Subtherapeutic Tylosin Phosphate in Broiler Feed Affects *Campylobacter* on Carcasses During Processing

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ABSTRACT Tylosin phosphate is an antimicrobial drug approved for use in broiler feed at subtherapeutic levels for growth promotion. Erythromycin is often the drug of choice for treating humans with campylobacteriosis. Both tylosin and erythromycin are classified as macrolide drugs and cross-resistance between these antimicrobials occurs. Commercial broiler chicks were placed in isolation grow-out chambers and colonized with *Campylobacter jejuni*. From 14 d of age through grow-out, broilers were fed ad libitum a diet that included 22 ppm of tylosin phosphate (20 g/ton). Control broilers received the same diet without tylosin phosphate. At 42 d of age, broilers were processed in a pilot plant with equipment that closely modeled commercial conditions. Carcass rinses were collected after feather removal, after inside and outside washing, and after immersion chilling. *Campylobacter* numbers recovered from carcasses after feather removal did not differ according to feed type (3.53 log cfu/mL of rinse for control carcasses, and 3.60 log cfu/mL of rinse for those fed medicated feed). Likewise, medicated feed did not affect *Campylobacter* numbers on carcasses after inside-outside washing (3.11 and 3.07 log cfu/mL of rinse). However, carcasses of broilers fed tylosin phosphate had lower numbers of *Campylobacter* after chilling (1.45 log cfu/mL of rinse) than control carcasses (2.31 log cfu/mL of rinse). No *Campylobacter* isolated from control carcasses were resistant to erythromycin; all *Campylobacter* recovered from carcasses fed tylosin phosphate were resistant to erythromycin. Application of tylosin phosphate in feed results in lower numbers of *Campylobacter* on chilled carcasses; however, the *Campylobacter* that do remain are resistant to erythromycin.

Key words: antibiotic, antimicrobial, *Campylobacter*, tylosin, resistance

INTRODUCTION *Campylobacter* is a human foodborne pathogen that has been associated with poultry and poultry meat products. The Centers for Disease Control and Prevention has identified campylobacteriosis as one of the most common foodborne bacterial diseases in recent years (CDC, 2006). *Campylobacter* generally enters the processing plant with the live broilers (Berrang et al., 2000). Although modern broiler processing is very effective in lessening the numbers of this pathogen, it can be found on fully processed carcasses postchill (Berrang and Dickens, 2000).

It was first noted in the 1940s that low doses of antimicrobial drugs could increase the rate of broiler growth (Jones and Ricke, 2003). This approach was adopted by much of the industry to maximize production and maintain a low-cost product. Many drugs are available for use as broiler feed additives without a prescription (Jones and Ricke, 2003). Some reports suggest that in addition to the production advantage, a subtherapeutic dosage of antimicrobial drugs may have a prophylactic effect, helping to prevent disease (Casewell et al., 2003). In cases when a bacterial disease outbreak does occur, broilers may be therapeutically dosed with antimicrobial drugs to combat the disease. Tylosin phosphate, a macrolide, has been approved for both therapeutic and subtherapeutic use in broilers (McEwen and Fedorka-Cray, 2002).

An argument can be made that subtherapeutic drug use fills a disease prevention role, thereby preventing the need for higher doses of drugs (Casewell et al., 2003). However, it is generally accepted that bacteria exposed to subtherapeutic levels of drugs can develop resistance to those drugs (McEwen and Fedorka-Cray, 2002; Singer and Hofacre, 2006). Because of widespread concern about the development of drug-resistant bacterial pathogens in food animals, subtherapeutic application of antimicrobial drugs in food animal production is banned in Europe.

Macrolide drugs work by binding to the bacterial ribosome, preventing protein synthesis (Payot et al., 2006). The result is lack of growth or cellular repair, resulting in eventual death of the cell. Resistance is usually due to a point mutation changing the ability of the drug to bind to the ribosome (Gibreel et al., 2005; Kim et al., 2006).
There is specific evidence that Campylobacter exposed to macrolide drugs can become resistant, gaining the ability to grow in the presence of the drug (Aarestrup et al., 1997). Macrolide resistance in Campylobacter is a very stable trait, being maintained for many generations even without continuous antimicrobial pressure (Gibreel et al., 2005; Kim et al., 2006). Campylobacter that have become resistant to macrolides are a concern for human health because the usual drug of choice to treat human campylobacteriosis is also a macrolide, erythromycin (Kim et al., 2006).

