Effects of subtherapeutic concentrations of antimicrobials on gene acquisition events in Yersinia, Proteus, Shigella, and Salmonella recipient organisms in isolated ligated intestinal loops of swine

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Objective—To assess antimicrobial resistance and transfer of virulence genes facilitated by subtherapeutic concentrations of antimicrobials in swine intestines.

Animals—20 anesthetized pigs experimentally inoculated with donor and recipient bacteria.

Procedures—4 recipient pathogenic bacteria (Salmonella enterica serotype Typhimurium, Yersinia enterocolitica, Shigella flexneri, or Proteus mirabilis) were incubated with donor bacteria in the presence of subinhibitory concentrations of 1 of 16 antimicrobials in isolated ligated intestinal loops in swine. Donor Escherichia coli contained transferrable antimicrobial resistance or virulence genes. After coincubations, intestinal contents were removed and assessed for pathogens that acquired new antimicrobial resistance or virulence genes following exposure to the subtherapeutic concentrations of antimicrobials.

Results—3 antimicrobials (apramycin, lincomycin, and neomycin) enhanced transfer of an antimicrobial resistance plasmid from commensal E coli organisms to Yersinia and Proteus organisms, whereas 7 antimicrobials (florfenicol, hygromycin, penicillin G, roxarsone, sulfamethazine, tetracycline, and tylosin) exacerbated transfer of an integron (Salmonella genomic island 1) from Salmonella organisms to Yersinia organisms. Sulfamethazine induced the transfer of Salmonella pathogenicity island 1 from pathogenic to nonpathogenic Salmonella organisms. Six antimicrobials (bacitracin, carbadox, erythromycin, sulfathiazole, tiamulin, and virginiamycin) did not mediate any transfer events. Sulfamethazine was the only antimicrobial implicated in 2 types of transfer events.

Conclusions and Clinical Relevance—10 of 16 antimicrobials at subinhibitory or subtherapeutic concentrations augmented specific antimicrobial resistance or transfer of virulence genes into pathogenic bacteria in isolated intestinal loops in swine. Use of subtherapeutic antimicrobials in animal feed may be associated with unwanted collateral effects. (Am J Vet Res 2013;74:1078–1083)

Subtherapeutic concentrations of antimicrobials have been used for decades as growth promotants and prophylactic agents. This practice has been scrutinized as a contributor to the dissemination of antimicrobial resistance. Specifically, subtherapeutic concentrations of chlortetracycline have been associated with an increase in resistance to multiple antimicrobials for swine intestinal microbes.1 Another study2 revealed that subtherapeutic concentrations of chloramphenicol have been associated with an increase in resistance to multiple antimicrobials for swine intestinal microbes.1 Another study2 revealed that subtherapeutic concentrations of antimicrobials can lead to the propagation of antimicrobial-resistant Enterococcus organisms in swine. A recent study3 indicated that intestinal bacteriophages are activated by subtherapeutic concentrations of antimicrobials, and this activation can lead to transfer of antimicrobial resistance genes. However, these studies have not addressed specific gene transfer events that precipitate resistance and virulence. Thus, little is known about the effects of these antimicrobials on specific gene transfer events that promote the dissemination of antimicrobial resistance and virulence genes in enteric pathogens.

The objective of the study reported here was to identify antimicrobials that at subtherapeutic concentrations augment the transfer of certain antimicrobial resistance and virulence genes. The main goal was to

ABBREVIATIONS
ESBL Extended-spectrum β-lactamase
SGI1 Salmonella genomic island 1
SPI1 Salmonella pathogenicity island 1
XLD Xylose lysine deoxycholate
assess 3 types of transfer events (plasmid transfer, integron transfer, and horizontal transfer of a pathogenicity island) mediated by subtherapeutic concentrations of antimicrobials in vivo. Specifically, the study was conducted to determine the relative rates of antimicrobial-mediated transfer events involving 3 representative antimicrobial resistance plasmids, 1 model integron, and 1 pathogenicity island in the presence of antimicrobials that have been approved for use (currently or in the past) at subtherapeutic concentrations as feed additives for swine.

Materials and Methods

Animals—Twenty juvenile swine were used to assess in vivo transfer events. Pigs were of mixed breeds and both sexes; pigs weighed between 5 and 10 kg. Animal experiments were approved by the Iowa State University Institutional Animal Care and Use Committee. Pigs were anesthetized with pentobarbital (40 mg/kg, intraperitoneal). Isolated ligated loops of intestine (9 loops/pig; each ligated loop was 10 cm in length) were created with 2-0 silk sutures. At the end of the experiments, anesthetized pigs were euthanized by intracardiac administration of an overdose of pentobarbital (100 mg/kg).

