AMERICAN UNIVERSITY OF BEIRUT

IDENTIFICATION OF THE MOLECULAR MECHANISMS OF HEAVY METALS DRIVEN ANTIMICROBIAL RESISTANCE IN *ACINETOBACTER BAUMANNII* ISOLATES ORIGINATING FROM CONFLICT AREAS

by
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AN ABSTRACT OF THE THESIS OF

Aya Hassan Nasser for Master of Science
Major: Microbiology and Immunology

Title: Identification of The Molecular Mechanisms of Heavy Metals Driven Antimicrobial Resistance in *Acinetobacter baumannii* Isolates Originating from Conflict Areas

Background: Wars have been a great burden on humanity for a long period of time. The high medical costs of wars particularly those incurred in treating patients with multi-drug resistant infections is probably one of its most adverse effects. *Acinetobacter baumannii* is one of those superbugs which has gained much notoriety during times of wars for causing multi-drug resistant infections among injured military and civilian personnel. The rapidly evolving resistance of this bacterium particularly during this incidence hints out the role of bacterial milieu in promoting the emergence of this highly resistant pathogen. Since military regions are considered hot spots for heavy metals contamination, we hypothesize that exposure of *A. baumannii* to heavy metals coming from shelling and use of ammunitions in war regions might be correlated with its increased levels of antimicrobial resistance (AMR). Therefore, herein we aim to investigate the effects of heavy metals on AMR of *A. baumannii* clinical isolates particularly those originating from war patients and determine the mechanisms implicated at the molecular level.

Methods: A total of 11 clinical isolates of *A .baumannii*, 7 obtained from non-war wounded patients living in conflict areas and 4 recovered from war injuries specifically from Iraqi patients, were screened for their susceptibility against a panel of 8 heavy metals (Zn^{2+}, Cu^{2+}, Cr^{6+}, Pb^{2+}, Ba^{2+}, Cd^{2+}, Hg^{2+}, and As^{5+}) commonly used in weapons, 5 antimicrobial agents (Meropenem, Colistin, Gentamicin, Ciprofloxacin, and Cefepime), and 40 different combinations of antimicrobial agents and heavy metals using the Broth Micro-Dilution (BMD) assay. Induction of resistance *in vitro* in the fully susceptible reference strain *A. baumannii* DSM 30008 to selected heavy metals (Copper, Lead, Cadmium, and Arsenate) and/or antimicrobial agents (Gentamicin, Cefepime, and Meropenem) followed by phenotypic resistance testing and Whole Genome Sequencing (WGS) of raised resistant mutants were performed to investigate the molecular mechanisms of heavy metals driven AMR in *A. baumannii* isolates. Moreover, quantitative reverse transcriptase polymerase chain reaction (qRT-PCR) measuring the relative expression levels of *adeIJK* and *adeFGH* genes encoding multi-drug (MDR) efflux pumps was performed on 3 selected isolates to investigate the mechanisms of Barium-induced Ciprofloxacin resistance shown in the combination testing.

Results: BMD susceptibility assay showed a wide range of resistance to almost all classes of antimicrobial agents in all tested isolates. Moreover, multi-heavy metal resistance phenotypes were observed in all isolates from both war injuries and non-war injuries. The antimicrobial susceptibility patterns obtained in combination testing showed potential positive association between some heavy metal ions and antimicrobial resistance. Through induction of resistance and WGS, heavy metals such as Copper, Cobalt, Zinc, and Cadmium were shown to have co-selection potential for Cefepime resistance. Lead was shown to have co-selection potential for Gentamicin resistance while Arsenate was shown to possess co-selection potential for Colistin resistance. WGS on "Arsenic and Gentamicin" mutant revealed a novel resistance mechanism to Arsenate which is reduced uptake through phosphate transporters. Through qRT-PCR, Barium was shown to significantly upregulate the expression levels of *adeI*, *adeJ*, and *adeH* genes in one isolate while in another isolate it was found to upregulate *adeF* and *adeG* genes.

Conclusion: This is the first study to describe the clinical impact of heavy metals use in military weapons on antimicrobial resistance of *A. baumannii*. This study has helped us understand better the mechanisms of emergence of antimicrobial resistance in bacteria and it has reaffirmed the hypothesis that heavy metal ions are potential and potent drivers of antimicrobial resistance. Most importantly, our study highlights that there is very high risk of co-selection of heavy metal and antimicrobial resistance to occur in war regions given the high concentrations of heavy metals in these regions which exceed significantly the concentrations we used to raise resistance *in vitro*. Our study calls for further research to better understand the mechanisms of co-selection by heavy metals. In addition, it prompts health organizations and policy makers to spread awareness and issue stringent legislations to reduce heavy metals contamination in the environment for effectively combating AMR.

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INTRODUCTION

"There is the danger", he said, "that the ignorant man may easily under-dose himself and by exposing his microbes to non-lethal quantities of the drug make them resistant.", said Professor Alexander Fleming (1). The danger of emergence of antimicrobial resistance (AMR) which was predicted 7 decades ago by the pioneer of the first discovered antimicrobial agent penicillin has now become a reality and one of the most formidable threats to the human health. According to a recent review published on AMR, the estimate global mortality from drug resistant bacterial infections is 700, 000 per year and is expected to reach 10 million deaths as of 2050 exceeding thereby the mortality rates of cancer and diabetes together (2). While there are number of bacterial pathogens which contribute to the burden of AMR, 6 pathogens grouped under the acronym "ESKAPE" were found to contribute the most to the staggering threat of AMR including *Enterococcus faecium*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa*, and *Enterobacter* spp. (3) .

A. baumannii is the most successful pathogen amongst all capable of escaping the action of several antimicrobial agents including the last lines of defense agents Carbapenems and Colistin (4). The high genomic plasticity, rapid acquisition of antimicrobial resistance genes via horizontal gene transfer, upregulation of inherently encoded resistance genes (efflux pumps and antimicrobial agents modifying enzymes), in addition to permeability defects (reduced expression of porins) are key factors which

contributed to its extensive resistance and dissemination (5, 6) . Several epidemiological studies have reported increasing rates of multi-drug resistant (MDR) and extensively drug resistant (XDR) *A. baumannii* infections in several countries across the world including the United States, Countries of the European Union (EU) such as Spain, Italy, and Greece in addition to countries of the Arab League like Iraq, Lebanon, Libya, Qatar, and Egypt (7-9). Despite the global spread of this pathogen nowadays, the first recorded isolation of MDR strains occurred in war regions specifically in Vietnam during the American-Vietnamese war in 1970 (10). Such observation brings us to the question whether wars have played a role in selecting and promoting the resistance of *A. baumannii*. So far, there are no systematic studies exploring the association between wars and AMR or exploring the causes of emergence of AMR during wartime.

Since there is evidence in the literature that heavy metals pollutants can indirectly select for AMR via co-selection mechanisms (11, 12) and there are recent reports documenting a rise in the levels of heavy metals during and post wars as a result of the massive bombardments and use of ammunitions (13-15), we hypothesize that exposure of *A. baumannii* to heavy metals coming from shelling and weapons in war regions might be correlated with its increased levels of antimicrobial resistance. Therefore, herein we aim to investigate the effects of heavy metals on AMR of *A. baumannii* clinical isolates particularly those originating from war regions and to discover the molecular mechanisms involved.

CHAPTER II

LITERATURE REVIEW

A. *Acinetobacter* Genus: Microbiology Characteristics, Species and Habitat

Acinetobacter is a genus of bacteria belonging to the family Moraxellaceae. Species of this genus are pleomorphic non-motile gram negative cocco-bacilli that grow on solid media used routinely in microbiology laboratories like blood and MacConkey agars. They are strictly aerobic, catalase positive, oxidase negative, and glucose non-fermenters (5, 16). To date, more than 30 genomic species have been identified using various genotypic and phenotypic methods. Members of the genus *Acinetobacter* are considered ubiquitous as they are widely distributed in the environment. They are commonly found in soil, water, food, sewage, on human skin and mucous membranes as normal flora as well as on hospital surfaces (16, 17). The most common and genetically related *Acinetobacter* species associated with human diseases are: *A. baumannii*, *A. calcoaceticus*, *A. genomic species 3*, and *A. genomic species 13TU* (18, 19) with *A. baumannii* being the most commonly reported pathogen amongst all accounting for 80% of all *Acinetobacter* infections (20).

B. *A. baumannii*: Resilience, Risk group and Clinical Manifestations

1. A. baumanni: A Robust Nosocomial Pathogen

A. baumannii is an opportunistic nosocomial pathogen which typically causes infections in health care facilities particularly in intensive care units (ICUs) (19, 20). The persistence of this bacterial pathogen in hospitals as a major cause of health care infections is attributed to its high resilience and adaptive capacity to wide range of pH values and temperatures (21, 22). Another key factor which aids in its spread in clinical settings is its cryptic ability to survive for prolonged times on both dry and moist hospital surfaces and equipments without being destroyed by disinfectants (16).

2. Risk Group

According to the Centers for Disease Control and Prevention (CDC), *A. baumannii* poses no threat to healthy individuals rather it primarily infects immunocompromised patients and patients with chronic lung diseases and diabetes (23). Critically ill patients who are on ventilators or using invasive instrumentations like endotracheal intubation or central venous catheters that might be carrying this pathogen are also at high risk to get infected or colonized with *A. baumannii*. Moreover, patients with a history of prolonged hospitalization or who have been on antimicrobial therapy that include antimicrobial agents with minimal or no activity against *Acinetobacter* are likely to be infected as well (24).

3. Clinical Manifestations

Clinically, there is a wide spectrum of diseases that are associated with *A. baumannii* ranging from skin, wound and soft tissues infections, gastrointestinal infections, urinary tract infections, respiratory infections including the ventilator-associated pneumonia and community-acquired pneumonia, eyes infections such as keratitis and endophthalmitis to infections like endocarditis, bacteremia and meningitis (18, 25). In severly immunocompromised patients, infections with *A. baumannii* can result in severe and critical complications such as: septic shock, extensive tissue necrosis, acute respiratory distress syndrome, dessiminated intravacular coagulation which can culminate in multi-organ failure/dysfunction and in extreme cases death (18).

C. Global Prevalence and Health Burden of *A. baumannii*

Over the past four decades, *A. baumannii* has emerged as a major nosocomial pathogen in hospitals throughout the world for causing multiple drug resistant (MDR) infections (26). The high mortality rates of this pathogen (can reach 35%) along with its rapid evolution and acquisition of antimicrobial resistant genes have raised much concerns among the public health community and led recently to its classification by the World Health Organization (WHO) as a critical priority pathogen necessitating new drug discovery (4, 27). The challenge posed by *A. baumannii* was previously addressed by the Infectious Diseases Society of America (IDSA) which grouped it among the ESKAPE pathogens: *Enterococcus faecium*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa*, and *Enterobacter* spp., a group of pathogens that have escaped the action of commonly used antimicrobial agents and

accounted for the majority of nosocomial infections worldwide (3). As a matter of fact, *A. baumannii* has acquired resistance to almost all clinically available antimicrobial agents including Penicillins, β-lactams combinations (Ampicillin-sulbactam), Cephalosporins, Aminoglycosides, and Fluoroquinolones, and recently resistance to Carbapenems and Polymyxins, last line agents for drug resistant infections, is increasingly reported worldwide (4). According to the data published by The Surveillance Network database (TSN) on *A. baumannii* resistance trends from respiratory and blood specimens between 2003 and 2012, resistance to Carbapenems (Imipenem, Meropenem and Doripenem) has increased from 21% in 2003-2005 to 47.9% in 2009-2012 as is the case of Colistin resistance which has increased from 2.8% in 2006-2008 to 6.9% in 2009-2012 (9). The European Centre for Disease Prevention and Control (ECDC) also reported the emergence of Carbapenems resistant *A. baumannii* in several south and southeastern countries across the European Union (EU) with prevalence rates reaching 50% or higher as in Spain, Italy, Greece, and Romania (7). The burden of Carbapenems resistant *A. baumannii* is even higher in countries of the Arab League reaching over 80% in Qatar, Iraq, Lebanon, Egypt, and Libya (8). The rising rates of antimicrobial resistance had greatly limited the therapeutic options for treatment of *A. baumannii* infections and led to an increase in mortality rates.

D. *A. baumannii*: The Pathogen of Battlefields

A. baumannii was particularly problematic during times of wars. In fact, it was the most notorious and prevalent pathogen during wars especially during the wars in Iraq, Afghanistan, and Vietnam where it caused multiple outbreaks of MDR infections among

combat troops (28-30). It was reported to be the most frequently recovered Gram-negative bacterium from war wounds and traumatic injuries during the Vietnamese war and the number one causative agent of blood stream infections among US soldiers during the operations in Iraq and Afghanistan (28, 31). The increased isolation of *A. baumannii* during these wars, its resistance to several antimicrobial agents along with its rapid spread throughout western Europe and US had raised alarming voices and prompted scientists to investigate the underlying mechanisms beyond its increased antimicrobial resistance.

Earlier studies showed that *A. baumannii* had acquired resistance to antimicrobial agents expeditiously. Before the end of 1970, almost all clinical isolates were completely susceptible to first line agents such as Ampicillin and Gentamicin (24). However, soon after resistance started to emerge, and scientists reported the first case of Multi-drug resistant (MDR) strain just at the end of the very same year (10). Then after around 15 years, Extensively-drug resistant (XDR) strains appeared in 1985 , and this was followed by the emergence of Pan-drug Resistant (PDR) strains in 2007 (10). The striking fact is that these antimicrobial resistant strains appeared exactly during and after wars and this was evidenced by the coincidence detection and isolation of several MDR, XDR, and PDR strains with multiple wars in the world such as the Vietnamese war (1955-1975) , Lebanese civil war (1975-1990), Iraq- Iran war (1980-1988), Afghanistan war (2001-2014), and Iraq-American war (2003-2011) (28-30, 32). In view of the above, studies were conducted to understand the relationship between wars and antimicrobial resistance (AMR) (28) and identify potential factors which aided in its spread and emergence (29, 33). Since there are reports which documented an increase in the levels of heavy metals pollutants post wars and military activities (13-15), we aimed to investigate the role and the effect of bacterial

milieu in war regions, specifically heavy metals pollutants released from weapons, shelling, and bombardments in selecting and promoting the antimicrobial resistance of *A. baumannii*.

E. Heavy Metals

1. Definition and Classification

Heavy metals are group of non-biodegradable metals and semi-metals (metalloids) with high atomic weight and density greater than 5 g/cm^3 (34, 35) They are often distinguished from non-metal chemical elements by their physical properties: high electrical conductivity and malleability, metallic luster, and ability to lose electrons to form cations (36, 37). There are around 53 elements in the periodic table classified as heavy metals (38, 39). Some examples include: Cobalt (Co), Copper (Cu), Chromium (Cr), Zinc (Zn), Lead (Pb), Arsenic (As), Cadmium (Cd), Nickel (Ni), Antimony (Sb) and Barium (Ba) (40).

Heavy metals are broadly divided into two categories: essential and non-essential/toxic metals. Essential heavy metals are nutritionally essential elements required for life at low concentrations. They serve as micronutrients for many biological systems (humans, plants, bacteria) and are required for maintenance of several cellular processes (35, 41). In fact, various biochemical reactions in living systems comprise metallo-enzymes which use heavy metals ions as co-factors (42). For instance, Copper is an integral cofactor for cytochrome oxidase and superoxide dismutase which are important enzymes involved in mitochondrial electron transport and oxidative stress (35, 43). Zinc is an essential trace element required for the catalytic activity of DNA and RNA polymerases

(44). Moreover, metal ions such as Ni and Cr are vital for the activity of urease and cytochrome enzymes respectively (19). In addition to their important metabolic roles, essential heavy metals play a vital role in maintaining redox balance by functioning as Lewis acids and Lewis bases in chemical reactions (26, 45). On the contrary, non-essential heavy metals are metals which do not have any biological role in living systems; as such, they are toxic at low concentrations. Examples of non-essential/toxic metals include: Mercury (Hg), Lead (Pb), Cadmium (Cd), Arsenic (As) etc. (35, 40, 41, 46).

2. Sources

Heavy metals are naturally occurring elements that have been found within the earth's crust 2.4 billion years ago (12, 47). They are present in water, soils, rocks, and living organisms at relatively tolerable concentrations. Natural phenomena such as volcanic emissions, earth crust weathering, soil and rocks abrasion continuously release heavy metals into the ecosystem (35, 48). Despite the natural occurrence of these elements, anthropogenic activities are thought to be the major contributors of heavy metals release into the environment. Industrial operations such as smelting, mining, petroleum combustion, and coal burning release considerable amounts of heavy metals pollutants into various environmental matrices. In addition, the use of heavy metals containing products in various fields such as in agriculture and animal husbandry (as pesticides, fungicides, antifouling agents, feed additives as growth promoters and antimicrobials), industry (in cosmetics, paints, textiles, plastics), and technology (in microelectronics, batteries) also adds significant amount of heavy metals in the ecosystem (12, 19, 35, 48). Besides the

industrial use of these chemical elements, heavy metals are widely used in medicine to treat number of diseases. They are used as antimicrobial agents (Silver Sulfadiazine antibiotic to treat skin infections), anti-inflammatory (calamine to treat itchiness), antiparasitic (pentavalent antimonial drugs to treat leishmaniasis), and antifungal agents (Selenium Sulphide) (12, 49). In addition, many metal-based compounds are used in chemotherapy to treat cancer. Platinum compounds such as cisplatin, carboplatin, and oxaliplatin are among the most commonly used anti-cancer agents nowadays (50).

3. Heavy Metals in Ammunitions and Military Equipments

Active military regions where armed operations and battles occur are considered hot spots for heavy metals contamination (13, 14, 51). In fact, heavy metals are extensively used in the defense sector to manufacture various weapon systems and military devices (52). For example, Lead (Lead styphnate) and Mercury (Mercury fulminate) are mainly used as explosives in primers and detonators (14, 53). Zinc (Zn), Copper (Cu), Nickel (Ni), Lead (Pb), and Chromium (Cr) are heavily used as coatings for bullets, missiles, gun barrels, and military vehicles (tanks, trucks, aircrafts) (54-56). Another metal with an important military application is Tungsten (W). Tungsten is often utilized in the kinetic bombardment weapons to destroy buildings without utilizing explosives owing to its high density (57). Metals such as Barium (Ba), Antimony (Sb), and Boron (B) can also be used in the military sector mainly as fuels and oxidizers in primers of weapons (53).

4. Mechanisms of Heavy Metals-Mediated Toxicity in Bacteria

Although some heavy metals are vital for bacterial survival, all become noxious when they exceed a threshold concentration (36, 45). Heavy metals have been reported to affect different cellular processes and structures in bacterial cells when they accumulate in toxic levels. Almost all cellular components in bacteria are susceptible to heavy metals mediated damage (58). Different mechanisms of heavy metal mediated toxicity have been described in various studies (26, 48, 58, 59). The first mechanism involves formation of Reactive Oxygen Species (ROS) that damage essential biomolecules and cellular organelles. Metals such as Lead (Pb), Cadmium (Cd), Copper (Cu), Arsenic (As), Silver (Ag), and Zinc (Zn) were shown to induce oxidative damage in bacterial cells when in excess by causing the production of free radicals that can alter the DNA and compromise the membrane integrity via lipid peroxidation (26, 48, 60). The second mechanism of metal toxicity is the complexation of metal ions with thiol containing enzymes thereby disrupting their functions (19, 45). Metal ions such as Hg^{2+}, Ag^{1+}, and Cd^{2+} are known to form strong covalent bonds with sulfhydryl groups of enzymes (58). Finally, toxic metal ions can disrupt cellular processes by acting as competitive inhibitors and displacing essential ions from their biological targets (45, 61).

