AMERICAN UNIVERSITY OF BEIRUT

THE EFFICACY OF COMBINATION THERAPY IN MDR, XDR AND PDR *ACINETOBACTER BAUMANNII*

by

DIANA ATA ABDULGHANI

A thesis
submitted in partial fulfillment of the requirements
for the degree of Master of Science
to the Department of Experimental Pathology, Immunology and Microbiology
of the Faculty of Medicine
at the American University of Beirut

Beirut, Lebanon
August 2019

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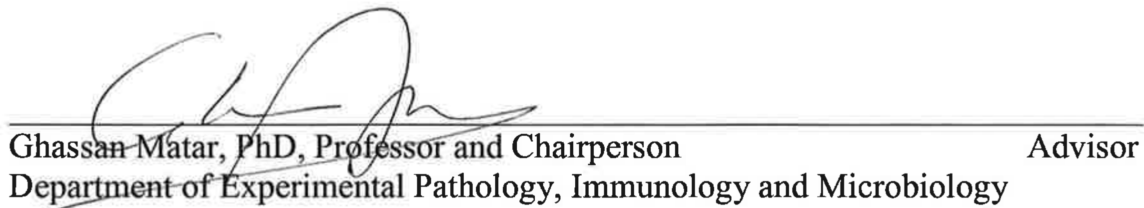
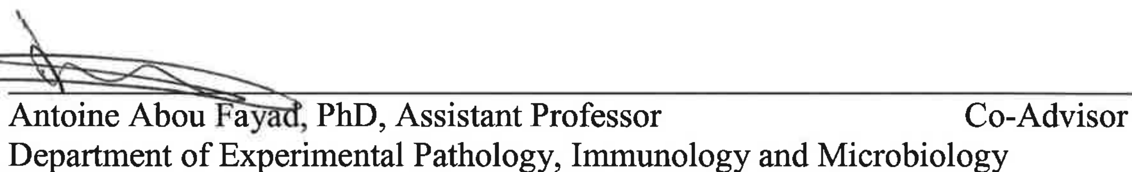
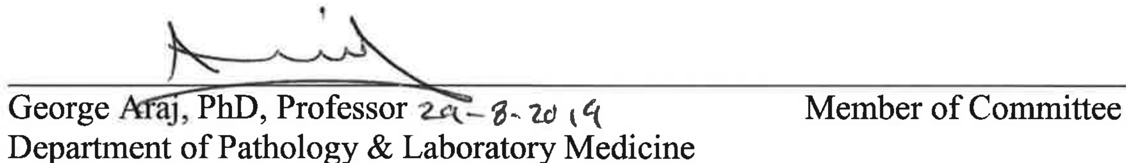


Ghassan Matar, PhD, Professor and Chairperson Advisor
Department of Experimental Pathology, Immunology and Microbiology

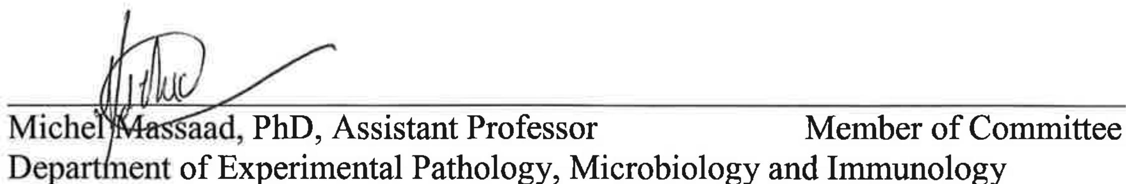


Antoine Abou Fayad, PhD, Assistant Professor Co-Advisor
Department of Experimental Pathology, Immunology and Microbiology



George Araj, PhD, Professor Member of Committee
Department of Pathology & Laboratory Medicine

Elias Rahal, PhD, Associate Professor Member of Committee
Department of Experimental Pathology, Immunology and Microbiology



Michel Massaad, PhD, Assistant Professor Member of Committee
Department of Experimental Pathology, Microbiology and Immunology

Date of thesis defense: August 27, 2019

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ACKNOWLEDGMENTS

I would like to express my deepest gratitude and appreciation to Prof. Ghassan Matar and Dr. Antoine Abou Fayad for their unlimited support, encouragement, guidance and for believing in my potentials throughout this great learning experience.

Second, I would like to thank my thesis committee members: Dr. George Araj, Dr. Elias Rahal and Dr. Michel Massaad for their insightful comments and encouragement.

My gratefulness goes to my supervisor Dr. Wael Bazzi. Thank you so much for your continuous help, support, encouragement and constructive criticism.

To my lab partners: Ms. Sara Issa and Mr. Mouayad Bakleh, thank you for your support and assistance and for being such amazing friends.

My appreciation goes to Mrs. Nour Sherri. Thank you for being an excellent guide and for helping me throughout the thesis.

To my amazing crazy friends: Joelle, Dana, Mirna and Zainab, I am so thankful and grateful for meeting you, for having you as friends and for sharing with you adventures, laughs and tears. You have made my heavy workload days bearable.

Finally, I would like to thank my beloved family for their continuous love and support and for believing in me. I couldn’t accomplish any of this without you by my side.

AN ABSTRACT OF THE THESIS

Diana AbdulGhani for Master of Science
Major: Microbiology and Immunology

Title: The Efficacy of Combination Therapy in MDR, XDR and PDR *Acinetobacter baumannii*

Background: Antimicrobial resistance has been emerging into alerting levels in the last few decades resulting in multi-drug resistance (MDR), extensive-drug resistance (XDR), and lately pan-drug resistance (PDR) in Gram negative bacteria such as *Acinetobacter baumannii*. Resistance to β-lactams, cephalosporins, and carbapenems has led to the re-emergence of polymyxin E (colistin) as a sole and last line solution to treat MDR and XDR cases. However, *A. baumannii* has acquired multiple resistance mechanisms to colistin, which in turn directed clinicians towards prescribing combination therapies as alternative options to improve the antibacterial activity of colistin. In Lebanon, no study addressed the *in vitro* combination of colistin with different antimicrobial agents against *A. baumannii* clinical isolates.

Methods: A total of 73 *A. baumannii* clinical isolates collected from AUBMC Clinical Microbiology Laboratory were screened for their susceptibility to colistin using the Broth Micro-dilution assay (BMD). Pulsed Field Gel Electrophoresis (PFGE) was performed to assess the genomic relatedness among the isolates. Checkerboard assays were performed on 7 colistin-resistant *A. baumannii* isolates to evaluate the effect of combining each of Tigecycline (TGC), Teicoplanin (TEC), Zerbaxa (ceftolozane/tazobactam; C_T), Meropenem (MEM), Levofloxacin (LVX) and Amikacin (AMK) with colistin (COL) and determine the possible synergistic effects between antibiotics. Induction of resistance was conducted on the reference strain (DSM30008) to generate various mutants, using a panel of antimicrobial agents (colistin, tigecycline, teicoplanin, zerbaxa, meropenem, levofloxacin and amikacin) to elucidate the resistance mechanisms that developed and establish a reference strain for Whole Genome Sequence (WGS) analysis.

Results: Our results revealed that n=8 (10.95 %) of isolates were resistant to colistin by the BMD and their MICs varied between 128 and 4096 μg/mL. PFGE analysis demonstrated that *A. baumanni* isolates were diverse and belonged to 8 clusters. Assessment of combination of colistin with various antimicrobials showed that only colistin-teicoplanin combination demonstrated synergistic effect when tested against ACN T17 and ACN T19 which are found to be genomically related by 64.2 %. However, all the tested combination displayed antagonistic or indifferent effect when tested against all the remaining isolates. Antagonism is demonstrated when levofloxacin and tigecycline was combined in the presence of 4 μg/mL of colistin against all the tested isolates. Interestingly, the raised mutants behaved as batches with high resistance profile observed in batch 2 (C_T, COL/C_T, MEM, COL/MEM, LVX, COL/LVX, AMK and COL/AMK mutants) when tested against colistin (MIC≥512 μg/mL), teicoplanin (MIC≥256 μg/mL), zerbaxa (MIC≥64 μg/mL), tazocin (MIC≥256 μg/mL), meropenem (MIC≥64 μg/mL), levofloxacin (MIC≥16 μg/mL) and amikacin (MIC >2048μg/mL).

Conclusion: This study was the first to show promising results between COL and TEC in treating *colR A. baumannii* isolates. In addition, we demonstrated reversion of resistance in several isolates assessed which needs to be further characterized upon performing fitness cost and frequency of resistance assays. Furthermore, we established *in vitro* resistant mutants to assess genome-wide modifications as compared to *colR A. baumannii* clinical isolates via WGS. This will pave the way for establishing novel antibiotics counteracting colistin-resistance mechanisms by better understanding the mode of resistance.

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CHAPTER I
INTRODUCTION

The alarming rise in antimicrobial resistance (AMR) is one of the major global health challenges of the 21st century. It is increasing world-wide into dangerously high levels which threatens our ability to treat common infectious diseases. The rise in AMR is mainly due to the continuous abuse and misuse of antimicrobial agents. A report by the UK government published in 2016 estimates that, by 2050, 10 million people will die every year due to AMR. *Acinetobacter baumannii* is a Gram-negative bacterium with a versatile genome which enables it to thrive in harsh environmental conditions. It is an endemic notorious superbug associated with world-wide nosocomial infections and accounts for about 20% of ICU patients, which makes it an important target for research. Moreover, *A. baumannii* is considered a critical pathogen with "Priority 1" classification for the need of new antimicrobial agents according the World Health Organization (WHO).

Increasing rates of multi-drug resistance (MDR) and extensive-drug resistance (XDR) in *A. baumannii* are being continuously reported. The lack of discovery of new antimicrobial agents have led scientists to resort to colistin to treat highly resistant infections. Colistin (polymyxin E) is a polycationic lipopeptide with a bactericidal mode of action that mainly targets the negatively charged lipid A of the lipopolysaccharide (LPS) of the Gram-negative bacteria. Unfortunately, *A. baumannii* has developed several mechanisms of resistance to overcome the effect of colistin by mainly modifying the LPS. The emergence of pan-drug resistance (PDR) including MDR, XDR, along with the resistance to tigecycline and colistin, and the lack of novel

antibiotics has led clinicians to suggest combination therapies as the final resort to treat various types of *A. baumannii* infections. In Lebanon, this study was the first to tackle the effect of combining colistin with different antimicrobial agents to treat *A. baumannii* clinical isolates.

Given the failure of monotherapy in treating *A. baumannii* infections, the emergence of MDR, XDR and lately PDR, the lack of novel antimicrobial agents along with the need of further understanding the underlying mechanisms of resistance to colistin in *A. baumannii* and the necessity of alternative antimicrobial options. Our present study mainly aims to:

- Evaluate *in vitro* the efficacy of combining colistin with antimicrobial agents belonging to different classes against colistin-resistant *A. baumannii* clinical isolates.
- Examine the underlying mechanisms of resistance to colistin in MDR, XDR and PDR *A. baummanii* clinical isolates by raising resistant mutants in comparison with the reference strain

CHAPTER II
LITERATURE REVIEW

A. General Characteristics of *Acinetobacter baumannii*

Acinetobacter spp. are non-fastidious, non-lactose fermenting, non-motile, oxidase-negative, catalase-positive, obligatory aerobic Gram-negative coccobacilli (1). they belong to the Moraxellaceae family which comprises more than fifty species, including *Acinetobacter baumannii*, *Acinetobacter haemolyticus*, *Acinetobacter nosocomialis*, *Acinetobacter lwoffii*, *Acinetobacter pittii* and *Acinetobacter calcoaceticus* (2). *A. baumannii* is considered the most pathogenic species in this family, as it is commonly associated with worldwide nosocomial infections (1). Infections caused by *A. baumannii* accounts for about 20% of ICU patients (3). It is mainly prevalent in various anatomical sites, such as the respiratory tract, bloodstream, skin and other soft tissues with different severity ranges, leading to high mortality rates in the ICU (4, 5). Due to the emergence of multi-drug resistant (MDR) and extensively-drug resistant (XDR) strains, WHO has considered *A. baumannii* as one of the most serious ESKAPE organisms (*Enterococcus faecium*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa*, and *Enterobacter* species) which have the ability to develop resistance to different classes of antimicrobial drugs and thus are the leading cause of nosocomial infections throughout the world (6, 7).

1. A. baumannii Virulence Factors and Pathogenesis

A. baumannii utilize several different virulence factors, such as capsular polysaccharides, lipopolysaccharides (LPS), outer membrane proteins, protein secretion systems, phospholipases as well as, iron-chelating system, where the latter plays a vital role in its pathogenicity (1, 6, 8).

a. Capsular Polysaccharides (CPS) and Lipopolysaccharides (LPS):

Capsular exopolysaccharides play a critical role in *A. baumannii* pathogenicity, it usually protects it from external threats, such as complement-mediated killing (9, 10). As reported by Miller et al., resistance to complement-mediated killing is mainly due to the CPS, where strains without capsule are getting killed easily by the complement system and thus are non-virulent (9). Capsular polysaccharides mutants are also found to have lower resistance to antimicrobials. Hence, they are involved in mediating *A. baumannii* antimicrobial resistance. Moreover, a relation between antibiotics exposure and CPS production has been noticed in a mouse model with systemic infection, where the presence of antimicrobials has induced the overproduction of the capsular polysaccharides which in turn, enhances the virulence of *A. baumanni* as well as the resistance to host-mediated complement killing (11).

The outer membrane of most Gram-negative bacteria is mainly composed of lipopolysaccharides (12), which are made up of three major components: the endotoxic Lipid A, the oligosaccharide core, and the O-antigen (13). LPS have a vital role in the *A. baumannii* virulence and viability. The most toxic region within the LPS is lipid A which provides *A. baumannii* with inflammatory properties associated with its virulence and pathogenesis. Thus, LPS is shown to have an effect on both the innate and acquired

host immunity, and this effect is mediated via its ability to escape the host immune response and to initiate a massive inflammatory response within the host cells, leading to high morbidity and mortality rates (14). Studies revealed that mutations in lipid A of the LPS, as well as modifications, can lead to resistance to various classes of antimicrobial agents, including colistin (14, 15).

b. Outer Membrane Proteins (Porins):

In *A. baumannii*, the outer membrane proteins, such as OmpA, have been found to be associated with virulence and cytotoxicity (6, 16). OmpA "a β-barrel porin", mediates its virulence by three proposed mechanisms. First, it causes the release of apoptosis-inducing factor and cytochrome c, which are proapoptotic molecules, leading to apoptosis of the epithelial cells after being localized in the mitochondria (17, 18). Second, OmpA induces cell death after being translocated in the nucleus (19). Third, it plays a major role in *A. baumannii* resistance to complement-mediated killing by the host cell through binding to human serum "factor H" and fibronectin In addition, it was found that OmpA plays an important role in *A. baumannii* antimicrobial resistance as the minimal inhibitory concentrations (MICs) of various antimicrobial agents including nalidixic acid, aztreonam and chloramphenicol have dropped after disrupting OmpA gene (15).

c. Protein Secretion System:

A. baumannii has been described to express many different Protein secretion systems (20), including the recently described Type II secretion system (T2SS). T2SS acts by translocating and secreting target proteins from the periplasmic space to the outside of the cell and in a two-step process. Researchers have found that the deletion of any of the genes responsible for encoding T2SS components such as *gspD* or *gspE*

prevents LipA secretion, which is considered essential for the breakdown of long-chain fatty acids. Thus, *gspD*, *gspE* and *lipA* mutants are incapable of surviving in a neutropenic mouse model with bacteremia due to the lack of its only carbon source (21).

d. Phospholipase:

Phospholipase is defined as a lipolytic enzyme needed for phospholipid metabolism. Scientists have described three classes of phospholipases depending on the cleavage site, phospholipase A (PLA) which breakdown glycerol backbone to produce fatty acids; phospholipase C (PLC) is responsible for the cleavage of the phosphorylated head groups from the phospholipids, while phospholipase D (PLD) has the ability to cleave the head group only and is considered a transphosphatidylase. These important characteristics of phospholipases, including the degradation of phospholipid and the cleavage of the head group, play a vital in *A. baumannii* virulence by affecting the host immune response. PLC and PLD are the only phospholipases identified in *A. baumannii*, where the inactivation of either PLC or PLD genes in two different *A. baummanii* strains, such as ATCC17978 and 98-37-09 affect its cytotoxic effect on host epithelial cells, thus mediating its pathogenicity (15).

e. Iron-chelating system:

Most aerobic bacteria suffer from iron limitation even if it is in a condition that allows it to survive in the best way. This is because ferric iron has poor solubility and can be easily chelated by transferrin, lactoferrin, or heme (15). Siderophores are compounds with high iron affinity, they are the only resort the bacteria have in order to cope with the problem of iron limitation (22). In *A. baumannii*, both siderophores and acinetobactin are present and act as virulence factors (6). Studies have shown that

mutations in genes involved in acinetobactin biosynthesis and transport, such as *entA* reduces its ability to survive within host epithelial cells, affecting its virulence (15). Another reported virulence factor in *A. baumannii* is the *nfuA* Fe-S scaffold protein, which is involved in Fe-S clusters formation, iron chelation and oxidative stress. *nfuA* mutants are unable to survive and grow well in host epithelial cells due to their sensitivity to reactive oxygen species, such as hydrogen peroxide (23).

