RESEARCH REPORT

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PUBLIC HEALTH/WORKER SAFETY

Title: Factors driving the emergence and persistence of multidrug resistance-encoding plasmids in the swine environment – NPB #12-080

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Date Submitted: February 16, 2014

Industry Summary.

This study examined the impact of subtherapeutic and therapautic levels of chlortetracycline in feed on the selection and dissemination of mobile genetic elements called IncA/C plasmids among *E. coli* in the pig gut. These plasmids are important to human and animal health because they enable bacteria to become resistant to multiple antibiotics, and they can be spread between bacteria through conjugation. Using oral inoculation of pigs with bacteria containing IncA/C plasmids, we showed that subtherapeutic use of chlortetracycline had no impact on the selection of these plasmids over the course of six weeks. In contrast, therapeutic use of the same drug significantly increased the proportion of plasmid-containing *E. coli* in the pig gut. Also, the mobility of IncA/C plasmids to different bacterial hosts was not evident from our work, suggesting that their movement is not as extensive as previously thought. This work indicates that short-term use of chlortetracycline as a growth promoter in feed does not necessarily select for multidrug resistance encoded by IncA/C-type plasmids in *E. coli*.

Keywords: antibiotics, growth promoters, chlortetracycline, plasmids, multidrug resistance, growth promotion

These research results were submitted in fulfillment of checkoff-funded research projects. This report is published directly as submitted by the project's principal investigator. This report has not been peer-reviewed.

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Scientific Abstract.

The contentious debate on the public health impact of antimicrobial use in food animals has perpetuated for over 40 years, and remains a front-burner issue for the swine industry. The failure to approach meaningful consensus on this question is largely a consequence of the void of detailed knowledge of the epidemiology and ecology of resistance determinants in bacteria populations under different conditions of antimicrobial exposure. The presence of multidrug resistance-encoding plasmids in the pig farm environment, within the animal, and on retail meat presents a plausible threat to both animal and human health. IncA/C plasmids pose a particular threat in this respect, because of their abundance in swine, their broad bacterial host range, and their apparent ability to rapidly disseminate through diverse bacterial communities [1]. In this study, we examined the influence of subtherapeutic and therapeutic levels of chlortetracycline in feed on plasmid selection in growing pigs. We found that subtherapeutic levels of chlortetracycline had no effect on the selection of IncA/C plasmids over the course of six weeks, but therapeutic levels of the drug resulted in a significant increase in plasmid-containing *Escherichia coli* populations in feces. Similarly, subtherapeutic levels of chlortetracycline did not affect the dissemination of this plasmid among bacteria in the feces, whereas therapeutic levels of this drug enhanced the ability of the plasmid to move between *E. coli* in the gastrointestinal tract. Finally, use of chlortetracycline was effective at significantly promoting the growth of pigs over the course of 8 weeks. Overall, this study demonstrates that the short term use of subtherapeutic levels of chlortetracycline in feed does not select for IncA/C plasmids or promote their dissemination in the pig gastrointestinal tract.

Introduction.

The plasmid incompatibility group IncA/C is a broad-host-range plasmid group that has emerged worldwide among a wide variety of animal-source and environmental bacteria, including those of fish [2,3], food animals [4-7], companion animals, humans [7-10], water [3,11] and soil. Concerns regarding IncA/C plasmid dissemination recently surfaced when it was realized that they have emerged among *Escherichia coli* and *Salmonella enterica* of humans and production animals, and that they harbor a remarkable number of

antimicrobial resistance genes [12-14]. IncA/C plasmids possess genes encoding resistance to multiple classes of antimicrobials, including aminoglycosides, carbapenems, newer-generation cephalosporins, penicillins, sulfonamides, tetracyclines, phenicols, and heavy metals. In addition to their unprecedented ability to acquire resistance modules, IncA/C plasmids are remarkable for their rapid emergence and their broad host range [15]. IncA/C plasmids have been increasingly identified among clinical *Salmonella* and *E. coli* in the United States, and circumstantial evidence suggests that these plasmids and strains originated from beef, poultry, and pork [16-18].

However, the emergence of IncA/C plasmids among *Enterobactericeae* of human importance is perplexing, since such bacterial species are not predicted to be efficient hosts of the IncA/C plasmid [15]. Certain varieties of these plasmids, such as some $bla_{\mathrm{CMY-2}}$-encoding IncA/C plasmids, are deficient in conjugative transfer [19]; and these plasmids impose a significant fitness cost to enterobacterial hosts and require selective pressure for their maintenance in populations [20]. Therefore, the rapid emergence of IncA/C plasmids among *Enterobacteriaceae* is surprising and implies that unexplained reasons exist for their success. This underscores the need to better understand their environmental sources, bacterial reservoirs, the factors driving their selection, and their basic biology. Overall, it is evident that IncA/C is a unique and emergent plasmid type, but its widespread dissemination in diverse biological systems remains unexplained, including the biological understanding of how these plasmids have disseminated despite their apparent burden to the bacterial hosts.

We have described the completed sequences of $bla_{\mathrm{CMY-2}}$-endcoding IncA/C plasmids from *E. coli* isolated from multiple animal sources, including a K88-positive *E. coli* from a case of post-weaning diarrhea in swine, pUMNK88_161 [12]. This work highlighted the propensity for IncA/C plasmids from porcine-source *E. coli* to acquire resistance-associated genes. In the case of pUMNK88_161, three different resistance-associated loci were identified containing genes associated with resistance to phenicols (*floR* and *cmlA*), tetracyclines (*tetAR*), aminoglycosides (*strAB*, *aacC*, *aadA*, and *aadA2*), sulfonamides (*sul2*), beta-lactams ($bla_{\mathrm{CMY-2}}$), quaternary

ammonium compounds (*qacEΔ1*), and heavy metals (*mer*). All IncA/C plasmids harbor these hotspots for resistance gene acquisition, but their content varies considerably between plasmids [12]. In contrast, the core backbone of these plasmids is highly conserved, with greater than 95% nucleotide sequence similarity within the core genes observed between all IncA/C plasmids. This is suggestive that IncA/C plasmids as a group share a recent ancestry but are under constant modification in their hotspots, likely due to selective pressures and/or the available mobile genetic element pool within an environment.

