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ETUDE EPIDEMIOLOGIQUE DE LA RESISTANCE AUX ANTIBIOTIQUES D'ISOLATS CLINIQUES AU LIBAN

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AVANT-PROPOS

Le format de présentation de cette thèse correspond à une recommandation à la spécialité Pathologie Humaine, Maladies infectieuses, à l'intérieur du Master des Sciences de la Vie et de la Santé qui dépend de l'Ecole Doctorale des Sciences de la Vie de Marseille.

Le candidat est amené à respecter les règles qui lui sont imposées et qui comportent un format de thèse utilisé dans le Nord de l'Europe et qui permet un meilleur rangement que les thèses traditionnelles. Par ailleurs, la partie introduction et bibliographie est remplacée par une revue envoyée dans un journal afin de permettre une évaluation extérieure de la qualité de la revue et de permettre à l'étudiant de commencer le plus tôt possible une bibliographie sur le domaine de cette thèse.

Par ailleurs, la thèse est présentée sur article publié, accepté, ou soumis associé d'un bref commentaire donnant le sens général du travail. Cette forme de présentation a paru plus en adéquation avec les exigences de la compétition internationale et permet de se concentrer sur des travaux qui bénéficieront d'une diffusion internationale.

Professeur Didier RAOULT

RÉSUMÉ

Les infections dues aux bactéries gram-négatif multi résistantes (MDR-GNB), telles que *Pseudomonas aeruginosa*, *Acinetobacter baumannii*, *Klebsiella pneumoniae* et *Escherichia coli*, en particulier la résistance aux carbapénèmes, représentent un problème majeur de santé publique. La hausse des taux de résistance à ces antibiotiques a conduit à la réutilisation de la colistine, comme alternative thérapeutique de dernier recours, engendrant une augmentation concomitante de la résistance à la colistine, chez ces microorganismes. Notre travail de thèse s'est concentré sur l'étude épidémiologique de la résistance aux antibiotiques d'isolats cliniques au Liban. Nos travaux se sont scindés en 4 chapitres, avec trois objectifs principaux; (i) l'étude des bactéries résistantes aux carbapénèmes dans les hôpitaux libanais, (ii) l'élucidation des mécanismes moléculaires de la résistance à la colistine des bactéries à Gram-négatif isolées chez les patients libanais et (iii) l'émergence de bactéries à Gram-positif résistantes à la vancomycine au Liban. Initialement, une revue de la littérature sur l'épidémiologie et les facteurs de risque associés à l'infection bactérienne au cours de conflits armés et catastrophes naturelles en Asie et au Moyen Orient a été rédigée. Il a été démontré qu'à travers le continent asiatique, des épidémies d'infection sont apparues sous l'effet direct de la guerre et à la suite de catastrophes naturelles. Tous ces changements affectant l'écosystème des pathogènes et leur environnement, ont facilité l'émergence et la transmission de maladies infectieuses post-catastrophes. Le deuxième chapitre consistait à effectuer la surveillance épidémiologique des souches bactériennes cliniques résistantes aux carbapénèmes collectées d'un hôpital libanais. Nous avons cherché à voir l'effet du changement de traitement de la combinaison colistine-carbapénème à la colistine en monothérapie sur la prévalence et la résistance d'*A. baumannii*, en plus de la détection du gène $bla_{\text{VIM-2}}$ codé par plasmide chez les bactéries résistantes au carbapénème. Le troisième chapitre consiste à étudier les mécanismes moléculaires génétiques des bactéries résistantes à

la colistine de divers échantillons cliniques prélevés chez des patients traités par la colistine et les carbapénèmes. Dans cette partie, nous avons détecté la propagation de bactéries gram-négatif résistantes à la colistine en raison de la mutation des systèmes à deux composants (TCR) (*pmrA /pmrB, phoP/phoQ*), ou de son régulateur négatif *mgrB.* Enfin, dans le dernier chapitre, nous détectons l'émergence du gène ***vanA*** d'*Enterococcus faecium* résistant à la vancomycine au Liban. Une étude phénotypique (culture, isolement, MALDI TOF, antibiogramme, CMI…) et génotypique (PCR en temps réel, PCR standard, MLST et séquençage) sur l'ensemble du matériel biologique déjà cité, ont été utilisés pour décrire la prévalence des organismes multirésistants et les mécanismes de résistance chez les souches cliniques isolées d'un Hôpital Libanais.

Nous pouvons ainsi conclure que le changement de la voie thérapeutique de l'association colistine-carbapénème à colistine en monothérapie des infections XDR *A. baumanii* a considérablement réduit la consommation d'antibiotiques, entraînant une baisse de la prévalence d'*A. baumanii* dans la culture des expectorations. Nous avons constaté une émergence d'entérobactéries résistantes à la colistine chez des patients traités par la colistine. Cette observation confirme en outre que la résistance à la colistine chez les bactéries à Gram négatif est en effet en augmentation, comme le montrent les rapports croissants de résistance à la colistine dans la littérature. En conclusion, il serait donc nécessaire et urgent de mettre en place des enquêtes de surveillance de l'usage des antibiotiques pour éviter la propagation de souches résistantes à ces antibiotiques chez les patients au liban.

Mots clés: colistine, carbapénémases, vancomycine, études épidémiologiques, études moléculaires, patients libanais.

ABSTRACT

Infections due to multidrug-resistant gram-negative bacteria (MDR-GNB), such as *Pseudomonas aeruginosa, Acinetobacter baumannii, Klebsiella pneumoniae,* and *Escherichia coli,* .especially the resistance to carbapenems, have become a major public health problem. This increase in resistance to antibiotics has led to the resuscitation of colistin, as a last-resort treatment option, which has been followed by an increase of resistance among gram-negative bacteria. Our PhD work focused on the epidemiological study of the antibiotic resistance of clinical isolates in Lebanon. This thesis is divided into 5 chapters with three main objectives; (1) the investigation of carbapenem-resistant bacteria in Lebanese hospitals. (2) the Elucidation of the molecular mechanisms of colistin-resistant bacteria in Lebanese patients, and (3) the emergence of vancomycin-resistant gram-positive bacteria in Lebanon. At the start of this thesis, we have prepared a literature review on the epidemiology and the risk factors associated with bacterial infection in conflict wounded and natural disaster in Asia and the Middle East. It has been shown that across the Asian continent, outbreaks of infection have arisen as a direct effect of war, and following natural disasters. All these changes in human situations, in the ecosystem of pathogens and in the environment, ease the emergence and transmission of these post-disaster infectious diseases. The second chapter was to carry out the epidemiological surveillance of carbapenem- resistant clinical samples collected from Lebanese Hospital. We aimed to see the effect of the shift of treatment from colistin-carbapenem combination to colistin monotherapy on the prevalence and resistance of *A. baumannii,* in addition to the detection of the plasmid-encoded $bla_{\text{VIM-2}}$ gene in carbapenem-resistant bacteria. The third chapter consists to study the genetic molecular mechanisms of colistin-resistant bacteria in a variety of clinical samples collected from patients treated with colistin and carbapenems. In this section, we have detected the spread of colistin-resistant gram-negative bacteria due to mutation of the two-component systems (TCR) (*pmrA /pmrB,*

phoP/phoQ), or its negative regulator *mgrB.* Finally, in the last chapter, we detect the emergence of *vanA* of *Enterococcus faecium* resistant to vancomycin in Lebanon.

A phenotypic study such as (culture, isolation, MALDI TOF, antibiogram, MIC ...) and genotyping approach such as (real-time PCR, standard PCR, MLST and sequencing) on all the biological material already mentioned were used to describe the prevalence of multidrug-resistant organisms and the mechanisms of resistance of clinical strains isolated from Lebanese patients.

Thus, we can conclude that changing the therapeutic pathway from colistin-carbapenem therapy to colistin monotherapy for XDR *A. baumanii* infections has significantly reduced antibiotic consumption, resulting in the decrease of prevalence of *A. baumanii* in the culture of sputum. We have also reported the emergence of colistin-resistant *Enterobacteriaceae* in patients treated with colistin. This observation further confirms that colistin resistance in Gram-negative bacteria in is indeed increasing, as shown by the increasing reports of colistin resistance in the literature. In conclusion, it appears necessary and urgent to set up surveys to monitor the use of antibiotics to prevent the spread of resistant strains in Lebanese patients.

Key words: colistin, carbapenemases, vancomycin, Epidemiological studies, Molecular studies, Lebanese patients.

INTRODUCTION

In the 20th century, the discovery of antibiotics have revolutionized modern medicine, which allow significant progress in the improvement of life expectancy and healthcare. In 1928, Alexander Fleming had his chance encounter with a petri dish of bacterial colonies that had been killed by mold contamination. This was the birth of penicillin, derived from the *Penicillium* genus of Ascomycetous fungi, which was called the miracle drug [1]**.** After that**,** Beta-lactams become the most prescribed antibiotics family in the world. In 1942, the first strain of *Staphylococcus aureus* resistant to penicillin by the production of penicillinase was described [2]. Therefore, a long-lasting race underwent between bacterial resistance and development of new molecules [2]. The emergence and the dissemination of new β-lactamases, which is the first mechanism involved in resistance of Gram-negative bacteria to β-lactamines, was parallel to the introduction of these antibiotics in therapy and the consumption of different β-lactamines. In the early 1980s, the introduction of third-generation cephalosporins (C3G) in therapy to fight against infections caused by penicillinase-producing bacteria, was followed, since 1983, by the description of the first expanded spectrum β-lactamase (ESBL) in *Klebsiella pneumoniae* in Germany [2]. Between the 1980s and 1990s the increasing prevalence of ESBLs among *K. pneumoniae* and *Escherichia coli* contributed to the increased consumption of carbapenems which became vital drugs in the treatment of healthcare-associated and severe community-acquired multidrug-resistant infections [3]. Unfortunately, resistance has eventually been seen to nearly all antibiotics that have been developed. Carbapenem-resistant bacteria are a group of bacteria that have become resistant to a large set of available antibiotics, including carbapenems, which are typically reserved as the "treatment of last resort" against drug-resistant pathogens [4]. Carbapenem resistance in Gram-negative bacilli poses a serious threat. The resistance might be achieved through selective loss of external membrane permeability (such as OprD porin loss in *Pseudomonas aeruginosa*), overexpressing of cephalosporinase type β-lactamase associated with the

acquisition of impermeability to β-lactamines, in particular to carbapenems. However, this phenomenon was not a worrying problem since the resistance was not transferable. The resistance might also be due to the presence of carbapenem-hydrolyzing enzymes such as the carbapenemases. The latter are of great concern because the respective encoding genes are carried by transmissible genetic elements that leads to a wide and rapid propagation. Due to the emergence of resistance to beta-lactams and carbapenems in multidrug-resistant (MDR) Gram-negative bacteria (GNB), such as *Pseudomonas aeruginosa, Acinetobacter baumannii*, *Klebsiella pneumoniae,* and *Enterobacter,* colistin was re-introduced into clinical medicine between the late 1990s and early 2000s, [5]. Polymyxin E (colistin) and polymyxin B have been used in humans and are bactericidal toward Gram-negative bacteria except for the bacteria that are intrinsically resistant to colistin such as *Burkholderia, Edwardsiella, Proteus, Providencia, Morganella* and *Serratia* [6]. This leads to the emergence of colistin-resistant bacteria among patients treated with this compound [7]. Several strategies are used by gram-negative bacteria to escape from polymyxin (colistin and polymyxin B). The major mechanism of resistance occurs by the alteration of the negatively charged lipopolysaccharide (LPS) due to chromosomal mutations in various genes, which is the initial site of mode of action of colistin [5]. Recently, *mcr-1*, *mcr-2*, *mcr-3*, *mcr-4* and *mcr-5* plasmid resistance genes have been described in many Enterobacteriaceae isolated from different country in the world such as Denmark, Italy, Dutch, Spain, Belgium, china, Germany, South-East Asia, America, and Africa. However, those strains have been mainly detected in animals [5].The emergence and dissemination of plasmid resistance is worrying, because they have the potential to be further transmitted and spread globally, including to and within regions with already high levels of antimicrobial resistance where multidrug-resistant bacteria represent a public health concern [8]. It has been shown that the increase and spread of antibiotic resistance (AR) in human is mainly due to the overuse and/or misuse of antibiotics. In

addition, there are many other sources leading to emergence of resistance, such as the use of antibiotics in agriculture and in food animals that are known to be drivers of antibiotic resistance [9]. Regarding this global concern on the emergence of these MDR pathogens observed, it is vital to conduct epidemiological, and molecular studies in addition to those already done to understand and to control the dissemination and the increase of antibiotic resistance in Lebanon. In a previous PhD work conducted in Lebanon by Dandachi et al, it has been shown that the predominance of ESBL and ampC producer in chicken and swine farms is high and there is an absence of carbapenemase producers. In addition, the prevalence of *mcr-1* colistin- resistant bacteria is high in chicken in Lebanese farm [10–12].

On the other hand, movement of people across borders, regardless of reason, has played a key role in the importation and spread of MDR pathogens. In the past decade, the Middle East and parts of Europe have witnessed an enormous movement of refugees and migrants due to the ongoing civil war in Syria but also due to conflicts, violence, and instability in other countries in Asia and Africa [13]. Syrian refugees have played a major role in the dissemination of bacteria in Lebanon. By March 2016, the United Nations reported more than 4.8 million refugees outside Syria, mainly divided between Turkey and Lebanon [14,15]. Lebanese hospitals have acted as referral centers for war casualties from Syria and Iraq as well as their own national soldiers and civilians. For example, in 2010 NDM-1-producing *K. pneumoniae* imported from Iraq were detected in Lebanon [16] and between 2011 and 2013 a 60% prevalence rate of carbapenem resistant *A. baumannii* was recorded in Tripoli, the largest Lebanese city bordering Syria [17]. Moreover, outbreaks of MDR *A. baumannii* infections have been reported previously during war, as shown by Scott et al. and Kusradze et al., who studied the bacteria isolated in US service members injured in Iraq [18,19]. Despite this fact, we aim to write a review that describe the microbiology of war-related wound infections and natural disasters and factors affecting their incidence in the country of Middle East and Asia.

It is in this context that this thesis project aim to study and to search for multidrug-resistant bacteria from clinical isolates in Lebanon through two main objectives:

1. Research and characterization of genes coding for resistance to carbapenems.
2. Research and characterization of the molecular mechanisms of colistin-resistant bacteria in Lebanese patients and identification of mechanisms and genetic supports of the resistance to other antibiotics.

Hence, this manuscript contain four chapters. Three main chapters and an annex section.

Chapter I is a literature review on the bacterial infections associated in conflict wounded and natural disaster in Asia and the Middle East (***Article N°1***). It has been shown that wars and conflicts around the world and natural disasters posed a major threat, which is the dissemination of infectious diseases. In addition, we summarize prevention and control measures to be considered in addressing challenges resulting from these two phenomena. The outline of this review is summarized as the following:

- Introduction
- Bacterial Infections during wars
    - Wars in the Middle East
    - Wars in Iraq and Afghanistan
    - Wars in East Asia
    - Wars in Central & South-East Asia
- Prevention of bacterial infections during wars
- Bacterial infections after natural disasters
    - Floods
    - Typhoons (hurricanes and cyclones)
    - Tsunamis
    - Earthquakes

- Prevention of bacterial infection after natural disaster
- Conclusion

In **Chapter II**, we studied the molecular epidemiology of carbapenemases producing bacteria in clinical patients in Lebanon. In this part, we focused on research and characterization of carbapenem-resistant *Acinetobacter baumannii* isolated from Saint Georges hospital in Lebanon from patients treated with colistin and carbapenem. This study shows a nosocomial spread of multidrug-resistant *A. baumannii* ST2 harboring the $bla_{\text{OXA-23}}$ carbapenemase encoding gene. (***Article N°2***). The objective was to evaluate the effect of the shift of treatment from colistin-carbapenem combination to colistin monotherapy on the prevalence and resistance of *A. baumannii* to antibiotics. Results showed that the change in therapeutic strategy for *A. baumannii* infections to colistin monotherapy dramatically decreased antibiotic consumption resulting in a greater drop of *A. baumannii* prevalence in sputum culture with successful elimination of $bla_{\text{OXA-23}}$ carrying ST2 clone ***(Article N°3)***. We also described in this work the first detection of the plasmid-encoded $bla_{\text{VIM-2}}$ gene in *P. aeruginosa* from Lebanon. This finding poses a serious public health problem since the plasmid harboring this β-lactamase is a major source of dissemination of this enzyme ***(Article N°4)***.

Chapter III intended to describe the mechanisms of resistance of clinical isolates to colistin in Lebanon. The use of colistin as a last line resort treatment was the last option to treat infections caused by multi-drug resistant bacteria. Unfortunately, the use of colistin therapy has been followed by an increase in reports of resistance among Gram-negative bacteria**.** Here, we report the prevalence of colistin-resistant *Enterobacteriaceae* isolated from rectal swabs from patients hospitalized in ICU at Saint-George Hospital in Beirut, Lebanon that were treated by carbapenem and colistin ***(Article N°5)***. In the same chapter, we described the presence of colistin resistant *Klebsiella pneumoniae*, due to mutations of the two component systems (*pmrA*/*pmrB*, *phoP*/*phoQ*), or its negative regulator *mgrB*, in addition to the presence

of the carbapenemase NDM-5 gene which is firstly reported in Lebanon. These isolates were collected from two soldiers admitted to Saint-George Hospital in Beirut and then transferred into another patients. ***(Article N°6).***

Chapter IV is an annex consisting of three articles. The first article showed the presence of vancomycin resistance gene *vanA* in *E. faecium* collected from rectal swabs in Lebanon, which is a major public health issue. Because we used the LBJMR medium that contains colistin and vancomycin, we report vancomycin-resistant enterococcus strains, isolated from patients without previous use of vancomycin. These isolates harbored the *vanA* gene and it is the first detection in Lebanon ***(Article N°7).*** The other articles of this chapter are devoted to other collaborative research works, in the team during my PhD. We participated in the establishment of the collection of animal reservoirs that can harbor bacteria carrying the *mcr-1* gene, through the detection of this gene in the feces of different animals, including cattle, sheep and goats, as well as in breeding surface swabs in Algeria ***(Article N°8).*** In this chapter, we also isolated colistin-resistant bacteria and characterized the molecular support of this resistance in gull droppings taken in the city of Marseille, France ***(Article N°9).***

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I - MICROBIOLOGY AND RISK FACTORS ASSOCIATED WITH BACTERIAL INFECTION DURING WAR AND CONFLICTS IN ASIA.

Human beings' coevolution with the microorganisms that infect them often progresses faster than does understanding of the resulting infectious diseases, especially during epidemics and armed conflicts [1]. Humans and microorganisms from different areas interact, increasing the dissemination capacity of various infectious agents globally, including antimicrobial-resistant pathogens. Movement of people across borders, regardless of reason, has played a key role in the importation and spread of carbapenem-resistant Enterobacteriaceae (CRE), multidrug-resistant (MDR), and extensively drug-resistant (XDR) tuberculosis (TB), with an impact on morbidity and/or mortality of affected patients and healthcare costs [2]. During the past 50 years, wars, and natural disasters have led to mass human movements and forced displacement of large numbers of people from their homes, and posed a major threat which is the dissemination of infectious diseases [3]. Across the world, outbreaks of infection have occurred as a direct effect of war, compounded by food and water shortages, displacement, and damage to infrastructure and health services [4]. Historically, disease and nonbattle injuries, such as cholera, dysentery, plague, smallpox, typhoid, and typhus, have been responsible for the vast majority of deaths during war [5]. Emerging and re-emerging infectious diseases are threats that military organizations have to guard against, as they cause substantial impact to operations and training. Many diseases, especially airborne, food and water borne, as well as vector borne diseases have been shown to spread readily in the military due to the close communal living and training quarters, operational constraints, and unique field hygiene conditions. Some outbreaks in military settings are linked to an increased incidence of disease in the local civilian population. Although the extent of interactions between military and civilian elements vary across militaries, some mixing is inevitable either during transit of personnel from one setting to another, or through socio-civic duties such as disaster relief or community programs [6]. Since the beginning of this century, developments have been recorded as regard the basic principles for the management of infectious diseases

caused by wars [7]. Moreover, in recent decades, the incidence and magnitude of natural disasters has grown resulting in substantial economic damages, affecting and killing millions of people. The risk of infectious disease outbreaks following natural disasters is well described [8]. An important contributory factor for such disease outbreaks may be the displacement of large numbers of people from their homes into over-crowded shelters where supplies may be limited. In addition, the availability and accessibility of medical services after such natural disasters is also a major concern. Epidemiological studies have reported that the increased incidence of infectious diseases after such disasters is associated with environmental changes, crowded shelter conditions and an inadequate or dirty water supply [9]. All these factors contribute to the transmission of these infectious disease following war and natural disaster.

The objective of this chapter was to describe potential infectious diseases occurring during wars and after natural disasters described in Asia. The chapter is entitled "**Bacterial infection during wars and conflicts and after natural disasters in Asia and the Middle East**"(***Article N°1***). This review was divided into two parts: **(1)** bacterial infections during wars, and **(2)** bacterial infections following natural disasters. Through this review, we reported the outbreaks of infections that arise as a direct effect of war, aggravated by food and water shortages, damage to infrastructure and health utilities and mainly by the displacement of large number of refugees. We first describe the microbiology of war-related wound infections that results from the war of the Middle East region that is afflicted with repeated armed conflicts affecting both civilians and soldiers. Second, we aimed to describe the large number of injuries among both civilians and the military population, in Iraq and Afghanistan that are affected by different wars and military conflicts, such as the Iran-Iraq war, the Operation Iraqi Freedom and war against terrorism in Afghanistan, and the war against the Islamic State of Iraq and Syria (ISIS) lately. Furthmore, we describe the bacterial infection

that occurs during wars in East Asia, central and South-East Asia. In the second part, we summarized the emergence and transmission of the post-disaster infectious diseases. We focused on the bacterial infections that affect the countries of Asia following earthquakes, tsunamis, floods, and hurricanes**.** In addition, we propose prevention and control measures in addressing challenges resulting from these two phenomena.

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LITERATURE REVIEW

Bacterial infection during wars and conflicts and after natural disasters in Asia and the Middle East.

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Bacterial infection during wars and conflicts and after natural disasters in Asia and the Middle East

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Abstract

During the past 50 years, Asia and the Middle East have witnessed several armed conflicts and wars. In addition, East-Asian countries are considered as the most natural disasters-prone countries in the world. Bacterial infections following wars, and natural disasters represent a major public health threat. These two phenomena have led to excessive mass movements of humans leading to the increase of bacterial transmission. During war, refugees and soldiers represent the two major sites of bacterial infection. Refugees coming from countries with a high prevalence of antimicrobial resistance in the community can spread these pathogens on their way to their final destination. In addition, these refugees living in overcrowded and non-adequate shelters may help on the spread of bacterial infection. Moreover, factors such as the presence of fixed foreign materials or fragments; environmental contamination and nosocomial transmission play a major role in the dissemination of bacteria among soldiers. As for the natural disasters, several factors are related to the increase in infectious disease transmission such as displacement of large numbers of people into over-crowded shelters, high exposure to disease vectors, poor water, sanitation, and sewage systems. Prevention of bacterial infections during wars, rely on the prevention of these infections in refugees and wounded citizens and soldiers. However, prevention of those infections after natural disasters depends on the rapid implementation of control measure strategy by the country affected by the disaster. This review summarizes potential infectious diseases occurring during wars and after natural disasters described in Asia and the Middle East. Moreover, it describes the prevention and control measures to be considered to face the challenges resulting from these two phenomena.

1- Introduction

During the past 50 years, wars, conflicts around the world and natural disasters have led to mass human movements and forced shifting of large numbers of people from their homes, but also posed a major threat which is the dissemination of infectious diseases [1]. Historically, diseases such as cholera, plague, typhoid and typhus have been linked with the majority of deaths during war. The mortality associated with infectious diseases during wars has significantly changed over the last century due to improvements in sanitation and the understanding of how to prevent infections during wars, with a mortality ratio dropping from 1:8 during the Spanish-American War in 1898 to 1:01 during the first Gulf War [2]. In the other side, global population growth, poverty and displacement have increased the number of people living in areas vulnerable to natural disasters which multiplied their public health impacts [3]. Over the past decade, the world has witnessed several natural disasters such as floods, typhoons, tsunamis and earthquakes, with an increasing rate where some sources cite a threefold increase from 2000 to 2009, when compared with 1980-1989 [4]. Increases in infectious disease transmission following natural disasters are related with several contributing factors. These contributing factors include displacement of large numbers of people into over-crowded shelters where supplies are limited [5], high exposure to and proliferation of disease vectors [3], poor water, sanitation, and sewage systems [6], low levels of immunity to vaccine-preventable diseases or inadequate vaccination coverage, and restricted access to healthcare services [3]. All these changes in human situations, in the ecosystem of pathogens and in the environment, ease the emergence and transmission of these post-disaster infectious diseases.

This review aims at describing potential infectious diseases occurring during wars and after natural disasters described in Asia and the Middle East. In addition, we summarize prevention

and control measures to be considered in addressing challenges resulting from these two phenomena.

2- Bacterial Infections during wars

War injuries are not restricted to military personnel and since 1945, more than 100 million civilian people, with 25 million deaths, have been noted in military conflicts worldwide [7]. The damages caused by war are many and complex. Death, injury, and displacement are the most evident, but infection is also closely interlaced with conflict and wars.

Bacterial infections, most importantly the multidrug resistant (MDR) one, have become an international public health problem during the past decades and now pose a serious challenge to the global healthcare [8]. Across the Asian continent, outbreaks of infection have arisen as a direct effect of war, aggravated by food and water shortages, damage to infrastructure and health utilities and mainly by the displacement of large number of refugees as reported from the Syrian war [9]. This enormous movement of refugees provides the opportunity for a variety of bacterial species to be transferred from one geographical location to another, thus increasing the dissemination capacity of several infectious agents globally, including antimicrobial-resistant pathogens. Migration during wars is an important factor in the spread of infectious diseases and MDR pathogens. Refugees coming from countries with a high prevalence of antimicrobial resistance in the community can spread these pathogens on their way to their final destination but also can acquire such pathogens from other refugees in crowded refugee camps with poor hygiene conditions. MDR pathogens include mainly extended spectrum β-lactamase and carbapenemase-producing *Enterobacteriaceae*, MDR *Acinetobacter baumannii*, methicillin-resistant *S. aureus* (MRSA) but also MDR tuberculosis (MDR-TB) [10].

Beside infection resulting from war refugees, that of conflict-related traumatic wounds has been a persistent feature throughout the history of wars, and types of traumatic wounds have changed as the weaponry of conflict and modality of wounding have changed [11]. Traumatic injuries secondary to high-velocity projectiles (shrapnel and gunshot), blast injuries (mines, mortars and improvised explosive devices) and burns, mainly to the extremities, account for the majority of wounds (65%), followed by head and neck (15%), thoracic (10%) and abdominal wounds (7%). Traumatic wounds have special characteristics that improve the development of infections. Factors influencing this include: wound type and stringency; the presence of devitalized tissue and fixed foreign materials or fragments, clots and fluid collections; environmental contamination; evacuation time from site of injury to health care institutes; launching of antimicrobial agents; the presence of nosocomial pathogens and may other factors [12,13].

