AMERICAN UNIVERSITY OF BEIRUT

UTILIZING NGS FOR AMR SURVEILLANCE IN NORTH LEBANON - A ONE HEALTH APPROACH

by
ZEINAB RIDA HODROJ

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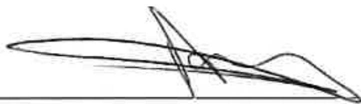

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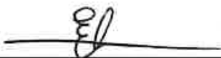

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ABSTRACT
OF THE THESIS OF

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Title: Utilizing NGS for AMR Surveillance in North Lebanon- A One Health Approach

Background: The global rates of antimicrobial resistance (AMR) are rising at an alarming rate with dreadful circumstances of more than 10 million deaths per year by 2050. In the Eastern Mediterranean Region (EMR), reported rates of resistance have reached dangerous levels both in healthcare settings and the community, threatening the hard-won gains in health and development, and the sustainability of public health response to many communicable diseases. AMR surveillance systems are the core component of infectious disease management and the foundation for a better understanding of the spread of antimicrobial resistance. In Lebanon, data on AMR surveillance is fragmented and lack representativeness. To this, we propose using Next-Generation Sequencing (NGS) in a One Health Approach to determine the spread of AMR and identify the threats in North Lebanon.

Methods: A total of 83 samples was received from wild animals, sewage, water, and soil samples. Samples were streaked on MacConkey agar plates supplemented with meropenem. Identification of bacterial spp. was primarily done by API20E for all isolated bacteria. Antimicrobial Susceptibility profile was determined by disk diffusion against 5 different antimicrobial agents. Carbapenem resistant Gram- negative bacteria were further characterized by Whole genome sequencing. Bacterial type, international clone, sequence type, resistance genes, and plasmids were detected using sequence data.

Results: We successfully isolated 76 bacterial isolates from different samples. The most common were *E. coli* (14 %), Pseudomonas spp. (33 %), and *Acinetobacter baumanii* (8.7 %). Disk diffusion results showed high resistance to meropenem among identified bacterial organisms which is about 75.5 %. Sequence results detected the presence of E. coli strains harboring NDM-5 along with IncFIA, IncFIB (AP001918), IncFII, IncI2(Delta), Incl (Gamma), IncY plasmids. *E. coli* strains were of ST 405 (n=1), ST361 (n=2), and ST648 (n=1). *Acinetobacter baumanii* detected by sequencing all belongs to ST2 in IC2. Some of these strains harbored both OXA-23 and OXA-66. A key finding in this study was the isolation of MDR *E. coli* strain harboring both NDM-5 and OXA-1 from wild animal (Otter).

Conclusion: This project effectively isolated Carbapenem resistant *Enterobacteriaceae*, *Acinetobacter baumanii*, and Pseudomonas spp. from animals and environmental samples (sewage, soil, water). The detection of similar clones in both environmental and clinical samples explain the possible transmission of ARG among spp. and across ecosystem which was further validated by the presence of plasmids that are common plasmids to humans.

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ABBREVIATIONS

AMR: Antimicrobial Resistance

ARB: Antimicrobial Resistant Bacteria

ARG: Antimicrobial Resistant Genes

GLASS: Global Antimicrobial Resistance and Use Surveillance System

WGS: Whole-Genome Sequencing

CRE: Carbapenem Resistant *Enterobacteriaceae*

CRAB: Carbapenem Resistant *Acinetobacter baumanii*

CRPA: Carbapenem Resistant *Pseudomonas aeruginosa*

E. coli: Escherichia coli

HGT: Horizontal Gene Transfer

MDR: Multi Drug Resistant

ST: Sequence Type

CARD: Comprehensive antibiotic resistance database

Inc: Incompatibility

CHAPTER I
INTRODUCTION

Antimicrobial resistance (AMR) is a global public health concern that poses a significant threat to human and animal health. In recent years, the emergence and spread of multidrug-resistant bacterial strains have become a major challenge for healthcare systems worldwide. The emergence and spread of AMR are driven by several factors, including overuse and misuse of antimicrobial drugs in humans and animals, inadequate infection prevention and control practices, and a lack of new antimicrobial drugs in development. Furthermore, there are multiple routes of transmission of ARB and ARG including movement of contaminated wastewater and soil through an environment ecosystem, direct contact between humans and animals, and through food chain. To combat AMR, One Health approach in surveillance programs should be implemented in which human, animal, and environmental health are integrated to gain a better understanding of the epidemiology of AMR. As part of One Health approach. Next-generation sequencing (NGS) technology has emerged as a powerful tool for identifying and characterizing bacterial pathogens and their resistance mechanisms

Carbapenems are a group of βlactam antibiotics used to treat serious infections, especially in hospital settings. Carbapenems have a very broad antimicrobial spectrum covering both Gram-positive and Gram- negative bacteria. Resistance to carbapenems is mediated by various factors such as the loss of outer membrane porins, production of carbapenemases and overexpression of efflux pumps. In recent years, the emergence of carbapenemase secreting bacteria has been reported worldwide including in many hospitals and in the environment of the Middle East countries.

In Lebanon, high prevalence of resistance strains was detected with some conferring resistance against Carbapenem. However, the extent of carbapenem in the country is not clear due to the lack of representative surveillance studies, limited national data, in addition to poor focus on environmental and animal health. To better understand and determine the extent of resistance to last resort antibiotics, one health approach should be implemented which is a gap in Lebanese studies. In this context, this project will mainly utilize NGS for AMR surveillance in North Lebanon, employing a One Health approach to combat AMR by focusing our research on carbapenem resistance since carbapenems are last resort treatment option for human. This project will mainly aim to:

- Investigate the extent of carbapenem resistant bacteria in wild animals, sewage, natural water sources, and soil in North Lebanon.
- Analyze the trends of this resistance and evaluate the threat on human health by examining for instance mobile genetic elements and their potential to spread to other pathogenic Gram-negative bacteria.

CHAPTER II

LITERATURE REVIEW

A. Antimicrobial Resistance

According to the World Health Organization (WHO) Antimicrobial Resistance (AMR) occurs when bacteria, viruses, fungi and parasites become non susceptible to antimicrobials they were able to respond to. Hence, infections caused by these microorganisms become harder to treat increasing the risk of disease spread, severe illness and death.

In the 21st century, AMR has been recognized as a major threat and one of the principle public health problems that arise the need for an improved and coordinated global effort to contain it. (1)

1. Causes of AMR

AMR is a natural phenomenon (1) that occurs as a result of microbial evolution and has widely emerged due to human practices (2). It is driven by a variety of factors that are complex, diverse, and cross- sectoral in nature. (3)

As for microbial causes, evolution plays a great role in antimicrobial resistance. In their article entitled "Antibiotic resistance in the environment", Larsson and Flack highlight the presence of resistance and the ability of bacteria to tolerate antibiotics long before humans' massive production of antibiotics. (2) In the pre- antibiotic era, similar to antibiotics , the natural production of secondary metabolites secreted by some microorganisms to compete for resources was an important driver for resistance mechanisms.(2) In 1928, Alexander Fleming discovered penicillin, 10 years later and upon the introduction of penicillin, resistant strains capable of inactivating the drug

became prevalent.[4] This highlight that the exposure of microorganisms to antibiotic, naturally produced or synthetic, confers their resistance and exerts a selective pressure favoring the survival and multiplication of resistant strains against susceptible ones.[1],[5] Resistance can be intrinsic, whereby the physiological properties of all members of a spp. allow it to resist the action of certain antibiotics; or acquired, either through de novo mutation or via the acquisition of Antimicrobial Resistant Genes (ARGs) via horizontal gene transfer. (HGT). [6],[4]

Intrinsic resistance is a naturally present resistant and it refers to the existence of genes in bacterial genomes that could generate a resistance phenotype.[4] It could be due to the lack of affinity of the drug for the bacterial target, inability of the drug to enter the bacterial cell, extrusion of the drug by chromosomally encoded efflux pumps, or presence of drug-degrading enzymes.[7] In addition to intrinsic resistance, bacteria can obtain the ability to resist antibiotics through acquired resistance. Acquired resistance occurs either through mutations in the DNA of the cell during replication, or via horizontal gene transfer whereby antibiotic resistant genetic material are disseminated across several bacterial spp. through transformation, transduction, or conjugation (Figure 1). [8]

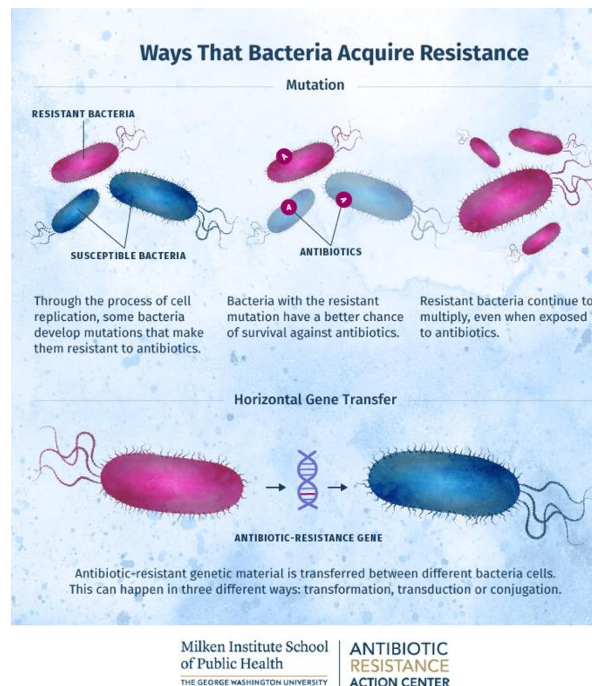

Figure 1. Acquired resistance through mutation or horizontal gene transfer. [8]

Besides microbial evolution, human practices in several domains play a major role in AMR. Indeed, these practices are one of the main drivers for microorganisms to evolve resistance as a way to adapt environmental stress. To start with, misuse and overuse of antibiotics in hospital settings and community are main causes of resistance. In clinical settings, clinicians overprescribe antibiotics either as an empirical treatment or by the serial application of antimicrobials. In case of the lack of laboratory testing or the long-time multiple tests could take to diagnose the case of patient, practitioners may adopt a random treatment or simultaneously apply different antimicrobials in the hope that one will aid to treat the unknown pathogen. As for the serial application of antibiotics, clinicians may adopt this type of practice based upon similar profile of patients, past experience, or according to local epidemiology. [1], [5] In other cases, the antimicrobial prescriptions are inappropriate such as giving wrong drug, wrong doses, or unnecessary

antibiotic. In a Lebanese study, it was shown that in 52 % of cases, the prescription dose was inappropriate while 63.7 % of physicians prescribed antibiotics with wrong duration of treatment. [9] These various cases of misuse of antibiotics by clinicians not only fails to efficiently cure the patient, but also encourage the development of AMR among non- pathogenic organisms in the patient's microbiota that can be transferred via HGT to susceptible pathogenic ones. [5]

In developing countries, misuse of antimicrobials is facilitated through self-medication as antibiotics are available without prescription and through unregulated supply chains. [9], [10]

Patients might also not complete the course of antibiotic or miss doses especially in cases where signs and symptoms begin to subside after an initial favorable therapeutic response.[9],[10] These actions result in the exposure of surviving pathogens to sub-therapeutic concentrations of antimicrobials thus increasing HGT of antimicrobial resistance genes (ARGs), and elevate mutation rates; all of which will increase the likelihood of resistance evolution.[3],[6],[9] Apart from the irrational use of antimicrobials, deprived sanitation in developing countries could also be considered as causes of AMR development as they contribute in the circulation and spread of resistant genes and microorganisms. [9], [10] Non humane use of Antimicrobials is another cause of AMR. Antimicrobials are used as prophylaxis and for treatment in animals; they are also used as growth promoters in animal breeding and agriculture. [3], [9]

2. *Transmission and spread of AMR*

The misuse and overuse of antibiotics in humans, animals, and environmental systems reflect the complexity in AMR spread and the diverse routes for its transmission across the ecosystem (Figure 2). (11)

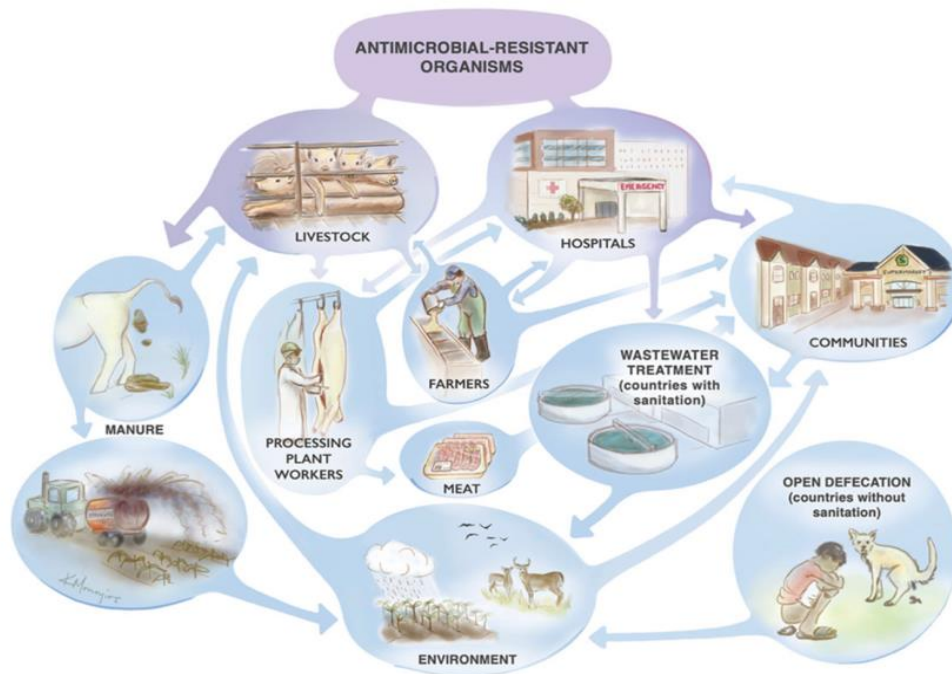

Figure 2. Routes of AMR transmission across ecosystem. (11)

Antibiotics reach environment through several routes such as municipal and hospital wastes, excretions (urine and feces) from humans and animals, improper disposal of unused or expired drugs, and agricultural soil and water. (2), (11), (13) Transmission of resistance is also facilitated by person-person contact, through contaminated water, food or by vectors. (9), (10)

Research has shown that high levels of ARGs and ARBs were detected in environmental samples collected from different types of wastewaters, including municipal sewage and hospital wastewater. (15) Among the most common ARB

identified in the wastewater samples belong to the family *Enterobacteriaceae*, in addition to high concentration of MDR bacteria and ARGs conferring resistance to varied classes of antimicrobial drug. [15] Despite the employment of waste water treatment, ARGs present in hospital wastewater were not effectively eliminated as no significant difference was recorded in ARG abundance between influent and effluent hospital wastewater samples. [15] Moreover, hospitals and other healthcare facilities are main sources of antimicrobial resistance not only by generation of wastewater, but also through discarded medicines. [13] Unused or expired antibiotics thrown in the garbage are not degraded and can enter the groundwater or aquatic system. [13]

