Changes in Antimicrobial Susceptibility of Native Enterococcus faecium in Chickens Fed Virginiamycin

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Received 28 January 2005/Accepted 23 March 2005

The extent of transfer of antimicrobial resistance from agricultural environments to humans is controversial. To assess the potential hazard posed by streptogramin use in food animals, this study evaluated the effect of virginiamycin exposure on antimicrobial resistance in Enterococcus faecium recovered from treated broilers. Four consecutive broiler feeding trials were conducted using animals raised on common litter. In the first three trials, one group of birds was fed virginiamycin continuously in feed at 20 g/ton, and a second group served as the nontreated control. In the fourth trial, antimicrobial-free feed was given to both groups. Fecal samples were cultured 1 day after chickens hatched and then at 1, 3, 5, and 7 weeks of age. Isolates from each time point were tested for susceptibility to a panel of different antimicrobials. Quinupristin/dalfopristin-resistant E. faecium appeared after 5 weeks of treatment in trial 1 and within 7 days of trials 2 to 4. Following removal of virginiamycin in trial 4, no resistant isolates were detected after 5 weeks. PCR failed to detect vat, vgb, or erm(B) in any of the streptogramin-resistant E. faecium isolates, whereas the msr(C) gene was detected in 97% of resistant isolates. In an experimental setting using broiler chickens, continuous virginiamycin exposure was required to maintain a stable streptogramin-resistant population of E. faecium in the animals. The bases of resistance could not be explained by known genetic determinants.

The enterococci are normal constituents of the intestinal flora of nearly all animals and are widely distributed in the environment. Enterococcal infections, caused almost exclusively by Enterococcus faecalis and Enterococcus faecium, are among the most common hospital-acquired infections. Over the past 2 decades, multiple-antimicrobial resistance in Enterococcus has increased, such that vancomycin was the only remaining effective treatment option in many cases. Subsequently, vancomycin-resistant E. faecium (VREF) appeared first in Europe in 1986 (12) and the United States in 1987 (21) and then later spread to other regions. VREF continues to be a major nosocomial problem, increasing 11% between 1997 and 2002 (15). In September 1999, the Food and Drug Administration (FDA) approved the streptogramin quinupristin/dalfopristin (Synercid) for use in treating patients infected with VREF (9). A related streptogramin, virginiamycin, has been used in animal production for 3 decades. It is approved as a feed supplement (alone or in combination with other agents) in cattle, swine, and poultry for disease prevention, growth promotion, and increased feed efficiency at concentrations ranging from 5 to 25g/ton (5.5 to 27.5 ppm).

Quinupristin/dalfopristin (Q/D) is a 30:70 mixture of semi-synthetic streptogramin B and streptogramin A compounds, which act synergistically at different sites on the bacterial ribosome to disrupt peptide elongation (1). Resistance to the streptogramin A component is associated with acetylases encoded by vat(D) and vat(E), first described in enterococcal isolates in Europe (17, 24). In the United States, vat(D) has not been seen to date; vat(E) has been reported in isolates from poultry farms (3) and in retail poultry meats (19), as well as in a single human clinical isolate (16). Resistance to streptogramin B is due principally to the erm gene products, which methylate an adenine residue in the 23S rRNA. M ethylation occurs in a region of overlapping binding sites for macrolides and lincosamides, resulting in the macrolides-lincosamides-streptogramin B resistance phenotype. Streptogramin B resistance may also be due to hydrolysis of the ring molecule via the lactonase encoded by vgb. The vgb genes were initially reported in Staphylococcus and are rarely present in Enterococcus (10). Streptogramin B (and macrolide) resistance has also been linked to the MsrC efflux pump, present in a majority of E. faecium isolates (23). Many streptogramin-resistant E. faecium (SREF) harbor none of the described resistance mechanisms (8).

Because virginiamycin induces cross-resistance to Q/D, it has been postulated that the use of virginiamycin in animal husbandry might influence the prevalence of Q/D resistance among human E. faecium isolates as a result of transmission through the food supply. SREF is common in the poultry production environment (22), including samples from litter and transport containers (7). SREF is also common on poultry meats at retail (6, 13, 19), suggesting that such meats serve as a continual source of resistant strains and/or their resistance genes. This raises the possibility that foodborne strains may transfer plasmid-borne resistance determinants to human native enterococci in vivo (11), which in turn may
donate these genes to other strains causing human infections. The food safety implications have prompted the FDA (http://www.fda.gov/cvm/index/updates/ra2.htm) and others (2, 20) to propose risk assessment models examining the potential public health consequences of virginiamycin use. To better understand the impact of virginiamycin use in the target animal species, this study examined the effects of continuous virginiamycin feeding on antimicrobial resistance of indigenous Enterococcus in broiler chickens over time.