2. Infections with A. baumannii

Reports have shown that *A. baumannii* infections occur on different anatomical sites with different ranges and severity (4). It usually infects organ systems which have high fluid levels (24), including the respiratory tract, bloodstream and other soft tissues (4). *A. baumannii* is known for its ability to form biofilms on catheter surfaces leading to hospital-acquired pneumonia (HAP). Scientists have isolated most of *A. baumannii* pathogens from the respiratory tracts of patients in the ICU (25, 26). This pathogen also has the ability to dwell easily within the tracheostomy sites causing an acute respiratory tract infection known as community-acquired pneumonia (CAP) in non-hospitalized patients (27, 28). It can cause bacteremia due to the intravascular and respiratory tract catheter where reports in the United States have shown that bloodstream infections caused by *A. baumannii* are responsible for a death rate ranged between 34% to 43.4% at the ICU. Nosocomial post-neurosurgical meningitis is a very serious and important disease caused by multi-drug resistant *A. baumannii*, it is responsible for about 70% mortality rates in adults (27).

3. Treatment for A. baumannii

Various antimicrobial classes have been used in the treatment of *A. baumannii* infections (29). The first line of antimicrobial agents used to treat infections caused by susceptible *A. baumannii* isolates are: cephalosporins such as ceftazidime or cefepime which have a broad spectrum of activity, ampicillin-sulbactam which is a combination of β-lactams/β-lactamase inhibitor, and carbapenems such as imipenem and meropenem which have the ability to overcome the activity of beta-lactamase, all of which having a good bactericidal effect against these isolates (30). Resistance to all the previously mentioned drugs have been reported, which introduce the terms of multi-drug resistance (MDR), extensively-drug resistance (XDR) and Pan-drug resistance (PDR) in *A. baumannii*. MDR bacteria are usually resistant to at least one agent in three or more antimicrobial categories, XDR bacteria are usually resistant to at least one agent in all but two or fewer antimicrobial categories and PDR bacteria are usually resistant to all agents in all antimicrobial categories (31).

Clinicians have used tigecycline as an alternative agent for the treatment of the MDR and XDR *A. baumannii* isolates. Tigecycline is a glycylcycline which has a broad spectrum of activity against both Gram-positive and Gram-negative bacteria (32). It inhibits protein synthesis via binding to the 30S subunit of the bacterial ribosome. However, resistance to tigecycline has been reported which led to the re-emergence of polymyxin E (colistin) to treat infections by MDR and XDR *A. baumannii* (33, 34).

B. Polymyxins

Polymyxins are a large family of cationic cyclic polypeptides which have a narrow spectrum of activity against Gram-negative bacteria. Polymyxin B and polymyxin E (colistin), are the only two agents among this family used by the clinicians to treat infections by MDR and XDR *A. baumannii.* The usage of these agents has stopped in the 1970s due to their toxic effect on patients, including neurotoxicity and nephrotoxicity. However, due to the extensive spreading of both MDR and XDR infections along with the increasing resistance to tigecycline, polymyxins became the last resort against such infections (34).

The structure of polymyxin B differs from that of polymyxin E by only one amino acid which is D-phenylalanine at the position 6 of the lipopeptide, replaced by D-leucine in colistin (Figure 1). Reports have shown that colistin has less common and severe nephrotoxicity than polymyxin B. Thus, it is more prescribed by clinicians and is considered as the last line of defense against MDR and XDR *A. baumannii* infections (34, 35).

Colistin is a bactericidal polycationic lipopeptide, divided into 3 parts: a heptapeptide ring consists of D-leucine along with 4 L-α,γ-diaminobutyric acid (L-Dab), an exocyclic tripeptide consists of 3 amino acids which are L-threonine and 2 L-Dab, and a fatty acyl chain. The L-α,γ-diaminobutyric acid provides colistin with multiple positive charges at physiological pH. Colistin is available in two commercial forms: colistin sulfate which can be administered orally, and colistimethate sodium (CMS) which is the inactive form of the drug and has less potent and toxic effects than

colistin sulfate. The latter can be administered intramuscularly and intravenously or by nebulization (35).

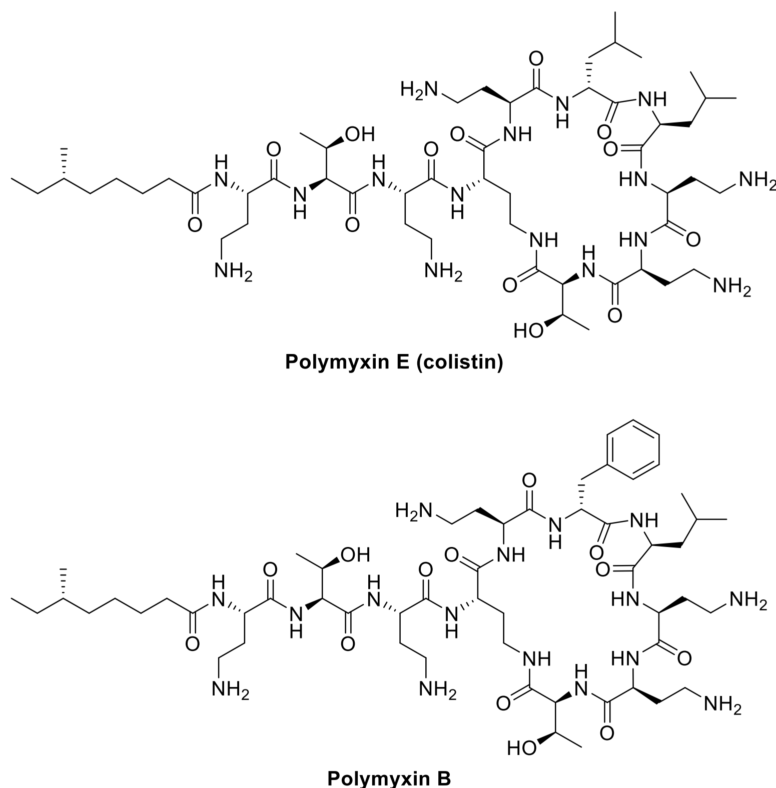


Figure 1: Chemical structures of polymyxin B and colistin.

1. *Mode of Action of Colistin*

Colistin has been described to have several modes of action against Gram-negative bacteria

a. <u>Permeabilization of the Outer Membrane (OM)</u>:

The bactericidal effect of colistin on Gram-negative bacteria occurs via two-step mechanisms: the first involves the initial binding via the electrostatic interactions between the polycationic ring of colistin and its negatively charged target lipid A of the lipopolysaccharide (LPS), leading to a competitive displacement of the magnesium

(Mg^{+2}) and calcium (Ca^{+2}) ions from the LPS phosphate groups which stabilizes the membrane. Therefore, bacterial cell death takes place through the disruption of both the inner and the outer cell membranes (Figure 2) (36)

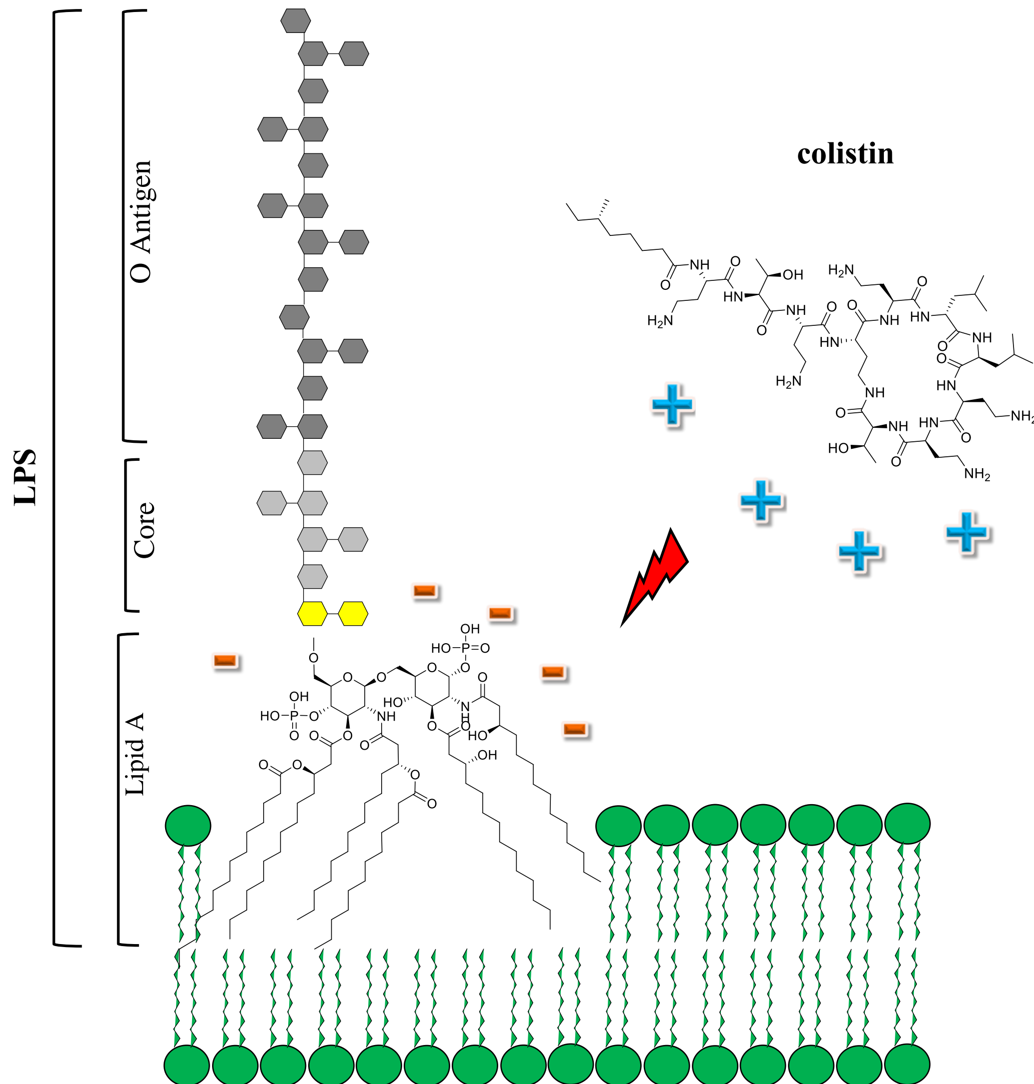


Figure 2: Mode of action of colistin targeting LPS of *A. baumannii* (36)

b. <u>Inhibition of NADH-quinone oxidoreductase activity</u>:

Scientists have reported another mode of action for colistin which takes place as it enters the bacterial cell (Figure 3). This mode of action is mediated via its binding to type II NADH-quinone oxidoreductase (NADH-II) which was proposed to be the

secondary binding site for colistin. Thus, colistin can contribute to the death of the bacterial cells by interrupting the action of NADH-II which is a vital enzyme for the function of the bacterial respiratory chain (37).

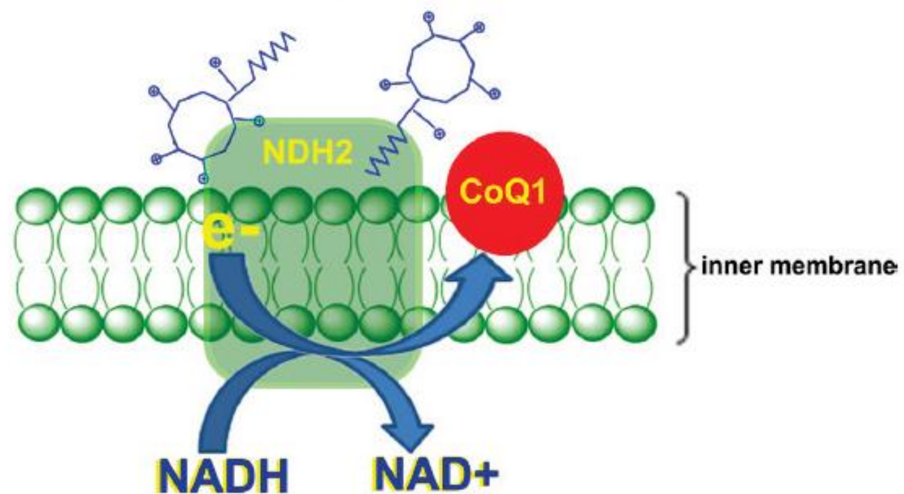


Figure 3: Mode of action of colistin targeting NADH-II of *A. baumannii* (37)

c. Hydroxyl Radical Death Pathway:

Another proposed mode of action for colistin is the hydroxyl radical death pathway which is independent of the cell lysis. It mediates the killing of the bacterial cell via the production of the hydroxyl radical which damages the DNA (Figure 4). As colistin enters the cell and reaches the inner membrane (IM), it causes the production of superoxide (O^{2-}) which through the effect of superoxide dismutase (SOD) is converted into hydrogen peroxide (H_2O_2). These two produced compounds O^{2-} and H_2O_2 lead to the inactivation of iron-sulfur (Fe-S) clusters via attacking them oxidatively, leading to iron leaking. The leaked ferrous ion (Fe^{2+}) and H_2O_2 then undergo a catalytic process called Fenton reaction which leads to the production of Fe^{3+} (ferric ion), and hydroxyl radical (•OH) respectively. Therefore, DNA, protein, and lipid damage take place (38).

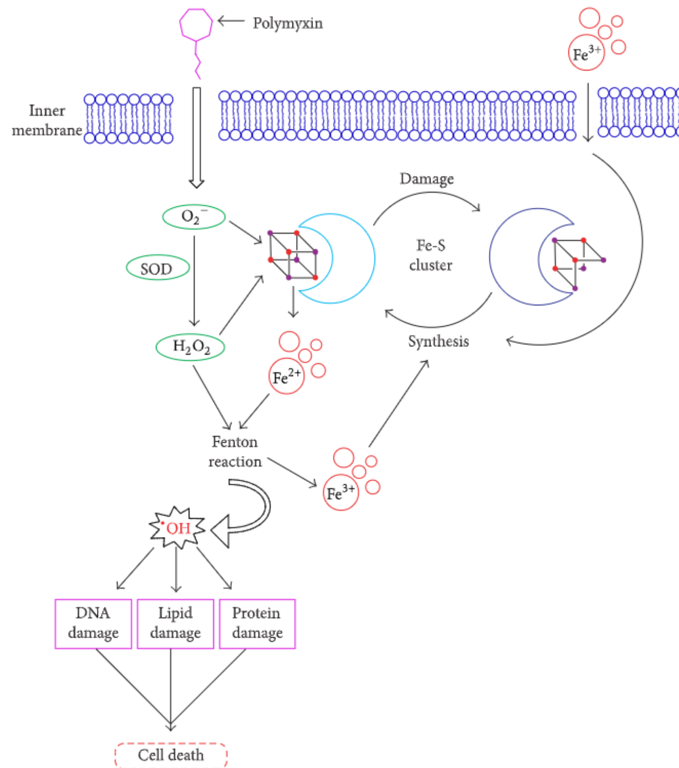


Figure 4: Mode of action of colistin inducing hydroxyl radical death mechanism in *A. baumannii* (38)

2. *Mechanisms of Colistin Resistance in A. baumannii*

A. baumannii has developed several mechanisms of resistance to overcome the effect of colistin, including lipid A modification by mutations in the two-component system pmrA/pmrB, LPS loss via a mutation in lipid A biosynthesis genes, outer membrane asymmetry, and efflux pumps (39).

a. <u>Lipid A modification via the two-component system pmrA/pmrB</u>:

Mutations in the two-component system pmrA/pmrB leading to lipid A modification is the most commonly reported mechanism of resistance to colistin by *A. baumannii* (Figure 5). This mutation occurs via amino acid substitution, such as the substitution of proline by serine at position 233(P233S), arginine by Cysteine at position 263(R263C), methionine by isoleucine at position 145(M145I). It usually takes place in the membrane-bound histidine kinase "pmrB", which may induce the continuous expression of pmrA leading to the overexpression of PmrC, a phosphoethanolamine transferase, with the consequent upregulation of pmrCAB operon. Therefore, phosphoethanolamine (PEtN) is synthesized and added to the 1′- or 4′-phosphate of lipid A of the LPS. This PEtN changes the negative charge of lipid A into a positive charge and thus lowers the affinity of the positively charged colistin (39, 40)

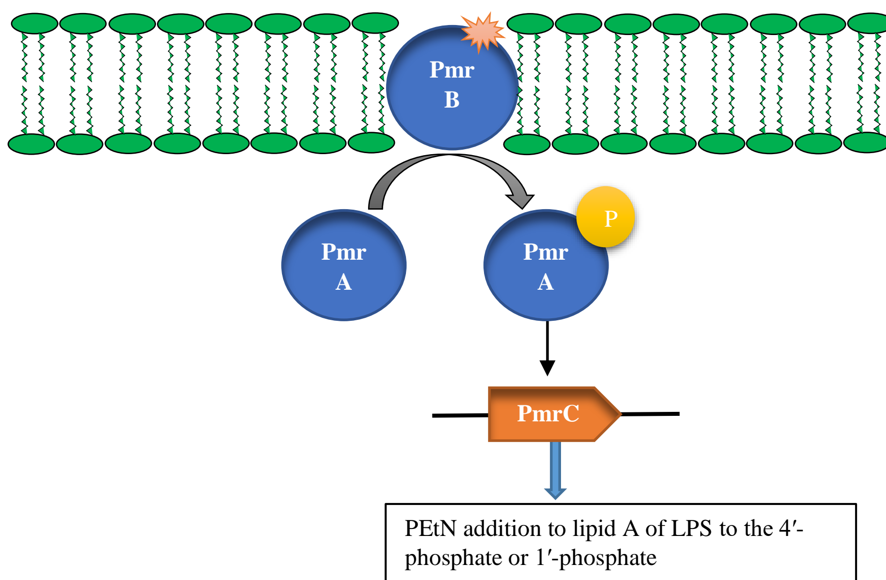


Figure 5: Mode of resistance to colistin via the two-component system pmrA/pmrB (39)

b. <u>Loss of LPS</u>:

Scientists have reported another mechanism for colistin resistance in *A. baumannii*, which is mediated via mutations by nucleotide substitution, deletion, or insertional inactivation by the insertional sequence "ISAba11" (Figure 6). These proposed mutations occur within *lpxA*, *lpxC* and *lpxD*, which are involved in the biosynthesis of lipid A of the LPS that takes place in the cytoplasm, along with mutations in the *lpsB* gene which encodes for glycosyltransferase, a vital enzyme for the synthesis of the LPS core, leading to the complete loss of the LPS and thus colistin resistance(41, 42). The loss of LPS might be due to mutations in a different gene that encodes for lptD an outer membrane protein needed for the translocation of LPS into the outer membrane (OM)(43).

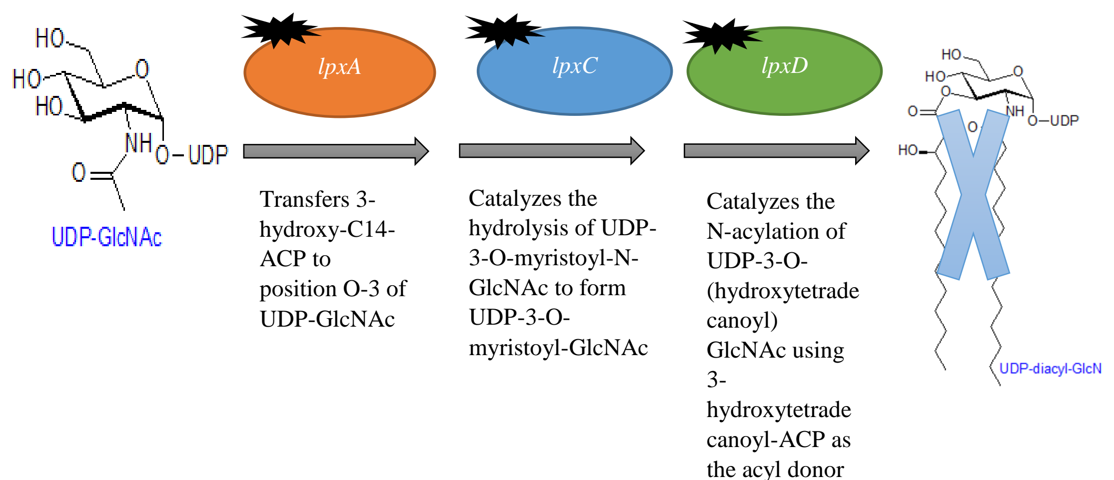


Figure 6: Mode of resistance to colistin via mutations in lipid A biosynthesis genes *lpxA*, *lpxC*, *lpxD* (39)

c. Outer-Membrane Asymmetry Genes:

Mutations in *vacJ* and *pldA* genes have been recently proposed to play a role in colistin resistance in *A. baumannii* (44). VacJ, a part of the Vps-VacJ ABC transporter system, is an outer membrane lipoprotein which plays a vital role in the maintenance of the OM asymmetry. This asymmetry is mediated via keeping the LPS in the OM outer leaflet and the phospholipid in the inner leaflet. In this way, the Gram-negative bacteria will be protected against any small toxic molecules since the asymmetric lipid distribution is vital for maintaining the cell's integrity and protection(45). On the other hand, PldA is a phospholipase which has a role in fixing any disruption in the OM via removing the phospholipids from the outer leaflet. Scientists have reported that the activity of PldA is found to be enhanced in case of bacterial membrane destabilization(46).

d. Efflux Pump:

The role of efflux pump in mediating colistin resistance in *A. baumannii* has been proposed in a few studies only. This role is demonstrated by the effect of cyanide 3-chlorophenylhydrazone (CCCP) which is an efflux inhibitor. Scientists have found a significant decrease in the minimal inhibitory concentrations (MICs) for colistin as a result of the usage of this inhibitor (47, 48). Another study has shown that tolerance to antimicrobial agents such as colistin is due to the upregulation of eighteen genes that encode for putative efflux transporters. Thus, colistin resistance in *A. baumannii* may be due to the effect of the efflux pump (47).

3. *Reported resistance to colistin in A. baumannii*

Studies on *A. baumannii* in Lebanon have addressed the carbapenem resistance. However, no study has tackled the colistin resistance in *A.baumannii*. colistin resistance has been reported by researchers in so many different *A. baumannii* isolates all over the world. Abdulzahra et al, 2018 have reported the first case of colistin resistance among carbapenem-resistant isolates from hospitalized patients in Egypt, suggesting that this resistance is due to mutations in *pmrCAB* genes (49) . Another study by Cafiso et al., 2019 has reported colistin resistance among *A. baumannii* strains isolated from two hospitalized patients being treated with colistin in two different ICU from the Sicilian hospital in Cannizzaro, Italy. After analyzing these isolates, they have found an over-expression of the *pmr*-operon along with a significant increase in the *pmrC* gene, they have also observed *lpxACD* under-expression in 1 resistant isolate (50). Colistin resistant *A. baumannii* has been isolated from hospitalized wounded patients in the US, scientists have linked resistance to colistin in these isolates to the over-expression of a novel *pmrC1A1B1* along with an increase in the expression in *pmrC* homologs: *eptA-1* and *eptA-2* (51).

C. Combination Therapy

The increased rates of antimicrobial resistance in *A. baumannii* were believed to be a result of monotherapies. The emergence of pan-drug resistance (PDR) including MDR, XDR, along with the resistance to tigecycline and colistin, and the lack of novel antibiotics has led clinicians to suggest combination therapies as the final resort to treat various types of *A. baumannii* infections. Scientists have shown that the usage of colistin in combination with other classes of antimicrobial agents, in case of colistin

resistance, may disrupt colistin resistance mechanisms such as efflux pumps which might play a role in preventing the other antimicrobial agent from mediating its action. This occurs through the ability of colistin to permeabilize the outer membrane of the bacteria. Therefore, the bacteria become more susceptible to the second antimicrobial agent used along with colistin and hence, no bacterial growth takes place (52-54). Previous studies have shown that colistin has been combined with various antimicrobial agents that mainly target Gram-negative bacteria including carbapenems and aminoglycosides and some results were found to be promising (53).

ACN Strain	Antibiotic combination of COL +	FICI	Interpretation	Mechanism of Resistance
MDR isolates	RIF	0.21-0.37	SYN (80%) IND (20%)	Not mentioned
	MEM	0.28-1	SYN (60%) IND (40%)	
	DOXY	0.62-2.5	IND (80%) ANT (20%)	
	AZM	0.37-1	SYN (60%) IND (40%)	
MDR isolate (AB210)	TLV	0.513	SYN	Production of $bla_{OXA\text{-}23\text{-}like}$ genes
	VAN	0.126	SYN	
MDR isolate (GN2231)	DAP	≤0.5	SYN	Production of $bla_{OXA\text{-}51\text{-}like}$ and $bla_{OXA\text{-}23\text{-}like}$ genes
MDR isolate (GN0624)	LVX	0.37	SYN	Production of $bla_{OXA\text{-}51\text{-}like}$ and $bla_{OXA\text{-}23\text{-}like}$ gene
MDR isolate (GN1115)		0.75	IND	
XDR isolates	TGC	0.708	IND	Not mentioned
MDR isolates	FOS	0.28-0.5 1-1.13	SYN (73.4%) IND (26.7%)	Not mentioned
	SUP	0.38-0.5 0.56-2.06	SYN (53.3%) IND (46.7%)	

XDR isolates	RIF	0.07-0.63	SYN (92%) IND (8%)	Production of $bla_{OXA-23-like}$, $bla_{OXA-24-like}$, $bla_{OXA-51-like}$, $bla_{OXA-58-like}$ and *metallo-β-lactamase* genes
	IMP	0.06-0.63	SYN (88%) IND (12%)	
	SUP	0.06-4.06	SYN (72%) IND (24%) ANT (4%)	
	LVX	0.06-1.25	SYN (64%) IND (36%)	
	FOS	0.14-18	SYN (50%) IND (44%) ANT (6%)	
MDR isolate	TEC	<0.5	SYN	Production of $bla_{OXA-23-like}$ gene
XDR isolates	AMK	N/A	SYN (66.6%) IND (33.3%)	Production of $bla_{OXA-23-like}$, $bla_{OXA-51-like}$ and *metallo-β-lactamase* genes

Table 1: colistin combination therapy results reported in the literature showing FICI and FICI ranges for MDR/XDR A. baumannii isolates. ACN: *A. baumannii*, FICI: fractional inhibitory concentration index, SYN: synergism, IND: indifference, ANT: antagonism. COL: colistin, RIF: rifampicin, MEM: meropenem, DOXY: doxycycline, AZM: azithromycin, TLV: telavancin, VAN: Vancomycin, DAP: daptomycin, LVX: levofloxacin, TGC: tigecycline, FOS: Fosfomycin, SUP: sulbactam, TEC: teicoplanin, AMK: amikacin. N/A: not applicable (55-64).

Antimicrobial agents' belonging to different classes have been tested in combination with colistin to treat MDR and XDR *A. baumannii* isolates. As shown in Table 1, colistin has been combined with rifampicin and synergism has been reported among most of the tested isolates (80% of MDR, 92% of XDR) with only 20% and 8% showing indifference, respectively. The combination of colistin with both meropenem and azithromycin showed synergism in 60% of the MDR *A. baumannii* isolates and indifference against the rest. However, no synergism was reported when the colistin-doxycycline combination was used (55, 56). In various studies, 100% synergism has been found when colistin was combined with telavancin, vancomycin, daptomycin, and teicoplanin to treat different types of MDR *A. baumannii* isolates. Resistance in these tested combinations has been linked to the production of $bla_{OXA-51-like}$ and $bla_{OXA-23-like}$

genes (57-60). Indifference has been shown in 100% of the XDR *A. baumannii* isolates when the colistin-tigecycline combination was used (61). The combination of colistin with levofloxacin, Fosfomycin, sulbactam, imipenem, and amikacin has shown to be synergistic in most of the tested isolates (MDR and XDR *A. baumannii*) and indifferent in others. The antagonistic effect has been reported only in case of colistin combination with sulbactam and Fosfomycin in a very small percentage. The mechanisms of resistance to the different antimicrobial agents are believed to be due to the production of $bla_{OXA\text{-}23\text{-}like}$, $bla_{OXA\text{-}24\text{-}like}$, $bla_{OXA\text{-}51\text{-}like}$, $bla_{OXA\text{-}58\text{-}like}$ and *metallo-β-lactamase* genes (62-64).

In the present study, various antimicrobial agents belonging to different classes which have not been used before, neither *in vitro* nor *in vivo*, against colistin-resistant isolates were selected, and these are teicoplanin, zerbaxa (ceftolozane–tazobactam), tigecycline, meropenem, levofloxacin, and Amikacin.

1. Teicoplanin

Teicoplanin is a naturally produced lipoglycopeptide (Figure 7). It has been used to treat Gram-positive bacteria, specifically Methicillin-resistant *Staphylococcus aureus* (MRSA). Teicoplanin induces bacterial death via binding to the D-Ala-D-Ala (Lipid II) moiety which links *N*-Acetyl muramine (NAM) and *N*-Acetyl glutamine (NAG) subunits of the peptidoglycan layer. Thus, blocking the peptidoglycan polymerization and inhibiting the synthesis of the bacterial cell wall (65, 66). Studies have shown that teicoplanin shares a similar structure and function with vancomycin, which is a glycopeptide, and that teicoplanin has less adverse effects than vancomycin. The combination of both colistin and vancomycin has been found to be effective against *A. baumannii* strains which are colistin resistant (67, 68). Therefore, the colistin-

teicoplanin combination can be considered a good option to treat colistin-resistant *A. baumannii* strains.

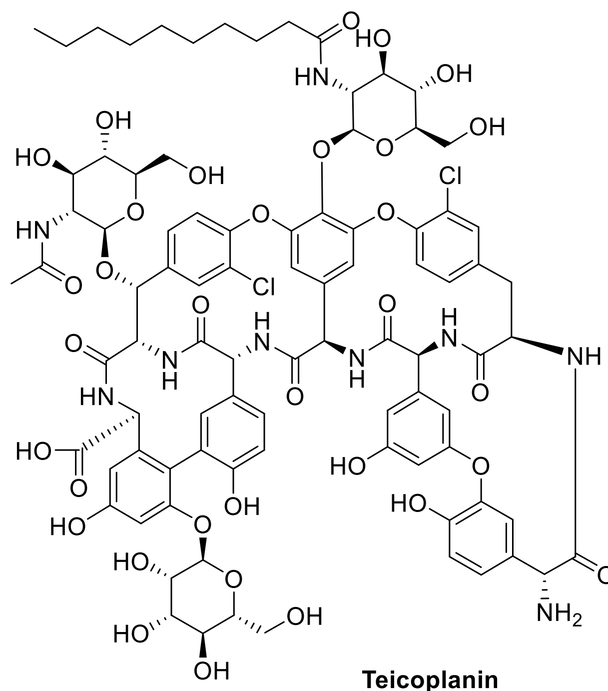


Figure 7: Chemical structure of teicoplanin

2. Zerbaxa

Zerbaxa is a combination of ceftolozane–tazobactam (Figure 8), which is a novel β-lactam/β-lactamase inhibitor combination used to treat complicated intra-abdominal infections (cIAIs), complicated urinary tract infections (cUTIs), and ventilator-associated bacterial pneumonia (VABP). Ceftolozane is a broad-spectrum cephalosporin which has a similar structure to ceftazidime. It is a β-lactam with a bactericidal activity that targets penicillin-binding proteins (PBPs), including PBP1b, PBP1c, and PBP3. Tazobactam is a β-lactamase inhibitor used to protect ceftolozane from the activity of β-lactamases. Zerbaxa is found to be active against a wide range of Gram-negative bacteria, including multidrug-resistant (MDR) *Pseudomonas aeruginosa,* extended-spectrum β-lactamase (ESBL)-producing Enterobacteriaceae,

Bacteroides fragilis, and several streptococcal species (69, 70). In this study, we are trying to test the effect of zerbaxa in combination with colistin in treating colistin-resistant *A. baumannii* infections for the first time.

