AMERICAN UNIVERSITY OF BEIRUT

MOLECULAR CHARACTERISTICS OF COLISTIN RESISTANCE IN GRAM-NEGATIVE BACILLI AND ASSESSMENT OF COMBINATION THERAPY

By

SEREEN NABIL IWEIR

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AN ABSTRACT OF THE THESIS OF

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Major: Microbiology and Immunology

Title: Molecular Characteristics of Colistin Resistance in Gram-negative Bacilli and Assessment of Combination Therapy

Background: Extensive and uncontrolled use of antibiotics, along with the natural evolution of bacteria, and the lack of discovery of new antimicrobial classes, has led to a rise in the number of multi-drug resistant (MDR) bacteria including *Acinetobacter spp.* and *Escherichia coli,* causing them to develop the ability to resist several classes of antimicrobials, including third generation cephalosporins and carbapenems. This rapidly growing phenomenon has led to increased reliance on the cyclic peptide antimicrobial agent colistin as a therapeutic option for MDR Gram-negative bacteria, making it the 'last-line' of defense against such infections.

In recent years, the rise of colistin resistant strains has been reported worldwide at an alarming rate; and with that, so did the demand on the scientific community to provide alternative options for treatment of colistin resistant *A. baumannii* and *E. coli*. In a previous study, molecular characteristics of colistin resistant isolates from the American University of Beirut Medical Center (AUBMC) was determined. This study aims to assess the efficacy of different antimicrobials agents, in combination with colistin, against resistant *A. baumannii* and *E. coli* isolates, and to correlate synergistic effect in relation to encoding colistin resistant gene profiles.

Methods: Broth microdilution in accordance with Clinical and Laboratory Standards Institute (CLSI) guidelines, was used to determine susceptibility to colistin on 33 *A. baumannii* and 6 *E. coli* MDR clinical isolates, obtained from the Clinical Microbiology laboratory at AUBMC, previously screened for colistin resistance by disc diffusion, E-test and Vitek®. Checkerboard and time-kill assays were performed on 5 resistant *A. baumannii* isolates to colistin, 2 CDC *mcr*-1 encoding *E. coli* strains, and 2 ATCC wild-type strains to investigate synergy between colistin and each of amikacin, rifampicin, and teicoplanin. Reverse transcription-quantitative PCR (RT-qPCR) was performed on colistin resistant and on colistin susceptible isolates to compare the relative levels of expression of the colistin resistance encoding genes that are associated with each resistance phenotype. Antimicrobial combinations were also assessed *in vivo* in a BALB/c *A. baumannii*-induced pneumonia model using a colistin resistant strain.

Results: *In vitro* assessment of the combinations of colistin with rifampicin, and colistin with teicoplanin demonstrated synergy against the colistin resistant *A. baumannii* isolates, with bactericidal effects of the combinations occurring before 4 hours of incubation. As for the *E. coli,* CDC and ATCC wild-type isolates, the combination of colistin and rifampicin showed indifferent activity when compared to the antibiotic monotherapies. The combination of colistin and amikacin proved to be indifferent as well against all the tested isolates of *A. baumannii* and *E. coli*.

In vivo assessment of the combinations of colistin with rifampicin showed a more efficacious effect than the combination of colistin and teicoplanin against a colistin resistant *A. baumannii* clinical isolate. Molecular testing of the relative levels of expression of the *pmr* genes and the lipid biosynthesis genes shows that colistin resistance was mediated by LPS loss for one of the isolates. On the other hand, resistance was mediated by a combination of both the reduced expression of some of the lipid biosynthesis genes and the over expression of the *pmr* genes in the remaining tested isolates. Both factors are associated with reduced affinity of colistin to its target lipid A portion of the LPS of the outer membrane.

Conclusion: The lack of the development of new classes of antimicrobials that can successfully target MDR Gram-negative bacteria, particularly colistin resistant isolates, has led physicians to utilize combination therapy as the only viable form of treatment for such pathogens. Our study demonstrated that the rifampicin-colistin and the teicoplanin-colistin combinations are effective options for treatment of colistin-resistant *A. baumannii*, with the rifampicin-colistin combination showing more promising results *in vivo.* Moreover, the study of the levels of expression of the lipid biosynthesis and the *pmr* signaling genes, revealed that in our *A. baumannii* isolates, resistance to colistin was either due to decreased expression of the lipid biosynthesis, or over expression of the *pmr* signaling genes.

TABLE OF CONTENTS

ILLUSTRATIONS

Figure ..... Page

TABLES

CHAPTER I

INTRODUCTION

One of the looming healthcare issues facing the world in the 21st century is the steep rise of antimicrobial resistance (AMR). A review commissioned by the British government in 2016 reported that the annual number of deaths attributed to AMR infections were of 700,000 lives, at the year of publication, and that they may rise to up to 10 million deaths per year by 2050 (1). The rise in AMR to third generation cephalosporins and carbapenems in Gram-negative bacilli, is largely due to the overuse and misuse of antibiotics both in clinical and agricultural settings. It is also accompanied by the lack of novel antibiotics with unexploited targets. These two factors, the emergence of resistance and the void in antibiotic discovery, have led to the re-use of colistin, an old nephrotoxic polymixin. Colistin is now the last resort for treatment of Gram-negative pathogens, and is losing its efficacy due to the emergence of colistin-resistant Gram-negative bacilli especially *Acinetobacter baumannii* and *Escherichia coli.*

A. baumannii, an opportunistic pathogen, is grouped by the World Health Organization (WHO) to be among the ESKAPE group of pathogens -*Enterococcus faecium, Staphylococcus aureus, Klebsiella pneumoniae, A. baumannii, Pseudomonas aeruginosa,* and *Enterobacter* species- that consist the majority of organisms that cause multi-drug resistant (MDR) nosocomial infections (2). While there have been multiple definitions for MDR *A. baumannii,* this study will follow the definition of Manchanda *et al.*, stating that multi-drug resistant *Acinetobacter* spp. isolates are those that are resistant to more than two of five classes of antimicrobials that include carbapenems,

cephalosporins (including inhibitor combinations), fluoroquinolones, and aminoglycosides (3). Epidemiological studies have shown that the prevalence of antimicrobial resistance is more severe in *A. baumannii* isolates that have been obtained from Asian, European, and Middle-Eastern ICUs than from American ICUs (4). In Lebanon for instance, surveillance data collected from 16 hospitals in the country revealed a dramatic drop in the susceptibility rates of *A. baumannii* to carbapenems from 49.2% in 2011 to only 15.1% in 2013 (5). This rise of MDR *A. baumannii* infections as well as extensively drug resistant (XDR) strains that are also resistant to carbapenems has caused physicians to promote the increased reliance on the cationic peptide antibiotic- colistin, as a therapeutic option for MDR isolates. This in turn provided conditions that fostered the rise of the devastating resistance to colistin particularly in nosocomial settings, with no alternative antimicrobial options for treatment (6).

E. coli on the other hand came in to the forefront of the discussion regarding colistin resistance when a 2015 study reported for the first time the ability for plasmid-mediated transfer of genetically encoded resistance to colistin, via the transfer of the mobile colistin resistance gene *mcr*-1 (7). The discovery of plasmid-encoded resistance is alarming due to the potential of interspecies transmission, as the study revealed that it was detected in both animal and human specimen (7). Subsequent screening and detection of the *mcr*-1 gene has been performed in many countries, including the Middle East (8-10). Moreover, following studies in Belgium revealed multiple forms of the *mcr* gene found on plasmids of different origin (11).

These findings have led to an interest in studying in depth the mechanisms of resistance to colistin and assessment of combination therapy against *A. baumannii* and

E. coli. A previous study done at the department of Experimental Pathology, Immunology and Microbiology provided insight pertinent to the mode of action of colistin and the molecular characteristics of resistance in Gram-negative bacteria (12). Due to the urgent demand for therapeutic options for treatment of infections that may be resistant to all classes of antimicrobials, including colistin, this study aimed at:

1. Assessing the synergistic effect of colistin with a number of antimicrobial agents against colistin resistant *A. baumannii* and *E. coli*
2. Correlating underlying mechanisms of resistance to colistin at the molecular level with phenotypic outcomes of colistin and other antimicrobial agents in combination.

This will provide potential therapeutic solutions to combat such infections and reduce their burden on the health of world-wide populations that may be affected by them.

CHAPTER II

LITERATURE REVIEW

A. General Characteristics of *A. baumannii*

A. baumannii is a strictly aerobic non-motile pleomorphic Gram-negative coccobacillus that is commonly associated with nosocomial infections and with infections in the immunocompromised. Infections caused by *A. baumannii* tend to be localized in the skin or in the oropharynx of the respiratory tract of the affected individual. As a member of the genus Acinetobacter, it is also characterized as being a catalase-positive and oxidase-negative non-fermenting coccobacilli, as well as having soil and water as its natural habitat (13).

1. Infections caused by A. baumannii

Routes of transmission of *A. baumannii* infections include person-to-person contact, or exposure to contaminated surfaces as well as colonized medical equipment (14). Having a natural affinity towards water environments, *A. baumannii* thrives in moist conditions provided by the mucous membranes of tissue that had been exposed to burn or injury, and such colonization can develop into fatal septicemia. This attribute of *A. baumannii* was underscored during the Iraq war, where the arid conditions may have contributed to it being the leading source of skin and soft tissue infections among injured troops deployed in the region as was reported by a study in 2003; giving it the notorious ‘Iraqibacter’ label (13). Moreover, A study in 2006 used Pulsed Field Gel Electrophoresis (PFGE) to compare the genetic profiles of *A. baumannii* outbreak strains in the US and the UK to those of repatriated infected soldiers revealed them to

be genetically indistinguishable; indicating that the return of the soldiers carrying the disease was a contributing factor to the outbreak in hospitals of both countries (15).

a) Hospital Acquired Pneumonia

The discovery of *A. baumannii* in a hospital setting is always alarming. Besides its ability to infect patients through open wounds, *A. baumannii* has a propensity for forming biofilms on the surfaces of catheters and breathing tubes, and thus poses a threat of causing what is referred to as hospital-acquired pneumonia (HAP).
A. baumannii is highly associated with bacteremic nosocomial pneumonias, as reported by a survey in 2012 of HAPs in a Thailand hospital, where *A. baumannii* was the source of 30.4% of studied cases. The outcome of HAP that is caused by *A. baumannii* is less favorable than a non- *A. baumannii* caused HAP (16). Moreover, HAP that is ventilator-associated, has been demonstrated to be associated with a higher mortality rate than non-ventilator associated *A. baumannii* HAP (17). This threat is especially exacerbated in the ICU, where patients that are immunocompromised, patients that have recently received antibiotic therapy and patients that require a prolonged stay (> 90 days) are especially at risk of developing the infection (13).

b) Community Acquired Pneumonia

Along with its ability to thrive in nosocomial settings, *A. baumannii* has been reported to cause community-acquired pneumonia (CAP), a form of acute respiratory infections that occur in people that hadn't been recently hospitalized (18). CAP caused by *A. baumannii* particularly affects tropical and subtropical Asia-pacific countries, including Taiwan and Australia, and has been shown to increase during the 'wet seasons' of those regions. In Australia, *A. baumannii* accounts for 10% of all cases of CAP and for 20% of deaths due to bacteremic CAPs. It has been shown to be more

significantly present in subjects with high levels of alcohol consumption (19). While less common than its hospital acquired counterpart, *A. baumannii* CAP usually presents with more severe symptoms and a mortality rate of 64% within 48 hours of admission, as reported in a study that was performed in Singapore in 2012 (20).

2. Treatment Options

Antimicrobials that are typically used as treatment options for *A. baumannii* infections include sulbactams, carbapenems, and aminoglycosides.

a) Sulbactam-ampicillin

Ampicillin is a broad-spectrum, bactericidal ß-lactam penicillin that is active against Gram-negative and Gram-positive aerobic and anaerobic bacteria. It acts by binding to and inhibiting the penicillin binding protein (PBP) present on the inner membrane of bacteria, thus inhibiting cell wall synthesis and causing bacterial cell lysis (21). Regimens containing sulbactam, a ß-lactamase inhibitor prescribed in combination with ampicillin, are able to combat the detrimental effect bacterial ß-lactamases can have on the action of penicillins, and can restore the activity of ampicillin. they have been documented to reduce mortality in subjects with MDR *A. baumannii* bloodstream infections in particular (22).

b) Carbapenems

Carbapenems such as imipenem and meropenem also act as ß-lactams and are considered to be the most essential therapeutic options when treating *A. baumannii* infections, since they have the ability to combat the effect of ß-lactamases and possess excellent bactericidal activity (4).

c) Amikacin

Aminoglycosides including amikacin, kanamycin and gentamicin, are another example of antimicrobials that can be used for treatment of *A. baumannii* infections. They however tend to be avoided by physicians due to their nephrotoxic and ototoxic side effects (23), unless resistance to safer drugs such as carbapenems is observed. Aminoglycosides exert their action by inhibiting protein synthesis in Gram-negative bacteria by preventing the elongation of peptides through binding to the 30S subunit of the bacterial ribosomes, which interferes with tRNA acceptor sites as well and leads to the production of non-functional toxic peptides (24).

d) Tigecycline

Tigecycline is a broad-spectrum glycycline that binds to the 30S subunit of the bacterial ribosome; and thus, inhibits protein synthesis in the target organism. When targeting organisms such as *E. coli, K. pneumoniae,* and *E. faecalis,* tigecycline tends to demonstrate a bacteriostatic effect (25).

3. Antimicrobial resistance in A. baumannii

When present, resistance against carbapenems and other ß-lactams such as ampicillin may be attributed to multiple mechanisms such as the production of metallo-β-lactamases and carbapenem-hydrolizing β-lactamases, the decreased expression of certain porins, the presence of multi-drug resistant efflux pumps, that also induce co-resistance to antibiotics such as tigecycline, and modifications in the penicillin binding protein (PBP), the target binding site for this class of antimicrobials (4). Resistance to amikacin is rising as well (24), and is mostly mediated by aminoglycoside-modifying enzymes (AMEs). AMEs include phosphotransferases,

acetyltransferases, and adenyltransferases that are encoded by transferrable genes that can be plasmid, integron, or transposon encoded. They have the ability to modify the chemical structure of amikacin thus preventing its activity (4).

B. General Characteristics of *E. coli*

As a member of the Enterobacteriaceae family of bacteria, *E.coli* is a facultative anaerobic, Gram-negative, lactose-fermenting bacilli that constitutes part of the normal flora of the lower intestines of animals and humans, it can also be found in water and soil due to fecal contamination (26). *E. coli* possesses peritrichous flagella that provides it motility as well as adhesion molecules known as intimins allow it to attach to the microvilli of the intestine. Certain serotypes of *E. coli* are pathogenic however, and are associated with food-borne illnesses worldwide (27).

1. Infections caused by E. coli

Pathogenic *E. coli* can cause several types of intestinal diarrheic infections as well as extra-intestinal systemic infections that include urinary tract infections, bacteremia, nosocomial pneumonia, cholecystitis, cholangitis, osteomyelitis, and infectious arthritis. *E. coli* is also a leading cause of neonatal meningitis (26).