Among plasmid types of *Enterobacteriaceae*, IncA/C plasmids are associated with resistance towards the greatest number of antimicrobial agents. In a study involving 2,002 *E. coli* isolates from poultry and humans, we sought associations between phenotypic resistance and plasmid content [21]. Of the 14 antimicrobials examined, we identified 12/14 that were strongly associated ($p<0.01$) with IncA/C plasmid carriage. The strong association of these resistance phenotypes with IncA/C plasmid carriage is suggestive that the genes encoding these resistances are harbored by these plasmids. IncA/C plasmids are also ubiquitous among *E. coli* in commercial swine [22]. We found that IncA/C plasmids were highly prevalent among both post-weaning diarrhea-associated and neonatal diarrhea-associated *E. coli* populations. This work suggests that pigs are colonized at a young age with bacteria harboring IncA/C and IncI1 plasmids, and that these plasmids are present in a wide array of bacterial clones and species in the swine environment. One could speculate that the high prevalence of IncA/C plasmids among porcine-source *E. coli* is due to the selection pressures resulting from antibiotic use. However, scientific evidence is lacking to demonstrate that this is the case.

Project Objectives.

The dissemination of multidrug resistance-encoding plasmids such as IncA/C among enterobacterial isolates of production animals is evident. However, in actuality they are not thought to be well suited to persist in *E. coli* and *Salmonella*, although it is clear that they have successfully disseminated among such bacteria in production animal environments. The principal reservoirs for these plasmids in the swine production environment and

surrounding ecosystems are unknown, as are the factors that drive their selection and dissemination. Important knowledge gaps exist limiting our understanding of how production practices might impact the emergence and persistence of these plasmids. In this study, we performed controlled animal experiments and in vitro experiments with the goal of better understanding the environmental sources of these plasmids and their possible selection. This work had two primary objectives:

1. **Determine the fate of the IncA/C plasmid in the pig gastrointestinal tract with and without exposure to chlortetracycline.**
2. **Identify reservoirs for the IncA/C plasmid in the pig gastrointestinal tract and in manure.**

Procedures to achieve these objectives.

Objective 1. Determine the fate of the IncA/C plasmid in the pig gastrointestinal tract with and without exposure to tetracyclines. Much debate and effort has addressed the selection of resistant bacterial organisms, but questions still remain as to which pressures actually do select for multidrug-resistant organisms [23]. This is particularly true in the case of plasmids such as IncA/C, with a broad host range enabling them to presumably exist in a variety of commensal bacterial species. No work has addressed the possible selection of such plasmids in the swine gut, nor has any work addressed the bacterial host range of these plasmids relative to their selection. We hypothesized that antibiotic selective pressures play no role in the persistence of IncA/C plasmids in the bacterial communities of the swine gut. That is, we predicted that their persistence is independent of antibiotic selective pressure. In Objective 1, we performed controlled animal experiments to examine the effects of subtherapeutic and therapeutic administration of chlortetracycline on the persistence of IncA/C plasmid-containing *E. coli* populations.

Bacterial strain construction. A single pig commensal *E. coli* strain (pig fecal *E. coli*, or PFEC) was used for these experiments. This strain was spontaneously mutated with chromosomally-encoded nalidixic acid resistance for its selection. To facilitate the tracking of our prototype IncA/C plasmid, pAR060302, plasmid labeling using red fluorescent protein (*rfp*) was used [24,25]. The system used allowed us to track pAR060302

in the pig gastrointestinal tract, and to differentiate between the presence of this plasmid in its original host or conjugative transfer to non-*E. coli* recipient bacteria. To develop this system, a mini-Tn$5_{A1\text{-}04/03}$::*rfp* cassette was inserted into pAR060302 using previously described procedure [24]. First, the mini-Tn$5_{A1\text{-}04/03}$::*rfp* cassette was mobilized into *E. coli* strain DH10B(pAR060302) using a helper plasmid and integrated into either the chromosome of DH10B or pAR060302 [26]. This mixture was mated with our PFEC strain and transconjugants were selected on media containing selective antibiotics for the recipient strain PFEC carrying pAR060302:*rfp*. The location of the mini-Tn$5_{A1\text{-}04/03}$::*rfp* cassette insertion in pAR060302 was verified via sequencing to ensure it was not inserted into any coding or regulatory regions of pAR060302. Because PFEC contains the *lac* operon, it encodes for LacI which represses the expression of RFP due to the mini-Tn$5_{A1\text{-}04/03}$::*rfp* being under the control of LacI [26]. The benefit of using this repressor is that non-*E. coli* cells that have acquired pAR060302:*rfp* fluoresce under blue light.

Animal experimental design. Our controlled animal experiments were conducted in isolation rooms at the University of Minnesota. Our experimental design involved four groups (Table 1). These groups included a negative control (group 1), a negative treatment control (group 2), and two treatment groups (groups 3-4). Each group used contained 5 clean-raised commercial pigs of 4 weeks of age that were purchased from a commercial vendor, verified prior to purchase to lack *E. coli* or *Salmonella* with resistance phenotypes typical of IncA/C plasmid carriage (ceftiofur, florfenicol, and kanamycin [CefFflKm]). Pigs were first housed (n=20) for one week with control feed to acclimate to their new environment. At week 5 of age, pigs were orally challenged with 10^8 CFU PFEC(pAR060302:*rfp*) (groups 2-4) or with PBS (group 1). After inoculation, groups 1-2 continued to receive control feed. Group 3 then received feed with 50 g/ton chlortetracycline (CTC) for the duration of the experiment (12 weeks of age). Group 4 then received 350 g/ton CTC for seven days, followed by treatment with 50 g/ton CTC for two weeks, then a second dose of 350 g/ton CTC for 7 days, then 50 g/ton CTC for the duration of the experiment. This represented multiple therapeutic doses of CTC followed by standard subtherapeutic treatment.