Plasmid transfer—To determine plasmid transfer from donor bacteria to recipient pathogenic bacteria, each loop was injected with approximately 10^9 to 10^11 CFUs of donor bacteria and 10^9 CFUs of recipient strain SL1344, and pathogenicity island in the presence of antimicrobials that were created with 2-0 silk sutures. At the end of the experiments, anesthetized pigs were euthanized by intracardiac administration of an overdose of pentobarbital (100 mg/kg).

Plasmid transfer—To determine plasmid transfer from donor bacteria to recipient pathogenic bacteria, each loop was injected with approximately 10^9 to 10^11 CFUs of donor bacteria and 10^9 CFUs of recipient bacteria in 1 mL of saline (0.9% NaCl) solution that contained 1 of 16 feed additive antimicrobials (ampicillin, bacitracin, carboxad, erythromycin, florfenicol, hygromycin, lincomycin, neomycin, penicillin G, roxarsone, sulfamethazine, sulfathiazole, tetracycline, tiamulin, tylosin, and virginiamycin); antimicrobial-free saline solution was used as a negative control treatment. Concentration of each antimicrobial was 1 µg/mL. This concentration was chosen because it was less than the established breakpoints for all of the antimicrobial-bacteria combinations, and preliminary experiments conducted by our laboratory group on the minimum inhibitory concentration of these antimicrobials revealed that this was the lowest common concentration that permitted growth of all donor and recipient bacteria for each of the 16 antimicrobials (data not shown).

Plasmid transfer from a donor commensal intestinal Escherichia coli (antimicrobial susceptible and lacking virulence genes) to each of 4 recipient pathogenic bacteria (Salmonella enterica serotype Typhimurium strain SL1344, Yersinia enterocolitica, Shigella flexneri, or Proteus mirabilis) was measured. All of the recipient bacteria were susceptible to most antimicrobials (except tetracycline and streptomycin), as determined by use of microdilution broth assays performed in accordance with standards established by the Clinical and Laboratory Standards Institute. The E coli donor strain was experimentally transformed with 1 of 3 conjugative plasmids encoding an ESBL, amikacin resistance (aac(C)-4), or fluoroquinolone resistance (via the qnr gene).

Bacteria were allowed to incubate in the isolated ligated intestinal loops for 1 hour, after which loops were excised. Total volume of intestinal content in each isolated loop was 1.1 to 1.2 mL; approximately 10% of the total content of each loop was removed for plating on media selective for each of the 4 pathogens (XLD agar for Salmonella and Shigella organisms, Yersinia selective agar base for Yersinia organisms, and phenylalanine agar for Proteus organisms); agar contained 1 of 3 antimicrobials (cefotin, 32 µg/mL for the ESBL plasmid; amikacin, 64 µg/mL; or enrofloxacin, 8 µg/mL) at their respective breakpoint concentrations. The content of each loop was cultured in triplicate (3 agar plates/loop). Control experiments revealed that the donor E coli did not grow on the selective media, except for the XLD agar; however, the E coli colonies that grew on XLD agar were biochemically distinct from the Salmonella and Shigella colonies.

Plates were incubated at 37 °C for 16 hours. Bacteria were then enumerated, and the number of translocants/10^9 recipients was calculated by multiplying the number of recovered colonies by 10 to account for the fact that only 10% of the intestinal contents were plated. The log_{10} number of the product was derived and used for final data analysis and statistical evaluation.

Transfer of the ESBL plasmid was assessed with a PCR assay with primers (5'-ATGATGAATACTCTATGCT-3' and 5'-TTATTGGACGTTTCAAGAAAT-3') specific to the blp_{392} gene present on the ESBL plasmid. Each transfer event was determined for 100 representative colonies.

