CAN THE USE OF OLDER-GENERATION BETA-LACTAM ANTIBIOTICS IN LIVESTOCK PRODUCTION OVER-SELECT FOR BETA-LACTAMASES OF GREATEST CONSEQUENCE FOR HUMAN MEDICINE?

A Dissertation

by

OLANREWAJU JAMES OGUNRINU

Submitted to the Office of Graduate and Professional Studies of
Texas A&M University
in partial fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

Chair of Committee,	H. Morgan Scott
Committee Members,	Keri N. Norman
	Sara D. Lawhon
	Virginia R. Fajt
Head of Department,	Ramesh Vemulapalli

December 2020

Major Subject: Biomedical Sciences

ABSTRACT

We tested the potential and extent to which β-lactam antimicrobials already approved for use in U.S. livestock operations may differentially select for resistance to unapproved highest priority antibiotics such as carbapenems. We did this using layered *in vitro* experimental approaches and by a mathematical pharmacokinetic-pharmacodynamic (PK-PD) model of β-lactam selection effects on a mixture of *Escherichia coli* (*E. coli*) population in the pig large intestine. To achieve this, host-adapted *E. coli* strains bearing a single β-lactamase gene (n=20 each) for $bla_{\text{TEM-1}}$, $bla_{\text{CMY-2}}$, and $bla_{\text{CTX-M-*}}$ or else $bla_{KPC/IMP/NDM}$ (due to limited availability, often in combination with other *bla* genes), were identified, along with 20 *E. coli* strains lacking beta-lactamase genes. Individual and group intrinsic bacterial fitness, as well as growth rates of resistant strains in corresponding β-lactams (e.g., ampicillin, ceftriaxone and meropenem) were estimated. Further, to estimate the relative impact of β-lactams on strain-groups, mixed-strain (n=10, 2 representatives/group) *in vitro* experiments in batch cultures and dynamic anerobic porcine chemostats were performed, with and without β-lactams. Similarly, a mathematical PK-PD model of the *E. coli* mixture in the pig colon, with and without routine ceftiofur therapy, was assessed.

Overall, *bla*-free strains demonstrated a fitness advantage over *bla*-positive strain-groups and strain-groups with lower resistance forms appeared better adapted to anerobic porcine culture medium; however, the persistence of higher resistance forms was increased with β-lactam introduction. In the presence of a 3rd generation

cephalosporin, ESBL-type strains consistently and notably out-competed the AmpC-type strains, even at relative lower starting densities. Furthermore, in experimental and modeled mixed-strain *E. coli* community, higher concentrations of routine use of β-lactams significantly elevated the relative proportions of carbapenemase-producing Enterobacteriaceae (CPE) in spite of their extremely low baseline composition. These findings suggest that currently approved β-lactams can increase the prevalence of newer resistance forms that are of greater public health consequence; further, it can be inferred that drug use restrictions alone may be insufficient to control the spread of CPEs in livestock and human settings. The currently extremely low prevalence levels of these strains in agriculture provides an opportunity for a proactive response rather than waiting to require a reactionary approach.

DEDICATION

In loving memory of my dearest mother; Mrs. Ayoka Victoria Ogunrinu, you left us too soon but your legacy shines on, illuminating the path of many that never met you. Continue to rest in power our generous queen.

A large share of who I am and what I am I owe to the big-brother and our epitome of leadership; Mr. Babatunde George Adebayo. It is a privilege and an honor for me to be able to dedicate this work to you.

ACKNOWLEDGEMENTS

I'll like to express my gratitude to the principal investigator and chair of my academic advising committee, Dr. H. Morgan Scott for providing the opportunity, capital and leadership required for my research work at the veterinary college. Similarly, I'll like to thank Dr. Keri Norman, Dr. Sara Lawhon and Dr. Virginia Fajt for their invaluable input and support as members of my academic advising committee.

I'll also like to thank Dr. Victoria Volkova of the Kansas State University, and her graduate student Mr. Tara Gaire for their support with rendering our mathematical systems model. The generosity of Dr. Thomas Wittum of the Ohio State University, in providing us with key start-up strains is greatly appreciated. My gratitude goes to Dr. Toni Poole and Dr. Kenneth Genovese of the United State Department of Agriculture, College Station for their facilitation of our continuous culture experiment. I will like to extend my appreciation to the CDC/FDA AR Isolate Bank for providing additional carbapenemase producing *E. coli* strains for our study; also, I must thank Dr. Paul Grunenwald and Ms. Bobbiejean Garcia of the Texas Department of State Health Services, for pointing me in the direction of the CDC/FDA AR Isolate Bank.

I'll like to acknowledge the kind contributions of Dr. Javier Vinasco and Ms. Roberta Pugh of the H.M Scott laboratory for their technical and logistic guidance. The support of fellow graduate students, student workers and summer interns- in particular Ms. Morgan Donsbach, in completing tedious tasks is greatly appreciated.

My sincere appreciation to Dr. Linda Logan, for being a pillar of support to many like myself at this great citadel of learning. To my dad and my wonderful brothers, I say thank you, for all the unflinching support through the years, I will not be half the man I am without you all.

CONTRIBUTORS AND FUNDING SOURCES

Contributors

This research work was supervised by a dissertation committee consisting of Dr. H. Morgan Scott [committee chair] and Dr. Sara Lawhon of the Department of Veterinary Pathobiology, also Dr. Keri Norman of the Department of Veterinary Integrative Biosciences and Dr. Virginia Fajt of the Department of Veterinary Physiology and Pharmacology. Fresh porcine chemostat set-up and maintenance was facilitated by Dr. Toni Poole and Dr. Kenneth Genovese and their team at the USDA/ARS. Ms. Morgan Donsbach performed the majority of chemostat sampling. Implementation of the inhibitory pharmacodynamics (Emax) model in Phoenix® software was facilitated by Mr. Tara Gaire and Dr. Victoria Volkova provided the overall expertise required for the set-up and execution of the mathematical PK/PD model in Vensim® software. All other work in the project was performed by the student independently.

Funding Sources

Throughout the duration of my research study, living stipend was provided by H. Morgan Scott Laboratory through the Texas A&M University Research Assistantship program. The data presented in this research were funded through Dr. Morgan Scott's start-up package and a grant by the USDA-NIFA-AFRI (2016-68003-24607) entitled "*Voluntary compliance in antimicrobial stewardship programs: a critical factor for effective intervention*". The findings or conclusions expressed in this dissertation are

those of the authors and do not represent the views of the United States Department of Agriculture.

NOMENCLATURE

AMR	Antimicrobial resistance
AR	Antibiotic resistance
ATCC	American Type Culture Collection
BHI	Brain heart infusion
BLI	Beta-lactamase inhibitor
CAMH-2	Cation-adjusted Mueller-Hinton II
CDC	U.S. Centers for Disease Control and Prevention
CFU	Colony forming unit
CLSI	Clinical Laboratory Standards Institute
CPE	Carbapenemase-producing Enterobacteriaceae
CRE	Carbapenem-resistant Enterobacteriaceae
DNA	Deoxyribonucleic acid
EDTA	Ethylenediaminetetraacetic acid
ESBL	Extended spectrum β-lactamase
ESC	Expanded spectrum cephalosporin
FDA	U.S. Food and Drug Administration
HAI	Healthcare-associated infection
MAC	MacConkey (agar or broth)
MIC	Minimum inhibitory concentration
MLST	Multi-Locus Sequence Typing

NARMS	National Antimicrobial Resistance Monitoring System
NCBI	National Center for Biotechnology Information
OD	Optical density
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PD	Pharmacodynamics
PFGE	Pulsed-field gel electrophoresis
PK	Pharmacokinetics
RPCF	Recombined-Porcine Continuous Flow
URT	Upper respiratory tract
UTI	Urinary tract infection
VRE	Vancomycin-resistant enterococci
WHO	World Health Organization
AMP	Ampicillin
AXO	Ceftriaxone
AXOCLAV	Ceftriaxone + clavulanic acid
FOX	Cefoxitin
MERO	Meropenem
PIME	Cefepime
TET	Tetracycline

TABLE OF CONTENTS

Page

LIST OF FIGURES

Page

LIST OF TABLES

Page

CHAPTER I

INTRODUCTION

Background

Antimicrobial resistance (AMR) is one of the leading challenges of modern medicine [1]. Bacterial resistance to antimicrobials has been increasing over the decades with the prevalence of AMR and its rate of expansion closely tied to the cumulative quantities and categories of antimicrobial agents being used [2]. Bacterial pathogens resistant to antimicrobials can significantly increase the morbidity and mortality of the diseases caused in infected humans and animals [3]. Further, resistance to available antimicrobials can lead to limited – or even complete lack of – treatment options for use by human medical and veterinary practitioners. To extend the clinical efficacy of currently available drugs long into the future, prudent use of antimicrobials is firmly and widely encouraged to help mitigate AMR [2-4].

Beta-lactams are the most widely used group of antimicrobial agents in human bacterial disease treatment [5]. Newer beta-lactam antibiotics, including third- and fourth-generation cephalosporins (3GC and 4GC, respectively), carbapenems and monobactams, are important contributors to human medical as well as veterinary companion animal antimicrobial prescriptions; in part, this is due to their broad spectrum of activity, low toxicity, reliable effectiveness, and relative affordability [5]. For instance, a review of the Intercontinental Medical Statistics (IMS) data by Bush & Bradford (2016) showed that in a ten-year period (2004 - 2014) newer beta lactam

antibiotics accounted for 65% of all parenteral antimicrobial prescriptions in the United States; of these, 47% were cephalosporins [6]. Depending on their molecular structure, beta-lactams can possess activity against either – or both – gram-positive and gram-negative bacteria [6]. Earlier generation penicillins and cephalosporins demonstrate activity against gram-positive bacteria. Potentiated aminopenicillins and later generations of cephalosporins show increased spectrum of gram-negative activity [7, 8].Carbapenems are the newest beta-lactams; these antibiotics, along with drugs belonging to the fourth and fifth generation of cephalosporins, are active against both gram-positive and gram-negative bacteria. The indications for beta-lactams are broad and include urinary tract infections, respiratory tract infections, peri-operative care, meningitis and septicemia [9, 10].

In United States (U.S.) animal agriculture, three types of beta-lactams are approved for use: 1) various penicillin and aminopenicillin preparations, 2) cephapirin, and 3) ceftiofur (a 3GC used only in animals and with a chemical structure and pharmacological properties similar to the human drug ceftriaxone). Their clinical indications include treatment and control of respiratory tract infections, acute metritis, mastitis and foot rot. More recently, the use of cephalosporins – and ceftiofur in particular – has become more restricted due to a prohibition order against certain types of extra-label antibiotic use issued by the U.S. Food and Drug Administration (FDA) in 2012 [11].

Most Enterobacteriaceae, including *Escherichia coli* (*E. coli*), *Enterobacter* spp., *Citrobacter* spp. and *Klebsiella* spp., are normal commensals of the mammalian large

intestine [12, 13]. When systemic antimicrobials are administered to treat infections in one organ system (e.g., respiratory tract, urinary tract), another well-documented but unintended consequence is the direct selection of resistant organisms in the host gastrointestinal tract [14]. Consequently, Enterobacteriaceae, and particularly *E. coli*, are commonly used indicators of bacterial resistance prevalence reflecting the immediate as well as the cumulative effects of antibiotic selection pressures over the decades [15]. Furthermore, most of these commensal species can cause opportunistic infections when host defense mechanisms are compromised; for instance, *Enterobacter cloacae* and *Klebsiella* spp. are major causes of healthcare-associated infections (HAI – also known as nosocomial infections) [16]. The public health importance of Enterobacteriaceae and related gram-negative bacteria (e.g., pseudomonads) is further magnified by their ability to readily exchange mobile genetic elements (MGEs) – e.g., plasmids and transposons – that carry various antimicrobial resistance genes, thus spreading antimicrobial resistance from intestinal commensals to more highly pathogenic strains [17].

Beta-lactam resistance among Enterobacteriaceae is largely mediated through beta-lactamase enzyme production [18]; of importance, the initial discovery of beta-lactam inactivating enzymes preceded the clinical introduction of the first beta-lactam: penicillin [19]. Two beta-lactamase classification schemes are commonly recognized, the more widely recognized Ambler structural classification (Groups A-D) [20, 21] and the Bush-Jacoby-Medeiros functional classification (Groups 1-4) [22, 23]. Ambler groups A, C & D are active site serine metabolizers while group B enzymes are metallo-beta-lactamases; as such, this latter class of enzymes requires a zinc ion co-factor for

hydrolytic activity [24]. Older recognized plasmid-borne beta-lactamase resistance genes of public health importance include: *bla*$_{\text{TEM-1/2}}$ [25], *bla*$_{\text{SHV-1}}$, and *bla*$_{\text{OXA-1}}$ [18], all of which are active against amino-penicillins and narrow spectrum cephalosporins. The *bla*$_{\text{CMY-*}}$ gene-bearing organisms (i.e., mobilized AmpC producers) are capable of inhibiting extended spectrum cephalosporins (3GC), cephamycins (2GC), many beta-lactam/beta-lactamase inhibitor (BLI) combinations (i.e., those with clavulanic acid or sulbactam), along with the older-generation beta-lactams [26]. Among others, enzymes encoded by *bla*$_{\text{TEM-3}}$ and higher subscript designations, *bla*$_{\text{SHV-2}}$ and higher subscript designations, and all of the *bla*$_{\text{CTX-M-*}}$ variant genes hydrolyze extended and broad spectrum cephalosporins (i.e., 3GC & 4GC) and are thus known as extended-spectrum beta-lactamases (ESBL). The *bla*$_{\text{KPC-*}}$, *bla*$_{\text{IMP-*}}$, *bla*$_{\text{VIM}}$, *bla*$_{\text{NDM-*}}$, and *bla*$_{\text{OXA-23+}}$ genes each encode carbapenemase enzyme production [27-31]. With the exception of the *bla* $_{\text{OXA-48/181}}$-type gene carriers, carbapenemase producers are potent neutralizers of almost all beta-lactams, including carbapenems [28, 29].

Antimicrobial use in animal agriculture has been reported to contribute to increased AMR prevalence, both in animals and in humans [32]. Some experts have suggested that uses in livestock production account for about two-thirds of the global antibiotic sales and consumption [33]. Although the risk posed to humans by direct acquisition of AMR organisms from food animals is difficult to quantify, ample evidence exists of human acquisition of resistant bacteria via food animal sources [34-39].

Carbapenems are the 'last line of defense' beta-lactams; as such, they have never been approved for use in food animals [40]. Nevertheless, carbapenemase-producing Enterobacteriaceae (CPE) have been isolated globally in a variety of food animal systems. The enzyme in *Acinectobacter baumannii* carrying *bla*$_{\text{OXA-32}}$ was the first carbapenemase-producing bacterium identified in food animals, and was isolated in dairy cattle in France [28]. In 2011, a year after the finding in France, the German Research Network (RESET) project found *E. coli* and multiple *Salmonella* isolates harboring the *bla*$_{\text{VIM-1}}$ gene in pig feces, the pig farm environment, and in poultry dust [41-43]. Other carbapenemase-producing bacteria have been found in food animals in places such as China (*A. baumannii*: *bla* $_{\text{NDM-1}}$) [44, 45] and Lebanon (*Pseudomonas aeruginosa*: *bla* $_{\text{VIM-2}}$, *A. baumannii*: *bla* $_{\text{OXA-23}}$) [46]. In the High Plains region of Texas and New Mexico, the first U.S. animal agricultural CPE were identified in 2016; of note, one *A. baumannii* from dairy cattle excreta carried a seemingly novel chromosomal gene *bla*$_{\text{OXA-497}}$ [47]. Later, plasmid-borne *bla*$_{\text{IMP-27/64}}$ genes were detected in multiple Enterobacteriaceae species from the environment and fecal samples of a farrow-to-finish swine operation in the Midwest region of the U.S. This latter finding included the observation that the CPE isolates were found in pig barns where the 3GC drug ceftiofur was used, but not in barns where its use was not recorded [48, 49].

Study significance

The increasing reports of isolation of CPEs from food animal agricultural settings poses somewhat of a conundrum. Mollenkopf et al. (2016) proposed that approved beta-

lactams in animal agriculture may differentially select for carbapenemase-producing strains in farm animals and their environment [49, 50]. Other authors such as Woodford et al., (2014) made a similar but broader suggestion that multiple classes of antimicrobials other than the beta-lactams may provide selection pressures for carbapenem-resistant Enterobacteriaceae (CRE) isolates in animal agriculture [51]. Multiple genes encoding resistance to different antimicrobial agents are frequently found together on mobile genetic elements – such as plasmids harbored by Enterobacteriaceae – and this can facilitate co-selection of resistance. However, no evidence exists to date to support assertions that early-generation beta-lactams might select disproportionately for higher-level beta-lactam resistance, including CPEs, in the absence of complementary selection mechanisms for these antimicrobials.

By simulating mammalian large intestinal commensal bacteria population dynamics in the presence of differing beta-lactam selection pressures through multiple *in vitro* methodologies and mammalian pharmacokinetics vis-à-vis bacterial pharmacodynamics systems modeling, our research can bridge this evidence gap. The use of *E. coli* strains (a representative Enterobacteriaceae) primarily obtained from swine (an exemplar mono-gastric livestock, similar to the human digestive tract), the findings of this study can be used to form the framework for understanding the risk of older generation beta-lactams selecting for more critical resistance to newer-generation beta-lactams, not only in populations of bacteria found in livestock, but potentially also in human enteric bacterial populations.

CPEs are currently largely identified as being associated with outbreaks of healthcare-associated infections (HAIs); however, if their spread is not controlled introduction into food animals and then expansion could lead to wider dissemination and community-acquired CRE infections through food products, direct animal contact and via the environment. Given the current low levels of CPEs in food animal agriculture, a window of opportunity remains available to investigate and mitigate potential risks. The findings of our study will provide the first direct evidence of the relative efficiency of differential ESBL and CPE selection by non-carbapenem beta-lactams, in the presence of competing bacteria with genes encoding varying degrees of resistance to older- through newer-generation beta-lactams. Understanding and managing these risks is essential for prolonging the clinical efficacy of carbapenems as drugs of last resort for severe gram-negative bacterial infections in humans.

Objectives

The primary objective of this research was to determine the extent to which older- and newer-generation β-lactam antibiotics approved in the U.S. for use in food animals (e.g., aminopenicillins and 3rd generation cephalosporins, respectively) can differentially select for highest priority antibiotic resistance (e.g., to 3rd and 4th generation cephalosporins and carbapenems, respectively) among representative Enterobacteriaceae. We aimed to characterize individual- and group-level fitness of 5-genotypic groups: *bla*$_{\text{TEM-1}}$, *bla*$_{\text{CMY-2}}$, *bla*$_{\text{CTX-M-*}}$, or *bla*$_{KPC/IMP/NDM}$, and a beta-lactamase-free group, of host-adapted *E. coli* strains in both antibiotic-containing and non-

antibiotic media. We aimed to choose strains within each genotype-group bearing one, but not a combination, of beta-lactamase genes, as far as possible. Furthermore, to narrow the existing knowledge gap concerning the potential for over-selection of ESBLs and CPEs in U.S. food animal production, we proposed to test the differential selection dynamics of the 5-genotypic groups, combined in one mixture through static and dynamic (chemostat) *in vitro* culture experiments, as well as, by mathematical simulation of in vivo intestinal Enterobacteriaceae selection in the presence of approved β-lactams- ampicillin and ceftiofur, respectively. Finally, we developed the framework and deployed a systems dynamic model of the porcine *in vivo* setting to mathematically explore the temporal dynamics not only of the interplay among five genotypic groups, but also their shifts over time in multiple metapopulations and in response to beta-lactam antibiotic treatment.

CHAPTER II

CRITICAL REVIEW OF THE LITERATURE

β-lactam antimicrobials have been one of the most widely used of the groups of antimicrobial agents in human and veterinary infectious disease therapy; however, their continued efficacy and utility is now uncertain due to the expanding threat of bacteria resistance. The following critical review of the literature highlights the nature and magnitude of enzyme-mediated resistance to expanded spectrum cephalosporins (ESC) and carbapenems in bacteria, and pertinent research approaches for estimating and predicting their spread.

The challenge of expanded-spectrum cephalosporin and carbapenem resistance among Enterobacteriaceae

AmpC β-lactamases

The emergence of group C β-lactamase at a scale leading to public health conseqneunce will be reviewed in this section. Many Enterobacteriaceae have previously been known to constitutively produce AmpC-type β-lactamase enzymes; often, these enzymes were produced at low levels. For instance, Honore et al. (1986) demonstrated that a regulatory gene- *ampR,* was missing in *Escherichia coli*; this was key knowledge for deciphering the constitutively low levels of AmpC enzyme production by *E. coli,* as well as understanding the absence of enzyme hyper-production through induction mechanisms in this organism [52]. After Honore et al. cloned the *ampR* gene from

Enterobacter cloacae strains into *E. coli* mini-cells, the mutant cells were subsequently exposed to the β-lactam antibiotic cefoxitin. Through SDS-polyacrylamide gel electrophoresis, a greater than 10-fold enzyme induction was observed in these cells. This observation demonstrated the potential impact of the presence or absence of such regulatory genes in clinical pathogens. *E. coli* is a leading cause of community acquired infections such as gastroenteritis, urinary tract infections (UTI) and healthcare-associated infections (HAI. Similarly, key Enterobacteriaceae in healthcare such as *Salmonella enterica* and *Klebsiella pneumoniae*, have previously been shown to lack, all together, a chromosomal *ampC* gene in their genome. Bergstrom et al. investigated two overlapping operon regions- *frd* and *ampC*, in *E. coli* and the extent of their conservation in the genome of other Enterobacteriaceae [53]. Their study found no similar chromosomal *ampC* region in tested *S.* Typhimurium and *K. pneumoniae*, suggesting this gene is likely missing and clinical infections from these strains are usually be treatable using a cephamycin β-lactam (2^{nd} generation cephalosporin). Through probes for both investigated genes, no significant hybridization was observed for *S.* Typhimurium and *K. pneumoniae*, as opposed to significant hybridizations noted for other Enterobacteriaceae such as *Citrobacter freundii*, *Enterobacter cloacae*, *Shigella sonnei* and *Shigella flexneri*. Their finding, perhaps inadvertently, also provided crucial data for monitoring future trends of AmpC-type resistance in *S.* Typhimurium and *K. pneumoniea.*

In 1989, a monumental shift in group C β-lactamase genetic coding patterns among Enterobacteriaceae was described. Bauernfeind and Chong (1989) identified,

characterized and demonstrated plasmid-borne cephamycin resistance in an Enterobacteriaceae (*K. pneumoniae*) [54]. The gene encoding for resistance, which was named *bla*$_{\text{CMY-1}}$ (or, beta-lactamase active against cephamycins) resided on a transferable plasmid (pMVP-1). This was groundbreaking due to the potential for dissemination of such resistance genes among bacterial populations; that is, within and among genera of commensals and pathogens alike.

Similar to chromosomally encoded AmpC-type β-lactamases, bacteria harboring the plasmid-borne enzyme exhibited elevated minimal inhibitory concentrations (MIC) for penicillins, cephalosporins (generations one through three) and monobactams; of interest and in contrast, observed activity against the third-generation cephalosporin ceftazidime was low. Also, this enzyme was not significantly inhibited by β-lactam inhibitors available at the time of their discovery and bacteria with this gene remained susceptible to temocillin, cefpirome and meropenem. The authors logically speculated that the emergence and transfer of a cephamycinase resistance gene onto plasmids was likely secondary to the spread of TEM-1 and SHV-1 type β-lactamases at the time and the consequent increased cephamycin prescription along with β-lactam/β-lactamase inhibitor therapies. [54].

Soon after the discovery by Bauernfeind and Chong, a number of additional transferable AmpC-type β-lactamases were described by these and other researchers. In 1995 Bauernfeind et al., described a mutant variant of their previously described plasmid-borne group *bla* C enzyme; of interest, this new enzyme attained even greater clinical importance than its predecessor, which continues to the present day: the CMY-2

β-lactamase enzyme was characterized by the investigators in a *K. pneumoniae* isolated from the urine of a pyelonephritis patient in Athens, Greece [55]. The new enzyme showed, in general, a similar β-lactam antimicrobial pattern to that of CMY-1-type AmpC β-lactamase and chromosomal AmpC β-lactamases; however, it exhibited a significantly elevated ceftazidime MIC, along with subdued cefotaxime MIC relative to the CMY-1 enzyme. Initially, the significance of this difference was not entirely clear, but it was observed that enzymes with similar genealogy -- that is, belonging to the C-1 (likely of *Citrobacter freundii* origin) sub-classification – were more likely to be observed in pathogenic strains. This distribution tendency, along with its level of ceftazidime resistance, may be key to understanding the clinical dominance of this enzyme variant relative to its peers.

A major difference observed between the plasmid-borne AmpC β-lactamases and the chromosomally-encoded AmpC β-lactamases was the constitutive levels of their enzyme expression. Bauernfeind et al. showed in their two studies that each of the *bla*$_{\text{CMY-1}}$ and *bla*$_{\text{CMY-2}}$ plasmid-encoded genes expressed consistently high levels of resistance enzymes. For example, their *bla*$_{\text{CMY-1}}$-trans-conjugated *E. coli* strain showed a cefoxitin MIC transformation of 4 to 256 μg/mL, cefotaxime of 0.03 to 64 μg/mL, ceftazidime of 0.13 to 4 μg/mL, and ceftriaxone of 0.03 to 32 μg/mL. Their *bla*$_{\text{CMY-2}}$-trans-conjugated *E. coli* strain showed an MIC transformation for cefoxitin of 4 to 256 μg/mL, cefotaxime of 0.03 to 16 μg/mL, and ceftazidime of 0.13 to 128 μg/mL.

When carried by disease-causing agents, the health impact of this can be severe given the broader spectrum of β-lactam resistance demonstrated by AmpC β-lactamases

and the resulting limitations in therapeutic antimicrobial options. Nakano et al. (2007), elucidated one of the mechanisms responsible for this observed high level enzyme expression [56]. A promoter integron IS*Ecp1* inserted onto a plasmid-borne *AmpR* gene binding site in a clinical *K. pneumoniae* strain (KU6500) isolated in Japan, was responsible for its high level CMY-4-type enzyme production. An *E. coli* DH5α strain with a clone of the plasmid bearing the IS*Ecp1* integron showed high levels of resistance to most β-lactams, while the *bla*$_{\text{CMY-4}}$-only bearing control remained susceptible to all β-lactam antimicrobials with the exception of cephalothin. Such revealing findings may provide opportunities for newer antimicrobial therapies and/or developing resistance-targeted control measures.

The acquisition of non-constitutive novel resistance mechanisms by micro-organisms has been shown to confer a metabolic burden (fitness) on the host cell. However, such deleterious effects do not last forever, and are generally lost after an extended period of time. The initial effect on bacterial fitness was documented by Morosini et al. (2000), in their study of the effect of acquisition of a plasmid-encoded AmpC resistance gene by a *Salmonella enterica* strain [57]. The strain's colony morphology, cellular size, growth rate estimates and eukaryotic cellular invasion rates were altered post-acquisition. Importantly, when the plasmid-encoded AmpC resistance gene was cloned from either *E. cloacae* or *E. coli* MC4100 into the *Salmonella* strain, along with a gene that regulates enzyme production upwards and downwards (that is, inducibility)- *ampR*, the observed fitness costs were reversed. This result demonstrates one of many described bacteria fitness cost adaptations in the antibiotic era,

underscoring the dynamism of bacterial genetics and the consequent challenge of increasing antimicrobial resistance prevalence.

Since their identification in Enterobacteriaceae, estimated prevalences of plasmid-encoded AmpC β-lactamases in clinical infections have steadily risen globally. In the United States, one of the first studies to estimate this prevalence was by Dunne et al (2000). They characterized resistance to ceftriaxone among human *Salmonella* isolates submitted to the U.S. National Antimicrobial Resistance Monitoring System (NARMS) from 17 state and community health departments between the years 1996 and 1998 [58]. The choice of indicator organism in the survey was well-informed, given the inherent lack of AmpC-type β-lactamases in *Salmonella*, detected group C-type resistance to ceftriaxone can be assumed to be secondary to horizontal gene acquisition. Estimated prevalence of ceftriaxone-resistant *Salmonella* was 0.1%, 0.4% and 0.5% for the consecutive years of study of 1996, 1997, and 1998, respectively. Of the 15 tested *Salmonella* isolates with MIC values equal to or above 16 µg/mL for ceftriaxone, 13 (87%) were positive by polymerase chain reaction (PCR) for an AmpC gene (*C. freundii ampC* gene or *bla*-$_{\text{CMY-2}}$ gene); importantly, local acquisition at 91% (10 out of 11) was confirmed through interviews regarding travel history. Though not often requiring antibiotic therapy, in instances where anti-infective treatment is indicated, ceftriaxone is one of the most commonly chosen antibiotics for treating salmonellosis in children. As a result, studies of this nature are of critical importance to AMR surveillance. Furthermore, to increase stakeholders' awareness of the growing challenge of ESC resistance in HAIs, Moland et al., (2000) studied *K. pneumoniae* isolates with initial

reduced susceptibility to cefoxitin or imipenem, collected from 24 healthcare facilities across the U.S and archived from the year 1996 [59]. The broad geographical and temporal coverage of the screened isolates in the study adds strength to the overall applicability of its findings and conclusions. Multiple test methodologies such as phenotypic testing (cefpodoxime, ceftriaxone, cefta-zidime, cefotaxime, cefepime, aztreonam, cefoxitin and imipenem), isoelectric focusing, β-lactamase inhibitor testing, spectrophotometric assays, induction and molecular assays were employed for characterizing groups of β-lactamase enzymes. Of the 24 healthcare facilities, 18 (75%) were positive for ESBL-producing strains, 10 (42%) for AmpC β-lactamase producers and 1 (4%) facility for carbapemase-producing strains. Although this study did not estimate actual resistance prevalence (that is among patient isolates) at the facilities, the facility level detection results would point to the need for in-depth studies at those individual facilities for which reduced susceptibility was detected.

Extended spectrum β-lactamases (ESBLs)

This section will highlight the discovery and inexorable rise of ESBL-type β-lactamases. In their review article, Ghafourian et al. (2015), defined extended spectrum β-lactamases as enzymes, produced by certain bacterial groups, that are able to hydrolyze expanded spectrum cephalosporins [60]. The initial plasmid-borne ESBL enzymes were found to be mutant variants of prevalent aminopenicillin- and penicillin-hydrolyzing enzymes. In 1985, Kliebe et al., described the first of these enzymes; through isoelectric point and heteroduplex analyses, extensive homology with the

penicillinase enzyme *bla*$_{\text{SHV-1}}$ was observed, and a novel enzyme from a clinical *Klebsiella ozaenae* strain was thereafter designated *bla*$_{\text{SHV-2}}$ [61]. Four years later, the first of these ESBL-type enzymes- *bla*$_{\text{TEM-10}}$ was described in the United States by Quinn et al. Described from two *K. pneumoniae* strains isolated from critically-ill patients, the enzyme conferred resistance to both ceftazidime and aztreonam and the penicillins. MICs to other cephalosporins were only mildly elevated, comparatively speaking [62].

A new epoch in plasmid-borne ESBLs began in 1988 when Matsumoto et al., described a highly-oxyimino-cephalosporin active β-lactamase- FEC-1 (now a member of the CTX-M-3 family of enzymes) from an *E. coli* isolate obtained from the feces of a dog that was previously treated with a β-lactam antimicrobial [63]. FEC-1 efficiently hydrolyzed cefuroxime, cefotaxime, cefmenoxime and ceftriaxone; however, no activity was recorded against cephamycins (e.g., cefoxitin) or imipenem, and inhibition by β-lactamase inhibitors (such as clavulanic acid and sulbactam) was notable. The observed MIC for ceftazidime was relatively low (25 μg/mL) when compared to 200 μg/mL for cefotaxime; of interest, this suggested that the novel enzyme hydrolyzed cefotaxime more efficiently than ceftazidime.

Through analyses of nationwide public health laboratory submissions to the U.S. Centers for Disease Control and Prevention (CDC)-NARMS program, Sjölund et al., detected and then characterized the first domestically acquired cefotaximase producing bacteria, in 2008 [64]. The *bla*$_{\text{CTX-M-5}}$ gene was described in a single *S.* Typhimurium isolated from the feces of a 3-month-old baby in the U.S. state of Georgia. Unlike prior descriptions of such an enzyme from Enterobacteriaceae in U.S patients, no history of

international travel (whether community or healthcare facility acquired) could be linked to this patient, suggesting a locally acquired resistance gene. This proved that evolutionary determinants similar to those responsible for the global spread of the CTX-M-type β-lactamase have a domestic presence, and a new CTX-M trend could have been emerging. The disproportionally elevated hydrolysis of cefotaxime relative to ceftazidime by the CTX-M-type β-lactamases has been an important source of its differentiation from the other penicillinase mutant ESBL enzymes like SHV and TEM. However, a novel pattern among the CTX-M-type β-lactamases' enzyme activity later emerged. Bonnet et al. (2001), described a pair of these enzymes CTX-M-9 and CTX-M-16 co-existing in three strains of Enterobacteriaceae [65]. The enzyme pair differed from each other by a single amino acid substitution (aspartate for glycine at position 240), signifying that CTX-M-16 was very likely a CTX-M-9 mutant. Both enzymes exhibited the expected high level of hydrolytic activity against cefotaxime; however, the mutant enzyme appeared also to possess enhanced hydrolysis of ceftazidime (MIC, 8 vs 1; and a 7.5-fold increase in ceftazidime affinity). This finding is an important indication of the evolutionary potential of Enterobacteriaceae within a short period of time; most likely, this change occurred in response to prevailing antimicrobial pressures imposed by physician choices of cephalosporin.

Further indication of this potential was described by Cartel et al., who observed an even greater ceftazidime hydrolytic activity (MIC >128μg/mL) from a replica mutation of a *bla*$_{\text{CTX-M-1}}$ gene to a *bla*$_{\text{CTX-M-32}}$ gene in a clinical *E. coli* strain [66]. Also, Kimura et al., observed a 4-32 times increase in ceftazidime hydrolysis in a mutant

compared to a 'wild-type' CTX-M producer following a single amino-acid substitution at position 167 (Pro167Ser). Conversely, they also observed a 2-4-fold reduction in cefotaxime metabolism in the mutant strains [67]. Of importance, these findings appear to demonstrate an evolutionary tendency towards a balanced hydrolysis of cefotaxime, ceftazidime, and their structural equivalents, two widely used subsets of ESC.

Since their initial description, the number and diversity of identified CTX-M-type β-lactamases have grown considerable. In general, the CTX-M family is now considered to comprise of five groups of enzymes: CTX-M-1, CTX-M-2, CTX-M-8, CTX-M-9 and CTX-M-25. Members of each group have a phylogenetic similarity score of >94% while the identity score across groups tends to be lower than 90% [68]. Along with their increase in diversity is their increased distribution in clinically encountered strains, one of the first studies to capture the clinical emergence of cefotaximase-mediated ESC resistance in the United States was Castanheira et al., in 2007 [69]. They screened 220 gram-negative bacteria isolates, from the 2007 national MYSTIC Program, for CTX-M-type ESBLs and other β-lactamases. Although previously considered rare in the United States, a prevalence rate of 38.8% (28/70) was observed for cefotaximases among the isolates phenotypically considered an ESBL. Further, CTX-M-type ESBLs were detected in 80% of the participating hospitals in the MYSTIC 2007 Program, with CTX-M-14 and -15 being the most frequently found enzyme variant.

Castanheira et al., followed this up in 2010 by testing Enterobacteriaceae (n=195) isolates from 20 states in the country, also obtained through the MYSTIC Program, for acquired β-lactamases [70]. Among these isolates, 85% (175/195) were positive for a β-

lactamase enzyme, 43.6% (85/195) harbored a *bla*$_{\text{CTX-M-*}}$ gene (33% of these were *bla*$_{\text{CTX-M-15}}$). SHV-type ESBLs were also detected in a total of 32 strains (16.4%, n=13 were of the SHV-12 variety). Plasmid-encoded AmpC β-lactamases were detected in 19 (9.7%) strains; of these, *bla*$_{\text{CMY-2}}$ was the predominant variety (n=12 strains). Of even greater importance, 33 strains were noted with reduced susceptibility to imipenem/meropenem (≥ 2 μg/mL); of these, a majority (n=28) carried a *bla*$_{\text{KPC}}$ gene (n=17 *bla*$_{\text{KPC-2}}$, and n=11 *bla*$_{\text{KPC-3}}$). These two studies unequivocally demonstrated the fairly rapid clinical emergence of CTX-M-type β-lactamases in the United States, long after their global domination of ESBL-type ESC resistance had previously occurred, with an inevitable further reduction in antimicrobial therapeutic options in healthcare settings.

Prior to the U.S. reports of Castanheira et al., a higher prevalence of cefotaximase-mediated resistance in community acquired infections had already been reported in other parts of the globe with high CTX-M-type β-lactamases prevalence in healthcare settings. The situation in the United States was initially undefined, likely due to the novel nature of this genotype in the country. In 2012 Doi et al., published their work on the occurrence of ESBL enzyme-producing *E. coli* isolates from patients initially presenting to five academic and community hospitals. Isolates were collected through the years 2009 and 2010 [71]. A stringent but reasonable definition of healthcare associated infection (HAI) was applied; that is, an outpatient or else a < 48 hours in-patient, with no history of the following: i) home intravenous (i.v.) therapy and specialized wound care in the home within 30 days of infection, ii) hospital attendance

or hemodialysis within 30 days of infection, or iii) residency in a nursing home or hospitalization for ≥ 2 days in 90 days prior to infection. Of the total isolated, 36.8% of identified ESBL strains were judged to be community acquired, of which 91.3% expressed a CTX-M-type ESBL enzyme. This demonstrates that a similar pattern of community acquired ESC resistant Enterobacteriaceae existed in the United States as previously described on other continents. Further investigation of attributable factors such as the food animal contamination in the community spread of AMR bacteria clearly is required.

Carbapenemase resistant Enterobacteriaceae (CRE)

Sometimes described as the 'big-five' carbapenemase enzymes, their discovery, importance and characteristics will be examined here. Efficient β-lactamase mediated carbapenem resistance was initially observed in obligate anerobic strains such as *Bacteroides fragilis, Aeromonas hydrophilia and Xanthomonas maltophilia* [72]; their enzyme products were encoded by chromosomal genes. Although, Watanabe et al. (1991) provided the first report of a transferable carbapenemase gene in a bacteria, the described genotype never rose to public health significance [73]. The first of the current five carbapenemase enzymes to gain public health significance, that is imipenemase-1 (IMP-1) was described by Osano et al. (1994) [74]. It was identified in a clinical *Serratia marcescens* isolate; of importance, the novel enzyme hydrolyzed imipenem, oxyimino-cephalosporins, 7-methoxy-cephalosporins and penicillins but not the monobactam drug aztreonam. Though not affected by β-lactamase inhibitors, the IMP-1 β-lactamase was

inhibited by Ethylenediaminetetraacetic acid (EDTA), thus implying a crucial role for metallic cations in enzyme activity (i.e., a metallo-β-lactamase enzyme). Horizontal transfer of the encoding gene was demonstrated through cloning and expression on the conjugant *E. coli* HB101 plasmid (pSMBNU24).

This finding was of critical importance to public health given the last resort status of the carbapenem drugs in infectious disease treatment and control. Sensibly, Ito et al. (1995), investigated the dissemination level of this genotype among clinical isolates in Japan [75]. Among 105 *S. marcescens* isolates obtained from general hospitals, at least four isolates produced the IMP-1-type enzyme, with three of these demonstrating resistance to imipenem at very high levels (MIC ≥ 64μg/mL). Numerous other follow-up studies have demonstrated spread of the genotype to other Enterobacteriaceae and endemicity of the *bla*$_{\text{IMP}}$ gene family in Japan and the Asian sub-region.

In the United States in 2004, the first described plasmid-borne carbapenem hydrolyzing metallo-β-lactamase was *bla*$_{\text{VIM-7.}}$ It was detected in a *P. aeruginosa* strain recovered from a cancer patient on admission to a hospital [76]. The Verona-Integron Metallo-β-lactamase (VIM) was initially described in Greece and shares a similar hydrolytic pattern with the IMP carbapenemases [77]. Although both the IMP and VIM metallo-carbapenemases have been described in clinical isolates from the United States, they have not been the dominant mediator of carbapenem resistance in the country. That position belongs to an Ambler group-A enzyme: the *Klebsiella pneumoniae* carbapenemase (KPC) enzyme.