Table 1. Experimental design for controlled pig studies.

Group	Pigs (n)	Challenge	Treatment	Room
1	5	None	None	1
2	5	PFEC(pAR060302)	None	2
3	5	PFEC(pAR060302)	CTC[C] subtherapeutic	3
4	5	PFEC(pAR060302)	CTC 2 therapeutic treatments	4

[C]CTC = chlortetracycline

Bacterial isolation and analysis. Rectal fecal collections were performed at days 3, 7, 14, 21, 28, and 35 post-inoculation. Feces from each animal were suspended in phosphate-buffered saline (PBS) and serial dilutions were performed. These dilutions were then plated on MacConkey agar to enumerate suspect *E. coli* bacteria. Our goal was to analyze the proportions of total *E. coli* in each group that harbored pAR060302. To achieve this goal, we compared the *E. coli* isolated on the following media: 1) media lacking antibiotics (for total *E. coli* counts), 2) media containing CefFflKm (for *E. coli* carrying pAR060302:*rfp*), 3) and media with CefFflKmNal (Nal = nalidixic acid, selecting for the donor strain PFEC carrying pAR060302:*rfp*). This yielded the recoverable PFEC(pAR060302:*rfp*), the proportion of transconjugant *E. coli* carrying pAR060302:*rfp*, and the proportions of these two populations relative to total *E. coli*. To futher characterize transconjugants (non-donor *E. coli*) that acquired pAR060302:*rfp in vivo*, *E. coli* phylogenetic typing was used to assess the clonal diversity following the interpretive approach described by Clermont et al. [27].

Some of the fecal samples collected above were also cultured on LB agar and Tryptic Soy Agar. These media were supplemented with CefFflKm. We then analyzed the plates under blue light to identify non-*E. coli* that acquired pAR060302:*rfp* since these bacteria will fluoresce. The proportions of transconjugants relative to total cultured microbiota were determined. A *t*-test of proportions was used to determine statistical differences between all groups examined.

Objective 2. Identify reservoirs for the IncA/C plasmid in the pig gastrointestinal tract and in manure. The circulation of IncA/C plasmids among swine-source *E. coli* and *Salmonella* spp. is well documented, but

the possible commensal bacterial reservoirs of this plasmid in the pig and its environment are not known. We hypothesized that non-*E. coli* commensal bacterial reservoirs sources exist for pAR060302 in the pig gastrointestinal tract and in swine manure, which would also lend credence to the concept that antimicrobial use (or the absence thereof) does not materially influence the persistence of these plasmids. In this objective, we performed conjugation experiments using pig feces and swine-source liquid manure to examine the potential of pAR060302:*rfp* to be acquired by such commensal bacteria. PFEC(pAR060302:*rfp*) was mixed with these samples and allowed to conjugate on R2A media, as previously described [28]. This media was used for the conjugation and culturing of total bacterial populations from swine fecal and liquid manure samples, and subsequently used to identify the gut microbial populations that have acquired pAR060302:*rfp*. We also performed in vitro experiments to assess the fitness cost of various *E. coli* and *Salmonella* strains for carrying IncA/C plasmids. Fitness cost experiments followed the protocols described in De Gelder et al [29].

Results.

Therapuetic chlortetracycline administration, but not subtherapeutic administration, selected for plasmid-containing *E. coli*. Fecal contents from 20 pigs were assessed at 6 time points for the proportion of IncA/C plasmid-containing *E. coli* following inoculation with a pig fecal *E. coli* strain harboring the MDR-encoding IncA/C plasmid, pAR060302 (Figure 1). At day 3 post-inoculation, no significant differences were found in the proportions of plasmid-containing E. coli relative to total *E. coli* counts in feces in either of the treatment groups. However, at all subsequent time points, group #4 (therapeutic-subtherapeutic pulse treatment group) had significantly higher proportions of IncA/C plasmid-containing *E. coli* compare to the non-treated inoculation groups ($P < 0.05$ using Student's t test). Treatment group #3 (subtherapeutic tetracycline administration in feed throughout experiment) never had significantly higher proportions of IncA/C plasmid-containing *E. coli* than those of the control group.

Tetracycline-resistant *E. coli* were indigenous to pigs. We also assessed the proportion of tetracycline-resistant *E. coli* at days 3, 7, and 21 of the experiment (Figure 2). At all time points examined, the proportion of

tetracycline-resistant *E. coli* approached the total *E. coli* counts and these counts did not differ significantly, indicating that pigs were colonized with tetracycline-resistant *E. coli* prior to inoculations and these proportions were maintained post-inoculation.

IncA/C plasmid transfer between *E. coli* occurred under therapeutic tetracycline treatment. At days 3, 7, 21, and 35 post-inoculation, we selected 96 *E. coli* colonies from each experimental group and time point (1,536 colonies total) that displayed resistance to kanamycin, ceftiofur, and florfenicol, indicating possession of an IncA/C plasmid. These colonies were replica plated to LB agar containing nalidixic acid to differentiate between the donor inoculum strain and possible recipients that occurred through conjugation in the pig gastrointestinal tract. At day 3 post-inoculation, no colonies were identified that were not donor inoculum (nalidixic acid resistant). However, at days 7, 21, and 35, colonies were identified from group #4 that were apparent transconjugants (Table 2). No colonies were identified from group #3 at any time point that were apparent transconjugants. We further characterized putative transconjugant *E. coli* from group #4 for *E. coli* phylogenetic type (Table 2). From this analysis, transconjugants were identified from all four phylogenetic groups. These isolates were further examined to confirm possession of an IncA/C plasmid using a plasmid-specific PCR for the IncA/C replication initiation gene [30]. All isolates examined were IncA/C positive using this approach.

