EMERGENCE AND MECHANISMS OF MULTI-DRUG RESISTANT MICROORGANISMS IN PATIENTS AT HIGH RISK FOR ANTIMICROBIAL RESISTANCE

By

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LAY ABSTRACT

Antimicrobial resistance (AMR) poses a substantial threat to public health and clinical medicine. By 2050, it's predicted that AMR will be responsible for a yearly mortality rate of 10 million people, surpassing the mortality of cancer. Despite this daunting future we face, there are many efforts currently employed to combat the growth of AMR. One significant effort involves surveillance and early identification of novel resistant bacteria circulating in high antibiotic exposure environments. The second chapter of this thesis focuses on sampling 25 patients from a hospital environment, rich with antibiotics, to build a collection of AMR bacteria that will be tested and added to surveillance efforts/future study. This chapter allowed for the identification of several worrying AMR bacteria that provide greater insights into circulating AMR in Canadian hospitals and their patients.

From the AMR collection created in chapter 2, we are also able to advance our scientific understanding of how antibiotic resistance develops within us and causes issues with treatment. In chapter 3, we looked at the effects of antibiotic administration routes on the level of AMR observed in our patient sample. We saw that current approaches to limit selection for AMR in the gut still resulted in clinically significant and concerning increases in AMR. Furthermore, this chapter allowed greater understanding of contributors to increased AMR in patients. AMR increases are not fully explained by exposure/colonization in hospital settings, but also by evolution of AMR originating from non-resistant bacteria in the gut. Additionally, analysis of these bacteria will inform expected AMR evolutionary trajectories and help us plan against them.

During analysis of patient data, we also came across evolution of a less understood resistance phenotype, hetero-resistance, to a very important antibiotic, colistin. We investigated a

commonly prescribed antifungal, fluconazole, for its ability to promote this resistance phenotype; however, it appeared that fluconazole did not promote this phenotype.

Ultimately, this thesis serves as a valuable reservoir of AMR bacteria for future study and contributes to a greater understanding of AMR development in patients, one day leading to more informed clinical decision making.

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TABLE OF CONTENTS

Descriptive Note …………………………………………………………………………….. ii
Lay Abstract ……………………………………………………………………………... iii
Acknowledgements ……………………………………………………………………….. v
List of Figures & Tables …………………………………………………………………... viii
Abbreviations ……………………………………………………………………………….. ix
CHAPTER 1: INTRODUCTION …………………………………………………...……. 1

- **Antimicrobial Resistance** …………………………………………………………… 2
- **Resistance Evolution and Transfer** ………………………………………………… 4
- **Emergence of MDR Gram-Negative Organisms and Common Mechanisms** ……... 7
- **Human Microbiome and Antibiotic Usage** ………………………………………….. 9
- **Knowledge Gap & Rationale** ………………………………………………………… 12
- **Central Hypothesis and Thesis Objectives** …………………………………………. 13

CHAPTER 2: CHARACTERIZING THE AMR STUDY ISOLATE COLLECTION ….. 14

- **Abstract** ……………………………………………………………………………….... 15
- **Background & Methodology** ………………………………………………………… 17
- **Results** ……………………………………………………………………………….... 20
- **Discussion** ……………………………………………………………………………... 28
- **Conclusion** …………………………………………………………………………….. 30

CHAPTER 3: THE EFFECTS OF PROPHYLACTIC ANTIBIOTIC THERAPY ON THE EMERGENCE AND MECHANISMS OF MDR IN GRAM-NEGATIVE BACTERIA …………………………………………………………………………………… 32

- **Abstract** ……………………………………………………………………………….... 33
- **Background** …………………………………………………………………………… 35
- **Methodology** …………………………………………………………………………... 36
- **Results** ………………………………………………………………………………… 39
- **Discussion** ……………………………………………………………………………... 47
- **Conclusion & Future Directions** ……………………………………………………. 50

CHAPTER 4: CHARACTERIZING THE ROLE OF FLUCONAZOLE IN COLISTIN HETERO-RESISTANCE ………………………………………………………………… 51

- **Abstract** ……………………………………………………………………………….... 52
- **Background & Methodology** ………………………………………………………… 53
- **Results** ………………………………………………………………………………… 56
- **Discussion & Conclusion** …………………………………………………………….... 57

SUMMARY ………………………………………………………………………………… 59
CONTRIBUTIONS TO THE FIELD ……………………………………………………….. 61
APPENDIX …………………………………………………………………………………. 62
REFERENCES ……………………………………………………………………………... 83

LIST OF FIGURES AND TABLES

Table 1: *Taxonomy of AMR Study Isolate Collection Isolates.* ……………………………….. 62
Table 2: *Species specific AMR phenotypes across the collection (n = 926).* …………………. 63
Table 3: *Resistance genes present in patient 5 TZP-susceptible and -resistant* E. coli *clonal isolates.* ………………………………………………………………………………… 64
Table 4: *Resistance genes present in patient 21 TZP-susceptible and -resistant* E. coli *clonal isolates.* ………………………………………………………………………………… 65
Table 5: *Descriptive statistics of antimicrobial therapy groups.* ……………………………... 65

Figure 1: *Protocol for isolation and characterization of resistant bacteria from clinical samples.* ……………………………………………………………………………………….. 66
Figure 2: *Descriptive Statistics of AML patient population*. …………………………………... 67
Figure 3: *PRISMA Patient Eligibility Assessment for AMR Analyses.* ……………………….. 68
Figure 4: *Heat map of antibiotic resistance across the AMR Study Isolate Collection.* ………. 69
Figure 5: *MDR gram-negative organism phenotypes across the AMR Study Isolate Collection.* ……………………………………………………………………………………….. 70
Figure 6: *Confirming colistin resistance in low ZOI* E. coli *from patient 18*. ………………… 71
Figure 7: *Antibiotic resistance across first patient sampled gram-negative organisms within the AMR Study Isolate Collection.* ……………………………………………………………… 72
Figure 8: *Prophylactic antibiotic treatment length significantly impacts gram-negative AMR phenotypes in a treatment dependent manner.* ………………………………………………… 73
Figure 9: *Observed gram-negative species over prophylactic antibiotic therapy.* …………… 74
Figure 10: *Longitudinal heatmaps of most resistant gram-negative species captured at each sampling day.* …………………………………………………………………………….. 75
Figure 11: *Evolution of TZP-resistant* E. coli *during TZP prophylaxis in patient -5* ………… 76
Figure 12: *Evolution of TZP-resistant* E. col*i during TZP prophylaxis in patient 21* ...…….. 77
Figure 13: *Effects of long-term prophylactic antibiotics on the microbiome.* ………………... 78
Figure 14: *Prophylactic treatment timeline of patient 6.* …………………………………….. 79
Figure 15: *Hetero-resistant phenotype of* E. bugandensis *isolated from patient 6.* ………….. 80
Figure 16: *Determining fluconazole's impact on antibiotic susceptibility in* Enterobacter spp. 81
Figure 17: *Role of fluconazole in evolution of colistin hetero-resistance over time.* ………… 82

ABBREVIATIONS

AML	Acute myeloid leukemia
AMR	Anti-microbial resistance
ARG	Antibiotic resistance gene
ATM	Aztreonam
BHI	Brain Heart Infusion Medium
BLBLI	β-lactam-β-lactamase inhibitor
CARSS	Canadian Antimicrobial Resistance Surveillance System
CAZ	Ceftazidime
COL	Colistin
CZCL	Ceftazidime-Clavulanic Acid
ESBL	Extended-spectrum beta-lactamase
FEP	Cefepime
IMP	Imipenem
LVX	Levofloxacin
MAC	MacConkey Medium
MALDI-ToF MS	Matrix assisted laser desorption ionization-time of flight mass spectrometry
MER	Meropenem
MDR	Multi-drug resistance
MH	Mueller Hinton Medium
MIC	Minimum inhibitory concentration
IM	Inner membrane
IV	Intravenous
OD	Optical Density
OM	Outer membrane
PBP	Penicillin binding proteins
PCR	Polymerase chain reaction
PFGE	Pulsed-field gel electrophoresis
PIP	Piperacillin
TET	Tetracycline
TMP/SMX	Co-trimoxazole (Sulfamethoxazole-Trimethoprim)
TZP	Piperacillin-Tazobactam
VAC	Vancomycin
VACC	Vancomycin, Amphotericin B, Ceftazidime, and Clindamycin Medium
XDR	Extensively drug resistance
ZOI	Zone of inhibition

CHAPTER 1: INTRODUCTION

Antimicrobial Resistance

Antimicrobial resistance (AMR) poses a substantial threat to public health and clinical medicine.[1] In 2018, it was reported that 14,000 Canadian deaths were resistance-related with 5,400 of those deaths directly caused by AMR.[2] The economic burden of AMR on the Canadian economy could rise from $2 billion (year 2018) to $13-$21 billion (year 2050).[2] By 2050, one estimate states that AMR bacterial infections will cause an estimated 10 million annual deaths worldwide, surpassing cancer mortality estimates.[3] As AMR infections continue to become more common, treatment becomes more difficult (increased duration and intensity) and more expensive.[2,4–6] If left undealt with, AMR has the potential to burden healthcare systems globally, both economically and in terms of patient volume.

Previous research has identified agricultural practices[7–11], antibiotic usage in healthcare[12], and wastewater treatment (includes agricultural and healthcare runoff)[8], as major contributors to AMR development. High volumes of antibiotics are utilized in agricultural practices to promote growth and prevent infection in livestock.[7–10] Over time, this functions to select for highly resistant bacteria that can carry antimicrobial resistance genes (ARGs) into the human population by food consumption or agricultural runoff.[8,10] Antimicrobial drug classes, such as arsenicals, polypeptides, glycolipids, tetracyclines, elfamycins, macrolides, lincosamides, polyethers, beta-lactams, quinoxalines, streptogramins, and sulfonamides, may be used as prophylactic or active care treatments during the lifecycle of poultry, cattle, and/or swine[13]; bacterial resistance to these antimicrobials threatens efficacy of clinical intervention due to significant crossover between veterinary and clinical antimicrobial therapies.[14]

Similar to agriculture, healthcare settings can function as hotspots for AMR development due to over prescription, misuse, and issues with compliance to antibiotic therapies.[12] Over prescription

occurs when physicians prescribe antibiotics for ailments that -by practice guidelines- do not indicate antibiotic prescription.[15] Misuse of antibiotics occurs when patients utilize previously prescribed antibiotics for treatment without consultation of a clinician, such as utilizing old antibiotics for treatment of cold symptoms.[16] Both over prescription and misuse constitute unnecessary exposure to antibiotics, which exert selective pressures on host microbial communities for resistant organisms.[12] Previous literature has shown that just one course of antibiotic treatment can significantly increase the abundance of antibiotic resistance genes in the GI tract of patients.[17]

Although similar to over prescription and misuse in its ability to select for AMR, issues with compliance differs from these by transiency of exposure.[18] Issues with compliance occur when patients do not complete their prescribed antibiotic treatment (ie: stop once symptoms improve), meaning this selective pressure is defined by transient antibiotic exposures.[18] This transiency allows survival of bacterial populations exposed to sub-inhibitory concentrations of antibiotics, allowing for bacterial adaptation.[19] *In vitro* evolution studies have shown that these conditions can lead to highly resistant bacteria, increasing rates of future treatment failure.[20–22] The most notorious example of compliancy issues remains to be the history of tuberculosis treatment and development of extensive drug resistance (XDR).[23] In certain countries, long-term TB treatments were difficult to obtain/afford, resulting in many patients starting treatment without completion and rapid TB AMR development.[23]

Although a large portion of research has gone into investigating selection for AMR within animal hosts, such as livestock and humans, a substantial amount of AMR development occurs in our environment, which inevitably recycles through our ecosystem. Antibiotics are not fully metabolized by humans or livestock and are excreted in bodily waste along with resistance genes

and bacteria.[8,19,24] With extensive usage of antibiotics in agriculture and clinical settings, waste water treatment systems experience significant turnover of waste containing conjugative bacteria, resistance plasmids, bacteriophage, and sub-lethal concentrations of antibiotics, creating a rich environment for resistance transfer and evolution.[8,19] Current research efforts have indicated that waste management practices are insufficient in controlling resistant bacteria and ARGs, posing a significant risk to workers and nearby residents.[25] With wastewater management facilities acting as AMR recycling bins, a grim reality of the AMR crisis is realized, with resistance development reaching far beyond the initial point of antibiotic administration.[13,25]

Resistance Evolution and Transfer

In the past, resistance evolution was mainly studied during MIC antibiotic exposures.[22] However, in recent decades, evolutionary study with broader concentrations of antibiotics have contributed to a greater understanding of complex resistance adaptation, where various antibiotic concentrations can select for highly resistant phenotypes. Currently, these conditions are defined as MIC and sub-MIC antibiotic selection.[22]

During exposure to bactericidal antibiotics, selection that occurs during MIC/lethal concentrations of antibiotics differs from sub-MIC concentrations in an all-or-nothing manner.[22] Lethal antibiotic concentrations will select for pre-existing resistant mutants within bacterial populations—arising randomly—while killing susceptible cells.[22] However, sub-MIC levels of antibiotics do not have such lethal effects on susceptible cells and instead provide conditions for survival of these cells and resistance evolution over time.[20–22] At low concentrations of antibiotics, evolution can proceed through successive mutations, each providing cumulative resistance, eventually producing highly resistant mutants.[26] *In vitro* evolution studies into *de novo* mutations under sub-MIC concentrations (0.1 x MIC) using ciprofloxacin and streptomycin

showed that—both *E. coli* and *S. typhimurium*—could evolve resistance several fold higher than the MIC after 600 generations of exposure.[22,26] Furthermore, due to the reduced bactericidal effects at sub-MIC concentrations, a larger diversity of evolutionary pathways to highly resistant mutants exist.[21] An *in vitro* evolution study showed that selection of streptomycin resistant *S. typhimurium,* using streptomycin (50xMIC), always resulted in point mutations in a common resistance gene, *rpsL*; whereas selection using 0.25xMIC resulted in a range of resistant mutants with mutations across five genes, *gidB, trkH, cyoB, nuoG*, and *znuA*, with resistant phenotypes containing all five mutations showing high resistance.[21] In this way, low levels of antibiotics can provide conditions allowing for complex evolutionary pathways towards highly resistant phenotypes that differ genotypically from MIC selected mutants.[20–22,26]

Not only does the complexity of evolutionary pathways leading to high levels of resistance broaden under sub-MIC conditions, but evolutionary processes are facilitated.[22] Sub-inhibitory concentrations of antibiotics can significantly increase rates of mutagenesis, recombination, and horizontal gene transfer (HGT), which can all contribute to evolution of resistant bacteria.[22]

Increased mutagenesis during sub-inhibitory antibiotic conditions has been linked to regulators of bacterial stress response, such as LexA, RecA, and the sigma factor, RpoS.[22,27,28] Proteins RecA and LexA regulate the bacterial SOS response to DNA damage.[29] In wild-type bacteria, LexA acts as a repressor protein, preventing downstream expression of the SOS regulon. When subjected to sub-inhibitory levels of antibiotics, or other environmental stressors, DNA damage functions to activate RecA protomers which assemble into filaments near LexA, promoting autocleavage of LexA and expression of SOS response genes.[22,29] The SOS response results in expression of translesion polymerases II & IV, which contribute to increased mutagenesis by high frequency of base-substitution errors.[27,29] Furthermore, this observed increase in

mutagenesis under sub-inhibitory levels of antibiotics has been shown to be ameliorated when the SOS response is impaired through inactivation of RecA[30] or addition of non-cleavable LexA.[31]

Also a regulator that responds to environmental stressors, RpoS is expressed during sub-MIC concentrations of bactericidal antibiotics and appears to influence mutagenesis.[22,28] A previous study showed that RpoS was induced by sub-MIC concentrations of amoxicillin and high concentrations of RpoS repressed *mutS* mRNA by induction of small-RNA, *sdsR*. MutS protein is responsible for mutation repair and its depletion results in an increased mutation rate.[28] As a result, LexA, RecA, and RpoS are all significant regulators of increased mutagenesis under antibiotic stress, which is thought to lead higher rates of mutant resistance genes.[19]

Contrary to mutagenesis, recombination promotes resistance to antibiotics via rearrangement of resistance genes within expressed cassettes rather than the expressed gene sequence.[22] Cassettes are small mobile genetic elements composed of a gene and recombination site, but can arrange with multiple genes behind a promoter. Within cassettes, composed of multiple genes in tandem, the genes closest to the promoter are expressed at the highest levels.[22] Thus, rearrangement events that result in resistance genes being assorted closer to the promoter exacerbates resistance phenotypes.[32] Interestingly, sub-inhibitory concentrations of fluoroquinolone antibiotics have been shown to stimulate intra- and inter-chromosomal recombination in *E. coli.*[33] As a result, sub-MIC levels of antibiotics may function to promote evolution of highly resistant phenotypes by recombination events.[32]

Once these genetic determinants and/or gene cassettes have evolved, they can be transferred between resistant and susceptible strains via horizontal gene transfer.[34] Horizontal transfer can be mediated by natural competence and transformation[35], bacteriophage[36], conjugative plasmids[37–

[39], and conjugative transposons[37,38]. Bacteriophage can exchange ARGs between a resistant donor bacterial cell and susceptible acceptor cell, in a contact-independent manner, by specialized or generalized transduction.[40] There is data indicating environmental phages carrying ARGs encoding for β-lactamases (TEM, CTX-M and SHV), PBPs, fluoroquinolone resistance, and resistance to many other antibiotics, including tetracycline, chloramphenicol, macrolides, lincomycin, clindamycin or erythromycin.[40] Furthermore, *in vitro* studies have demonstrated the ability of DNA phages to transfer resistance to susceptible strains.[40] One study indicated that the presence of antibiotics, such as fluoroquinolones, can facilitate resistance transfer in *E. coli* by triggering the SOS response, resulting in prophage induction and transduction.[22] Furthermore, fluoroquinolones have also been shown to induce prophage-mediated gene transfer in multi-drug resistant (MDR) *Salmonella sp.*[41]

Differing from bacteriophage transduction, transfer of ARGs through conjugative plasmids and transposons requires intimate contact between donor and acceptor bacteria.[37–39] Intimate interaction is established by complex extracellular filaments, known as sex pili.[39] Transposons containing ARGs can excise to form circular intermediates which can migrate through sex pilis and reintegrate in susceptible organisms.[39] Furthermore, transposons can also excise and reintegrate within plasmids of the same cell, contributing to the formation of plasmids containing multiple ARGs.[38] Interestingly, the presence of antibiotics, and concomitant activation of the SOS response, derepresses integrated conjugative elements (ICE) in *Vibrio cholerae*, allowing expression of conjugative, transfer, and integrase genes.[22,42]

Emergence of Multi-drug resistant (MDR) Gram-Negative Organisms and Common Mechanisms

Due to frequent antibiotic exposure *in vivo* and *ex vivo,* MDR bacteria have emerged and continue to become more common.[43] Furthermore, healthcare settings frequently deal with high

volumes of sick patients and frequent use of antibiotics, making this setting a particularly troubling breeding ground for multi-, extensive- (XDR), or pan-drug resistance (PDR) development.[5,12,37,43] Although both gram-positive and gram-negative MDR pathogens exist, gram-negatives with an MDR phenotype are of particular concern as their outer membrane limits the permeability to many antibiotics.[5,44] Increasing prevalence of MDR and XDR gram-negative bacteria are associated with increased complications of antibiotic therapy and treatment failure.[43] Specifically, the rising rates in gram-negative MDR pathogens, such as *Acinetobacter spp., Burkholderia cepacia* complex*, Pseudomonas aeruginosa, Legionella spp.*, *Escherichia coli, Stenotrophomonas maltophilia, Proteus mirabilis, Salmonella enterica, Serratia marcescens* and *Klebsiella pneumoniae*, are responsible for increased rates of mortality worldwide.[43,45–47]

These high risk MDR organisms differ significantly in microbial behaviour; however, many share similar characteristics in MDR phenotype development and exacerbation in clinical settings.[48–51] A common feature of MDR among these organisms is an inherent resistance to several classes of antibiotics with phenotypes further exacerbated by gained resistance to relevant clinical treatments, often through inheritance of mobile resistance elements.[48] *Acinetobacter sp.*, intrinsically resistant to several classes of antibiotics and typically treated with carbapenem antibiotics, can inherit carbapenemases via HGT.[49,50] Increasing frequency of carbapenemase-producing *Acinetobacter sp.* has led to reliance on last-line therapies, tigecycline and colistin, associated with higher mortality.[49] Similarly, *Enterobacteriaceae* species., including *E. coli* and *Klebsiella sp.*, are typically treated with beta-lactam antibiotics, however, high frequency of transmissible environmental extended-spectrum beta-lactamases (ESBLs) have complicated beta-lactam use as a therapeutic option.[51]

Although inheritance of mobile resistance elements have been shown to be a major contributor to increasing AMR phenotypic severity, MDR bacteria often have MDR efflux pumps which allow for increased survivability, mutagenesis, and likelihood of developing de novo resistance.[52–54] A recent study has shown that overexpression of the efflux pump AcrAB-TolC can increase frequency of resistant mutants via downregulation of MutS.[55] Efflux pumps can provide sub-inhibitory concentrations of antibiotics within the bacterial cell, allowing for survival of susceptible cells while resistance can develop.[55] Several different types of efflux pumps exist, including multi-drug and toxin extrusion (MATE), small multidrug resistance (SMR), major facilitator superfamily (MFS), resistance nodulation cell division (RND), and ATP-binding cassette (ABC), each with different spectrums of antibiotics coverage and unique evolutionary capability.[53,54]

Human Microbiome and Antibiotic Usage

The human microbiome comprises more than 100 trillion micro-organisms, occupying many niches on and within the body.[56] Of these niches, the GI tract remains a rich community complexly interlinked with human health.[57] The GI tract comprises the mouth, stomach, small and large intestines, and anus.[57] It is an open system that experiences frequent turnover, affecting the composition of species colonizing gut mucosal surfaces.[56,57] The GI microbiome has been shown to be heavily influenced by antibiotic exposure.[37,57–59] Specifically, antibiotic therapy has been shown to influence microbial composition, diversity and ARG carriage.[37]

Different antibiotics are shown to perturb the healthy microbiome in different ways.[17,60] One systematic review indicated that penicillins, cephalosporins, lipopolyglycopeptides, macrolides, ketolides, clindamycin, tigecycline, quinolones and fosfomycin all increased abundance of *Enterobacteriaceae*, however, penicillins, cephalosporins, macrolides, clindamycin, quinolones

and sulphonamides decreased abundance of *E. coli*.[60] In contrast to general beta-lactam impacts on the microbiome, penicillin combinations increase abundance of *E. coli*. The review also indicated that penicillins and combinations were typically associated with outgrowth of *Enterococcus sp*.[60] Despite these observed differences of impact between antibiotics, antimicrobial treatments are generally shown to decrease overall microbial diversity of the microbiome and decrease beneficial bacteria, such as *Bifidobacterium sp.*, *Ruminococcus sp.,* and other *Lachnospiraceae* and butyrate producing bacteria.[61]

Compositional microbiome changes mediated by antibiotic therapy are accompanied by changes in ARG abundance within microbial populations inhabiting the gastrointestinal (GI) tract.[17] Several studies have identified significant increases in abundance of ARGs following antibiotic treatment, which decreases over weeks following exposure.[61–63] The diversity of resistance genes that are enriched within the gut during antibiotic therapy is influenced by baseline gut resistome and local resistomes.[61] In areas with more extensive antibiotic exposure, such as parts of southern Asia and Africa, there exist a higher prevalence of environmental AMR bacteria linked to higher rates of AMR carriage and greater ARG diversity.[64–66]

The severity of antibiotic effects on the microbiome is directly impacted by route of administration.[67] When the gut microbiome is directly exposed to oral antibiotics, abundance of antibiotic resistance increases in the gut. However, gut microbiome exposure to intravenous (IV) antibiotics relies on drug elimination routes. IV antibiotics eliminated by biliary excretion, such as tetracycline, will still have significant effect on the microbiome compared to drugs eliminated parenterally (ex: kidneys).[67] Depending on route of elimination, certain IV drugs are excreted into the GI tract at sub-inhibitory concentrations and contribute to AMR selection.[19,67] Mouse studies have linked IV antibiotic administration to increased ARG prevalence in the GI tract

following prolonged exposure.[67] Furthermore, prolonged exposure to various antibiotics can increase risk of MDR colonization and subsequent carriage.[68,69] However, the impacts of long-term IV antibiotic use on MDR gram-negative emergence—and mechanisms through which they emerge clinically—still remains to be fully elucidated.

