RESEARCH REPORT
PUBLIC HEALTH/WORKER SAFETY

Title: Antibiotic resistance gene diversity and mobility in bacterial communities from post-weaning swine fed antibiotics or alternatives **(NPB #18-184)**

Investigator: Heather K. Allen (Crystal Loving)

Institution: USDA-ARS-National Animal Disease Center

Date Submitted: 9/3/2020

Industry Summary:
Antibiotic-resistant bacterial infections are a growing health crisis, which has prompted calls for decreased antibiotic use in human medicine and animal agriculture. With increased restrictions on antibiotics in animal agriculture, alternatives products are utilized. Heavy metals, including zinc and copper, can have antimicrobial properties at high enough concentrations and are used as one alternative product. However, the impacts of dietary zinc or copper on antibiotic resistance genes (ARGs) abundance and transmission are not completely understood. The proposed research project identified and compared the ARG carriage and dissemination potential in one of the groups of swine gut bacteria most relevant to public health, the Gram-negative bacteria (mostly *Enterobacteriaceae*; this group includes *Escherichia coli*), in groups of nursery-age pigs that were fed either antibiotics or dietary heavy metals (zinc and copper).
When the presence of ARGs in swine gut bacteria was examined, no statistically significant differences among treatment groups was observed. However, limitations in study design may be responsible for this result, as the sequencing data suggested that the *Enterobacteriaceae* sequenced may be an incomplete representation of the *Enterobacteriaceae* in the gut due to how the organisms were cultured. Nevertheless, *Enterobacteriaceae* present in swine carried an average of 9.2 ARGs. These ARGs encoded resistances to a wide range of antibiotics, which may have clinical implications. Thus, despite the dietary additive, a number of ARGs were detected in Enterobacteriaceae cultured from pig fecal samples. Bacterial plasmids, as opposed to the chromosome, encode ARGs with other mobile genetic elements. In the Enterobacteriaceae cultured from pigs, certain ARGs were associated with specific types of plasmids. One of these plasmid types, IncI1, is a narrow host-range plasmid of *Escherichia coli* and *Salmonella enterica* species. Thus, carriage of ARGs on IncI1 can readily transfer to organisms associated with disease in humans or animals.
The relative abundance of ARGS in feces tested were not impacted by the addition of therapeutic zinc oxide and copper chloride or chlortetracycline when compared to ARG abundance in feces of pigs on unamended diet. The qPCR targets examined included genes which encoded

resistance to different classes of antibiotics, the beta-lactams (*blaCMY-2 and blaTEM)*, tetracyclines (*tet32*, *tetA*, and *tetW*), aminoglycosides (*aph2'-id*) as well as the IncF plasmid replicon. Again, ARGS were detected in fecal DNA of all the pigs in the trial, and there was a wide range in the abundance of ARGs within a treatment group.
Collectively, swine gut bacteria encoded a number of different ARG in *Enterobacteriaceae,* and the abundance of the ARGs in swine fecal bacteria varies widely between pigs. Methodology used in the study limited the interpretation of the impact of dietary heavy metals on ARG mobility, and a larger number of samples may be required to fully appreciate the impact of heavy metals on the abundance of ARGs given the range in abundance in pigs in the same treatment group.

Crystal Loving, PhD
Research Microbiologist
USDA-ARS-National Animal Disease Center
Crystal.Loving@usda.gov

Key Findings:
Enterobacteriaceae cultured from swine feces carried an average of 9 antimicrobial resistance genes, including tetracycline (*tetA*, *tetB*, *mdfA*), doxycycline (*tetA*, *tetB*), minocycline (*tetB*), streptomycin (*aph*(3')-Ib and *aph*(6)-Id), erythromycin (*mdfA*), fluoroquinolones (*mdfA*), amoxicillin (*blaCMY-2*, *blaTEM-1b*), ampicillin (*blaCMY-2*, *blaTEM-1b*), cefoxitin (*blaCMY-2*), ceftazidime (*blaCMY-2*), piperacillin (*blaCMY-2*, *blaTEM-1b*), ticaracillin (*blaCMY-2*, *blaTEM-1b*), cephalothin (*blaTEM-1b*), rifampin (*mdfA*), kanamycin (*mdfA*), chloroamphicol (*mdfA*), and ciprofloxacin (*mdfA*).

IncFIB replicons were the most prevalent plasmid replicon type detected, but there was little conservation in antibiotic resistance gene patterns amongst this plasmid type.

There were no significant differences AMR gene abundance across dietary treatment groups, which included *aph2'-id, blaCMY-2*, *blaTEM*, IncF, *tetA*, *tet32*, *tetW*.

Keywords: antibiotic resistance, dietary zinc, gene diversity, antimicrobial, plasmid, post-weaning.

Scientific Abstract:

Antibiotic-resistant bacterial infections are a global health crisis, which has resulted in calls for improved antibiotic prudency both in human medicine and animal agriculture. One way to improve antibiotic prudency in agriculture is through the use of alternatives to antibiotics to improve animal health and decrease the need for antibiotic use pigs. In addition to testing the efficacy of non-antibiotic feed additives for improving animal health, evaluating the impacts of these additives on antibiotic resistance gene diversity in the gut bacteria is essential to defining whether the non-antibiotic additive is effectively mitigating the antibiotic resistance problem. Heavy metals, including therapeutic levels of zinc and copper, have emerged in the US as one alternative to antibiotics, though the impact on antibiotic resistance is not well defined. To

examine the impact of therapeutic heavy metals on gut bacteria and antibiotic resistance genes, samples collected from two different animal trials experiments were assessed. For the first experiment, pigs fed an unamended diet, amended diet with chlortetracycline, or amended diet with zinc oxide and copper chloride. For the second experiment, pigs were fed an unamended diet, amended diet with chlortetracycline, or amended diet with zinc oxide. On day 14 (experiment 1) and day 23 (experiment 2), rectal swabs were performed for feces collection. Swabs were cultured in MacConkey media to enrich for *Enterobacteriaceae* and DNA extracted from bacteria. The DNA was sequenced using both short (n=54) and long (n=21) read technology to assess antimicrobial resistance and mobility gene profiles. No treatment effect was observed in the sequenced material, but the IncFIB, IncI1, and Col(MG828) plasmid replicons were present in a majority of samples (83%, 64%, and 60%, respectively). Antibiotic resistance genes present in a majority of samples included *tetB* (85%), *aph(3')-Ib* (74%), *mdfA* (74%), *aph(6)-Id* (72%), *blaCMY-2* (70%), *tetA* (64%), and *blaTEM-1B* (53%). The *blaCMY-2* gene co-occurred with the IncI1 plasmid replicon, which suggests this plasmid type may be of special interest when assessing the potential for resistance to beta-lactams. Additionally, DNA was extracted directly from feces to evaluate gene abundance by qPCR, and no differences were detected in gene abundance among treatment groups for the genes examined. However, these genes represent a small selection of genes responsible for antibiotic resistance and antimicrobial resistance gene transfer. Further work should examine different culturing techniques and *Escherichia* retrieval, as zinc may reduce *Escherichia coli* strain diversity.