It was shown that with the progress made in the treatment of conflict-related traumatic injuries, the bacteriology of war wounds has also evolved over time. One of the earliest descriptions of war wound bacteriology was by Fleming in 1919 [14]. Experiences from World War I led to early descriptions of the evolution of the pathogens leading to wound infections through three phases. Initial infection involved sporulating anaerobes (such as *clostridium* species) and *Streptococci* that transitioned to non-sporulating bacteria of faecal origin after approximately 7 days (e.g. *Escherichia coli* and *klebsiella* species) and then to pyogenic organisms (including *Staphylococcus* species and *Streptococcus pyogenes*) during the third phase after approximatively 20 days [13,14]. The introduction of aggressive surgical debriment probably led to the eradication of clostridial gas gangrene during World War I, and the use of penicillin in World War II likely contributed to the diminution of wound infections caused by *S. pyogenes* [15]. Simultaneously, the expanded use and broader spectra of antimicrobial agents have led to the appearance of increasingly resistant bacteria [16,17].

Since the Vietnam War, the organisms now occasionally associated with combat-related trauma wounds include *Staphylococcus aureus, S. pyogenes* and Gram-negative bacteria such as *Pseudomonas aeruginosa, Enterobacter* spp., *E. coli* and *Klebsiella* spp. [18]. Wounds infections during wars have always had a great impact on both morbidity and mortality whether in civilians or military causalities [12,19,20]. The infectious diseases outbreaks that occurs following war are summarized in Table 1

a- Wars in the Middle East

The Middle East region is afflicted with repeated armed conflicts affecting both civilians and soldiers. The Arab-Israeli wars and recent uprisings (Syria and Yemen) are very good examples.

Syria

One of the most accurate examples of a military conflict that affects both civilians and soldiers is the Syrian civil war. This war, now in its seventh year, has resulted in more than 200 000 deaths, 500 000 wounded, and more than 9 million refugees [19,21]. Prior to the onset of the crisis, Syria's health care system was comparable with other neighboring countries. By 2016 the healthcare infrastructure was greatly diminished in all sectors of the country by destruction of hospitals and clinics, reduction in the numbers of healthcare personnel and shortages of medical supplies [19].

A study conducted by Doctors Without Borders in 2014 on 61 Syrian orthopedic patients with suspected infections showed that 74% of these patients had at least one positive wound culture and 13% had polymicrobial results. Gram-negative organisms represented 56% of cultures with *P. aeruginosa* being the most dominant bacteria (56%), followed by *E. coli*

(19%) and *A. baumannii* (14%). Gram-positive bacteria, including MRSA, represented 44% of the isolates. Overall, 69% of infected patient had MDR organisms with MRSA representing 42% of staphylococcal isolates [22].

The Syrian health system has declined since the beginning of the civil war. It has been noted that many Syrian hospitals and medical institutes have been destroyed by fighter jet strikes and bombing, which caused the death of many doctors and other medical staff. Since 2013, Syrian patients, primarily those wounded in the fighting, have been receiving care in Israel, Lebanon and Turkey.

The Syrian war has resulted in an efflux of 1300 wounded, both fighters and civilians, into Israeli borders seeking medical treatment [23]. Among such patients, the incidence of MDR isolates ranged from 47% to 66%, the most common being ESBL-producing and/or carbapenem-resistant Enterobaceriaceae (CRE), MRSA and *Acinetobacter baumannii-calcoaceticus* complex (ABC); two of the CRE isolates produced New Delhi metallo-β-lactamase [24,25]. Another study has been conducted in four hospitals in northern Israel receiving patients evacuated from the Syrian territory. A total of 595 Syrian patients were admitted to the study hospitals during the period studied in each hospital. Thirty (6.5%) were found to be CPE carriers. The bla_{NDM} gene was detected in 19 of the isolates, while the $bla_{\text{OXA-48}}$ gene was detected in 13 [26]. CPE acquisition might have occurred in Syria in the community, but may also have occurred during the patients' stay in Syrian healthcare settings, during evacuation to Israel, in the Israeli medical facility operational along the border [27], or during transfer to one of the Israeli civilian hospitals. The finding of some new clones in this study from Syrian patients in the Israeli hospitals implies a common source of acquisition of these clones that predates the hospitalization in northern Israel [26]. Another study described the emergence of $bla_{\text{NDM-1}}$-producing *A. baumannii* isolated in Lebanon from Syrian civilians wounded [28]. Outbreaks of MDR A. baumannii infections have been reported previously

during war, as shown by Scott et al. and Kusradze et al., who studied the bacteria isolated in US service members injured in Iraq [29,30]. Because of the impossibility to obtain information on the date of injury, conditions of care, or on the treatment administrated in Syria, they conducted that the infection may have been acquired from environmental sources on the battlefield, during the patient's stay in Syrian clinics, or during evacuation to Lebanon.

Beside infections caused by the direct effect of war, Syrian refugees have played a major role in the dissemination of bacteria. By March 2016, the United Nations reported more than 4.8 million refugees outside Syria, mainly divided between Turkey and Lebanon [31,32]. Mass movement of the Syrian refugees has contributed to a rise in the number of tuberculosis (TB) cases across the region but also between refugees because they generally live in crowded and unsanitary conditions. TB had been declining in Lebanon until 2011, according to the World Health Organization (WHO) and Lebanese Ministry of Public Health, when the refugee influx into the country began [32]. In 2012, there was a 27% increase in TB cases in Lebanon compared to 2011. The main factor contributing to the dissemination of the disease is that there are no formal refugees camps in Lebanon as in Turkey; rather there are hundreds of informal tents settlements scattered around the country where refugees live in crowded and unfavorable hygiene conditions [32]. In a study done in Turkey, 10,589 Syrian refugees were screened for TB, and the prevalence was found to be 18.7/100,000. In 2015, 558 new cases among Syrian refugees were diagnosed and treated [33,34].

Lebanon

Lebanon has been harassed by wars and military conflicts since 1975 and the country has witnessed, on several occasions, armed battles whether civil war inside Lebanon or war with the Israeli army. However, few reports have been published describing war injuries in Lebanon during the civil war (1975-1978) and the war with Israel (1982-2006) [35–38].

During the civil war, a study was conducted on 1021 Lebanese patients over a 10-year period (1975-1984) at a tertiary hospital. The study showed an infection rate of 12% with *S. aureus*, *P. aeruginosa*, and *E. coli* being the most common organisms, respectively [38]. A similar study from the same center on the outcome of 1500 patients, showed a 2.1% incidence rate of sepsis as the second most common cause of death after hemorrhage (3.7%) [38].

Intracranial infections, including brain abscesses, following missile injuries to the brain were reported by Taha et al. at the American University of Beirut medical Centre in patient treated between 1981 and 1988 [38]. Lately, the 2006 war with Israel, resulted in the release of more than four million sub-munitions over Lebanese soil including one million unexploded duds [39]. Fares et al. conducted a study on 350 injured casualties in 2013 and showed an infection rate overall of 19.4% with bacterial infections accounting for 86.8%. *P. aeruginosa* was the most common isolate identified (30.5%) followed by *E. coli* [40].

As mentioned before, the Lebanese hospitals have acted as referral centers for war casualties from Syria but also from Iraq. This explain the emergence of $bla_{\text{NDM-1}}$ producing *A. baumannii* [40], *E. coli* and *Klebsiella pneumonia* [41] in Lebanon.

Israel

Israel has been always in continuous conflict with bordering Arab countries. During the Yom Kippur war, infection rates from 4.9% to 58.3% were reported with *P. aeruginosa* being the most common pathogen [42–44], as seen also in the ensuing 1982 war with Lebanon [45]. These studies showed a low frequency of clostridial infections, in contrast to finding during the Korean War, due to the early and adequate surgical treatment including debridement, and the routine early administration of penicillin G to all cases with tissue destruction [44]. In these two conflicts, the increased incidence of infection was associated with penetrating abdominal wounds involving the colon, extensive soft tissue loss, burns involving >25% of

the body surface, multiple operations, open drains inserted in the first operation, and wounds located below the diaphragm [17,43,44]. Simchen et al [44] recommended the prompt administration of clindamycin and gentamicin in combination for all abdominal injuries and treatment was continued for 48-72h if colonic involvement was shown at laparotomy. This was applied because the infections due to penetrating abdominal wounds involving the colon can be attributed to heavy colonic bacterial flora mainly *Bacteroides* spp. and Enterobacteriaceae.

During the Yom Kippur war, the administration of prophylactic antibiotics did not have a significant impact in preventing wound infection [17]. Moreover, this practice possibly contributed to an increase in infections due to Gram-negative rods [19,46].

Kingdom of Saudi Arabia

Diarrheal disease has long been a serious problem for military forces, especially during combat [47,48]. During operation desert shield, the US troops slept on cots in tents, or in sleeping bags on the sand or atop equipment. In addition toilet facilities ranged from open trenches to covered wooden latrines and indoor flush toilets [49]. Under combat conditions, despite extensive efforts to secure a safe supply of food and water and a high level of sanitation, infectious disease can become a major threat to military forces especially when large numbers of soldiers are deployed. Between September and December 1990, stool cultures for enteric pathogens were obtained from 432 military personnel who presented with diarrhea, cramps, vomiting, or hematochezia. A bacterial enteric pathogen was identified in 49.5% of the troops with gastroenteritis, *E. coli* and *Shigella sonnei* being the most common bacterial pathogens [49].

Yemen

Yemen, a country with a population of approximatively 25 million located at the southern tip of the Arabian peninsula, have experienced one of the largest cholera outbreaks in recent history. Even before the conflict between Houthi rebels and the Yemeni regime, Yemen was among the poorest of the Arab countries, and considered among the most water-stressed countries in the world [50]. According to the WHO-UNICEF statistics, in 2014 only 53% of the population used improved sanitation facilities and only 55% had access to clean drinking water [51]. Since the beginning of the conflict, the situation has worsened markedly. Millions of people have been displaced and now live under conditions with inadequate shelter, water, sanitation and food. The outbreak, which began in October, 2016, appeared to peak in December, before abating by April 2017, with around 25 000 cases suspected [9]. Cholera is easily treated, but access to basic healthcare is also blocked by war, in addition to the destruction of these centers which become more common in recent years, particularly in Syria and Yemen.

b- Wars in Iraq and Afghanistan

During the last years, Iraq and Afghanistan has been plagued by wars and military conflicts, from the Iran-Iraq war, to the Operation Iraqi Freedom and war against terrorism in Afghanistan, to the war against the Islamic State of Iraq and Syria (ISIS) lately. These wars resulted in a large number of injuries among both civilians and the military bur also to the displacement of millions of refugees to zones with poor sanitation conditions.

Iraq

During the Iran-Iraq war, missile was the main weapon used. Contamination, presence of retained bone and metal fragments serving as a nidus for growth of microorganisms, make treating missile wounds to the head challenging [52]. From February 1981 to August 1987, 379 patients with missile wounds to the head and dural penetration were evacuated to

Nemazee Hospital in Fhiraz for primary debriment and closure. Infections occurred in 18 patients, 16 of them had meningitis, with *k. pneumonia*e and *Enterobacter* species being the most common pathogen isolated. The study showed that the chance of infection was increased 20 times in patients with cerebrospinal fluid fitsulas [52].

War related orthopedic injury is frequently complicated by environmental contamination and delays in management, placing patients at high risk for long-term infectious complications. A retrospective analysis was performed on the patients from Iraq with suspected war-related chronic osteomyelitis admitted to the Médecins Sans Frontières Reconstructive Surgery Project in Amman netween October 1, 2016, and June 30,2009. 107 patients were considered to have osteomyelitis with Gram-negative organisms representing 63% of isolates, most commonly *E. coli* (20%), *P. aeruginosa* (18%), *K. pneumonia* (12%), *Proteus* spp (9%), *Enterobacter* spp (9%) and *A. baumannii* (4%). *S. aureus* was the most common individual organism, representing 21% of all isolates. The study showed a nosocomial infection in Iraqi health facilities [53].

In 2015, Iraq faced an outbreak of cholera that started in September along the Euphrates valley of the country. According to WHO, nearly 2800 cases of *Vibrio cholera* 01 Inaba infection were reported in the country which was controlled by the ISIS. The large number of cholera cases was attributed both to low water levels in the Euphrates river and deteriorating infrastructure. These two main problems in addition to the difficulties associated with displacement and conflict made the outbreak a serious problem [54].

Iraq was ranked 44th out of 212 countries and territories by estimated number of tuberculosis cases (WHO 2012). It is considered among the nine ‘high tuberculosis burden’ countries in the World Health Organization Eastern Mediterranean Regional Office (WHO-EMRO) Region, contributing 3% of total tuberculosis cases worldwide. The prolonged recent war

especially that against the ISIS, has resulted in reductions in the infrastructural and human capital capacity of the National Tuberculosis Program (NTP), to deliver effective diagnosis, treatment and prevention campaigns in the majority parts of the country. In addition, this war has led to a huge human man displacement, which under unfavorable hygiene conditions, has contributed to this high prevalence of TB [55].

Afghanistan

War of more than two decades has left dramatic effects on Afghanistan. Mortality, morbidity, and disabilities due to TB are alarming [56]. A study conducted in 2002, showed that the incidence of active TB cases in Afghanistan is 278 per 100,000 and mortality mounts to 15,000 cases per year [57]. Furthermore, increasing number of TB cases among war prisoners is another aspect of war. The situation has worsened due to the disruption of TB control activities during the war. Overcrowded refugee camps and lack of treatment facilities has increased manyfold the risk of further transmission [57]. Since September 11, and especially after the start of the US attacks on October 7, hundreds of thousands of Afghans, many of whom are infected with TB, have rushed the border with Pakistan where they lived in disastrous conditions which led to the increased incidence of TB [58].

Military operations in Iraq and Afghanistan

Since the beginning of military operations in Afghanistan in 2001 and Iraq in 2003, amongst the predominant multinational coalition forces, over 46 000 US military personnel and 2000 British troops have been wounded in action [13]. Following initial emergency treatment in the combat field, approximatively 30% of conflict-related trauma casualties have required aeromedical evacuation to military medical facilities in their host nation for further care. The evacuation route from the fields of operations in Afghanistan and Iraq is complex, representing a continuum of medical care of wounded soldiers that operates over several

thousands of miles through a diversity of treatment facilities in different countries over variable durations. Wounded US casualties are typically evacuated from the combat field within 1-3 days following injury, are transferred through an average of four facilities, including spending a short period in Germany, before reaching their final US medical treatment facility within 6-8 days from the point of injury [59]. Therefore, the potential for nosocomial colonization and infection of combat-injured personal with organisms and or MDR organisms during the complex process of casualty treatment and evacuation is important.

An observable increase in infections with *A. baumannii*, particularly with MDR isolates, was first reported in US military casualties injured in Iraq and Afghanistan in 2004. Injured soldiers were first treated at combat field medical facilities before evacuation to Germany and USA. At these facilities between January 2012 and August 2004, 102 *A. baumannii* bloodstream infections were identified, 83% of which occurred in casualties injured in Iraq and Afghanistan, and the majority of the isolates showed high profile of resistance to an array of antimicrobial agents [60]. Since initial reports, MDR *Acinetobacter* has been shown repeatedly to be a cause of deep-wound infections, osteomyelitis, respiratory infections and bacteremia in trauma-related combat casualties in Iraq and Afghanistan [61].

A retrospective study of 211 trauma casualties evacuated from Iraq to the US Navy hospital ship (USNS) comfort in early 2013 revealed a total of 56 infections. Wound infections accounted for 84% of cases, followed by bloodstream infections. *Acinetobacter* species (36%) were the predominant organisms followed by *E. coli* and *P. aeruginosa* (14% each) [62].

Another retrospective review over a 7-year period evaluating changes in the incidence of MDR organisms isolated from overseas combat casualties and local military and civilian casualties in a US military medical centre not only showed an increase in the incidence of

MDR *A. baumannii* from 4% to 55% (essentially recovered from the respiratory tract), but also that the majority of these isolates originated from the deployed casualties (52%) rather than local patients (20%) [63]. A study conducted in 2011, aimed to molecularly analyze the Imipenem-resistant *A. baumannii* isolated from US service members wounded in Iraq and Afghanistan from 2003 to 2008. A total of 298 *A. baumannii* isolates were collected of which 46 were identified as resistant to imipenem. These strains collected from different overseas and domestic military treatment facilities, shared over 90% genetic similarity by PFGE. These findings provide a potential cross-transmission of bacteria through environmental contamination of treatment facilities [64]. Another studies confirmed these findings and reported significant increases in the rate of MDR *Acinetobacter* complex infections in military casualties evacuated from Iraq and Afghanistan, resulting in osteomyelitis, extremity soft-tissue deep-wound infections and burn infections [65]. Furthermore, Other studies of osteomyelitis and deep-wound infections in military patients with conflict-related orthopedic injuries have confirmed the predominance of Gram-negative organisms, especially *Aconetobacter* spp., in polymicrobial infections and that whilst early infections are more commonly associated with these organisms [66].

It has been shown that the mechanism of injury sustained during operations in Iraq and Afghanistan includes burn in 5-10% of combat casualties [67,68]. A recent study assessing burn infections amongst deployed and non-deployed civilian patients receiving care at a US military burn centre has shown the pre-eminence of *A. baumannii* as the most prevalent organism recovered from military burn patients injured during operations in Iraq and Afghanistan (58%), contrasting with *S. aureus* in civilian patients (46%) [69]. A similar retrospective cohort study was performed in the US Army Institute of Surgical Research burn center from January 2003 to May 2006 to evaluate bacteremia in burn-patient population. 1,258 patients admitted to the burn center became bacteremic during their hospitalization. Of

these, 92 had bacteremia with the top four pathogens in this burn center, ie, *P. aeruginosa*, *K. pneumonia*, *A. calcoaceticus-baumannii* complex, and *Staphylococcus aureus* [70].

In response to the observed increase in the number of infections in US military casualties, several studies to determine the source of infections have been conducted. Several potential sources of infection have been considered and investigated, including pre-injury skin colonization, environmental contamination (soil) with introduction of infection at the time of traumatic injury, and nosocomial transmission and acquisition of infection after injury in healthcare facilities. It has been shown that up to 17% of a population of 102 healthy active-duty US soldiers, prior to deployment to Iraq or Afghanistan, had skin colonisation with *Acinetobacter* complex [71]. Furthermore, these strains were not related, either genotypically by ribotyping or phenotypically by antimicrobial susceptibility testing, to strains isolated from injured soldiers admitted to military medical facilities.

A. baumannii species are ubiquitous in nature and that therefore environmental contamination may be a source of infection at the time of injury. These presumptions arose prior to species differentiation within the genus and are probably incorrect. In their study, Scott et al.[30] included environmental soil sampling and sampling from deployed healthcare facilities. Of 49 soil samples collected from in and around seven field hospitals in Iraq and Kuwait (18 collected during environmental sampling and 31 archived soil samples), *A. baumanii-A. calcoaceticus* (ABC) complex isolates were recovered from only 1 sample. The remainders of the 36 isolates were obtained from samples of the field hospital environment. Studies have consistently failed to show any natural habitat of these organisms outside medical facilities and they have only rarely been isolated from soil and water samples, likely reflecting the hospital, rather than the natural, environment as an innate habitat.

Other studies showed that non-US patients, who have experienced prolonged hospital admissions and have often been transferred between US and Iraqi hospitals, may have served not only to introduce *A. baumannii* and gram-negative bacilli into military treatment facilities but also as a potential reservoir for nosocomial transmission of infection [72–75].

Apart from common gram-negative bacilli and *A. baumannii*, data from recent conflicts in Iraq and Afghanistan related to war wounds and obligate anaerobes are limited. It has long been postulated that anaerobes complicating war wounds are from the environment, particularly the soil and *Bacteroides* spp. have also been shown to be well-adapted and present in soil [76]. A study by White et al. [77], showed that from a total of 4 180 US combat casualties evacuated to Germany during the study period, 59 had growth of obligate anaerobes on culture, *Bacetroides* spp. being the most organism isolated, which was a shift from previous findings, indicating that *Clostridium* species were the predominant obligate anaerobes among war trauma (WWI to the Korean War). Patients infected with obligate anaerobes were noted to be more severely injures, with a high mortality rate.

Furthermore, a lot of studies have described infrequent bacterial infection during wars in Iraq and Afghanistan. A study conducted in 2011, described a *Brucella melitensis* infection following military duty in Iraq [78]. These infections which are primarily acquired via consumption of high-risk foods or travel to endemic areas, are less prevalent in the US, but common in the Middle East. The case-patient was a 23-year-old who was returned to the US following his second deployment in Iraq, where he was involved in combat and patrol operations.

Rickettsial and rickettsial-like diseases have played a considerable role in military activities throughout much of recorded history [79]. These diseases, which have worldwide distribution and cause a high number of deaths and illnesses, include the select agents *Rickettsia*

prowazekii and *Coxiella burnetii*, the causative agents of epidemic typhus and Q fever, respectively. The outbreak of Q fever and brucellosis in local residents in the Bamyan province of Afghanistan during 2011 and the historic presence of spotted fever group rickettsiae (SFGR) and vectors known to carry SFGR highlight the inherent risk of contracting rickettsial-like diseases in Afghanistan. A recent study done to reveal seroconversions for *Coxiella* and Rickettsial pathogens among US marines deployed to Afghanistan from 2001–2010, showed that the rate of *C. burnetii* and SFGR serocoversions were 3.4% and 0.5% respectively [80]. This study highlighted the risk of contracting Q fever and the need for Q fever diagnostics in military engagements in Central Asia.

Troop disease including acute respiratory disease (ARD) poses a serious public health to deployed army. In November 2006, a significant increase of ARD was detected in soldiers of different nationalities, with a 10-fold increase among French troops at week 51 in Kabul, Afghanistan. This outbreak which was mainly due to pertussis, highlights the importance of nontraumatic illness in wartime when military field conditions enhance exposure to, and incidence of, endemic diseases [80].

c- Wars in East Asia

Few studies have described bacterial infection during wars in the eastern zone of the continent. During the Korean War from June 25, 1950 to July 27, 1953, the prisoners of war (POWs) who fought for the communist side of North Korea and the People's Republic of China were held captive in United Nations-administered POW camps. The camps were built on Geoje-do (Geoje island), Jeju-do, and several mainland areas of the southern part of the Korean peninsula under US direction, and the largest camp was set up in Geoje-do.

Several studies have described the causes of death of prisoners of war during the Korean War [81,82]. A recent study showed that the most common category of causes of death of POW's

was infectious disease, tuberculosis and dysentery/diarrhea were the most common or leading causes of death, followed by tetanus [83]. The study revealed that the death of prisoners by infection disease was due mainly to two reasons. First, the general population in Korea during the war experienced poor health, with low immunity and resistance to the causative agents of disease because of an inadequate diet and semi-starvation conditions. Second, the concept of sanitation in Korean society was unfamiliar and poor [83]. Furthermore, overcrowding of the POW's camps could allow infectious disease to spread rapidly, exacerbate sanitation problems at the camps, and result in a decline in individual hygiene.

Another study in Japan, described a cutaneous melioidosis in a man who was taken as a prisoner of war by the Japanese during World War II [84]. This infection caused by the gram-negative bacillus *Burkholderia pseudomallei*, is endemic to Southeast Asia and Northern Australia. Human infection is acquired through contact with contaminated water via percutaneous inoculation which explains the infection in this prisoner who presented with a nonhealing ulcer on his right hand.

d- Wars in Central & South-East Asia

Vietnam was considered truly war-torn for a long time, with an ever-moving populace ad innumerable refugee camp. Several studies have described bacterial infection during the Vietnam War. A study from the Vietnam conflict reported findings from112 initial wound cultures, 2 of which yielded *Alcaligenes* (possible *Acinetobacter* species). The other gram-negative pathogens identified included *Aerobacter aerogenes* and *Pseudomonas* species, but no *Acinetobacter* species or Mimeae-Herellea-Bacterium-*Alcaligenes* group were described [16]. An analysis of 1531 initial wound cultures performed in Japan from US soldiers wounded in Vietnam during 1967 and 1968 revealed that the most common gram negative bacteria were *P. aeruginosa*, *Proteus species*, *E. coli*, *Aerobacter aerogenes*, and *Klebsiella*

pneumonia [85]. Among orthopedic war wounds evaluated at Brooke General Hospital (now Brooke Army Medical Center) during the Vietnam conflict, 100 tissue samples revealed that *P. aeruginosa*, *Proteus species*, *Klebsiella-Enterobacter* group, and *E. coli* were the predominant gram-negative bacteria identified [86]. Unlike other wars, *A. baumannii* didn't have a role in the Vietnam conflict.

Bacteriological studies were performed on 45 craniocerebral missile wounds incurred in Vietnam within 2 to 4 hours of occurrence. All missiles had penetrated into the brain. Aerobic and anaerobic cultures were taken of the skin wound, brain, and indriven bone fragments. Forty-four of the skin wounds were contaminated, predominantly with staphylococcus. Only five brain wounds showed bacterial contamination 2 to 4 hours after wounding, indicating that many missile tracks within the brain are initially sterile. Of the patients who had early debridement, 45% had contaminated bone within the brain; possibly up to 75% of all indriven bone chips were sterile. This study shows that eventual bacterial invasion of the brain parenchyma from the skin wound or from contaminated indriven bone may occur and account for the rising incidence of brain infection as the time interval lengthens between wounding and definitive neurosurgical debridement with dural and skin closure [87].

Vietnam was one of the regions of the world where plague was endemic, and since 1962 the number of reported cases has steadily increased especially during the Vietnam War. The situation in Vietnam was conducive to transmission of the disease: villages and towns damaged by military action provide ample breeding places for rats, and unmoved garbage provides a food supply close to human beings [88]. Crowded conditions, such as prevail among refugees and those rendered homeless have worsened the situation. In January and February 1966, 44 instances of plague occurred in Hoa Do in Vietnam with 36 deaths [89]. This was the first instance of plague to be diagnosed in the city.

As mentioned earlier, TB is an increasingly important cause of morbidity and mortality in refugee and displaced populations. Between June and December 1998, TB was diagnosed in 178 people in Churachandpur district in India during the civil conflict in that region [90]. TB treatment and control were possible in that region unlike other conflict setting and WHO targets for cure were attainable. The factors associated with the success of the programme were strong local community support, the selection of outreach workers from each ethnic group to allow access to all areas and patients, the use of directly observed therapy three times a week instead of daily in the interest of increased safety, and the limiting of distances travelled by both outreach workers and patients.

Wracked by civil war, Tajikistan has experienced the highest reported rates of diphtheria observed in the epidemic that swept across the Newly Independent States (NIS) of the former Soviet Union. In 1992, because of the civil war, many factors contributed to this situation including an increase in the number of persons who were not fully immunized, a breakdown of health care services and disease surveillance, civil war, an increase in migration, shortages of qualified medical personnel, and shortages of products, resources, and services [91].

3- Prevention of bacterial infections during wars

Prevention of bacterial infections during wars, rely on the prevention of these infections in refugees and wounded citizens and soldiers. Refugees are a major public health thread and play a crucial role in the dissemination of bacterial infections. Currently, there is no consensus regarding screening and infection control measures for refugees. Guidelines for screening upon admission of refugees have been released in Germany and Israel so far, although no specific guidelines for pediatric refugees exist. However, many reports mention that the risk of importation of MDR pathogens through refugees and migrants in non-endemic or low-

endemic host countries is real and represents a serious public health matter [92,93]. Routine carriage testing for MDR pathogens in refugees and migrants, including children and adolescents, upon admission to a healthcare facility should be performed and hospitalization under contact precautions should be implemented until the screening results become available, thus preventing MDR cross-infection in the healthcare settings [10].

In addition to the screening for MDR pathogens, that of TB remains essential. Adequate coordination of TB care services across borders can lead to increased quality of care and consequently to a rupture in transmission. Recently, the TB Advocacy ad-hoc Working Group of the European Respiratory Society and the WHO Regional Office for Europe have proactively launched a new branch of the TB Consilium (a free Internet-based platform) to allow better cross-border TB control among refugees and migrants [94]. Furthermore, easy access to continuous healthcare services is of great importance for the prevention and control of TB among refugees and should be part of an holistic approach to their healthcare needs [95].