Knowledge Gap & Rationale

A major component to addressing AMR-related mortality involves surveillance of circulating ARGs and characterizing mechanisms of resistance.[70,71] Past surveillance efforts have been focused on identifying circulating ARGs to guide hospital infection control and contribute to management of resistant pathogen outbreaks.[71] These surveillance strategies were accomplished by utilizing culture-based and metagenomic approaches.[37,72] However, these strategies focus solely on ARG identification and fail to identify novel mechanisms of resistance, which may inform novel pharmacological intervention and clinical decision making.[37,72] Furthermore, these studies looked at single-time point samples, leaving little known about mechanisms surrounding resistance development within patients over time. Thus, the effects of prolonged antibiotic exposure on AMR and MDR development within patients remains to be explored.

Optimal AMR surveillance strategies focus on sampling rich GI tract microbial communities (i.e. feces collection/rectal swabs) from patient populations experiencing substantial selective pressure imposed by high antibiotic exposure, such as those who are immunocompromised and require prophylactic antibiotics.[71,73] Acute myeloid leukemia (AML) patients are an example of such patients as chemotherapy ablates the immune system, which necessitates the extensive use of prophylactic antibiotics.[74] Previous work by the Surette lab in collaboration with clinicians, Dr. Mark Loeb and Dr. Dominik Mertz, was focused on collecting rectal swabs up to 8 weeks from an AML patient cohort (n = 25), receiving prophylactic antibiotics (Figure 1). Rectal samples were grown on media selective for susceptible or resistant gram-negative bacteria. Colonies were selected from each patient sample and stored to create a library of 926 clinical isolates of interest, which will be used to track AMR development over prophylactic treatment time.

Central Hypothesis and Thesis Objectives

Previous efforts show that sub-MIC selective pressure increases survival and diversity of evolutionary trajectories capable of producing high level antibiotic resistance. These conditions have been shown to be common within the gut during IV therapy and may promote evolution in addition to selection for pre-existing resistant gram-negatives within the gut.[67,75–77] The overarching hypothesis of this thesis is that prophylactic IV antibiotics provide cumulative selection over time and resistance phenotypes will continue to evolve broader spectrum resistances over long periods of treatment. This thesis aims to first identify unique MDR gram-negative phenotypes in order to contribute to surveillance efforts of novel resistance phenotypes arising in nosocomial pathogens **(Chapter 2)**. Second, these MDR gram-negatives were assessed in the context of time on prophylactic therapy in order to elucidate mechanisms of colonization, overgrowth and/or evolution **(Chapter 3)**. Third, an interesting trend between fluconazole used and emergence of colistin hetero-resistance was observed, suggesting a link between antifungal therapy and bacterial AMR. Thus, the effects of fluconazole on colistin hetero-resistance development were investigated **(Chapter 4)**.

As a result, this thesis has two primary aims.

Aim 1 Characterization of antibiotic resistance and bacterial taxonomy within the Surette Lab AMR gram-negative bacterial isolate collection **(Chapter 2)**

Aim 2 Examine the effects of prophylactic antibiotic therapy on the emergence and mechanisms of MDR in gram-negative bacteria **(Chapter 3 & 4)**

Chapter 2: CHARACTERIZATION OF THE AMR STUDY ISOLATE COLLECTION

ABSTRACT

Background: Surveillance efforts continue to stand at the forefront of combat against AMR. To remain effective, these efforts require constant input from a variety of populations and environments. Furthermore, these strategies rely on discovery of novel circulating resistance mechanisms, which continue to be characterized.

Objectives: 1) Characterize the AMR Study Isolate Collection in order to identify novel gram-negative MDR phenotypes using 10 antibiotics (LEV, COL, TET, PIP, TZP, CAZ, CZ/CL, ATM, FEP, & IMP), **2)** create a comprehensive list of high importance AMR/MDR gram-negative strains for future molecular study.

Methodology: Rectal swabs from 25 prophylactic antibiotic users, were cultured on BHI, MAC, VACC, and ESBL plates. Gram-negative colonies were picked and stored in the AMR Study Isolate Collection. Each gram-negative colony was taxonomically identified using MALDI-ToF MS and challenged with 10 antibiotics (fluoroquinolones, beta-lactam and combinations, polymyxins, and tetracycline) by disk diffusion.

Results: A total of 926 colonies were picked and characterized. The majority (79.3%) of the collection was composed of *E. coli* (50.8%), *K. pneumoniae* (18.0%), and *K. variicola* (10.5%). The most common resistance phenotypes within the database were PIP (55.2%) and tetracycline (23.9%). Average baseline AMR carriage within the study population did not differ between therapy types [TZP IV, CAZ IV, trimethoprim (TMP) + sulfamethoxazole (SMX) PO, ceftriaxone (CRO) IV + cephalexin (LEX) PO, & TMP-SMX PO + TZP IV] and antibiotic history. Several MDR gram-negatives were identified, including *Citrobacter sp., Morganella morganii, Raoultella ornithynolytica, Stenotrophomonas maltophilia, E. coli, Enterobacter cloacae*, and *K. pneumoniae*, with broadest spectrum resistance phenotypes characterized by extended spectrum beta-lactam resistance.

Conclusion: Broad spectrum MDR phenotypes were detected in high priority gram-negative opportunistic pathogens, including *C. braakii, C. freundii, E. coli, K. pneumoniae, M. morganii, R. ornythinolytica,* and *S. maltophilia*. Future molecular study with these strains may hold novel resistance determinants and inform surveillance efforts.

BACKGROUND

A major component to addressing AMR-related mortality involves surveillance of circulating ARGs and characterizing mechanisms of resistance.[70,71] Additionally, effective surveillance relies upon updated samples of resistant organisms within high exposure environments in order to identify novel troublesome phenotypes as they arise. Past surveillance efforts have been focused on identifying circulating ARGs to guide hospital infection control and contribute to management of resistant pathogen outbreaks.[71] These surveillance strategies were accomplished using culture-based and/or metagenomic approaches.[37,72] However, these strategies focus on previous ARG identification and fail to identify novel phenotypes as well as their mechanisms of resistance, which may inform novel pharmacological intervention, improved screening, and clinical decision making.[37,72]

In this chapter, I characterized the AMR Study Isolate Collection comprising 926 gram-negative isolates isolated from longitudinal samples of 21 AML patients receiving prophylactic antibiotics. As such, these samples capture baseline AMR carriage as well as how MDR carriage is influenced by long term exposures to prophylactic treatment. This database is intended to be used to identify novel MDR phenotypes among gram-negatives as well as serve as a reservoir of AMR/MDR gram-negative strains for future molecular study.

METHODOLOGY

Sample collection

The study design and sample collection is summarized in Figure 1. Patients diagnosed with AML were recruited and the research study nurse obtained 2-3 rectal swabs/week for up to 8 weeks. Samples were received by the Surette lab on the day of collection. Rectal swabs were vortexed in

universal transport media (UTM) to dislodge bacteria. UTM from the rectal swab was diluted from 10^{-1} to 10^{-6} and 5µL was spotted onto Brain Heart Infusion (BHI) and MacConkey (MAC) agar for total bacterial counts and gram-negative counts, respectively. Then 100µL of diluted UTM was plated on vancomycin, amphotericin B, ceftazidime, and clindamycin (VACC) and CHROMID (Biomerieux) agar and all plates were grown overnight to 48hrs at 37°C. From VACC and CHROMID (Biomerieux) plates, up to 10 morphologically different colonies were picked and streaked on MAC plates to confirm as gram-negative bacteria before being frozen in 10% skim milk at -80°C. For the first rectal sample, 5µL of UTM dilutions (10^{-2},10^{-4}, & 10^{-6}) were spotted on all 4 plates [5µL spot dilutions for BHI and MAC, 100µL for VACC and CHROMID (Biomerieux)]. However, this was adjusted to 100µL of UTM dilutions (10^{-1},10^{-2}, & 10^{-4}) plated onto VACC and CHROMID (Biomerieux) for the remaining rectal samples for better resolution.

Taxonomic identification

Clinical isolate frozen stocks were grown on BHI agar and streaked to purity. Colonies were then picked from pure overnight BHI agar plates and spread on a matrix assisted laser desorption ionization-time of flight mass spectrometry (MALDI-ToF MS) sample target. Once colony smears were dry, HCCA matrix was added to each sample target well slot. Prepared matrix-colony sample targets were read using the MALDI Biotyper® Sirius CA System (Bruker Ltd).

Kirby-Bauer disk diffusion assay

Clinical isolates were inoculated from frozen stocks onto BHI agar and streaked to purity. Pure cultures were used to inoculate liquid BHI broth. Inoculi were grown overnight at 37°C. Overnight cultures were standardized to an optical density at 600nm (OD_{600}) of 0.1 using a

SpectraMax M3 plate reader (Molecular Devices). Standardized cultures were swabbed on Mueller-Hinton (MH) agar plates and stamped with levofloxacin [LVX (5µg)], ceftazidime [CAZ (30µg)], ceftazidime/clavulanic acid [CZ/CL (30µg:10µg)], colistin-sulphate [COL (10µg)], cefepime [FEP (30µg)], aztreonam [ATM (30µg)], tetracycline [TET (30µg)], piperacillin [PIP (100µg)], piperacillin/tazobactam [TZP (100µg:10µg)], and imipenem [IMP (10µg)] disks. Plates were incubated overnight at 37°C. Zone sizes were measured using the BIOMIC V3 microbiology system (Giles Scientific). Resistance classifications were assigned in accordance with CLSI guidelines. ZOIs not standardized for some gram-negative species, such as *Stenotrophomonas maltophilia* and colistin ZOI for *Enterobacteriaceae*, were assigned as projected-resistance. Projected-resistance was predicted from the closest related species for which CLSI guidelines were available. *S. maltophilia* was classified using breakpoints for *Pseudomonas sp*. Colistin projected-resistance for *Enterobacteriaceae* was classified as ZOI ≤ 10mm and further characterized as resistant by broth dilution assay.

Minimum inhibitory concentration (MIC) broth dilution assay

Stock concentrations were made using colistin-sulphate powder solubilized in sterilized milliQ water. Liquid MH broth was inoculated with *E. cloacae* and *E. coli* isolates and incubated overnight at 37°C. Overnight cultures were diluted 1/1000 in MH broth and grown to an OD_{600} of 0.1. Liquid cultures (OD_{600}=0.1) were further diluted 1/200 in MH broth and pipetted in 100µL volumes within a 96-well plate. Stock concentrations were diluted 1/100 in each well to final serial dilution concentrations of 0-128µg/mL or 0-256µg/mL. Plates were incubated overnight at 37°C. Well optical densities were measured at 600nm using a SpectraMax M3 plate reader (Molecular Devices). Antibiotic concentrations producing optical densities equal to sterile media controls (OD_{600}=0.049) were considered as inhibitory and used to determine MIC values.

RESULTS

Descriptive statistics of clinical sample

Our clinical sample is composed of 44% male and 56% female, with a mean age of 61 years old (Figure 2). Four patients were sampled for the full 8 weeks, with an average collection time of 41.75 days. Fourteen patients were discharged before 8 weeks. On average, time until discharge was around 25 days. Finally, seven patients withdrew from the study. On average these patients withdrew after 9 days of study involvement.

Overview of Collection

A broad approach was taken in characterizing all gram-negative isolates from patient rectal swabs. The rationale for this approach was to cast a wide net and identify novel resistance phenotypes and MDR bacteria. From the 25 patients that were initially recruited, the rectal swab samples from 3 patients showed no growth on MAC, VACC, or CHROMID plates, indicating a lack of gram-negative bacteria at the level of detection from these samples. Additionally, samples from 1 patient showed no growth on any media type (BHI, MAC, VACC, or CHROMID). As a result, these patients did not contribute any gram-negative bacteria to the collection and were excluded from further analysis (Figure 3). Colonies were picked from the remaining 21 patient rectal swabs grown on VACC, and/or CHROMID plates to generate the AMR gram-negative collection (926 cultured gram-negative bacteria).

All 926 bacterial isolates were grown from frozen stock and analyzed for taxonomic identification by matrix-assisted laser desorption ionization time of flight (MALDI-ToF) mass spectrometry (MS). A positive identification was obtained for all isolates. The most dominant taxa were identified as *E. coli*, *Klebsiella pneumoniae*, and *Klebsiella variicola*, composing

50.8%, 18%, and 10.5% of the collection, respectively (Table 1). Other gram-negatives—typically associated with nosocomial infection—such as *Stenotrophomonas maltophilia, Enterobacter spp., Citrobacter spp., Pseudomonas spp.,* and *Morganella morganii*, comprise the remaining ~21% of the collection.

The isolates were screened against 10 antibiotics, relevant to clinical treatment of gram-negative infections, to determine AMR phenotypes for each organism. An AMR heatmap was generated to highlight AMR phenotypes of all 926 isolates (Figure 4). At a first glance, PIP non-susceptibility was most common resistance phenotype (55.2%), followed by TET non-susceptibility (23.9%). On the other hand, LVX and IMP resistance was least observed. Resistance to beta-lactams and combinations, such as CAZ, CZ/CL acid, and FEP, was observed less frequently than COL projected-resistance or TZP non-susceptibility; however, these phenotypes were more frequent than IMP resistance.

Resistance phenotypes differed among gram-negatives isolated from different patients. Most patients (5-8, 10, 13, & 16-23) harbored gram-negative bacterium that were characterized as non-susceptible to 3 or more antibiotics (MDR) at some time during their study involvement. However, patients 3, 4, 11, 12, 14, 15, 24, and 25 did not harbor any MDR gram-negatives and isolates from these patient samples were characterized as non-susceptible to 2 or less antibiotics. Furthermore, no gram-negatives from any patients were characterized as extensively-drug resistant (XDR) or pan-drug resistance (PDR).

When looking at the y-axis, there was a significant difference between the number of bacterial isolates included within each patient. This was due to several factors within the collection protocol. First, patients had different lengths of involvement in the study, meaning some patients had more samples cultured and more colonies picked. Second, diversity of colony morphology

can significantly differ across cultured patient samples, resulting in some patients having 20 morphologically unique colonies picked within a cultured sample vs. 2 morphologically unique colonies from another patient sample. Both of these factors contributed to the differences in length of y-axis data points within the heatmap (Figure 4).

Gram-negative AMR and MDR characteristics

Within the collection, there appear to be certain species associated with higher levels of resistance, and of broader spectrum (Table 2).

Citrobacter sp.

In our collection, there was broader spectrum of resistance displayed by *Citrobacter braakii* compared to resistance among *Citrobacter freundii* isolates. *C. braakii* displayed an extended spectrum of beta-lactam resistance, with isolates commonly resistant to ATM (95.8%), PIP (100%), CAZ (100%), as well as PIP (79.2%) and CAZ (100%) combinations (Table 2). This extended spectrum resistance to beta-lactams is also observed in *C. freundii* isolates, but much less frequently. Additionally, *Citrobacter braakii* isolates display some resistance to TET as well as projected-resistance towards COL. These resistance phenotypes are not observed in *C. freundii* isolates. Furthermore, all *C. braakii* and *C. freundii* isolates remained susceptible to LVX, FEP, and IMP.

Several *C. braakii* isolates were classified as MDR (Figure 5) and characterized by extended-spectrum beta-lactam resistance, including resistance towards PIP, TZP, CAZ, CZ/CL, and ATM. Some MDR isolates also displayed TET resistance and COL projected-resistance. In these isolates, COL resistance was deemed more likely as there was no visible inhibition when challenged with 10μg COL disks (ZOI = 6mm) while susceptible isolates typically show ZOI ≥

11mm. Although less extensive, *C. freundii* also demonstrated extended-spectrum beta-lactam resistance, mainly PIP, CAZ, and CZ/CL. Intermediate resistance to ATM was inconsistently observed across these MDR isolates. Additionally, one of the MDR *C. freundii* isolates displayed extended spectrum beta-lactam resistance similar to *C. braakii* MDR isolates cultured from the same patient.

Enterobacter sp.

All *Enterobacter sp.* were identified as part of the *Enterobacter cloacae complex* (ECC). Only one isolate of both *Enterobacter asburiae* and *Enterobacter kobei* were identified. These species showed susceptibility to all antibiotics, except for *E. asburiae*'s resistance to PIP and intermediate resistance to TZP (Table 2). The vast majority of ECC complex species were identified as *E. cloacae* (Table 1). Some *E. cloacae* isolates show resistance towards PIP and TZP along with TET resistance and most of these isolates display resistance towards COL (Table 2). Resistance towards COL was characterized by growth within a defined zone of inhibition, indicating a phenotypic similarity to hetero-resistance. Thus, *E. cloacae* COL hetero-resistance was further characterized (See Chapter 4).

MDR was identified within isolated *E. cloacae* isolates. Two MDR phenotypes were observed, differing mainly by the presence and absence of TET resistance (Figure 5). Both display some level of resistance toward PIP and TZP as well as extensive growth within a definable ZOI when challenged with COL and are suspected to be hetero-resistant.

E. coli

The dominance of *E. coli* within the collection coincides with significant diversity in resistance phenotypes. The most common phenotypes observed in *E. coli* are PIP and TET resistance,

remaining in line with observed resistance within the total collection (Figure 4). Resistance to other beta-lactams, such as TZP, CAZ, CZ/CL, and ATM, was infrequent among *E. coli* isolates, and resistance to IMP was not observed. Interestingly, resistance against FEP (3.4%) was more common than resistance to CAZ (0%). This is an atypical observation as FEP was originally introduced to combat CAZ resistance and is more effective against *E. coli*.[5,78] Additionally, low frequency LVX resistance and COL projected-resistance was observed.

Being the most frequently recovered organism, *E. coli* had the most diversity in MDR phenotypes (Figure 5). MDR *E. coli* picked from patients 5, 7, and 20 was characterized by PIP, TZP, and TET resistance or low zone diameters when challenged with COL (ZOI = 6mm). *E. coli* from patients 18 and 19 displayed similar MDR phenotypes, with some isolates showing broader spectrum resistance to beta-lactams, such as intermediate resistance to CAZ and CZ/CL. Additionally, MDR *E. coli* from patient 18 and 19 displayed low zone diameters to COL disks and were further investigated using broth dilution assays, indicating COL resistance (MIC ≥ 2µg/mL) (Figure 6). *E. coli* from patient 13 had a unique MDR phenotype with prominent resistance observed to PIP, ATM, and FEP (4th generation cephalosporin). Additionally, one MDR isolate had resistance to LVX, uncommonly observed throughout *E. coli* isolates within the database.

Klebsiella sp.

Among all *Klebsiella sp.*, PIP resistance was commonly observed (Table 2). Of the four *Klebsiella* species. identified in the collection, including *K. pneumoniae, K. variicola, K. aerogenes,* and *K. oxytoca,* broader spectrum resistance phenotypes are associated with *K. pneumoniae*. Some *K. pneumoniae* isolates was more resistant to TZP, as well as a small proportion of isolates displayed resistance to ATM (3%), CAZ (1.8%) and CZ/CL (1.2%).

Additionally, several isolates displayed low zone diameters when challenged with COL disks. These have been characterized as projected-resistance and have yet to be characterized as resistant by broth microdilution.

K. pneumoniae was the only *Klebsiella* species to display MDR phenotypes (Figure 5). MDR phenotypes characterized by resistance to PIP, TZP, and projected-resistance to COL were detected in patients 6, 7, and 17. Other *K. pneumoniae* isolates—isolated from patients 7, 8, and 17—differed by their spectrum of beta-lactam resistance as well as TET resistance. The broadest spectrum of resistance appears in MDR *K. pneumoniae* isolated from patient 8. Here one MDR isolate displayed extended spectrum resistance, including PIP, TZP, CAZ, CZ/CL, and ATM resistance; whereas the other isolate has additional resistance to TET and projected-resistance to COL while remaining ATM susceptible.

Morganella morganii

Of the bacteria characterized, *M. morganii* isolates had the broadest spectrum resistance profiles. Most were resistant to seven of the ten panelled antibiotics (Table 2). MDR isolates were mainly characterized by extended-spectrum beta-lactam resistance, TET resistance, and COL projected-resistance (Figure 5). However, these MDR isolates remained susceptible to LVX, FEP, and IMP.