Introduction:

Antibiotic-resistant bacterial infections are a growing global health crisis, which has resulted in calls for improved antibiotic prudency both in human medicine and animal agriculture. One way to improve antibiotic prudency in agriculture is through the use of alternatives to antibiotics to improve animal health and decrease the need for antibiotic use. The various alternatives to antibiotics are expected to have different impact on antibiotic resistance genes because of unlike modes of action. Co-selection of antibiotic resistance genes by other agents, such as heavy metals, is likely a contributing factor to antibiotic resistance gene dissemination and persistence [1]. The effects of in-feed heavy metal use on the development, dissemination, and spread of antimicrobial resistance (AMR) in commensal and zoonotic bacterial agents is largely unknown. There is a risk that co-selection of AMR genes by heavy metals can contribute significantly to AMR gene dissemination and persistence [1]. Particularly as some heavy metals, including copper and mercury, have been shown to select AMR genes [2-4]. The impact of therapeutic zinc in AMR development and spread in zoonotic organisms is less clear, but zinc has been shown to select for multi-drug resistant *Enterobacteriaeceae* [5-7] and methicillin-resistant *Staphylococcus aureus* (MRSA) [8, 9].

The European Medicines Agency recently determined that the risk associated with in-feed zinc administration and subsequent selection for antibiotic resistance is greater than the benefits that zinc confers to swine health [10], suggesting that the use of zinc in the European Union might be limited in the near future. However, in the United States, there is currently a lack of reliable data to evaluate the effects of in-feed use of zinc on AMR in nursery pigs. Currently, over 80% of nursery-aged swine are administered at least one antibiotic [11]. A shift towards heavy metals as an alternative to antibiotics may see widespread application of such products in compensation for antibiotic removal. As a result, validation of heavy metals for the nursery phase of swine production and the potential impact its use may have on public health is urgently needed.

Information of this kind will also be required to adequately inform policy generation surrounding the use of in-feed application of heavy metals like zinc in the swine production sector in the U.S.

Historically, the detection of AMR has relied on large scale surveillance studies that culture specific groups of bacteria and measure their resistance to antibiotics in culture. This strategy targets, at most, a few indicator organisms pertinent to either human or animal health, neglecting the vast majority of bacterial species present in complex ecosystems. In addition, it typically only screens for resistance against a handful of antimicrobials relevant to either human or veterinary medicine. High-throughput qPCR can increase the scalability of AMR gene screening at modest cost, and it has become a powerful way to broadly survey ARG in complex bacterial communities such as feces [12-15]. Use of this culture-independent tool has provided valuable insights into the existence and dynamism of ARG in bacterial communities from agroecosystems at large, not simply in pathogens, including how they are impacted by in-feed antibiotics [12, 14, 15]. However, culture-independent methods have a limited ability to assign resistance genes to specific bacteria and often fail to capture information in regards to how the genes that confer antibiotic resistance are moving.

ARG are often found on mobile genetic elements, such as plasmids, that can be transferred among bacteria. *Enterobacteriaceae*, such as *E. coli* and *Salmonella*, are particularly adept at sharing plasmids carrying antibiotic resistance genes [16, 17], facilitating a rise in antibiotic resistance. Consequently, characterizing and quantifying plasmids is an important factor in defining the risk for AMR spread because plasmids can dictate how broadly and stably the resistance gene can be shared among bacteria.

Resistance genes to certain heavy metals have been shown to co-occur on antibiotic resistant plasmids, which can facilitate the continued transfer of antibiotic resistance genes in the absence of antibiotic use. However, not all heavy metals have equal potential to co-select ARG. A recent study by Fang *et al.* (2016) identified co-selection of plasmids carrying both ARG and and resistance genes for heavy metals including copper, silver, arsenic and mercury, but notably not zinc [18]. Another study specifically looking at zinc supplementation found increased diversity of *E. coli* within the treated group without any increase in overall antibiotic resistance; however, their data suggested that the zinc-treated group may more frequently be carrying plasmids encoding resistance to multiple drugs, referred to as multi-drug resistant plasmids [6]. Data to specifically define and compare the antibiotic resistance gene carriage and dissemination potential in one of the groups of swine gut bacteria most relevant to public health, the Gram-negative bacteria (mostly *Enterobacteriaceae*; this group includes *Escherichia coli*), in groups of nursery-age pigs that were fed either antibiotics or alternatives, including dietary heavy metals (zinc and copper) can inform judicious use practices to limit AMR.

Objectives:

The objective of this study was to determine whether the use of heavy metals and other feed additives affects antibiotic resistance gene abundance and mobility within the microbiota of nursery-aged swine. This will be evaluated two ways: 1) qualitative DNA sequence and analysis of plasmids and 2) by high-throughput quantitative PCR (qPCR) of hundreds of antibiotic resistance genes.

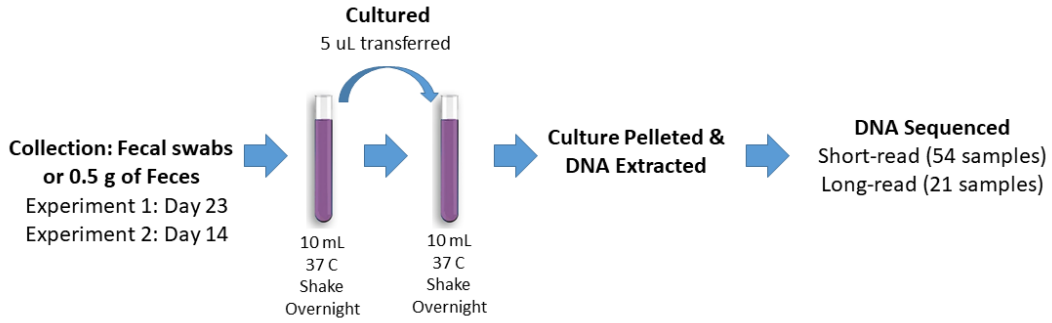
Materials & Methods:

Sample collection. The samples used in this study originate from two previously conducted animal trails; the first a nursery production feed trial that took place in a commercial-like setting, and the second, a nursery trial evaluating the improved feed efficiency of antibiotics compared to dietary zinc. For Experiment 1 three treatment groups were examined, (1) an unamended diet (negative control), (2) an unamended diet with chlortetracycline (50 g/ton) and tiamulin (35 g/ton) (positive control), and (3) an unamended diet with zinc oxide (2,000 mg/kg) and copper chloride (200 mg/kg). Fecal swabs or feces was collected on days 14 and 23 and used for this study. For Experiment 2, three treatment groups were examined, (1) an unamended diet (negative control), (2) an unameneded diet with chlortetracycline (40 ppm), and (3) an unamended diet with zinc oxide (3,000 ppm). Fecal swabs and/or feces was collected on day 14 and used for this experiment.

Part 1: Evaluate antibiotic resistance gene presence and mobility by qualitative DNA sequencing and plasmid analysis

Culturing and DNA extraction. In order to enrich for Enterobacteriaceae in fecal material, samples were subjected to culturing as follows. Experiment 1: Fecal samples (0.5 g) collected on day 23 were cultured in 10 mL of MacConkey broth overnight shaking at 37 °C. Experiment 2: Fecal swabs collected on day 14 were cultured in 10 mL of MacConkey broth overnight at 37 °C. From each of these starting cultures, 5 uL was used to inoculate fresh 10 mL of MacConkey broth and incubated at 37 °C shaking overnight. Bacteria in the final, second culture was pelleted and washed twice with phosphate-buffered saline (PBS) prior to DNA extraction. DNA from each culture was extracted using the Qiagen CompactPrep Plasmid Maxi kit kit. Figure 1 illustrates the overall workflow.