E. coli Serotypes

E. coli can be classified into different serotypes by using the Kaufmann-White scheme for classification according to differences in the structure of their surface antigens; the oligosaccharide “O” or antigen, the capsular “Ki” antigen, and the flagellar “H” antigen (28).

a) Shiga Toxin Producing *E. coli* (STEC)

Also referred to as verocytotoxic *E. coli* (VTEC) or enterohemorrhagic *E. coli* (EHEC), The O157:H7 (STEC) serotype, is a common pathogenic form of *E. coli* in the U.S. and is usually contracted from raw or undercooked food. It causes a severe form of hemorrhagic diarrhea, the CDC reports that around 5–10% of people diagnosed with *E. coli* O157 infection can develop a life-threatening type of kidney failure referred to as hemolytic uremic syndrome (HUS) (29).

b) Enterotoxigenic *E. coli* (ETEC)

ETEC is a major cause for traveler's diarrhea, predominantly in third-world countries. It can be transmitted through feces-contaminated food and water (29).

c) Enteropathogenic E. coli (EPEC)

EPEC is associated with infantile diarrhea, in developing countries, EPEC is commonly isolated from stools of healthy carriers; indicating colonization is more common than pathogenicity (30).

d) Uropathogenic *E. coli* (UPEC)

UPEC cause extra intestinal infections affecting the urinary tract (UTIs). UTIs caused by UPEC can be sexually transmitted, and have a higher incidence rate among women. UPEC is a member of the KEEs *(K.pneumoniae, E.coli,* and *Enterobacter ssp.)* which account for 80-85% of outpatient UTI cases (29).

e) *coli* K1

The *E. coli* serotype containing the capsular K1 antigen is the most commonly isolated Gram-negative organism from infants with neonatal meningitis. Neonatal meningitis is due to *E. coli* K1's ability to invade the intravascular space, where it heavily multiplies, reaching a bacteremic threshold of $>10^3$

colony forming units (CFUs)/mL of blood, which allows it to in turn penetrate the blood brain barrier (BBB) and invade the central nervous system (CNS) (31).

2. Treatment Options

Several classes of antimicrobials can be used for treatment of *E. coli* infections, ß-lactams, trimethoprim-sulfamethoxazole (TMP-SMX), and aminoglycosides are common options for empiric treatment. Nevertheless, *E. coli* has displayed resistance to these antimicrobials, promoting increased reliance on carbapenems and third-generation cephalosporins (26).

a) TMP-SMX

Trimethoprim-sulfamethoxazole is also known as co-trimoxazole is a combination of two antimicrobials that affect Gram-positive and Gram-negative organisms. TMP binds to the dihydrofolate reductase of the bacteria, while SMX, a sulfonamide, competitively inhibits the enzyme dihydropteroate synthase (DHPS), both enzymes are essential in folate synthesis in bacteria, a metabolite that is essential for the growth and reproduction of susceptible bacteria (32).

b) Third-Generation Cephalosporins

Third-generation cephalosporins are broad-spectrum antimicrobials that are used for the treatment of drug resistant infections. Ceftriaxone is an example of a cephalosporin used for the treatment of *E. coli*. Ceftriaxone exerts its bactericidal activity by binding to the PBP and inhibiting cell

wall synthesis in the bacteria. Ceftriaxone is stable against the action of many ß-lactamases (33).

c) Fluoroquinolones

Fluoroquinolones (FQ) are a class of broad-spectrum antibiotics as well that exerts its bactericidal action by inhibiting the bacterial DNA gyrase. They are highly potent on the treatment of Gram-negative bacteria, with variable activity against Gram-positive organisms. Ciprofloxacin is the most widely prescribed antibiotic of this class (34).

d) Fosfomycin

Fosfomycin is a bactericidal antibiotic that prevents cell wall synthesis by inhibiting the action of the bacterial phosphoenolpyruvate synthetase in Gram-positive and Gram-negative bacteria and blocking the production of *N*-acetylmuramic acid as a consequence. Fosfomycin is a popular choice for treatment of MDR UTIs in particular due to its good distribution rates as well as its low propensity for adverse effects (35).

3. Antimicrobial Resistance in E. coli

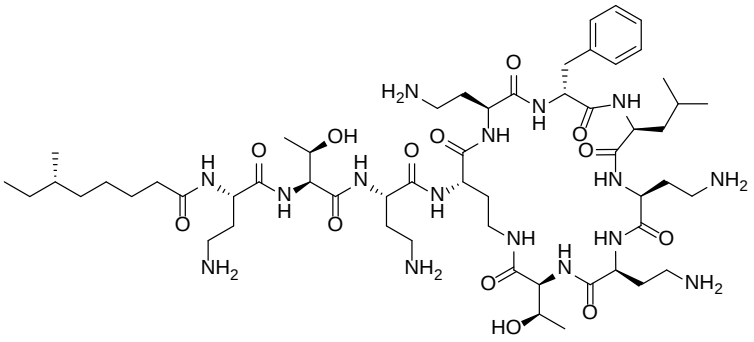
In *E. coli*, resistance to TMP-SMX is very common and often correlates with the presence of dihydrofolate reductase (DHFR) and dihydropteroate synthase (DHPS) genes in class I integrons, which also carry genes encoding for resistance to multiple antibiotic, such as streptomycin and ampicillin (36). Resistance to third-generation cephalosporins on the other hand can be mediated by a diverse group of plasmid-encoded enzymes termed as extended-spectrum ß-lactamases (ESBLs). ESBL-producing organisms present major challenges for

treatment assignment, particularly due to the co-transfer of resistance to other classes of antimicrobials such as aminoglycosides, trimethoprim, sulfonamides, tetracyclines and chloramphenicol (37). Additionally, mutations DNA topoisomerase II (DNA gyrase) and topoisomerase IV, the targets of the action of FQ, are considered to be the main mechanism by which E. coli develops resistance to FQs such as ciprofloxacin (38).

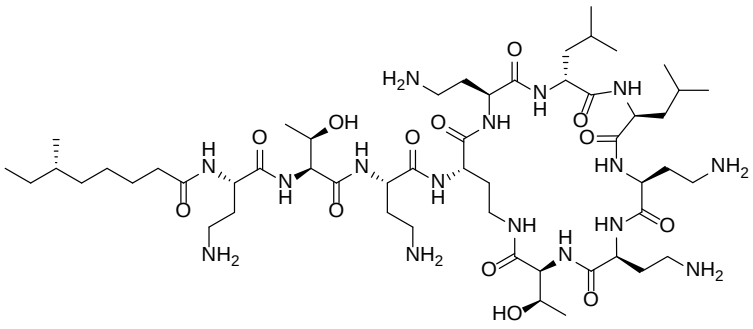
E. Polymixins

Polymixins are cationic cyclic polypeptides, of which, only polymixin B and polymixin E, or colistin, are clinically utilized. Polymixins have a narrow spectrum of activity against Gram-negative bacteria mainly. Their use declined in the 1970s due to their association with neurotoxicity and nephrotoxicity; however, the recent increase in MDR infections, along with recent studies downplaying its toxicity (39, 40), have led to it becoming the 'last-line' of defense against such infections (23).

The main structural difference between polymixin B and E is due to the presence of a D-phenylalanine residue at the position 6 of the lipopeptide portion of polymixin B, as opposed to a D-leucine residue found in that position in colistin (41). Colistin is more frequently prescribed by physicians due to studies showing that nephrotoxicity due to polymixin B tends to be more common and more severe. Moreover, there are two commercial variants of colistin: colistin sulfate, administered orally, and colistimethate sodium (CMS), for intravenous and intramuscular administration or by nebulization. CMS is the inactive pro-drug form of polymixin E, it has been established to be less potent and less toxic than colistin sulfate, and is more commonly prescribed (42).



Polymyxin B



Polymyxin E (colistin)

3. Mode of Action of Colistin

Several modes of action for colistin have been described.

a) Permeabilization of the Outer Membrane (OM) of Gram-negative Bacteria

Labeled as the 'self-promoted uptake' pathway, the consensus is that colistin's main binding target is the Lipid A moiety of the lipopolysaccharide (LPS) portion of the outer membrane of Gram-negative bacteria. Due to its cationic nature at physiological pH, colistin has a high affinity towards the anionic LPS, and is able to displace the magnesium (Mg^{+2}) and calcium (Ca^{+2}) molecules at the surface of the bacterial cell. This impairs the integrity of the OM, leading to aggregate pore formation, eventual lysis and cell death (41).

b) Inhibition of NADH-quinone oxidoreductase activity

A secondary binding site for the action of colistin was proposed to be the type II NADH-quinone oxidoreductases (NDH-II) enzyme. Colistin been reported to have the ability to interrupt the action of NADH-II, an enzyme that is vital to the function of the respiratory chain of bacteria, contributing to its death (43).

c) Hydroxyl Radical Death Pathway

Colistin has been thought to exhibit bactericidal action independent of cell lysis through inducing the massive production of lethal hydroxyl radicals from within *A. baumannii* cells that lead to irreversible damage to the bacterial DNA (44).

4. Mechanisms of Colistin Resistance in A. baumannii and E. coli

a) Multidrug Efflux Pumps

Multidrug resistant efflux pumps are capable of transporting antibiotics outside of the bacterial cell, allowing it to survive the clinically effective concentration of the drug. Efflux pumps in *A. baumannii* have been associated with increased resistance against several classes of antimicrobials including imipenem, tigeycline and colistin (4, 45).

b) Porins

Porins are outer membrane protein channels (OMPs) that function in transporting molecules across the bacterial membrane. Mutations reducing the expression of certain porins such as *Omp*W in *A. baumannii* for instance have been linked with reduced susceptibility to colistin (46).

c) LPS Alterations

Deletion of the LPS structures on the membrane of *A. baumannii* and/or alteration of its Lipid A portion can induce resistance to polymixins.

i. LPS Loss Through Mutations in Lipid Biosynthesis Genes

Mutations in *lpx*A, *lpx*C and *lpx*D lipid biosynthesis genes in *A. baumannii* isolates, movement of insertion sequence IS*Aba*11 in Acinetobacter and deactivation of *lpx*A, *lpx*C and *lpx*D trigger the complete loss of LPS which in its turn accounts for colistin resistance (47).

ii. Lipid A modification through Mutations in the Two-Component system

Mutations in the *pmr*A/B two-component signaling system can reduce the net negative charge of the anionic LPS, thus reducing the affinity of colistin to its target. This is caused by the addition of 4-amino-4-deoxy-L-arabinose (L-Ara4N) and/or phosphoethanolamine due to the upregulation of *pmr*C or *eptA* phosphoethanolamine transferase genes that shield the anionic phosphate groups of the lipid A component of the LPS (48).

d) Acquired Resistance (Plasmid mediated)

In 2015, a study done in China revealed for the first time that bacteria can gain resistance to colistin through horizontal transfer of plasmids containing the Mobile Colistin Resistant (*mcr*-1) phosphoethanolamine transferase gene that has the ability to alter the net charge of the outer membrane of *E. coli* strains of animal and human origin (7). Screening for the presence of the *mcr*-1 plasmid-encoded gene in animal and human isolates was performed in various countries with discovery of its world-wide dissemination (49). A novel form of plasmid-encoded resistance gene dubbed *mcr*-2 was identified in 2016 in

porcine and bovine *E. coli* samples that did not contain *mcr*-1 in Belgium (50). More recently, a Chinese study identified a third colistin resistance plasmid associated gene, *mcr*-3 in *E.coli* of a pig origin (51). An *mcr*-4 gene was also found in *E. coli* of porcine origin in Belgium in 2017 (11).

F. Combination Therapy

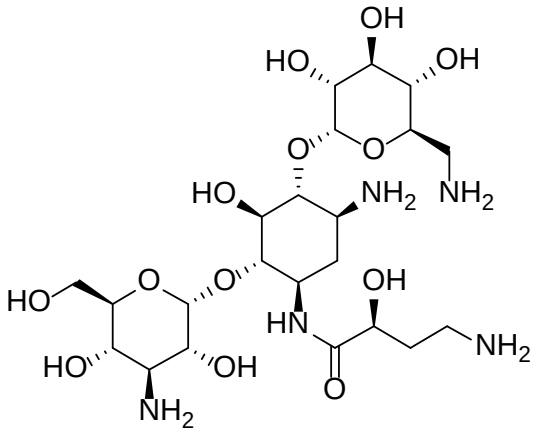
With the rising rate of resistance to all antibiotics traditionally used to treat Gram-negative bacterial infections including colistin, along with the lack of novel antimicrobials the only method for combatting such infections is to resort to combination therapy. In the case of colistin resistant organisms, combining colistin with another class of antimicrobial can successfully inhibit the growth of the bacteria. Combinations that have been extensively studied can be of colistin and another Gram-negative targeting antimicrobial, such as carbapenems and aminoglycosides, with varying results (52-54). The combinations of meropenem in particular, fosfomycin or, tigecycline have shown promising results; a study conducted in 2018 at AUBMC demonstrated the potency of both combinations in targeting carbapenem-resistant *E. coli*, albeit the 82%-84% of the isolates used in the study were susceptible to colistin (55). Alternatively, the presence of colistin has been hypothesized to increase the permeability of the outer membrane of Gram-negative organisms, causing it to allow the uptake of antimicrobials that it had been previously impermeable towards (56).

For this study, we aimed to assess the utility of antimicrobial combinations for potential therapeutic treatment of *A. baumannii* and *E. coli* isolates that are resistant to colistin. The antibiotics that we selected for that purpose were those that had not been previously studied in the literature for their efficacy in targeting colistin resistant

isolates (whether *in vitro* or *in vivo*), and had different mechanisms of action in targeting the bacteria.

1. Amikacin

As an aminoglycoside, amikacin's spectrum of bactericidal activity includes Gram-negative bacteria, and have therefore been studied for their effect in combination with colistin, which resulted in varying success. A 2017 study for instance showed significant synergistic activity between the two antimicrobials against *E. coli* co-producing *mcr*-1 and NDM-5, a type of ß-lactamase (57). While another study demonstrated limited synergy between colistin and amikacin against XDR *A. baumannii* strains (58).

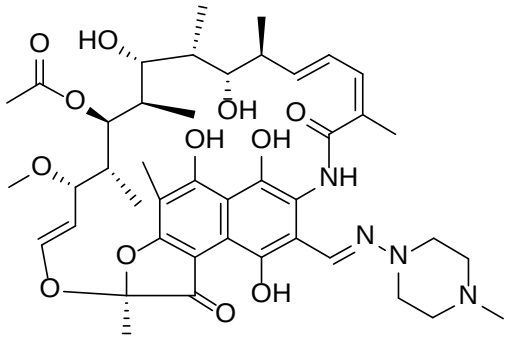


Amikacin

2. Rifampicin

Rifampicin is a semi-synthetic naphthoquinone that is most commonly used in combination with other antimicrobials for the treatment of *Mycobacterium tuberculosis*. Rifampicin inhibits the initiation of RNA synthesis by forming a stable complex with the bacterial DNA-dependent RNA polymerase. Toxicity to rifampicin is minimal, nausea and unconsciousness may result after chronic

exposure (59). Various studies have demonstrated successful synergy between rifampicin and colistin at low concentrations of both drugs against *A. baumannii* isolates *in vitro* (58); however, more studies should be performed *in vivo* to better assess the success of this combination.