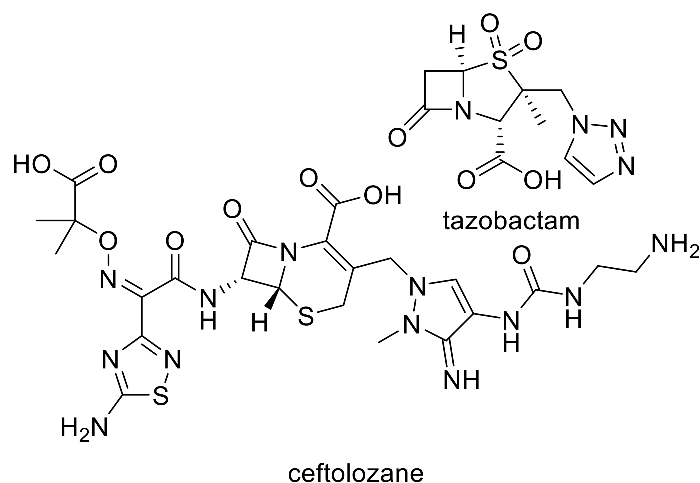


Figure 8: Chemical structure of zerbaxa

3. Tigecycline

Tigecycline is considered the first antimicrobial agent belonging to the class glycylcycline (Figure 9). It mediates its bactericidal function against both Gram-positive and Gram-negative bacteria via binding to the 30S bacterial ribosomal subunit, thus inhibiting protein synthesis. Scientists have reported various adverse effects for tigecycline, including nausea, vomiting, and diarrhea. Tigecycline had been used for the treatment of both MDR and XDR *A. baumannii* infections. However, resistance has emerged, thus tigecycline as a monotherapy is not a promising choice anymore (32, 33). Colistin-tigecycline combination has been studied for its effect against colistin-resistant *A. baumannii* isolates. In some studies, this combination was successful and showed synergistic effects in combating bacterial growth, while antagonistic effects were documented in others (71, 72). In our study, we are trying to test the colistin-tigecycline combination to show that this is not the best option to be used to treat such infections.

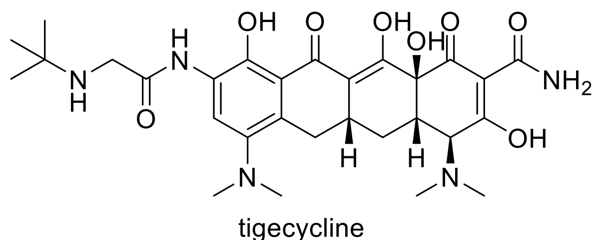


Figure 9: Chemical structure of tigecycline

4. Meropenem

As a carbapenem, meropenem is a β-lactam which has a broad-spectrum bactericidal activity against both aerobic and anaerobic Gram-negative and Gram-positive bacteria (Figure 10). It binds to penicillin binding protein and interferes with the major bacterial cell wall components causing bacterial death (73). In most of the previously reported studies, colistin-meropenem combination was found to be successful with synergistic effects. However, only few studies have shown that this combination has additive/indifferent effects against XDR *A. baumannii* strains (53, 74). In the present study, we are trying to check if this combination is a good choice to be used to treat colistin-resistant *A. baumannii* infections.

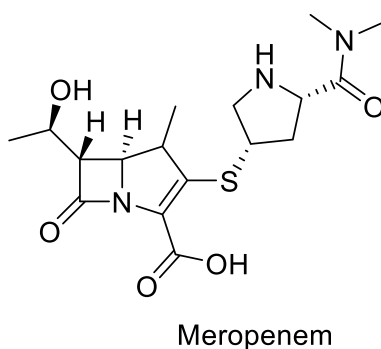


Figure 10: Chemical structure of meropenem

5. Levofloxacin

Levofloxacin is a broad-spectrum antimicrobial agent which belongs to the class fluoroquinolone (Figure 11). As a fluoroquinolone, Levofloxacin mediates its bactericidal effect via inhibiting the bacterial DNA gyrase, thus preventing bacterial DNA replication during bacterial growth. Its broad spectrum of activity includes Gram-positive, Gram-negative, aerobic and atypical bacteria (75). In Several studies, the combination of colistin and levofloxacin has been tackled and it was shown to exert either synergistic or indifferent effects when tested against XDR *A. baumanni* isolates (62, 76). It is good to try to see the effect of combining levofloxacin with colistin in order to treat colistin-resistant *A. baumannii* infections.

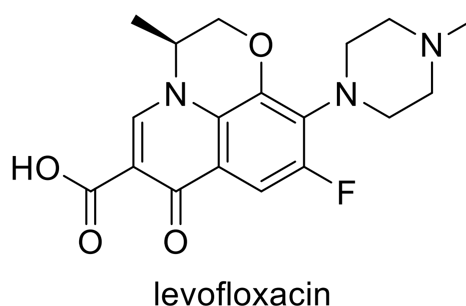


Figure 11: Chemical structure of levofloxacin

6. Amikacin

Amikacin is an aminoglycoside antimicrobial agent (Figure 12) which has a broad spectrum of bactericidal activity against Gram-negative bacteria. It acts through binding to 30S bacterial ribosome, and thus, protein synthesis inhibition and ultimately bacterial cell death (77). Previous study has reported limited synergism when colistin-amikacin combination was used against XDR *A. baumannii* strains (78). Using

amikacin in combination with colistin in this study, will give us the chance to examine its effect against colistin-resistant *A. baumannii* infections.

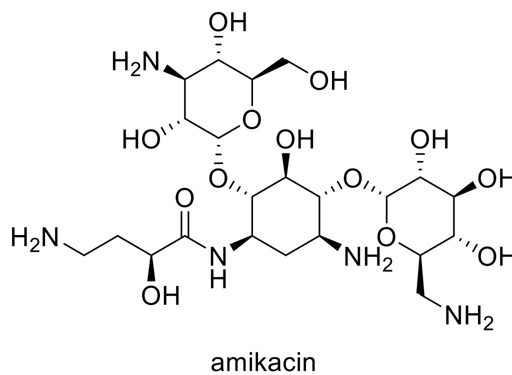


Figure 12: Chemical structure of amikacin

CHAPTERIII
MATERILAS AND METHODS

A. Source of *A. baumannii* Isolates

A total of 73 *A. baummannii* clinical isolates were obtained from the Clinical Microbiology Laboratory at the American University of Beirut Medical Center (AUBMC).

B. Bacterial Identification

1. Phenotypic Identification

a) <u>General Cultivation Conditions</u>:

Cultivation conditions such as media type and growth phase have minimal effect on the identification. For Acinetobacter species identification the colonies are usually isolated on MacConkey plates. Fresh grown isolates (overnight culture) should be used, unless it is a slow growing organism. Plates are then stored at room temperature incase needed later.

b) <u>Direct Transfer Method (DT)</u>:

Smear biological material, a small amount from a single colony, using a toothpick as a thin film directly onto a spot on a MALDI target plate. Overlay the spot with 1 ul of HCCA (Matrix) solution within 1 hour and allow to dry at room temperature.

c) <u>Extended Direct Transfer Method (eDT)</u>:

Smear biological material, a small amount from a single colony, using a toothpick as a thin film directly ontoa spot on a MALDI target plate. Overlay the spot with 1 ul of 70% Formic Acid (FA) and allow to dry at room temperature. Immediately overlay the spot with 1ul of HCCA (Matrix) solution and allow to dry at room temperature.

C. Antimicrobial agents' preparation

Colistin (Sigma-Aldrich, spruce street, St. Louis, USA), Tigecycline (Tygacil®, Wyeth Lederle S.r.L Via Franco gorgone Z.I, Catania, Italy), Teicoplanin (Sanofi Aventis, France), Zerbaxa (ceftolozane/tazobactam; Merck Sharp & Dohme Ltd Hertford road, Hoddesdon Hertfordshire EN119BU, UK), Meropenem (ACS Dobfar SpA, Italy for AstraZeneca UK Limited, Macclesfield, UK), Levofloxaacin (Sigma-Aldrich, spruce street, St. Louis, USA), Amikacin (Anfarm Hellas, Greece) and Tazocin (Wyeth Lederle S.p.A, Catania, Italy) were freshly prepared and stored at -20 °C. All antimicrobial agents used were dissolved as powder in 1mL of autoclaved Milli Q water except for Amikacin which was purchased as a liquid of concentration 500 mg/2 ml, thus it requires further dilution to achieve stock solution concentrations of 10 mg/mL.

D. Broth Microdilution Antimicrobial Susceptibility Testing

Broth microdilution susceptibility testing was established in order to determine the minimum inhibitory concentration (MIC) of the 7 tested antimicrobial agents, including colistin (COL), Tigecycline (TGC), Teicoplanin (TEC), Zerbaxa (C_T), Meropenem (MEM), Levofloxacin (LVX) and Amikacin (AMK) against the 7 ColR *A. baumannii* isolates. A volume of Cation-adjusted Mueller-Hinton Broth (CA-MHB) (BBL™, BD, Frankin Lakes, NJ, USA) equal to 0.1 mL was added to all the wells of a

sterile 96-well microtiter plate (Costar® Inc, NY, USA), and the volume needed in the first well was optimized to 200 μL to include the initial volume of the antimicrobials. For each row, a two-fold serial dilution was made between well 1 and 11, starting from a concentration of 2048 μg/mL to reach 2 μg/mL which is the lowest dilution for all the antimicrobials used. Well 12 served as a positive control. Bacterial inoculum was then prepared where each of the tested isolates were cultured on MacConkey agar plates with incubation overnight at 37 °C. A 0.5 McFarland turbidity standard was prepared in order to obtain a bacterial concentration of 2×10^8 CFU/mL. Using the 0.5 McFarland tube within 15 minutes, 789 μL of the bacterial suspension was added to 14.211 mL of CAMHB to dilute it before adding 10 μL to each well of the 96-well plate, so that the final concentration of the bacteria in the wells was 5×10^5 CFU/mL. Finally, the plates were covered to prevent contamination and left on the shaker overnight at 37 °C. Each plate was used to determine the MIC against 4 different bacterial isolates since all the tested susceptibility profiles were done in duplicates in order to provide accurate representation of the MIC. Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH (DSM) strain for *A. baumannii* which is DSM 30008 was used and ran in parallel with the clinical isolates in order to provide a reference for the initial MIC. This procedure was performed in accordance with the Clinical and Laboratory Standard Institute (CLSI) guidelines (79). However, minor changes were applied in order to adjust with the presence of the antimicrobial agents which were not mentioned in the CLSI guidelines. All adjustments were made to ensure that all the constituents were coherent to the CLSI recommendations.

E. Checkerboard Assay

Checkerboard assay has been established to check for synergism between colistin and other antimicrobial agents, including: tigecycline, teicoplanin, zerbaxa, meropenem, levofloxacin and amikacin. A volume of 100 µL CA-MHB was added into wells A_2 to A_{12} and wells C_{12} to H_{12} of a sterile 96-well microtiter plate (Figure 13). "X" µL of the CA-MHB was added into well A_1 and "Y" µL into well B_{12} (depending the MIC of each antimicrobial agent which is multiplied either by 4 or 8). "K" µL of colistin was pipetted into well A_1 and Z µL of the other antimicrobial agent into well B_{12} to reach a total volume of 200 µL. A two-fold serial dilution was performed from well A_1 to A_{10} and well B_{12} to H_{12} to reach a total volume of 100 µL in each well. Wells A_{11} and A_{12} served as negative and positive controls, respectively. 50 µL of CA-MHB was added into wells B_2 to H_{11} and "Q" µL of CA-MHB into wells B_1 to H_1 (column 1). "V" µL of colistin were added into wells B_1 to H_1 to reach a total volume of 100 µL, a two-fold serial dilution was also performed between wells in column 1 to 11 (B_1 to H_{11}) to reach a total volume of 50 µL in all wells between B_1 and H_{11} . In a different 96-well microtiter plate, a certain volume of CA-MHB was added into row 2 (B_1 to B_{11}) followed by the addition of "H" µL of the second antimicrobial agent to be tested. A total of 50 µL of the broth was added into wells C_1 to H_{11} and a two-fold serial dilution was performed by pipetting 50 µL from wells in the 2nd row (B_1 to B_{11}) to the 8th row (H_1 to H_{11}) to reach a total volume of 50 µL in all wells. The content (50 µL) of the second 96-well microtiter plate (which contains the dilution of the 2nd antimicrobial agent) were transferred into the first plate (which contains the dilution of colistin), to reach a total volume of 100 µL in wells B_1 to H_{11}. Bacterial inoculum was then prepared where each of the tested isolates were cultured on MacConkey agar plates with

incubation overnight at 37 °C. A 0.5 McFarland turbidity standard was prepared in order to obtain a bacterial concentration of 2×10^8 CFU/mL. Using the 0.5 McFarland tube within 15 minutes, 789 µL of the bacterial suspension was added to 14.211 mL of CA-MHB followed by adding 10 µL to each well in the 96-well plate, so that the final concentration of the bacteria in the wells was 5×10^5 CFU/mL. Well A_{10} served as a negative control. Finally, the plates were left on the shaker at 37 °C for 24-48 hrs. Following incubation, the turbidity (bacterial growth) 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 $\Sigma FIC \geq 2$. This procedure was performed in accordance with the Clinical and Laboratory Standard Institute (CLSI) guidelines (79). All modifications were applied in coherence with the CLSI recommendations.

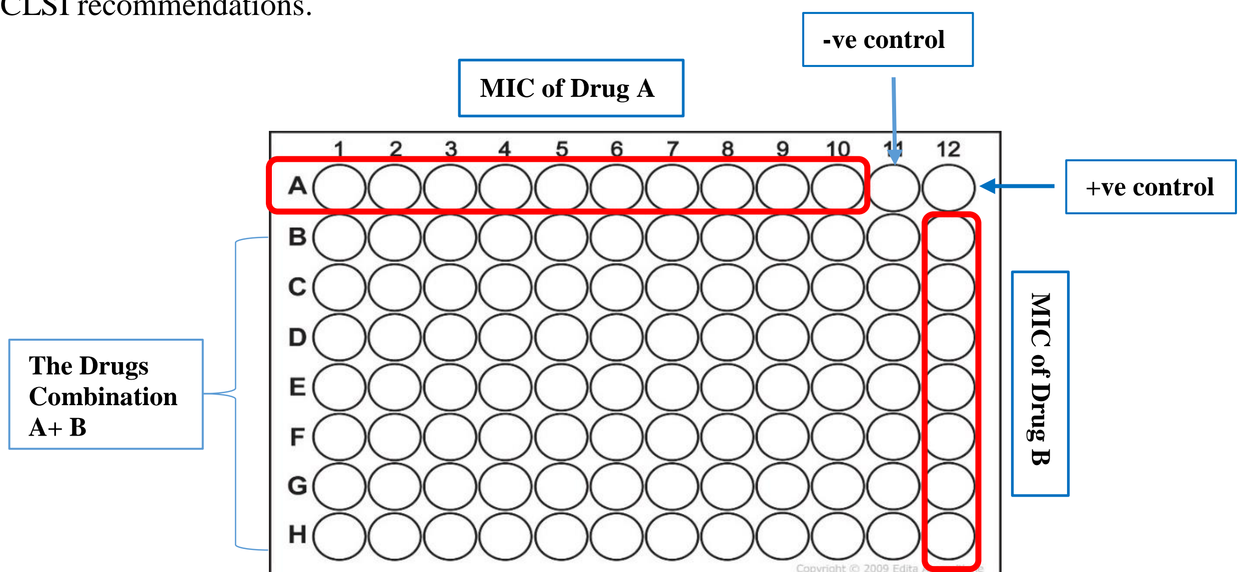


Figure 13: 96-well plate showing double therapy-checkerboard assay

1. Triple Combination therapy

Synergy between levofloxacin and tigecycline in the presence of 4 µg/mL of colistin has been assessed. Same protocol was applied as above (See Checkerboard Assay) with some modifications, where in this case antimicrobial agent A is levofloxacin, B is tigecycline and the volumes used are different depending on the MIC of each antimicrobial agent against the 7 tested isolates (Figure 14). For example: in the case of (ACN1) 0.1 mL of the broth was added into wells A_2 to A_{12} and wells C_{12} to H_{12} of a sterile 96-well microtiter plate. a volume of CA-MHB equals to194.88 µL was added into well A_1 and 5.12 µL of levofloxacin from a stock of 5 mg/mL was pipetted into the same well, 193.9 µL of the broth was added into well B_{12} followed by pipetting 6.4 µL of tigecycline from a stock of 1 mg/mL to reach a total volume of 0.2 mL. a two-fold serial dilution was made between well A_1 and A_{10} starting from a concentration of 128 µg/mL down to 0.25 µg/mL and between well B_{12} and H_{12} starting from a concentration of 32 µg/mL to 0.5 µg/mL to reach a total volume of 0.1 mL in each well. wells A_{11} and A_{12} served as negative and positive control, respectively. 0.05 mL of CA-MHB was added into wells B_2 to H_{11} and 97.44 µL of CA-MHB into wells B_1 to H_1 (column 1). 2.56 µL of levofloxacin were pipetted into wells B_1 to H_1 to reach a total volume of 0.1 mL, a two-fold serial dilution was also made between wells in column 1 to 11 (B_1 to H_{11}) to reach a total volume of 0.05 mL in all wells between B_1 and H_{11} . In another sterile 96-well microtiter plate, a 96.8 µL of CA-MHB was added into row 2 (B_1 to B_{11}) followed by the addition of 3.2 µL of the tigecycline. A total of 0.05 mL of the broth was added into wells C_1 to H_{11} and a two-fold serial dilution was made by pipetting 0.05 mL from wells in the 2nd row (B_1 to B_{11}) to the 8th row (H_1 to H_{11}) to reach a total volume of 0.05 mL in all wells. The content (0.05 mL) of the second 96-

well microtiter plate (which contains the dilution of tigecycline) were added into the first one (which contains the dilution of levofloxacin), to reach a total volume equals to 0.01 mL in wells B_1 to H_{11}. 5 µL of colistin (4 µg/mL) was then added into all wells from B_1 to H_{11} (Figure z). The preparation of colistin was performed by diluting 160 µL (10 mg/mL) in 19.84 mL of autoclaved Milli Q water to reach a total volume of 20 mL as a stock to be used in the assay. The range of concentrations used in the remaining isolates are as follows: ACN2 and ACN3 (LVX: 256-0.5 µg/mL; TGC: 32-0.5 µg/mL), ACN12 (LVX: 512-1 µg/mL; TGC: 16-0.25 µg/mL), ACN13 (LVX: 128-0.25 µg/mL; TGC: 16-0.25 µg/mL), ACN T17 (LVX: 512-1 µg/mL; TGC: 32-0.5 µg/Ml) and ACN T19 (LVX: 128-0.25 µg/mL; TGC: 64-1 µg/mL).