Yigit et al. (2001) first described the KPC-1 enzyme in a *K. pneumoniae* isolate recovered from a patient in a North Carolina hospital [78]. The isolate demonstrated an elevated MIC (16µg/mL) to both imipenem and meropenem; in addition, elevated hydrolytic activities against penicillins, cephalosporins and aztreonam were also observed. Unlike the metallo-β-lactamases, KPC-1 enzyme activity was inhibited by β-lactamase inhibitors such as clavulanic acid and tazobactam; further, the metal chelator-EDTA had no impact on KPC-1 enzyme activity. It was insightful of the authors to interpret the observed carbapenem MICs in their study with much care, and to seek out other resistance mechanisms that may play contributory roles in the acutely elevated MIC values. Analyses of bacterial membrane proteins showed that the study strain lacked most but one outer membrane porin protein (OmpK36), indicating significant intrinsic resistance by the strain. The existence of this adaptation in the presence of acquired resistance mechanisms can confer exaggerated MIC estimates on strains; this is particularly important when estimating carbapenem resistance levels in carbapenemase-enzyme producing strains due to their constitutive low production levels.

To appraise the prevailing dissemination level of this novel gene in the United States healthcare system soon after its discovery, a study was performed by Kaiser et al in 2015 [79]. From the 2007 – 2009 database of the SENTRY Antimicrobial Surveillance Program (n=42 medical centers), a total of 2,049 *K. pneumoniae* isolates was screened for the bla_{PKC} gene; their findings showed a combined detection rate of 6.1% for carbapenem non-susceptibility (imipenem/meropenem ≥ 2µg/mL) isolates, and 5.5% of these were confirmed positive for the bla_{PKC} gene using PCR. Annual KPC-type

carbapenemase detection rates were 5.9% in 2007, 4.9% in 2008 and 5.7% in 2009; further, annual frequencies by region were put at 29%, 23%, 33% for 2007 – 2009 respectively in the Mid-Atlantic region, and 0%, 3.1%, 3.8% for the same years respectively in the East North Central region suggesting a strong regional affinity, at least at first. The conspicuous absence of estimates from regions distant from the east coast in this study may underscore the emergence and initial spread of this resistance profile on the Atlantic coast of the country.

As the prevalence of the *bla*$_{\text{PKC}}$ gene in *K. pneumonia* in the United States continued to rise and propagate to new regions, a new challenge arose; on June 25, 2010 the CDC's weekly Morbidity and Mortality Report published the detection of the first metallo-β-lactamase enzyme in an Enterobacteriaceae within the U.S. (VIM was reported in a *Pseudomonas*) [80]. The New Delhi metallo-β-lactamase-1 gene (NDM-1) was detected in three organisms: *E. coli, K. pneumoniae* and *E. cloacae*; of importance, all were isolated from patients with a positive history of healthcare facility visits on the Indian subcontinent. NDM-1 was first described by Yong et al., in *K. pneumoniae* isolated from a Swedish patient of Indian descent with an analogous travel history as the U.S cases [81]; clearly, these initial observations pointed to India as the likely origin of the enzyme.

Although very little homology (32.4% to VIM and much less for IMP) was recorded when compared with known metallo-β-lactamases, the antimicrobial profile of the carbapenemase NDM-1 was similar to that of the preceding metallo-β-lactamase enzymes. The NDM-type metallo-β-lactamase is unique in its importance to public

health; unlike prior carbapenemase enzymes that are often described in HAI-implicated organisms such as *Klebsiella* and *Enterobacter*, NDM-1 appeared to be well adapted to *E. coli* species. *E. coli* is a widely distributed intestinal commensal and an important agent of community acquired infections. The prospect of carbapenem resistance in common community acquired infections through environmental dissemination of *E. coli* strains outside of hospitals was daunting. Consequently, in 2010 Walsh et al. (2011), investigated this theory in the environs of New Delhi [82]. The researchers collected 171 seepage (street puddle), 50 tap water and 70 sewage samples across central New Delhi. Among these, *bla*$_{\text{NDM-1}}$ positive organisms were detected in two tap water samples and twelve seepage samples while none was found in sewage samples. Most of the positive strains, particularly Enterobacteriaceae, bore the *bla*$_{\text{NDM-1}}$ gene on transferable plasmids; however, *Vibro cholerae* and *Aeromonas caviae* each had a chromosomally encoded *bla*$_{\text{NDM-1}}$ gene. This finding by Walsh et al. is poignant and highlights the imminent challenge of community associated carbapenem resistance if adequate mitigation measures are not taken.

Expanded spectrum β-lactam resistance in Enterobacteriaceae of food animal origin in the U.S

This section of the dissertation will focus on the extent and health impact of newer generation cephalosporin resistance in Enterobacteriaceae of food animal origin within the U.S. Fey et al. (2000)), concluded from their study, including an investigation of a Nebraska child with a *Salmonella* infection exhibiting ESC resistance (due to a

CMY-2 gene), that this resistance originated from livestock production as a result of prevalent use of β-lactam antimicrobials [83]. At the time of their report, acquired resistance to ESC was largely unknown in the country; meanwhile, the paltry number of described human cases were associated with foreign travel.

In the study of Fey et al., five isolates of *S. enterica* serotype Typhimurium variant Copenhagen were analyzed for relatedness by susceptibility testing, isoelectric focusing, pulsed field gel electrophoresis (PFGE), conjugation experiments and sequence hybridization. One isolate was recovered from the feces of a clinically ill 12-year old boy (the son of a veterinarian), and the others were isolated from fecal samples of sick cattle from his family's herds. Results showed that all the isolates were related; in fact, the isolate from the child as well as one of the animal isolates were basically replicas of each other. The two isolates appeared to share a plasmid (IncA/C) that accounted for the multi-drug resistance including resistance to cefoxitin, ceftriaxone and aztreonam; later, the presence of the plasmid-borne *bla*$_{\text{CMY-2}}$ gene was confirmed in both isolates [83]. This study's findings were truly significant because they confirmed definitively the potential for spread of resistant bacteria strains from livestock to humans via direct contact. Further, given the lack of direct contact with a sick animal by the child, a secondary acquisition of the resistant strain via the father by the child was deemed highly probable, indicating the potential for propagation and distant spread of resistant Enterobacteriaceae of animal origin. This case highlights the need for evaluation, monitoring and control of food animal AMR.

Consequently, the role and impact of ceftiofur (an animal-only third generation cephalosporin with molecular and antibacterial properties similar to ceftriaxone and cefotaxime) administration on ceftriaxone resistance in *E. coli* recovered from fecal samples of dairy cows was investigated by Tragesser et al (2006) [84]. Surprising to some, their study did not demonstrate an increase in ceftriaxone resistance along with ceftiofur usage at the individual animal level; importantly however, increased ceftriaxone resistance was observed at the herd level. Resistance to this class of antibiotics was observed more often in herds where the drug was used, versus where it was not. This observation demonstrates the importance of population clustering among livestock AMR bacteria spread through their shared local environment.

Similar to isolates obtained from human sources, the primary mechanism of Enterobacteriaceae ESC resistance in United States animal agriculture at the time, and as confirmed by Fey et al., was due to a plasmid-borne AmpC β-lactamase enzyme. However, in 2010 Wittum et al. described the first ESC resistance mediated by a CTX-M-type ESBL recovered from isolates of both healthy and sick dairy cattle in Ohio [85]. One hundred fecal samples and 18 banked surveillance isolates (*Salmonella* (n=16) and *E. coli* (n=2)) were tested for phenotypic resistance to cefotaxime and cefepime. Three fecal samples were positive for ESC-resistant Enterobacteriaceae, all positive isolates were *E. coli.* An *E. coli* strain from the banked isolates also was resistant; of greatest importance, the resistant isolates all showed susceptibility to cefoxitin and cefotaxime/BLI combinations. Resistance genotypes (*bla*$_{\text{CTX-M-1}}$ and *bla*$_{\text{CTX-M-79}}$ genes)

were determined by PCR, sequencing (of the PCR product) and trans-conjugation experiments.

Since the time of this initial description, evidence of increased CTX-M-type ESBL frequency among Enterobacteriaceae of animal origin in the United States has emerged. Tadesse et al. (2018) characterized a number of ESBL *E. coli* isolates: five isolates each of cattle and chicken (breast meat) origin, six isolates obtained from ground turkey and one isolate each from ground beef and pork chops, collected between the years 2011 and 2015 by NARMS [86]. Whole genome sequencing results showed that all isolates bore at least one variety of *bla*$_{\text{CTX-M}}$ gene; most frequently annotated was *bla*$_{\text{CTX-M-27,}}$ particularly from cattle and turkey isolates, while *bla*$_{\text{CTX-M-1,}}$ *bla*$_{\text{CTX-M-14}}$ and *bla*$_{\text{CTX-M-15}}$ also were encountered.

Although the challenge of ESC resistance in animal-origin Enterobacteriaceae appears to be expanding, given the increasing variety of described resistance mediators, the use of a carbapenem antimicrobial in livestock to combat this challenge has so far not been reported globally. This is no doubt in part because the carbapenems themselves have never been approved for use in animals. Nevertheless, gram-negative bacterial strains bearing transferable genes that modulate reduced susceptibility to carbepenems have been detected in livestock operations. In 2017, Mollenkopf et al. described the first recovery of carbapenemase-producing Enterobacteriaceae (CPE) from a livestock production facility in the United States [49]. Three *E. coli* and one *Proteus mirabilis* isolate that bore the *bla*$_{\text{IMP-27}}$ gene (later designated *bla*$_{\text{IMP-64}}$) on an IncQ1 plasmid were recovered from two out of 30 (7%) pig nursery environmental samples. Further, from

two farrowing rooms 11 of 24 environmental samples yielded multiple genera of Enterobacteriaceae bearing the *bla*$_{\text{IMP-27}}$ (*bla*$_{\text{IMP-64}}$) gene on the same plasmid. Of interest, finishing barn environmental samples, harvest-ready pig fecal samples and piglet anal swabs yielded no CPE strains.

The then common practice (extra-label, and arguably banned by the U.S. FDA) administration of ceftiofur on the first day of life to piglets, a second dose of ceftiofur to male piglets at castration and the labeled therapeutic use of the drug in sows with infectious conditions such as metritis was postulated to be the driver of the elevated CPE frequency in the farrow barn versus the absence in the settings with little ceftiofur use. These initial findings were supplemented with additional environmental sampling, sow fecal samples and piglet fecal swab sampling [48]. The follow up sampling results (published in 2018) showed sample prevalence of the *bla*$_{\text{IMP-64}}$ gene on an IncQ1 plasmid for farrow-barn environment, sow feces and piglet swabs as 64%, 14% and 18%, respectively. Of the samples from the nursery pen, only one yielded a CPE while no carbapenemase-producing strain was recovered from the finishing ban.

The authors reasonably opined that the ESC regularly administered in the farrow barn may be providing the selection pressure for the carbapenem resistance. Although plausible, empirical evidence of this as well as the extent of this selection effect, particularly in the absence of an ESBL-enzyme co-selection factor in the bacteria, is not available. Our present study is focused on bridging this evidence gap through a series of experimental and mathematical modeling methodologies. This approach is the subject of the review in next section.

Experimental *in vitro* approaches to the challenge of beta-lactam resistance in Enterobacteriaceae

Innovative applications of *in vitro* culture methodologies in bacteria AMR research, both static and dynamic, will be explored in this section. The classic *in vitro* batch culture method has been applied in AMR research perhaps from its inception. Many clever modifications of traditional culture protocols have been developed to mimic the *in vivo* microbial ecology and to estimate expected changes based on perturbations of the ecosystem.

Negri et al. (1993) tested the effect of varying concentrations of amoxicillin, cefixime, cefuroxime and cefotaxime on a mixture of *S. pneumoniae* strains [87]. The strains differed by their resistance levels (recorded as MICs) to β-lactam antimicrobials. Of the four clinical isolates (RYC28551, RYC28057, RYC09982, and RYC28543) employed, one was generally susceptible to β-lactams. The creative protocol adopted for the study included creating a 10mL mixed-culture, from suspensions (~10^8 CFU/mL) of individual strains; 8.89mL of strain RYC28551 (the most susceptible strain) suspension, 1ml of strain RYC28057 suspension, 0.1ml of a strain RYC09982 suspension, and 0.01 mL of a strain RYC28543 suspension. The resulting effective strain proportions were 90%, 9%, 1% and 0.1% respectively, similar to expected strain prevalence values in human upper respiratory tracts (URT). Aliquots of the mixture were subsequently incubated under usual experimental conditions in plain broth (brain heart infusion (BHI)); as well as in varying concentrations of antimicrobials: 0.015, 0.5, 1, and 2 μg/mL amoxicillin; 0.03, 0.5, 1, and 4 ug/mL of cefuroxime; 0.25, 1, 4, and 16 ug/mL of

cefixime; and 0.015, 0.25, 0.5, and 1 μg/mL of cefotaxime. A commercial β-lactamase enzyme was added to antimicrobial supplemented cultures at four hours to abort their effects; next, a fresh BHI broth subsequently was inoculated with an aliquot of each culture and carried on to a 24-Hour incubation.

This was a clever approach by the researchers aimed at addressing the challenge of variable antimicrobial deterioration rates in extended culture environments; further, it aided in preventing a carry-over antimicrobial effect on phenotypic estimation when the mixed cultures were re-plated. In their findings, the composite strains were found to lack mutual interference or competitive inhibition when cultured absent an antibiotic selection pressure. This was important to discern before attributing negative effects on any given strain to antibiotics themselves. Higher starting concentrations, as well as more potent β-lactam antimicrobials, exhibited more effective selection for bacteria with higher MIC values, on occasion depleting susceptible strains to below the limit of detection.

The study of Negri et al. provided evidence that different generations of β-lactam antimicrobials can favorably select for potentially pathogenic and resistant gram-positive commensal strains in the URT of humans. Later, this same team of researchers employed a similar *in vitro* model in their study of resistant *Enterobacter cloacae* selected from a mixed-culture by ceftazidime and cefepime [88]. They prepared a mixture comprising a wild-type ESC susceptible strain (99.75%) and an AmpC derepressed strain (0.25%). The range of antimicrobial selection concentrations for resistant strains was: 1 – 4096 μg/mL for ceftazidime and 0.12 – 16 μg/mL for cefepime. Negri and Baquero concluded

that *in vivo* therapeutic dosages such as a 2g, thrice daily, cefepime treatment may be adequate for deselecting mutant derepressed AmpC strains. Plasmid-encoded enzymes have been the prominent mediator of AmpC-type resistance in clinical strains, importantly, these enzymes generally demonstrate low cefepime hydrolysis. Nonetheless, the value of the suggested cefepime regimen in derepressed chromosomally-mediated AmpC-type resistance may be significant for preserving the clinical efficacy of carbapenem β-lactams. The inexpensive and relatively uncomplicated nature of *in vitro* batch culture methodologies makes them of great utility in microbial research. The static nature of this method is, however, an obvious limitation particularly regarding modeling the reality of much more complex *in vivo* conditions.

A dynamic (media) anerobic culture system (chemostat) may better model *in vivo* peristalsis, metabolism, acid-base levels and pharmacokinetic changes. Wu and Livermore (1990) applied a chemostat in their study of: i) bacteria growth rate and antimicrobial killing, ii) antimicrobial selection of β-lactamase enzyme producers, and iii) post antimicrobial effects [89]. Five varieties of clinically acquired *P. aeruginosa* strains: an inducible chromosomal β-lactamase (M2297P) strain, a derepressed AmpC enzyme producer (M2297) strain, an intrinsic imipenem 'resistant' mutant (M2297I) strain, and a '2-pre' and a '4-pre' imipenem sensitive strain, were employed. When equal volumes of M2297 and M2297P active cultures were mixed in a single chemostat system for two weeks, absent a β-lactam antimicrobial, no population advantage by either strain was observed. Furthermore, continuous infusion of imipenem at 10 μg/mL uniformly suppressed the M2297I strain and its parent strain in a mixed-culture. These

outcomes connote that the genetic adaptions observed in these strains do not impose a fitness cost on them; such an observation is consistent with expected results for chromosomally determined antimicrobial adaptations which are believed to be well-evolved.

In a single-strain chemostat experiment, cefoxitin induction of β-lactamase enzyme production by the M2297P strain correlated well with bacterial growth rate. This underscores the efficiency of cefoxitin at inducing AmpC-type β-lactamase in strains with such capabilities, and the consequent therapeutic failure likely to follow *in vivo*. In this study, antimicrobials were added to cultures intermittently or by automated continuous drug infusions; these adaptations can be of great value in simulating representative *in vivo* pharmacokinetics. Researchers have also utilized the chemostat apparatus in the study of long-term homeostatic *in vivo* systems and adaptive changes that follow a perturbing event. For instance, Poole et al. (2001) using the chemostat system studied the establishment (colonization) rate of a highly resistant exogenous bacterium in an ongoing recombined pig fecal culture (known as RPCF) [90]. This continuous maintenance of mammalian normal bacteria flora replica *in vitro*, affords researchers a unique opportunity to investigate the characteristics of stable dynamic systems and the impact of novel variables on these systems for prolonged periods of time. When Poole et al. challenged the RPCF with a vancomycin-resistant *Enterococcus* (VRE) strain (7.0 $\log_{10}$ CFU/mL), the challenge strain was cleared from the system within seven days. Introduction of various concentrations of vancomycin in the RPCF showed reduced VRE clearance in parallel with drug concentrations; for example,

clearing rates of 0.94 log_{10}/day, 0.52 log_{10}/day and 0.53 log_{10}/day were recorded at 0.001 μg/mL, 0.01 μg/mL, 0.1 μg/mL vancomycin concentrations, respectively. These results demonstrate the potential protective effect of established intestinal commensals, in particular the obligatory anaerobes, against potentially deleterious exogenous organisms.

Mathematical PK-PD modeling of *in vivo* antimicrobial effects on bacterial populations

Systems models that couple pharmacokinetic and pharmacodynamic data have been successfully used in both AMR and clinical drug therapy research; in this section the most pertinent of such research to our own study will be reviewed. Ahmad et al. (2016), implemented a PK-PD mathematical model in their study of intra-muscular ampicillin dosing regimens and their effect on intestinal *E. coli* populations (β-lactam susceptible and resistant) among nursery pigs [91]. Fifty *E. coli* strains with known MIC values were included in their study, data for their inhibitory PD (Emax) model was obtained via *in vitro* bacteria growth/kill rates in ampicillin media cultures (ampicillin concentrations: 0.125 – 1024 μg/mL) relative to plain broth. Published pig plasma ampicillin concentrations were utilized as surrogate PK data for intestinal β-lactam concentrations. In their study, baseline ampicillin resistance was set at 30%, starting intestinal density per strain varied between 10^6 and 10^9, and the peak intestinal carrying capacity was set at 10^{10}. Fractional bacterial excretion and uptake were accounted for by the model; lastly, the intramuscular ampicillin treatment regimen varied by dosing frequency and treatment duration.

The multi-scenario adaptability of this approach, as demonstrated in their study, may be quite beneficial owing to its cost effectiveness. Although it may be argued that intestinal ampicillin concentration is likely over estimated from the plasma concentration surrogates employed by the study, this is standard practice when data availability is limited. The outcome of the model suggests that longer treatment durations increases resistant bacteria prevalence and quantity; in contrast, the frequency of antimicrobial dosing was not associated with elevated resistance. Increased intestinal excretion by the pig during antimicrobial therapy was associated with increased levels of resistance and a quicker return of resistance prevalence to pre-treatment levels when treatment was discontinued. Based on the model, it was concluded that when effective as a treatment option, antimicrobial therapy is preferably kept short to reduce the extent to which AMR bacteria prevalence remains elevated and thus reduce adaption of the resistant bacteria and increased baseline prevalence.

Volkova et al. (2011) similarly investigated cattle intestinal commensal bacteria and those factors affecting third generation cephalosporin resistance using a related mathematical model [92]. Unlike Ahmad et al., the PD data for ceftiofur susceptible and resistant *E. coli* were retrieved from published literature along with plasma PK data. This study uniquely modeled actual intestinal PK values through gall bladder excretion fraction and excretion patterns, intestinal transit time and intestinal volume. This detailed approach may further increase model outcome accuracy and better predict observed live animal values. Additionally, variables such as plasmid transfer rate, fractional bacteria inflow/outflow rates, susceptible/resistant bacteria ratio and maximum bacteria carrying

capacity were accounted for in this study. Common parenteral ceftiofur treatment regimens were modeled (using two formulations commonly employed in beef and dairy production); the model output suggested that absent a β-lactam antimicrobial a stable ESC-resistant bacteria fraction would be maintained by horizontal plasmid sharing, clonal expansion and continued environmental ingestion of resistant strains (e.g., from the manure pack). All tested regimens of ceftiofur therapy in Volkova et al. showed absolute and relative increases in the resistant bacterial population. From the model, the proportion of intestinal 3GC resistant strains did not return to pre-treatment levels until five weeks after the commencement of a 5-day short-duration formulation ceftiofur therapy.

These findings are akin to results from observational studies, thereby highlighting the utility of such approaches. As an illustration, Græsbøl et al. (2014), developed a PK-PD model of multiple animals, their environment and their bacterial microbiota [93]. The researchers asked a number of questions in their study such as: 1) how are resistant bacteria levels sustained in pig intestine despite reduced fitness, 2) what is the nature of co-existence of resistant bacteria varieties, 3) does strain variety influence bacterial populations in a pig pen, and 4) what is the effect of antimicrobial introduction on the prevailing microbial ecology? The outlined inquiries required not only a PK-PD model of bacterial population in a pig intestine, but also a multi-animal gut model, as well as incorporation of the pen environment that functions as a connecting reservoir of bacteria exchange among animal populations. Consequently, a multi-strain multi-animal PK-PD model was constructed; of note, the environmental

component of the model was defined as the sum of bacterial excretion by the pen population reflecting the role of animal manure as a component of the pen. A fixed amount of these, fractioned by pig population was defined as bacteria intake. Similar to Volkova et al. (2011), estimates of model inputs were sourced from existing literature. Model execution indicated that factors of bacterial excretion and subsequent ingestion (by self and others) played highly important roles in ensuring spread and sustainability of resistant strains among animals. Initial constituent strain diversity and population were also important. Most importantly, antimicrobial use was found to significantly increase resistant bacteria prevalence and to promote its sustainability in the pens and animal populations.

In conclusion, the characteristics of the foremost enzymes that mediate ESC resistance among Enterobacteriaceae, the scope of the challenge and the primary contributory factors to their spread were reviewed in this chapter. Efforts to get ahead of this challenge, in particular, using experimental approaches for improved understanding of the driving forces were also critically reviewed. These approaches (i.e., experimental static and dynamic *in vitro* models, as well as PK/PD mathematical models) will be combined in this dissertation to explore the potential role of older beta-lactam antibiotics classes in selecting for newer-generation higher importance beta-lactamase-producing Enterobacteriaceae.

CHAPTER III

BACTERIAL FITNESS ESTIMATIONS AND MIXED-STRAIN BATCH CULTURE ASSAYS

Introduction

The CDC has identified bacterial resistance to antimicrobials as an urgent public health crisis [3]. AMR among bacteria has increased over the years; it has been noted that this trend directly correlates with the total quantity of antimicrobials in general use [94]. Infections due to resistant bacteria are major causes of clinical treatment failures, reduced therapeutic options, increased morbidity and mortality and economic loss. For instance, an estimated 2.8 million cases of antibiotic resistant (AR) bacterial infections occur in the United States each year, with an associated mortality of about 35,000 persons [95] and a collective annual economic loss of approximately $55 billion [3].

β-lactam antibiotics are a widely used, broadly categorized group of clinically important antimicrobials; in fact, the World Health Organization (WHO) classifies most members of these group as either highly important or critically important for human medicine [96]. Penicillin compounds and a handful of cephalosporins are approved in the U.S. for treating, controlling and preventing livestock infections such as bovine respiratory disease, mastitis and metritis and porcine respiratory disease and metritis. The use of carbapenems in livestock has never been approved; however, carbapenem-resistant strains of the Enterobacteriaceae family are increasingly being isolated in livestock operations worldwide. Although it has been suggested as a contributing factor, no data yet exist to support the hypothesis that already approved β-lactams might increase the prevalence of CRE/CPE bacteria in animal agriculture [41-45].

The objective of this study was to determine the extent, if any, to which older-generation β-lactam antibiotics can differentially select for the highest priority antibiotic resistance (e.g., ESC and carbapenems, respectively) among representative Enterobacteriaceae. We pursue this through characterization of individual strain growth and fitness in antibiotic and non-antibiotic media. Additionally, we aimed to assess the selection patterns of ampicillin and ceftiofur at different *in vitro* concentrations on a 5-group mixture of host-adapted *E. coli* strains; each strain ideally bearing one, but not a combination, of beta-lactamase genes: *bla*$_{\text{TEM-1}}$, *bla*$_{\text{CMY-2}}$, *bla*$_{\text{CTX-M-}*}$, or *bla*$_{KPC/IMP/NDM}$, as well as comparing to beta-lactamase-free strains.

Materials and methods

Bacterial strain selection

Bacteria used in this study were sourced either from our own strain collection , from the Tom Wittum lab at The Ohio State University, or from the jointly sponsored U.S. Centers for Disease Control and Prevention (CDC) and Food and Drug Administration (FDA) Antimicrobial Resistance (AR) Isolate-Bank [97]. Isolates from our own collection and those of Dr. Wittum were characterized as to antimicrobial resistance, both phenotypically and genotypically, in previous research projects. Detailed methodologies employed in those characterizations have previously been published [48, 49, 98]. For carbapenem-producing Enterobacteriaceae (CPE), initial isolation of the bacterial strains from field samples was through supplemented MacConkey broth containing meropenem at 0.5 µg/mL and zinc sulfate heptahydrate 70 µg/mL [48].

Briefly, minimum inhibitory concentrations (MIC) of antimicrobial susceptibility which are routinely monitored in Enterobacteriaceae were determined for the isolates using the broth micro-dilution method. The Sensititre^{TM} system (TREK, Thermo Scientific Microbiology,

Oakwood Village, OH) was employed, using gram-negative CMV3AGNF custom panels designed for the U.S. National Antimicrobial Resistance Monitoring System (NARMS); for some isolates, extended-spectrum beta-lactamase ESB1F panels (TREK, Thermo Scientific Microbiology, Oakwood Village, OH) also were used to further characterize the beta-lactam phenotypic susceptibility (i.e., AmpC versus ESBL versus carbapenemase). Bacterial antimicrobial testing was performed in accordance with published NARMS protocols [99]. Outcomes expressed as antimicrobial minimum inhibitory concentrations (MIC) were interpreted according to the clinical interpretative human breakpoint values recommended for *E. coli* by the Clinical Laboratory Standards Institute (CLSI) as listed below, or else as their raw values befitting the type of the statistical analysis performed [100].

Bacterial genotypes were determined in our laboratories through short-read whole genome sequencing. In brief, bacterial genomic DNA was extracted with the QIAamp DNA extraction kit on the QIAcube HT automated platform (QIAGEN, Valencia, CA) while library preparation was with the Illumina Nextera XT or DNA Flex Kits (Illumina Inc, San Diego, CA). Sequencing runs were performed with the MiSeq Reagent Kit v3 paired-end reads (2 x 300 bp) on the Illumina MiSeq instrument (Illumina Inc., San Diego, California). Post-run bioinformatic analyses were performed on the BaseSpace Sequence Hub (Illumina Inc., San Diego, California). Depending on the source of the isolate, nucleotide sequence reads were assembled with either Velvet *de novo* or SPAdes genome assembler software [101, 102]; bacteria sequence types were determined with the SRST2 Basespace application (Illumina Inc., San Diego, California).

Initial antimicrobial resistant gene annotation was with the Antibiotic Resistance Gene-ANNOTation database (ARG-Annot) or ResFinder [103] and plasmid type annotation was via PlasmidFinder [104]. Genotypic and phenotypic characterizations of isolates obtained from the

CDC/FDA AR Isolate Bank were as published on the agency website and accessible from the National Center for Biotechnology Information (NCBI) portal [97]. For the purpose of uniformity in data presentation, and to further ascertain prior strain annotations from the aforementioned sources, raw reads of strains used in this study were again, either pulled from NCBI (National Center for Biotechnology Information, U.S. National Library of Medicine) SRA (sequence read archive) [105] or from our archive. Nucleotide sequence reads were assembled using SPAdes ver.3.11.1 genome assembler softwares [102]; bacteria sequence types were determined with the SRST2ver.0.2.0 [106] and Multi-Locus Sequence Typing (MLST) database of *Escherichia coli* (accessed on May, 2020) [107]. Antimicrobial resistant gene and plasmid annotation was performed using ABRicate ver.0.8.7 [108] and ResFinder [103] and PlasmidFinder [109], databases (both accessed May, 2020). Corresponding sequence type, genotypic and phenotypic antimicrobial resistance profile, and plasmidal information of the isolates included in this study are provided under the given bioproject and biosample accession numbers in document appendix (Appendix A).

When possible, the choice of *E. coli* strains of swine origin was prioritized over other bacterial sources, swine being our exemplar food animal host. When this was not possible, strains from other livestock, human patients, or else the environment were selected. *E. coli* strains were initially selected according to the presence of plasmid-borne genes encoding beta-lactamases, or else a complete lack thereof. Isolates identified from this screening process were then stratified by the presence of their beta-lactamase genes; that is, $bla_{\text{TEM-1}}$ or $bla_{\text{CMY-2}}$ or $bla_{\text{CTX-M-*}}$, or $bla_{KPC/IMP/NDM}$, or none, but avoiding to the extent possible those strains with a combination of *bla* genes. Each group of the selected resistance genes corresponded to a known spectrum of hydrolytic activity against beta-lactam antibiotics.

Twenty isolates were selected per *bla*-positive group or *bla*-negative control group. Ideally, 30 strains of each group would have been the sample size to achieve near-normal $\log_{10}$ transformed distribution of target quantitative outcomes under the Central Limit Theorem; however, strains with only a single *bla* gene were limited in the sources accessible to us. Even more so for the CPEs; consequently, presence of a single *bla* gene was achieved only for ten percent of the carbapenemase producing strains (see Appendix B). Study strains were allowed to possess genes encoding resistance to other classes of antimicrobials. These genes were not expected to impact the beta-lactam antimicrobial resistance of the strains in the absence of co-selection pressures; for example, genes conferring tetracycline, aminoglycoside and sulfonamide resistance were commonly identified among all strain groups.

Bacterial growth curve estimation

To assess the growth rates of each bacterial strain absent and under different antibiotics and their concentrations, and to estimate the within- and among-*bla*-group growth fitness parameter differences, bacterial growth curves were estimated with the Bioscreen C™ Automated Microbiology Growth Curve Analysis System (Growth Curve Ltd, Helsinki, Finland). Bacteria from pure culture and preserved on cryobeads at -80ºC were streaked onto Trypticase™ soy agar with 5% sheep blood (Becton, Dickinson and Company, Sparks, MD) and incubated at 37ºC overnight. A 0.5 McFarland standard (Sensititre™ Thermo Fisher Scientific, Waltham, MA) bacterial suspension was made for each isolate (i.e., to a bacterial concentration of ~1.5 x 10^8 CFU/mL), by suspending one or two colonies selected from the overnight plate growth in demineralized water (Thermo Fisher Scientific, Waltham, MA). A 1:10 mixture of the bacterial suspension (120 ul) in cation-adjusted Mueller-Hinton II (CAMH-2) broth (Thermo

Fisher Scientific, Waltham, MA) (1,080 ul) in a 1.5 mL black sample tube was then made (final bacterial concentration of ~1.5 x 10^7 CFU/mL). From this bulk mixture, 300ul aliquots in triplicate were dispensed into each 10*10 honeycomb plate well (Growth Curves USA, Piscataway, New Jersey, USA) for each strain; thereafter, automated optical density (OD) estimates were obtained at 420-580nm (wideband) over 48 hours at 37^{o}C. Measurements (OD) were taken every 10 minutes following moderate agitation of the incubating cultures. For each experiment, a single QC strain *E. coli* ATCC 25922 (American Type Culture Collection, Manassas, VA) in triplicate wells, and two wells with plain CAMH-2 broth, were included as positive and negative controls, respectively.

The effects of ampicillin (an aminopenicillin), ceftriaxone (a third-generation cephalosporin (3GC)), and meropenem (a carbapenem) on the growth parameters of each strain in each of the corresponding *bla*-groups were estimated. The tested concentration of each antimicrobial corresponded to the human clinical resistance breakpoint value for the drug-bacteria species MIC as recommended by the CLSI [100]. Specifically, the concentrations were 32 µg/mL of ampicillin sodium, 4 µg/mL of ceftriaxone disodium, or 4µg/mL of meropenem trihydrate; to achieve this, high purity forms of the antimicrobials (Sigma-Aldrich Inc., St. Louis, MO) dissolved in CAMH-2 broth were used to prepare the tested concentrations. Ceftriaxone (a commonly used 3GC in human medicine) was chosen for this *in vitro* assay to represent potential selection risks in the human host, as opposed to utilizing ceftiofur which is only approved for use in food animals.

Bacterial competition assays

From each resistance group (i.e., no *bla* genes, – but with a tetracycline resistance gene, *tet*(B) – *bla*$_{\text{TEM-1}}$, *bla*$_{\text{CMY-2}}$, *bla*$_{\text{CTX-M-*}}$, or *bla*$_{\text{KPC/IMP/NDM}}$), two representative strains were selected for competition studies. A 0.5 McFarland (Sensititre™ Thermo Fisher Scientific, Waltham, MA) standardized bacterial suspension was prepared for each selected isolate as described above. An equal mixture of all ten strains (1 mL of each suspension) was prepared (expected density of each strain in the mixture was ~1.5 x 10^7 CFU/mL). A 1:10 dilution of the mixture in CAMH-2 broth, as well as in CAMH-2 broth with various antibiotic concentrations (expected density of each strain ~1.5 x 10^6 CFU/mL), was made. The antimicrobial concentrations were ampicillin sodium (Sigma-Aldrich, St. Louis, MO) at 2, 4, 8, 16 or 32 µg/mL and ceftiofur hydrochloride (Zoetis Inc., Parsippany-Troy Hills, NJ) at 0.5, 1, 2, 4, or 8 µg/mL. Ceftiofur was chosen for this assay to represent the 3GC actually used in veterinary medicine; that is, to directly mimic the selection pressure occurring *in vivo* in farm animals.

Triplicates of the mixed cultures were incubated at 37°C for 24 hours in the Bioscreen C™ Automated Microbiology Growth Curve Analysis System, as previously outlined. The post-growth density of each strain group was estimated phenotypically by the spiral-plate method (Eddy Jet 2™ Spiral-Plater, Neutec Group Inc., NY); to achieve this, after 24 hours of incubation the mixed cultures were spiral-plated onto MacConkey agar plates infused with one of tetracycline - 16 µg/mL, ampicillin - 32 µg/mL, cefoxitin - 32 µg/mL, ceftriaxone - 4 µg/mL, ceftriaxone - 4 µg/mL with clavulanate - 4 µg/mL, cefepime - 8 µg/mL, or meropenem - 4 µg/mL [100]. The bacterial culture dilutions for spiral-plating were performed as necessary on ice beds (to halt bacterial growth). The agar plates were read on an automated colony counter (Flash & Go™, Neutec Group Inc., NY) to obtain estimates of the bacterial density in colony

forming units (CFU)/mL of the mixed culture as of 24 hours of incubation. The strain group(s) whose density was assessed using each of the selective agars is listed in Table 1. A fresh preparation of experimental bacterial mixture was also plated as outlined above.

Table 1. Antibiotic supplemented media with presumed *E. coli* genotypes and phenotypes selectively grown on each type.

MacConkey agar with antibiotic plates[a]	***E. coli* resistance genotype expected to grow on the plates**	***E. coli* resistance phenotype expected to grow on the plates**
Tetracycline (16 µg/mL) – (MAC+TET)	*tet*(B)	All tetracycline resistant strains
Ampicillin (32 µg/mL) – (MAC+AMP)	*bla*$_{\text{TEM-1}}$, *bla*$_{\text{CMY-2}}$, *bla*$_{\text{CTX-M}}$, *bla*$_{\text{KPC/IMP/NDM}}$	All beta-lactamase producing strains
Cefoxitin (32 µg/mL) – (MAC+FOX)	*bla*$_{\text{CMY-2}}$, *bla*$_{\text{KPC/IMP/NDM}}$	AmpC + CPE
Ceftriaxone + clavulanic acid (4 µg/mL + 4 µg/mL) – (MAC+AXOCLAV)	*bla*$_{\text{CMY-2}}$, *bla*$_{\text{KPC/IMP/NDM}}$	AmpC + CPE
Ceftriaxone (4 µg/mL) – (MAC+AXO)	*bla*$_{\text{CMY-2}}$, *bla*$_{\text{CTX-M}}$, *bla*$_{\text{KPC/IMP/NDM}}$	AmpC + ESBL + CPE
Cefepime (8 µg/mL) – (MAC+PIME)	*bla*$_{\text{CMY-2}}$, *bla*$_{\text{CTX-M}}$, *bla*$_{\text{KPC/IMP/NDM}}$	ESBL + CPE
Meropenem (4 µg/mL) – (MAC+MERO)	*bla*$_{\text{KPC/IMP/NDM}}$	CPE

AmpC, molecular class C-type beta-lactamase; ESBL, extended spectrum beta-lactamase; CPE, carbapenemase-producing Enterobacteriaceae.
[a]The antibiotic concentrations correspond to human clinical interpretive breakpoints, as recommended by the CLSI as of the date of experimentation [100]

Growth curve analyses

Data from the EZexperiment™ software (Growth Curves Ltd, Helsinki, Finland) were transformed from wide to long format based on the time of incubation (i.e., in 10-minute intervals from time=0) in Stata version 15.1 software (Stata Corp, College Station, TX). Triplicate OD measurements were collapsed to their mean by strain at each time point to obtain a single representative value. Raw and mean measured OD of each bacterial strain were plotted against time. The isolates were stratified by resistance group and the maximum bacterial population growth rates were estimated, as well as the growth rates in the presence of each of the antibiotic concentrations.

To more precisely estimate the growth parameters, four nonlinear regression models were fitted to the OD-based growth curve of the bacterial groups grown in non-selective media, using the least squares method implemented in the Stata® 15.1 software (Stata Corp., College Station, TX). The four models were: 3-parameter Gompertz, 4-parameter Gompertz [110][65], 3-parameter logistic [111] and 3-parameter exponential [112]. Further, the relative fit of the models across all the bacterial resistance groups and experimental conditions (i.e., with and without antibiotics) was explored. The 3-parameter Gompertz (Equation 1) was found to most consistently provide the best fit (highest adjusted coefficient of determination (R^2)) for the growth curves, across all antibiotic concentration/bacteria-group combinations. Consequently, the bacterial growth rate (OD/Hour) estimates, after transforming time units from minutes to hours, were extracted from the 3-parameter Gompertz models fitted to these growth curves:

$$OD = \beta_1 * e^{(-e^{(-\beta_2*(t-\beta_3))})} \quad (1)$$

Where, t is time in hours, β_1 is the peak bacterial density (OD), β_2 is the estimated maximum growth rate (Δ OD per Hour) and β_3 is the estimated bacterial growth lag period (in

hours). Models were stratified by resistance gene group and the type of growth media (i.e., without and with different antibiotics at breakpoint concentrations). To obtain graphical representations of modeled growth rates, post-analysis non-linear OD marginal predictions were generated. Predicted OD were subsequently graphed against time in hours.

Strain group growth in the mixed cultures

Mixed-effect nonlinear regression models were fitted to the estimated density of each resistance group after 24 hours of incubation of the mixed-strain culture (the CFU/mL readings from the selective antibiotic MAC plates were $\log_{10}$ transformed to normalize the data prior to the analysis). Modeled fixed effects were the antibiotic type, the various concentrations of each antibiotic used in the growth media for the mixed culture, and the selective antimicrobial plate type. The experimental replicate was modeled as a random effect factor. Marginal mean predictions of the strain group densities from the model output, along with 95% confidence intervals, were determined and represented graphically. The analysis also was performed in Stata® version 15.1 software (Stata Corp., College Station, TX).