5. Heavy Metals Resistance Mechanisms

Since the origin of earth 4.5 billion years ago, microorganisms have been exposed to metal ions in their surroundings (62).The earliest studies and the more recent ones show that heavy metal resistance determinants are very ancient and ubiquitous in almost all

bacterial species and can be detected in nearly all environmental matrices (62). Many studies reported the presence of heavy metal resistant bacteria in pristine environments not previously contaminated with heavy metals. Farias and his colleagues (63) isolated 35 strains of bacteria belonging to 8 different species with multi-metal resistant phenotypes from deep sea hydrothermal vents, sites which are known to be protected from anthropogenic sources of heavy metals. Equally true, bacteria recovered from anthropogenically contaminated sites (industrial effluents, diesel fuel contaminated soil, agricultural soil, aquaculture sediments, sewage) belonging to various species including *A. baumannii* were also shown to exhibit resistance to various metals (63-66). Such resistance reflects an evolutionary adaptation of bacterial species to metal ions which occurred through certain resistance/protection mechanisms. In general, there are 6 proposed mechanisms of heavy metal resistance in bacteria reported in the literature (19, 45, 58, 62, 67): (1) Exclusion of metal ions by extracellular barriers such as capsule, cell wall, and plasma membrane (2) Extrusion of metal ions by efflux pumps or chemiosmotic transporters (3) Intracellular sequestration of metal ions (4) Extracellular sequestration of metal ions (5) Bio-transformation/detoxification of toxic ions into less toxic forms (6) Reducing sensitivity of cellular targets to metal ions (**Figure 1**). The ultimate aim of these detoxification mechanisms is to provide protection for cellular components and molecules while keeping the intracellular concentrations of heavy metals within homeostatic ranges (38). Its noteworthy to mention that resistance mechanisms to heavy metals are very diverse and heterogenous at the genetic level. Genes encoding metal resistance can be either localized on mobile genetic elements such as plasmids and transposons or can be carried on chromosomes (58).

a. Exclusion of Metal Ions by Natural Barriers

Extracellular structures such as the capsule, cell wall and plasma membrane can serve as natural barriers which limit the entry of metal ions into bacterial cells and thus, conferring protection against metal toxicity (45). Such limitation is largely due to the net negative charges of these components imparted by the negatively charged groups present in their structures (carboxyl, hydroxyl, and phosphate groups) (45, 68). Several bacterial species were shown to adsorb metal ions on their capsule and exopolysaccharides (EPS). Peleg and his colleagues reported Copper (Cu), Lead (Pb) and Chromium (Cr) adsorption on EPS of *Acinetobacter* spp. which conferred protection to these species against metal toxicity (16). Biosorption of metal ions was also reported in *Pseudomonas aeruginosa*. In their study to investigate the effects of heavy metals stress on biofilms and planktonic cells of *P. aeruginosa*, Teitzel and Parsek (69) found that biofilm cells were more resistant to metal ions (Cu^{2+}, Pb^{2+}, Zn^{2+}) compared to planktonic cells and attributed this to the adsorption of these metal ions on extracellular polymeric substances (EPS) that encase the biofilms. Besides the EPS, the bacterial cell membrane can also function as a natural barrier against heavy metals entry owing to its high lipid composition.

b. Extrusion of Metal Ions by Efflux Pumps or Chemiosmotic Transporters

This is the most prevalent and effective mechanism of heavy metals resistance in bacteria (45, 48). Export of metal ions by efflux systems is an energy consuming process. Bacteria expel metal ions out by utilizing energy coming either from Adenosine triphosphate (ATP) hydrolysis or from the electrochemical gradient of protons (45). There are around 5 major families of efflux systems in microorganisms: (1) ABC family (ATP Binding Cassettes) (2) RND family (Resistance, Nodulation, Cell Division) (3) SMR

family (Small Multi-Drug Resistance) (4) MATE (Multi-Drug and Toxic Compounds Efflux) (5) MFS family (Major Facilitator Superfamily). These pumps differ in their amino acid sequence, substrate specificity, and energy they use to transport metal ions (62, 70, 71). **Table 1** summarizes the 5 major families of efflux systems in bacteria: their function, substrate spectrum, and the energy they utilize to drive this transport.

c. Intracellular Sequestration of Metal Ions

In addition to extracellular barriers and efflux pumps, metal ions can be detoxified by sequestration intracellularly on metal ions binding proteins (Metallothioneins, Glutathione, and Metallochaperones) or in the form of inorganic salts (45, 59). Nickel (Ni) and Lead (Pb) are examples of metals that can be precipitated intracellularly by complexation with phosphate ions (38, 48). Several microorganisms such as *S. aureus*, *Providencia* spp., *Vibrio harveyi*, *Shewanella* spp., and *Bacillus* spp., were shown to precipitate Lead (Pb) in the form of phosphate salts as a way to lower its free toxic concentration (72-75). On the contrary, thiophilic metals or sulfur loving metals such as Cadmium (Cd), Mercury (Hg), Silver (Ag), Lead (Pb), and Zinc (Zn) can be precipitated by trapping them on small cysteine rich polypeptides known as Metallothionein (MT). The role of MTs in providing tolerance to heavy metals has been documented in various studies. Bacteria belonging to the genus *Synechococcus*, *Pseudomonas*, and *Anabaena* were shown to withstand high concentrations of thiophilic metals by trapping them on MTs (76-78). To limit the concentration of free thiophilic metals, some bacteria resort alternatively to the synthesis of a specific chelator similar to MT known as Glutathione which scavenges and detoxifies metals through its thiol (SH) group. In a study conducted on Rhizobium leguminosaru bacterium, (79) Lima et al. demonstrated the role of Glutathione in mediating

tolerance to Cadmium in Cadmium resistant Rhizobium leguminosaru where it was shown to chelate most of the intracellular Cd^{2+} ions thereby enabling the bacteria to endure toxic concentrations. Another class of metal-binding proteins which protect bacterial cells from metals associated toxicity is Metallochaperones. In fact, Metallochaperones are thought to play a role in metallostasis in bacteria. Their function is to bind metal ions and deliver them to target proteins to decrease their free toxic concentrations and protect sensitive cellular targets (80). Examples of Metallochaperones include: Copper chaperones (Cu^{1+} binding chaperone CusF, Cu^{1+} and Cu^{2+} periplasmic chaperones PcoC and PcoE) and Arsenite and Antimonite chaperone ArsD (12, 81).

d. Extracellular Sequestration of Metal Ions

Another strategy bacteria use to detoxify the effects of heavy metals is extracellular sequestration. It involves precipitating metal ions with certain microbially produced substances such as Siderophores, Oxalate, Phosphate, and Sulfide (82, 83). It is an efficient resistance mechanism which not only confers protection for intracellular targets but also for the whole bacterial cell. However, despite its high level of protection, it is only possible in relatively static environments which contain constant concentrations of heavy metals and when enough amounts of sequestering molecules are present (58). Extracellular sequestration of metal ions has been demonstrated in various studies. Dimkpa et al. reported Nickel (Ni) and Iron (Fe) precipitation on Hydroxamate Siderophores produced by *Streptomyces acidiscabies* (84). Daryl and Leon (1993) reported Cadmium precipitation with Sulfide in *Clostridium thermoaceticum* (83). More recently, a phosphatase producing bacterium *Achromobacter xylosoxidans* was shown to precipitate Lead (Pb) with Phosphate through phosphatase catalyzed reaction (85).

e. Bio-transformation/Detoxification of Toxic Ions into Less Toxic Forms

Some bacteria resort to enzymatic transformations of toxic metal ions to reduce their toxicity. This can be carried out via different chemical reactions such as oxidation, reduction, and methylation (58, 62). Mercury, for example, in its bioavailable form Hg^{2+} is very toxic to all microorganisms. To reduce its toxicity, bacteria use mercuric reductase encoded by *MerA* gene which detoxifies Hg^{2+} by reducing it to a less toxic elemental form Hg^{0} (12, 19) Similarly, Chromium (Cr) in its hexavalent form "chromate" is very deleterious to microbial cells. Once it enters the latter, it is rapidly reduced to Cr^{3+}, a non-toxic trivalent cation (38, 62, 86). Other metal ions such as Arsenite (As^{3+}) are more toxic in their reduced forms and thus, require oxidation to lessen their toxic effects (87, 88). Alternatively, heavy metal ions can be detoxified by methylation (62). Biomethylation of heavy metal ions has been reported in many bacteria including *Pseudomonas*, *Acinetobacter*, *Aeromonas*, and *Flavobacterium* which were shown to methylate Lead ions (Pb^{2+}) (48, 89). Mercury (Hg) methylation, on the other hand, has only been documented in anaerobic bacteria (90).

f. Reducing Sensitivity of Cellular Targets to Metal Ions

In addition to the above-mentioned resistance mechanisms, bacteria might resort to reduce the target's sensitivity to metal ions as a way to minimize their toxicity (58). This can be achieved through different means: 1- Introducing mutations in the metal's target to decrease the bacterial susceptibility to the detrimental metal effect while retaining its function. 2- Increasing the expression of the metal's target site thus, rescuing the metabolic pathway involved. 3- Finding an alternative way to synthesize a resistant form of the metal's target such as activating the expression of a plasmid encoded gene that produces a

modified and more resistant form of the metal's target and thus, bypassing the less resistant form encoded by the chromosomal DNA. 4- Repairing DNA damage caused by the metal which is the case of Chromium (Cr) resistance via activating SOS response which is a regulatory mechanism in bacteria induced by DNA damage and aims to repair DNA mutagenic lesions (58, 91, 92)

6. Occurrence, Biological role, and Resistance Mechanisms to Selected Heavy Metals

a. Copper (Cu)

Copper (Cu) is a naturally occurring metal which exists as free metallic element or in the form of compounds and often alternates between 2 oxidation states Cu^{1+} and Cu^{2+}. Copper is an essential element for all living organisms (plants, animals, humans, bacteria) (93). In human beings, Copper (Cu) plays various key roles ranging from maintenance of heart and blood vessels elasticity, brain development, neurotransmitters synthesis to skin formation and maintenance of strong immunity (94). In bacteria, Copper (Cu) is a vital cofactor for several enzymes and is important for fundamental processes such as cellular respiration and redox balance (26, 67). However, despite its physiological role, Copper (Cu) can become very toxic at high concentrations. To avoid Copper (Cu) toxicity, microorganisms have evolved detoxification and homeostasis mechanisms allowing them to survive Copper (Cu) stress (67, 95). The most studied Copper (Cu) resistance systems are those identified in Gram negative bacteria specifically in *Escherichia coli* and *Pseudomonas syringae* (96). There are 4 Copper (Cu) homeostatic systems that have been identified in these bacteria: Cue, Cus, Pco and Cop. Cue and Cus are chromosomally encoded Copper (Cu) efflux systems while Pco and Cop are plasmid encoded Copper (Cu)

resistance systems (12). The 2 main detoxification mechanisms used by these systems are efflux and charge modification through oxidation (12, 67).

i. Cue System

Cue system, also known as Cu efflux system, provides resistance to Copper (Cu) at low or moderate levels and is only active under aerobic conditions (12, 67). It consists of an inner membranous Cu^{1+} trans-locating P-type ATPase CopA and a periplasmic multi-Copper oxidase CueO. The genes encoding CopA and CueO are activated by a cytoplasmic transcriptional regulator known as CueR. CueR, which also functions as a Copper (Cu) sensor protein, induces the expression of *CopA* and *CueO* upon detecting an increase in cytoplasmic Copper (Cu) concentrations (12, 67, 97).

Copper Resistance via the Cue System

- First, Copper (Cu) in the form of Cu^{1+}/Cu^{2+} enters the bacterial cells via non-specific uptake systems such as porin proteins.
- Once CueR senses an increase in the intracellular levels of Copper (Cu), it gets activated and stimulates in turn the expression of *CopA* and *CueO*.
- Then, Cu^{1+} translocating P-type ATPase CopA translocates Cu^{1+} ions into the periplasm to protect the cytoplasmic Copper (Cu) sensitive cellular components.
- In the periplasm, CueO will oxidize Cu^{1+} ions (Cuprous) into Cu^{2+} ions (Cupric), the less toxic ionic form of Copper (12, 67) (**Figure 2**).

ii. Cus System

The Cus system is another efflux system which is responsible for detoxifying both Copper (Cu) and Silver (Ag) ions (97). Unlike the Cue system, the Cus efflux system provides resistance at high concentrations of Copper (Cu) and Silver (Ag) and is only active under anaerobic conditions (62, 98). In addition, the Cus system plays major roles in detoxifying Copper (Cu) in the periplasmic compartment contrary to the Cue system which detoxifies Copper (Cu) both in the periplasm and the cytoplasm (96). This system consists of 4 genes which together form an operon called *CusCFBA*. The latter is thought to be regulated by a two-component regulatory system CusRS (99). CusS is a histidine kinase which gets activated upon sensing Copper (Cu) or Silver (Ag) ions while CusR is a DNA-binding transcriptional activator which induces the expression of *CusCFBA* operon upon CusS activation (99). The protein products of *CusC*, *CusB*, and *CusA* genes combine to form a multi-Copper/Silver efflux pump CusCBA which expels out Copper (Cu) and Silver (Ag) ions by functioning as proton-ion antiporter (12, 62, 99). *CusF* gene, on the contrary, encodes a small periplasmic metallochaperone CusF which binds and scavenges Cu^{1+}/Ag^{1+} ions and recruit them to the CusCBA efflux pump (97).

Copper and Silver Resistance via the Cus System

- First, Copper and silver ions enter the periplasm and induce the activation of CusS.
- The activated CusS then phosphorylates and activates CusR.
- Then, CusR binds to *CusCFBA* promoter and induces the transcription of its cognate operon *CusCFBA*.

- The encoded proteins then assemble to form a multi-Copper/Silver efflux pump CusCBA which pumps out Copper (Cu) and Silver (Ag) ions recruited by CusF Metallochaperone (12, 97, 99) (**Figure 2**).

iii. Pco system

Pco system is a plasmid-borne-Copper resistance system which was first detected on plasmids of *E. coli* isolated from guts of pigs fed on Copper Sulfate supplemented diet (100). This system is encoded by a cluster of 9 to 10 genes forming 2 operons, *pcoGFE,* and *pcoABCDRS* in addition to a single gene, *PcoE* (12). Based on sequence homology studies, *PcoABCDGFE* genes are predicted to be under the regulation of the two-components regulatory system, *PcoRS* (12, 101). Studies have shown that the Pco system cannot function independently, but rather requires the activity of CopA of the Cue system to confer resistance to Copper (Cu) ions. As such, CopA has to transport first Copper ions (Cu^{1+}) from the cytoplasm to the periplasm, and then the Pco system will handle the transported Copper (Cu) ions in the periplasm (96, 102). PcoA and PcoC are thought to be the main key players in the Pco system. PcoA is a multi-Copper oxidase similar to CueO (96, 102). Its main function is to detoxify Copper (Cu) by oxidizing the periplasmic Cu^{1+} into the less toxic Cu^{2+} form (96). On the contrary, PcoC is a Copper-binding protein located in the periplasm and is speculated to contribute to Copper (Cu) resistance by playing different roles. It either assists CopA by functioning as a Copper chaperone that binds to Copper (Cu) ions and delivers them to CopA for oxidation , or it delivers Copper to PcoD, an inner membranous Copper (Cu) transporter which its exact role in Copper (Cu) homeostasis is still unclear, but is predicted to be involved in the uptake of Copper (Cu) across the inner membrane (96, 103). Another possible role of PcoC

is electron transportation (104). PcoB is a putative outer membrane transporter predicated to interact with PcoA (12, 102). PcoE is another important Metallo-chaperone of the Pco system which is believed to provide initial resistance to Copper (Cu) by acting as a sequestering agent before the complete activation of the Pco system (96, 102), and unlike *PcoABCDGFE* genes *PcoE* is regulated by CusRS (96) (**Figure 3**).

iv. Cop system

The Cop system is the least studied Copper (Cu) resistance system which was first identified in the plant pathogenic strain *P. syringae* pv. -*Tomato* PT23 which infects tomato (96, 105). It is encoded by a cluster of 6 plasmid borne genes arranged in two operons, *CopABCD* and *CopRS* even though they can be occasionally localized on chromosome (12, 105). The Cop determinants are genetically related to their counterparts in the Pco system of *E. coli* indicating similar function (105). The expression of *CopABCD* operon is induced by CopRS in response to elevated Copper (Cu) levels (105). Moreover, the protein products of this operon were shown to possess strong Copper sequestering/binding activities enabling them to sequester high concentrations of Copper (Cu) in the periplasm and outer membrane (105, 106). *CopA* and *CopC* genes encode periplasmic Copper-binding proteins which are responsible for sequestering Copper in the periplasm. On the contrary, *CopB* and *CopD* encode outer and inner membranous proteins respectively. The role of CopB in Copper (Cu) detoxification has not been determined yet, but it is believed to be involved in Copper (Cu) uptake together with CopD (96, 105).

b. Mercury (Hg)

Mercury (Hg) is another heavy metal with natural occurrence. It exists in 3 main forms: metallic, organic, and inorganic (35, 107). While metallic Mercury (Hg) is the

elemental form of Mercury (Hg) which is liquid at room temperature, inorganic Mercury (Hg) is the ionic form which can have a valence of +1 (Mercurous) or +2 (Mercuric). Conversely, organic Mercury (Hg) is the compound form of Mercury (Hg) which is combined to carbon atoms (107). Despite having different toxicology profiles, all forms of Mercury (Hg) are extremely toxic to human beings (35, 107). Exposure to Mercury (Hg) was reported to affect different organ systems including the brain, the kidneys, the respiratory and gastrointestinal tracts (107).

Unlike Copper (Cu), Mercury (Hg) does not have any cellular function in bacteria. As such, it is toxic at low concentrations (38, 67). Mercury (Hg) is bioavailable to bacterial cells in 2 forms: inorganic mercuric ion (Hg^{2+}) and organic Mercury, both of which are toxic (67). Despite their toxicity, several bacterial species (environmental and clinical) were shown to tolerate high levels of Mercury (Hg) (67, 108, 109). Resistance to Mercury (Hg^{2+} and organic Mercury) is encoded by the *mer* operon which is often localized on plasmids and transposons (62). *Mer* operon consists of a cluster of 8 genes *MerTPCAGBDE*, 4 of which are always present on Mercury operon of Gram negative bacteria (21). The main key player in Hg^{2+} detoxification is MerA, Mercuric Ions Reductase, encoded by *merA* gene. MerA detoxifies toxic Hg^{2+} ions by reducing it to a non-toxic elemental form Hg^{0}. Since the latter is volatile at room temperature, it gets evaporated readily and diffuses out of the bacterial cell thereby allowing the bacterial cell to escape its toxicity (21, 38, 62, 67). The *mer* operon, also, encodes a network of proteins that chaperone mercuric ions in the periplasm to deliver them ultimately to MerA while protecting the periplasmic compartment (38). The first protein to bind Hg^{2+} after it enters through porin proteins is MerP, a small periplasmic mercuric ions chaperone (12, 38). After binding to Hg^{2+}, MerP

then delivers the bound cations to MerT or MerC or MerF, inner membranous mercuric ions-binding proteins, to be transported to the cytoplasm. While *MerT* is mostly present on the *mer* operon, *MerC* and *MerF* can occasionally exist as well (62). Once in the cytoplasm, MerA will detoxify Hg^{2+} ions through reduction-catalyzed volatilization (67). MerE is an additional protein of currently unknown function (62). In Gram negative bacteria, *mer* operon is primarily regulated by MerR, Hg^{2+} responsive activator which is inducible by Hg^{2+} ions and to a lesser extent by MerD, co-repressor (21, 67, 110). On the other hand, resistance to organic Mercury is conferred by *MerB* gene which encodes an organomercurcial lyase located in the cytoplasm designated as MerB. By cleaving the Mercury-carbon bond, MerB releases Hg^{2+} ions into the cytoplasm, and MerA then reduces it to Hg^{0} (12, 38, 67) (**Figure 4**).

c. Arsenic (As)

Arsenic (As) is a ubiquitous metalloid which is generally found at low levels in nature, but its concentration can reach very high levels in point sources such as mining, volcanic eruption, soil erosion and industrial activities (35, 111, 112). Generally speaking, Arsenic is regarded as a toxic metal to all living systems but its degree of toxicity is mostly determined by its chemical form (113). In nature, Arsenic (As) exists in two chemical forms: inorganic and organic (88, 113). Inorganic Arsenic occurs in 4 oxidation states: pentavalent Arsenate (As^{5+}), trivalent Arsenite (As^{3+}), elemental Arsenic (As^{0}), and Arsenide (As^{3-}) with the trivalent Arsenic (As^{3+}) being the most toxic inorganic form (35, 38, 112). Alternatively, Arsenic (As) may occur in nature in the form of organic compounds which are generally less toxic than inorganic Arsenicals (113). These include methylated metabolites such as monomethylarsonic acid (MMA), dimethylarsinic acid

(DMA) and trimethylarsine oxide (35). Human exposure to any chemical form of Arsenic (As) has been associated with various deleterious health effects (cardiovascular diseases, respiratory disorders, hematological disorders, neurological and developmental anomalies) in addition to skin and lung cancers (34, 35, 113). Given the intrinsic toxicity of Arsenic (As) to almost all living systems, it is not surprising that some microorganisms have evolved different resistance mechanisms to this metal including oxidation, reduction, methylation, efflux, and intracellular sequestration on cysteine rich peptides (62, 88, 112). Microbial Arsenic (As) resistance is very ancient and has been documented in various bacterial species isolated from different environmental matrices (63, 87, 88, 114). Resistance to Arsenic (As) is mainly encoded by the *ars* operon which can be plasmid or chromosomally derived even though it can occasionally be encoded by other genetic determinants such as *arr* genes and *aox* genes (62, 88, 112). The *ars* operon carries 3 co-transcribed core genes which confer resistance not only to Arsenic (As^{3+} and As^{5+}) but also to Antimony (Sb^{3+}): *ArsR* (encodes a transcriptional repressor), *ArsC* (encodes a cytoplasmic arsenate reductase), and *ArsB* (encodes a membrane bound Arsenite efflux pump) (38, 62, 112). Sometimes, *ars* operon may carry additional genes, *ArsA* and *ArsD*. ArsA is an intracellular ATPase which binds to ArsB to form an ArsA-ArsB ATPase efflux pump. The latter is thought to be more effective than ArsB in extruding Arsenite (As^{3+}) and Antimonite (Sb^{3+}). ArsD is a Metallo-chaperone which scavenges (As^{3+}) and (Sb^{3+}) and delivers them to ArsA-ArsB complex for efflux (62, 67, 112). In addition, ArsD is predicated to function as a trans-activating co-repressor of *ars* operon along with ArsR (62, 112). Aside from reduction and efflux mechanisms, some microorganisms escape the toxicity of Arsenic (As) by methylating and converting it into less toxic and easily volatile

derivatives such as MMA and DMA (112). It is noteworthy to mention that microorganisms do not express any specific uptake systems for Arsenic (As) as it does not have any metabolic role. It usually enters bacterial cells using non-specific transporters mainly phosphate transporters Pit and Pst in the case of Arsenate (As^{5+}) and aquaglycerolporin in case of Arsenite (As^{3+}) (112). On the contrary, some bacterial species were shown to possess genes that enable them to utilize Arsenic (As) for their cellular processes. Bacteria belonging to the genus *Shewanella*, *Sulfurospirillum*, *Clostridium*, and *Bacillus* were reported to utilize Arsenate (As^{5+}) as a final electron acceptor during anaerobic respiration by reducing it into Arsenite (As^{3+}) (112). Such activity is mediated by an enzyme called Respiratory Arsenate Reductase which is encoded by *arrAB* operon (62, 112). Moreover, other bacteria were found to rely on Arsenite (As^{3+}) oxidation as electron donor during their aerobic respiration. Respiratory oxidation of Arsenite (As^{3+}) is carried out by an enzyme named Respiratory Arsenite Oxidase which role is to use Arsenite (As^{3+}) as an electron donor during respiration by oxidizing it to Arsenate (As^{5+}). This enzyme is encoded by *asoAB* genes or alternatively by *aoxBA* and *aroAB* (62, 112) (**Figure 5**).

d. Chromium (Cr)

Chromium is the seventh most abundant element in the earth's crust (115). It exists in various oxidation states with the trivalent (Cr^{3+}) and the hexavalent Chromium (Cr^{6+}) being the two most stable and prevalent states in nature (86, 91, 115). While Cr^{3+} is naturally present in the environment, Cr^{6+} is mostly produced by industrial processes (91, 116). Cr^{6+} is 1000 x more toxic to human beings compared to Cr^{3+} (115). The International Agency for Research on Cancer (IARC) has classified Cr^{6+} as human mutagen and carcinogen mainly associating it with nasal and bronchogenic cancers (117). In

microorganisms, Chromium (Cr) does not have any metabolic role and its toxicity has been confirmed in a number of bacterial species such as *Pantoea* spp., *Aeromonas* spp., and *E. coli* (38, 118, 119). To avoid Chromium (Cr) toxicity, bacteria have evolved 5 different mechanisms of resistance to Cr^{6+} that are mostly plasmid encoded (91, 115). The first mechanism involves reducing the uptake of Cr^{6+} (91). Cr^{6+} exists largely in the form of oxyanions chromate (CrO_4^{2}) and dichromate ($Cr_2O_7^{2-}$). These anions have similar structures to sulfate and thus, are transported to bacterial cells by utilizing the latter uptake system (38, 86, 91, 115). So, one way bacterial cells prevent Cr^{6+} toxic accumulation is by limiting its entry via the sulfate transport system (91). The second mechanism of resistance is efflux of Cr^{6+} via Cr^{6+} specific efflux pumps (91). Chromate resistance via efflux pumps has been first identified in *Pseudomonas aeruginosa* and *Alcaligenes eutrophus* species. These bacteria were shown to extrude Chromate by active efflux through ChrA pump (120). The third Chromate resistance mechanism is activation of oxidative stress related enzymes, catalase and superoxide dismutase as a way to counteract Cr^{6+} induced oxidative stress (91, 115). In fact, Cr^{6+} is a very strong oxidizing metal which is known to induce intense oxidative stress in bacterial species (115). After it enters bacterial cells, it interacts readily with reducing agents such as Glutathione, NADH, NADPH, Ascorbate, and Pentoses to generate free radicals, unstable intermediates Cr^{4+} and Cr^{5+}, and Cr^{3+} as an end-product. Since all the products of such reaction can cause oxidative damage to both DNA and proteins, bacteria might resort to up-regulating the genes of antioxidants enzymes to scavenge ROS and protect cellular components (86, 91, 121). The fourth Chromate resistance mechanism involves repairing DNA damage induced by Cr^{6+} and its derivatives (Cr^{3+}, Cr^{4+} and Cr^{5+}) through activating SOS response enzymes RecA, RecG, and RuvAB.