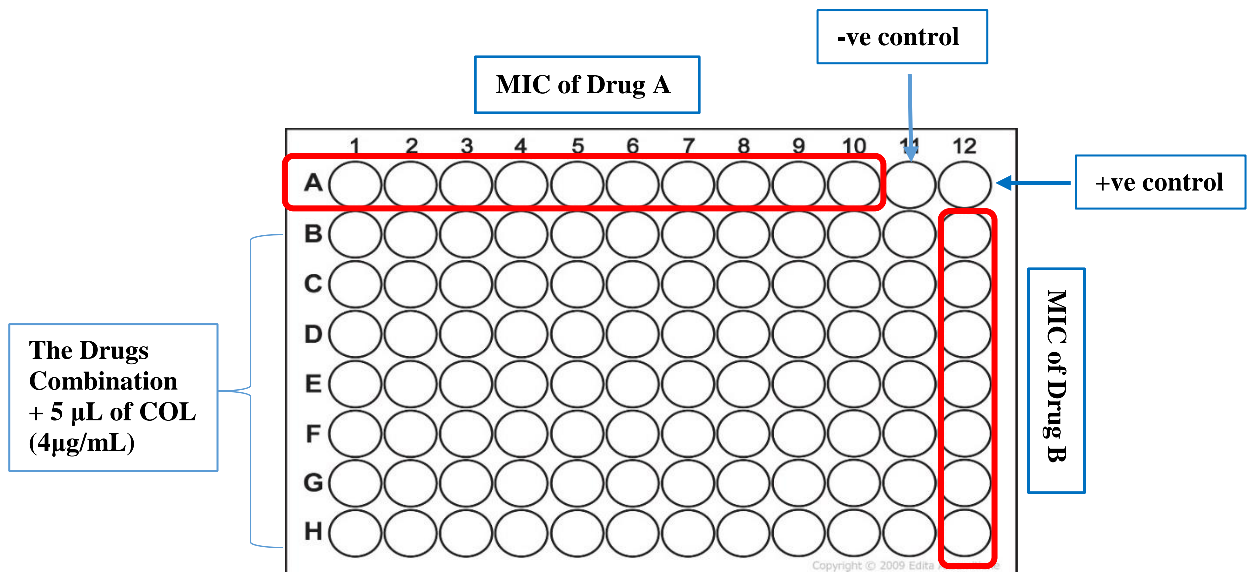


Figure 14: 96-well plate showing triple therapy-checkerboard assay

F. Pulse Field Gel Electrophoresis (PFGE)

Relatedness between the clinical isolates were assessed by using the PFGE method. This procedure was performed in accordance with the Centers for Disease Control and Prevention (CDC) guidelines (80). However, minor changes were applied in order to adjust with *A. baumannii* (81).

Day 1:

The clinical isolates were cultured on MacConkey agar plates and the BAA which is the ladder used was cultured on SS agar followed by overnight incubation at 37 °C. a total volume of 100 mL cell-suspension buffer (CSB) was prepared by mixing 100 mL of 1M Tris pH 8.0 (VWR, Life Science, AMRESCO®), 20 mL 0.5 M EDTA pH 8.0 (VWR, Life Science, AMRESCO®), and 70 mL sterile distilled H_2O (autoclaved) in a 500 mL autoclaved glass bottle. A 500 mL of cell-lysis buffer (CLB) was prepared by mixing 25 mL 1 M Tris pH 8.0, 50 mL 0.5 M EDTA pH 8.0, 425 mL sterile distilled H_2O and 5 g N-Lauryl sarcosine sodium salt (Sigma-Aldrich, spruce street, St. Louis, USA) into 1000 mL sterile (autoclaved) glass bottle. The 10X TBE of volume 500 mL was prepared by adding 500 mL of distilled H_2O into1000 mL autoclavable glass bottle followed by 60.55 g Tris Base (Trizma® base, Sigma-Aldrich, spruce street, St. Louis, USA), 30.99 g Boric Acid (VWR, Life Science, AMRESCO®) and1.85 g Disodium EDTA (VWR, Life Science, AMRESCO®), the mixture was then autoclaved. TE buffer of volume 500 mL was then prepared by mixing 5 mL 1 M Tris Base pH 8.0, 1 mL 0.5 M EDTA pH 8.0 and 494 mL sterile distilled H_2O in a 1000 mL autoclaved glass bottle.

Day 2:

Cell suspension was prepared, where 2 mL CSB were added into the 15 labeled 5 mL tubes (isolates IDs and 2 tubes for BAA) and the bacterial colonies were inoculated in the labeled tubes to reach 2 McFarland for samples and 2.5 MCF for BAAs. A 20 µL of proteinase K (Sigma-Aldrich, spruce street, St. Louis, USA) was added into the 15 labeled 1.5 microcentrifuge tubes and 400 µL of the suspension were transferred into each tube. 1 % liquid agarose gel was then prepared by adding 0.5 g Seakem Gold Agar Gel to 50 mL TE buffer in a 200 mL autoclaved Erlenmeyer flask, microwaving the mixture to dissolve and become clear and incubating it in the water bath of 50 °C. A volume of 400 µL of liquid agarose was added to the samples in the microcentrifuge tubes and the mixture was then pipetted up and down. 400 µL of the pipetted mixture was then transferred to the mold with the 1st and the 7th molds for BAA, the mold were incubated in the fridge at 4 °C to solidify. A 20 µL of proteinase K was then added to the 15 labeled 50 mL conical tubes (Avenida Industrial del Norte S/N, Reynosa, Tamaulipas, Mexico) followed by the addition of 5 mL of CLB into each tube. Molds were slided into its corresponding tubes and the tubes were incubated at 54 °C for 2 hours in shaker incubator with shaker on. The conical tube caps were replaced by the green sieved caps and each tube were then emptied, 5 mL sterile distilled water were added into each tube and incubated for 15 minutes on the shaker incubator (2 times). The sterile distilled water from each tube were emptied and 5 mL of TE buffer were added and incubate for another 15 minutes (4 times). When the last wash ended, the content of each tube was emptied and 5 mL of TE were added again, the tubes were recapped and stored in the fridge for the next day.

Day 3:

300 µL 10X Tango buffer were diluted in 2700 µL sterile distilled H_2O to form 1X tango buffer. The mixture was then vortexed, and 200 µL were added into 15 microcentrifuge tubes (labeled with samples IDs and BAA). After turning on the heating block at 37 °C, 50 mL conical tubes were taken out of the fridge and the plugs were taken out from each tube using a spatula and placed on the cutting plate were the edges of each plug dried using kimwipes (Kimberly-Clark Professional, Canada). Each plug was cut to 2x6mm using a razor and placed in its microtube having Tango buffer. The remaining of the plugs were returned into the conical tubes then into the fridge. The tubes were then incubated for 15 minutes on the heating block at 37 °C. Meanwhile, restriction mix for the *A. baumannii* isolates and BAA were prepared. The restriction enzyme used in *A. baumannii* is ApaI (Thermo Fisher Scientific, USA), while in BAA it is XbaI (Thermo Fisher Scientific, USA). For this purpose, 2447.12 µL of nuclease free water (AGUETTANT, France), 128.4 µL of tango buffer (Thermo Fisher Scientific, USA), 12.48 µL of Bovine Serum Albumin (BSA) (Bio-RAD, USA) were added into 5 mL, the mixture was then vortexed and 15.6 µL of ApaI were added and pipetted up and down to mix. 331.36 µL of nuclease free water, 52.8 µL of tango buffer and 2.64 µL of BSA were added to a different 5 mL tube, a brief vortexing were applied, followed by the addition of 13.2 µL of XbaI which was pipetted up and down to mix. The microcentrifuge tubes were taken out of the heating block and the tango buffer was discarded and 200 µL of the restriction mix were added into each tube and incubated at 37 °C for 2 hours. In the meantime, 1 % Seakem Gold Agar (SKG) (SeaKem® Gold Agarose, Lonza, USA) were prepared by diluting 5 mL of 10X TBE into 95 mL sterile distilled H_2O to get 100 mL of 0.5X TBE and adding 1 g SKG

agarose to 100 mL of 0.5X TBE. The gel was then microwaved to make sure it dissolved and incubated in the water bath for few minutes at 56 °C, 1 mL of the gel was transferred into a microtube and kept in the water bath for 20 minutes. The liquid gel was poured into the cast, it took about 20-30 mins to solidify.

After 2 hours, the plugs were removed from the microtubes and slided using a spatula into the wells of the solidified gel and 50 µL of the aliquot liquid gel from the water bath were added to cover each well and left to solidify. In order to run the gel in the machine (CHEF MAPPER, BIORAD), 2500 mL 0.5X TBE were poured into the machine, the power switch, pump switch, and cooling module were tuned on, the actual temperature was set at 14 °C, the gel was inserted into the machine after removing the white borders along with excess gel on the edges. The program was then inserted as follows:

a) Auto Algorithm
b) 30 kb – Low MW / 700 kb – High MW
c) Select default values except where noted by pressing Enter
d) (Initial switch time: 5 sec, Final switch time: 30 sec)
e) Change Run Time to 20 hrs

Day 4:

When the run time finish, the machine was turned off, the gel was then added into a tray containing 400 mL of distilled water and 40 µL of 10 mg/mL ethidium bromide (BIO-RAD, USA), the tray was placed on the rocker machine at 15 MPH for 20 mins (staining). The water from the tray was then removed and 500 mL distilled water were added to the gel and the tray was placed back to on the rock (de-staining).

The gel was visualized under UV light using the Gel Doc™ EZ Imager (BIO-RAD) and the DNA fingerprints were analyzed using BioNumerics 6.6.

G. Induction on Resistance

Induction of resistance was performed as indicated in previous studies with some modifications in order to generate resistant mutants against several antimicrobial agents with different modes of action (82). This has been done for the purpose of understanding the molecular mechanisms underlying the induced antimicrobial resistance in comparison with the reference strain. A total of 12 mutants from the reference strain (DSM 30008) were raised using the induction of resistance assay. The antimicrobial agents used for this purpose are: colistin, colistin/teicoplanin, tigecycline, colistin/tigecycline, zerbaxa, colistin/zerbaxa, amikacin, colistin/amikacin, meropenem, colistin/meropenem, levofloxacin and colistin/levofloxacin. 5 mL tubes (Avenida Industrial del Norte S/N, Reynosa, Tamaulipas, Mexico) have been used and the volume in each tube has been adjusted to be 2 mL/tube (1.9 mL of CA-MHB with the antimicrobial combination and 100 µL of bacterial suspension). For the bacterial suspension preparation, the DSM 30008 was cultured on MacConkey agar plates followed by overnight incubation at 37 °C and a 0.5 McFarland turbidity standard was prepared in order to obtain a bacterial concentration of 2×10^8 CFU/mL. For each tube, a gradual increase in the concentration was performed from 0.1MIC up to 0.5MIC, MIC, 2MIC and 4MIC. A volume of 100 µL of the bacterial suspension was added to all tubes which were incubated with constant shaking at 160 rpm overnight at 37 °C. The following day, turbidity in the broth was measured using an optical densitometer (BioMerieux DENSIMAT) and the bacteria were cultured on MacConkey Agar plate to check for bacterial growth. The tubes which showed low turbidity were left for another

24-hour incubation in the same conditions. From each tested concentration, we conducted 4 passages to ensure that resistance has been induced. Tubes that showed heavy turbidity were considered for the gradual increase in concentrations by transferring 100 μL to a new test tube harboring augmented antimicrobial concentrations. A tube containing only CA-MHB served as a negative control.

Broth Microdilution susceptibility testing against the following antimicrobial agents: COL, TGC, TEC, C_T, TZP, MEM, LVX and AMK (2048 μg/mL-2 μg/mL) has been performed on the resistant mutants that reached the highest concentration. DSM 30008 served as a reference strain.

1. Induction of Colistin Resistance on MIC-Fluctuating Isolates

For the purpose of determining the reason behind the fluctuation of the MIC in the clinical isolates ACN T12, ACN T17 and ACN T20, three passages in the presence and absence of 5 μg/mL of colistin was performed. The same experiment was conducted on DSM 30008. Same protocol was applied as above (See Induction of Resistance). Broth Microdilution susceptibility testing against colistin has been performed on the mutants, in comparison with the previous MICs.

H. Genomic DNA Extraction

Genomic DNA extraction has been performed to extract the DNA from the resistant isolates as well as the resistant mutants and send for whole-genome sequencing. We have extracted the DNA using 2 different methods, including: QIAamp® DNA Mini Kit and Phenol.

1. Genomic DNA Extraction by QIAamp® DNA Mini Kit (QIAGEN)

Bacterial suspension has been prepared as each of the tested isolates were cultured on MacConkey agar plates with incubation overnight at 37 °C. after suspending bacterial colonies in CA-MHB and visualize the turbidity, 1 mL of the bacterial suspension has been added to a sterile 1.5 mL microcentrifuge tubes (Corning Incorporated, USA) and centrifuged at 5,000 x g for 5 minutes. The supernatant was then discarded and 170 µL of buffer ATL was added to the pellet followed by 20 µL of proteinase K. the mixture was then vortexb0ed for 10 seconds and incubated in a heating block at 56 ˚C for 1 hour with brief vortexing every 15 minutes. The microcentrifuge tubes were centrifuged briefly to collect the droplets from inside the lid. A volume of 200 µL of AL buffer was then added to the tubes and vortexed for 15 seconds followed by incubation in the heating block at 70 ˚C for 10 minutes. Spin down was then applied on the microcentrifuge tubes to collect the droplets from inside the lid. 95 % ethanol of volume 200 µL was added to the microcentrifuge tubes which were then vortexed for 15 seconds, the mixture was then added to the center of QIAamp® Mini spin columns with the 2 mL collection tubes without touching the rim. The spin columns were centrifuged at 6,000 x g for 1 minute. After centrifugation was done, the spin columns were transferred into a new 2 mL collection tubes and 500 µL of AW1 buffer were added to the center of the column without touching the rim followed by centrifugation at 6,000 x g for another 1 minute. The spin columns were also transferred into new 2 mL tubes and 500 µL of AW2 buffer were added to the center of the spin column, 3 minutes centrifugation at 20,000 x g was then applied. The spin columns were then transferred into new 2 mL collection tubes and centrifuged at 20,000 x for 1 minute to get rid of the residual buffer AW2. Finally, the spin columns were then

transferred into sterile 1.5 mL microcentrifuge tubes and a maximum of 65 μL AE buffer which were incubated for few minutes on the heating block at 70 ˚C were added to the center of each microcentrifuge tube, followed by incubation at room temperature for 5 minutes. The spin columns were discarded after centrifuging the microcentrifuge tubes containing the spin columns at 6,000 x g for 1 minute. The DNA extracts were left in the microcentrifuge tubes with the AE buffer and the concentration and purity were measured using the Spectrophotometry. The microcentrifuge tubes were then stored at – 20 ˚C until use. This protocol was provided by QIAGEN specific for isolation of genomic, mitochondrial, bacterial, parasite or viral DNA.