Water also plays an important role in the transmission of many infectious and non-infectious agents to humans [14] specially in developing countries with inadequate consideration of sanitation and poor hygiene. [11] In Lebanon for example, the Central Administration of Statistics indicates that only 37 % of the buildings are connected to a sewer networks, while others either use cesspools or septic tanks, or release raw sewage directly into the environment. [16] Worldwide, diverse countries reported the presence of Carbapenemase and extended-spectrum beta lactamase (ESBL) genes such as blaNDM and blaCTX-M-type respectively, in drinking water (Figure 3).[17] Furthermore, animal agricultural process enrich ground and surface water with ARBs and ARGs especially if animals receive antibiotics in areas close to soil and water resources.[11] As for irrigation water, less evidence compared to manure application is present on the ability of ARBs and ARGs to be transmitted to crops tissues. [11] However, findings on the effect of irrigation water in mediating resistance documented the high abundance of ESBL-producing E. coli in investigated irrigation water samples which might be spread via

irrigation into foods that are consumed raw and thus posses a potential health risk on humans or/and animals. [18]

In case of animals, application of animal waste in the form of manure onto agricultural land results in propagation of antibiotic resistance among soil bacteria.[12] Significant amount of antibiotic residue, about 30 % to 90 %,[3] is excreted in feces and urine due to incomplete absorption of these antibiotics in animals gut.[12],[14] These wastes are then used as manure in the aim to improve the fertility of soil, however, they also become responsible for the transfer of traces of antibiotics from animals to the soil ecosystem and ultimately to plants and humans. [12] In addition, the higher density of microbes in the soil environment encourages genetic exchange contributing to the development of microbial resistance in the presence of antibiotics. [14] ARBs and ARGs could also be spread through direct and indirect contact between humans and animals. As for domestic animals, both modes are involved either by farmers being in contact with animals and animal products, or indirectly through food chain. Companion animals could also transmit resistant bacteria as they are in intimate contact with their owners. [11] Although there is no direct contact between humans and wildlife, wild animals have also the capacity to spread resistance across the environment. A review on the complexities of AMR spread across humans, animals, and environmental systems suggested the possible wildlife mediation in ARG movement after it found that blaNDM-1 (carbapenemase gene) and other clinically important ARGs were elevated in Arctic soils near birds, reindeer, and arctic fox-watering areas. [11] In another study done on seagulls in Alaska, results revealed the presence of carbapenemase genes such as blaKPC-2 or blaOXA-48 in *E. coli* strains isolated from the feces of these wild animals.

Indeed, some of these resistant strains belong to a clone with reported interspecies transmission between wildlife, humans, and companion animals. [32]

All these routes that drain into the environment provide numerous and diverse resistant genes that potentially could be acquired and used by pathogens to counteract the effect of antibiotics. [2] These genes are often passed on to pathogenic bacteria from native and non pathogenic bacteria in the environment that act as as a reservoir for ARGs by HGT. [3],[15]

Figure 3. Examples of ARGs and plasmids present in drinking water worldwide. [17]

3. *Impact of AMR on health*

WHO has listed antibiotic resistance among the biggest threat for global health, food safety, and development.[18] It is estimated that infections caused by antibiotic-resistant bacteria are responsible for around 700,000 deaths per year worldwide and for over 10 million deaths per year in 2050 .[21] In hospital settings, patients with serious illness due to infections with ARB are left for prolonged duration in hospitals and thus can act as a

reservoir of infection for a longer period putting at high risk members of community and health care workers.[19],[20] In addition, high mortality and morbidity rates are correlated with AMR. A study has shown that patients with bacteremia caused by *Enterobacteriaceae* with extended-spectrum β-lactamase (ESBL) show higher rates of treatment failure and mortality compared to patients with bacteremia caused by non-ESBL producers.[22] In the same study, researchers mentioned that infections caused by Carbapenem Resistant Enterobacteriaceae (CRE) are associated with hospital mortality of 48% – 71%, and transplantation failure due to CRE colonization among stem cell transplant patients; whereas carbapenem-resistant Acinetobacter baumannii bacteremia was associated with a 14-day mortality of 45 %.[22] Due to the emergence of multi drug resistant (MDR) Gram-negative bacteria, infections are treated by last-resort classes of antibiotics such as carbapenems and polymyxins which show diverse side effects and with time bacteria are also getting resistant to them .[22],[19]

B. AMR Surveillance: Core Element in AMR Control

Surveillance is defined by the WHO as the ongoing, systematic collection, analysis and interpretation of health-related data required to plan, implement and asses the public-health practice. [28] AMR surveillance plays an integral role in infectious disease management and it is the cornerstone to better understand and control the spread of antimicrobial resistance. [23] This section will shed light on different surveillance systems present globally, in high income countries, and in low- middle income countries within hospital and non- hospital settings. It will mainly highlight the case of Lebanon and clarify the topic of one health approach.

1. ***Global AMR Surveillance***

In 2014, WHO in collaboration with the Food and Agriculture Organization of the United Nations (FAO) and the World Organization for Animal Health (WOAH) have established an agreement to develop a joint action plan for AMR as a reflection to the global threat AMR has posed to human health.(27) Using the official recommendations of WHO, FAO and WOAH, many high income countries have instituted surveillance programs or systems to monitor AMR, while most low–middle economies have little to no programs to address this issue.(27)

In May 2015, the Sixty-eighth World Health Assembly (top decision-making body of WHO) adopted the Global Action Plan on Antimicrobial Resistance. And one of the five strategic objectives of the Global Action Plan is to enhance global surveillance and research on AMR. (24) In accordance with this objective and within the same year, WHO established the Global Antimicrobial Resistance and Use Surveillance System (GLASS) to monitor AMR in common bacteria at the national and global level. (25) To date, 127 countries are enrolled in this system. (25)

In their last report released in 2022, GLASS highlighted the global spread of AMR and the presence of high rates of AMR in several low- and middle-income countries (LMICs) compared to rates in high-income countries. The report also document based on clinical specimens the presence of resistance to third-generation cephalosporins in K. pneumonia, the emergence of carbapenemase- producing Enterobacterales and carbapenem and aminoglycoside resistant Acinetobacter spp. (25) In partnership with Glass, national and international AMR surveillance systems include the Central Asian and European Surveillance of Antimicrobial Resistance (CAESAR), the European Antimicrobial Resistance Surveillance Network (EARS-Net), the Latin American

Network for Antimicrobial Resistance Surveillance (ReLAVRA), industry-funded surveillance platforms and, more recently, the UK's Fleming Fund programme. [28]

In Europe, an annual report on AMR surveillance is generated based on data reported to CAESAR and EARS-Net. The annual report of antimicrobial resistance surveillance in Europe published in 2022 showed widespread of AMR in European region with higher percentages in the southern and eastern parts of the country. This report also cited the AMR pattern of different bacterial spp. conferring resistance. For example, *E. coli* which is the most common cause of community-acquired bloodstream infections and urinary tract infections showed high resistance to fluoroquinolones and 3rd generation cephalosporins with the emergence of carbapenem-resistant strains. Carbapenem resistant *Pseudomonas aeruginosa* and Acinetobacter spp. which are main causes of hospital- acquired infections were also widely present in the region. [26] Besides, surveillance on food and animals was also documented in governmental and non-governmental organization in Europe as an attempt to implement the one health approach[29] highlighted by WHO Global Action Plan as a need in monitoring AMR.[27] In addition to countries in the European region, Cornejo et al pointed out that countries with high economic levels such as Australia, Canada, the United States, Hong Kong, Japan, and New Zealand have developed AMR surveillance systems for animal health. In contrary, low–middle economies have minimal or nonexistent programs or systems to monitor antibiotic use in nonhuman settings. [27]

Apart from one health approach, whole-genome sequencing (WGS), molecular biology tool used to obtain the (nearly) complete DNA sequence of an organism, [30] is considered as a valuable addition to national and international surveillance systems to better surmise transmission events between humans and animals and trace the origin of

AMR strains. [31] WGS can also determine pathogen characteristics such as virulence factors and resistance genes, as well as clonal relatedness and abundance. [31] This knowledge helps to manage disease outbreaks and epidemics caused by resistance strains. In the report "whole-genome sequencing for surveillance of antimicrobial resistance" published in 2020, GLASS mentioned different cases in which WGS is incorporated in surveillance systems of some high-income countries at the local, national, and international levels. For instance, at the national level, WGS was used in a one health approach to detect mcr-1 positive *E. coli* strains in hospital settings in Latin America and chicken farms in Argentina. Comparative sequence analysis of the data showed that the same group of mcr-1 plasmid was present in both human and animal specimens. [30]

On the contrast, most low- medium income countries lack WGS and fails to achieve the one health approach in surveillance systems present in some of these countries. In the Eastern Mediterranean Region, diverse factors (such as wars and conflicts, poor sanitation and control, limited capacities of laboratories, and self medication) contribute to the emergence and spread of AMR in light of weak health and limited surveillance systems.[32] In the article , "Increasing Antimicrobial Resistance in World Health Organization Eastern Mediterranean Region, 2017–2019", the authors claim based on analysis of bloodstream infections the emergence of highly critical pathogens with highest percentage of carbapenem resistant *Acinetobacter baumanii* (70.3%) and lowest for carbapenem resistant *E. coli (4.6%)* based on 14 countries reported bloodstream infections to Glass as for 2019.[32] Besides, Sleiman et al. emphasized based on a review of 192 studies the prevalence of carbapenem-resistant Gram-negative bacilli in all countries of the eastern Mediterranean region (EMR). He pointed out the spread and

increase of carbapenem resistance in Enterobacteriaceae, *A. baumannii* and *P. aeruginosa* in addition to the presence of high variety of carbapenem resistance-encoding genes. Most of these isolates were collected from hospital settings. [(33)]

2. *AMR Surveillance in Lebanon*

Lebanon is a developing country, classified as a middle-income country by the WHO. [(34)] Studies on AMR status in Lebanon have showed how complex the AMR issue is as numerous uncontrollable factors contribute to the emergence and spread of multidrug-resistant (MDR) strains.[(36)] In April 2017, Lebanon has been enrolled in the WHO's GLASS [(36)] and in March 2019, the Lebanese Ministry of Public Health has developed the National Action Plan for combating AMR in Lebanon.[(34)] AMR surveillance has been improved during the last years ,however, there is only significant number of published studies targeting the epidemiology of AMR among the human population in Lebanese hospital settings with no enough clear data on the epidemiological situation of antibiotics usage in animals, food and the environment. [(36)] In his review tackling AMR in the Middle East region, Sleiman et al. summarized the prevalence of carbapenem resistant Enterobacteriaceae (CRE), A. baumannii (CRAB) and P. aeruginosa (CRPA) in Lebanese hospital settings. [(33)] At the country level, data showed an increase in carbapenem resistance during the last decade. Among the most predominantly detected genes, bla_{OXA-48} (48 %) was the main carbapenemase gene present in CRE, bla_{VIM-2} (21.27 %) in CRPA, and bla_{OXA-23} (100 %) in CRAB. [(33)] In addition, CRE (mainly *E. coli* and *K. Pneumonia*) were mostly prevalent in north Lebanon in contrast to CRAB and CRPA which didn't show specific prevalent location. [(33)] In accordance with this review, a recent study was done to evaluate the current state

of carbapenem resistant in GNB mainly CRE, CRAB, and CRPA within hospitals in northern Lebanon. [38] This study utilized WGS to determine imipenem resistance in clinical isolates. Results showed similar data of rising rates of carbapenem resistance in GNB mainly GNAB isolated over a 5-year period in three hospitals in the northern part of Lebanon (figure 4). [38] Resistance genes detected in this study were in line with previous data with new variant genes discovered such as NDM-19 and OXA-162 (in E. coli), and VIM-62 (in P. aeruginosa). Another study was performed on clinical isolates in two hospitals in north Lebanon showed ertapenem-resistant E. coli causing urinary tract infection and carbapenem resistant GNB among cancer patients. [36]

Despite the large number of publications done on clinical specimens, AMR is not only confined to clinical settings, but also widely present in the environment which act as a significant reservoir for ARB and ARGs. [36] In continuous to data extrapolated from studies done on clinical specimens, studies on environmental samples have detected similar genes. Regarding carbapenemases, blaVIM-2 carrying Pseudomonas aeruginosa and blaOXA-23- carrying Acinetobacter baumannii strains were isolated from Lebanese livestock and poultry, E. coli OXA-48 was also detected in fowl. [36] As mentioned previously, water is a major route in AMR transmission specially in developing countries. In the case of Lebanon, water has been under an increasing pollution threat, mainly due to population growth, wastewater and solid waste mismanagement, lack of monitoring and surveillance programs, in addition to insufficient number of wastewater treatment plants. [39] A study done in 2021 aimed to detect the diversity and dissemination of WHO priority antibiotic-resistant pathogens in Lebanese estuaries observed a high relative abundance of ARGs in North Lebanon. [37] Another nationwide study on the quality of water in Lebanese rivers pointed out that the

majority of unacceptable water samples were collected from rivers in the North (71.4%) in compared with 18% of the samples collected in the South and 9% in Mount Lebanon. [(39)] The same study also mentioned that carbapenem resistance was only identified in few isolates from the North region only. [(39)] Following this, it is clear that North Lebanon records the highest rates in AMR in both clinical and non clinical settings and this corresponds to the high density of refugees' camps, poverty, and infrastructure challenges present in this region compared with the rest of the country. [(36), (37), (39)]

In short, studies indicate that the spread of antibiotic-resistant bacteria in Lebanon could possibly come from the nature due to the discovery of the same resistant strains in both clinical and non-clinical specimens. This is a significant concern for public health because the presence of resistant bacteria in the environment implies contamination from either human or animal sources. To combat this issue, it is crucial to implement a One Health approach which involves the use of whole-genome sequencing for monitoring microbes in humans, animals, and the surroundings. This approach will also help develop comprehensive preventive and control methods to address antibiotic resistance. [(39)]

3. *Challenges: One Health Approach*

AMR is characterized by complex interactions involving various microbial populations that affect the health of humans, animals, and the environment. To address

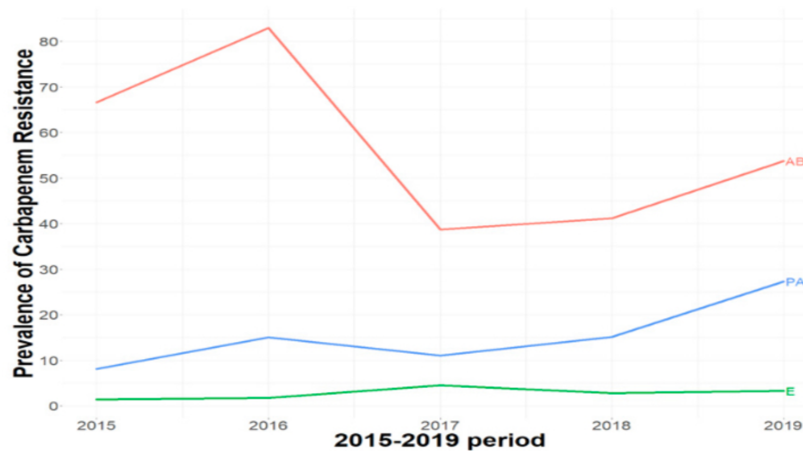

Figure 4. The prevalence of carbapenem resistance among E, PA, AB isolates in northern Lebanon from 2015 to 2019. (38)

AMR threat, it is imperative for surveillance systems to implement a coordinated, multisectoral approach, such as One Health.(13),(40) 'One Health' is a public health program approach launched by the WHO which seeks to enhance antimicrobial stewardship through monitoring antimicrobial usage in various sectors (humans , animals and the surrounding environment) as a way to ensure improved prescribing and utilization practices while preserving the effectiveness of antimicrobial medications for both humans and animals.(13),(35),(40)

In addition to what have previously mentioned about the role of environment and animals in the transmission of AMR, many other factors further highlight the need of one health approach in surveillance. To illustrate, humans and animals not only share the same environment, but also different infectious diseases. It has been estimated that 75 % of human infections are of zoonotic origins (they first originated in animals). (40) For instance, some *E. coli* strains appear to be specific pathogens for animals, while others are capable of infecting multiple spp., including humans. (40), (35) Worldwide, the volume of antimicrobials used in animals is estimated to be greater than in humans. Although few antimicrobial cl asses are reserved more or less exclusively for humans,

and few others are limited to veterinary use, the great majority of antimicrobial classes are used in both humans and animals, including broad-spectrum beta-lactams and quinolones. [(40), (35)]

This data implies that AMR is not confined only to humans and is not only acquired by strains infecting them, animals and other environmental sectors can also act as great reservoirs for ARBs and ARGs which can be transmitted across both clinical and non-clinical settings to humans.

