<tr>
<td>2</td>
<td>1.14 A* (0.63)</td>
</tr>
<tr>
<td>3</td>
<td>0.44 A (0.44)</td>
</tr>
<tr>
<td>4</td>
<td>2.90 A (1.10)</td>
</tr>
<tr>
<td>5</td>
<td>5.82 B (1.11)</td>
</tr>
<tr>
<td>6</td>
<td>5.05 B (0.98)</td>
</tr>
</tbody>
</table>

* Medication treatments were initiated after sample collection at 2 weeks of age.

Values with different letters within a column are significantly different (P < 0.05) by Tukey’s honest significant difference.
acquisition of resistance has led to speculation that resistance in Campylobacter differs, depending on both the species and host animal (20).

Limited data are available regarding the development of resistance from the subtherapeutic administration of antimicrobials in livestock production. Inglis et al. (19) reported that the administration of tylosin at subtherapeutic concentrations did not influence the carriage rates of erythromycin-resistant Campylobacter in feedlot cattle. The prevalence of erythromycin resistance observed in C. coli from broilers administered tylosin at subtherapeutic concentrations is similar to that commonly observed in swine isolates (2, 20, 39). Although the relatively low prevalence of macrolide-resistant Campylobacter in broilers typically reported in survey data compared with swine isolates may reflect substantially less macrolide use in poultry production, this is difficult to establish. In the United States, published estimates of antimicrobial use in food animal production differ markedly (24). Nevertheless, our findings are consistent with the majority of data indicating that a significantly higher proportion of C. coli isolates are macrolide resistant than are C. jejuni, regardless of the source of isolates.

In addition, we observed significantly higher frequencies of macrolide resistance when tylosin was administered at subtherapeutic concentrations (Table 5). The administration of tylosin at therapeutic concentrations significantly reduced cecal Campylobacter counts (Tables 3 and 4) and the prevalence of Campylobacter (Table 2). These trends were less obvious when tylosin was administered at subtherapeutic concentrations, in which case, the dose administered in combination with the rate of feed consumption appeared to be inadequate in inhibiting Campylobacter growth, with an end result of emerging resistance under selective pressure. Further studies are needed to determine the factors involved in the apparent difference in the acquisition of macrolide resistance in C. coli compared with C. jejuni.

REFERENCES

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