Table 2. Summary of transconjugants analyzed from pig inoculation experiments.

				Percent transconjugants belonging to:			
Days post-inoculation	Experimental group	Colonies examined	Percent transconjugant colonies	Phylotype A	Phylotype B1	Phylotype B2	Phylotype D
3	2	96	0.0%	N/A	N/A	N/A	N/A
3	3	96	0.0%	N/A	N/A	N/A	N/A
3	4	96	0.0%	N/A	N/A	N/A	N/A
7	2	96	0.0%	N/A	N/A	N/A	N/A
7	3	96	0.0%	N/A	N/A	N/A	N/A
7	4	96	24.0%	52.20%	34.80%	4.40%	8.60%
21	2	96	0.0%	N/A	N/A	N/A	N/A
21	3	96	0.0%	N/A	N/A	N/A	N/A
21	4	96	29.2%	46.40%	35.70%	10.70%	7.20%

35	2	96	0.0%	N/A	N/A	N/A	N/A
35	3	96	0.0%	N/A	N/A	N/A	N/A
35	4	96	32.3%	41.90%	25.80%	12.90%	19.40%

IncA/C plasmid transfer did not occur outside of *E. coli*. At each timepoint, we also examined colonies on LB agar and TSA agar containing kanamycin, ceftiofur, and florfenicol for potential non-*E. coli* transconjugants that had acquired the inoculated IncA/C plasmid. Colonies were present on both types of media at all timepoints. However, none of these colonies fluoresced, indication that the IncA/C plasmid had not transferred outside of *E. coli*. We selected some of the non-fluorescing colonies and restreaked them on MacConkey agar, and this confirmed that colonies growing on these media were likely *E. coli*.

Figure 1. Proportions of IncA/C plasmid-containing *E. coli* in pig feces over 35 days post-inoculation. A "*" indicates a proportion of plasmid-containing *E. coli* significantly higher than non-treated control group #2 ($P < 0.05$).

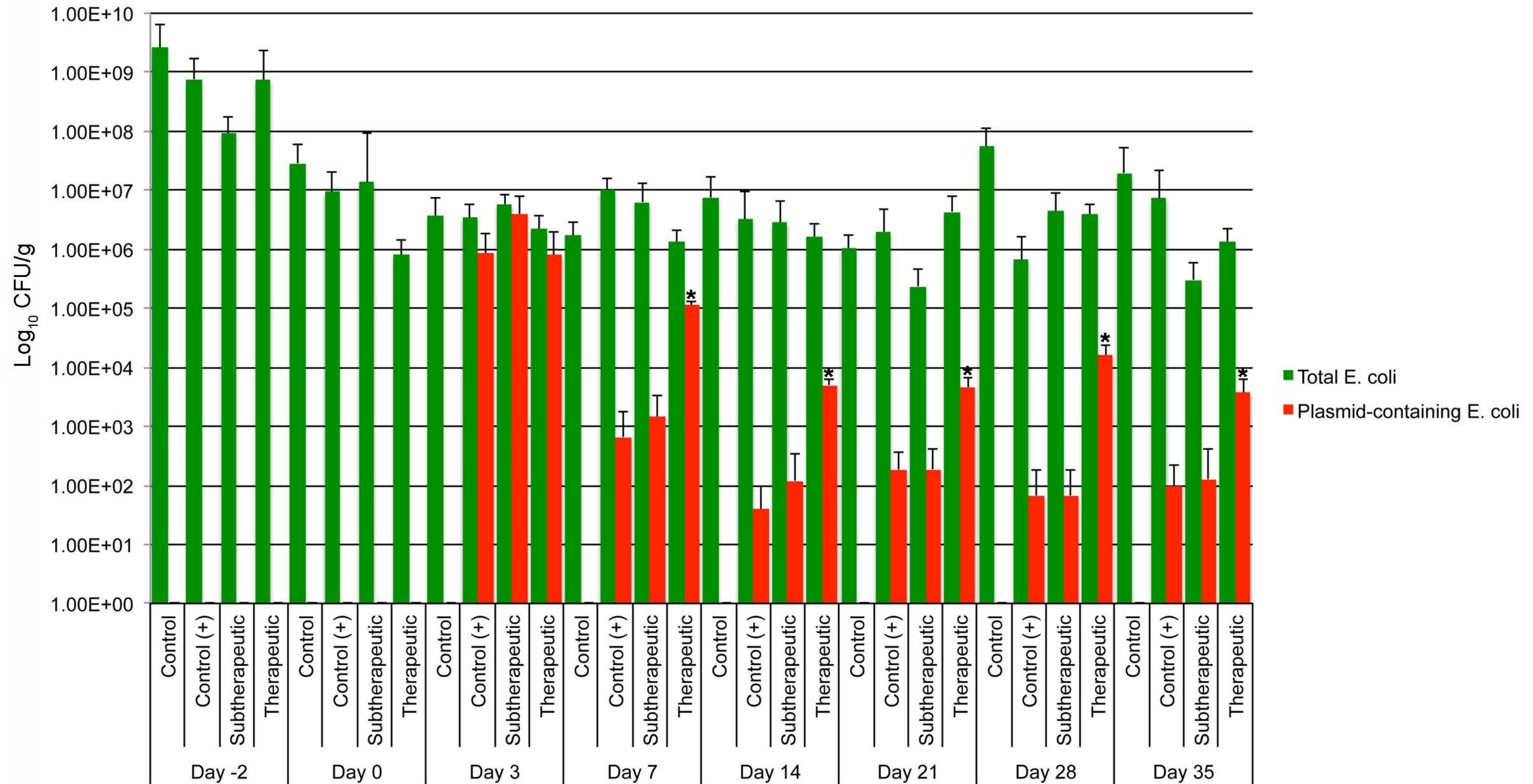
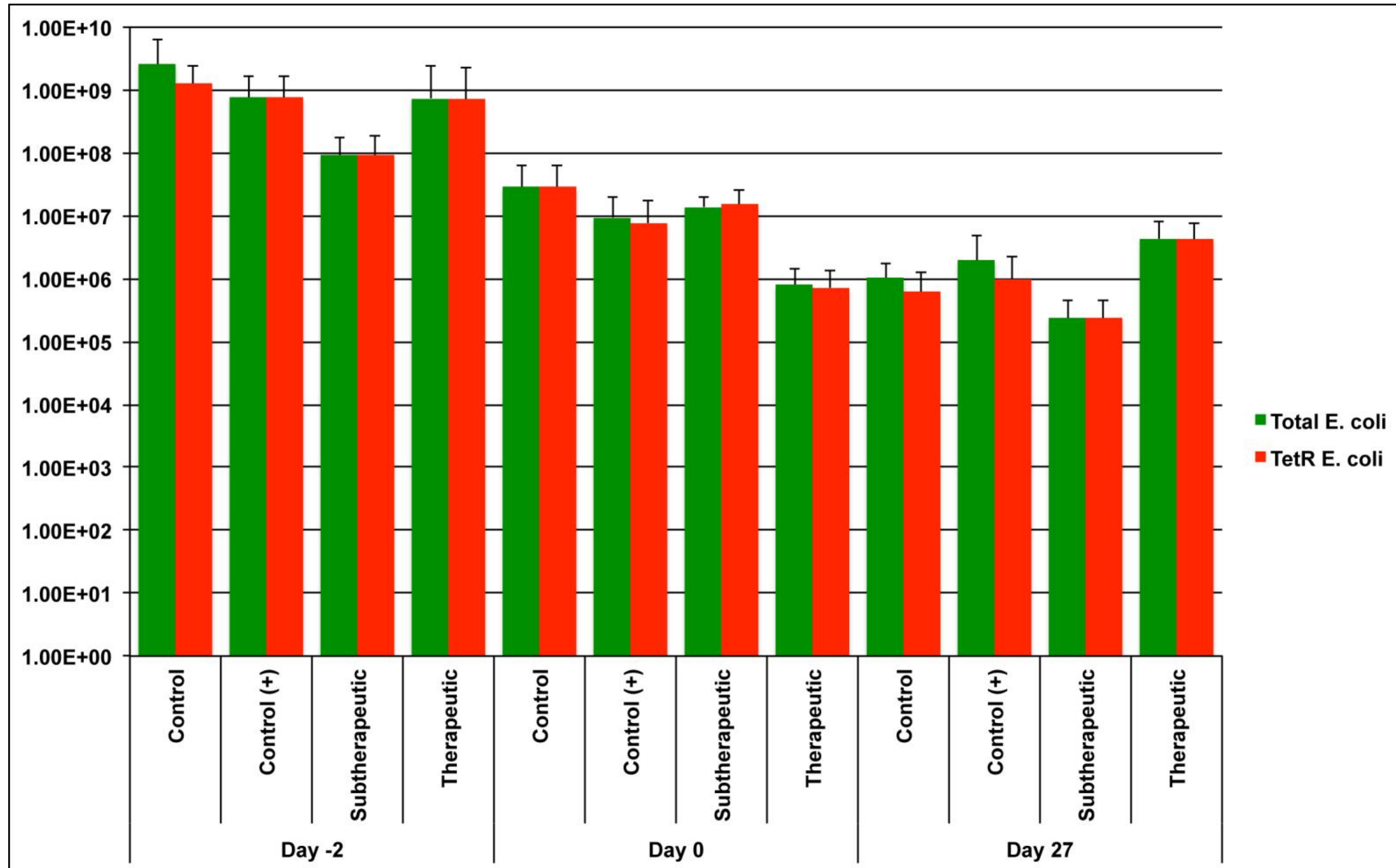