C. Gram-negative Bacteria

In the WHO's Global Priority Pathogens List, carbapenem-resistant *Enterobacteriaceae* (CRE), carbapenem-resistant *Pseudomonas aeruginosa* (CRPA), and carbapenem- resistant *Acinetobacter baumannii* (CRAB) were listed in the critical priority pathogens group. [(33)]

1. *General Characteristics of Escherichia coli*

Escherichia coli is a gram-negative rod-shaped bacterium that belong to the family Enterobacteriaceae, a large family which also includes Salmonella, Klebsiella, Proteus, and Enterobacter spp.[(45)] *E. coli* is facultative anaerobic, non-sporulating and oxidase negative coliform bacterium that commonly inhabits the environment, foods, and the distal end of the intestinal tract of humans and warm-blooded animals, where it is part of the gut microbiota.[(41) ,(42), (43)] *E. coli* are usually motile through the action of peritrichous flagella. Many E. coli cells have capsules made of acidic polysaccharides while some mucoid strains produce an extracellular slime. [(42)] On MacConkey agar media, colonies of *E. coli* are pink in color due to lactose fermentation, which is

important for distinguishing *m* from other non– lactose fermenters bacteria that produce colorless colonies. [43]

a. Infections and Complications

Escherichia coli strains can be classified as: non- pathogenic commensal organisms that are normal residents of the gastrointestinal tract, strains that cause diarrheal intestinal disease, and strains that cause extra-intestinal infections. [45] Away from the intestinal tract, *E. coli* is a major cause of serious bacterial infections, including urinary tract infections (UTIs), enteritis, meningitis, and bloodstream infections (BSI).[41] *E. coli* is a also common cause of community acquired infections and nosocomial infections including catheter-associated UTIs and ventilator-associated pneumonia .[44] The variety of intestinal and extra intestinal diseases associated with *E. coli* are dependent on the virulence factors this bacteria express such as fimbrial adhesins, capsules, toxins (exotoxins, hemolysins, and enterotoxins), and iron up-take systems.[43]

E. coli are transmitted primarily through the fecal–oral route, contaminated food or water ingested by humans can thus cause intestinal illness. [45] Research has reported various cases of E. coli infections associated with different sources of contaminated water (drinking water, irrigation water, rivers) specifically in developing countries with poor sanitation. [42]

b. Treatment

The course of treatment for patients with *E. coli* associated infections varies according to the specific strain and the nature of the illness. When dealing with intestinal illnesses the initial focus of treatment is based on relieving symptoms. While antibiotics are not the preferred treatment for most patients, particularly due to the

potential side effects and links to antibiotic resistance, they may be necessary in severe cases.[44] As such, β -lactams are commonly used in the treatment of BSI caused by E. coli.[41] However, with the emergence of ESBL producing *E. coli* which exhibit resistance to the majority of the b-lactam antibiotics including penicillin, monobactams and most cephalosporins, carbapenem is recommended as the first-line treatment for infections outside of the urinary tract caused by ESBLs-producing *E. coli*. Unfortunately, resistance of E. coli to carbapenems is also emerging. [41]

2. *General characteristics of Pseudomonas spp.*

Pseudomonas spp. are aerobic, non-spore-forming, gram-negative bacilli found in diverse ecosystems, including water, soil, and the rhizosphere. [46],[49] They are motile due to the presence of one or more polar flagella. On MacConkey agar, they produce smooth colorless colonies as they are non-lactose fermenters. [46] Unlike other fluorescent pseudomonas including *P. aeruginosa, P. fluorescens*, and *P. putida* which are oxidase positive, *P. luteola* is oxidase negative and produce yellow- pigmented smooth colonies on MacConkey agar that may become rough or wrinkled after 48 hours of incubation. [50],[48]

a. Infections and Complications

From a clinical perspective, *Pseudomonas aeruginosa* is the most important and extensively characterized spp. in the Pseudomonas genus. [49] *Pseudomonas aeruginosa* is an opportunistic pathogen that cause both community-acquired and hospital-acquired infections. Community-acquired infections include ulcerative keratitis, otitis externe, dermatitis and soft tissue infections. As for nosocomial infections, *P. aeruginosa* causes

pneumonias, UTIs, bloodstream infections, and a variety of systemic and recurrent infections, particularly in immunocompromised hosts and patients with cystic fibrosis. (47)

Complications with *P. aeruginosa* are related to several virulence mechanisms such as secreting toxins, ability to evade cells via its pili and flagellum, biofilm formation, and a type III secretion system that alters host cell functions. (47)

Although no enough data is present on the pathogenicity of other members of the genus Pseudomonas, some may act as opportunistic pathogens, causing infections mainly in immunosuppressed patients or individuals subjected to invasive medical procedures.(49) For instance, *P. fluorescens* has been reported to cause bloodstream, urinary, respiratory, and soft-tissue infections.(49) In few case reports, *P. luteola* was also found to be related to some human infections including bacteremia, pneumonia, surgical site infections, and infections associated with the presence of prosthetic devices. (50) Findings have suggested the ability of *P. putida* to colonize patients, and also to persist in fluids and in water-associated hospital settings. (46) To illustrate, P. putida has been detected in multiple instances in both intensive care units (ICU) and non-ICU. Indeed, some of these cases were caused by the spread of contaminated fluids. Several reports of bacteraemia, keratitis, UTIs, pneumonia, and soft tissue infections caused by P. putida has been also indicated. (46) *P. fluorescens* and *P. putida* aw well represents a serious concern in the spread of ARGs to more pathogenic organisms as they act as reservoirs for clinically important ARGs. (46),(49)

b. Treatment

With the emergence of multi drug resistant strains among Pseudomonas spp., treatment options become limited. Worldwide, multidrug-resistant *P. putida* harboring metallo-β-lactamase (MBL) genes and multidrug-resistant (MDR) and extensively drug-resistant (XDR) P. aeruginosa have been reported. [46],[47] In 2017, the WHO reported CRPA as one of the pathogens in the "critical priority" group for which new antibiotics are urgently required, accordingly new antibiotics and antibiotic combinations have been used.[52]

In the case of nosocomial infections with low risk of MDR *P. aeruginosa* and low mortality risk, narrow-spectrum antibiotic such as ceftriaxone, ertapenem or levofloxacin should be introduced. However, if MDR *P. aeruginosa* is encountered ceftolozane-tazobactam and ceftazidime-avibactam are used as novel combination antimicrobial with antipseudomonal activity. [47] Cefiderocol, a novel siderophore cephalosporin, could be a treatment option when more complex mechanisms of resistance interact together as in XDR phenotypes and MBL-producer strains. [52]

3. *General characteristics of Acinetobacter baumanii*

Acinetobacter spp. are strictly aerobic, encapsulated, non-motile, non-fastidious, oxidative-negative, gram-negative coccobacilli. [55] They are non- lactose fermenters producing light lavender color colonies on MacConkey agar. [54] Acinetobacter spp. are commonly distributed in the environment as free living saprophytes. [54] Different spp. of the genus is associated with various habitats such as soil, water, sewage, human, foods and animals. [54] Among the most clinically significant spp. are *A. baumannii*, *A. haemolyticus* and *A. calcoaceticus*.

a. Infections and Complications

A. baumannii is an opportunistic pathogen responsible for many hospitals acquired infections across several sites in patient's body. It has been most frequently isolated from wounded skin and tissues, respiratory system, bloodstream and central nervous system.[(54),(57)] *A. baumanii* has the ability to survive on dry surfaces with limited nutrition thus increasing its association with infections that involve organ systems with high levels of fluids such as the urinary and respiratory tract and peritoneal cavity.[(53),(54)] Besides, colonized medical devices serve as reservoirs for *A. baumanii* in prolonged hospital outbreaks and account for a wide variety of local and systemic infections, including pneumonia, bacteremia and wound infections.[(54)] Bacteremia incidences due to *A. baumanii* infections have been widely reported and associated with a mortality rate that can reach 58.6%.[(56)] Patients who rely on mechanical ventilation are at high risk of pneumonia due to the potential formation of biofilms on their endotracheal tube by *A. baumannii.* This may lead to the overgrowth and colonization of this bacteria in their lower respiratory tract.[(57)] In addition to nosocomial infections, Acinetobacter easily inhabit tracheostomy sites and result in community acquired infections such bronchiolitis and tracheobronchitis[(54)] and to lesser extent bacteraemia and pneumonia.[(57)]

In conflict regions, *A. baumannii* is one the most frequently isolated organism and the major causative agent of multi-drug resistant infections among injured military and civilian soldiers. Several cases of *A. baumanii* infections has been reported from battle victims with open tibia fractures in both Iraq and Afghanistan war zones.[(55)] MDR *A. baumannii* can spread from injured military patients who are brought back to civilian

hospitals. This type of infection is most common in critically ill patients in the ICU, and it can account for up to 20% of infections in ICUs around the world. [55], [57]

b. Treatment

Carbapenem is the most effective antibiotic recommended to treat infections caused by Acinetobacter spp. [54], [55] However, the rate of carbapenem-resistant *A. baumannii* is increasing gradually with few effective antibiotic options are available to treat resistant strains. [54] Sulbactam is a beta-lactamase inhibitor with bactericidal action against a number of resistant strains. It is used in combination with a beta-lactam antibiotic such as ampicillin- or cefoperazone. [56] In case of carbapenem and sulbactam-resistant *A. baumannii* strains, tetracyclines, such as minocycline and doxycycline are used as a treatment option.[57] As a more effective treatment especially for patients with CRAB bloodstream infection, tigecycline, derivative of minocycline , is used in combination with a second agent such as carbapenem or expanded- spectrum cephalosporin.[54] Highly drug-resistant *A. baumannii* infections are also treated with optimized doses of colistin as a part of combination regimen with a second agent such as carbapenem , tigecycline, or sulbactam.[57] Studies have stated that the cure or improvement rates among patients infected with multidrug-resistant *A. baumannii* and treated with colistin is about 57–77%.[54] Cefiderocol and Fosfomycin as well are currently implemented as novel treatments for *A. baumannii* infections that are resistant to previously mentioned antibiotics. [57]

D. Carbapenem: ß-lactam antibiotic

Carbapenem is a semi-synthetic β-lactam antibiotic.[57] As all β-lactams, it has a 4 member beta-lactam ring.[59] However, in contrast to penicillins, the 4:5 fused ring system in carbapenems is unsaturated and has no ring sulphur; instead, sulphur is a substituted by carbon atom.[60] In carbapenems, the side chain is in the trans position instead of the cis position, commonly found in other β-lactams, which made them insensitive to the effects of β-lactamases (figure 5).[59]

Among the wide variety of β-lactam antimicrobials, carbapenem has the most extensive antibacterial spectrum with the strongest activity against both Gram-positive and Gram- negative bacteria.[57] It has a good stability against many b-lactamases and are usually successful in treating severe nosocomial infections generated by extended-spectrum (ESBL) and AmpC β-lactamases producing strains that are resistant to other members of the b-lactam antibiotic group.[58] Indeed, carbapenem is prescribed as one of the last-line antibiotic in treating infections caused by the most critical resistant bacteria including *Acinetobacter baumannii, Pseudomonas aeruginosa*, and *Enterobacteriaceae*.[59]

Among the carbapenem family, the WHO lists meropenem as an essential antibiotic used to target serious hospital-acquired infections.[60] Meropenem is one of the smallest β-lactam antibiotics with a very broad-spectrum due to its compact size that enables it to easily penetrate the cell membrane of multiple Gram- negative bacilli.[57] Unlike Imipenem, meropenem has a higher activity against Gram-negatives ; and cilastatin, renal dehydropeptidase inhibitor, does not need to be administered simultaneously as it has a 1-b-methyl group which makes it unsusceptible to the hydrolysis by the enzyme dehydropeptidase (figure 6).[60]

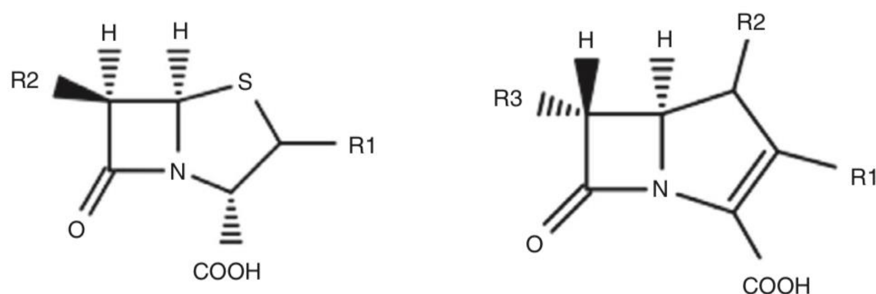

Figure 5. Core chemical structures of penicillin and carbapenem from left to right. (57)