Rifampicin

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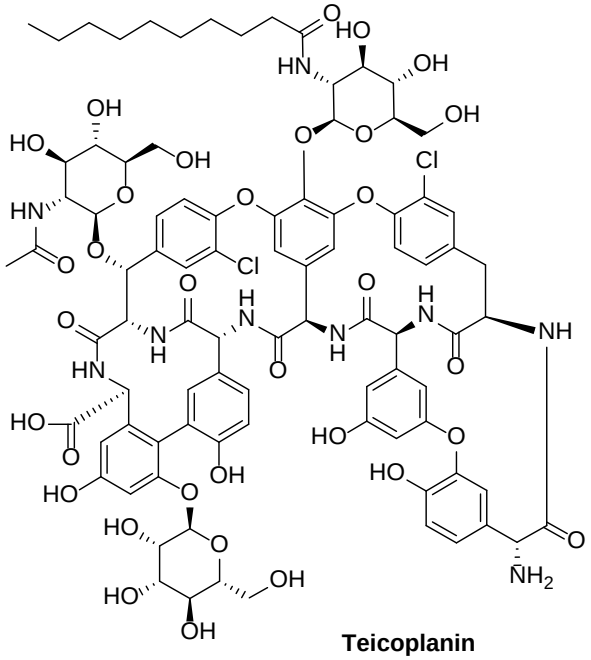
3. Teicoplanin

Teicoplanin is a naturally produced lipoglycopeptide that is used for the treatment of Gram-positive organisms, such as Methicillin-resistant *Staphylococcus aureus* (MRSA) and *Enterococcus faecalis*. It is very similar to vancomycin (a glycopeptide) both structurally and functionally, although studies show that adverse effects of teicoplanin, including nephrotoxicity, are less frequent (60). Teicoplanin binds to the D-Ala-D-Ala (Lipid II) moiety linking the *N*-Acetyl muramine (NAM) and *N*-Acetyl glutamine (NAG) subunits of the peptidoglycan layer of the bacterial cell wall, this prevents the polymerization of the peptidoglycan, inhibiting cell wall synthesis and leading to cell death (61). Several studies tackled the combination of vancomycin and colistin successfully against colistin resistant *A. baumannii* strains (62, 63). Since teicoplanin exerts

its antimicrobial activity in a similar process to vancomycin, and is proven to be significantly safer, it may be considered as a viable treatment alternative.



Teicoplanin

CHAPTER III

MATERIALS AND METHODS

A. Source of *A. baumannii* and *E. coli* strains

Twenty-six non-duplicate MDR *A. baumannii* (ACN) isolates were collected between 2012 and 2015, and 4 *E. coli* (ECOL) isolates were collected in 2016 and 2017, from the Clinical Microbiology Laboratory at the Department of Pathology and Laboratory Medicine, American University of Beirut Medical Center (AUBMC). Seven Isolates of ACN were obtained from Al Makased General Hospital and one MCR-1 expressing ECOL isolate was isolated from poultry. In addition, 2 MCR-1 expressing *E. coli* isolates were obtained from the Antimicrobial Resistance (AR) Isolate Bank (AR #0346, AR #0349) from the CDC, and 2 wild-type strains were obtained from the American Type Culture Collection (ATCC); 1 being an ACN wild-type isolate (ATCC15308), and the other an ECOL (ATCC225922). The isolates were stored in Brucella Broth (BD BBL™, USA) with 10% glycerol at -80 ˚C.

B. Antimicrobial Agents

This study was designed to evaluate colistin in combination with rifampicin, teicoplanin and amikacin as combination therapy options for the treatment of infections caused by colistin resistant ACN and ECOL. Stocks of the antimicrobials were freshly prepared in appropriate solvents, and stored at -20 ˚C. Teicoplanin (Sanofi Aventis, France) was prepared in dimethyl sulfoxide (DMSO) (Malinckrodt AR®, UK), rifampicin (Sigma, USA) was prepared using methanol, while amikacin sulfate (Amfarm Hellas, Greece) and colistin sulfate powders (Sigma, USA) were dissolved in sterile water.

C. Antimicrobial Susceptibility Testing

Antimicrobial susceptibility testing was performed in accordance with the Clinical and Laboratory Standard Institute (CLSI) guidelines (64). Broth microdilution (BMD) was performed on all strains to determine their susceptibility to colistin, rifampicin, teicoplanin and amikacin in duplicates. 100 μL of Mueller Hinton Cation-adjusted broth (MHCAB) (BD BBL™, USA) was added to each well in a 96-well U-bottom microtiter plate (CoStar®, US). Next, 100 μL of the drug solution was then added to the first well in each of the test rows, and a series of 2-fold serial dilutions was performed from a highest drug concentration of 256 μg/mL to the lowest concentration of 0.125 μg/mL. Bacterial inoculum was prepared by adjusting bacterial suspension in MHCAB to 0.5 McFarland using a turbidometer (Densimat®, USA). It was then diluted in MHCAB so that after inoculating each well with 10 μL of the bacterial suspension, it would contain approximately 10^5 CFU/mL. A row in each plate had neither antimicrobial agent added nor bacterial inoculum, only 100 μL of MHCAB, and thus served as a negative or sterility control. Another row contained 100 μL of MHCAB and 10 μL of the bacterial inoculum, and served as a positive, or growth control. ECOL ATCC25922 or ACN ATCC15308 were used as quality control strains with each run of BMD. The plates were incubated overnight at 37 ˚C in a shaker-incubator. Results were recorded after 20 h of incubation, and confirmed by measuring the optical density of the wells using the Berthold Technologies® (USA) TriStar^{2}S multimode plate reader. The minimum inhibitory concentration (MIC) was defined as the highest dilution of the antimicrobial with no visible growth or turbidity. Breakpoints for susceptibility and resistance to the tested antimicrobials were applied in accordance with CLSI MIC interpretive standards; whereby resistance to colistin was defined as MIC $\geq$ 4 μg/mL and susceptibility as

MIC ≤ 2 μg/mL. Resistance to rifampicin was defined as MIC ≥ 4 μg/mL, intermediance as MIC = 2 μg/mL, and susceptibility as MIC ≤ 1μg/mL. While resistance to amikacin was defined as MIC ≥ 64 μg/mL, intermediance as MIC ß= 32 μg/mL, and susceptibility as MIC ≤ 16 μg/mL. Nonetheless, there are no interpretive MIC criteria published by the CLSI for teicoplanin in treatment of Gram-negative organisms, since it is a Gram-positive targeting antimicrobial.

D. *In-vitro* Antimicrobial Synergy Testing

1. Checkerboard Assay

Synergy between colistin and rifampicin, colistin and teicoplanin, or colistin and amikacin at a range of concentrations was assessed by using the checkerboard method. Ninety-six-well microtiter plates containing MHCAB were incubated with vertically doubling concentrations of colistin, and horizontally doubling concentrations of the other drug, such so that each combination well contained 50 μL of the colistin solution in sterile water, and 50 μL of the solution of the other drug (drug B). In the case of rifampicin and teicoplanin, the drug B solutions were prepared by adding 5 μL of the stock to 45 μL of MHCAB. The first column in a microtiter well plate contained 50 μL of serially diluted colistin alone, and the first row only contained 50 μL of the drug B solution. Fifty μL of bacterial suspension was added to the wells such so that the final bacterial concentration in each well was approximately 10^5 CFU/mL. One well was designated as a negative sterility control and contained broth only, another was a positive growth control and contained broth with the bacterial inoculum. The concentration gradients of each antimicrobial were chosen such so that they showed the exact MIC of each drug alone, as well as the MIC of the drug when combined with colistin, on one 96-well microtiter plate. This was achieved when utilizing a

concentration ranging from 4096 µg/mL to 64 µg/mL of colistin, or from 2048 µg/mL to 2 µg/mL of teicoplanin, amikacin and rifampicin, when assessing the ACN isolates. On the other hand, colistin concentrations for the ATCC and CDC isolates ranged between 16 µg/mL and 0.25 µg/mL. Each colistin resistant (ColR), ATCC and CDC isolate was tested with the three combinations in duplicates. The plates were incubated overnight in a 37 ˚C shaker-incubator. Turbidity was observed visually and confirmed by measuring the optical density of the wells. The Fractional Inhibitory Concentration Index (FICI) of each of the combinations was then determined by obtaining the sum of the MIC of drug A in the combination/MIC of drug A alone and the MIC of drug B in the combination/MIC of drug B alone. Synergism was defined as $\Sigma FIC \leq 0.5$, indifference as a $\Sigma FIC > 0.5$ and < 2, and Antagonism as a $\Sigma FIC \geq 2$.

2. Time-Kill Assay

Time-kill assays were performed in accordance with CLSI guidelines (64) on the ColR, CDC, and ATCC isolates using concentrations based on the MICs showing synergy as determined by the checkerboard assays. For each tested isolate, 5 (15-mL) reaction tubes were used, each consisting of:

- Positive control: 4.5 mL MHCAB + 0.5 mL ~10^6 CFU/mL bacterial inoculum
- Colistin monotherapy: 4 mL MHCAB + 0.5 mL COL + 0.5 mL inoculum
- Rifampicin/Teicoplanin monotherapy: 4.45 mL MHCAB + 0.05 mL Rifampicin/Teicoplanin + 0.5 mL inoculum
- Negative control: 5 mL MHCAB

The reaction tubes were incubated in a shaker-incubator at 37 ˚C in ambient air for 24 h. During which, 0.1 mL aliquots were obtained from each of the reaction tubes at times 0

and 0.5, 1, 2, 4 and 24 h post-inoculation, and were serially diluted in 0.85% sodium chloride solution for the purpose determination of viable colony counts; whereby counts that exceeded 300 CFU, or were less 30 CFU per plate were excluded. Diluted samples, 0.02 mL, were cultured on Mueller-Hinton agar (Conda Pronadisa, Germany) plates. Total bacterial counts were determined after 18 h of incubation at 37˚C and reported as CFU/mL. The log10 of the CFU/mL was calculated and charted against time, with synergism being defined as a > 2 x log10 decrease in colony counts with the antimicrobial combination when compared to the most active single agent. A > 2 x log10 increase in counts with the antimicrobial combination was interpreted to be antagonistic, while a < 2 x log10 increase or decrease indicated that the combination was indifferent.

E. Limulus Amebocyte Lysate Endotoxin Test

The E-TOXATE™ (Sigma-Aldrich®, USA) Limulus Amebocyte Lysate (LAL) test was used to assess the production of bacterial LPS by three *A. baumannii* isolates: ACN1, ACN2, and ATCC15308

1. ***Materials used:***
    - E-TOXATE Reagent (with kit)
    - E-TOXATE Endotoxin Standard (with kit)
    - E-TOXATE Water, endotoxin free (with kit)
    - Sterile (10 x 75 mm) glass tubes
    - Sterile water
    - Heat block
2. ***Protocol:***

- Each of the test isolates was grown in 3 mL of TSB broth overnight in a shaker-incubator at 37 °C
- The bacterial cultures were then centrifuges for 20 minutes at 3500 rpm, the supernatant was discarded and the bacteria was re-suspended in TSB broth
- 9 (10 x 75 mm) glass tubes were labeled and the Endotoxin Standard was prepared using serial dilution according to the table below:

Tube No.	Endotoxin	E-TOXATE Water (mL)	Final Concentration (EU/mL)
1	0.2 ml Endotoxin Std. Stock Soln.	1.8	400
2	0.2 ml from Tube No. 1	1.8	40
3	0.2 ml from Tube No. 2	1.8	4
4	0.3 ml from Tube No. 3	2.1	0.5
5	1 ml from Tube No. 4	1.0	0.25
6	1 ml from Tube No. 5	1.0	0.125
7	1 ml from Tube No. 6	1.0	0.06
8	1 ml from Tube No. 7	1.0	0.03
9	1 ml from Tube No. 8	1.0	0.015

- For each of the test isolates, 4 (10 x 75 mm) glass tubes were prepared according to the table below:

Tube		Sample	E-TOXATE Water	Endotoxin Std. Dilution	E-TOXATE Reagent working solution
A	test for endotoxin in sample	0.1 mL	-	-	0.1 mL
B	test for inhibitor in sample	0.1 mL	-	0.01 mL of 4 EU/ml (Tube no. 3)	0.1 mL
C	negative control	-	0.1 mL	-	0.1 mL
D	standard	-	-	0.1 ml of 0.5 EU/ml (Tube no. 4)	0.1 mL

- The tubes were swirled gently, covered with Parafilm, and then placed in a hot plate for 1 hour at 37 °C

- The tubes were then removed, held horizontally and observed for evidence of gelation, indicating a positive reaction. The results were interpreted according to the following criteria:

Tube				Interpretation
A	B	C	D	
−	+	−	+	No endotoxin, or endotoxin is below detection level
+	+	−	+	Positive result; sample contains endotoxin ≥ the amount present in the most dilute Endotoxin Standard.
+	+	+	+	Possible contamination; sample result not valid.
−	−	−	+	Absence of hard gel in Tube B and presence of hard gel in Tube D show that sample contains an inhibitor of the E-TOXATE Reagent. Test is not valid.
±	±	−	−	E-TOXATE Reagent or Endotoxin Standard has deteriorated. Sample results are not valid.

F. **RNA extraction**

RNA extraction was performed using the iIustra^{TM} RNAspin Mini RNA Isolation Kit (GE healthcare, UK) according to the manufacturer's specifications for bacterial cells. RNA was extracted from ColR isolates ACN1, ACN12, ACN13, and colistin susceptible (ColS) isolates ACN6, ACN27, and ATCC15308.

It was also performed on 6 samples incubated *in vitro* according to the following protocol to assess the effect antimicrobial monotherapy and combination therapy on ACN2:

Tube 1: contained 3 mL 10^6 ACN2 bacterial suspension in MHCAB

Tubes 2, 3, 4: 1.5 mL bacterial suspension + 1.5 mL of COL, RIF and TEC, respectively at a concentration equivalent to the MICs obtained from the checkerboard assays

Tubes 5 and 6: 1 mL bacterial suspension + 1 mL COL + 1mL RIF, TEC, respectively.

RNA was also extracted from the pooled serum of the dissected mice from each of the *in vivo* test groups infected with ACN 2, after centrifugation at 3500 rpm for 20 minutes at 4 ºC.

1. ***Materials used:***

- Lysis Solution (RA1) (with kit)
- Desalting Buffer (MDB) (with kit)
- DNase I (with kit)
- Wash Buffer I (RA2) (with kit)
- Wash Buffer II (RA3) (with kit)
- RNase-free water (with kit)
- TE Buffer (AMRESCO®, USA)
- Lysozyme from chicken egg white (Sigma®, USA)
- β-Mercaptoethanol (β-ME)
- 70% Ethanol
- RiboLock RNase Inhibitor (Thermo Scientific™, USA)

2. ***Protocol:***

a. <u>Cell lysis and Homogenization</u>

- Cultures of the bacterial isolates were grown overnight in TSB broth at 37 ˚C. For each of the isolates, a bacterial suspension was prepared by a 1:100 dilution of 0.5 McFarland, to reach a total of 1×10^6 CFU/mL.
- 1.5 mL of culture broth was transferred to a microcentifuge tube, and then centrifuged at maximum speed for 15 minutes.

- The supernatant from each sample was discarded and the bacterial cell pellet was re-suspended in 100 μL of TE buffer containing 0.2 mg/mL lysozyme and was vortexed vigorously. The tubes were then incubated at 37 ˚C for 10 minutes.
- Afterwards, 350 μL of RA1 buffer and 3.5 μL of β-ME were added to each tube.

b. Filtration of lysate

- The mixture was transferred to a violet RNAspin Mini filter unit placed in a collection tube and was centrifuged for 1 minute at 11,000 x *g*.
- The filtrate was then transferred to a new 1.5 mL RNase-free microcentrifuge tube and the RNAspin Mini filter unit was discarded.

c. RNA binding in adjusted conditions

- 350 μL of previously prepared 70% ethanol was added to each filtrate and mixed by pipetting up and down prior to the transfer to a blue RNAspin Mini column placed in a new collection tube.
- The samples were centrifuged for 30 seconds at 8,000 x *g* and the column was placed in a new collection tube.

d. Desalting of silica membrane and DNA digestion

- To each column, 350 μL of Membrane Desalting Buffer (MDB) was added. Next, the samples were centrifuged for 1 min at 11,000 x *g* to dry the membrane.
- Afterwards, the filtrate was discarded and the column was returned to the same collection tube.