Results

Strain group fitness

In all strain groups, numerous strains demonstrated biphasic exponential growth patterns – suggesting a switch in energy source utilization from glucose to a different compound [113-115] when nutrient supplies became limited – while others did not (Figure 1). All groups exhibited a lag period prior to the exponential amplification of the OD value. Within-group similarity in the first phase of exponential growth was observed for strains with and without a

diauxic growth pattern. The onset of the second phase of exponential growth – when present – exhibited within- and among-group variation, as seen in Fig 1 with selected representative strains. The maximum OD value attained by any strain across all resistance gene groups was roughly the same (≈1.4), but the time taken to reach this point differed within and among groups.

The 4-parameter Gompertz and the 3-parameter exponential models showed the lowest R^2 values when fitted to the data (0.34 to 0.84 and 0.34 to 0.85 across the strains, respectively). The 3-parameter logistic (0.85 – 0.93) and 3-parameter Gompertz (0.86 – 0.93) models demonstrated the highest R^2 values. Thus, the 3-parameter Gompertz model was fitted to all the growth curves, and the model predictions were plotted and evaluated. When modeled in the absence of beta-lactam antibiotics, the estimated peak growth rate of the beta-lactam susceptible group [0.159 (OD/Hour), 95% CI: 0.152 – 0.166] was significantly higher than for the *bla*-positive groups (see Table 2 and Figure 2). Among the *bla* producers, the estimated growth rate of the carbapenemase producing *E. coli* in non-antibiotic media was highest [0.142 (OD/Hour), 95% CI: 0.136 – 0.149]; however, this estimate was not statistically significantly different from that of the TEM-type beta-lactamase group [0.139 (OD/Hour), 95% CI: 0.135 – 0.143], as indicated by the overlap of their respective 95% CIs. The AmpC-type beta-lactamase group and the ESBL producers exhibited statistically similar peak growth rate estimates [0.120 (OD/Hour), 95% CI: 0.116 – 0.124] and [0.127 (OD/Hour), 95% CI: 0.121 – 0.132]. Although the beta-lactam susceptible group showed a higher growth rate, among the beta-lactam resistant groups no fitness cost pattern along the gradient of the encoded resistance (from non-extended-to-extended or from extended-to-non-extended beta-lactam resistance) was observed.

In a similar fashion, the combined growth rates of the representative pair of strains of each group used in the mixed cultures were predicted (Table 3). Select representative raw data

line plots of OD-based growth curves from cation-adjusted Mueller-Hinton broth for 10 study strains (color-coded by the 5 resistance gene groups) are shown in Figure 1. These estimates show a 95% CI overlap among multiple group-based strain pairs; the pair of beta-lactam susceptible strains demonstrated the highest predicted growth rates [0.16 (OD/Hour), 95% CI: 0.15 – 0.17], an estimate non-statistically different from that of the carbapenemase producers [0.13 (OD/Hour), 95% CI: 0.12 – 0.15]. The predicted growth rates of the ESBL-producing pair [0.12 (OD/Hour), 95% CI: 0.12 – 0.12] were observed to be non-statistically different from that of the carbapenemase producers but statistically different from the beta-lactam susceptible strains. The lowest growth rate estimates were those of the *bla*$_{\text{TEM-1}}$ encoding strain pair [0.10 (OD/Hour), 95% CI: 0.08 – 0.11], and the AmpC-type beta-lactamase encoding strain pair [0.10 (OD/Hour), 95% CI: 0.09 – 0.11]. In general, the maximum growth rates of the representative pairs were not far removed from one another, suggesting a relatively similar intrinsic fitness absent an extrinsic antibiotic pressure. Notably, the estimated maximum bacterial density (peak OD) attained by the pairs appeared to differ across the groups. The ESBL-producers showed a significantly higher upper asymptote [1.49 (OD), 95% CI: 1.48 – 1.50], as did the beta-lactam susceptible strains [1.41 (OD), 95% CI: 1.40 – 1.43]. In decreasing order, the peak density estimates for the AmpC-type beta-lactamase pair, the carbapenemase producing pair and the *bla*$_{\text{TEM-1}}$ encoding pair were: 1.34 (OD), 95% CI: 1.31 – 1.37, 1.24 (OD), 95% CI: 1.22 – 1.27, and 1.15 (OD), 95% CI: 1.11 – 1.19, respectively.

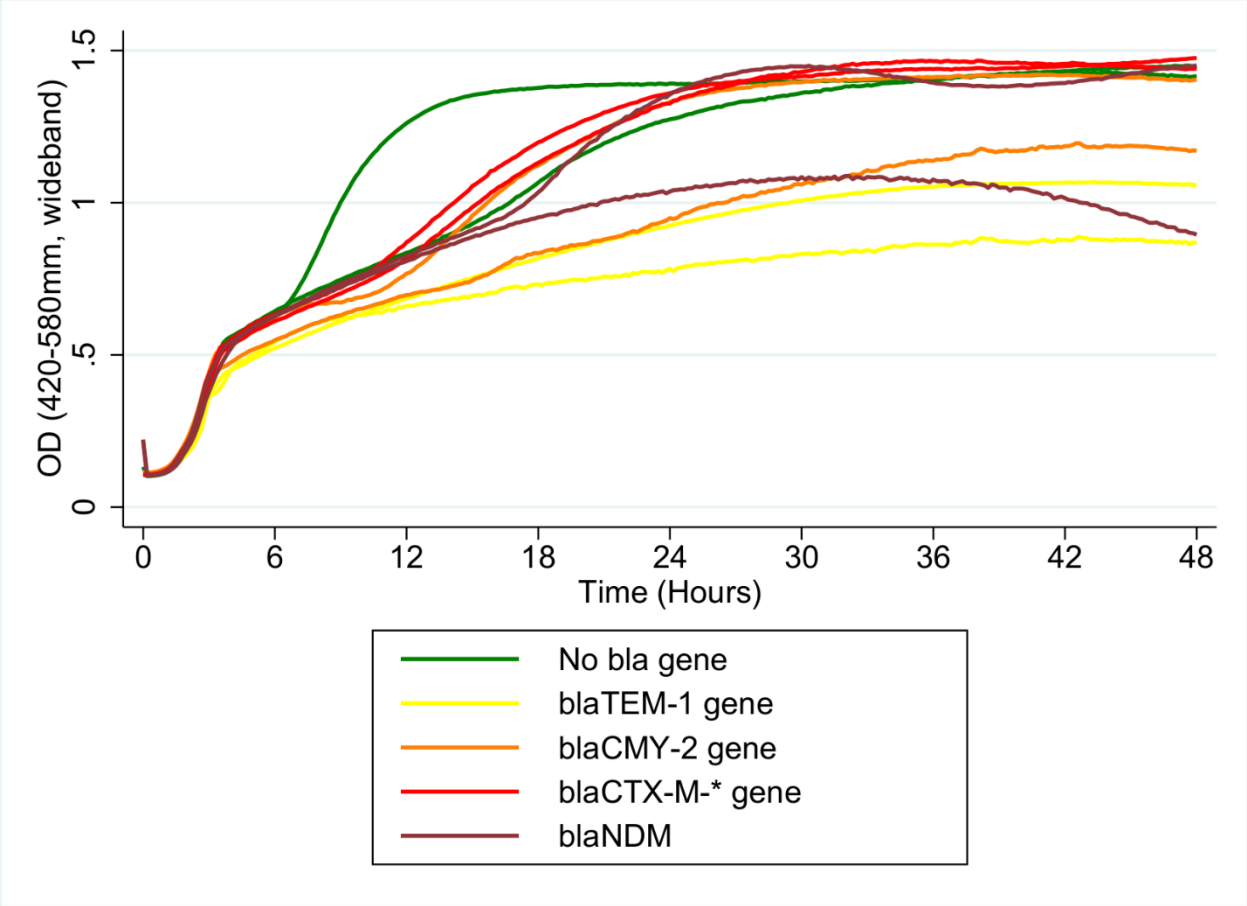


Figure 1. Raw optical density line plots for 10 individual strains[a] grown in cation-adjusted Mueller-Hinton II broth.
(Green) *E. coli* strains lacking any beta-lactamase encoding genes, (Yellow) *bla*$_{\text{TEM-1}}$ beta-lactamase encoding gene strains, (Orange) *bla*$_{\text{CMY-2}}$ beta-lactamase encoding gene strains, (Red) *bla*$_{\text{CTX-M-*}}$ beta-lactamase encoding gene strains, and (Maroon) *bla*$_{\text{NDM/IMP/KPC}}$ carbapenemase encoding gene *E. coli* strains.
[a]These same two selected strains per *bla*-gene group (color) were used in the multi-strain mixed-culture batch growth experiments.

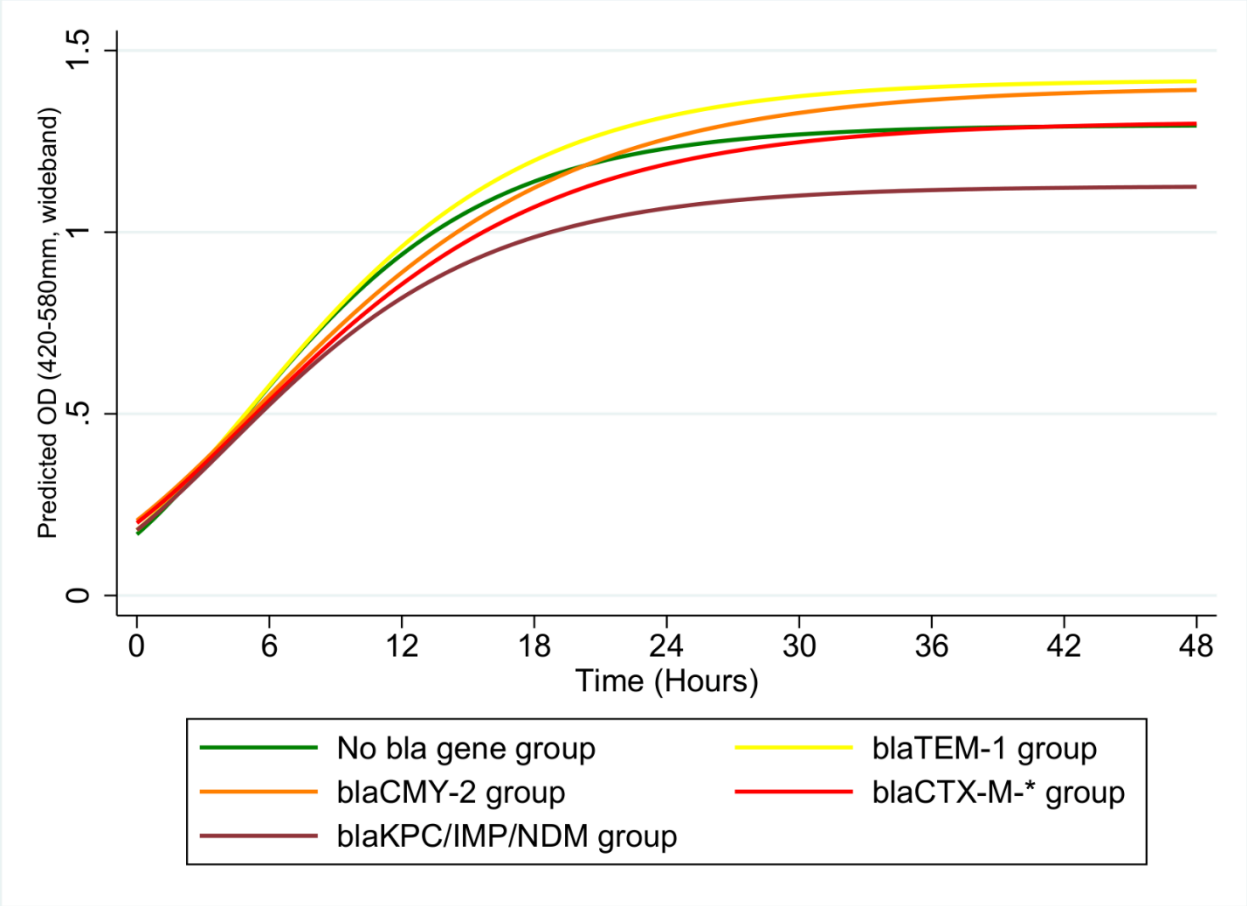


Figure 2. *E. coli* strains (n=20 / *bla*-gene group) 3-Parameter Gompertz fitted growth curves grown in CAMH-2 broth.
CAMH-2 (Cation-adjusted Mueller-Hinton II). (Green) *E. coli* strains lacking any beta-lactamase encoding genes, (Yellow) $bla_{\text{TEM-1}}$ beta-lactamase encoding gene strains, (Orange) $bla_{\text{CMY-2}}$ beta-lactamase encoding gene strains, (Red) $bla_{\text{CTX-M-*}}$ beta-lactamase encoding gene strains, and (Maroon) $bla_{\text{NDM/IMP/KPC}}$ carbapenemase encoding gene *E. coli* strains.

Table 2. Bacterial growth curve parameter values with 95% confidence intervals as estimated by a 3-parameter Gompertz non-linear model, across resistance gene group and by antibiotic type.

Growth Media		*NBL			*TEM-1			*CMY-2			*CTX-M-*			*CPE		
		coeff	***95% CI***		***coeff***	***95% CI***		***Coeff***	***95% CI***		***coeff***	***95% CI***		***coeff***	***95% CI***	
			Lower	Upper		Lower	Upper		Lower	Upper		Lower	Upper		Lower	Upper
No Antibiotic	[a]**b1**	1.305	1.296	1.315	1.419	1.412	1.426	1.400	1.391	1.409	1.318	1.307	1.329	1.094	1.085	1.103
	[b]**b2**	0.159	0.152	0.166	0.139	0.135	0.143	0.120	0.116	0.124	0.127	0.121	0.132	0.142	0.136	0.149
	[c]**b3**	4.676	4.487	4.865	5.225	5.099	5.352	5.414	5.261	5.567	5.089	4.876	5.302	4.043	3.828	4.257
Ampicillin (32 µg/mL)	**b1**				1.278	1.262	1.294	1.365	1.356	1.374	1.214	1.199	1.229	1.081	1.071	1.123
	b2				0.144	0.135	0.154	0.122	0.118	0.125	0.125	0.117	0.133	0.142	0.135	0.155
	b3				5.289	4.980	5.598	5.629	5.471	5.786	4.967	4.648	5.286	3.935	3.693	4.176
Ceftriaxone (4 µg/mL)	**b1**							1.011	0.968	1.054	1.161	1.143	1.179	1.087	1.077	1.096
	b2							0.059	0.052	0.066	0.133	0.122	0.144	0.135	0.128	0.142
	b3							5.608	4.784	6.432	4.703	4.303	5.102	4.056	3.809	4.303
	b1													1.024	1.002	1.045

Table 2. Continued

Growth Media		*NBL			*TEM-1			*CMY-2			*CTX-M-*			*CPE		
		coeff	*95% CI*		*coeff*	*95% CI*		*Coeff*	*95% CI*		*coeff*	*95% CI*		*coeff*	*95% CI*	
			Lower	Upper		Lower	Upper		Lower	Upper		Lower	Upper		Lower	Upper
Meropenem (4 µg/mL)	**b2**													0.083	0.078	0.089
	b3													8.944	8.523	9.365

Where growth parameters were not estimable using the models, such as for gene groups susceptible to the antibiotic in question, results are represented as missing data.
[a]Peak bacterial density (OD at 420-580mm, wideband).
[b]Estimated growth rate (OD/Hour).
[c]Estimated growth lag (hours).
*NBL – No beta-lactamase gene present, TEM-1 – *bla*$_{\text{TEM-1}}$ gene present, CMY-2 – *bla*$_{\text{CMY-2}}$ gene present, CTX-M-* – *bla*$_{\text{CTX-M-*}}$ gene present, CPE – carbapenemase-producing Enterobacteriaceae gene present (e.g., *bla*$_{\text{KPC}}$, *bla*$_{\text{NDM}}$, *bla*$_{\text{IMP}}$).

Table 3. Bacterial growth parameter values with 95% confidence intervals estimated by a 3-parameter Gompertz model, for strain pairs used in the batch competition assay.

bla *gene group*[1]	*Predicted growth rate (OD/Hour)*	*95% CI* Lower; Upper	*Estimated lag (Hour)*	*95% CI* Lower; Upper	*Peak density (OD)*	*95% CI* Lower; Upper
NBL	0.16	0.15; 0.17	4.79	4.55; 5.02	1.41	1.40; 1.43
TEM-1	0.10	0.08; 0.11	4.50	3.67; 5.34	1.15	1.11; 1.19
CMY-2	0.10	0.09; 0.11	5.82	5.33; 6.30	1.34	1.31; 1.37
CTX-M-*	0.12	0.12; 0.12	6.09	5.96; 6.25	1.49	1.48; 1.50
NDM	0.13	0.12; 0.15	4.56	4.09; 5.04	1.24	1.22; 1.27

*NBL – No beta-lactamase gene present, TEM-1 – *bla*$_{\text{TEM-1}}$ gene present, CMY-2 – *bla*$_{\text{CMY-2}}$ gene present, CTX-M-* – *bla*$_{\text{CTX-M-*}}$ gene present, NDM – carbapenemase-producing Enterobacteriaceae gene present (i.e., *bla*$_{\text{NDM}}$).

Effect of beta-lactam antibiotics on bacterial growth rates

The relative growth rates of the resistance-gene groups in the presence of beta-lactam antibiotics of different generations (i.e., at concentrations corresponding to the human clinical interpretive breakpoints for MICs of these drugs for *E. coli*) were assessed, to predict preferential selection by the antibiotics for the strains with studied resistance gene groups. As expected, the beta-lactamase-free strains registered no discernable growth in media with any of the three tested beta-lactam antibiotics. All strains from the four beta-lactam resistance gene groups showed no significant growth impairment in 32 μg/mL ampicillin (though with substantive 95% CI overlap of ampicillin-containing versus plain CAMH-2 broth growth rates, respectively: [0.139 (OD/Hour), 95% CI: 0.135 – 0.143] compared to [0.144 (OD/Hour), 95% CI: 0.135 – 0.154] for the TEM-1 beta-lactamase group; [0.120 (OD/Hour), 95% CI: 0.116 – 0.124] compared to [0.122 (OD/Hour), 95% CI: 0.118 – 0.125] for the AmpC-type beta-lactamase group; [0.127 (OD/Hour), 95% CI: 0.121 – 0.132] compared to [0.125 (OD/Hour), 95% CI: 0.117 – 0.133] for the ESBL group; and [0.142 (OD/Hour), 95% CI: 0.136 – 0.149]compared to [0.142 (OD/Hour), 95% CI: 0.135 – 0.155] for the CPE group (see Table 2 and Figure 3).

Correspondingly, the CPE group and the ESBL producers both demonstrated comparable robust growth in 4 μg/mL ceftriaxone and in plain CAMH-2 broth [0.127 (OD/Hour), 95% CI: 0.121 – 0.132] compared to [0.133 (OD/Hour), 95% CI: 0.122 – 0.144] for ESBL producers; and [0.142 (OD/Hour), 95% CI: 0.136 – 0.149] compared to [0.135 (OD/Hour), 95% CI: 0.128 – 0.142] for the CPE group; however, AmpC producers (i.e., harboring only $bla_{\text{CMY-2}}$) showed significantly reduced growth rates in the ceftriaxone-containing media. Comparing growth rates of the AmpC-type beta-lactamase group in plain CAMH-2 versus 4 μg/mL ceftriaxone broth, the predicted maximum growth rate dropped from 0.120 (OD/Hour), 95% CI: 0.116 – 0.124 to 0.059

(OD/Hour), 95% CI: 0.052 – 0.066 (see Table 2 and Figure 3). As expected, only CPE strains registered growth in 4 μg/mL meropenem; however, the presence of the drug at this concentration significantly altered the estimated peak growth rate of the strains from 0.142 (OD/Hour), 95% CI: 0.136 – 0.149 to 0.083 (OD/Hour), 95% CI: 0.078 – 0.089. The estimated duration of the lag phase of bacterial population growth in that media was also considerably prolonged [8.944 (hours), 95% CI: 8.523 – 9.365] when compared with such estimates in other tested beta-lactams (Table 2); so too was the peak OD value affected, significantly reduced to 1.024 (OD), 95% CI: 1.002 – 1.045 from 1.094 (OD), 95% CI: 1.085 – 1.103 (Table 2 and Figure 3d).

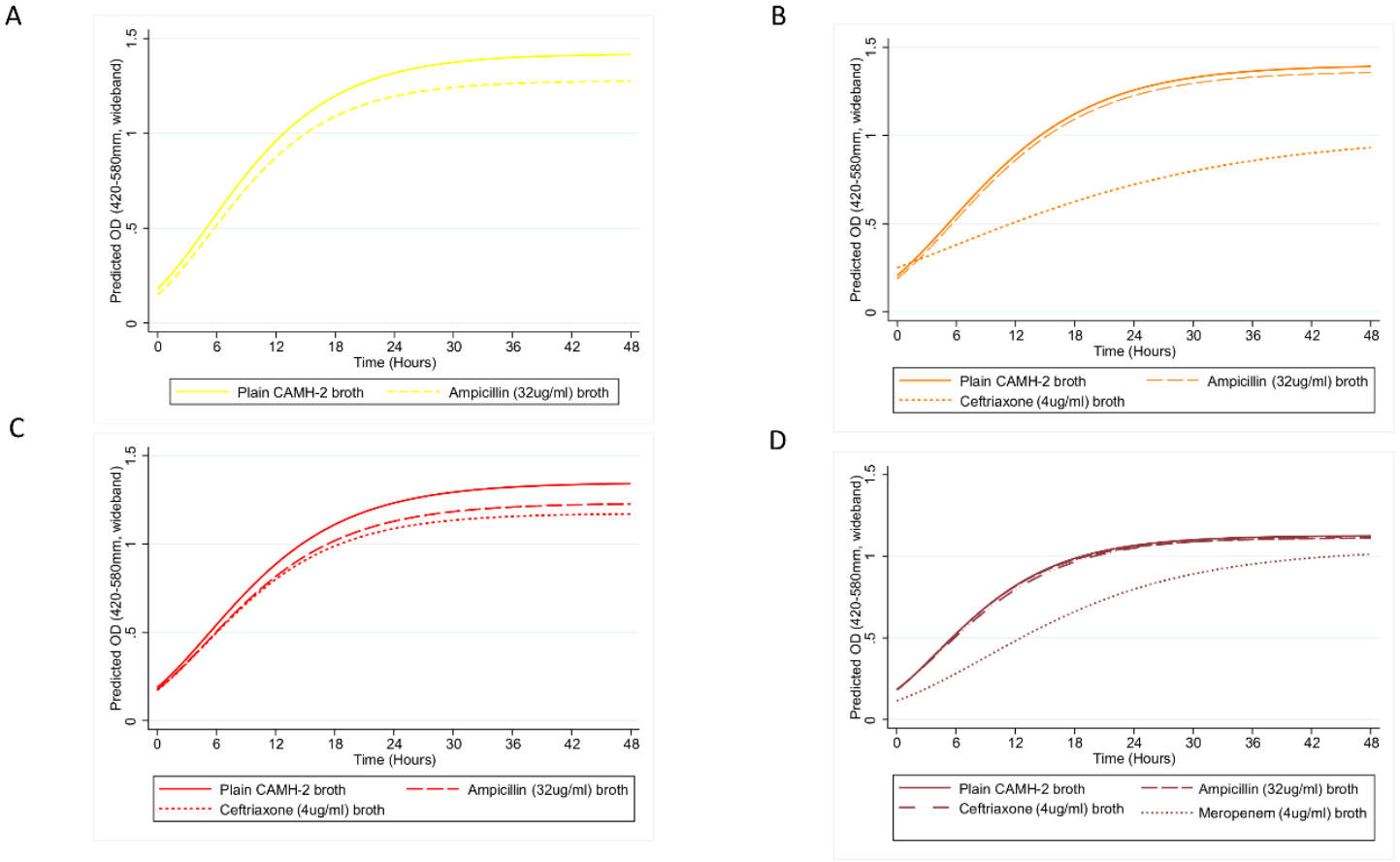


Figure 3. Growth curves of *bla*-gene positive *E. coli* resistance gene groups grown in beta-lactam antimicrobial broth, as estimated with a 3-parameter Gompertz model.

(a) *bla*$_{\text{TEM-1}}$ gene encoding strains in plain CAMH-2 broth and ampicillin (32 µg/mL) broth, (b) *bla*$_{\text{CMY-2}}$ gene encoding strains in plain CAMH-2 broth, ampicillin (32 µg/mL) and ceftriaxone (4 µg/mL) broths, (c) *bla*$_{\text{CTX-M-*}}$ gene encoding strains in plain CAMH-2 broth, ampicillin (32 µg/mL) and ceftriaxone (4 µg/mL) broths, (d) *bla*$_{\text{KPC/IMP/NDM}}$ encoding strains in plain CAMH-2 broth, ampicillin (32 µg/mL), ceftriaxone (4 µg/mL) and meropenem (4 µg/mL) broths. CAMH-2 (Cation-adjusted Mueller Hinton II).

Effect of beta-lactam antibiotics on mixed bacterial populations

Initial resistance-gene group populations grown on the selective antimicrobial MAC plates (Table 1) using the starting mixture of select representative strains (see Table 1 for legend) provided CFU estimates for MAC+TET, MAC+AMP, MAC+FOX, MAC+AXO, MAC+AXOCLAV, MAC+PIME and MAC+MERO plates equivalent to 91.5%, 95.3%, 19.4%, 64.7%, 37.8%, 14.7%, and 10.7%, respectively, of the total CFU estimate from the non-selective MAC plate (Table 4). Assuming a simple substitution model with no compensatory growth through to nutrient capacity, and no negative antibiotic effects on growth of resistant strains, the expected proportion of the CFU estimate on each plate type – relative to those on the plain MAC– would have been: MAC+TET plate (~ 100%), the MAC+AMP plate (~ 80%), MAC+FOX (~40%), MAC+AXO (~60%), MAC+AXOCLAV (~40%), MAC+PIME (~40%) and MAC+MERO (~20%). The observed data provide an experimental baseline reference for our phenotypic quantification methodology. Upon incubation of the starting 10-strain (5-gene groups x 2 strains per group) bacterial consortium for 24 hours in plain CAMH-2 broth, the relative proportions of CFU on the selective antibiotic plates were: MAC+TET plate (~ 105%; note, likely a counting artifact), the MAC+AMP plate (~ 27.7%), MAC+FOX (~7.2%), MAC+AXO (~23.1%), MAC+AXOCLAV (~10.6%), MAC+PIME (~4.4%) and MAC+MERO (~0.05%) (see Table 4).

Table 4. Within-column relative quantities (%) of estimated bacterial counts from various antibiotic broth concentrations subsequently grown on plain versus selective MacConkey agar plates.

MacConkey plate type	Input Mixture (no broth incubation)	Plain CAMH-2 broth (0 µg/mL)	Ampicillin CAMH-2 broth (2 µg/mL)	Ampicillin CAMH-2 broth (4 µg/mL)	Ampicillin CAMH-2 broth (8 µg/mL)	Ampicillin CAMH-2 broth (16 µg/mL)	Ampicillin CAMH-2 broth (32 µg/mL)	Ceftiofur CAMH-2 broth (0.5 µg/mL)	Ceftiofur CAMH-2 broth (1 µg/mL)	Ceftiofur CAMH-2 broth (2 µg/mL)	Ceftiofur CAMH-2 broth (4 µg/mL)	Ceftiofur CAMH-2 broth (8 µg/mL)
	Column % Log_{10} CFU	Column % Log_{10} CFU	Column % Log_{10} CFU	Column % Log_{10} CFU	Column % Log_{10} CFU	Column % Log_{10} CFU	Column % Log_{10} CFU	Column % Log_{10} CFU	Column % Log_{10} CFU	Column % Log_{10} CFU	Column % Log_{10} CFU	Column % Log_{10} CFU
MAC	**100 7.16**	**100 9.58**	**100 9.60**	**100 9.55**	**100 9.32**	**100 9.25**	**100 9.32**	**100 9.17**	**100 9.24**	**100 9.11**	**100 8.87**	**100 8.93**
MAC+TET	91.48	105.59	85.89	94.37	92.68	94.80	82.28	94.12	80.31	69.59	52.50	59.69
MAC+AMP	95.34	27.66	29.11	34.72	59.15	100.37	89.87	115.84	108.11	98.45	86.67	107.75
MAC+FOX	19.36	7.18	6.81	5.93	28.05	40.52	27.85	43.44	37.45	64.43	70.83	86.82
MAC+AXO	64.73	23.14	27.50	30.53	51.52	96.28	86.71	120.36	98.07	86.08	94.17	103.10
MAC+AXOCLAV	37.80	10.64	6.77	5.76	23.78	43.87	48.73	56.56	52.90	60.31	78.33	108.53
MAC+PIME	14.69	4.43	3.63	4.71	10.37	14.13	13.29	28.96	11.58	41.24	65.83	103.88
MAC+MERO	10.71	0.05	0.04	0.02	0.10	0.16	0.13	0.15	0.10	0.30	0.82	1.92

MAC (Plain MacConkey), +TET (Tetracycline - 16 µg/mL), +AMP (Ampicillin - 32 µg/mL), +FOX (Cefoxitin - 32 µg/mL), +AXO (Ceftriaxone - 4 µg/mL), +AXOCLAV (Ceftriaxone - 4 µg/mL and clavulanate -4 µg/mL), +PIME (Cefepime - 8 µg/mL) and +MERO (Meropenem -4 µg/mL and 1 µg/mL); CAMH-2 (Cation-adjusted Mueller Hinton II); CFU (Colony forming unit).

Ampicillin

No significant difference was observed (Table 4) between the estimated (via plain MacConkey agar plates) total bacteria CFU in the mixed population culture after 24-Hour incubation in non-selective CAMH-2 broth [9.58 (log_{10}CFU/mL) 95% CI: 9.44 – 9.72] compared to the lowest ampicillin broth concentration (2 μg/mL) that we tested [9.60 (log_{10}CFU/mL) 95% CI: 9.50 – 9.69]. In direct contrast to an increasing concentration of ampicillin, a steady decrease in estimated total bacteria population CFU in the mixed-strain culture was observed. Across the tested concentration range, estimated bacterial density decreased from 9.60 (log_{10}CFU/mL) [95% CI: 9.50 – 9.69] to 9.32 (log_{10}CFU/mL) [95% CI: 9.21 – 9.43]. The magnitude of reduction in estimated total CFU between the two lowest ampicillin broth concentrations (relative to total CFU in plain broth, the maximum carrying capacity) was slight (5.3%). The largest decrease (40.5%) was observed between ampicillin broth concentrations of 4 μg/mL and 8 μg/mL. A further 9.8% reduction was observed with the next 2-fold increase in broth antibiotic concentration, though this reduction was unstable as reflected in final concentration of bacteria grown at 32 μg/mL.

The constituent proportions of beta-lactamase producers in the ampicillin containing cultures, as estimated by the CFU counts on the MAC+AMP agar plates relative to the non-selective plate, were 29%, 34.7%, 59.1%, and 100% for 2, 4, 8, and 16 μg/mL, respectively, in ascending order of ampicillin concentration. In contrast to this trend, the beta-lactamase producers were estimated to grow only to 90% of the total expected CFU in the 32 μg/mL ampicillin broth culture, a substantial drop from the preceding concentration. Nonetheless, the absolute count of the beta-lactamase producers increased steadily across ampicillin

concentrations from 9.07 (log_{10}CFU/mL) [95% CI: 8.97 – 9.17] in the 2 μg/mL ampicillin broth culture to 9.28 (log_{10}CFU/mL) [95% CI: 9.16 – 9.39] in the 32 μg/mL ampicillin broth culture. Although this result exhibits a seeming paradoxical increase in relative proportions of non-beta-lactamase producers in 32 μg/mL ampicillin broth culture, the overall trend observed was a steep decline in the susceptible bacterial sub-population and a steady increase in the component beta-lactamase producing strains' population as *in vitro* ampicillin concentrations increased. This trend suggests that reductions in estimated total bacteria count in ampicillin broth cultures are primarily due to the suppression of the non-beta-lactamase producing sub-population component.

The estimated proportion of CPE strains in the mixed culture quadrupled from the 2 μg/mL ampicillin broth to the 16 μg/mL ampicillin broth, increasing from 0.04% of the estimated total density to 0.16% (peak). Bacterial counts on the MAC+AXOCLAV plates also generally increased in proportion along with ampicillin broth concentration: 6.8%, 5.8%, 23.8%, 43.9% and 48.7% (for 2, 4, 8, 16, and 32 μg/mL, respectively). Likewise, bacterial count estimates on the MAC+AXO plates increased in proportion with doubling ampicillin concentrations, peaking at ampicillin concentrations of 16 μg/mL: 27.5%, 30.5%, 51.5%, 96.3% and 86.7%, and in ascending order of ampicillin concentration. The marginal mean estimates from the MAC+AXO plates, MAC+AXOCLAV plates and the MAC+AMP plates suggest the ESBL-producing strains constitute the bulk of beta-lactamase producers following selective pressures of ampicillin in a competitive mixed culture (see Table 4 and Figure 4).

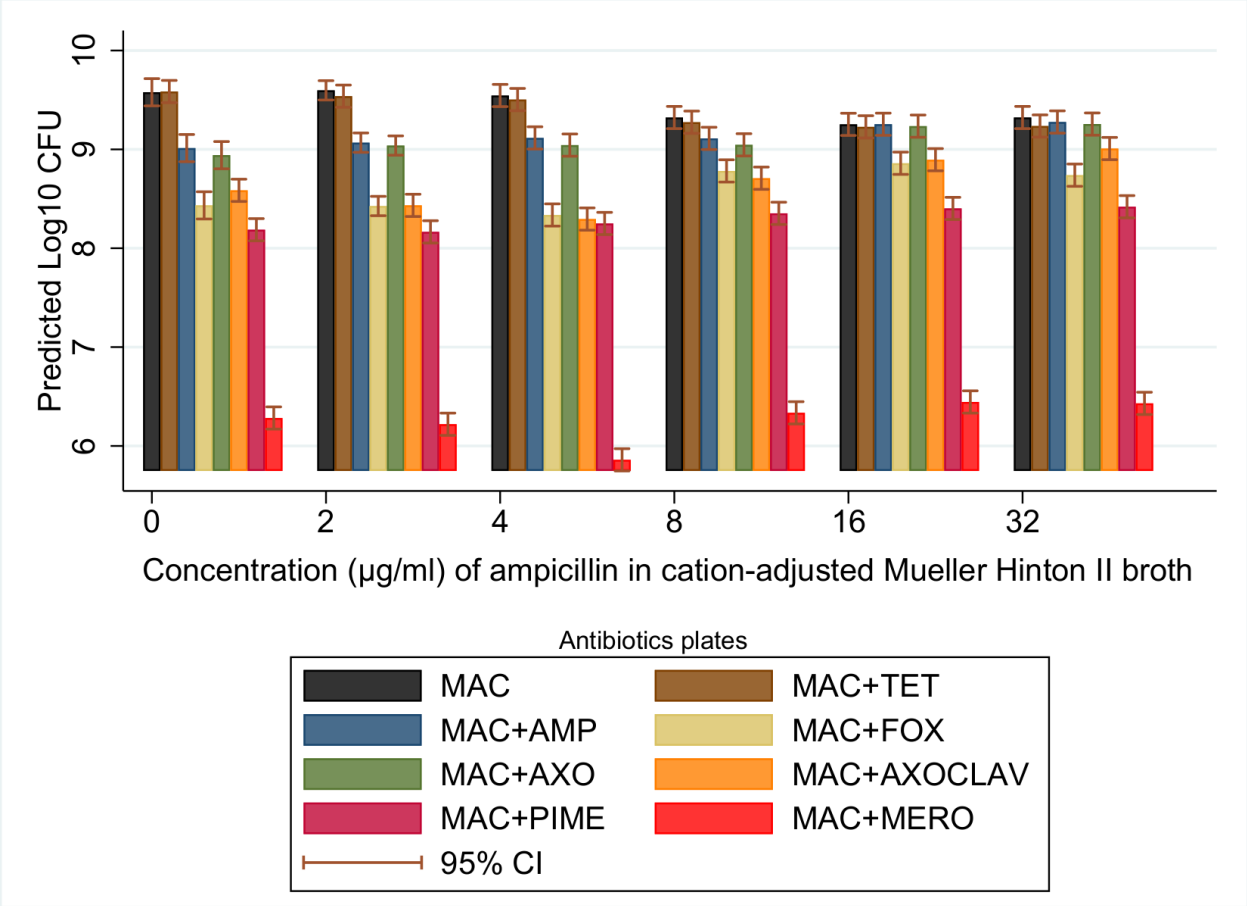


Figure 4. Effects of varying ampicillin concentrations on 10-strain mixed-bacterial culture. Predicted marginal mean counts (log_{10}CFU) with 95% CI following 24-Hour incubation in ampicillin (at 2, 4, 8, 16, 32 µg/mL) in cation-adjusted Mueller-Hinton II broth.
*The selective MacConkey agar plate antibiotic concentrations were: ampicillin (32 µg/mL), tetracycline (16 µg/mL), cefoxitin (32 µg/mL), ceftriaxone (4 µg/mL), ceftriaxone (4 µg/mL) + clavulanic acid (4 µg/mL), cefepime (8 µg/mL), and meropenem (4 µg/mL); CFU (Colony forming unit).

Ceftiofur

In contrast to the starting ampicillin broth concentration (2 μg/mL), the starting ceftiofur broth concentration (0.5 μg/mL) demonstrated a significant suppression of the estimated total bacterial count relative to estimates from the non-selective broth culture [9.17 (log_{10}CFU/mL) 95% CI: 9.08 – 9.26 compared to 9.58 (log_{10}CFU/mL) 95% CI: 9.44 – 9.72], respectively (Table 4). Further significant ($P < 0.05$) suppressions of the estimated total bacterial CFU in the mixed-strain culture were not observed until the broth concentration doubled from 2 μg/mL to 4 μg/mL of ceftiofur [9.11 (log_{10}CFU/mL) 95% CI: 9.02 – 9.20 compared to 8.87 (log_{10}CFU/mL) 95% CI: 8.78 – 8.96], respectively. The proportion of the maximum capacity (that is, in absence of the antibiotic) achieved by beta-lactamase producers in the ceftiofur broth culture (as estimated by the CFU counts on the MAC+AMP agar plates relative to the non-selective plate) were 115%, 108%, 98%, 87% and 108%, in ascending order of ceftiofur concentration. The proportions above or near 100% suggest dominance of the culture by such strains and are likely artifacts introduced by bacterial counting methods when they exceeded 100% of growth on plain media.

The absolute count of the beta-lactamase producers as estimated on these plates also decreased; most notably, they were suppressed in the broths with 4 μg/mL and 8 μg/mL of ceftiofur. This suggests a ceftiofur-concentration-dependent inhibition of growth of low potency beta-lactamase producing strains (i.e., TEM-1-type and potentially AmpC-type beta-lactamase producers). The observed drop in total estimated bacteria population counts in ceftiofur-containing broth was more likely to be due to the suppression of component beta-lactamase producing strains, distinct from the ampicillin experimental assay findings. The overlap in estimated CFU proportions on the MAC+AMP and MAC+AXO selective agar plates suggests the TEM-1-type beta-lactamase strains constituted a less important component of the

community. The estimated proportion of CPE strains isolated via the MAC+MERO selective media plates showed a steady increase from 0.2%, through 0.1%, 0.3%, 0.8%, and 1.9%, along the ceftiofur concentration gradient outlined in Table 1. The CFUs selected by the MAC+AXOCLAV plates also increased in proportion along with ceftiofur concentrations: 56.6%, 52.9%, 60.3%, 78.3% and 108.5% (see Table 4 and Figure 5).