Infection control in the war wounded includes several stages from evacuation to the post-operative wound care (Figure 1).

a- Evacuation time and Nosocomial transmission

Evacuation time plays a major role in the nosocomial transmission during evacuation of injured soldiers to their home nations. Kaspar *et al*. found a link between war wounds, the evacuation chain and nosocomial transmission of pathogens [59]. Nosocomial transmission of hospital-acquired organisms remains a significant threat to all patients. Healthcare associated infections may be associated with transmission from other patients, medical attendants or the physical environment itself. During the Iraq conflict, local civilians and detainees accounted for up to 50 per cent of patients in deployed field hospitals [96]. While union troops may stay

in deployed hospitals for a few hours before evacuation, the time spent by local troops, civilians and detainees may be considerably longer. Limited treatment and medical infrastructure may even necessitate these individuals staying for a long duration. This situation represents an 'open reservoir of pathogenic bacteria' as detailed by Miles *et al.* [97]. These studies concluded that more the evacuation time last, more the nosocomial infection increase. Infection prevention and control within the medical chain is now known as a high priority in medical facilities, this include:

- Up to dated design and construction of healthcare facilities
- Suitable infrastructure
- Strong surveillance of both infections and 'alert' organisms
- Education at all levels
- Trained infection control personnel, both as part of the deployed hospital strength and 'reach-back' capability to home country subject matter experts (SMEs) [20].

b- Pre-hospital antibiotics

The fact behind the administration of military prehospital antibiotics is based on limited animal studies [98]and on timeframes suggested in a study of only 49 cases [99]. More robust animal models should be developed to investigate both the efficacy of antimicrobial agents and their delivery systems. These models need to cover all kind of injuries and all the various species of bacteria associated with wound infection. Until such time, standard practice with narrow spectrum agents only for delayed evacuation remains best practice.

c- Irrigation as an adjunct to debridement

Animal studies have shown that wound irrigation can reduce post-operative contamination and subsequent infection when compared with those wounds created in a similar way in which no irrigation is done [100]. Irrigant delivery by high pressure systems has been under discussion for over 30 years. The use of pulsatile lavage (PL) for irrigating contaminated wounds has been publicized after initial preclinical work [101]. Several studies assumed that

PL was a more effective and efficient method of irrigation to remove bacteria than a conventional irrigation technique [102]. Svoboda *et al*. [103]compared the use of water against normal saline using PL. However, there was no difference in bacterial counts between groups. A subsequent study compared both the delivery mechanism of low versus high-pressure irrigation and the use of different solutions [104]. The authors concluded that none of the tested solutions performed better than sodium chloride, and that a low pressure device using saline solution to irrigate wounds was the best choice. A study done by Owens & Wenke [105], showed that earlier irrigation was more likely to have an effect. It seems probable on the basis of the studies performed to date that wound irrigation does have an effect in reduction of the rates of wound infection.

d- Hydrosurgery as an adjunct to debridement

Hydrosurgery is the new addition to the armamentarium of the military surgeon. This technique uses high pressure irrigation in combination with conventional sharp debridement in a single hand tool and has an important role in combat wounds particularly for repeat debridement at reconstructive surgery. Although this technique has demonstrated a reduction in initial bacterial counts, there was a decrease in overall operative time and cost, making it likely invalid in forward surgical care at least in present.

e- Post-operative wound care and infection: Tropical Negative Pressure Wound Therapy (TNPWT)

TNPWTexposes the wound bed to a negative pressure environment and through deformation of the wound edge, a signaling cascade is initiated which eventually leads to granulation tissue formation and wound healing [106]. It has been shown that TNPWT decrease time from injury to definitive wound coverage, which may have an indirect effect on wound infection rates [107]. In an animal model, Lalliss et *al.* [108] showed a reduced bacterial counts in TNPWT when compared to a standard dressings in a contaminated open fracture

model. Waterman *et al.* [109] also studied the performance of different TNPWT systems in the contaminated animal wound model and found no significant difference in bacterial count reduction between different pieces of equipment. They concluded that it was the technique rather than specific equipment which was significant.

f- Post-operative wound care and infection: Use of dressings in combat wounds

Military soldiers providing far-forward surgical care cannot benefit from the TNPWT technology which is suitable for established hospital facilities. Optimizing wound care is assured by initial dressings applied to the wound. However, no evidence exists regarding the best dressing to use in the initial care of the contaminated military wound, although research is currently in progress [20].

4- Bacterial infections after natural disasters

Natural disasters are always considered as major worldwide problem and pose a threat to humans through increased morbidity and mortality. Climatic changes such as global warming have been suggested as the main triggers for increased severity of these disasters [5]. Natural disasters impose a burden on public health due to outbreaks of infectious diseases such as cholera, typhoid fever, bacillary dysentery, leptospirosis, melioidosis and malaria. These disasters can be divided to water related disaster such as floods, tsunamis, typhoons (hurricanes and cyclones) and non water related disaster such as earthquakes and volcanoes.

Environmental water exposure increases the risk of skin infections and problems; even in recreational marine or fresh water environments with no known source of domestic sewage contamination. During water related disaster and due to contact with flotsam and with sharp objects lying unseen in murky waters, and by clinging to trees or climbing structures in

attempts at self rescue, injuries may occur and infections may subsequently develop [4]. Even in absence of water contamination by sewage, infections can occur in saltwater such that of *Vibrio vulnificus* and atypical mycobacteria or in freshwater such that of *Aeromonas hydrophila*, *Burkholderia pseudomallei* (melioidosis) and *Leptospira interrogans* (leptospirosis) [4]. Furthermore water related disasters may cause disruption of water purification and sewage disposal systems, rupture of underground pipelines and storage tanks, and overflowing of toxic waste. This can lead to increased exposure to contaminated waste, food and more pathogens which can lead to gastrointestinal disease [110].

Several diseases may also occur in non water related disasters (earthquakes and volcanoes) such as cholera, TB and other respiratory disease. An important contributory factor for such disease outbreaks may be the displacement of large numbers of people from their homes into over-crowded shelters where supplies may be limited [5]. The availability and accessibility of medical services after such natural disasters is also a major concern [111]. The infectious diseases outbreaks that occurs after natural disaster are summarized in Table 2

a- Floods

Flood is one of the most common and most severe forms of natural disasters, accounting for up to one half of all natural disasters in the world [112,113]. Flooding events are expected to increase in frequency and intensity due to rising sea levels and more frequent and extreme precipitation events [114].

China is one of the most flood-prone countries in the world. Large population, complicated topography and climate conditions, and rapid urbanization promote a high risk of exposure to flood [110]. During the flood event of Huai river in 2007, the most commonly notified diseases were malaria (incidence rate =17.867/100,000), diarrhea (incidence rate =

8.113/100,000), and bacillary dysentery (incidence rate = 3.474/100,000) [110]. A quantitative analysis of burden of bacillary dysentery associated with floods in Hunan, China showed that floods were significantly associated with an increased risk of the number of cases of bacillary dysentery (OR = 3.270, 95% CI: 1.299–8.228 in Jishou; OR = 2.212, 95% CI: 1.052–4.650 in Huaihua) [115].

During July–August 2010, Pakistan experienced extreme flooding that affected approximately 18 million persons. A surveillance study was conducted for outbreak detection and response. The most common diseases found were acute watery diarrhea, bloody diarrhea and acute respiratory infection. This surveillance contributed to the fast detection of cholera, bacillary dysentery and severe pneumonia respectively [116].

Almost every year many districts of Assam, India witnesses flood during Monsoon (rainy season) which leads to plenty of extra water received in glacier fed Himalayan River system (Brahamputra). Often after flood, the state faces the challenge of diarrheal diseases including cholera. In a study conducted on different sites from the states showed that before monsoon 40 % sites were found positive for *V. cholerae* whereas during monsoon 86% sites were observed positive [117]. Several other reports described cholera outbreaks in India after natural disaster especially floods [118,119] but also in South East Asia as Bangladesh [120].

b- Typhoons (hurricanes and cyclones)

Typhoon (hurricane or cyclone, depending on the geographic location) is a storm system categorized by wind speeds ranging from 119 km/h to over 252 km/h on the Saffir-Simpson scale. Between 1980 and 2009, typhoons caused an estimated 412,644 deaths and 290,654 injuries and affected 466 million people [121]. The impact of typhoons goes beyond immediate deaths and injuries. The dissemination of bacterial infections and the damage of public health infrastructures, lead to long-term health effects on the population.

In Asia, the Philippines is well known as an incredibly disaster-prone country. On September 26, 2009, a typhoon caused serious flooding in Metro Manila. After that typhoon, an outbreak of leptospirosis occurred; 471 patients were hospitalized and 51 (10.8%) died [122]. On 8 November 2013, a storm surge caused by Super Typhoon Haiyan (Yolanda) inundated the entire coastal areas of Tacloban and Palo in Leyte, Philippines. A study was conducted on soil samples along the coastal areas of Tacloban and Palo 2 months after the storm surge. Leptospires were isolated from primary cultures of 22 out of 23 samples. When these isolates were experimentally mixed with soil, they were found to survive in seawater for 4 days. These results show the possibility that leptospires living in soil survived after the storm surge. These findings may serve as a warning that when saltwater covers the land during a disaster, an outbreak of leptospirosis could occur in the disaster-stricken area [123]. After the same typhoon, a retrospective, descriptive analysis of admissions to Ormoc District Hospital (ODH) in Leyte, Philippines, for October to February 2013 was performed. Gastroenteritis and pneumonia were the main medical causes of admission prior to the typhoon for adults and children. Afterwards, there was a statistically significant increase in their incidence. An outbreak of gastroenteritis was detected mainly caused by the contaminated water from the municipal water system. Furthermore, an increase in TB cases was noted likely due to the interruption of treatment but also to the lack of access to healthcare facilities [124].

On 25 May 2009, a major cyclone named Aila at a speed of 120-140 km per hour hit the coastal islands of the Sunderbans, the largest delta islands in the world, situated in the southern part of West Bengal, eastern India. Several cholera outbreak have been reported related to disruption of the water distribution system and inadequate hygiene situation after this cyclone [119,125,126]. Another severe cyclonic storm hit the area of Pondicherry, Southern India on 7 January 2012, and was the reason of another cholera outbreak. A study

672 was performed to investigate the outbreak, which suggested that it was due to ingestion of
673 water contaminated by drainage following the cyclone [6].

674 In 2009, the moderate-strength Typhoon Morakot, with a maximum cumulative rainfall
675 amount up to 3,059.5 mm, damaged Taiwan. Several studies has described that more
676 leptospirose and melioidosis cases were observed after the typhoon period in 2009 [127,128].
677 Furthermore, the positive rates for *Clostridium tetanus* and *Clostridium chauvoei* in Pingtung
678 county after the severe floods caused by the typhoon Morakot increased significantly from
679 13.73 and 7.84% to 53.85 and 50.00%, respectively. This results were obtained from a study
680 conducted on soil samples collected from different areas of Taiwan, which are the regions that
681 are most frequently damaged by the typhoon. This study shows that there are changes in the
682 environmental distribution of *Clostridium* spp. after water-related disaster and indicates that
683 screening for soil-related zoonotic pathogens is a potential strategy that may help to control
684 the spread of these diseases [129].

685 **c- Tsunamis**

686 Tsunamis are considered a nightmare to all coastal lands. East (Japan) and South-East
687 (Indonesia, Thailand) Asian countries are the most frequent countries touched by tsunamis in
688 the world. Ordinary bacterial pathogens, such as pyogenic *Staphylococcus* and *Streptococcus*,
689 are common causes of skin infections in tsunamis and flooding and should be treated with
690 appropriate antibiotics. However, other less common bacterial infections associated with
691 marine (e.g. *Vibrio vulnificus*) or aquatic (e.g. *Aeromonas hydrophila*) habitats may also
692 occur, many of which are naturally present in the soil, vegetation, and waters of certain
693 geographic areas. However, tsunamis increase exposure to these pathogens, consequently
694 increasing infection rates [4].

On 26 December 2004, a tsunami devastated the west coast of Thailand and caused 8457 injuries and 5395 deaths. A large number of *Aeromonas* infections were reported after the 2004 Asian tsunami. Among 777 patients transferred from southern Thailand to four Bangkok hospitals, 515 (66.3 %) had Skin and Soft Tissue Infections (SSTIs) and *Aeromonas* was the most common isolated organism, accounting for 145 (22.6 %) of 641 bacterial isolates [130]. Furthermore, the Bangkok Hospital in Phuket, Thailand, reported that approximately 25 % of the isolates from hundreds of patients with prolonged water exposure contained *Aeromonas* [131].

Atypical mycobacteria are nontuberculous, mildly acid-fast bacilli that are ubiquitous in soil and water in various natural environments around the world. They can cause lazy primary infections in healthy individuals, particularly when traumatic wounds are exposed to contaminated water, but they are often more virulent in immunocompromised patients. Several reports of patients diagnosed with these atypical mycobacterial infections followed the 2004 Asian tsunami have been published [132,133]. In one report of 15 tsunami survivors who suffered traumatic injuries and subsequently developed late-onset SSTIs, mycobacterial isolates included seven cases of *M. abscessus*, six cases of *M. fortuitum*, and one case each of *M. peregrinum* and *M. mageritense* [133].

Burkholderia pseudomallei Infects humans and animals, causeing disease mainly in Southeast Asia and coastal areas of Australia's Northern Territory. The disease is acquired after exposure to contaminated soil or water, either directly. This disease has an increased incidence during rainy or monsoon seasons, tsunamis and floods. In addition to the numerous reported cases of severe post immersion, aspiration pneumonic melioidosis after the 2004 Asian tsunami [134,135], many cases of *B. pseudomallei* cutaneous wound infections were also reported [136–138].

This same tsunami affected other neighboring countries such as Sri Lanka, India and Indonesia which beared the huge portion of the losses. A study was performed to determine the health impact of this tsunami in Indonesia [139]. In the first month after the tsunami, up to 96 cases of tetanus were reported in Aceh with an epidemic peak between 8 and 17 January 2015. This was due to the low coverage rates of tetanus vaccination in Aceh which posed a major post-tsunami risk of the disease. Furthermore, wound infections accounted for 16.9% of all diagnoses in a clinic of the Indonesian army. In addition, during the first five months following the tsunami, 37,492 cases of acute respiratory infections were reported to the WHO [139]. 19 months after the tsunami, another study investigated pathogens in natural aquatic habitats in the affected area in Banda Aceh. At the same time, interviews with tsunami survivors were performed to determine the influential factors that facilitated wound infections after the tsunami. From the 49 water samples tested, *Aeromonas* sp., *Vibrio* sp., *Klebsiella* sp., and *Proteus* sp. were isolated from 24, 16, 15, and six samples, respectively [140]. This confirms the result of previous studies done which showed that bacteria in natural aquatic environment are an important source of infection.

On March 11, 2011, an earthquake occurred off the coast of eastern Japan. This earthquake, which has been named the Great East Japan Earthquake, caused a massive tsunami that resulted in approximately 20,000 deaths and left 23 million tons of debris. Sludge brought by the tsunami could contain pathogens with the potential to harm workers. A study was conducted to identify bacteria in sludge brought by the 2011 tsunami in Japan to determine the necessary precautions for workers who handle the sludge [141]. The study showed the detection of 51–61 genera in sludge samples and 14 and 17 genera in water samples collected in the tsunami-affected areas. In sludge samples collected in the tsunami-affected areas, more genera belonged to *Proteobacteria* than to *Bacteroidetes*, but in water samples collected in these areas, more genera belonged to *Bacteroidetes* than to *Proteobacteria*. They concluded

744 that sludge brought by the tsunami contained some pathogens; therefore, frequent hand
745 washing is recommended for workers who have direct contact with the sludge to minimize
746 their risk of infection.

747 Within 3 weeks of the same tsunami, an increased number of pneumonia admissions and
748 deaths occurred in local hospitals. A multicentre survey was conducted at three hospitals in
749 Kesennuma City (population 74 000), northern Miyagi Prefecture [142]. A total of 550
750 pneumonia hospitalisations were identified, including 225 cases during the post-disaster
751 period. A marked increase in the incidence of pneumonia was observed during the 3-month
752 period following the disaster due to the aspiration of seawater [142]. Other studies have also
753 highlighted the same type of infection during this tsunami [143,144].

754 **d- Earthquakes**

755 Earthquake disasters are found to be the second most reported natural disaster (after floods)
756 and the first among the geophysical disasters. They are specifically reported in regions with
757 high seismic activity such as in America (central and south) and Asia (southeast and central
758 Asia). Outbreaks of infectious diseases may be reported when the earthquake disasters result
759 in substantial population displacement into unplanned and overcrowded shelters, with limited
760 access to food and safe water. Furthermore, disease outbreaks may also result from the
761 destruction of water/sanitation systems and the degradation of sanitary conditions directly
762 caused by the earthquake [3]. Epidemics of diarrheal diseases among victims are frequently
763 related to fecal contamination and contamination of water during transportation and storage.
764 Outbreaks have also been related to shared water containers and cooking pots, scarcity of
765 soap and contaminated food. Following the 2005 earthquake in Pakistan, an estimated 42%
766 increase in diarrheal infections was reported in an unplanned and poorly equipped refugee
767 camp [145]. This was due to poor hygiene, crowding, lack of potable water and ineffective

768 sanitation. Another study after the same earthquake showed that wound infection following
769 trauma was the commonest complication in the earthquake patients mainly due to the
770 contamination of wounds by debris resulted from the earthquake [146].

771 TB is a major concern in refugee settings, especially in post-conflict situation. Factors such as
772 population displacement, poor access to healthcare services and interruption of on-going treat-
773 ment or control programs may increase the disease burden. Several reports have described the
774 increasing incidence of TB after the 2011 Great East Japan Earthquake [147–149].
775 Overcrowded shelters and poor hygiene and sanitation were the main factors of that increased
776 incidence.

777 Increased TB rates were also described in China after the earthquake that hits Wenchuan in
778 2008 [150]. Several other reports have described wound infections among patients who
779 survived that earthquake [151–153]. The most common bacteria isolated were *A. baumannii,*
780 *E. coli* and *S. aureus*. Most patients in the Wenchuan earthquake were buried under ruins with
781 soil, brick or stone. The subsequent rainstorm and high temperatures made conditions even
782 worse and most wounds were heavily contaminated. Cases of gas gangrene caused by
783 earthquakes were rarely reported, and no hospital has reported admitting suspected cases of
784 patients with gas gangrene in the short term after earthquake. A study was designed to
785 investigate clinical characteristics, appropriate therapy, and effective control of nosocomial
786 cross-infection of gas gangrene in Wenchuan earthquake victims [154]. Sixty-seven cases of
787 suspected gas gangrene were found, in which 5 cases were confirmed by culture of
788 *Clostridium perfringens*. This result may be related to the following factors: serious injury
789 and long duration of trauma (which promotes infection) and poor sanitation in Healthcare
790 centers.

791

5- Prevention of bacterial infection after natural disaster

Several reports have described the direct impact of natural disasters on public health increasing mortality and morbidity. Bacterial infection and diseases outbreaks are a major concern in disaster settings. The consequences of devastating disasters such as Hurricane Katrina in the USA (2005) and the Great Eastern Japan Earthquake and tsunami (2011) have shown that even the most developed countries are vulnerable to natural disasters. Risk assessment is essential in post-disaster situations and the rapid implementation of control measures delivery should be given high priority to prevent the dissemination of bacterial infections (Figure 1).

a- Organization of shelters

Shelters should be organized according to the existing international guidelines [155]. This includes providing 3.5 m2 of shelter space per person, building one latrine for every 20 persons and locating the latrines at 30 m distance from shelters and 100 m distance from water supplies [156]. This good organization of shelters helps preventing water-borne and air-borne diseases.

b- Water supply, hygiene and sanitation

Good supplies of water per person (minimum agreed standard of 20 l per person per day) for drinking, bathing, washing and for excreta disposal, as well as management of solid wastes, are primordial in preventing outbreaks of diarrheal diseases and other vector-borne diseases. Adequate and sufficient water containers, cooking pots should also be provided. People should be aware that water storage containers are well protected and that the food is well cooked. It is necessary to provide sufficient amounts of soap (minimum of 250 g per person per month) and to educate the community on personal hygiene and circumstances in which

hand washing is essential [157]. Chlorine remains the standard disinfectant for drinking water and the most affordable one especially where no alternative supply of safe water exists [158].

c- Health education and disease management

Besides a sufficient level of sanitation, medical supplies should be provided and training of healthcare personnel on appropriate case management should be conducted. Health workers and volunteers can play an important role by informing people about the risk of the on-going outbreaks as well as advising them on preventive measures to be taken.

Furthermore, every country should have an emergency plan to be adopted especially in developing countries where outbreaks may go unnoticed due to the lack of basic medical facilities. Moreover these countries still live in unstable conditions which make the settlement of emergency plan impossible.

6- Conclusion

In the past decade, wars and natural disasters have become a serious public health problem. Bacterial infections and infectious diseases outbreaks resulting from these phenomena represent a major global challenge. This review described bacterial infections and outbreaks occurring during wars and after natural disasters in Asia. Furthermore, it sheds the light on prevention and control measures to be considered to limit the dissemination of bacterial infections during and after these two phenomena.

It has been shown in this review that bacterial infections during wars are not restricted to military persons but also to refugees who represent a major source for this dissemination. In addition to wars, natural disasters will continue to be a threat to our global community. They are continuously occurring globally, leading to population movement and to the exacerbation of factors that enhance the spread bacterial infection.

Therefore, the major challenge now is to prevent wars and natural disasters from transmitting bacterial infection. Prevention of bacterial infections during wars rely on the prevention of these infections in refugees (Screening for specific pathogen, organization of shelters, adequate water supply…), but also in wounded citizens and soldiers, (quick evacuation, wound care, prevent nosocomial transmission….). As for the natural disasters, rapid implementation of prevention and control measures should be a priority in community displaced by disasters. Management protocols should be implemented according to the national guidelines.

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Table 1: Infectious diseases outbreaks following war

War	Country	Year(s)	Infectious disease outbreak following the war	Ref
Wars in the Middle East	Syria	2014	*P. aeruginosa*, *E. coli*, *A. baumannii*, and MRSA,	22
		2013-2014	ESBL-producing and/or carbapenem-resistant *Enterobaceriaceae* (CRE), MRSA and *Acinetobacter baumannii-calcoaceticus* complex (ABC)	24,25
		2014	Tuberculosis (TB)	32
	Lebanon	1975-1984	*S. aureus*, *P. aeruginosa*, and *E. coli*	38
		1991	sepsis, and hemorrhage	38
		1981-1988	Intracranial infections	38
		2013	*P. aeruginosa*, and *E. coli*	40
		2012-2013	*A. baumannii*, *E. coli* and *Klebsiella pneumoniae*	40, 41
	Israel	1982	*P. aeruginosa*	42, 43, 44
		1977-1991	clostridial infections, *Bacteroides spp*. and *Enterobacteriaceae*.	44, 45

	Kingdom of Saudi Arabia	1978-1982	Diarrheal disease	47,48
		1991	*E. coli* and *Shigella sonnei*	49
	Yemen	2017	cholera	50
Wars in Iraq and Afghanistan	Iraq	1981-1987	*k. pneumonia*e and *Enterobacter*	52
		2009-2016	*S. aureus, E. coli, P. aeruginosa, K. pneumonia, Proteus* spp, *Enterobacter* spp and *A. baumannii*	53
		2015	*Vibrio cholera*	54
		2014	Tuberculosis (TB)	55
	Afghanistan	2002	Tuberculosis (TB)	56, 57
	Military operations in Iraq and Afghanistan	2003-2008	*A. baumannii*	60,64 65,66 , 69
		2013	*Acinetobacter* species, *E. coli* and *P. aeruginosa*	62
		2010	*S. aureus*	69
		2008	*P. aeruginosa, K. pneumonia, A. calcoaceticus-baumannii* complex, and *Staphylococcus aureus*	70
		2011-2016	*Bacetroides spp., Clostridium* species, and *Brucella melitensis.*	77, 78

		2001-2010	*Rickettsia prowazekii* and *Coxiella burnetii*	79-80
		2006	*Bordetella pertussis*	80
Wars in East Asia	Korea	1950 - 1953	infectious disease, tuberculosis and dysentery/diarrhea	83
	Japan	2005	*Burkholderia pseudomallei*	84
Wars in Central & South-East Asia	Vietnam	1968	*Alcaligenes, Aerobacter aerogenes and Pseudomonas species*	16
		1969	*P. aeruginosa*, *Proteus species*, *E. coli*, *Aerobacter aerogenes*, and *Klebsiella pneumonia*	85, 86
		1971	staphylococcus	87
		1962	plague	88, 89
	India	1998	Tuberculosis (TB)	90
	Tajikistan	1992	diphtheria	91

Table 2: Infectious diseases outbreaks following natural disasters

Natural Disaster	**Country**	**Year(s)**	**Infectious disease outbreak following natural disaster**	**Ref**
Floods	Huai river, China	2007	malaria, diarrhea, and bacillary dysentery	110
	Hunan, China	2016	bacillary dysentery	115

	Pakistan	2010	cholera, bacillary dysentery and severe pneumonia	116
	India	2009, 2010, 2016	*V. cholerae*	117, 118, 119
	Bangladesh	2008	*V. cholerae*	120
Typhoons	Philippines	2009	leptospirosis	122, 123
	Philippines	2013	Gastroenteritis, pneumonia, and Tuberculosis (TB)	124
	India	2009, and 2012	cholera	6, 119, 125, 126
	Taiwan	2009, 2012, 2013	leptospirose, melioidosis and Clostridium spp	127, 128, 129
Tsunamis	Thailand	2004	*Aeromonas*	130, 131
	Asian tsunami	2004	*M. abscessus, M. fortuitum, M. peregrinum, M. mageritense Burkholderia pseudomallei, aspiration pneumonic melioidosis* , and *B. pseudomallei*	132-138
	Indonesia	2015	Clostridium tetani, acute respiratory infections , *Aeromonas sp., Vibrio sp., Klebsiella sp.,* and *Proteus sp*	139, 140
	East Japan	2011	*Proteobacteria, Bacteroidetes,* and *K. pneumonia*	141-144
Earthquakes	Pakistan	2005	diarrheal infections, and wound infection	145, 146
	East Japan	2011	Tuberculosis (TB)	147-149
	China	2008	Tuberculosis (TB) , *A. baumannii, E. coli, S. aureus* and gas gangrene caused by *Clostridium perfringens*	150-154

Figure 1: Prevention of bacterial infections during wars and after natural disasters