Proteus mirabilis

COL resistance is intrinsic to *P. mirabilis*[79], which explains a high frequency of COL resistant isolates (78.6%) (Table 2). TET resistance (92.9%) was commonly observed in *P. mirabilis*. Resistance to beta-lactams was rare, with only 17.9% and 3.6% of all *P. mirabilis* isolates displaying PIP and CAZ resistance, respectively. Multiple resistance phenotypes were observed

within several *P. mirabilis,* indicating MDR (Figure 5). MDR *P. mirabilis* isolates picked from patients 19, 20, and 23, displayed similar resistance phenotypes. MDR towards TET, PIP, and COL was the most common MDR phenotype across these 3 patients. A unique phenotype emerged in patient 20 characterized by resistance to CAZ and susceptibility to PIP.

Pseudomonas sp.

P. aeruginosa and *P. citronellolis* were the two species identified (Table 1). Few isolates of *P. aeruginosa* were identified and only 1 in 3 showed intermediate resistance to TET and ATM (Table 2). *P. citronellolis* was more frequently picked. These isolates were resistant to ATM (94.1%), with some intermediate resistance to PIP (41.2%) and CAZ (11.8%). Many of these resistance phenotypes appeared alone or in pairs, with only one MDR phenotype being observed among *Pseudomonas sp.* This MDR phenotype was characterized by intermediate resistance towards PIP and CAZ as well as resistance to ATM (Figure 5). However, the MDR *P. citronellolis* remained susceptible to beta-lactam combinations TZP and CZ/CL.

Raoultella ornithinolytica

This species was very rare within the collection, with only 2 isolates recovered (Table 1). Of these two isolates, one was susceptible to all antibiotics, except PIP (Table 2); while the other colony displayed MDR characterized by non-susceptibility to COL, TET, PIP, TZP, CAZ, and CZ/CL (Figure 5).

Salmonella

Similar to *R. ornithinolytica*, *Salmonella enterica* were rarely isolated within the collection, with only 2 colonies picked (Table 1). These isolates were susceptible to most antibiotics, but 1

colony displayed resistance to both TET and PIP antibiotics (Table 2). No MDR was observed among sampled *Salmonella.*

Stenotrophomonas maltophilia

S. maltophilia was sampled somewhat frequently (5.1%) compared to other gram-negative species (Table 1). From the picked isolates, resistance to PIP (100%), ATM (100%), FEP (89.4%), and IMP (100%) was observed (Table 2). Some level of resistance to PIP tazobactam was observed. However, COL projected-resistance was only observed in 2.1% of isolates. Despite minor resistance fluctuations between *S. maltophilia* isolates, all were characterized as MDR (Figure 5). Furthermore, all isolates remained susceptible to LVX and third-generation cephalosporin, CAZ and its combination (CZ/CL).

Assessing first sampled gram-negative characteristics across all patients

When looking at the collection of microbes present within the first sampling periods for each patient, *E. coli* appears to be the most commonly observed gram-negative species, remaining in line with the observed abundance of *E. coli* within the collection (Table 1). Generally, *E. coli* was isolated from all patients, but was isolated with other Enterobacteriaceae (*K. pneumoniae* or *C. braakii* and *Enterobacter sp*) in several patients (Figure 7A). For some patients *E. coli* was not recovered from the first sample, such as patients 06, 10, 11, 17, 20, and 23. Patient 06 had only *K. variicola* and *C. freundii* isolated from their first sampling. Patient 10 appeared to have *P. citronellolis* isolated from the first sample. Patients 11 and 17 only had *K. pneumoniae* isolated from their first sampling. Additionally, patients 20 and 23, appear to start their sampling period with *P. mirabilis* alone. There do not appear to be major differences across observed

species when comparing baselines on prophylactic treatment; however, sample sizes are not large enough to draw conclusions past speculation.

The majority of samples came from patients on TZP as their first round of IV prophylactic therapy. Consistent with our observations of dominant PIP resistance across the entire collection, PIP resistance appears to be the most common gram-negative resistance phenotype across first samples from all patients, followed by TET resistance. (Figure 7B). Gram-negative organisms isolated from first samples of patients on TZP IV prophylaxis, appear to be resistant to a broader spectrum of antibiotics; some gram-negative organisms even appeared to show resistance to LVX, COL, and FEP, which are generally uncommon within the overall collection. Interestingly, the TZP IV prophylaxis group with history of antibiotic use in the past 6 months did not appear to have equivalent or greater spectrum of gram-negative resistance phenotypes.

DISCUSSION

The composition of major gram-negative taxa detected in this collection matches rectal swab sampling observations in previous research.[80] The most dominant gram-negative taxa within the collection were *E. coli* and *Klebsiella sp.*. These organisms, as well as other *Enterobacteriaceae,* are commonly found within the colon environment, especially during antibiotic prophylaxis.[61] Furthermore, a notable number of *Pseudomonas* species were isolated within the collection. However, only one patient, patient 10, was observed to carry this at baseline sampling, indicating a carriage rate of 1/21 (~4.8%) (Figure 7A); similar to reported carriage rates among healthy patients.[81]

In terms of the resistance detected across the AMR collection, the most common resistance phenotypes were PIP and TET non-susceptibility (Figure 4). It has been reported that both beta-

lactamase and TET pump resistance genes are very common throughout our environment.[82] Since it is typical that local resistomes contribute to the resistance seen within the gut, these high frequencies in our gut are likely a reflection of environmental contributions.[17,64,82] However, several species within the collection appeared to have broader spectrum resistances, which are thought to be less common in our environment and typically associated with nosocomial exposure.[43,45–47]

These MDR phenotypes of interest were observed in *Citrobacter sp., M. morganii, R. ornithynolytica, S. maltophilia, E. coli, E. cloacae,* and *K. pneumoniae.* Most MDR phenotypes within this collection are defined by broad beta-lactam resistance, with most concerning extended spectrum resistance phenotypes arising in *Citrobacter sp., K. pneumoniae, R. ornithynolytica,* and *M. morganii.* Past research has identified extended-spectrum beta-lactamases (ESBL) as being the most common primary determinant of this type of resistance.[5,83] Past studies on an extensive *E. coli* database indicates that reduced outer membrane protein function and overexpression of beta-lactamases, such as AmpC, can mimic beta-lactamase inhibitor resistance seen in this collection.[84] Isolates of particular interest include those displaying extended-spectrum beta-lactam resistance in addition to non-susceptibility to COL (Figure 5). Due to an increasing reliance on COL as a last resort therapy in treatment of XDR and PDR gram-negatives, these isolates may serve as ideal candidates in the study of common genetic determinants of COL resistance in GI-resident MDR gram-negatives.

In addition to the extensive spectrum beta-lactam resistance phenotypes observed, narrower spectrum resistance was found in *E. cloacae, C. freundii, E. coli, K. pneumoniae, Pseudomonas spp.* and *P. mirabilis*, often coexisting with resistance in other antibiotic categories. MDR *E. cloacae, E. coli, K. pneumoniae*, and *P. mirabilis* often displayed narrow spectrum beta-lactam

resistance towards PIP and TZP in addition to TET and COL projected-resistance. As previously mentioned, beta-lactamases are a common determinant of beta-lactam resistance.[5] In these species, CAZ resistance is not observed, indicating a lack of ESBLs.[77] However, overexpression of certain beta-lactamases, such as $bla_{TEM\text{-}1b}$, can provide this narrow spectrum phenotype, resistant to tazobactam combination.[85] Another interesting feature of these narrow spectrum isolates is the presence of COL non-susceptibility (ZOI ≤ 10mm). Aside from *P. mirabilis* and *Serratia marcescens*, COL resistance is not intrinsic to *Enterobacteriaceae.*[79] It is not uncommon for organisms to inherit COL resistance, often attributed to mutations in *mgrB* genes and these MDR isolates are candidates for further study.[79]

In recent years, clinical concern has started to grow regarding the increasing prevalence of carbapenem resistance among gram-negative species.[4,45,46,83] Here we report a lack of carbapenem resistance among all isolated MDR gram-negatives within the collection, with the exception of *S. maltophilia.* This gram-negative bacteria has many chromosomally encoded efflux pumps and beta-lactamases, which provide a wide array of intrinsic resistance, including resistance to carbapenems.[44,86]

CONCLUSION

The gram-negative AMR collection from this study holds a wide variety of organisms and resistance phenotypes, including many MDR organisms of interest for future study. MDR phenotypes were detected in high priority gram-negative opportunistic pathogens, including *Citrobacter braakii, C. freundii, Enterobacter cloacae, Escherichia coli, Klebsiella pneumoniae, Morganella morganii, Proteus mirabilis, Pseudomonas citronellolis, Raoultella ornythinolytica,* and *Stenotrophomonas maltophilia*. Resistance to last resort antibiotics, such as COL and carbapenems, was infrequent. COL projected-resistance was more common among MDR

phenotypes, whereas carbapenem resistance was completely absent among MDR *Enterobacteriaceae.* This collection houses many phenotypes previously identified by surveillance efforts, but introduces some interesting gram-negative isolates to the Surette lab and IIDR for future microbiological study.

CHAPTER 3: THE EFFECTS OF PROPHYLACTIC ANTIBIOTIC THERAPY ON THE EMERGENCE AND MECHANISMS OF MDR IN GRAM-NEGATIVE BACTERIA

ABSTRACT

Background: IV antibiotics are commonly used in antibiotic prophylaxis. Although this route of administration has low penetrance into the GI tract, it has been shown to have a significant selective impact on AMR in the microbiome. However, little is known about the mechanisms through which long-term antibiotic prophylaxis impacts MDR gram-negative emergence over time.

Objectives: Identify the impacts of long-term antibiotic prophylaxis on rectal MDR gram-negative carriage and elucidate mechanisms of AMR.

Methodology: Patient eligibility was limited to those with long-term antibiotic exposure and rectal sampling (>14 days). Gram-negative AMR phenotypes were assessed longitudinally. Resistance evolution was further investigated using pulsed-field gel electrophoresis and comparative genomics. Microbiome diversity and composition was assessed.

Results: AMR in gram-negatives appeared to significantly increase over time on antibiotic prophylaxis (p-value < 0.0001) with MDR carriage being identified after 13 to 23 days on therapy. Changes in AMR over treatment time were primarily characterized by increased detection of resistance to a broader spectrum of beta-lactam antibiotics. Increased AMR in gram-negatives was associated primarily with IV TZP/VAC/MER treatment (adj. p-value = 0.008). Increase MDR gram-negative carriage was contributed to both colonization and de novo evolution. De novo evolution of TZP resistance in *E. coli* occurred in two distinct lineages from two different patients, 5 and 21. EnvZ osmolarity regulator mutants and a 20kb mobile element deletion was observed in *E. coli* from patients 5 and 21, respectively. Microbiome analysis showed that therapy groups, associated with increased MDR, appeared to be associated with increased levels of microbial dysbiosis and loss of beneficial bacterial species.

Conclusion: Both antibiotic therapy characteristics and treatment length serve as risk factors for MDR gram-negative carriage. Furthermore, increased carriage is defined by both MDR colonization and de novo resistance evolution. Increased MDR may be linked to increased microbial dysbiosis, indicating complex interactions in addition to selective pressures of administered antibiotics

BACKGROUND

Over the past 20 years, it has become extensively understood that increased antibiotic exposure is a major facilitator of AMR development in patients.[12,16,61] Specifically, patients receiving prophylactic antibiotics—commonly prescribed to those with compromised immune systems—constitute a substantial portion of clinical antibiotic exposure.[71,73] These patient populations have been shown to carry higher abundances of ARGs and have greater risk of AMR nosocomial pathogen colonization/carriage.[69,77,87] More pronounced increases in AMR have been linked with oral prophylactic therapy users.[63,67] Thus, IV antibiotic treatments have become standard due to their characteristically low penetrance into the gastrointestinal system, reducing the antibiotic selective pressure on patient microbiomes.[67] However, despite this low penetrance into the gut, long-term prophylactic IV exposure still has an effect on AMR selection within the gut.[20,67]

Popular clinical IV therapies, such as PIP/tazobactam and carbapenems, have been shown to have significant impacts on the microbiome.[75,76] Studies have identified significant biliary excretion of TZP as well as low concentrations in stool of patients receiving these therapies intravenously; these pharmacokinetic observations corresponded with decreased *Enterobacteriaceae, Bifidobacterium, Eubacteria, Lactobacillus* after only four to eight days of IV therapy.[75,76] Carbapenem IV therapies also appear to have significant biliary excretion generally resulting in decreased abundance of *Enterobacteriaceae.*[76] In addition to effects on relative microbial abundances, IV prophylactic therapies have been shown to increase abundance antibiotic resistant genes (ARGs) within the gut of mice.[67] These results were echoed by clinical observations of increased abundance of resistant *Enterobacteriaceae* following IV antibiotic prophylaxis.[76,77]

Although there remains an undeniable contribution of IV antibiotic prophylaxis to AMR development in the gut, the mechanism of this contribution requires further definition. To date, it has been shown that prophylactic therapies, and consequential sub-MIC concentrations within the gut during administration, may contribute to increased resistance within the gut by promoting resistance evolution of commensal organisms[20,21], selecting for pre-existing resistant mutants[22], and/or depleting beneficial micro-organisms that normally repel colonization of MDR bacteria[69,88,89]. However, the relative contributions of these AMR-related phenomena to increased AMR status within the gut remain to be further elucidated. Little is known about the dominant factors that determine both AMR and MDR status within gram-negatives of the gut, during IV antibiotic prophylaxis. As a result, this chapter aims to **1)** address the potential of prophylactic antibiotic prophylaxis in promoting increased MDR gram-negative status within the gut, **2)** identify causes of increased MDR gram-negatives (i.e.: colonization or evolution), and **3)** characterize resistance determinants in evolved MDR gram-negatives.

METHODOLOGY

Patient Eligibility

Although patient eligibility was assessed for the analysis in chapter 1, eligibility was reassessed with additional criteria catering to this chapter's aim (Figure 3). Patients were assessed for the length of sampling time. Patients that withdrew less than 14 days after initiation were excluded as this study aim requires a long-term picture of prophylactic antibiotic exposure.

Whole genome sequencing and Mutation Analysis

Fresh MH broth was inoculated with isolates of interest and grown overnight at 37°C. Genomic DNA was isolated from overnight cultures using the Wizard genomic DNA purification kit

(PROMEGA). Final product concentrations were determined using a Qubit 4 fluorometer (Invitrogen). DNA sequencing libraries were generated using a modified NEB-Next UltraII FS DNA library prep protocol and sequenced on an Illumina HiSeq platform with 2 x 250 paired end reads. Genome assembly was carried out using *unicycler*.[90] Library prep and genome assembly were carried out by Dr. Hooman Derakhshani.

Assemblies were then analyzed using the comprehensive antibiotic resistance database (CARD) resistance gene identifier (RGI) to predict resistance genes within each assembly and assess gained resistance genes.[91] Only strict and perfect matches within the RGI output were considered. Additionally, genome assemblies were annotated using PROKKA software[92] and clonal strain genomes, isolated from different time points, were compared using BreSeq software.[93] Mutations of interest were identified and tabulated.

16S rRNA sequencing

Genomic DNA was extracted as described by Stearns et al.[94] Briefly, 300µL of universal transport medium from each rectal swab was transferred to a screw cap tube containing 2.8 mm ceramic beads, 0.1mm glass beads, GES solution and sodium phosphate buffer (178 mM, pH = 8). Samples were mechanically lysed in a homogenizer for 3 minutes at 3000 rpm for 2 cycles. Samples were then incubated at 37C° for 1 hour after the addition of 50µL lysozyme (100 mg/mL) and 10µL RNase A (10 mg/mL in H_2O), followed by a second incubation at 65C° for 1 hr after the addition of 25µL SDS (25% w/v, diluted in ddH2O, filter sterilized), 62.5µL NaCl (5M, sterilized) and 25µL Proteinase K (20 mg/mL). Samples then underwent centrifugation at 13000 rpm for 5 min. The supernatant was mixed with 200uL of DNA binding buffer as part of the Zymo DNA Clean and Concentrator-25 kit. The DNA was purified as per the kit instructions and finally eluted with 50uL of ultrapure water.

Purified DNA was used to amplify the v34 region of the 16S rRNA gene by PCR. 50 ng of DNA was used as template with 1U of Taq, 1x buffer, 1.5 mM $MgCl_2$, 0.4 mg/mL BSA, 0.2 mM dNTPs, and 5 pmoles each of 341F (CCTACGGGNGGCWGCAG) and 806R (GGACTACNVGGGTWTCTAAT) with added Illumina adaptors primers as described in Bartram et al.[95] The reaction was carried out at 94C° for 5 minutes, 5 cycles of 94C° for 30 seconds, 47C for 30 seconds and 72C° for 40 seconds, followed by 25 cycles of 94C° for 30 seconds, 50C for 30 seconds and 72C° for 40 seconds, with a final extension of 72C° for 10 minutes. Resulting PCR products were visualized on a 1.5% agarose gel. Positive amplicons were normalized using the SequalPrep normalization kit (ThermoFisher#A1051001) and sequenced on the Illumina MiSeq platform at the McMaster Genomics Facility.

Raw reads were filtered and trimmed of PCR primer sequences using Cut adapt with a minimum quality score of 30 and a minimum read length of 100bp.[96] Sequence variants were then resolved from the trimmed raw reads using DADA2.[97] DNA sequences were filtered and trimmed based on the quality of the reads for each Illumina run separately, error rates were learned, and sequence variants were determined by DADA2. Sequence variant tables were merged to combine all information from separate Illumina runs. Bimeras were removed and taxonomy was assigned using the SILVA database version 1.3.8.

Pulsed-field gel electrophoresis (PFGE)

PFGE was used to determine strain relatedness within and between patients, and 15 isolates from patient 18 were profiled. PFGE was performed according to the Standard Operating Procedure for PulseNet PFGE of *E. coli* O157:H7, *E. coli* non-O157, *Salmonella* serotypes, *Shigella sonnei* and *Shigella flexneri* (https://www.cdc.gov/pulsenet/pdf/ecolishigella-salmonella-pfge-protocol-508c.pdf). Isolates were streaked on BHI agar for overnight growth at 37C° and chromosomal

DNA was digested using XbaI. Digested fragments were separated by electrophoresis on a 1% agarose gel using the BioRad CHEF-DR III system. Running conditions were as follows: 0.5X TBE buffer, 14ºC, initial switch time 6.76 s, final switch time: 35.58 s, voltage: 6V, included angle: 120º, run time: 18 hours. Gel images were contrast adjusted and DNA patterns were analyzed in order to identify possible clonal strain populations.

Statistical analysis

Statistical analyses were performed using Rstudio and PRISM GraphPad software. Rstudio's 'lmer' package was utilized to generate a linear mixed model across 10 eligible patients and assess effects of treatment time on AMR. PRISM GraphPad software was used to compute all other statistical tests in this chapter.

RESULTS

Prophylactic antibiotic treatment increases MDR gram-negative carriage

In order to address the effects of prophylactic antibiotic exposure on multidrug resistance over time, the patient cohort needed to be further narrowed down to those that were consistently sampled during their long-term antibiotic exposure (>14 days). Thus, patient eligibility was revisited to exclude patients that had short term sampling (Figure 3). From the 21 patients contributing to the isolate collection and chapter 2 analysis, 11 patients were excluded due to short term sampling and lack of cultured samples to provide long-term resistance profiling. A final cohort of 10 patients was used to assess the impact of long-term prophylactic antibiotics on AMR and MDR in gram-negative bacteria (Figure 3).

To assess AMR changes overtime within gram-negatives isolated from longitudinal rectal swabs, we devised an AMR index. This index measures non-susceptibility to 10 antibiotic categories

represented by the panel of 10 tested antimicrobials relevant to clinical treatment of gram-negative bacteria. For example, an *E. coli* that was measured as non-susceptible (resistance or intermediate resistance) to both PIP and TET would be non-susceptible to 2 out of the 10 panelled antibiotics, providing an AMR index of 0.2 (i.e. 2 non-susceptible divided by 10 tested antibiotic categories). Across our sample of 10 patients receiving long-term prophylactic antibiotics (>14 days), we measured changes in AMR index from isolated gram-negative bacteria over time.

For each patient sample/timepoint the gram-negative colony that displayed the broadest spectrum of activity was assigned an AMR index to represent gram-negative AMR carriage. This approach was utilized in order to capture MDR gram-negatives and the most resistant gram-negative carried at each timepoint. When plotting AMR index of each patient over their treatment course (in days), a statistically significant upward linear trend was modelled (p-value < 0.0001), indicating that gram-negative AMR increases with greater length of prophylactic antibiotic treatment (Figure 8A). This linear trend is modelled by the following linear equation, AMRIndex = 0.01(Time) + 0.132. Thus, patients harbor gram-negative bacteria that generally show increased resistance to one additional antibiotic drug class with every ~10 days of prophylactic treatment. Furthermore, it is seen that MDR is associated with longer prophylactic treatment lengths, with this model displaying MDR gram-negative status after ~13 to ~23 days of antibiotic prophylaxis.

After concluding a significant positive relationship between MDR gram-negative carriage and treatment length, we looked to ascertain which antibiotic drug classes were being resisted. For this section of the analysis, patient sample AMR indexes were then grouped by week. The first week contains the average AMR index (avg. resistance spectrum) across the first samples of all

participants while subsequent weeks display the average of the largest AMR indexes (avg. of broadest resistance spectrums) taken from the patient's samples during that week. Over 4 weeks of prophylactic treatment, increased COL projected-resistance and resistances towards TET, and all beta-lactam antibiotics were observed (Figure 8A). From week one to week two, there was a noticeable increase in TET and PIP resistance. Weeks two to three, further increases in COL, TET, PIP, TZP, and IMP non-susceptibility. The noted increase in IMP non-susceptibility was observed in patient 16. Upon week four of treatment, patient samples displayed the broadest spectrum of resistance, with the greatest frequency of COL projected-resistance and resistance to other antibiotics, including TET, PIP, TZP, CAZ, CZ/CL, ATM, and IMP.

Despite an overall increasing trend in AMR Index, it was clear that some patients comprised larger increases in AMR carriage compared to others. It was suspected that different prophylactic antibiotic treatment regimens may explain these differences in AMR carriage between patients. Thus, patients were stratified by treatment type to observe resistance over time in these individual groups. Higher increases in AMR overtime appeared to be more prevalent in the group receiving three IV antibiotics, TZP, vancomycin, and meropenem, in sequential order during their treatment (Figure 8B). All five patients receiving this treatment regimen eventually carried MDR gram-negative bacteria during their sampling period. The remaining patients received either TZP IV along with double strength co-trimoxazole per oral (PO) or TZP IV alone (Figure 8B). Patients in these groups generally did not develop MDR during their study sampling period. In fact, as seen with patient 13, 19, & 20, some patients started with resistant organisms during their first sampling, which were lost over the course of their treatment (Figure 8B). Additionally, a unique case was noted in patient 17, carriage displayed an opposite trend where AMR carriage increased above the MDR threshold during their treatment. Despite this unique

case, prophylactic antibiotic regimen did appear to alter carriage with the presence of VAC + MER appearing to promote increased MDR.