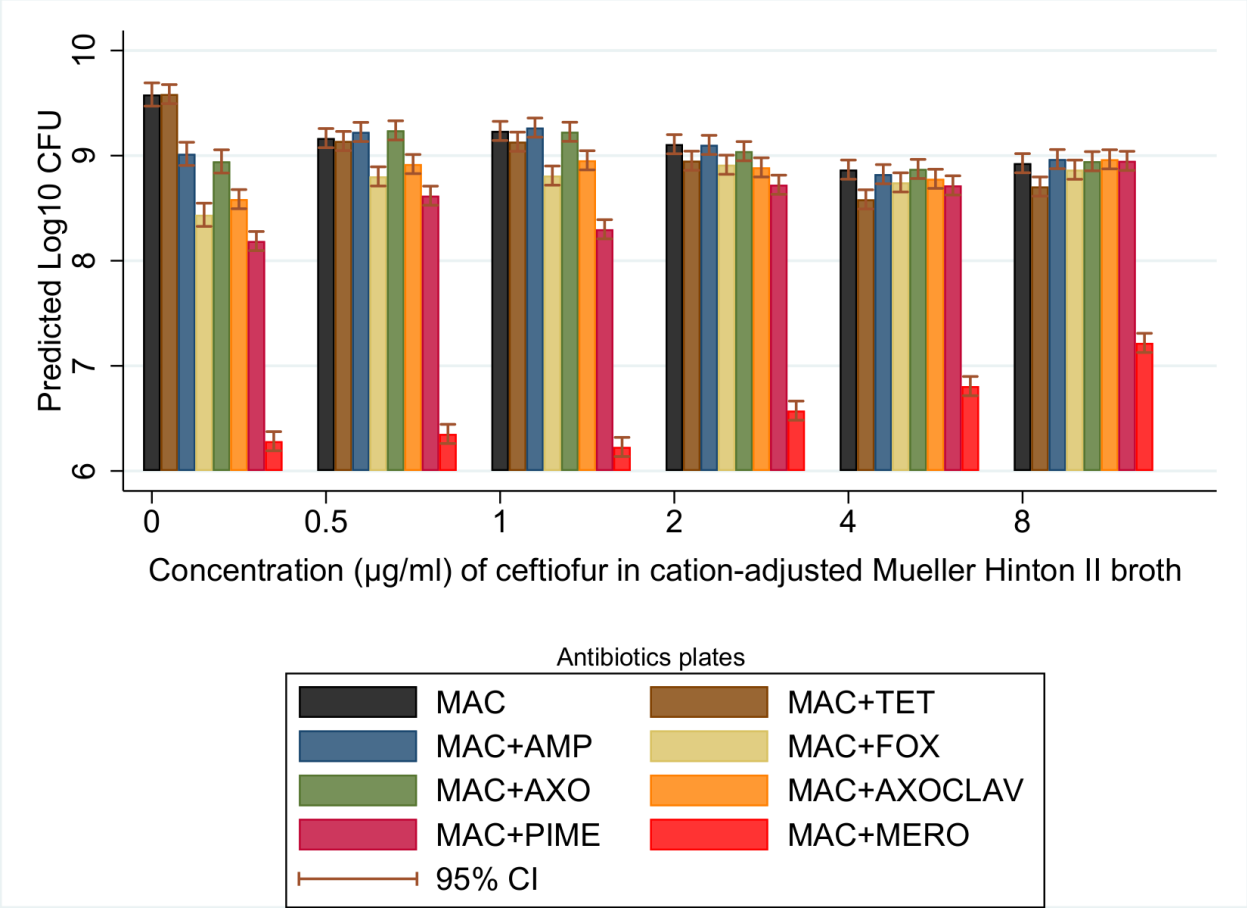


Figure 5. Concentration effects of ceftiofur on 10-strain mixed-bacterial culture.
Predicted marginal mean CFU with 95% CI following 24-Hour incubation in ceftiofur (at 0.5, 1, 2, 4, 8 µg/mL) in cation-adjusted Mueller-Hinton II broth.
*MacConkey agar plate antibiotic concentrations: ampicillin (32 µg/mL), tetracycline (16 µg/mL), cefoxitin (32 µg/mL), ceftriaxone (4 µg/mL), ceftriaxone (4 µg/mL) + clavulanic acid (4 µg/mL), cefepime (8 µg/mL), and meropenem (4 µg/mL); CFU (Colony forming unit).

Discussion

Often, and for good reasons, regulatory and policy approaches to the challenge of AMR tend to be reactive rather than proactive. These are exemplified by restrictions on the use of specific antimicrobial drugs, classes, or drug indications for specific diseases, often long after they have been approved and labeled for such use. The current absence of carbapenems and 4GC use in the U.S. in livestock production has offered unique opportunities for assessing the value of proactive policies for addressing the question of the roles of direct and indirect selection of bacterial resistance types to these antimicrobials in the presence and absence of co-selection by other antimicrobials.

Though more consistently seen with chromosomally located genes, the carriage of AMR genes on horizontally transferable genetic materials has also been shown to impose a fitness cost (often estimated using the maximal growth rate as a surrogate fitness measure) on bacterial strains [116, 117]. In our study, mammalian-derived commensal *E. coli* strains with similar beta-lactamase genotypes were found to possess a spectrum of fitness levels as reflected in the growth rates. This underscores the importance of a global genotypic evaluation across all AMR profiles, virulence profiles and other stress adaptation mechanisms, in order to estimate the fitness impact of a particular variable. In this study, unlike in prior studies [87], we characterized the growth of a large number of bacterial strains for each gene group to assess and adjust for this variation among strains of the same species exhibiting similar genotypic and phenotypic characteristics. When analyzed based on the *bla*-gene classifications (n=20 per gene group), our study found the *bla*-negative group to be the most fit in the absence of antibiotic selection pressures, suggesting a fitness advantage may still exist in association with the lack of a *bla* gene. A similar model comparing beta-lactamase producers, surprisingly, showed a similar level of fitness between the

high-potency carbapenemase *bla* gene group and the lower potency *bla* gene group (*bla*$_{\text{TEM-1}}$). Consequently, our results do not suggest a trend of increasing fitness cost (as estimated by the maximum growth rate) in lock step with increasing resistance 'severity' of the *bla* gene groups. On the other hand, a relatively impaired maximum bacterial density (as implied by the highest OD as a surrogate measure) was observed for the carbapenemase-producing group; taken together, this may constitute a more important indicator of relative fitness cost in an enteric environment with limited nutrient-based carrying capacity and intense competition with other strains utilizing the same resource. Diminished group total bacterial density estimates in the antibiotic-supplemented media may indicate a different form of impaired bacterial fitness that could also impact population dynamics. Indeed, the biphasic exponential growth phase and the prolong lag phase duration seen in raw data plots (Figure 1) were not reflected in the model predictions nor the fitted plots of the growth curves respectively.

When bacteria of mixed origin are in a community, competition is known to occur, either due to limitations in resource availability or else as strains adapt and obtain an evolutionary advantage [118, 119]. For instance, Ushijima and Seto (1991) demonstrated in an *in vitro* study that a group of normal intestinal commensals – *E. coli, E. aerogenes, Enterococcus faecalis, Bacteroides ovatus* and *Fusobacterium varium* – are capable of suppressing the growth of an intestinal pathogen – *Salmonella* Typhimurium – under certain conditions [120]. In addition, bacterial ecologists have noted that when members of a bacterial community are genetically similar, antagonism/inhibition is less likely to occur; in contrast, altruistic cooperation may ensue [121]. Given the similarity (same species) of our limited number of study strains, it is reasonable to expect that the fitness differential would predict the population dynamics of the bacterial community, especially given the similar profile of nutrient requirements and ecological niche.

The fitness pattern of the five sets of representative pairs of strains from the five *bla*-gene-based groups in the mixed populations followed an overall similar trend to the estimates with twenty strains per group, each grown as mono-culture (Figures 1 and 2). That said, the margins of fitness advantage exhibited by the *bla*-negative pair and the pair of carbapenemase producers over the other pairs chosen for the competitive assay were slightly more pronounced (Tables 2 and 3). Our results showed a preponderance of the *bla*-negative strains in the bacterial community after 24-Hour incubation in broth without a selection pressure, an expected outcome in the absence of mutual growth interference by the component *E. coli* strains. The data also showed a seeming suppression of the carbapenemase-producers beyond the limitations conferred by their growth rates; however, this may instead reflect CPE density under-estimation using the agar plates supplemented with 4 μg/mL meropenem (and perhaps point to a need for additional micro-nutrient supplementation such as zinc). Negri et al. (1994), in their *in vitro* mixed culture study of beta-lactam resistant and susceptible *Streptococcus pneumoniae*, similarly found no interference among *S. pneumoniae* strains with different profiles of resistance to beta-lactams [87].

Mollenkopf et al. (2017), in their CPE surveillance study of a swine production facility, found a 16.5% detection rate across environmental and fecal samples [49]. Notably, virtually all (~100%) of their CPE (*bla*$_{\text{IMP-64}}$/IncQ1) positive samples originated from the farrowing barns; importantly, those authors attributed this finding to the use of ceftiofur in the sows (on-label treatments as needed) and in the piglets (extra-label use for infection prevention and control, such as following castration of male piglets) [48]. As mentioned earlier, when estimated across twenty strains, the carbapenemase-producers did not appear to exhibit a high fitness cost relative to other *bla*-positive strains; importantly, this suggests that even absent beta-lactam selection

pressure and with a sufficiently high initial population, CPEs could persist at a low prevalence along with other beta-lactam-resistant Enterobacteriaceae. Instead, the relatively lower maximum OD of the CPE cultures may represent the real factor associated with lower levels of CPE currently found in food animal production environments.

The introduction into the bacterial growth media of different generations of beta-lactam antibiotics, and at increasing concentrations, was observed to change the population dynamics of the *in vitro* bacterial consortium. Increasing concentrations of ampicillin sodium gradually inhibited the *bla*-negative sub-population while selecting for the beta-lactamase resistant strains, including the full spectrum from TEM-1-type beta-lactamase producers through CPE strains. In contrast, low concentrations of ceftiofur hydrochloride (0.5 μg/mL) completely suppressed the susceptible populations of *bla*-negative and TEM-1-type beta-lactamase producers, thus effectively selecting for the CMY-2, CTX-M, and CPE resistant sub-populations. Increasing concentrations of ceftiofur hydrochloride suppressed the total bacterial density while further increasing the proportions of the higher-level beta-lactamase producing strains; for example, the carbapenemase producers showed about a forty-fold jump in relative proportion from non-selective broth through to 8 μg/mL of ceftiofur hydrochloride broth.

Our results show that a 3rd generation cephalosporin (3GC), such as ceftiofur, provides a more than adequate selection advantage for carbapenemase producers, even in the absence of direct selection (i.e., carbapenem use) and minimal indirect co-selection. The presence of co-selected *bla* genes on the same plasmids could further aggravate the selection observed, given that carriage of many plasmid borne resistance genes has been observed to impose little or no additional fitness cost to the organism [122, 123]. In our own study, it was difficult to find strains harboring either AmpC or ESBL genes that did not also harbor *bla*$_{\text{TEM-1}}$. Among the CPE strains,

it was even more difficult to find strains that lacked not only *bla*$_{\text{TEM-1}}$, but also any AmpC or ESBL genes. This was perhaps not surprising given that genes are generally added to an existing arsenal of resistance and virulence factors in the strains. These findings agree with the observations in the surveillance study of the swine production facility by Mollenkopf et al. (2017) that ceftiofur provided adequate selection advantage for carbapenemase-producing Enterobacteriaceae to emerge into detectable levels [48].

In both ampicillin- and ceftiofur-supplemented CAMH-2 broth, the ESBL sub-population appeared to increase in dominance with increasing antimicrobial concentrations. This was likely facilitated by their relatively high minimum inhibitory concentrations (MIC) for these antimicrobials [124] and their fitness advantage over other *bla*-positive strains (Table 3). Overall, our findings among Enterobacteriaceae are remarkably similar to those found by Negri et al. in gram-positive bacteria, which tested the effect of varying concentrations of amoxicillin, cefixime, cefuroxime and cefotaxime on a mixture of *S. pneumoniae* strains with MIC values ranging from susceptible to resistant across the antimicrobial agents. The newer generation beta-lactams completely suppressed their susceptible strains while selecting for higher resistance strains; in contrast, the less potent amoxicillin, at lower concentrations, mildly suppressed the susceptible strains while selecting effectively for the low-level beta-lactamase producing strains [87].

Ambler class C beta-lactamase enzymes, such as encoded by the plasmid-borne *bla*$_{\text{CMY-2}}$ gene and the Ambler class A enzyme encoded by the plasmid-borne *bla*$_{\text{CTX-M}}$ gene have both been determined to be effective against 3GC antibiotics [26, 124]. However, in 4 μg/mL ceftriaxone broth the CMY-2 type beta-lactamase strains demonstrated impaired group growth rates compared to the CTX-M* type beta-lactamase strains (Table 2 and Figure 3). This observed

difference in their growth potential suggests that within any given mixed bacterial community exposed to a similar or higher concentration of ceftriaxone, the strains harboring the *bla*$_{\text{CTX-M}}$ genes would be favored over the CMY-2 type beta-lactamase producing strains. Despite this theoretical outcome, in our mixed-strain cultures grown in ceftiofur broth, the relative proportion of the AmpC-type *bla* strains continued to increase along with the concentration of ceftiofur all the way to the highest experimental concentration. This occurred, rather than a plateauing or reduction in the relative proportion of these strains at the highest ceftiofur concentrations.

In the 10-strain competition assay, there was a preponderance of susceptible strains in the bacterial community in the absence of antibiotics, when compared with the beta-lactamase producers, as would be expected giving the superior fitness of the susceptible strains (Table 3). This is also consistent with contemporary estimates of beta-lactam resistance prevalence among indicator organisms such as *E. coli,* especially absent antibiotic selection-pressure. In contrast to estimated fitness values, the proportion of carbapenemase-producers, as measured by way of the MAC+MERO plates, was not comparable with the AmpC-type beta-lactamase strains. It should be noted that the component CPE population was estimated at 1 μg/mL meropenem (MAC+MERO) agar plates for the starting mixture and 4 μg/mL meropenem (MAC+MERO) agar plates for the post-incubation mixture, due to serious strain inhibition below the limits of detection at the higher meropenem concentration for the starting mixture; consequently, the CPE strain population was likely to be underestimated at the higher meropenem concentration. However, as the resistance breakpoint established by CLSI [100] is at 4 μg/mL meropenem (MAC+MERO) and so this was how the study was designed.

Importantly, the limitations inherent in models such as employed in this research must be considered in evaluating their usefulness. For instance, it should be noted that in nature the

starting populations of far more diverse bacteria with various resistance profiles are unlikely to be equal as was modeled in this study; therefore, the post-exposure changes in the prevalence of the resistant bacterial strains may not be as remarkable as we determined. Furthermore, *in vivo* antimicrobial concentrations are not constant, as was the case with our *in vitro* model; rather, the drug and active drug metabolite concentrations dynamically rise and fall, based on the dosing regimen and the drug distribution, metabolism and excretion. Still, recommended antimicrobial regimens are known to expand resistant coliform populations in livestock intestinal flora, sometimes for several weeks after the final drug administration [14]. Also, in nature, a distinct separation of bacterial groups by beta-lactamase enzyme profile is unlikely; that is, resistant bacteria frequently harbor multiple resistance determinants against a single class of antimicrobial agent, as well as to different classes of agents. Overlapping sets of resistance genes, as was frequently encountered during our isolate selection for this study, would be expected to add layers of complexity to the selection dynamics in nature.

Our study did not factor in the role of innate bacteria resistance mechanisms, such as non-specific efflux pumps and membrane-porin down regulation [125], because the presence of such mechanisms can be expected to exert a relatively uniform and non-specific effect across study strains and in some case antibiotics; however, for beta-lactam antibiotics this is not likely to interfere with interpretations since most resistance is enzymatic and the damage inflicted by the antibiotic is to the cell wall. Lastly, the influence of far more abundant and niche-competing anerobic intestinal commensals, or free-living environmental strains, in antimicrobial selection was not modeled in this study. Although the conditions of these *in vitro* models may not perfectly approximate *in vivo* or environmental realities, they nonetheless constitute a reasonable first step in a systematic and order approach to this challenge.

Overall, our hypothesis that older generation beta-lactam antibiotics of lesser priority – such as ampicillin – can also provide a selection advantage to highest priority resistance types – such as 3rd/4th generation cephalosporins and carbapenems – albeit less efficiently when compared to 3rd generation cephalosporins, was supported by the results of these two *in vitro* experimental studies. Currently, beta-lactam antimicrobial resistance due to carbapenemase producing Enterobacteriaceae is not a known existential therapeutic threat in animal agriculture; in contrast, human health care infections caused by the *K. pneumoniae* producing KPC-type *bla* enzyme are both a U.S. and a global concern [1]. These strains, along with other Enterobacteriaceae bearing less prevalent carbapenemase encoding genes (e.g., bla_{NDM}, bla_{VIM} & bla_{IMP}) have been reported in many U.S. states. Presently, the challenge posed by carbapenemase-producing bacteria is limited to specific settings in the human healthcare system. Although *E. coli* strains with carbapenemase-encoding genes (including the bla_{KPC} gene) have been identified [126, 127], community acquired infections with strains bearing this order of beta-lactam resistance are still relatively rare [128].

Our study suggests that if introduced into food-animal populations, perhaps through surface water downstream from hospital and wastewater treatment plant effluent discharge [129], CPEs along with other high priority beta-lactamase resistance profiles could be expanded due to lower-priority beta-lactam use in food animals, and subsequently spread back to the human community through food-animal products and via the livestock environment. To further clarify and characterize these findings, we suggest additional studies, both observational and experimental. For example, an *in vitro* continuous anerobic medium such as employed by Ushijima and Seto (1991) might provide a better model of the selection dynamics expected in a monogastric mammalian bowel compared to the 10-strain batch culture we used [120]. Further,

pharmacokinetics-pharmacodynamics (PK-PD) mathematical modeling of these selection dynamics and ultimately *in vivo* animal studies would be logical next steps. In conclusion, this study showed that the absence of direct carbapenem selection pressure in food-animal production cannot be relied upon alone to reduce the spread of bacterial strains with reduced carbapenem susceptibility. Use of commonly prescribed older-generation beta-lactams such as ampicillin and ceftiofur can expand both ESBL Enterobacteriaceae and CPE prevalence in commensal and pathogenic bacterial communities.

CHAPTER IV

MIXED-STRAIN COMPETITION IN PORCINE INTESTINAL MICROFLORA CHEMOSTAT EXPERIMENTS

Introduction

β-lactam antibiotics are one of the most widely used groups of antimicrobials in human and veterinary medicine [5]. Bacterial AMR currently threatens their continued efficacy, including the class of last resort β-lactams: the carbapenems. Although the use of carbapenems in food animal production has never been approved, bacteria with transferable resistance to this class of β-lactams are now being reported in agricultural settings in many parts of the world [41, 42, 44, 47]. Approved β-lactams such as the penicillins and cephalosporins, commonly used in livestock operations have been ascribed the blame for this observation [49].

The objective of our research project was to test this hypothesis and to determine the potential extent of this selection pressure among representative Enterobacteriaceae; we pursued this through multi-layer experimental and modeling methods, including *in vitro* approaches. *In vitro* batch culture methods have been the mainstay of bacterial research for several decades due to their affordability, reproducibility and robustness. Although very valuable in morphology and viability research, they are disadvantaged when used as *in vivo* simulation studies. In this study, a dynamic *in vitro* culture system (i.e., a chemostat) was employed. The dynamic *in vitro* culture system incorporates and realistically mimics natural *in vivo* phenomena such as nutrient and metabolite exchange, anerobic respiration and the presence of competitive gram-positive anaerobes of swine gut origin. Consequently, we aimed to better model relative Enterobacteriaceae populations in mammalian (swine) hindgut, through a continuous anerobic *in*

vitro porcine-flora culture system and in the presence/absence of β-lactams such as ampicillin and ceftiofur that are approved for food animal therapeutic indications in the U.S.

Methods

A triad of continuous-flow porcine intestinal flora anerobic culture systems (RPCF chemostat) were utilized for this experiment. Each chemostat system consisted of a 500 mL electronically stirred, on-going anerobic culture of porcine cecal microbiota, and maintained with Viande Levure (VL) broth medium at 37^{o}C and with a pH that ranged between 6.0-6.4 [130]. The cecal microbiota is mainly a collection of various gram-positive bacteria. In brief, as estimated by metagenomic assay, the component population is dominated by *Moryella indoligenes* (20.67%), *Sporanaerobacter* spp. (17.56%), *Pyramidobacter piscolens* (15.86%), *Bacteroides* spp. (8.83%), *Bacteroides uniformis* (8.04%), *Prevotella stercorea* (5.96%), *Clostridium* sp. (5.84%), *Porphyromonas somerae* (4.38%), *Solobacterium* spp. (1.78%), *Faecalicoccus clostridiales bacterium* (1.36%), *Lachnoclostridium clostridium bolteae* (1.32%), *Parabacteroides distasonis* (1.19%), *Rummeliibacillus stabekisii* (1.13%); other identified bacteria genus existed in progressively lower proportions (see appendix C for complete list and proportions).

The same pair of representative isolates per *bla* genotype group as previously employed in the batch culture competition assay were again selected for this study. From overnight incubated blood agar plates, a 5.0 McFarland (1.5 x 10^9 CFU/mL) (Sensititre^{TM} Thermo Fisher Scientific, Waltham, MA) standardized bacteria suspension in phosphate-buffered saline (PBS) was prepared for each strain.

A mixed suspension (1 mL each) of the test strains was then prepared (that is, effective bacteria density of 1.5 x 10^8 CFU/mL). To achieve a density of 10^8 CFU/mL, 5 mL sterile PBS was added to the mixture. After active mixing, three 5 mL aliquots of the suspension were prepared as test samples for each chemostat system; in a three-cycle experiment, each of the three recombined porcine continuous flow (RPCF) systems (chemostat systems) served once as a control (no antibiotic), once as an ampicillin sodium test medium (32 μg/mL) (Sigma-Aldrich, St. Louis, MO), and once as a ceftiofur sodium (8 μg/mL) (Zoetis Inc., Parsippany-Troy Hills, NJ) test medium. Prior to inoculation with test strains, each chemostat system was sampled to identify any background *E. coli* population.

Once inoculated (1:10 dilution ratio *ab initio*), a one Hour mixing time of the test sample in the chemostat was observed prior to antimicrobial injection and subsequent sampling. Chemostat sampling was at hours: 0 (just prior to antimicrobial introduction), 1, 2, 4, 6, 8, 12, 24 for Day 1 of the experiment; later still, samples were collected at 12-Hour intervals for Day 2 through Day 5. Samples were collected in 5mL sterile sample tubes and transported on ice for storage at -80ºC until processing. The experiment was performed in three trials for data robustness and for adjustment of chemostat-system (RPCF culture selection) specific bias; consequently, assignment of chemostat systems was rotated in a clockwise fashion (control – ampicillin – ceftiofur – control, etc) with each run (see Figure 6). After appropriate sample dilution with CAMH-2 (on ice pebbles to halt bacteria multiplication), the *E. coli* strain-type population structure was estimated phenotypically by spiral-plating (Eddy Jet 2™ Spiral-Plater, Neutec Group Inc., NY) on antimicrobial supplemented MacConkey agar plates (see Table 1). CFU counts were performed with an automated colony counting device (Flash & Go™, Neutec Group Inc., NY).

Estimated CFU densities were $\log_{10}$ transformed to achieve a normalized data distribution. For the second and third experimental replicates, effective proportions of pre-inoculation carry-over CFUs were estimated from the Hour-0 CFU counts by selective agar plate-type. These proportions were deducted from all subsequent CFU estimates per selective plate type. A mixed-effect linear regression model was then fitted to the data; the model fixed effects were RPCF culture type (by antimicrobial), time (Hour) and type of selective antimicrobial plate and replicate runs were random effects. Marginal mean predictions of the strain group densities from the model output with 95% confidence intervals were determined and represented graphically. Proportions of *E. coli* strain-type(s) on selective agar plates relative to the non-selective plate were determined with un-transformed bacteria count data and subsequently graphed. These analyses were performed in Stata® version 15.1 software (Stata Corp., College Station, TX).

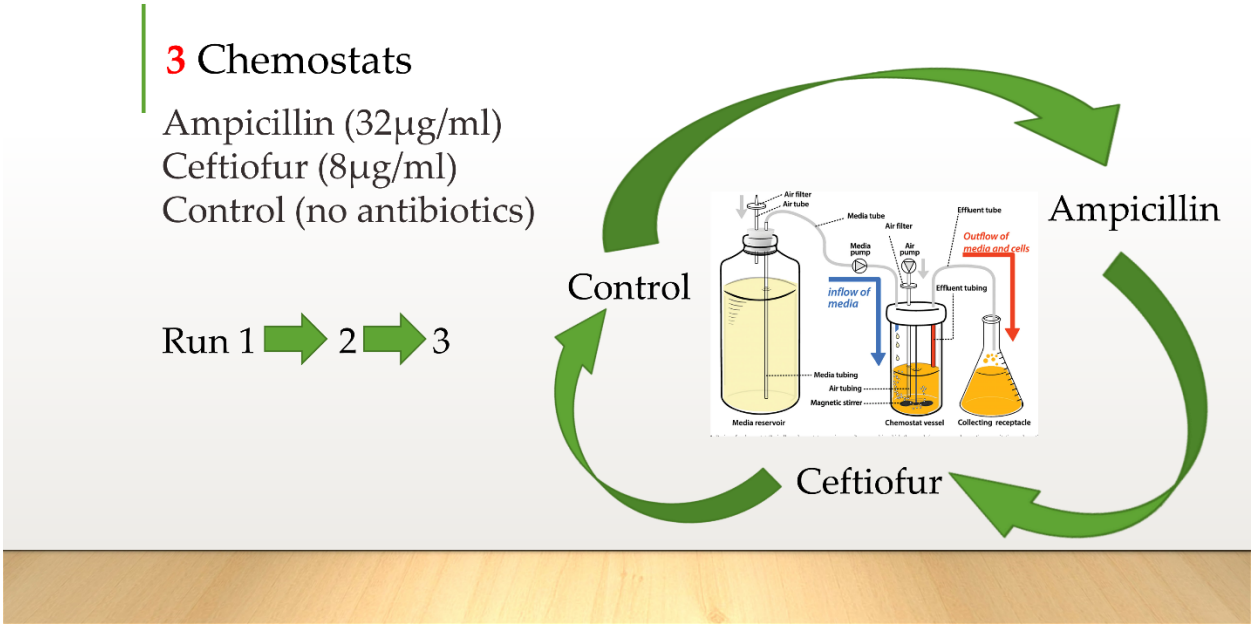


Figure 6. Porcine chemostat rotation pattern with experimental trial cycling, adapted from orbitbiotech.com. [131]

Results

For all ten strains, individual bacterial densities (attained with the 5.0 McFarland standard) in suspension before equal volume mixing were estimated by spiral plating onto MacConkey agar. Estimated concentrations of *E. coli* per strain ranged between 7.5 $\log_{10}$CFU/mL and 8.3 $\log_{10}$CFU/mL; across all trials, the estimated mean concentration was 8.1 $\log_{10}$CFU/mL per suspension. At the start of the experiment, that is Run-1, all three chemostat systems tested negative via the plating method for the presence of *E. coli*, which was both the expected and desired result

In the control RPCF culture, predicted population densities of total *E. coli* (as estimated by plain MacConkey), as well as the *E. coli* sub-groups reduced progressively over the course of

the study (Figure 7). Estimated total *E. coli* density in this culture medium (averaged across three trials) at the start of the experiment, Hour 0, was 6.26 $\log_{10}$CFU/mL (6.1 – 6.4, 95% CI), and 5.1 $\log_{10}$CFU/mL (95% CI: 4.8 – 5.50 at the conclusion of the trial, Hour 108. The densities of *E. coli* sub-groups, at commencement and conclusion were: 6.28 $\log_{10}$CFU/mL (95% CI: 6.2 – 6.4) and 5.0 $\log_{10}$CFU/mL (95% CI: 4.6 – 5.3), respectively, for any β-lactamase producers (MAC+AMP plates); 5.9 $\log_{10}$ CFU/mL (95% CI: 5.8 – 6.0) and 3.6 $\log_{10}$CFU/mL (95% CI: 3.3 – 4.0), respectively, for ESC resistant strains (MAC+AXO plates); 5.6 $\log_{10}$CFU/mL (95% CI: 5.4 – 5.7) and 3.0 $\log_{10}$CFU/mL (95% CI: 2.7 – 3.4), respectively, for AmpC-enzyme and carbapenemase producing strains (MAC+FOX plates) and; 5.5 $\log_{10}$CFU/mL(95% CI: 5.3 – 5.6) and 2.8 $\log_{10}$CFU/mL (95% CI: 2.3 – 3.0) respectively for CPEs (MAC+MERO plates).

In antimicrobial-infused RPCF cultures, experimental Enterobacteriaceae appeared to persist at relatively higher densities and for longer compared with the control culture over the duration of the experiment (see Figures 8 and 9). In the ampicillin RPCF culture, the estimated densities of total *E. coli,* β-lactamase producers, ESC resistant strains, AmpC-enzyme and carbapenemase producing strains, and CPEs were: 6.6 $\log_{10}$CFU/mL (95% CI: 6.0 – 7.3) and 6.3 $\log_{10}$CFU/mL (95% CI: 5.6 – 7.0) at Hour 0 and Hour 108; 6.6 $\log_{10}$CFU/mL (95% CI: 6.0 – 7.3) and 6.3 $\log_{10}$CFU/mL (95% CI: 5.6 – 7.0) at Hour-0 and Hour-108; 6.1 $\log_{10}$CFU/mL (95% CI: 5.4 – 6.8 and 5.4 $\log_{10}$CFU/mL (95% CI: 4.7 – 6.0) at Hour-0 and Hour-108; 5.6 $\log_{10}$CFU/mL (95% CI: 5.0 – 6.3) and 5.2 $\log_{10}$CFU/mL (95% CI: 4.5 – 5.9) at Hour-0 and Hour-108; 5.5 ($\log_{10}$CFU/mL) (95% CI: 4.8 – 6.1) at Hour-0 and Hour-108 and 4.8 $\log_{10}$CFU/mL (95% CI: 4.2 – 5.5) at Hour-0 and Hour-108, respectively (Figure 3). The estimated densities in ceftiofur inoculated RPCF, at commencement (Hour 0) and conclusion (Hour 108) of experiment were: 6.5 $\log_{10}$CFU/mL (95% CI: 5.8 – 7.1) and 5.5 $\log_{10}$CFU/mL (95% CI: 4.9 – 6.1) respectively for

all *E. coli*; 6.4 log_{10}CFU/mL (95% CI: 5.7 – 7.0) respectively and 5.4 log_{10}CFU/mL (95% CI: 4.7 – 6.0) respectively for β-lactamase producers; 6.2 log_{10}CFU/mL (95% CI: 5.6 – 6.9) and 4.9 log_{10}CFU/mL (95% CI: 4.2 – 5.5) f respectively or ESC resistant; 5.6 log_{10}CFU/mL (95% CI: 5.0 – 6.3) and 3.5 log_{10}CFU/mL (95% CI: 2.9 – 4.1) respectively for AmpC-enzyme and carbapenemase producing strains, and; 5.5 log_{10}CFU/mL (95% CI: 4.9 – 6.1) and 3.3 log_{10}CFU/mL (95% CI: 2.6 – 3.9) respectively for CPEs (Figure 9).

Relative to estimated total bacterial density, the proportion of *bla*-gene positive *E. coli* strains (estimated with MAC+AMP plates) at Hour-0 and Hour-108 in the three chemostat systems; control, ampicillin and ceftiofur were: 100% and 71%, 88% and 100%, and 74% and 49%, respectively (Table 5 and Figure 10). In the control (no antibiotic) porcine culture, estimates of *bla*-positive strain proportions straddled that of total Enterobacteriaceae over the course of the experiment, indicating a dominant presence of these strains relative to the β-lactamase negative strains. In the ampicillin porcine culture, an unexplained decrease in the proportion of *bla*-positive *E. coli* strains was observed between Hour 8 and Hour 24; subsequently, a steady rise to the apex proportion (by Hour 84) was observed (Figure 11). A similar decline in *bla* proportion that commenced at Hour 8 was seen for the ceftiofur inoculated culture; however, unlike with the ampicillin culture only a small rise in proportion was observed by the conclusion of the experiment (Figure 12).

ESC resistant strains (MAC+AXO selective plates) maintained a stable fraction in the control porcine culture for about two days; however, a downward trend was observed from about the second day of the experiment until the end – the estimated proportion at Hour 0 was 49% and was 3% by Hour 108. In the ampicillin porcine culture, proportions of ESC resistant strains were generally stable *ab initio*; subsequently, a steady rise in proportion was observed onwards from

about Hour 60 -- the estimated proportion at Hour 0 was 20% and was 50% by Hour108. In contrast, for the ceftiofur porcine culture, ESC resistant strains demonstrated a drastic reduction in proportion in the first 36 hours of the experiment, later, the the initial steep reduction was replaced by subtle drops in proportions over remaining course of the culture experiment -- the estimated proportion at Hour 0 was 58% and 14% by Hour 108. The combined proportion of AmpC-enzyme producers and CPEs (MAC+FOX plates), as well as the estimated proportion of CPE strains (MAC+MERO plates) in the three varieties of culture media, followed a similar temporal pattern as the ESC resistant strains. Briefly, in control culture, at Hour 0 and Hour 108, strains on MAC+FOX plates constituted 21% and 0% (limit of detection was 20 CFU/mL) of the population; strains on MAC+MERO plates were 17% and 0% at a similar sampling frame. In ampicillin culture, strains on MAC+FOX plates constituted 7% and 45% at Hour 0 and Hour 108, respectively, while strains on MAC+MERO plates were 5% and 39%. In ceftiofur culture, strains on MAC+FOX plates were 14% and 1% at Hour-0 and Hour-108 and strains on MAC+MERO plates constituted 10% and 1%.

A closer examination of post-antimicrobial inoculation *E. coli* growth (that is, during the first 24 hours), revealed an early relative increase in β-lactamase producer proportions, particularly the ESBL-enzyme producers in the test cultures compared to the control culture (Figures 13-15). Antimicrobial selection effects were generally observed within six hours post inoculation, while the peak effect was frequently seen at about Hour 4. In the ampicillin inoculated chemostat, the proportion of ESBL-type β-lactamase strains (as estimated by MAC+PIME plates) increased from 4% *ab initio* to an initial peak of 14% at Hour 4; in contrast, the control culture showed a marginal reduction in ESBL proportions estimated across the same time period (Figure 9). The ceftiofur-infused chemostat showed a similar selection pattern:

ESBL-type β-lactamase strain proportions increased from 11% at the commencement of the experiment to a peak of 43% at Hour 4, thus suggesting a disproportionate selection preference for this *E. coli* sub-group (Figure 15). Spikes in estimated *E. coli* proportions on MAC+AMP plates, MAC+FOX plates and MAC+AXO plates paralleled that of the MAC+PIME plates, suggesting a cumulative effect of ESBL-enzyme producers over time.

Although not statistically significant, both ampicillin- and ceftiofur-infused chemostats showed a minimal increase in CPE proportions at Hour 4, with a change of 5% to 7% and 10% to 13%, respectively; in contrast, a reduction (17% to 13%) in CPE proportion was recorded for the control RPCF culture. The lack of increased estimated proportions of AmpC-enzyme producers and CPEs early on in the antibiotic-infused chemostats, suggested a primary favorable over-selection for the ESBL-type enzyme producers under the experimental conditions.

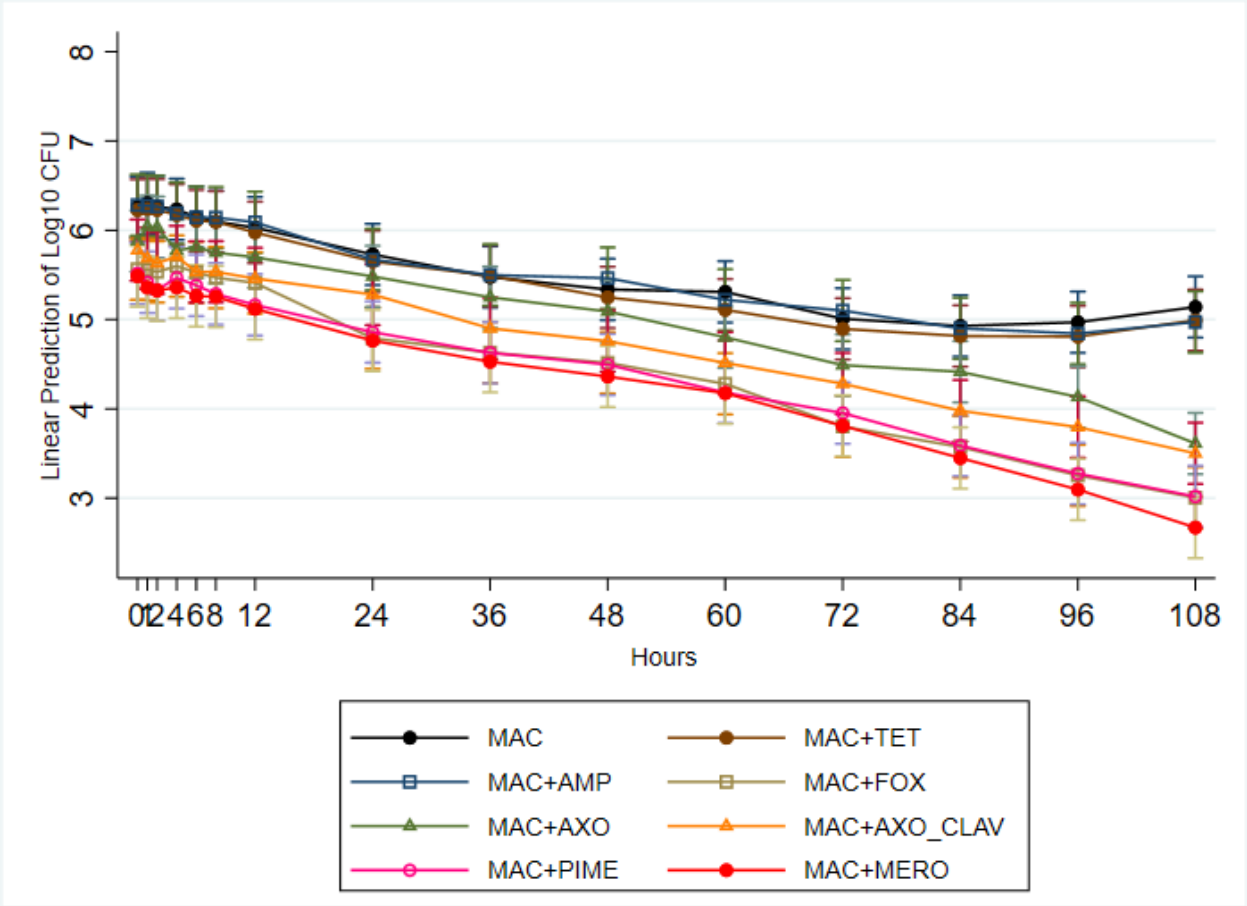


Figure 7. Temporal quantitative estimates of the 10-strain mixture in control (i.e., no antibiotic) porcine chemostat with 95% CIs.