2. Phenol method

For the purpose of preparing TrisCl-saturated Phenol, a volume of 100 mL of 50 mM TrisCl (VWR, Life Science, AMRESCO®) of molecular weight 157.64 g/mol and of pH 8 were added into100 g of phenol crystals (VWR Chemicals BDH®) in a 500 mL beaker in a fume hood, the beaker was then covered tightly by aluminum foil and the mixture was shaken gently. The mixture within the beaker was left to stand for 1-2 hours until phenol liquifies and the phases were separated (this step can be fastened by using a sonicator to help the 2 phases to be mixed easily. The supernatant was then removed via a pipette and treated as phenolic contaminant. Another 100 mL of 50 mM TrisCl pH 8 were then added and shaken gently. The mixture was also left to stand for 15 min-1 hr until the two faces were totally separated or separated via sonicator. 100 mL of 50 mM TrisCl pH 8 were added again for 2-3 times until the pH of supernatant reach 7 or 8 (using the pH strips). After reaching the required pH, the phenol phase which is in the bottom of the beaker were transferred into aliquots of 20 mL falcon

tubes and 10 mL of 50 mM TrisCl pH 8 were added to each aliquot. The aliquots were then covered by aluminum foil, labelled and stored at -20 ˚C. Bacterial inoculum was prepared after culturing each of the tested isolates on MacConkey agar plates with incubation overnight at 37 °C and suspending bacterial colonies in CA-MHB until turbidity was observed. A volume of 500 µL of the suspension was added into 1.5 mL microcentrifuge tubes and followed by another 500 µL of TrisCl- saturated phenol, the mixture was then vortexed and centrifuged for 15 min at 13,000 rpm. The supernatant was then transferred into another 1.5 mL tube, and a volume equivalent to 1/10 of the supernatant's volume of COLD sodium acetate (3 M, pH 5.2) was then added and mixed followed by the addition of 3 times the supernatant's volume of COLD 70 % which was stored at -20 ˚C ethanol, for example: if the supernatant volume was 400 µL, then 40 µL of cold sodium acetate was added and mixed by pipetting up and down and 1000 µL of cold 70 % ethanol was also added to reach a total volume which wouldn't exceed the size of the microcentrifuge tubes. The tubes were stored at -80 °C overnight. In the following day, the microcentrifuge tubes were incubated for few minutes at room temperature and then centrifuged for 15 minutes at 13,000 rpm (at 4 °C). The supernatant was then discarded, and the obtained DNA pellets undergo washing with 70 % ethanol for three times via adding of 1 mL of 70 % ethanol and centrifuging the tubes at 13,000 rpm for 15 minutes each time and discarding the supernatant. The pellet was then left to air-dry for 1-2 minutes and re-suspended in a maximum of 60 µL TE buffer (depending on the size of the pellet). The concentration and purity were measured using the Spectrophotometry. The microcentrifuge tubes were then stored at –20 ˚C until use.

CHAPTER IV
RESULTS

A. Broth Microdilution Antimicrobial Susceptibility Testing

Eighty *A. baumannii* clinical isolates were obtained from the Clinical Microbiology Laboratory at AUBMC and 73 isolates out of 80 have been tested for their susceptibility to colistin as well as different antimicrobial agents, including: tigecycline, teicoplanin, zerbaxa (ceftolozane-tazobactam), meropenem, levofloxacin and amikacin. Out of all the tested isolates, 8 isolates (ACN T17, ACN T19, ACN T31, ACN1, ACN2, ACN3, ACN12 and ACN13) were found to be resistant to colistin in accordance to CLSI guidelines (79) with MICs ≥ 128 µg/mL. The MIC values for the other tested antimicrobial agents are shown in Table 2: ACN T17, ACN1 and ACN3 were found to be resistant to all tested antimicrobial agents except for tigecycline (MIC: ≤4 µg/mL), and hence extensively-drug resistant (XDR). The remaining isolates, ACN T19, ACN T31, ACN2, ACN12 and ACN13 were found to be resistant to all the tested antimicrobial agents and hence pan-drug resistant (PDR). The MIC break point for zerbaxa against *A. baumannii* is not mentioned in the CLSI guidelines, so it was determined in advance in DSM 30008 to serve as a reference for comparison. DSM 30008 was shown to be susceptible to all the tested antimicrobial agents except for teicoplanin (TEC) with MIC: 1024 µg/mL.

Table 2: Minimum Inhibitory concentrations (MIC) of COL: colistin, TGC: tigecycline, TEC: teicoplanin, C_T: zerbaxa (ceftolozane-tazobactam), MEM: meropenem, LVX: levofloxacin, and AMK: amikacin against 7 ACN clinical isolates: ACN T17, ACN T19, ACN T31, ACN 1, ACN 2, ACN 3, ACN 12, and ACN 13 using Broth Microdilution assay (BMD). B.P: susceptibility break point.

Isolates	MIC (µg/mL)						
	COL B.P: ≤2	**TGC B.P: ≤4**	**TEC -**	**C_T B.P: ≤8**	**MEM B.P: ≤2**	**LVX B.P: ≤2**	**AMK B.P: ≤16**
DSM 30008	≤2	≤2	1024	8	≤2	≤2	≤2
ACN T17	512	4	512	256	32	32	>2048
ACN T19	512	8	1024	64	16	16	256
ACN T31	2048	64	512	256	64	64	>2048
ACN1	512	4	256	32	128	16	>2048
ACN2	128	8	128	64	64	64	>2048
ACN3	512	4	256	64	64	64	>2048
ACN12	2048	8	256	64	64	16	>2048
ACN13	4096	8	512	32	64	16	>2048

B. Checkerboard Assay

For the purpose of determining the effect of combination therapy on the colistin resistant isolates (colR): ACN T17, ACN T19, ACN1, ACN2, ACN3, ACN12 and ACN13, colistin was combined with six antimicrobial agents belonging to different classes and the results are shown in Table 3. Antagonism (ANT) was observed against all the tested isolates when COL + C_T, COL + LVX and COL + AMK combinations were used with FICI ≥2. Further antagonism was observed when COL + TGC combination was used against all the tested

isolates except for ACN T17 which displayed an indifferent effect (FICI =1.5). The combination of COL and TEC displayed an antagonistic effect when tested against ACN2, ACN3, ACN12 and ACN13 and indifferent effect when tested against ACN1. Interestingly, this combination displayed a synergistic effect when tested against ACN T17 and ACN T19 with an FICI of 0.156 and 0.0351, respectively. Indifference was observed against all the tested isolates when COL + MEM combination was used with 0.5< FICI <2.

Table 3: Checkerboard assay results showing synergy (FIC Index ≤0.5), indifference (2> FICI >0.5) and antagonism (FICI ≥2). All MIC values are in µg/ml. COL: colistin, FOS: Fosfomycin, TGC: tigecycline, TEC: teicoplanin, C_T: zerbaxa (ceftolozane/tazobactam), MEM: meropenem, LVX: levofloxacin, AMK: amikacin, COMB1: COL+ TGC, COMB2: COL+ TEC, COMB3:COL+ C_T, COMB4: COL+ MEM, COMB5: COL+ LVX, COMB6: COL+ AMK. I: interpretation, SYN: synergism, IND: indifference, ANT: antagonism.

Isolate	**ACN T17**	**ACN T19**	**ACN 1**	**ACN 2**	**ACN 3**	**ACN 12**	**ACN 13**
MIC COL	512	2048	256	32	512	2048	4029
MIC TGC	2	8	4	8	4	8	8
MIC COL IN COMB 1	512	>2048	256	32	512	>2048	8192
MIC TGC IN COMB 1	≤1	8	16	8	8	16	16
FICI COL+ TGC	1.5	>2	5	2	3	4	4
I	**IND**	**ANT**	**ANT**	**ANT**	**ANT**	**ANT**	**ANT**
MIC COL	512	512	512	64	512	2048	4096
MIC TEC	512	1024	256	128	256	256	512
MIC COL IN COMB 2	16	≤2	512	128	1024	2048	2048
MIC TEC IN COMB 2	64	≤32	128	128	128	256	1024
FICI COL+ TEC	0.156	0.0351	1.5	3	2.5	2	2.5

I	SYN	SYN	IND	ANT	ANT	ANT	ANT
MIC COL	256	512	256	32	256	512	4096
MIC C_T	256	64	32	64	64	64	32
MIC COL IN COMB 3	512	1024	1024	32	512	1024	8192
MIC C_T IN COMB 3	256	16	32	64	64	64	64
FICI COL+ C_T	3	2.25	5	2	3	3	4
I	**ANT**	**ANT**	**ANT**	**ANT**	**ANT**	**ANT**	**ANT**
MIC COL	64	1024	1024	128	512	2048	4096
MIC MEM	32	16	128	64	64	64	64
MIC COL IN COMB 4	16	≤2	1024	32	32	128	4096
MIC MEM IN COMB 4	16	≤8	64	64	32	32	32
FICI COL+ MEM	0.75	0.5019	1.5	1.25	0.5625	0.5625	1.5
I	**IND**	**IND**	**IND**	**IND**	**IND**	**IND**	**IND**
MIC COL	128	1024	512	32	512	1024	4096
MIC LVX	32	16	16	64	64	16	16
MIC COL IN COMB 5	126	1024	1024	64	512	1024	8192
MIC LVX IN COMB 5	32	32	32	64	64	32	32
FICI COL+ LVX	2	3	4	3	3	3	4
I	**ANT**	**ANT**	**ANT**	**ANT**	**ANT**	**ANT**	**ANT**
MIC COL	128	512	512	32	256	512	8192
MIC AMK	>8192	256	>8192	>8192	>8192	>8192	>8192
MIC COL IN COMB 6	1024	2048	>1024	64	1024	1024	>8192
MIC AMK IN COMB 6	>8192	512	>8192	>8192	>8192	>8192	>8192
FICI COL+ AMK	9	6	3	3	5	3	2
I	**ANT**	**ANT**	**ANT**	**ANT**	**ANT**	**ANT**	**ANT**

1. Triple-combination therapy

For the purpose of determining the effect of using colistin as a surfactant in combination with levofloxacin and tigecycline against all the tested colR isolates, and since all combinations used were shown to be antagonistic in most of the cases when tested against all colR isolates, LVX + TGC combination was used in the presence of 4µg/mL of COL and the results are shown in Table 4. Antagonism was observed when this triple-combination therapy was used against all the tested isolates (FICI ≥2), except for ACN 17 which displayed indifferent effect with FICI =1.5.

Table 4: Checkerboard assay results showing synergy (FIC Index ≤0.5), indifference (2> FICI ≥0.5) and antagonism (FICI ≥2). All MIC values are in µg/ml. (4 µg/ml) of COL was added in all combinations.

Isolate	**ACN T17**	**ACN T19**	**ACN 1**	**ACN 2**	**ACN 3**	**ACN 12**	**ACN 13**
MIC $_{LVX}$	64	32	32	16	16	32	32
MIC $_{TGC}$	8	8	8	4	8	4	8
MIC $_{LVX}$ IN COMB (COL:4µg/mL)	32	32	32	16	32	32	32
MIC $_{TGC}$ IN COMB (COL:4µg/mL)	8	16	16	8	16	4	16
FICI LVX + TGC	1.5	3	3	3	4	2	3
I	**IND**	**ANT**	**ANT**	**ANT**	**ANT**	**ANT**	**ANT**

C. Induction of resistance

For the purpose of understanding the molecular mechanisms beyond the induced antimicrobial resistance in comparison with the reference strain, a total of 12 mutants from the reference strain (DSM 30008) were raised using the following antimicrobial agents and combinations: colistin, colistin / teicoplanin, tigecycline, colistin / tigecycline, zerbaxa, colistin / zerbaxa, amikacin, colistin / amikacin, meropenem, colistin / meropenem, levofloxacin and colistin / levofloxacin. The MICs and the highest concentrations reached with each induced antimicrobial agent and combinations are shown in Table 5. Four-passages at 4MIC were reached in all antimicrobial agents and combinations with concentrations of 2 µg/mL for COL and 8 µg/mL for the rest of the antimicrobial agents mentioned in Table 5, except in the case of COL/TEC combination where four-passages at the MIC have been reached with concentrations of 0.5 / 1024 µg/mL as no growth was observed when higher concentrations were used.

Table 5: Induction of resistance to COL, COL+ TEC, TGC, COL+ TGC, C_T, COL+ C_T, AMK, COL+ AMK, MEM, COL+ MEM, LVX, COL+ LVX on the ACN DSM 30008 strain.

ACN Strain	Antibiotics	Concentration (µg/mL)
ACN DSM 30008	Colistin	4MIC: 2
	Tigecycline	4MIC: 8
	Zerbaxa (C_T)	4MIC: 8
	Meropenem	4MIC: 8
	Levofloxacin	4MIC: 8
	Amikacin	4MIC: 8
	Colistin / Teicoplanin	MIC: 0.5 / 1024
	Colistin / Tigecycline	4MIC: 2 / 8
	Colistin / Zerbaxa	4MIC: 2 / 8
	Colistin / Meropenem	4MIC: 2 / 8
	Colistin / Levofloxacin	4MIC: 2 / 8
	Colistin / Amikacin	4MIC: 2 / 8

D. Minimal Inhibitory Concentration (MIC) determination of Resistant Mutants

The minimal inhibitory concentrations (MICs) of different antimicrobial agents, including: COL, TGC, TEC, C_T, TZP (piperacillin-tazobactam), MEM, LVX and AMK have been determined against the 12 *A. baumannii* mutants (DSM 30008 mutants), the results are shown in Table 6. All the resistant mutants have revealed high resistance profile against colistin with MIC ≥16 µg/mL, except for TGC mutant which remained susceptible to COL with MIC ≤2 µg/mL similar to that of the reference strain DSM 30008. 4 out of 12 resistant mutants, including: TGC, COL/TGC, COL/TEC and AMK mutants revealed high resistance profile against TGC with MIC ≥8 µg/mL, the rest of the mutants were found to be susceptible with MIC ≤2 µg/mL similar to that of DSM 30008 reference strain. All the resistant mutants along with DSM 30008 have showed high resistance profile against TEC with MIC values between 32 and > 2048 µg/mL. For the rest of the tested antimicrobial agents, including: C_T, TZP, MEM, LVX and AMK, the mutants have showed similar susceptibility patterns against these agents, including: COL, TGC, COL/TGC and COL/TEC mutants which showed high susceptibility profiles similar to that of the reference strain with MIC values of: ≤8 µg/mL for C_T, ≤16 µg/mL for TZP, ≤2 µg/mL for both MEM and LVX and ≤ 16 µg/mL for AMK except for COL/TGC mutants which show resistance to AMK with MIC of 32 µg/mL. However, C_T, COL/C_T, MEM, COL/MEM, LVX, COL/LVX, AMK and COL/AMK mutants revealed high resistance profile with MIC values of: ≥64 µg/mL for C_T, : ≥256 µg/mL for TZP, : ≥64 µg/mL for MEM, : ≥ 16 µg/mL for LVX and >2048 µg/mL for AMK.

Table 6: Minimum Inhibitory Concentrations (MIC) of COL, C_T, TEC, TGC, TZP: tazocin (piperacillin/tazobactam), MEM, LVX, and AMK against ACN DSM30008 strain and ACN DSM30008 with induced resistance to COL, TGC, COL/TGC, and COL/TEC, C_T, COL/C_T, MEM, COL/MEM, LVX, COL/LVX , AMK and COL/AMK.