- A DNase reaction mixture was prepared by adding 10 µL reconstituted DNase I to 90 µL DNase reaction buffer (per sample). The solution was mixed by flicking the tube several times.
- For each sample, 95 µL of the DNase I reaction mixture was added directly onto the center of the column. The samples were incubated at room temperature for 15 minutes.

e. Washing and Drying

- To each of the RNA spin Mini column, 200 µL of buffer RA2 was added, followed by centrifugation for 1 minute at 11,000 x *g*. The column was placed into a new collection tube.
- A 600 µL of buffer RA3 was added to each RNA spin Mini column, and then centrifuged for 1 minute at 11,000 x *g*. The filtrate was discarded and the column was returned to the same collection tube.
- A 200 µL of buffer RA3 was added to each RNA spin Mini column, the samples were centrifuged for 2 minute at 11,000 x *g*. The column of each sample was transferred into a nuclease free 1.5 mL microcentrifuge tube.

f. Elution and Aliquoting

- A 100 µL RNase free water was added to the samples, followed by centrifugation for 1 minute at 11,000 x *g* to elute the RNA.
- The tubes were placed on ice to prevent potential degradation.
- Next, 1 µL Ribolock RNase inhibitor was added to each sample. Aliquots of 20 µL were prepared and stored at -80 ˚C for further use.

Extracted RNA concentrations and purity were measured using the NanoDrop 1000

spectrophotometer (Thermo Scientific™, USA).

G. Reverse Transcription and cDNA synthesis

The iScript™ cDNA Synthesis Kit (Bio-Rad, USA) was used, according to the manufacturer's specifications, for the production of cDNA from the previously extracted RNA. The cDNA was synthesized to be used in the subsequent Reverse Transcriptase – quantitative PCR (RT-qPCR) experiments.

1. Materials used:

- Extracted RNA
- Nuclease free water (with kit)
- iScript™ Reaction Mix, containing oligo-dT dissolved in water (with kit)
- iScript™ Reverse Transcriptase (RNase H+) (with kit)

2. *Protocol*:

All the reagents and the RNA samples were kept on ice while performing the procedure.

- The preparation of a master mix which contained 4 μL of iScript™ Reaction Mix (per sample), 1 μL of iScript reverse Transcriptase (per sample), and 4 μL of nuclease-free water (per sample), and was carried out on ice.
- Next, 6 μL of the master mix was mixed with the 14 μL mixture of each sample of extracted RNA, producing a total reaction volume for each tested sample of 20 μL.
- The samples were then placed in a thermal cycler to manage incubation conditions of: 5 minutes at 25 ˚C followed by 20 minutes at 46 ˚C,

1 minute at 95 ˚C. Lastly, the resulting cDNA were stored at -80 ˚C until further use.

Resulting cDNA concentrations and purity were measured using the NanoDrop 1000 spectrophotometer (Thermo Scientific™, USA).

H. Real Time Polymerase Chain Reaction (RT-qPCR)

The iTaq™ Universal SYBR® Green Supermix kit (Bio-Rad, USA) was used to examine and compare the levels of expression of the lipid biosynthesis genes *lpxA*, *lpx*C and *lpx*D, and phosphoethanolamine transferase *pmr*A, *pmr*B and *pmr*C genes, which are associated with colistin resistance in ColR and ColS ACN isolates. The primers that were used (Table 1) were reconstituted in specific volumes of TE according to the manufacture's guidelines.

1. Materials used:

- cDNA templates
- Nuclease free water (with kit)
- Real Time forward and reverse primers
- iTaq™ Universal SYBR® Green Supermix (with kit) including:
    a) dNTP mix
    b) SYBR® Green I Dye
    c) Antibody-mediated hot-start iTaq DNA Polymerase
    d) $MgCl_2$, enhancer, stabilizers, and passive reference dyes

2. *Protocol*:

- All the samples and reagents used in this experiment were thawed on ice to room temperature.

- A reaction mix was prepared by combining: 5 µL of iTaq™ Universal SYBR® Green Supermix, 2 µL of nuclease free water, 1 µL forward primer, and 1 µL reverse primer, per sample, producing a total volume 9 µL.
- A qPCR plate was used for each run. Whereby into each well, 9 µL from the reaction mix for the corresponding gene was transferred.
- Next, 1 µL of the cDNA samples was added into the wells, for a total volume of 10 µL. 1 µL of nuclease-free water was added to some of the wells, which served as negative controls
- The plate was then sealed, vortexed for 30 seconds, and spun to get rid of air bubbles.
- The Bio-Rad CFX96 Real Time System C1000 Thermal Cycler (Bio-Rad, USA) was used for the Real time runs; and the cycling condition steps for each primer were as follows:
    1. 1 cycle of 95 ˚C for 30 seconds (for polymerase activation and initial DNA denaturation)
    2. 45 cycles of 95 ˚C for 5 seconds (DNA denaturation)
    3. 45 cycles of 55.9 ˚C for 30 seconds (annealing) for *lpx*A, *lpx*C and *lpx*D primers, or 58.4 ˚C for *pmr*A, *pmr*B and *pmr*C primers
    4. 45 cycles of 72 ˚C for 20 seconds (elongation/extension)
    5. Melt curve 65 ˚C to 95 ˚C, increments of 0.5 ˚C at 5 seconds/step (melt curve analysis)
    6. Hold at 12 ˚C for 5 minutes.

The expression levels of the genes in question were calculated for the samples that are ColR and compared to the ColS samples, employing the reference housekeeping gene *rpo*B as a standard, using the Bio-Rad (Bio-Rad, USA) CFX manager software.

3. Statistical analysis:

The unpaired student's t-test was used for statistical analysis, in which p-values ≤ 0.05 were considered statistically significant.

I. *In-vivo* Antimicrobial Synergy Testing

1. *Induction of Neutropenia in the BALB/c Mice:*

The mice used in this study were obtained from the Animal Care Facility, after the approval from the Institutional Animal Care and Use Committee (IACUC) at AUB. The mice in use were BALB/c, adult male, ranging between 6-8 weeks old, and weighing between 30-40 g. A total number of 116 mice were used. The mice were cared for and handled according to "Guide for the Care and Use of Laboratory Animals" prepared by the Institute of Laboratory Animal Resources Committee on Life Sciences Nation Research Council. In addition, the mice were allowed to consume food and water without any restrictions.

All the mice used in these experiments were rendered neutropenic using a previously described cyclophosphamide (CPM) regimen (65). CPM powder (Baxter, USA) was dissolved in fresh saline according to package instructions and then dissolved to a create the first dose of CPM; equivalent to a concentration of 150 mg/kg, 0.4 mL of the CPM solution was administered to each mouse intraperitoneally (Day1). On Day 4, the mice were intraperitoneally injected with 0.4 mL of the second dose of CPM solution; equivalent to 100 mg/kg CPM solution. A group of 6 mice that were rendered

neutropenic were monitored for 7 days prior to start of further experimentation to ensure that the dose was not lethal. The number of granulocytes were determined through microscopic examination of blood that was withdrawn from one mice per group to confirm neutropenia. Any bacterial inoculations were performed 24 h after the 2nd dose of CPM was administered (Day 5).

2. *Treatment of Infected BALB/c Mice Using Antimicrobial Agents for Assessment of Efficacy of Combination Therapy:*

a) Preparation of bacterial suspension for infection:

Prior to the start of the experiment, a pilot study was conducted whereby 20 mice were intranasaly infected with the ColR ACN2 in incremental doses of the bacterial inoculum. 4 h post-infection, 1 mouse from each group was dissected, the blood was collected from the lungs, and cultured on MacConkey agar plates to check for the presence of the respective isolate. After that, the colonies grown on the plates were identified to the species level using API®20NE kits (Biomérieux, France), which allowed the verification of the induction of pneumonia in the animals.

A sub-lethal bacterial dose of 1.5 x 10^9 CFU/mL of ACN2 was determined to be infective and was administered to the mice intranasaly using a micropipette (25µL per nostril). To prepare the bacterial iocula, freshly isolated colonies were inoculated in 3 mL TSB and incubated overnight at 37 ˚C. On the next day, a turbidity meter was used to determine the concentration (CFU/mL) of the bacterial suspension. A volume was transferred to a centrifuge tube and spun for 20 minutes at 3500 rpm. Following that, the supernatant was discarded, and the pellet was re-suspended in a volume of TSB corresponding to the total volume of bacterial inocultation needed (50 µL/mouse).

b) Preparation of antibacterial agent injections:

The therapeutically relevant *in vivo* doses of the antimicrobial agents were prepared in accordance with their previously published pharmacokinetic and pharmacodynamic properties in *in vivo* mouse models. Doses of 20 mg/kg of CMS and 12 mg/kg of teicoplanin were prepared in sterile water, and the injections were carried out twice daily. Rifampicin, on the other hand, was dissolved in DMSO then slowly diluted in sterile water to produce doses of 25 mg/kg, which were administered once every 24 h.

c) Mice groups:

A total number of 96 mice were used in this experiment, and divided into 8 groups containing 12 mice each, with each group being assigned different types of injections. Table 2 shows the distribution of mice into various groups with different treatment regimens for each group. The injections were calculated so that the maximum total volume to be administered per mouse would not exceed 0.5 mL. The antibacterial agents that were used were colistimethate sodium (CMS), rifampicin, and teicoplanin. The mice were anesthetized with Isoflurane (Forane ®, Baxter, USA), and bacterial inoculations were performed intranasaly using a 100-µL pipet, whereby 25 µL of the bacterial suspension was administered to each nostril, drop by drop at t = 0 hr. Followed by intraperitoneal treatment regimens, which were administered at t =2 h. The antimicrobial agent volume administered was 0.1 mL for each antibacterial agent. Six of the mice in each group were monitored for a period of 7 days.

d) Mice Dissection:

Six mice from each group were sacrificed and dissected at 24 h of induction of pneumonia. Blood was collected from the heart, diluted in fresh saline and 20 µL were cultured on MacConkey agar plates, the rest of the blood collected from each of the test

groups was centrifuged for 20 minutes at 3500 rpm, in order to separate the serum that was later used for RNA extraction. Lungs were also collected, homogenized, diluted in fresh saline and then cultured on MacConkey agar plates to compare the colony counts from each groups in CFU/mL. API®20NE kits were used to confirm that the species of the bacteria isolated from the mice was indeed *A. baumannii.*

4. Statistical analysis:

The unpaired student's t-test was used for statistical analysis, in which p-values ≤ 0.05 were considered statistically significant.

CHAPTER IV

RESULTS

A. Antimicrobial Susceptibility Testing:

Thirty-three ACN and four ECOL isolates obtained from different sources and testing of their susceptibility to COL was previously conducted at the Department of Pathology and Laboratory Medicine at AUBMC using disk diffusion, the E-test method, and Vitek® system (Table 3). For our study, we confirmed the results by performing BMD, the results for which are indicated in Table 3. Of all the tested isolates, 5 isolates (ACN1, ACN2, ACN3, ACN12, and ACN13) showed resistance to colistin (COL) in accordance with CLSI guidelines (64), with MICs that were ≥ 128 μg/mL. The minimum inhibitory concentrations (MIC) of rifampicin (RIF), amikacin (AMK), and teicoplanin (TEC) were also obtained for these isolates, as well as for the CDC isolates (346 and 349) and the two ATCC strains (ATCC25922 and ATCC15308) (Table 3). Additionally, the MICs of COL against ACN4, ECOL4, and ATCC25922 which were chosen for the induction of COL resistance study are indicated in Table 4, with first column showing the susceptibility of the isolates to COL at the start of the experiment. As the serial passages were performed, susceptibility to COL gradually decreased, until resistance was achieved by the fourth run.

B. *In-vitro* Assessment of Combination Therapy

1. Checkerboard assay results:

Checkerboard (CB) assays were performed to test the combinations of COL + RIF, COL + TEC, and COL + AMK against the ColR ACN1, ACN2, ACN3, ACN12, ACN13, as well as ATCC25922, ATCC15308 (for quality assurance), and CDC isolates 346 and 349. Results of the CB assays for the ACN isolates showed that synergy was achieved ($\sum$ FICI < 0.5) between COL and RIF, and COL and TEC even at the lowest tested COL concentration of 64 μg/mL. However, ACN13 required relatively high concentrations of all antimicrobials and was excluded from subsequent assays (Table 5). Moreover, due to the fact that the $\sum$ FICI was relatively low for all the ACN isolates, additional CB assays were performed on ACN1, ACN2, ACN3, and ACN12 using a lower set of COL concentrations ranging between 0.25 μg/mL to 16 μg/mL, and a higher set of RIF and TEC concentration ranging between 4 μg/mL to 4096 μg/mL. Synergy was observed at a COL concentration that was as low as 4 μg/mL when combined with 16 μg/mL of RIF, and when combined with 512 or 1024 μg/mL TEC (Table 6). As for the CDC and ATCC control isolates, they all displayed synergy with RIF, and indifference with TEC, except for ATCC25922. Furthermore, the combination of AMK and COL displayed an indifferent effect against all the tested isolates ($2 > \sum FIC \geq 0.5$); thus, AMK was excluded from further assessments (Table 5).

2. Time-kill assay results:

Time-kill assays (TK) were performed to evaluate the effect of synergy at the concentrations obtained from the checkerboard assays over a period of 24 hours against the growth of ACN1, ACN2, ACN3, ACN12, CDC346, CDC349, ATCC25922, and ATCC15308. Our results show that, when combining 4 μg/mL COL with 16 μg/mL RIF, synergy (> 2 x Log10 decrease in the CFU/mL of the combination compared to the

most active agent) occurred between 1 and 2 hours of incubation with the ACN bacterial inoculums, with complete eradication of growth occurring after 2 or 4 hours of incubation (Figure 1, Table 7).

As for the COL + TEC combination, when testing against the ACN isolates, TEC monotherapy of 1024 μg/mL was demonstrated to be as potent as the combination of 4 μg/mL COL and 1024 μg/mL TEC. However, when a TEC concentration of 128 μg/mL was attempted in combination with 4 μg/mL of COL, the TK results of that combination against the ACN isolates was demonstrated to be synergistic at 2 or 4 hours of incubation with the bacterial inoculum, at which times complete eradication of bacterial growth was also observed (Figure 1).

As for the CDC isolates, since no synergy was observed between COL and TEC in the checkerboard assays, only RIF was assessed for its effect in combination with COL. The results show that for CDC346, while the combination of the two antimicrobials was more efficacious, the significant decrease of > 2 x log10 in CFU/mL only occurred towards the end of the 24 h incubation period. Alternatively, in the case of CDC349, monotherapy of RIF displayed almost identical results to the combination of COL and RIF, indicating that the combination is indifferent over time against the *mcr*-1 harboring CDC ECOL isolates (Figure 2). The time-kill assays performed on the wild-type ATCC samples 25922 (ECOL), and the 15308 strain (ACN) demonstrated bactericidal effect for both COL monotherapy and combination therapy, against both isolates, with RIF monotherapy being efficacious against ATCC25922 as well (Figure 4, Table 7).

C. Limulus Amebocyte Lysate Endotoxin Test

The LAL test results demonstrated clot formation in the liquid cultures of all the three tested isolates (ACN1, ACN2, ATCC15308), indicating the possible production of LPS by the three isolates.

D. *In vitro* Relative levels of expression by RT-qPCR:

The results of the RT-qPCR demonstrate the levels of expression of the *pmr*A, *pmr*B, and *pmr*C genes, and of the lipid biosynthesis genes *lpx*A, *lpx*C, and *lpx*D, when compared to the wild-type ATCC15308 strain, as indicated in Figures 5 and 6. They show that for ColR ACN12 and ACN13, the levels of expression of the *pmr* genes were relatively high. This was not the case for the ACN1 isolate, which exhibited a decrease in the level of expression for the *pmr* genes, as well as a reduction in the expression of all the tested lipid biosynthesis genes.