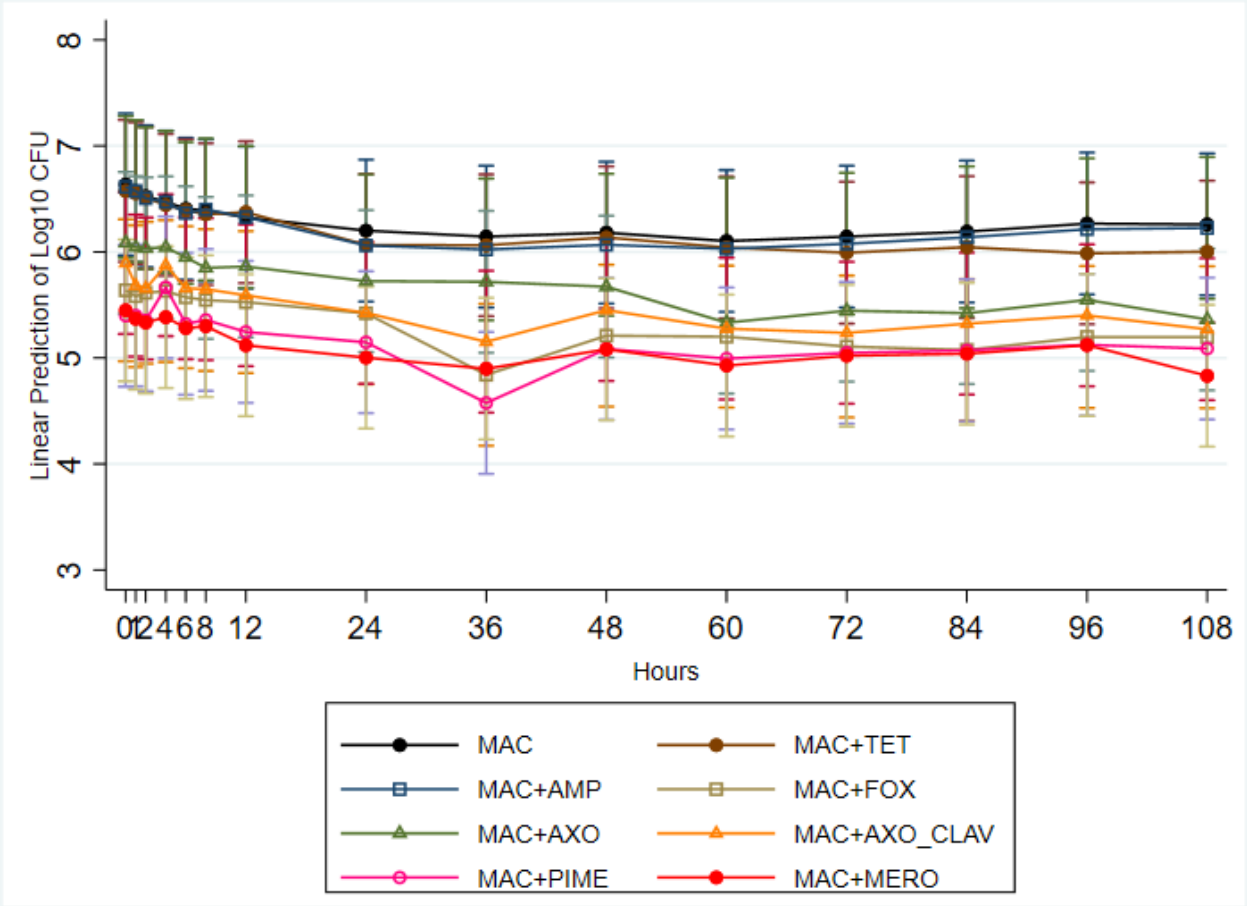


Figure 8. Temporal estimates of the 10-strain mixture in the ampicillin-infused porcine chemostat with 95% CIs.

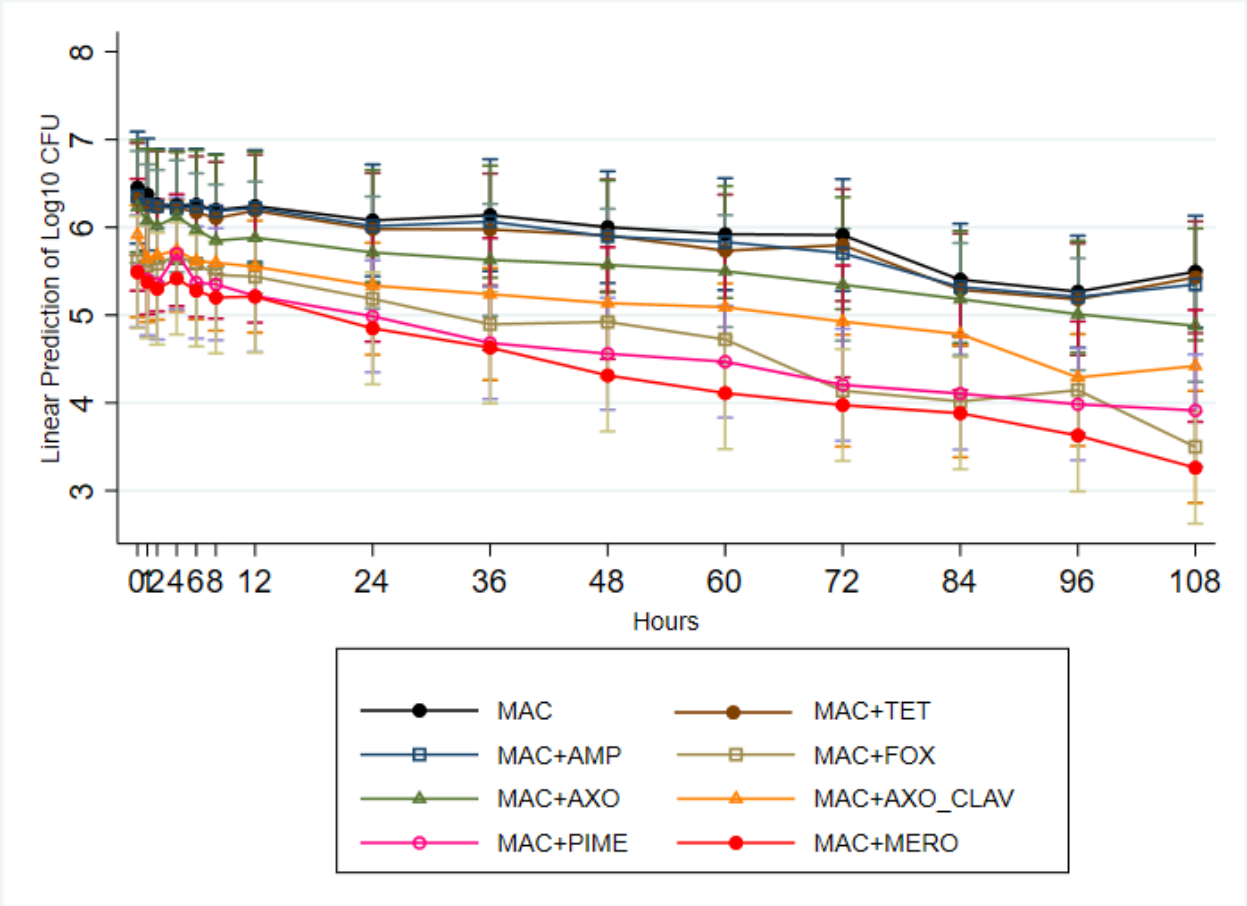


Figure 9. Temporal estimates of the 10-strain mixture in the ceftiofur-infused porcine chemostat with 95% CIs.

Table 5. Within-column relative quantities (%) of estimated bacterial counts at increasing hours post-inoculation and subsequently grown on plain versus selective antibiotic MacConkey agar plates.

HOUR/ PLATE	0	1	2	4	6	8	12	24	36	48	60	72	84	96	108
Control															
	% CFU	% CFU	% CFU	% CFU	% CFU	% CFU	% CFU	% CFU	% CFU	% CFU	% CFU	% CFU	% CFU	% CFU	% CFU
MAC	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
MAC+TET	0.99	0.90	0.92	0.91	0.90	0.99	0.85	0.80	1.04	0.86	0.76	1.18	0.84	0.86	0.98
MAC+AMP	1.08	0.95	1.02	0.98	1.01	1.11	1.09	0.87	1.05	1.10	0.82	1.45	0.92	0.93	0.71
MAC+FOX	0.21	0.19	0.19	0.23	0.23	0.24	0.23	0.11	0.14	0.13	0.10	0.08	0.02	0.01	0.00
MAC+AXO	0.49	0.55	0.57	0.39	0.46	0.44	0.44	0.54	0.57	0.50	0.32	0.35	0.18	0.11	0.03
MAC+AXOCLAV	0.32	0.24	0.23	0.30	0.24	0.26	0.25	0.35	0.26	0.22	0.15	0.17	0.06	0.04	0.01
MAC+PIME	0.18	0.13	0.11	0.17	0.17	0.15	0.13	0.13	0.14	0.12	0.08	0.11	0.03	0.01	0.00
MAC+MERO	0.17	0.12	0.12	0.13	0.13	0.14	0.11	0.10	0.11	0.10	0.08	0.08	0.02	0.01	0.00
Ampicillin															
	% CFU	% CFU	% CFU	% CFU	% CFU	% CFU	% CFU	% CFU	% CFU	% CFU	% CFU	% CFU	% CFU	% CFU	% CFU
MAC	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
MAC+TET	0.85	1.01	0.93	1.04	1.04	0.96	1.28	0.58	0.73	0.91	0.88	0.77	0.79	0.53	0.57
MAC+AMP	0.88	0.99	0.93	1.03	0.88	0.99	0.93	0.50	0.50	0.65	0.83	0.88	1.03	0.97	1.01
MAC+FOX	0.07	0.08	0.09	0.13	0.13	0.12	0.11	0.09	0.04	0.08	0.12	0.16	0.21	0.29	0.45
MAC+AXO	0.20	0.22	0.23	0.33	0.31	0.23	0.25	0.19	0.20	0.21	0.17	0.24	0.28	0.44	0.50
MAC+AXOCLAV	0.13	0.10	0.10	0.22	0.17	0.15	0.14	0.12	0.11	0.16	0.15	0.16	0.24	0.41	0.40
MAC+PIME	0.04	0.05	0.05	0.14	0.07	0.08	0.06	0.05	0.03	0.07	0.08	0.11	0.12	0.19	0.24
MAC+MERO	0.05	0.05	0.05	0.07	0.07	0.07	0.05	0.04	0.03	0.07	0.08	0.12	0.16	0.22	0.39

Table 5. Continued

HOUR/ PLATE	0	1	2	4	6	8	12	24	36	48	60	72	84	96	108
Ceftiofur															
	% CFU	% CFU	% CFU	% CFU	% CFU	% CFU	% CFU	% CFU	% CFU	% CFU	% CFU	% CFU	% CFU	% CFU	% CFU
MAC	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
MAC+TET	0.72	0.75	0.88	0.89	0.83	0.81	0.85	0.71	0.62	0.70	0.55	0.74	0.76	0.77	0.75
MAC+AMP	0.74	0.75	0.91	0.86	0.95	0.94	0.91	0.79	0.77	0.68	0.68	0.42	0.75	0.79	0.49
MAC+FOX	0.14	0.16	0.20	0.25	0.21	0.18	0.15	0.11	0.04	0.08	0.09	0.01	0.01	0.20	0.01
MAC+AXO	0.58	0.48	0.54	0.67	0.52	0.46	0.42	0.39	0.23	0.28	0.32	0.16	0.19	0.17	0.14
MAC+AXOCLAV	0.28	0.18	0.24	0.28	0.22	0.24	0.20	0.16	0.09	0.13	0.18	0.09	0.12	0.02	0.08
MAC+PIME	0.11	0.10	0.12	0.43	0.13	0.14	0.09	0.07	0.02	0.02	0.03	0.01	0.01	0.02	0.01
MAC+MERO	0.10	0.10	0.10	0.13	0.10	0.10	0.09	0.05	0.02	0.01	0.01	0.01	0.01	0.01	0.01

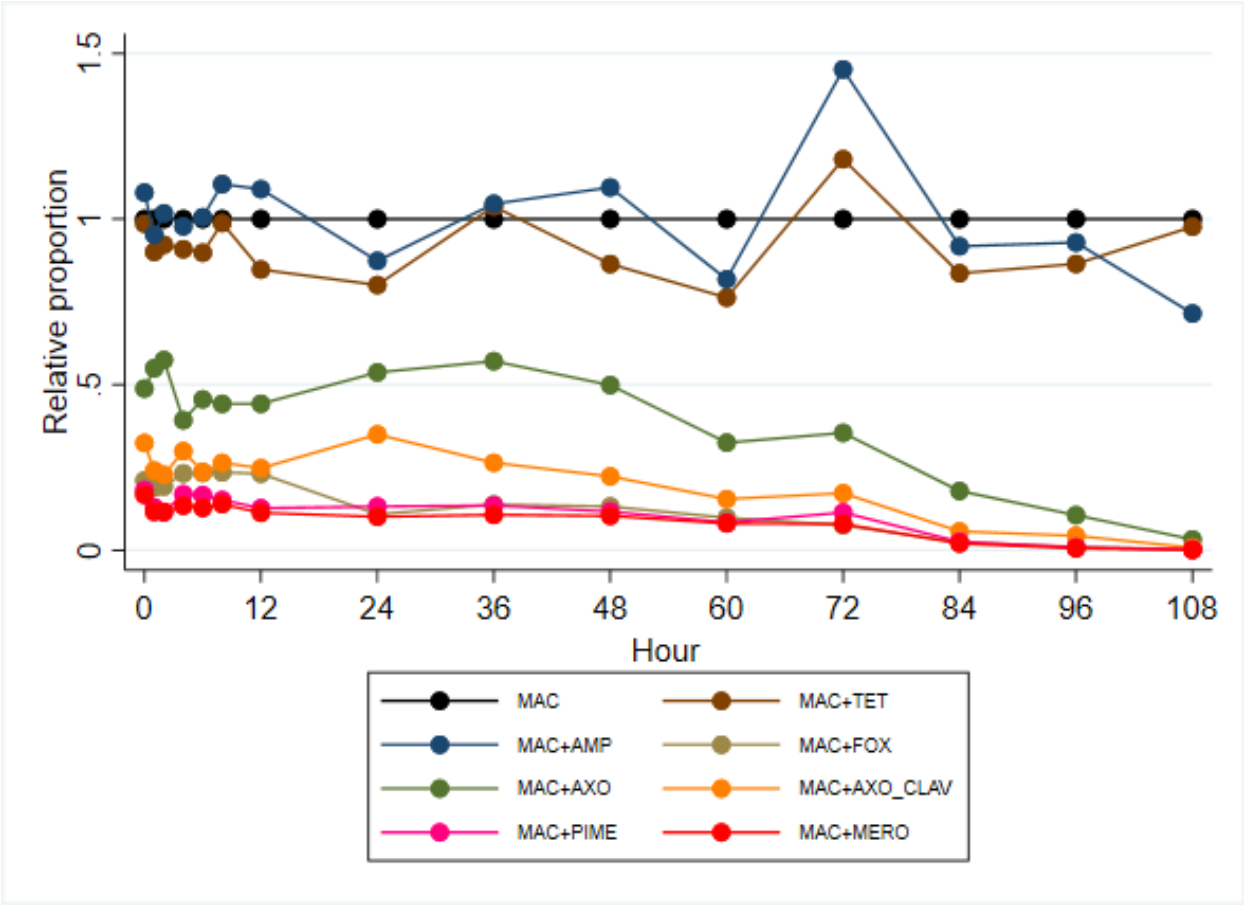


Figure 10. Relative proportions of the 10-strain *E. coli* mixture in the control (i.e., no antibiotic) porcine chemostat over 108 hours. Comparison is the growth on plain MacConkey agar at each time point.

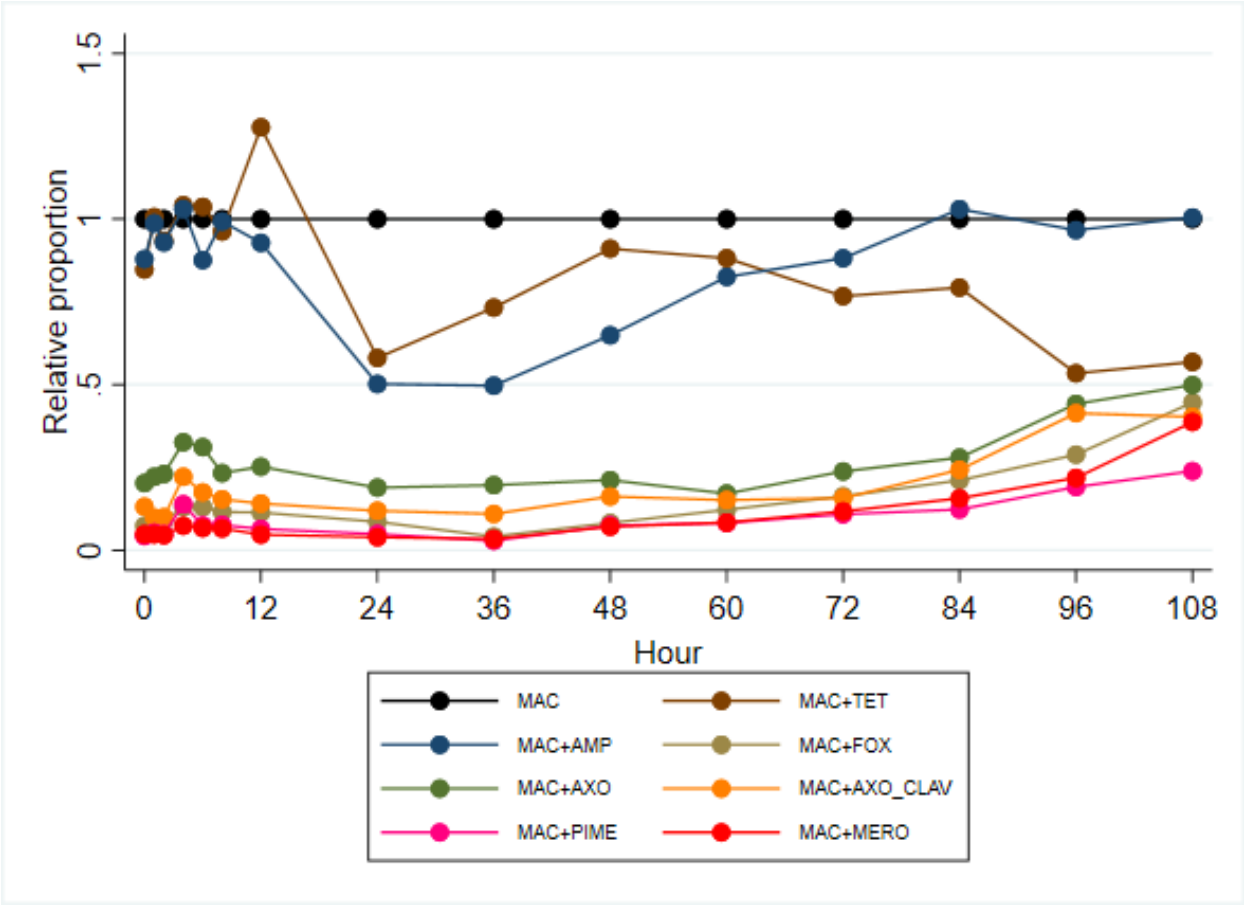


Figure 11. Relative proportions of the 10-strain *E. coli* mixture in the ampicillin-infused porcine chemostat over 108 hours. Comparison is to the growth on plain MacConkey agar at each time point.

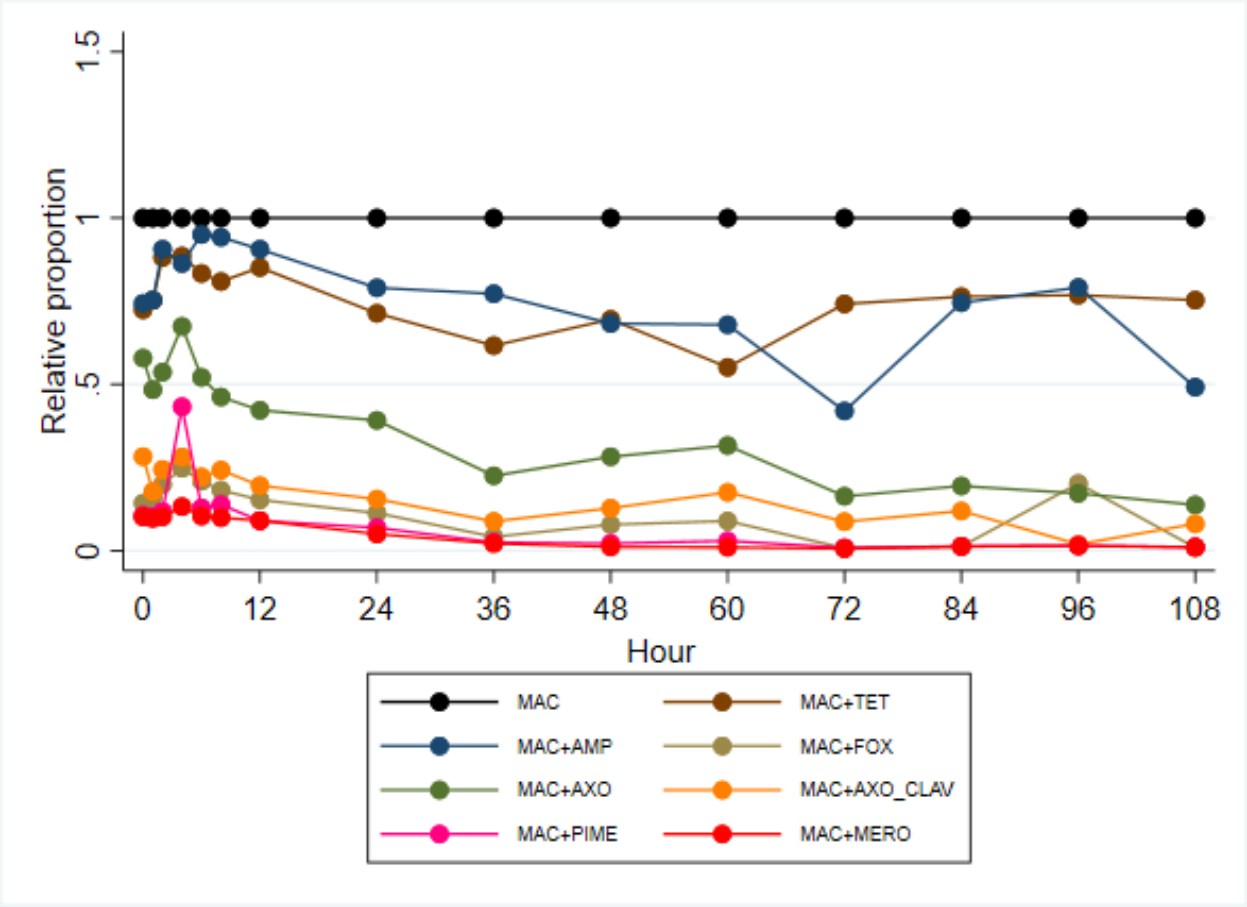


Figure 12. Relative proportions of the 10-strain *E. coli* mixture in the ceftiofur-infused porcine chemostat over 108 hours. Comparison is to the growth on plain MacConkey agar at each time point.

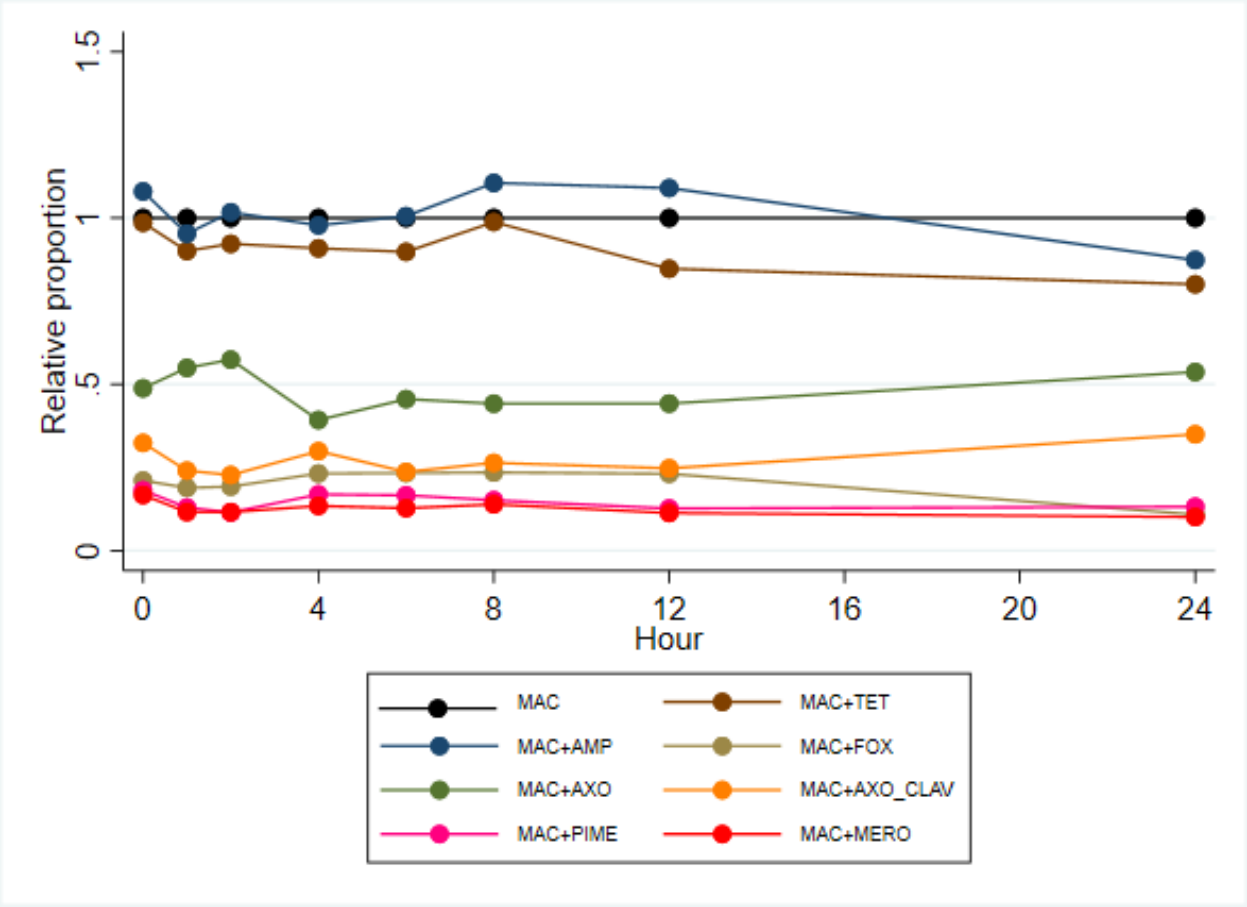


Figure 13. Relative proportions of the 10-strain *E. coli* mixture in the control (i.e., no antibiotic) porcine chemostat first 24 hours. Comparison is the growth on plain MacConkey agar at each time point.

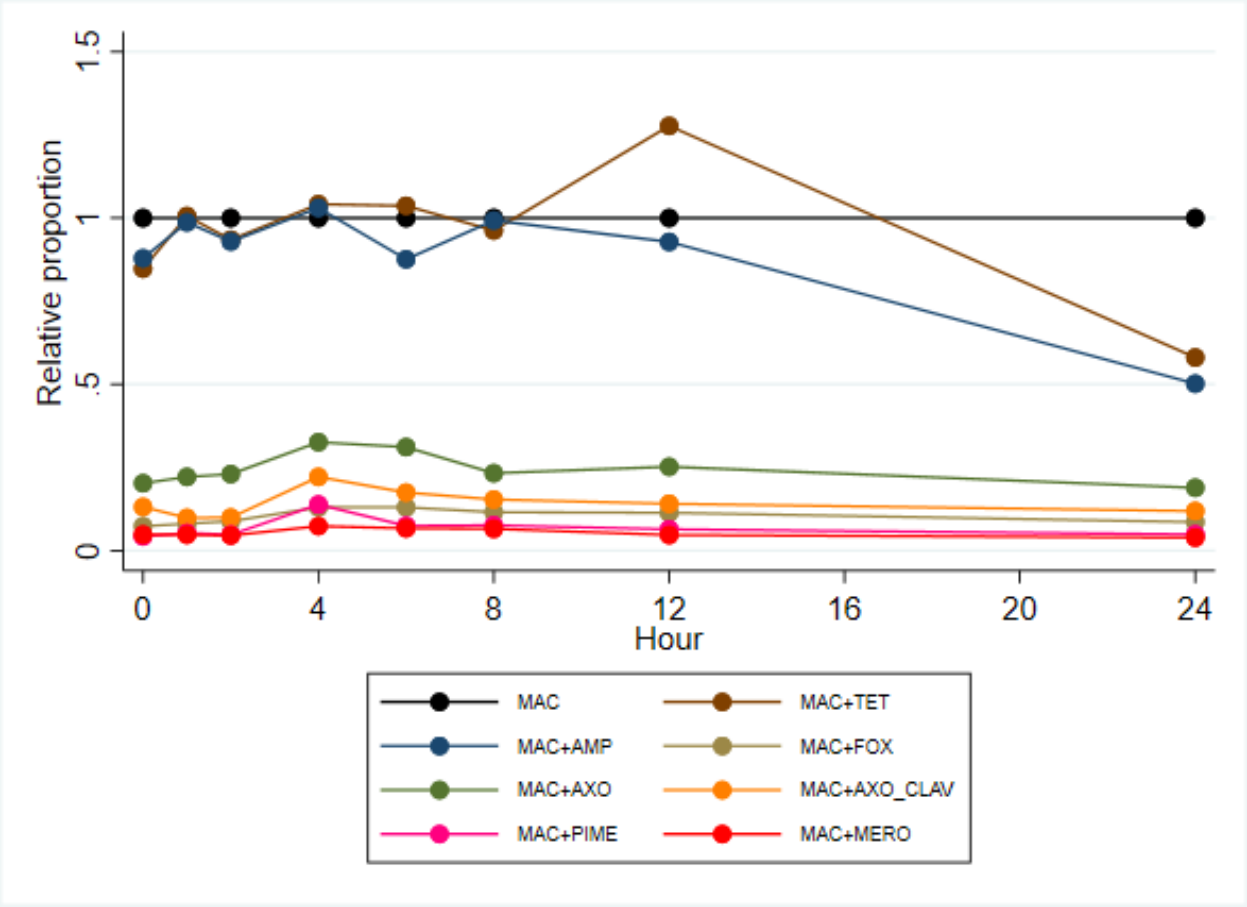


Figure 14. Relative proportions of the 10-strain *E. coli* mixture in the ampicillin-infused porcine chemostat first 24 hours. Comparison is to the growth on plain MacConkey agar at each time point.

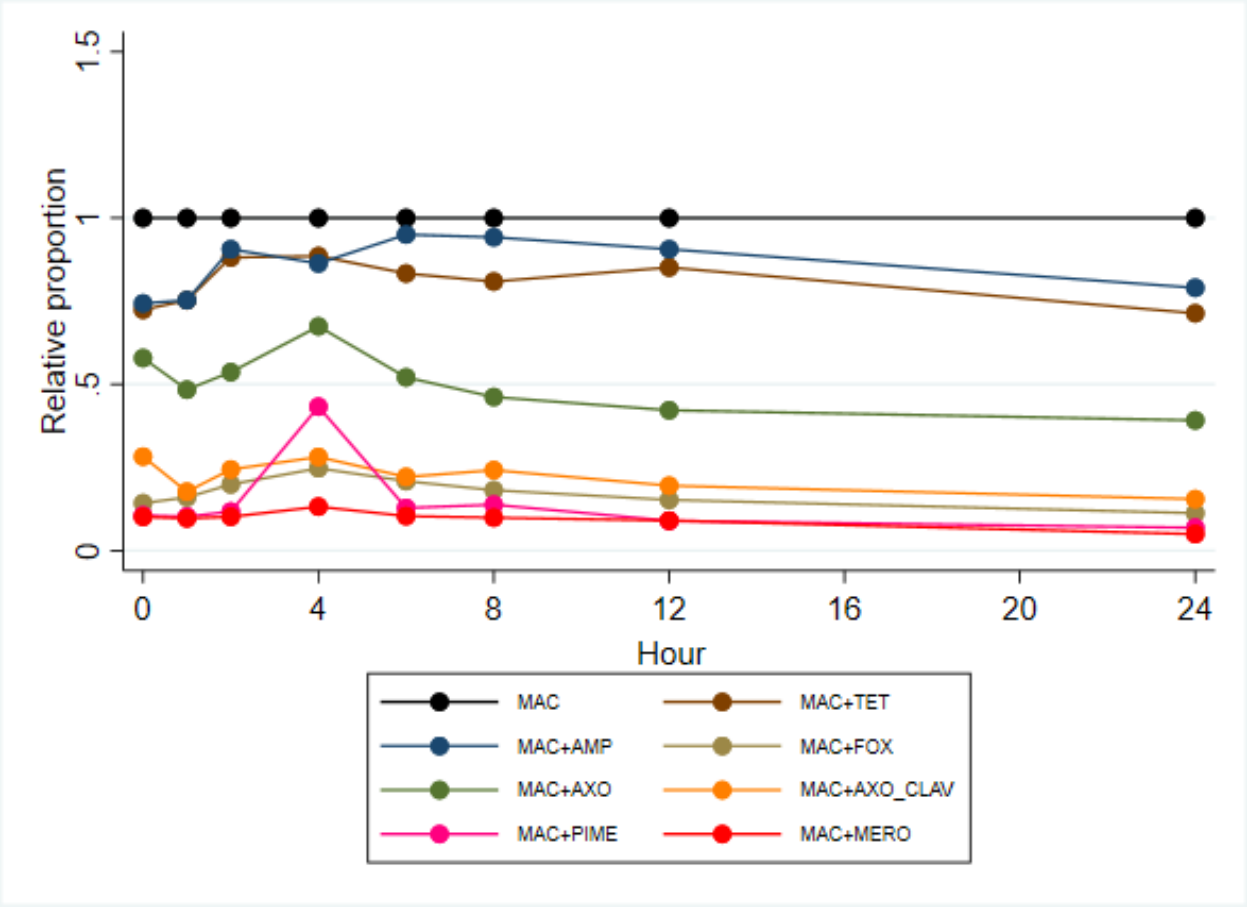


Figure 15. Relative proportions of the 10-strain *E. coli* mixture in the ceftiofur-infused porcine chemostat first 24 hours. Comparison is to the growth on plain MacConkey agar at each time point.

Discussion

To examine the extent to which older-generation β-lactams (e.g., penicillins and third-generation cephalosporins) can disproportionally select for broader-spectrum β-lactamase enzymes (e.g., cephamycinase, cefotaximase, and carbapenemase) producing Enterobacteriaceae, our prior set of experiments (Plos One, revision under review)

employed a series of mono- and mixed-strain batch culture experiments using MacConkey broth as the medium. Although these batch trials are expedient for exploring numerous phenotypic characteristics and the dynamics among pure culture strains of bacteria, they are limited for studying the much more dynamic components of a complex *in vivo* bacterial community. To better the simulate mammalian lower intestinal environment using an *in vitro* experimental model, competitive anerobic commensal microorganisms were incorporated into our current experiment. Further improvements to prior *in vitro* simulations in this study included culture media dynamism, anerobic respiration and temporal estimation of inoculated Enterobacteriaceae over an extended period.

To simulate the behavior of inoculated strains in a mammalian intestinal milieu, growth patterns of *E. coli* inoculum in antimicrobial-free control porcine culture were assessed. In our study, all strain groups in the inoculum, regardless of their individual fitness parameters, largely declined in density and trended towards elimination from the culture over the course of the experiment. Poole et al. (2001), had earlier shown that an exogenous vancomycin-resistant *Enterococcus faecium* of the ATCC 700221 variety introduced into a similar anerobic continuous-flow culture was largely eliminated from the culture system within a seven-day period [90]; in comparison, our own findings agree with this prior observation even though there were still viable *E. coli* at the beginning of the next experimental cycle (~ 7 days). Given that the experimental strains: *E. coli* and *E. faecium,* in these two studies are highly prevalent mammalian intestinal commensals, such findings perhaps underscore the exclusivity and well-balanced

microbiome of *in vivo* normal microbiota communities and the peculiar adaptability required for long-term colonization by novel ingested strains.

As another example, Zambrano et al. (1993), studied Enterobacteriaceae survival in a stressful environment; specifically, they studied *E. coli* growth in stationary phase for extended periods. Their results showed that, when mixed with a fresh culture (~ one day old) of the same strain, even in small quantities, aged *E. coli* strains (over 10 days old) out-competed and overtook the fresh culture after two weeks incubation [132]. The peculiar survival ability of the aged strains was accounted for by a mutation in the *rpoS* gene, a protein-synthesis regulator gene. The capacity and conditions for such adaptations may be essential for fresh inoculum to survive in normal mammalian intestinal flora.

When supplemented with antibiotics, inoculated *E. coli* were noted to have persisted longer in the porcine culture; the observed persistence overall was more pronounced for ampicillin sodium than for ceftiofur sodium. The determinants of the observed superior persistence of inoculated *E. coli* in ampicillin chemostat long-term over ceftiofur porcine culture are not presently known; however, the possibility of a disparity in pharmacokinetic characteristics playing a role cannot be ruled out. It is likely this experiment would need to be replicated to ensure the observed results were not an artifact of the experimental design. In their study, Poole et al. (2001) also observed a reduction in clearance of the exogenous VRE from the RPCF when vancomycin was added to the culture; as examples, the clearance rate of VRE from the culture in the absence of vancomycin reduced from 1.44 $\log_{10}$CFU/day to 0.94 $\log_{10}$CFU/day, 0.52

log_{10}CFU/day and 0.53 log_{10}CFU/day for concentrations of 0.0 μg/mL (negative control), 0.001 μg/mL, 0.01 μg/mL and 0.1 μg/mL of vancomycin, respectively [90]. This observation suggests a likely correlation of antimicrobial concentrations and reduced resistant bacterial clearance in the intestine when selection pressure in the form of antibiotic is applied. These *in vitro* models of human and animal hindgut bacterial competition suggest that antimicrobial exposure may increase mammalian shedding of antimicrobial resistant commensals through post-therapy persistence of such strains; thus, further signifying the diverse ways antimicrobial therapy may impact intestinal microbial ecology.

Absent an antimicrobial, the representative pan-β-lactam susceptible strains demonstrated measurable relative growth advantage in broth-based growth curve experiments; however, in antibiotic-free anerobic continuous porcine culture (chemostats), their estimated proportions were rendered almost negligible for the most of experimental duration. Penicillinase-producing strains appeared most dominant in this culture medium, especially with beta-lactam antibiotics. Unexpectedly, in β-lactam-infused porcine intestinal microbiota cultures, estimated proportions of these strains were significantly higher after an initial period of suppression post-antimicrobial exposure (i.e., post 24 hours). This paradoxical and unexplained fitness of *bla*-negative *E. coli* growth in antimicrobial-free porcine culture relative to β-lactam supplemented cultures may indicate the presence of additional selection parameter(s) that are as yet unidentified; clearly, replication and further investigations are warranted.

In ampicillin-infused porcine culture, the constituent proportions of penicillinase-producers expectedly increased, although marginally, in the first six to eight hours of the study before declining with time. Along with penicillinase-producers, the estimated proportions of ESBL-enzyme producing strains in this medium also increased over similar time periods. This finding indicates that ampicillin can over-select for ESC resistance in an *in vitro* fecal microbiota culture. Observations, as such, may indicate that the impact of direct antimicrobial selection on *E. coli* sub-groups relative to other competing stress parameters in a continuous anerobic porcine microbiota culture system may be limited to a brief window of time post-antimicrobial exposure.

Similar to the ampicillin-infused porcine culture, the introduction of ceftiofur into the chemostat produced an acute increase in estimated proportions of ESBL-enzyme producing strains. In contrast, the observed increase in ESBL-strain proportion was distinctly more marked in the ceftiofur-infused porcine chemostat when compared to the ampicillin-infused culture medium. This observation suggests a clearly superior selection for ESBL-enzyme producing strains by the third-generation cephalosporin ceftiofur than the aminopenicillin β-lactam. A similar observation was seen by Negri et al., in their mixed culture experiment of gram-positive bacteria. Although theirs was an *in vitro* batch experiment without competitive intestinal commensals, higher concentrations of ampicillin favorably selected for *S. pneumoniae* strains with ESC resistance over those with merely aminopenicillin resistance [87].

Notably, in our study, no measurable positive selection for AmpC-enzyme producers or carbapenemase-producing strains was recorded in either ampicillin- or

ceftiofur-infused porcine-chemostat. The observed selection of plasmid-encoded ESBL-enzyme producers over the plasmid-encoded AmpC enzyme producing *E. coli* in the ceftiofur-infused chemostat culture supports our prior finding that AmpC-type strains demonstrated diminished fitness in the presence of a 3rd-generation cephalosporin (e.g., ceftriaxone at 4 μg/mL) compared to ESBL-type strains (see Figure 3 and Table 2). Wu and Livermore (1990), in their study of a wild-type *P. aeruginosa* strain and its mutants, both in pure and mixed strain chemostat cultures, found no selection of the imipenem resistant mutant over the parent strain when challenged with imipenem infusions. Although their mutant strain exhibited imipenem resistance (MIC= 8 μg/mL) in static *in vitro* cultures, relative selection was not seen in the dynamic culture [89].

It seems plausible that the inflow and outflow of fresh versus old culture broth likely continuously modulates constituent bacteria density and drug concentrations (that is, low drug-bacteria contact time). The observed differences in static and dynamic culture findings may be partly attributed to these parameters. Additionally, the potential competition impact of fastidious gram-positive anaerobes on effective density of inoculated strains may also play a modulatory role.