To test the significance of IV VAC+MER in promoting AMR, patients were grouped by the presence or absence of IV VAC+MER (Figure 8C). Pairwise comparison of patient AMR Index within the TZP + VAC + MER IV group showed an average increase in AMR to four classes of antimicrobials from their first sample to our linear model predicted time window of MDR (adj. p-value = 0.008) (Figure 8C). Conversely, TZP with co-trimoxazole or alone did not appear to have a significant impact on AMR carriage (adj. p-value = 0.820). Furthermore, these trends were largely explained by fluctuations in susceptibility to beta-lactam antibiotics (Figure 8D). Increases and decreases in AMR Index were mainly characterized by broadening and narrowing of resistance spectra, respectively. Here it is shown that patients receiving TZP + VAC + MER IV antibiotics gain broader spectrum resistance across many beta-lactams by week 4 while other treatment types lose resistance. Lastly, we compared the median magnitudes of AMR change between these groups to identify points of significant AMR uptake or loss (Figure 8E). Patients receiving TZP + VAC + MER IV appeared to have greater median increases in AMR (max. ΔAMR Index = 0.5) than TZP alone or TZP + TMP/SMX (max. ΔAMR Index = 0) during their study involvement (p-value = 0.0119) (Figure 8E).

Given these interesting trends noted in a small patient sample, it was also recognized that shorter sampling windows could provide a bias against therapies, such as TZP + OTHER. However, when comparing the median sampling time of TZP + OTHER patients to the median time taken until the first major ΔAMR detection in TZP + VAC + MER IV patients, there was no significant difference (Figure 8F). Thus, variation seen in AMR over time cannot be significantly explained by differences in sampling time alone and can be attributed to therapy effects.

Current literature suggests that low level penetrance of antibiotics into the gut, as seen with prophylactic IV therapy, may cause these observed increases in AMR in two ways: 1) by providing sub-MIC antibiotics and promoting resistance evolution within the gut, 2) by impacting colonization resistance and promoting increased colonization of MDR organisms. Thus, we investigated the cause of increased gram-negative AMR within our study population by analyzing changes in resistance profiles and observed gram-negative species over time.

Evidence of MDR gram-negative colonization and carriage (Patients 5, 7, & 16)

Among patients displaying increases in AMR over time, patients 5, 7, and 16 appeared to have AMR increases attributed to gained MDR organisms (Figure 9&10). Patient 5 gained a MDR *Morganella morganii* species, resistant to 7 of the 10 screened ABX categories (COL, TET, PIP, TZP, CAZ, CZ/CL, and COL), after 23 days of prophylactic antibiotic therapy. Patient 7 gained MDR *Morganella morganii* and MDR *Citrobacter freundii*, both resistant to 7 of the 10 screened ABX categories (COL, TET, PIP, TZP, CAZ, CZ/CL, and COL), after 22 days of prophylactic therapy. Patient 16 gained a MDR *Stenotrophomonas maltophilia*, resistant to 6 of the 10 screened ABX categories (COL, PIP, TZP, COL, FEP, and IMP), after 16 days of prophylactic therapy. Furthermore, the resistance profile for *Stenotrophomonas maltophilia* appears to lose COL resistance after day 16 and transition between TET intermediate resistance and susceptibility (Figure 10).

Evidence of resistance evolution and MDR (Patients 5 & 21)

In both patients 5 and 21, TZP resistance appeared to develop in *E. coli* over each patients prophylactic therapy window (Figure 10). Both patients were observed to have stable *E. coli* populations that were not lost during long-term sampling (Figure 9). Furthermore, these *E. coli*

appeared to have similar resistance profiles, suggesting strain similarity (Figure 10). Patient 5 carried PIP and TET resistant *E. coli* on D3 of therapy while the same resistance pattern remained throughout sampling and was observed at D16 with additional resistance to TZP (Figure 10). Similarly, patient 21 carried PIP resistant *E. coli* isolated at D0 (not shown), which transitioned to carriage of *E. coli* that was TZP resistant and intermediately CAZ resistant (Figure 10). Since TZP resistance developed within the same species while the patients were receiving long-term TZP IV therapy, we investigated the possibility of TZP evolution within the same strain of *E. coli.*

Pulsed-field gel electrophoresis (PFGE) was used to identify differences in banding patterns between longitudinal *E. coli* isolates within each patient (Figure 11&12). PFGE showed similar digestion patterns between longitudinal isolates within each patient (Figure 11B & 12B). In patient 5, earlier *E. coli* isolates displayed identical banding patterns with only the addition of a band in later TZP-resistant isolates (Figure 11B). In patient 21, earlier *E. coli* isolates had identical banding patterns with later isolates showing the appearance of two bands in TZP-resistant *E. coli* (Figure 12B). Since later TZP-resistance *E. coli* isolates displayed similar digestion patterns with few variations (1 or 2 bands), isolates from each patient were reasoned as likely being clonal populations. Further analysis of suspected ancestral populations—using whole genome sequencing—provided additional evidence for strain similarity (>97% similar). For each patient's set of longitudinal *E. coli* isolates, BreSeq software[93] comparison between the first isolated *E. coli* species and subsequently sampled *E. coli* indicated no major genomic differences. As a result, the remaining analysis treats these *E. coli* isolates as clonal populations which evolve TZP resistance, with the first isolated *E. coli* within each patient labelled as the ancestral strain.

Next, we looked to identify the determinants of TZP-resistance in these independent cases of *E. coli* TZP evolution. Whole genomes assemblies of all isolates were analyzed using the comprehensive antibiotic resistance database (CARD) in order to identify inherited or lost resistance genes that may explain changes in susceptibility to TZP. After predicting resistance genes using CARD RGI, both TZP-susceptible and -resistant *E. coli* were shown to have similar predicted resistance genes related to beta-lactam resistance, such as *AmpC*, *AmpH*, and *TEM-1* (Table 3&4). Since there were no observed differences in presence of beta-lactam resistance genes between TZP-sensitive and -resistant *E. coli* clonal isolates, mutation analysis was conducted using BreSeq software[93] to further investigate the mechanisms of evolved TZP-resistance.

Different mutations appeared to evolve in TZP-resistant *E. coli* clonal populations from each patient. In patient 5 *E. coli* clonal isolates, mutations were found in various hypothetical proteins as well as EnvZ, an osmolarity regulator protein. An EnvZ E212V mutation was found in P3A7, an *E. coli* isolated after 23 days of IV TZP therapy, while an EnvZ A175E mutation was observed in P3D4, an *E. coli* isolated after IV TZP, VAC, and MER therapy (Figure 11). Both amino acid substitutions are characterized as non-polar to polar shifts or vice versa, resulting in significant impacts on structure of EnvZ.

In patient 21 *E. coli* clonal isolates, two deletions of Δ14,417bp and Δ5,374bp were noted in TZP-resistant isolates, P9G12 and P9H6 (Figure 12). These segments contained various genes common to plasmids, such as *traA, traJ_2, traM, traV, traY, flmC, vapB, parM*, and *stbB*. As a result, it is suspected that a plasmid may be lost and effect chromosomal expression of beta-lactam resistance genes. However, further bioinformatic analysis and *in vitro* methods will need to be utilized to investigate this possibility.

Stable and Lost resistance over prophylactic therapy (Patients 3, 13, 19, & 20)

Patients 3, 19, and 20, show stable resistance phenotypes that do not change extensively over the period of sampling. Interestingly, patients 19 and 20 had *Proteus mirabilis* isolated from their samples, but did not appear to develop resistance to the main prophylactic agent used, IV TZP (Figure 9&10). In patient 13, we observe an interesting phenomenon where the first gram-negatives isolated grew on CHROMID plates and were resistant to strong/last-line antibiotics, IMP and FEP. However, over treatment time, these microbes were lost, with only *Klebsiella variicola* being isolated after day 10 (Figure 9&10).

Patients with significant antibiotic-mediated microbial dysbiosis appear at greater risk for developing MDR carriage

When assessing the microbiome, it became clear that IV TZP/VAC/MER therapy—associated with increased MDR carriage—may promote a greater degree of microbial dysbiosis (Figure 13). First, microbial diversity appeared to decrease in patients receiving either TZP/TMP/SMX or TZP/VAC/MER therapies over time (Figure 13A). However, a greater decrease in diversity was observed in TZP/VAC/MER patients overtime compared to those receiving TZP/TMP/SMX. Second, expansion/overgrowth of *Enterococcaceae, Bacteroidaceae*, and *Enterobacteriaceae spp.* populations, are more commonly seen in patients receiving IV TZP/VAC/MER (patients 5, 6, 7, 16, & 21) (Figure 13B&C). Both *Bacteroidaceae*, and *Enterobacteriaceae spp.* expansions were commonly seen during TZP administration (typically the first administered antibiotic) while *Enterococcaceae spp.* expansion was observed during IV VAC and MER treatment (given later in therapy regimen). Additionally, these expansions typically occurred in tandem with the depletion of beneficial bacterial species (Figure 13B). In patients 5, -6, and 7, beneficial *Rumminococcus* group, *Faecalibacterium,* and *Lachnoclostridium* species are lost after 16, 14,

and 12 days on antibiotic therapy, respectively (Figure 13B). In patient 16, both *Pediococcus* and *Lactobacillus spp.* are lost over the patient's treatment duration. Furthermore, in patient 21, a prominent *Akkermansia spp.* population is lost prior to *Enterococcus* and *Escherichia/Shigella spp.* expansion.

Comparatively, patients receiving TZP alone or in combination with TMP/SMX appeared to maintain beneficial bacterial populations, despite some expansion of *Enterococcus spp.* in patients3 and 17 as well as *Escherichia/Shigella spp.* in patient 19 (Figure 13B). In patient 3, *Blautia, Lachnoclostridium,* and *Ruminococcus* group species remain despite expansion. Similarly in patient 17, *Lachnoclostridium* and *Ruminococcus gnavus* groups species remain stable during antibiotic therapy. In patient19, *Ruminococcus torques* group and *Lachnoclostridium* species remain stable during *Escherichia/Shigella* expansion. The remaining patients, patients 13 and 20, appeared to have more unique microbial compositions, with no noted overgrowth of *Enterococcaceae* or *Enterobacteriaceae spp.* Furthermore, these patient microbiomes appear to retain significant relative abundance of beneficial bacteria throughout their time on therapy.

DISCUSSION

Previous research has shown that extensive antibiotic usage is positively associated with higher rates of AMR carriage.[69] This chapter builds on this previous work to show that MDR gram-negative carriage increases with time on IV antibiotic prophylactic therapy (Figure 8). Furthermore, it was seen that broader spectrum IV therapy groups—such as patients receiving TZP/VAC/MER therapy—experienced greater significant increases in AMR, eventually conferring MDR carriage. Increases in MDR gram-negative carriage were shown to be mediated by a combination of MDR colonization as well as de novo evolution of resistance towards

administered IV antibiotics, such as TZP. Furthermore, increased MDR carriage was often characterized by broader spectrum resistance to beta-lactams, indicating the significant selective roles of IV TZP and MER on intestinal microbiota. Interestingly, these phenotypic resistance observations were coupled with more severe microbial dysbiosis in patients receiving TZP/VAC/MER treatments (Figure 13).

In all TZP/VAC/MER patients expansion of *Bacteroidaceae*, *Enterobacteriaceae*, or *Enterococcaceae* species were observed, which is a sign of destabilization of the healthy microbiome community under antibiotic pressure.[59] Furthermore, in these patients, beneficial species—implicated in colonization resistance through their contributions to a competitive GI environment—were lost, including *Faecalibacterium*, *Lachnoclostridium*, *Blautia*, *Ruminococcus* groups, *Akkermansia*, *Lactobacillus*, and *Pediococcus spp.*[87,102–109] *Faecalibacterium, Lachnoclostridium*, *Blautia*, *Ruminococcus* groups, and *Akkermansia spp.*, provide protection through their production of SCFAs, particularly butyrate.[87,102–105] *In vitro* experiments demonstrate that adequate concentrations of burtyate can directly impede growth of *E. coli* and *Salmonella sp.*[110] Additionally, butyrate promotes aerobic respiration in colonocytes, limiting oxygen and nitrate from *Enterobacteriaceae* populations looking to expand or colonize.[106] In addition to their roles as SCFA producers, *Ruminococcus* groups and *Akkermansia spp.* are prominent mucin degraders, freeing up nutrients for other beneficial species.[107] The bacterial species, *Lactobacillus* and *Pediococcus spp.*, contribute to acidification of the colon by production of lactic acid, which contributes to pH necessary for other colonisation resistance mechanisms.[108,109] Lastly, bacterial families, such as *Lachnospiraceae* and *Ruminococcaceae* are implicated in secondary bile acid metabolism, which can effectively reduce survival, colonization, and germination of MDR gram-negative pathogens.[87]

Over long-term antibiotic prophylaxis, loss of these beneficial microbial species can lead to a less competitive GI environment with greater ease of colonization and expansion by MDR gram-negative organisms.[104,111] However, this chapter's results indicates that the effects of a less competitive and more fertile GI environment may extend beyond increased MDR colonization; these conditions likely also provide greater survivability to compensate fitness costs in evolved AMR gram-negative mutants.[112,113] Evolution of clonal resistance was only apparent in two patients from the IV TZP/VAC/MER group (Figure 11&12). It was clear that these patients experienced a loss of beneficial butyrate producing bacteria, which could lead to decreased colonic respiration and increased oxygen/nitrate availability allowing TZP-resistant mutants to propagate freely.

Although observed infrequently, evolution of TZP resistance was observed in *E. coli.* Of great interest were the EnvZ mutants isolated from patient 5. EnvZ is a osmolarity sensor protein that regulates expression of OmpF and OmpC.[98,99] EnvZ consists of a periplasmic domain, two transmembrane domains, and a cytoplasmic histidine kinase. Previous studies have found several structure altering EnvZ mutations—within these domains—to cause constitutive expression of OmpC while repressing OmpF, impacting outer membrane permeability.[98,99] Harlocker et al. displayed an EnvZ mutant in the linker region connecting the second transmembrane domain to the cytoplasmic histidine kinase (E212K) which preferentially expressed OmpC over OmpF.[99] Tokishita et al. found that mutagenesis of EnvZ within the transmembrane domains also had a significant impact on outer membrane protein expression.[98] Furthermore, the EnvZ mutant (A175E) has been identified as contributing to carbapenem resistance in *E. coli*[100] echoing the importance of these mutants on membrane permeability and beta-lactam resistance.[101]

CONCLUSION

This chapter demonstrated that IV antibiotic prophylaxis length contributes to clinically relevant MDR gram-negative carriage. This contribution is highly dependent on the coverage and diversity of antibiotics taken during prophylaxis. Furthermore, it was shown that increased MDR was explained by both colonization and de novo evolution of resistance within GI resident gram-negative species. Different therapies appeared to perturb the microbiome to differing degrees, possibly explaining the difference between colonization and evolution rates.

FUTURE DIRECTIONS

Little investigation was done into the roles of anti-cancer drugs on AMR development within the gut. It was considered that some anti-cancer drugs may promote survival of certain AMR mutants. Some of the chemotherapy drugs in our sample (Table 5) have been tested on gram-negative bacteria and do not show significant bactericidal activity.[114] However, anti-neoplastic drugs can significantly impact the microbiome and drug metabolism.[115,116] In this way, certain chemotherapy drugs may have previously unexplored effects on microbial competition in the GI environment, possibly leading to higher propensities of colonization and opportunist expansion.

In this chapter, the significance of components of the GI environment is discussed. Future study should focus on the hypothesis that beneficial bacterial protective products impact AMR evolution under sun-MIC concentrations. By investigating SCFA & pH, secondary bile acids, and nutrient availability, one may be able to highlight a bottleneck phenomenon with implications on AMR evolutionary trajectories and fitness costs.

Chapter 4: CHARACTERIZING THE ROLE OF FLUCONAZOLE IN COLISTIN HETERO-RESISTANCE

ABSTRACT

Background: Hetero-resistance towards colistin has been increasing in prevalence, resulting in higher rates of gram-negative treatment failure and threatening the efficacy of this last-line therapy. Colistin hetero-resistance, like colistin resistance, can occur in response to outer membrane destabilization. Due to the membrane destabilizing effects of fluconazole, this antifungal was investigated for its impacts on promoting colistin hetero-resistance.

Objectives: Explore the impact of fluconazole on colistin hetero-resistance in *Enterobacter cloacae* complex (ECC).

Methodology: A set of colistin hetero-resistant *Enterobacter sp.* isolates were used from the AMR collection. Broth microdilutions were used for MIC determination and checkboard assays. Serial evolution experiments were done using high dose fluconazole and/or low dose colistin.

Results: Colistin hetero-resistance appeared to build during fluconazole administration in patient 6 samples from the AMR collection. Disk diffusion and broth microdilution assays were used to identify the presence of a colistin resistant sub-population, indicating hetero-resistance. This hetero-resistance appeared to increase in size of sub-population and MIC while the patient received fluconazole. Checkerboard assays using clindamycin, vancomycin, rifampicin, and PIP paired with fluconazole displayed no significant effect of acute fluconazole exposure on membrane permeability. Furthermore, serial evolution experiments indicated no significant impact of chronic fluconazole exposure on colistin hetero-resistance.

Conclusion: Although fluconazole does appear to have *in vitro* membrane destabilizing capabilities, this does not appear to affect the gram-negative outer membrane or promote colistin hetero-resistance.

BACKGROUND

With increasing rates of MDR, XDR, and PDR, first- and second-line therapy options are becoming obsolete. As a result, colistin is becoming increasingly relied upon as a clinical antimicrobial therapy.[4,49,51] Resistance to this antimicrobial peptide has been growing in frequency over the past decades.[37] In particular, *Enterobacter sp.* are of particular concern as these opportunistic pathogens been identified as colistin resistant in several clinical studies.[117,118] Furthermore, *Enterobacter spp.* are associated with rapid colistin resistance development, known as hetero-resistance.[118]

Colistin hetero-resistance is a novel phenotype associated with high rates of last-line antibiotic treatment failure.[118,119] Unique from traditional resistance, hetero-resistance is an unstable phenotype, which can be strengthened or lost rapidly within a clonal population.[120,121] Alike other colistin resistance mechanisms, they respond to outer membrane destabilization.[122] It has been shown that fluconazole, a very popular antifungal, can destabilize lipid membranes.[123] Due to the frequency of fluconazole use in prophylactic therapies, this chapter investigates the ability of fluconazole to promote colistin hetero-resistance in *Enterobacter spp.* isolated from a chemotherapy patient.

METHODOLOGY

Checkerboard minimum inhibitory concentration (MIC) broth dilution assay

Stock concentrations were made using clindamycin, vancomycin, rifampicin, and PIP powders solubilized in milliQ water and fluconazole solubilized in DMSO. All stocks were filter sterilized. Liquid MH broth was inoculated with *Enterobacter spp.* and incubated overnight at 37°C. Overnight cultures were diluted 1/1000 in MH broth and grown to an OD_{600} of 0.1. Liquid

cultures (OD_{600}=0.1) were further diluted 1/200 in MH broth and pipetted in 100µL volumes within a 96-well plate. Stock concentrations were diluted 1/100 in each well to final serial dilution concentrations of 0-16µg/mL (rifampicin and PIP), 0-128µg/mL (clindamycin), or 0-512µg/mL (vancomycin). Additionally, down the columns of the plate, fluconazole was serially diluted to concentrations of 0-512µg/mL. Plates were incubated overnight at 37°C. Well optical densities were measured at 600nm using a SpectraMax M3 plate reader (Molecular Devices).

Isolation of sensitive hetero-resistant strain (P3A3-P9)

MH broth was inoculated with an early *Enterobacter* isolate (P3A3) and was grown overnight at 37°C. Overnight cultures were standardized to OD_{600}=1 and serially diluted to OD_{600}=1x 10^{-7}. Dilute culture was spread on MH agar and grown overnight at 37°C. Using a RapidPick MP: 20 Pin Colony Picker (Hudson Robotics), 344 isolates were picked and grown in MH broth overnight at 37°C. Overnight cultures were stamped into MH broth supplemented with 2µg/mL colistin and grown for 2 days at room temperature. Wells that showed no growth were determined as sensitive. A sensitive strain was picked and labelled as P3A3-P9.

Spot dilution assay (Hetero-resistance Test)

Overnight liquid cultures were serially diluted to 1:10^8. Serial dilutions of 10^{-3}-10^{-8} were plated on MH agar plates and dilutions 10^{-1}-10^{-6} were plated on 4µg/mL & 8µg/mL colistin MH agar plates. Plates were grown at room temperature for 48 hrs and CFUs counted. CFU/mL were calculated and used determine the size of hetero-resistance subpopulations present at different breakpoints.

Fluconazole evolution experiment (P3A3-P9)

MH broth was inoculated with a sensitive colistin-hetero-resistant *Enterobacter* isolate (P3A3-P9) and was grown overnight at 37°C. Overnight culture was standardized to OD_{600}=0.1 and diluted 1:50 in four different experimental conditions: control (MH broth + 0.4% DMSO), colistin condition (MH broth + 0.4% DMSO + 0.125µg/mL), fluconazole condition (MH broth + 0.4% DMSO + 128µg/mL), and colistin & fluconazole condition (MH broth + 0.4% DMSO + 0.125µg/mL + 128µg/mL). Each condition was cultured in 25mL triplicates. Inoculated test media were grown overnight at 37°C with shaking (140 rpm) and diluted 1:1000 every 23-25hrs. Isolates were passaged for 16 days with hetero-resistance measured every 3-4 days by spot dilution assay (4µg/mL & 8µg/mL colistin MH agar plates).

Whole genome sequencing and Mutation Analysis

Fresh MH broth was inoculated with isolates of interest and grown overnight at 37°C. Genomic DNA was isolated from overnight cultures using the Wizard genomic DNA purification kit (PROMEGA). Final product concentrations were determined using a Qubit 4 fluorometer (Invitrogen). DNA sequencing libraries were generated using a modified NEB-Next UltraII FS DNA library prep protocol and sequenced on an Illumina HiSeq platform with 2 x 250 paired end reads. Library prep and genome assembly were carried out by Dr. Hooman Derakhshani.

Assemblies were then analyzed using the comprehensive antibiotic resistance database (CARD) resistance gene identifier (RGI) to predict resistance genes within each assembly and assess gained resistance genes.[91] Only strict and perfect matches within the RGI output were considered. Additionally, genome assemblies were annotated using PROKKA software[92] and

clonal strain genomes, isolated from different time points, were compared using BreSeq software.[93] Mutations of interest were identified and tabulated.