In their study on the highly Chromate resistant bacterium *Ochrobactrum tritici*, Morais et al. found that SOS response related protein , RuvB, is implicated in Chromate resistance and is one of the different resistance mechanisms this bacterium use to overcome Chromate stress (122). Miranda et al. also established the role of RuvB along with RecG helicases in mediating Chromate resistance in *P. aeruginosa* through repairing Cr^{6+} induced DNA damage (123). Another resistance mechanism reported in bacteria is Cr^{6+} reduction (86, 91). Microbial Chromate reduction is an efficient resistance mechanism which enables bacterial cells to escape Cr^{6+} toxicity through converting it to the less toxic form Cr^{3+} (86). Reduction of Cr^{6+} has been described in different bacteria belonging to different genera such as *Pseudomonas*, *Bacillus*, *Staphylococcus*, and *Acinetobacter* (124-127). While the end products of such reaction are the same in all bacterial species, the mechanism of Cr^{6+} reduction varies between species and is strictly dependent on oxygen presence (115). In general, aerobic bacteria reduce Cr^{6+} by soluble enzymes located in the cytoplasm while using NADH or NADPH as sources of electrons. On the contrary, anaerobes reduce Cr^{6+} by utilizing membrane associated enzymes often involving those of the electron transport chain (cytochrome b, cytochrome c, flavin reductases) while using glucose or H_2 among other molecules as sources of electrons (86, 91, 115). In addition to enzymes-catalyzed Cr^{6+} reduction, hexavalent Chromium (Cr^{6+}) can be reduced non-enzymatically by interacting with several cellular reductants such as Cysteines, Glutathione, Pentoses, Ascorbate or with metabolic end products such as H_2S and Fe (II) produced by sulfate and iron reducing bacteria respectively (86, 115) (**Figure 6**).

e. Cadmium (Cd)

Like Chromium, Cadmium (Cd) is a toxic heavy metal which is normally found in the environment (35, 128). It shares similar chemical properties with Zinc (Zn) and Mercury (Hg). In human beings, a wide range of target organs were reported to be susceptible to Cadmium damage. According to the International Agency for Research on Cancer (IARC), Cadmium (Cd) is categorized as a human carcinogen primarily associated with lung cancer (128). As in human beings, Cadmium (Cd) is extremely toxic to microorganisms and what aids in its toxicity is its ability to enter bacterial cells by exploiting uptake transporters of other structurally similar and essential cations such as Manganese (Mn^{2+}), Magnesium (Mg^{2+}), and Zinc (Zn^{2+}) (38, 71). It is believed to induce damage by binding to several enzymes and disrupting their functions, inducing oxidative stress, interfering with Zinc (Zn) and Calcium (Ca) metabolisms, and inhibiting DNA repair (38, 129). In spite of its toxicity, various bacterial species were shown to tolerate high Cadmium (Cd) levels. Abbas et al. (130) isolated a *Pseudomonas* spp. from wastewater resistant to Cadmium (Cd) at a concentration of 550 µg/mL. In another study, Khan et al. (131) reported Cadmium (Cd) resistance at 10.6 mM in *E. coli* isolated from industrial wastewater. Generally, microbial Cadmium (Cd) resistance occurs via 2 main mechanisms: active efflux and sequestration (129). Efflux of Cd^{2+} involves 3 main classes of pumps: P-type ATPase (CadA), CBA/RND chemiosmotic antiporter Cobalt, zinc and Cadmium efflux system (czcCBA), and Cation Diffusion Facilitator (CDF) transporter (czcD) (62, 129). The best studied Cadmium efflux systems are czcCBA of *R. metallidurans* CH34 and CadA ATPase pump of *S. aureus* (62). While czc efflux system encodes resistance to several divalent metal ions such as Cobalt (Co^{2+}), Zinc (Zn^{2+}), and

Cadmium (Cd^{2+}), CadA was shown to encode resistance preferentially to Cadmium ions (Cd^{2+}) ((62). On the contrary, czcD is a CDF transporter reported to mediate Cobalt (Co^{2+}), Zinc (Zn^{2+}) and Cadmium (Cd^{2+}) efflux and was also shown to regulate the expression of czcCBA efflux system in *R. metallidurans* CH34 (132). Another resistance mechanism bacteria use to detoxify Cd^{2+} is sequestration. Sequestration of Cadmium can occur intracellularly on metallothionein proteins (MT) as reported in *Synechococcus* spp. (133) or extracellularly on capsular polysaccharides or EPS as demonstrated in *Pseudomonas*, *Arthrobacter*, and *Klebsiella aerogenes* (134-136).

f. Lead (Pb)

Lead is a bluish metal which is predominantly coming from human activities (137). In fact, Lead (Pb) has many industrial usages. It is used to manufacture pipes, storage batteries, pigments, x-ray shields, ammunitions, bullets etc. (48, 137). Over the past decades, there has been a growing concern regarding the rapid increase in Lead (Pb) levels in the environment owing to its hazardous nature (137). Lead (Pb) is a systemic toxin which can affect any organ in the human body (35). According to the Agency for Toxic Substances and Disease Registry (ATSDR), Lead (Pb) exposure was shown to be correlated with several diseases including neurodevelopmental disorders, cardiovascular diseases, renal impairment, and adverse hematological effects (137). Various mechanisms of Lead (Pb) toxicity were described in human beings, but the most important ones involve inducing cellular damage through ROS formation, binding to enzymes and disrupting their configurations, as well as interfering with Calcium (Ca) metabolism (35). Similarly, Lead (Pb) is toxic to microorganisms and its toxicity stems mainly from lacking any physiological or metabolic role in cellular processes (138). Several research studies were

conducted and had identified Lead (Pb) tolerant bacteria expressing different resistance mechanisms (48, 72, 139). The first discovered mechanism involves adsorption of Lead (Pb) on extracellular polymeric substances (EPS) and cell wall of resistant bacteria (48). Structures like cell wall and extracellular polymers often serve as natural barriers to metal ions as they contain functional groups that readily adsorb them as mentioned previously (48). In their study to assess the biosorption property of the *Bacillus* spp. ATS-2, Cabuk and his co-workers reported the involvement of hydroxyl and carbonyl groups of EPS in adsorption to Pb^{2+} ions (140). More recent evidence on *P. putida* showed that other functional groups such as carboxyl and phosphate are also involved in Pb^{2+} chemisorption (141). The second mechanism of resistance to Lead (Pb) is based on reducing its accumulation through intracellular and extracellular precipitation. Many microorganisms were shown to precipitate Lead (Pb) in the form of insoluble Lead phosphate salts including *S. aureus* (72), *Providencia* (142), and *Pseudomonas* (143). Other bacteria such as *Bacillus* and *Brevibacterium* were reported to precipitate Lead (Pb) in the form of Lead sulfide (PbS) rather than Lead phosphate (144). In *Citrobacter freundii*. Lead (Pb) precipitation was shown to occur extracellularly and was thought to be mediated by an acid phosphatase (145). The third Lead (Pb) resistance mechanism consists of adsorbing Lead (Pb) to the intracellular proteins, Metallothioneins which are often encoded by plasmid borne genes namely *smtAB,* and *bmtA* (48, 146). Lead binding-Metallothioneins have been detected in Lead (Pb) resistant *P. aeruginosa* strain WI-1.(76). DNA analysis of this strain revealed the presence of a plasmid borne metallothionein encoding gene, *bmtA* responsible for Lead (Pb) sequestration. Recently, the first report on Lead (Pb) precipitation in the periplasm of *Providencia vermicola strain SJ2A* aided by bmtA metallothionein was

released (147). Other microorganisms detoxify Lead (Pb) compounds by methylating and converting them to less toxic volatile forms (48). Biomethylation of Lead (Pb) was documented in several bacterial species including *Acinetobacter* spp., *Pseudomonas* spp., *Aeromonas* spp. among others (148). Finally, Lead (Pb) can be detoxified by efflux systems, the most common and effective heavy metal resistance mechanisms reported in bacteria (48, 62). Efflux of Lead (Pb) is largely mediated by P-type ATPases such as CadA of *S. aureus*, ZntA of *E. coli*, and PbrA of *C. metallidurans* and to a lower extent by RND/CBA chemiosmotic transporters. CadA, ZntA, and PbrA are homologous P-type ATPases which are involved in efflux of not only Pb^{2+} ions but also Zn^{2+} and Cd^{2+} (48). In addition to these ATPases, Pb^{2+} can be exported by other transporters such as those belonging to the CBA superfamily (48). Up till now, the CBA transporter of *P. Putida*, czcCBA1 is the only transporter of this family which was shown to extrude Pb^{2+} ions outside the bacterial cell (48, 149) (**Figure 7).**

g. Zinc (Zn)

Zinc (Zn) is an essential trace element which is required for life in all living systems (38, 150, 151). It is one of the most widely distributed elements in the earth's crust (150). In humans, Zinc (Zn) is a vital component for a broad range of metalloenzymes and Zn^{2+} dependent transcription factors (38, 59, 150). Despite its fundamental physiological role, excess intake of Zinc (Zn) was shown to induce acute toxicity mainly affecting the GI tract and the respiratory tracts (150). Likewise, Zinc (Zn) is used as a cofactor for metalloenzymes in bacterial species. It is estimated that around 5-6% of the total bacterial proteome binds Zn^{2+} ions (152). Bacteria have evolved very tight regulatory mechanisms to keep Zinc (Zn) levels within homeostatic ranges and avoid its toxic accumulation: (1)

Regulation of Zinc (Zn) import (2) Regulation of Zinc (Zn) export and (3) Regulation of its sequestration on Metallochaperones. Uptake of Zinc (Zn) in bacteria is mediated by several importers depending on the energy availability, Zinc (Zn) levels, and the bacterial species. In *S. cerevisiae*, Zinc (Zn) enters the bacterial cell using the Magnesium ions uptake transporter CorA MIT (38). Whereas, in *E. coli*, uptake of Zinc (Zn) is mediated by different transporters depending on its concentration: by the energy dependent znuABC complex under Zinc (Zn) limiting conditions, by the less energy consuming ZupT under Zinc sufficient levels or by phosphate transporters under toxic Zinc levels (153). Extrusion of Zinc occurs via 3 major families of efflux pumps: P-type ATPases, CBA/RND chemiosmotic antiporters, and CDF transporters (38, 59). So far, there are 3 discovered P-type ATPases that were shown to pump out Zinc ions (Zn^{2+}): Znt A, ZiaA, and CadA. While the first 2 pumps are implicated in Zinc efflux, CadA was shown to extrude out both Zinc (Zn) and Cadmium (Cd) ions (62). Alternatively, Zinc (Zn) ions can be pumped out by broader substrate spectrum efflux pumps of the RND family, also known as "transenvelope transporters". The best characterized RND efflux system conferring resistance to Zinc (Zn) is czcCBA efflux system from *C. metallidurans* CH34. Not only does this system encode resistance to Zinc (Zn), but also to Cadmium (Cd) and Cobalt (Co) ions (38). Another efflux system encoding resistance to Zinc (Zn) is czcD. The latter is a heavy metal transporter of the CDF family responsible for both Zinc (Zn) and Cadmium (Cd) expulsion (62). In addition to regulating the uptake and export of Zinc (Zn), bacteria also control its intracellular levels by regulating its binding and release from Metallothioneins as was demonstrated in cyanobacteria, *Synechococcu*s PCC 7942 (154).

h. Barium (Ba)

Barium is a alkaline metal which exists in nature at low levels exclusively in the form of compounds (155, 156). It is a very reactive element which has similar chemical properties to Calcium (Ca) and Magnesium (Mg) but has different solubility depending on the compound it forms (155). Insoluble Barium (Ba) compounds such as Barium sulfate ($BaSO_4$) are in general non-toxic to human beings, whereas soluble compounds were shown by several reports to cause deleterious health effects (155). In microbial population, on the contrary, little is known about the metabolic role and toxicity of Barium (Ba) compounds and much of the data on Barium (Ba) is based on very old studies. Barium (Ba) in the form Barite/Barium sulfate ($BaSO_4$) was shown to be utilized by sulfate reducing bacteria as a source of Sulfur (S) during anaerobic respiration (156, 157). In a more recent study to investigate the bacterial susceptibility to Ba^{2+} ions, Sivolodskii examined the susceptibility of different *Pseudomonas* spp. to Barium chloride ($BaCl_2$) and found that most of the species were able to tolerate Ba^{2+} up to 6 g/L (158). So far, no published date is available on the mechanisms of Barium (Ba) resistance in bacteria.

F. Association between Heavy Metals and Antimicrobial Resistance (AMR)

After the industrial revolution and the increased use of heavy metal in various fields, increasing concerns were raised about heavy metals contamination and their ability to induce AMR in bacterial populations. These concerns were principally based on 4 major facts: (1) Heavy metals use is on the rise worldwide. (2) They are non-biodegradable and tend to persist for long time in the environment. (3) Many bacterial species have evolved

resistance mechanisms against heavy metals. (4) Genes encoding resistance to heavy metals are often physically linked to one or more AMR genes on mobile genetic elements (159).

Since 1970's a growing body of literature has examined the association between heavy metals and AMR in different environmental matrices and has found that these chemical elements can act as selective pressures on microbial population and induce indirectly antibiotics resistance via a process called co-selection (19, 160). Co-selection of metal and AMR was shown to occur via three main mechanisms (**Figure 8**):

1. Co-resistance

Co-resistance to metal ions and antibiotics occurs when genes encoding resistance to these toxins are physically linked/located in close proximity to each other on mobile genetic elements such as on plasmids, transposons or integrons (11, 19, 159). Such genetic linkage of resistance has been documented in several bacteria originating from different natural environments. For example, a genetic linkage on conjugative plasmid between Copper (Cu) resistance encoded by *tcrB* gene and Erythromycin and Vancomycin resistance was reported in *Enterococcus faecium* isolated from pigs (161). Another study by Gilmour et al. (162) reported the isolation of a plasmid carrying Chloramphenicol, Kanamycin and Tetracycline resistance genes along with heavy metal encoding genes namely Arsenic (As), Copper (Cu), Mercury (Hg), Silver (Ag), and Tellurium (Te) resistance encoding genes in the opportunistic pathogen, *Serratia marcescens*. In a study conducted in 2001, Whole Genome Sequencing (WGS) of the MDR *S. typhi* (CT18) revealed a linkage between Mercury resistance genes and several antibiotics resistance genes (Chloramphenicol, Ampicillin, Streptomycin, Sulfonamide, and Trimethoprim) on a

conjugative plasmid confirming previous findings on Mercury (Hg) and its association with increased resistance to antibiotics (163, 164). This molecular mechanism of co-selection is of special interest as it highlights the possibility of combined dissemination of metals and antibiotics resistance genes via horizontal gene transfer in metal contaminated environments.

2. Cross-resistance

Cross-resistance is when one resistance system/mechanism confers resistance to metals ions and antibiotics at the same time. Cross-resistance can occur via different mechanisms including alteration in cell membrane, mutations, and active efflux via multi-drug efflux pumps. The latter are considered the most common mechanisms responsible for cross resistance (11, 12). Several multi-drug efflux systems were discovered in bacteria such as MdrL efflux pump in *Listeria monocytogenes* which was to encode resistance to Zinc (Zn), Cobalt (Co), Chromium (Cr), Erythromycin, Josamycin, and Clindamycin (165). Another described multi-drug efflux system is DsbA–DsbB disulphide bond formation system in *Burkholderia cepacia* which was reported to be involved in cross resistance to several antibiotics (β lactams, Kanamycin, Erythromycin, Novobiocin, Ofloxacin) and metal ions (Zn^{2+} and Cd^{2+}) (166). In addition, studies on antibiotics efflux systems in *Campylobacter jejuni* identified the presence of a multi-drug efflux pump designated as CmeABC which was shown to mediate resistance to several classes of antimicrobial agents and heavy metals namely Cobalt (Co) and Copper (Cu) (167).

3. Co-regulatory resistance

Co-regulation is a less common mechanism of co-selection. It occurs when resistance genes to antibiotics and heavy metals are controlled by a common regulatory protein (12). A very well characterized regulatory system involved in co-regulation resistance mechanism is CzcS-CzcR, two component regulatory system of *P. aeruginosa*. This system was shown to confer resistance to Zn^{2+}, Cd^{2+} and Co^{2+} by activating the expression of *czc*CBA efflux pump and to Carbapenems (Imipenem) by suppressing the expression of *OprD* porin encoding gene (168).

CHAPTER III

MATERIALS AND METHODS

A. Identification of Bacterial Samples Obtained from Syria

1. Purification of Bacterial Samples

A total of 43 mixed bacterial samples were obtained from war wounded patients from Tishreen University Hospital in Latakia, Syria. Samples were first cultured on MacConkey agar plates (Sharlau S.L., Spain) and incubated at 37 °C for 24 hrs. After 24 hrs., cultures were checked for purity and non-pure cultures showing different colonial morphologies and colors were purified by repeated streaking on MacConkey agar plates. Pure cultures were then maintained as frozen stocks at -80 °C in brucella broth supplemented with 15% glycerol (BD BBL ™, France).

2. Identification of Acinetobacter spp. by CHROMagar

To identify *Acinetobacter* spp., pure stocks of bacterial isolates were thawed and plated onto CHROMagar™ Acinetobacter (CHROMagar, Paris, France) which is a selective agar used for the detection and isolation of *Acinetobacter* species. CHROMagar was prepared following the manufacturers' instructions. In general, bacteria belonging to the genus *Acinetobacter* grow as red creamy colonies while bacteria belonging to other genera might either grow as non-red colonies or they might not grow at all.

3. Identification of Acinetobacter spp. by Multiplex PCR

A multiplex PCR based on the amplification of *gyrB* gene which encodes the subunit B of DNA gyrase was performed to determine the species of the suspected *Acinetobacter* grown on CHROMagar following the protocol described by Higgins and his co-workers (169).

a. Total DNA Extraction

Total DNA was extracted from the six suspected *Acinetobacter* isolates using the Qiagen QIAmp DNA mini kit (Qiagen, Germany) and was later used for PCR analysis.

i. Materials needed

- ATL buffer
- Proteinase K
- RNAse A
- AL buffer
- 95% ethanol
- QIAamp Mini spin column
- 2 mL collection tubes
- AW1 buffer
- AW2 buffer
- AE buffer
- 1.5 mL sterile microcentrifuge tubes
- Filter tips
- Centrifuge

- Heating block
- Vortexer (Thermo Fisher Scientific, USA)

ii. Protocol

- Few colonies of overnight cultures of each bacterial isolate were suspended in Cation Adjusted Mueller-Hinton broth (CAMHB) in sterile 5 mL test tubes and were put in the incubator at 37 °C for 24hrs.
- On the second day, 1 mL of each bacterial suspension was transferred to a sterile 1.5 mL microcentrifuge tube and centrifuged at 5000 x g for 5 minutes.
- After the centrifugation, the supernatant was discarded and 170 µL of ATL buffer was added to each pellet.
- 20 µL of Proteinase K was then added to each pellet/ATL buffer mix and the microcentrifuge tubes were briefly vortexed and incubated in a heating block at 56 °C for 1hr with vortexing every 15 minutes.
- After the incubation, tubes were briefly spun down to remove any buildup droplets on the lid.
- Next, 4 µL of RNase A was added to each tube and the tubes were then vortexed for 15 seconds and incubated at room temperature for 2 minutes.
- Then, 200 µL of another lysis buffer (AL buffer) was added to each tube followed by vortexing for 15 seconds then incubating the tubes at 70 °C in a heating block for 10 minutes.