(This work was presented in part at the International Conference on Emerging Infectious Diseases, Atlanta, Ga., February 2004).

**MATERIALS AND METHODS**

**Animals.** Day-old broiler chicks were purchased from a commercial supplier and were raised on corncob bedding at a population density of 1.5 ft² per bird. Fresh corncob litter was placed in the pens at the beginning of the study. In each trial, birds were randomly assigned to two groups (treatment and control) and placed in adjacent floor pens separated by about 3 feet. The birds were provided feed and water ad libitum. Both groups were fed a standard broiler starter/finisher diet that was prepared on site for each new flock. The treatment group received 20 g/ton of virginiamycin (Stafac 20) in the feed, and the control group received no medicated feed. Between trials, litter was manually turned and supplemented with fresh litter but was not removed from the pens. The animal research protocol was approved by the FDA Center for Veterinary Medicine institutional animal care and use committee.

**Experimental design.** We conducted four consecutive feeding trials with groups of broiler chickens raised on the same litter. In trial 1, birds placed on the original litter were allowed to acclimate for 5 days before virginiamycin was introduced into the feed of the treatment group. In trials 2 and 3, day-old broiler chicks in the treatment group were fed virginiamycin-supplemented feed upon arrival and continuously throughout the production cycle. In the fourth and final trial, virginiamycin was excluded from the feed of both treatment and control pens so that the persistence of SREF could be examined in the absence of drug.

In each feeding trial, broiler fecal samples were collected from birds at 1 day of age and again at approximately 1, 3, 5, and 7 weeks of age. Litter was cultured prior to placement and between each successive group to determine the susceptibility profiles of Enterococcus in the animal environment. Between flocks, the litter was allowed to stand for 2 to 4 weeks to mimic common production practices. For litter analysis, 500 g samples were collected from five areas of the pen and mixed to eosin methylene blue (EMB) agar plates (Beckton Dickinson) and incubated at 35°C for 24 h. Colonies typical of Enterococcus on EMB were subcultured to brain heart infusion agar for catalase, Gram stain, and typical gram-positive characteristic. Antimicrobial susceptibility testing was performed by the Clinical and Laboratory Standards Institute (CLSI; formerly NCCLS) MIC Interpretive Standards, where available (14). Sensititre susceptibility testing was performed according to the manufacturer’s instructions.

**Enterococcus species distribution.** As expected, Enterococcus spp. were recovered from every fecal, feed, and litter sample analyzed, resulting in a total of 1,542 isolates (Table 1). Among these, 1,065 (69%) were Enterococcus faecium. Enterococcus faecalis was the second most common species isolated (n = 366), followed by Enterococcus gallinarum (n = 46), Enterococcus hirae (n = 46), Enterococcus

### TABLE 1. Overall distribution of Enterococcus species

<table>
<thead>
<tr>
<th>Species</th>
<th>No. of isolates from broilers</th>
<th>No. of isolates from feed of</th>
<th>Total no. of isolates</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Hatchings Control Treated</td>
<td>Control Treated</td>
<td></td>
</tr>
<tr>
<td>E. faecium</td>
<td>66</td>
<td>150</td>
<td>134</td>
</tr>
<tr>
<td>E. faecalis</td>
<td>65</td>
<td>180</td>
<td>140</td>
</tr>
<tr>
<td>E. gallinarum</td>
<td>5</td>
<td>17</td>
<td>24</td>
</tr>
<tr>
<td>E. hirae</td>
<td>0</td>
<td>18</td>
<td>25</td>
</tr>
<tr>
<td>E. munditii</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>E. casseliflavus</td>
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<td>0</td>
</tr>
<tr>
<td>E. durans</td>
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<td>4</td>
<td>4</td>
</tr>
<tr>
<td>Unidentified</td>
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</tr>
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</table>

**RESULTS**

Enterococcus species distribution. As expected, Enterococcus spp. were recovered from every fecal, feed, and litter sample analyzed, resulting in a total of 1,542 isolates (Table 1). Among these, 1,065 (69%) were Enterococcus faecium. Enterococcus faecalis was the second most common species isolated (n = 366), followed by Enterococcus gallinarum (n = 46), Enterococcus hirae (n = 46), Enterococcus...
Enterococcus mundtii (n = 2), Enterococcus casseliflavus (n = 1), and Enterococcus durans (n = 1). Fifteen isolates, recovered from various sources, could not be identified to species level.