As for the ColS isolates (ACN6 and ACN27), the levels of expression of *pmr*B and *pmr*C were relatively low in ACN6 when compared to the positive control (the ATCC strain). Their expression in ACN27 was higher than in the wild-type strain but nonetheless reduced when compared to the ColR ACN12 AND ACN13. *lpx*A was only expressed in ACN27 according to our data, and with that an overexpression of *lpx*D was observed in all the tested isolates. The fold-increase in the expression levels of *lpx*D was higher in the ColS isolates than in ColR ones (Table 9).

Moreover, the *in vitro* RT-qPCR results of the ACN2 isolate incubated with COL, RIF, and TEC separately and in combination revealed varied expression levels in comparison to the positive control of ACN2 that was incubated sans antimicrobial solutions (Figure 9). All the lipid biosynthesis genes were under-expressed upon incubation with the

COL-RIF combination (COMB1), including a 7-fold reduction in the expression of *lpx*C. The *pmr* genes however appeared to be overexpressed as a response to COMB1 resulting in a 7-fold increase in the level of expression of *pmr*A. The COL-TEC combination on the other hand caused the over-expression of the pmr genes and the under-expression of the lipid biosynthesis genes showing an 8-fold increase in *lpx*A and a 5-fold decrease in *pmr*B (Table 10).

E. *In-vivo* Assessment of Combination Therapy

The combinations that showed synergy *in vitro* were tested in a neutropenic *A. baumannii* pneumonia BALB/c mouse model. During the 7-day monitoring period, none of the mice in each of the test groups died as a result of the infection, indicating a sub-lethal infection, which is expected due to the use of a clinical colistin resistant strain as opposed to a hyper virulent standard strain (66). Our data showed that the bacterial load in each of the COL, RIF, and TEC monotherapy test groups was comparable to that of the untreated bacterial control group in both the respiratory tract and in the blood of the dissected animals. The data also demonstrated a relatively low level of bacterial dissemination into the circulatory system in all the test groups in association with the low level of virulence of the chosen strain (Figure 7).

A 3.6 Log_{10} reduction in the bacterial counts obtained from the lung homogenates of the mice that were treated with COMB1 (COL + RIF) was observed when compared to the bacterial control group, as well as to the COL and RIF monotherapy groups (Figure 7), indicating a synergistic effect in combating the bacterial load in the respiratory tract ($p < 0.05$). Moreover, the mice in the group treated with COMB1 showed a complete eradication of bacterial dissemination to the blood. On the other hand, the fold reduction

in the bacterial load of the animals treated with COMB2 (COL + TEC) was a 1.3-fold reduction in the lung homogenates, and a 1.7-fold reduction in their collected blood, indicating a less efficacious combination *in vivo* (Table 12).

The survival of all the mice in groups 2 and 3 that only received the COMB1 and COMB2 treatments and were not infected with the bacterial strain, along with the lack of physiological changes to the mice upon dissection after receiving the treatment over the monitoring period indicated that the combinations were not toxic to the animals.

The RT-qPCR results of the collected serum pooled from the dissected mice of each of the groups infected with ACN2 revealed different responses to the antimicrobial administrations. For the animal groups receiving antimicrobial monotherapies, the level of expression of the lipid biosynthesis genes was lower than the level of expression *in vitro*, especially for the in the case of *lpxD* (Figure 10). However, both groups receiving antimicrobial combination therapy displayed a significant overexpression of *lpxD* ($p < 0.05$), with a resultant 14-fold increase in the relative level of expression of *lpxD* of ACN2 in the animals receiving COMB1 treatment (Table 11). On the other hand, the *pmr*C gene in particular appeared to be overexpressed in all of the mice groups, with the highest expression level occurring in the COMB2 group that displayed a 7-fold increase.

Table 1: Primers used in the RT-qPCR for evaluation of the expression levels of colistin resistance genes (47).

Gene	Primer sequence (5'-3')	Amplicon Size
***lpx*A$_{AB}$**	F: TAGCATGATTGGTGGGGCTT R: GCATGTGCAGGGTTACCAGA	1180 bp
***lpx*C$_{AB}$**	F: TGAGTGCGATTGCCGGTTTA R: AGGAGCATCTTGTTCACGCA	1164 bp
***lpx*D$_{AB}$**	F: TATTCTAGCTGGGGCGTGTG R: CGCAAGCGTACAATCGTCTT	1502 bp
***pmr*A$_{AB}$**	F: TGGTATGCAAGTTTTGAAGCA R: GTAATTGATCTCGAGCAGAAATAAT	120 bp
***pmr*B$_{AB}$**	F: TGCTTACAAGGTTGCACTTCA R: TTCTTTCCGCTAAATCTTCATTT	150 bp
***pmr*C$_{AB}$**	F: GGTCGGTGTTTTACTTTTTACCTA R: CATCCCTTTTAAATCACGATGT	129 bp
***rpo*B**	F: GTGCTGACTTGACGCGTGAT R: AGCGTTCAGAAGAGAAGAACAAGTT	184 bp

Table 2: BALB/c mice distribution for *in-vivo* evaluation of combination therapy. CMS = colistimethate sodium, RIF = rifampicin, TEC = teicoplanin.

Group 1	Group 2	Group 3	Group 4	Group 5	Group 6	Group 7	Group 8
ACN	Combination1 (CMS + RIF)	Combinantion2 (CMS + TEC)	ACN + Combination1	ACN + Combination2	ACN + CMS	ACN + RIF	ACN + TEC

Table 3: Minimum inhibitory concentrations of COL against ACN and ECOL isolates obtained using the disk diffusion (DD), E-test, Vitek and BMD methods.
INT = interpretation, S = sensitive, R = resistant.

Code	Isolate #	DD(mm)	E-test (ug/ml)	Vitek	BMD (ug/ml)	INT
ACN1	2090	0	>256	≥16	128	R
ACN2	2285	0	32	4	>256	R
ACN3	2273	0	>256	≥16	>256	R
ACN12	2209	12	>256	4	>256	R
ACN13	R4570	0	>256	≥16	>256	R
ACN4	2493	0	0.75	≤0.5	1	S
ACN6	3630	14	2	≤0.5	1	S
ACN7	R4018KM	14	1	≤0.5	1	S
ACN8	TKM352	15	1	0.5	1	S
ACN9	R4020 LA	13	1	0.5	0.25	S
ACN10	HAR 168	15	0.5	≤0.5	1	S
ACN11	SAU 492	14	0.5	≤0.5	0.25	S
ACN5	R1314	12	1	≤0.5	1	S
ACN25	Hfnew R697	15	0.75	≤0.5	1	S
ACN14	PFU9	13	1	≤0.5	0.5	S
ACN15	R4139NC	13	2	≤0.5	0.25	S
ACN16	ZHIEK1	11	1	≤0.5	0.25	S
ACN17	EHR489	12	1	≤0.5	0.25	S
ACN18	R1334	14	0.75	≤0.5	<0.25	S
ACN19	UMMM99	12	0.5	≤0.5	0.5	S
ACN20	I3993JK	14	0.5	≤0.5	1	S
ACN21	M1561	14	0.75	≤0.5	0.5	S
ACN22	R4034MR	15	0.25	≤0.5	0.25	S
ACN23	R1681	14	1	≤0.5	1	S
ACN24	SGR1663	15	0.5	≤0.5	<0.25	S
ACN26	R1497	14	1	≤0.5	0.5	S
ACN27	B3091	14	0.5	≤0.5	0.5	S
ACN28	B5259	0	256	≥16	<0.25	S
ACN29	M3965	0	256	≥16	0.25	S
ACN30	U1357	14	0.5	≤0.5	<0.25	S
ACN31	I1136	14	0.25	≤0.5	0.25	S
ACN32	R0069	14	2	≤0.5	0.25	S
ACN33	R2730	14	0.75	≤0.5	0.25	S

Table 4: Antimicrobial susceptibility profiles for the colistin resistant ACN isolates, the ATCC control strains, and the *mcr*-1 harboring CDC isolates, which were obtained using BMD. All values are in μg/mL. COL = colistin, RIF = rifampicin, TEC = teicoplanin, AMK = amikacin, I = Interpretation, R = resistant, S = sensitive.

Isolate	RIF MIC	I	TEC MIC	I	AMK MIC	I	COL MIC	I
ACN1	256	R	128	-	>256	R	128	R
ACN2	128	R	128	-	>256	R	>256	R
ACN3	128	R	128	-	>256	R	>256	R
ACN12	64	R	128	-	>256	R	>256	R
ACN13	256	R	128	-	>256	R	>256	R
CDC346	16	R	>256	-	>256	R	4	R
CDC349	8	R	>256	-	2	S	2	S
ECOL ATCC25922	128	R	>256	-	4	S	0.5	S
ACN ATCC15308	256	R	>256	-	0.5	S	0.5	S

Table 5: Checkerboard assay results showing synergy (∑ FIC < 0.5) at the lowest concentration of colistin on the microtiter plate, as well as the concentrations showing the minimum value of indifference (2 > ∑ FIC ≥ 0.5). All MIC values are in μg/mL.
COL = colistin, RIF = rifampicin, TEC = teicoplanin, AMK = amikacin, COMB1= COL + RIF, COMB2 = COL +TEC, COMB3 = COL + AMK. I = Interpretation, SYN = synergy, IND = indifference.

Isolate	MIC $_{COL}$	MIC$_{COL}$ IN COMB1	MIC$_{RIF}$ IN COMB1	FIC$_{COL}$ +RIF	I	MIC$_{COL}$ IN COMB2	MIC $_{TEC}$ IN COMB2	FIC$_{COL}$ +TEC	I	MIC COL IN COMB3	MIC AMK IN COMB3	FIC$_{COL+AMK}$	I
ACN 1	1536	64	4	0.057	**SYN**	64	192	0.391	**SYN**	1024	1024	1.5	**IND**
ACN 2	512	64	2	0.156	**SYN**	64	128	0.187	**SYN**	128	1024	0.75	**IND**
ACN 3	2048	64	4	0.033	**SYN**	64	256	0.25	**SYN**	1024	512	0.75	**IND**
ACN 12	4096	64	2	0.017	**SYN**	64	256	0.141	**SYN**	1024	16	0.517	**IND**
ACN 13	2048	64	128	0.14	**SYN**	64	384	0.406	**SYN**	2048	1024	1.5	**IND**
CDC 349	4	0.25	16	0.313	**SYN**	0.25	2048	1.01	**IND**	2	1	1	**IND**
CDC 346	4	0.5	128	0.375	**SYN**	0.5	1024	0.625	**IND**	1	512	0.75	**IND**
ATCC 25922	2	0.25	16	0.25	**SYN**	0.125	128	0.188	**SYN**	0.5	2	0.75	**IND**
ATCC 15308	1	0.125	16	0.375	**SYN**	0.25	1024	0.75	**IND**	0.5	0.5	1.5	**IND**

Table 6: Results of the checkerboard assays using a lower range of colistin concentrations for ACN1, ACN2, ACN3, and ACN12. MIC values are in μg/mL.

Isolate	**MIC COL**	**MIC$_{COL\ IN\ COMB1}$**	**MIC$_{RIF\ IN\ COMB1}$**	**FIC$_{COL+RIF}$**	**I**	**MIC$_{COL\ IN\ COMB2}$**	**MIC $_{TEC}$ IN COMB2**	**FIC$_{COL+TEC}$**	**I**
ACN1	1536	4	16	0.003	**SYN**	4	1024	0.254	**SYN**
ACN2	512	4	16	0.039	**SYN**	4	1024	0.297	**SYN**
ACN3	2048	4	16	0.127	**SYN**	4	512	0.251	**SYN**
ACN12	4096	4	16	0.0169	**SYN**	4	512	0.2509	**SYN**

Table 7: Correlation between the tested combinations of antimicrobial agents against the ACN isolates used in this study, and the PCR results of the genes associated with COL resistance that were obtained from a previous study conducted at the Bacteriology and Molecular Microbiology Lab (12). MIC values are in μg/mL. I = Interpretation, SYN = synergy, IND = indifference.

Isolate	MIC_{COL}	$FIC_{COL+RIF}$	I	$FIC_{COL+TEC}$	I	$FIC_{COL+AMK}$	I	*lpxD*	*lpxA*	*lpxC*	*pmrA*	*pmrB*	*pmrC/eptA*
ACN 1	128	0.003	**SYN**	0.254	**SYN**	1.5	**IND**	-	-	-	-	-	+/+
ACN 2	>256	0.039	**SYN**	0.297	**SYN**	0.75	**IND**	+	-	+	-	-	+/+
ACN 3	>256	0.127	**SYN**	0.251	**SYN**	0.75	**IND**	+	-	+	-	+	+/+
ACN 12	>256	0.0169	**SYN**	0.2509	**SYN**	0.517	**IND**	+	-	+	-	+	+/+
ACN 13	>256	0.14	**SYN**	0.406	**SYN**	1.5	**IND**	-	+	+	+	+	+/+

Table 8: The PCR profiles of the samples that were selected for the RT-qPCR experiment. MIC values are in μg/mL. INT = interpretation, R = ColR, S = ColS. MIC values are in μg/mL.

Isolate	MIC_{COL}	INT	*lpxD*	*lpxA*	*lpxC*	*pmrA*	*pmrB*	*pmrC*
ACN 1	128	R	-	-	-	-	-	+
ACN 12	>256	R	+	-	+	-	+	+
ACN 13	>256	R	-	+	+	+	+	+
ACN 6	1	S	+	-	+	-	+	+
ACN27	0.5	S	-	+	+	+	+	+

Table 9: Relative expression of the *pmr*A, *pmr*B, and *pmr*C signaling genes and of the *lpx*A, *lpx*C, and *lpx*D lipid biosynthesis genes compared to the ACN ATCC strain. PC = positive control.

	Relative Gene Expression						Fold Increase (+) or decrease (-)						P value					
	***pmr*A**	***pmrB***	***pmr*C**	***lpx*A**	***lpx*C**	***lpx*D**	***pmr*A**	***pmrB***	***pmr*C**	***lpx*A**	***lpx*C**	***lpx*D**	***pmr*A**	***pmr B***	***pmr*C**	***lpx*A**	***lpx*C**	***lpx*D**
ATCC	1	1	1	1	1	1	-	-	-	-	-	-	-	-	-	-	-	-
ACN6	1.231	0.379	0.0544	-	0.543	4.084	+ 1.2	- 2.6	- 18	-	- 1.8	+ 4	0.0549	0.078	0.242	-	0.317	0.2075
ACN27	1	1.516	1.516	1.454	2.55	8.456	-	+ 1.5	+ 1.5	+ 1.5	+ 2.6	+ 8.5	0.1174	0.077	0.5301	0.554	0.849	0.586
ACN1	0.536	0.1015	0.134	-	0.13	2.35	- 2	- 10	- 7.5	-	- 7.7	+ 2.4	0.1502	0.0868	0.0015	-	0.0992	0.08
ACN12	1.624	0.435	1.624	-	0.732	3.81	+ 1.6	- 2.3	+ 1.6	-	- 1.4	+ 3.8	0.0746	0.0483	0.353	-	0.0655	0.524
ACN13	0.812	0.536	2.143	-	0.688	3.81	- 1.3	- 2	+ 2	-	- 1.5	+ 3.8	0.0077	0.678	0.0003	-	0.17	0.245

Table 10: *In vitro* relative gene expression of *pmr*A, *pmr*B, and *pmr*C signaling genes and of the *lpx*A, *lpx*C, and *lpx*D lipid biosynthesis genes of ACN2 in response to antimicrobial mono- and combination therapy combared to a positive control (PC) of ACN2 without treatment. COMB1 = COL+ RIF, COMB2 = COL + TEC.