	Antibiotics	MIC (µg/mL)							
		COL **B.P: ≤2**	**TGC** **B.P: ≤4**	**TEC** **-**	**C_T** **B.P: ≤8**	**TZP** **B.P: ≤16**	**MEM** **B.P: ≤2**	**LVX** **B.P: ≤2**	**AMK** **B.P: ≤16**
ACN DSM 30008	**DSM 30008**	≤2	≤2	1024	8	16	≤2	≤2	≤2
	Colistin 4MIC:2µg/mL	16	≤2	128	≤2	16	≤2	≤2	≤2
	Tigecycline 4MIC:8 µg/mL	≤2	32	32	8	16	≤2	≤2	4
	Col / Tige 4MIC:2 / 8 µg/mL	512	64	128	8	16	≤2	≤2	32
	Col / Teicoplanin MIC:0.5 / 1024 µg/mL	128	64	128	8	16	≤2	≤2	16
	Zerbaxa(C_T) 4MIC:8 µg/mL	1024	4	512	64	256	64	32	>2048
	Col / Zerbaxa 4MIC:2 / 8 µg/mL	1024	≤2	256	64	256	128	32	>2048
	Meropenem 4MIC:8 µg/mL	512	≤2	1024	64	512	64	32	>2048
	Col, Meropenem 4MIC:2 / 8 µg/mL	512	4	512	64	512	128	32	>2048
	Levofloxacin 4MIC: 8 µg/mL	1024	≤2	512	64	512	128	32	>2048
	Col, Levofloxacin 4MIC:2 / 8 µg/mL	2048	4	512	64	512	128	16	>2048
	Amikacin 4MIC:8 µg/mL	1024	8	>2048	512	512	256	32	>2048
	Col, Amikacin 4MIC:2 / 8 µg/mL	2048	4	512	128	512	64	32	>2048

E. Induction of Colistin Resistance on MIC-Fluctuating Isolates

1. *MIC determination:*

For the purpose of investigating the reason beyond the fluctuation in the MICs of the three ACN clinical isolates: T12, T17 and T20 against COL, these isolates along with DSM 30008 reference strain were incubated with 0. 5 µg/mL of COL and repassed on the same concentration for 3 times followed by 3 passages without COL. The MIC values were then determined using BMD and the results are shown in Table 6. ACN T17, T20 and DSM 30008 behaved similarly when incubated and repassed for three times with and without COL, where the MICs increased up to 512,1024 and 2048 µg/mL, respectively after 3 passages with COL. The MICs of these isolates and the reference strain have decreased after 3 passages without COL down to 8, 4 and 256 µg/mL, respectively. ACN T12 behaved differently in comparison with the rest of the tested isolates and the reference strain, where the MIC decreased to 2 µg/mL and became susceptible after 3 passages in the presence of COL. However, its MIC has increased to 4 µg/mL and the isolate has returned to be resistant to COL after 3 passages without COL.

Table 7: Induction of COL resistance on the ACN DSM 30008 strain, T12, T17, and T19 showing MICs to COL after 3 passages with 0.5 µg/ml of COL, and after 3 passages deprived of COL on the 0.5µg/ml induced isolates.

Isolates	Previous MIC to COL	MIC after 3 passages with COL (0.5µg/ml)	MIC after 3 passages without COL (0.5µg/ml induced isolates)
DSM 30008	0.5	2048	256
ACN T12	2048	2	4
ACN T17	512	512	8
ACN T20	512	1024	4

F. Pulse Field Gel Electrophoresis (PFGE) Dendrogram

For the purpose of investigating the level of relatedness between the *A. baumannii* clinical isolates, PFGE was performed using bacterial suspension. The gel images are shown in Figures 15, 16 and 17 and the dendrogram results are shown in Figure 18. Using ≥75% similarity cut-off as a threshold (83), PFGE classified our *A. baumannii* isolates into 8 clusters (Figure 18). Cluster 1 comprises 2 isolates: ACN T31 and ACN T33 with percentage of relatedness: 77.8%, cluster 2 comprises 22 isolates: ACN T37, ACN T40, PLMB 234, PLMB 273, ACN T36, ACN30, ACN T46, ACN T47, ACN T45, ACN T38, ACN T39, ACN T41, ACN T35, ACN T6, ACN T8, ACN T5, ACN T9, ACN T32, ACN T34, ACN26, ACN27 and ACN29 with percentage of relatedness: 78.4%. Cluster 3 comprises 9 isolates: ACN T10, ACN T17, ACN T3, ACN T4, ACN T14, ACN T16, ACN T12, ACN T18 and ACN T1, with 76.8%

relatedness. ACN T15 is related to the 3rd cluster by 72.3%. The 4th cluster is related by 75% and comprises 2 isolates: ACN T21 and ACN T26. The 5th cluster comprises 12 isolates: ACN T22, ACN T23, ACN T28, ACN T30, ACN T31, ACN T29, ACN T24, ACN T25, ACN T19, ACN T27, ACN T7 and ACN T7, and the percentage of relatedness were shown to be 75.2%. The 6th cluster comprises 2 isolates: ACN T11 and ACN T13 with 97.1% relatedness. ACN T2 is related to the 6th cluster by 70.7%. The 7th cluster is related by 78.8% and comprises 4 isolates: ACN14, ACN32, ACN13 and ACN33. The 8th and final cluster is related by 84.9% and comprises 12 isolates: ACN1, ACN2, ACN12, ACN3, ACN7, ACN10, ACN11, ACN9, ACN4, ACN T48, ACN5 and ACN28. Within the 2nd cluster, ACN T46 and ACN T47, ACN T39 and ACN T41, ACN26 and ACN27 were shown to be identical with 100% relatedness. Within the 3rd cluster, ACN T14 and ACN T16 were found to be identical. Within the 5th cluster, 3 isolates were found to be identical, including: ACN T22, ACN T23 and ACN T28. The 8th cluster also consists of identical isolates, including: ACN1 and ACN2, ACN7 and ACN10. The percentage of relatedness between all isolates is 58.7%.

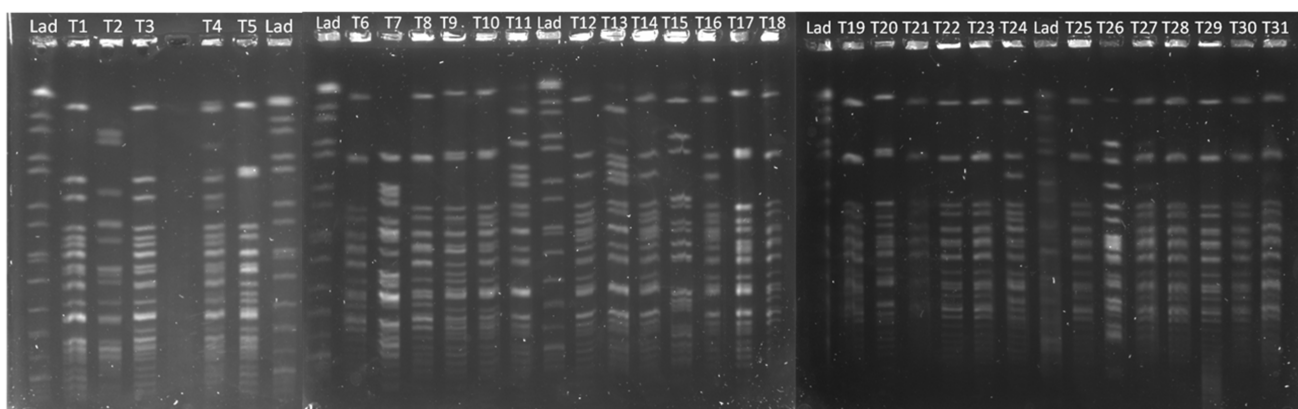


Figure 15: PFGE gel images for 31 *A. baumannii* isolates: T1 to T31, showing DNA fingerprints.

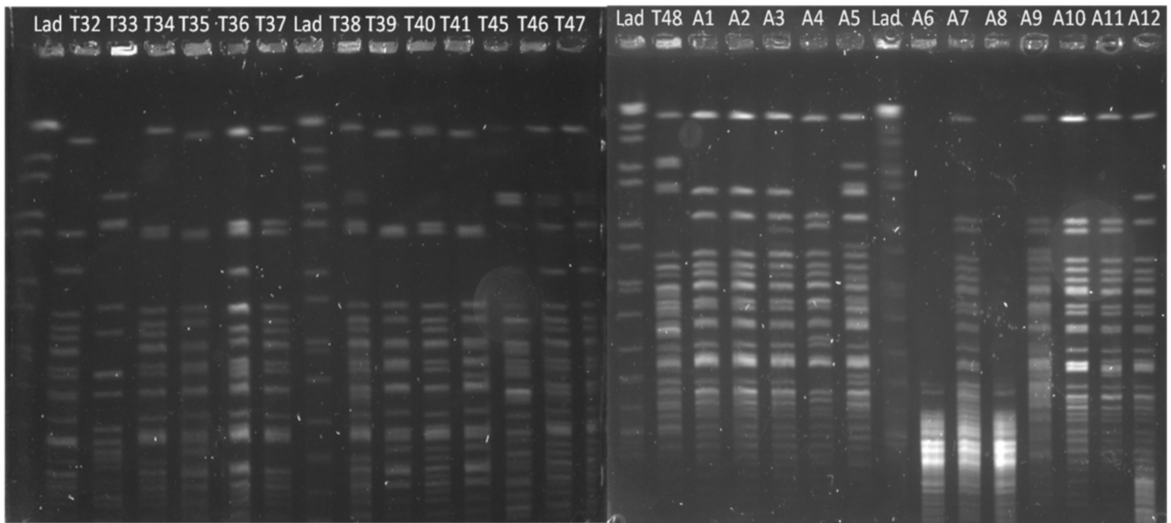


Figure 16: PFGE gel images for 26 *A. baumannii* isolates: T32-T48 and ACN1-ACN12, showing DNA fingerprints.

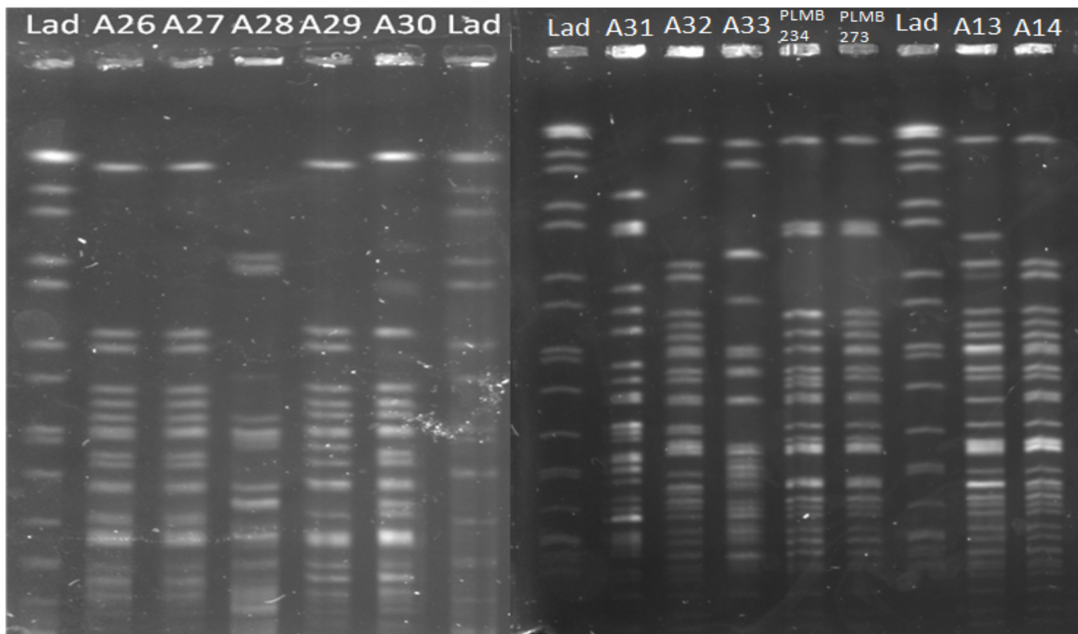
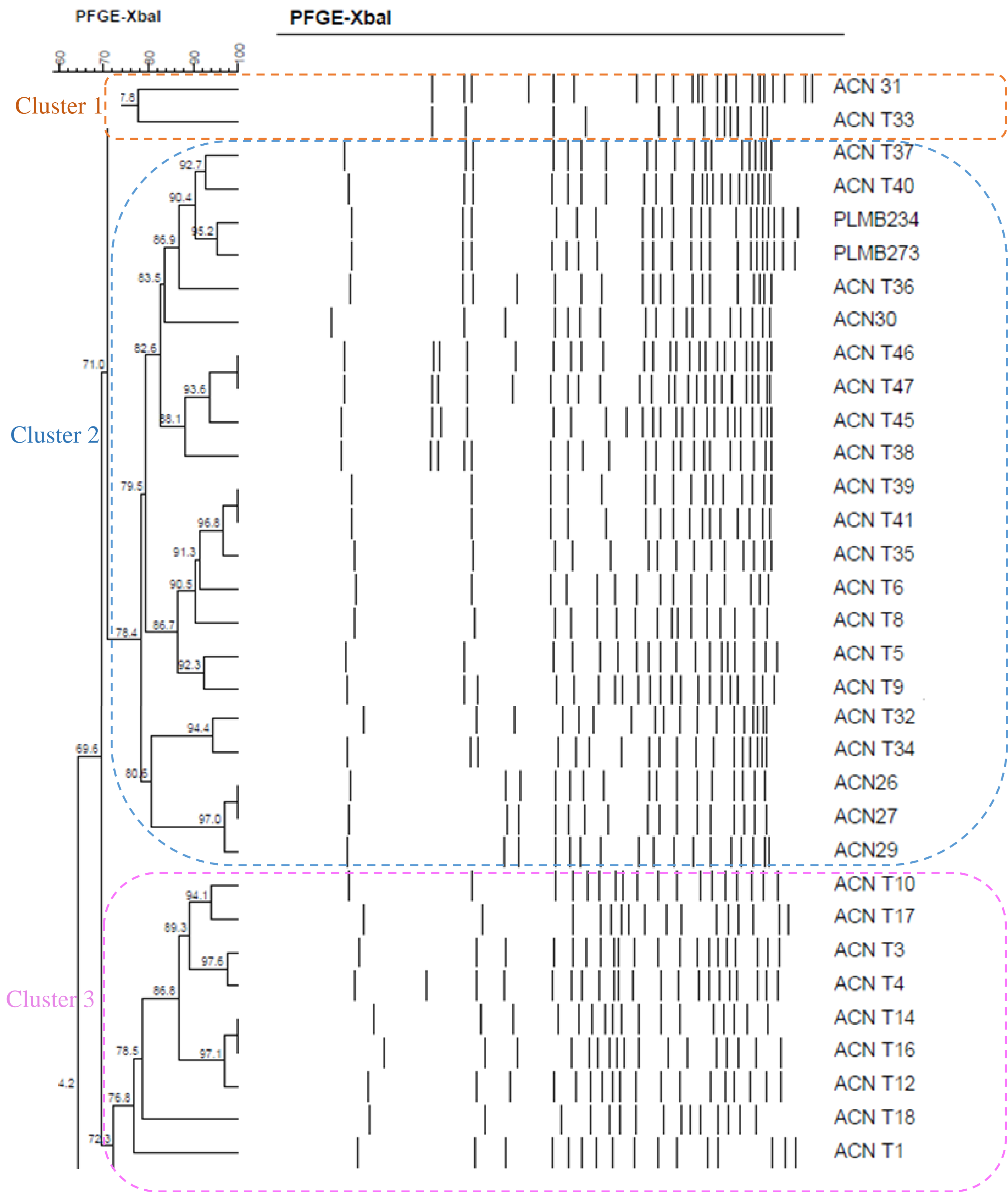


Figure 17: PFGE gel images for 12 *A. baumannii* isolates: ACN13, ACN14, ACN26-33 and PLMB234, PLMB273, showing DNA fingerprints.