- After this step, tubes were briefly spun down to collect the droplets formed on the inside of the lid.
- To precipitate the DNA, 200 µl of 95% ethanol was added to each tube followed by vortexing for 15 seconds.
- Afterwards, the entire content of each tube was transferred to a labeled QIAmp Mini spin column placed in 2 mL collection tube and the spin columns were then centrifuged at 6000 x g for 1 minute.
- Collection tubes were discarded, and the spin columns were placed in new 2 mL collection tubes.
- To remove any contaminants or debris adsorbed to the silica membranes of the spin columns, 2 washing steps were performed:
    - The first washing step was done by adding 500 µL of the washing buffer AW1 to each spin column followed by centrifugation at 6000 x g for 1 minute.
    - Afterwards, the filtrate was discarded, and each spin column was transferred into a new collection tube.
    - A second washing step using AW2 buffer was done by adding the same volume as AW1 to each spin column followed by centrifugation at higher speed 20,000 x g for 3 minutes.
    - A second centrifugation step at the same speed for 1 minute was done to remove any residual AW2 buffer adsorbed to the silica

membrane after placing the spin columns in new 2 mL collection tubes.

- To elute the adsorbed DNA, spin columns were transferred to new labeled sterile 1.5 mL microcentrifuge tubes and 100 µL of elution buffer AE was added to each tube and the latter was incubated at room temperature for 5 minutes.
- Finally, tubes were centrifuged at 6000 x g for 1 minute and the DNA extracts were collected in the microcentrifuge tubes suspended in AE buffer.
- The concentration and purity of DNA extracts were measured using the NanoDrop (Denovix ®, Wilmington, DE, USA).

b. The gyrB Multiplex PCR

Since *Acinetobacter baumanniii* can't be distinguished from other *Acinetobacter* spp. using phenotypic and biochemical tests, we opted to use a PCR-based approach previously described by Higgins and his co-workers for that purpose (169). The method involves amplifying a gene called *gyrB* gene, encoding DNA gyrase subunit B, as it was shown to be conserved and unique to each *Acinetobacter* spp.

Amplification of *gyrB* gene was performed using 3 primers: sp4F, sp4R and sp2F (Macrogen, Inc., South Korea) as indicated by Higgins and his colleagues, each diluted to a working concentration of 10 µM (169). The first 2 primers are universal primers to both *A. baumannii* and *A. genomic species 13TU*. Whereas sp2F primer is specific for *A. baumannii* (169). The nucleotides sequence of each primer, its length and the amplicon size

it yields are summarized in **Table 2.** A PCR reaction mixture was prepared for *gyrB* amplification (**Table 3**).

The PCR assay was carried out in C1000^{TM} Thermal Cycler (Bio-Rad Laboratories, Inc., USA). The cycling conditions consisted of 2 minutes of initial denaturation at 94 °C, followed by 25 cycles involving 1 minute denaturation at 94 °C, 30 seconds annealing at 60 °C, and 1 minute extension period at 72 °C with a final extension at 72 for 10 minutes. The amplification products were visualized on Gel Doc^{TM} EZ Imager – Bio- Rad after electrophoresis on 1.2 % agarose Invitrogen E-Gel.

c. DNA Extraction Using Tris-Cl Saturated Phenol

To further confirm the results, PCR amplification of *gyrB* gene was repeated on the same bacterial isolates grown on CHROMagar but using an alternative DNA extraction protocol employing Tris-Cl saturated phenol.

i. Materials needed

- Tris-Cl saturated phenol
- Cold sodium acetate of concentration 3M and PH 5.2 (AMRESCO®, USA)
- Cold 70% ethanol
- TE (Tris-EDTA) buffer (AMRESCO®, USA)
- Sterile 1.5 mL microcentrifuge tubes
- Vortexer

Tris-Cl Saturated Phenol Preparation

First, 700 mL of Tris-Cl (PH=8, concentration 50 mM) was prepared by dissolving 5.52 grams in 700 mL autoclaved distilled water and adjusting the PH to 8 using a PH meter (Thermo Scientific, USA). Second, 100 mL of the prepared Tris-Cl was added to 100 grams of phenol crystals (Sigma-Aldrich, USA) in a 500 mL beaker in the fume hood and the beaker was allowed to stand for 1-2 hrs. until the phenol liquified and 2 separated phases were seen. Next, the upper phase was removed, 100 mL of Tris-Cl was added, and the beaker was allowed to stand for an additional 15 minutes to 1 hr. until 2 separated phases were seen. Then, this step was repeated for 4 times until the PH of the upper phase reached about 7 or 8 as measured by the PH meter. Finally, the phenol bottom layer was pipetted up and aliquoted into 15 ml falcon tubes and 3 mL of Tris-Cl were added to each tube and the tubes were stored at -20 °C sealed with aluminum foil.

ii. Protocol

- 500 µL suspensions in CAMHB of each bacterial isolate were prepared in 1.5 mL microcentrifuge tubes and an equivalent volume of Tris-Cl saturated phenol (aliquoted from the bottom layer) was then added to each bacterial suspension.
- After adding the phenol, tubes were centrifuged for 15 minutes at 13,000 rpm and the supernatants were retained and transferred to new 1.5 mL microcentrifuge tubes.
- Next, 1/10 of the supernatant's volume of sodium acetate (PH 5.2 and concentration 3M), previously stored at – 20 °C, was added to each tube with mixing.

- Then, an equivalent of 3 times the supernatant's volume of cold 70 % ethanol, previously stored at – 20 °C, was added to each microcentrifuge tube and the tubes were placed in the freezer at -80 °C for 3hrs.
- After the 3 hrs. incubation, tubes were centrifuged at 4 °C for 15 minutes at 13,000 rpm and supernatants were discarded.
- Next, DNA pellets were washed 3 times with 70% ethanol such that each washing step involved adding 1 mL of 70% ethanol to each DNA pellet, centrifuging at 13,000 rpm for 15 minutes and discarding the supernatant.
- Finally, the DNA pellets were dried, suspended in TE buffer and their concentrations and purity were then measured using the nanodrop (Denovix ®, Wilmington, DE, USA).

4. Identification of Acinetobacter spp. using API NE kit

To identify bacterial samples down to the species level, API NE test (Biomérieux, France) was used. Bacterial suspensions for the API NE test were first prepared in 0.9 % NaCl and adjusted to 0.5 McFarland. The suspensions were then added to the microtubes of the API strips following the manufacturer's instructions. After incubating the strips for 24 hrs. at 37 °C, reactions were read and recorded to obtain a 7-digit number. This number was then used to identify the bacterial isolates using the analytical profile index (1990 edition).

B. Antimicrobial and Heavy Metal Susceptibility Testing of *A. baumannii* Isolates

A total of 11 *Acinetobacter baumannii* clinical isolates (ACN), which were previously obtained from the Clinical Microbiology Laboratory at the American University of Beirut Medical Center (AUBMC) and Al Makased General Hospital, were used in this assay. 4 out of the 11 isolates tested were recovered between 2007 and 2013 from war-wounded Iraqi patients admitted to AUBMC and the rest were isolated from non-war wounded patients admitted to the ICUs of Al Makased and AUBMC hospitals (**Table 5**). Broth microdilution susceptibility testing (BMD) was performed on these isolates to examine their susceptibility against a panel of 8 heavy metals (Zn^{2+}, Cu^{2+}, Cr^{6+}, Pb^{2+}, Ba^{2+}, Cd^{2+}, Hg^{2+}, and As^{5+}) commonly used in weapons, 5 clinically utilized antimicrobials covering the major classes (Meropenem, Colistin, Gentamicin, Ciprofloxacin, and Cefepime) and 40 different combinations of antimicrobials and heavy metals. The BMD procedure was performed in accordance with CLSI guidelines with slight modifications. ACN DSM 30008 was used as a reference strain and isolates were tested in duplicates.

1. Materials needed for BMD

- 96-wells microtiter plates (Corning®, USA)
- Cation Adjusted Mueller-Hinton broth (CAMHB)
- Antimicrobials: Meropenem (MEM), Colistin Sulfate (CST), Gentamicin Sulfate (GEN), Ciprofloxacin (CIP), and Cefepime HCL (FEP)

- Heavy metals salts: Zinc Sulphate Heptahydrate ($ZnSO_4.7H_2O$) (Merck and Co, USA), Anhydrous Cupric Sulphate ($CuSO_4$) (Fisher Scientific, USA), Potassium Dichromate ($K_2Cr_2O_7$) (Fisher Scientific, New Jersey, USA), Barium Chloride Dihydrate ($BaCl_2.2H_2O$), Cadmium Acetate Dihydrate ($Cd(C_2H_3O_2)_2.2H_2O$), Mercuric Acetate $Hg(C_2H_3O_2)_2$, (BDH Chemicals Ltd., Poole, England), Lead Acetate Trihydrate ($Pb(C_2H_3O_2)_2.3\ H_2O$) (Fisher Scientific, New Jersey, USA) and Sodium Arsenate Dibasic Heptahydrate ($Na_2HAsO_4.\ 7H_2O$) (Sigma-Aldrich, USA)
- Densitometer (Densimat, Biomérieux Biotechnology,USA)
- Pipettes
- Sterile loops
- Shaker (Daihan, Scinetific Co, South Korea)
- Incubator (Thermo Scientific, USA)

2. Procedure

a. Antibiotics Stock Solutions Preparation

10 mg/mL stock solutions of the powdered antimicrobials (MEM, CST, GEN, and FEP) were prepared in accordance with the CLSI guidelines and stored at -20 °C. MEM (AstraZeneca UK limited, United Kingdom), CST (Sigma-Aldrich, USA) and GEN

(Sigma-Aldrich, USA) were dissolved in autoclaved Milli-Q water while FEP was dissolved in 10X phosphate buffer saline (PBS) (Lonza, Walkersville, MD USA) concentration 0.1 M. As for CIP (HIKMA Pharmaceuticals, Amman-Jordan), a ready to use liquid vial of concentration 2 mg/mL was used.

b. Heavy Metals Stock Solutions Preparation

Heavy metals stock solutions of concentration 1 M were prepared by dissolving an appropriate mass of each metal salt in 50 mL autoclaved Milli-Q water and were then filtered using 0.22 µm sterile syringe filters. See **Table 4** for detailed information on the heavy metals' salts used.

i. Materials needed

- Weighing balance
- Weighing boat
- Spatula
- 50 mL falcon tubes (BD Falcon ™)
- Digital pipet
- Hotplate stirrer (Cornings ®, USA)
- 50 mL Erlenmeyer flask
- Sterile syringe filter (Cornings ®, USA)
- Sterile syringes (Becton Dickinson, USA)
- Heavy metals salts
- Autoclaved Milli-Q water

ii. Procedure

- An appropriate mass of each heavy metal salt was weighed on a weighing boat using a balance and transferred to a 50 mL Erlenmeyer flask.
- 50 mL of autoclaved Milli-Q water was then added to the flask using a digital pipet and the flask was swirled gently until the salt completely dissolved.
- If the salt didn't dissolve, the flask was placed on a hotplate stirrer until a homogenous solution was obtained.
- After complete dissolution of the metal salt, the prepared solution was drawn using a sterile syringe and decanted through a sterile syringe filter placed on a properly labeled 50 mL falcon tube for filtration.
- Filtered heavy metal solutions were then stored at room temperature.

Note:

-Not all heavy metals solutions required a heating step to dissolve. Some were readily soluble in their corresponding solvents at room temperature. See table 2.

-All steps were performed under the fume hood.

-A vapor respirator was worn during the process of preparing heavy metals solutions.

c. Bacterial Inoculum Preparation

Bacterial isolates' stocks, which were previously stored at -80 °C, were streaked on MacConkey agar plates (Sharlau S.L., Spain) and put in the incubator at 37 °C

overnight. On the next day, colonies of each bacterial isolate were picked up and suspended in CAMHB (Simga-Aldrich Co, St. Louis, USA) to yield a standard bacterial inoculum of density 0.5 McFarland using a densitometer.

d. Microdilution in 96-wells Microtiter Plates

90 µL of CAMHB was dispensed in wells 2 through 12 of each of the microtiter plates.

i. Antimicrobial agents Dilution

38.9 µL of the prepared antimicrobials solutions (MEM, CST, GEN, and FEP) was added to 151.1 µL of CAMHB in the first well of each row to achieve concentrations equal to 2048 µg/mL. In case of CIP, 97.3 µL was added to 92.7 µL of CAMHB in the first well to get a working concentration equal to 1024 µg/mL. Next, the added antimicrobial agents were diluted in a serial 2-folds dilution using a multichannel pipette starting from the first wells until wells 11 yielding thereby gradient concentrations of antimicrobials, from the highest concentrations: 2048 µg/mL for MEM, CST, GEN, and FEP and 1024 µg/mL for CIP, to the lowest concentrations: 1 µg/mL for CIP and 2 µg/mL for the rest of the antimicrobials. Well 12 of each row of the microtiter plate was used as a growth control containing bacterial inoculum suspended in broth.

ii. Heavy Metals Dilution

121.6 µL of each prepared heavy metal solution and 68.4 µL of CAMHB were added to wells 1 of each column to achieve working concentrations of 640 mM. Then, 2-folds serial dilution of each heavy metal was carried out using a multichannel pipette starting from the first wells until wells 11 to obtain a gradient concentration; from the

highest concentration 640 mM to the lowest concentration 0.625 mM (**Figure 9**). Well number 12 of each row of the microtiter plate was used as a growth control.

iii. Antimicrobial agents and Heavy Metals Dilution

For every "metal-non-Ciprofloxacin combination" tested, 38.9 µL of each antimicrobial stock solution and 121.6 µL of each heavy metal solution were added to 29.5 µL of CAMHB in wells 1 to achieve working concentrations equal to 2048 µg/mL and 640 mM of antimicrobials and heavy metals respectively. Whereas for each "metal-Ciprofloxacin combination" tested, 97.3 µL of Ciprofloxacin and 60.8 µL of each heavy metal solution were added to 31.9 µL of CAMHB in wells 1 to obtain concentrations equal to 1024 µg/mL and 320 mM of CIP and heavy metals respectively. Metals and antimicrobials were then diluted in a serial 2-folds dilution using a multichannel pipette starting from the first wells until wells 11. Wells 12 of each plate were used as growth controls.

Finally, all plates were inoculated with 10 µL bacterial suspension adjusted to 0.5 McFarland so that each well after the inoculation would contain a bacterial concentration equal to 5* 10^5 CFU/mL and were then incubated at 37 °C for 16-24 hrs. with shaking at 160 rpm. On the next day, plates were inspected for visual growth and the MICs, defined as the lowest concentrations of antimicrobial agents/heavy metals required to inhibit bacterial growth, were determined and interpreted in reference to the CLSI susceptibility breakpoints.

Lead Acetate Trihydrate susceptibility testing, on the contrary, was performed in 5 mL test tubes rather in microtiter plates due to its precipitation property in liquid media (See **Figure 10** and **11**). So, a larger volume of culture media was used to distinguish

between Lead precipitation and actual bacterial growth/turbidity. The exact same procedure described above was followed but with a total volume equal to 2 mL instead of 100 µL. Hence, the volumes of antimicrobials and heavy metals added were adjusted accordingly.

C. Induction of Resistance by Serial Passage

To assess the effect of heavy metals on antimicrobial resistance of *A. baumannii*, we raised resistance *in vitro* in the fully susceptible reference strain *A. baumannii* DSM 30008 to selected heavy metals (Copper, Lead, Cadmium, and Arsenic) and/or antimicrobial agents (Gentamicin and Cefepime) by serial passage of the bacterium in CAMHB containing increasing concentrations of heavy metals and/or antimicrobials at a total volume of 2 mL. The starting concentrations of antimicrobial agents were chosen below their MICs, i.e., the first passage was carried out at a concentration equivalent to 0. 5 µg/mL of GEN and FEP. As for heavy metals, the starting concentrations were variable and were mainly dependent on their toxicity: [Copper] = 5 mM, [Lead] = 2 mM, [Arsenic] = 1 mM, and [Cadmium] = 1mM. After every passage, resistant mutants were recovered and transferred to a fresh CAMHB containing higher concentrations of heavy metals and/or antimicrobials (See **Table 6** for the list of induced mutants).

1. Materials needed

- Cation Adjusted Mueller Hinton Broth (CAMHB)
- 5 mL sterile glass test tubes
- Heavy metals solutions (previously prepared and used in BMD)
- Antimicrobials solutions

- Densitometer
- Pipettes

2. Procedure

First, stock solutions of GEN and FEP, concentration 2 mg/mL, were freshly prepared by dissolving 2 mg of antimicrobials' powders in 1 mL autoclaved Milli-Q water. Second, a standardized inoculum adjusted to 0.5 McFarland of the strain ACN DSM 30008 was prepared using the direct colony suspension method. Next, 5 tubes were used and were labeled as follows (**Figure 12**):

- Tube 1 contained ACN DSM 30008 + one antimicrobial agent
- Tube 2 contained ACN DSM 30008 + one heavy metal
- Tube 3 contained ACN DSM 30008 + a combination of one heavy metal and one antimicrobial
- Tube 4 served as a growth control
- Tube 5 contained broth only

To raise resistance to GEN or FEP at concentration equal to 0.5 µg/mL and heavy metals at concentration 5 mM singly or in combination, the following volumes were added in each tube:

- Tube 1: 1900 µL of CAMHB, 0.475 µL of antimicrobial agent
- Tube 2: 1890 µL of CAMHB, 9.5 µL of heavy metal solution

- Tube 3: 1890 µL of CAMHB, 0.475 µL of antimicrobial, and 9.5 µL of heavy metal solution
- Tube 4: 1900 µL of CAMHB

After the addition of antimicrobial agents and heavy metals, 100 µL of the prepared 0.5 McFarland suspension was dispensed in each tube except tube 5 and all tubes were subsequently incubated overnight at 37 °C with shaking at 160 rpm. On the next day, tubes were inspected visually for bacterial growth/turbidity. For tubes showing bacterial growth after 24 hrs., 2 glycerol stocks of the induction generation were prepared and stored at – 80 °C. In addition, 100 µl of the mutant culture was transferred to a fresh media containing higher concentrations of antimicrobials and/or heavy metals. On the contrary, tubes which didn't show any visual turbidity were kept in the incubator for an additional 24 hrs. and a subculture onto Mac plate was performed to ensure that there was truly no bacterial growth. In case of positive subculture after 24 hrs. several passages of the mutants at the same concentration of antimicrobial and/or heavy metal were carried out to further enrich their growth and enhance their resistance phenotypes.

D. Antimicrobial Susceptibility Testing of Induced Mutants

To examine whether heavy metals exposure in ACN DSM 30008 has altered its susceptibility to antimicrobial agents, all induced resistant mutants (listed in **Table 6**) were checked for their susceptibility to antimicrobial agents namely Meropenem, Colistin,

Gentamicin, Cefepime, and Ciprofloxacin by broth microdilution. The experimental procedure followed is the same as previously described.

E. Whole Genome Sequencing (WGS) of Induced Mutants

In order to identify the molecular mechanisms underlying the increased resistance observed in heavy metals and/or antimicrobials mutants, whole genome sequencing was performed on the 12 generated resistant mutants. Genomic DNA extractions were performed using Wizard genomic DNA purification kit (Promega, WI, USA) with a few modifications to the manufacturer's protocol, such as adding 5 µl of RNase solution during cell lysis as well as incubating the supernatant carrying the DNA at −20° C for 1 h after addition of isopropanol. DNA concentration was measured using Qubit dsDNA BR assay kit (Molecular Probes, OR, USA) before sequencing. Whole genome libraries were prepared using Ion plus fragment library kit, and Emulsion PCR was carried out using Ion one touch 200 Template kit v2 DL (Life technologies, CA, USA). Sequencing of the amplicon libraries was carried out on a 318 chip, using Ion Personal Genome Machine Ion Torrent sequencer through Ion Sequencing 200 kit (Life technologies, CA, USA). The resultant reads were assembled using MIRA plug-in (version 4.0) of Torrent suite software. Genome assemblies were annotated using RAST annotation pipeline, and further validated with Geneious pro 8.0 (Aziz et al., 2008; Kearseet al., 2012). Genes encoding the efflux systems, porins, and virulence factors of the panel strains were aligned using Clustal Omega, and verified for the polymorphisms (Sieverset al., 2011). Resistance genes were

identified using Resfinder (Zankari et al., 2012), and manually curated using NCBI BLAST.

F. Incubation Conditions of *A. baumannii* Clinical Isolates Selected for qRT-PCR

Three *A. baumannii* isolates ACN I4785, ACN I4789, and ACN U4388 designated as isolates 1, 3 and 4, which showed a remarkable decrease in their susceptibility to Ciprofloxacin (CIP) upon combination testing with Barium (Ba) (MIC increased from 64 to 512 µg/mL) were selected for quantitative reverse transcriptase-polymerase chain reaction (qRT-PCR). Analysis was carried out on these isolates to measure the impact of Barium on the expression levels of two (RND) type multidrug efflux systems, AdeFGH and AdeIJK which are very prevalent and ubiquitous in almost all *A. baumannii* isolates (170, 171). Briefly, each isolate was cultured with Ciprofloxacin alone and Ciprofloxacin in combination with Barium at half their MICs in CAMHB at 37 °C for 48hrs. Three positive controls (PCs) containing bacterial isolates suspended in CAMHB without any treatment were used (1 PC, 3 PC, and 4 PC).

The volumes added in each treatment condition are summarized in **Table 7**.