	Relative Gene Expression						Fold Increase (+) or Decrease (-)						P value					
	***pmr*A**	***pmrB***	***pmr*C**	***lpx*A**	***lpx*C**	***lpx*D**	***pmr*A**	***pmrB***	***pmr*C**	***lpx*A**	***lpx*C**	***lpx*D**	***pmr*A**	***pmr B***	***pmr*C**	***lpx*A**	***lpx*C**	***lpx*D**
PC	1	1	1	1	1	1	-	-	-	-	-	-	-	-	-	-	-	-
COL	1.19	0.51	0.56	0.42	0.96	0.88	+ 1	- 2	- 2	- 2.4	- 1	- 1	0.1538	0.4388	0.245	0.17	0.0031	0.0124
RIF	0.53	0.37	0.21	1.79	0.59	0.88	- 2	- 2.6	- 5	+ 2	- 2	- 1	0.0669	0.043	0.0881	0.0017	0.154	0.0092
TEC	0.68	0.99	1.49	1.18	0.29	0.82	- 1.5	- 1	+ 1.5	+ 1	- 3.4	- 1	0.205	0.0535	0.0127	0.0024	0.204	0.0696
COMB1	7.14	4.17	3.4	0.16	0.15	0.21	+ 7	+ 4	+3	- 6	- 7	- 5	0.0461	0.0384	0.0319	0.0513	0.0954	0.0026
COMB2	0.53	0.2	0.28	8.11	4.17	5.88	- 2	- 5	- 3.5	+ 8	+ 4	+ 6	0.0378	0.0495	0.0241	0.0385	0.0012	0.0028

Table 11: *In vivo* relative gene expression of *pmr*A, *pmr*B, and *pmr*C signaling genes and of the *lpx*A, *lpx*C, and *lpx*D lipid biosynthesis genes of ACN2 in response to antimicrobial mono- and combination therapy combared to a positive control (PC) of ACN2 without treatment. COMB1 = COL+ RIF, COMB2 = COL + TEC.

	Relative Gene Expression						Fold Increase (+) or Decrease (-)						P value					
	***pmr*A**	***pmrB***	***pmr*C**	***lpx*A**	***lpx*C**	***lpx*D**	***pmr*A**	***pmrB***	***pmr*C**	***lpx*A**	***lpx*C**	***lpx*D**	***pmr*A**	***pmr B***	***pmr*C**	***lpx*A**	***lpx*C**	***lpx*D**
PC	1	1	1	1	1	1	-	-	-	-	-	-	-	-	-	-	-	-
COL	2.83	-	0.76	1.83	0.96	-	+ 4	-	-1	+ 2	-1	-	0.547	-	0.0319	0.173	0.0031	-
RIF	4.02	0.66	4.47	1.93	5.96	-	+ 3	- 1.5	+ 4.5	+ 2	+ 6	-	0.767	0.478	0.2058	0.0017	0.0092	-
TEC	-	0.7	2.26	0.38	-	-	-	- 1.4	+ 2	- 2.6	-	-	-	0.0856	0.117	0.0024	-	-
COMB1	-	0.18	1.98	0.536	1.04	13.96	-	- 5.5	+ 2	- 2	+ 1	+ 14	-	0.0646	0.359	0.0513	0.0027	0.0022
COMB2	3.29	0.34	7.64	1.56	0.199	6.26	+ 3	- 2.9	+ 7.6	+ 1.6	- 5	+ 6	0.481	0.0021	0.8975	0.0385	0.0017	0.0063

Table 12: Bacterial counts obtained from the blood and lung homogenates of each of the *in vivo* experimental groups infected with ACN2 in CFU/mL, and the fold change relative to the bacterial control group. COMB1 = COL+ RIF, COMB2 = COL + TEC.

Group	Lung Counts	Log	p-value	Fold Δ	Blood Counts	Log	p-value	Fold Δ
ACN2 control	2.87×10^5	5.46	-	-	300	2.52	-	-
ACN2 + COL	2.23×10^5	5.35	0.0391	-1	290	2.46	0.293	-1
ACN2 + RIF	2.1×10^5	5.32	0.0429	-1	100	1.98	0.0071	-1.2
ACN2 + TEC	2.02×10^5	5.3	0.0932	-1	250	2.38	0.088	-1
ACN2 + COMB1	79	1.89	0.0082	-3	0	-	0.0002	-
ACN2 + COMB2	1.1×10^4	4.05	0.0006	-1.3	53	1.5	0.0393	-1.7

Figure 1: Time-kill synergy assay results of ACN 1, ACN 2, ACN 12, ACN 23 testing the combination of colistin and rifampicin over time (in hours). COMB1 = combination 1 (RIF + COL), POS CTRL = positive control.

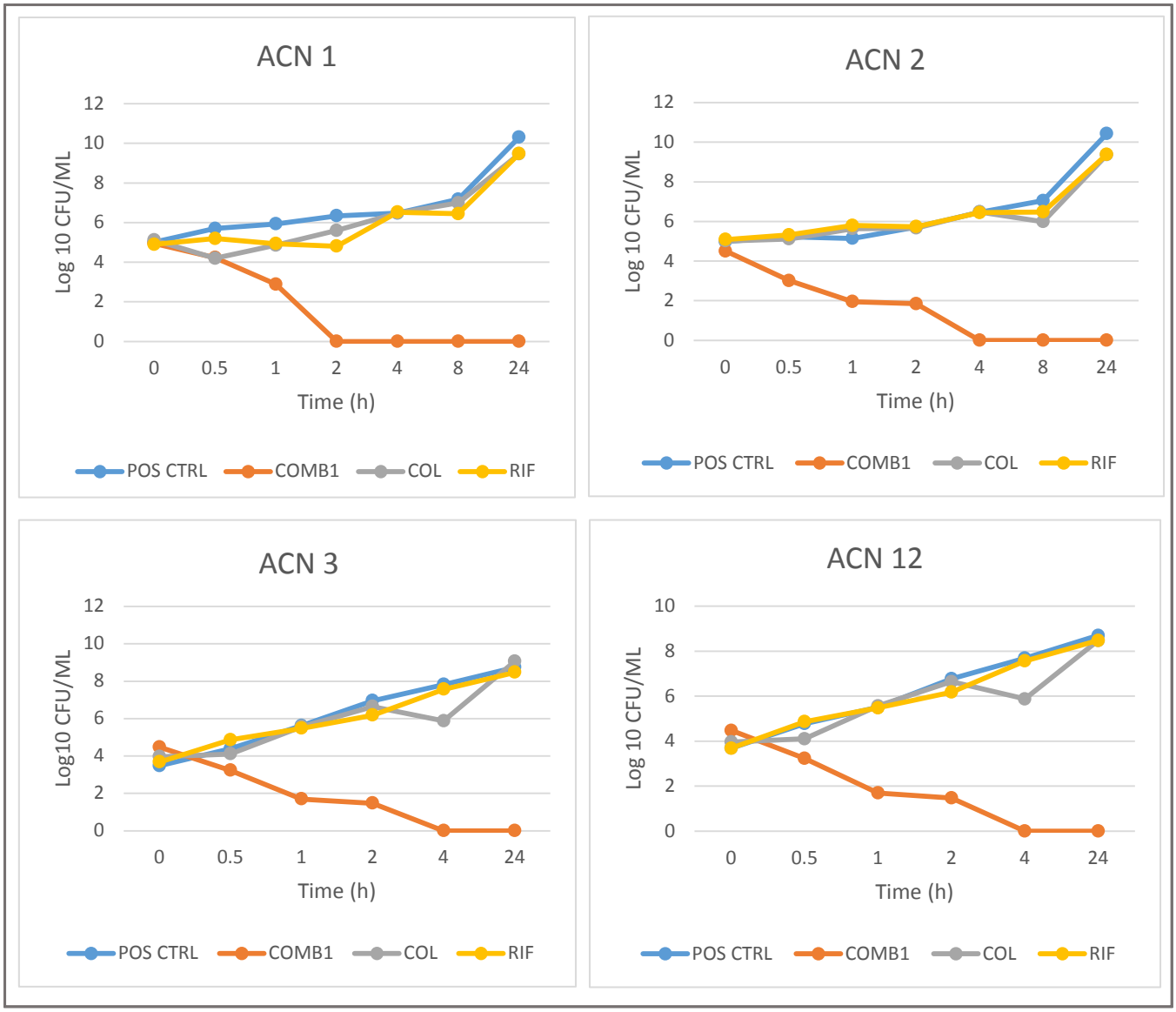


Figure 2: Time-kill synergy assay results of ACN 1, ACN 2, ACN 12, and ACN 13 testing the combination of colistin and teicoplanin over time (in hours).
COMB2 = combination 2, (TEC + COL), POS CTRL = positive control.

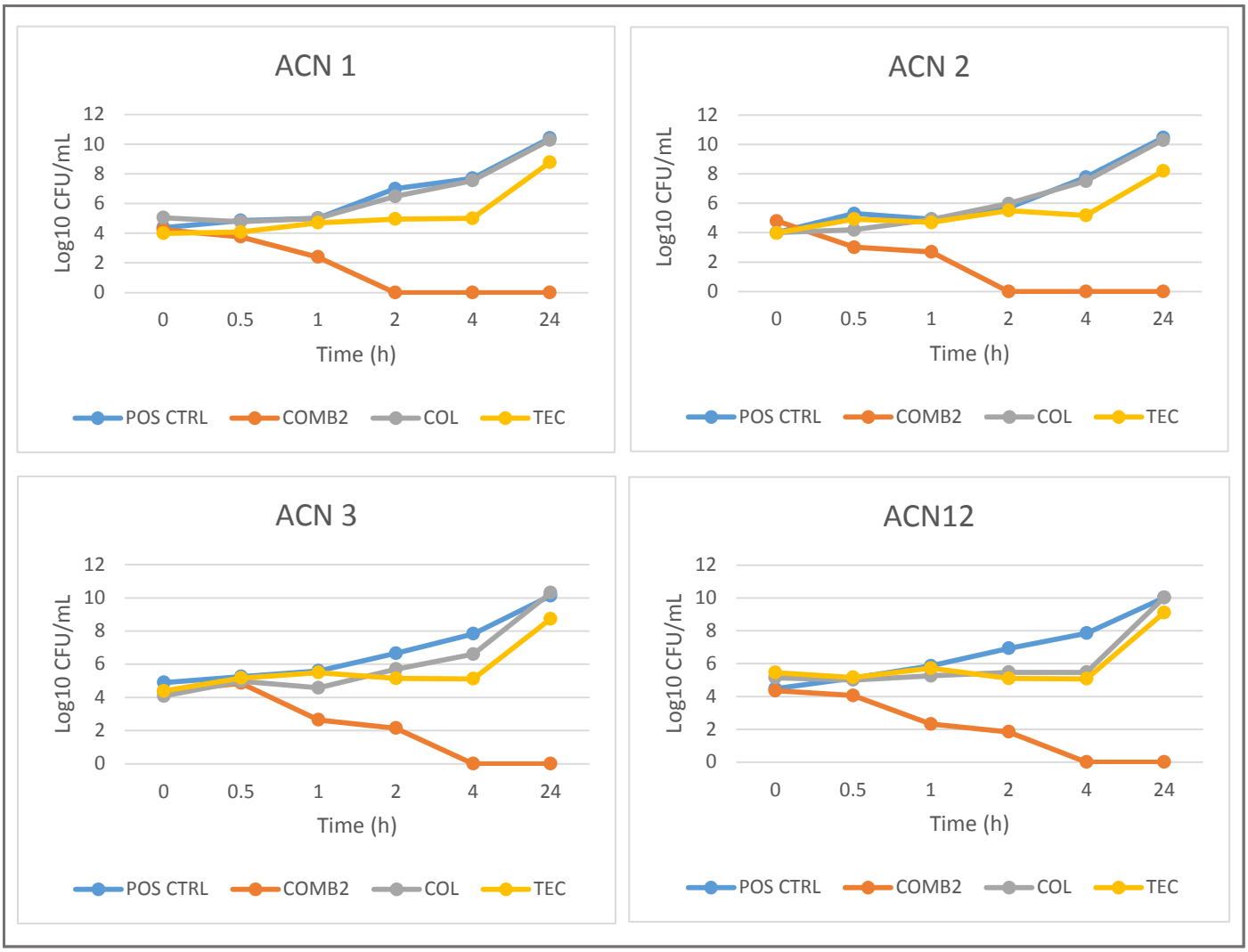


Figure 3: Time-kill synergy assay results of CDC isolates 346 and 349 testing the combination of colistin and rifampicin over time (in hours).

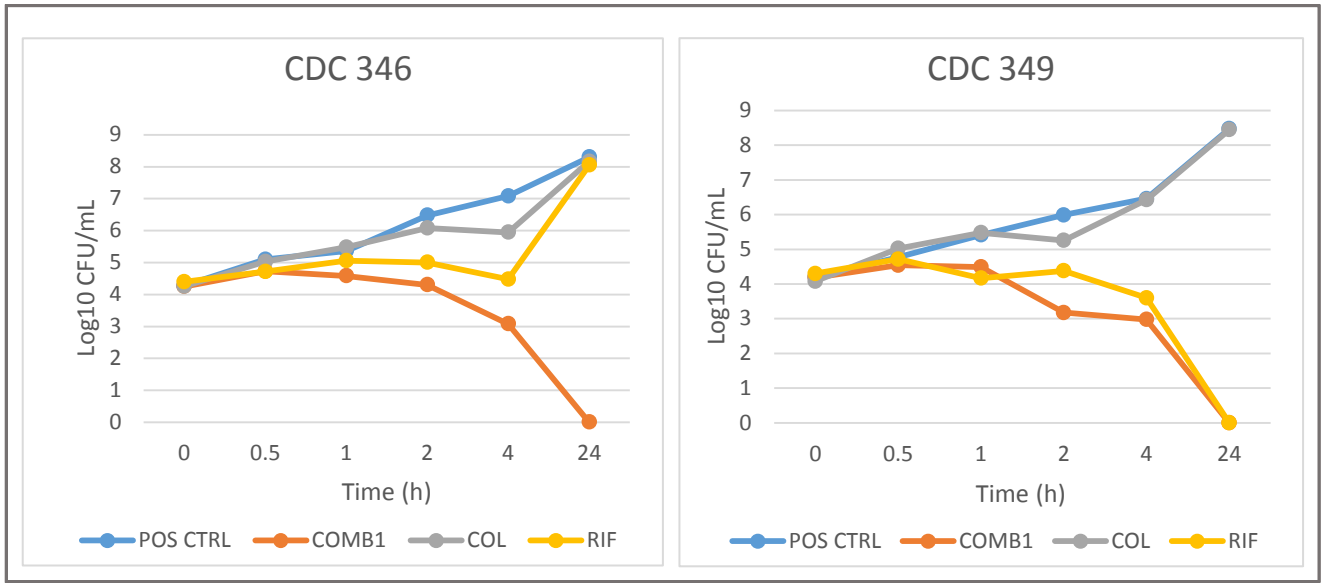


Figure 4: Time-kill synergy assay results of ATCC15308 and ATCC2522 testing the combination of colistin and teicoplanin over time (in hours). COMB1 = combination 1 (RIF + COL), COMB2 = combination 2 (TEC + COL), POS CTRL = positive control.