Among the 105 Enterococci recovered from feed, 93 (89%) belonged to E. faecium, and none belonged to E. faecalis. In contrast, E. faecalis was usually more common in day-old hatchlings. After chicks were on feed for 5 days, however, E. faecium was consistently isolated more frequently than E. faecalis in the feces from all four broiler groups, suggesting that enterococci from feed quickly supplanted the intestinal flora established in the chicks after hatching or, alternatively, that E. faecium more readily colonizes birds as they age. In addition, no E. faecalis isolates were found in litter from the control pens, whereas 10 isolates (12.8% overall) from the litter of the treated flocks were E. faecalis isolates, 9 of which appeared at the end of trial 4. E. gallinarum was recovered from fecal samples sporadically throughout the experiment, and E. hirae was recovered only in trial 4.

Changes in streptogramin susceptibility. Q/D MICs of 4 µg/ml (CLSI resistance breakpoint) for E. faecium were frequently observed in nonselected environments in this study, including the feed and the litter prior to drug exposure. Q/D MICs of 4 µg/ml for E. faecium were observed in 20% (4/20) of fresh litter samples and in 22% (21/94) of isolates from feed samples. In addition, for 6.6% (6/90) of fecal E. faecium isolates from day-old chicks, sampled on arrival, the Q/D MICs were 4 µg/ml (data not shown). Prior to streptogramin use, no isolates were recovered with Q/D MICs of >4 µg/ml. Because of this background resistance at the level of 4 µg/ml, we used a Q/D breakpoint of ≥8 µg/ml to assess changes in virginiamycin susceptibility following exposure to the antimicrobial.

Fecal isolates. Virginiamycin was administered in the feed at 20 g/ton for the first three trials, and then drug-free feed was given in the fourth trial. Figure 1 shows the dynamics of SREF over the four time periods. In trial 1, resistant strains (MIC of 16 µg/ml) were first observed in 3.4% (1/29) of isolates 3 weeks after introducing virginiamycin-supplemented feed. This increased to 90% (20/22) of E. faecium isolates from day-old chicks (MICs of 8 to 16 µg/ml). In trials 2 and 3, 80 to 95% of E. faecium were resistant within 1 week. This shorter lag time may have been due to acquisition from the litter of resistant strains remaining from the previous group(s). After 5 weeks of virginiamycin use, 100% of E. faecium were Q/D-resistant in both trial 2 and trial 3 (MICs of 8 to 16 µg/ml). In trial 4, birds were placed on litter from previously treated animals and fed antimicrobial-free feed. After 3 weeks, 36% of fecal isolates were
Q/D resistant (MIC of 16 to 32 μg/ml); this declined to 4% at week 5. By the time this last flock reached market age (7 weeks of age), no isolates for which the Q/D MIC was >4 μg/ml were detected in the feces.

*E. hirae* isolates, which appeared only in trial 4, were detected in both treatment and control groups. Interestingly, nearly all were streptogramin resistant. Isolates for which the MICs were ≥8 μg/ml were found in 94% (17/18) of the control pen isolates and 96% (24/25) of the treatment pen isolates. Three isolates recovered from feed were susceptible (data not shown).

**Feed and litter isolates.** Litter samples were collected prior to placement of the first groups of chicks, between each successive group, and at the end of the study. In total, 163 litter and 93 feed *E. faecium* isolates were recovered and tested for antimicrobial susceptibility (Table 1). No isolates for which the Q/D MIC was >4 μg/ml were detected in any of the feed samples (data not shown). In general, Q/D resistance among litter *E. faecium* paralleled the fecal profiles. At the end of trial 1, 44% (4/9) of litter *E. faecium* isolated from the treated flocks were Q/D resistant (MICs of 8 μg/ml), and 100% of litter isolates (n = 20) were resistant after trial 2 (MICs of 8 to 16 μg/ml) and trial 3 (MICs of 16 to 32 μg/ml; n = 20). At the end of trial 4, 7 weeks after discontinuing virginiamycin, no SREF was detected in the litter (n = 19). On four occasions, a few SREF isolates (7 isolates in total) were detected in the control animals. However, resistant isolates only persisted in the local environment in which the drug was present.

**Resistance to nonstreptogramin antimicrobials.** Resistance to nonstreptogramin antimicrobials (see Materials and Methods) was also monitored. No isolates were resistant to chloramphenicol, vancomycin, or linezolid. Among the 93 *E. faecium* feed isolates, overall resistances were as follows: tetracycline, 49%; lincomycin, 26%; nitrofurantoin, 11%; ciprofloxacin, 7%; and kanamycin, 6%. Among 20 *E. faecium* isolates from fresh litter, resistance was as follows: tetracycline, 45%; lincomycin, 26%; nitrofurantoin, 20%; ciprofloxacin, 3%. Among 72 isolates from day-old hatchlings, nitrofurantoin resistance was most common (43%), followed by penicillin (29%), gentamicin (28%), lincomycin (28%), tetracycline (26%), and ciprofloxacin (7%).