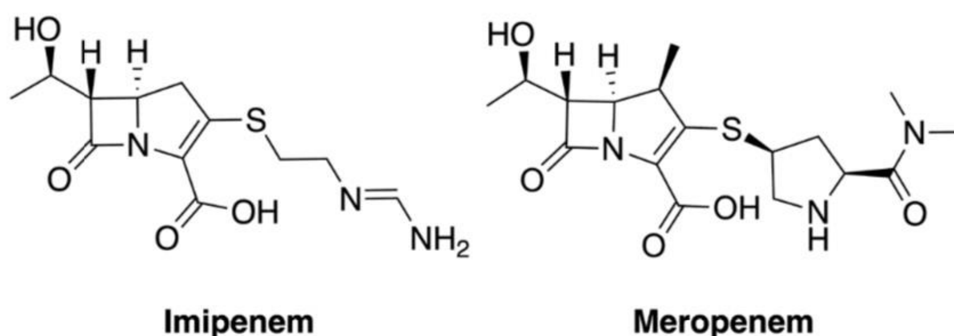

Figure 6. Chemical structures of imipenem and meropenem. (60)

1. ***Mode of Action***

Carbapenem as other β-lactam antibiotics are inhibitors of cell-wall biosynthesis. (59) Carbapenems have a bactericidal action. (60) They specifically inhibit transpeptidation, the last step in peptidoglycan synthesis, which is essential to conserve the structural integrity of the bacterial cell wall. Inhibiting peptidoglycan cross linking disrupt cell wall biosynthesis leading to cell lysis and death. (59),(60)

Carbapenems show limited permeability to the outer membrane of Gram-negative bacteria. (60) They enter the envelope of bacterial cell through outer membrane proteins

known as porins in which some are specific for the entry of carbapenems. [57] Once inside the cell, carbapenem interact with penicillin-binding-proteins, family of enzymes needed in the formation of peptidoglycan, and irreversibly bind to its active site inhibiting its action and preventing the completion of transpeptidation. [57] The basis of this inhibition is the structural similarity of the β-lactam ring to the d-Ala–d-Ala terminus of the peptidoglycan substrate. [60] As a result of obstruction of cell wall formation by carbapenems, the cell membrane becomes too weak to prevent the hypertonic cell from bursting by osmotic shock, thus the cell eventually ruptures due to osmotic pressure (figure 7). [57]

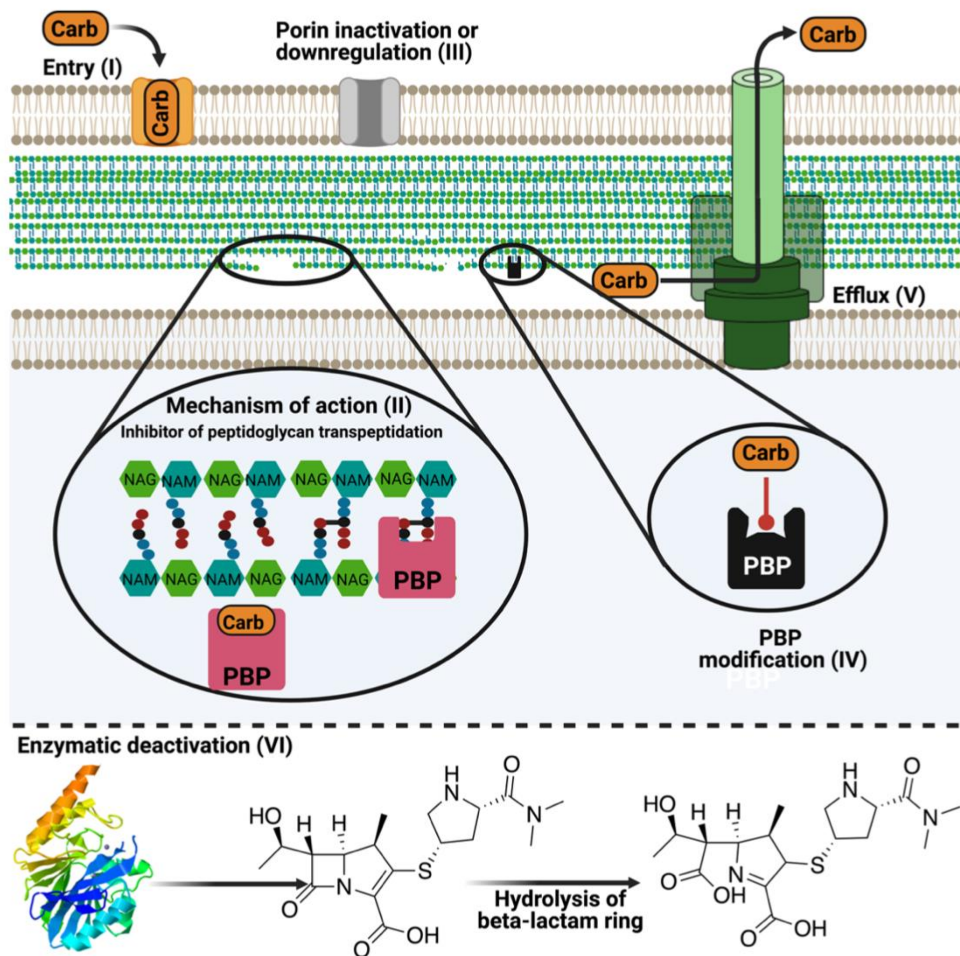

Figure 7. The mode of action and mechanisms of resistant of carbapenem. (I) Entry of carbapenem into bacterial cell. (II) inhibition of transpeptidation by irreversible interaction with PBPs. (III) loss of Porins prevents antibiotic from entering the cell. (IV) Production of low-affinity or mutated PBPs. (VI) Enzymatic hydrolysis of the β-lactam ring. (V) overexpression of efflux pumps extrudes carbapenem outside the bacterial cell. [(60)]

2. *Mechanism of resistance in Gram-negative bacteria*

Carbapenem resistance refers to the ability of bacteria to live and proliferate in the presence of clinically significant concentrations of carbapenems. [(61)] In recent years, carbapenem resistance emerged mainly among GNB such as non-fermenters *Acinetobacter baumannii* and *Pseudomonas aeruginosa*, and fermenters Enterobacterales. [(58)] Mechanism of carbapenem resistance can be intrinsically mediated

or acquired and it can be typically sorted into four categories, (as summarized in figure 7). [60] This section will shed light on the three main mechanism of carbapenem resistance present in *Enterobacteriaceae*, Pseudomonas spp., and *Acinetobacter baumanii* and they include: production of carbapenemases, overexpression of efflux pumps, and porin loss or mutation. As for target modification, no clear data is present on the impact of this mechanism on carbapenem resistance specially in GNB. It is suggested that downregulation of PBPs may lead to lower affinity to drug, but not resistance. [57]

a. Production of β-lactamases (carbapenemases)

The modification of antibiotics by hydrolysis is a major mechanism of antibiotic resistance mediated by β-Lactamases located within the periplasmic space of bacteria. [59],[61] Beta-lactamases are classified into four main groups based on their amino acid sequences (classes A, B, C and D), and exist as two different structural classes based on their modes of action, serine-dependent (Ambler Class A ,C, and D) and metal-dependent (Ambler Class B, also named metallo-β-lactamase). [41] The production of all four classes of beta-lactamases is generally chromosomally encoded although many carbapenemases are recently identified as plasmid- mediated and have been reported in Enterobacteriaceae, P. aeruginosa and A. baumannii. [59] This section will mainly consider carbapenemases within classes A, B and D β-lactamases. [41] As for class C β-lactamases, they are not considered carbapenemases. However, when combined with diminished outer-membrane permeability or efflux pump overexpression, AmpC may show resistance to carbapenem. [41], [59]

i. Class A Carbapenemases

Class A carbapenemases can be chromosomally encoded such as SME (Serratia marcescens enzyme), SHV (sulfhydryl variable lactamase), NMC-A (non-metallo-carbapenemase-A), and SFC (Serratia fonticola carbapenemase). Plasmid-encoded carbapenemases include GES (Guiana extended- spectrum β-lactamase), KPC (Klebsiella pneumoniae carbapenemase), and IMI (imipenemase) which is also chromosomally encoded. [59],[63] Among these, KPC is the most clinically relevant and occurs among diverse bacterial spp. such as E. coli[64] P. aeruginosa, and A. baumannii.[59] In addition, GES was isolated from A. baumannii [57] and P. aeruginosa. [63]

ii. Class B Carbapenemases

Class B β-lactamases are metallo-β-lactamases (MBLs) that require zinc in their active site for catalysis. [59] The most clinically relevant metallo-β-lactamase families include the (NDM) New Delhi metallo-β-lactamase, IMP (Imipenem-resistant Pseudomonas), VIM (Verona integron-encoded metallo-β-lactamase), GIM (German imipenemase) and SIM (Seoul imipenemase).[64] IMP and VIM are mainly included in the integron structure and are integrated into chromosomal DNA or plasmid DNA. They are mostly reported in *Acinetobacter baumanii* [57] and Pseudomonas spp. such as *Pseudomonas aeruginosa* and *Pseudomonas putida*. [64] In contrast, NDM gene is present on the plasmid without an integral structure and its mainly detected in Klebsiella and *E. coli* isolates. [63] Noteworthy, mutations present in these genes affect enzymes activity against different carbapenems. To illustrate, substitution of serine to glycine in IMP-6 from IMP-1 enhanced the resistance to meropenem. [63]

iii. Class D Carbapenemases

Class D enzymes are oxacillinases. [59], [63] These enzymes have been widely identified among Acinetobacter, Pseudomonas, and Burkholderia spp. [63] Most oxacillinases are chromosomally encoded with some reported in plasmids such as OXA-23 and OXA-48. [63] Among the various number of oxacillinases present, OXA-48 and OXA-23 are the most prevalent in *Enterobacteriaceae* and *Acinetobacter baumannii*, respectively. [62] In addition to their presence in clinical isolates, class D carbapenemases were also reported in environmental samples. A study done on environmental samples from north Lebanon detected the presence of OXA-48 and OXA-244 mainly in *Enterobacteriaceae* and OXA-23, OXA-24, OXA-58, OXA-72 and OXA-143 in *Acinetobacter baumanii*. [38], [64]

b. Overexpression of efflux pumps

Efflux pumps are membrane proteins [65] that cross both the inner and outer membranes of Gram-negative bacteria [59] and play a major role in multidrug resistance. [57] They are involved in the active extrusion of a number of antimicrobial agents including carbapenems out of the cell. [61] Genes encoding efflux pumps can be found on bacterial chromosomes or mobile genetic elements such as plasmids. [62] Among the six families of efflux pumps discovered so far, the resistance-nodulation-division (RND) family is the most clinically significant.[65] Efflux pumps of this family exist as tripartite system which includes a transporter protein found in the inner membrane, a membrane fusion protein (MFP), and an outer membrane channel.[57] The best known efflux pumps among critically pathogenic bacteria include AcrAB-TolC in

Enterobacteriaceae, MexAB- OprM, MexCD-OprJ, and MexXY-OprM in Pseudomonas spp., and AdeABC in *Acinetobacter baumannii*. (65)

c. Loss or mutation in the outer membrane porins

Antibiotic resistance can be affected by changes in envelope permeability. (55) To illustrate, antibiotics enter the bacterial cell mainly through porins; loss or mutations in these channels affect the permeability of the cell to antibiotics thus preventing them from reaching their target. (61) Studies reported that resistance of *Pseudomonas aeruginosa* to carbapenem is mostly due to mutations in carbapenem specific porin, OprD. (62) Decreased susceptibility to meropenem was also identified in *Acinetobacter baumanii* with low expression of CarO. (57)

CHAPTER III
MATERIALS AND METHODS

A. Sample collection

10 wild animal samples in the form of fecal matter from bats, rodents, otters, and foxes were received from Lebanese Wildlife Organization. Different sources of water samples (62) and sewage samples (6) were received from the Ministry of Health. 5 soil samples were obtained from gardens farms in Beddawi. All samples were received during the year of 2022 from different regions in North Lebanon (Figure 9, Table 1). Samples were processed once received or stored at 4˚C and analyzed within 24 hours (h).

B. Isolation and purification of meropenem resistant Gram-negative bacteria

Raw environmental samples were pre-enriched with 6 ml sterilized peptone water broth. The mixture was then incubated overnight at 37 °C on the shaker. After 18-24 h, an aliquot (30 µL) from each mixture was spread on MacConkey agar plate (Neogen, USA) supplemented with 2 mg/L meropenem (Sigma–Aldrich, USA). The agar plate was left in the incubator for 24-72 h. If growth was not detected on the plate, the isolate was recorded as meropenem susceptible, and the plate was discarded. If growth was observed, each colony with a different morphology was sub-cultured on separate MacConkey agar plates. Each plate was twice or more sub-cultured until pure cultures are observed. Two to three bacterial colonies are then added to 3 ml Luria Bertani Broth (Bio-Rad, USA) in polystyrene tubes and left in

the incubator for 24 h. After 24 h, if turbidity was detected, transfer 1000 µL of bacterial mixture to 60 % glycerol tubes. Isolates are stored at -80 °C.

C. Gram staining

One bacterial colony is fixed on a microscopic slide using Bunsen burner. Crystal violet is first added as a primary stain for 1minute, then excess stain is washed using tap water. Second, iodine is added as a mordant to fix the stain. After 2 minutes, the slide is washed using tap water. A few drops of decolorizer are then added to the slide and rinsed with water after 10 seconds. The smear is finally stained with safranin as a counter stain and rinsed with water after 1 minute. Under light microscope, Gram-positive bacteria are stained with purple while Gram-negative bacteria are stained with pink. All the Gram-negative isolates were subjected to primary and secondary biochemical identification tests.

D. Antimicrobial susceptibility testing

An antibiotic susceptibility test was performed using the Kirby-Bauer disk diffusion method. The following antibiotic discs at the final concentrations that are indicated were used: Meropenem (MEM) 10 µg, Ciprofloxacin (CIP) 5 µg, Gentamicin (GMN) 10 µg, Ceftazidime (CAZ) 30 µg, Ceftazidime-avibactam (CZA) 50 µg.

For each isolate, a bacterial suspension was prepared by suspending the freshly grown bacteria in 6ml LB, and the turbidity was adjusted to that of a 0.5 McFarland standard. Then, this suspension was spread over the entire surface of a round Mueller-Hinton agar plate using a cotton swab to produce confluent growth. The

plate was left for around 10 minutes closed on the bench, followed by the addition of the 5 tested antimicrobials. The plate was then incubated at 37 °C for 18-24 hours. After incubation period, the diameter for the zone of inhibition, the area around the disk without bacterial growth, was measured. The results were interpreted according to CLSI M100 guidelines.

E. Oxidase Test

This test was performed using the oxidase disc (70439-50DISKS-F) from Millipore in accordance with the manufacturer's published protocol. A well-isolated pure colony was spread on an oxidase disc using a loop. Within 2 minutes, a color change is observed with oxidase positive isolates producing a purple color and oxidase negative isolates were colorless or produce pink color.