After adding Barium and Ciprofloxacin in their corresponding treatment tubes, a standard bacterial inoculum of density equal to 0.5 McFarland was prepared for each isolate and 200 µL of the prepared suspension was added to each tube. Tubes were then incubated at 37 °C for 48 hrs. and used afterwards for RNA extraction.

G. RNA Extraction

Total RNA was extracted from the three *A. baumannii* isolates (ACN I4785, ACN I4789, and ACN U4388), using the Illustra™ RNAspin Mini RNA Isolation Kit (GE healthcare, UK) following the manufacturer's instructions.

1. Materials needed

- Lysis Solution (LS)
- Desalting Buffer (DB)
- DNase I solution
- DNase Reaction Buffer
- Wash Buffer I (WB I)
- Wash Buffer II (WB II)
- RNase-free water
- RNAspin Mini Filter
- RNAspin Mini Column
- RNAspin Mini Collection Tubes
- 70 % Ethanol
- TE Buffer (AMRESCO®, USA)
- Lysozyme from chicken egg white (Sigma®, USA)
- β-Mercaptoethanol (β-ME) (AMRESCO®, USA)
- mL RNase-free microcentrifuge tubes

2. Protocol

a. Cells Pelleting and Lysis

- First, 1.5 mL of each culture broth was transferred to an RNase-free 1.5 mL microcentrifuge tube and centrifuged at maximum speed for 15 minutes.
- Second, the supernatant of each culture broth was discarded and 100 µL of 0.2 mg/mL Lysozyme dissolved in TE buffer was added to each tube.
- Third, tubes were vortexed for few seconds and incubated at 37 °C for 10 minutes.
- Next, 350 µL of Lysis Solution (LS) and 3.5 µL of β-ME were added to each tube followed by vortexing for few seconds.

b. Filtration of Lysates

- The lysates were then added to RNAspin Mini Filters placed in collection tubes, and the latter were centrifuged at 11,000 x g for 1 minute.
- After centrifugation, the RNAspin Mini Filters were discarded, and the filtrates were retained and transferred to new RNase-free 1.5 mL microcentrifuge tubes.

c. Adjustment of RNA binding conditions

- To favor the adsorption of RNA to silica membranes in the coming steps, 350 µL of 70 % Ethanol was added to each microcentrifuge tube, and the tubes were vortexed for 5 seconds.

d. RNA binding

- Subsequently, solutions were mixed by pipetting up and down 3 times then loaded onto the blue colored RNAspin Mini Columns with collections tubes before centrifugation at 8,000 x g for 30 seconds.
- Following centrifugation, collections tubes were discarded, and columns were transferred to new collections tubes.

e. Desalting

- 350 µL of Desalting Buffer (DB) was added to each column to remove salts adsorbing to silica membranes.
- After the addition of Desalting Buffer, spin columns were centrifuged at 11,000 x g for 1 minute, filtrates were discarded, and columns were placed back into their corresponding collection tubes.

f. DNA digestion

- DNase I reaction mixture was prepared by adding 10 µL of reconstituted DNase I to 90 µL of DNase reaction buffer in a microcentrifuge tube for each sample. The prepared mixture was mixed afterwards by flicking and 95 µL was added to the center of each spin column before incubating columns at room temperature for 15 minutes.

g. Washing

- 200 µL of Wash Buffer I (WB I) was added to each spin column followed by centrifugation at 11,000 x g for 1 minute.
- Next, collection tubes were discarded, and the spin columns were placed into new ones.

- Then, 600 µL of Wash Buffer II (WB II) was added to each column followed by centrifugation at 11,000 x g for 1 minute.
- Subsequently, filtrates were discarded, and each spin column was placed back into its corresponding collection tube.
- A final wash using Wash Buffer II (WB II) was performed by adding 250 µL to each column and centrifuging the columns at 11,000 x g for 2 minutes. Afterwards, columns were transferred to properly labeled 1.5 mL RNase-free microcentrifuge tubes for elution.

h. Elution

- To elute the adsorbed RNA, 100 µL of RNase-free water was added to each column followed by centrifugation at 11,000 x g for 1 minute.
- Then, eluted RNA was immediately put on ice to prevent its degradation and its concentration and purity were measured using the NanoDrop (Denovix ®, Wilmington, DE, USA).

H. Reverse Transcription and cDNA Synthesis

Reverse transcription and cDNA synthesis were carried out on the previously extracted RNA using the iScript™ cDNA synthesis kit (Bio-Rad, Hercules, CA, USA). RNA extracts and reagents used were kept on ice throughout the experimental procedure to avoid any potential degradation.

1. Materials needed

- Extracted RNA
- 5x iScript™ Reaction Mix
- iScript™ Reverse Transcriptase
- Nuclease-free water
- 0.2 mL microcentrifuge tubes
- T100™ Thermal Cycler (Bio-rad, USA)
- DS-11 FX Spectrophotometer

2. Protocol

First, a master mix containing nuclease-free water, 5x iScript™ Reaction Mix and iScript™ Reverse Transcriptase and is sufficient for all RNA samples was prepared. For each RNA sample, 4 µL of 5x iScript™ Reaction Mix was mixed with 1 µL iScript™ Reverse Transcriptase and 5 µL nuclease-free water. Next, 10 µL of the prepared master mix was mixed with 10 µL RNA to obtain a total volume of 20 µL per each reaction mixture. Then, reaction tubes were incubated in the Thermal cycler at the following cycling conditions as per the manufacturer's recommendations:

- Priming at 25 °C for 5 minutes
- Reverse transcription at 46 °C for 2 minutes

- Reverse transcription inactivation at 95 °C for 1 minute

After the run was completed, the concentrations and purity of synthesized cDNA samples were measured using DS-11 FX Spectrophotometer (Denovix®, Wilmington, DE, USA).

I. Relative Expression Levels of *adeIJK* and *adeFGH* Genes by qRT-PCR

To quantify the relative expression levels of genes encoding AdeFGH and AdeIJK MDR efflux pumps (**Table 8**) in Barium and Ciprofloxacin treated cultures, qRT-PCR was performed using iTaq™ Universal SYBR® Green Supermix kit (Bio-Rad, USA) on the Bio-Rad CFX96 Real Time System C1000 Thermal Cycler (Bio Rad, USA) using gene-specific primers listed in **Table 8**. The expression levels of the *ade* genes were calculated using the threshold cycle (CT) values and normalized to the expression levels of the housekeeping gene *rpoB* which encodes the β subunit of the bacterial RNA polymerase. Differences in the expression levels of the target genes were analyzed by Student's unpaired t-test and were considered significant if the P-values were ≤ 0.05. All reactions were carried out in triplicates.

1. Materials needed

- iTaq™ Universal SYBR® Green Supermix (Bio-Rad, Hercules, CA, USA)
- Forward and Reverse primers
- Nuclease-free water (AMRESCO, Solon, OH, USA)

- 0.2 mL 8-tubes PCR strips (Bio-Rad, Hercules, CA, USA)
- cDNA samples

2. Protocol

All reagents used were kept on ice through the experiment.

- Primers were first reconstituted in nuclease-free water according to the manufacturer's instructions to obtain stock primers' concentrations equal to 100 µM.
- Different aliquots of working primers solutions of concentrations 20 µM were then prepared and stored at -20 °C.
- Next, cDNA templates were diluted to a final concentration of 500 ng/µL.
- A real time PCR rmaster mix containing 5 µL iTaqTM Universal SYBR® Green Supermix, 3.5 µL nuclease free water, 0.25 µL forward primer, and 0.25 µL reverse primer per PCR tube was prepared for each primer set/gene and 9 µL of the prepared mix was added to each tube in the PCR strip.
- Finally, 1 µL of each diluted cDNA sample was added to the PCR strip tubes to obtain a total volume of 10 µL. Whereas, in the No Template Controls (NTCs) 1 µL of nuclease-free water was added instead to make up the volume to 10 µL.

- After adding all reaction components, PCR strips were sealed, spun down at 1000 rpm for 1 minute, and loaded onto the CFX96 Real Time System C1000 Thermal Cycler which was set at the following cycling conditions:
    - cycle 1: at 95 °C for 10 minutes for polymerase activation and initial DNA denaturation
    - cycle 2: repeated 40 times and consisted of 3 steps:

        step 1: 1 cycle at 95 °C for 15 seconds for further DNA denaturation

        step 2: 1 cycle at 58 °C for 10 seconds for primers' annealing

        step 3: 1 cycle at 72 °C for 30 seconds for extension/elongation
    - Melt curve analysis: 65 °C to 95 °C, increments of 0.5 °C at 5 seconds/step
    - Hold at 12 °C for 5 minutes.

Chapter IV

RESULTS

A. Identification of Bacterial Samples Obtained from Syria

Bacterial samples obtained from Syria were identified using 3 different tests: (1) phenotypic (growth on CHROMagar™), (2) biochemical (API NE), and (3) molecular (*gyrB* multiplex PCR) tests. Out of the 80 purified bacterial samples, 6 were presumptively identified as *Acinetobacter* species by giving red creamy colonies on CHROMagar™ (**Figures 13** and **14**). To identify the exact species of *Acinetobacter* isolates, a multiplex PCR approach utilizing 3 different primers (sp4F, sp4R, and sp2F) was used to amplify the *gyrB* gene encoding the B subunit of the DNA gyrase as it was shown to be unique to each species. (**Figure 15**). As shown in **Figure 15**, PCR amplification of the *gyrB* gene was negative in all the suspected *Acinetobacter* spp. contrary to what we expected (samples: 2,7A, 9, 18A, 19A, and 76) as no bands were observed compared to the positive control (ACN DSM 30008) which showed the expected 2 bands of size 490 and 294 base pairs.

B. Susceptibility Profiles of *A. baumannii* Isolates from Non-War Wounded Patients

All *Acinetobacter baumannii* isolates (listed in **Table 5**) recovered from non-war wounded patients, displayed a wide range of resistance to nearly all tested antimicrobial agents (**Figure 16** and **Table 9**). 5 (71.4%) isolates showed extensively drug resistant profiles (resistant to Meropenem, Gentamicin, Ciprofloxacin, and Cefepime) and 2 isolates (28.5%) namely ACN 4785 and ACN 2209 were pan drug resistant, where they were

resistant to all tested classes including the last resort antimicrobial agent, Colistin with MICs equal to 8 and > 2048 respectively (**Figure 16** and **Table 9**).

In addition, equal metal resistance profiles were observed in nearly all isolates. All showed very high levels of resistance to Barium (MIC: 640 mM), Arsenate (MIC: 40-80 mM) and Lead (MIC: 20-40 mM), a moderate resistance to Zinc (MIC: 5-10 mM), Copper (MIC: 10 mM), and Dichromate (MIC: 1.25-5 mM), and very low resistance to Cadmium (MIC: ≤ 0.625 mM) and Mercury (MIC: ≤ 0.625 mM) (**Figure 17** and **Table 10**).

C. Effect of Heavy Metals on Antimicrobial Susceptibility of *A. baumannii* Isolates from Non-War Wounded Patients

To assess the impact of heavy metals exposure on antimicrobial susceptibility of *A. baumannii* isolates, broth microdilution susceptibility testing involving 40 different combinations of heavy metals and antimicrobial agents was performed on each isolate. Different metals were shown to elicit different responses in bacterial isolates when tested in combination with antimicrobial agents.

1. Zinc (Zn) and Copper (Cu)

Zinc in the form of Zinc Sulphate Heptahydrate ($ZnSO_4.7H_2O$) and Copper in the form of Anhydrous Cupric Sulphate ($CuSO_4$) were shown to exert the same effect on the susceptibility of *A. baumanii* isolates to all tested antimicrobial agents except Colistin. Both significantly enhanced the isolates' susceptibility to Meropenem, Gentamicin, Ciprofloxacin, and Cefepime which was shown by the remarkable drop in their MICs when

tested in combination with these metals (**Figures 18** and **19** compared to **Figure 16** and **Tables 11** and **12** compared to **Table 9**). On the contrary, Zinc exerted different effects on different bacterial isolates when tested along with Colistin. In 4 isolates (ACN I4789, ACN I4785, ACN R4020 LA, ACN PFU9), it increased the MICs of Colistin by an average of 6.5 folds while it had no effect on the MIC in 2 isolates (ACN I4388 and ACN 2493). Interestingly, ACN 2209, the most resistant isolate to Colistin with a MIC of > 2048, was the only isolate to display a significant increase in its susceptibility to Colistin in the presence of Zinc, where it showed at least 128 folds decrease (> 2048 to 16 µg/mL) in its MIC against Colistin when tested in combination with Zinc versus when tested alone (**Figure 18** compared to **Figure 16** and **Table 11** compared to **Table 9**). Unlike Zinc, Copper changed the susceptibility of Colistin resistant isolates only (ACN 2209 and ACN I4785) with no effect on other isolates. Interestingly, the MIC of Colistin decreased in ACN 2209 by more than 32 folds while it increased in ACN I4785 by 4 folds (**Figure 19** compared to **Figure 16** and **Table 12** compared to **Table 9**).

2. Chromium (Cr)

Chromium in the form of Potassium Dichromate ($K_2Cr_2O_7$) was shown to indiscriminately enhance the susceptibility of bacterial isolates to all antimicrobial agents. It substantially decreased the MICs of Meropenem, Gentamicin, and Ciprofloxacin and restored the susceptibility to Cefepime in all the 7 tested isolates with an average drop in Cefepime's MIC from 201 µg/mL to 4.5 µg/mL (**Figure 20** compared to **Figure 16** and **Table 13** compared to **Table 9**).

3. Lead (Pb)

The most notable change in the MICs of antimicrobial agents was observed with Lead Acetate Trihydrate ($Pb(C_2H_3O_2)_2.3\ H_2O$). It demonstrated the greatest increase in Colistin MICs (4 to 32 folds increase) as compared to other tested heavy metals and invariably reduced Gentamicin resistance in all tested isolates (16 to 32 folds decrease in Gentamicin MICs). All isolates evolved resistance to Colistin when used in combination with Lead except ACN 2209. Combining Lead with Colistin was shown to restore partially its susceptibility to the latter which was revealed by the remarkable drop in MIC from > 2048 to 64 µg/mL. The impact of Lead on the susceptibility of isolates against other antimicrobial agents such as Cefepime, Meropenem, and Ciprofloxacin was variable and strain specific. Lead was found to restore bacterial susceptibility to antimicrobial agents in some isolates while in other isolates it either decreased the antimicrobials MICs or had no effect at all (compare **Figure 21** to **Figure 16** and **Table 14** to **Table 9**).

4. Barium (Ba)

The most significant observation that emerged from combination testing is the effect of Barium Chloride Dihydrate ($BaCl_2.2H_2O$) on antimicrobial susceptibility. Even though Barium (Ba) was shown to be very well tolerated and relatively non-toxic to all tested isolates (MIC = 640 mM), it however displayed antibacterial activity when combined with certain antimicrobial agents such as, Meropenem and Gentamicin as shown in **Table 15** and **Figure 22**. Surprisingly, Barium increased resistance to Ciprofloxacin (in 6 out of the 7 tested isolates) with an average increase in Ciprofloxacin MIC from 110 to 402

µg/mL. Like Chromium, Barium was shown to have synergistic activity when tested along with Colistin. It substantially enhanced the susceptibility of ACN 2209 to Colistin as documented by the decrease in Colistin's MIC from > 2048 to 128 µg/mL in combination testing. Similarly, ACN I4785, which is also a Colistin resistant isolate, became more susceptible to Colistin in the presence of Barium (MIC decreased from 8 to 4 µg/mL) (compare **Figure 22** to **Figure 16** and **Table 15** to **Table 9**).

5. Cadmium (Cd) and Mercury (Hg)

Unlike all tested heavy metals, Cadmium in the form of Cadmium Acetate Dihydrate ($Cd(C_2H_3O_2)_2.2H_2O$) and Mercury in the form of Mercuric Acetate ($Hg(C_2H_3O_2)_2$) were shown to be the most toxic heavy metals to *A. baumannii* isolates. They invariably restored isolates susceptibility to all antimicrobial agents when tested in combination even the susceptibility to the last resort antimicrobial agent, Colistin as was the case in ACN 2209 and ACN I4785 (**Figures 23** and **24** and **Tables 16** and **17**).

6. Arsenic (As)

The effect of Arsenic (Sodium Arsenate Dibasic Heptahydrate; $Na_2HAsO_4.7H_2O$) on the antimicrobial susceptibility was highly comparable to that of Barium and Copper. Like Barium, Arsenic improved the susceptibility to Meropenem and Gentamicin and increased Ciprofloxacin resistance in 3 out of 7 isolates. In addition, Arsenic altered the susceptibility to Colistin only in the Colistin resistant isolates, ACN 2209 and ACN I4785 similar to Copper. It decreased Colistin MIC by at least 32 folds in ACN 2209; whereas, it increased that of ACN I4785 by 2 folds (**Figure 25 and Table 18**).

D. Susceptibility Profiles of *A. baumannii* Isolates from War Wounded Patients

To investigate whether heavy metals exposure in context of war has increased the antimicrobial resistance of *A. baumannii*, 4 isolates previously collected from Iraqi patients wounded during the war were tested for their levels of resistance to antimicrobial agents and heavy metals using the same panel described above. Susceptibility profiles of tested isolates were then compared to those coming from non-war wounded patients living in conflict areas.

The antimicrobial susceptibility profiles of *A. baumannii* isolates from war wounded patients were very comparable to those from non-war wounded patients. All were extensively drug resistant showing broad resistance to all tested antimicrobial agents (Meropenem, Gentamicin, Cefepime, and Ciprofloxacin) except Colistin (**Figure 26** and **Table 19**).

In case of heavy metals, isolates were shown to exhibit similar susceptibility patterns to those from non-war wounded patients except for Dichromate, Barium, and Arsenic (**Figure 27** and **Table 20**). Of the 4 tested isolates, 2 (ACN M0180 and ACN M4210) were very susceptible to Dichromate with MICs ≤ 0.625 µg/mL while 2 were moderately resistant with MICs ranging from 1.25 to 2.5 µg/mL. On the contrary, resistance to Barium was relatively high in all tested isolates with MICs equal to 160 mM, but the overall level of resistance to Barium in these isolates was lower than in isolates from non-war wounded patients (MIC = 640 mM). As for Arsenic, it was shown to be very well tolerated by bacterial isolates. While most of the isolates were resistant to Arsenic up

to a concentration of 40 mM, one isolate which is ACN B0910 was extremely resistant with a MIC reaching 320 mM.

E. Effect of Heavy Metals on Antimicrobial Susceptibility of *A. baumannii* Isolates from War Wounded Patients

1. Zinc (Zn) and Copper (Cu)

Zinc ($ZnSO_4.7H_2O$) and Copper ($CuSO_4$) were shown to improve the susceptibility of bacterial isolates to all tested antimicrobial agents except Colistin (**Figures 28 and 29** compared to **Figure 26** and **Tables 21 and 22** compared to **Table 19**). When Colistin was tested in combination with Zinc, 2 isolates (ACN M0180 and ACN B1211) previously determined to be susceptible to Colistin evolved resistance (4-8 folds increase in Colistin MIC) while the other 2 (ACN M4210 and ACN B0910) maintained their susceptibility with MIC ≤ 2 μg/mL. Conversely, there was no detectable change in the MIC of Colistin when tested in combination with Copper.

2. Chromium (Cr)

All isolates demonstrated significant increase in their susceptibility to antimicrobial agents when tested in combination with Chromium ($K_2Cr_2O_7$) similar to what has been previously observed in isolates from non-war wounded patients. This was shown by the remarkable drop in the MICs of antimicrobial agents when tested along with

Chromium versus when tested alone (**Figure 30** compared to **Figure 26** and **Table 23** compared to **Table 19**).

3. Lead (Pb)

The antimicrobial susceptibility profiles of war wounded isolates were slightly comparable to those of non-war wounded patients in combination testing with Lead. Both isolates evolved resistance to Colistin and partially regained susceptibility to Gentamicin (> 16 folds reduction in Gentamicin MIC) in the presence Lead. On the contrary, increased resistance to Meropenem and Ciprofloxacin was invariably detected in all tested isolates from war wounded patients when tested in combination with Lead unlike isolates from non-war wounded patients which showed diverse susceptibilities in response to this metal (**Figure 31** compared to **Figure 26** and **Table 24** compared to **Table 19**).

4. Barium (Ba)

The effect of barium on Ciprofloxacin resistance in isolates from war wounded patients was consistent with our previous findings in that it increased Ciprofloxacin MIC in all tested isolates by an average of 3.5 folds (**Figure 32** compared to **Figure 26** and **Table 25** compared to **Table 19**). However, Barium was found to exert antibacterial activity when tested in combination with Gentamicin which is also in line with what we have previously reported. As for Colistin, no correlation was found between Barium exposure and Colistin resistance in bacterial isolates where all retained their susceptibility to Colistin with MICs ≤ 2 µg/mL (**Figure 32** compared to **Figure 26** and **Table 25** compared to **Table 19**).

5. Cadmium (Cd) and Mercury (Hg)

As reported earlier, Cadmium ($Cd(C_2H_3O_2)_2.2H_2O$) and Mercury ($Hg(C_2H_3O_2)_2$) were shown to reverse the susceptibility of all tested isolates to antimicrobial agents, a finding which might be attributed to the highly toxic nature of these heavy metals (**Figures 33** and **34**, **Tables 26** and **27**).

6. Arsenic (As)

Arsenic ($Na_2HAsO_4.\ 7H_2O$) was shown to improve moderately the susceptibility of all tested isolates to nearly all antimicrobial agents (Meropenem, Gentamicin, and Cefepime). Even though it displayed an antibacterial activity when combined with different antimicrobial agents, it increased however Ciprofloxacin resistance in 2 out of 4 isolates (MIC increased from 64 to 128 µg/mL) which is consistent with what we previously observed in isolates from non-war wounded patients (**Figure 35** and **Table 28**). Such finding is of special interest as it suggests a positive association between Arsenic exposure and Ciprofloxacin resistance in *A. baumannii* isolates.