RESULTS

During analysis of resistant gram-negatives overtime, patient 6 appeared to have several isolates identified as *Enterobacter cloacae* which displayed increased colistin hetero-resistance overtime (Figure 15A). In order to determine if these species were the same strain or more resistant isolates inherited overtime, isolates were sent for whole genome sequencing and identified as the same strain of *Enterobacter bugandensis*, another subspecies of the *Enterbacter cloacae complex* (ECC). As a result, it was determined that these strains were evolving resistance over sampling period, sample 4, 7, and 8 (Figure 15). Furthermore, this evolved resistance appeared to coincide with consistent administration of fluconazole (Figure 14).

Since fluconazole was received consistently during this period of increased hetero-resistance, it was thoroughly investigated as a possible agent to promote colistin hetero-resistance (Figure 16 & 17). However, high doses of fluconazole (<512µg/mL) did not appear to effect susceptibility to PIP, clindamycin, vancomycin, or rifampicin in *Enterobacteriaceae spp.*, indicating weak impact of high dose acute exposure on permeability (Figure 16). Still curious about the effects of chronic fluconazole exposure on outer membrane modification, an evolution experiment was conducted with a sensitive *Enterobacter bugandensis* colony (P3A3-P9); this evolution experiment was performed with multiple conditions in serial fashion (Figure 17A). However, this evolution with fluconazole (128µg/mL) showed minimal effect in increasing hetero-resistance to colistin (Figure 17B&C). This suggests that maintenance and promotion of hetero-resistance in the GI tract is likely independent of fluconazole usage. Interestingly, low concentrations of

colistin (0.125µg/mL) alone were shown to increase hetero-resistant CFU proportions approx. 500-fold over 13 days.

DISCUSSION

Despite interest in fluconazole as a possible promoter of colistin hetero-resistance, here we prove that fluconazole has little effect on the development of colistin hetero-resistance in the *Enterobacter spp., Enterobacter bugandensis*. Instead, this phenotype may be heavily influenced by the immune environment created by chemotherapy and subsequent antibiotic/antifungal prophylaxis. Following chemotherapy, immune processes are suppressed and take some time to recover following their administration.[115] Furthermore, this period of recovery is defined by prophylactic antibiotics and antifungals, often leading to overgrowth of resistant and harmful organisms in the colon.[59] During rebound, immune pathways begin to function and synthesize antimicrobial peptides with the purpose of controlling outgrown populations of either gram-positive or negative organisms.[124] Previous literature has found that colistin hetero-resistance in *Enterobacter spp.* can be promoted by host mucosal immune functions, such as CRAMP.[118] Studies in other gram-negative organisms suggest that cross-resistance occurs strongly between some human antimicrobial peptides, such as LL-37 and PR-39, and colistin.[125] Since colistin hetero-resistance began to develop some time after administration of chemotherapy in patient 6 that then became worse with increased antibiotic therapy length and microbial dysbiosis (Figure 13).

CONCLUSION

Colistin hetero-resistance is a significant clinical resistance phenotype in *Enterobacter spp.*, resulting in MDR treatment failure. A patient from the AMR collection (Chapter 2), Patient 6,

presented with multiple isolates of *Enterobacter bugandensis* which appeared to evolve prominent colistin hetero-resistance overtime. The patient was receiving several chemotherapeutic agents in their treatment regimen, however, fluconazole's ability to destabilize bilayers *in vitro* made it a candidate in facilitating colistin hetero-resistance. Investigations into acute and chronic exposure to fluconazole indicated an insignificant impact on hetero-resistance development. From these results, it is suspected that the *Enterobacter spp.* colistin hetero-resistance stems from other therapeutic agents or host immune processes.

SUMMARY

AMR continues to grow and threaten efficacy of antibiotic interventions. Specifically, MDR gram-negative infections increase likelihood of treatment failure. However, very little is known about how these MDR bacteria develop and emerge within patients receiving antibiotics. As a result, a collection of 926 gram-negative isolates were cultured from rectal swabs of an AML patient population receiving IV and oral prophylactic antibiotics.

In this study, resistance and taxonomy of gram-negatives carried in these patients over time was surveyed. The majority of the collection was identified as *E. coli* (50.8%), *K. pneumoniae* (18.0%), and *K. variicola* (10.5%). Furthermore, the most common resistance was to piperacillin (55.2%) and tetracycline (23.9%). Upon surveying the entire database, MDR phenotypes were identified in gram-negatives of interest, including *C. braakii, C. freundii, E. coli, K. pneumoniae, M. morganii, R. ornythinolytica,* and *S. maltophilia*. Some MDR *Citrobacter spp., K. pneumoniae, M. morganii, P. mirabilis* and *R. ornythinolytica* isolates were found to be extended spectrum beta-lactam resistant. Extended spectrum beta-lactam resistance was observed to co-occur with colistin projected-resistance in some *C. braakii* and *R. ornythinolytica* isolates. Cefepime resistance was observed in several *E. coli* and *S. maltophilia* isolates. Our surveillance efforts indicate that among rectal isolated MDR gram-negatives, carbapenem and fluoroquinolone resistance is uncommon.

Since cultured swabs were obtained longitudinally, gram-negative AMR and MDR was assessed overtime on prophylactic therapy. Across the patient sample ($n = 10$), AMR increased overtime on prophylactic IV and/or oral therapy (p-value < 0.0001). On average, patients gained resistance to 1 category of antibiotic every 10 days receiving prophylactic therapy. Furthermore, patients conferred positive MDR gram-negative status after 13-23 days receiving prophylactic therapy.

Patients receiving TZP + VAC + MER IV therapy appeared to explain the majority of AMR increase observed within the patient sample (adj. p-value = 0.008).

When comparing the microbiomes of patients from different therapy groups, gram-negative AMR was associated with a higher degree of microbial dysbiosis. Microbiome alpha-diversity decreased over time on prophylactic antibiotics, with patients from the TZP + VAC + MER IV group displaying a sharper decrease over time. Decreases in microbial diversity occurred in combination with overgrowth of *Enterobacteriaceae* and/or *Enterococcaceae* as well as decreases in relative abundance of beneficial bacteria, including *Faecalibacterium*, *Lachnoclostridium*, *Blautia*, *Ruminococcus* group, *Akkermansia*, *Lactobacillus*, and *Pediococcus spp.* Thus, there exists a link between the composition of the microbiome and susceptibility to increased AMR.

Next, increases in AMR was investigated further. Colonization and/or growth of resident MDR *M. morganii, C. braakii, S. maltophilia,* and *K. pneumoniae* explained resistance increases in patients 5, 6, 7, and 16. However, *de novo* evolution of resident *E. coli* explained AMR increase and MDR carriage in patients, 5 and 21. PFGE was used to confirm strain similarity and clonal evolution of TZP-resistance in patient 5 and 21 *E. coli* lineages. Using BreSeq, comparative genomic analysis of TZP-resistance evolution lineages indicated two separate evolutionary pathways. The *E. coli* lineage from patient 5 showed the evolution of two independent EnvZ mutations, A175E and E212V, indicating a possible reduction in outer membrane permeability. The *E. coli* lineage from patient 21 showed the loss of several contigs containing mobile genetic element genes, including *traA, traJ_2, traM, traV, traY, flmC, vapB, parM*, and *stbB*, indicating the loss of plasmid or integrated genetic. Since CARD RGI analysis of all genome assemblies

indicated no gained beta-lactam resistance genes, both of these *E. coli* lineages are thought to impact expression of harboured beta-lactam resistance genes.

While completing the study, an interesting colistin hetero-resistance phenotype was noticed in isolated *E. bugandensis* from patient 6. This phenotype was noted to increase while the patient was receiving fluconazole prophylactically. Since previous work has identified fluconazole as a membrane destabilizing agent, it was investigated as a possible promoter of colistin hetero-resistance. However, both acute and chronic exposure to fluconazole appeared to have no impact on colistin hetero-resistance development.

CONTRIBUTIONS TO THE FIELD

This study serves as the first extensive longitudinal characterization of the microbiome of AML patients receiving prophylactic antibiotics. Furthermore, this study indicates that compositional microbiome data is linked with rectal MDR gram-negative carriage, contributing to a new view of the relationship between gastrointestinal interbacterial composition and AMR development. Additionally, this study provides two examples of unique *de novo* TZP-resistance evolutionary pathways that occur in two lineages of *E. coli*. To our knowledge, this serves as the first time that rectal gram-negative commensals evolve TZP-resistance *de novo* in patients receiving TZP IV therapy.

APPENDIX:

Table 1: *Taxonomy of AMR Study Isolate Collection Isolates.*

Species	Count (n, %)
Escherichia coli	470 (50.8)
Klebsiella pneumoniae	167 (18.0)
Klebsiella variicola	97 (10.5)
Stenotrophomonas maltophilia	47 (5.1)
Proteus mirabilis	28 (3.0)
Enterobacter cloacae	25 (2.7)
Citrobacter braakii	24 (2.6)
Citrobacter freundii	22 (2.4)
Pseudomonas citronellolis	17 (1.8)
Hafnia alvei	7 (0.8)
Morganella morganii	7 (0.8)
Klebsiella aerogenes	5 (0.5)
Pseudomonas aeruginosa	3 (0.3)
Salmonella sp	2 (0.2)
Raoultella ornithinolytica	2 (0.2)
Enterobacter kobei	1 (0.1)
Enterobacter asburiae	1 (0.1)
Klebsiella oxytoca	1 (0.1)
Total	926

Rows display the number of each species identified by MALDI-ToF MS within the AMR Study Isolate Collection.

Table 2: *Species specific AMR phenotypes across the collection (n = 926).*

Organism	Resistance phenotype (n , %)	Antibiotics									
		LVX	COL	TET	PIP	TZP	CAZ	CZ/CL	ATM	FEP	IMP
Citrobacter braakii	Susceptibile	24 (100)	21 (87.5)	11 (45.8)	0	0	0	0	0	24 (100)	24 (100)
	Intermediate Resistance	0	0	0	0	4 (16.7)	0	0	1 (4.2)	0	0
	Resistance	0	3 (12.5)	13 (54.2)	24 (100)	19 (79.2)	24 (100)	24 (100)	23 (95.8)	0	0
Citrobacter freundii	Susceptibile	22 (100)	22 (100)	22 (100)	10 (45.5)	21 (95.5)	7 (31.8)	8 (36.4)	18 (81.8)	22 (100)	22 (100)
	Intermediate Resistance	0	0	0	8 (36.4)	0	6 (27.3)	2 (9.1)	3 (13.6)	0	0
	Resistance	0	0	0	4 (18.2)	1 (4.5)	9 (40.9)	12 (54.5)	1 (4.5)	0	0
Enterobacter asburiae	Susceptibile	1 (100)	1 (100)	1 (100)	0	0	1 (100)	1 (100)	1 (100)	1 (100)	1 (100)
	Intermediate Resistance	0	0	0	0	1 (100)	0	0	0	0	0
	Resistance	0	0	0	1 (100)	0	0	0	0	0	0
Enterobacter cloacae	Susceptibile	25 (100)	13 (52.0)	20 (80.0)	12 (48.0)	19 (76.0)	25 (100)	25 (100)	25 (100)	25 (100)	25 (100)
	Intermediate Resistance	0	0	1 (4.0)	5 (20.0)	5 (20.0)	0	0	0	0	0
	Resistance	0	12 (48.0)	4 (16.0)	8 (32.0)	1 (4.0)	0	0	0	0	0
Enterobacter kobei	Susceptibile	1 (100)	1 (100)	1 (100)	1 (100)	1 (100)	1 (100)	1 (100)	1 (100)	1 (100)	1 (100)
	Intermediate Resistance	0	0	0	0	0	0	0	0	0	0
	Resistance	0	0	0	0	0	0	0	0	0	0
Escherichia coli	Susceptibile	460 (97.9)	416 (88.5)	321 (68.3)	166 (35.3)	420 (89.4)	451 (96.0)	464 (98.7)	415 (88.3)	445 (94.7)	470 (100)
	Intermediate Resistance	0	0	3 (0.6)	54 (11.5)	32 (6.8)	19 (4.0)	6 (1.3)	42 (8.9)	9 (1.9)	0
	Resistance	10 (2.1)	54 (11.5)	146 (31.1)	250 (53.2)	18 (3.8)	0	0	13 (2.8)	16 (3.4)	0
Hafnia alvei	Susceptibile	7 (100)	7 (100)	7 (100)	7 (100)	7 (100)	7 (100)	7 (100)	7 (100)	7 (100)	7 (100)
	Intermediate Resistance	0	0	0	0	0	0	0	0	0	0
	Resistance	0	0	0	0	0	0	0	0	0	0
Klebsiella aerogenes	Susceptibile	5 (100)	5 (100)	5 (100)	3 (60.0)	5 (100)	5 (100)	5 (100)	5 (100)	5 (100)	5 (100)
	Intermediate Resistance	0	0	0	1 (20.0)	0	0	0	0	0	0
	Resistance	0	0	0	1 (20.0)	0	0	0	0	0	0
Klebsiella oxytoca	Susceptibile	1 (100)	1 (100)	1 (100)	1 (100)	1 (100)	1 (100)	1 (100)	1 (100)	1 (100)	1 (100)
	Intermediate Resistance	0	0	0	0	0	0	0	0	0	0
	Resistance	0	0	0	0	0	0	0	0	0	0
Klebsiella pneumoniae	Susceptibile	167 (100)	155 (92.8)	152 (91.0)	88 (52.7)	135 (80.8)	163 (97.6)	165 (98.8)	162 (97.0)	167 (100)	167 (100)
	Intermediate Resistance	0	0	1 (0.6)	47 (28.1)	17 (10.2)	1 (0.6)	0	0	0	0
	Resistance	0	12 (7.2)	14 (8.4)	32 (19.2)	15 (9.0)	3 (1.8)	2 (1.2)	5 (3.0)	0	0
Klebsiella variicola	Susceptibile	97 (100)	97 (100)	97 (100)	90 (92.8)	94 (96.9)	97 (100)	97 (100)	97 (100)	97 (100)	97 (100)
	Intermediate Resistance	0	0	0	2 (2.1)	3 (3.1)	0	0	0	0	0
	Resistance	0	0	0	5 (5.2)	0	0	0	0	0	0
Morganella morganii	Susceptibile	7 (100)	0	0	0	0	0	0	0	7 (100)	7 (100)
	Intermediate Resistance	0	0	0	0	2 (28.6)	0	0	3 (42.9)	0	0
	Resistance	0	7 (100)	7 (100)	7 (100)	5 (71.4)	7 (100)	7 (100)	4 (57.1)	0	0
Proteus mirabilis	Susceptibile	28 (100)	6 (21.4)	1 (3.6)	23 (82.1)	28 (100)	27 (96.4)	28 (100)	28 (100)	28 (100)	28 (100)
	Intermediate Resistance	0	0	1 (3.6)	0	0	0	0	0	0	0
	Resistance	0	22 (78.6)	26 (92.9)	5 (17.9)	0	1 (3.6)	0	0	0	0
Pseudomonas aeruginosa	Susceptibile	3 (100)	3 (100)	2 (66.7)	3 (100)	3 (100)	3 (100)	3 (100)	2 (66.7)	3 (100)	3 (100)
	Intermediate Resistance	0	0	1 (33.3)	0	0	0	0	1 (33.3)	0	0
	Resistance	0	0	0	0	0	0	0	0	0	0
Pseudomonas citronellolis	Susceptibile	17 (100)	17 (100)	17 (100)	10 (58.8)	17 (100)	15 (88.2)	17 (100)	1 (5.9)	17 (100)	17 (100)
	Intermediate Resistance	0	0	0	7 (41.2)	0	2 (11.8)	0	0	0	0
	Resistance	0	0	0	0	0	0	0	16 (94.1)	0	0
Raoultella ornithinolytica	Susceptibile	2 (100)	1 (50.0)	1 (50.0)	0	1 (50.0)	1 (50.0)	1 (50.0)	2 (100)	2 (100)	2 (100)
	Intermediate Resistance	0	0	0	1 (50.0)	1 (50.0)	0	0	0	0	0
	Resistance	0	1 (50.0)	1 (50.0)	1 (50.0)	0	1 (50.0)	1 (50.0)	0	0	0
Salmonella sp.	Susceptibile	2 (100)	2 (100)	1 (50.0)	1 (50.0)	2 (100)	2 (100)	2 (100)	2 (100)	2 (100)	2 (100)
	Intermediate Resistance	0	0	0	0	0	0	0	0	0	0
	Resistance	0	0	1 (50.0)	1 (50.0)	0	0	0	0	0	0
Stenotrophomonas maltophilia	Susceptibile	47 (100)	46 (97.9)	45 (95.7)	0	23 (48.9)	47 (100)	47 (100)	0	0	0
	Intermediate Resistance	0	0	2 (4.3)	0	9 (19.1)	0	0	0	5 (10.6)	0
	Resistance	0	1 (2.1)	0	47 (100)	15 (31.9)	0	0	47 (100)	42 (89.4)	47 (100)

Each column represents a tested antibiotic [levofloxacin (LVX, 5µg), colistin (COL, 10µg), tetracycline (TET, 30µg), piperacillin (PIP, 100µg), piperacillin-tazobactam (TZP, 100µg:10µg), ceftazidime (CAZ, 30µg), ceftazidime-clavulanic acid (CZ/CL, 30µg:10µg), aztreonam (ATM, 30µg), cefepime (FEP, 30µg), imipenem (IMP, 30µg)]. In accordance with CLSI guidelines, zone diameters were classified as resistance, intermediate resistance, or susceptible.

Table 3: *Resistance genes present in patient 5 TZP-susceptible and -resistant E. coli clonal isolates.*

	AMR Gene	TZP-susceptible P2B7	TZP-susceptible P2E3	TZP-resistant P2H3	TZP-resistant P3A7	TZP-resistant P3D4	Beta-lactam Resistance Coverage
Antibiotic Inactivation	*ampH**	+	+	+	+	+	cephalosporins, penicillins (penams)
	*ampC1**	+	+	+	+	+	cephalosporins, penicillins (penams)
	*ampC**	+	+	+	+	+	cephalosporins, penicillins (penams)
	TEM-1 $^{\Omega}$	+	+	+	+	+	monobactams, cephalosporins, penicillins (penams), carbapenems (penems)
Efflux pumps/Reduced Permeability	*KpnE* $^{\alpha}$	+	+	+	+	+	cephalosporins
	KpnF $^{\alpha}$	+	+	+	+	+	cephalosporins
	H-NS $^{\alpha\gamma}$	+	+	+	+	+	cephalosporins, penicillins (penams)
	evgA $^{\alpha\gamma}$	+	+	+	+	+	penicillins (penams)
	evgS $^{\alpha\gamma}$	+	+	+	+	+	penicillins (penams)
	CRP $^{\gamma}$	+	+	+	+	+	penicillins (penams)
	mdtE $^{\gamma}$	+	+	+	+	+	penicillins (penams)
	mdtF $^{\gamma}$	+	+	+	+	+	penicillins (penams)
	gadX $^{\gamma}$	+	+	+	+	+	penicillins (penams)
	acrB $^{\gamma}$	+	+	+	+	+	cephalosporins, penicillins (penams)
	acrA $^{\gamma}$	+	+	+	+	+	cephalosporins, penicillins (penams)
	AcrF $^{\gamma}$	+	+	+	+	+	cephalosporins, penicillins (penams)
	AcrE $^{\gamma}$	+	+	+	+	+	cephalosporins, penicillins (penams)
	AcrS $^{\gamma}$	+	+	+	+	+	cephalosporins, penicillins (penams)
	Mutant *acrR* $^{\gamma}$	+	+	+	+	+	cephalosporins, penicillins (penams)
	Mutant *marR* $^{\gamma}$	+	+	+	+	+	cephalosporins, penicillins (penams)
	marA $^{\gamma\varepsilon}$	+	+	+	+	+	monobactams, cephalosporins, penicillins (penams), carbapenems (penems)
	TolC $^{\delta\alpha\gamma}$	+	+	+	+	+	cephalosporins, penicillins (penams), carbapenems (penems)
	Mutant *soxR* $^{\delta\alpha\gamma}$	+	+	+	+	+	cephalosporins, penicillins (penams)
	Mutant *soxS* $^{\delta\alpha\gamma\varepsilon}$	+	+	+	+	+	monobactams, cephalosporins, penicillins (penams), carbapenems (penems)
Target Alteration	*Haemophilus influenzae* PBP3 $^{\lambda}$	+	+	+	+	+	cephalosporins, penicillins (penams)

* = ampC-type beta-lactamase
Ω = TEM beta-lactamase
α = Major facilitator superfamily (MFS) antibiotic efflux pump
γ = Resistance-nodulation-cell division (RND) antibiotic efflux pump
δ = ATP-binding cassette (ABC) antibiotic efflux pump
ε = General Bacterial Porin with reduced permeability to beta-lactams
λ = Penicillin-binding protein mutations conferring beta-lactam resistance

The "+" represents the presence of the resistance gene as either a strict or perfect hit by CARD.