F. Analytical Profile Index (API) 20E Test

The API 20E test was performed in accordance with the manufacturer's protocol (BioMe´Rieux, 69280, Marcy I'Etoile, France) and the organisms were identified to spp. level using API software.

G. DNA Extraction

Bacterial strains were cultured on MacConkey or LB agar plates in order to perform DNA extraction using Zymo Quick-DNA™ Fungal/Bacterial Miniprep Kit (D6005). According to the manufacture's protocol, a loop full of bacterial cells is suspended in up to 200 µL of nuclease free water and added to ZR Bashing Bead™ Lysis Tube (0.1 mm & 0.5 mm). 750 µL Bashing Bead™ Buffer is added a to the

tube. Then, the tube is secured in a bead beater fitted with a 2 ml tube holder assembly and process at maximum speed for 30 minutes using Disruptor Genie ™. The ZR Bashing Bead™ Lysis Tube (0.1 & 0.5 mm) is centrifuged in a micro-centrifuge at 10,000 x g for 1 minute. Supernatant is transferred to a Zymo-Spin™ III-F Filter in a Collection Tube and centrifuged at 8,000 x g for 1 minute. 1,200 µL of Genomic Lysis Buffer is added to the filtrate in the Collection Tube. 800 µL of the mixture is added to a Zymo-Spin™ IICR Column® in a Collection Tube and centrifuged at 10,000 x g for 1 minute. The flow through from the Collection Tube is discarded and the previous step is repeated. 200 µL DNA Pre-Wash Buffer is then added to the Zymo-Spin™ IICR Column in a new Collection Tube and centrifuged at 10,000 x g for 1 minute. Followed by the addition of 500 µL g-DNA Wash Buffer to the Zymo-Spin™ IICR Column and centrifuged at 10,000 x g for 1 minute. The Zymo-Spin™ IICR Column is transferred to a clean 1.5 ml microcentrifuge tube and 35 µL minimum DNA Elution Buffer is added directly to the column matrix and centrifuged at 10,000 x g for 30 seconds to elute the DNA.

H. DNA Clean and Concentrate

This is done using Zymo Genomic DNA Clean & Concentrator™ (D4010, D4011) kit and according to the manufacture's protocol. In a 1.5 ml microcentrifuge tube,70 µL of DNA Binding Buffer is added to each volume of DNA sample and mixed briefly by vertexing. The mixture is transferred to a provided Zymo-Spin ™ Column in a Collection Tube and centrifuged for 30 seconds at 13,000 x g. The flow-through is discarded. 200 µL DNA Wash Buffer is added to the column and centrifuge for 30 seconds at 13,000 x g. This wash step is repeated. 22 µL DNA

Elution Buffer is added directly to the column matrix and incubated at room temperature for one minute. The column is transferred into a 1.5 ml microcentrifuge tube and centrifuged for 30 seconds to elute the DNA. Ultra-pure DNA is now ready for use. Recovered DNA was quantified using Nanodrop technology and stored at -20°C.

I. Oxford nanopore and Illumina library preparation and sequencing

Based on the antimicrobial susceptibility test results and API20E identification test, a total of 28 meropenem resistant Gram-negative isolates (Pseudomonas spp., Acinetobacter baumanii, Enterobacteriaceae) were sequenced by Illumina and among them 4 isolates were also sequenced by Minion. Illumina sequencing libraries were prepared using the Nextera XT library prep kit (Illumina GmbH, Munich, Germany) and sequenced on Illumina MiSeq sequencer, 2 × 150 bp. Minion sequencing libraries was done using Rapid Barcoding Kit 96 (SQK-RBK110.96) and sequenced using R9.4.1 flow cells (FLO-MIN106) and Flow Cell Wash Kit (EXP-WSH004).

1. *Illumina DNA Preparation:*

Tagmentation of the genomic DNA, this step uses the Bead-Linked Transposomes (BLT) to tagment DNA, which is a process that fragments and tags the DNA with adapter sequences. First, 2–30 µl DNA was added to each well of a 96-well PCR plate so that the total input amount is 100–500 ng. To prepare the Tagmentation Master Mix, equal volumes of BLT and TB1 (Tagment Buffer 1) (10 µl of each per sample) was added. The Mix was vortexed and 20 µl was transferred to each well of the plate

containing a sample and mixed well. Then, the plate was placed on the preprogrammed thermal cycler and the TAG program was run.

Post Tagmentation Cleanup, this step washes the adapter-tagged DNA on the BLT before PCR amplification. Thus, 10 µl TSB (Tagment Stop Buffer) was added to the plate and slowly each well was pipetted 10 times to resuspend the beads, and then sealed. The plate was placed on the preprogrammed thermal cycler and the PTC program was run. When the program ends, the plate is placed on the magnetic stand until liquid is clear. Using a multichannel pipette, supernatant was removed and discarded. Then double washes were done by removing the sample plate from the magnetic stand and adding 100 µl TWB (Tagment Wash Buffer) directly onto the beads to fully resuspend them. The plate was then placed on magnetic stand to remove and discard supernatant. After that, TWB was added, and the plate was kept on the magnetic stand until the next step.

Amplify Tagmented DNA, this step amplifies the tagmented DNA using a limited-cycle PCR program. The PCR step adds Index 1 adapters, Index 2 adapters, and sequences required for sequencing cluster generation. To prepare the PCR Master Mix, equal volumes of EPM (Enhanced PCR Mix) and Nuclease-free water (10 µl of each per sample) was added. With the plate still on the magnetic stand, supernatant was removed and discarded. After removing the plate from the magnet, 40 µl PCR Master Mix was added immediately and directly onto the beads in each sample well. Pipetting is done to mix until the beads are fully resuspended and then appropriate index adapters are added to each sample. Alternatively, the plate was sealed and placed on the preprogrammed thermal cycler and the BLT PCR program was run.

Clean Up Libraries, this step uses double-sided bead purification procedure to purify the amplified libraries. First, the plate was centrifuged and placed on the magnetic stand. Then, 45 µl supernatant was transferred from each well of the PCR plate to the corresponding well of a new plate. Next, Illumina purification master Mix was prepared by mixing 45 µl IP and 40 µl nuclease-free water per sample. The plate was placed on the magnetic stand and then 125 µl supernatant was transferred from each well of the first plate to a new plate and 15 µl of undiluted IPB (Illumina Purification Beads) was added to each well. The plate was incubated at room temperature for 5 minutes then placed on the magnetic stand until the liquid is clear. Without disturbing the beads, supernatant was removed and discarded. After that, double washes were done while the plate was on the magnetic stand with the addition of 200 µl fresh 80 % EtOH without mixing, and then incubation took place for 30 seconds. Supernatant was removed and discarded. Air-dry on the magnetic stand was done for 5 minutes. The plate was removed from the magnetic stand and 32 µl RSB (Resuspension Buffer) was added to the beads and resuspended by pipetting. Incubation is done at room temperature for 2 minutes then the plate is placed on the magnetic stand until the liquid is clear. Finally, 30 µl supernatant was transferred to a clean 1.5 ml microcentrifuge tube that can be stored at -25 °C to -15 °C for up to 30 days.

Pool Libraries, when the DNA input is 100-500 ng, quantifying and normalizing individual libraries generated in the same experiment is not necessary. However, the final yield of libraries generated in separate experiments can vary slightly. To achieve optimal cluster density, equal library volumes were pooled and quantified before sequencing. 5 µl of each library was combined in a 1.7 ml microcentrifuge

tube. Vortexing is done to mix, and then centrifuged. The library pool was quantified using a dsDNA fluorescent dye method, such as Qubit.

2. *Minion library preparation:*

The extracted DNA was prepared in nuclease-free water by transferring 50 ng of genomic DNA per sample into a 1.5 ml Eppendorf and adjusting the volume to 9 µl with nuclease-free water. Second, in 0.2 ml thin-walled PCR tubes, 9 µl of template DNA and 1 µl of Rapid Barcodes (RB01-96, one for each sample) were mixed thoroughly by pipetting. The tubes were incubated at 30°C for 2 minutes and then at 80°C for 2 minutes. Then, they were put briefly on ice to cool. All barcoded samples were pooled noting the total volume. After resuspending the AMPure XP Beads (AXP, or SPRI) by vortexing, an equal volume to the entire pooled barcoded sample of it was added and mixed by flicking the tube. Then, it was incubated on a Hula mixer (rotator mixer) for 5 minutes at room temperature. The sample and pellet were span down on a magnet and the supernatant was pipetted off. While the tube was kept on the magnet, the beads were washed with 1.5 ml of freshly prepared 80% ethanol in nuclease free water without disturbing the pellet. The ethanol was removed using a pipette and the previous step was repeated. Next, the tube was briefly span down and placed back on the magnet. Any residual ethanol was pipetted off. The pellet was allowed to dry for 30 seconds, but not to the point of cracking. After that, the tube was removed from the magnetic rack and the pellet was resuspended in 15 µl Elution Buffer (EB) and incubated for 10 minutes at room temperature. The beads were pelleted on a magnet until the eluate was clear. 15 µl of eluate which contains the DNA library was removed and retained in a clean 1.5

ml Eppendorf DNA LoBind tube and the pelleted beads were disposed. 11 µl of the sample was transferred into a clean 1.5 ml Eppendorf DNA LoBind tube, and 1 µl of Rapid Adapter F (RAP F) was added to 11 µl of barcoded DNA. The tube was gently mixed by flicking and span down. Finally, the reaction was incubated for 5 minutes at room temperature and the prepared library was used for loading into the flow cell.

J. Bioinformatics Analysis

Reads quality control and trimming was done using Trimmomatic (v.1.2.14) after which assembly of the genome was performed using Unicycler on Galaxy (https://usegalaxy.org/). Antimicrobial resistance genes were acquired through CARD (https://card.mcmaster.ca/). Plasmids harbored in each isolate were determined using Plasmid Finder on CGE (https://cge.food.dtu.dk/services/PlasmidFinder/). Sequence types were identified using MLST on Galaxy.

Long reads generated by minion sequencing technique were analyzed using EPI2ME platform(https://epi2me.nanoporetech.com/)

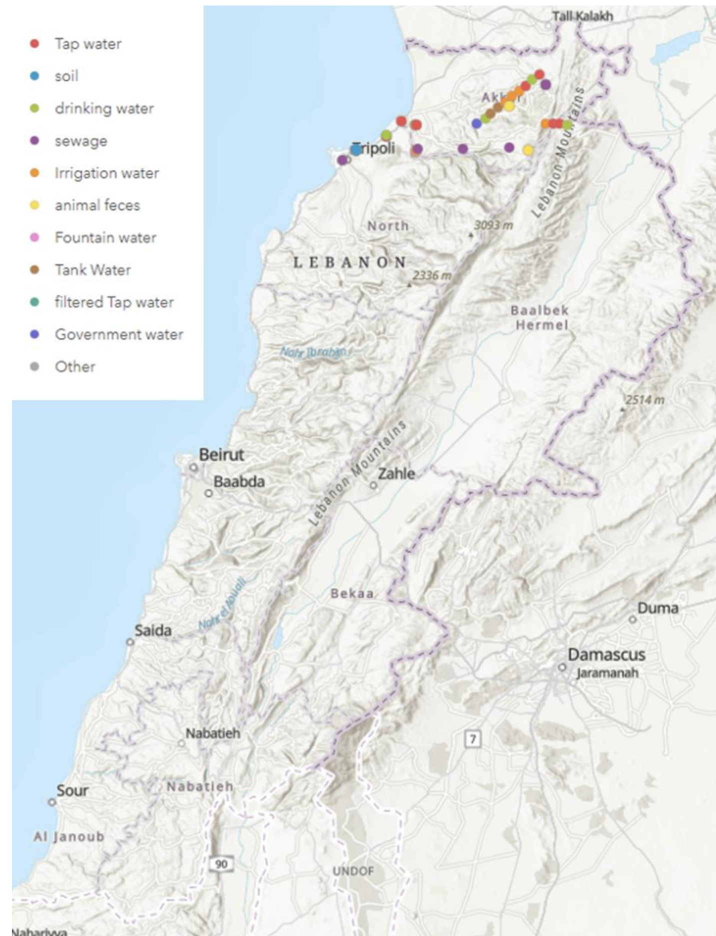

Figure 8. Geographical distribution of the samples collected from wild animals and environment (water, sewage, soil).

Table 1. Distribution of samples based on sample type and province.

Sample	Type of Sample	Code	Province
Water	Tap water	W1	Bebnine -Akkar
	Drinking water	w3	Bebnine -Akkar
	Fountain water	w5	Bebnine -Akkar
	Irrigation water	w8	Bebnine -Akkar
	Drinking water	A38	Saadine - Akkar
	Irrigation water	A60	Mhamarra- Akkar
	Tap water	A61	Mhamarra- Akkar
	Tank water	A81	Akkar (Lea)
Wate r	Irrigation water	A82	Akkar -Bebnine
	Drinking water	A86	Akkar (Nisrine)
	Tank water	A69	Akkar

	Tank water	A69	Akkar
	Government water	A125	Akkar
	Drinking water	A133	Akkar
	Tank Water	A134	Akkar
	Irrigation water	A137	Batoul Edawi - Akkar
	Irrigation water	A138	Batoul Edawi - Akkar
	Tap water	A141	Sundus Bitar-Akkar
	Drinking water	A142	Sundus Bitar-Akkar
	Tap water	A144	Rahma El Sheikh-Akkar
	Irrigation water	A145	Rahma El Sheikh-Akkar
	Tap Water	A147	Rukayya Sweid -Akkar
	Drinking water	A148	Rukayya Sweid -Akkar
	Tap water	A149	Akkar
	Tap water	A210	Menieh well- Akkar
	Tap water	A212	Menieh well- Akkar
	Filtered Water for using	A213	Menieh - Akkar
	Drinking Filtered Water	A214	Menieh - Akkar
	Tap water	A224	Thakanat Al werwar - Akkar
	Drinking Water	A225	Thakanat Al werwar - Akkar
	Drinking Water	A227	Beddawi - Akkar
	Tap water	A228	Beddawi - Akkar
	Tap water	A229	Akkar
	Tap water	A231	Gov Well - Akkar
	Filtered Water for using	A232	Gov Well - Akkar
	Drinking Water	A239	Nabiha Dahdah - Akkar
	Drinking water	A80	Akkar (Lea)
	Drinking water	A83	Akkar (Mahmoud Skeif)
	Fountain water	A84	Akkar
	Tank water	A85	Akkar
	Tap water	A87	Akkar (Nisrine)
	Tank water UN 012 ITS	A124	Akkar
	Tank Water	A127	Akkar
	Tank Water (Khodor Soufan)	A128	Akkar
	Drinking water	A131	Akkar
	Drinking water	A132	Akkar
	Drinking water filtered	A135	Akkar
Water	Drinking Water	Z211	Quob Elias (Al- Sabil)
	Tank water	Z212	Quob Elias
	Tap Water	Z238	Morghan Ahmad -Akkar