Figure 2. Proportions of tetracycline-resistant *E. coli* in pig feces at selected time points. At no time points were viable counts of tetracycline-resistant *E. coli* significantly different than total *E. coli* counts.



IncA/C plasmid transfer to indigenous flora did not occur in liquid manure or soil. We used microcosm experiments in an effort to better understand the host range of the IncA/C plasmid in non-*E. coli* hosts using liquid manure and soil samples. This approach was first optimized by spiking these samples with a known *E. coli* recipient, followed by inoculation with PFEC(pAR060302:*rfp*). The same approach was performed with a spiked recipient *Salmonella* strain to confirm that fluorescing transconjugant colonies could be identified using this approach. These approaches verified that conjugation to non-*E. coli* hosts is capable of being measured in these microcosm experiments. However, we were unable to identify any fluorescing transconjugants using the indigenous microflora of liquid manure and soil samples, plated on R2A agar and TSA agar. We also attempted to mate PFEC(pAR060302:rfp) in vitro with several recipient bacterial hosts, including *Aeromonas hydrophila*, *Geobacter sulfurreducens*, and *Shewanella oneidensis*. None of these matings produced successful transconjugants.

IncA/C plasmid fitness cost varies within, and between, *E. coli* and *Salmonella*. Fourteen *E. coli* strains and 21 *Salmonella* strains were examined for the cost of IncA/C plasmid carriage using fitness cost calculations obtained via competition experiments. For each competition, the wild type bacterial strain was competed with its IncA/C plasmid-containing derivative. For comparison purposes, we also included comparisons of the cost of carriage of an IncI1 plasmid, and the cost of carriage for both plasmids together (Figure 3). In *E. coli*, a distinct pattern was observed were strains belonging to *E. coli* phylogenetic group A actually benefited from carriage of any plasmid combination, whereas other phylogenetic groups (B1, B2, and D) generally had cost associated with plasmid carriage. Notably, in several strains, the fitness cost for carriage of both IncA/C and IncI1 plasmids together was less than the cost for carriage of either plasmid

alone. In general, the cost of IncA/C plasmid carriage was greater than the cost of IncI1 plasmid carriage. In *Salmonella* strains, we also observed differential costs for carriage of the IncA/C plasmid that were related to serovar (Figure 4). Specifically, *S*. Enteritidis had the highest cost for IncA/C plasmid carriage, while *S*. Typhimurium had the lowest cost for IncA/C plasmid carriage.