Figure 1: Experimental method for sequencing to select for *Enterobacteriacae*.



Sequencing. From Experiment 1 (n=13) and Experiment 2 (n=41), a total of 54 DNA samples were sequenced using an Illumina short-read sequencing platform (Table 1). Libraries were generated using NEB Ultra II library kits and the samples divided across two HiSeq3000 lanes to maximize sequence coverage.

A subset of samples were selected for long-read sequencing using an Oxford Nanopore MinION instrument. In total 21 DNA samples were selected (Table 1). Sequencing libraries were prepared with the rapid barcoding sequencing kit, SQK-RBK004 according to the manufacturer's instructions. Three sequencing libraries were run on FLO-MIN106 flow cells for 48 hours at a time. Sequencing data was basecalled and de-multiplexed with Guppy v. 3.1.5 [19].

Data analysis. HiSeq reads were trimmed and filtered with tools in the bbtools packages [20]. The resulting sequences were assembled with MegaHit, and contigs shorter than 1,000 bases were removed from the analysis. The genomic classification of each contig (Figure 2) was classified by the sendsketch.sh script from bbtools [20]. Resistance and replicon gene presence was determined by screening with the ResFinder and PlasmidFinder databases (Figures 3 and 4) [21-23]. Screening results with greater than 90% coverage and identity were further analyzed in RStudio using the tidyverse package (Figures 3 and 4) [24].

Long-read (Nanopore) sequences were filtered and quality reads (Q≥7) were assembled with Flye assembler [25, 26]. The 21 Flye assemblies were polished with the HiSeq data, using as many as 20 Pilon iterations [27]. Contig with less than 90% coverage of HiSeq data mapping were excluded from the analysis. Co-occurrences of antibiotic resistance and replicons on the contigs was determined by screening with the ResFinder, PlasmidFinder, and VirulenceFinder databases [21-23, 28]. Gene targets with greater than 90% identity and coverage were maintained. Data was visualized using the pheatmap package [29].

Part 2: Evaluate abundance of antibiotic resistance genes and mobile elements by quantitative (q) PCR

High-throughput qPCR. DNA from feces was extracted using the Qiagen MagAttract PowerSoil DNA kit and normalized to 5 ng/uL. All samples were screened in duplicate for 54 different antibiotic resistance, plasmid replicon, and insertion sequence targets using the Takara SmartChip Real-Time PCR System **(**previously WaferGen Bio-systems**)** at the Michigan State University Genomics Core Facility. Due to insurmountable technical difficulties via the high-throughput qPCR screening method, data obtained by this method was unable to reliably contribute to this study and was not included in this analysis. As the samples sent for analysis were finite, study alterations were made to survey genes of interest in-house via qPCR.

Quantitative PCR. To evaluated the difference in abundance of AMR genes between treatment groups, fecal DNA from day 14 of Experiment 1 was extracted (n=30; 10-NC, 10-CTC, and 10-ZnO+CuCl) using the QIAamp BiOstic Bacteremia DNA kit (Qiagen, Germantown, MD). qPCR targets examined to date included tetracycline (*tetA*, *tetW*, *tet32*), aminoglycoside (*aph2'-id*), beta-lactam (*blaCMY-2* and *blaTEM*) antibiotic resistance genes, as well as the IncF plasmid replicon. The *tetW*, *tet32*, and *aph2'-id* gene targets were chosen because they were previously found at a higher abundance in swine administered therapeutic oxytetracylcine in-feed for 7 days compared to a control group (Ricker *et al.*, in publication). The other targets listed were selected as they were prevalent within the sequencing data (Figures 3 & 4) and may hold treatment relevance for both human and veterinary medicine. Primer sequences are listed in Table 2

Table 1: Treatment groups and number of samples for each analysis platform used in this study.

Treatment	Treatment Name[a]	Samples evaluated via qPCR	Short-Read Sequencing Samples	Long-Read Sequencing Samples
Unamended Diet (Negative Control)	1-NC	10	6	3
CTC (Positive Control)	1-CTC	10	3	3
ZnO + CuCl	1-ZnO+CuCl	10	4	3
Unamended Diet (Negative control)	2-NC	0	10	3
CTC (Positive Control)	2-CTC	0	10	3
Zinc Oxide (3,000 ppm)	2-ZnO3	0	20[b]	6

[a]Number refers to Experiment

[b]Twenty-one zinc oxide (2-ZnO3) DNA samples were sequenced, but one was excluded due to poor sequencing depth.

Table 2. Primers used in this study.

Primer	Sequence (5'—3')	Reference
tet32-F	CCATTACTTCGGACAACGGTAGA	Ricker, In publication
tet32-R	CAATCTCTGTGAGGGCATTTAACA	Ricker, In publication
tetW-F	ATGAACATTCCCACCGTTATCTTT	Ricker, In publication
tetW-R	ATATCGGCGGAGAGCTTATCC	Ricker, In publication
aph(2')-Id-F	TGAGCAGTATCATAAGTTGAGTGAAAAG	Ricker, In publication
aph(2')-Id-R	GACAGAACAATCAATCTCTATGGAATG	Ricker, In publication
tetA-qfw	CCGCGCTTTGGGTCATT	[30, 31]
tetA-qrv	TGGTCGCGTCCCAGTGA	[30, 31]
blaTEM-F	TCCGCTCATGAGACAATAACC	[32]
blaTEM-R	TTGGTCTGACAGTTACCAATGC	[32]
blaCMY-F	AAAGCCTCATGGGTGCATAAA	[32]
blaCMY-R	ATAGCTTTTGTTTGCCAGCATCA	[32]
IncF-F	CACGGTATGTGGGARATGCC	[31, 33]
IncF-R	TCCGGCGGCAGYATVCCRAC	[31, 33]

qPCR assays were carried out using the iTaq Universal SYBR Green Supermix (Bio-Rad, Hercules, CA) and the QuantStudio 5 Real-Time PCR System (ThermoFisher Scientific, Waltham, MA) with a relative standard curve method used for quantification. The relative standard was generated using a series of six 10-fold dilutions of fecal bacterial DNA

(concentration ranging from 20ng/µl to $2x10^{-5}$ng/µl) prepared from a previous study that was tested positive for the presence of *tet32, tetW,* and *aph2* genes. For *IncF, blaCMY, blaTEM,* and *tetA-q* genes, the DNA relative standard was prepared using a series of six 10-fold dilutions (concentration ranging from 20ng/µl to $2x10^{-5}$ng/µl) from a pool of specific fecal DNA samples from day 14 of Experiment 1 where the genes were found prevalent based on sequencing data (Figures 3 & 4). All DNA samples were run in duplicate with reaction volumes of 20 µl according to manufacturer's recommendations, with 10 nM of each primer. Thermal cycling conditions for *tet32, tetW,* and *aph2*-specific qPCR assays consisted of: 95°C hold for 2 m, followed by 40 cycles of 95 °C for 15 s and 60.8 °C for 60 s/cycle. Thermal cycling conditions for *IncF, blaCMY, blaTEM,* and *tetA-q*-specific qPCR assays consisted of: 95 °C hold for 2m53s, followed by 40 cycles of 95°C for 34s and an annealing temperature of 56.4°C (*blaCMY*), 62.1°C (*tetA-q, IncF*), or 63.9°C (b*laTEM*) for 1m4s/cycle. Each specific target amplicon was verified by presence of a single melting temperature peak. Control reactions with no DNA template were run with each primer set to ensure the absence of non-specific primer dimers.