	Irrigation-Tap-Water	A139	Batoul Edawi - Akkar
	Irrigation-Tap-Water	A140	Batoul Edawi - Akkar
	Tap Water	A156	Abed Muwati-Akkar
	Drinking water	A157	Abed Muwati-Akkar
	Tap Water	A158	Takla Akiki - Akkar
	Drinking water	A159	Takla Akiki-Akkar
	Irrigation water	A207	Bqaatouta- Akkar
	Drinking water	A143	Rahma El Sheikh-Akkar
	Irrigation water	A209	Bqaatouta- Akkar
	Tap water	A211	Menieh well- ITS029- Akkar
	Drinking Water	A230	Aakkar
	Filtered Water	A233	Gov Well - Akkar
Sewage	Sewage	QOB_W40	Quob-akkar
	sewage	TRP_W40	Tripoli
	sewage	QOBW41	Akkar- Qobayat
	Sewage	S2-1	Bebnine -Akkar
	Sewage	S2-2	Bebnine -Akkar
	Sewage	S3	Bebnine -Akkar
Wild animals	Bat feces	M-001	Aakkar El Aatiqa
	Bat feces	M-002	Aakkar El Aatiqa
	Otter feces	M-004	Aakkar El Aatiqa
	Otter feces	M-005	Aakkar El Aatiqa
	Rodent feces	M-007	Aakkar El Aatiqa
	Fox feces	M-009	Aakkar El Aatiqa
	Bat Feces	M-011	Aakkar El Aatiqa
	Bat Feces	M-013	Aakkar El Aatiqa
	Bat Feces	M-014	Aakkar El Aatiqa
	Bat Feces	M-015	Aakkar El Aatiqa
Soil	Soil	SO 001	beddawi gardens farm
	Soil	SO 002	beddawi gardens farm
	Soil	SO 003	beddawi gardens farm
	Soil	SO 004	beddawi gardens farm
	Soil	SO 005	beddawi gardens farm

CHAPTER IV

RESULTS

A. Number of bacterial strains isolated from animals and environmental samples

Out of 83 samples, 57 samples produced one or more different colonies on meropenem supplemented MacConkey agar plates to produce a total of 76 bacterial isolates. These were distributed as: 45 isolates from water samples, 9 isolates from sewage samples, 10 isolates from soil samples, and 12 isolates from animal samples. (Figure 10, Table 3)

B. Gram staining

Of all isolated organisms 92 % (70/76) were identified as Gram-negative. Gram positive only represent 8 % (6/76).

C. Identification of Gram- negative strains by API20E

Among the recovered organisms, 75.7 % (53/70) were able to be identified by API as an acceptable profile. The most common were *E. coli* (10/70; 14.3 %), *Pseudomonas spp.* (23/70; 33%), and *Acinetobacter baumanii* (6/70; 8.7 %). The remaining 20.3 % (14/70) were distributed between *Burkholderia cepacia, Stenotrophomonas maltophilia, Kluyvera spp., Citrobacter freudii, Citrobacter baraaki, K. pneumonia, Pasteurella spp.., Enterobacter spp., vibrio fluvialis, and Aeromonas spp.* (Figure 11, Table 4).

D. Antibiotic Resistance Profiles of identified Gram-negative isolates

Disk diffusion results showed that out of the 53 identified isolates, 75.47 % (40/53) were resistant to meropenem, 47.2 % (25/53) to Aztreonam, 45.3 % (24/53) to Ceftazidime, 37.7 % (20/53) to Ciprofloxacin, and 35.8 % (19/53) to Gentamicin (Table 5). Moreover, 23 isolates (43.4 %) were identified as MDR as they are resistant to at least one agent in three or more antimicrobial categories with 56.5% of them resistant to all the selected agents (Table 5).

E. Whole Genome Sequencing

Library preparation and sequencing was done for 28 isolates which were identified by API20E and AST as meropenem resistant- *Enterobacteriaceae*, Pseudomonas *spp.*, and *Acinetobacter baumanii*. Analysis was done for only 12 of them distributed as follow: 2 sewage samples, 1 soil sample, 8 water samples, 1 animal sample.

F. Multi Locus Sequence Typing (MLST)

Sequence types and international clones were detected by MLST on Galaxy. Seven housekeeping gene loci were chosen for MLST analysis for *Acinetobacter baumanii* and *E. coli* isolates and eight housekeeping gene loci were chosen for MLST analysis for Pseudomonas spp. (Table 6).

G. Resistant Genes

The antibiotic susceptibility testing results were further validated in silico using CARD. 67 different genes were detected by *E. coli* isolates, 29 different genes were

detected by Acinetobacter baumanii isolates, and 4 different genes were detected by Pseudomonas putida. Each gene confers resistance to one or more of the 22 categories of antimicrobial agents detected by CARD (macrolide, fluoroquinolone, peptide, nitroimidazole, monobactam, carbapenem, cephalosporin, glycylcycline, cephamycin, penam, tetracycline, rifamycin, phenicol, penem, aminoglycoside, aminocoumarin, phosphonic acid, nucleoside, diaminopyrimidine, glycopeptide, sulfonamide, lincosamide). (Table 7)

As for Carbapenems, 9 different genes were detected and among them 5 different genes were detected in *E. coli* isolates (MarA, SoxS, TolC, NDM-5, OXA-1), and 5 different genes were present in *Acinetobacter baumanii* isolates *(*adeI, adeK, adeJ, OXA-66, OXA-23).

H. Plasmids

Plasmids were detected in silico by Plasmid Finder 1.3. Only plasmids carried by *Enterobacteriaceae* spp. were able to be detected by this platform. Successfully, 6 different plasmids were detected that belong to the Inc group. IncFIA, IncFIB (AP001918), IncFII, IncI2(Delta) plasmids were present in *E. coli* strain isolates from irrigation water. IncFIA, IncFIl, Incl (Gamma), IncY plasmids were present in *E. coli* strain isolated from sewage samples. IncFIA, IncFIB (AP001918), IncFIl (pRSB107) plasmids we're present in *E. coli* strain isolated from otter.

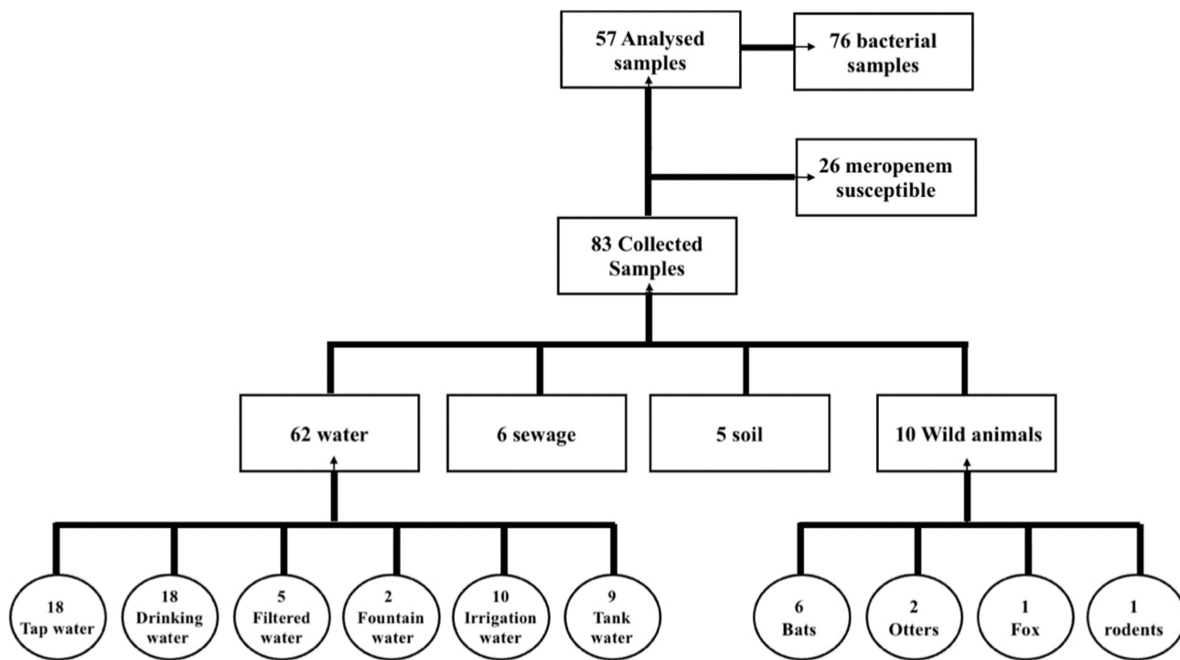

Figure 9. Flowchart of sample collection and Process.

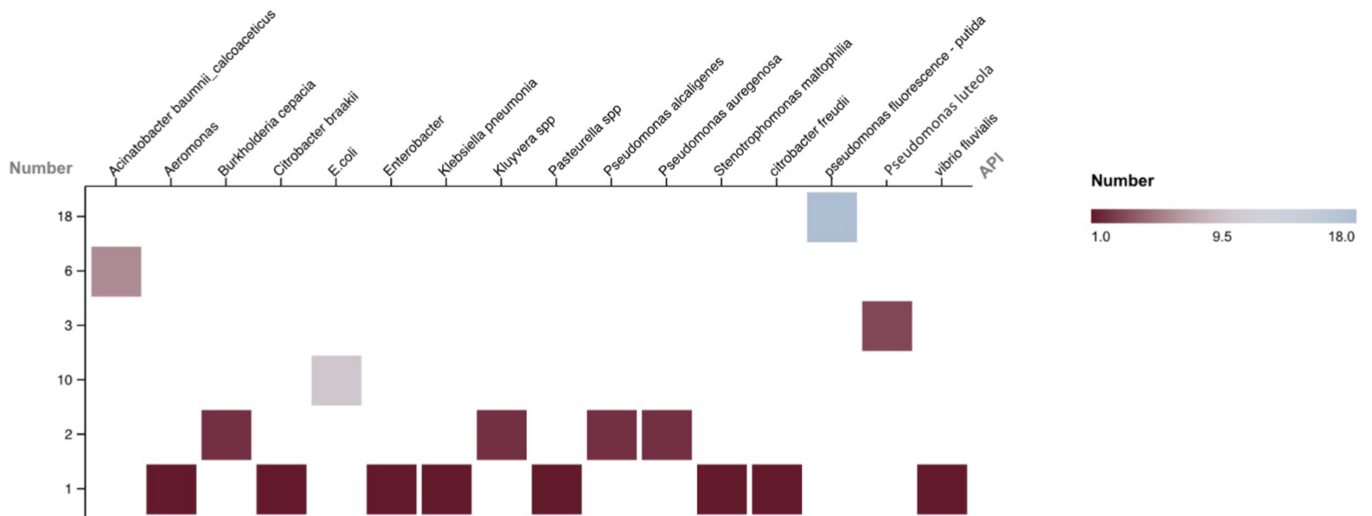

Figure 10. Total number of bacterial strains identified by API-20E

Table 2. Bacterial strains isolated on MacConkey agar plates supplemented with meropenem distributed based on sample type

Sample type	Strain
Water	W1
Water	w3-1
Water	w5-1
Water	w5-2
Water	w8-1
Water	w8-2
Water	A38
Water	A60-1
Water	A60-2
Water	A61
Water	A81
Water	A82
Water	A86-1
Water	A86-2
Water	A69
Water	A69
Water	A125
Water	A133
Water	A134-1
Water	A134-2
Water	A137-1
Water	A137-2
Water	A138
Water	A141

Sample type	Strain
Water	A142
Water	A144
Water	A145
Water	A147-1
Water	A147-2
Water	A148
Water	A149
Water	A210
Water	A212
Water	A213
Water	A214
Water	A224
Water	A225
Water	A227
Water	A228-1
Water	A228-2
Water	A229
Water	A231
Water	A232
Water	A239
Sewage	QOB_W40-1
Sewage	QOB_W40-2
Sewage	TRP_W40-1
Sewage	TRP_W40-2
Sewage	QOB_W41-1

Sample type	Strain
Sewage	QOB_W41-3
Sewage	S2-1
Sewage	S2-2
Sewage	S3
Soil	SO 001 -1
Soil	SO 002 -1
Soil	SO 003
Soil	SO 004 -1
Soil	SO 004 -3
Soil	SO 004 -4
Soil	SO 004 -5
Soil	SO 005-4
Soil	SO 005-6
Wild animals	M005 a1
Wild animals	M011
Wild animals	M013
Wild animals	M014
Wild animals	M015 a3
Wild animals	M015 a5
Wild animals	M015 a6
Wild animals	M002
Wild animals	M009
Wild animals	M001
Wild animals	M-004
Wild animals	M007

Table 3. Bacterial strains identified by API-20E

Sample Type	Code	API-20E
Tap water	W1	*Pseudomonas fluorescence – putida*
	A228-1	*Acinetobacter baumnii_calcoaceticus*
	A228-2	*Pseudomonas fluorescence – putida*
	A229	*Acinetobacter baumnii_calcoaceticus*
	A231	*pseudomonas luteola*
	A149	*Pseudomonas fluorescence – putida*
	A212	*Acinetobacter baumanii*
	A224	*pseudomonas luteola*
	A69	*Aeromonas*
	A69	*Kluyvera spp*
	A133	*Citrobacter freudii*
	A147-1	*Stenotrophomonas maltophilia*
	A61	*Pseudomonas aeruginosa*
Drinking water	w3-1	*Pseudomonas fluorescence – putida*
	A225	*Acinetobacter baumnii_calcoaceticus*
	A227	*Acinetobacter baumnii_calcoaceticus*
	A148	*vibrio fluvialis*
	A142	*Pseudomonas fluorescence – putida*
Irrigation water	A82	*E. coli*
	w8-2	*Burkholderia cepacia*
	A60-1	*E. coli*
	A60-2	*Pseudomonas fluorescence – putida*
	A137-1	*E. coli*
	A137-2	*Enterobacter*
Tank water	A81	*Pseudomonas fluorescence – putida*
	A134-1	*Acinetobacter baumnii_calcoaceticus*
	A134-2	*Klebsiella pneumonia*

Sample Type	Code	API-20E
Filtered water	A214	*Pseudomonas luteola*
	A232	*Pseudomonas fluorescence - putida*
Fountain water	w5-2	*Burkholderia cepacia*
Sewage	QOB_W40-1	*Pseudomonas aeruginosa*
	QOB_W40-2	*E. coli*
	TRP_W40-1	*E. coli*
	TRP_W40-2	*Pseudomonas fluorescence - putida*
	QOB_W41-1	*E. coli*
	QOB_W41-3	*E. coli*
	S3	*E. coli*
	S2-1	*Pasteurella spp*
Soil	S0-001	*Pseudomonas alcaligenes*
	S0-002	*Pseudomonas alcaligenes*
	S0-004-2	*Pseudomonas fluorescence - putida*
	S0-004-5	*Pseudomonas fluorescence - putida*
	S0-005-3	*Pseudomonas fluorescence - putida*
	S0-005-4	*Pseudomonas fluorescence - putida*
	S0-005-6	*Pseudomonas fluorescence - putida*
Wild animals	M001	*Enterobacteriaceae*
	M004	*Pseudomonas fluorescence - putida*
	M005-a1	*E. coli*
	M011	*Kluyvera*
	M013	*Citrobacter braakii*
	M014	*E. coli*
	M015-a5	*Pseudomonas fluorescence - putida*
	M015-a6	*Pseudomonas fluorescence - putida*