SGI1 transfer—The possibility that subinhibitory concentrations of antimicrobials can modulate the transfer of SGI1 from Salmonella organisms to Yersinia recipients, Shigella recipients, or Proteus recipients was evaluated. The SGI1 is a multiresistance integron that encodes resistance to 5 antimicrobials in Salmonella organisms. Integrons are mobile genomic elements putatively transferred by bacteriophages, and studies have indicated that Yersinia spp, Shigella spp, and Proteus spp are capable of receiving integrons. To evaluate SGI1 transfer, approximately 10^9 CFUs of SGI1-free recipient (Yersinia enterocolitica, Shigella flexneri, or Proteus mirabilis) were inoculated with 10^9 CFUs of SGI1-bearing donor Salmonella enterica serotype Typhimurium phage type DT104 strain LNWI into ligated intestinal loops. Coincubations included 1 of the 16 aforementioned antimicrobials (concentration, 1 µg/mL) and the negative control treatment (saline solution without an antimicrobial).

Bacteria were allowed to incubate in the isolated ligated intestinal loops for 1 hour, after which loops were excised. Total volume of the intestinal content in each isolated loop was 1.1 to 1.2 mL; approximately 10% of the total content of each loop was removed for plating on media selective for each of the 3 pathogens (XLD agar for Salmonella and Shigella organisms, Yersinia selective agar base for Yersinia organisms, and phenylalanine agar for Proteus organisms); agar contained 1 of 2 antimicrobials relevant for SGI1 (ampicillin, 32 µg/
ml; chloramphenicol, 32 µg/mL at their respective breakpoint concentrations. The content of each loop was cultured in triplicate (3 agar plates/loop).

Plates were incubated at 37°C for 16 hours. Bacteria then were enumerated, and the number of translocants/109 recipients was calculated by multiplying the number of recovered colonies by 10 to account for the fact that only 10% of the intestinal contents were plated. The log_{10} of the product was derived and used for final data analysis and statistical evaluation.

A PCR assay specific to the floR-tetR sequence in SGI1 was used to assess the presence of the SGI1 integron in 10 representative recipient bacteria that grew on selective media containing ampicillin and chloramphenicol. Additionally, translocants were assessed for the absence of a Salmonella virulence gene segment (sipB/C). Control experiments revealed that the donor Salmonella organisms were not able to grow on the media selective for Yersinia spp and Proteus spp, whereas Salmonella organisms were distinguishable from Shigella organisms on XLD agar.

SP11 transfer—Transfer of SP11 from pathogenic to nonpathogenic Salmonella organisms was evaluated. The SP11 is a major determinant of virulence in Salmonella organisms, and avirulent Salmonella organisms lack SP11. The genomic structure of SP11 suggests that this island is transferrable. In vivo coinoculations with the 16 antimicrobials and the negative control treatment (saline solution without an antimicrobial) were performed as described previously for the plasmid and integron transfer experiments. The SP11-bearing S enterica Typhimurium (antimicrobial-susceptible strain SL1344) was incubated with 1 of 4 SP11-free strains (S enterica serotypes Litchfield, Senftenberg, Seminole, or Betiocky), all 4 of which were transformed with a nonconjugative plasmid encoding green fluorescent protein. For this experiment, 1011 CFUs of donor bacteria and 1011 CFUs of recipient bacteria were used for the incubations.

Bacteria were allowed to incubate in the ligated loops for 1 hour. Total volume of each loop was 1.1 to 1.2 mL; approximately 10% of the total content of each loop was removed and used in a large-volume tissue culture invasion assay in which recovered bacteria were plated on XLD agar that contained 50 µg of zeocin/mL (zeocin is the selective marker for the fluorescence plasmid). The content of each loop was cultured in triplicate (3 agar plates/loop). Fluorescent colonies that were invasive (ie, recovered from inside tissue culture cells) were individually expanded in fresh nutrient broth and then subjected to a second invasion assay. Amount of invasion was compared with that of strain SL1344. Serotype analysis was conducted at another laboratory, and a PCR assay that detected the sipB-sipC sequence in SP11 was performed on each clone for which invasion was indistinguishable from that of strain SL1344 (approx 1% invasion).

Statistical analysis—Statistical differences were assessed with an ANOVA. There were 51 combinations of antimicrobials and transfer events (16 antimicrobials plus 1 antimicrobial-free treatment) times 3 transfer events), each assessed in 3 separate ligated loops (153 total loops) and 3 agar plates/loop (459 total loops). Frequency data for all transfer events were analyzed en masse to allow for interantimicrobial and intraevent comparisons and intra-antimicrobial and interevent comparisons (ie, comparisons were made among the antimicrobials within the 3 transfer events [plasmid, SGI1, and SP11] and among the 3 transfer events within a specific antimicrobial). The Scheffe F test was chosen as the ad hoc test because our research group has empirically found it to be the most conservative for detecting differences, in contrast to the Bonferroni test. Values of P < 0.05 were considered significant.