Figure 3. Relative fitness cost of plasmid carriage on various *E. coli* recipients. A positive value indicates a cost for plasmid carriage, whereas a negative value indicates a benefit for plasmid carriage. pAC = IncA/C plasmid, pAR060302; pI1 = IncI1 plasmid, pCVM29188_101. Phylogenetic group of each isolate is displayed above the isolate designation.

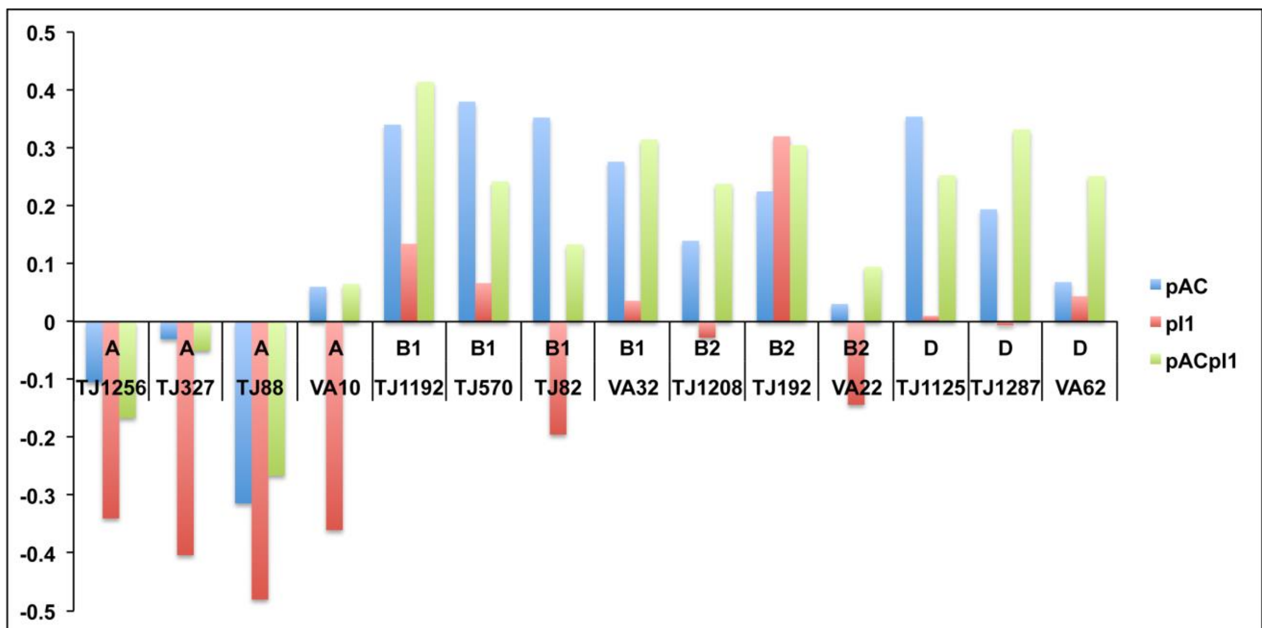
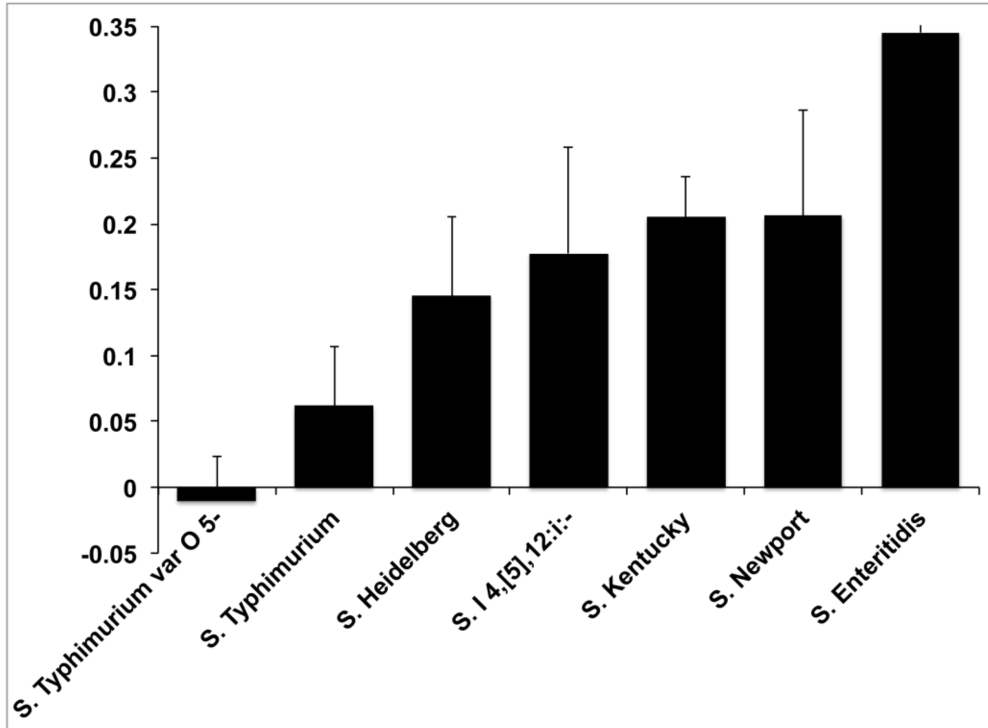
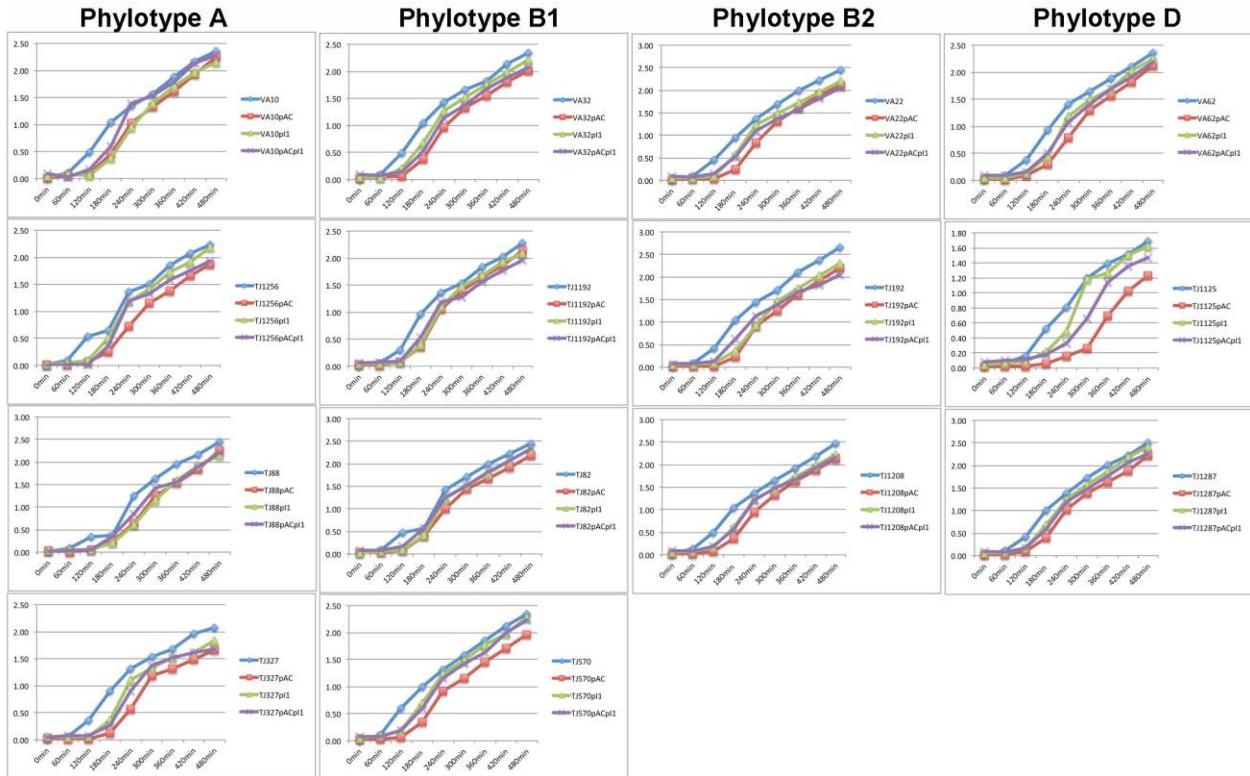