Table 4. Antibiotic susceptibility results for all identified Gram-negative organisms using 5 different antimicrobial agents covering 5 different classes. (MDR strains marked in light yellow color)

MEM= meropenem, ATM= Aztreonam, CAZ= Ceftazidime, GMN= Gentamicin, CIP= Ciprofloxacin, R= Resistant, S= Susceptible, I= Intermediate

PP= *Pseudomonas putida*, PA= *Pseudomonas aeruginosa*, PL= *Pseudomonas Luteola*, PG= *Pseudomonas alcaligenes*, BC= *Burkholderia cepacia*, SM= *Stenotrophomonas maltophilia*, AB= *Acinetobacter baumanii*, PS=*Pasteurella spp.*, KL= *Kluyvera spp.*, CF= *Citrobacter freudii*, CB= *Citrobacter baraaki*, EB= *Enterobacter spp.*, KP= *Klebsiella pneumoniae*, AE= *Aeromonas spp.*, VF= *Vibrio fluvialis*, EC= *E. coli*,

			Disc Diffusion				
Type of Specimen	**Code**	**Isolate**	**MEM**	**ATM**	**CAZ**	**GMN**	**CIP**
Tap water	W1	PP	21- S	22- S	20- S	23- S	30- S
	A228-1	AB	6- R	9- R	6- R	6- R	6- R
	A228-2	PP	15- R	22- S	25- S	22- S	30- S
	A229	AB	6- R	13- R	6- R	6- R	6- R
	A231	PL	6- R	8- R	6- R	6- R	6- R
	A149	PP	17- I	27- S	29- S	29- S	35- S
	A212	AB	6- R	6- R	6- R	6- R	6- R
	A224	PL	6- R	13- R	6- R	6- R	6- R
	A69	AE	7-R	12-R	6-R	20-S	6-R
	A69	KL	10 - R	15- R	6-R	19- S	6- R
	A133	CF	14- R	28- S	27- S	26- S	36- S
	A147-1	SM	6- R	19- I	33- S	30- S	39- S
	A61	PA	18- I	26- S	26- S	25- S	32- S
Drinking water	w3-1	PP	10- R	18- I	13- R	29- S	35- S
	A225	AB	6- R	12- R	6- R	6- R	6- R
	A227	AB	6- R	9- R	6- R	6- R	6- R
	A148	VF	9-R	12-R	20- S	24- S	26-S
	A142	PP	6- R	8- R	20- S	26- S	30- S
Irrigation water	A82	EC	9- R	21- S	6-R	20- S	6- R
	w8-2	BC	6- R	6-R	20- I	9- R	22- I
	A60-1	EC	30 - S	32- S	27- S	20- S	30- S
	A60-2	PP	14- R	18- I	20- S	18- S	25- S
	A137-1	EC	14- R	15- R	17- R	20- S	26- S

	A137-2	EB	20- I	19- I	23- S	25- S	30- S
Tank water	A81	PP	19- S	19- I	22- S	22- S	29- S
	A134-1	AB	6- R	8- R	6-R	6-R	6-R
	A134-2	KP	29- S	31- S	28- S	20- S	29- S
Filtered water	A214	PL	6- R	9- R	6- R	6- R	6- R
	A232	PP	6- R	10- R	6- R	6- R	6- R
Fountain water	w5-2	BC	6- R	6-R	6-R	21- S	25- I
Sewage	QOB_W40-1	PA	12 - R	24- S	24- S	12- R	25- S
	QOB_W40-2	EC	7- R	10- R	6- R	20- S	6- R
	TRP_W40-1	EC	12 - R	10-R	18- I	20- S	25- I
	TRP_W40-2	PP	18- I	16- I	17- R	20- S	25- I
	QOB_W41-1	EC	7- R	6- R	6-R	19- S	6-R
	QOB_W41-3	EC	10- R	12- R	6-R	20- S	6-R
	S3	EC	11- R	11- R	6-R	12- R	6-R
	S2-1	PS	20-S	35-S	37-S	32-S	47-S
Soil	S0-001	PG	6- R	25- S	27-S	6- R	36- S
	S0-002	PG	6- R	23- S	25- S	6- R	35- S
	S0-004-2	PP	20- S	23- S	25- S	23- S	25- S
	S0-004-5	PP	6- R	25- S	23- S	6- R	34- S
	S0-005-3	PP	9- R	16- I	25- S	25- S	26- S
	S0-005-4	PP	16- I	18- I	21- S	20- S	25- S
	S0-005-6	PP	10- R	17- I	21- S	18- S	24- I
Wild animals	M001	EC	21- S	27- S	18- I	21- S	27- S
	M004	PP	19- S	20- I	28- S	35- S	6- R
	M005-a1	EC	8- R	6- R	6- R	6- R	6- R
	M011	KL	10- R	6- R	6- R	10- R	19- I
	M013	CB	6- R	6- R	6- R	6- R	6- R
	M014	EC	14- R	31- S	20- I	20- S	36- S
	M015-a5	PP	12- R	22- S	25-S	21- S	21- S
	M015-a6	PP	13- R	20- I	22- S	21- S	25- S

Table 5. Bacterial type, bacteria identification, and sequence type (ST) of bacterial isolates identified by MLST based on specific housekeeping genes

ID	Bacteria Identification	ST	Housekeeping Genes							
A231	abaumannii_2	2	Pas_cpn60(2)	Pas_fusA(2)	Pas_gltA(2)	Pas_pyrG(2)	Pas_recA(2)	Pas_rplB(2)	Pas_rpoB(2)	
A 82	ecoli_achtman_4	405	adk(35)	fumC(37)	gyrB(29)	icd(25)	mdh(4)	purA(5)	recA(73)	
A137-1	pputida	-	argS(~62)	gyrB(~74)	ileS(23)	nuoC(~15)	ppsA(~21)	recA(~36)	rpoB(7)	rpoD(31)
A148	pputida	-	argS(10)	gyrB(22)	ileS(24)	nuoC(18)	ppsA(~27)	recA(32)	rpoB(~8)	rpoD(~110)
A214	abaumannii_2	2	Pas_cpn60(2)	Pas_fusA(2)	Pas_gltA(2)	Pas_pyrG(2)	Pas_recA(2)	Pas_rplB(2)	Pas_rpoB(2)	
A228	abaumannii_2	2	Pas_cpn60(2)	Pas_fusA(2)	Pas_gltA(2)	Pas_pyrG(2)	Pas_recA(2)	Pas_rplB(2)	Pas_rpoB(2)	
Quab 41-1	ecoli_achtman_4	361	adk(10)	fumC(99)	gyrB(5)	icd(91)	mdh(8)	purA(7)	recA(2)	
A212	abaumannii_2	2	Pas_cpn60(2)	Pas_fusA(2)	Pas_gltA(2)	Pas_pyrG(2)	Pas_recA(2)	Pas_rplB(2)	Pas_rpoB(2)	
A 227	abaumannii_2	2	Pas_cpn60(2)	Pas_fusA(2)	Pas_gltA(2)	Pas_pyrG(2)	Pas_recA(2)	Pas_rplB(2)	Pas_rpoB(2)	
Quab40-2	ecoli_achtman_4	361	adk(10)	fumC(99)	gyrB(5)	icd(91)	mdh(8)	purA(7)	recA(2)	
SO005-6	pputida	-	argS(69)	gyrB(~87)	ileS(~120)	nuoC(83)	ppsA(~104)	recA(~103)	rpoB(45)	rpoD(~105)
M005 a1	ecoli_achtman_4	648	adk(92)	fumC(4)	gyrB(87)	icd(96)	mdh(70)	purA(58)	recA(2)	

Table 6. AMR Genes identified by CARD for 12 different isolates toward 22 different agents.

ID	AMR Genes																					
	macrolide	fluoroquinolone	peptide	nitroimidazole	monobactam	carbapenem	cephalosporin	glycylcycline	cephamycin	penam	tetracycline	rifamycin	phenicol	penem	aminoglycoside	aminocoumarin	phosphonic acid	nucleoside	diaminopyrimidine	sulfonamide	glycopeptide	lincosamide
A231	AmvA, adeJ, adeI,adeK, abeS, mphE	gyrA , parC , AbaQ, adeF, adeH, adeG, adeL, adeJ, adeI,adeK	LpsB	–	–	adeJ, adeI,adeK	adeJ, adeI,adeK, ADC-73	adeR, adeA, adeC	–	adeJ, adeI, adeK	adeF, adeH, adeG, adeL, adeJ, adeI,adeK, adeR, adeA, adeC, tet(B), tetR	adeJ, adeI,adeK	adeJ, adeI,adeK	–	ANT(3")-IIc, APH(6)-Id, APH(3")-Ib, armA	abeS	AbaF	–	adeJ, adeI,adeK	–	–	adeJ, adeI,adeK
A 82	TolC, evgA, H-NS, emrE, CRP, mdtE, mdtF, gadX	TolC, emrR, emrB, mdtH, emrA, gyrA , parC, marA, evgA, H-NS, AcrS, rsmA, evgS, acrA, acrB, CRP, mdtE, mdtF, gadX, soxS, MarR, AcrR , soxR	TolC	msbA	marA, TEM-1, soxS	TolC, marA, NDM-5, soxS	TolC, marA, H-NS, TEM-1, NDM-5, AcrS, evgS, acrA, acrB, soxS, EC-8, PBP3, MarR, AcrR , soxR	TolC, marA, AcrS, acrA, acrB, soxS, MarR, AcrR , soxR	TolC, marA, H-NS, AcrS, soxS, PBP3	TolC, marA, evgA, H-NS, TEM-1, NDM-5, AcrS, acrA, acrB, CRP, mdtE, mdtF, gadX, soxS, PBP3, MarR, AcrR	TolC, marA, evgA, emrK, emrY, H-NS, AcrS, mdfA, acrA, acrB, soxS, MarR, AcrR , soxR	TolC, marA, AcrS, acrA, acrB, soxS, MarR, AcrR , soxR	TolC, marA, AcrS, rsmA, acrA, acrB, soxS, MarR, AcrR , soxR	TolC, marA, TEM-1, acrB, soxS	TolC, baeR, kdpE	TolC, baeR, baeS, mdtB, mdtC, mdtA	mdtG, GlpT,cyaA, baeS	mdtO, mdtP, mdtN, leuO	rsmA	–	BRP(MBL)	–

										, soxR												
A137-1	–	AbaQ, adeF, YajC	–	–	–	–	YajC	YajC	–	YajC	adeF, YajC	YajC	YajC	–	–	–	–	–	–	–	vanT, YajC	–
A148	–	AbaQ, YajC	–	–	–	–	YajC	YajC	–	YajC	YajC	YajC	YajC	–	–	–	–	–	–	–	YajC	–
A214	mphE, msrE, abeS, adeI, adeK, adeJ, AmvA	adeL, adeG, adeF, adeH, adeI, adeK, adeJ, AbaQ, gyrA, parC	LpsB	–	–	adeI, adeK, adeJ, OXA-66, OXA-23	adeI, adeK, adeJ, ADC-73, OXA-66, OXA-23	adeA, adeC, adeR	–	OXA-66, OXA-23	adeL, adeG, adeF, adeH, adeI, adeK, adeJ, adeA, adeC, adeR, tetR	adeI, adeK, adeJ	adeI, adeK, adeJ	adeI, adeK, adeJ	armA, APH(6)-Id, APH(3")-Ib, ANT(3")-IIc	abeS	AbaF	–	adeI, adeK, adeJ	–	–	adeI, adeK, adeJ
A228-1	abeS, msrE, mphE, adeK, adeI, adeJ, AmvA	adeL, adeG, adeF, adeH, adeI, adeK, adeJ, AbaQ, gyrA, parC	LpsB	–	–	adeK, adeI, adeJ, OXA-66, OXA-23	adeK, adeI, adeJ, ADC-73, OXA-66, OXA-23	adeC, adeA, adeR	–	OXA-66, OXA-23	adeG, adeL, adeH, adeF, adeK, adeI, adeJ, adeC, adeA, adeR, tet(B), tetR	adeK, adeI, adeJ	adeK, adeI, adeJ	adeK, adeI, adeJ	armA, APH(6)-Id, APH(3")-Ib, ANT(3")-IIc	abeS	AbaF	–	adeK, adeI, adeJ	–	–	adeK, adeI, adeJ
Ouab 41-1	evgS, evgA, TolC, H-NS, mdtE, CRP, gadX, mdtF, Mrx, emrE, mphA, KpnE	evgS, evgA, marA, acrA, acrB, soxS , MarR, emrR, emrB, mdtH, gyrA ,parC, emrA, TolC, H-NS, AcrS, AcrE, AcrF, mdtE, CRP, gadX, mdtF, rsmA, mdtM	PmrF, bacA, TolC, YojI, ep	msbA	marA, soxS, MarR	marA, soxS , MarR, TolC, NDM-5	marA, acrA, acrB, soxS , MarR, TolC, H-NS, AcrS, AcrE, AcrF, NDM-5, KpnE, ampC	marA, acrA, acrB, soxS, MarR, TolC, AcrS	marA, soxS, MarR, TolC, H	evgS, evgA, marA, acrA, acrB, soxS, MarR , TolC, H-NS, AcrS, AcrE, AcrF	evgS, evgA, emrK, marA, acrA, acrB, soxS , MarR, TolC, H-NS, AcrS, emrY, tet(A), mdfA, KpnE	marA, acrA, acrB, soxS , MarR, TolC, AcrS, KpnE	marA, acrA, acrB, soxS , MarR, TolC, AcrS, rsmA, mdtM	marA, soxS , MarR, TolC	cpxA, TolC, kdpE, aadA2, baeS, baeR, KpnE	cpxA, TolC, mdtB, acrD, mdtA, mdtC, baeS, baeR	mdtG, GlpT	mdtN, mdtP, leuO, mdtO, mdtM	dfrA12, rsmA	sul1	BRP(MBL)	mdtM