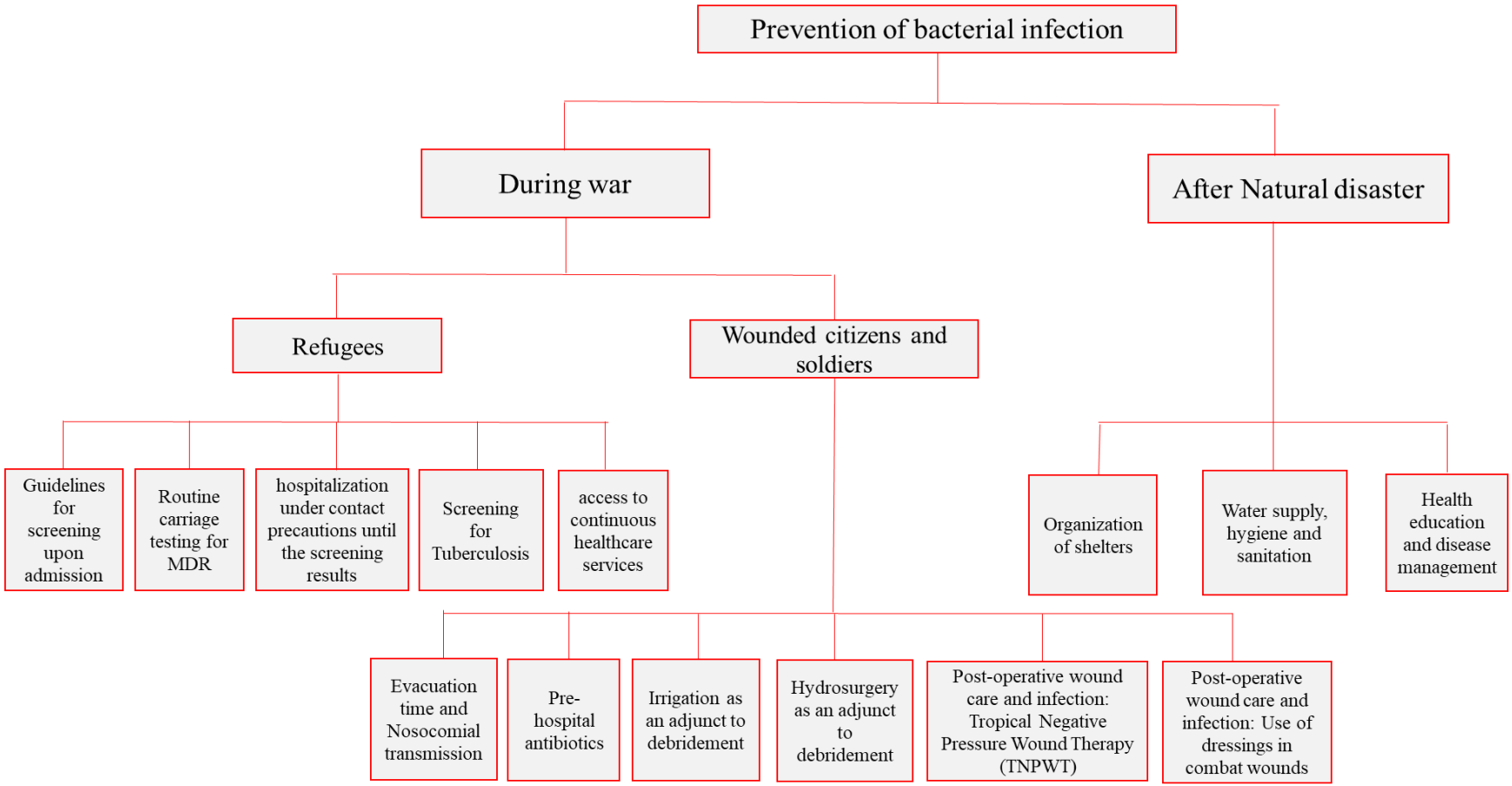
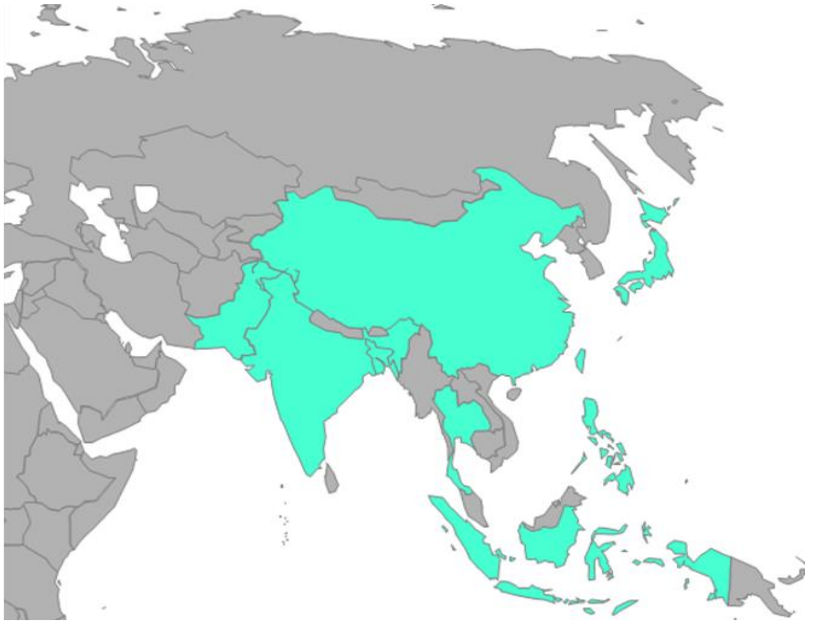


Figure 2: Distribution of bacterial infections in countries affected by wars (**A**), and by natural disasters (**B**)

(A) Countries affected by war



(B) Countries affected by natural disasters

II- INVESTIGATION OF CARBAPENEM-RESISTANT BACTERIA IN LEBANESE HOSPITALS

Resistance to carbapenems in Gram-negative bacteria such as Enterobacteriaceae, *Pseudomonas*, and *Acinetobacter* species over the last decade has become a major problem worldwide because of their rapid spread and the lack of development of new antimicrobial drugs. Since the description of a metallo-β-lactamase IMP-1 in *Pseudomonas aeruginosa*, and a serine carbapenemase OXA-23 in *Acinetobacter baumannii,* carbapenemase encoding genes have spread worldwide and are now distributed through the main Gram-negative multidrug resistant bacteria, responsible of a large number of hospital-acquired and nosocomial infections [1]. These infections often led to higher morbidity and mortality, higher healthcare costs and, in particular, can limit therapeutic options [2].

Since the early 1980s, *A. baumannii* outbreaks have been observed worldwide, and an investigation of these outbreaks using molecular typing methods demonstrated the relative ease of transmission of this organism between hospitals via the transfer of colonized patients [3]. MDR *A. baumannii* are associated with a wide spectrum of infectious diseases, ranging from nosocomial and community acquired infections to those acquired in natural disasters or wars [4]. Several mechanisms contribute to carbapenem resistance in *A. baumannii* such as the expression of β-lactamases, alteration of cell membrane permeability, and over expression of efflux pumps [5]. The most important mechanism of carbapenem resistance in *A. baumannii* is the enzymatic hydrolysis mediated by carbapenem-hydrolyzing β-lactamases, belonging to class D ($bla_{\text{OXA-23}}$, $bla_{\text{OXA-24}}$, $bla_{\text{OXA-51}}$, $bla_{\text{OXA-58}}$, $bla_{\text{OXA-104}}$, $bla_{\text{OXA-143}}$, $bla_{\text{OXA-164}}$, and $bla_{\text{OXA-182}}$) [4]. On the other hand, *Pseudomonas aeruginosa* is resistant to carbapenem due to the production of three classes of carbapenemase enzymes: Ambler class A, B, and D. The main mechanism of resistance

occurs by the production of metallo-β-lactamases (MBLs) such as VIM and IMP, or by the alteration or the loss of the outer membrane porin protein *oprD*, and by the expression of the efflux pumps [6].

In the first study of this chapter (***Article N °2***), we have investigated the molecular epidemiology and the genetic support of genes that code for the production of β-lactamases and carbapenemases in multidrug-resistant *A. baumannii* clinical isolates collected from Saint-Georges Hospital in Lebanon. In total, 31 *A. baumannii* were isolated from 31 sputum samples collected from patients infected with ventilator-associated pneumonia (VAP) and receiving colistin-carbapenem combination therapy. We searched for the presence of ESBLs ($bla_{\text{CTX-M}}$, bla_{TEM}, bla_{SHV}) and carbapenemases ($bla_{\text{OXA-23}}$, $\text{bla}_{\text{OXA-24}}$, $bla_{\text{OXA-58}}$, $bla_{\text{VIM,}}$ bla_{NDM}, and bla_{SHV}) by phenotypic (culture, isolation and identification) and genotypic approaches (standard PCR, RT-PCR and sequencing). Most of the isolates exhibited multidrug-resistant phenotypes. Results of RT-PCR, and standard PCR showed that 30/31 of the isolates harbored the acquired OXA carbapenemase $bla_{\text{OXA-23-like}}$ and one isolate expressed the $bla_{\text{OXA-24-like}}$ gene. In addition, the $bla_{\text{TEM-1}}$ gene was detected in all isolates. MLST results revealed three sequence types, namely ST2, ST699, and ST627. Isolates having ST2 were the most prevalent clone (29/31, 93.5%). This study showed that the circulating *A. baumannii* found in Saint George hospital in Lebanon belonged to international clone II lineage and harbored the OXA-23 gene.

The second study (***Article N °3***) aimed to study the effect of the shift of treatment of multi-drug resistant *A. baumannii* at our institution from colistin-carbapenem combination to colistin

monotherapy. We intended to assess the effect of this change in treatment protocol on *A. baumannii* prevalence and resistance. In total, 17 *A. baumannii* were isolated from 17 sputum samples collected from patients infected with ventilator-associated pneumonia (VAP) and received colistin monotherapy. We searched also for the presence of ESBLs ($bla_{\text{CTX-M}}$, bla_{TEM}, bla_{SHV}) and carbapenemases ($bla_{\text{OXA-23}}$, bla$_{\text{OXA-24}}$, $bla_{\text{OXA-58}}$, $bla_{\text{VIM,}}$ bla_{NDM}, and bla_{SHV}) by phenotypic (culture, isolation and identification) and genotypic approaches (standard PCR, RT-PCR and sequencing). Isolates cultured during this period were less resistant compared to the first study (***Article N °2***), with 64.8 % sensitivity to ceftazidime and cefepime and improvement toward piperacillin-tazobactam and carbapemen sensitivity (17.6%, 3/17). Results of RT-PCR, and standard PCR showed that 6 out of 17 of the isolates carried the class D carbapenemase $bla_{\text{OXA-23-like,}}$ 5 out of 17 of the isolates carried the $bla_{\text{OXA-24-like}}$ gene and 3 out of 17 of the isolates carried the $bla_{\text{OXA-23-like,}}$ and $bla_{\text{OXA-24-like}}$ gene at the same time. In addition, all the isolates carried the $bla_{\text{TEM-1}}$ gene. Results of MLST showed that 10/17 have ST25, one isolate harbored ST99, and 6 isolates have new sequence types ST (1200, 1201, 1202, 1203, 1204, and 1205). This study showed that outbreaks of MDR infection might be sustained and amplified by overconsumption of antibiotics. Results showed that the prevalence of *A. baumannii* in sputum cultures decreased with a positive correlation with the carbapenem fall. Finally, we can conclude that therapy should not be based on “more is better”. Minimizing the antimicrobial selective pressure can affect positively the resistance profile of *A. baumannii*. There is near elimination of AB $bla_{\text{oxa-23}}$-carrying ST2 clone. On the other hand, the rate of non-AB MDR infections remained stable over the study period.

The third study (***Article N °4***) aimed to investigate the molecular mechanism of carbapenem resistance in *P. aeruginosa* collected from ICU patients treated with carbapenem. Four *Pseudomonas aeruginosa* were isolated. They were all resistant to imipenem, with MIC greater than 256 μg/ml. Results of Real-time PCR, and standard PCR showed that 3/4 isolates carried the $bla_{\text{VIM-2}}$ gene. Moreover, all the isolates showed mutations in the OprD gene. MLST analysis reveals that three *P. aeruginosa* harbored the sequence types ST357 and one isolate carried ST233. Conjugal transfer showed that the metallo-β-lactamases $bla_{\text{VIM-2}}$ were plasmidic for ST357 and chromosomally encoded for the clone ST233. This study reports the first detection of the plasmid-encoded $bla_{\text{VIM-2}}$ gene in Lebanon. This finding poses a serious public health problem since the plasmid harboring this β-lactamase is a major source of dissemination of this enzyme through our country.

ARTICLE 2

Title: Investigation of multidrug-resistant ST2 *Acinetobacter baumannii* isolated from Saint Georges hospital in Lebanon.

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Investigation of multidrug-resistant ST2 *Acinetobacter baumannii* isolated from Saint George hospital in Lebanon

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Abstract

Background: *Acinetobacter baumannii* is an opportunistic pathogen causing various nosocomial infections. The spread of multidrug-resistant *A. baumannii* is a major public health problem. The aim of this study was to investigate the molecular epidemiology and the genetic support of multidrug-resistant *A. baumannii* isolates collected from Saint-Georges Hospital in Lebanon.

Methods: Between January and August 2016, 31 *A. baumannii* were isolated from sputum samples collected from patients receiving colistin-carbapenem combination therapy. Antibiotic susceptibility testing was performed using the disk diffusion method. Carbapenemases, extended spectrum β-lactamases encoding genes and mcr-1/2 genes were investigated by RT-PCR and standard PCR. The epidemiological relatedness of the strains was studied using MLST analysis.

Results: Most of the isolates exhibited multidrug-resistant phenotypes. All the isolates were carbapenem-resistant and among them, 30 carried the class D carbapenemase $bla_{\text{oxa-23}}$ gene while one isolate carried $bla_{\text{oxa-24}}$ gene. In addition, $bla_{\text{TEM-1}}$ gene was detected in all isolates. MLST results revealed three sequence types, namely ST2, ST699, and ST627. Isolates having ST2 were the most prevalent clone (29/31, 93.5%).

Conclusions: This study showed the spread of the international clone II lineage *A. baumannii* carrying $bla_{\text{oxa-23}}$ in Saint-George hospital in Lebanon. Monitoring and control measures need to be adopted to avoid the spread of *A. baumannii* to patients.

Keywords: *Acinetobacter baumannii*, $bla_{\text{oxa-23,}}$ ST2 clone.

Background

Acinetobacter baumannii is a glucose non-fermentative, gram-negative, opportunistic pathogen and is one of the leading causes of nosocomial and community infections [1, 2]. These features make *A. baumannii* capable of causing a wide variety of clinical complications such as pneumonia, particularly ventilator-associated pneumonia (VAP), bloodstream and urinary tract infections, meningitis, surgical site and wound infections especially in intensive care units [2, 3]. Carbapenems are the first choice in the treatment of severe *A. baumannii* infections [4]. Carbapenem resistant *A. baumannii* nosocomial outbreaks have become a major concern worldwide since they lead to limited treatment options [5]. Several mechanisms contribute to carbapenem resistance in *A. baumannii* such as the expression of β-lactamases, alteration of cell membrane permeability, increased expression of efflux pumps, DNA gyrases, and topoisomerases [1]. In addition, the presence of three different types of β-lactamases in *A. baumannii* leads to β-lactame resistance such as: (1) $bla_{\text{GES-14}}$, bla_{TEM}, bla_{SHV}, $bla_{\text{CTX-M}}$ and bla_{KPC} belonging to Ambler class A β-lactamases; (2) $bla_{\text{IMP-like}}$, $bla_{\text{VIM-like}}$, $bla_{\text{SIM-1}}$, and $bla_{\text{NDM-1}}$ belonging to metallo-β-lactamases and (3) $bla_{\text{OXA-23-like}}$, $bla_{\text{OXA-24-like}}$, $bla_{\text{OXA-51}}$-like, $bla_{\text{OXA-58-like}}$, $bla_{\text{OXA-104}}$, $bla_{\text{OXA-143}}$, $bla_{\text{OXA-164}}$ and $bla_{\text{OXA-182}}$ genes belonging to oxacillinases [6, 7]. The oxacillinases are considered as the main mechanism responsible for carbapenem resistance in *A. baumannii*.

The global dissemination of carbapenem-resistant *A. baumannii* is a major challenge in public health, especially in South and Southeast Asia, where these strains are predominant in nosocomial infections [8].

Regarding Europe, a lower rate of carbapenem resistance was shown in France, Germany and Sweden (10–20%, 8% and 4%, respectively) whereas the rates increase up to 50–80% in Turkey, 85% in Greece, 60% in Italy and 45% in Spain [9]. It has been noticed that in many cases, one or two epidemic strains were perceived in a certain epidemiological setting. This is due to the transfer of colonized patients who transmit these strains between hospitals [10]. To date, in Lebanon, there has been a higher level of carbapenem-resistant *A. baumannii* strains [11].

The aim of the current study was to investigate the molecular epidemiology and the genetic support of multidrug-resistant (MDR) *A. baumannii* isolates collected from Saint-George Hospital in the capital of Lebanon, Beirut.

Results

Antimicrobial susceptibility testing of isolated strains

A total of 31 strains isolated from Saint-George Hospital in Beirut were identified by MALDI-TOF as *A. baumannii*. These isolates were collected from the sputum of the respiratory tract (Table 1). Antibiotic susceptibility testing results revealed high levels of resistance rates of all isolates to ticarcillin, ticarcillin clavulanic acid, piperacillin tazobactam, ceftazidime, cefepime, cefotaxime, imipenem, meropenem, ciprofloxacin, and levofloxacin. In addition, 3.2% of the isolates were resistant to gentamicin, tobramycin, and amikacin. E-tests showed high-level of resistance to imipenem, with MIC greater than 256 μg/ml for all the isolates. None of the isolates was resistant to colistin (MIC < 2 μg/ml).

Detection of beta lactamase genes

Results of PCR for carbapenemase-encoding genes showed that 30/31 of the isolates harbored the acquired OXA carbapenemase *bla*$_{\text{OXA-23-like}}$ and one isolate expressed the *bla*$_{\text{OXA-24-like}}$ gene.

In addition the ß-lactamases gene *bla*$_{\text{TEM}}$ was detected in all isolates. All the sequences of the *bla*$_{\text{TEM}}$ gene were identified as *bla*$_{\text{TEM-1}}$. None of the isolates harbored *bla*$_{\text{NDM-1}}$, *bla*$_{\text{oxa-58}}$, *bla*$_{\text{VIM}}$ gene, *bla*$_{\text{SHV}}$, *bla*$_{\text{CTX-M}}$ and mcr-1/2 genes.

MLST analysis

MLST analysis showed that 93.5% (29/31) of the *A. baumannii* isolates belonged to ST2 sequence type, whereas two isolates were assigned to ST699 and ST627, respectively. The most common clone (ST2), harboring the *bla*$_{\text{OXA-23}}$ and *bla*$_{\text{TEM-1}}$ genes, was found to be circulating in the hospital. The isolate belonging to ST627 was associated with the production of OXA-24 and TEM-1 (Table1).

Discussion

Acinetobacter baumannii has been identified as one of the most successful pathogens responsible for nosocomial infections especially for patients admitted to intensive care units (ICUs) [12]. *A.baumannii* is able to acquire resistance to broad types of antibiotics including carbapenems. Carbapenem-resistant *A. baumannii* has been reported worldwide and has become a significant health problem due to the limited options for antibiotic treatment [13, 14].

Between 1999 to 2009, carbapenem-resistant *A. baumannii* harboring the bla$_{OXA-58}$ gene were predominant in the hospital flora of many Mediterranean countries such as Lebanon, Italy, Greece, and Turkey [15]. After 2009, a huge shift from OXA-58 *A. baumannii* to OXA-23 producing belonging to the international clonal I and II lineages has been observed globally [15].

An outbreak of MDR *A. baumannii* has been observed in Saint George Hospital in Beirut, Lebanon between November 2004 and October 2005 [16].

In our study, the *bla*$_{OXA-23}$ gene was found in 30 carbapenem-resistant *A. baumannii* isolates (96.7%) recovered from Saint George hospital in Beirut. In 2015, a study done in Lebanon showed the predominance of Imipenem-resistant *A. baumannii bla*$_{OXA-23}$ and *bla*$_{GES-11}$ gene -among the majority of *A. baumannii*. This dissemination of OXA-23 carbepenemase in Lebanon is consistent with the worldwide epidemiology of OXA-23 [17]. Also in 2016, Al Atrouni et al. showed the high dissemination of carbapenem-resistant *A. baumannii* harboring the *bla*$_{OXA-23}$ gene and belonging to the international clone II lineage [18] (Table2).

All the isolates in our study were resistant to imipenem. In Lebanon in 2012, it has been shown that 88% of *A. baumannii* were imipenem-resistant. This number is closely related to the rates of 70% in Egypt, 24-72% in Turkey, 25-75% in Spain, and approximating 100% in Italy [17].

In addition to the *bla*$_{OXA-23}$ gene, we identified the presence of the *bla*$_{OXA-24}$ gene in one isolate. A study in Lebanon reported the occurrence of *bla*$_{OXA-24}$ gene in two isolates that harbored also the *bla*$_{OXA-23}$ gene [19]. Moreover, Rafei et al reported that among 31 carbapenem-resistant strains collected from different hospitals in Beirut and Northern Lebanon, 28 isolates carried the *bla*$_{OXA-23}$ gene, 1 strain the *bla*$_{OXA-24}$ gene and 2 strains the *bla*$_{OXA-58}$ gene [20]. She also reported in 2015

the spread of the international clone II lineage with high incidence of *bla*$_{\text{OXA-23}}$ carbapenemase, in addition to the presence of *bla*$_{\text{NDM-1}}$, *bla*$_{\text{OXA-51}}$, *bla*$_{\text{OXA-66}}$ and *bla*$_{\text{OXA-69}}$ in different hospitals in Tripoli, Lebanon [21].

Moreover, we found that the majority of our strains harbored the ß-lactamases *bla*$_{\text{TEM-1}}$ gene. A study in Egypt in 2017 showed that *bla*$_{\text{TEM}}$ is the most frequent gene for ESBL[22]. In Saudi Arabia, Aly et al revealed that some of the isolates harbored the *bla*$_{\text{TEM}}$ resistance genes as well as the *bla*$_{\text{PER-1}}$ gene[23] . Also in Turkey, a study by Beris showed that *bla*$_{\text{TEM}}$ was the most prevalent ESBL type amongst *A.baumannii* strains isolated from different regions [24].

In our study, the analysis of MLST showed that the strains belonged to three different clones, ST2, ST699, and ST627, where the ST2 was the most common clone (29/31). The ST2 and ST699 clones were associated with the production of OXA-23 carbapenemase, and the clone ST627 was associated with OXA-24. The ß-lactamases (ESBL) *bla*$_{\text{TEM-1}}$ gene was found in all STs clones (Table1). The ST2 clone has been reported in several Mediterranean countries. From 1999 to 2009, a study in four Mediterranean countries (Greece, Italy, Lebanon and Turkey) showed that *A.baumannii* outbreaks were caused by the spread of strains belonging in particular to ST2 and, to a lesser extent to ST1, ST25, ST78 and ST20. These clones harbored the *bla*$_{\text{OXA-58}}$, *bla*$_{\text{OXA-23}}$ and *bla*$_{\text{OXA-72}}$ genes [25].

In Greece, it has been observed that the ST2 was the most common clone circulating in Greek hospitals. These clones harbored the *bla*$_{\text{OXA-23}}$ gene that was displacing the *bla*$_{\text{OXA-58}}$ gene, which was the only carbapenemase found among carbapenem-resistant *A.baumannii* isolates until 2009 [26].

Conclusion

In conclusion, this study described the spread of the international clone II lineage *A. baumannii* carrying $bla_{oxa\text{-}23}$ in the hospital. The resistant *A. baumannii* isolates found in Saint George hospital may due to an increase in the usage of carbapenem to treat ICU patients. An urgent strategy needs to be adopted to control the spread of such resistant microorganisms among patients as well as appropriate infection control measures.

Methods

Bacterial isolates

Between January and August 2016, 31 *A. baumannii* isolates were collected from the sputum of the respiratory tract of patients infected with ventilator-associated pneumonia (VAP) and treated with colistin-carbapenem combination therapy in Saint-George Hospital in Beirut. 29/31 samples were collected from the sputum of the upper respiratory tract and 2/31 from the sputum of the lower respiratory tract from hospitalized patients and kept at -80°C before being transported to the laboratory in Marseille. Once arrived, the isolates were cultivated for 24h at 37°C on Trypticase Sodium Agar medium (TSA). Colonies growing on this medium were identified using matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) (Microflex; Bruker Daltonics) as previously described [27].

Antibiotic susceptibility testing

The antibiotic susceptibility testing was performed using the disk diffusion method on Mueller-Hinton agar as recommended by the European Committee of Antimicrobial Susceptibility Testing (EUCAST) 2017. Fourteen different antibiotics were tested: ticarcillin, ticarcillin clavulanic acid, piperacillin tazobactam, ceftazidime, cefepime, imipenem, meropenem, gentamicin, amikacin, tobramycin, colistin, ciprofloxacin, levofloxacin, and cefotaxime. Interpretations of the results of antibiotic sensitivity testing were made according to EUCAST recommendations. In addition, E-test was performed to determine the minimal inhibitory concentration (MIC) of imipenem as recommended by the 2017 European Committee of Antimicrobial Susceptibility Testing (EUCAST) (http://www.sfmmicrobiologie.org/UserFiles/files/casfm/CASFM%20V2_0_Mai2017.pdf). Moreover, the minimal inhibitory concentration (MIC) of colistin was determined using the broth microdilution method according to EUCAST 2017.

DNA extraction

Bacterial DNA was extracted using the automatic robot EZ1 (Qiagen BioRobot EZ1-, Tokyo, Japan), with the extraction kit (EZ1 DNA, Qiagen, Hilden, Germany), following the manufacturer's instructions. The extracted DNA was eluted in 200mL of elution buffer and was stored at -20°C.

Screening of samples by real-time PCR and molecular characterization of beta lactamase genes.

Real-time PCR was performed to screen for the presence of carbapenemase-encoding genes using specific primers previously described for *bla*$_{\text{NDM-1}}$, *bla*$_{\text{OXA-23}}$, bla$_{\text{OXA-24}}$, *bla*$_{\text{OXA-58}}$, *bla*$_{\text{VIM}}$ and *bla*$_{\text{SHV.}}$ All MDR bacteria were also screened for β-lactamases (*bla*$_{\text{CTX-M}}$, *bla*$_{\text{TEM}}$, *bla*$_{\text{SHV)}}$ genes and for the mcr1-2 gene as described previously [28–30]. Negative and positive controls were used in each assay. The positive PCR products for any gene tested were sequenced using BigDye1 terminator chemistry on an automated ABI 3130 sequencer (PE Applied Biosystems, Foster City, CA). The sequences of the genes obtained were analyzed using the ARG-ANNOT database [31] (http://en.mediterranee-infection.com/article.php?laref=283&titre=arg-annot), and compared to other genes using the BlastN and BlastP of the National Center for Biotechnology Information (NCBI) database (http://www.ncbi.nlm.nih.gov).

Multilocus sequence typing

Molecular typing of the isolates was done to determine the genetic relationship among the clinical isolates by using the seven housekeeping genes (*cpn60, fusA, gltA, pyrG, recA, rplB,* and *rpoB*) as described on Institute Pasteur's MLST Web site (https://pubmlst.org/abaumannii). Each single locus has different allele and the allelic profile or sequence types (ST) of the seven loci were given a specific identification number.

List of abbreviations

MDR-AB: multidrug-resistant *Acinetobacter baumannii*, **MALDI-TOF**: matrix-assisted laser desorption and ionization time-of-flight mass spectrometry, **AST**: antibiotic susceptibility testing,

MLST: multilocus sequence typing, **ST:** sequence type. **ESBL**: extended spectrum beta lactamase, **PCR**: polymerase chain reaction, **RT-PCR**: real time polymerase chain reaction.

Ethics approval and consent to participate

Not applicable

Consent for publication

Not applicable

Availability of Data and materials

Not applicable

Competing Interests

No conflicts of interest or financial disclosure for all authors.

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Authors' Contribution

TND and SC wrote the manuscript, performed experiments, and analyzed the data. ED and NA provided the strains and helped draft the manuscript. CB and SD helped draft the manuscript. JMR conceived the study, participated in its design and coordination, and helped draft the manuscript. All authors read and approved the final manuscript.