In SREF isolates from medicated animals, the prevalence and MICs for most antimicrobials were not significantly different from strains derived from the nontreated control animals. A notable exception was gentamicin resistance (Fig. 2). Except for trial 1 in which no resistance was seen, gentamicin resistance (MIC of >1,024 μg/ml) ranging from 22% to 88% was detected in *E. faecium* (but not *E. faecalis*) from each group of day-old chicks sampled upon arrival. Ninety-two percent (48/52) of the gentamicin-resistant isolates were also resistant to...
penicillin, whereas only 12.9% (7/54) of gentamicin-susceptible isolates were penicillin resistant. As shown in Fig. 2, gentamicin resistance was transient in each group of broilers but was more prevalent and persisted longer with each successive feeding trial.

Among other antimicrobials, resistance to tetracycline was more prevalent in enterococci from the treated pen than the control. Tetracycline resistance (MIC of >32 μg/ml) increased from 29% of isolates in the day-old hatchlings and reached 100% resistance in the final sampling day of first feeding trial. In subsequent trials, as was seen with streptogramin resistance, 100% of isolates were tetracycline resistant within only 1 week. Using a weighted repeated measures analysis of variance on the arcsine-transformed percentages, pair-wise comparisons between the treatment groups at each week within each experiment showed significantly more tetracycline resistance (P = 0.02 to 0.06) in strains from animals exposed to virginiamycin on all but one sampling day (data not shown). Differences between groups were not statistically significant after virginiamycin was removed from the feed.

Resistance genes. PCR was used to screen for the carriage of the known enterococcal streptogramin resistance genes, vat, msr(C), vgb, and erm, in all isolates for which the streptogramin MICs were >4 μg/ml. High-level (≥16 μg/ml) streptogramin resistance has been linked to the activity of acetylases encoded by vat(D) or vat(E). Neither gene was detected in any of the resistant isolates. Among the other targeted resistance genes, msr(C) was identified in 97% of Q/D-resistant E. faecium isolates. No other resistance genes were detected.

Changes in antimicrobial susceptibility of E. coli. On each sampling day, feed, fecal, and litter specimens were also cultured for E. coli to explore whether exposure to virginiamycin had any effect on the susceptibility profile of a representative gram-negative organism. Because E. coli and Enterococcus are known for their capacity to exchange resistance determinants, we postulated that long-term virginiamycin exposure might affect susceptibility in E. coli, either by directly influencing population structure or by promoting movement of mobile genetic elements between genera.

A total of 945 E. coli isolates were recovered and tested for antimicrobial susceptibility: 431 from control animals, 399 from treated animals, 50 from day-old chicks, 18 from feed, and 49 from litter samples collected between trials. Among the feed isolates, the only resistance observed was to tetracycline (n = 7) and nalidixic acid (n = 6). (As noted above, feed also contained ciprofloxacin-resistant enterococci.) The ciprofloxacin MICs of the nalidixic acid-resistant E. coli (MIC of >32 μg/ml) were from 0.125 to 0.5 μg/ml. Testing of the fecal E. coli isolates showed that the quinolone resistance was present only in the first two groups of broilers. For E. coli from animals, the proportion of fecal isolates resistant to a given drug changed most evidently following placement of a new group of animals. Changes in susceptibility within each experimental trial did not differ significantly between treated animals and controls and could not be linked to the prevalence of resistances among the Enterococcus isolates recovered in the same feeding trials.

DISCUSSION

The widespread use of antimicrobials in food animals, with the subsequent spread of antimicrobial-resistant zoonotic bacteria to humans via the food supply, is an ongoing public health concern. It is recognized that the use of antimicrobials in food animals promotes the evolution of resistant bacteria. The temporal dynamics of resistance development is dependent on the antimicrobial and pathogen in question. Inasmuch as the farm use environment plays a role in the ecology of resistance, attempts should be made to quantify the effects of agricultural antimicrobial use in the target animal species. This study was done to help assess the risks arising from food animal antimicrobial use by examining the temporal relationship of resistance development following virginiamycin exposure and the persistence of resistance in fecal isolates once the selective pressure of the antibiotic was removed from the environment.