Table 4: *Resistance genes present in patient 21 TZP-susceptible and -resistant E. coli clonal isolates.*

	AMR Gene	TZP-susceptible				TZP-intermediate resistant			TZP-resistant		Beta-lactam Resistance Coverage
		P9B7	P9C4	P9D2	P9F1	P9D7	P9F3	P9G4	P9G12	P9H6	
Antibiotic Inactivation	*ampH**	+	+	+	+	+	+	+	+	+	cephalosporins, penicillins (penams)
	*ampC1**	+	+	+	+	+	+	+	+	+	cephalosporins, penicillins (penams)
	*ampC**	+	+	+	+	+	+	+	+	+	cephalosporins, penicillins (penams)
	TEM-1 $^{\Omega}$	+	+	+	+	+	+	+	+	+	monobactams, cephalosporins, penicillins (penams), carbapenems (penems)
Efflux pumps/Reduced Permeability	*KpnE* $^{\alpha}$	+	+	+	+	+	+	+	+	+	cephalosporins
	KpnF $^{\alpha}$	+	+	+	+	+	+	+	+	+	cephalosporins
	H-NS $^{\alpha\gamma}$	+	+	+	+	+	+	+	+	+	cephalosporins, penicillins (penams)
	evgA $^{\alpha\gamma}$	+	+	+	+	+	+	+	+	+	penicillins (penams)
	evgS $^{\alpha\gamma}$	+	+	+	+	+	+	+	+	+	penicillins (penams)
	CRP $^{\gamma}$	+	+	+	+	+	+	+	+	+	penicillins (penams)
	mdtE $^{\gamma}$	+	+	+	+	+	+	+	+	+	penicillins (penams)
	mdtF $^{\gamma}$	+	+	+	+	+	+	+	+	+	penicillins (penams)
	gadX $^{\gamma}$	+	+	+	+	+	+	+	+	+	penicillins (penams)
	acrB $^{\gamma}$	+	+	+	+	+	+	+	+	+	cephalosporins, penicillins (penams)
	acrA $^{\gamma}$	+	+	+	+	+	+	+	+	+	cephalosporins, penicillins (penams)
	AcrF $^{\gamma}$	+	+	+	+	+	+	+	+	+	cephalosporins, penicillins (penams)
	AcrE $^{\gamma}$	+	+	+	+	+	+	+	+	+	cephalosporins, penicillins (penams)
	AcrS $^{\gamma}$	+	+	+	+	+	+	+	+	+	cephalosporins, penicillins (penams)
	Mutant *acrR* $^{\gamma}$	+	+	+	+	+	+	+	+	+	cephalosporins, penicillins (penams)
	Mutant *marR* $^{\gamma}$	+	+	+	+	+	+	+	+	+	cephalosporins, penicillins (penams)
	marA $^{\gamma\varepsilon}$	+	+	+	+	+	+	+	+	+	monobactams, cephalosporins, penicillins (penams), carbapenems (penems)
	TolC $^{\delta\alpha\gamma}$	+	+	+	+	+	+	+	+	+	cephalosporins, penicillins (penams), carbapenems (penems)
	Mutant *soxR* $^{\delta\alpha\gamma}$	+	+	+	+	+	+	+	+	+	cephalosporins, penicillins (penams)
	Mutant *soxS* $^{\delta\alpha\gamma\varepsilon}$	+	+	+	+	+	+	+	+	+	monobactams, cephalosporins, penicillins (penams), carbapenems (penems)
Target Alteration	*Haemophilus influenzae* PBP3 $^{\lambda}$	+	+	+	+	+	+	+	+	+	cephalosporins, penicillins (penams)

* = ampC-type beta-lactamase
Ω = TEM beta-lactamase
α = Major facilitator superfamily (MFS) antibiotic efflux pump
γ = Resistance-nodulation-cell division (RND) antibiotic efflux pump
δ = ATP-binding cassette (ABC) antibiotic efflux pump
ε = General Bacterial Porin with reduced permeability to beta-lactams
λ = Penicillin-binding protein mutations conferring beta-lactam resistance

The "+" represents the presence of the resistance gene as either a strict or perfect hit by CARD.

Table 5: *Descriptive statistics of antimicrobial therapy groups.*

		TZP & TZP + OTHER						TZP + VAC + MER IV					
		P-03	P-13	P-17	P-19	P-20	Average Use	P-05	P-06	P-07	P-16	P-21	Average Use
ABX	PiPeracillin/Tazobactam	1	1	1	1	1	1	1	1	1	1	1	1
	COTRIMOXAZOLE	1		1	1		0.6						0
	NYSTATIN SUSP-ENSION	1			1		0.4						0
	VANCOMYCIN						0	1	1	1	1	1	1
	CEFTAZIDIME						0			1			0.2
	MEROP-ENEM						0	1	1	1	1	1	1
	DAP-TOMYCIN						0	1					0.2
	TOTAL ABX	3	1	2	3	1	2	4	3	4	3	3	3.4
ANTI-CANCER DRUG	Busulfan	1		1	1		0.6						0
	FluDARAbine	1		1	1	1	0.8					1	0.2
	Methotrexate	1		1	1		0.6						0
	Cytarabine		1			1	0.4	1	1	1	1	1	1
	DAUNOrubicin		1			1	0.4	1	1	1	1	1	1
	hydroxyUREA					1	0.2		1				0.2
	IDArubicin						0					1	0.2
	TOTAL ABX	3	2	3	3	4	3	2	3	2	2	4	2.6

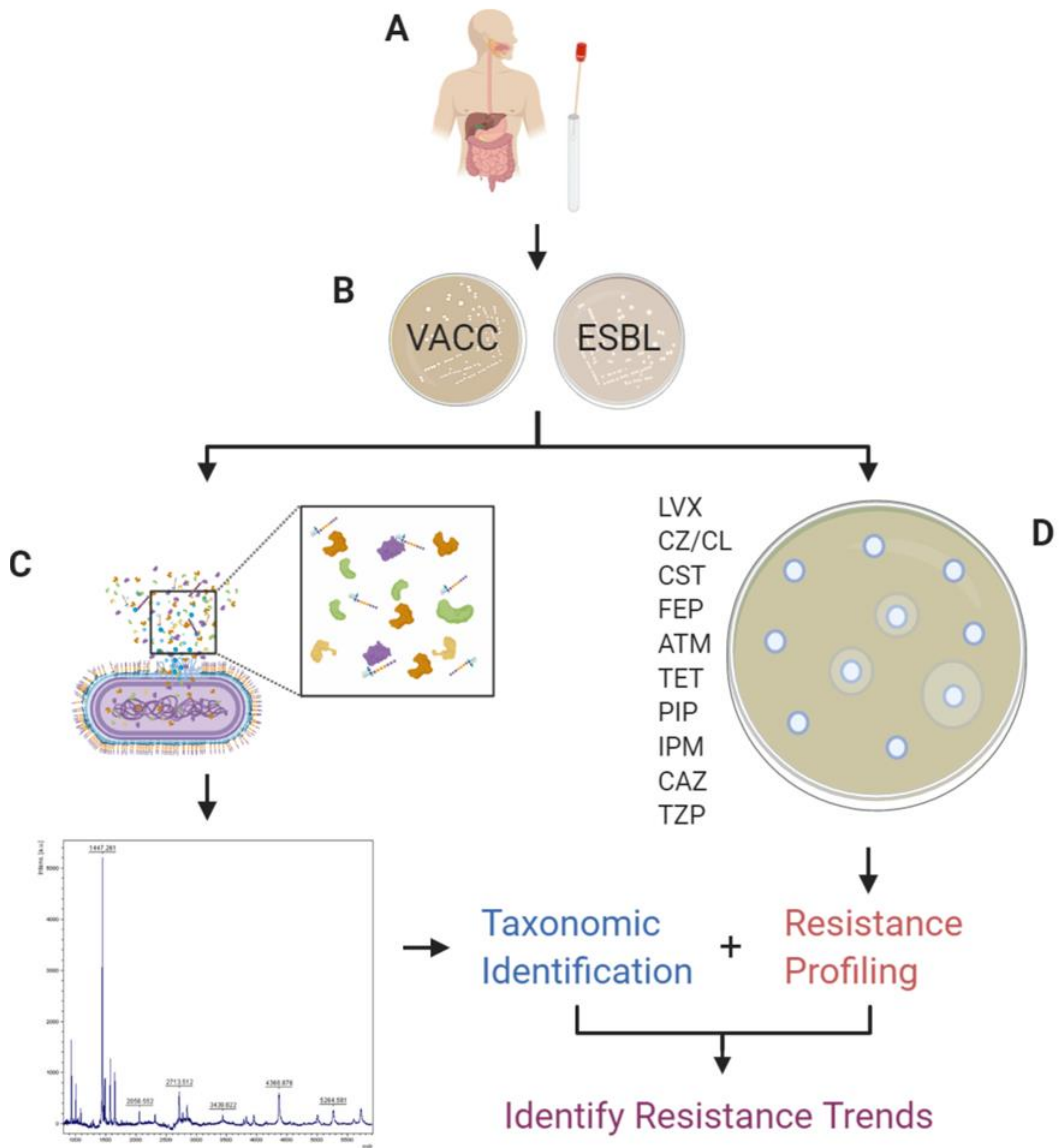


Figure 1: *Protocol for isolation and characterization of resistant bacteria from clinical samples.* **A)** Rectal swabs were taken from 25 chemotherapy patients over an 8-week period. **B)** Samples were streaked onto BHI, MAC, VACC, and ESBL plates. Colonies of interest, growing on VACC and ESBL plates, were picked and stored at -80°C. **A-B)** Indicates previous work done by the Surette lab. **C)** Clinical isolates were characterized by MALDI-ToF MS. **D)** Clinical isolates were tested for carbapenem, 3rd & 4th cephalosporins, fluoroquinolones, monobactams, and combination susceptibility. **C&D)** Taxonomic identification and resistance profiling were combined to identify resistance trends and highlight patients of interest for further investigation into resistance mechanisms.

	N		
Sample Size	25		
	Male [count (%)]	**Female [count (%)]**	
Sex	11 (44)	14 (56)	
	Mean	**Median**	**Q1, Q3 (IQR)**
Age	61.8	62	57.5, 69 (11.5)
	Count (%)		
Patients Discharged	14 (56)		
	Mean	**Median**	**Q1, Q3 (IQR)**
Time to Discharge (Days)	25.1429	24.5	20.75, 28.75 (8)
	Count (%)		
Patients Withdrawn	7 (28)		
	Mean	**Median**	**Q1, Q3 (IQR)**
Time to Withdraw (Days)	9	9	0, 12 (12)
	Count (%)		
Patients Completed Study	4 (16)		
	Mean	**Median**	**Q1, Q3 (IQR)**
Time to Completion (Days)	41.75	41	40.25, 44 (3.75)

Figure 2: *Descriptive Statistics of AML patient population*.

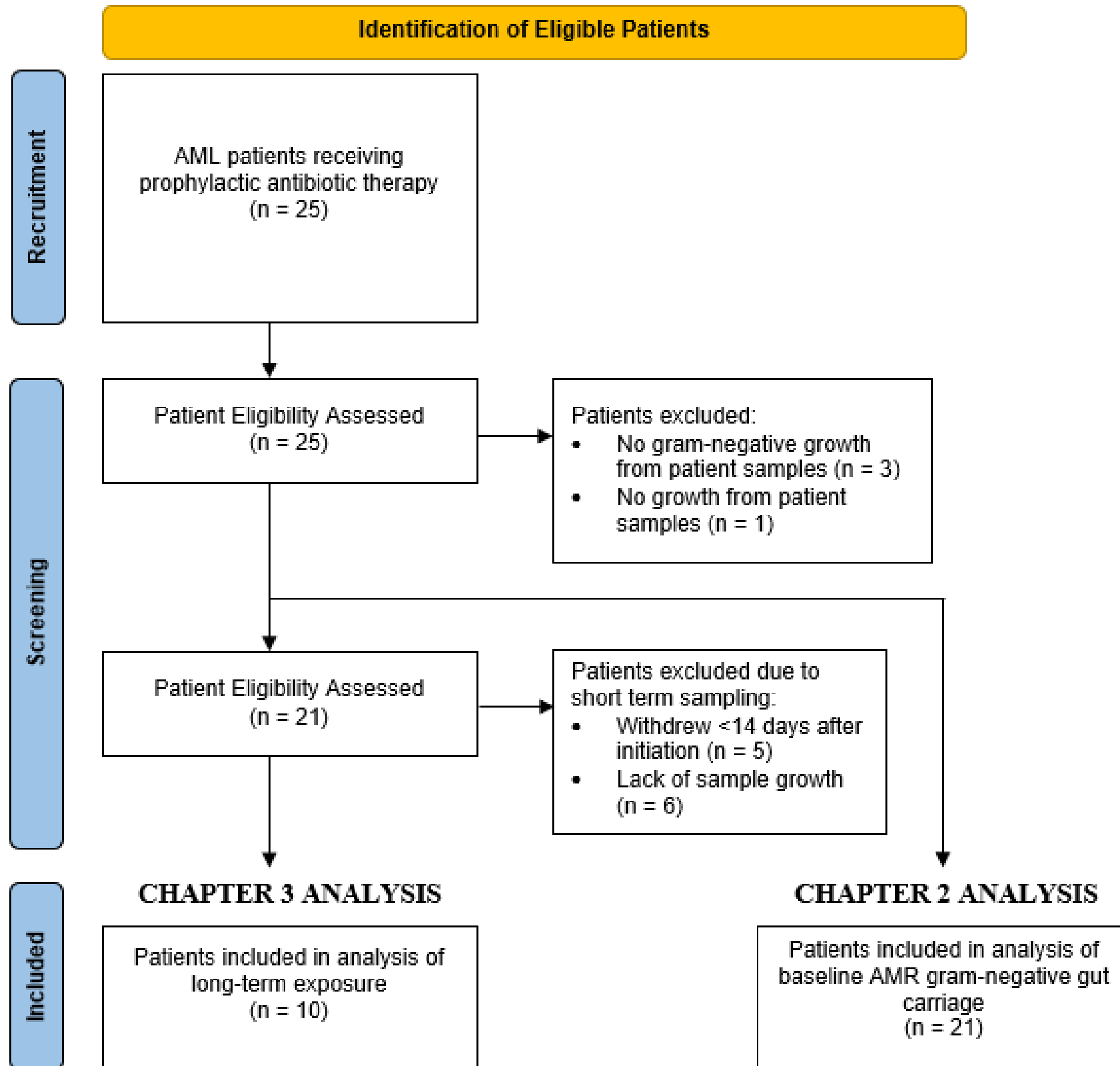


Figure 3: *PRISMA Patient Eligibility Assessment for AMR Analyses.*

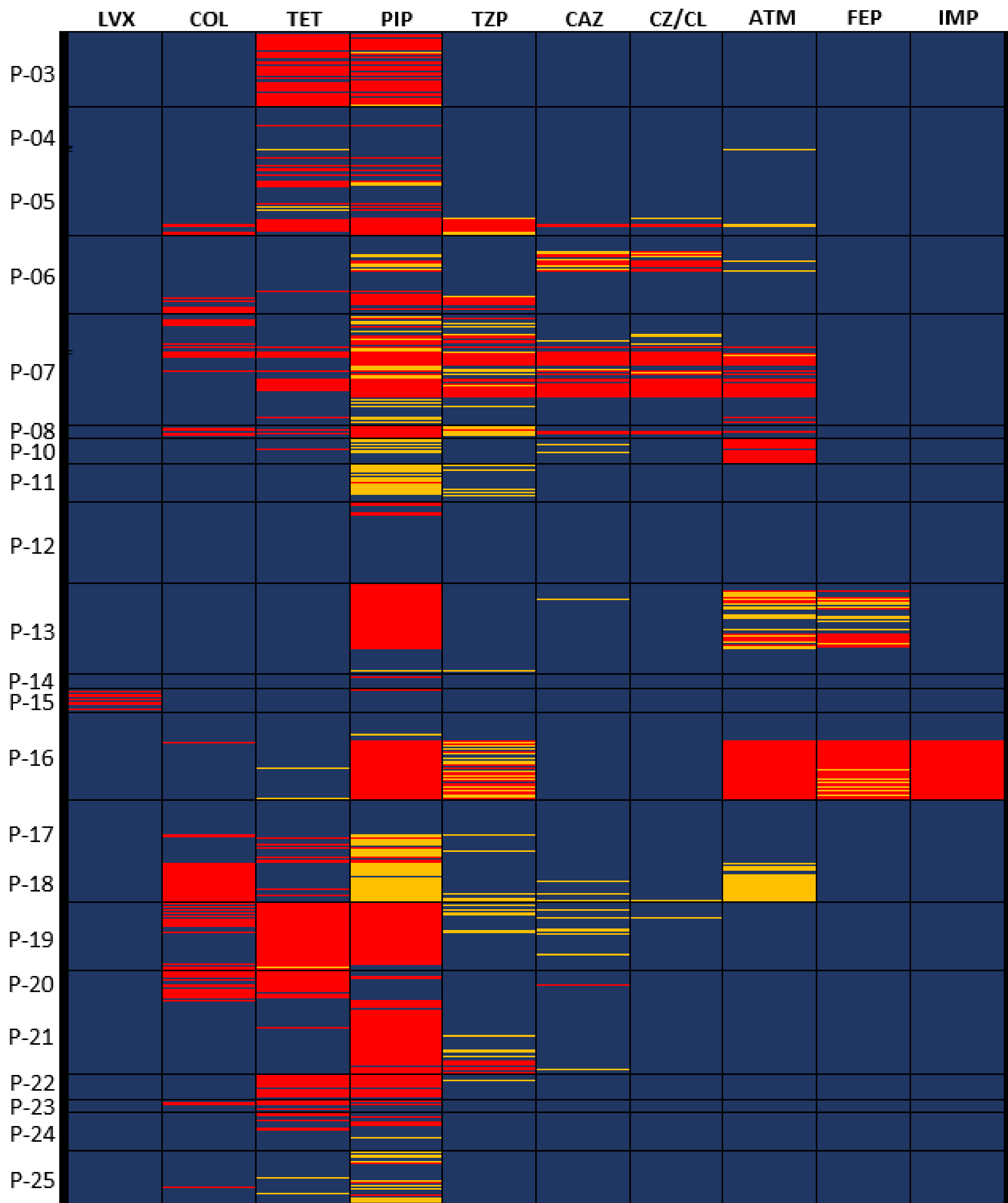


Figure 4: *Heat map of antibiotic resistance across the AMR Study Isolate Collection.* Each column represents a tested antibiotic [levofloxacin (LVX, 5µg), colistin (COL, 10µg), tetracycline (TET, 30µg), piperacillin (PIP, 100µg), piperacillin-tazobactam (TZP, 100µg:10µg), ceftazidime (CAZ, 30µg), ceftazidime-clavulanic acid (CZ/CL, 30µg:10µg), aztreonam (ATM, 30µg), cefepime (FEP, 30µg), imipenem (IMP, 30µg)] with each row/line representing a clinical isolate with disk diffusion zone diameters filled along the row. In accordance with CLSI guidelines for *Enterobacteriales*, zone diameters were classified as resistance, intermediate resistance, or susceptible shown in red, orange, and blue, respectively.

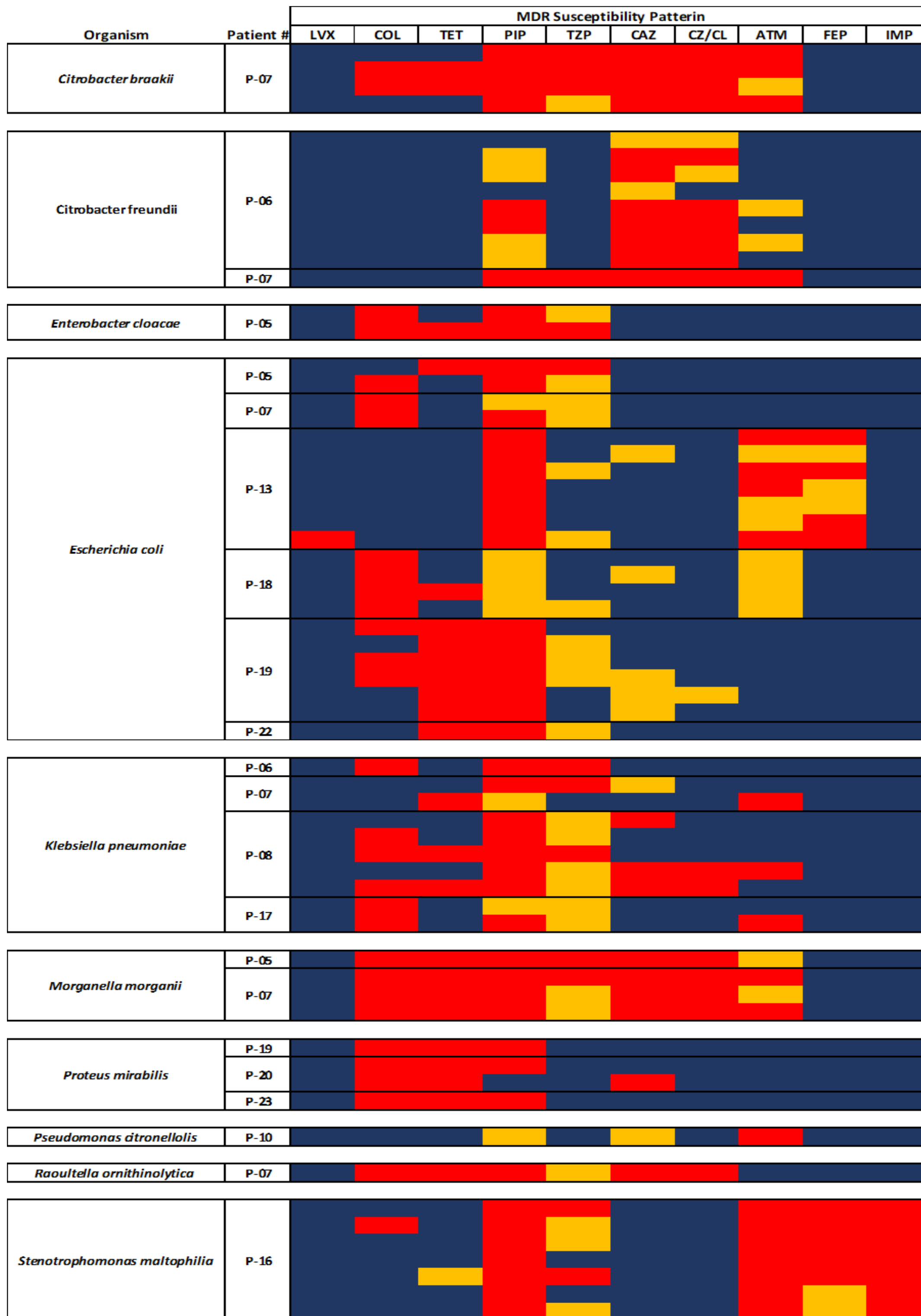


Figure 5: *MDR gram-negative organism phenotypes across the AMR Study Isolate Collection.* Each column represents a tested antibiotic [levofloxacin (LVX, 5µg), colistin (COL, 10µg), tetracycline (TET, 30µg), piperacillin (PIP, 100µg), piperacillin-tazobactam (TZP, 100µg:10µg), ceftazidime (CAZ, 30µg), ceftazidime-clavulanic acid (CZ/CL, 30µg:10µg), aztreonam (ATM, 30µg), cefepime (FEP, 30µg), imipenem (IMP, 30µg)]. In accordance with CLSI guidelines, zone diameters were classified as resistance, intermediate resistance, or susceptible represented by red, orange, and blue, respectively.