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Table 1-Phenotypic and genotypic features of 31 *Acinetobacter baumannii* Isolated from Saint Georges hospital in Lebanon

Isolate	M / F	Collection Date	Types of Sputum	IPM	IMP MIC (µg/ml)	Carbapenemases	MLST
1	M	10-06-16	Upper Respiratory Tract	R	>256	OXA-23	ST2
2	M	06-07-16	Upper Respiratory Tract	R	>256	OXA-23	ST2
3	M	08-06-16	Upper Respiratory Tract	R	>256	OXA-23	ST2
4	F	23-07-16	Upper Respiratory Tract	R	>256	OXA-23	ST2
5	M	10-06-16	Upper Respiratory Tract	R	>256	OXA-23	ST2
6	M	08-08-16	Upper Respiratory Tract	R	>256	OXA-23	ST2
7	M	20-06-16	Upper Respiratory Tract	R	>256	OXA-23	ST2
8	F	01-07-16	Upper Respiratory Tract	R	>256	OXA-23	ST2
9	M	15-06-16	Upper Respiratory Tract	R	>256	OXA-23	ST2
10	F	07-07-16	Upper Respiratory Tract	R	>256	OXA-23	ST2
11	F	06-02-16	Upper Respiratory Tract	R	>256	OXA-23	ST2
12	F	30-03-16	Upper Respiratory Tract	R	>256	OXA-23	ST2
13	M	16-03-16	Upper Respiratory Tract	R	>256	OXA-23	ST2
14	F	15-02-16	Upper Respiratory Tract	R	>256	OXA-23	ST2
15	M	11-03-16	Upper Respiratory Tract	R	>256	OXA-23	ST2
16	M	02-05-16	Upper Respiratory Tract	R	>256	OXA-23	ST2
17	M	14-01-16	Upper Respiratory Tract	R	>256	OXA-23	ST2
18	M	28-01-16	Upper Respiratory Tract	R	>256	OXA-23	ST2
19	M	06-02-16	Upper Respiratory Tract	R	>256	OXA-23	ST2
20	M	08-02-16	Upper Respiratory Tract	R	>256	OXA-23	ST2
21	M	24-03-16	Upper Respiratory Tract	R	>256	OXA-23	ST2
22	F	28-01-16	Upper Respiratory Tract	R	>256	OXA-23	ST2
23	M	31-01-16	Upper Respiratory Tract	R	>256	OXA-23	ST2
24	M	08-03-16	Upper Respiratory Tract	R	>256	OXA-23	ST2
25	M	08-03-16	Upper Respiratory Tract	R	>256	OXA-23	ST2
26	F	27-01-16	Upper Respiratory Tract	R	>256	OXA-23	ST699
27	M	31-01-16	Upper Respiratory Tract	R	>256	OXA-23	ST2
28	F	30-01-16	Upper Respiratory Tract	R	>256	OXA-23	ST2
29	M	16-02-16	Upper Respiratory Tract	R	>256	OXA-23	ST2
30	M	12-01-16	Lower Respiratory Tract	R	>256	OXA-23	ST2
31	M	12-01-16	Lower Respiratory Tract	R	>256	OXA-24	ST627

M, male; F, female; ESBL, extended spectrum ß-lactamases; MLST, multilocus sequence typing; ST, sequence type.

Table 2-Study of carbapenemase *Acinetobacter baumannii* in Lebanon

Origine	Carbapenemase genes	Other Resistant genes	ST	Ref
Clinical isolates	OXA-23,OXA-24,OXA-58, NDM-1,		ST2,ST25,ST46, ST85, ST193, ST424, ST570, ST85, ST600, ST622, ST636, ST690, ST702, ST715, ST706,ST707, ST1, ST708, ST713, ST807, ST808, ST809,ST810, ST811, and ST812	[18]
Clinical isolates	OXA-23,OXA-24		ST2, ST4, ST10, and ST14	[19]
Clinical isolates	OXA-23,OXA-24, OXA-58,			[20]
Clinical isolates	OXA-23,OXA-51, OXA66, OXA69, NDM-1,		ST2,ST1,ST460, ST85, ST6, ST25, ST103,ST154, ST3, ST158, ST146,ST459,ST284, ST150, ST108, ST461, ST462	[21]
Clinical isolates	MBL, OXA.			[32]
Clinical isolates	OXA-143		ST286 to ST296 and ST464 to ST476	[33]
Clinical isolates	OXA-71			[34]
Clinical isolates	OXA-23,OXA-24		ST2	[35]
Clinical isolates	OXA-58	GES-5		[36]
Clinical isolates	OXA-23,OXA-24	GES-11		[11]
Clinical isolates	OXA-23,OXA-24	GES-11		[37]
Livestock	OXA-23,OXA-58,		ST491, ST492, ST493, ST2 and ST20	[38]

ST, Sequence Type; Ref, reference

ARTICLE 3

Title: Successful control and elimination of XDR *A. baumanii* ST-2 at a tertiary care center ICU by changing standard of care: colistin monotherapy carbapenem sparing regimen.

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Successful control and elimination of XDR A. baumanii ST-2 at a tertiary care center ICU by changing standard of care: colistin monotherapy carbapenem sparing regimen

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Abstract 50 words

Dispatch 1064 words

Running Title: Near- elimination of XDR A. baumanii ST-2 at a tertiary care center ICU

Abstract

The change in therapeutic pathway of XDR *Acinetobacter baumanii* (AB) infections to colistin monotherapy at our ICU dramatically decreased antibiotic consumption resulting in 78% drop of AB prevalence in sputum culture with near elimination of $bla_{\text{oxa-23}}$-carrying ST2 clone. The rate of non-AB MDR infections remained stable over the one-year study period.

The antimicrobial stewardship (AMS) program at Saint Georges Hospital University Medical Center (SGHUMC) in Lebanon requires pre-authorization by an Infectious Disease specialist for the use of restricted broad-spectrum antibiotics and regularly monitors the rate of nosocomial infections including central line associated blood stream infections (CLABSI) and the total hospital antimicrobial consumption. In the first quarter of 2015, the incidence of extensively drug-resistant *Acinetobacter baumannii* (XDR-AB) blood stream infections reached its highest level of 0.47/1,000 PD (*1*) while the monthly carbapenem consumption increased steadily to 130 DDD/1,000 PD, an absolute increase of a 30 DDD/1000PD since 2012. AB became a nosocomial threat due to limited therapeutic options in severe infections leading to the common practice of combination therapy of colistin and carbapenem, for possible synergetic effect despite carbapenem resistance(*2–8*) . We evaluated 100 non-duplicate XDR-AB isolates collected at SGHUMC and found no synergy between colistin and carbapenem by the checkerboard technique(*9*). The lack of convincing data regarding the combination therapy and the uncontrollable surge of carbapenem consumption led us to change the standard of care of all XDR-AB infections at SGHUMC to colistin mono therapy (loading dose 270mg IV and maintenance dose of 135mg twice daily with normal GFR) or in combination with tigecycline. The AMS office, infectious disease team and the intensive care unit (ICU) physicians approved implementation of this new standard of care.

The Study

Our aim was evaluating the impact of carbapenem sparing regimen on antimicrobial consumption, clinical outcome, and ICU microbiological flora. To note, a long-established protocol at our ICU requires a routine every-third-day sputum culture of intubated patients all throughout the intubation period.

We retrieved data from the hospital's computerized ordering system and retrospectively examined the medical records of patients admitted to the ICU between February 2016 and January 2017. In the first period, February-June 2016, patients received colistin-carbapenem combination therapy for AB infections. The new standard of care was applied during the second period from July 2016 to January 2017. The total number of all bacterial cultures collected from the ICU with note to site and time (in relation to day of admission) of sampling was recorded with exclusion of duplicates. Clinical data included patient demographics, admission diagnosis and presence of mechanical ventilation (MV). The occurrence of ventilator-associated pneumonia (VAP) during the study period was reported, and we defined our variables according to CDC/WHO guidelines for XDR-AB(*10*), case-fatality rate and according to ATS/IDSA for VAP(*11*). Prevalence density is the number of prevalent cases of clinical isolates per 1000 ICU patient days (PD) in a given month. For result analysis we grouped antibiotics in the following: Group 1: Antibiotics not requiring pre approval like third generation cephalosporin , amoxicillin –clavulanic acid and quinolones, Group 2: *Clostridium difficile* (C.diff) therapy (oral vancomycin; metronidazole) , Group 3: imipenem and meropenem, Group 4: Broad spectrum carbapenem-sparing regimens (piperacillin/tazobactam, cefepime, ceftazidime, amikacin), and Group 5:the XDR-AB-active antibiotics (colistin and tigecycline) (Table 1). Antibiotic consumption was measured by defined daily dose (DDD) per 1000 patient days.

Forty-eight laboratory AB isolates, 31 collected from period 1 and 17 from period 2, were sent to IHU-Mediterranee Infection, Marseille for the following microbiological studies: matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) (Microflex; Bruker Daltonics), antibiotic susceptibility testing using disk diffusion method interpreted according to the European Committee of Antimicrobial Susceptibility Testing (EUCAST) 2017, real-time PCR to screen for carbapenemase-encoding genes, and molecular typing to determine genetic relationship among the isolates.

The Results

A total of 537 patients were admitted to the ICU during the study period. In Table 1 demographic and clinical characteristics, all cause ICU mortality and length of ICU stay, types of therapy protocol and number of courses given for XDR-AB VAP and ICU antimicrobial consumption are reported. The patient characteristics between the 2 periods were statistically similar. Throughout the study period, the incidence of AB VAP decreased from 154.9 to 38/1000 PD ($p = 0.0074$) and the rate of AB-VAP case fatality ratio dropped significantly from 79/1000PD to 12/1000PD. ICU all-cause mortality rates between periods 1 and 2 remained unchanged.

In group 1 and group 4 antimicrobials, there was no significant difference in consumption between the 2 periods (Table 1). As expected, carbapenem consumption decreased by a dramatic 59%, a total of 318DDD/1000PD, as well as a 637DDD/1000 PD of total restricted antimicrobial consumption ($p = 0.0045$). Colistin consumption decreased by 55% (20DDD/1000PD in Period 1 to 9DDD/1000PD in Period 2, $p = 0.0185$), due to the significant decrease in the prevalence of AB (Figure 2). Tigecycline consumption remained the same (84 vs 62 DDD/1000PD). Interestingly, group 2 C.diff active therapy consumption significantly dropped by 231DDD/1000PD ($p = 0.0424$), a 51% decrease in period 2 that likely mirrors reduced rate of *Clostridium difficile* infections.

The prevalence of AB in sputum cultures decreased by 53.6% from 82/1000PD to 38/1000PD with a positive correlation with the carbapenem fall (p = 0.004) (Figure 1), without an increase of non-AB MDR isolate cultures (Figure 2).

The microbiological characteristics of AB isolates are listed in Table 2, along with a summary of antimicrobial susceptibility testing. All isolates in the study periods carried ESBL $bla_{\text{TEM-1}}$ genes. During periods 1, all strains were XDR, of which 30/31 strains carried class D carbapenemase $bla_{\text{oxa-23}}$ gene and 1/31 carried $bla_{\text{oxa-24}}$ gene. MLST revealed three sequence types ST2, ST699, and ST627 with ST2 being the most prevalent clone *(29/31, 93.5%).* Overall, the rate of isolation of XDR-AB decreased from 100% in Period 1 to 35.3% in Period 2. On the other hand, isolates from Period 2 had a 64.8% sensitivity to ceftazidime and cefepime (11/17) and an improved piperacillin-tazobactam (17.6%, 3/17) and carbapenem susceptibility (17.6%, 3/17). Six strains carried class D carbapenemase $bla_{\text{oxa-23}}$ gene, 5/17 carried $bla_{\text{oxa-24}}$ gene and 3/17 carried both $bla_{\text{oxa-23}}$ and $bla_{\text{oxa-24}}$ genes. MLST revealed three sequence types, *ST25* (10/17, 58.8%), *ST99* (1/17, 5.9%) and six new sequence types were assigned as: ST 1200,1201,1202,1203,1204, and 1205 (35.2%).

Conclusion

In the era of severely ill, MDR-colonized patient management, relying on existing guidelines is not enough. A creative multidisciplinary approached based on local epidemiology is key. Our work affirms that the added carbapenem pressure sustained a survival advantage for ST2 XDR-AB. Our success in near elimination of the ST2 XDR-AB clone, remarkably reducing AB disease, and the total antimicrobial consumption at our institution was only possible by active surveillance program of antibiotic consumption and resistance profiles as well as trusted collaboration with the ICU and microbiology department.

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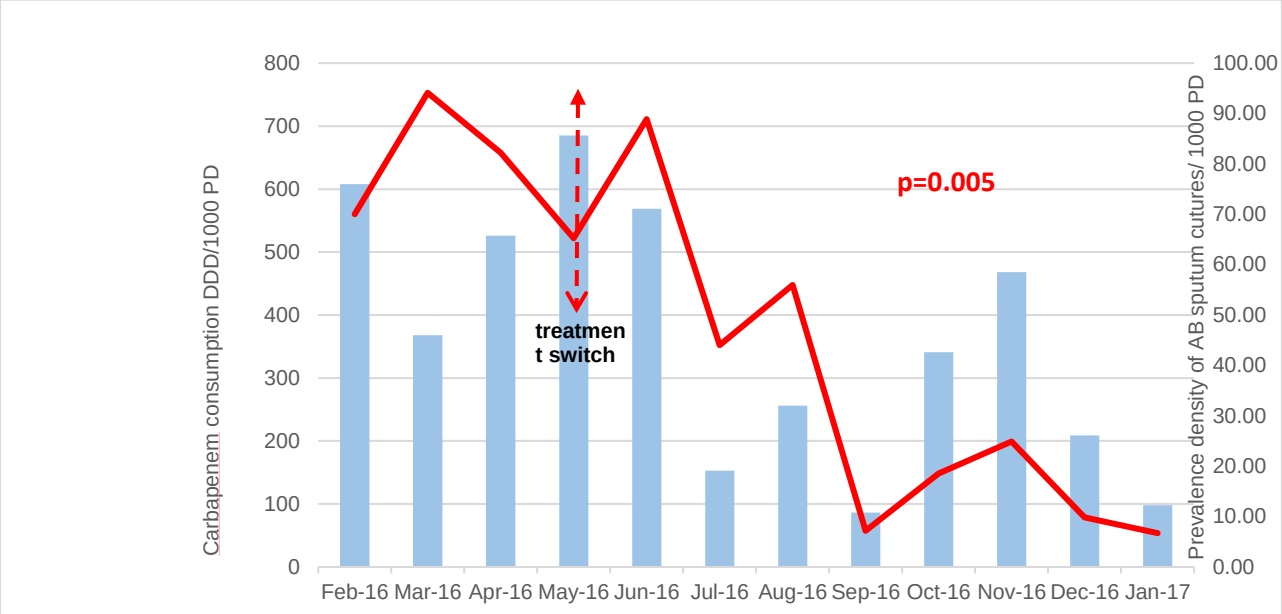
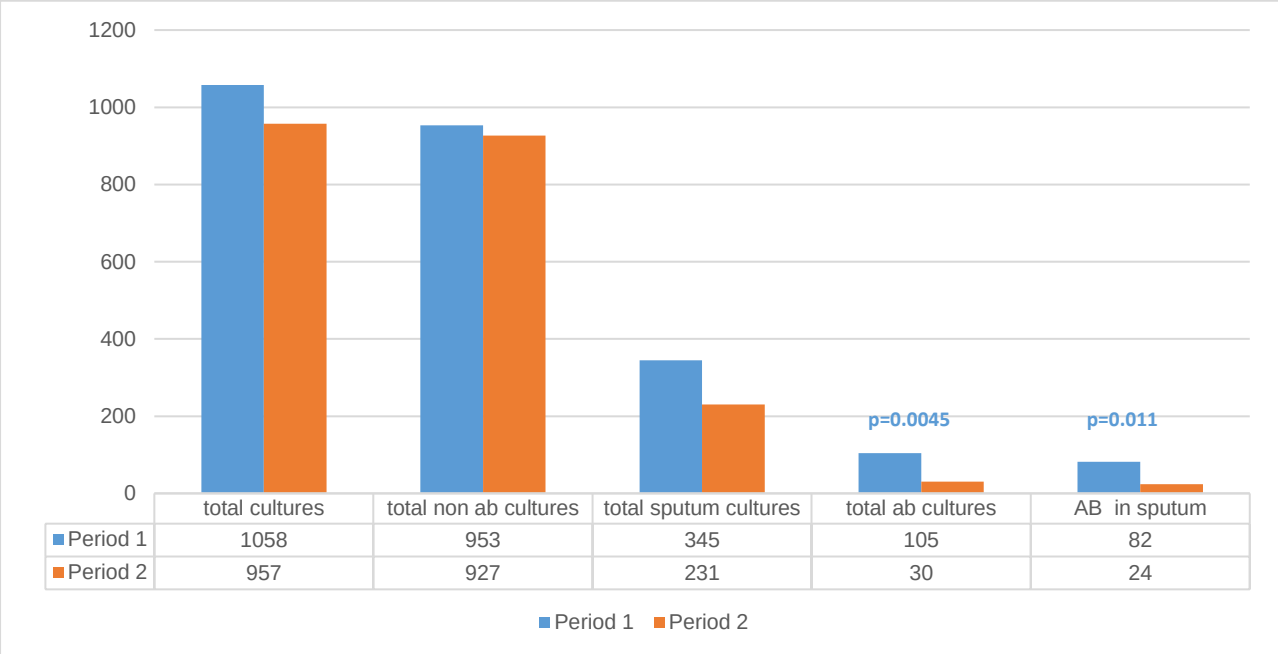
Figure 1: Prevalence Density of AB isolates in sputum cultures/1000 patient days vs. carbapenem consumption by DDDs/1000 patient days



	Feb-16	Mar-16	Apr-16	May-16	Jun-16	Jul-16	Aug-16	Sep-16	Oct-16	Nov-16	Dec-16	Jan-17
Carbapenem/1000 PD	608	368	526	685	569	153	256	86	341	468	209	98
AB Prevalence/1000 PD	70.00	94.12	82.19	65.22	88.89	44.05	55.94	7.17	18.52	24.90	9.85	6.71

Figure 2: Prevalence culture per 1000 ICU patient days in period 1 and 2



	total cultures	total non ab cultures	total sputum cultures	total ab cultures	AB in sputum
Period 1	1058	953	345	105	82
Period 2	957	927	231	30	24

Table 1: Patient Demographics, VAP descriptive and treatment courses, Antibiotic Consumption

Table 1. Patient Demographics, VAP descriptive and treatment courses, Antibiotic Consumption	Period 1 (Feb-Jun16)	Period 2 (Jul 16-Jan 17)	p-value
Number of Patients	213	324	nonsignificant
Female	79	144	
Male	134	180	
Mean Age	69	68	
Mean LOS (days)	6.8	6	
ICU days	1128	1804	
Type of Admission			
Medical admission	163	253	
Surgical admission	50	71	
Admitted from home/ER	73	114	
Transferred from ward	79	141	
Transferred from another hospital	10	16	
Post-operative	51	57	
Intubated on admission	64	85	
Intubated during admission	14	16	
Outcome			p-value
AB VAP incidence	15%	3.70%	0.0074
Discharged	170	259	
Deceased	43	64	
Total AB VAP events	32	12	
Deceased during VAP	17	4	
ICU Mean Mortality rate per month	20.40%	19.30%	0.1675
AB VAP case fatlity ratio	7.9%	1.20%	0.0058
Number of XDR-AB VAP courses received			
Colistin and carbapenem	17	2	
Colistin and tigecycline	6	2	

Colistin monotherapy	6	6	
Tigecycline	3	2	
Consumption DDD/1000 ICU PD	Period 1 (Feb-Jun16)	Period 2 (Jul 16-Jan 17)	p-value
Group 1 Unrestricted:(3rd GC, amoxi-clav, quinolones)	333	320	0.4649
Group 2 C.diff active: vancomycin PO, metronidazole	455	224	**0.0424**
Group 3 Carbapenems: meropenem, imipenem	541	223	**0.0045**
Group 4 Broad spectrum carbapenem sparing	165	145	0.8075
Group 5: XDR-AB Therapy			
colistin	20	9	**0.0185**
tigecycline	184	62	0.5698
Total restricted antibiotics	1214	577	**0.0045**

Table 2: AB specimen type, site of collection and microbiologic characteristics

	Period 1	**Period 2**
A. Type of culture and site of collection		
Sputum	29	11
Blood	0	3
Wound/catheter tip	0	3
ICU	21	12
Regular Floor	10	5
Total Cultures	31	17
B. Percent Antibiotic Susceptibility (by disc diffusion)		

Cefepime/Ceftazidime	0%	**64.70%**
Piperacillin/tazobactam	0%	**17.65%**
Imipenem	0%	**17.65%**
Colistin	100%	100%
XDR %	100%	35.30%
C. PCR carbapenemase genes tested		
ESBL bla_{TEM-1}	31/31 (100%)	17/17 (100%)
bla_{oxa-23} gene	30/31 (96.8%)	6/17 (35.3%)
bla_{oxa-24} gene	1/31 (3.2%)	5/17 (29.4%)
bla_{oxa-23} & blaoxa-24	0/31	3/17 (17.6%)
D. MLST sequence types		
ST2	**29/31 (93.5%)**	**0/17**
ST699	**2/31 (6.5%)**	0/17
ST627	0/31	0/17
ST 25	0/31	**10/17 (58.9%)**
ST 99	0/31	**1/17 (5.8%)**
New ST types (1200-1206)	0/31	**6/17 (35.3%)**

ARTICLE 4

Title: Emergence of Plasmid-Encoded VIM-2 Producing *Pseudomonas aeruginosa* Isolated from Clinical Samples in Lebanon

Tania Nawfal Dagher, Charbel Al-Bayssari, Eid Azar, Seydina. M. Diene, and Jean-Marc Rolain.

Submitted to ***New Microbes and New Infections***.

Emergence of Plasmid-Encoded VIM-2 Producing *Pseudomonas aeruginosa* Isolated from Clinical Samples in Lebanon.

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Text word count = 700

Number of references =5

Number of Tables = 1

Number of Figures = 1

Running Title: Carbapenem-Resistant gram-negative bacteria

Keywords: VIM-2, *oprD* gene, imipenem, *P. aeruginosa*, *E. coli*.

Sir

Pseudomonas aeruginosa is an important pathogen that is the leading cause of acute nosocomial infections especially in immune-compromised patients [1]. Resistance of *Pseudomonas aeruginosa* to carbapenem becomes a major global threat and is worsened by the excessive use of carbapenems [1,2]. This resistance is mainly due to the production of three classes of carbapenemase enzymes: Ambler class A, B, and D [1]. *Pseudomonas aeruginosa* is resistant to carbapenem due to the production of metallo-β-lactamases (MBLs) such as VIM and IMP, or by the alteration or the loss of the outer membrane porin protein *oprD*, and by the increased expression of the efflux pumps [2].

The purpose of the present study was to characterize the molecular mechanism of carbapenem-resistant *Pseudomonas aeruginosa* collected from rectal swabs of 23 ICU patients treated with carbapenem and colistin combination therapy between October 2016 and February 2017 from Saint-George Hospital in Lebanon. The 23 rectal swabs were cultured on MacConkey agar plates supplemented with Ertapenem (2 µg/ml) for the screening of carbapenem-resistant organisms. Four *Pseudomonas aeruginosa* were isolated and were identified using the matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectrometry. Antimicrobial susceptibility testing was performed on Mueller-Hinton agar for 16 antibiotics using the disk diffusion method, and E-test was performed to determine the minimal inhibitory concentration (MIC) of imipenem as recommended by the 2017 European Committee of Antimicrobial Susceptibility Testing (EUCAST). (http://www.sfmmicrobiologie.org/UserFiles/files/casfm/CASFM%20V2_0_Mai2017.pdf). The phenotypic detection of carbapenemase was confirmed using the carbaNP test [3].

The carbapenemase encoding genes, and the extended-spectrum β-lactamase were screened by real-time PCR, and standard PCR and were sequenced. Molecular characterization of the *oprD* gene was performed using PCR amplification and sequencing [4]. Analysis of the

sequenced *oprD* gene was compared against the reference strain *Pseudomonas aeruginosa* PAO1 using the npsa_clustalwan software (https://npsa-prabi.ibcp.fr/cgi-bin/npsa_automat.pl?page=/NPSA/npsa_clustalwan.htm.). Multilocus Sequence Typing was performed to determine the genetic relationship among the clinical isolate as described on Institute Pasteur's MLST Web site (www.pasteur.fr/mlst).

P. aeruginosa isolates were resistant to all antibiotics tested except to colistin and fosfomycin. E-tests showed high-level of resistance to imipenem, with MIC greater than 256 µg/ml for all the isolates (Table 1). The results of Real-time PCR showed that all *P. aeruginosa* isolates harbored $bla_{\text{VIM-2}}$ gene, except *P. aeruginosa* (PA-4) (Table 1). All the *P. aeruginosa* isolates had mutations of the *oprD* gene (Figure 1). MLST analysis reveals that three *P. aeruginosa* (PA-3, PA-6, PA-16), and one *P. aeruginosa* (PA-4) isolates harbored the sequence types (ST357, ST233) respectively (Table 1). Conjugal transfer between carbapenemase producing *P. aeruginosa* and *E. coli* (J35), succeeded to yield *E. coli* transconjugants harboring an ~45-kb plasmid, except for the clone ST233, suggesting that these metallo-β-lactamases $bla_{\text{VIM-2}}$ were plasmid encoded for ST357 and chromosomally encoded for ST233.

In this study, we describe the emergence of carbapenem-resistant *P. aeruginosa* in Saint-Georges Hospital due to the presence of $bla_{\text{VIM-2}}$ gene and mutations of the *oprD* gene. These results are in concordance with those reported previously in Lebanon where Al Bayssari *et al*. have reported the emergence of VIM-2 producing *P. aeruginosa* in human [2]. In addition *P. aeruginosa* harboring $bla_{\text{VIM-2}}$ gene have been also reported in the Mediterranean basin [2]. Our study showed also that mutations leading to a premature stop codon resulting in a defective protein *oprD* was the main cause of resistance of *P. aeruginosa* to imipenem as described previously [2]. The main finding in our study is the emergence of *P. aeruginosa*

harboring the VIM-2 plasmid. *P. aeruginosa* harboring the VIM-2 plasmid has never been detected in Lebanon previously where all the isolates detected had the chromosomic *bla*$_{\text{VIM-2}}$ gene [2]. MLST analysis showed that the three *P. aeruginosa* isolates harboring the plasmid-encoded *bla*$_{\text{VIM-2}}$ gene belonged to ST357 clone which have been found in different countries of the central Europe [5]. However, the *P. aeruginosa* ST233 which has the chromosomic *bla*$_{\text{VIM-2}}$ gene have been previously described in Lebanon and different countries of the Mediterranean basin [2].

Finally, this study reports the first detection of the plasmid-encoded *bla*$_{\text{VIM-2}}$ gene in Lebanon. This finding poses a serious public health problem since the plasmid harboring this β-lactamase is a major source of dissemination of this enzyme. An urgent strategy must be adopted to control the spread of these resistant microorganisms among hospitalized patients.

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Competing interests: No conflicts of interest or financial disclosure for all authors

Ethical approval: Not required.