It has been shown that Campylobacter in broilers can develop macrolide resistance caused by feeding low levels of tylosin phosphate (S. R. Ladley, P. J. Fedorka-Cray, M. E. Berrang, M. D. Englen, and R. J. Meinersmann, USDA-ARS, Athens, GA, and M. A. Harrison, University of Georgia, Athens, unpublished data). The objective of the current study was to determine the effect of broiler processing on the presence, number, and macrolide resistance of Campylobacter from broilers fed subtherapeutic levels of tylosin phosphate.

**MATERIALS AND METHODS**

**Experimental Overview**

Day-of-hatch commercial broiler chicks were exposed to penmates previously inoculated with macrolide-susceptible Campylobacter. At 14 d of age, treated birds began to receive feed containing a subtherapeutic level of tylosin phosphate (20 g/ton). Control birds received a nonmedicated feed. At 42 d of age, all birds were slaughtered and processed in a pilot processing plant that closely simulated commercial conditions. Ten whole carcass rinses were collected after defeathering, after inside-outside washing, and after immersion chill. Campylobacter resistant to and sensitive to erythromycin were enumerated from rinses, and the numbers from treated birds were compared with those from carcasses of birds that had been fed the control diet. Three replications were conducted.

**Broilers and Housing**

In each of 3 replicate trials, straight run day-of-hatch chicks were obtained from a commercial broiler hatchery and were allotted to 1 of 2 groups of 35 birds each. Chicks were placed in separate isolation rooms that had been sanitized and outfitted with fresh pine shaving litter. Chicks were provided a standard nonmedicated broiler starter-grower diet and water ad libitum. All procedures were administered in accordance with protocols approved by an institutional animal care and use committee.

**Inoculum and Campylobacter Colonization**

At placement, birds were exposed to Campylobacter jejuni by comingling with 2 seeder chicks that had been challenged by oral gavage with $10^7$ cfu of a 3-strain cocktail of C. jejuni. Seeder birds were marked at challenge and were not included in sample collection.

All strains used in these studies were selected from the National Antimicrobial Resistance Monitoring System-Enteric Bacteria (NARMS) Campylobacter collection at the USDA- Agricultural Research Service Russell Research Center in Athens, Georgia. The strains used were originally isolated from poultry carcass rinses. Previous antimicrobial resistance testing had determined that the isolates were susceptible to azithromycin, erythromycin, ciprofloxacin, clindamycin, chloramphenicol, gentamicin, nalidixic acid, and tetracycline. Challenge cultures were prepared by subculturing frozen stock cultures of each strain onto blood agar (tryptic soy agar with 5% sheep blood, Becton-Dickinson, Sparks, MD) and incubating at 22°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 3 strains were suspended as a mixture in sterile PBS (0.9%, pH 7.2) and adjusted to a final concentration of 10⁸ cfu/mL using an absorbance of 0.45 at 540 nm (Spectronic 20, Spectronics Instruments Inc., Rochester, NY). Inoculum concentrations were confirmed by spread plating serial dilutions of each inoculum in duplicate.

**Feed Treatments**

At 14 d of age, tylosin phosphate (Tylan 10, Elanco Animal Health, Indianapolis, IN) was administered in the diet of experimental birds at a subtherapeutic concentration of 22 ppm (20 g/ton). This is an FDA-approved level for increased rate of weight gain and improved feed efficiency in broilers and is in accordance with the manufacturer’s label directions. Tylosin medicated feed was provided ad libitum to this group of broilers for the remainder of the study (4 wk). Untreated control broilers continued to receive nonmedicated feed for the remainder of grow-out.