Results

Plasmid transfer—In vivo transfer events of 3 clinically relevant antimicrobial resistance plasmids were assessed by use of ligated intestinal loops of swine. Loops were coinoculated with donor commensal E coli bearing 1 of 3 antimicrobial resistance plasmids, 1 of 4 recipient pathogenic Enterobacteriaceae, and an antimicrobial (1 µg/mL) approved as a feed additive in swine. Commensal E coli and recipient Enterobacteriaceae were chosen because these microbes are highly representative of enteric bacteria that transfer genetic information. Three antimicrobials mediated a significant increase in the frequency of a specific plasmid transfer event in vivo (Figure 1). An increased frequency of transfer of the ESBL plasmid from E coli to Yersinia recipients and from E coli to Proteus recipients was evident in the presence of apramycin, lincomycin, or neomycin (Table 1). None of the antimicrobials caused significant changes in the frequencies of ESBL plasmid transfer from E coli to Salmonella recipients and from E coli to Shigella recipients. Additionally, none of the antimicrobials caused significant changes in transfer of amikacin resistance plasmids or fluoroquinolone resistance plasmids.
The PCR assay of 100 representative ceftiofur-resistant colonies from each of the aforementioned transfer events revealed that 96% to 97% of the putative transconjugates contained \( \text{bla}^{\text{CMY-2}} \). Colonies that lacked this sequence were numerically discounted as transconjugates in the final calculations (Figure 1).

**SGI1 transfer**—In vivo transfer events of an integron were assessed with ligated intestinal loops coinoculated with donor *Salmonella* organisms bearing SGI1,11 of 4 recipient pathogenic Enterobacteriaceae, and an antimicrobial (1 \( \mu \)g/mL) approved as a feed additive in swine. Seven antimicrobials mediated a significant increase in the frequency of in vivo transfer of a specific integron (Figure 2). An increased frequency of transfer of the SGI1 integron from *Salmonella* organisms to *Yersinia* organisms was evident in the presence of florfenicol, hygromycin, tetracycline, and tylosin (Table 1). None of the antimicrobials caused significant changes in the frequencies of SGI1 transfer events from *Salmonella* organisms to either of the other 2 pathogens examined. The PCR assay of 100 representative colonies from each of the 16 aforementioned transfer events revealed that 98% to 99% of the putative SGI1 recipients contained the \( \text{floR-tetR} \) sequence indicative of SGI1.16 Colonies that lacked this sequence were numerically discounted as translocants in the final calculations.

<table>
<thead>
<tr>
<th>Antimicrobial</th>
<th>ESBL plasmid transfer</th>
<th>SGI1 transfer</th>
<th>SPI1 transfer</th>
<th>Ratio of total transfer frequencies</th>
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<tr>
<td>Saline solution</td>
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<td>NA</td>
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<td>—</td>
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<td>Roxarsone</td>
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<td>3 recipient nonpathogenic serovars of <em>Salmonella enterica</em>†</td>
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<tr>
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<td>Tetracycline</td>
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<td>—</td>
<td>—</td>
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</table>

Mean transfer frequency in the absence of an antimicrobial was approximately \( 1.4 \times 10^{-8} \) transconjugates/recipient for the ESBL plasmid, \( 2.6 \times 10^{-9} \) transconjugates/recipient for SGI1, and \( 3 \times 10^{-11} \) invasive clones/noninvasive recipient for SPI1; these frequencies were calculated by dividing the total number of all recovered recipients by the total number of donor bacteria added to all of the coincubations in the absence of an antimicrobial. For each antimicrobial, the ratio of total transfer frequencies = (total number of all bacteria that received the new genetic element when coincubated with an antimicrobial/total number of recipients coincubated with an antimicrobial)/total number of all bacteria that received the new genetic element when coincubated without an antimicrobial/total number of recipients coincubated without an antimicrobial).

*Transfer frequency was significantly (\( P < 0.05 \)) greater than the transfer frequency for the antimicrobial-free (negative control) treatment.

†Salmonella enterica serotypes Betocky, Litchfield, and Seminole.