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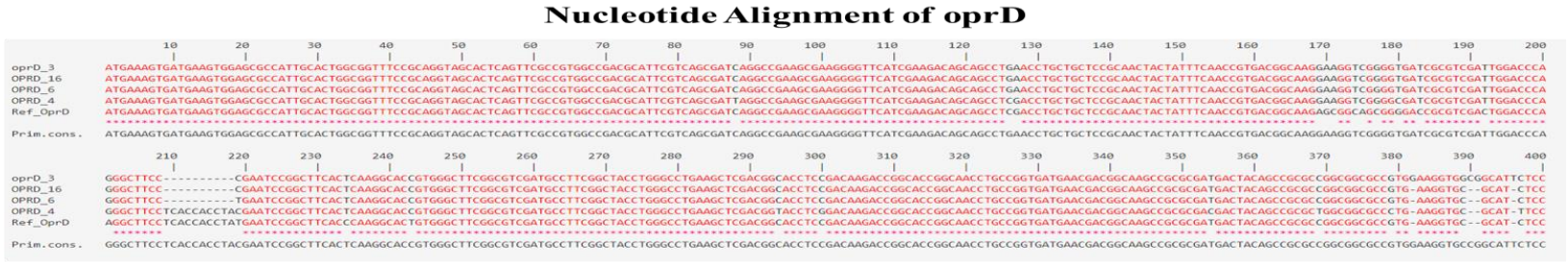
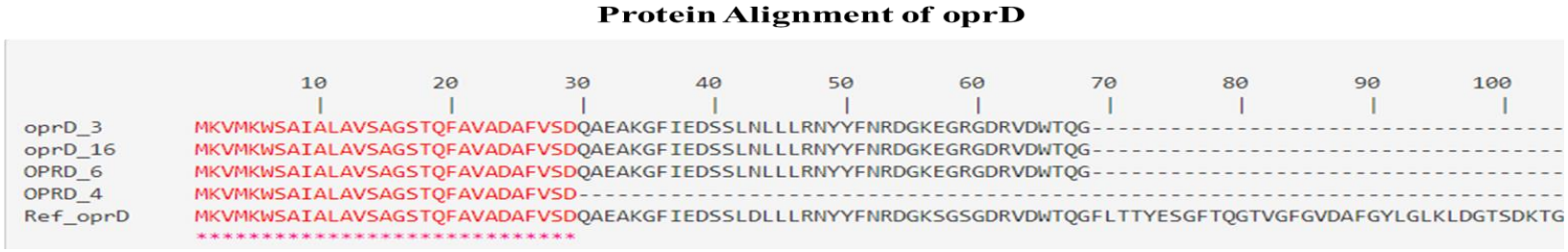
Figure 1: Genetic representation of the *oprD* gene.

(i)



(ii)



(i) Nucleotide alignment of the *oprD* gene and **(ii)** Protein alignment of the *oprD* gene

- For *P. aeruginosa* PA-3, PA-16, PA-6: several substitutions mutations between positions 169 to 201, in addition to a large deletion of 10 nucleotides from position 209 to 218, leading a defective protein resulting in a truncated polypeptide made of 68 amino acid residues.
- For *P. aeruginosa* PA-4: C to T substitution in nucleotide position 88 leading to the premature stop codon TAG in oprD resulting in a truncated polypeptide made of 29 amino acid residues.

Table 1. Phenotypic and genotypic features of the imipenem-resistant clinical isolates

Strain name	Source	Antibiotic resistance profile	IMP MIC (µg/ml)	Carba NP test	VIM-2	TEM	ST
P. aeruginosa PA-3	Rectal swab	TIC, TCC, TZP, CAZ, FEP, IMP, ERT, AK, TOB, CIP, F, DO, SXT, R	>256	+	+	TEM-1	357
P. aeruginosa PA-16	Rectal swab	TIC, TCC, TZP, CAZ, FEP, IMP, ERT, AK, TOB, CIP, F, DO, SXT, R	>256	+	+	-	357
P. aeruginosa PA-6	Rectal swab	TIC, TCC, TZP, CAZ, FEP, IMP, ERT, AK, TOB, CIP, F, DO, SXT, R	>256	+	+	-	357
P. aeruginosa PA-4	Rectal swab	TIC, TCC, TZP, CAZ, FEP, IMP, ERT, AK, TOB, CIP, F, DO, SXT,R	>256	+	-	-	233

Ticarcillin (**TIC**), Ticarcillin-clavulanic acid (**TCC**), piperacillin tazobactam (**TZP**), Ceftazidime (**CAZ**), cefepime (**FEP**), imipenem (**IPM**), ertapenem (**ERT**), amikacin (**AK),** tobramycin (**TOB**), ciprofloxacin (**CIP**), fosfomycin (**FF**), nitrofurantoin (**F**), doxycycline (**DO**), trimethoprim sulfamethoxazole (**SXT**), rifampicin (**R**), sequence type (**ST**).

Chapter Conclusion

The history of antimicrobial resistance dates back to antimicrobial discovery and parallels its use. The emergence of extended-spectrum-β-lactamases (ESBLs) compromised the effect of most β-lactam antibiotics. In this situation, carbapenem antibiotics remain the drug of choice and have been increasingly used to treat infections. However, the increasing use of carbapenems, triggers bacterial resistance against this class of antimicrobial agents. Reports of carbapenem-resistant *Enterobacteriaceae* have been published from different regions, including Europe, America, Turkey, Greece, Israel, Egypt, Kuwait and Saudi Arabia [7].

Thus, in a strategy to control hospital infections with multidrug resistant bacteria, the identification of patients carrying these bacteria is essential. For example, many Lebanese hospitals have adopted the systematic search for human multidrug-resistance reservoirs using rectal and nasal swabs when patients are admitted to a hospital center, in particular in an intensive care unit. Patients identified as colonized by multidrug-resistant bacteria will be reinforced with technical and geographic isolation measures (with the mention of "Patient to be isolated"). Moreover, some Lebanese institutions have opted for different strategies to control the overuse of antibiotics, including restriction, rotation or combination of antibiotics. However, in the absence of a national plan, each hospital has adopted its own strategy within the local nosocomial infection control committee. Through this chapter, we reported the detection of carbapenem *A. baumannii* isolates that belonged to international clone II lineage and ST25, which have been reported in several Mediterranean countries such as Greece, Italy, and Turkey [8]. We are able to proof at the end of this study that the shift of treatment from colistin-carbapenem combination therapy to colistin monotherapy leads to a decrease in the prevalence of *A. baumannii* in sputum culture. On the other hand, it has been shown that *P. aeruginosa* isolates

harboring the plasmid-encoded *bla*$_{\text{VIM-2}}$ gene and belonged to ST357 clone are found in different countries of the central Europe [9]. However, the *P. aeruginosa* ST233 that has the chromosomic *bla*$_{\text{VIM-2}}$ gene have been previously described in Lebanon and different countries of the Mediterranean basin [6]. In addition, bacterial resistance is closely associated with the use of antimicrobial agents in clinical practice [10]. Management is difficult, as the strains often display resistance to multiple classes of antibiotics, including extended-spectrum cephalosporins, aminoglycosides, and fluoroquinolones, severely limiting therapeutic options [11].

In Lebanon, many actions have been undertaken both in the surveillance of resistance and in the prevention of transmission of resistant bacteria in Lebanese health institutions. In parallel, the Lebanese system should constitute a database of the evolution of the susceptibility profiles, as well as the emergence of resistant strains. This data bank can be enhanced by integrating data from molecular and epidemiological studies. Indeed, the molecular characterization of resistance gives valuable information regarding bacterial strains circulating in a specific hospital or national environment and above all makes it possible to identify homologies between human, animal and environmental strains, as it has been done by some Lebanese teams. Furthermore, WHO has created a set of strategies to combat rising antibiotic resistance, which include improving sanitation and hygiene to reduce overall infection rates, and optimising the use (and preventing the overuse) of antibiotics in both humans and animals [12].

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III– ELUCIDATION OF THE MOLECULAR MECHANISMS OF COLISTIN-RESISTANT IN *ENTEROBACTERIACEAE* ISOLATED FROM CLINICAL LEBANESE PATIENTS.

Antimicrobial resistance is recognized as one of the most serious global threats to human health in the 21st century. These serious concerns have been catalyzed by the rapid increase in carbapenemase producing Enterobacteriaceae expressing enzymes such as KPC-2 (*Klebsiella pneumoniae* carbapenemase-2) and NDM-1 (New Delhi metallo-β-lactamase-1) [1]. This growing infection caused by multidrug-resistant (MDR) Gram-negative bacteria has led to the renewal of colistin as a last-resort treatment option [2]. Colistin was first introduced to treat infections in 1959, but became unpopular by the 1980s because of its renal toxicity profile. Between the late 1990s and early 2000s, the need to treat MDR Gram-negative bacteria in human beings has led to the reintroduction of colistin use, primarily in critically ill patients in intensive care units and patients with decreased immunity due to cancer chemotherapy or organ transplantation [3]. Unfortunately, colistin resistance has emerged recently worldwide especially among patients treated with colistin [4]. Bacteria employ several strategies to protect themselves from adverse environmental stimuli. These strategies include alterations of the negatively charged lipopolysaccharide (LPS), by the addition of phosphoethanolamine (PEtN) or 4-amino-4-deoxy-L-arabinose (L-Ara4N), to the lipid A moiety of the LPS. This can be accomplished by specific mutations of the two-component systems (TCSs) (*pmrA*/*pmrB*, *phoP*/*phoQ*), or its negative regulator *mgrB* [5]. It has been shown that until November 2015, colistin resistance was mainly known to be mediated by chromosomic mutations, but after that Liu et al reported the first detection of a plasmid mediated colistin-resistant *mcr-1* gene [1]. Since this has raised many concerns worldwide, we have devoted ourselves in this chapter to characterize the emergence of colistin-resistant Enterobactariaceae from patients hospitalized at Saint-George Hospital in Beirut, Lebanon.

In the first study of this chapter (***Article N °5***), we decipher the mechanisms of resistance to colistin and β-lactam antibiotics in clinical isolates in Lebanon. In this study, 23 rectal swabs were collected from 23 patients hospitalized in the ICU of Saint-George Hospital in Beirut who received colistin and carbapenem. Twelve colistin-resistant *Enterobacteriaceae* strains were isolated. Among the isolates, 8 colistin-resistant strains were identified as: (5 *Escherichia coli*, 2 strains as *Enterobacter cloacae,* and 1 strain as *Klebsiella pneumoniae*). In addition, 4 strains that were intrinsically resistant to colistin were also isolated (1*Proteus mirabilis* and 3 *Morganella morganii)* but were excluded from this study. All colistin-resistant strains have MICs for colistin that ranged from 8 mg/L to 32 mg/L. Resistance to colistin is defined as MIC > 2 mg/L. In addition, all the isolates were resistant to amoxicillin, amoxicillin clavulanic acid, and to the cephalosporins except *E. cloacae*, that was sensitive to ceftriaxone and cefepime. Real-time PCR and standard PCR results showed that 5 strains harbored $bla_{\text{TEM-1}}$ and one strain harbored $bla_{\text{TEM-163}}$. Moreover, 4 strains were positive for $bla_{\text{CTX-M-15}}$, $bla_{\text{CTX-M-103}}$, and $bla_{\text{CTX-M-189}}$, and *K. pneumoniae* harbored $bla_{\text{SHV-1}}$. Due to the absence of the *mcr* type genes, the resistance of the isolates to colistin was linked to mutations of two-component regulatory genes (*pmrA/pmrB* and *phoP/phoQ*) as well as its negative regulator *mgrB* gene. Interestingly, we report by analogy the detection of mutation in *mgrB* regulator in colistin resistant *E. cloacae.* Furthermore, in the context of colistin and carbapenem-resistance bacteria, in (***Article N °6***), two soldiers with gunshots wounds were transferred to Saint-George Hospital in Beirut, after being hospitalized in another Lebanese hospital. Two multidrug-resistant *Klebsiella pneumoniae* were isolated. During their hospitalization, another three isolates were collected from three different patients. All the isolates were carbapenem-resistant, and two out of five isolates were colistin-resistant due to mutations in the amino-acid sequences of proteins (PmrA/B, PhoP/Q, and

MgrB). Our results revealed also that those strains carried several genes that are involved in this resistant phenotype, in addition to the presence of NDM-5 gene, which is firstly reported in our country. Molecular and epidemiological studies must be done to understand the risk factors, and the mode of transmission of these microorganisms. Therefore, an urgent strategy must be taken to prevent the spread of these resistant microorganisms among hospitalized patients.

ARTICLE 5

Title: Emergence of colistin resistant gram-negative bacteria in clinical isolates at Saint Georges hospital Lebanon.

Tania Nawfal Dagher, Sophie Baron, Eid Azar, Selma Chabou, Linda Hadjadj, Seydina. M. Diene, and Jean-Marc Rolain.

Submitted in *Antimicrobial Agents and Chemotherapy*.

Emergence of colistin resistant Enterobacteriaceae in clinical isolates at Saint Georges hospital Lebanon.

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Keywords: Colistin resistance, gram-negative bacteria, *pmrAB*, *phoPQ*, *mgrB*, Lebanon.

Abstract:

Background: The increase in resistance to carbapenem has led to the revival of colistin, as a last option for treatment that has led to an increase detection of colistin-resistant gram-negative bacteria. In this study, we report the presence of clinical colistin-resistant *Enterobacteriaceae* isolated from Lebanese hospital.

Methods: From twenty-three rectal swabs, eight colistin-resistant clinical strains (5 *Escherichia coli*, 2 *Enterobacter cloacae*, and 1 *Klebsiella pneumoniae*) were isolated. Antibiotic susceptibility testing was performed using disk diffusion method and E-test. Broth microdilution method was performed to determine colistin susceptibility. RT-PCR and standard PCR were used to investigate the genes encoding for extended spectrum β-lactamases, carbapenemases encoding genes, and colistin resistance genes; and were sequenced. Genotyping of these isolates was conducted by MLST.

Results: Results of antibiotic susceptibility testing revealed that all isolates were resistant to colistin. MICs for colistin ranged from 8 mg/L to 32 mg/L. Real-time PCR results showed that 5 strains harbored $bla_{\text{TEM-1}}$ and one strain harbored $bla_{\text{TEM-163}}$. Moreover, 4 strains were positive for the for the $bla_{\text{CTX-M-15}}$, $bla_{\text{CTX-M-103}}$, and $bla_{\text{CTX-M-189}}$, and *K. pneumoniae* harbored $bla_{\text{SHV-1}}$. Resistance to colistin was linked to mutations in the amino-acid sequences of proteins (PmrA/B, PhoP/Q, and MgrB). Interestingly, we report by analogy here the first detection of mutation in *mgrB* regulator in colistin resistant *E. cloacae*.

Conclusions: This study highlights the presence of colistin-resistant gram-negative bacteria in a Lebanese hospital, which is worrisome. An urgent strategy need to be adopted in order to avoid the spread of such bacteria.

Introduction

Resistance to antibiotics in Gram-negative bacteria such as *Acinetobacter baumannii*, *Pseudomonas aeruginosa* and *Enterobacteriaceae* has become a field of interest over the last decade. This has led to a new fear of resistance to many antibiotics that seems to be not justifiable (1). Those bacteria develop a high resistance to most available antibiotics such as beta-lactams, aminoglycosides, and fluoroquinolones (2, 3). This growing infection caused by multidrug-resistant (MDR) Gram-negative bacteria has led to the renewal of colistin as a last-resort treatment option especially for patients in intensive care units (ICUs) (4, 5). Unfortunately, the resistance to polymyxins has been increasingly reported worldwide (2). Polymyxin E (colistin) and polymyxin B have been used in humans and are bactericidal toward Gram-negative bacteria except for the bacteria that are intrinsically resistant to colistin such as *Burkholderia*, *Edwardsiella*, *Proteus, Providencia, Morganella* and *Serratia,* (3, 6). From 1959, colistin was available to treat infections caused by Gram-negative bacteria (7). Conversely, its utilization is limited due to the daily dose that cannot be increased because it will lead to nephrotoxicity in almost 60% of patients (8). Between the late 1990s and early 2000s, colistin was re-introduced into clinical medicine due to the emergence of resistance to beta-lactams and carbapenems (2). This leads to the emergence of colistin-resistant bacteria among patients treated with this compound (9). The bacterial cell membrane is the main target for the antimicrobial activity of colistin: colistin binds to the anionic lipid A moiety of lipopolysaccharide (LPS) and disrupts the cell membrane (2, 6). Several strategies are used by gram-negative bacteria to escape from polymyxin (colistin and polymyxin B). The major mechanism of resistance occurs by the alteration of the negatively charged lipopolysaccharide (LPS), by the addition of phosphoethanolamine (PEtN) or 4-amino-4-deoxy-L-arabinose (L-Ara4N), to the lipid A moiety

of the LPS. This can be accomplished by specific mutations of the two-component systems (TCSs) (*pmrA*/*pmrB*, *phoP*/*phoQ*), or its negative regulator *mgrB* (2). The modified LPS with this positive charge reduces its binding to polymyxins and produces this resistance (2, 10). Moreover, it has been demonstrated that resistance to colistin can also be due to the loss of LPS production due to mutations in the *lpxACD* genes (11), in addition to the use of efflux pumps, the capsules formation, and overexpression of OprH , which are all efficiently regulated at the molecular level (2). Finally, colistin resistance can also be due to the presence of plasmid-mediated *mcr-1* gene that encodes the enzyme phosphoethanolamine transferase which is capable of modifying the lipid A moiety of LPS with the addition of phosphoethanolamine (12, 13). Finally, the increased use of colistin as a last-resort therapeutic medication to treat patients infected with multidrug-resistant (MDR) Gram-negative bacteria, has been followed by an increase in the number of Gram-negative bacteria resistant to colistin (2). The present study aims to investigate the prevalence of colistin-resistant Enterobacteriaceae isolated from rectal swabs from patients hospitalized in ICU at Saint-George Hospital in Beirut, Lebanon that were treated by carbapenem and colistin and to decipher the molecular support of resistance to these antibiotics.

Materials and methods

Microbiology procedure:

A- Study Design

Between October 2016 and February 2017, a rectal swab was collected for each patient hospitalized in the ICU of Saint-George Hospital in Beirut who received colistin and carbapenem during their stays in ICU. Rectal swab was collected after 7 days of therapy for analysis of

colistin and carbapenem-resistant bacteria. They were kept at -80°C before being transported to the laboratory in Marseille, France.

B- Microbiological procedures

Screening of colistin-resistant enterobacteriaceae

Rectal swabs were transferred to an enrichment Tryptic Soy Broth (TSB) medium and incubated overnight at 37°C. After incubation, 100 µL of the enrichment medium were cultivated for 24 hours at 37°C on the selective medium LBJMR for the screening of colistin- resistant organisms (14), and on MacConkey agar plates supplemented with Ertapenem (2 µg/ml) for the screening of carbapenem-resistant organisms. Species intrinsically resistant to colistin were excluded from this study. Bacterial identification at the species level was performed using the matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF) method (Microflex; Bruker Daltonics), as previously described (15).

Antibiotic susceptibility testing

The standard disk diffusion method on Mueller-Hinton agar was performed to determine the antibiotic susceptibility testing of the strains as recommended by the European Committee of Antimicrobial Susceptibility Testing (EUCAST) 2017 (http://www.sfm-microbiologie.org/UserFiles/files/casfm/CASFM%20V2_0_Mai2017.pdf). The minimal inhibitory concentration (MIC) of colistin was determined using the broth microdilution method according to EUCAST 2017. Each strain with an MIC > 2 mg/L for colistin was considered as resistant to this antibiotic.

DNA extraction

The automatic robot EZ1 (Qiagen BioRobot EZ1-, Tokyo, Japan) was performed to extract the DNA of the bacteria, with the extraction kit (EZ1 DNA, Qiagen, Hilden, Germany), according to the manufacturer's guidelines and eluted in 200 µL of elution buffer and stored at -20°C.

Multilocus sequence typing (MLST)

To determine the genetic relationship between the clinical isolates, genotyping analysis was done using seven housekeeping genes for *E. coli*, *K. pneumoniae* and *E. cloacae*, as described on Institute Pasteur's MLST Web site (www.pasteur.fr/mlst).

Screening of isolates by real-time PCR, standard PCR and sequencing of the antibiotic resistance genes

To detect the presence of carbapenemase encoding genes, real-time PCR assay was done, using specific primers for $bla_{\text{OXA-48}}$, $bla_{\text{OXA-58}}$, bla_{KPC}, bla_{NDM} and bla_{VIM} genes. All colistin-resistant bacteria were also screened for the presence of extended-spectrum β-lactamase (bla_{TEM}, bla_{SHV}, $bla_{\text{CTX-M}}$,) genes and for the *mcr*-1, *mcr*-2, *mcr*-3, *mcr*-4, and mcr-5 genes. Probes and primers used were described previously (16–19). PCR amplification products that were positive for the antibiotic resistance genes tested were sequenced using BigDye terminator chemistry on an automated ABI 3130 sequencer (PE Applied Biosystems, Foster City, CA). ARG-ANNOT database was used to analyze the sequenced genes (20), and were compared to other genes using the BlastN and BlastP tool from the National Center for Biotechnology Information (NCBI) database (http://www.ncbi.nlm.nih.gov). The described colistin-resistant genes including *pmrA*, *pmrB*, *phoP*, *phoQ* and *mgrB* were amplified and sequenced (21–25). Sequenced genes were compared with those of the reference strains including *E. coli* K-12 MG 1655 (NCBI GenBank

accession no.CP000647), *K. pneumoniae* MGH 78578 (NCBI GenBank accession no.CP000647) and *E. cloacae* ATCC 13047 (NCBI GenBank accession no.CP000647) using the nps alignment software (https://prabi.ibcp.fr/htm/site/web/home). We used PROVEAN (Protein Variation Effect Analyser) software (http://provean.jcvi.org/seq_submit.php), to predict whether the identified amino acids substitutions that results from missense mutations would affect the function of the proteins (21). If the protein variant has a score below or equal to a predefined threshold (-2.5), it is predicted to have a "deleterious" effect. If it is above this threshold, it is predicted to have a "neutral" effect.

Results

23 rectal swabs were collected from 23 patients treated with colistin and carbapenem. No carbapenem-resistant *Enterobacteriaceae* have been isolated from MacConkey agar medium supplemented with Ertapenem. Of the 23 patients, 12 patients have no colistin-resistant enterobacteria whereas 11 patients carried at least one CRE. The mean carriage of CRE was 1 /sample except one patient carries 2CREs/sample. In summary, twelve CRE were collected with the LBJMR medium. Among the isolates, 8 colistin-resistant strains were identified as: (5 *Escherichia coli*, 2 strains as *Enterobacter cloacae,* and 1 strain as *Klebsiella pneumoniae*). In addition, 4 bacteria that were intrinsically resistant to colistin were also isolated (1*Proteus mirabilis* and 3 *Morganella morganii)* but were excluded from this study. The antibiotic resistance profile of all the isolates is presented in Table 1. Results showed that all isolates were resistant to amoxicillin, amoxicillin/clavulanic acid, cefalotin, doxycycline and colistin. Colistin MICs ranged from 8 to 32 mg/L. Inversely, all isolates were susceptible to ertapenem, imipenem, amikacin, gentamicin, and nitrofurantoin. In addition, six strains were resistant to the third generation cephalosporins. Real-time PCR and standard PCR results showed that *E. coli* EC-5,

EC-10, and EC-23, *E. cloacae* Eclo-14A and Eclo-14B, and *K. pneumoniae* KP-16, were positive for the penicillinase enzyme bla_{TEM} gene. All the sequences of the bla_{TEM} gene were recognized as $bla_{\text{TEM-1}}$ except for *E. coli* EC-23 which was identified as $bla_{\text{TEM-163}}$. Moreover, 4 out of the 5 *E. coli* strains harbored the $bla_{\text{CTX-M-A}}$ gene, especially the $bla_{\text{CTX-M-15}}$ in two isolates, the $bla_{\text{CTX-M-103}}$ in one isolate, and the $bla_{\text{CTX-M-189}}$ in another one (Table 1). The single *K. pneumoniae* KP-16 harbored bla_{SHV-1}. None of the strains harbored the *mcr*-1, *mcr*-2, *mcr*-3, *mcr*-4, and *mcr*-5 genes. MLST analysis reveals that the five *E. coli* belonged to five different sequence types (STs) including ST131, ST6174, ST405, ST162 and ST1451. *K. pneumoniae* has ST45, and the two *E. cloacae* have new sequence types ST924 and ST925 according to Pasteur web site (Table 1). Due to the absence of *mcr-1/2/3/4/5* genes, the associated colistin resistance genes including *mgrB, pmrA, pmrB, phoP* and *phoQ* were amplified and sequenced as appropriate. As shown in Table 2, sequence analysis of these latter revealed that the two *E. cloacae* isolates exhibited non-synonymous mutations in *mgrB*, *pmrA, pmrB,* and *phoP* genes leading to amino acid changes. These mutations in *mgrB* were detected for the first time and were considered as deleterious by the software PROVEAN. Moreover, sequence analysis of *E. coli* strains revealed that the colistin resistance of *E. coli* EC-5 was due to different missense in *pmrAB* resulting in amino acids substitutions; for *E. coli* EC-10, the missense mutations were occurred in *phoPQ*; for *E. coli* EC-12, missense, and deletion mutations were observed in *pmrB* and *phoPQ*; for *E. coli* EC-21, the *pmrA* and *phoP* genes were affected by missense mutations*;* and for *E. coli* EC-23 colistin resistance was due to missense and deletions mutations in *pmrA* and *phoPQ*. Analysis of *K. pneumoniae* KP-16 revealed that there were two genetic changes linked to colistin resistance: first, a missense mutations in addition to a mutation that creates a premature stop codon of the *mgr*B ; and second, a missense in the *pmrB* gene that results in

amino acid substitutions (Table 2). All these mutations were predicted as deleterious by the software PROVEAN.

Discussion

The increase of multidrug resistance Gram-negative bacteria, especially carbapenem-resistant bacteria, is a worldwide clinical problem. Indeed, this has led to the re-introduction of colistin into clinical treatment against infections caused by MDR bacteria (11, 26). This study describes the presence of intestinal carriage of colistin- and carbarpanem-resistant enterobacteriae in patients who received colistin and carbapenem for treatment. We found that these patients carried only colistin-resistant bacteria but no carbapenem resistant bacteria, suggesting that the use of these molecules may select colistin resistance but not carbapenem resistance. One limit of our study is that no sample were collected before the instauration of the treatment. It is possible that these patients already carried these colistin-resistant bacteria, before the use of colistin. Different studies, in different countries such as France, Columbia, Spain, Laaos, Thailand, USA, Nigeria, and Saudi Arabia have described, the presence of colistin resistant enterobacteriae in humans without prior colistin exposure (9). Moreover, a study done by Nakayama T et al. showed that even a short-term trip to some countries may result in the spread of strains that harbored *mcr-1* and that are carried by international travelers where their numbers continues to increase, thereby increasing the risk of spreading of colistin resistant to the developed countries (27).

The genotyping results showing that our strains belonged to different sequence types (ST), suggest that there is no link between the strains isolated in the same hospital. It has been shown that the clone ST131 is a globally important pathogen among multidrug resistance *E. coli* and is linked to nosocomial and acquired infections (28). Moreover, the clones of the other

Enterobacteriaceae ST405, ST162, ST1451 and ST45 have been reported in hospitalized patients in different counties, such as Brazil, Spain, Sweden and Uruguay (29–32). In addition, ST405 has also been reported in animals in Algeria (33).

In addition, all the strains in this study were resistant to amoxicillin, amoxicillin clavulanic acid, and to the cephalosporins antibiotics cefalotin, cefepim, and ceftriaxone except the *E. cloacae,* which are sensitive to ceftriaxone and cefepime. All the isolates harbored the β-lactamase genes such as TEM, SHV, and CTX-M. In Lebanon, previous studies have reported the presence of $bla_{\text{TEM-1}}$ and $bla_{\text{CTX-M-15}}$ (34–36), whereas the $bla_{\text{TEM-163}}$, $bla_{\text{CTX-M-103}}$, $bla_{\text{CTX-M-189}}$, and $bla_{\text{SHV-1}}$ detected in this present study have never been previously reported.