PFGE-XbaI
PFGE-XbaI
60
70
80
90
100
Cluster 1
Cluster 2
Cluster 3
ACN 31
ACN T33
ACN T37
ACN T40
PLMB234
PLMB273
ACN T36
ACN30
ACN T46
ACN T47
ACN T45
ACN T38
ACN T39
ACN T41
ACN T35
ACN T6
ACN T8
ACN T5
ACN T9
ACN T32
ACN T34
ACN26
ACN27
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ACN T14
ACN T16
ACN T12
ACN T18
ACN T1

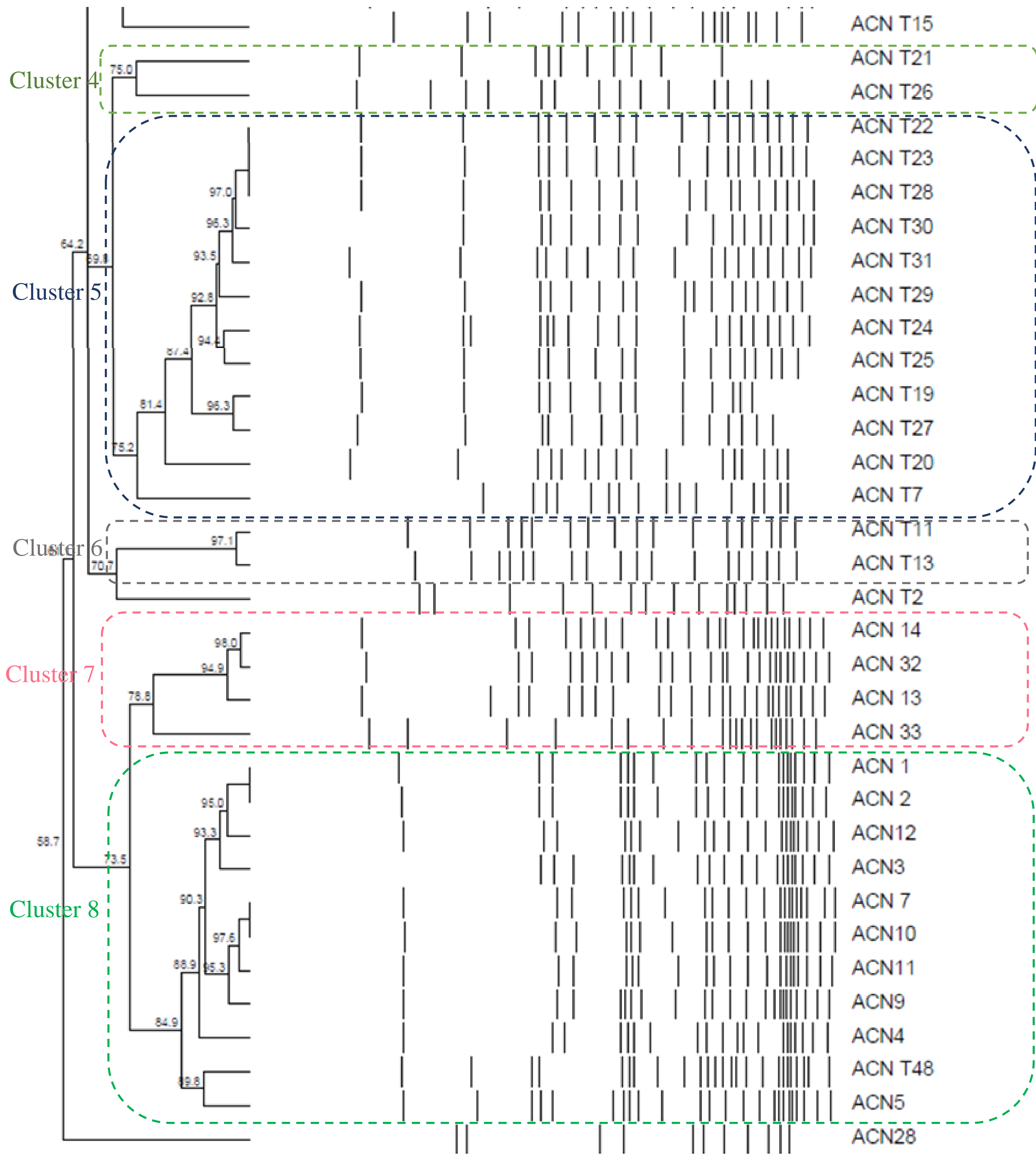

Figure 18: PFGE dendrogram for *A. baumannii* isolates showing percentage of relatedness. 8 clusters are identified with a cut-off level ≥75%.

CHAPTER V
DISCUSSION

Antimicrobial resistance (AMR) is rising into dangerously high levels in all parts of the world which threatens our ability to treat common infectious diseases. This increase in AMR is due to the continuous misuse and abuse of antimicrobial agents. *A. baumannii* is considered one of the notorious superbugs which is associated with world-wide nosocomial infections, it is classified by the World Health Organization as a "Priority 1 for research of new antibiotics" and a "critical" pathogen. Scientists have reported world-wide increasing rates in MDR, XDR and lately PDR *A. baumannii*, where the lack of discovery of new antimicrobial agents, have led scientists to re-use colistin to treat highly resistant infections, which in turn led to the emergence of colistin resistance (84-86).

According to Vijayakumar et al., 2019, MALD-TOF is not considered an accurate method for *Acinetobacter* species identification, where species within the *Acinetobacter calcoaceticus-Acinetobacter baumannii* (ACB) complex are found difficult to be distinguished. Variations in the specificity and sensitivity have been reported for ACB complex identification by MALD-TOF (87). Thus, PCR based assay of the *gyrB* gene was performed on all isolated to reconfirm their identity as *A. baumannii* at the molecular level according to Higgins et al., 2007 and Higgins et al, 2010. All isolates previously tested were reconfirmed to be *A. baumannii* as shown in the electrophoresis gel images in Apendix I, Figures 19, 20 and 21 (88, 89), except for ACN T29, ACN T30, ACN6, ACN7 and ACN15; these isolates might be different Acinetobacter species. ACN T47 was repeated by PCR and found to be *gyrB* positive as shown in Figure 21.

For the purpose of assessing the effect of combination therapy on MDR, XDR and PDR *A. baumannii* clinical isolates and to understand the mode of resistance to the antimicrobial agents belonging to different classes, 73 *A. baumannii* clinical isolates have been assessed for their susceptibility to colistin. Eight isolates showed resistance to colistin and thus, were tested for their susceptibility to other antimicrobial agents, including: tigecycline, teicoplanin, zerbaxa, meropenem, levofloxacin and amikacin using broth microdilution assay. The colR isolates in Table 2 were found to be XDR including: ACN T17, ACN1 and ACN3 and PDR including: ACN T19, ACN T31, ACN2, ACN12 and ACN13. Based on the MIC values, the variations in the resistance profile of these isolates is attributed to different mechanisms of resistance, such as: efflux pumps, porin loss and LPS modification (29, 39).

Interestingly, some isolates among the 73 assessed were found to have fluctuating MIC values, including: ACN T12, ACN T17 and ACN T20. For the purpose of understanding the reason beyond this fluctuation, these isolates along with the reference strain (DSM 30008) were incubated in the presence of 0.5 µg/mL of colistin and repassed for three times followed by 3 passages without COL, BMD was then performed on these isolates against COL. The fluctuations in MICs presented in Table 7 might be due to an important phenomenon known as “Fitness Cost”. In brief, fitness cost reflects how costly is the mutation for a bacterium; according to Andersson et al., 2010, most resistance mechanisms were found to be associated with fitness cost and this is manifested by the reduction in the bacterial growth rate which affects the resistance development rate, the stability of the resistance as well as “the frequency of resistance accompanied with antibiotics usage” (39, 90). This aspect might explain the fluctuations in the MICs observed in the assessed isolates. To further confirm this hypothesis, fitness

cost assays should be performed in order to understand the reason beyond the observed MIC variations which in turn might be due to energy (ATP) consumption (91).
The variations in MICs of these isolates after 3 passages with and without COL reflects that these isolates might have a high fitness cost which affects the growth rate of the bacteria; thus, the bacteria find it easier to revert than to preserve the mutation.

Checkerboard assay has been performed to assess the effect of combination therapy on the colR isolates. Interestingly, some results were found to be promising (Table 3), where the combination of COL and TEC shows synergy when tested against ACN T17 and ACN T19 only. Interestingly, these two isolates were collected at the same year "2019" and our molecular analysis by PFGE dendrogram shows that the percentage of genomic relatedness between these two isolates is 64.2 %, which reflects that within this genome similarity possible identical resistant genes might be present reflecting similar mechanisms of resistance. ACN T17 and ACN T19 behaved similarly when tested against the other combinations and displayed antagonistic effects when COL + TGC, COL + C_T, COL + LVX and COL + AMK combinations were used and indifferent effects when COL + MEM combinations were assessed. In order to further understand the similarity and the mechanisms of resistance between T17 and T19 such as the lipid A modification via the two-component system pmrA/pmrB and the loss of LPS (39), whole genome sequencing (WGS) should be performed to check genome-wide modifications. This will give insights and confirm the presence of similar resistant genes and thus, similar resistance mechanisms. This approach could be applied on other assessed isolates such as ACN1, ACN2, ACN3, ACN12 and ACN13, that behaved similarly and displayed antagonistic effect when tested against all the combinations except for MEM (indifferent effect). This is further supported by our dendrogram

analysis where these isolates belong to clusters 7 and 8 that are related by more than 70%.

Triple combination therapy was also assessed to determine the effect of using colistin as a surfactant (4 µg/mL) in combination with levofloxacin and tigecycline against all the tested colR isolates. Based on the results in Table 4, all tested isolates behaved similarly and displayed antagonistic effect when tested against this combination, where the usage of colistin as a surfactant was found to have no effect in allowing other antimicrobial agents to exert their function; Furthermore, the percentage of relatedness between all tested isolates is 58.7 % which might be the reason behind the similar behavior against the triple- combination. The usage of a low concentration of COL might also be the reason behind the negative impact on the combination therapy. Nevertheless, all tested isolates are colR, so it would be useful to try using higher concentrations of colistin to check for possible synergy.

For the purpose of understanding the molecular mechanisms beyond the induced antimicrobial resistance, 12 mutants have been raised using different antimicrobial agents and combinations: COL, TGC, COL/TGC, COL/TEC, C_T, COL/C_T, MEM, COL/MEM, LVX, COL/LVX, AMK and COL/AMK. 4 MIC has been reached in all antimicrobials and combinations used except for COL/TEC. BMD was then performed to assess the effect of inducing resistance on the DSM 30008 reference strain. Based on the results presented in Table 6, the 12 raised mutants have affected the susceptibility to COL, where all were found to be COL resistant except for TGC-induced mutants (MIC ≤2 µg/mL). Sensitivity to COL and other antimicrobial agents in TGC-induced mutants is due to the fact that the mechanism of resistance to tigecycline in this case, is the modification of the 30S ribosomal subunit which is the main target for TGC (33, 39).

The behavior of the 12 mutants is different when tested against TGC, where only 4 out of the 12 resistant mutants, including: TGC, COL/TGC, COL/TEC and AMK mutants revealed high resistance profile to TGC with MIC ≥8 μg/mL; this might be due to the fact that TGC is an antimicrobial agent which is found not to be affected by most of the resistant mechanisms that usually affect tetracycline, where it exhibits enhanced ribosomal binding and it is hard to be removed by most bacterial efflux pumps, unless overexpression in the multidrug efflux pumps is present (92). All the resistant mutants along with DSM 30008 have showed high resistance profile against TEC since it is a Gram-positive targeting antimicrobial agent; However, in some cases: COL, TGC, COL/TGC and COL/TEC mutants, the MICs to TEC have decreased in comparison with the reference strain and other mutants. This might be due to the difference in the triggered resistance mechanisms. Interestingly, the susceptibility and the resistance patterns of the 12 resistant mutants against C_T, TZP, MEM, LVX and AMK are proved to be similar. This might be attributed to the fact that by using these antimicrobial agents , we have triggered several mechanisms and most importantly efflux pumps, which is the most prevalent resistance mechanism against the assessed antimicrobial agents (COL, TGC, C_T, MEM, LVX and AMK) (29). This could be further confirmed via WGS which allows us to understand the effect of inducing resistance at the genome level in addition to Fluorescence Assays upon establishing fluorescein-tagged antimicrobial agents to track them within the cell.

In conclusion, this project was the first to show synergistic effects between COL and TEC in treating colR *A. baumannii* isolates. In addition, we demonstrated reversion of resistance in several isolates assessed which needs to be further characterized upon performing fitness cost and frequency of resistance assays. Furthermore, we established

in vitro resistant mutants to assess genome-wide modifications as compared to colR *A. baumannii* clinical isolates via WGS. Future perspectives include WGS, and fluorescence assays via tracking fluorescein-tagged antimicrobial agents within the bacterial cells and performing *in vivo* studies on colR *A. baumannii* isolates to assess the impact of combination therapies on survival.

APPENDIX I

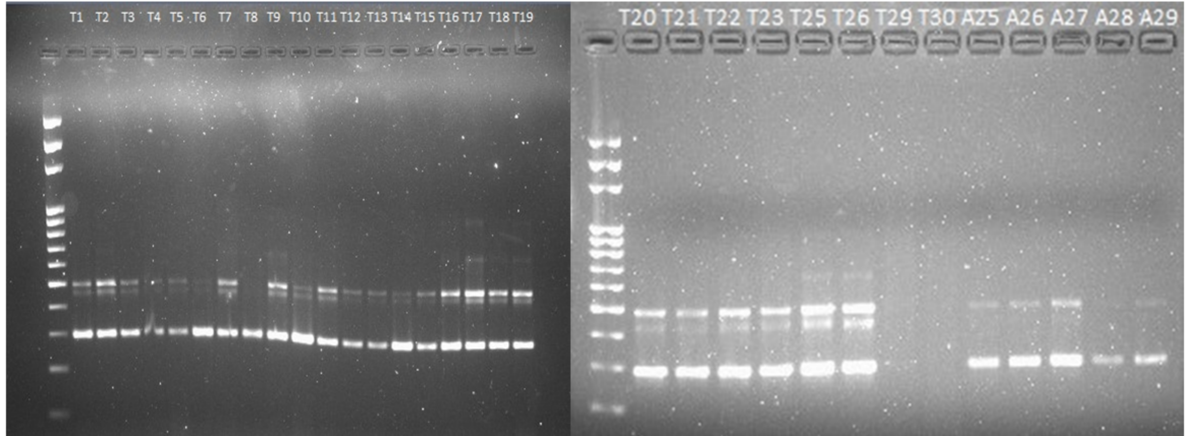

Figure 19: Electrophoresis gel image showing the expression of *gyrB* in the *A. baumannii* clinical isolates except for T29 and T30.

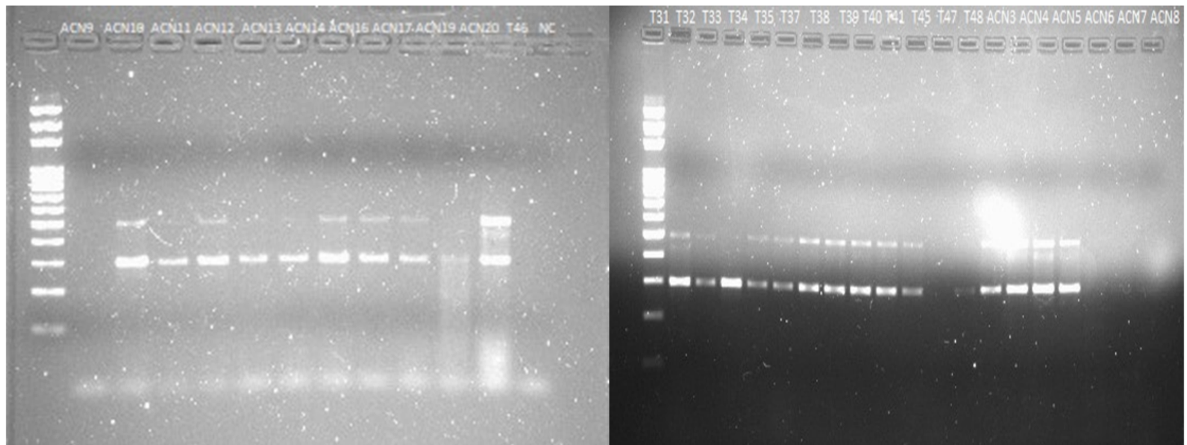

Figure 20: Electrophoresis gel image showing the expression of *gyrB* in the *A. baumannii* clinical isolates except for ACN6, ACN7 and ACN8.

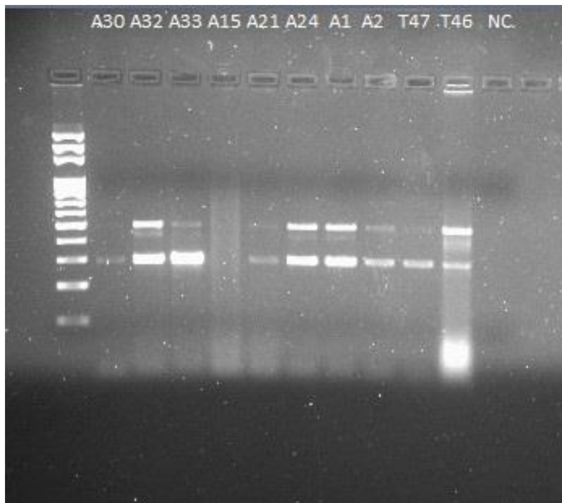

Figure 21: Electrophoresis gel image showing the expression of *gyrB* in the *A. baumannii* clinical isolates except for ACN15.

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