The detection limit of the qPCR assay was approximately 34 cycles for *tet32*, 31 cycles for *tetW*, 33 cycles for *aph2,* 30 cycles for *tetA-q*, 34 cycles for *IncF*, 31 cycles for *blaTEM*, and 27 cycles for *blaCMY* genes. Standard curve coefficient correlation was 0.99 for all seven genes, and average efficiencies of qPCR reactions were 100%, 95%, 106%, 100%, 88%, 74% , and 91% for *tet32*, *tetW*, *aph2, tetA-q*, *IncF, blaTEM,* and *blaCMY* standard curves, respectively. GraphPad Prism v. 8 (GraphPad Software, San Diego, CA) was used for data analysis. DNA concentrations were converted to log values using the formula C = log(X) + 5 – log(2), where X is the DNA concentration, and C is the assigned log value. The Kruskal-Wallis test followed by Dunn's multiple-comparisons test (for nonparametric data) was applied using the mean log values of the unknowns from each group.

Results:

Objective I:

When sequenced, the DNA extracted from the samples cultured in MacConkey broth to enrich for Gram-negative bacteria were primarily composed of genomic material identified as *Enterobacteriaceae*, a family encompassing the *Enterobacter*, *Escherichia*, *Salmonella*, and *Shigella* species. The single exception to this was a sample from a pig in the unamended diet group in study 2, which was composed of three different genera, *Enterobacter*, *Enterococcus*, and *Fusobacterium* (Figure 2). Except for the outlying sample, the assembled contigs were all primarily annotated as *Escherichia.*

When the *Escherichia*-majority assemblies were examined, the assembly sizes were smaller than expected (Figure 2, 4.7-7.06 Mb). As a single *Escherichia coli* genome can range from 4.5-5.5 Mb in size [34], we anticipated to see larger assembly sizes, which would reflective higher strain

diversity within the samples. The smaller than expected assemblies likely indicate that the *Escherichia* strain diversity *in vivo* for all samples, regardless of treatment, was originally low, or that, although the enrichment method was successful in promoting the culture of our targeted population of *Enterobacteriaceae*, a potential bias may have occurred towards promoting the growth of a few particular strains. However, the enrichment component remains a necessary step for the acquisition of *Enterobacteriaceae* sequencing data, if fecal DNA were to be sequenced directly only a small fraction of that data would likely have been associated with *Enterobacteriaceae*, the vast majority likely contaminating host DNA and DNA from commensal microbes. Although an enrichment step was required to examine our target population (*Enterobacteriaceae*) within the complex gut community, the potential for bias in strain composition upon re-culturing is an important consideration identified during this project and may need to be examined if the method is employed in future research.

Figure 2: Contigs identified as *Escherichia* species were enriched when samples were cultured as shown in Figure 1. Striations demarcate a different contig within the genomic data for each sample.

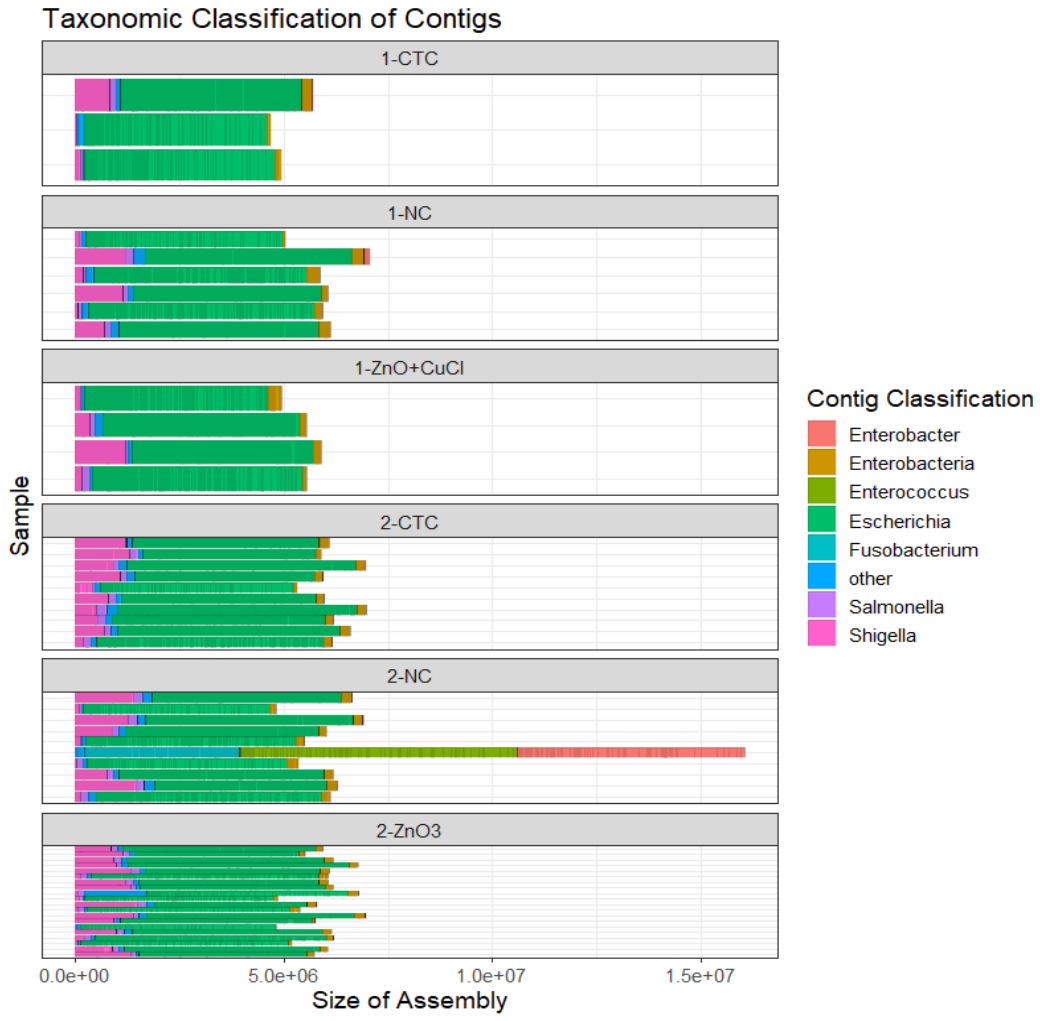
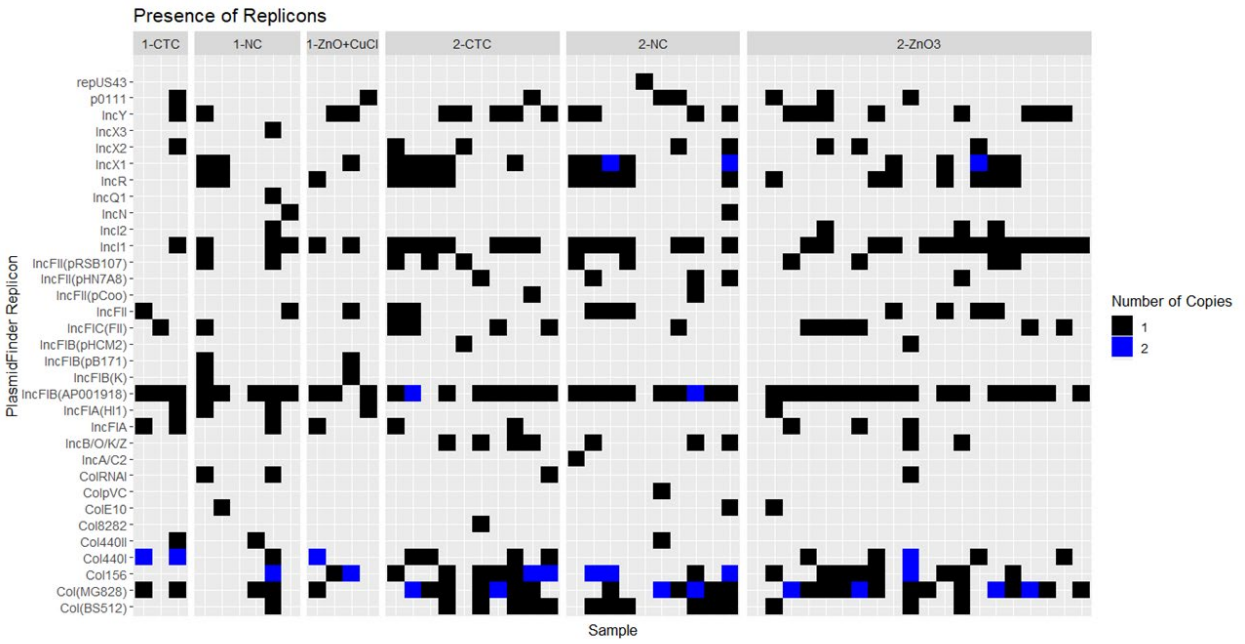