Interestingly, many different gene mutations including missenses and deletions have been identified in genes associated to colistin resistance in our clinical isolates. However, some of these mutations have been reported earlier such as R81S found in PmrA of colistin-resistant strain isolated from animal samples under the Spanish Surveillance Network of Antimicrobial Resistance in Bacteria (24, 37). R81S is also similar to the missense mutation R81H which was found in PmrA of *S. Typhimurium* (38, 39). The D150W mutation in PhoQ of our *E. coli* EC-10 isolate, was previously reported in Taiwan in a *K. pneumoniae* isolate collected from patient at the Taipei Veterans General Hospital (40). The mutation T92H found in *pmrB* of *E. coli* EC-12 isolate, were similar to T92A described in PmrB of *S. Typhimurium* (11, 38). The D191Y mutation in PhoP of *E. coli* EC-21, was reported in South Africa in clinical *K. pneumoniae* isolate (41). Olaitan et al. have also identified the mutation L96P in *K. pneumoniae* isolated from patients and healthy individuals in Nigeria, Thailand, Lao PDR, and France (4) which is similar to L96S found in the PhoQ of our study. Regarding the colistin-resistant *K. pneumoniae* KP-16, different missense mutations have been found, such as G37M, C39P and N42Y that were similar

to the mutation identified in the *mgrB* of *K. pneumoniae* described by Poirel et al. In Lebanon, there is no report describing the mechanism of colistin resistance of *Enterobacteriaceae* clinical isolates in Lebanon except a study reported recently by one of our group that describe three colistin-resistant *K. pneumoniae* isolated from Lebanese Hospital in Beirut in 2015. The resistant of those strains was due to mutations of *mgrB, pmrA, pmrB,* and *phoQ* genes (13). Those strains were isolated from patients without previous use of colistin. On the other hand, two studies were done by Dandachi et al., which described the mechanism of colistin resistance in animals. In these studies, colistin resistance was due to the presence of the plasmid mediated *mcr-1* gene isolated from *E. coli* strain from poultry in Lebanon (42) and from 23 *E. coli* strains isolated from Lebanese swine farms (43). In fact, the carriage of *mcr-1* in farms could constitute a potential key for the introduction of this gene into the community as well as to the clinical settings in Lebanon by horizontal transfer from animals to humans. Due to the spread of carbapenemase producers in hospitals in Lebanon, it is expected that once *mcr-1* would be introduced, this latter will be selected by the frequent use of colistin.

The mechanism of colistin resistance in *E. cloacae* is actually not known. Among the few studies that have occurred on that topic, it seems that the PhoP/PhoQ two component system may play a role in colistin resistance in *E. cloacae.* The *mgrB* gene acts as a repressor of this two-component system in *K. pneumoniae* and inactivation of this gene leads to colistin resistance in this species. However, a previous study on colistin-resistant *E. cloacae* has detected no alteration of the *mgrB* gene, leading the mechanism still unknown (25). In our study, a G insertion between G37 and V38 leads to a frameshift mutation. Moreover, different missense mutations have been found, such as C39G, N42S, I45Y, W47V, W47S, *48K and *48Y that were similar to the mutation identified in the *mgrB* of *K. pneumoniae* described by Poirel et al (37).

In conclusion, this study reports the first detection of *mgrB* mutations in colistin resistant *E. cloacae* and the emergence of colistin resistance in gram-negative bacteria in Lebanon. The use of polymyxins to treat patients has probably contributed to the emergence of colistin-resistant strains in this hospital since no relationship between our strains has been observed. An urgent strategy must be implemented to prevent the spread of these resistant microorganisms among hospitalized patients.

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Transparency declarations and conflicts of interest:

Authors have no conflicts of interest to declare.

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TND and SMD wrote the manuscript, performed experiments, and analyzed the data. ED provided the strains and helped draft the manuscript. SC and LH helped draft the manuscript. JMR conceived the study, participated in its design and coordination, and helped draft the manuscript. All authors read and approved the final version of the manuscript.

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Table 1: Phenotypic and genotypic features of the eight colistin-resistant strains isolated at the Saint Georges hospital in Lebanon

Strain name	AMX	AMC	TZP	CF	CRO	FEP	ERT	IPM	AK	GEN	CIP	FF	F	DO	SXT	CS	Colistin MIC (μg/ml)	*bla*$_{SHV}$	*bla*$_{TEM}$	*bla*$_{CTX-M-A}$	ST
E. coli **EC-5**	R	R	S	R	R	R	S	S	S	S	S	S	S	R	S	R	16	-	TEM-1	-	1451
E. coli **EC-10**	R	R	S	R	R	R	S	S	S	S	R	S	S	R	S	R	16	-	TEM-1	CTX-M-15	131
E. coli **EC-12**	R	R	S	R	R	R	S	S	S	S	S	S	S	R	S	R	16	-	-	CTX-M-189	6174
E. coli **EC-21**	R	R	R	R	R	R	S	S	S	S	R	S	S	R	S	R	16	-	-	CTX-M-15	405
E. coli **EC-23**	R	R	S	R	R	R	S	S	S	S	R	S	S	R	R	R	8	-	TEM-163	CTX-M-103	162
E. cloacae **Eclo-14A**	R	R	S	R	S	S	S	S	S	S	S	R	S	R	S	R	8	-	TEM-1	-	924
E. cloacae **Eclo-14B**	R	R	S	R	S	S	S	S	S	S	S	R	S	R	S	R	16	-	TEM-1	-	925
K. pneumoniae **KP-16**	R	R	R	R	R	R	S	S	S	S	R	S	S	R	R	R	32	SHV-1	TEM-1	-	45

amoxicillin (**AMX**), amoxicillin clavulanic acid (**AMC**), piperacillin tazobactam (**TZP**), Cefalotin (**CF**), ceftriaxone (**CRO**), cefepime (**FEP**), ertapenem (**ERT**), imipenem (**IPM**), amikacin (**AK**), gentamicin (**GEN**), ciprofloxacin (**CIP**), fosfomycin (**FF**), nitrofurantoin (**F**), doxycycline (**DO**), trimethoprim sulfamethoxazole (**SXT**), colistin (**CS**). **R,** resistant; **S,** sensitive, sequence type (**ST**)

Table 2: Mutations of the associated colistin-resistance proteins

Strain names	MgrB	PmrA	PmrB	PhoP	PhoQ
E. coli **EC-5**	Not tested	L11Q R81S	A65del	No mutation	No mutation
E. coli **EC-10**	Not tested	No mutation	No mutation	R163P, N165del	I88T, L95P, P111T, W113R, L114S, S116W, R145W, D150W, H157P, L218F
E. coli **EC-12**	Not tested	No mutation	V77A, L81P, T92H R93P, L95A, E97G L98A, Q99A, L102S E103W	Q113P, K171R, H198del	L231R, N235I, E246del
E. coli **EC-21**	Not tested	G15E, R81S D82del, D86R, K87del	No mutation	K171R, R185P, S187I, D191Y, K200R, I210S, V213G, Y218H	L96S, Q99A, I109H, W113M, F119S
E. coli **EC-23**	Not tested	R81S	No mutation	N119I, E154del	K46I, del_{56-70}
K. pneumoniae **KP-16**	S36G, G37D, I38A, C39A, I41T, N42Y	No mutation	D150N, S205I, G207del	No mutation	No mutation
E. cloacae **Eclo-14A**	G37_V38insG V38S, C39G, A40K I41M, N42S, K43G, I45Y, P46G W47V, ins48K	S64C, L216W, E217I	L38S	No mutation	No mutation
E. cloacae **Eclo-14B**	V38I, W47S, ins 48V	No mutation	No mutation	D46V, I47F, I49F, E140del, F141del, I143D, N144A, $del_{148-163}$	No mutation

ARTICLE 6

Title: First Detection of NDM-5 Carbapenemase in colistin-resistant *Klebsiella pneumoniae* isolated from clinical Lebanese patients.

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First Detection of NDM-5 Carbapenemase in colistin-resistant *Klebsiella pneumoniae* isolated from clinical Lebanese patients.

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Abstract:

Here, we report the first description of NDM-5 producing carbapenem-resistant *Klebsiella pneumoniae* ST383, isolated from hospitalized patients in Lebanon. In addition to carbapenem resistance, two out of five isolates were colistin-resistant due to mutations in the amino-acid sequences of proteins (PmrA/B, PhoP/Q, and MgrB). Therefore, screening for such isolates may be effective in limiting the spread of these resistant microorganisms among hospitalized patients and within community.

Introduction

Klebsiella pneumoniae is one of the main gram-negative bacteria known to cause severe nosocomial infections [1]. Carbapenemase-producing *K. pneumoniae* infections constitute a major clinical problem worldwide because of the few remaining therapeutic options, since the renewal of colistin as a last-resort treatment option may have resulted in colistin-resistant carbapenemase-producing *K. pneumonia* [1]. Numerous resistance genes have been found in multidrug-resistant (MDR) Gram-negative bacteria such as the extended-spectrum β- lactamase, the carbapenemase genes, and the colistin resistance genes [1]. The beta-lactamase, NDM-1 (New Delhi Metallo-enzyme) was first reported in 2008 in New Delhi, India, from Swedish patient with urinary tract infection caused by carbapenem-resistant *K. pneumoniae* [2]. Bacteria harboring NDM-1 subsequently captured attention worldwide because it is, in turn, associated with additional resistance genes, found in nosocomial bacteria [3]. The NDM-5 are variant of NDM-1, which was first described in the United Kingdom from patient infected with MDR *Escherichia coli* strain who was previously hospitalized in India [4]. On the other hand, it has been shown that colistin resistance in *K. pneumoniae* can be induced by mutation of the two components system (*pmrA*/*pmrB*, *phoP*/*phoQ*), or its negative regulator *mgrB* [5]. Here, we report the presence of NDM-5 in carbapenem and colistin-resistant *K. pneumoniae* isolated from Lebanese patients.

Materials and methods

In November 2017, two soldiers with gunshots wounds were transferred to Saint-George Hospital in Beirut, after being hospitalized in another peripheral Lebanese hospital. At the date of admission, two multidrug resistant *Klebsiella pneumoniae* were isolated from sputum and

abdominal surgical wounds. One transferred to floor and discharged, another stayed in ICU for about one month. During their hospitalization, another three isolates were collected from three different patients from various clinical specimens and kept at -80°C before being transported to the laboratory in Marseille, France. The strains were cultivated on Trypticase Sodium Agar medium (TSA) for 24h at 37°C. They were identified using the matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) (Microflex; Bruker Daltonics) as previously described [6]. To determine the antibiotic susceptibility testing of the strains, the standard disk diffusion method was performed on Mueller-Hinton agar as recommended by CLSI and EUCAST. Thirty-two different antimicrobial agents were tested. Minimal inhibitory concentration (MIC) of imipenem was determined using E-test, and the minimal inhibitory concentration (MIC) of colistin was done for isolates resistant to colistin using the broth microdilution method as recommended by the European Committee of Antimicrobial Susceptibility Testing (EUCAST) (http://www.sfm-microbiologie.org/UserFiles/files/casfm/CASFM%20V2_0_Mai2017.pdf). Strain was considered resistant to colistin when the MIC > 2 mg/L. All strains were subjected to real-time PCR assay to detect the presence of carbapenemase encoding genes using specific primers for *bla*$_{\text{OXA-48}}$, *bla*$_{\text{OXA-58}}$, *bla*$_{\text{OXA-23}}$, *bla*$_{\text{OXA-24}}$, *bla*$_{\text{KPC}}$, *bla*$_{\text{NDM}}$ and *bla*$_{\text{VIM}}$ genes. All bacteria were also screened for the presence of extended-spectrum β-lactamase (*bla*$_{\text{TEM}}$, *bla*$_{\text{SHV}}$, *bla*$_{\text{CTX-M}}$) genes and for the *mcr*-1, 2, 3, 4 and 5 genes. Probes and primers used were described previously [7–10]. PCR products that were positive for the antibiotic resistance gene tested were sequenced using BigDye amplification terminator chemistry on an automated ABI 3130 sequencer (PE Applied Biosystems, Foster City, CA). ARG-ANNOT database was used to analyze the sequenced genes [11], and then they were compared to other genes using the BlastN and BlastP tool from the

National Center for Biotechnology Information (NCBI) database (http://www.ncbi.nlm.nih.gov). The colistin resistance genes (PmrA/B, PhoP/Q, and MgrB) were amplified and sequenced. Sequenced genes were compared against the reference strains *K. pneumoniae* MGH 78578 (NCBI GenBank accession no.CP000647) using the nps alignment software (https://prabi.ibcp.fr/htm/site/web/home). To predict, whether the identified amino acids substitutions would affect the function of the proteins, we used PROVEAN (Protein Variation Effect Analyser) software (http://provean.jcvi.org/seq_submit.php). The genetic relationship among isolates was determined by multilocus sequence typing (MLST) using the seven housekeeping genes for *K. pneumoniae* as described on Institute Pasteur's MLST Web site (http://bigsdb.pasteur.fr/klebsiella/primers_used.html).

Results

Results of the antibiotic resistance showed that all bacteria have high resistance to the major antibiotics tested including: amoxicillin, amoxicillin clavulanic acid, piperacillin tazobactam, Cefalotin, ceftriaxone, cefepime, ertapenem, imipenem, meropenem, aztreonam, tobramycin, amikacin, gentamicin, cefoxitin, cefotaxime, tigecycline, ciprofloxacin, minocycline, nitrofurantoin, doxycycline, rifampicin, sulfadiazine, fusidic acid, mupirocin, oxacillin, ticarcillin-clavulanic acid, teicoplanin. E-tests showed high-level of resistance of all isolates to imipenem, with MIC greater than 256 µg/ml. In addition, two out of five isolates had MICs for colistin ranging from 8 to 32 mg/L (Table 1). Real-time PCR and sequencing results showed that all the five strains harbored the carbapenemase genes $bla_{\text{OXA-48}}$ and $bla_{\text{NDM-5}}$, and the ESBL genes $bla_{\text{TEM-135}}$, $bla_{\text{SHV-145}}$, $bla_{\text{CTX-M-14}}$, and $bla_{\text{CTX-M-15}}$ genes (Table 1). None of the strains contained the $bla_{\text{OXA-23}}$, $bla_{\text{OXA-24}}$, $bla_{\text{OXA-58}}$, bla_{KPC}, bla_{VIM}, *mcr-1*, *mcr-2*, *mcr-3*, *mcr-4*, and *mcr-5* gene. Due to the absence of

the *mcr* genes, sequence analysis of the colistin-resistant isolates KP-2 and KP-4 showed that the colistin resistant phenotypes observed was due to mutation of *mgrB*, *pmrB* and *phoQ* genes. For KP-2, analysis showed that there was two genetic changes conferring resistance to colistin: first, a nucleotide insertion (C340) in the *pmrB* gene leading to a frameshift mutation resulting in a defective protein, and second, an insertion of T nucleotide in the *phoQ* gene at position 461 leads to a frameshift mutation resulting in a defective protein. For KP-4, analysis revealed that *mgrB* has many missenses mutations (T21P, N25T, C28Y, S36G, I38F, N42K), some of them were described previously, and another has never been identified but were verified as deleterious. Moreover, this strain showed also a mutation in the *pmrB* gene that was due to a three nucleotide insertion (CCC) at position (271-273) leading to a frameshift mutation and resulting in a defective protein, In addition, several nucleotides insertion in the *phoQ* genes between the nucleotide 100 to 135 leads to a frameshift mutation resulting also in a defective protein (Figure 1). Results of MLST analysis showed that all isolates have the same sequence types (STs) ST383 (Table 1). This finding suggests that there is a link between the isolates collected in the same hospital.

Discussion

This study describes the emergence of NDM-5 and OXA-48 carbapenem-resistant *klebsiella pneumoniae* carrying different types of genes that are involved in this phenotype, in addition to the identification of colistin-resistant strains due to mutations in *mgrB, pmrB,* and *phoQ* genes. In Lebanon, studies revealed the presence of *Klebsiella pneumoniae* ESBL (+) carrying different types of genes and carbapenem genes such as $bla_{\text{OXA-48}}$, and $bla_{\text{CTX-M-15}}$ [12–16] whereas the $bla_{\text{NDM-5}}$, and the ESBL genes $bla_{\text{TEM-135}}$, $bla_{\text{SHV-145}}$, and $bla_{\text{CTX-M-14}}$ genes have never been previously reported. On the other hand, it has been reported that the β-lactamase

encoding gene *bla*$_{\text{NDM-5}}$ was found in *K. pneumoniae* and *E. coli* in different countries in the world such as India, Algeria, Denmark, Spain, South Korea, Japan, and the United Kingdom [17]. In addition, our groups reported the presence of *bla*$_{\text{NDM-5}}$ in *E. coli* collected from the rectal swab of a pilgrim after Hajj [17]. Previous study revealed that the bacteria, that harbored the New Delhi metallo-enzyme (NDM), is resistant to all β-lactams antibiotics except to aztreonam [4], which is not the case in our results because all our strains confer resistant to all β-lactams tested which is worrisome. Interestingly, we report here the detection of colistin-resistant *K. pneumoniae* isolated from patients without previous use of colistin. Many different gene mutations have been identified in genes responsible for colistin resistance in our clinical isolates. However, some of these mutations have been reported earlier such as C28Y, and N42K found in *mgrB* gene of *K. pneumoniae* described by Poirel et al.[18]. A previous study reported the colistin resistance in three *K. pneumoniae* isolated from clinical samples in Lebanon. The resistant of those isolates was due to mutations in *mgrB, pmrA, pmrB,* and *phoQ* genes [16]. Genotyping revealed that the *K. pneumoniae* strains belong to the same sequence types (ST383), which has been reported in *K. pneumoniae* in different counties, such as UK, China and Greek [19–21]

In conclusion, the presence of MDR *K. pneumoniae* in hospital settings is highly concerning. Several genes of resistance are implicated in this *K. pneumoniae* ST383 resistance phenotype, with a more concerning carriage of *NDM-5* gene associated with colistin resistance; the first report in Lebanon. Molecular and epidemiological studies are thus essential to understand the risk factors, the reservoirs, and the mode of transmission of this microorganism. Therefore, an urgent strategy must be taken to limit the spread of this MDR *K. pneumoniae* clone between hospitals and the community.

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Disclosure Statement

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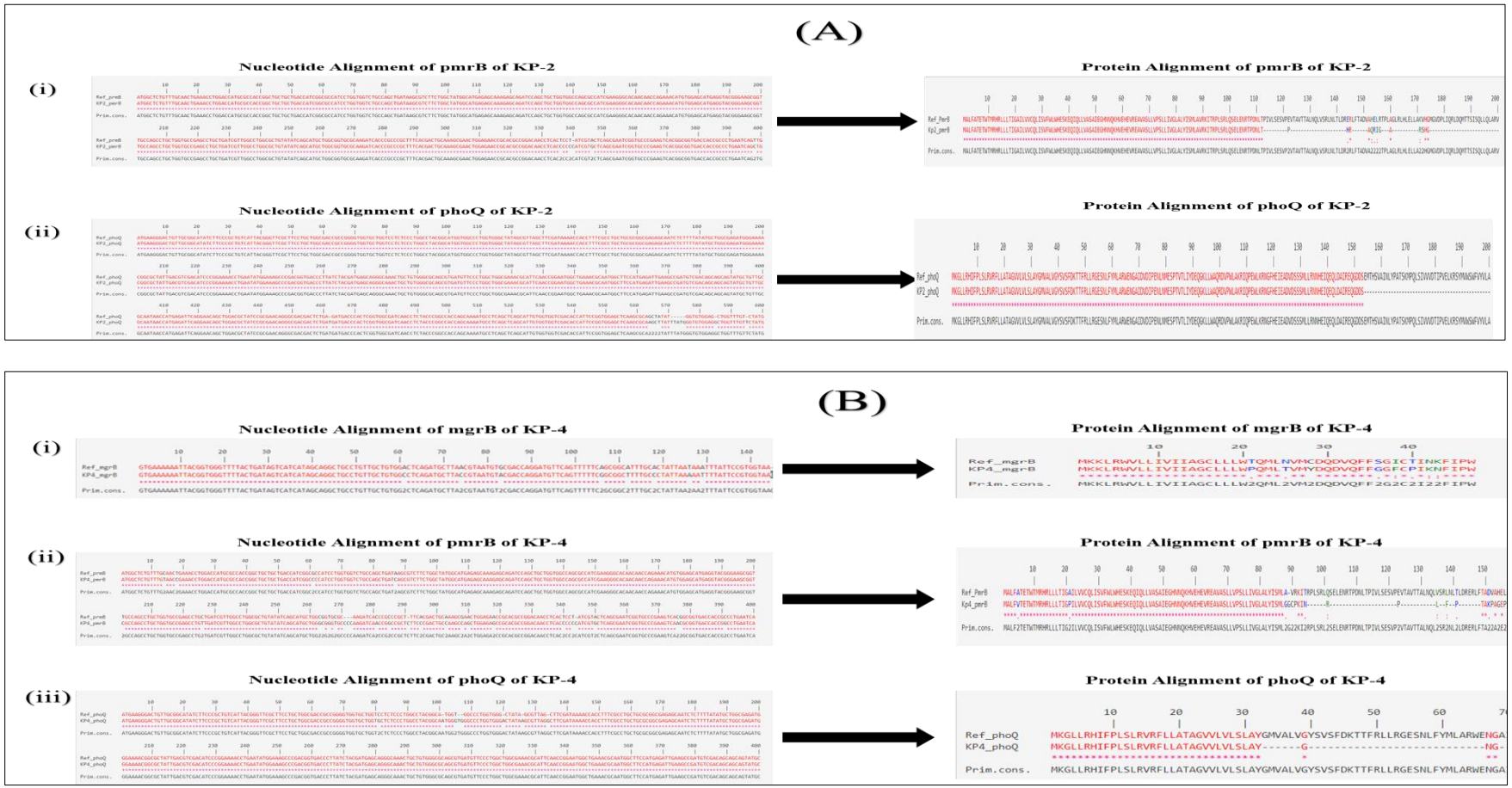
Figure 1. Genetic representation of the *pmrB /phoQ*, and the negative regulator, *mgrB*.



(A) (i) *K. pneumoniae* KP-2 with a defective protein *pmrB* due to nucleotide insertion (C340) and **(ii)** *K. pneumonia* KP-2 with a defective protein *phoQ* due to an insertion of T nucleotide at position 461 **(B) (i)** *K. pneumoniae* KP-4 with a defective protein *mgrB* due to many missenses mutations **(ii)** *K. pneumoniae* KP-4 with a defective protein *pmrB* due to a three nucleotide insertion (CCC) at position (271-273), and **(iii)** *K. pneumoniae* KP-4 with a defective protein due to several nucleotides insertion between the nucleotide 100 to 135

Table 1: Phenotypic and genotypic features of the five-*kebsiella pneumoniae* isolated from Lebanese hospital

Strain name	Source	IPM	ERT	CS	IMP MIC (µg/ml)	Colistin MIC (µg/ml)	*bla*$_{OXA-48}$	*bla*$_{NDM}$	*bla*$_{SHV}$	*bla*$_{TEM}$	*bla*$_{CTX-M-1}$	*bla*$_{CTX-M-9}$	ST
K. pneumoniae KP-1	urine	R	R	S	>256		+	NDM5	SHV-145	TEM135	CTX-M-15	CTX-M-14	383
K. pneumoniae KP-2	sputum	R	R	R	>256	8	+	NDM5	SHV-145	TEM135	CTX-M-15	CTX-M-14	383
K. pneumoniae KP-3	abdominal surgical wounds	R	R	S	>256		+	NDM5	SHV-145	TEM135	CTX-M-15	CTX-M-14	383
K. pneumoniae KP-4	urine	R	R	R	>256	32	+	NDM5	SHV-145	TEM135	CTX-M-15	CTX-M-14	383
K. pneumoniae KP-5	urine	R	R	S	>256		+	NDM5	SHV-145	TEM135	CTX-M-15	CTX-M-14	383

Resistant strains (**R**), imipenem (**IPM**), colistin (**CS**), sequence type (**ST**)

Chapter Conclusion

This work clearly supports the ongoing emergence of colistin and carbapenem resistance among Enterobacteriaceae in Lebanon. Colistin-resistant Enterobacteriaceae are now widely reported in many continents including Americas, Europe, Asia, Africa and the countries of Arabian Peninsula [6]. In the first part of this work, the screening of fecal stool samples led to the recovery of colistin-resistant Gram-negative bacteria that have mutations in the *mgrB* gene as well as mutations in two-component regulatory genes (*pmrA/pmrB* and *phoP/phoQ*). Similarly, the isolation of carbapenem and colistin-resistant *K. pneumoniae* clinical isolate represents the first report of the presence of NDM-5 gene associated to colistin resistance in Lebanon. However, multiclonal outbreaks with OXA-48-, NDM-, and both OXA-48- and NDM-producing *K. pneumoniae* are currently ongoing in Turkey [7]. All our results showed the emergence of colistin resistance among humans in lebanon. Our studies showed that specific mutations in both *pmrA/pmrB, phoP/phoQ* and *mgrB* genes are responsible for colistin resistance. In Lebanon, a previous study reported by Okdah et al showed that colistin resistance in three *K. pneumoniae* strains isolated from clinical samples, was due to mutations in *mgrB, pmrA, pmrB,* and *phoQ* genes [8]. In recent years, colistin currently retain significant activity against most Gram-negative organisms, multiple studies have indicated that the prevalence of colistin resistance has increased rapidly among clinical isolates [7]. In the countries of the Middle East, such as Turkey and Israel, low rates of resistance to colistin among CR-KP isolates have been reported (2.7% and 4.5%, respectively). In Lebanon, colistin-resistant bacteria begins to spread among Lebanese patients. This emergence is worrisome and necessitates the re-evaluation of colistin use in clinical settings. There was no detection of *mcr* genes. Colistin-resistant was due to chromosomal mutation in genes associated to colistin resistance. Ultimately, reinforcing the

detection of polymyxin-resistant isolates must be encouraged in our Lebanese hospitals. Prospective epidemiological surveys are needed, since the current knowledge on this issue is very rare. Actually, the recent development of a rapid diagnostic test for detection of polymyxin resistance, along with the development of a screening agar medium, will contribute to facilitating those surveys. In addition, surveillance studies targeting the spread of colistin resistant Gram-negative bacilli are warranted in the clinical and community settings of Lebanon in order to quantify the magnitude of this emerging problem. Moreover, future work should rely on the possible infection control measures that can be taken at the national level in order to limit the dissemination of colistin resistance especially the transfer from animals to humans.

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IV ANNEX AND COLLABORATIVE STUDIES- DETECTION OF VANCOMYCIN-RESISTANT GRAM-POSITIVE BACTERIA IN LEBANON AND COLISTIN RESISTANT-GRAM NEGATIVE BACTERIA IN ANIMALS IN ALGERIA AND MARSEILLE.

Antimicrobial agents are at least partially responsible for the development of serious infections by *Staphylococcus aureus*, vancomycin-resistant enterococci, extended-spectrum B-lactamase producing Enterobacteriaceae, and other infectious agents [1]. In particular, vancomycin-resistant *Enterococcus* (VRE) are known as common nosocomial pathogens that cause serious human diseases especially within hospitals [2]. Enterococci are facultative anaerobic gram-positive cocci in pairs/chains that live in the gastrointestinal (GI) tract and ordinarily function commensally with humans. However, they can cause a variety of infections, most commonly urinary tract infection (UTI), intraabdominal infection, bacteremia, or endocarditis. Additionally, VRE often exist as colonizing organisms that does not always contribute to infection, making it more difficult to determine when and how to treat these infections [3]. Despite these difficulties, the prevalence of VRE in any hospital or health facilities has been associated with higher treatment costs, prolonged morbidity and greater mortality rates [4] . Resistance to vancomycin is typically mediated by one of the nine van genes: vanA, vanB, vanC, vanD, vanE, vanG, vanL, vanM, and vanN. Among the vancomycin-resistant enterococci (VRE) vanA and to a lesser extent vanB are the most prevalent genes reported [2]. VanA VRE exhibit resistance to both vancomycin and teicoplanin whereas VanB VRE are resistant to vancomycin but susceptible to teicoplanin. This is due to the presence of an additional *vanZ* gene that is present on the *vanA* operon, conferring this resistance by an unknown mechanism [5].