F. Antimicrobials Susceptibility of Induced *A. baumannii* Mutants

To further confirm the role of heavy metals in selecting and inducing antimicrobial resistance in *A. baumannii*, we performed 2 additional tests. First, we induced resistance *in vitro* in the fully susceptible reference strain ACN DSM 30008 to selected heavy metals, which are reported in the literature to have antimicrobial co-selection potential (Copper (Cu), Lead (Pb), Cadmium (Cd), and Arsenic (As)), and antimicrobial agents (Gentamicin and Cefepime) by performing successive cultures of the bacterium in a liquid media

(CAMHB) with increasing concentrations of heavy metals and/or antimicrobial agents. Secondly, the generated bacterial mutants (listed in **Table 6**) were tested for their susceptibility profiles to a panel of antimicrobial agents like Meropenem (MER), Colistin (CST), Gentamicin (GEN), Ciprofloxacin (CIP), and Cefepime (FEP) using the Broth Microdilution Assay (BMD). **Table 29** illustrates the susceptibility profiles of the 12 generated mutants.

Induced resistant mutants were shown to exhibit different antimicrobial susceptibility profiles. Some retained their susceptibility to antimicrobial agents despite their growth under antimicrobials/heavy metals stress such as the Copper-induced mutant (Copper 13 mM) and Gentamicin and Cadmium-induced mutant (Cadmium 0.5 mM + Gentamicin 0.5 µg/mL). On the contrary, other mutants evolved resistance to specific antimicrobial agents. Inducing resistance to Copper at a concentration of 9.5 mM in combination with Gentamicin at a concentration of 8 µg/mL induced resistance to Cefepime (MIC increased from $\leq$ 2 to 64 µg/mL), which is above the susceptibility breakpoint established by Clinical Laboratory Standard Institute (CLSI) (8 µg/mL). Paradoxically, such resistance was not observed upon inducing resistance to Copper alone. Instead, it was observed in Gentamicin resistant mutant which showed borderline resistance to Cefepime with an MIC = 8 µg/mL.

Likewise, raising resistance to Cefepime in ACN DSM 30008 resulted in resistance to Gentamicin which signifies that both antimicrobials might be inducing the same resistance mechanism. Like Copper, exposure of DSM 3008 to Lead alone did not alter the susceptibility to any antimicrobial agent except when combined with Cefepime. This combination was shown to induce resistance to Gentamicin (see susceptibility profile

of Cefepime 6µg/mL +Lead 6 mM in **Table 29**) To determine if Lead is playing role in increasing the resistance to Gentamicin, we induced resistance to Lead in the previously generated Cefepime mutant (Cefepime 200 µg/mL) by serially passing it in a broth media containing increasing concentrations of Lead. The obtained mutant (Cefepime 200 µg/mL +Lead=8 mM) was found to exhibit the same susceptibility profile as Cefepime 6µg/mL +Lead 6 mM mutant which indicates that resistance to Gentamicin in both mutants is dependent on the presence of both compounds, Cefepime and Lead.

Inducing resistance to Cadmium at 1mM concentration increased moderately Cefepime's MIC from ≤ 2 to 8 µg/mL which is a very interesting finding due to the fact that Cadmium is highly toxic as previously indicated. Resistance to Colistin was surprisingly detected in Arsenic resistant mutant only (Arsenic 13 mM) as shown by the increase in Colistin's MIC from ≤ 2 to 4 µg/mL. Nevertheless, when we induced resistance to Arsenic in combination with Gentamicin, resistance to Cefepime was detected and no change in Colistin's MIC was noticed.

G. Whole Genome Sequencing (WGS) of Induced Resistant Mutants

WGS analysis on induced resistant mutants revealed the presence of various mutations including mutations in antimicrobial agents/heavy metals resistance genes, in genes with unknown functions (hypothetical genes), and in genes encoding enzymes essential for bacterial survival and metabolism. Moreover, some induced resistant mutants were shown to lack any genetic mutations and their genomes were very similar to that of the wild type strain (ACN DSM 30008). See **Table 30** for detailed information on the genetic mutations identified in the resistant mutants by WGS.

1. Cefepime Resistant Mutant: New Insights into Co-Selection of Metals and Antimicrobial Resistance

The most marked finding in the WGS data of the induced mutants is the potential association between Cefepime resistance and Cobalt, Zinc, and Cadmium (CZC) resistance. Such association was extrapolated from the WGS data obtained from the Cefepime resistant mutant (Cefepime 200 µg/mL). In fact, it was found to harbor a gene encoding a RND type efflux pump known as czcCBA which is involved in Cobalt (Co^{2+}), Zinc (Zn^{2+}), and Cadmium (Cd^{2+}) efflux even though it was not grown in the presence of any of the mentioned metal ions. The fact that this heavy metal efflux pump was induced by Cefepime and not by Cobalt (Co^{2+}), Zinc (Zn^{2+}), or Cadmium (Cd^{2+}) is a very interesting finding which suggests that czcCBA efflux system can be a putative Cefepime efflux pump. This highlights the importance of cross-resistance between heavy metals and antimicrobial agents where both might be extruded via the same efflux pump or one antimicrobial/heavy metal resistant gene can induce resistance to both simultaneously (11, 12). It is noteworthy to mention that prior to this study; there was no experimental evidence documenting this association.

2. "Lead and Cefepime" Resistant Mutants

(Cefepime 200 µg/mL +Lead=8 mM) and (Cefepime 6µg/mL +Lead 6 mM) mutants were shown to possess different genetic mutations even though they showed the same antimicrobial susceptibility profiles (both resistant to Gentamicin at MIC = 16

µg/mL) and were exposed to the same toxic compounds (Lead and Cefepime) (**Tables 29** and **30**). While the latter was shown to harbor a mutation in the gene encoding D -alanyl- D -alanine carboxypeptidase involved in cell wall synthesis and often mutated in isolates resistant to β-lactams, the former revealed no mutations in known antimicrobial resistance genes (**Table 30**). Such difference can be attributed to the different exposure patterns used to raise these mutants. Cefepime 200 µg/mL+ Lead=8 mM mutant was raised by exposing it to Cefepime first then Lead while Cefepime 6µg/mL +Lead 6 mM was raised by exposing it to Cefepime and Lead simultaneously. Based on this, we can infer that mutations induced by toxic compounds (heavy metals and antimicrobial agents) not only depend on their nature but also on the order of exposure to them: simultaneous vs sequential.

3. Arsenic: A Potential Inducer of Colistin Resistance

Arsenic resistant mutant Arsenic 13 mM which was previously reported to be resistant to Colistin with a MIC = 4 µg/mL was found to possess mutations in the gene encoding Glucosamine 6-phosphate synthetase involved in glucosamine 6-phosphate synthesis, an essential building block of the bacterial cell wall. No mutations were found in the lipid A biosynthesis genes (*lpxA*, *lpxC*, and *lpxD)* nor in genes involved in lipid A modification (*pmrA*, *pmrB*, and *pmrC*) which are the most common mutations responsible for Colistin resistance in *A. baumannii* (172). On the basis of the above findings, we hypothesize that Colistin resistance in this mutant resulted from 2 possible mechanisms. Either it was more of a phenotypic resistance resulting possibly from the increase in the net

positive charge of the lipopolysaccharides (LPS) due to the physical adsorption of the positively charged Arsenic to its phosphate groups or other new and unidentified resistance mechanisms accounted for Colistin resistance in this mutant which remain to be identified.

4. Arsenic Resistance due to Reduced Uptake: A Novel Resistance Mechanism in "Arsenic and Gentamicin" Mutant

Arsenic and Gentamicin resistant mutant (Arsenic 10 mM + Gentamicin 8 µg/mL) was found to harbor mutations in the response regulator encoding gene, *PhoB*. PhoB is a transcriptional activator for the genes involved in inorganic phosphate transport and is induced under phosphate starving conditions (173). Given that bacterial cells don't express specific transporters for Arsenate owing to its toxicity and the latter usually gains entry by utilizing non-specific transporters particularly phosphate transporters due to structural similarities between the two ions (112), it is very likely that the bacterium mutated *phoB* as a way to limit the entry of the toxic Arsenate via the phosphate transporters. This is an important finding highlighting a previously unreported mechanism of resistance to Arsenate which is reduced uptake via phosphate transporters. On the contrary, Arsenic and Gentamicin resistant mutant was not shown to possess any efflux pumps, β-lactamases, or aminoglycosides modifying enzymes that could explain their increased resistance towards Cefepime and Gentamicin.

5. No Detected Antimicrobial Resistance Genes in Copper Resistant Mutants

Although Copper is documented in the literature to have high co-selection potential, it however did not increase resistance to any antimicrobial agent as shown in Copper resistant mutant (Copper 13 mM) (**Table 29**), a finding which was also validated by WGS as no mutations in known antimicrobial resistance genes were detected (**Table 30**). Genes encoding Ferredoxin, a small protein involved in electron transport were the major genes found to be mutated. This correlates with previous findings on the effect of Copper stress/toxicity on Ferredoxin activity in yeast cells (174) .

6. Gentamicin Induces Accumulation of Several Mutations

Inducing resistance to Gentamicin resulted in the accumulation of several mutations most of which were not previously correlated with AMR such as mutations in genes encoding: (1) NADH ubiquinone oxidoreductase, complex I of the bacterial respiratory chain, (2) Glucose/Sorbosone dehydrogenase, involved in carbohydrates metabolism, (3) Catalase enzyme, anti-oxidative stress enzyme, (4) PhoB response regulator, (5) Signal transduction histidine kinase, (6) Per-Arnt-Sim(PAS)/PAC domain, a signaling domain present in many proteins, and (7) Taurine transporter (**Table 30**). In addition to these mutated genes, Gentamicin resistant mutant was shown to possess mutations in the gene encoding the 50S ribosomal protein L6 as well as expressing the adeB, a RND-type multi-drug (MDR) efflux pump. Since resistance to Gentamicin was previously reported to be associated with mutated L6 protein (175), and many *Acinetobacter* species expressing *adeB* gene were found to be resistant to Gentamicin (176), it is very likely that this mutant developed resistance to Gentamicin by selecting one

of the above mentioned genes or both of them. Moreover, adeB could also be involved in the observed resistance to Cefepime (as shown in **Table 29**) due to its broad substrate spectrum.

7. Meropenem Resistant Mutant

In addition to mutations in the *PhoB* gene, Meropenem resistant mutant (Meropenem 10 µg/mL) was shown to possess unique mutations in the gene encoding UDP-glucose 4-epimerase which were not documented in any of the sequenced mutants. UDP-glucose 4-epimerase is an enzyme involved in the synthesis of activated sugars such as UDP-galactose and UDP-glucose and required for multiple polysaccharides biosynthesis pathways. Another important gene which was found in this mutant is *adeJ*. This gene encodes a MDR efflux pump of the RND superfamily known as adeJ which is very ubiquitous in *Acinetobacter* species and often contributes to the intrinsic resistance of MDR isolates. In addition, AdeJ pump has a broad substrate spectrum as it was shown to mediate resistance to wide range of antimicrobial agents including β-lactams, Fluoroquinolones, Tetracycline, Chloramphenicol among others (177). AdeJ expression could be one of the induced resistance mechanisms to Meropenem in this mutant.

8. No Known Resistance Genes in "Copper and Gentamicin" Resistant Mutant

Although this mutant evolved resistance to Gentamicin and Cefepime with MICs equal to > 2048 and 64 µg/mL respectively, no known antimicrobial resistance genes were detected by WGS analysis. The only two known genes which were found to be mutated are

Glucose/Sorbosone dehydrogenase and *Histidine kinase*. The rest of the mutations were detected in hypothetical genes with unknown function.

9. "Mutants" with No Known Mutations

Cadmium (Cadmium 1 mM), Lead (Lead 6.5 mM), and Gentamicin and Cadmium (Cadmium 0.5 mM + Gentamicin 0.5 µg/mL) resistant mutants were shown to lack any genetic mutations when compared to the wild type strain (ACN DSM 30008) which correlates with their antimicrobial susceptibility profiles obtained by the Broth Microdilution Assay (BMD) where all the three mutants were shown to be susceptible to all tested antimicrobial agents including Meropenem, Colistin, Gentamicin, Ciprofloxacin, and Cefepime (see **Table 29**). One possible explanation for this might be attributed to the highly toxic nature of these metal ions especially Cadmium, a finding which was previously observed in the combination testing. Another possible explanation might be referred to the limited number of passages performed on these induced mutants as compared to other mutants. In fact, Cadmium is among the metals which is well documented in the literature to have co-selection potential especially for Methicillin resistance (through co-resistance mechanism) and for Carbapenems resistance (through co-regulatory mechanism).

Since none of the heavy metal resistant mutants or heavy metal and antimicrobial resistant mutants were shown to harbor any known genetic determinants encoding resistance to metal ions, it is crucial to determine whether any of the hypothetical genes they carry encode putative heavy metals resistance proteins. This can be achieved by

determining the crystal structures of these hypothetical proteins and comparing them to the structures of known proteins present in the databases to detect structural homologies. Based on this, we can further determine functional properties and their impact on the resistant phenotypes. In addition to structural homology studies, gene knockout can be performed on these hypothetical genes to study their association with antimicrobial and heavy metals resistance.

It is important to mention here that we cannot rule out the contribution of the mutated genes, which were not previously reported to be associated with antimicrobial resistance, such as Glucosamine 6-phosphate synthetase, Glucose/Sorbosone dehydrogenase, UDP-glucose 4-epimerase among others to the increased resistance phenotypes of the mutants as this requires further investigations.

H. Relative Expression Levels of *adeIJK* and *adeFGH* Genes by qRT-PCR

All the assayed isolates (isolates 1, 3 and 4) were shown to express AdeIJK and AdeFGH efflux pumps but at relatively different levels (**Figures 36-38**). The relative expression levels of AdeIJK pump were higher in isolates 3 and 4 as compared to isolate 1 with AdeK being the most expressed subunit amongst all. Similarly, the relative expression levels of AdeFGH pump were higher in isolates 3 and 4 as compared to isolate 1 which showed the least expression of this pump (**Figures 36-38**).

This validates previous studies on the wide distribution of the Ade multi-drug efflux pumps in *Acinetobacter* species and provides additional evidence on the cryptic

nature of *A. baumannii* in preferentially expressing certain subunits of the Ade efflux pumps while suppressing the expression of the rest (177, 178).

The relative expression levels of *adeI* and *adeJ* genes in isolate 1 were significantly higher in Barium and Ciprofloxacin treated Bactria (1 Ba+ Cip) as compared to Ciprofloxacin treated (1 Cip) and untreated bacteria (1 PC) (**Figure 36**) signifying that Barium exposure is upregulating the expression levels of the AdeIJ efflux pump in this isolate. On the contrary, Barium exposure didn't upregulate the expression levels of *adeK*, encoding the third subunit of the tripartite efflux pump AdeIJK since it was equally expressed in both Ciprofloxacin treated (1 Cip) and Barium and Ciprofloxacin treated (1 Ba+ Cip) bacteria (**Figure 36**). This result shows that the increased resistance towards Ciprofloxacin which was observed in the combination testing with Barium in isolate 1 might be due to the upregulation of the MDR efflux pump AdeIJ in response to Barium exposure.

On the contrary, no significant increase in the expression levels of *adeI*, *adeJ*, and *adeK* genes was observed in isolate 3 in response to Ciprofloxacin (3 Cip) or in response to Barium in combination with Ciprofloxacin (3 Ba+ Cip) as shown in **Figure 37**. Unlike isolates 1 and 3, Barium exposure in isolate 4 was shown to downregulate the expression levels of the three genes *adeI*, *adeJ*, and *adeK* (**Figure 38**) contrary to the expectations which signifies that the increased resistance to Ciprofloxacin which was previously observed in combination testing with Barium is not mediated by AdeIJK efflux pump. Barium exposure in isolate 1 was shown to nonsignificantly downregulate the expression levels of *adeF* and *adeG* genes and significantly increase the expression levels of *adeH* relative to the positive control (1 PC) (**Figure 36**). Contrary to isolate 1, isolate 3

demonstrated a non-significant increase in the expression levels of the three gene *adeF*, *adeG*, and *adeH* when treated with Barium and Ciprofloxacin (3 Ba+ Cip) relative to the positive control (3 PC) (**Figure 37**). As for isolate 4, a significant increase in the relative expression levels of *adeF* and *adeG* genes was observed when the isolate was grown in the presence of Barium and Ciprofloxacin (4 Ba+ Cip) as compared to when grown alone (4 PC) (**Figure 38**). Unlike isolates 1 and 3, Barium exposure in isolate 4 was shown to significantly downregulate the expression levels of the gene *adeH* (**Figure 38**). This suggests that the increased resistance towards Ciprofloxacin which was observed in isolate 4 upon combination testing with Barium didn't result from the activity of AdeH pump.

Table 1: The five major efflux pumps in bacteria (179-181).

Efflux Pumps Families	Function	Substrate	Energy Source
ABC	Importers and exporters	Broad substrate spectrum: lipid, sugars, peptides, ions, metal ions, antibiotics etc.	ATP hydrolysis
RND	Efflux pumps	Broad substrate spectrum: MDR, monovalent and divalent heavy metal cations	Chemiosmosis/Proton motive force
SMR	Efflux pumps	MDR	Chemiosmosis/Proton motive force
MATE	Efflux pumps	MDR	Proton motive force and/or Na^+ gradient
MFS	Efflux pumps	Broad substrate spectrum: antibiotics, ions, sugars, phosphate esters etc	Proton motive force

Table 2: Sp4F, sp4R, and sp2F primers used in gyrB multiplex PCR for *A. baumannii* identification.

Target gene	Primer	Sequence	Length	Amplicon size
gyrB	sp4F	(5′-CACGCCGTAAGAGTGCATTA)	20 bp	294 bp
	sp4R	(5′-AACGGAGCTTGTCAGGGTTA)	20 bp	
	sp2F	(5′-GTTCCTGATCCGAAATTCTCG)	21 bp	490 bp

Table 3: The components of the PCR reaction mixture used for *gyrB* amplification.

Amount/sample	Component	Final concentration
4 µL	5x FIREPoL Master Mix	1 x
0.5 µL	sp4F Forward primer	0.2 µM
0.5 µL	sp4R Reverse primer	0.2 µM
0.5 µL	Sp2F Forward primer	0.2 µM
2 µL	DNA template	
17.5 µL	Nuclease-free water	
PCR reaction total volume/sample = 25 µL		

Table 4: Heavy metals salts used for broth microdilution susceptibility assay (BMD).

Chemical name	**Molecular formula**	**Molar mass(g/mol)**	**Solubility**	**Heating step**	**Mass (g) to prepare 50 mL solution, concentration 1 M**
Zinc Sulphate Heptahydrate	$ZnSO_4.7H2O$	287.541	Soluble in H_2O: 1 g/0.6 mL	Yes	14.37
Anhydrous Cupric Sulphate	$CuSO_4$	159.602	Highly soluble in H_2O 20.3 g/100 mL at 20°C	No	7.98
Potassium Dichromate	$K_2Cr_2O_7$	294.185	Soluble in H_2O: 13 g/100 mL at 20 °C	Yes	14.71
Lead Acetate Trihydrate	$Pb(C_2H_3O_2)_2.3\ H_2O$	379.33	Good solubility in H_2O	No	18.966
Barium Chloride Dihydrate	$BaCl_2.2H_2O$	244.257	Good solubility in H_2O: 37.5 g/100mL at 26°C	No	12.212
Cadmium Acetate Dihydrate	$Cd(C_2H_3O_2)_2.2H_2O$	266.49	Very soluble in H_2O	No	13.32
Mercuric Acetate	$Hg(C_2H_3O_2)_2$	318.678	Good solubility in H_2O: 40 g/100 mL at 20°C	yes	15.93
Sodium Arsenate Dibasic Heptahydrate	$Na_2HAsO_4.7H_2O$	312.01	Solubility in H_2O 39 g/100 mL at 21°C	No	15.601

Table 5: Labels of *A. baumannii* clinical isolates used in broth microdilution (BMD).

Isolates from conflict zone of non-war wounded patients	**Isolates from conflict zone of war-wounded patients**
ACN I4789	ACN M0180
ACN I4785	ACN M4210
ACN U4388	ACN B0910
ACN 2493	ACN B1211
ACN 2209	
R4020 LA	
PFU9	

Table 6: Labels of resistant mutants and their selection conditions.

Resistant mutants ID ACN DSM 30008	**Selection conditions**	**Selection concentrations**	**Number of passages**
Gentamicin 3000 µg/mL	Gentamicin	3000 µg/mL	25
Copper 13 mM	Copper	13 mM	15
Gentamicin 8µg/mL + Copper 9.5 mM	Gentamicin and Copper	-Gentamicin: 8 µg/mL -Copper: 9.5 mM	16
Cefepime 200 µg/mL	Cefepime	200 µg/mL	23
Lead 6.5 mM	Lead	6.5 mM	10
Cefepime 6µg/mL +Lead 6 mM	Cefepime and Lead	-Cefepime: 6 µg/mL -Lead: 6 mM	13
(Cefepime 200 µg/mL) +Lead=8 mM	Cefepime followed by Lead	-Cefepime: 200 µg/mL -Lead 8 mM	5
Cadmium 1 mM	Cadmium	1 mM	4
Cadmium 0.5 mM + Gentamicin 0.5 µg/mL	Cadmium and Gentamicin	-Cadmium: 0.5 mM -Gentamicin: 0.5 µg/mL	3
Arsenic 13 mM	Arsenic	13 mM	6
Arsenic 10 mM + Gentamicin 8 µg/mL	Arsenic and Gentamicin	-Arsenic: 10 mM -Gentamicin: 8 µg/mL	6
Meropenem 10 µg/mL	Meropenem	10 µg/mL	12

Table 7: Incubation conditions of ACN I4785, ACN I4789, ACN U4388 selected for qRT-PCR analysis.