**Processing**

At 42 d of age, all broilers were subjected to a feed withdrawal period of 12 h. Broilers were then caged in plastic coops, transported to a pilot processing facility, and hung in groups of 10 in commercial-style shackles. Control broilers were processed first. All broilers were stunned electrically with 12 V DC (Stunner model SF-7000, Simmons Engineering Co., Dallas, GA) and killed by cutting blood vessels in the neck with an automated killing machine (Killer model SK.5, Simmons Engineering Co.). Carcasses were scalded in a set of 3 triple-pass scald tanks (Scald Tank model SGS-3CA, Stork Gamco, Gainesville, GA) set at 56°C. Shackle speed was set so that carcasses spent 30 s in each scald tank with 30 s in between. Carcasses then proceeded into a commercial defeathering machine (Picker model d-8, Stork Gamco) operated with a tap water spray (average total chlorine of 0.5 ppm). Carcasses were removed from the kill line, the feet were removed, necks were broken, and carcasses were rehung on an evisceration line. Carcasses proceeded through a commercial style venter-opener (model v/o 164, Stork
Gamco), evisceration machine (model PNT-24, Stork Gamco), and inside-outside washer (model MBW-16, Stork Gamco) using tap water set at 80 psi. Carcasses were removed from shackles and placed in pilot-scale agitated chill tanks (one tank for control carcasses and another for treated carcasses) filled with ice and tap water. Carcasses remained in the chill tank with agitation for 45 min. In between the control and treated broilers, the processing equipment was thoroughly cleaned using a hose and 52°C water. Broilers fed medicated feed were processed using the same methods and equipment as described above, except that a separate pilot-scale chiller was used.

**Sampling and Campylobacter Culture**

Ten carcasses from each treatment were collected for sampling after feather removal, after inside and outside washing, and after chilling. Carcasses were placed in sterile plastic bags and subjected to a 60-s low volume whole-carcass rinse procedure (Cox et al., 1981) using PBS as the diluent. Serial dilutions were prepared in PBS and platted onto duplicate Campy-Cefex agar (CCA; Stern et al., 1992) and Campy-Cefex agar supplemented with 8 μg/mL of erythromycin (CCAE). All plates were incubated at 42°C for 48 h in a microaerobic atmosphere. Total and resistant populations of *Campylobacter* were estimated by plate counts on CCA and CCAE, respectively. Colonies characteristic of *Campylobacter* were counted.

Presumptive *Campylobacter* colonies for each sample were selected from CCA and CCAE plates for confirmation and susceptibility testing. All colony types found in each sample were confirmed as members of the genus *Campylobacter* by observation of cellular morphology and motility under phase-contrast microscopy. All colony types were further confirmed using a latex agglutination serological test (Microgen Bioproducts Ltd., Camberly, UK).

**Antimicrobial Resistance Measurement**

The minimum inhibitory concentrations (MIC) of erythromycin for all *Campylobacter* isolates recovered from individual carcass rinse samples were determined using the agar dilution method recommended by the Clinical and Laboratory Standards Institute (NCCLS, 2002). Erythromycin is recommended for macrolide susceptibility testing of *Campylobacter*, because interpretive 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) using 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 (NCCLS, 2002). *Campylobacter jejuni* ATCC 33560 was used as a quality-control strain, and its erythromycin MIC remained 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 (NCCLS, 2002).

**Statistical Analysis**

*Campylobacter* counts were transformed to log colony-forming units per milliliter of carcass rinse. A GLM analysis was conducted using a complete randomized block design with replication as the block. Means were separated with a Tukey's honest significant difference test. Student's t-test was used to compare MIC values. All analyses were conducted using the Statistica software package (StatSoft Inc., Tulsa OK).

**RESULTS**

The numbers of *Campylobacter* recovered from whole-carcass rinses collected during processing are shown in Table 1. Feed containing tylosin phosphate had no effect on the number of *Campylobacter* detected per milliliter of rinse for carcasses examined immediately following defeathering or the inside-outside washer. However, carcasses from the broilers fed tylosin had lower numbers of *Campylobacter* per milliliter of rinse after chilling than did control carcasses from nonmedicated broilers.