Figure 4. Relative fitness cost of IncA/C plasmid pAR060302 in various *Salmonella* hosts. Twenty-one *Salmonella* isolates were examined (3 from each serovar). A positive value indicates a cost for plasmid carriage, whereas a negative value indicates a benefit for plasmid carriage.



Because we observed that the combination of co-infecting IncA/C and IncI1 plasmids incurred a lesser fitness cost than some plasmids infected alone, we examined the growth curves of each *E. coli* strain with and without IncA/C and IncI1 plasmids (Figure 5). In all cases, the same patterns of growth were observed. That is, the wild type strain in each case grew the fastest in LB broth, and the IncA/C plasmid-containing strain grew the slowest. However, when both IncA/C and IncI1 plasmids were co-infected into the same strain, they always grew better than strains singly infected with an IncA/C plasmid.

Figure 5. Growth curves for IncA/C and IncI1 plasmid carriage in 14 different E. coli hosts. X-axis depicts time in minutes, Y-axis depicts A600 values at each timepoint. For each chart, the blue line depicts the wild type strain, the red line depicts the strain containing an IncA/C plasmid, the green line depicts the strain containing an IncI1 plasmid, and the purple line depicts the strain containing both plasmids.



Discussion.

The purpose of this study was to better understand the role of subtherapeutic and therapeutic tetracycline administration on IncA/C plasmid transfer in commercial pigs. IncA/C plasmids are important because they are a predominant plasmid type among porcine *Escherichia coli* and *Salmonella*, they harbor remarkable combinations of drug resistance genes, they have recently emerged in human and animal pathogens, and they have an apparently broad host range through self-conjugation. We sought to determine if two different types of chlortetracycline treatment in pig feed had an impact of the prevalence and dissemination of an IncA/C plasmid. Several notable observations came from this study. First, subtherapeutic administration of chlortetracycline in feed at a concentration of 50 g/ton had no observable impact on the proportions of IncA/C plasmid-containing *E. coli* over the course of 35 days after inoculation with an IncA/C plasmid-containing commensal strain. In all inoculated groups, IncA/C plasmid-containing *E. coli* were found post-inoculation throughout the experiment, but the proportions found in subtherapeutic chlortetracycline-treated pigs never significantly exceeded that of the control groups. These proportions were also relatively low, ranging from approximately 0.01%-0.1% of total *E. coli* present in feces. In contrast, inclusion of therapeutic pulses of chlortetracycline at levels of 350 g/ton in feed significantly increased the proportion of IncA/C plasmid-containing *E. coli* at all time points post-inoculation.

It does not appear that increases in the proportions of IncA/C plasmid-containing *E. coli* were due to direct selection, since nearly all of the indigenous *E. coli* in the pigs were already resistant to tetracycline. Had this experiment been performed in pigs lacking tetracycline-resistant *E. coli*, the results may have been different. However, data from the Minnesota Veterinary Diagnostic

Laboratory indicates that *E. coli* isolates in pigs are almost always resistant to tetracycline (personal communication with Simone Oliveira), as does other work [31]. Therefore, this experiment likely reflects a more realistic scenario where tetracycline-resistant *E. coli* are already dominant in the pig gastrointestinal tract. It can be concluded, then, that subtherpeutic administration of chlortetracycline in pigs is not likely to exert selection or co-selection for IncA/C plasmids. Because there are many proteins of unknown function encoded on the IncA/C plasmid core backbone, we have speculated in the past that these core proteins may provide additional selective advantages for bacteria harboring IncA/C plasmids in the animal gastrointestinal tract. While this work cannot conclusively rule out that possibility, there was no selective advantage for IncA/C plasmid-containing strains in the face of subtherapeutic chlortetracycline administration.