Overall, resistance appeared more quickly and reached higher levels with prolonged environmental exposure. In the virginiamycin-treated groups, a low proportion of Q/D-resistant isolates appeared within 3 weeks following introduction of drug into the feed. In the next two feeding trials, where medicated broilers were raised on litter from previously treated birds, nearly all recovered E. faecium isolates were resistant within the first week. Although occasional resistant isolates appeared in the nearby nontreated pen, resistant isolates predominated and persisted only in the immediate environment in which animals were continuously exposed to virginiamycin. Similarly, the maximum observed MICs of 32 μg/ml did not emerge until the end of the grow-out period of the third trial. These strains were acquired by the following (fourth) group, presumably from the litter, and shed for another 3 weeks in the absence of drug use. After 3 weeks on nonmedicated feed, resistant fecal strains were no longer detected. Exposure to virginiamycin had no obvious effect on susceptibility among fecal E. coli.

In this study, we used a resistance breakpoint MIC of ≥8 μg/ml to monitor the impact of virginiamycin exposure in this study. The CLSI Q/D-resistant breakpoint for E. faecium is ≥4 g/ml. Among human clinical strains of E. faecium examined as part of the Synercid clinical trials, resistance to Q/D (MIC of ≥4 g/ml) was rare (4). In this study, however, MICs of 4 μg/ml for E. faecium were frequently observed in fresh feed and the litter, as well as in day-old chicks. In contrast, Q/D MICs were ≥8 μg/ml for all of the isolates recovered from drug-exposed environments. Isolates for which the MICs were 4 μg/ml may represent the normal MIC distribution of environmental isolates (accounting for variations in dilution susceptibility testing), independent of anthropogenic selection pressure. In surveys of feed components, the Q/D MICs were 4 μg/ml for a substantial number of E. faecium isolates recovered from whole grains, plant protein commodities, and oil seeds (D. Wagner, unpublished data). Alternatively, these strains may represent isolates that have spread over the decades of macrolide, lincosamide, and/or streptogramin B selection to become a common microbial constituent of materials used for feed and litter manufacture. The latter explanation is supported by the MICs we observed for other antimicrobials. For example, tetracyclines and aminoglycosides also have been used for decades in agriculture. Resistance to these agents, including high-level resistance phenotypes, was also observed in feed and litter prior to animal placement. Similarly, resistance in E. coli was common for these and other “older” agents (data not shown). In any case, the susceptibility phenotypes...
seen in this study, including isolates at the CLSI Q/D-resistant breakpoint, were associated with nonsupplemented feed commodities and components of corncob litter.

Some E. faecium resistance phenotypes were observed predominantly or exclusively in the cloacal flora of day-old chicks. The level and persistence varied by antimicrobial with each new group of birds. Gentamicin resistance was unique to hatchlings and may reflect exposure to gentamicin incorporated into the Marek's vaccine (18). While gentamicin resistance disappeared in each group over time, the frequency of resistance was longer, and the lag time to susceptibility was higher, in bacterial populations from trials 3 and 4. Gentamicin resistance in day-old chicks was highly associated with penicillin resistance; however, it was not obviously influenced by virginiamycin exposure.

The potential for streptogramin resistance genes to migrate from foodborne enterococcal isolates to those causing disease in humans is largely unknown. This is due in part to an incomplete understanding of the array of genes underlying resistance. PCR was used to investigate the prevalence of known streptogramin resistance genes. The putative efflux pump encoded by msr(C) was the only gene detected in the Q/D-resistant isolates, including those for which the MICs were high (≥32 µg/ml). These findings, along with those reported in other studies (5, 8, 19), indicate that most mechanisms of streptogramin resistance in Enterococcus (including their capacity to transfer horizontally) remain undiscovered.

In summary, our results suggest that virginiamycin resistance is slow to develop in a naı̈ve broiler environment but emerges quickly in animals raised on litter from a previously treated flock. Maintaining streptogramin resistance in the broiler gut requires continuous exposure to the drug. Following removal of virginiamycin from the feed, susceptible fecal E. faecium, presumably entering via the feed, displaced within 3 weeks resistant strains acquired from the litter. Over the course of successive feeding trials, MICs reached a maximum value of 32 µg/ml (MIC levels associated with the vat genes) only in the third medicated flock. Despite the appearance of strains for which the streptogramin MICs were high, resistance could not be explained by known mechanisms of resistance. Additional genetic studies to characterize the mechanisms of streptogramin resistance in these and other isolates lacking the vat gene are ongoing. Estimations of the potential health risks to humans resulting from agricultural streptogramin use will need updating as more information on the genetics of resistance becomes available.

ACKNOWLEDGMENTS

We thank Robert Walker for critical review of the manuscript. PCR control plasmid pPf808 was generously provided by N. El Solh, Institut Pasteur.

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