No *Campylobacter* were detected on the CCAE plates from carcasses of broilers fed nonmedicated feed. However, the numbers of *Campylobacter* detected on carcasses fed tylosin phosphate were the same on CCAE as on plain CCA. This suggests that virtually all the *Campylobacter* detected on carcasses from medicated birds were in fact resistant to erythromycin, whereas none of the *Campylobacter* from control birds were.

Minimum inhibitory concentrations of erythromycin and tylosin were determined for *Campylobacter* from control and treated broilers. All *Campylobacter* isolates from carcasses of control broilers were susceptible to erythromycin, with MIC values of 1 μg/mL. Tylosin phosphate MIC values for *Campylobacter* from control broilers ranged from 2 to 16 μg/mL; 8 μg/mL was the most common MIC.

*Campylobacter* isolates from carcasses of broilers fed the medicated feed showed resistance to both drugs. *Campylobacter* from test carcasses were isolated on both CCA and CCAE plates. Isolates from CCA plates had a range of MIC values for erythromycin ranging from 8 (3 isolates) to >256 (16 isolates), with most of the isolates (36) having MIC values of 128. Tylosin MIC ranged from 32 to >256 (16 isolates), with most of the isolates (36) having MIC values of <0.01. For isolates from CCAE, an MIC of 256 was the most common value for erythromycin; for tylosin, all isolates from CCAE had MIC values of >256.

**DISCUSSION**

Reports in the literature suggest that there may be an overall fitness cost associated with antimicrobial resistance (Andersson and Levin, 1999). In other words, in the
process of becoming drug resistant, a bacterial cell may gain or lose other traits, resulting in less vigor under conditions other than drug pressure. The current data show that compared with wild-type Campylobacter on control carcasses, there were lower numbers of resistant Campylobacter on chilled carcasses of broilers fed tyllosin. Further studies are warranted to determine whether resistance to erythromycin carries a fitness price, changing the ability of Campylobacter to adhere to broiler carcasses or survive immersion chilling.

In this study, CCAE included 8 μg of erythromycin/mL, which is the break point at which an organism is considered resistant to this drug. Therefore, anything that grows on CCAE would be considered resistant to erythromycin. There is no established break point for tyllosin; however, these 2 drugs are closely related, and when Campylobacter is resistant to erythromycin, it would be expected to be resistant to tyllosin also. The current data show that Campylobacter-positive broilers fed subtherapeutic levels of tyllosin will result in processed carcasses with erythromycin-resistant Campylobacter. Because erythromycin is often used to treat human campylobacteriosis, these results may be cause for concern.

Some researchers, although fully recognizing the development of drug resistance in bacterial pathogens, feel that this represents a low risk to the human consumer of meat animals. Phillips et al. (2004) suggest that because cooking kills human pathogens associated with food animals, the risk of disease or complicated treatment is low. A published deterministic model suggests that tyllosin use in food animals would lead to a very low probability of treatment failure (1 out 10 million; Hurd et al., 2004). Another study reports that removing macrolides from animals would actually result in more human disease caused by increased disease in animals (Cox and Popken, 2006).

The question of subtherapeutic antimicrobial use in meat animal production is complex. Macrolide resistance is a stable trait, likely to be maintained by Campylobacter for many generations, even without continued exposure to the drug. The possibility exists that resistant Campylobacter from poultry could contaminate kitchens, maintain its resistant trait, and become increasingly prevalent in the environment in general. Treatment of human campylobacteriosis is relatively rare, because most people with diarrhea do not seek medical attention. Nevertheless, in those instances when the most susceptible members of the population become ill and treatment is sought, resistance could have an impact on the likelihood of success. Indeed, infection with drug-resistant Campylobacter has been found to be related to a higher incidence of invasive disease or death than infection with susceptible Campylobacter (Helms et al., 2005). Industry and consumers alike will benefit from continued studies and discussion to clarify the impact of subtherapeutic antimicrobial use in poultry production.

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REFERENCES