Figure 3: Presence of Antibiotic Resistance Genes identified through sequencing. When samples were cultured as described in Figure 1, no statistically significant differences were observed for antibiotic resistance genes (black) in the ResFinder database among treatment types for the positive controls (CTC), negative controls (NC), or treatment groups for experiment 1 or 2.

Detection of AMR genes via sequencing. A number of antibiotic resistance genes were detected within the sequenced data when screened with the ResFinder database. However, no statistically significant differences were detected between treatment groups in the number of different AMR genes detected between groups. The range of antibiotic resistance genes annotated per sample was zero to 22 genes with a mean of 9.2 genes per sample. Resistance genes present in more than half of the samples included *tetB* (85%), *aph(3')-Ib* (74%), *mdfA* (74%), *aph(6)-Id* (72%), *blaCMY-2* (70%), *tetA* (64%), and *blaTEM-1B* (53%). These genes alone encode resistances for large number of antibiotics, including tetracycline (*tetA*, *tetB*, *mdfA*), doxycycline (*tetA*, *tetB*), minocycline (*tetB*), streptomycin (*aph*(3')-Ib and *aph*(6)-Id), erythromycin (*mdfA*), fluoroquinolones (*mdfA*), amoxicillin (*blaCMY-2*, *blaTEM-1b*), ampicillin (*blaCMY-2*, *blaTEM-1b*), cefoxitin (*blaCMY-2*), ceftazidime (*blaCMY-2*), piperacillin (*blaCMY-2*, *blaTEM-1b*),

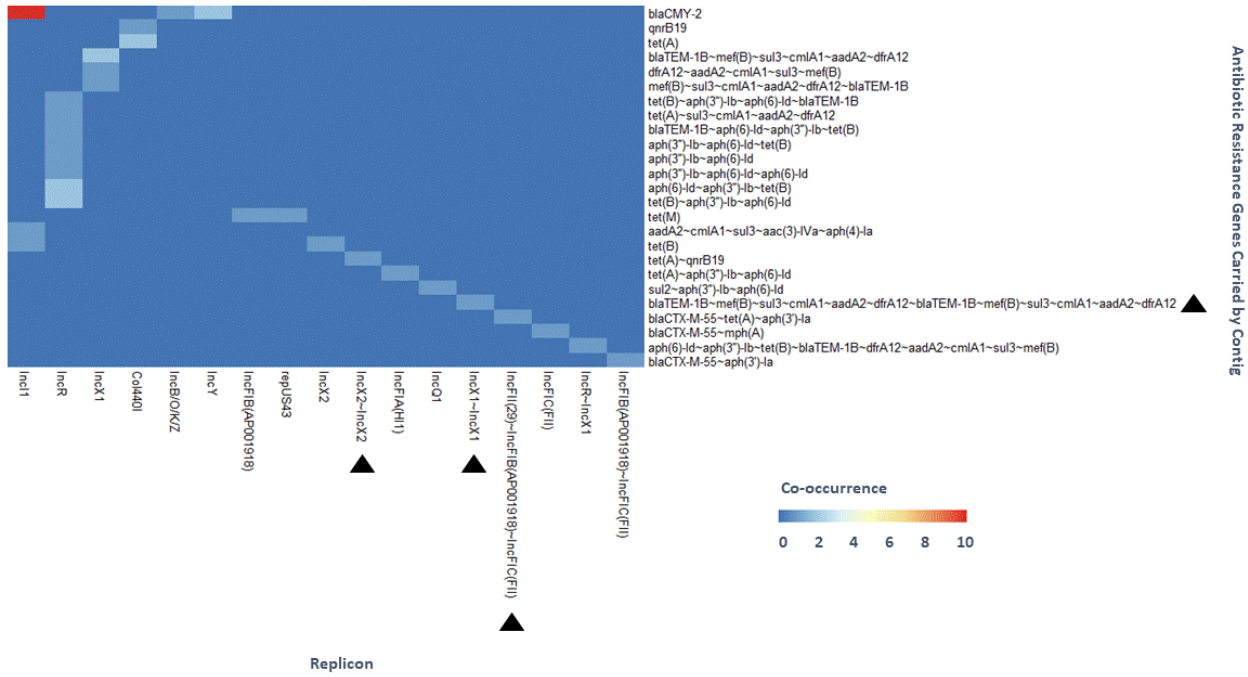
ticaracillin (*blaCMY-2*, *blaTEM-1b*), cephalothin (*blaTEM-1b*), rifampin (*mdfA*), kanamycin (*mdfA*), chloroamphicol (*mdfA*), and ciprofloxacin (*mdfA*).

Figure 4: Replicon Genes Present in the Samples. When samples were cultured as described in Figure 1, no observable differences occurred for replicons (black or blue) in the PlasmidFinder database among treatment types for the positive controls (CTC), negative controls (NC), or treatment groups for experiment 1 or 2.