Treatment conditions	**Ciprofloxacin (32 µg/mL)**	**Ba (80 mM) and Ciprofloxacin (256 µg/mL)**
Volumes (µL)	V Cip = 28.8 V Broth = 1771.2	V Ba = 144 V Cip = 230.4 V Broth = 1425.6

Table 8: list of primers used for qRT-PCR.

genes	**Primers sequences (5'-3')**	**Primers size**	**Amplicon size (bp)**
adeI	F 5'-CAAATGCAAATGTAGATCTTGG-3'	22	211
	R 5'- AAACTGCCTTTACTTAGTTG-3'	20	
adeJ	F 5'- GGTCATTAATATCTTTGGC-3'	19	222
	R 5'- GGTACGAATACCGCTGTCA-3'	19	
adeK	F 5'- TTGATAGTTACTTGACTGTTC-3'	21	163
	R 5'- GGTTGGTGAACCACTGTATC-3'	20	
adeF	F 5'- GGTGTCGACCAAGATAAACG-3'	20	208
	R 5'- GTGAATTTGGCATAGGGACG-3'	20	
adeG	F 5'- GTGTAGTGCCACTGGTTACT-3'	20	203
	R 5'- ATGTGGGCTAGCTAACGGC-3'	19	
adeH	F 5'- CGATCAGCAAATTCAGGCTC-3'	20	181
	R 5'- GCTTGCAATGATTTGGCTGC-3'	20	

Table 9: The Minimum Inhibitory Concentrations (MICs) of 5 antimicrobial agents Meropenem, Colistin, Gentamicin, Ciprofloxacin, and Cefepime (µg/mL) against *A. baumannii* isolates recovered from non-war wounded patients as determined by the Broth Microdilution Assay (BMD).

	Antimicrobial agents MIC Values (µg/mL)				
Isolate ID	Meropenem	Colistin	Gentamicin	Ciprofloxacin	Cefepime
ACN DSM 30008	≤ 2	≤ 2	≤ 2	≤ 2	≤ 2
ACN I4789	64	≤ 2	> 2048	64	128
ACN I4785	128	8	> 2048	64	128
ACN I4388	64	≤ 2	> 2048	64	256
ACN 2493	128	≤ 2	> 2048	64	512
ACN 2209	128	> 2048	> 2048	128	256
R4020 LA	32	≤ 2	> 2048	128	64
PFU9	32	≤ 2	> 2048	256	64

Table 10: The Minimum Inhibitory Concentrations (MICs) of 8 heavy metals (Zinc, Copper, Dichromate, Lead, Barium, Cadmium, Mercury, and Arsenate) against *A. baumannii* isolates recovered from non-war wounded patients as determined by the Broth Microdilution Assay (BMD).

	Heavy Metals MIC Values (mM)							
Isolate ID	Zinc	Copper	Dichromate	Lead	Barium	Cadmium	Mercury	Arsenate
ACN DSM 30008	5	10	2.5	40	640	≤ 0.625	≤ 0.625	40
ACN I4789	5	10	2.5	40	640	≤ 0.625	≤ 0.625	40
ACN I4785	5	10	1.25	40	640	≤ 0.625	≤ 0.625	40
ACN I4388	5	10	2.5	20	640	≤ 0.625	≤ 0.625	80
ACN 2493	5	10	2.5	40	640	≤ 0.625	≤ 0.625	40
ACN 2209	10	10	5	20	640	≤ 0.625	≤ 0.625	40
ACN R4020 LA	5	10	2.5	40	640	≤ 0.625	≤ 0.625	40
ACN PFU9	5	10	2.5	40	640	≤ 0.625	≤ 0.625	40

Table 11: The Minimum Inhibitory Concentrations (MICs) of Zinc and antimicrobial agents (Meropenem, Colistin, Gentamicin, Ciprofloxacin, and Cefepime) against *A. baumannii* isolates from non-war wounded patients in combination testing.

	MICs of Zinc (in mM) and antimicrobials agents (in µg/mL) in combination testing									
Isolate ID	Zn+ Meropenem		Zn+ Colistin		Zn+ Gentamicin		Zn+ Ciprofloxacin		Zn+ Cefepime	
	Zn	Meropenem	Zn	Colistin	Zn	Gentamicin	Zn	Ciprofloxacin	Zn	Cefepime
ACN DSM 30008	≤ 0.625	≤ 2	≤ 0.625	≤ 2	≤ 0.625	≤ 2	≤0.3125	≤ 1	≤ 0.625	≤ 2
ACN I4789	5	16	5	16	5	16	5	16	5	16
ACN I4785	5	16	5	16	5	16	5	16	5	16
ACN I4388	5	16	≤ 0.625	≤ 2	5	16	5	16	5	16
ACN 2493	5	16	≤ 0.625	≤ 2	5	16	5	16	5	16
ACN 2209	5	16	5	16	5	16	10	32	5	16
R4020 LA	5	16	5	16	5	16	5	16	5	16
PFU9	5	16	5	16	5	16	5	16	5	16

Table 12: The Minimum Inhibitory Concentrations (MICs) of Copper (Cu) and antimicrobial agents (Meropenem, Colistin, Gentamicin, Ciprofloxacin, and Cefepime) against *A. baumannii* isolates from non-war wounded patients in combination testing.

	MICs of Copper (in mM) and antimicrobials agents (in µg/mL) in combination testing									
Isolate ID	Cu+ Meropenem		Cu+ Colistin		Cu+ Gentamicin		Cu+ Ciprofloxacin		Cu+ Cefepime	
	Cu	Meropenem	Cu	Colistin	Cu	Gentamicin	Cu	Ciprofloxacin	Cu	Cefepime
ACN DSM 30008	≤ 0.625	≤ 2	≤ 0.625	≤ 2	≤ 0.625	≤ 2	≤0.3125	≤ 1	≤ 0.625	≤ 2
ACN I4789	5	16	≤ 0.625	≤ 2	10	32	10	32	5	16
ACN I4785	5	16	10	32	10	32	10	32	5	16
ACN I4388	5	16	≤ 0.625	≤ 2	10	32	10	32	5	16
ACN 2493	5	16	≤ 0.625	≤ 2	10	32	10	32	5	16
ACN 2209	5	16	10	32	10	32	10	32	5	16
R4020 LA	5	16	≤ 0.625	≤ 2	10	32	10	32	5	16
PFU9	2.5	8	≤ 0.625	≤ 2	10	32	10	32	5	16

Table 13: The Minimum Inhibitory Concentrations (MICs) of Chromium (Cr) and antimicrobial agents (Meropenem, Colistin, Gentamicin, Ciprofloxacin, and Cefepime) against *A. baumannii* isolates from non-war wounded patients in combination testing.

	MICs of Chromium (in mM) and antimicrobials agents (in µg/mL) in combination testing									
Isolate ID	Cr + Meropenem		Cr + Colistin		Cr + Gentamicin		Cr + Ciprofloxacin		Cr + Cefepime	
	Cr	Meropenem	Cr	Colistin	Cr	Gentamicin	Cr	Ciprofloxacin	Cr	Cefepime
ACN DSM 30008	≤ 0.625	≤ 2	≤ 0.625	≤ 2	≤ 0.625	≤ 2	≤0.3125	≤ 1	≤ 0.625	≤ 2
ACN I4789	2.5	8	≤ 0.625	≤ 2	2.5	8	2.5	8	≤ 0.625	≤ 2
ACN I4785	2.5	8	≤ 0.625	≤ 2	2.5	8	1.25	4	≤ 0.625	≤ 2
ACN I4388	2.5	8	≤ 0.625	≤ 2	≤ 0.625	≤ 2	1.25	4	≤ 0.625	≤ 2
ACN 2493	2.5	8	≤ 0.625	≤ 2	1.25	4	1.25	4	2.5	8
ACN 2209	2.5	8	2.5	8	2.5	8	2.5	8	1.25	4
R4020 LA	1.25	4	≤ 0.625	≤ 2	5	16	5	16	2.5	8
PFU9	1.25	4	≤ 0.625	≤ 2	≤ 0.625	≤ 2	5	16	1.25	4

Table 14: The Minimum Inhibitory Concentrations (MICs) of Lead (Pb) and antimicrobial agents (Meropenem, Colistin, Gentamicin, Ciprofloxacin, and Cefepime) against *A. baumannii* isolates from non-war wounded patients in combination testing.

	MICs of Lead (in mM) and antimicrobials agents (in µg/mL) in combination testing									
Isolate ID	Pb + Meropenem		Pb + Colistin		Pb + Gentamicin		Pb + Ciprofloxacin		Pb + Cefepime	
	Pb	Meropenem	Pb	Colistin	Pb	Gentamicin	Pb	Ciprofloxacin	Pb	Cefepime
ACN DSM 30008	10	32	2.5	8	5	16	2.5	8	10	32
ACN I4789	40	128	2.5	8	40	128	20	64	40	128
ACN I4785	40	128	40	128	40	128	20	64	40	128
ACN U4388	40	64	20	64	20	64	20	64	20	64
ACN 2493	40	128	2.5	8	40	128	20	64	40	128
ACN 2209	20	64	20	64	20	64	20	64	20	64
R4020 LA	40	128	5	16	40	128	20	64	40	128
PFU9	40	128	10	32	40	128	20	64	40	128

Table 15: The Minimum Inhibitory Concentrations (MICs) of Barium (Ba) and antimicrobial agents (Meropenem, Colistin, Gentamicin, Ciprofloxacin, and Cefepime) against *A. baumannii* isolates from non-war wounded patients in combination testing.

	MICs of Ba (in mM) and antimicrobials agents (in µg/mL) in combination testing									
Isolate ID	Ba + Meropenem		Ba + Colistin		Ba + Gentamicin		Ba + Ciprofloxacin		Ba + Cefepime	
	Ba	Meropenem	Ba	Colistin	Ba	Gentamicin	Ba	Ciprofloxacin	Ba	Cefepime
ACN DSM 30008	≤ 0.625	≤ 2	≤ 0.625	≤ 2	≤ 0.625	≤ 2	≤0.3125	≤ 1	1.25	4
ACN I4789	10	32	≤ 0.625	≤ 2	160	512	160	512	40	128
ACN I4785	20	64	1.25	4	160	512	160	512	40	128
ACN U4388	20	64	≤ 0.625	≤ 2	160	512	160	512	40	128
ACN 2493	10	32	≤ 0.625	≤ 2	160	512	80	256	40	128
ACN 2209	10	32	40	128	160	512	80	256	40	128
ACN R4020 LA	5	16	≤ 0.625	≤ 2	160	512	160	512	40	128
ACN PFU9	5	16	≤ 0.625	≤ 2	160	512	80	256	40	128

Table 16: The Minimum Inhibitory Concentrations (MICs) of Cadmium (Cd) and antimicrobial agents (Meropenem, Colistin, Gentamicin, Ciprofloxacin, and Cefepime) against *A. baumannii* isolates from non-war wounded patients in combination testing.

	MICs of Cd (in mM) and antimicrobials agents (in µg/mL) in combination testing									
Isolate ID	Cd + Meropenem		Cd + Colistin		Cd + Gentamicin		Cd + Ciprofloxacin		Cd + Cefepime	
	Cd	Meropenem	Cd	Colistin	Cd	Gentamicin	Cd	Ciprofloxacin	Cd	Cefepime
ACN DSM 30008	≤ 0.625	≤ 2	≤ 0.625	≤ 2	≤ 0.625	≤ 2	≤0.3125	≤ 1	≤ 0.625	≤ 2
ACN I4789	≤ 0.625	≤ 2	≤ 0.625	≤ 2	≤ 0.625	≤ 2	0.625	2	≤ 0.625	≤ 2
ACN I4785	≤ 0.625	≤ 2	≤ 0.625	≤ 2	≤ 0.625	≤ 2	0.625	2	≤ 0.625	≤ 2
ACN U4388	≤ 0.625	≤ 2	≤ 0.625	≤ 2	≤ 0.625	≤ 2	≤0.3125	≤ 1	≤ 0.625	≤ 2
ACN 2493	≤ 0.625	≤ 2	≤ 0.625	≤ 2	≤ 0.625	≤ 2	≤0.3125	≤ 1	≤ 0.625	≤ 2
ACN 2209	≤ 0.625	≤ 2	≤ 0.625	≤ 2	≤ 0.625	≤ 2	≤0.3125	≤ 1	≤ 0.625	≤ 2
ACN R4020 LA	≤ 0.625	≤ 2	≤ 0.625	≤ 2	≤ 0.625	≤ 2	0.625	2	≤ 0.625	≤ 2
ACN PFU9	≤ 0.625	≤ 2	≤ 0.625	≤ 2	≤ 0.625	≤ 2	0.625	2	≤ 0.625	≤ 2

Table 17: The Minimum Inhibitory Concentrations (MICs) of Mercury (Hg) and antimicrobial agents (Meropenem, Colistin, Gentamicin, Ciprofloxacin, and Cefepime) against *A. baumannii* isolates from non-war wounded patients in combination testing.

	MICs of Hg (in mM) and antimicrobials agents (in µg/mL) in combination testing									
Isolate ID	Hg + Meropenem		Hg + Colistin		Hg + Gentamicin		Hg + Ciprofloxacin		Hg + Cefepime	
	Hg	Meropenem	Hg	Colistin	Hg	Gentamicin	Hg	Ciprofloxacin	Hg	Cefepime
ACN DSM 30008	≤ 0.625	≤ 2	≤ 0.625	≤ 2	≤ 0.625	≤ 2	≤0.3125	≤ 1	≤ 0.625	≤ 2
ACN I4789	≤ 0.625	≤ 2	≤ 0.625	≤ 2	≤ 0.625	≤ 2	≤0.3125	≤ 1	≤ 0.625	≤ 2
ACN I4785	≤ 0.625	≤ 2	≤ 0.625	≤ 2	≤ 0.625	≤ 2	≤0.3125	≤ 1	≤ 0.625	≤ 2
ACN U4388	≤ 0.625	≤ 2	≤ 0.625	≤ 2	≤ 0.625	≤ 2	≤0.3125	≤ 1	≤ 0.625	≤ 2
ACN 2493	≤ 0.625	≤ 2	≤ 0.625	≤ 2	≤ 0.625	≤ 2	≤0.3125	≤ 1	≤ 0.625	≤ 2
ACN 2209	≤ 0.625	≤ 2	≤ 0.625	≤ 2	≤ 0.625	≤ 2	≤0.3125	≤ 1	≤ 0.625	≤ 2
ACN R4020 LA	≤ 0.625	≤ 2	≤ 0.625	≤ 2	≤ 0.625	≤ 2	≤0.3125	≤ 1	≤ 0.625	≤ 2
ACN PFU9	≤ 0.625	≤ 2	≤ 0.625	≤ 2	≤ 0.625	≤ 2	≤0.3125	≤ 1	≤ 0.625	≤ 2

Table 18: The Minimum Inhibitory Concentrations (MICs) of Arsenic (As) and antimicrobial agents (Meropenem, Colistin, Gentamicin, Ciprofloxacin, and Cefepime) against *A. baumannii* isolates from non-war wounded patients in combination testing.

	MICs of As (in mM) and antimicrobials agents (in µg/mL) in combination testing									
Isolate ID	As + Meropenem		As + Colistin		As + Gentamicin		As + Ciprofloxacin		As + Cefepime	
	As	Meropenem	As	Colistin	As	Gentamicin	As	Ciprofloxacin	As	Cefepime
ACN DSM 30008	≤0.625	≤ 2	≤0.625	≤ 2	≤0.625	≤ 2	≤0.3125	≤1	125	4
ACN I4789	10	32	≤0.625	≤ 2	40	128	40	128	20	64
ACN I4785	20	64	5	16	80	256	40	128	20	64
ACN U4388	20	64	≤0.625	≤ 2	80	256	40	128	20	64
ACN 2493	10	32	≤0.625	≤ 2	40	128	20	64	20	64
ACN 2209	10	32	20	64	40	128	40	128	10	32
ACN R4020 LA	5	16	≤0.625	≤ 2	80	256	20	64	20	64
ACN PFU9	5	16	≤0.625	≤ 2	80	256	40	128	20	64

Table 19: The Minimum Inhibitory Concentrations (MICs) of antimicrobial agents (Meropenem, Colistin, Gentamicin, Ciprofloxacin, and Cefepime) in µg/mL against *A. baumannii* isolates recovered from war wounded patients as determined by the Broth Microdilution Assay (BMD).

	Antimicrobial agents MIC Values (µg/mL)				
Isolate ID	Meropenem	Colistin	Gentamicin	Ciprofloxacin	Cefepime
ACN M0180	32	≤ 2	> 2048	64	128
ACN M4210	64	≤ 2	> 2048	64	128
ACN B0910	64	≤ 2	> 2048	32	512
ACN B1211	128	≤ 2	> 2048	64	128

Table 20: The Minimum Inhibitory Concentrations (MICs) of 8 heavy metals (Zinc, Copper, Dichromate, Lead, Barium, Cadmium, Mercury, and Arsenate) against *A. baumannii* isolates recovered from war wounded patients as determined by the Broth Microdilution Assay (BMD).

	Heavy Metals MIC Values (mM)							
Isolate ID	Zinc	Copper	Dichromate	Lead	Barium	Cadmium	Mercury	Arsenate
ACN M0180	10	10	≤ 0.625	40	160	≤ 0.625	≤ 0.625	40
ACN M4210	5	10	≤ 0.625	40	160	≤ 0.625	≤ 0.625	20
ACN B0910	2.5	10	2.5	40	160	≤ 0.625	≤ 0.625	320
ACN B1211	5	10	1.25	40	160	≤ 0.625	≤ 0.625	20

Table 21: The Minimum Inhibitory Concentrations (MICs) of Zinc and antimicrobial agents (Meropenem, Colistin, Gentamicin, Ciprofloxacin, and Cefepime) against *A. baumannii* isolates from war wounded patients when tested in combination.

	MICs of Zinc (in mM) and antimicrobials agents (in µg/mL) in combination testing									
Isolate ID	Zn + Meropenem		Zn + Colistin		Zn + Gentamicin		Zn + Ciprofloxacin		Zn + Cefepime	
	Zn	Meropenem	Zn	Colistin	Zn	Gentamicin	Zn	Ciprofloxacin	Zn	Cefepime
ACN M0180	5	16	5	16	10	32	10	32	10	32
ACN M4210	5	16	≤ 0.625	≤ 2	5	16	5	16	5	16
ACN B0910	2.5	8	≤ 0.625	≤ 2	2.5	8	2.5	8	2.5	8
ACN B1211	5	16	2.5	8	5	16	5	16	5	16

Table 22: The Minimum Inhibitory Concentrations (MICs) of Copper (Cu) and antimicrobial agents (Meropenem, Colistin, Gentamicin, Ciprofloxacin, and Cefepime) against *A. baumannii* isolates from war wounded patients when tested in combination.

	MICs of Copper (in mM) and antimicrobials agents (in µg/mL) in combination testing									
Isolate ID	Cu + Meropenem		Cu + Colistin		Cu +Gentamicin		Cu + Ciprofloxacin		Cu + Cefepime	
	Cu	Meropenem	Cu	Colistin	Cu	Gentamicin	Cu	Ciprofloxacin	Cu	Cefepime
ACN M0180	5	16	≤ 0.625	≤ 2	10	32	10	32	5	16
ACN M4210	5	16	≤ 0.625	≤ 2	10	32	10	32	5	16
ACN B0910	5	16	≤ 0.625	≤ 2	10	32	10	32	10	32
ACN B1211	5	16	≤ 0.625	≤ 2	10	32	10	32	5	16

Table 23: The Minimum Inhibitory Concentrations (MICs) of Chromium (Cr) and antimicrobial agents (Meropenem, Colistin, Gentamicin, Ciprofloxacin, and Cefepime) against *A. baumannii* isolates from war wounded patients when tested in combination.

	MICs of Chromium (in mM) and antimicrobials agents (in µg/mL) in combination testing									
Isolate ID	Cr + Meropenem		Cr + Colistin		Cr + Gentamicin		Cr + Ciprofloxacin		Cr + Cefepime	
	Cr	Meropenem	Cr	Colistin	Cr	Gentamicin	Cr	Ciprofloxacin	Cr	Cefepime
ACN M0180	≤0.625	≤2	≤0.625	≤2	≤0.625	≤2	0.625	2	≤0.625	≤2
ACN M4210	≤0.625	≤2	≤0.625	≤2	≤0.625	≤2	≤ 0.3125	≤ 1	≤0.625	≤2
ACN B0910	≤0.625	≤2	≤0.625	≤2	≤0.625	≤2	≤ 0.3125	≤ 1	2.5	8
ACN B1211	≤0.625	≤2	≤0.625	≤2	1.25	4	0.625	2	≤0.625	≤2

Table 24: The Minimum Inhibitory Concentrations (MICs) of Lead (Pb) and antimicrobial agents (Meropenem, Colistin, Gentamicin, Ciprofloxacin, and Cefepime) against *A. baumannii* isolates from war wounded patients when tested in combination.

	MICs of Lead (in mM) and antimicrobials agents (in µg/mL) in combination testing									
Isolate ID	Pb + Meropenem		Pb + Colistin		Pb + Gentamicin		Pb + Ciprofloxacin		Pb + Cefepime	
	Pb	Meropenem	Pb	Colistin	Pb	Gentamicin	Pb	Ciprofloxacin	Pb	Cefepime
ACN M0180	40	128	20	64	40	128	40	128	40	128
ACN M4210	40	128	2.5	8	40	128	40	128	40	128
ACN B0910	40	128	2.5	8	40	128	40	128	40	128
ACN B1211	40	128	5	16	40	128	40	128	40	128

Table 25: The Minimum Inhibitory Concentrations (MICs) of Barium (Ba) and antimicrobial agents (Meropenem, Colistin, Gentamicin, Ciprofloxacin, and Cefepime) against *A. baumannii* isolates from war wounded patients when tested in combination.