Although the continuous-flow porcine cultures provided a more representative simulation of mammalian intestinal conditions, the rotation of assigned antimicrobial treatments among the same bioreactor systems, even with a 48-Hour rest period, may have impacted experimental findings due to residual carry-over effects of both antibiotics and bacteria. In this study, equal starting proportions of all study genotypic-groups were utilized and this is likely to vary significantly among strain-groups in

nature; consequently, the impact of varied starting densities of these strain-groups was not assessed. It is noteworthy that while the dynamic *in vitro* system simulates bacteria outflow and nutrient replacement, the usual ongoing bacteria ingestion that is seen in animal production environments was not accounted for; therefore, the strain-group population dynamics would be expected vary in a more natural setting on account of this. Hence, supplementary studies such as PK-PD mathematical modeling or with live animal trials, each with an inherent capacity to either model or empirically estimate additional predictive *in vivo* variables remain necessary and are highly recommended.

Overall, this study showed that older-generation β-lactams can positively select for higher priority resistance types such as the cefotaximase ESBL-enzyme in the absence of a co-selecting gene for that antimicrobial. Although a preferential selection for carbapenemase producers was not recorded in this study, such as was seen with our batch culture study, the observed expansion of CTX-M-type β-lactamase producers by ampicillin sodium supports the theory that, upward over-selection for higher-order transferable resistance, even in the absence of antimicrobial specific co-factors, may account for some observed findings in field studies. A pertinent example is the survey of a farrow-to-finish swine operation by Mollenkopf et al. (2017), which showed that commonly used β-lactams such as ceftiofur may be maintaining, and likely expanding the CPE population in that environment [49]. The prevalence of CTX-M-type enzyme producing strains in human and veterinary settings has been increasing in the U.S and indeed globally since initial descriptions [14, 71]; of particular importance, the *bla*-$_{\text{CTX-M*}}$ gene and related genes that encode broad-acting β-lactamase enzymes are major

contributors to increased morbidity and mortality from AMR infections in clinical and community settings [133,134]. The result of this study suggests that, once present in a bacterial community, both the judicious and especially less-judicious uses of ampicillin may increase ESBL strain prevalence; hence, further investigations into this risk, as well as ways to alleviate it are required.

In conclusion, this study demonstrated that an older-generation β-lactam (ampicillin) can positively select for broader spectrum β-lactamase (ESBL) producing strains, even in anerobic mammalian fecal microbiota environments and absent a co-selecting gene for that antimicrobial. Given the high-priority critical importance of 3rd generation and 4th generation cephalosporins to human medicine [96], a complete switch of antimicrobial group (i.e., away from beta-lactams) may be beneficial in particular high-risk situations for mitigating the risk of further ESC resistance expansion.

CHAPTER V

PHARMACOKINETIC/PHARMACODYNAMIC (PK/PD) MATHEMATICAL MODELING OF CEFTIOFUR EFFECTS ON PIG INTESTINAL *E. COLI*

Introduction

Bacterial resistance to antimicrobials threatens the basis of modern clinical response to infectious diseases [15]. Since antimicrobials were first introduced into clinical practice, the frequency and variety of microbial resistance to these agents have also increased; thus, suggesting a direct response of bacteria to the antimicrobials. This is further aggravated by indiscriminate and excessive use of these antimicrobial agents [2]. Currently, certain bacterial species can develop resistance to multiple combinations of antimicrobial therapy, even approaching pan-drug resistance. Hence, the use of newer, often reserved and/or more toxic antimicrobials, including carbapenems, in treating such infections is now necessitated [135].

Although no use in livestock has been reported, and no drugs in the class have ever been approved for such uses, resistance to carbapenems among intestinal commensals of livestock origin is now being globally reported; this suggests that previously approved antimicrobials may also be selecting for this resistance type. When therapeutic antimicrobials are administered systemically they exert a collateral effect on susceptible intestinal commensals; consequently, intestinal bacteria may serve as major reservoirs for varieties of transferable resistance types that benefit from periods of antibiotic-induced stress favoring conjugation and other events.

We have utilized multiple *in vitro* methodologies in our explorations of the extent to which older-generation β-lactam antibiotics (e.g., penicillins and cephalosporins) can differentially select for highest priority antibiotic resistance (e.g., ESC and carbapenems) among representative Enterobacteriaceae. To subject this hypothesis to a comprehensive array of additional experimental assessments, this current study involved exploratory mathematical modeling of intestinal bacterial populations in the presence of a 3rd generation cephalosporin. In this population, we introduced a carbapenemase-producing *E. coli*, something that cannot presently be reliably observed in natural field settings.

Pharmacokinetic/pharmacodynamic (PK-PD) mathematical models have been previously deployed in both AMR and gastrointestinal research [93, 136]; this study uniquely applied this approach to test a hypothesis of upward resistance selection by β-lactam antimicrobials of older generations. Unlike many antimicrobial selection studies where static *in vitro* methodologies (e.g., MIC) form the mainstay of a pharmacodynamic assessment, this study employs a multi-dose temporal estimation of drug pharmacodynamics in its integrated model to simulate *in vivo* antimicrobial effects more accurately. We specifically aimed to explore the resistance selection pattern of ceftiofur sodium at standard prescription label-dose values on a mixed-collection of *E. coli* strains in a simulated pig large intestine.

Methods

Bacterial-antimicrobial pharmacodynamics (Time-kill) assay

One strain each from the pair of representative strains in the mixed-culture experiments was further selected (randomly) as representative of each study strain-genotype. For each strain, 20 mL CAMHB was inoculated with two colonies from fresh blood agar plates and cultured overnight with continuous shaking (200 rpm) at 37°C. In the morning, 115µL of the culture was added to a fresh 20mL CAMH-2 broth and cultured for 60 minutes under similar conditions (to obtain exponential phase bacteria); from this, an experimental mixture of bacterial culture and antibiotic (ceftiofur sodium) broth (CAMH-2) (1:20, that is, effective bacterial concentration of 5×10^5) was prepared. A similar mixture, without antimicrobial, was also prepared for baseline growth rates.

For uninterrupted incubation at sampling times, a one milliliter aliquot of experimental cultures was dispensed into light-proof 1.5 mL sample tubes at each sampling time: 0, 1, 2, 4, 6, 8, 12 and 24 hours, in triplicates. Final antimicrobial concentrations in experimental cultures were equivalent to 0.5 MIC, 1 MIC, 2 MIC, 5 MIC and 10 MIC for each test strain/antimicrobial combination (see strain MIC values in Table 7). Samples were diluted with CAMH-2 broth as required for accurate CFU estimation, while being maintained on ice to slow continued bacteria growth. Bacterial densities were estimated phenotypically on MacConkey agar by spiral plating (Eddy Jet 2™ spiral-plater, Neutec Group Inc., NY.

Strain ceftiofur MIC values were retrieved from previous broth micro-dilution antimicrobial susceptibility testing (AST) (Sensititre ™ Thermo Fisher Scientific, Waltham, MA); that is, provided the strain MIC was covered by the antimicrobial range of the commercial test plates. Otherwise, in-house broth macro-dilution antimicrobial susceptibility tests (AST) were performed. Briefly, suspensions of each bacterial strain were adjusted with 0.5 McFarland standard, cultures with effective bacteria concentration of ~10^5 in the antimicrobial media were subsequently prepared (1:1000 bacteria / antimicrobial media). Adjusted for bacteria suspension volume, ceftiofur CAMH-2 broth media were prepared in two-fold incremental concentrations; cultures were grown with the Bioscreen C™ Automated Microbiology Growth Curve Analysis System (Growth Curve Ltd, Helsinki, Finland) at 37°C for 18 hours without agitation as recommended by the CLSI protocol. MIC values were then determined visually and by OD analyses (less than ten-fold increase in OD value compared with baseline).

The pharmacodynamic relationship between ceftiofur and each experimental strain was estimated with the inhibitory sigmoid pharmacodynamics (Emax) model.

$$E(C) = E_0 - \frac{E_{max} \times C^H}{EC_{50} + C^H}$$

Where: E(C) is the effect (in this case bacterial inhibition) of antimicrobial concentration C; EC_{50} is the antimicrobial concentration that achieves 50% maximal bacteria inhibition; E_{max} is the maximum bacteria inhibition and Hill's coefficient (H) is an estimate of growth rate change with antimicrobial concentration. Statistical operations

were performed in Microsoft Excel™ (Microsoft Corp., Redmond, Washington) and in Phoenix® (Phoenix Software, El Segundo, California).

Model pharmacokinetics

Concentration estimates of ceftiofur and ceftiofur active metabolites (both designated, ceftiofur equivalent (CE)) in swine plasma were obtained from the literature [137-139]. Reported estimates were obtained from 3-4 month-old male and female pigs of between 28 and 78 kg body weight. Animals received intramuscular ceftiofur for three days at a daily dose of 3-5 mg/kg. Plasma drug concentrations are frequently employed as surrogates for tissue/intestinal drug concentrations [91]. In this study, a two-compartment pharmacokinetics model was fitted to the estimated CE plasma concentrations; in addition, reported biliary excretion fraction of ceftiofur [140] was adopted as the effective drug transfer ratio from the central compartment (plasma) to the peripheral compartment (intestinal lumen). Swine upper and lower intestinal CE concentrations were modeled with intestinal transit time estimates [141, 142], intestinal CE bio-degradation quotient [143] and the drug fecal elimination rate [141].

Pharmacokinetics/pharmacodynamics (PK/PD) mathematical models

Lower intestinal density of each group representative *E. coli* strain (*i)*, was estimated by simple integration. Effects of fresh bacterial strain ingestion, bacterial excretion and ceftiofur treatment were simultaneously simulated as represented below:

$$\frac{dN_i}{dt} = r_i\left(1 - \frac{N}{N_{max}}\right)E_iN_i + v_i\gamma N_{max} - \gamma N_i$$

Where, N_i denotes the strain-specific population density (e.g., per gram ingesta); N is the sum total of all *E. coli* strain density and t is a measure of model time. In parenthesis is the density-dependent model inhibitor as determined by the *E. coli* carrying capacity, N_{max}, of the milieu. The parenthetical model component effectively provides for the logarithmic growth curve, slowing as capacity is neared. E_i is the strain-specific inhibitory pharmacodynamics effect; γ signifies the fractional ingestion/excretion constant and v_i represents the strain specific fraction of ingested bacteria.

Initially, to estimate the luminal steady state of *E. coli* population density achieved at reported ingestion and excretion rates of constituent strains [144-146], Enterobacteriaceae population dynamics were modeled without antimicrobial influence. Subsequently, a once daily, three-day ceftiofur treatment was applied to the test population at the same ingestion and excretion rates, strain fractions and peak carrying capacity *ab initio* (see Table 6). Given the absence of experimental data on CPE prevalence in livestock, an initial constituent fraction of 0.001% was assigned to these strains. Later, the ingesta fractions of the CTX-M-type and the NDM-type strains were serially increased by two-fold (at the expense of the β-lactamase free strains) in the presence of ceftiofur treatment. Model simulations were deterministic in nature and steady-state runs were started at varying total *E. coli* densities. Along with estimating the treatment effects of ceftiofur, simulation duration was set at ten days to assess the post-treatment temporal population dynamics of experimental strains.

A list of model parameters as defined in the published literature is provided in Table 6. Large intestinal *E. coli* carrying-capacity (N_{max}) was set at 10^{10}; the established *E. coli* ingestion/excretion constant (γ) was 0.01. Adopted baseline constituent fraction of experimental strain-types for intestinal and ingesta populations were: β-lactamase-free strains (84%); TEM-type enzyme producers (15%); cephamycinase producers (0.89%); cefotaximase producers (0.1%) and carbapenemase producers (0.01%). Pig ceftiofur biliary excretion fraction was modeled at 11%; the estimated intestinal transit times for the model were, 3.5 and 17 hours for small and large intestines respectively. Reported intestinal bio-degradation constant of ceftiofur in cattle feces (0.2 μg/Hour) was also used for the pigs (Figure 16). System modeling was executed in Vensim® software (Ventana Systems, Inc, Harvard, Massachusetts).

Table 6. PK-PD model parameters and values

Parameters	Symbol	Value	Reference
Swine gastric emptying		2.5-5.5 hours	140
Swine *SI transit time		3-4 hours	139
Swine *LI transit time		10-24 hours	139
E. coli carrying capacity	N_{max}	10^{10}	89, 90
Strain-type fraction *ab initio*	$v_{i\ (i=\ 1\text{-}5)}$		
	(1) *NBL	84%	142
	(2) TEM	15%	142
	(3) AmpC	0.89%	143, 144
	(4) ESBL	0.10%	
	(5) CRE	0.01%	
Fractional inflow/outflow	γ	0.01	89, 90
Ceftiofur biliary excretion fraction		11% [+/-5]	138
*CE biodegradation rate		0.2 µg/hour	141

*SI, Small intestine; LI, Large intestine; CE, Ceftiofur equivalent, NBL, No β-lactamase gene.

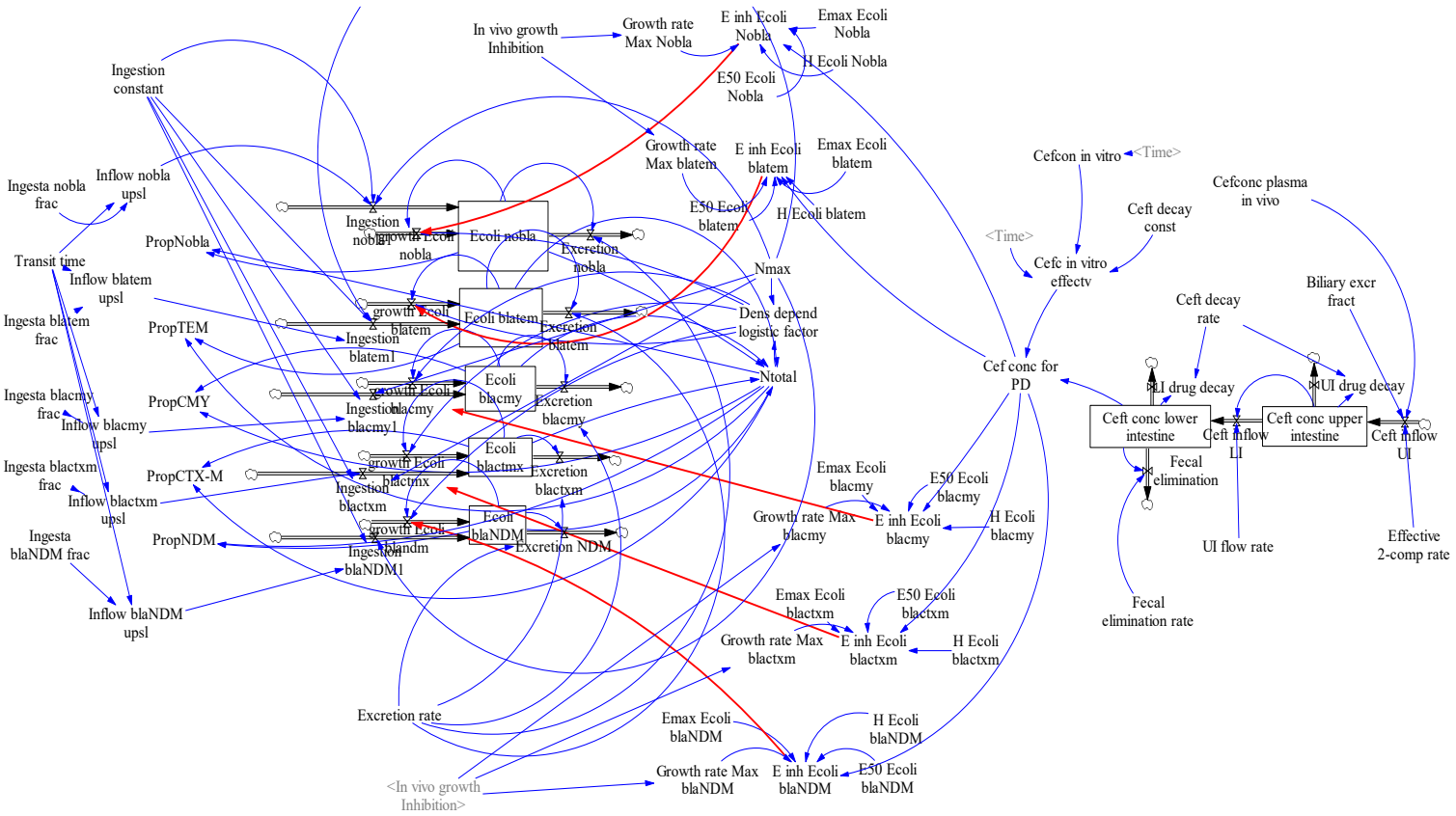


Figure 16. Vensim schematic of PK-PD mathematical model

Results

Estimated *in vitro* ceftiofur MICs were 0.5 µg/mL, 0.5 µg/mL, 16 µg/mL, 512 µg/mL and 8000 µg/mL for *bla*-negative strains, TEM-type enzyme producers, cephamycinase producers, cefotaximase producers and carbapenemase producers, respectively. Predicted EC_{50} values generally followed a similar pattern as the estimated MIC values. As expected, the magnitude of strain growth inhibition with increasing ceftiofur concentrations was greatest with the most susceptible strains as expected (Table 7).

At set ingestion/excretion rates and strain fractions of Enterobacteriaceae, without antimicrobial influence, the attained maximum density (*N*) at steady-state was ~10^9 (Figure 17a), regardless of the starting value; that is, the value of *N* dropped when initiated at carrying capacity and increased when begun at much lower densities. The constituent proportion of the β-lactamase-free strains marginally increased progressively from a starting value of 84% to ~87% by simulation end, usually at the expense of other strains. Estimated fractions of penicillinase producers dropped from 15% to ~12%; cephamycinase producing strains from 0.0089% to ~0.008%, CTX-M-type strains from 0.001% to ~0.0008% and CREs from 0.0001% to ~0.000067% (Figure 18a).

In the presence of experimental antibiotics, an initial steep drop in total bacterial density was observed (~45 x 10^6 CFU/mL at minimum density); subsequently, total population irregularly increased with time. Notably, the total bacterial density did not attain pre-treatment levels until several hours post antimicrobial therapy (Figure 19). Similarly, a precipitous drop in density and proportion of the *bla*-negative strains was

observed, likely accounting for the initial total density drop; the lowest density recorded for this strain-type was ~45 x 10^6 CFU/mL at 8 hours post-treatment initiation, while its least constituent proportion (1.5%) was at 20.5 hours post-treatment. A measured but continuous increase in strain density was subsequently observed; later, steeper increases in density towards pre-treatment levels were seen from about 112 hours onwards. In a like manner, penicillinase producers also showed a sharp decline in population numbers at the initiation of ceftiofur therapy; that is, from a starting density of 15 x 10^8 CFU/mL to a minimum value of ~8.6 x 10^6 CFU/mL (at 8.4 hours post-treatment). Meanwhile, its constituent fraction did not reach the lowest value (0.3%) until 20.5 hours post-treatment. Subsequent growth patterns of penicillinase producers paralleled that of the *bla*-negative strains.

CMY-type β-lactamase producers increased in strain density and proportion almost immediately post-antimicrobial introduction. A rapidly achieved maximum value in estimated density and proportion of 5.28 x 10^9 CFU/mL and 76%, respectively, were each attained at 27 and 9 hours, respectively. Subsequent drops in density and proportional estimates occurred in a step-ladder fashion in direct contrast to daily drug administration and a consequent relative rise in ESBL strain-type population numbers and proportions. A steeper drop towards pre-treatment levels was seen at a similar time as the prior strain types. Observed increases in density and proportion of constituent ESBL strains seemingly mirrored the effective drug concentration. Constituent ESBL strains demonstrated a stepwise increase in density and proportion with estimated peak density and proportions, presumably alongside peak drug concentration, at 5.28 x 10^9

CFU/mL and 65%, respectively, at 92 hours post treatment. A decline towards pre-treatment levels was not observed until about 112 hours as with other strains.

Estimated density and proportions of carbapenemase-producing strains also amplified in response to ceftiofur therapy. Peaks of both estimates (quantity and proportion) were observed between experimental hours 78.1 and 78.6, and at 4.7×10^8 CFU/mL strain density and 5.5% constituent proportion, respectively. A decline towards *ab initio* levels was observed similar to the other *bla* strains (Figures 17b and 18b).

Serial two-fold increases in the ESBL strain ingesta fraction showed corresponding increases in peak density and relative proportions as follows: 5.28×10^9 CFU/mL and 65%; 5.65×10^9 CFU/mL and 65%; 5.77×10^9 CFU/mL and 67%; 5.97×10^9 CFU/mL and 69%; and 6.25×10^9 CFU/mL and 72%. Concurrent two-fold increases of CRE strain fraction in the ingesta yielded a similar pattern as the ESBL strains as follows: 4.7×10^8 CFU/mL and 5.5%; 4.77×10^8 CFU/mL and 5.6%; 4.89×10^8 CFU/mL and 5.7%; 5.08×10^8 CFU/mL and 5.9%; and 5.36×10^8 CFU/mL and 6.2%. Conversely, the CMY-type strain component appeared to decrease in the bacteria community with the increases in the ingesta fraction of ESBL- and CRE-type strains. This was indicated by a reduction in peak density and relative proportions at baseline: 5.28×10^9 CFU/mL and 76% to 4.21×10^9 CFU/mL and 68% respectively, at apex ingesta fractions of the two strains. The relative proportions of the TEM-type component strains appeared unchanged with the two-fold incremental ingesta fraction experiment (Figures 17c-f and 18c-f).

Table 7. Inhibitory pharmacodynamics parameter estimates

Strain-group	E_0	E_{max}	EC_{50}	***H***	EC_{50} **: MIC**	**MIC**
			(µg/mL)			**(µg/mL)**
NBL	0.98	2.38	0.29	4.94	0.58	0.5
TEM	0.92	2.23	0.28	3.78	0.55	0.5
AmpC	0.95	3.01	10.59	1.85	0.66	16
ESBL	0.91	3.41	156.91	1.21	0.31	512
CRE	0.86	1138	12000	0.39	1.50	8000

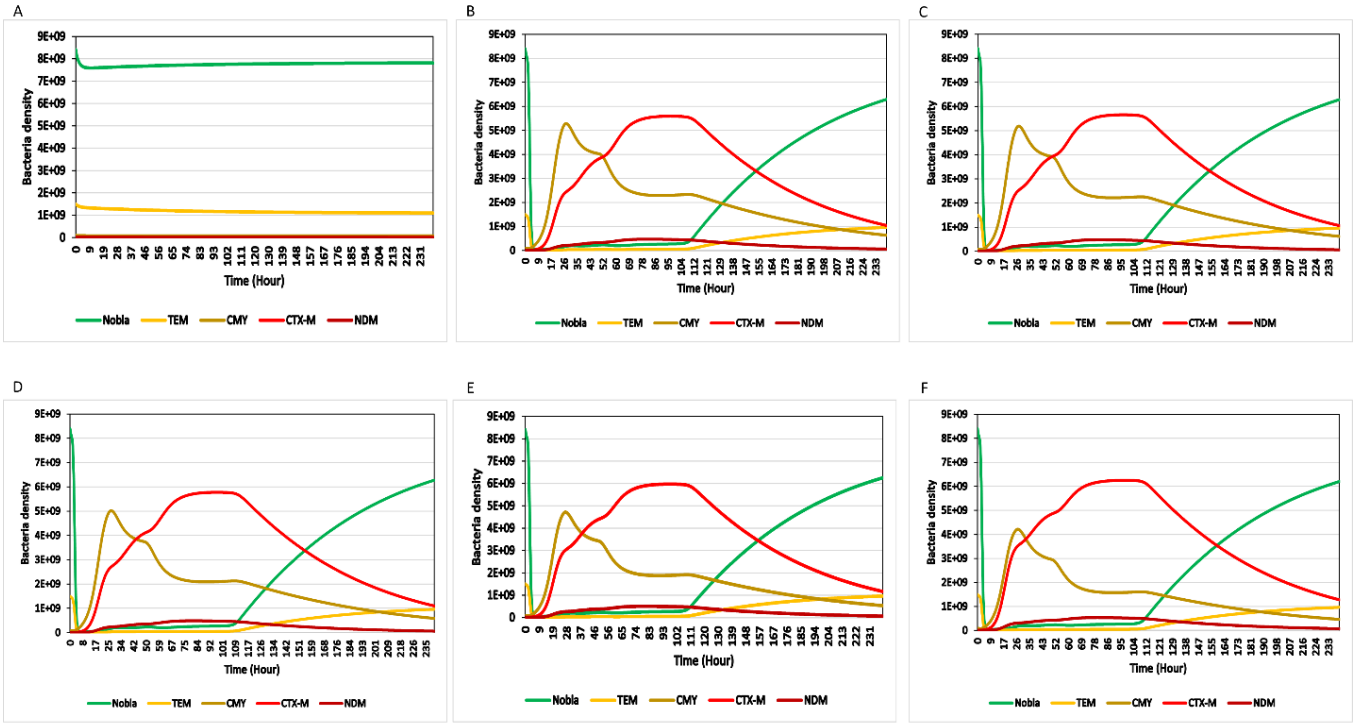


Figure 17. Absolute *E. coli* population densities by strain type in pig intestinal model
(a) strain population absent antimicrobial selection, (b) strain population with three-day ceftiofur treatment at baseline ingestion fractions, (c) strain population with first 2-fold increase of ESBL-type strains and CREs at fixed ceftiofur treatment, (d) strain population with second 2-fold increase of ESBL-type strains and CREs at fixed ceftiofur treatment, (e) strain population with third 2-fold increase of ESBL-type strains and CREs at fixed ceftiofur treatment, (f) strain population with fourth 2-fold increase of ESBL-type strains and CREs at fixed ceftiofur treatment.

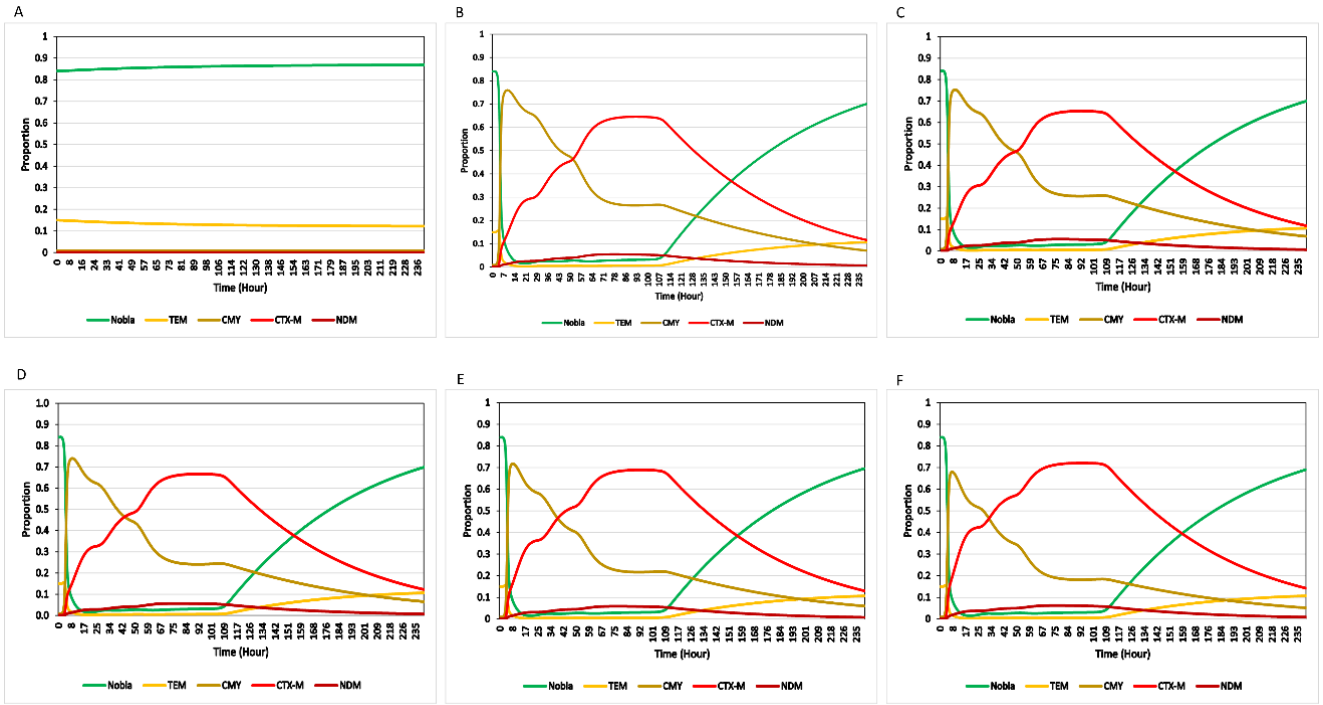


Figure 18. Relative *E. coli* proportions by strain type in pig intestinal model
(a) relative strain proportion absent antimicrobial selection, (b) relative strain proportion with three-day ceftiofur treatment at baseline ingestion fractions, (c) relative strain proportion with first 2-fold increase of ESBL-type strains and CREs at fixed ceftiofur treatment, (d) relative strain proportion with second 2-fold increase of ESBL-type strains and CREs at fixed ceftiofur treatment, (e) relative strain proportion with third 2-fold increase of ESBL-type strains and CREs at fixed ceftiofur treatment, (f) relative strain proportion with fourth 2-fold increase of ESBL-type strains and CREs at fixed ceftiofur treatment.

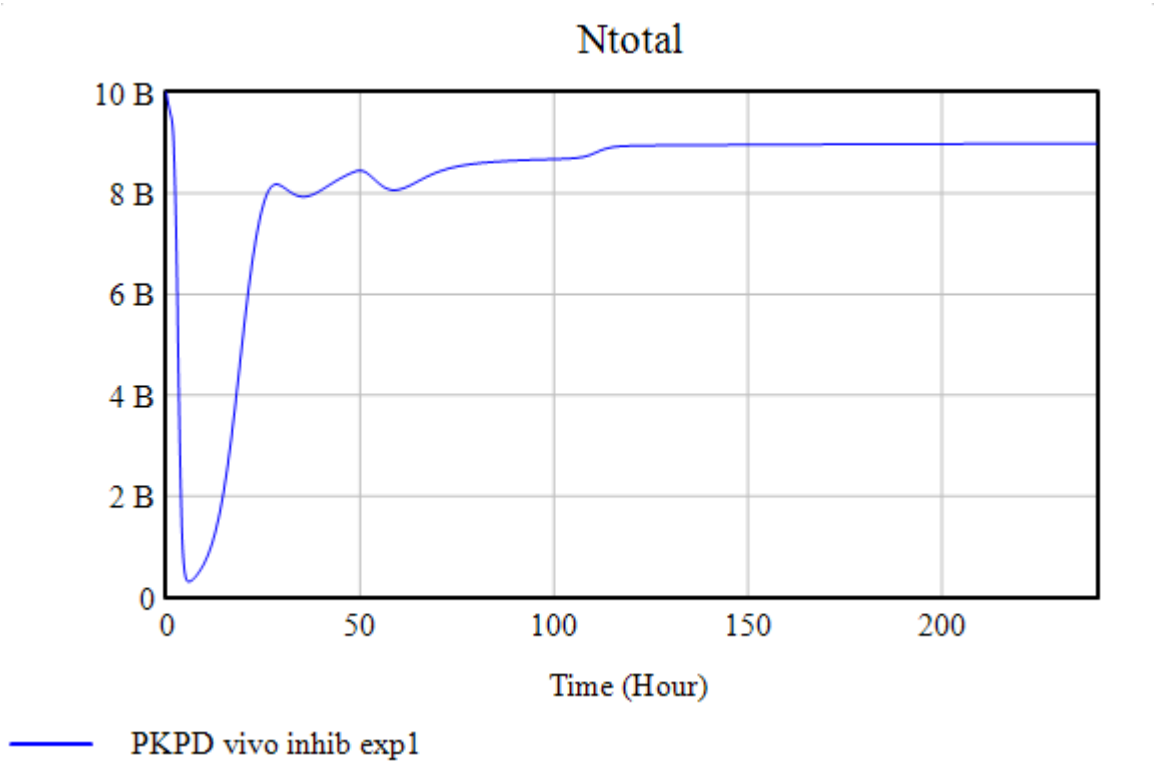


Figure 19. Variations of total *E. coli* population in ceftiofur treated pig model

Discussion

Most mathematical PK-PD modeling research relies on surrogate drug pharmacodynamic data for model implementation; in this study, we uniquely estimated the antimicrobial pharmacodynamic parameters of representative strains of actual host-adapted *E. coli* species. Consequently, the results of this study may be expected to more likely predict *in vivo* Enterobacteriaceae population dynamics as observed in field studies. Relatedly, our study determined fine-tuned MIC values of ceftiofur for

individual representative strains beyond the limited drug concentration ranges provided on commercial sensitivity test kits so as to better ascertain true strain MIC values. Highly elevated MIC values were determined for both ESBL and CRE representative strains; much more so for the CRE strain (8 mg/mL) than for the ESBL strain. This finding with the CRE strain clearly suggest a near absolute resistance to ceftiofur by this strain and the MIC value itself takes on almost comical values such as for a saline solution, or of a nutrient, rather than a toxic agent. At least one study in the literature has reported similarly elevated MIC estimates for CPE/CRE strains among Enterobacteriaceae; Stachyra et al. (2009), in their study of a new pharmacologic agent, reported estimated MIC values for many β-lactam antimicrobials including ceftazidime and ceftriaxone. Ceftriaxone MIC for four strains- an *E. coli,* a *K. pneumoniae* and two *E. cloacae* strains that harbored $bla_{\text{KPC-2}}$ genes were estimated at >2 mg/mL, indicating that the true MIC value may indeed be much higher [147].

To test the reproducibility of reported relative levels of β-lactam resistant *E. coli* strains in the swine large intestine using our mathematical model, a model simulation absent the impact of an antimicrobial agent was performed. The model produced a similar dynamic balance of constituent strains as seen in field studies; that is, according to the model, factors such as strain-specific growth rates, strain ingesta abundance and overall excretion rates play key roles in determining intra-intestinal abundance of strain types. Not often considered in many mathematical PK-PD models, but of equal importance, is the reported difference in the growth rates of bacteria *in vitro* versus *in vivo*. Rubin (1986), compared the growth rates of *Haemophilus influenza* b in different

rat tissues with *in vitro* growth. The study showed that test strain generation time was at least three times as long in rats with intact spleens compared with *in vitro* values. Asplenic rats showed less than twice the *in vitro* generation time *in vivo* [148]. These findings support the imposition of *in vivo* growth restrictions in our model and its direct relevance to improvements attained with *in vivo* mathematical modeling. Furthermore, although trending downwards, the recorded elevated levels of resistant strains did not return to pre-treatment levels by the final (tenth) model day, indicating that antimicrobial selection effect may extend well beyond the treatment duration. Other studies have reported similar findings; for example, Volkova et al. (2011), reported a 5-week duration for attainment of pretreatment levels in their model [92] while Ahmad et al. (2016), reported a drug-dose-dependent duration that was not less than 30 days at all tested doses [91].

The introduction of daily intramuscular short-duration ceftiofur therapy for three days as seen in some livestock operations, into the mathematical PK-PD model, showed that ESCs can readily alter gram-negative bacteria population balance in the large intestine. Model outputs showed that ESC resistant strains (such as AmpC-, ESBL- and CRE-type strains) were preferentially expanded at the expense of strains susceptible to 3rd generation cephalosporins. In our model, AmpC and ESBL strains showed dominance due to their relative population starting densities. Although the AmpC-type strains showed an initial population advantage upon ceftiofur introduction, a reversal of this observed trend was seen with a time-dependent increase in ceftiofur concentration and a cumulative rise in ESBL strain population. Much of this is compatible with what is

being seen in observational studies with ESBLs overtaking AmpC bacteria as the dominant ESC resistant types. The results of our pig intestinal modeling experiment are similar to the findings of Volkova et al. (2011), in their mathematical model of ceftiofur resistant *E. coli* commensals in the cattle intestine. Their results showed that resistant strain types almost completely filled the model carrying capacity when either regular or slow-release ceftiofur treatments were administered [92]. Even further, an *in vivo* experimental study of the impact β-lactam antimicrobials including amoxicillin, ceftiofur and cefquinome on the prevalence of CTX-M-enzyme producing *E. coli* in pig intestines by Cavaco et al. (2008), also demonstrated an expansion of these strain types in all treatment groups [149]. Their observed increase in ESC resistance was most pronounced in the 3rd and 4th generation cephalosporin treatment groups and at levels similar to those reported in our models. Also, the observed overtaking of CMY-2-type strains in terms of dominance of the system by the CTX-M-type strains (despite their lower starting population density) supports the findings of our strain group-fitness assay, which indicated that AmpC-type strains show diminished relative growth rates in a 3-GC broth at certain concentrations (specifically, 4 μg/mL ceftriaxone).

Modeled increments of ingestion fractions of the ESBL-type strains and the CREs showed a relative increase in their absolute population densities and their relative proportions in the bacteria community, even at fixed ceftiofur concentrations. This finding highlights the feedback effect of environmental AMR bacteria prevalence in the face of continued *in vivo* antimicrobial selection pressure. Some studies such as Muller et al. (2018), have demonstrated high prevalence of antimicrobial resistance in gram-

positive commensals in the absence of immediate antibiotic treatment, thus suggesting that environments with prolonged exposure to resistant bacteria can serve as cumulative reservoirs of such strains for new hosts [150]. The proportion of CRE-type strains in the bacterial community was expanded along with the AmpC-type and ESBL-type strains after ceftiofur therapy. Though upon first glance the reported peak proportions of this strain-type may appear relatively small (5.5%), it must be noted that this peak estimate amounted to a 550-fold increase in proportion from the starting values. Increases in ingesta fraction further expanded these values, indicating that ESC can effectively select for this strain-type even if initially present at very low prevalence. Consequently, it can reasonably be expected that with increased dissemination, further selection by ESC and re-ingestion of fecal bacteria, the relative prevalence of this group of Enterobacteriaceae in food animals could soon rise to levels of public health importance.

One notable limitation to consider in the interpretation and application of this research work is the estimated 'absolute' resistance of the representative CRE strain to the antimicrobial ceftiofur. Given the extreme values of the recorded MIC for the strain it was difficult to accurately estimate other properties of ceftiofur pharmacodynamics and to distinguish this from a concentration more related to toxicity. Although commonly used in academic and industry research as surrogates when direct data are lacking, the estimation of large intestinal ceftiofur concentrations through plasma levels of the drug results in a likely limitation of accurate *in vivo* representation by the model. Furthermore, the deterministic nature of the current report precludes all variability that exists in true animal populations and among all of the parameter values utilized in Table

6. Consequently, a stochastic analytic approach is recommended as a next step to account for expected distributions of variable values that might be observed in real populations.

Overall, the results of this modeling effort support the assertion that older-generation β-lactam antimicrobials commonly approved for use in food animal production can differentially select for highest priority antibiotic resistance types among representative Enterobacteriaceae. Expanded spectrum cephalosporins are classified as highest priority critically important antimicrobials for human medicine by the WHO [96]; therefore, expansion of resistance determinants, particularly *bla*$_{\text{CTX-M*}}$ genes, among gram-negative bacteria represents a major threat to infectious disease treatment and control, as well as the continued efficacy of this drug class. Our work further indicates the potential for the spread of this resistance profile and the need for improved and consistent prudent use of critically important antimicrobials.

Equally important is the potential of this commonly used class of antimicrobials to select for carbapenem-resistant bacteria, both in humans and animals; of particular concern, the expansion of CRE among food animal commensal microbiota may increase the risk of community dissemination into the human population through fecal contamination of food products. Similarly, given the common use of ESC in treatment of humans with community acquired infections and HAI alike, our study suggest that such therapy may select for and invariably disseminate CRE strains from unrecognized carriers to contacts in the community and healthcare facilities and later into the environment via wastewater treatment.

In conclusion, our study findings show that the use of commonly prescribed and critically important expanded-spectrum cephalosporin β-lactam antimicrobials can preferentially expand the population of gram-negative strains bearing broad-spectrum high impact resistance profiles, including that of carbapenemases.