			tA, KpnE						-NS, CMY-145, AcrS, AcrE, AcrF, NDM-5	, mdtE, CRP, gadX, mdtF, NDM-5, ampC												
A212	mphE, msrE, abeS, adeI, adeK, adeJ, AmvA	adeL, adeG, adeF, adeH, adeI, adeK, adeJ, AbaQ, gyrA, parC	LpsB	–	–	adeI, adeK, adeJ, OXA-66, OXA-23	adeI, adeK, adeJ, ADC-73, OXA-66, OXA-23	adeA, adeC, adeR	–	OXA-66, OXA-23	adeL, adeG, adeF, adeH, adeI, adeK, adeJ, adeA, adeC, adeR, tetR	adeI, adeK, adeJ	adeI, adeK, adeJ	adeI, adeK, adeJ	armA, APH(6)-Id, APH(3'')-Ib, ANT(3'')-IIc	abeS	AbaF	–	adeI, adeK, adeJ	–	–	adeI, adeK, adeJ
A 227	abeS, msrE, mphE, adeK, adeI, adeJ, AmvA	adeL, adeG, adeF, adeH, adeI, adeK, adeJ, AbaQ, gyrA, parC	LpsB	–	–	adeK, adeI, adeJ, OXA-66, OXA-23	adeK, adeI, adeJ, ADC-73, OXA-66, OXA-23	adeC, adeA, adeR	–	OXA-66, OXA-23	adeG, adeL, adeH, adeF, adeK, adeI, adeJ, adeC, adeA, adeR, tet(B), tetR	adeK, adeI, adeJ	adeK, adeI, adeJ	adeK, adeI, adeJ	armA, APH(6)-Id, ANT(3'')-IIc, APH(3'')-Ib, APH(3')-VIa	abeS	AbaF	–	adeK, adeI, adeJ	–	–	adeK, adeI, adeJ
Ouab40-2	evgS, evgA, TolC, H-NS, mdtE, CRP, gadX, mdtF, Mrx, emrE, mphA, KpnE	evgS, evgA, marA, acrA, acrB, soxS , MarR, emrR, emrB, mdtH, gyrA ,parC, emrA, TolC, H-NS, AcrS, AcrE, AcrF, mdtE, CRP, gadX,	PmrF, bacA,	msbA	marA, soxS, MarR	marA, soxS , MarR, TolC, NDM-5	marA, acrA, acrB, soxS , MarR, TolC, H-NS, AcrS, AcrE, AcrF, NDM-5, KpnE, ampC	marA, acrA, acrB, soxS, MarR,	marA, soxS,	evgS, evgA, marA, acrA, acrB, soxS	evgS, evgA, emrK, marA, acrA, acrB, soxS , MarR, TolC, H-NS, AcrS, emrY, tet(A), mdfA, KpnE	marA, acrA, acrB, soxS , MarR,	marA, acrA, acrB, soxS , MarR,	marA, soxS , MarR, TolC	cpxA, TolC, kdpE, aadA2, baeS, baeR, KpnE	cpxA, TolC, mdtB, acrD, md	mdtG, GlpT	mdtN, mdtP, leuO, mdtO, mdtM	dfrA12, rsmA	sul1	BRP(MBL)	mdtM

		mdtF, rsmA, mdtM	TolC, YojI, eptA, KpnE					TolC, AcrS	MarR, TolC, H-NS, CMY-145, AcrS, AcrE, AcrF, NDM-5	, MarR , TolC, H-NS, AcrS, AcrE, AcrF, mdtE, CRP, gadX, mdtF, NDM-5, ampC		TolC, AcrS, KpnE	TolC, AcrS, rsmA, mdtM			tA, mdtC, baeS, baeR						
SO005-6	–	adeF, YajC, soxR	–	–	–	–	YajC	YajC, soxR	–	YajC, soxR	adeF, YajC, soxR	YajC, soxR	YajC, soxR	–	–	–	–	–	–	–	YajC	–

M005a1	TolC, evgA, H-NS, emrE, CRP, mdtE, mdtF, gadX, Mrx	TolC, emrR, emrB, mdtH, emrA, gyrA , parC, marA, evgA, H-NS, AcrS, rsmA, evgS, acrA, acrB, CRP, mdtE, mdtF, gadX, soxS, MarR, AcrR , soxR	TolC	msbA	marA, TEM-1, soxS	TolC, marA, NDM-5, soxS, OXA-1	TolC, marA, H-NS, TEM-1, NDM-5, AcrS, evgS, acrA, acrB, soxS, EC-8, PBP3, MarR, AcrR , soxR, CTX-M-15	TolC, marA, AcrS, acrA, acrB, soxS, MarR, AcrR , soxR	TolC, marA, H-NS, AcrS, soxS, PBP3	TolC, marA, evgA, H-NS, TEM-1, NDM-5, AcrS, acrA, acrB, CRP, mdtE, mdtF, gadX, soxS, PBP3, MarR, AcrR, soxR, CTX-M-15	TolC, marA, evgA, emrK, emrY, H-NS, AcrS, mdfA, acrA, acrB, soxS, MarR, AcrR , soxR	TolC, marA, AcrS, acrA, acrB, soxS, MarR, AcrR , soxR	TolC, marA, AcrS, rsmA, acrA, acrB, soxS, MarR, AcrR , soxR	TolC, marA, TEM-1, acrB, soxS	TolC, baeR, kdpE	TolC, baeR, baeS, mdtB, mdtC, mdtA	mdtG, GlpT,cyaA, baeS	mdtO, mdtP, mdtN, leuO	rsmA		BRP(MBL)	

CHAPTER V

DISCUSSION

Antimicrobial resistance (AMR) is widely recognized as a major public health threat that occurs at the animal–human–environment ecosystem. [67] Despite global efforts to minimize the emergence and spread of AMR, studies conducted in clinical, veterinary, and environmental settings consistently show an increase in AMR levels and the emergence of new antimicrobial resistance genes in important pathogens in which some were reported in Lebanon. [66] However, the extent of AMR in the country remains unclear due to the lack of representative and well-established surveillance systems and limited national studies on AMR. [66]

As addressed by WHO, the use of antimicrobial agents to treat various infectious diseases in animals is often the same or similar to those used in humans. This means that the transmission and spread of ARBs in humans is closely linked to that occurring in animals and the environment. Furthermore, the increasing use of antimicrobial agents in human, animal, and environmental contexts is recognized as a potential driver of AMR selection. [67] As a result, the spread of AMR bacteria, genes, and mobile genetic elements across human, animal, and environmental compartments is a complex process that occurs through numerous pathways. [67,68]

Thus, to effectively address AMR issue and to better understand the dynamics influencing the selection and transmission of AMR, a One Health Approach should be implemented. [66] To this purpose, 83 different samples were collected from soil, sewage, water, and wild animals in North Lebanon. As North Lebanon corresponds to

the area with the highest number of inhabitants, high density of refugees' camps, and particularly shows high levels of ARGs. [69]

A key Finding in this study was the isolation of meropenem resistant *E. coli*, *Acinetobacter baumanii* and Pseudomonas spp. from environmental and animal samples knowing that these pathogens are grouped among the critical priority list by WHO. [33] Various factors can explain the occurrence of carbapenem resistance. These factors include overcrowding, unsanitary conditions, polluted water sources, and inappropriate antibiotic use, which together promote the spread of carbapenemase-producing bacteria across ecosystem. [71] This is further validated by the fact that about only 11.65% of the population in the North of Lebanon were connected to serviceable sewage networks. [39] Thus, these bacteria in sewage and water may contaminate the wider environment and spread resistance genes into many species.

The second noteworthy finding was the detection of *E. coli* isolates harboring NDM-5 gene isolated from irrigation water and sewage samples with ST361(n=2), ST405 (n=1), respectively. In addition to the isolation of *E. coli* strain from an otter harboring both NDM-5 and OXA-1 and of ST648. NDM-5 is a variation of NDM-1 that has two different amino acids (Val-88-Leu and Met-154-Leu) and shows greater resistance to extended-spectrum cephalosporins and carbapenems. NDM-5 encodes for carbapenemases and act by inhibiting the action of carbapenem. Such strains have been reported previously in clinical isolates and was first detected in 2011 in a patient in the United Kingdom with *E. coli* infection. [72] In Lebanon, blaNDM-5 was found in *K. pneumoniae* with clinical origin which suggest its possible transmission between bacterial species. [74] In addition, NDM-5 positive *E. coli* with ST361 was recovered from water sample in El Qa'a refugee camp. This finding is inconsistent with our

sequence results that detected the presence of NDM-5 positive *E. coli* of ST361 in 2 sewage samples in Quab Akkar.

As for the *E. coli* sample detected in irrigation water, it belongs to ST405 and harbors similarly NDM-5 gene. [(72)] This strain was also detected in companion animal samples (dogs) and humans in Finland. The presence of same strain in water, animals, and human suggest the possible transmission of this strain across ecosystem.

To our knowledge, this project was the first to detect *E. coli* ST648 isolate co-producing NDM-5 and OXA-1 in wild animals in Lebanon. In 2022, Corbellini *et al,* mentioned his first detection of *E. coli* isolates co-producing NDM-5 and OXA-1 in Italy; these strains belonged to ST44, ST405 and ST167. [(82)] As for this ST, it is also mentioned by another recent study the first detection of of ST648 *E. coli* in wild birds, however, it harbored ESBL and pAmpC. [(81)] The international clone ST648 was found to be predominantly MDR and virulent, and one of the most commonly reported international sequence types (STs) in the human–animal–environmental interface worldwide.[(81)] In this study, the isolated E. coli strain of ST648 showed resistance to all tested antimicrobials by disk diffusion, and through sequence data 67 different genes conferring resistance against wide range of agents were also detected along with the co-occurrence of OXA-1 and NDM-5. The presence of MDR *E. coli* strain in Otters could be a reflect of water contamination and digestion of contaminated seafood. This finding also highlights the need to monitor AMR in wildlife as this sector was underestimated.

Further analysis for isolate harboring NDM-5 with/without OXA-1 detected the presence of different plasmids in both strains including IncFIA, IncFIB (AP001918), IncFII, IncI2(Delta), Incl (Gamma), IncY. These plasmids facilitate the spread and transmission of resistance genes among species and are identified as common plasmids

in human pathogens. Similar findings were present in a study done on immunocompromised patient in Lebanon were blaNDM-5 gene appeared to coincide with the presence of the IncFII plasmid. [75] IncFII plasmids are highly mobile and carry several blaNDM variants in *Enterobacteriaceae*. In a recent study, blaNDM bearing IncFII plasmid was able to be transferred to different members of *Enterobacteriaceae* along with the other ARGs.[73] Moreover, IncFIA-FIB plasmids carrying carbapenem resistance NDM genes were detected in samples collected from river and sewage treatment plants in India.[76] Indeed, some of these isolates with plasmids were carrying more than one resistance gene as present by our study in which E. coli isolates were resistant to 21 different agents including Carbapenem. In addition to IncF plasmids, IncY and IncI plasmids were also detected in this study in NDM-5 positive *E. coli* isolates. Although less data is present on strains carrying NDM-5 gene and IncY, IncI plasmid, further analysis should be done to validate on which plasmid NDM-5 gene is present. According to the literature, these plasmids were linked with MDR strains harboring mcr-1 gene, a colistin resistance gene in *E. coli*, and they were detected in chickens in studies done in both China and Lebanon suggesting the ability to spread to humans through food chain. [77,78] In this study, the defection of these plasmids in irrigation water, sewage, and wild animals is of added value as it illustrates the possible transmission of resistance genes on plasmids between humans, animals, and the environment.

Furthermore, these *E. coli* strains along with NDM-5 gene and OXA-1 expressed 3 different genes (MarA, SoxS, TolC) that encodes for active efflux pump which is common among multidrug-resistant and carbapenem-resistant *E. coli*. [71] The resistance-nodulation-division (RND) family of efflux pumps is the most clinically

significant, as it is associated with multidrug resistance. The best-characterised RND system is AcrAB- TolC, which is present in *Enterobacteriaceae*. (70) In *E. coli*, expression of the acrAB and tolC genes is primarily controlled by MarA and SoxS and this was clearly highlighted in this study. (70)

Another key finding in this study is the isolation of Carbapenem resistant *Acinetobacter baumnaii* of ST2 from water samples of diverse types (tap water, drinking water, tank water, filtered water). This suggests the lack of proper water treatment systems in addition of poor water quality and water contamination. The presence of such clinically critical pathogen in drinking water is of great impact on health as it can be easily transmitted to humans through water consumption. Analysis on resistance genes present in these strains detected the co-presence of 5 different genes conferring resistance to carbapenem including (adeI, adeK, adeJ) which encode for efflux pumps and (OXA-66, OXA-23) which encode for class D beta-lactamases (OXA-type) that inactive antibiotics including Carbapenem. Carbapenem resistant *Acinetobacter baumanii* strains harboring OXA- 23 isolated in this study resemble clinical strains reported among the majority of samples collected from different hospitals in Northern Lebanon. (78) Indeed, it has been shown that A. *baumannii* outbreaks were caused by the spread of strains belonging in particular to ST2. These strains mainly harbored OXA-23. (78) In addition, OXA-23 and OXA-66 genes were also detected in *Acinetobacter baumannii* isolates from companion animals and these strains belonged to ST2 shared with clinical strains. (79)

In addition to oxacillinases, all analyzed *A. baumanii* strains showed expression of AdeIJK effluent pumps. AdeIJK efflux pumps belongs to RND family. AdeIJK is constitutively expressed in *A. baumanii* and provides intrinsic levels of resistance to

various classes of antibiotics including chloramphenicol, tetracycline, fluoroquinolones, trimethoprim as well as beta lactams, erythromycin, lincosamides, fusidic acid, novobiocin and rifampicin. [80]

An important fact to mention is the failure in detecting *Acinetobacter baumanii* by biochemical identification tests such as API20E. 2 Isolates were falsely detected by API20E as *Pseudomonas luteola* with good identification and they were recognized by WGS as *Acinetobacter baumanii* isolates. This emphasize the need to rely on precise and efficient techniques such as WGS to detect critical organisms specially in clinical settings where correct diagnosis is essential for targeted treatments.

As for Pseudomonas spp. detected in this study, WGS validate the presence of *Pseudomonas putida* in drinking water, irrigation water, and soil samples. No specific genes were perfectly present that encodes for carbapenem resistant. However, efflux pumps were detected that might clarify the results obtained by Disk diffusion which showed meropenem resistance. Hence, further investigation should be done in this case.

In conclusion, this project effectively isolated Carbapenem resistant *Enterobacteriaceae, Acinetobacter baumanii*, and Pseudomonas spp. from animals and environmental samples (sewage, soil, water). All the environmentally isolated *E. coli* and *Acinetobacter baumanii* are known to be human pathogens that cause severe infections with high rate of mortality in some cases. The detection of similar clones in both environmental and clinical samples explain the possible transmission of ARG among spp. and across ecosystem. This fact was further validated by the presence of clinically relevant plasmids in E. coli strains harboring NDM-5 that are able to be transmitted to different bacterial spp. and spread resistance. A key finding in this project was the isolation of MDR *E. coli* strain of ST648 from an Otter; this strain harbored

both OXA-1 and NDM-5. This study also highlights the urgent need to control the spread of such resistant microorganisms, the need to adopt appropriate infection control measures and implement effective surveillance programs that implement a One health approach.

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