Our data suggest that therapeutic administration of chlortetracycline resulted in increased movement of the IncA/C plasmid to other indigenous *E. coli* hosts. We were unable to observe any evidence of such plasmid movement in our inoculated control or subtherapeutic-treated groups. It is possible that plasmid transfer did occur within these groups but was below our limits of detection. Nevertheless, there was certainly evidence of plasmid transfer in the subtherapeutic-therapeutic pulsed treatment group. There is previous evidence for the enhancement of conjugal transfer upon exposure to tetracyclines [32]. Further work will be required to determine if chlortetracycline enhances IncA/C plasmid transfer, and at which concentrations it does so.

We hypothesized that other bacterial reservoirs exist for IncA/C plasmids in the gastrointestinal tract, in manure, and in soil. However, we were unable to detect any evidence of conjugal

transfer of the IncA/C plasmid between our donor strain and the indigenous flora of these samples. Because appropriate controls were included in each experiment, it is unlikely that these failures were due to an inability of the plasmid to transfer. The most likely explanation for our results is that *E. coli* is not an ideal donor for the IncA/C plasmid to other non-*E. coli* strains. Alternatively, it is possible that the sublineage of IncA/C plasmids to which pAR060302 belongs has adapted to *E. coli* and *Salmonella* and is no longer suitable for efficient transfer and/or stable replication in other bacterial taxa. Whatever the case, our hypothesis of promiscuity of this plasmid once infected into a microbial community such as the gut is likely not true. Instead, it appears that conjugal transfer may play less of a role in IncA/C plasmid dissemination, as compared to clonal expansion.

Another notable finding from this study was that bacterial host background substantially impacts the cost of plasmid carriage in *E. coli* and *Salmonella*. There was a distinct pattern in *E. coli*, using both IncA/C and IncI1 plasmid types, where strains belonging to phylogenetic group A were better suited to carry plasmids than other *E. coli* types. It has previously been shown that *E. coli* belonging to phylogenetic group A are more amenable to acquiring drug resistance genes and phenotypes than other phylogenetic groups [33]. The PFEC strain used in this study was also a phylogenetic group A strain. However, the mechanisms enabling *E. coli* belonging to phylogenetic group A to be more amenable to plasmid and resistance gene acquisition are currently unknown. Also, few studies have examined the effects of plasmid acquisition on multiple strains belonging to the different *E. coli* phylogenetic groups [34]. Therefore, this study validates previous work in the context of IncA/C plasmids using multiple strains from each *E. coli* phylogenetic group.

We also found that, in many of the strains studied, co-infection with two plasmids did not confer an additive fitness cost effect on the host. In fact, many strains actually benefited from carrying two plasmids versus one single plasmid. This form of epistasis has been previously described for plasmid carriage [35,36]. Given that most porcine-source *E. coli* isolates appear to harbor multiple plasmids [30], it suggests that multiple plasmid carriage compared to single plasmid carriage may be a beneficial trait. Again, the mechanisms driving positive epistasis related to co-infecting plasmids are unknown, but certainly deserve greater attention.

In summary, this work resulted in several interesting findings that extend our understanding of the mechanistic nature of IncA/C plasmid-encoded multidrug resistance in *E. coli*. First, we found no evidence of IncA/C plasmid selection in pig feces in the face of subtherapeutic use of chlortetracycline, which is among the most widely used growth promoters in commercial pigs. We cannot conclude from this work that extended use of growth promoters, combined with other management practices, does not result in the selection or co-selection of resistance-encoding plasmids. However, we can conclude that, in pure form, the use of a subtherapeutic level of chlortetracycline in pig feed does not result in the selection of IncA/C plasmid-containing *E. coli* in short-term experiments. While a great deal of literature has focused on the selection of drug resistant bacteria in animal agriculture, surprisingly few experiments have been undertaken that aim to dissect the impact of different drugs, different concentrations, different lengths of application, and the effects of such on the selection of different types of genetic elements. More work of this nature is greatly needed to better understand the mechanistic aspects of selection of plasmid-encoded multidrug resistance in commensal bacteria of production animals.

Secondly, we can conclude from this study that IncA/C plasmid host range may indeed be predicted to be broad [1], but such predictions do not take into account the required event of bacterial conjugation for plasmid spread to occur. We believe that IncA/C plasmids are indeed broad-host-range plasmids, and the existing literature supports this notion [14,37-39]. However, the results of this project suggest that actual IncA/C plasmid transfer between bacterial hosts of differing species may be quite rare. This differs from other plasmid types, such as IncP plasmids [24,29], and it has important implications regarding the basic biology of these plasmids and their dissemination.

Lastly, even within a bacterial species such as *E. coli* or *S. enterica*, the impact of plasmid carriage is very complex and cannot be generalized to a single plasmid or single bacterial strain. It appears that IncA/C plasmids are better suited for some *E. coli* and *Salmonella* hosts than others, and this is reflected in the clinical literature where these plasmids are more prevalent among some serovars of *Salmonella* than others [40-45]. Also, multiple plasmid carriage appears to be beneficial for *E. coli* and *Salmonella* harboring IncA/C plasmids. This form of positive epistasis may be driving the evolution of porcine-source bacterial populations.

Acknowledgements.

The authors wish to extend their heartfelt gratitude to the National Pork Board for supporting this study. We also thank Drs. Richard Isaacson and Randy Singer at the University of Minnesota for their enthusiastic and helpful discussions related to the study results.

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