CHAPTER VI

CONCLUSION

Often, increased incidence and prevalence of specific resistance phenotypes are associated with increased use of corresponding antimicrobial agents. Despite the absence of carbapenem usage in food animal production, intestinal commensals bearing resistance to this class of agents are being found increasingly in livestock operations worldwide. Consequently, it was suggested that β-lactam antimicrobials such as cephalosporins approved for use in U.S livestock operation may be responsible for the observed trend, although empirical evidence of this assertion remained elusive. The aim of our study was to bridge this evidence gap through layered *in vitro* experimental approaches, as well as via a mathematical pharmacokinetic-pharmacodynamic (PK-PD) model of β-lactam selection effects on a mixture of *E. coli* populating the pig large intestine. We hypothesized that older-generation β-lactam antimicrobials (e.g., aminopenicillins and 3rd generation cephalosporins) can differentially select for highest priority antibiotic resistance (e.g. to 3/4-GC and carbapenems, respectively) among representative Enterobacteriaceae.

To test this hypothesis, 5-genotype groups: *bla*$_{\text{TEM-1}}$, *bla*$_{\text{CMY-2}}$, *bla*$_{\text{CTX-M-*}}$, or *bla*$_{\textit{KPC/IMP/NDM}}$, and a group of beta-lactamase-free strains of host-adapted *E. coli* were assembled. As much as possible, each genotype possessed one variant, and not a combination of β-lactamase genes. Individual and group (n=20) strain fitness parameters were characterized with and without antimicrobials (e.g., ampicillin, ceftriaxone and meropenem) to assess the fitness cost of resistance genes and the impacts of β-lactams

on the growth rate parameters of resistant strains. Furthermore, the differential selection effects of ampicillin and ceftiofur sodium, at varied concentrations, were tested on a mixture of representative strains from each genotypic group in batch cultures. Collectively, the β-lactamase-free strain group showed a significant fitness advantage over β-lactamase producer strains. In 4 μg/mL ceftriaxone, AmpC-type strain-group demonstrated less robust fitness compared with the ESBL-type strain. The mixed-strain *in vitro* culture experiment showed that higher concentrations of both ampicillin and ceftiofur can expand CPE proportions relative to controls in a community of strains. The observed increase in CPE proportions was more pronounced for ceftiofur when compared with ampicillin. These findings provided strong preliminary evidence that approved β-lactams can select for resistance to newer generations of antimicrobial of major clinical importance.

In our quest to thoroughly evaluate the challenge at hand, the primary findings of our growth rate and mixed-group batch culture experiments were followed up with an *in vitro* dynamic culture assay. The continuous culture system (chemostat) incorporated multiple dynamic parameters such as nutrient supply, metabolite removal, anerobic respiration and natural swine fecal microbiota, for a representative model of the mammalian intestinal environment. To assess the relative growth dynamics of the strain groups in the presence of competitive gram-positive anerobic bacteria, the very same ten-strain mixture of representative *E. coli* from each of the same 5 *bla* groups used in the batch culture experiment was introduced into the porcine culture. Furthermore, to measure the selection impact of tested β-lactams on the strain groups in this milieu,

single doses of ampicillin and ceftiofur (both at CLSI breakpoint values) were introduced into replicate chemostat systems. Strain-groups with lower resistance forms appear better adapted to the porcine culture environment. β-lactam introduction showed differential selection for ESBL-type strains early (Hour 4) in the course of the experiment. A clear relative expansion of CPE components was not recorded. This experiment further confirmed that commonly used β-lactams such as aminopenicillins can disproportionally select for higher priority antibiotic resistance (e.g., AmpC and ESBL resistance) even when co-selection genes for these antimicrobials are absent.

Lastly, we applied previously utilized PK-PD mathematical models of bovine Enterobacteriaceae populations to pig intestines to further our inquiry into the resistance selection effects of commonly used β-lactams on newer resistance profiles of greater clinical impact. In the absence of experimental animal studies, informed and sound mathematical simulations can provide useful approximations of *in vivo* experimental findings. Time-kill assays for one representative strain per *bla*-strain group were performed. Estimated growth/death rate values at varied drug concentrations provided the input data for the inhibitory pharmacodynamic (E_{max}) model parameter estimates per strain.

Multi-animal studies of β-lactam plasma concentration values after intramuscular injection in pigs provided the pharmacokinetics data for the system model. Integrations of individual strain-type population and combined strain populations in the pig large intestine were performed; further, the relative levels of each *E. coli bla*-strain group in pigs within United States agriculture were obtained from the published peer-reviewed

literature and set as baseline. Values of relevant physiological processes such as intestinal transit times, drug excretion fraction of the gall bladder and biodegradation of the antimicrobial in large intestine, were also obtained from published literature. To estimate the impact of ceftiofur on the Enterobacteriaceae population in the pig colon, a once daily, three-day treatment regimen of ceftiofur sodium was applied to the model. Furthermore, given their assigned low baseline proportions, the ingestion fractions of CTX-M-type strains and CREs were serially doubled to assess the influence of starting proportions on strain relative intestinal prevalence at fixed doses of ceftiofur therapy.

The results of this study showed that the labeled dose and regimen of ceftiofur clearly expands constituent proportions of AmpC-type strains, ESBL strains and CPEs. Despite its low baseline constituent fraction, the ESBL-type strains dominated the population for larger periods of drug treatment and immediately post-treatment. Even more paltry was the baseline proportions of the CPEs; nonetheless, a 550-fold increase in their constituent proportion was recorded at peak drug selection effect. Observed increases in the constituent proportions were subtly increased further by increasing the ingestion fraction of these strains. The dominance of the ESBL strains over the AmpC-type strains corroborates our earlier observation of relative impaired fitness when grown in ceftriaxone. The results also support the assertion that prior-generations of β-lactams can differentially select for resistance profile to newer-generations of β-lactams, absent a co-selection gene for those agents.

A notable theme of our PK-PD mathematical model was the deterministic nature. This approach fails to account for natural variance in data estimates and data distribution

patterns; a potential source of bias for assessments. Consequently, model sensitivity analysis that accounts for these variations is recommended and a newer approach using stochastic modeling is likely warranted.

Additionally, *in vitro* approaches were the backbone of microbiological studies for many decades and were clearly the source of many expedient data utilized in the field. Yet, there are clear inherent limitations to replicating *in vivo* conditions with this approach. We suggest further live animal experiments with mixed-strains, as with *in vitro* studies, to further test the selection patterns of these widely used antimicrobials and perhaps under varied animal conditions (e.g., with healthy vs sick animals). Clearly, it would be unethical to introduce CREs into food animals and send them to slaughter, so such experimentation would be expensive and likely limited to younger and smaller pigs. As a result of these cost and ethical limitations, though less classical compared to *in vitro* methods, mathematical models have risen in prominence and application over the years. Of course, similar, limitations also abound with this approach; for example, pertinent aspects of normal physiology may be impossible to replicate or else lack reliable data for mathematical simulation.

Given the set of outcomes from our experiment series, we conclude that older-generation β-lactam antimicrobials can differentially select for highest priority antibiotic resistance among representative Enterobacteriaceae. Increased prevalence and spread of this resistance profile in food animals may pose enormous public health risks due to the potential for community spread via contaminated food products. The results of the study also indicate that avoidance/limitation of carbapenem antibiotic use alone may be

insufficient to prevent the spread of CREs into the community through expansion of these strain types in food animal settings. Consequently, we believe that with the currently low prevalence levels of CREs in agriculture there exists an opportunity to respond proactively rather than react later.

REFERENCES

1. Logan, L.K. and R.A. Weinstein, *The epidemiology of carbapenem-resistant Enterobacteriaceae: the impact and evolution of a global menace.* The Journal of Infectious Diseases, 2017. **215**(suppl_1): p. S28-S36.
2. *Antimicrobial resistance: implementing the global action plan in the Region.* East Mediterr Health J, 2016. **22**(2): p. 156-157.
3. CDC, *Antibiotic Resistance Threats in the United States, 2013*. U.S. Department of Health and Human Services, Center for Disease Control and Prevention, 2013.
4. O'Neill, J., *Tackling Drug-resistant Infections Globally: Final Report and Recommendations. The Review on Antimicrobial Resistance. London: HM Government and the Wellcome Trust. 2016.*
5. Wilke, M.S., A.L. Lovering, and N.C. Strynadka, *β-Lactam antibiotic resistance: a current structural perspective.* Current Opinion in Microbiology, 2005. **8**(5): p. 525-533.
6. Bush, K. and P.A. Bradford, *β-Lactams and β-lactamase inhibitors: an overview.* Cold Spring Harbor Perspectives in Medicine, 2016. **6**(8): p. a025247.
7. El-Shaboury, S.R., et al., *Analysis of cephalosporin antibiotics.* Journal of Pharmaceutical and Biomedical Analysis, 2007. **45**(1): p. 1-19.
8. Nightingale, C.H., D.S. Greene, and R. Quintiliani, *Pharmacokinetics and clinical use of cephalosporin antibiotics.* Journal of Pharmaceutical Sciences, 1975. **64**(12): p. 1899-1927.

9. Peechakara, B.V., H. Basit, and M. Gupta, *Mechanism of Action.* Europepmc.org. https://europepmc.org/books/n/statpearls/article-17481/?extid=29083631&src=med

10. Roche, *Rocephin (ceftriaxone sodium) for injection*. 2015, Genentech USA, Inc. South San Francisco, CA.

11. Administration, U.S.F.a.D., *New Animal Drugs; Cephalosporin Drugs; Extralabel Animal Drug Use; Order of Prohibition*, in *FR Doc No: 2012-35*, D.O.H.A.H. SERVICES, Editor. 6/1/2012, Food and Drug Administration: https://www.govinfo.gov/content/pkg/FR-2012-01-06/html/2012-35.htm.

12. Dethlefsen, L., et al., *Assembly of the human intestinal microbiota.* Trends in Ecology & Evolution, 2006. **21**(9): p. 517-523.

13. Xu, J. and J.I. Gordon, *Honor thy symbionts.* Proceedings of the National Academy of Sciences, 2003. **100**(18): p. 10452-10459.

14. Taylor, E.A., et al., *Effects of two-dose ceftiofur treatment for metritis on the temporal dynamics of antimicrobial resistance among fecal Escherichia coli in Holstein-Friesian dairy cows.* Plos One, 2019. **14**(7): p. e0220068.

15. WHO, *Integrated surveillance of antimicrobial resistance in foodborne bacteria: application of a one health approach: guidance from the World Health Organization Advisory Group on Integrated Surveillanec of Antimicrobial Resistance (AGISAR).* 2017.

16. CDC, *Tracking CRE in the United States.* Centers40 for Disease and Control and Prevention, United States Department of Health and Human Services, 2019. https://www.cdc.gov/hai/organisms/cre/trackingcre.html

17. McCuddin, Z., et al., *Klebsiella to Salmonella gene transfer within rumen protozoa: implications for antibiotic resistance and rumen defaunation.* Veterinary Microbiology, 2006. **114**(3-4): p. 275-284.

18. Livermore, D.M., *beta-Lactamases in laboratory and clinical resistance.* Clinical Microbiology Reviews, 1995. **8**(4): p. 557-584.

19. Abraham, E.P. and E. Chain, *An enzyme from bacteria able to destroy penicillin.* Nature, 1940. **146**(3713): p. 837-837.

20. Hall, B.G. and M. Barlow, *Revised Ambler classification of β-lactamases.* Journal of Antimicrobial Chemotherapy, 2005. **55**(6): p. 1050-1051.

21. Ambler, R.P., *The structure of β-lactamases.* Philosophical Transactions of the Royal Society of London. B, Biological Sciences, 1980. **289**(1036): p. 321-331.

22. Bush, K., G.A. Jacoby, and A.A. Medeiros, *A functional classification scheme for beta-lactamases and its correlation with molecular structure.* Antimicrobial Agents and Chemotherapy, 1995. **39**(6): p. 1211.

23. Bush, K. and G.A. Jacoby, *Updated functional classification of β-lactamases.* Antimicrobial Agents and Chemotherapy, 2010. **54**(3): p. 969-976.

24. Page, M.I., *The chemistry of β-lactams*. 2012: Springer Science & Business Media.

25. Sanders, C.C. and W.E. Sanders Jr, *β-Lactam resistance in gram-negative bacteria: global trends and clinical impact.* Clinical Infectious Diseases, 1992. **15**(5): p. 824-839.

26. Jacoby, G.A., *AmpC β-lactamases.* Clinical Microbiology Reviews, 2009. **22**(1): p. 161-182.

27. Nordmann, P., G. Cuzon, and T. Naas, *The real threat of Klebsiella pneumoniae carbapenemase-producing bacteria.* The Lancet Infectious Diseases, 2009. **9**(4): p. 228-236.

28. Poirel, L., et al., *Emergence of oxacillinase-mediated resistance to imipenem in Klebsiella pneumoniae.* Antimicrobial Agents and Chemotherapy, 2004. **48**(1): p. 15-22.

29. Castanheira, M., et al., *Early dissemination of NDM-1-and OXA-181-producing Enterobacteriaceae in Indian hospitals: report from the SENTRY Antimicrobial Surveillance Program, 2006-2007.* Antimicrobial Agents and Chemotherapy, 2011. **55**(3): p. 1274-1278.

30. Bush, K. and J.F. Fisher, *Epidemiological expansion, structural studies, and clinical challenges of new β-lactamases from gram-negative bacteria.* Annual Review of Microbiology, 2011. **65**: p. 455-478.

31. Patel, G. and R. Bonomo, *"Stormy waters ahead": global emergence of carbapenemases.* Frontiers in Microbiology, 2013. **4**: p. 48.

32. McEwen, S.A. and P.J. Fedorka-Cray, *Antimicrobial use and resistance in animals.* Clinical Infectious Diseases, 2002. **34**(Supplement_3): p. S93-S106.

33. Aarestrup, F., *Get pigs off antibiotics.* Nature, 2012. **486**(7404): p. 465-466.

34. Johnson, J.R., et al., *Similarity between human and chicken Escherichia coli isolates in relation to ciprofloxacin resistance status.* The Journal of Infectious Diseases, 2006. **194**(1): p. 71-78.

35. Tacket, C.O., et al., *An outbreak of multiple-drug-resistant Salmonella enteritis from raw milk.* JAMA, 1985. **253**(14): p. 2058-2060.

36. Alexander, T., et al., *Farm-to-fork characterization of Escherichia coli associated with feedlot cattle with a known history of antimicrobial use.* International Journal of Food Microbiology, 2010. **137**(1): p. 40-48.

37. Sørensen, T.L., et al., *Transient intestinal carriage after ingestion of antibiotic-resistant Enterococcus faecium from chicken and pork.* New England Journal of Medicine, 2001. **345**(16): p. 1161-1166.

38. Price, L.B., et al., *Elevated risk of carrying gentamicin-resistant Escherichia coli among US poultry workers.* Environmental Health Perspectives, 2007. **115**(12): p. 1738-1742.

39. Levy, S.B., G.B. Fitzgerald, and A.B. Macone, *Spread of antibiotic-resistant plasmids from chicken to chicken and from chicken to man.* Nature, 1976. **260**(5546): p. 40-42.

40. OIE, *OIE list of antimicrobials of veterinary importance*. 2007, World Organisation for Animal Health: https://www.oie.int/fileadmin/Home/eng/Internationa_Standard_Setting/docs/pdf/OIE_list_antimicrobials.pdf.

41. Fischer, J., et al., *Escherichia coli producing VIM-1 carbapenemase isolated on a pig farm.* Journal of Antimicrobial Chemotherapy, 2012. **67**(7): p. 1793-1795.

42. Fischer, J., et al., *Salmonella enterica subsp. enterica producing VIM-1 carbapenemase isolated from livestock farms.* Journal of Antimicrobial Chemotherapy, 2013. **68**(2): p. 478-480.

43. Fischer, J., et al. *Carbapenemases are present in enterobacteria from non-domestic animals... more frequently than we thought* in *Twenty-third European Congress of Clinical Microbiology and Infectious Diseases. Berlin, Germany*. 2013.

44. Wang, Y., et al., *Identification of New Delhi metallo-β-lactamase 1 in Acinetobacter lwoffii of food animal origin.* Plos One, 2012. **7**(5): p. e37152.

45. Zhang, W.-J., et al., *Complete sequence of the bla NDM-1-carrying plasmid pNDM-AB from Acinetobacter baumannii of food animal origin.* Journal of Antimicrobial Chemotherapy, 2013. **68**(7): p. 1681-1682.

46. Al Bayssari, C., et al., *Emergence of carbapenemase-producing Pseudomonas aeruginosa and Acinetobacter baumannii in livestock animals in Lebanon.* Journal of Antimicrobial Chemotherapy, 2015. **70**(3): p. 950-951.

47. Webb, H.E., et al., *Carbapenem-resistant bacteria recovered from faeces of dairy cattle in the high plains region of the USA.* Plos One, 2016. **11**(1): p. e0147363.

48. Mollenkopf, D.F., et al., *Maintenance of Carbapenemase-producing Enterobacteriaceae in a farrow-to-finish swine production system.* Foodborne Pathogens and Disease, 2018. **15**(6): p. 372-376.

49. Mollenkopf, D.F., et al., *Carbapenemase-producing Enterobacteriaceae recovered from the environment of a swine farrow-to-finish operation in the United States.* Antimicrobial Agents and Chemotherapy, 2017. **61**(2).

50. Poirel, L., et al., *Carbapenemase-producing Acinetobacter spp. in cattle, France.* Emerging Infectious Diseases, 2012. **18**(3): p. 523.

51. Woodford, N., et al., *Carbapenemase-producing Enterobacteriaceae and non-Enterobacteriaceae from animals and the environment: an emerging public health risk of our own making?* Journal of Antimicrobial Chemotherapy, 2014. **69**(2): p. 287-291.

52. Honore, N., M.H. Nicolas, and S.T. Cole, *Inducible cephalosporinase production in clinical isolates of Enterobacter cloacae is controlled by a regulatory gene that has been deleted from Escherichia coli.* The EMBO journal, 1986. **5**(13): p. 3709-3714.

53. Bergström, S., et al., *Comparison of the overlapping frd and ampC operons of Escherichia coli with the corresponding DNA sequences in other gram-negative bacteria.* Journal of Bacteriology, 1983. **155**(3): p. 1297-1305.

54. Bauernfeind, A., S. Schweighart, and Y. Chong, *Extended broad spectrum β-lactamase in Klebsiella pneumoniae including resistance to cephamycins.* Infection, 1989. **17**(5): p. 316-321.

55. Bauernfeind, A., et al., *Characterization of the plasmidic beta-lactamase CMY-2, which is responsible for cephamycin resistance.* Antimicrobial Agents and Chemotherapy, 1996. **40**(1): p. 221-224.

56. Nakano, R., et al., *Resistance to gram-negative organisms due to high-level expression of plasmid-encoded ampC β-lactamase blaCMY-4 promoted by insertion sequence ISEcp1.* Journal of Infection and Chemotherapy, 2007. **13**(1): p. 18-23.

57. Morosini, M., et al., *Biological Cost of AmpC Production forSalmonella enterica Serotype Typhimurium.* Antimicrobial Agents and Chemotherapy, 2000. **44**(11): p. 3137-3143.

58. Dunne, E.F., et al., *Emergence of domestically acquired ceftriaxone-resistant Salmonella infections associated with AmpC beta-lactamase.* JAMA, 2000. **284**(24): p. 3151-6.

59. Moland, E.S., et al., *Occurrence of newer beta-lactamases in Klebsiella pneumoniae isolates from 24 U.S. hospitals.* Antimicrob Agents Chemother, 2002. **46**(12): p. 3837-42.

60. Ghafourian, S., et al., *Extended spectrum beta-lactamases: definition, classification and epidemiology.* Curr Issues Mol Biol, 2015. **17**(1): p. 11-22.

61. Kliebe, C., et al., *Evolution of plasmid-coded resistance to broad-spectrum cephalosporins.* Antimicrobial Agents and Chemotherapy, 1985. **28**(2): p. 302-307.

62. Quinn, J.P., et al., *Novel plasmid-mediated beta-lactamase (TEM-10) conferring selective resistance to ceftazidime and aztreonam in clinical isolates of Klebsiella pneumoniae.* Antimicrobial Agents and Chemotherapy, 1989. **33**(9): p. 1451-1456.

63. Matsumoto, Y., et al., *Novel plasmid-mediated beta-lactamase from Escherichia coli that inactivates oxyimino-cephalosporins.* Antimicrobial Agents and Chemotherapy, 1988. **32**(8): p. 1243-1246.

64. Sjölund, M., et al., *Human Salmonella infection yielding CTX-M β-lactamase, United States.* Emerging Infectious Diseases, 2008. **14**(12): p. 1957.

65. Bonnet, R., et al., *Novel cefotaximase (CTX-M-16) with increased catalytic efficiency due to substitution Asp-240-->Gly.* Antimicrob Agents Chemother, 2001. **45**(8): p. 2269-75.

66. Cartelle, M., et al., *High-level resistance to ceftazidime conferred by a novel enzyme, CTX-M-32, derived from CTX-M-1 through a single Asp240-Gly substitution.* Antimicrob Agents Chemother, 2004. **48**(6): p. 2308-13.

67. Kimura, S., et al., *Predictive analysis of ceftazidime hydrolysis in CTX-M-type beta-lactamase family members with a mutational substitution at position 167.* Int J Antimicrob Agents, 2007. **29**(3): p. 326-31.

68. Bonnet, R., *Growing group of extended-spectrum beta-lactamases: the CTX-M enzymes.* Antimicrob Agents Chemother, 2004. **48**(1): p. 1-14.

69. Castanheira, M., et al., *Rapid emergence of bla CTX-M among Enterobacteriaceae in US medical centers: molecular evaluation from the MYSTIC Program (2007).* Microbial Drug Resistance, 2008. **14**(3): p. 211-216.

70. Castanheira, M., et al., *Prevalence of beta-lactamase-encoding genes among Enterobacteriaceae bacteremia isolates collected in 26 U.S. hospitals: report from the SENTRY Antimicrobial Surveillance Program (2010).* Antimicrob Agents Chemother, 2013. **57**(7): p. 3012-20.

71. Doi, Y., et al., *Community-associated extended-spectrum beta-lactamase-producing Escherichia coli infection in the United States.* Clin Infect Dis, 2013. **56**(5): p. 641-8.

72. Livermore, D.M., *Carbapenemases.* Journal of Antimicrobial Chemotherapy, 1992. **29**(6): p. 609-613.

73. Watanabe, M., et al., *Transferable imipenem resistance in Pseudomonas aeruginosa.* Antimicrobial Agents and Chemotherapy, 1991. **35**(1): p. 147-151.

74. Osano, E., et al., *Molecular characterization of an enterobacterial metallo beta-lactamase found in a clinical isolate of Serratia marcescens that shows imipenem resistance.* Antimicrobial Agents and Chemotherapy, 1994. **38**(1): p. 71-78.

75. Ito, H., et al., *Plasmid-mediated dissemination of the metallo-beta-lactamase gene blaIMP among clinically isolated strains of Serratia marcescens.* Antimicrobial Agents and Chemotherapy, 1995. **39**(4): p. 824-829.

76. Toleman, M.A., et al., *blaVIM-7, an evolutionarily distinct metallo-beta-lactamase gene in a Pseudomonas aeruginosa isolate from the United States.* Antimicrob Agents Chemother, 2004. **48**(1): p. 329-32.

77. Lauretti, L., et al., *Cloning and characterization of bla VIM, a new integron-borne metallo-β-lactamase gene from a Pseudomonas aeruginosa clinical isolate.* Antimicrobial Agents and Chemotherapy, 1999. **43**(7): p. 1584-1590.

78. Yigit, H., et al., *Novel carbapenem-hydrolyzing beta-lactamase, KPC-1, from a carbapenem-resistant strain of Klebsiella pneumoniae.* Antimicrob Agents Chemother, 2001. **45**(4): p. 1151-61.

79. Kaiser, R.M., et al., *Trends in Klebsiella pneumoniae carbapenemase-positive K. pneumoniae in US hospitals: report from the 2007-2009 SENTRY Antimicrobial Surveillance Program.* Diagn Microbiol Infect Dis, 2013. **76**(3): p. 356-60.

80. CDC, *Morbidity and Mortality Weekly Report News Synopsis for June 24, 2010*. Morbidity and Mortality Weekly Report, 2010. Centers for Disease and Control and Prevention. https://www.cdc.gov/media/mmwrnews/2010/n100624.htm#5

81. Yong, D., et al., *Characterization of a new metallo-beta-lactamase gene, bla(NDM-1), and a novel erythromycin esterase gene carried on a unique genetic structure in Klebsiella pneumoniae sequence type 14 from India.* Antimicrob Agents Chemother, 2009. **53**(12): p. 5046-54.

82. Walsh, T.R., et al., *Dissemination of NDM-1 positive bacteria in the New Delhi environment and its implications for human health: an environmental point prevalence study.* The Lancet Infectious Diseases, 2011. **11**(5): p. 355-362.

83. Fey, P.D., et al., *Ceftriaxone-resistant Salmonella infection acquired by a child from cattle.* New England Journal of Medicine, 2000. **342**(17): p. 1242-1249.

84. Tragesser, L.A., et al., *Association between ceftiofur use and isolation of Escherichia coli with reduced susceptibility to ceftriaxone from fecal samples of dairy cows.* American Journal of Veterinary Research, 2006. **67**(10): p. 1696-1700.

85. Wittum, T.E., et al., *CTX-M-type extended-spectrum β-lactamases present in Escherichia coli from the feces of cattle in Ohio, United States.* Foodborne Pathogens and Disease, 2010. **7**(12): p. 1575-1579.

86. Tadesse, D.A., et al., *Whole-Genome Sequence Analysis of CTX-M Containing Escherichia coli Isolates from Retail Meats and Cattle in the United States.* Microb Drug Resist, 2018. **24**(7): p. 939-948.

87. Negri, M.C., et al., *In vitro selective antibiotic concentrations of beta-lactams for penicillin-resistant Streptococcus pneumoniae populations.* Antimicrobial Agents and Chemotherapy, 1994. **38**(1): p. 122-125.

88. Negri, M.-C. and F. Baquero, *In vitro selective concentrations of cefepime and ceftazidime for AmpC β-lactamase hyperproducer Enterobacter cloacae variants.* Clinical Microbiology and Infection, 1999. **5**: p. S25-S28.

89. Wu, P.J. and D.M. Livermore, *Response of chemostat cultures of Pseudomonas aeruginosa to carbapenems and other β-lactams.* Journal of Antimicrobial Chemotherapy, 1990. **25**(6): p. 891-902.

90. Poole, T.L., et al., *Persistence of a vancomycin-resistant Enterococcus faecium in an anaerobic continuous-flow culture of porcine microflora in the presence of subtherapeutic concentrations of vancomycin.* Microbial Drug Resistance, 2001. **7**(4): p. 343-348.

91. Ahmad, A., et al., *Modeling the growth dynamics of multiple Escherichia coli strains in the pig intestine following intramuscular ampicillin treatment.* BMC Microbiology, 2016. **16**(1): p. 1-10.

92. Volkova, V.V., et al., *Mathematical model of plasmid-mediated resistance to ceftiofur in commensal enteric Escherichia coli of cattle.* Plos One, 2012. **7**(5): p. e36738.

93. Græsbøll, K., et al., *How fitness reduced, antimicrobial resistant bacteria survive and spread: a multiple pig-multiple bacterial strain model.* Plos One, 2014. **9**(7): p. e100458.

94. Mendelson, M. and M.P. Matsoso, *The World Health Organization Global Action Plan for antimicrobial resistance.* S Afr Med J, 2015. **105**(5): p. 325.

95. CDC, *Antibiotic Resistance Threats in the United States, 2019*, Centers for Disease Control Prevention, U.S. Department of Health and Human Services, 2019.

96. WHO, *Critically important antimicrobials for human medicine.* 2019. World Health Orgazation. https://www.who.int/foodsafety/areas_work/antimicrobial-resistance/cia/en/

97. CDC, *CDC-FDA Antimicrobial Resistance Isolate Bank; Enterobacteriaceae Carbapenem Breakpoint Panel, Gram Negative Carbapenemase Detection (Active) Panel, Gram Negative Carbapenemase Detection Panel, Enterobacteriaceae Carbapenemase DiversityPanel, Ceftazidime/avibactam Panel, Ceftazidime/avibactam (Active) Panel, Cefepime/ zidebactam (Active) Panel, Meropenem/vaborbactam Verification (Active) Panel.*, U.S.D.o.H.a.H. Services, Editor. 2019, CDC: https://wwwn.cdc.gov/ARIsolateBank/.

98. Chalmers, G., et al., *Distribution of the PCO gene cluster and associated genetic determinants among swine Escherichia coli from a controlled feeding trial.* Genes, 2018. **9**(10): p. 504.

99. *National Antimicrobial Resistance Monitoring System. Interagency Laboratory Manual Third Edition*, F. U.S. Department of Health and Human Services, Editor. 2016, U.S Food and Drug Administration: https://www.fda.gov/media/101423/download.

100. Clinical and L.S. Institute, *Performance standards for antimicrobial susceptibility testing*. 2017, Clinical and Laboratory Standards Institute Wayne, PA.

101. Zerbino, D.R., *Using the velvet de novo assembler for short-read sequencing technologies.* Current Protocols in Bioinformatics, 2010. **31**(1): p. 11.5. 1-11.5. 12.

102. Bankevich, A., et al., *SPAdes: a new genome assembly algorithm and its applications to single-cell sequencing.* Journal of Computational Biology, 2012. **19**(5): p. 455-477.

103. Zankari, E., et al., *Genotyping using whole-genome sequencing is a realistic alternative to surveillance based on phenotypic antimicrobial susceptibility testing.* Journal of Antimicrobial Chemotherapy, 2013. **68**(4): p. 771-777.

104. Carattoli, A., et al., *In silico detection and typing of plasmids using PlasmidFinder and plasmid multilocus sequence typing.* Antimicrobial Agents and Chemotherapy, 2014. **58**(7): p. 3895-3903.

105. NCBI, *Sequence Read Archive*, N. U.S. Department of Health and Human Services, Editor. 2020, U.S. National Library of Medicine: https://www.ncbi.nlm.nih.gov/sra.

106. Inouye, M., et al., *SRST2: rapid genomic surveillance for public health and hospital microbiology labs.* Genome Medicine, 2014. **6**(11): p. 90.

107. Larsen, M.V., et al., *Multilocus sequence typing of total-genome-sequenced bacteria.* Journal of Clinical Microbiology, 2012. **50**(4): p. 1355-1361.

108. Seemann, T., *ABRicate: mass screening of contigs for antimicrobial and virulence genes.* Department of Microbiology and Immunology, The University of Melbourne, Melbourne, Australia. Available online: https://github.com/tseemann/abricate (accessed on 28 February 2019), 2018.

109. Carattoli, A., et al., *Novel plasmid-mediated colistin resistance mcr-4 gene in Salmonella and Escherichia coli, Italy 2013, Spain and Belgium, 2015 to 2016.* Eurosurveillance, 2017. **22**(31): p. 30589.

110. Tjørve, K.M. and E. Tjørve, *The use of Gompertz models in growth analyses, and new Gompertz-model approach: An addition to the Unified-Richards family.* Plos One, 2017. **12**(6): p. e0178691.

111. Fujikawa, H., A. Kai, and S. Morozumi, *A new logistic model for Escherichia coli growth at constant and dynamic temperatures.* Food Microbiology, 2004. **21**(5): p. 501-509.

112. Baty, F. and M.-L. Delignette-Muller, *Estimating the bacterial lag time: which model, which precision?* International Journal of Food Microbiology, 2004. **91**(3): p. 261-277.

113. Aduse-Opoku, J. and W.J. Mitchell, *Diauxic growth of Clostridium thermosaccharolyticum on glucose and xylose.* FEMS Microbiology Letters, 1988. **50**(1): p. 45-49.

114. George, S.E., C.J. Costenbader, and T. Melton, *Diauxic growth in Azotobacter vinelandii.* Journal of Bacteriology, 1985. **164**(2): p. 866-871.

115. Lee, I.H., A. Fredrickson, and H. Tsuchiya, *Diauxic growth of Propionibacterium shermanii.* Applied Microbiology, 1974. **28**(5): p. 831-835.

116. Gillespie, S.H. and T.D. McHugh, *Antibiotic resistance protocols*. 2010: Springer.

117. Andersson, D.I. and B.R. Levin, *The biological cost of antibiotic resistance.* Current Opinion in Microbiology, 1999. **2**(5): p. 489-493.

118. Birch, L.C., *The meanings of competition.* The American Naturalist, 1957. **91**(856): p. 5-18.

119. Case, T.J. and M.E. Gilpin, *Interference competition and niche theory.* Proceedings of the National Academy of Sciences, 1974. **71**(8): p. 3073-3077.

120. Ushijima, T. and A. Seto, *Selected faecal bacteria and nutrients essential for antagonism of Salmonella typhimurium in anaerobic continuous flow cultures.* Journal of Medical Microbiology, 1991. **35**(2): p. 111-117.

121. Cornforth, D.M. and K.R. Foster, *Competition sensing: the social side of bacterial stress responses.* Nature Reviews Microbiology, 2013. **11**(4): p. 285-293.

122. Levin, B.R., V. Perrot, and N. Walker, *Compensatory mutations, antibiotic resistance and the population genetics of adaptive evolution in bacteria.* Genetics, 2000. **154**(3): p. 985-997.

123. Kugelberg, E., et al., *Reduction of the fitness burden of quinolone resistance in Pseudomonas aeruginosa.* Journal of Antimicrobial Chemotherapy, 2005. **55**(1): p. 22-30.

124. Walther-Rasmussen, J. and N. Høiby, *Cefotaximases (CTX-M-ases), an expanding family of extended-spectrum β-lactamases.* Canadian Journal of Microbiology, 2004. **50**(3): p. 137-165.

125. Cox, G. and G.D. Wright, *Intrinsic antibiotic resistance: mechanisms, origins, challenges and solutions.* International Journal of Medical Microbiology, 2013. **303**(6-7): p. 287-292.

126. Kim, Y.A., et al., *Features of Infections Due to Klebsiella pneumoniae Carbapenemase–Producing Escherichia coli: Emergence of Sequence Type 131.* Clinical Infectious Diseases, 2012. **55**(2): p. 224-231.

127. Naas, T., et al., *When carbapenem-hydrolyzing β-lactamase KPC meets Escherichia coli ST131 in France.* Antimicrobial Agents and Chemotherapy, 2011. **55**(10): p. 4933-4934.

128. Temkin, E., et al., *Carbapenem-resistant Enterobacteriaceae: biology, epidemiology, and management.* Annals of the New York Academy of Sciences, 2014. **1323**(1): p. 22-42.

129. Mathys, D.A., et al., *Carbapenemase-producing Enterobacteriaceae and Aeromonas spp. present in wastewater treatment plant effluent and nearby surface waters in the US.* Plos One, 2019. **14**(6): p. e0218650.

130. Genovese, K.J., et al., *Competitive exclusion treatment reduces the mortality and fecal shedding associated with enterotoxigenic Escherichia coli infection in nursery-raised neonatal pigs.* Canadian Journal of Veterinary Research, 2000. **64**(4): p. 204.

131. Obpl, *Chemostat, a continuous culture*. Orbitbiotech.com, 2018. https://orbitbiotech.com/chemostat-a-continous-culture-culture-microbial-growth-chemostat/; accessed in 2019

132. Zambrano, M.M., et al., *Microbial competition: Escherichia coli mutants that take over stationary phase cultures.* Science, 1993. **259**(5102): p. 1757-1760.

133. Valverde, A., et al., *Dramatic increase in prevalence of fecal carriage of extended-spectrum β-lactamase-producing Enterobacteriaceae during nonoutbreak situations in Spain.* Journal of Clinical Microbiology, 2004. **42**(10): p. 4769-4775.

134. Ben-Ami, R., et al., *Influx of extended-spectrum β-lactamase—producing Enterobacteriaceae into the hospital.* Clinical Infectious Diseases, 2006. **42**(7): p. 925-934.

135. Guh, A.Y., et al., *Epidemiology of Carbapenem-Resistant Enterobacteriaceae in 7 US Communities, 2012-2013.* JAMA, 2015. **314**(14): p. 1479-87.

136. Tedeschi, L.O., A. Cannas, and D.G. Fox, *A nutrition mathematical model to account for dietary supply and requirements of energy and other nutrients for domesticated small ruminants: The development and evaluation of the Small Ruminant Nutrition System.* Small Ruminant Research, 2010. **89**(2-3): p. 174-184.

137. Lin, Z., C.I. Vahl, and J.E. Riviere, *Human food safety implications of variation in food animal drug metabolism.* Scientific Reports, 2016. **6**(1): p. 1-10.

138. Brown, S., et al., *Comparison of plasma pharmacokinetics and bioavailability of ceftiofur sodium and ceftiofur hydrochloride in pigs after a single intramuscular injection.* Journal of Veterinary Pharmacology and Therapeutics, 1999. **22**(1): p. 35-40.

139. Yein, F., et al. *Disposition and metabolism of 14C-ceftiofur sodium in swine*. in *Proceedings, International Pig Veterinary Society, 11th Congress, July 1-5, 1990, Lausanne, Switzerland.* 1990. Swiss Association of Swine Medicine.

140. Beconi-Barker, M.G., et al., *Ceftiofur sodium: absorption, distribution, metabolism, and excretion in target animals and its determination by high-performance liquid chromatography*. 1996, ACS Publications.

141. Gardner, N., et al., *Development and validation of a pig model for colon-specific drug delivery.* Journal of Pharmacy and Pharmacology, 1996. **48**(7): p. 689-693.

142. Davis, S., L. Illum, and M. Hinchcliffe, *Gastrointestinal transit of dosage forms in the pig.* Journal of Pharmacy and Pharmacology, 2001. **53**(1): p. 33-39.

143. Gilbertson, T.J., et al., *Environmental fate of ceftiofur sodium, a cephalosporin antibiotic. Role of animal excreta in its decomposition.* Journal of Agricultural and Food Chemistry, 1990. **38**(3): p. 890-894.

144. Funk, J.A., et al., *The effect of subtherapeutic chlortetracycline on antimicrobial resistance in the fecal flora of swine.* Microbial Drug Resistance, 2006. **12**(3): p. 210-218.

145. Davis, M.A., et al., *Recent emergence of Escherichia coli with cephalosporin resistance conferred by bla CTX-M on Washington State dairy farms.* Applied and Environmental Microbiology, 2015. **81**(13): p. 4403-4410.

146. Daniels, J.B., et al., *Role of ceftiofur in selection and dissemination of blaCMY-2-mediated cephalosporin resistance in Salmonella enterica and commensal*

Escherichia coli isolates from cattle. Applied and Environmental Microbiology, 2009. **75**(11): p. 3648-3655.

147. Stachyra, T., et al., *In vitro activity of the β-lactamase inhibitor NXL104 against KPC-2 carbapenemase and Enterobacteriaceae expressing KPC carbapenemases.* Journal of Antimicrobial Chemotherapy, 2009. **64**(2): p. 326-329.

148. Rubin, L.G., *Comparison of in vivo and in vitro multiplication rates of Haemophilus influenzae type b.* Infection and Immunity, 1986. **52**(3): p. 911-913.

149. Cavaco, L., et al., *Selection and persistence of CTX-M-producing Escherichia coli in the intestinal flora of pigs treated with amoxicillin, ceftiofur, or cefquinome.* Antimicrobial Agents and Chemotherapy, 2008. **52**(10): p. 3612-3616.

150. Müller, H.C., et al., *Effects of intermittent feeding of tylosin phosphate during the finishing period on feedlot performance, carcass characteristics, antimicrobial resistance, and incidence and severity of liver abscesses in steers.* Journal of Animal Science, 2018. **96**(7): p. 2877-2885.