Detection of plasmid replicon genes via sequencing. A large number of replicaon genes were detected when screening sequences through PlasmidFinder database. More specifically, 51 of the 53 samples (96%) contained at least one replicon gene (Figure 4). However, no statistically significant differences were detected between treatment groups. The number of different replicon genes detected in each sample ranged from zero to 13 with a mean of 6.2 genes. Replicon genes detected in more than half the samples included IncFIB(AP001918) (83%), IncI1 (64%), and Col(MG828) (60%). IncFIB plasmids are plasmids associated with *Gammaproteobacteria*, particularly *Enterobacteriaceae* [35], but IncI1 plasmids have a narrower host range, as they are associated primarily with *Salmonella enterica* and *Escherichia coli* [36]. Both IncFIB and IncI1 are plasmid replicons associated with the *E. coli* of poultry [37, 38]. The IncFIB replicon is associated with plasmids encoding virulence genes and iron acquisition and transport operons [38]. Certain replicons also occurred in two copies per assembly, including IncFIB (n=2) and Col(MG828) (n=8). Additional replicons which occurred twice in certain samples included Col(156) (n=8 strains), IncX1 (n=3), and Col440I (n=4).

Figure 5. Long-read sequencing (n=21, Table 1) was employed to detect the co-occurrence of plasmid replicon and antibiotic resistance genes. Contigs with identified replicons (x-axis) and antibiotic resistance genes (y-axis) suspected of potential misassembly are indicated with triangles.



To determine the genetic context of antibiotic resistance genes and replicons, the co-occurrence of these genes was examined among 21 of the cultured samples (Table 1). The IncI1 plasmid co-occured with the *blaCMY*-2 gene. This result concurs with other work from the human clinical setting where 96% of *bla*CMY-2 genes identified in *E. coli* were carried by IncI1 plasmids [39].

IncFIB replicons may have been the most prevalent replicon type (Figure 4), but there was little conservation in antibiotic resistance gene patterns amongst this plasmid type. Only 17% percent of plasmids with an IncFIB replicon carried at least one resistance gene. The most prevalent resistance gene combination for IncFIB plasmids was *bla*CTX-M55 and *aph*(3")-ia (7%). However, a majority of plasmids with an IncFIB contig carried virulence-associated genes (76%), most frequently encoding ToxB cytotoxin (38%) and hemolysin toxin (17%), aerobactin siderophore (10%), salmochelin receptor (7%), and EAST-1 toxin (7%).

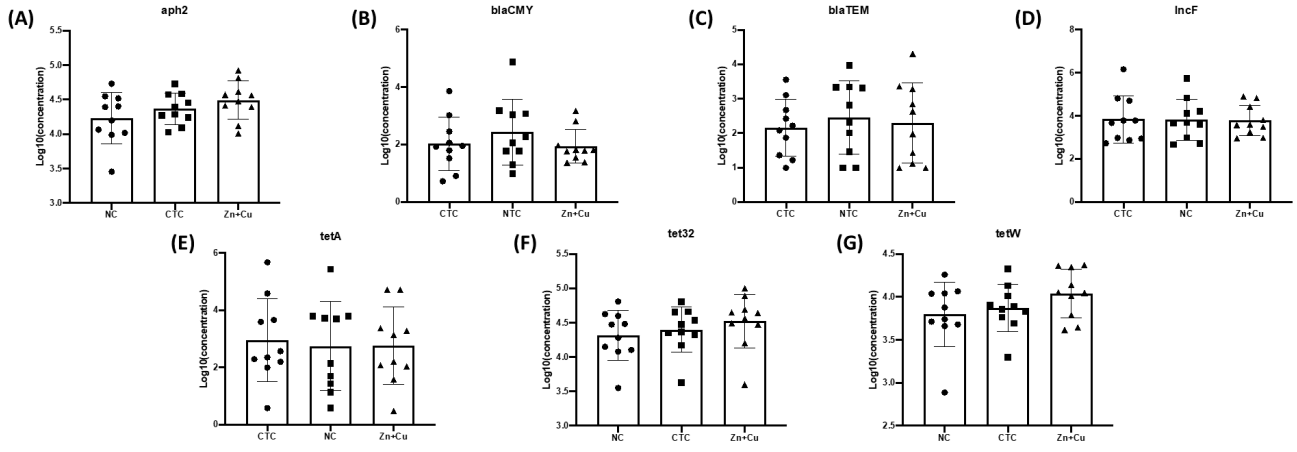
However, the co-occurrence data for antibiotic resistance and virulence genes is only as reliable as current sequencing and assembly techniques allow. Similarities between gene arrangements among plasmids as well as repeat genes present on both plasmids and within bacterial chromosomes were found to complicate assembly, and unlikely constructs were flagged during this analysis (Figure 5). Pairing short-read sequencing data with long-read sequencing data did not allow this study to circumvent this phenomenon, but the work highlights some of the major challenges researchers face when looking to fill in knowledge gaps surrounding mobile genetic

elements as it relates to antibiotic resistance. Based on our experience here, additional culture-based methods involving cloning, subsequent isolation, and sequencing of individual plasmids of interest may be required to obtain additional resolution of the plasmids.

Part II:

Abundance of AMR genes evaluated by qPCR. The intial intent was to use high-throughput qPCR methods (Wafergen) to assess AMR gene abundance in DNA from fecal samples, but technical errors and limited DNA quantity led to inability to obtain reliable data for analysis. To assess if there was an effect of treatment on AMR gene abundance, real-time PCR using a relative standard curve was instead performed. Genes were slected based on detection in sequencing, but was only a small subset of AMR genes. Fecal DNA, as opposed to DNA from cultured bacteria (Figure 1) were used in the analysis. There were no significant difference among treatment groups for the relative abundance of queried genes, which included *aph2'-id (A)*, *tetW (B)*, *tet32* (C), *tetA* (D), *blaCMY-2* (E), and the IncF replicon (F) (Figure 6). Although no treatment effect was observable, these genes are a small subset of those examined in the genomic data (Figures 3 and 4).

Figure 6: Relative abundance of AMR genes in DNA isolated from feces of pigs in indicated treatment group. There were no significant differences in abundance of AMR genes queried, which included *aph2'-id (A)*, *blaCMY-2* (B), *blaTEM* (C), IncF (D), *tetA* (E), *tet32* (F), *tetW* (G). Treatment groups include diets that were unamended (NC), amended with chlortetracycline (CTC), or zinc oxide and copper chloride (Zn+Cu). qPCR was performed on fecal DNA samples from experiment 1 only (Table 1).



Discussion:

This study demonstrated that culturing in MacConkey broth could be used to successfully select for members of the *Enterobacteriaceae* family from swine feces for the majority of samples (Figure 2). However, the same selection pressure and our culturing techniques likely biased the sequencing outcome and limited the data available for analysis. Indeed, recent work by Peto *et al*. demonstrated that after enrichment in metronidazole and vancomycin media, a resistant subpopulation of *Enterobacteriaceae* spiked into fecal samples could not be detected. In addition, the study found relatively small differences in growth rates could produce different results, as culturing for six hours could lead to a 12-fold difference in abundance of their spiked in sample [40]. These results may partially explain the outcome of this study, as the amount of sequencing data for each MacConkey culture was roughly the size of a single genome for an *E. coli* or *Salmonella* species (Figure 2). Furthermore, this study likely amplified the problem presented by Peto *et al.*, as samples were cultured twice in MacConkey overnight, with a minimal amount of broth (5 uL) spiked into the second culture (Figure 1). Therefore, the culturing technique used may have promoted the fastest growing organism or the fastest growing organism of a stochastic selection due to the transfer of only 5 uL. Becerra-Castro *et al.* (2015) found that at concentrations greater than 1 mM, zinc produces a strong inhibitory effect on the culturability of *Enterobacteriacae*, and in a non-selective culture media, zinc or copper select for mono-species populations [5]. However, this study found that the *Escherichia* diversity was low regardless of treatment, as indicated by assembly size (Figure 2). Clearly, further research is needed to develop techniques to limit bias and to answer questions into plasmid content and antimicrobial resistance in subpopulations of the gut. Although we do not have sequencing results that can tell us about the potential global *Enterobacteriaceae* community in each sample, the sequenced results can provide a small window of insight into *Enterobacteriaceae* of the swine feces.