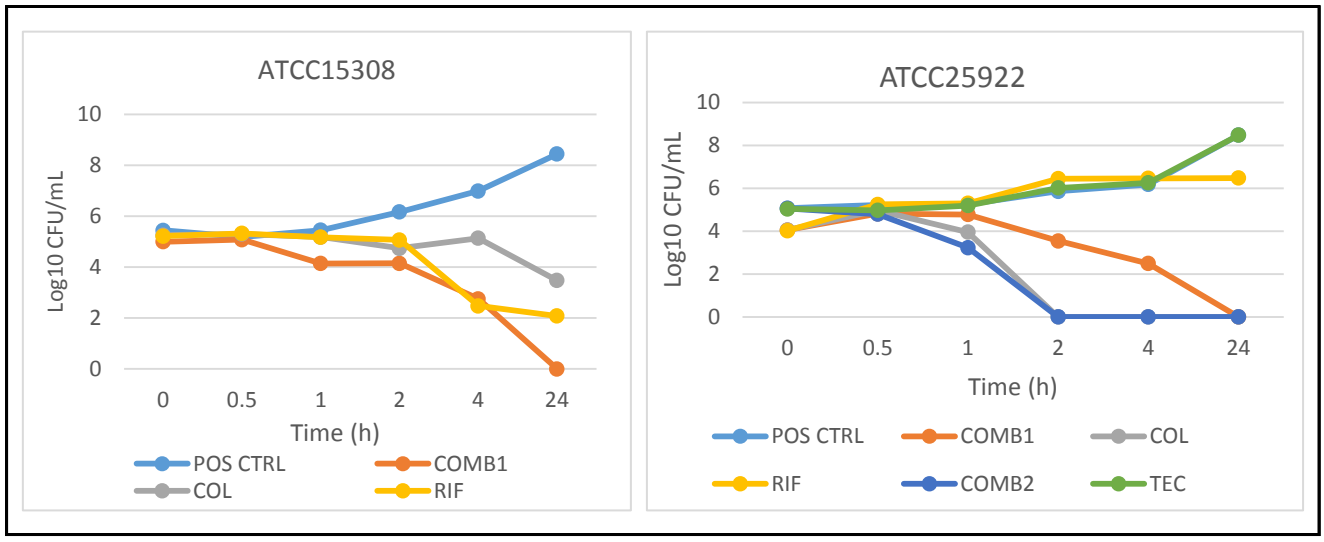


Figure 5: Relative gene expression of *pmr*A, *pmr*B, and *pmr*C genes in ColS ACN6 and ACN27 and ColR ACN1, ACN12 and ACN13, compared to a positive control (ATCC15308). Statistically significant = * ($p \leq 0.05$).

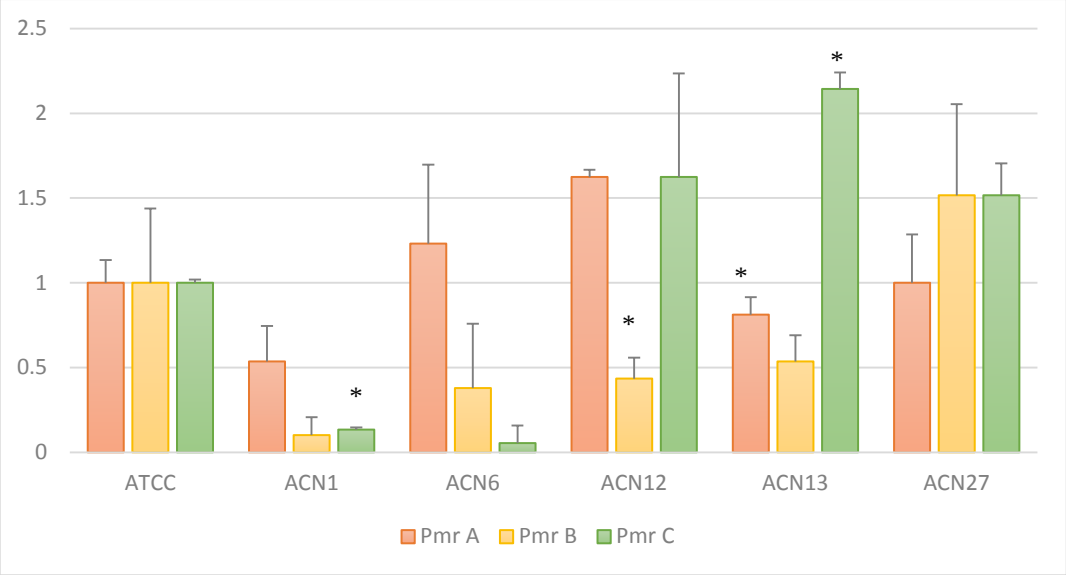


Figure 6: Relative gene expression of *lpx*A, *lpx*C, and *lpx*D genes in ColS ACN6 and ACN27 and ColR ACN1, ACN12 and ACN13, compared to a positive control (ATCC15308). Statistically significant = * ($p \leq 0.05$).

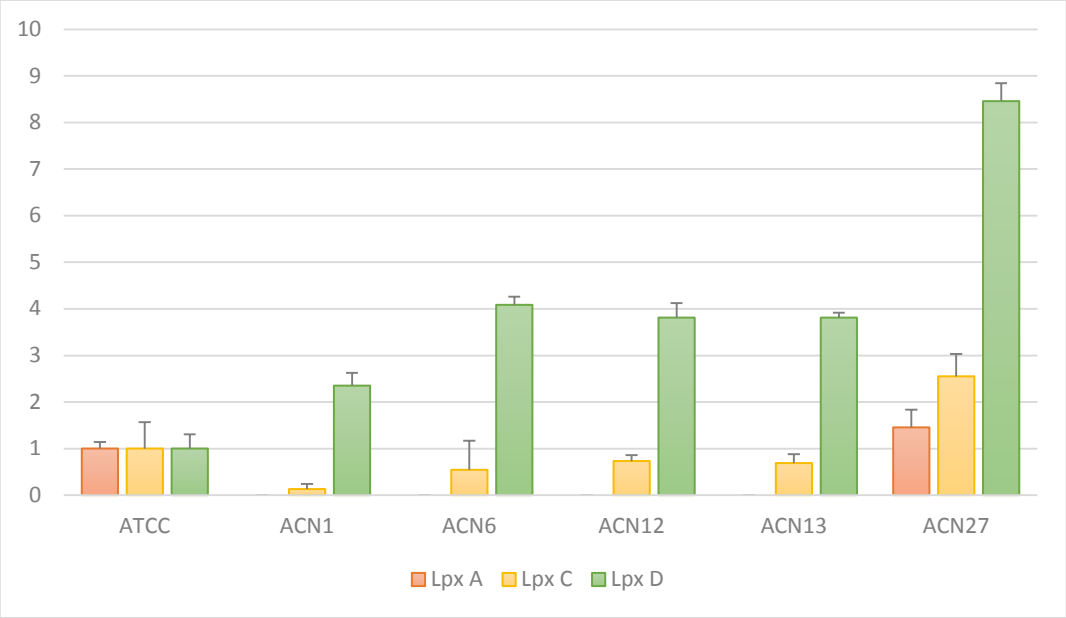


Figure 7: Bacterial counts obtained from the blood and lung homogenates of the *in vivo* experimental groups that were infected with ACN2 represented in Log 10 CFU/mL. COMB1 = COL+ RIF, COMB2 = COL + TEC. Statistically significant = * ($p \leq 0.05$).

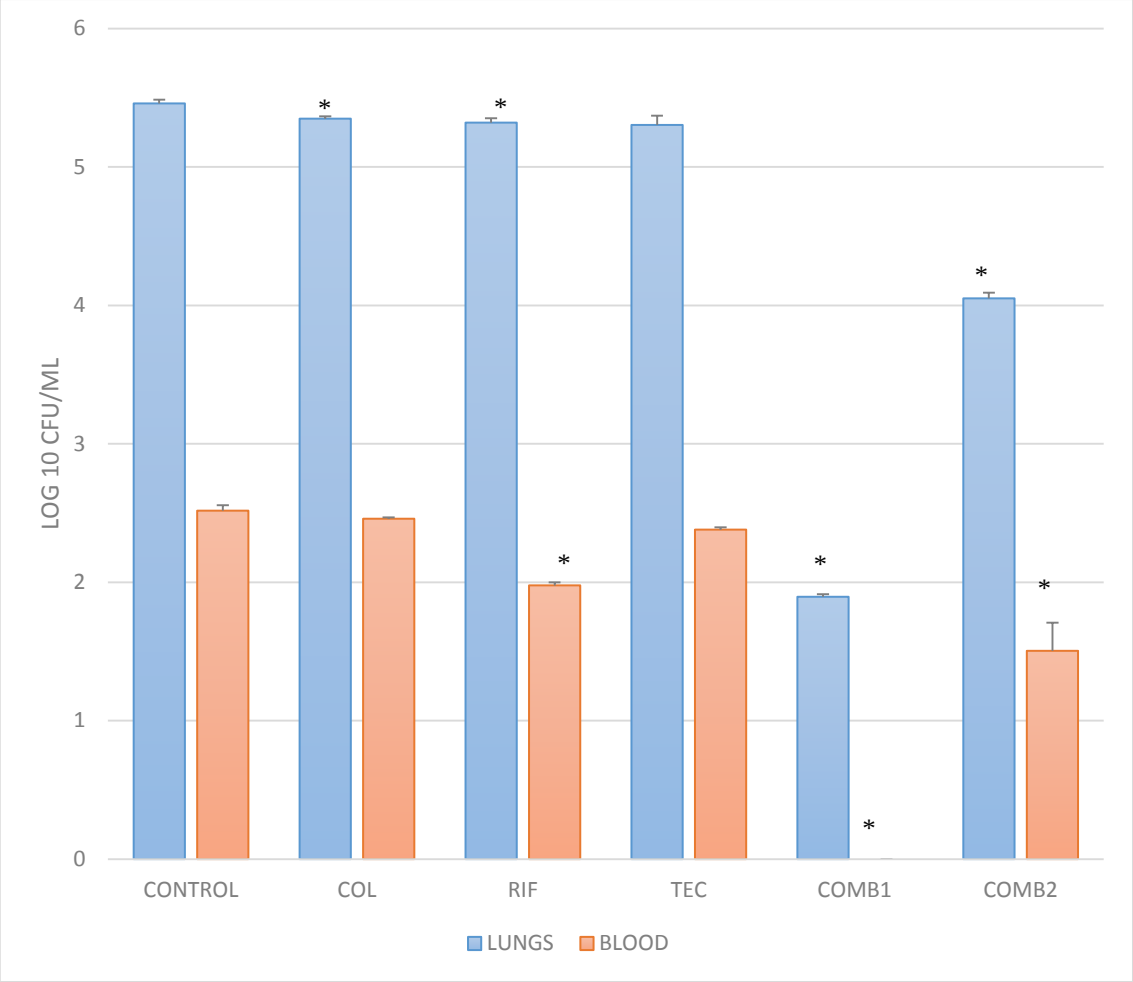


Figure 8: *In vitro* Relative gene expression of *pmr*A, *pmr*B, and *pmr*C genes of ACN2 in response to antimicrobial mono- and combination therapy combared to a positive control of ACN2 without treatment. COMB1 = COL+ RIF, COMB2 = COL + TEC. Statistically significant = * ($p \leq 0.05$).

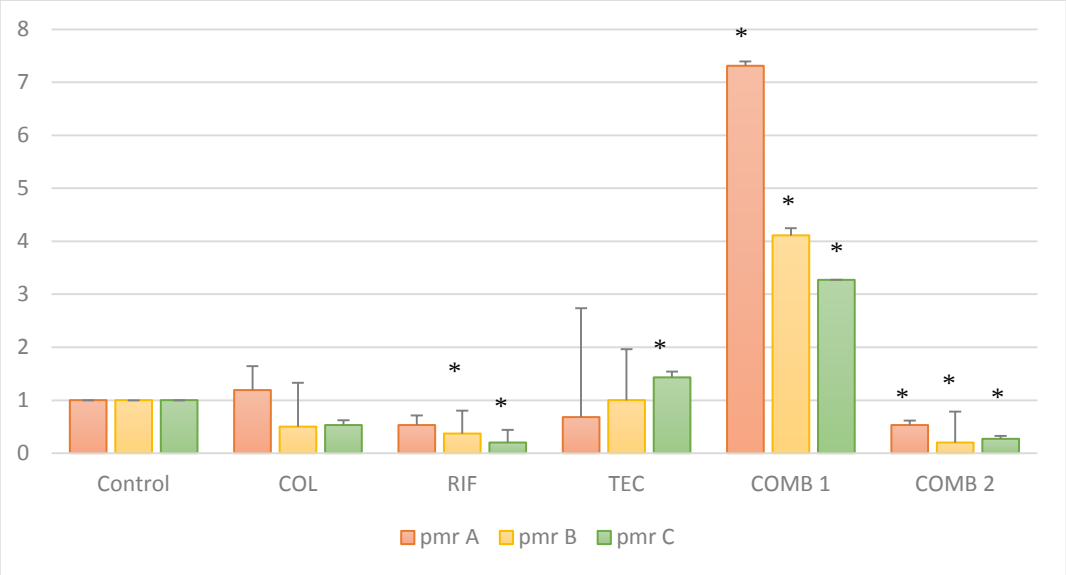


Figure 9: *In vitro* Relative gene expression of *lpx*A, *lpx*C, and *lpx*D genes of ACN2 in response to antimicrobial mono- and combination therapy combared to a positive control of ACN2 without treatment. COMB1 = COL+ RIF, COMB2 = COL + TEC. Statistically significant = * ($p \leq 0.05$).

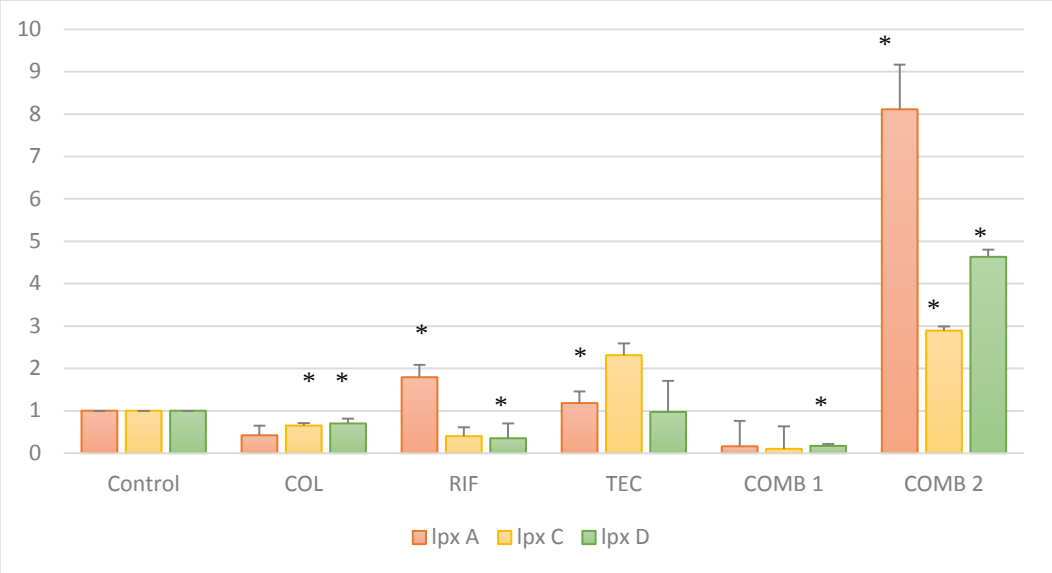


Figure 10: *In vivo* relative gene expression of *pmr*A, *pmr*B, and *pmr*C genes in serum obtained from the experimental groups that were infected with ACN2 and different treatment regimens in comparison with the control group that did not receive any treatment. Statistically significant = * ($p \leq 0.05$).