	MICs of Ba (in mM) and antimicrobials agents (in µg/mL) in combination testing									
Isolate ID	Ba + Meropenem		Ba + Colistin		Ba + Gentamicin		Ba + Ciprofloxacin		Ba + Cefepime	
	Ba	Meropenem	Ba	Colistin	Ba	Gentamicin	Ba	Ciprofloxacin	Ba	Cefepime
ACN M0180	20	64	≤ 0.625	≤ 2	160	512	40	128	40	128
ACN M4210	20	64	≤ 0.625	≤ 2	160	512	160	512	40	128
ACN B0910	20	64	≤ 0.625	≤ 2	80	256	20	64	40	128
ACN B1211	80	256	≤ 0.625	≤ 2	80	256	40	128	80	256

Table 26: The Minimum Inhibitory Concentrations (MICs) of Cadmium (Cd) and antimicrobial agents (Meropenem, Colistin, Gentamicin, Ciprofloxacin, and Cefepime) against *A. baumannii* isolates from war wounded patients when tested in combination.

	MICs of Cd (in mM) and antimicrobials agents (in µg/mL) in combination testing									
Isolate ID	Cd + Meropenem		Cd + Colistin		Cd + Gentamicin		Cd + Ciprofloxacin		Cd + Cefepime	
	Cd	Meropenem	Cd	Colistin	Cd	Gentamicin	Cd	Ciprofloxacin	Cd	Cefepime
ACN M0180	≤ 0.625	≤ 2	≤ 0.625	≤ 2	≤ 0.625	≤ 2	≤ 0.3125	≤ 1	≤ 0.625	≤ 2
ACN M4210	≤ 0.625	≤ 2	≤ 0.625	≤ 2	≤ 0.625	≤ 2	≤ 0.3125	≤ 1	≤ 0.625	≤ 2
ACN B0910	≤ 0.625	≤ 2	≤ 0.625	≤ 2	≤ 0.625	≤ 2	≤ 0.3125	≤ 1	≤ 0.625	≤ 2
ACN B1211	≤ 0.625	≤ 2	≤ 0.625	≤ 2	≤ 0.625	≤ 2	≤ 0.3125	≤ 1	≤ 0.625	≤ 2

Table 27: The Minimum Inhibitory Concentrations (MICs) of Mercury (Hg) and antimicrobial agents (Meropenem, Colistin, Gentamicin, Ciprofloxacin, and Cefepime) against *A. baumannii* isolates from war wounded patients when tested in combination.

	MICs of Hg (in mM) and antimicrobials agents (in µg/mL) in combination testing									
Isolate ID	Hg + Meropenem		Hg + Colistin		Hg + Gentamicin		Hg + Ciprofloxacin		Hg + Cefepime	
	Hg	Meropenem	Hg	Colistin	Hg	Gentamicin	Hg	Ciprofloxacin	Hg	Cefepime
ACN M0180	≤ 0.625	≤ 2	≤ 0.625	≤ 2	≤ 0.625	≤ 2	≤ 0.3125	≤ 1	≤ 0.625	≤ 2
ACN M4210	≤ 0.625	≤ 2	≤ 0.625	≤ 2	≤ 0.625	≤ 2	≤ 0.3125	≤ 1	≤ 0.625	≤ 2
ACN B0910	≤ 0.625	≤ 2	≤ 0.625	≤ 2	≤ 0.625	≤ 2	≤ 0.3125	≤ 1	≤ 0.625	≤ 2
ACN B1211	≤ 0.625	≤ 2	≤ 0.625	≤ 2	≤ 0.625	≤ 2	≤ 0.3125	≤ 1	≤ 0.625	≤ 2

Table 28: The Minimum Inhibitory Concentrations (MICs) of Arsenic (As) and antimicrobial agents (Meropenem, Colistin, Gentamicin, Ciprofloxacin, and Cefepime) against *A. baumannii* isolates from war wounded patients when tested in combination.

	MICs of As (in mM) and antimicrobials agents (in µg/mL) in combination testing									
Isolate ID	As + Meropenem		As + Colistin		As + Gentamicin		As + Ciprofloxacin		As + Cefepime	
	As	Meropenem	As	Colistin	As	Gentamicin	As	Ciprofloxacin	As	Cefepime
ACN M0180	5	16	≤ 0.625	≤ 2	40	128	20	64	10	32
ACN M4210	10	32	≤ 0.625	≤ 2	40	128	40	128	20	64
ACN B0910	20	64	≤ 0.625	≤ 2	160	512	10	32	80	256
ACN B1211	10	32	≤ 0.625	≤ 2	20	64	40	128	10	32

Table 29: Antimicrobials susceptibility profiles of the 12 generated resistant mutants

Resistant mutants ID **ACN DSM 30008**	**Antimicrobial agents MIC Values (in µg/mL)**				
	Meropenem	Colistin	Gentamicin	Ciprofloxacin	Cefepime
Gentamicin 3000 µg/mL	≤ 2	≤ 2	>2048	≤ 1	8
Copper 13 mM	≤ 2	≤ 2	≤ 2	≤ 1	≤ 2
Gentamicin 8µg/mL + Copper 9.5 mM	≤ 2	≤ 2	2048	≤ 1	64
Cefepime 200 µg/mL	≤ 2	≤ 2	32	≤ 1	512
Lead 6.5 mM	≤ 2	≤ 2	≤ 2	≤ 1	4
Cefepime 6µg/mL +Lead 6 mM	≤ 2	≤ 2	16	≤ 2	16
(Cefepime 200 µg/mL) +Lead=8 mM			16		
Cadmium 1 mM	1	1	1	≤ 0.25	8
Cadmium 0.5 mM + Gentamicin 0.5 µg/mL	0.5	0.5	1	≤ 0.25	2
Arsenic 13 mM	≤ 2	4	≤ 2	≤ 0.25	≤ 2
Arsenic 10 mM + Gentamicin 8 µg/mL	≤ 2	≤ 2	32	0.5	16
Meropenem 10 µg/mL	32	≤ 2	≤ 2	≤ 1	1024

Table 30: Summary of all the genetic mutations identified in the induced resistant mutants compared to the wild type strain (ACN DSM 30008)

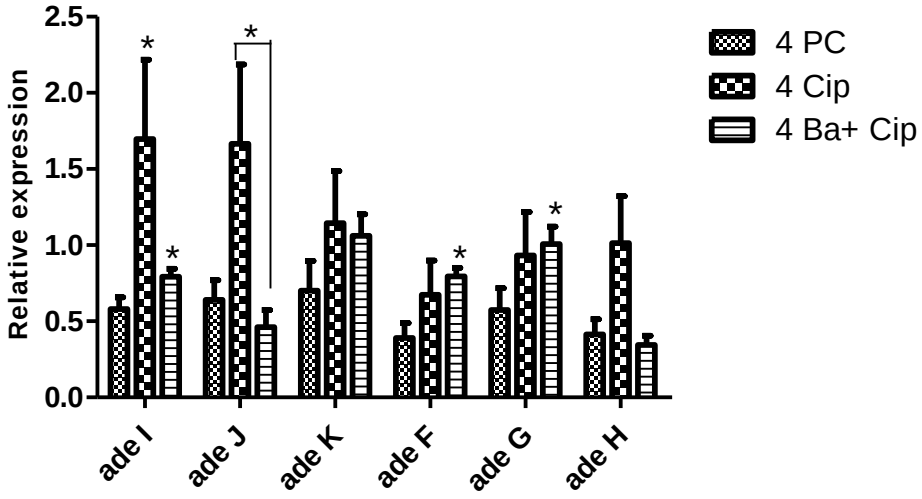
Mutants ID ACN DSM 30008	**Selection Conditions**	**# of passages**	**Mutations detected**	**Encoded protein function**
Cefepime 200 µg/mL	Cefepime 200 µg/mL	23	1-Co/Zn/Cd efflux system component 2-Glucose/sorbosone dehydrogenase 3- Hypothetical protein 4-Hypothetical protein 5-Hypothetical protein	1-RND type chemiosmotic antiporter involved in Cobalt, Zinc, and Cadmium efflux 2-Oxidoreducatase enzyme involved in carbohydrates metabolism. Its main function is to oxidize glucose into gluconolactone. 3-Unknown function 4-Unknown function 5-Unknown function
(Cefepime 200 µg/mL) +Lead=8 mM	Cefepime then Pb	5	1-Glucose/sorbosone dehydrogenase 2-Hypothetical protein 3-Hypothetical protein 4-Signal transduction histidine kinase	1-Oxidoreductase enzyme involved in carbohydrates metabolism. Its main function is to oxidize glucose into gluconolactone. 2-Unknown function 3-Unkonwn function 4-Important enzyme involved in signal transduction which is present upstream of several pathways. It senses environmental stimuli and transmits it to response regulators.

Cefepime 6µg/mL +Lead 6 mM	Cefepime & Pb	13	1-D-alanyl-D-alanine carboxypeptidase 2-Hhypothetical protein 3-Hypothetical protein 4-Hypothetical protein 5-Signal transduction histidine kinase	1-Important enzyme involved cell wall biosynthesis. It catalyzes the last step in cell wall synthesis namely transpeptidation which cross links the peptide chains of the peptidoglycan layer together. 2-Unknown function 3-Unknown function 4-Unknown function 5-See above
Lead 6.5 mM	Pb 6.5 mM	10	No known mutations	
Cadmium 1 mM	Cd 1 mM	4	No known mutations	
Arsenic 13 mM	As 13 mM	6	Glucosamine 6-phosphate synthetase	Important enzyme involved in cell wall synthesis. It synthesizes Glucosamine 6-P which is an essential structural component of cell wall
Copper 13 mM	Cu 13 mM	15	Ferredoxin	Small proteins involved in electron transport Reported in the literature as targets for Copper toxicity.
Arsenic 10 mM + Gentamicin 8 µg/mL	As 10 mM & Gentamicin 8 µg/mL	6	PhoB response regulator consisting of CheY-like receiver domain and a winged-helix DNA binding domain	Phosphate regulon transcriptional regulatory protein. It functions as a transcriptional activator for the genes involved in phosphate transport and utilization.

Cadmium 0.5 mM + Gentamicin 0.5 µg/mL	Cd 0.5 mM & Gentamicin 0.5 mM	3	No known mutations	
Gentamicin 8µg/mL + Copper 9.5 mM	Cu & Gentamicin	16	1-Hypothetical protein 2-Glucose/sorbosone dehydrogenase 3-Hypothetical protein 4-Hypothetical protein 5-Hypothetical protein 6-Signal transduction histidine kinase	1-Unknown function 2-See above 3-Unkown function 4-Unkown function 5-Unkown function 6-See above
Gentamicin 3000 µg/mL	Gentamicin	25	1-NADH: ubiquinone oxidoreductase 24 kD subunit 2-Catalase 3-AdeB RND exporter 4-PAS/PAC domain protein 5-Glucose/sorbosone dehydrogenase 6-PhoB response regulator consisting of CheY- like receiver domain and a winged-helix DNA binding domain 7-ABC-type taurine transport system 8-Signal transduction histidine kinase 9-50S ribosomal protein L6	1-The first complex of the respiratory chain in bacteria. Involved in electron transfer. 2-Anti-oxidative stress enzyme. Involved in H_2O_2 degradation 3-A RND exporter. It often forms tripartite efflux pump, adeABC with adeA and adeC proteins. A major mechanism of resistance to wide range of antimicrobial agents in *A. baumannii* isolates. 4-Singaling domain present in many proteins 5-See above 6-See above 7-Taurine transporter. Taurine is a β amino acid, an essential structural component of the capsular

				polysaccharides and cell wall in bacteria 8-See above 9-A component of the large ribosomal subunit in bacteria. It is a key component for its assemblage.
Meropenem 10 µg/mL	Meropenem	12	1-PhoB response regulator consisting of CheY-like receiver domain and a winged-helix DNA binding domain 2-AdeJ RND exporter 3-UDP-glucose 4-epimerase	1-See above 2-a MDR exporter of the superfamily RND. Widely distributed in *A. baumannii* isolates. Confers resistance to many classes of antimicrobial agents. Often forms tripartite efflux pump adeIJK with adeJ and adeK. 3-An enzyme involved in the synthesis of activated sugars such as UDP-galactose and UDP-glucose which are required for multiple polysaccharides biosynthesis pathways.

Figure 38: Relative expression levels of *adeI*, *adeJ*, *adeK*, *adeF*, *adeG*, and *adeH* in isolate 4 (ACN U4388) under Ciprofloxacin treatment and Barium and Ciprofloxacin treatment. 4 PC: Positive control, untreated bacteria.



CHAPTER V

DISCUSSION

With the alarming increase in the number of infections caused by multi-drug resistant bacteria worldwide and the slow pace of discovery of new and effective classes of antimicrobial agents (2) , there is an ineluctable need to deeply understand all the mechanisms and drivers of antimicrobial resistance (AMR). For a long period of time, the overuse and misuse of antimicrobial agents have been considered the major drivers of antimicrobial resistance in bacterial population. While the excessive use of antimicrobial agents clearly drives the evolution of antimicrobial resistance by selecting the growth of drug resistant bacteria, other compounds such as antibacterial biocides and heavy metals may also induce and promote AMR via co-selection mechanisms (11, 12). Heavy metals can induce AMR by three main mechanisms: (1) when heavy metals resistance genes are physically linked to antimicrobial resistance genes on mobile genetic elements, (2) when resistance to both compounds is conferred by a single resistance mechanism, (3) and when both compounds share the same regulatory mechanisms (11, 12). Although the association between heavy metals and antimicrobial resistance has been studied decades ago, it was only explored in few sectors namely in agriculture, aquaculture, and industry (19, 65, 159). Since high rates of multi-drug resistant (MDR) bacteria particularly *A. baumannii* have been noted in wars and conflict areas (28-30, 32) and many reports are currently coming out from war regions in the middle east particularly from Yemen, Libya, and Syria showing increasing trends of resistance in *A. baumannii* isolates from war injuries (182-185) , it is

crucial to explore whether exposure of this bacterium to heavy metals in this setting might have played a role in promoting its resistance to antimicrobial agents.

To explore the potential association between heavy metals and antimicrobial resistance in *A. baumannii*, 7 *A. baumannii* isolates collected from non-war wounded patients living in conflict areas and 4 recovered from war-wounded Iraqi patients were screened for their antimicrobial and heavy metals susceptibility against a panel of 5 antimicrobial agents and 8 heavy metals commonly used in weapons.

All *A. baumannii* isolates displayed similar resistance patterns. 9 isolates were extensively drug resistant (XDR) showing resistance to all tested antimicrobial agents except Colistin and 2 were pan drug resistant (PDR) exhibiting resistance to all antimicrobial agents including Colistin (**Tables 9** and **19**). The obtained resistance profiles match the regional resistance trends of *A. baumannii* isolates (8, 186). Moreover, resistance to heavy metals was very prevalent in all tested isolates. All exhibited high levels of resistance to Barium, Lead, and Arsenate, moderate resistance to Copper, Zinc, and Dichromate, and very low resistance to Cadmium and Mercury (**Tables 10** and **20**). Given that all isolates were able to tolerate high concentrations of heavy metals in the range of "mM" despite their toxicity, it is very likely that these isolates have been exposed to high concentrations of heavy metals in their surroundings and thus, have become more tolerant. The high level of Arsenate resistance displayed by all isolates might be attributed to the ubiquitous distribution of *ars* operon among bacteria (187). While most of the isolates were resistant to Arsenate up to 40 mM concentration, only one isolate which is ACN B0910 recovered from war wounded Iraqi patient was extremely resistant with a MIC = 320 mM

(**Tables 10** and **20**). This can most probably be related to the usage of Arsenate as a chemical warfare in conflict areas including Iraq (188).

To investigate whether exposure to heavy metals in context of wars increased the antimicrobial resistance of *A. baumannii*, we performed 2 sets of experiments: antimicrobial and heavy metals combination testing using the Broth Microdilution (BMD) Assay and induction of resistance by serial passage.

Based on the broth microdilution combination testing results, it appears that certain metals can co-select for resistance against one or more antimicrobial agent. For example, Lead was shown to be strongly associated with Colistin resistance since it rendered all isolates resistant to Colistin when tested in combination and demonstrated the greatest increase in Colistin MIC compared to other metals (**Tables 14** and **24**, **Figures 21** and **31**). In addition, our data suggests that Lead might be also linked to Meropenem resistance since it invariably increased Meropenem MIC in all *A. baumannii* isolates from war wounded patients (**Table 24** and **Figure 31**). As far as we know, this is the first time that Lead shows co-selection potential for Colistin and Meropenem resistance in bacteria although it was previously reported to be associated with Tetracycline resistance in bacteria isolated from soil (189). Another metal which showed positive association with AMR is Barium. Surprisingly, Barium was found to increase resistance to Ciprofloxacin in nearly all tested isolates (**Tables 15** and **25**, **Figures 22** and **32**). Given that no published data is available on Barium and its role in bacteria, it is essential to conduct research studies to discover its exact metabolic role in bacterial species, its resistance mechanisms and the molecular mechanisms by which it induces resistance to antimicrobial agents, particularly Ciprofloxacin. Increased resistance to Ciprofloxacin was also detected when tested in

combination with Arsenate (**Tables 18** and **28**, **Figures 25** and **35**). Since Arsenic resistance has been previously associated with resistance to several classes of antimicrobial agents including Chloramphenicol, Tetracycline, Streptomycin, Kanamycin, and β-lactams due to genetic linkage of resistance genes on plasmids (162, 190), it is very likely that isolates which showed increased resistance to Ciprofloxacin in the presence of Arsenate might have plasmids carrying resistance genes to both compounds (Arsenic and Ciprofloxacin). Other metals such as Cadmium, Mercury, Copper, Zinc, and Dichromate appear to be toxic to bacterial isolates at low concentrations since combining antimicrobial agents with any of these metals was shown to enhance remarkably their antimicrobial susceptibility (**Tables 11-13**, **16-17**, **21-23**, **26-27**). There are two possible scenarios which might explain this result. One scenario is that these metals owing to their inherent toxicity might have induced cellular damage in bacterial isolates and thereby rendered them more susceptible to antimicrobial agents. Another valid scenario is the possibility of occurrence of reversed mutations. Since these heavy metals are known to cause the production of ROS which induce damages to DNA, it could be that these damages/mutations occurred in antimicrobial resistance genes and led to genetic reversion in susceptibility.

Through induction of resistance and Whole Genome Sequencing (WGS) of induced mutants, we demonstrated that metals ions such as Cu^{2+}, Pb^{2+}, Co^{2+}, Zn^{2+}, Cd^{2+}, and As^{5+} can induce antimicrobial resistance at minimal concentrations. Copper, Cobalt, Zinc, and Cadmium were shown to have co-selection potential for Cefepime resistance (**Tables 29** and **30**). Lead was shown to have co-selection potential for Gentamicin resistance while Arsenate was shown to possess co-selection potential for Colistin resistance (**Table 29**). Since none of the heavy metals mutants and “antimicrobial and

heavy metals" mutants were shown by WGS to harbor any heavy metals resistance genes despite their selective growth under heavy metals stress and most of the mutants lacked known antimicrobial resistance genes (**Table 30**) even though they evolved phenotypic resistance as indicated in **Table 29**, we cannot confirm the potential association between the heavy metals and the antimicrobial agents mentioned above at the genetic level. One important thing that must be done to understand the genetic basis of this association is to figure out the function of the hypothetical proteins identified by WGS analysis (**Table 30**) as they might be potential heavy metals or antimicrobial agents resistance proteins. As for the association between Cefepime resistance and Cobalt, Zinc, and Cadmium resistance revealed by WGS of Cefepime resistant mutant (**Table 30**), a knock out mutant lacking *czc* gene must be generated to check if Cefepime is a substrate for this heavy metal efflux pump.

Our qRT-PCR results have strengthened our confidence that Barium is associated with Ciprofloxacin resistance in *A. baumannii* isolates and it mediates its effect by upregulating the expression of the Ade MDR efflux pumps (**Figures 36-38**) a finding which was not previously described in any study. While the exact mechanisms by which it is increasing resistance to Ciprofloxacin is still unclear owing to the very limited data available on Barium role in prokaryotes, it is inevitable that such finding has widened our knowledge on the impact of the Barium metal on AMR and may pave the way for the discovery of potential therapeutic targets to combat Ciprofloxacin resistance in *A. baumannii* isolates.

Future work will focus on performing WGS on all the screened isolates used in this project to identify all the genetic determinants encoding resistance to heavy metals and antimicrobial agents and to figure out potential association. In addition, we believe that it is important to compare the genomes of both clinical isolates and induced resistant mutants as this will not only help identify common resistance mechanisms but might also lead to the discovery of novel mechanisms of resistance in *A. baumannii*. Also, we hope to validate our results by screening a larger sample size in the future. This can be achieved by collecting a larger number of *A. baumannii* isolates especially those coming from war injuries. Moreover, we are planning in the near future to assess the phenotypic stability of the induced resistant mutants to check if the mutations induced by heavy metals are stable and not transient. Also, we are planning to measure the fitness cost of these mutations by comparing the growth rates of the induced mutants to that of the wild type strain as an indication of their persistence and risk of dissemination to other bacteria.

This study has helped us understand better the mechanisms of emergence of antimicrobial resistance in bacteria and it has reaffirmed the hypothesis that heavy metal ions are potential and potent drivers of antimicrobial resistance just like antimicrobial agents. Most importantly, our study highlights that there is very high risk of co-selection of heavy metal and antimicrobial resistance to occur in war regions given the high concentrations of heavy metals in these regions which exceed significantly the concentrations we used to raise resistance *in vitro*. In other words, if using 6 mM of Lead in combination with Cefepime and 10 mM of Arsenic in combination with Gentamicin was sufficient enough to increase resistance to both antimicrobial agents by a minimum of 8 folds despite the few numbers of passages performed (MIC increased from ≤ 2 to 16), then

by considering the high concentrations present in war regions and the long term exposure of bacteria to such concentrations, it is very likely that co-selection of AMR by heavy metals have taken place, a hypothesis that generates alarm calls if turns out to be true.

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