APPENDIX A

E. COLI (N=100) SEQUENCING DATA

Number	Laboratory source	Isolate name	Source	Organism	Bioproject	Biosample	ST	*Resistance gene (cov 97%, ID 97%)	*Plasmid (cov 80%, ID 97%)
1	HM Scott	9_D0_Mac	Swine	*E. coli*	PRJNA 355857	SAMN09381034	101	mdf(A)_1,tet(B)_2	IncFIA(HI1)_1_HI1,IncFIB(AP001918)_1
2	HM Scott	15_D0_Mac	Swine	*E. coli*	PRJNA 355857	SAMN09381036	88	mdf(A)_1,sul2_2,tet(A)_6	Col156_1,IncFIB(AP001918)_1
3	HM Scott	24_D0_Mac	Swine	*E. coli*	PRJNA 355857	SAMN09381039	1307	mdf(A)_1,tet(B)_2	IncFIA_1,IncFIB(AP001918)_1
4	HM Scott	32_D0_Mac	Swine	*E. coli*	PRJNA 355857	SAMN09381042	75	mdf(A)_1,mph(B)_1,tet(A)_6,tet(B)_1	IncFIB(AP001918)_1,IncY_1
5	HM Scott	90_D0_Mac	Swine	*E. coli*	PRJNA 355857	SAMN09381056	154	mdf(A)_1,tet(B)_2	IncFIB(AP001918)_1
6	HM Scott	95_D0_Mac	Swine	*E. coli*	PRJNA 355857	SAMN09381058	641	mdf(A)_1,sul2_2,tet(B)_2	IncN_1,IncX1_1
7	HM Scott	101_D0_Mac	Swine	*E. coli*	PRJNA 355857	SAMN09381062	1244	mdf(A)_1,tet(A)_6	IncFII(29)_1_pUTI89,IncN_1
8	HM Scott	119_D0_Mac	Swine	*E. coli*	PRJNA 355857	SAMN09381066	1307	mdf(A)_1,tet(B)_2	IncFIA_1,IncFIB(AP001918)_1
9	HM Scott	188_D0_Mac	Swine	*E. coli*	PRJNA 355857	SAMN09381096	2509	aac(3)-IVa_1,aadA2_1,aph(4)-Ia_1,aph(6)-Id_1,cmlA1_1,mdf(A)_1,strA_1,sul3_2,tet(B)_2	IncFIB(AP001918)_1
10	HM Scott	207_D0_Mac	Swine	*E. coli*	PRJNA 355857	SAMN09381099	2144	mdf(A)_1,sul1_5,tet(B)_2	IncFIA_1,IncFIB(AP001918)_1,IncHI2_1,IncHI2A_1

Number	Laboratory source	Isolate name	Source	Organism	Bioproject	Biosample	ST	*Resistance gene (cov 97%, ID 97%)	*Plasmid (cov 80%, ID 97%)
11	HM Scott	208_D0_Mac	Swine	*E. coli*	PRJNA355857	SAMN09381100	10	aph(6)-Id_1,mdf(A)_1,strA_1,tet(B)_2,tet(M)_5	IncFIA(HI1)_1_HI1,IncHI1A_1,IncHI1B(R27)_1_R27
12	HM Scott	841_D28_Mac	Swine	*E. coli*	PRJNA355857	SAMN09381103	1244	mdf(A)_1,tet(A)_6	IncFII(29)_1_pUTI89
13	HM Scott	844_D28_Mac	Swine	*E. coli*	PRJNA355857	SAMN09381104	1244	mdf(A)_1,tet(A)_6	IncFII(29)_1_pUTI89
14	HM Scott	851_D28_Mac	Swine	*E. coli*	PRJNA355857	SAMN09381107	10	mdf(A)_1,tet(B)_2	IncFII_1
15	HM Scott	852_D28_Mac	Swine	*E. coli*	PRJNA355857	SAMN09381108	10	mdf(A)_1,tet(B)_2	IncFII_1
16	HM Scott	857_D28_Mac	Swine	*E. coli*	PRJNA355857	SAMN09381111	154	mdf(A)_1,tet(B)_2	IncFIB(AP001918)_1
17	HM Scott	861_D28_Mac	Swine	*E. coli*	PRJNA355857	SAMN09381114	2509	aac(3)-IVa_1,aadA2_1,aph(4)-Ia_1,aph(6)-Id_1,cmlA1_1,mdf(A)_1,strA_1,sul3_2,tet(B)_2	IncFIB(AP001918)_1
18	HM Scott	925_D28_Mac	Swine	*E. coli*	PRJNA355857	SAMN09381118	1244	mdf(A)_1,tet(A)_6	IncFII(29)_1_pUTI89_CP003035
19	HM Scott	939_D28_Mac	Swine	*E. coli*	PRJNA355857	SAMN09381123	101	mdf(A)_1,tet(B)_2	IncFIB(AP001918)_1,IncN_1
20	KN Norman	3fy2-tsp-qd2-mtet	Feedlot dust	*E. coli*	PRJNA625742	SAMN14842449	58	aph(6)-Id_1,mdf(A)_1,strA_1,sul2_2,tet(B)_1	none
21	HM Scott	3_D0_Mac	Swine	*E. coli*	PRJNA355857	SAMN09381032	8975	aph(6)-Id_1,blaTEM-1B_1,mdf(A)_1,strA_1,tet(B)_2,tet(M)_5	Col(BS512)_1__NC_010656_dupe,IncFIA(HI1)_1_HI1,IncFIC(FII)_1,IncHI1A_1,IncHI1B(R27)_1_R27
22	HM Scott	41_D0_Mac	Swine	*E. coli*	PRJNA355857	SAMN09381043	8954	aph(6)-Id_1,blaTEM-1B_1,mdf(A)_1,strA_1,tet(B)_2,tet(M)_5	IncFIA(HI1)_1_HI1,IncFIB(AP001918)_1,IncFIC(FII)_1,IncHI1A_1,IncHI1B(R27)_1_R27
23	HM Scott	64_D0_Mac	Swine	*E. coli*	PRJNA355857	SAMN09381050	154	aph(6)-Id_1,blaTEM-1B_1,mdf(A)_1,strA_1,tet(B)_2,tet(M)_5	IncFIA(HI1)_1_HI1,IncFIC(FII)_1,IncHI1A_1,IncHI1B(R27)_1_R27
24	HM Scott	72_D0_Mac	Swine	*E. coli*	PRJNA355857	SAMN09381051	8954	aph(6)-Id_1,blaTEM-1B_1,mdf(A)_1,strA_1,tet(B)_2,tet(M)_5	IncFIA(HI1)_1_HI1,IncFIB(AP001918)_1,IncFIC(FII)_1,IncHI1A_1,IncHI1B(R27)_1_R27

Number	Laboratory source	Isolate name	Source	Organism	Bioproject	Biosample	ST	*Resistance gene (cov 97%, ID 97%)	*Plasmid (cov 80%, ID 97%)
25	HM Scott	110_D0_Mac	Swine	*E. coli*	PRJNA355857	SAMN09381063	8972	aph(6)-Id_1,blaTEM-1B_1,mdf(A)_1,strA_1,tet(B)_2	IncFIB(AP001918)_1,IncFIC(FII)_1,IncFII_1
26	HM Scott	118_D0_Mac	Swine	*E. coli*	PRJNA355857	SAMN09381065	34	aph(6)-Id_1,blaTEM-1B_1,mdf(A)_1,strA_1,tet(B)_2	Col(BS512)_1,Col(BS512)_1__NC_010656_dupe,IncFIB(AP001918)_1,IncX1_1,p0111_1
27	HM Scott	121_D0_Mac	Swine	*E. coli*	PRJNA355857	SAMN09381068	744	aadA5_1,aph(3'')-Ib_5,aph(6)-Id_1,blaTEM-1B_1,dfrA17_1,mdf(A)_1,mph(A)_2,sul1_5,sul2_3,tet(B)_2,tet(D)_1	IncFIA_1,IncFIB(AP001918)_1,IncQ1_1,IncX3_1
28	HM Scott	131_D0_Mac	Swine	*E. coli*	PRJNA355857	SAMN09381074	48	aadA2_1,blaTEM-1B_1,cmlA1_1,mdf(A)_1,sul3_2,tet(B)_2,tet(M)_5	IncFIB(AP001918)_1,p0111_1
29	HM Scott	143_D0_Mac	Swine	*E. coli*	PRJNA355857	SAMN09381078	6031	blaTEM-1B_1,mdf(A)_1,tet(B)_2	IncFIA(HI1)_1_HI1,IncFIA_1,IncFIB(AP001918)_1,IncFII_1,IncHI1A_1,IncHI1B(R27)_1_R27
30	HM Scott	152_D0_Mac	Swine	*E. coli*	PRJNA355857	SAMN09381080	48	aadA2_1,blaTEM-1B_1,cmlA1_1,mdf(A)_1,sul3_2,tet(B)_2,tet(M)_5	Col8282_1,IncFIB(AP001918)_1
31	HM Scott	153_D0_Mac	Swine	*E. coli*	PRJNA355857	SAMN09381081	48	aadA2_1,blaTEM-1B_1,cmlA1_1,mdf(A)_1,sul3_2,tet(B)_2,tet(M)_5	Col8282_1,IncFIB(AP001918)_1
32	HM Scott	172_D0_Mac	Swine	*E. coli*	PRJNA355857	SAMN09381087	1642	aac(3)-IId_1,aph(3'')-Ib_5,aph(6)-Id_1,blaTEM-1B_1,mdf(A)_1,sul2_3,tet(B)_2	IncFIA_1,IncFIB(AP001918)_1,IncQ1_1,IncY_1
33	HM Scott	176_D0_Mac	Swine	*E. coli*	PRJNA355857	SAMN09381089	744	aadA5_1,aph(3'')-Ib_5,aph(6)-Id_1,blaTEM-1B_1,dfrA17_1,mdf(A)_1,mph(A)_2,sul1_5,sul2_3,tet(B)_2,tet(D)_1	IncFIA_1,IncFIB(AP001918)_1,IncQ1_1,IncX3_1
34	HM Scott	181_D0_Mac	Swine	*E. coli*	PRJNA355857	SAMN09381092	48	aadA2_1,blaTEM-1B_1,cmlA1_1,mdf(A)_1,sul3_2,tet(B)_2,tet(M)_5	IncFIB(AP001918)_1,p0111_1
35	HM Scott	209_D0_Mac	Swine	*E. coli*	PRJNA355857	SAMN09381101	6694	aph(3'')-Ib_5,aph(6)-Id_1,blaTEM-1B_1,mdf(A)_1,tet(B)_2	IncFIA_1,IncFIB(AP001918)_1
36	HM Scott	847_D28_Mac	Swine	*E. coli*	PRJNA355857	SAMN09381106	8954	aph(6)-Id_1,blaTEM-1B_1,mdf(A)_1,strA_1,tet(B)_2,tet(M)_5	IncFIA(HI1)_1_HI1,IncFIB(AP001918)_1,IncFIC(FII)_1,IncHI1A_1,IncHI1B(R27)_1_R27
37	HM Scott	858_D28_Mac	Swine	*E. coli*	PRJNA355857	SAMN09381112	10	aac(3)-IId_1,blaTEM-1B_1,mdf(A)_1,tet(B)_2	IncFII_1
38	HM Scott	929_D28_Mac	Swine	*E. coli*	PRJNA355857	SAMN09381120	10	aph(6)-Id_1,blaTEM-1B_1,mdf(A)_1,strA_1,tet(M)_5	IncFIA(HI1)_1_HI1,IncFIB(AP001918)_1,IncHI1A_1,IncHI1B(R27)_1_R27

Number	Laboratory source	Isolate name	Source	Organism	Bioproject	Biosample	ST	*Resistance gene (cov 97%, ID 97%)	*Plasmid (cov 80%, ID 97%)
39	HM Scott	1019_D28_Mac	Swine	*E. coli*	PRJNA355857	SAMN09381138	154	aph(6)-Id_1,blaTEM-1B_1,mdf(A)_1,strA_1,tet(B)_2,tet(M)_5	IncFIA(HI1)_1_HI1,IncFIC(FII)_1,IncHI1A_1,IncHI1B(R27)_1_R27
40	HM Scott	1033_D28_Mac	Swine	*E. coli*	PRJNA355857	SAMN09381145	154	aph(6)-Id_1,blaTEM-1B_1,mdf(A)_1,strA_1,tet(B)_2,tet(M)_5	IncFIA(HI1)_1_HI1,IncFIC(FII)_1,IncHI1A_1,IncHI1B(R27)_1_R27
41	HM Scott	5_D0_Mac	Swine	*E. coli*	PRJNA355857	SAMN09381033	1640	aac(3)-VIa_1,ant(3'')-Ia_1,aph(3')-Ia_1,aph(3'')-Ib_5,aph(6)-Id_1,blaCMY-2_1,floR_2,mdf(A)_1,sul1_5,tet(A)_6,tet(B)_2	IncA/C2_1,IncI1_1_Alpha,IncI2_1_Delta,p0111_1
42	HM Scott	11_D0_Mac	Swine	*E. coli*	PRJNA355857	SAMN09381035	189	aac(3)-VIa_1,ant(3'')-Ia_1,aph(3'')-Ib_5,aph(6)-Id_1,blaCMY-2_1,floR_2,mdf(A)_1,sul1_5,sul2_2,tet(A)_6,tet(B)_2	IncA/C2_1
43	HM Scott	17_D0_Mac	Swine	*E. coli*	PRJNA355857	SAMN09381037	2500	aac(3)-VIa_1,ant(3'')-Ia_1,aph(3'')-Ib_5,aph(6)-Id_1,blaCMY-2_1,floR_2,mdf(A)_1,sul1_5,sul2_2,tet(A)_6,tet(B)_2	IncA/C2_1
44	HM Scott	26_D0_Mac	Swine	*E. coli*	PRJNA355857	SAMN09381040	349	aph(6)-Id_1,blaCMY-2_1,mdf(A)_1,strA_1	Col156_1,IncFIB(AP001918)_1,IncI1_1_Alpha
45	HM Scott	27_D0_Mac	Swine	*E. coli*	PRJNA355857	SAMN09381041	101	blaCMY-2_1,mdf(A)_1,tet(B)_2	IncFIB(AP001918)_1,IncI1_1_Alpha
46	HM Scott	42_D0_Mac	Swine	*E. coli*	PRJNA355857	SAMN09381044	278	blaCMY-2_1,mdf(A)_1,tet(B)_2	IncFIA_1,IncFIB(AP001918)_1,IncI1_1_Alpha
47	HM Scott	44_D0_Mac	Swine	*E. coli*	PRJNA355857	SAMN09381045	58	aph(6)-Id_1,blaCMY-2_1,mdf(A)_1,strA_1,tet(B)_2	IncFIB(AP001918)_1,IncI1_1_Alpha,p0111_1
48	HM Scott	48_D0_Mac	Swine	*E. coli*	PRJNA355857	SAMN09381047	1640	aac(3)-VIa_1,ant(3'')-Ia_1,aph(3')-Ia_1,aph(3'')-Ib_5,aph(6)-Id_1,blaCMY-2_1,floR_2,mdf(A)_1,sul1_5,tet(A)_6,tet(B)_2	IncA/C2_1,IncI1_1_Alpha,p0111_1
49	HM Scott	52_D0_Mac	Swine	*E. coli*	PRJNA355857	SAMN09381048	12	aph(3'')-Ib_5,aph(6)-Id_1,blaCMY-2_1,floR_2,mdf(A)_1,sul2_2,tet(A)_6	IncA/C2_1
50	HM Scott	77_D0_Mac	Swine	*E. coli*	PRJNA355857	SAMN09381054	48	aph(3'')-Ib_5,aph(6)-Id_1,blaCMY-2_1,floR_2,mdf(A)_1,sul2_2,tet(A)_6	IncA/C2_1
51	HM Scott	94_D0_Mac	Swine	*E. coli*	PRJNA355857	SAMN09381057	90	blaCMY-2_1,cmlA1_1,mdf(A)_1,sul3_2,tet(B)_2	IncFIB(AP001918)_1,IncFIC(FII)_1,IncFII_1,IncI1_1_Alpha,IncI2_1_Delta
52	HM Scott	97_D0_Mac	Swine	*E. coli*	PRJNA355857	SAMN09381059	58	aph(6)-Id_1,blaCMY-2_1,mdf(A)_1,strA_1,tet(B)_2	IncFIB(AP001918)_1,IncI1_1_Alpha,p0111_1

Number	Laboratory source	Isolate name	Source	Organism	Bioproject	Biosample	ST	*Resistance gene (cov 97%, ID 97%)	*Plasmid (cov 80%, ID 97%)
53	HM Scott	100_D0_Mac	Swine	*E. coli*	PRJNA355857	SAMN09381061	189	aac(3)-VIa_1,ant(3'')-Ia_1,aph(3'')-Ib_5,aph(6)-Id_1,blaCMY-2_1,floR_2,mdf(A)_1,sul1_5,sul2_2,tet(A)_6,tet(B)_2	IncA/C2_1
54	HM Scott	115_D0_Mac	Swine	*E. coli*	PRJNA355857	SAMN09381064	154	blaCMY-2_1,mdf(A)_1,tet(B)_2	IncFIB(AP001918)_1,IncI1_1_Alpha
55	HM Scott	120_D0_Mac	Swine	*E. coli*	PRJNA355857	SAMN09381067	3759	aac(3)-VIa_1,ant(3'')-Ia_1,aph(3'')-Ib_5,aph(3')-IIa_2,aph(6)-Id_1,blaCMY-2_1,floR_2,mdf(A)_1,sul1_5,sul2_2,tet(A)_6,tet(M)_5	IncA/C2_1,IncFIA(HI1)_1_HI1,IncHI1A_1,IncHI1B(R27)_1_R27,IncN_1,IncY_1
56	HM Scott	126_D0_Mac	Swine	*E. coli*	PRJNA355857	SAMN09381070	75	aac(3)-VIa_1,ant(3'')-Ia_1,aph(3'')-Ib_5,aph(6)-Id_1,blaCMY-2_1,floR_2,mdf(A)_1,sul1_5,sul2_2,tet(A)_6,tet(B)_1	IncA/C2_1,IncY_1
57	HM Scott	130_D0_Mac	Swine	*E. coli*	PRJNA355857	SAMN09381073	2509	aac(3)-IVa_1,aadA2_1,aph(4)-Ia_1,aph(6)-Id_1,blaCMY-2_1,cmlA1_1,mdf(A)_1,strA_1,sul3_2,tet(B)_2	IncFIB(AP001918)_1,IncI1_1_Alpha
58	HM Scott	138_D0_Mac	Swine	*E. coli*	PRJNA355857	SAMN09381076	58	aph(6)-Id_1,blaCMY-2_1,mdf(A)_1,strA_1,tet(B)_2	IncFIB(AP001918)_1,IncI1_1_Alpha
59	HM Scott	149_D0_Mac	Swine	*E. coli*	PRJNA355857	SAMN09381079	75	blaCMY-2_1,mdf(A)_1,mph(B)_1,tet(A)_6,tet(B)_1	IncFIB(AP001918)_1,IncI1_1_Alpha,IncY_1
60	HM Scott	156_D0_Mac	Swine	*E. coli*	PRJNA355857	SAMN09381082	12	aph(3'')-Ib_5,aph(6)-Id_1,blaCMY-2_1,floR_2,mdf(A)_1,sul2_2,tet(A)_6	IncA/C2_1
61	HM Scott	2_D0_Mac	Swine	*E. coli*	PRJNA355857	SAMN09381031	744	blaCTX-M-27_1,erm(B)_18,mdf(A)_1,tet(B)_2	IncFII_1
62	HM Scott	45_D0_Mac	Swine	*E. coli*	PRJNA355857	SAMN09381046	744	blaCTX-M-27_1,mdf(A)_1,tet(B)_2	IncFII_1
63	HM Scott	75_D0_Mac	Swine	*E. coli*	PRJNA355857	SAMN09381052	744	blaCTX-M-27_1,erm(B)_18,mdf(A)_1,tet(B)_2	IncFII_1
64	HM Scott	76_D0_Mac	Swine	*E. coli*	PRJNA355857	SAMN09381053	744	blaCTX-M-27_1,erm(B)_18,mdf(A)_1,tet(B)_2	IncFII_1
65	HM Scott	87_D0_Mac	Swine	*E. coli*	PRJNA355857	SAMN09381055	744	blaCTX-M-27_1,erm(B)_18,mdf(A)_1,tet(B)_2	IncFII_1
66	HM Scott	124_D0_Mac	Swine	*E. coli*	PRJNA355857	SAMN09381069	744	blaCTX-M-27_1,mdf(A)_1,tet(B)_2	IncFIA_1,IncFII_1,IncN_1

Number	Laboratory source	Isolate name	Source	Organism	Bioproject	Biosample	ST	*Resistance gene (cov 97%, ID 97%)	*Plasmid (cov 80%, ID 97%)
67	HM Scott	128_D0_Mac	Swine	*E. coli*	PRJNA355857	SAMN09381071	744	blaCTX-M-27_1,erm(B)_18,mdf(A)_1,tet(B)_2	IncFII_1
68	HM Scott	129_D0_Mac	Swine	*E. coli*	PRJNA355857	SAMN09381072	744	blaCTX-M-27_1,erm(B)_18,mdf(A)_1,tet(B)_2	IncFII_1
69	HM Scott	140_D0_Mac	Swine	*E. coli*	PRJNA355857	SAMN09381077	744	blaCTX-M-27_1,erm(B)_18,mdf(A)_1,tet(B)_2	IncFII_1
70	HM Scott	178_D0_Mac	Swine	*E. coli*	PRJNA355857	SAMN09381090	744	blaCTX-M-27_1,mdf(A)_1,tet(B)_2	IncFIA_1,IncFII_1,IncN_1
71	HM Scott	D99-56-4339-7-Ec	Cattle	*E. coli*	PRJNA625741	SAMN14774805	1725	aph(3')-Ia_1,blaCTX-M-55_1,floR_2,mdf(A)_1,sul3_2,tet(B)_2	IncFIB(AP001918)_1
72	HM Scott	D99-9-4243-5-Ec	Cattle	*E. coli*	PRJNA625741	SAMN14774806	11081	blaCTX-M-32_2,mdf(A)_1,tet(B)_2	IncN_1
73	HM Scott	D56-58-4330-3-Ec	Cattle	*E. coli*	PRJNA625741	SAMN14774800	17	blaCTX-M-27_1,mdf(A)_1	IncB/O/K/Z_3,IncFII_1,p0111_1
74	HM Scott	D56-9-4234-4-Ec	Cattle	*E. coli*	PRJNA625741	SAMN14774801	Not Found	blaCTX-M-32_2,mdf(A)_1	IncFII(pSE11)_1_pSE11
75	HM Scott	D28-57-4329-4-Ec	Cattle	*E. coli*	PRJNA625741	SAMN14774796	306	aph(3'')-Ib_5,aph(6)-Id_1,blaCTX-M-1_1,floR_2,mdf(A)_1,mph(A)_2,qnrB19_1,sul2_2,tet(A)_6	IncFII(pSE11)_1_pSE11,IncR_1,IncX1_1
76	HM Scott	D28-57-4315-3-Ec	Cattle	*E. coli*	PRJNA625741	SAMN14774795	306	aph(3'')-Ib_5,aph(6)-Id_1,blaCTX-M-1_1,floR_2,mdf(A)_1,mph(A)_2,qnrB19_1,sul2_2,tet(A)_6	IncFII(pSE11)_1_pSE11,IncR_1,IncX1_1
77	KN Norman	1-fy3-qd-3_._maccef	Feedlot dust	*E. coli*	PRJNA625742	SAMN14842428	8828	blaCTX-M-27_1,erm(B)_18,mdf(A)_1,tet(C)_3	IncFIB(AP001918)_1,IncFII_1
78	KN Norman	2-fy3-d-2_ecpc_maccef	Feedlot dust	*E. coli*	PRJNA625742	SAMN14842435	2536	blaCTX-M-32_2,mdf(A)_1	IncFIA_1,IncFIC(FII)_1
79	HM Scott	17-A1-13286-28-1-MACFEP-Ecoli	Cattle	*E. coli*	PRJNA625290	SAMN14596660	56	blaCTX-M-32_2,mdf(A)_1	IncFIA_1,IncFIB(AP001918)_1,IncFIC(FII)_1

Number	Laboratory source	Isolate name	Source	Organism	Bioproject	Biosample	ST	*Resistance gene (cov 97%, ID 97%)	*Plasmid (cov 80%, ID 97%)
80	HM Scott	25-A1-13332-28-1-ESBL-Ecoli	Cattle	*E. coli*	PRJNA625290	SAMN14596656	301	blaCTX-M-32_2,mdf(A)_1,tet(A)_6	IncFII(pSE11)_1_pSE11,IncR_1,IncX1_1
81	CDC/FDA	0451	Human	*E. coli*	PRJNA391513	SAMN07291544	131	aac(6')-Ib_1,blaKPC-3_1,blaOXA-9_1,blaTEM-1A_1,dfrA14_5,mdf(A)_1,qnrS1_1	IncFIA_1,IncN_1
82	TE Wittum	S2	Sewage	*E. coli*	PRJNA635418	SAMN15074832	361	aadA2_1,blaNDM-5_1,dfrA12_8,mdf(A)_1,mph(A)_2,sul1_5,tet(A)_6	IncFIA_1,IncFII_1,IncY_1
83	TE Wittum	S3	Sewage	*E. coli*	PRJNA635418	SAMN15074833	167	aadA2_1,aph(3'')-Ib_5,aph(6)-Id_1,blaCMY-2_1,blaNDM-5_1,blaTEM-1B_1,dfrA12_8,erm(B)_18,mdf(A)_1,mph(A)_2,rmtB_1,sul1_5,sul2_2	IncFIA_1,IncFIB(AP001918)_1,IncFII_1
84	TE Wittum	S4	Sewage	*E. coli*	PRJNA635418	SAMN15074834	617	aac(6')-Ib-cr_1,aadA2_1,aadA5_1,aph(3'')-Ib_5,aph(6)-Id_1,blaCTX-M-15_1,blaNDM-5_1,blaOXA-1_1,catA1_1,dfrA12_8,dfrA17_1,dfrA5_1,erm(B)_18,mdf(A)_1,mph(A)_2,qnrS1_1,sul1_5,tet(B)_2	Col(BS512)_1,IncB/O/K/Z_2,IncFIA_1,IncFIB(AP001918)_1
85	TE Wittum	S5	Sewage	*E. coli*	PRJNA224116	SAMN09289752	607	aadA2_1,ant(2'')-Ia_1,blaKPC-3_1,dfrA12_8,dfrA16_2,mdf(A)_1,mph(A)_2,mph(E)_1,msr(E)_4,qnrA1_1,sul1_2,tet(G)_2	IncA/C2_1,IncW_1
86	TE Wittum	S6	Swine environment	*E. coli*	PRJNA635418	SAMN15074835	218	aac(3)-IVa_1,aph(3')-Ia_3,aph(4)-Ia_1,aph(6)-Id_1,blaCMY-2_1,blaIMP-64_1,blaTEM-1B_1,mdf(A)_1,strA_1,sul2_14	IncA/C2_1,IncI1_1_Alpha,IncX1_1,IncX4_1
87	CDC/FDA	0001	Human	*E. coli*	PRJNA224116	SAMN04014842	131	aac(6')-Ib-cr_1,aadA5_1,blaKPC-3_1,blaOXA-1_1,dfrA17_1,mdf(A)_1,mph(A)_2,sul1_5,tet(A)_6	IncFIB(pQil)_1_pQil,IncFII_1
88	CDC/FDA	0048	Human	*E. coli*	PRJNA224116	SAMN04014889	101	aadA2_1,aph(3')-Ia_1,aph(6)-Id_1,blaCMY-6_1,blaCTX-M-15_1,blaNDM-1_1,blaOXA-2_1,blaTEM-1A_1,blaTEM-1B_1,catA1_1,dfrA12_8,dfrA29_1,mdf(A)_1,rmtC_1,strA_1,sul1_5,tet(B)_2	IncA/C2_1,IncFIB(pB171)_1_pB171,p0111_1
89	CDC/FDA	0055	Human	*E. coli*	PRJNA224116	SAMN04014896	131	aac(3)-IIa_1,aac(6')-Ib-cr_1,aadA5_1,blaCMY-6_1,blaNDM-1_1,blaOXA-1_1,dfrA17_1,mdf(A)_1,mph(A)_2,rmtC_1,sul1_5,tet(A)_6	IncA/C2_1,IncFIA_1,IncFII_1
90	CDC/FDA	0061	Human	*E. coli*	PRJNA224116	SAMN04014902	1408	aac(6')-Ib_1,aadA2_1,aph(3'')-Ib_5,aph(6)-Id_1,blaKPC-3_1,blaOXA-9_1,blaTEM-1A_1,blaTEM-1B_1,dfrA12_8,dfrA14_5,mdf(A)_1,sul1_5,sul2_2,sul3_2,tet(A)_6	IncN_1,IncX1_1
91	CDC/FDA	0069	Human	*E. coli*	PRJNA224116	SAMN04014910	1809	aph(3'')-Ib_5,blaCMY-6_1,blaNDM-1_1,blaTEM-1B_1,dfrA8_1,sul1_5,sul2_2,tet(A)_6	IncA/C2_1
92	CDC/FDA	0114	Human	*E. coli*	PRJNA224116	SAMN04014955	617	ant(2'')-Ia_1,aph(3'')-Ib_5,aph(6)-Id_1,blaKPC-3_1,blaTEM-1B_1,cmlA1_1,dfrA5_1,mdf(A)_1,sul1_5,sul2_2	IncFIA_1,IncFIB(AP001918)_1,IncN_1,IncX4_1,IncY_1

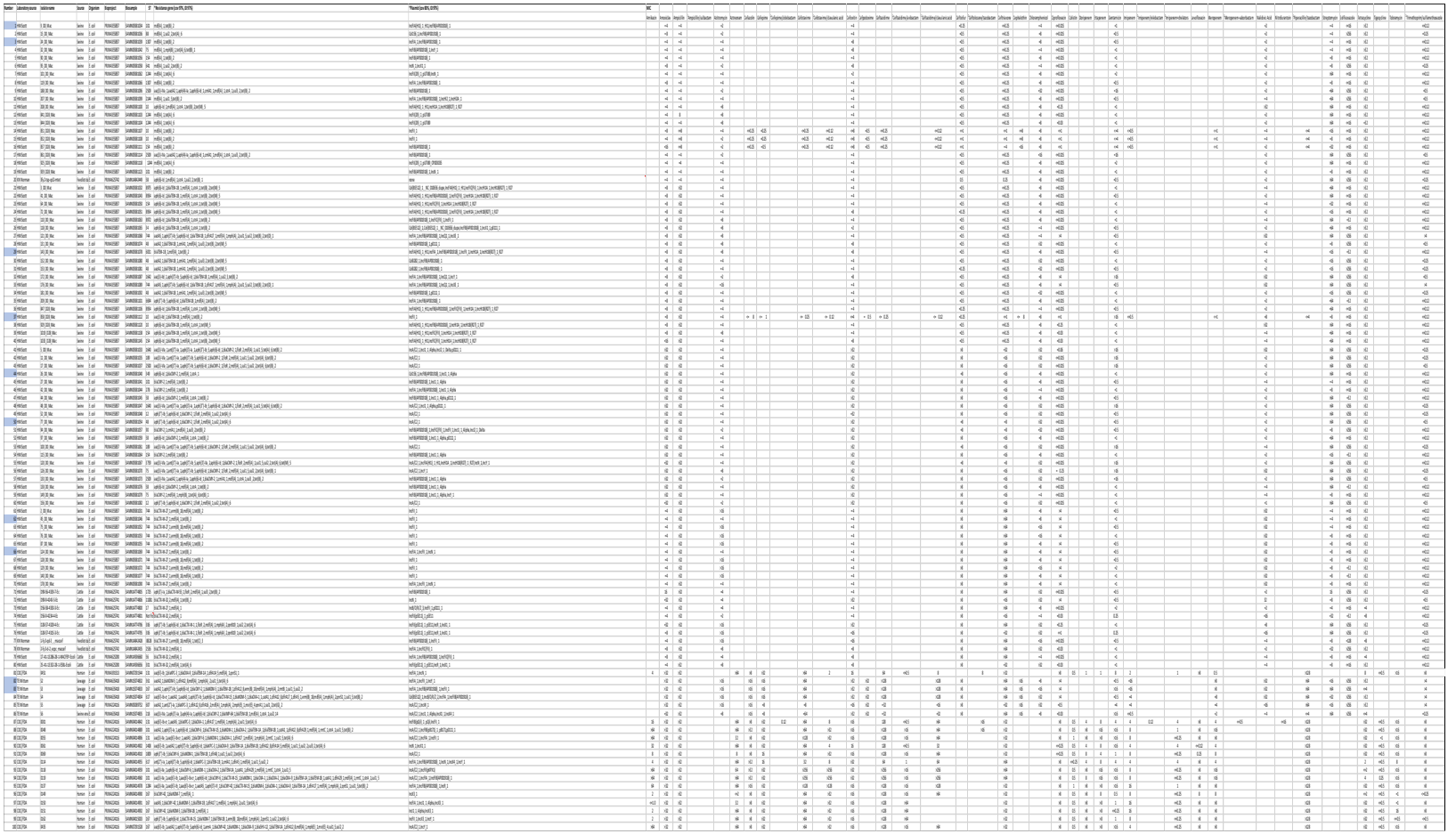
Number	Laboratory source	Isolate name	Source	Organism	Bioproject	Biosample	ST	*Resistance gene (cov 97%, ID 97%)	*Plasmid (cov 80%, ID 97%)
93	CDC/FDA	0118	Human	*E. coli*	PRJNA224116	SAMN04014959	101	aac(3)-IIa_1,aph(6)-Id_1,blaCMY-6_1,blaNDM-1_1,blaOXA-2_1,blaTEM-1A_1,catA1_1,dfrA29_1,mdf(A)_1,rmtC_1,strA_1,sul1_5	IncA/C2_1,IncFII(pKPX1)
94	CDC/FDA	0119	Human	*E. coli*	PRJNA224116	SAMN04014960	101	aac(3)-IIa_1,aac(6')-Ib_1,aac(6')-Ib-cr_1,aph(6)-Id_1,blaCMY-6_1,blaCTX-M-15_1,blaNDM-1_1,blaOXA-1_1,blaOXA-2_1,blaOXA-9_1,blaTEM-1A_1,blaTEM-1B_1,catA1_1,dfrA29_1,mdf(A)_1,rmtC_1,strA_1,sul1_5	IncA/C2_1,IncFIA_1,IncFIB(AP001918)_1
95	CDC/FDA	0137	Human	*E. coli*	PRJNA224116	SAMN04014978	1284	aac(3)-IIa_1,aac(6')-Ib_1,aac(6')-Ib-cr_1,aadA5_1,aph(3')-VI_1,blaCMY-42_1,blaCTX-M-15_1,blaNDM-6_1,blaOXA-1_1,blaOXA-9_1,blaTEM-1A_1,dfrA17_1,mdf(A)_1,mph(A)_2,qnrS1_1,sul1_5,tet(B)_2	IncFIA_1,IncFIB(AP001918)_1,IncR_1
96	CDC/FDA	0149	Human	*E. coli*	PRJNA224116	SAMN04014990	167	blaCMY-42_1,blaNDM-7_1,mdf(A)_1	IncX3_1
97	CDC/FDA	0150	Human	*E. coli*	PRJNA224116	SAMN04014991	167	aadA5_1,blaCMY-42_1,blaNDM-5_1,blaTEM-1B_1,dfrA17_1,mdf(A)_1,mph(A)_2,sul1_5,tet(A)_6	IncFIA_1,IncI1_1_Alpha,IncX3_1
98	CDC/FDA	0151	Human	*E. coli*	PRJNA224116	SAMN04014992	167	blaCMY-42_1,blaNDM-5_1,blaTEM-1B_1,mdf(A)_1	IncI1_1_Alpha,IncX3_1
99	CDC/FDA	0162	Human	*E. coli*	PRJNA224116	SAMN04015003	167	aph(3'')-Ib_5,aph(6)-Id_1,blaCTX-M-15_1,blaNDM-7_1,blaTEM-1B_1,erm(B)_18,mdf(A)_1,mph(A)_2,qnrS1_1,sul2_2,tet(A)_6	IncFII_1,IncX3_1,IncY_1
100	CDC/FDA	0435	Human	*E. coli*	PRJNA224116	SAMN07291528	167	aac(6')-Ib_1,aadA2_1,aph(3'')-Ib_5,aph(6)-Id_1,armA_1,blaCMY-42_1,blaNDM-1_1,blaOXA-9_1,blaSHV-12_1,blaTEM-1A_1,dfrA12_8,mdf(A)_1,mph(E)_1,msr(E)_4,sul1_5,sul2_2	IncA/C2_1,IncY_1

*Raw reads of the WGS sequences were obtained and re-assembled using SPAdes ver.3.11.1 assembler for all (n=100) isolates and later searched against MLST, ResFinder and PlasmidFinder databases using bioinformatic tools of SRST2ver.0.2.0 and ABRicate ver.0.8.7 to provide a uniform presentation of the sequence types, resistance genes and plasmids.

Group representative strains for mixed-strain experiments are in color boxes.

APPENDIX B

E. COLI (N=100) SEQUENCING DATA AND PHENOTYPE RESULTS



Please magnify to view contents of embedded spreadsheet. The embedded Excel workbook is also provided as supplementary data with this dissertation document.

*Raw reads of the WGS sequences were obtained and re-assembled using SPAdes ver.3.11.1 assembler for all (n=100) isolates and later searched against MLST, ResFinder and PlasmidFinder databases using bioinformatic tools of SRST2ver.0.2.0 and ABRicate ver.0.8.7 to provide a uniform presentation of the sequence types, resistance genes and plasmids. Serial numbers of group representative strains for mixed-strain experiments are color highlighted.

APPENDIX C

RECOMBINED PORCINE CONTINUOUS FLOW (RPCF) STRAIN COMPOSITION AND ESTIMATED PROPORTIONS SYSTEM

S/N	Strain type	%*
1	*Moryella indoligenes*	20.672
2	*Sporanaerobacter* spp.	17.562
3	*Pyramidobacter piscolens*	15.859
4	*Bacteroides* spp.	8.831
5	*Bacteroides uniformis*	8.038
6	*Prevotella stercorea*	5.958
7	*Clostridium* sp.	5.843
8	*Porphyromonas somerae*	4.376
9	*Solobacterium* spp.	1.777
10	*Faecalicoccus clostridiales bacterium*	1.355
11	*Lachnoclostridium clostridium bolteae*	1.318
12	*Parabacteroides distasonis*	1.194
13	*Rummeliibacillus stabekisii*	1.128
14	*Olsenella umbonata*	0.929
15	*Tannerella* spp.	0.873
16	*Phascolarctobacterium* spp.	0.686
17	*Lachnoclostridium clostridium symbiosum*	0.666
18	*Prevotella* spp.	0.624
19	*Alistipes senegalensis*	0.262

20	*Ruminococcus* spp.	0.245
21	*Anaerovorax* spp.	0.198
22	*Megasphaera* spp.	0.192
23	*Christensenella minuta*	0.146
24	*Eubacterium* sp.	0.145
25	*Syntrophococcus* spp.	0.137
26	*Lachnoclostridium clostridium scindens*	0.109
27	*Porphyromonas* spp.	0.098
28	*Enterococcus faecalis*	0.072
29	*Intestinimonas butyriciproducens*	0.068
30	*Prevotella histicola*	0.066
31	*Acetanaerobacterium* spp.	0.066
32	*Prevotella* sp.	0.063
33	*Selenomonas sputigena*	0.063
34	*Clostridium* spp.	0.050
35	*Bacteroides pyogenes*	0.032
36	*Bacteroides* sp.	0.031
37	*Prevotella buccae*	0.028
38	*Slackia isoflavoniconvertens*	0.027
39	*Bulleidia* spp.	0.025
40	*Tissierella praeacuta*	0.021
41	*Bacteroides acidifaciens*	0.020
42	*Lachnoclostridium clostridium hathewayi*	0.018
43	*Olsenella* sp.	0.016
44	*Succiniclasticum* spp.	0.014
45	*Prevotella ruminicola*	0.012
46	*Anaerorhabdus* spp.	0.011

47	*Bacteroides denticanoris*	0.009
48	*Pseudoflavonifractor bacteroides capillosus*	0.009
49	*Prevotella veroralis*	0.008
50	*Succiniclasticum ruminis*	0.008
51	*Sporanaerobacter acetigenes*	0.008
52	*Erysipelatoclostridium clostridium ramosum*	0.007
53	*Peptoniphilus* sp.	0.006
54	*Moryella* spp.	0.005
55	*Lachnoclostridium [clostridium] saccharolyticum*	0.005
56	*Mitsuokella jalaludinii*	0.004
57	*Fusobacterium naviforme*	0.004
58	*Lachnoclostridium clostridium aminophilum*	0.003
59	*Catabacter hongkongensis*	0.003

*Percentage of Sequences from which each Taxonomic designation was derived (02/18/16)