A

Sample ID	Organism	COL ZOI (mm)	COL MIC (µg/mL)
P7E1	*Escherichia coli*	9	8
P7E6	*Escherichia coli*	9	8
P7E10	*Escherichia coli*	9	8
P7F9	*Escherichia coli*	9	16
P7F12	*Escherichia coli*	8	8
P7G5	*Escherichia coli*	9	8
P7G6	*Escherichia coli*	9	8

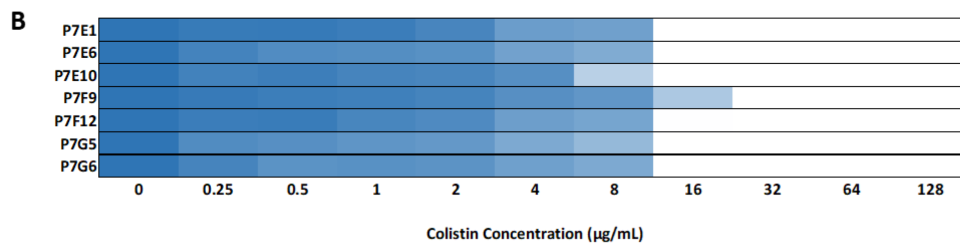


Figure 6: *Confirming colistin resistance in low ZOI E.coli from patient 18*. **A)** displays ZOI of a random sample of colistin projected-resistant *E. coli* along with tested MIC by broth microdilution. **B)** shows growth of several *E. coli* P7E1, E6, E10, F9, F12, G5, and G6 with increasing concentrations of colistin. Intensity of blue is proportional to OD_{600} bacterial growth.

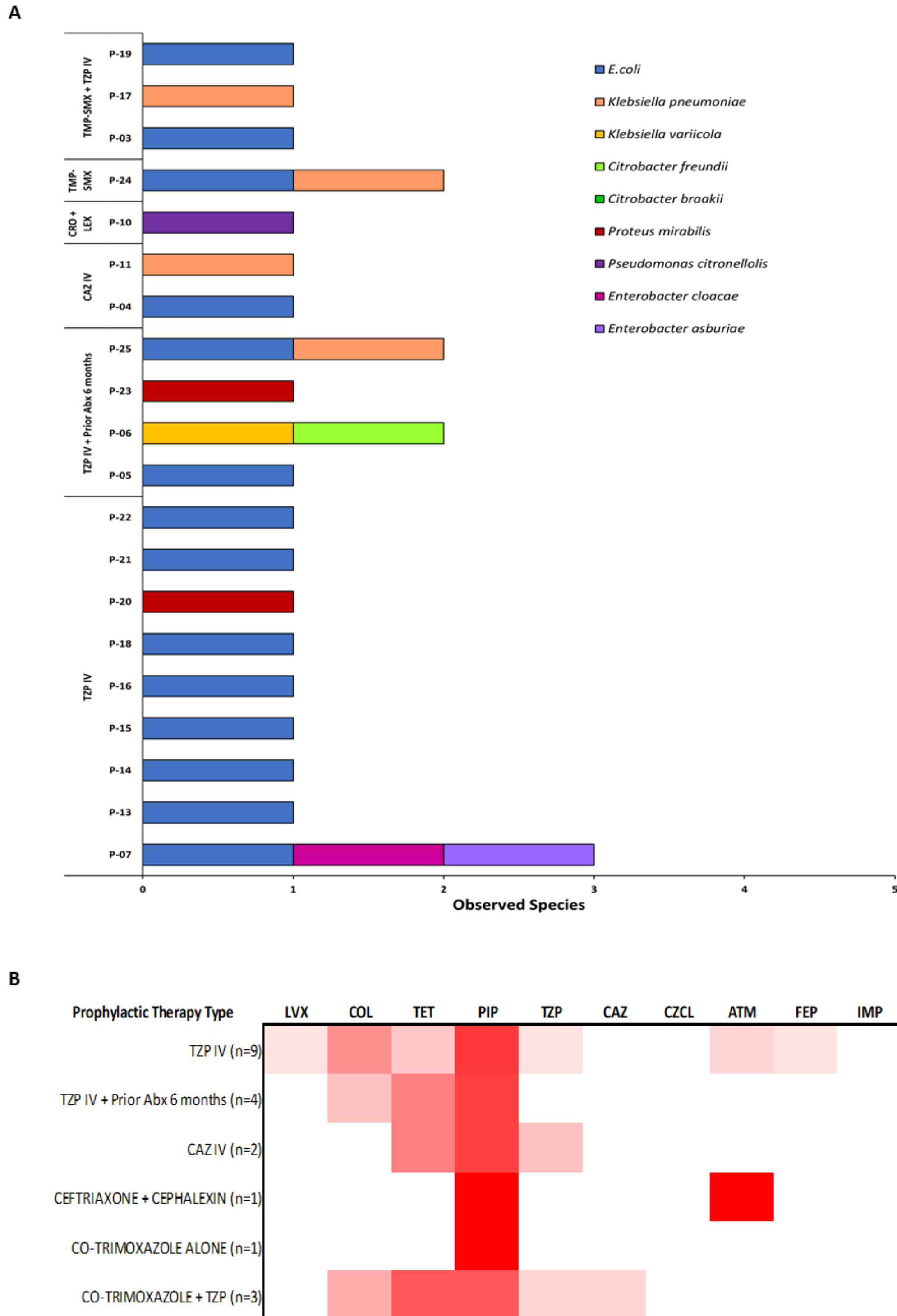


Figure 7: *Antibiotic resistance across first patient sampled gram-negative organisms within the AMR Study Isolate Collection.* **A)** Displays observed gram-negative organisms across first patient samples by prophylactic therapy type. **B)** shows a heatmap representing gram-negative non-susceptibility phenotypes averaged across patients receiving similar antibiotic prophylactic treatments. Red indicates the presence of non-susceptibility, with greater intensities of red representing higher frequency of non-susceptibility to a certain drug class across first patient samples from a certain therapy group. For example, patient 15 (P-15) first sample contained two *E. coli* phenotypes, one resistant to levofloxacin and the other to piperacillin. This alone would have contributed to a heatmap intensity of 1 of 9 within the levofloxacin and piperacillin columns.

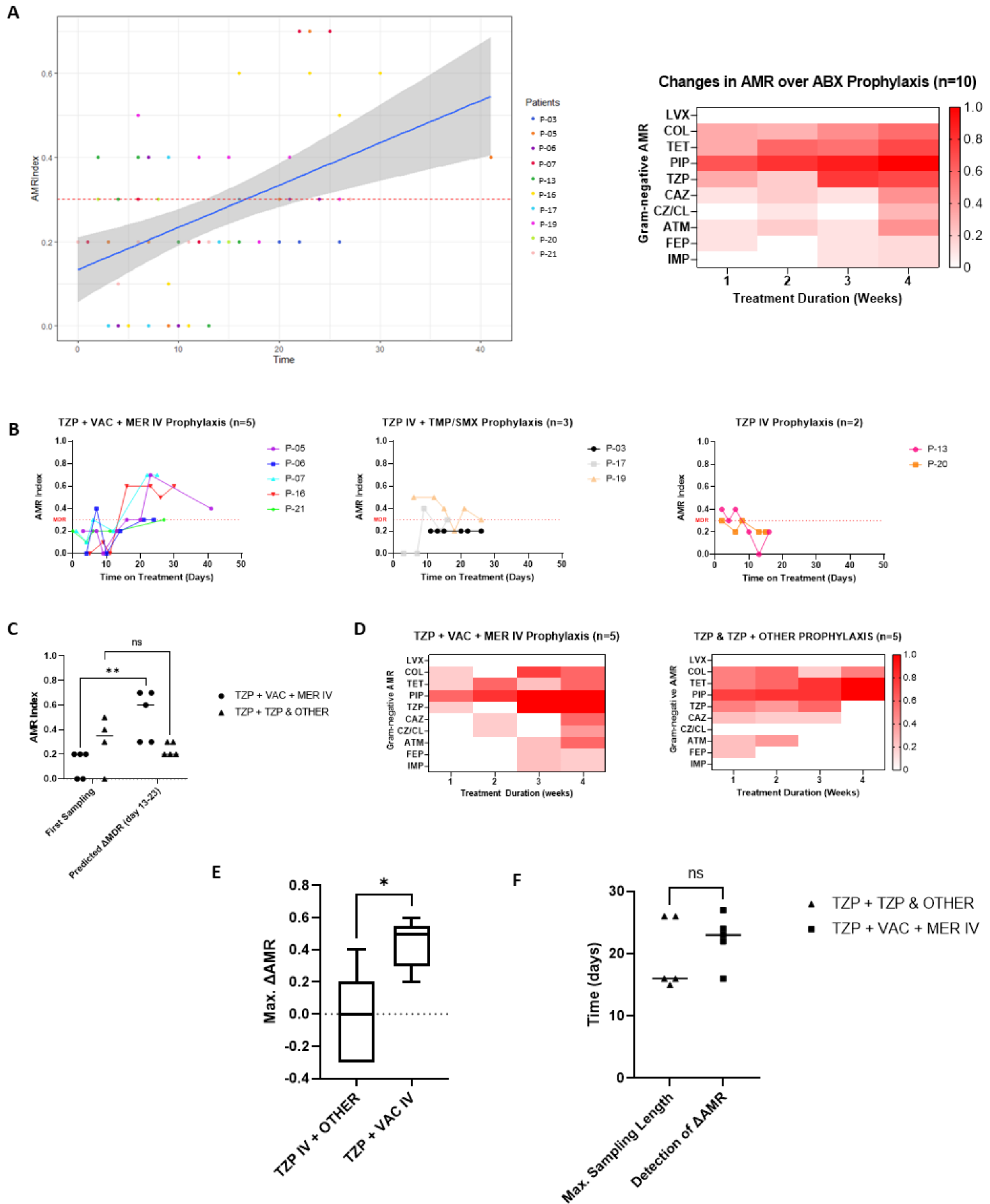


Figure 8: *Prophylactic antibiotic treatment length significantly impacts gram-negative AMR phenotypes in a treatment dependent manner.* **A)** displays a linear mixed model of AMR Index by treatment time (days) along with a heatmap describing features of resistance changes (weeks). Linear model line indicated by the following equation: AMRIndex = 0.01(Time) + 0.132. The heatmap shows the avg. AMR Index with intense red indicating an avg. AMR Index of 1. **B)** shows AMR Index mapped over time in patients from different treatment groups. **C)** mixed effects pairwise ANOVA comparison between baseline (first sampling) AMR Index and predicted MDR window from linear model (13-23 days). For this section, highest AMR Indexes sampled within the MDR window were plotted. **D)** heatmap of resistance change by drug class over time. **E)** Mann-Whitney U test comparison between max. change in AMR Index of either treatment group. **F)** Mann-Whitney U test comparison between max. sampling length and detection of significant AMR increase.

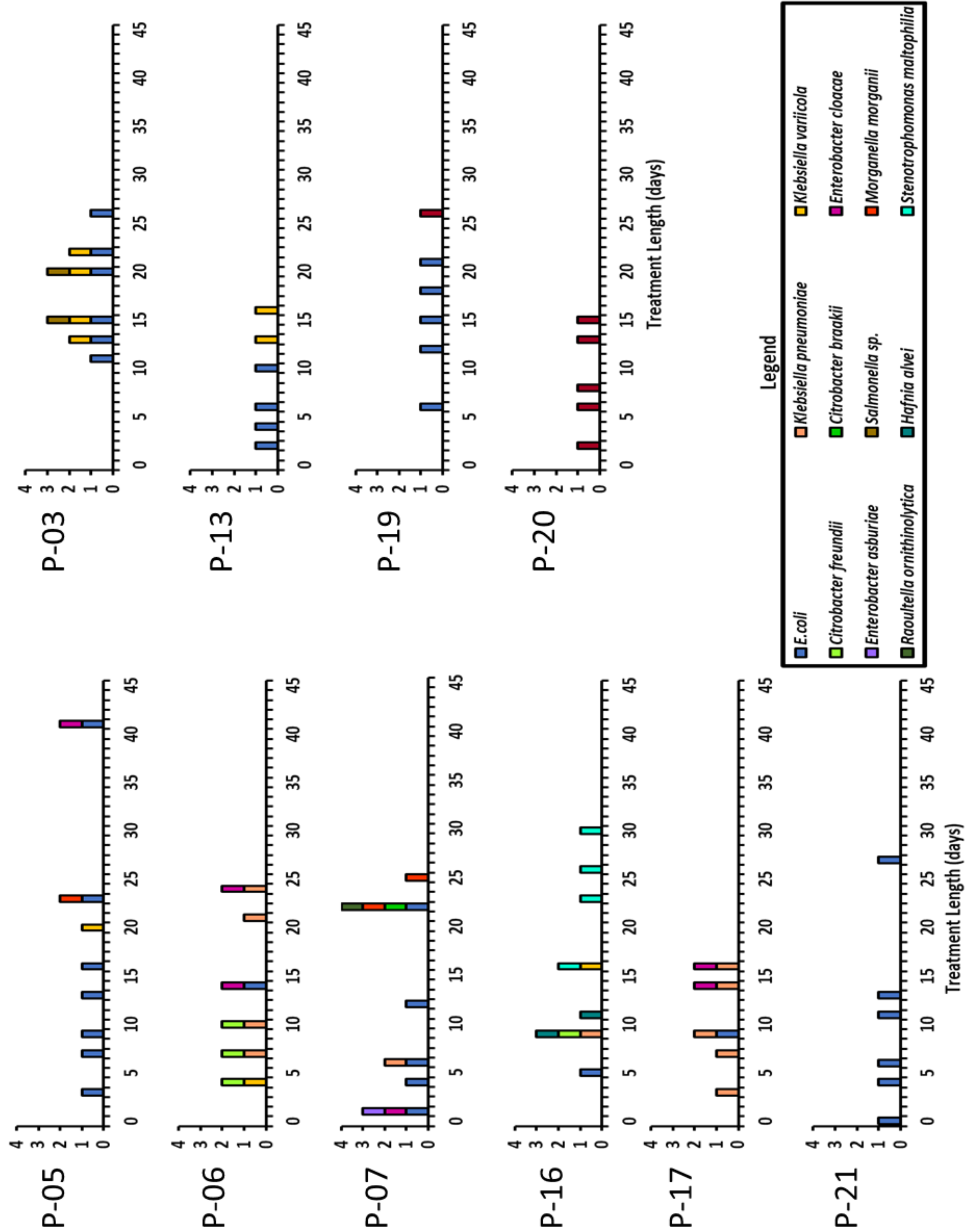


Figure 9: *Observed gram-negative species over prophylactic antibiotic therapy.* The y-axis represents the number of observed gram-negative species and the x-axis is the timepoint at which they were sampled. Patients grouped on the left experienced increases in AMR while those on the right did not.

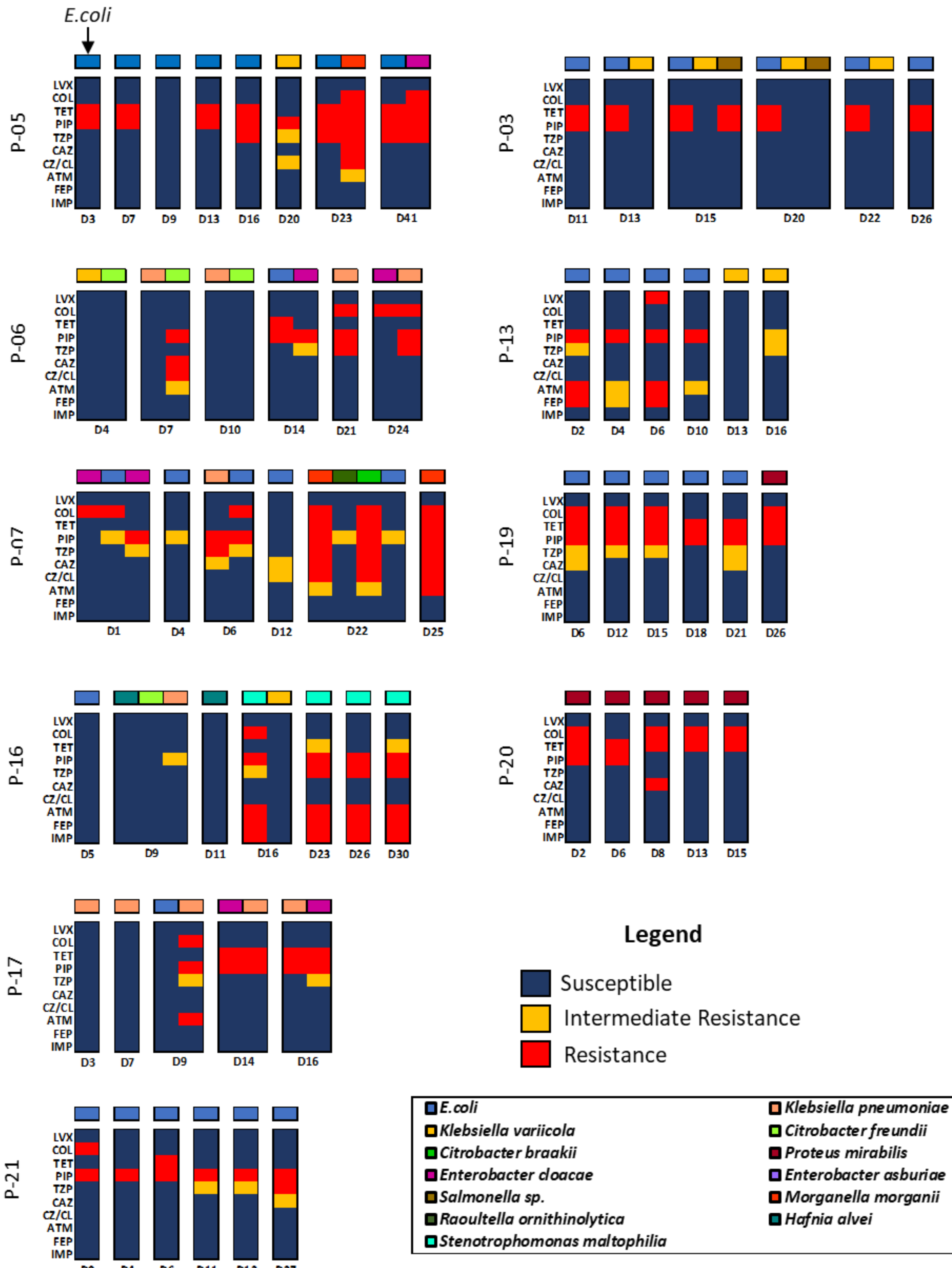
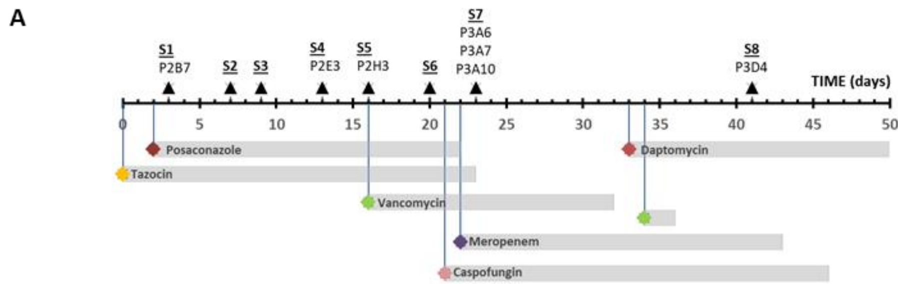
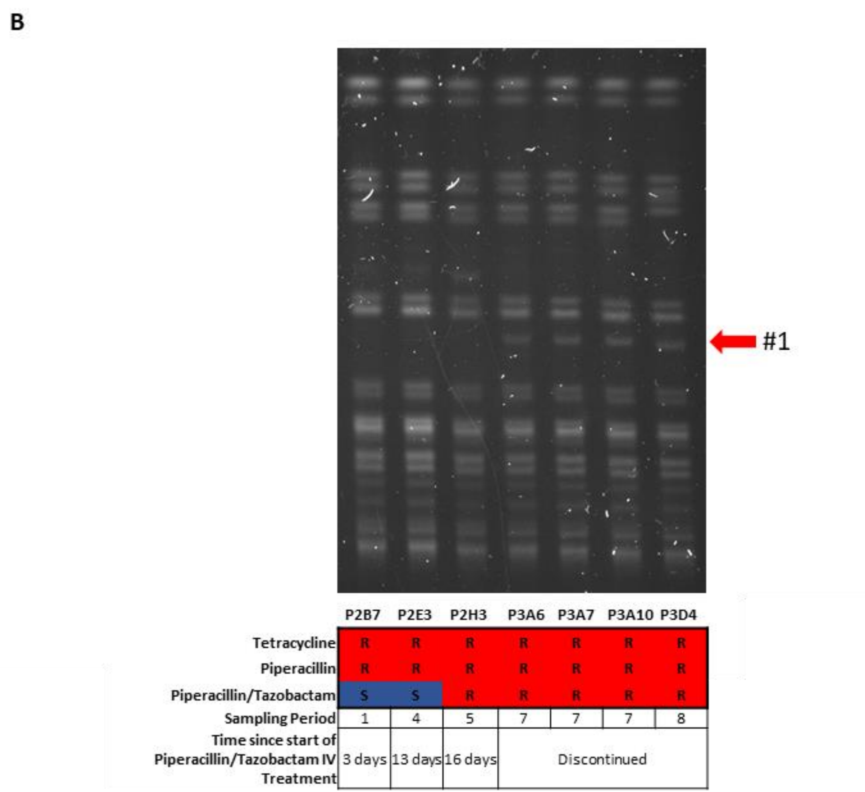


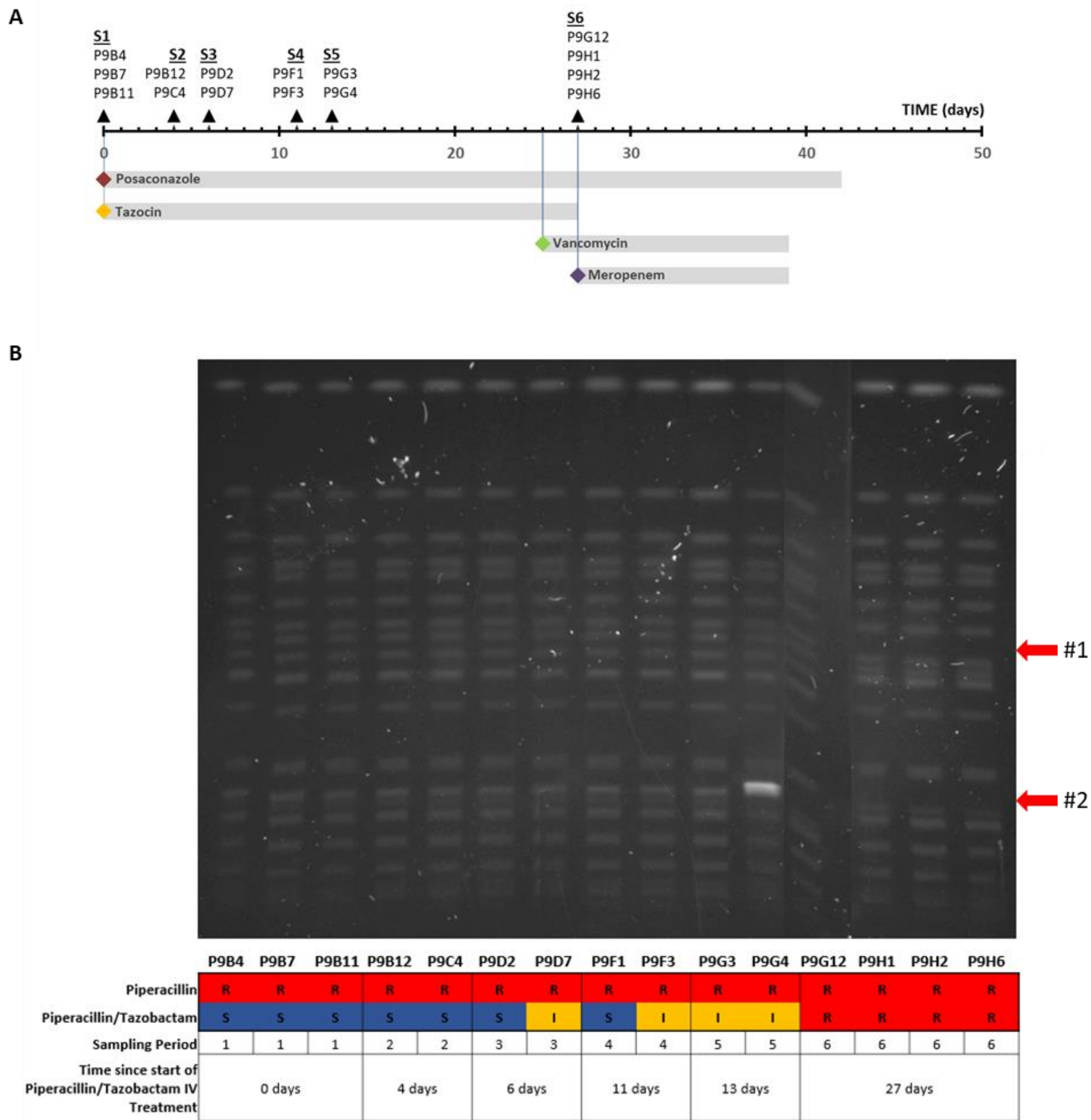
Figure 10: *Longitudinal heatmaps of most resistant gram-negative species captured at each sampling day.* The heatmaps show resistance, intermediate resistance, and susceptibility to the 10 listed antibiotics by the colours red, orange, and blue, respectively. Above each column, a colour is shown which corresponds to the taxonomy assigned to that resistance profile.