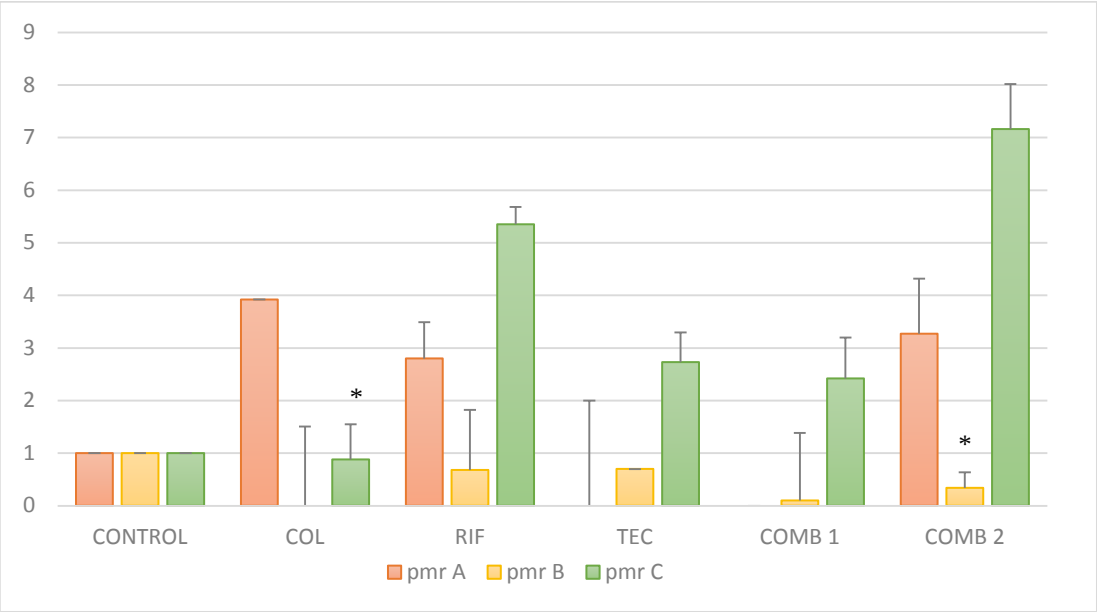
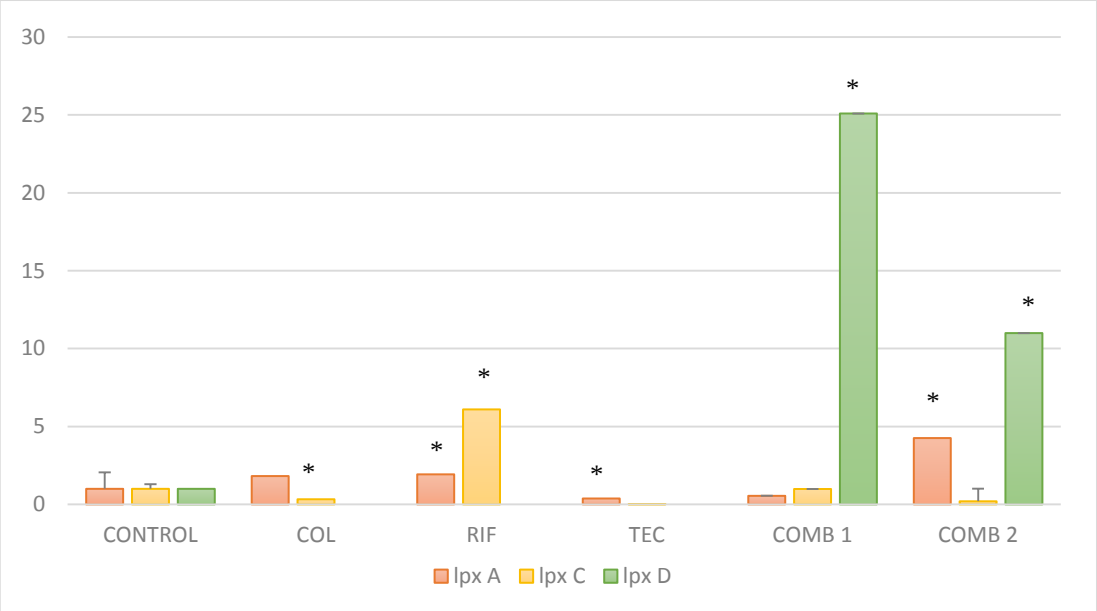


Figure 11: *In vivo* relative gene expression of *lpx*A, *lpx*C, and *lpx*D genes in serum obtained from the experimental groups that were infected with ACN2 and different treatment regimens in comparison with the control group that did not receive any treatment. Statistically significant = * ($p \leq 0.05$).



CHAPTER V

DISCUSSION

Reports of increasing rates of MDR, XDR and pan-drug resistant (PDR) strains of *A. baumannii* and *E. coli* have been rapidly increasing world-wide (67). Both types of bacteria are now grouped by the World Health Organization as "Priority 1" group of pathogens requiring investigations on new forms of treatment (68). Increasing trends of AMR have been reported at AUBMC in a study reflecting bacterial resistance in Lebanon over a decade (2000-2010), including a rise in ESBL producing and carbapenems resistant *E. coli* isolates (69). The lack of discovery of new antimicrobial agents has led to the increased utilization of COL for treatment of highly resistant infections, and consequently, increased reports of COL resistance (23). The annually conducted antibiogram brochure published by Araj *et al.* at the Department of Pathology & Laboratory Medicine reported that susceptibility to COL in *Acinetobacter spp*. clinical isolates has dropped from 93% in 2016 to 56% in 2017 (70). Such reports triggered the initiation of studies to identify mechanisms of resistance at the molecular level and evaluate viable options for treatment of COL resistant infections (55, 71).

Detection of resistance to COL was undertaken by disk diffusion, E-test, and Vitek® assays and the currently used broth microdilution experiments, the recommended method of testing for determination of COL susceptibility according to the CLSI (72). Five ACN isolates showed a high level of resistance to COL, matching their disk diffusion, E-test, and Vitek® results (Table 3). Moreover, the *mcr*-1 encoding *E. coli* samples that were obtained from the CDC showed MIC values that were between 2-4 μg/mL, matching the published results by CDC (73), with the CDC349

isolate being considered susceptible due to its MIC of 2 μg/mL. BMD was done to obtain the MIC of AMK, RIF, and TEC. Understandably, all the isolates exhibited resistance to TEC since it is a Gram-positive targeting antimicrobial agent, while resistance to the RIF and AMK was observed with the exception of the wild-type ATCC and *E. coli* CDC 349 strains, matching previously published data of susceptibility to AMK (73).

For the purpose of assessing combination therapy for the treatment of COL resistant infections *in vitro*, we chose to start with the checkerboard assay in order to assess for potential use of synergistic combinations of antimicrobials against the ColR ACN isolates, the ATCC, and the *mcr*-1 encoding *E. coli* CDC strains. The AMK + COL combination proved to be indifferent against all of the tested isolates, and was therefore excluded from further testing. Our results showed that synergy was obtained against 100% of the ACN strains with the COL + RIF and COL + TEC combinations. This was confirmed by performing the time-kill assay which is considered to be the gold standard for determination of synergy according to CLSI guidelines (64). It is important to confirm checkerboard results by conducting time-kill assays since the effect of the antimicrobial combinations can change over time (74); in addition, this assay provides a time-point as to when bactericidal activity of the combination can be best achieved, which can have important therapeutic applications.

The success of the TEC + COL combination is interesting due to the fact that TEC is a Gram-positive targeting antimicrobial. In such cases, the presence of COL has been hypothesized to reduce the permeability of the outer membrane, and as a consequence, facilitate TEC penetration and expose its lipid II target in the bacterial cell wall (75). Recent studies have reported similar effect with more commonly used

glycopeptides, such as vancomycin, but this combination was reported to be a risk factor for the development of renal failure in patients (76). Alternatively, TEC has been reported to have a comparable efficacy to vancomycin, with less adverse effects and reduced levels of toxicity (77). Our assessment of COL and TEC therefore provides a safer viable mode of potential alternative treatment.

As for the *E. coli* CDC and ATCC strains, COL + RIF appeared to be a more successful combination in targeting the organisms. However, the time-kill assays revealed that over-time, RIF or COL monotherapy appeared to be as efficacious as the combination therapy. This might be explained by their increased levels of susceptibility to COL and RIF when compared to the ACN isolates.

Due to the success of the COL + RIF and COL + TEC combinations *in vitro* against the ColR ACN isolates, both combinations were assessed in an *A. baumannii*-induced neutropenic pneumonia murine model, in an attempt to mimic the common human conditions of contracting the infection, and to avoid spontaneous healing by the animals. We chose the ACN2 ColR clinical isolate for the purpose of inducing pneumonia in the BALB/c mice due to its high level of resistance to COL, as well as its response to both antibiotic combinations (Table 5). Although the administered dose was relatively high 1.5×10^9 CFU/mL, the infection proved to be sub-lethal, with minimal dissemination to the circulation in all of the tested mice groups. The significant reduction of the bacterial load in the mice treated with COL + RIF in treating the highly resistant ACN isolate is promising; however more *in vivo* studies should be conducted using multiple ColR *A. baumannii* isolates to confirm these results. Moreover, the option of using higher dose of TEC in combination with COL

should be evaluated in more comprehensive studies assessing the pharmacokinetic and pharmacodynamics properties of the antimicrobial combination.

For the characterization of the molecular aspects of COL resistance in our isolates, we referred to a previous study that was conducted in the Bacteriology and Molecular Microbiology Lab at AUB that utilized the same isolates included in our experiments (12). The study consisted of PCR amplification of specific genes encoding for COL resistance and associated with target modification in ACN, in order to compare the results with their degree of susceptibility to COL (12). Table 7 correlates PCR data with outcomes of the synergy assessment using a combination of COL with antimicrobial agents.

We selected ACN1; a ColR isolate that appeared to be LPS deficient both phenotypically and genomically according to the findings of the aforementioned study (12). We also selected 2 ColR ACN strains (ACN12 and ACN13) and 2 ColS strains (ACN6 and ACN27) that appeared to have comparable PCR profiles, but significantly different response to COL (Table 8), in order to assess the relative levels of expression of the same genes through the use of RT-qPCR in relation to levels of susceptibility/resistance to COL. The wild-type ACN ATCC15308 strain served as a reference strain and a positive control.

Our data showed that when compared to the ColS isolates, COL resistance in ACN1 could be in fact associated with the reduced expression of the *lpx*A, *lpx*C, and *lpx*D genes (Figure 6). These genes encode multiple enzymes that are essential for the first three steps of lipid A biosynthesis pathway, a fundamental component that is required for the anchoring of the LPS to the bacterial membrane (47). Of particular interest is the observed 7.7-fold decrease of the level of expression of the *lpx*C gene in

ACN1 when compared to the wild-type strain (Table 9), since *lpx*C is considered to be the committed step for lipid A biosynthesis in *A. baumannii* (78). The observed expression of the *lpx*D gene might account for the positive LAL test result for this isolate. However, the levels of expression of the *pmr* genes that are implicated in the other major mechanism of resistance to COL appeared to be normal, further corroborating the possibility that some type of LPS defeciency might have contributed to the observed resistance to COL in ACN1. Studies assessing the chemical structure of the outer membrane of the isolate might be beneficial in understanding the mechanisms this isolate utilizes to resist the action of COL. There appeared to be some discrepancies between the PCR results from the referenced study (12), and our RT-qPCR results, particularly in the case of ACN1 (Table 8). The change in the genetic profiles might be due to the acquirement of new mutations in these genes in response to frequent sub-culturing of the isolates, or due to errors in the annealing of the primers that were used in that study. The use of more specific primers could provide a clearer picture of the mutations in ACN1; additionally, analysis of the whole genome sequences (WGS) of the ColR isolates might provide information that is crucial to identifying the specific alterations in each of the isolates and to how they relate to their antimicrobial susceptibility profiles.

Interestingly, *lpx*A in particular did not appear to be expressed in any of the tested isolates, regardless of their levels of COL susceptibility. Nonetheless, *lpx*D was overexpressed when compared to the reference strain, mainly in the ColS isolates. This increased expression of *lpx*D raises the question of it possibly having compensatory mechanisms in the absence of *lpx*A due to the significant sequence homology between the two genes (79). Moreover, overexpression of *pmr*A and *pmr*C in ACN12 and the

overexpression of *pmr*C in ACN13 might be the associated with their ColR phenotypes, since previous studies have reported that mutations in the *pmr*A/B signaling system can lead to the overexpression of *pmr*C, encoding the addition of phosphoethanolamine or L-Ara4N sugar to the LPS, and reducing their overall negative charge, and their affinity COL as a consequence (47).

As for the *in vitro* and *in vivo* gene expression results of ACN2 in response to the mono- and combination therapies of COL, RIF and TEC, the results showed a highly varied genetic response to the antimicrobial treatment options. The notable rise in the expression level of the *pmr*C gene in particular might be a defensive mechanism employed by ACN2 *in vivo* to combat the permeabilization effect of the presence of COL. The *in vivo* reduction of the expression of the lipid biosynthesis genes, particularly *lpx*A and *lpx*C, indicates that both combinations of COL + RIF, and COL + TEC may have reduced the production of LPS, one of the virulence factors of *A. baumannii*, in the animals' serum. Combined with the observed reduction of the bacterial counts in the animals, this demonstrates an additional benefit to the use of these combinations, as they appear to have the potential ability of decreasing the virulence of the infection.

Our RT-qPCR results also demonstrated that the expression levels of the *lpx* lipid biosynthesis genes and, even more so, the *pmr* signaling genes of the ACN2 isolate appear to be highly sensitive to the presence of the different antimicrobials in addition to the conditions of incubation, highlighted by the difference between the responses *in vitro* and *in vivo* (Figures 8-11). These divergences may be due to the numerous factors pertaining to the host-pathogen interactions in the animal model that could not be accounted for *in vitro*. For instance, previous studies have reported that changes in the

acidity of the environment as was well as the presence of certain cationic molecules might induce modulations to the gene expression level of the PmrA/PmrB two-component systems in Gram-negative bacteria (80, 81). Further studies may be conducted using larger sample sizes and on multiple ColR *A. baumannii* strains with varied genetic profiles to account for the variability of the genetic responses both *in vitro* and *in vivo.*

In conclusion, due to the rising phenomenon of MDR, and more alarmingly, COL resistance in *A. baumannii,* the current only viable treatment option is the use of combination therapy. This study demonstrated that synergy between COL and RIF or TEC is achievable when targeting highly resistant ACN isolates *in vitro*. The efficacy of COL + RIF combination seems to have a promising potential as an alternative choice for treatment of life-threatening highly resistant infections. The combination of COL + TEC on the other hand requires further adjustments to increase its *in vivo* efficacy. As for the *mcr*-1 encoding *E. coli* isolates, more studies should be conducted on alternate forms of treatment, either by evaluating more antimicrobial combinations, developing inhibitors of antimicrobial-hydrolyzing enzymes, or by isolating new classes of antimicrobial agents from natural products or synthetic origin.

Moreover, concerning the molecular aspect of COL resistance in our study, our results indicate that the relative levels of expression of certain genes, as opposed to their presence or absence, may have contributed to the levels of resistance in our ColR ACN isolates. This resistance seemed to be generally associated with either the over expression of the *pmr*A/B signaling genes, or the under expression of the lipid biosynthesis genes, or with a combination of both mechanisms. Ultimately, the use of

WGS can provide a clearer picture of the underlying mechanisms of resistance to COL which might be valuable in creating a more targeted therapeutic approach in the future.

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