Long-read sequencing data revealed co-occurrences between plasmid replicons and antibiotic resistance and certain virulence genes. Of particular interest is the association between IncI1 and *blaCMY-2*, an antibiotic resistance gene present in 70% of samples that encodes resistance to ampicillin and amoxicillin (Figure 2 & 4). A previous study has indicated that *blaCMY-2* is carried by 41.8% and 25.3% of *E. coli* strains implicated in post-weaning diarrhea (PWD) and neonatal diarrhea in production pigs, respectively [36]. Previous work has found plasmids with the IncI1 replicon to be associated with a number of resistance genes including *sul1*, *sul2*, *bla(CTX)*, *bla(SHV)*, *bla(CMY)*, and *bla(TEM)* genes [36, 39, 41-46], and our long-read sequencing data suggests that *blaCMY-2* co-occurs on IncI1 plasmids in swine most frequently. Although IncI1 plasmids may have clinical implications for the swine industry for PWD, the IncFIB replicon was the most prevalent replicon (Figure 3). The IncFIB replicon was not always associated with any particular resistance genes, but it was associated with certain virulence genes. 78% of contigs carrying an IncFIB replicon encoded at least one virulence gene; the most common gene associated with IncFIB plasmids was *toxB*, a gene required for the full O157:H7 enterohemorrhagic *E. coli* epithelial cell adherence phenotype [47]. Although the co-occurrence between the IncFIB replicon and *toxB* is not well-known, there are reports of IncFIB plasmids carrying the *toxB* gene [48, 49]. As IncFIB plasmids are well-known to carry bacteriocins [38, 50], the high prevalence of IncFIB plasmids in our sequencing data (83%) presents the possibility that these plasmids may have contributed a competitive advantage against other *Enterobacteriaceae* when cultured (Figure 1).

Limitations of the sequencing data of this study include not only the culturing and enrichment methodology (Figure 1) but also current genomic and metagenomics assembly technologies. To account for these limitations, when sequencing data was suspicious, this study attempted to indicate questionable data. For example, for two plasmids with the same incompatibility group, both are unlikely to be stably maintained [51]. Replication and stability are highly questionable when incompatibility genes of the same type (e.g. IncX1 and IncX1 or IncX2 and IncX2) are encoded by the same contig after quality control filtering of the data.

The DNA from swine feces allowed an analysis of the relative abundance of AMR genes to examine for any treatment effect. Several qPCR targets (*tetW*, *tet32*, and *aph2'-id*) were selected for analysis as they were previously found at a higher abundance in swine fed oxytet in-feed for 7 days formulated to 10 mg/lb of body weight daily compared to a control group (Ricker *et al.*, in publication). This study found no significant differences for these genes, but different in-feed treatment groups (chlortetracycline or zinc oxide and copper chloride) were used and different experimental time points were chosen. Similar to *tetW*, *tet32*, and *aph2'-id*, other qPCR targets chosen displayed no significance difference among the treatment groups.

With no observable treatment effect and few co-occurrences between replicon and antibiotic resistance genes found, many questions still remain unanswered. The complete antibiotic resistance profile of *Enterobacteriacae* of swine remains one such question. However, this study has shown a wide diversity and high number of antibiotic resistance genes in the cultured samples (Figure 3). The diversity of AMR genes is concerning and may result in negative treatment effects in swine in the future if not mitigated.

References

1. Doyle, M.P., et al., *Antimicrobial Resistance: Challenges and Perspectives.* Comprehensive Reviews in Food Science and Food Safety, 2013. **12**(2): p. 234-248.
2. Baker-Austin, C., et al., *Co-selection of antibiotic and metal resistance.* Trends Microbiol, 2006. **14**(4): p. 176-82.
3. Skurnik, D., et al., *Is exposure to mercury a driving force for the carriage of antibiotic resistance genes?* Journal of Medical Microbiology, 2010. **59**(7): p. 804-807.
4. Pal, C., et al., *Co-occurrence of resistance genes to antibiotics, biocides and metals reveals novel insights into their co-selection potential.* BMC Genomics, 2015. **16**: p. 964.
5. Becerra-Castro, C., et al., *Assessment of copper and zinc salts as selectors of antibiotic resistance in Gram-negative bacteria.* Science of the Total Environment, 2015. **530**: p. 367-372.
6. Bednorz, C., et al., *The broader context of antibiotic resistance: zinc feed supplementation of piglets increases the proportion of multi-resistant Escherichia coli in vivo.* International Journal of Medical Microbiology, 2013. **303**(6-7): p. 396-403.
7. Hölzel, C.S., et al., *Heavy metals in liquid pig manure in light of bacterial antimicrobial resistance.* Environmental research, 2012. **113**: p. 21-27.
8. Aarestrup, F.M., L. Cavaco, and H. Hasman, *Decreased susceptibility to zinc chloride is associated with methicillin resistant Staphylococcus aureus CC398 in Danish swine.* Veterinary microbiology, 2010. **142**(3-4): p. 455-457.
9. Cavaco, L., et al., *Cloning and occurrence of czrC, a gene conferring cadmium and zinc resistance in methicillin-resistant Staphylococcus aureus CC398 isolates.* Antimicrobial agents and chemotherapy, 2010. **54**(9): p. 3605-3608.