NA = Not applicable. — = Transfer frequency was not significantly greater than the transfer frequency for the antimicrobial-free (negative control) treatment.
SPI1 transfer—In vivo transfer events of a pathogenicity island were assessed with ligated intestinal loops coincoculated with donor Salmonella enterica serovars that lacked SPI1, and an antimicrobial (1 μg/mL) approved as a feed additive in swine. Sulfamethazine mediated the horizontal transfer of SPI1 in vivo (Figure 3). Three of 4 SPI1-free S enterica serovars (Betiocky, Seminole, and Litchfield) had transfer events in the presence of sulfamethazine (Table 1). Transfer of SPI1 was detected in the absence of an antimicrobial for 1 clone of S enterica serovar Betiocky did not acquire SPI1 in this experiment. Results of the PCR assays confirmed the presence of the sipB-sipC genomic segment in all clones that had invasion indistinguishable from that of strain SL1344.

Discussion

The use of subtherapeutic concentrations of antimicrobials in livestock feed is a controversial practice that is being scrutinized. Of concern are the unknown collateral effects on bacterial gene transcription, plasmid transconjugation from commensals to pathogens, and viral-mediated transduction of genes from commensals to pathogens and from one pathogen to another. Specifically, these collateral effects can activate molecular processes culminating in gene transfer events that yield pathogenic bacteria with multiple antimicrobial resistance or pathogens with new virulence capabilities.

The experiments in the present study involved the use of a low concentration (1 μg/mL) of antimicrobials to mimic field conditions without directly harming the donor or recipient bacteria. Although this concentration may be lower than concentrations in the intestinal tract of swine fed feed that contains antimicrobials, this concentration was considered relevant for swine in which the antimicrobial is removed from the diet prior to slaughter.

The present study revealed that there were transfer events in the absence of an antimicrobial and that certain antimicrobials mediated gene transfer events at a higher frequency than did other antimicrobials (Table 1). Sulfamethazine mediated 2 separate transfer events (SGI1 transfer and SPI1 transfer), whereas a related drug, sulfathiazole, did not mediate any transfer events. Bacitracin, carbadox, erythromycin, tiamulin, and virginiamycin were also not implicated in transfer events. Transfer events were confirmed with a PCR assay, although a few (1% to 4%) transconjugates and transloconts did not harbor the transferrable element, which suggested that efflux systems may have been activated in these few clones.

Three classes of antimicrobials were implicated in transfer of the ESBL plasmid. Lincomycin is a lincosamide, apramycin is an aminocyclitol, and neomycin is an aminoglycoside (although the latter 2 are sometimes grouped in the same class). Lincomycin is an inhibitor of the 50S ribosome in bacteria, yet 3 other 50S inhibitors (erythromycin, tiamulin, and virginiamycin) did not mediate transfer events. Apramycin and neomycin are inhibitors of the 30S ribosome, but another 30S inhibitor, tetracycline, did not exacerbate transfer of the ESBL plasmid. It is possible that apramycin, lincomycin, and neomycin can selectively alter protein synthesis that impacts sex pheromones and conjugation.

Seven antimicrobials (lorfenicol, hygromycin, penicillin G, roxarsone, sulfamethazine, tetracycline, and tylosin) from 7 antimicrobial classes were implicated in the transfer of SGI1. Tylosin and lorfenicol are 50S inhibitors, but that is the extent of the similarities among these 7 antimicrobials. Because movement of SGI1 may be a phage-mediated event,12 it is possible that those 7 antimicrobials activated phage recombinase in SGI1-bearing Salmonella organisms, similar to that recently reported for sulfamethazine, chlorotetraacycline, and penicillin.1 It is also possible that naked SGI1 DNA was transferred from Salmonella organisms to Yersinia organisms, although to our knowledge this process has not been described in the literature.

Analysis of results of the present study indicated that certain antimicrobials at subtherapeutic concentrations are more likely to mediate unwanted gene transfer into pathogenic bacteria in ligated intestinal loops in swine. Sulfamethazine mediated 2 types of transfer, whereas apramycin, lincomycin, and neomycin exerted the greatest quantitative effect on a single transfer event into 2 genera of Enterobacteriaceae. Bacitracin, carbadox, erythromycin, sulfathiazole, tiamulin, and virginiamycin did not significantly influence any of the 3 transfer events evaluated. No antimicrobial class-specific patterns were observed in the 3 transfer events. Transfer of the ESBL plasmid was detected at the highest frequency. Protein synthesis irregularities may underlie transfer of the ESBL plasmid, and activation of bacteriophages may be involved.
in SG11 transfer or SPI1 transfer (or both). Regardless of the mechanisms involved, subtherapeutic concentrations of antimicrobials have potential ecologic impacts that involve the dissemination of antimicrobial resistance and virulence genes.

References