	P2B7	P2E3	P2H3	P3A6	P3A7	P3A10	P3D4
Tetracycline	R	R	R	R	R	R	R
Piperacillin	R	R	R	R	R	R	R
Piperacillin/Tazobactam	S	S	R	R	R	R	R
Sampling Period	1	4	5	7	7	7	8
Time since start of Piperacillin/Tazobactam IV Treatment	3 days	13 days	16 days	Discontinued			

Figure 11: *Evolution of TZP-resistant E. coli during TZP prophylaxis in patient5.* **A)** Displays a patient's antibiotic prophylaxis timeline for patient 5. Multiple *E. coli* isolates (P2B7, E3, & H3 and P3A6, A7, A10, & D4) were picked from different rectal swab samples (S1-S6). **B)** Pulsed-field gel electrophoresis (PFGE) of *E. coli* isolates, digested with *xbaI*, paired with piperacillin and piperacillin-tazobactam susceptibility (R = resistance, I = intermediate resistance, and S = susceptibility). Arrow #1 highlights an additional band in isolates P3A6, A7, A10, & D4.



	P9B4	P9B7	P9B11	P9B12	P9C4	P9D2	P9D7	P9F1	P9F3	P9G3	P9G4	P9G12	P9H1	P9H2	P9H6
Piperacillin	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R
Piperacillin/Tazobactam	S	S	S	S	S	S	I	S	I	I	I	R	R	R	R
Sampling Period	1	1	1	2	2	3	3	4	4	5	5	6	6	6	6
Time since start of Piperacillin/Tazobactam IV Treatment	0 days			4 days		6 days		11 days		13 days		27 days			

Figure 12: *Evolution of TZP-resistant E. coli during TZP prophylaxis in patient 21.* **A)** Displays a patient's antibiotic prophylaxis timeline. Multiple *E. coli* isolates (P9B4, B7, B11, B12, C4, D2, D7, F1, F3, G3, G4, G12, H1, H2, & H6) were picked from different rectal swab samples (S1-S6). **B)** Pulsed-field gel electrophoresis (PFGE) of *E. coli* isolates, digested with *xbaI*, paired with piperacillin and piperacillin-tazobactam susceptibility (R = resistance, I = intermediate resistance, and S = susceptibility). Arrow #1 highlights a banding difference in isolates P9G12, H1, H2, & H6. Arrow #2 indicates a banding difference in isolates P9H1, H2, &H6.

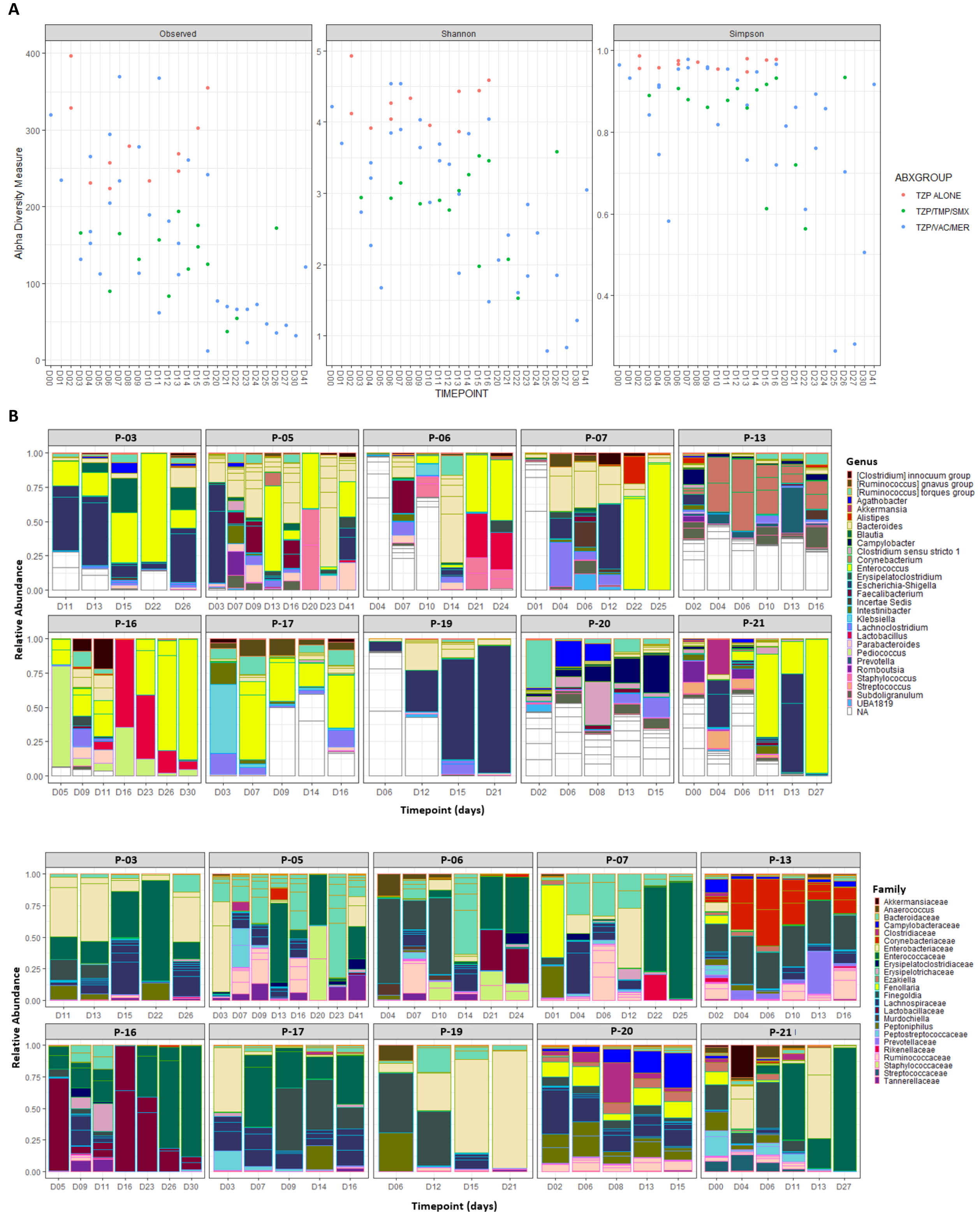


Figure 13: *Effects of long-term prophylactic antibiotics on the microbiome.* **A)** displays microbial diversity across patient samples over time (days). **B)** shows relative abundance of microbial species at both family- and genus-level across patient samples by timepoint (days).

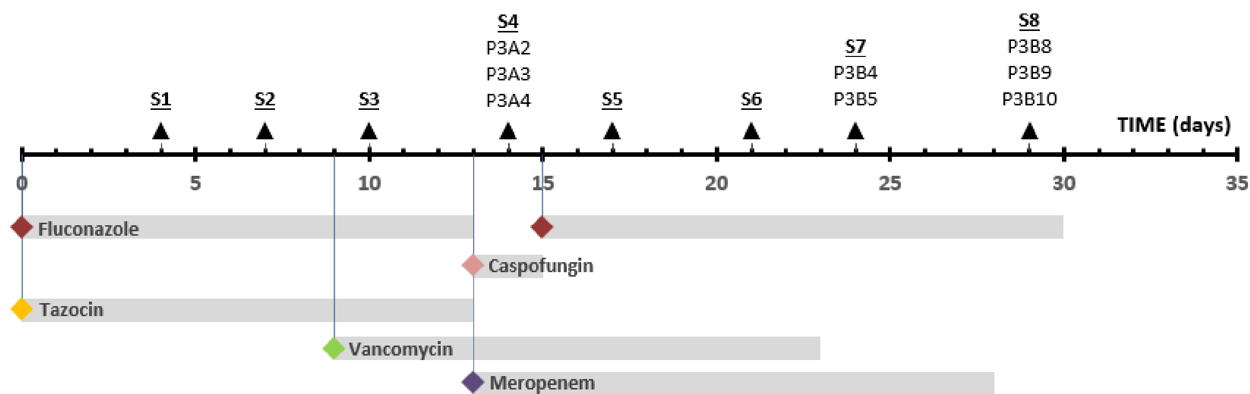


Figure 14: *Prophylactic treatment timeline of Patient 6.* Blue bars are sized proportional to the duration of treatment. Dotted lines connected to triangles designate sampling times for each rectal swab (ex: S1=Swab1).

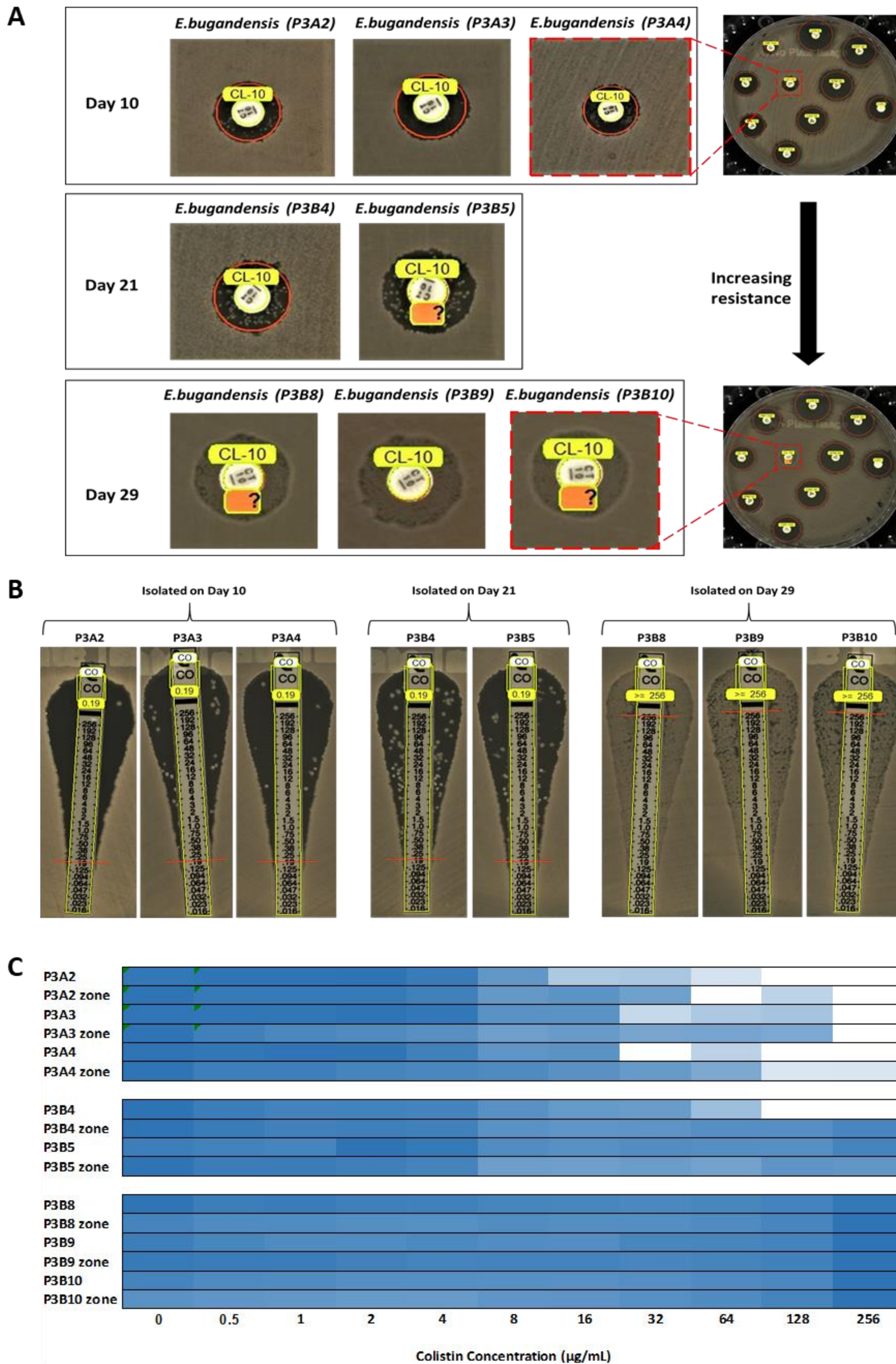


Figure 15: *Hetero-resistant phenotype of E. bugandensis isolated from patient 6.* Isolation days refer to the specific day during study involvement that the bacteria was sampled from patient 6. **A)** depicts the initial antibiotic screening and detection of possible hetero-resistance phenotypes over treatment time. **B)** shows repeated assays characterizing hetero-resistance by the presence of a highly colistin resistant sub-population. **C)** colistin MICs for *Enterobacter bugandensis* isolates tested against base population and sub-population within ZOI from **A)** and **B)**.

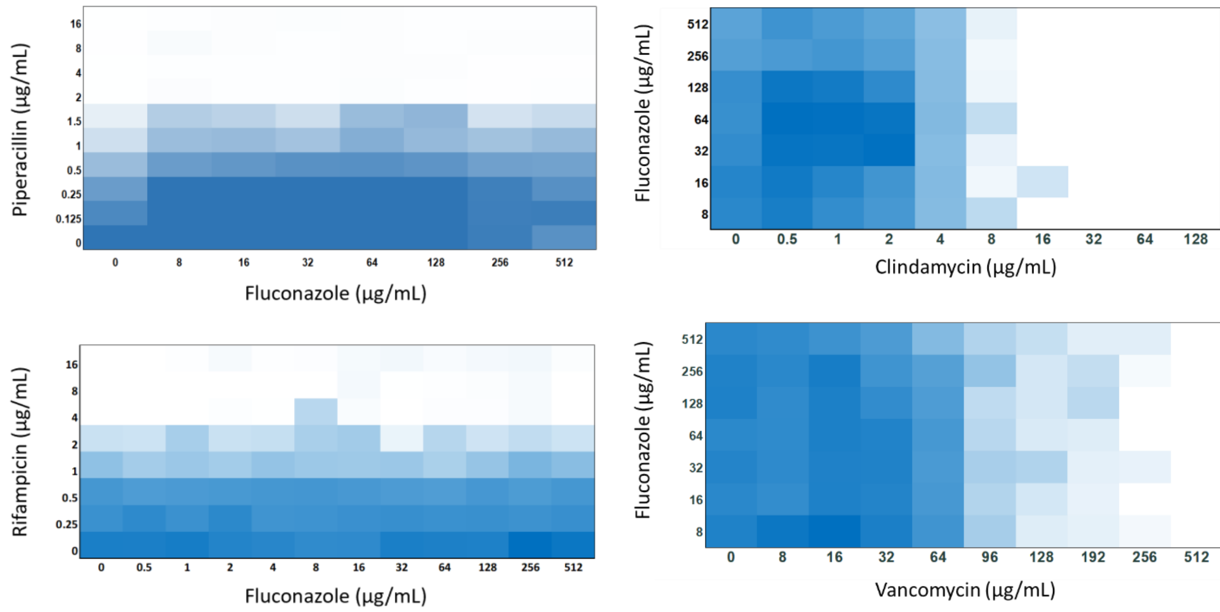


Figure 16: *Determining fluconazole's impact on antibiotic susceptibility in Enterbacter spp.* Varrying concentrations of fluconazole were tested with varying concentrations of antibiotics of different classes in a checkboard fashion.

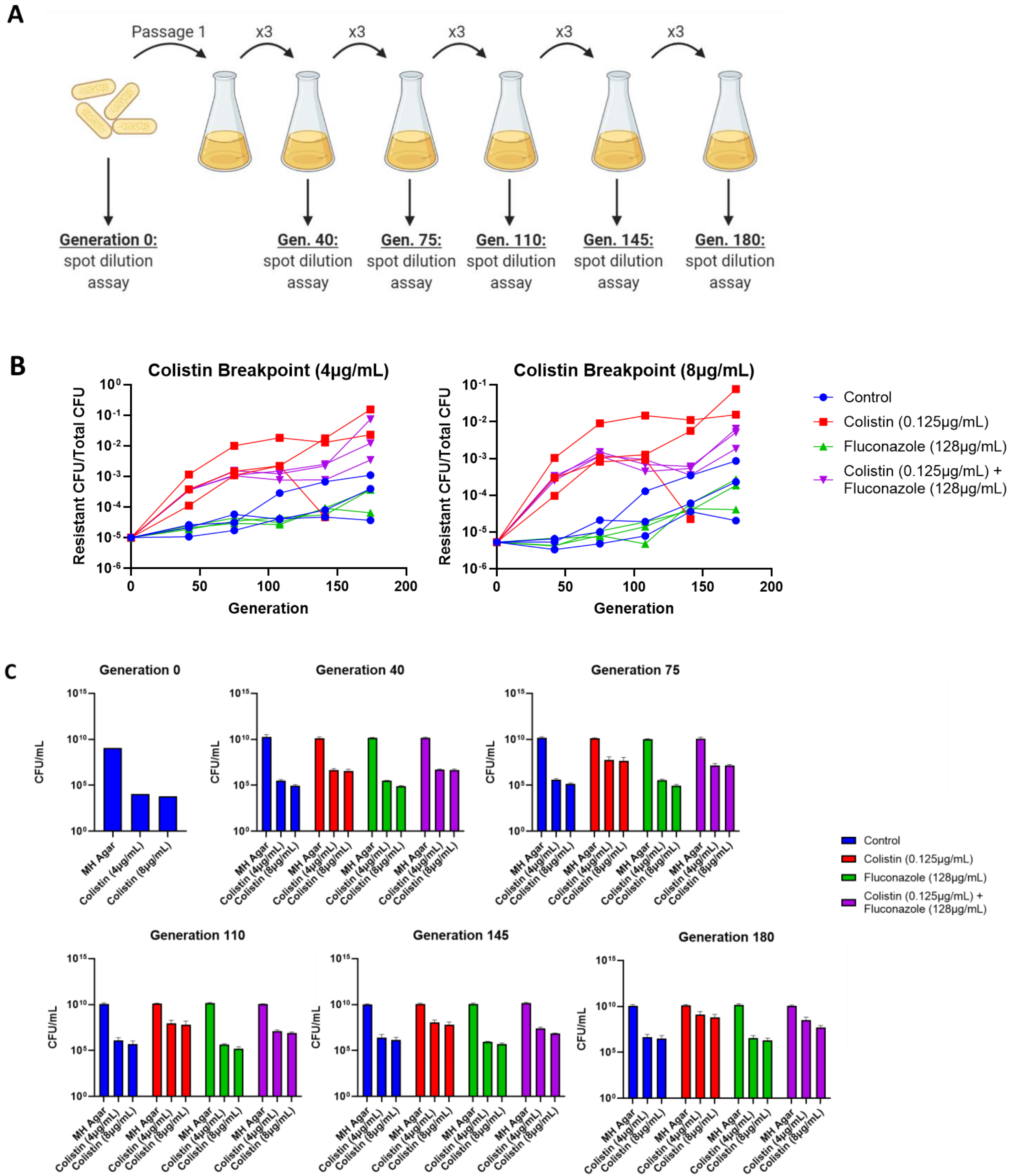


Figure 17: *Role of fluconazole in evolution of colistin hetero-resistance over time.* **A)** shows a visual overview of the evolution protocol, with spot dilutions representing colistin hetero-resistance testing and "x3" indicating 3 serial passages. Schematic **A** was performed in triplicate with each of the control and 3 test medias (colistin, fluconazole, and colistin + fluconazole). **B**) displays the log-scale increase in proportion of colistin resistance CFUs relative to total CFUs by generation number. A colistin replicate became contaminated before measurement and thus only 2 replicates are present at generation 180. **C**) shows log-scale average CFU counts on 3 different agar types [MH, MH + colistin (4µg/mL), and MH + colistin (8µg/mL)] with increasing generation. **B&C**) For all graphs, generation 0 indicates the parental culture (P3A3-P9 strain) used to inoculate all 4 growth conditions. Legend indicates 4 growth conditions in which bacteria were serially cultured.

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