10. Directorate, V.M. *Veterinary medicines containing zinc oxide: European referral outcome*. 2016 [cited 2018 May 14]; Available from: https://www.gov.uk/government/news/veterinary-medicines-containing-zinc-oxide-european-referral-outcome.
11. USDA, *Swine 2012, Part II: Reference of swine health and health management practices in the United States*, USDA:APHIS:VS and CEAH, Editors. 2012: Fort Collins, CO. p. 1-140.
12. Johnson, T.A., et al., *Clusters of Antibiotic Resistance Genes Enriched Together Stay Together in Swine Agriculture.* mBio, 2016. **7**(2): p. e02214-15.
13. Karkman, A., et al., *High-throughput quantification of antibiotic resistance genes from an urban wastewater treatment plant.* FEMS microbiology ecology, 2016. **92**(3).
14. Zhu, Y.-G., et al., *Diverse and abundant antibiotic resistance genes in Chinese swine farms.* Proceedings of the National Academy of Sciences, 2013. **110**(9): p. 3435-3440.
15. Looft, T., et al., *In-feed antibiotic effects on the swine intestinal microbiome.* Proceedings of the National Academy of Sciences of the United States of America, 2012. **109**(5): p. 1691-1696.
16. Carattoli, A., *Plasmids and the spread of resistance.* Int J Med Microbiol, 2013. **303**(6-7): p. 298-304.
17. Rozwandowicz, M., et al., *Plasmids carrying antimicrobial resistance genes in Enterobacteriaceae.* J Antimicrob Chemother, 2018.
18. Fang, L., et al., *Co-spread of metal and antibiotic resistance within ST3-IncHI2 plasmids from E. coli isolates of food-producing animals.* Sci Rep, 2016. **6**: p. 25312.
19. Wick, R.R., L.M. Judd, and K.E. Holt, *Performance of neural network basecalling tools for Oxford Nanopore sequencing.* Genome Biol, 2019. **20**(1): p. 129.
20. Bushnell, B., *BBTools software package.* URL http://sourceforge. net/projects/bbmap, 2014.
21. Carattoli, A. and H. Hasman, *PlasmidFinder and In Silico pMLST: Identification and Typing of Plasmid Replicons in Whole-Genome Sequencing (WGS).* Methods Mol Biol, 2020. **2075**: p. 285-294.
22. Carattoli, A., et al., *In silico detection and typing of plasmids using PlasmidFinder and plasmid multilocus sequence typing.* Antimicrob Agents Chemother, 2014. **58**(7): p. 3895-903.
23. Zankari, E., et al., *Identification of acquired antimicrobial resistance genes.* J Antimicrob Chemother, 2012. **67**(11): p. 2640-4.
24. Wickham, H., et al., *Welcome to the Tidyverse*. Journal of Open Source Software, 2019. **4**(43): p. 1686.
25. Kolmogorov, M., et al., *Assembly of long, error-prone reads using repeat graphs.* Nat Biotechnol, 2019. **37**(5): p. 540-546.
26. Lin, Y., et al., *Assembly of long error-prone reads using de Bruijn graphs.* Proc Natl Acad Sci U S A, 2016. **113**(52): p. E8396-E8405.
27. Walker, B.J., et al., *Pilon: an integrated tool for comprehensive microbial variant detection and genome assembly improvement.* PLoS One, 2014. **9**(11): p. e112963.
28. Kleinheinz, K.A., K.G. Joensen, and M.V. Larsen, *Applying the ResFinder and VirulenceFinder web-services for easy identification of acquired antibiotic resistance and E. coli virulence genes in bacteriophage and prophage nucleotide sequences.* Bacteriophage, 2014. **4**(1): p. e27943.
29. Kolde, R. and M.R. Kolde, *Package 'pheatmap'*. R Package, 2015. **1**(7).
30. Guarddon, M., et al., *Real-time polymerase chain reaction for the quantitative detection of tetA and tetB bacterial tetracycline resistance genes in food.* Int J Food Microbiol, 2011. **146**(3): p. 284-9.
31. Gonzalez-Plaza, J.J., et al., *Antibiotic-manufacturing sites are hot-spots for the release and spread of antibiotic resistance genes and mobile genetic elements in receiving aquatic environments.* Environ Int, 2019. **130**: p. 104735.

32. Stedtfeld, R.D., et al., *Primer set 2.0 for highly parallel qPCR array targeting antibiotic resistance genes and mobile genetic elements.* FEMS Microbiol Ecol, 2018. **94**(9).
33. Blau, K., et al., *The Transferable Resistome of Produce.* mBio, 2018. **9**(6).
34. Ohnishi, M., K. Kurokawa, and T. Hayashi, *Diversification of Escherichia coli genomes: are bacteriophages the major contributors?* Trends Microbiol, 2001. **9**(10): p. 481-5.
35. Ravi, A., et al., *Transmission and persistence of IncF conjugative plasmids in the gut microbiota of full-term infants.* FEMS Microbiol Ecol, 2018. **94**(1).
36. Johnson, T.J., et al., *Comparative genomics and phylogeny of the IncI1 plasmids: a common plasmid type among porcine enterotoxigenic Escherichia coli.* Plasmid, 2011. **66**(3): p. 144-51.
37. Johnson, T.J., et al., *DNA sequence of a ColV plasmid and prevalence of selected plasmid-encoded virulence genes among avian Escherichia coli strains.* J Bacteriol, 2006. **188**(2): p. 745-58.
38. Johnson, T.J., et al., *Plasmid replicon typing of commensal and pathogenic Escherichia coli isolates.* Appl Environ Microbiol, 2007. **73**(6): p. 1976-83.
39. Sidjabat, H.E., et al., *Expansive spread of IncI1 plasmids carrying blaCMY-2 amongst Escherichia coli.* Int J Antimicrob Agents, 2014. **44**(3): p. 203-8.
40. Peto, L., et al., *Selective culture enrichment and sequencing of feces to enhance detection of antimicrobial resistance genes in third-generation cephalosporin resistant Enterobacteriaceae.* PLoS One, 2019. **14**(11): p. e0222831.
41. Hansen, K.H., et al., *Host-Specific Patterns of Genetic Diversity among IncI1-Iγ and IncK Plasmids Encoding CMY-2 β-Lactamase in Escherichia coli Isolates from Humans, Poultry Meat, Poultry, and Dogs in Denmark.* Applied and Environmental Microbiology, 2016. **82**(15): p. 4705.
42. Bortolaia, V., et al., *High Diversity of Extended-Spectrum β-Lactamases in Escherichia coli Isolates from Italian Broiler Flocks.* Antimicrobial Agents and Chemotherapy, 2010. **54**(4): p. 1623.
43. Literak, I., et al., *Antimicrobial-resistant faecal Escherichia coli in wild mammals in central Europe: multiresistant Escherichia coli producing extended-spectrum beta-lactamases in wild boars.* Journal of Applied Microbiology, 2010. **108**(5): p. 1702-1711.
44. Cloeckaert, A., et al., *Dissemination of an Extended-Spectrum-β-Lactamase bla_{TEM-52} Gene-Carrying IncI1 Plasmid in Various Salmonella enterica Serovars Isolated from Poultry and Humans in Belgium and France between 2001 and 2005.* Antimicrobial Agents and Chemotherapy, 2007. **51**(5): p. 1872.
45. Bean, D.C., D.M. Livermore, and L.M.C. Hall, *Plasmids Imparting Sulfonamide Resistance in Escherichia coli: Implications for Persistence.* Antimicrobial Agents and Chemotherapy, 2009. **53**(3): p. 1088.
46. Mąka, Ł., et al., *Resistance to Sulfonamides and Dissemination of sul Genes Among Salmonella spp. Isolated from Food in Poland.* Foodborne Pathogens and Disease, 2015. **12**(5): p. 383-389.
47. Tatsuno, I., et al., *toxB gene on pO157 of enterohemorrhagic Escherichia coli O157:H7 is required for full epithelial cell adherence phenotype.* Infect Immun, 2001. **69**(11): p. 6660-9.
48. Valat, C., et al., *Absence of co-localization between pathovar-associated virulence factors and extended-spectrum β-lactamase (blaCTX-M) genes on a single plasmid.* Veterinary Microbiology, 2016. **192**: p. 163-166.
49. Carter, M.Q., et al., *Genomic Insight into Natural Inactivation of Shiga Toxin 2 Production in an Environmental Escherichia coli Strain Producing Shiga Toxin 1.* Foodborne Pathog Dis, 2020.

50. Abraham, S., et al., *Molecular Characterization of Escherichia coli Strains That Cause Symptomatic and Asymptomatic Urinary Tract Infections.* Journal of Clinical Microbiology, 2012. **50**(3): p. 1027-1030.
51. Novick, R.P., *Plasmid incompatibility.* Microbiological reviews, 1987. **51**(4): p. 381-395.