The first article of this chapter (***Article N °7***), focused on the detection and description of vancomycin resistant *Enterococcus faecium* strains (VREfm) isolated from clinical samples of asymptomatic patients in Lebanon. Because we used the LBJMR medium that contains colistin and vancomycin, we isolated four *E. faecium* from 23 rectal swabs of patients treated with colistin-carbapenem and without previous use of vancomycin. Antibiotic susceptibility testing

revealed resistance of all isolates to vancomycin, and teicoplanin. E-tests showed that the MIC of vancomycin was greater than 256 μg/ml. All the isolates harbored the vancomycin resistance gene *vanA* and none of them carried *vanB* gene (Table 1). Result of MLST showed that the four *E. faecium* isolates belonged to new different sequence types. This means that there was no relationship between the strains isolated from the same hospital. This study demonstrates the presence of VREfm strains in patients in Lebanon, which is a major public health problem. To the best of our knowledge, there is no report describing VREfm clinical isolates in Lebanon. In addition, we report vancomycin-resistant enterococci strains isolated from patients without previous use of vancomycin. Monitoring and control measures must be adopted to prevent the spread of such bacteria in hospitals.

During my thesis, I was able to participate in collaborative work with other PhD students. One work (***Article N °8***), consisted of constitution of a map summarizing the spread of mcr-1 in Algeria and identifying other potential reservoir of mcr-1 in animals. In this study, we worked on a collection of 20 feces of cattle, 20 feces of sheep, 20 feces of goats and 20 samples of surface swabbing of a farm in El Tarf, Algeria, whose occurrence of mcr-1 were tested in these samples. Among these samples, 8 strains of *E. coli* carrying the mcr-1 gene were isolated from 4 feces of goats, 2 from cattle and 2 strains from swabbing the surface of a barn and 2 *Enterobacter cloacae* from the surface of a barn.

A second collaborative study (***Article N °9***), was devoted to the research of colistin resistance genes in gulls at the Frioul island of Marseille. We collected in this investigation 38 droppings of gulls. Three colistin resistant strains (EC3, EC35 and KP15) were isolated from LBJMR medium and identified by MALDI-TOF. No mcr-1 strain was identified. The resistance of the isolates to

colistin was likely due to mutations in the *mgrB* gene as well as mutations in two-component regulatory genes (*pmrA/pmrB* and *phoP/phoQ*).

ARTICLE 7

Title: Emergence of Vancomycin-Resistant *Enterococcus faecium* Isolated from Clinical Samples in Lebanon.

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Submitted **to *International journal of Infectious diseases***

Emergence of Vancomycin-Resistant *Enterococcus faecium* Isolated from Clinical Samples in Lebanon

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Abstract:

Objective: Infections due to vancomycin-resistant *enterococci* have become a major problem in hospital settings. In the present study, we aim to describe the presence of vancomycin resistance genes in *Enterococcus faecium* collected from rectal swabs of patients treated with colistin-carbapenem from Lebanese hospital.

Methods: Twenty three rectal swabs were cultivated on selective medium LBJMR. Antibiotic susceptibility testing was performed using the disk diffusion method and E-test. RT-PCR, standard PCR and sequencing were used for the screening of vancomycin resistance genes. MLST was done to determine the genetic relationship among the clinical strains isolated.

Results: Due to the usage of LBJMR medium that contains vancomycin, four vancomycin-resistant *E. faecium* were isolated from 23 rectal swabs of asymptomatic patients without previous use of vancomycin. Antibiotic susceptibility testing revealed that all isolates were resistant to vancomycin, have MICs greater than 256 μg/ml and harbored the vancomycin resistance gene *vanA*. MLST analysis revealed that the four *E. faecium* strains belonged to new sequence types.

Conclusion This study showed the presence of vancomycin resistance gene *vanA* in *E. faecium* collected from Lebanese patients. This emergence is worrisome and reemphasizes the importance of implementing surveillance programs in order to avoid the spread of such bacteria.

Keywords: *Enterococcus faecium*, vancomycin, *vanA*.

Sir

Infections due to vancomycin-resistant enterococci (VRE) have been recently reported in many countries. There is a high incidence of nosocomial infections that is due to the presence of VRE in intensive care units (Faron et al. 2016). In 1988, the first vancomycin-resistant *Enterococcus faecium* (VREf) was isolated in UK (van Hal et al. 2016). In Europe, according to the 2012 report of the European Antimicrobial Resistance Surveillance Network (EARSNet), that covers invasive isolates only, the prevalence of VRE infections ranged from 0% in the Netherlands and Sweden to 44% in Ireland (Abat et al. 2016). In the North of France, outbreaks of VRE infections have occurred between 2004 and 2008, meanwhile in the South of France no VRE had ever been detected till now (Abat et al. 2016). In a study done in the Middle East and Africa, 32 VREf isolates were reported in Israel, Oman, Pakistan and Saudi Arabia (Kanj et al. 2014). In the present study, we report the detection of vancomycin resistance genes in *E. faecium* collected from rectal swabs in Lebanon. To the best of our knowledge, there is no report describing VREf clinical isolates in Lebanon.

Between October 2016 and February 2017, 23 rectal swabs were collected for each patient hospitalized in the ICU for more than one week of Saint-George Hospital in Beirut who received colistin and carbapenem combination therapy. Patients were asymptomatic for vancomycin resistance *E. faecium* during their stays. They were shipped to Marseille. Once they arrived, they were transferred to an enrichment broth, Tryptic Soy Broth medium (TSB), and incubated at 37°C. After an overnight incubation, 100 µL of the enrichment medium was cultivated for 24h at 37°C on the selective medium LBJMR that contains colistin and vancomycin (Bardet et al. 2017). Colonies growing on this medium were identified using the matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectrometers as described previously

(Abat et al. 2016). Antimicrobial susceptibility testing was performed using the disk diffusion method on Mueller-Hinton agar as recommended by the 2017 European Committee of Antimicrobial Susceptibility Testing (EUCAST). E-test was performed to validate the resistance phenotype of VRE (Abat et al. 2016). Bacterial DNA was extracted using the automatic robot EZ1 (Qiagen BioRobot EZ1-, Tokyo, Japan) with the extraction kit (EZ1 DNA, Qiagen, Hilden, Germany), following the manufacturer's instructions. Molecular detection of vancomycin resistance genes *vanA* and *vanB* was performed using PCR amplification. Positive PCR products of the *vanA* gene were sequenced. To determine the genetic relationship among the clinical isolates, MLST was done by using the seven housekeeping genes *adk*, *atpA*, *ddl*, *gdh*, *gyd*, *pstS* and *purK*, according to MLST Pasteur (www.pasteur.fr/mlst).

Because we used the LBJMR medium that contains vancomycin, four vancomycin-resistant *E. faecium* were collected from patients without previous use of vancomycin. Antibiotic susceptibility testing revealed resistance of all isolates to the major antibiotics tested except to pristinamycin and linezolid. In addition, two out of four strains showed sensitivity to fosfomycin (Table 1). E-tests showed high-level of resistance to vancomycin, with MIC greater than 256 µg/ml. All the isolates harbored the vancomycin resistance gene *vanA* and none of them carried *vanB* gene (Table 1). MLST analysis revealed that the four *E. faecium* isolates belonged to new sequence types (ST1327, ST1328, ST1329 and ST1330) according to Pasteur web site. This means that there was no relationship between the strains isolated from the same hospital.

The MDR *enterococci* have become a major public health issue due to an increase in VRE. In the Middle East and Africa, 32 VREf isolates were collected and had MIC greater than 64 only (Kanj et al. 2014). In Lebanon, there was no detection of vancomycin-resistant isolates except for one *E. gallinarum* (*vanC* phenotype resistance) that had a MIC of 16 mg/l and was recovered

from an infected catheter (Zouain and Araj 2001). In contrast, the Middle Eastern countries presented a wide range of enterococcal glycopeptide resistance rates (0.8–75%) (Zouain and Araj 2001). Throughout the world, *E. faecium* carrying the gene *vanA* are the dominant VRE (Faron et al. 2016). Results showed that all the strains in this study were highly resistant to teicoplanin in addition to vancomycin, and harbored *vanA* gene. It has been shown by Faron et al. that bacteria harboring *vanA* phenotype are characterized as having a high resistance level to vancomycin, while the *vanB* phenotype has various levels of resistance to vancomycin (Faron et al. 2016). Moreover, the strains harboring the *vanA* gene conferred resistance to the glycopeptide teicoplanin (Te) in addition to vancomycin. This is due to the presence of an additional *vanZ* gene that is present on the *vanA* operon, conferring this resistance by an unknown mechanism (Faron et al. 2016).

In conclusion, this is the first study showing the presence of VREf strains in patients in Lebanon, which is a major public health issue. Vancomycin resistant isolates were isolated from asymptomatic patients and without previous use of vancomycin. An urgent strategy must be implemented to prevent the spread of such bacteria among hospitalized patients.

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Ethical approval

We have read the policy on ethical consent and ethical approval is not required for this work.

Consent for publication

Not applicable

Availability of Data and materials

Not applicable

Conflicts of interest

No conflicts of interest or financial disclosure for all authors.

Authors' Contribution

TND wrote the manuscript, performed experiments, and analyzed the data. ED provided the strains and helped draft the manuscript. SC and SD contributed to the literature review, and helped draft the manuscript. JMR conceived the study, participated in its design and coordination, and helped draft the manuscript. All authors read and approved the final manuscript.

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Table 1: Phenotypic and genotypic features of the four vancomycin-resistant *E. faecium* clinical isolates.

Isolate no.	**Species**	**Source**	**M / F**	**B-Lactams**			**Glycopeptides**		**Macrolides**				**Others**					**VA**	***VanA***	***VanB***	**ST**
				OXA	AMX	CRO	VA	TEC	DA	E	PT	GEN	LNZ	FF	F	DO	RA	**MIC (µg/ml)**			
5	***E. faecium***	Rectal swab	M	R	R	R	R	R	R	R	S	R	S	R	R	R	R	>256	+	-	1327
7	***E. faecium***	Rectal swab	F	R	R	R	R	R	R	R	S	R	S	S	R	R	R	>256	+	-	1328
8	***E. faecium***	Rectal swab	M	R	R	R	R	R	R	R	S	R	S	R	R	R	R	>256	+	-	1329
15	***E. faecium***	Rectal swab	M	R	R	R	R	R	R	R	S	R	S	S	R	R	R	>256	+	-	1330

MIC, minimum inhibitory concentration; **ST**, sequence type; **M**, Male; **F**, Female; **R**, resistant; **S**, sensitive; **OXA**, oxacillin; **AMX**, amoxicillin; **CRO**, ciprofloxacin; **VA**, vancomycin; **TEC**, teicoplanin; **DA**, daptomycin; **E**, erythromycin; **PT**, pristinamycin; **GEN**, gentamicin; **LNZ**, linezolid; **FF**, fosfomycin; **F**, nitrofurantoin; **DO**, doxycycline; **RA**, rifampicin.

ARTICLE 8

Title: First report of the plasmid-mediated colistin resistance gene *mcr-1* in *Escherichia coli* isolated from feces of livestock and farm surface, Algeria.

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To be submitted to ***Veterinary research journal***

Title: First report of the plasmid-mediated colistin resistance gene *mcr-1* in *Escherichia coli* isolated from feces of livestock and farm surface, Algeria.

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Keywords:*mcr-1*, livestock, *E. coli*, Algeria.

To editor

Colistin is extensively used as the last-line antibiotic for treating infections caused by multiresistant Gram-negative bacteria (1). Colistin resistance was previously linked only to chromosomal mutations on target genes and was not transferable (1). Recently, a novel plasmid-mediated colistin resistance gene (*mcr-1)* that encodes a phosphoethanolamine transferase enzyme was described (2), which was identified in *Escherichia coli* and *Klebsiella pneumoniae* strains isolated from animals and humans (2). Nowadays, several studies have revealed a wide geographically spread of *mcr-1* gene in humans and animals worldwide (1). It seems that the uncontrolled use of colistin in veterinary medicine is one of the main cause of the emergence and dissemination of this resistance worldwide. To determine the exact animal role as a possible *mcr-1* gene reservoir, several investigations were concentrated on this item.

Therefore, this study was conducted to evaluate the occurrence of plasmid- mediated colistin resistance *mcr-1* in livestock from Algeria.

In 2017, feces of cattle, goats and sheep were collected from El Tarf, eastern of Algeria. The feces was collected directly from animals using a rectal swabs, as well as, we have collected swabs from surfaces of a farm. The sampling was done following the permission of the animals' owners. The samples were kept at -80°C before being forwarded to the laboratory in Marseille, France. All samples were transferred to an enrichment broth Tryptic Soy Broth medium (TSB) (BioMérieux, Marcy l'Étoile, France) and incubated at 37°C. After that, 100 µL of this medium were cultivated on the selective medium LBJMR (3) that contains colistin for 24h at 37°C. The colonies were selected and cultured individually on Trypticase soy agar. All colonies were selected and identified by MALDI-TOF (matrix-assisted laser desorption and ionization time-of-flight mass spectrometry) (Microflex, Bruker Daltonics, Bremen, Germany).

Antibiotic susceptibility was determined on Mueller–Hinton agar using the standard disc diffusion procedure as recommended by the European Committee on Antimicrobial Susceptibility Testing (EUCAST) (www.eucast.org). The following Sixteen antimicrobial agents were tested: amoxillin, amoxillin clavulanic acid, piperacilline tazobactam, cefalotin, ceftriaxone, cefepime, ertapenem, imipenem, amikacin, gentamicin, ciprofloxacin, fosfomycin, nitrofurantoin, doxycycline, trimethropine sulphamethoxazole, and colistin (Bio-Rad, Marnes-la-Coquette, France). Minimum inhibitory concentrations (MICs) of colistin was determined by using the E-test and broth microdilution method. The microdilution plates were incubated at 37 °C for 24h. The results were interpreted according to the EUCAST guidelines (www.eucast.org).

All strains identified from each selective plate, were subjected to a real time PCR analysis for the detection of *mcr* variants genes (*mcr-1*, *mcr-2*, *mcr-3*, *mcr-4* and *mcr-5*). DNA extraction was performed for all isolates using the DNA extraction kits EZ1 (Qiagen, Courtaboeuf, France). The primers and probes used in this study have been designed in our laboratory (data not published). Standard PCR and sequencing were performed to confirm the presence of *mcr* genes. The sequences obtained were assembled using ChromasPro 1.7 (Technelysium Pty Ltd., Tewantin, Australia) and compared with *mcr-1* sequences found in the GenBank database using BLAST (http://blast.ncbi.nlm.nih.gov/Blast.cgi). The MLST was done based on allelic profiles to determine their genetic relationship among seven housekeeping genes (adk, fumC, gyrB, icd, mdh, purA and recA) and from the MLST database (http://mlst.warwick.ac.uk/mlst/dbs/Ecoli). Each single locus has different allele and a specific identification number that was given by adding the sequence types (ST) of the seven loci.

Strains were tested for their ability to transfer resistance. Conjugation was tested with azide-resistant *E. coli J53*. Trans-conjugants were selected on MacConkey agar (Beckton

Dickinson, Le Pont de Claix, France) added with 120 µg/mL sodium azide and 4 µg/mL colistin, as described (4). If conjugation assay failed, transformation assay was done (4).

A total of 80 samples was collected in the extrem East of Algeria (El Tarf), 20 fecal samples from goat, 20 fecal samples from sheep, 20 fecal samples from cattle and 20 samples from swab of the farm surface. Overall the 80 samples, 4 fecal samples from goats, 2 fecal samples from cattles and 3 samples from the farm surface have growth in the LBJMR medium. Eight *E.coli* and one *E. cloacae* were isolated and identified by MALDI-TOF MS, Microflex LT spectrometer (**Table1**).

Disk diffusion susceptibility test showed that a total of 10 strains (eight *E.coli* and one *E. claocae)* were resistant to colistin with a disc diameter between 9 and 13 mm (**table1**). Minimum inhibitory concentrations (MICs) of colistin confirmed resistance with MIC ranging from 4 to 8 mg/l of *E. coli and* >256 mg/l of *E. cloacae.* The antibiotic resistance profile of these isolates is shown in Table 1.

Overall strains, five *E.coli* out of eight (four *E.coli* from goat and one *E.coli* from surface farm) were harboring *mcr-1* and were positive in qPCR. The results were confirmed by standard PCR and sequencing. The obtained sequences were 100% identical to the *mcr-1* gene sequence reported by Liu and colleagues (2). The conjugation results showed that the strains plasmid was not conjugative. The analysis of the Multi Locus Sequence Typing showed that the four *E. coli* harboring *mcr-1* gene isolates from the goat belonged to the same genotype that was a new sequence types (ST submitted). The *E. coli* harboring *mcr-1* gene isolated from the farm surface as belonging to ST 48 which has already been reported in Algeria (Chabou et al., submitted in MDR). The two *E. coli* colistin resistant isolated from the cattle belonged to the same genotype (ST 164) which has already been reported in worldwide

livestock. This means that there is no relationship between the different strains isolated from the same farm. The *E. cloacae* isolates belonged to new sequence types (ST submitted).

The uncontrolled use of colistin in veterinary medicine has a highly important part in the global emergence of *mcr-1* in animals. The World Health Organization has newly included polymyxins as critically important antibiotics (5). In France, the prevalence of *mcr-1* was 5.9% in turkeys, 1.8% in broilers and did not exceed 0.5% in pigs (6). These findings support that *mcr-1* gene has spread in French livestock (6). In Germany, 79.8% of colistin resistant isolates harbored the *mcr-1* gene. The highest prevalence was detected in the turkey food chain (10.7%), followed by broilers (5.6%). A low prevalence was determined in pigs, veal calves and laying hens (7). The prevalence of *mcr-1* was higher in compared with France (7). That confirm the European Summary Report for 2014 an EU-level prevalence of colistin resistance of 0.9% for *E. coli* from broilers and 7.4% in *E. coli* from turkeys was reported (8). In Algeria, the first *mcr-1* was detected in *E. coli* from feces of chickens in 2016 (9). Recently, our team was confirmed the presence of *mcr-1* gene in poultry from another region from Algeria (under rev). As well as, *mcr-1* colistin resistant *E. coli* was also isolated from wildlife in Bejaia, Algeria (4). This isolate also carried the *mcr-1*, $bla_{CTX\text{-}M\text{-}15}$, $bla_{TEM\text{-}1}$, and *qnrB19* genes (4).

The fact that *mcr-1* gene is detected in poultry and also in livestock farm, confirm the rapid spread of the plasmid-mediated colistin resistance, not only in Europe but also in Algeria livestock. To the best of our knowledge, *mcr-1* isolates from livestock have been previously reported in Africa country including Tunisia, Egypt and South Africa (1) but never in Algeria. Furthermore, this plasmid was detected in a clinical *E. coli* isolate from University Hospital from Algeria (1).

Emergence of *mcr-1* gene in Algeria is probably related to the wide use of colistin in veterinary medicine (6) that should be banned in the future. This work shows that there is a

need for monitoring programs to avoid the spread of *mcr-1* gene, as well as to implement colistin susceptibility testing for Gram negative bacteria in clinical microbiology laboratories.

CONFLICT OF INTEREST AND FINANCIAL DISCLOSURE

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Table 1: Phenotypic and genotypic results of all strains.

	AMC	AX	FEP	TPZ	CRO	CN	AK	CIP	KF	SXT	DO	F	FF	ETP	IMP	CT (mm)	CMI (mg/l)	Mcr-1	MLST
E. coli_ 29GT	R	R	S	S	S	S	S	S	S	S	S	S	S	S	S	12	8	+	New
E. coli_ 30GT	S	R	S	S	S	S	S	S	S	S	S	S	S	S	S	13	4	+	New
E. coli_ 32GT	R	S	S	S	S	S	S	S	S	S	S	S	S	S	S	13	8	+	New
E. coli_ 34GT	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	13	4	+	New
E. coli_ 11CT	S	S	S	S	S	S	S	S	R	S	S	S	S	S	S	9	4	-	ST164
E. coli_ 15CT	R	R	S	S	S	S	S	S	S	S	R	S	S	S	S	9	8	-	ST164
E. coli_ 10S	R	R	S	S	S	S	S	S	R	S	R	S	S	S	S	11	8	-	ST48
E. coli_ 9S	R	R	S	S	S	S	S	R	R	R	R	S	S	S	S	13	4	+	ST48
E. cloacae_ 12S	R	R	S	S	S	S	S	S	R	S	S	S	S	S	S	6	>256	-	New

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ARTICLE 9

Title: First report of colistin resistance GNB in Yellow-legged Gull from Marseille.

Selma Chabou, Tania Nawfal Dagher, Sophie Baron and Jean Marc Rolain.

To be submitted to *J Glob Antimicrob Resist.*

Title: First report of colistin- resistant bacteria in Yellow-legged Gulls from Marseille, France

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- Table: 1

Sir;

The increased global incidence of multidrug-resistant (MDR) bacterial pathogens, particularly carbapenem-resistant Gram-negative bacteria, has led to the reactivation of old and abandoned antibiotics, polymyxin (polymyxin B and colistin), for the treatment of severe MDR bacterial infections (Olaitan et al., 2014). It is currently used as a last-line drug against multidrug-resistant Gram negative bacteria and is an effective antibiotic to treat severe bacterial infections (Baron et al., 2016a). Recently, resistance to colistin has emerged among pathogens, but there is still a dearth of information on the comprehensive mechanisms of colistin resistance in Gram-negative bacteria (Baron et al., 2016b; Olaitan et al., 2014). Various modifications leading to colistin resistance have been described including modification of lipopolysaccharide (LPS) such as the of Loss of LPS production due to mutations in the lpxACD genes or the addition of phospho-ethanolamine or 4-amino-4-arabinose mediated by mutations in the two-component systems *pmrA/pmrB, phoP/phoQ* (Baron et al., 2016a). More recently a new plasmid mediated colistin resistance mechanism due to *mcr-1* gene that encodes a phospho-ethanolamine transferase has been described (Liu et al., 2016).

Migratory birds appear to be reservoirs of multi-resistant bacteria and could therefore play an important epidemiological role in the spread of resistance (Bouaziz et al., 2017). The objective of this study was to investigate the presence of colistin resistance Gram-Negative bacteria in fecal samples collected from a group of yellow-legged gulls (*Larus michahellis*) in Marseille, France.

In August 2017, feces from Yellow-legged gull (*Larus michahellis*) were collected in Frioul iles Marseille. Samples were collected directly on site and placed in a single sterile, identified tube. For the selection of colistin resistant Gram-negative bacteria, samples were incubated on

a selective culture medium LBJMR for 24h à 37°C as described (Bardet et al., 2017). Isolated colonies from each sample were identified by MALDI-TOF. Antibiotic susceptibility testing was determined on Mueller–Hinton agar using the standard disc diffusion method against 16 antibiotics. Colistin susceptibility was determined by broth microdilution. DNA extraction was carried out subsequently in an automatic robot EZ1. Colistin resistance genes (*pmrA, pmrB, phoP, phoQ, mgrB, mcr-1, mcr-2, mcr-3, mcr-4* and *mcr-5)* were screened by real time PCR and/ or standard PCR, and were then sequenced(Chabou et al., 2016; Yassin et al., 2017). Results of the sequenced genes were compared against the reference strain *K. pneumoniae* MGH 78578, and *E. coli* K-12 MG 1655 (NCBI GenBank accession no.CP000647). Translated amino acid sequences were analysed using npsa_clustalwan software (https://npsa-prabi.ibcp.fr/cgi-bin/npsa_automat.pl?page=/NPSA/npsa_clustalwan.htm.)PROVEAN software (http://provean.jcvi.org/seq_submit.php) was used to verify whether the mutation would affect the function of the proteins. Multi-locus Sequence Typing (MLST) was performed for genotyping using seven housekeeping genes.

Thirty-eight samples of feces from Yellow-legged gulls were collected in Iles Frioul, Marseille in August 2017. Three (7,8%) out of thirty-eight samples, colistin-resistant strains were isolated from LBJMR meduim and identified by MALDI-TOF, two *E.coli* (EC3, EC35) and one *k. pneumonia* (KP15). The three isolates were resistant to colistin and amoxicillin and sensitive to the other antibiotics tested. Minimum inhibitory concentrations (MICs) for colistin ranged from 8 to 16 mg/ l. To study the clonality of our isolates, a Multi Locus Sequence Typing (MLST) analysis was performed and revealed the same type of sequence for *E. coli* (ST 126). The MLST identified KP15 as belonging to ST 248. All qPCR for *mcr* genes (*mcr-1, mcr-2, mcr-3, mcr-4*and *mcr-5)* were negative. Results of sequence analysis of colistin-resistant genes are summarized in Table 1.

Analysis of *E. coli* (EC3) showed that the resistance was due to many mutations in the *pmrB* and *phoQ* genes) that have not previously been described. Olaitan et al showed that L140H (table 1) was similar to T140P found in the *pmrB* of *K. pneumoniae* (Olaitan et al., 2014). In addition, *E. coli* (EC35) had deletions and substitutions mutations in the *pmrA* and *phoP/Q* genes that have not been previously reported and were considered as deleterious using PROVEAN software (work in progress).In addition, *K. pneumoniae* was resistant due to large deletion in phoQ of 240 amino acid resulting in the formation of a truncated protein made of 113 amino acid.

The migratory birds play a potential role in the transport of resistant GNB. Most studies have previously shown the presence of ESBL-producing Gram-negative bacteria in wild birds (Bouaziz et al., 2017). However, the plasmid mediated colistin resistance gene *mcr-1* has been identified in *E. coli* isolates from infected migratory Magellanic penguins (*Spheniscus magellanicus*) (Sellera et al., 2016).

Our results showed the potential for GNB to resist colistin transmission by yellow-legged gulls and could pose a public health risk. This requires wildlife and human studies to improve our knowledge of resistant bacterial transmission pathways and to determine the source of bacterial contamination. Although *mcr* genes were not detected in our study, the presence of colistin resistant Enterobacteriaceae in urban gulls is worrisome since they are living in close contact with humans and could be a source of zoonotic transmission of multi drug resistant bacteria.

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Table 1: Mutation of colistin resistance genes

Strains	*mgrB*	*pmrA*	*pmrB*	*phoP*	*phoQ*	*Mcr genes*
E.coli_3G	-	No mutation	V78G,L79S, T80P, L84Y, Q88P, A90G, V91T, R96A, P97R, L98W, A99R, E100S, L101C, L140H	No mutation	A22H, L28C, T129C, H157P, L218F	Negative
E. coli_15G	-	L11Q	No mutation	I44L, K171R, R185P, E186D, S187I, K200R, I210S	L26R, del_{30-40}, F44I, D45del, S116W, F119S, G441R, E451G, A470G	Negative
K. pneumonia_35G	No mutation	No mutation	T10P, E37D, L146X, G147X, V219I, L222P, Q223A, D224G, E225R, L226R, E227D, A228S, M229L, L230C, A231C, Q232R, R233X.	No mutation	A large deletion of 240 amino acid	Negative

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Chapter Conclusion

Via this chapter, we reported the presence of VREfm strains in patients in Lebanon, which is a major public health problem. The prevalence of vancomycin resistance amongst enterococci differs widely among different countries including from one hospital to another in the same country. VRE is an important health concern not only because its infections are difficult to treat in healthcare settings but also because the VRE clones can spread within hospitals as well as between regions or countries [4]. The ability of enterococci to grow and persist in hostile conditions and their transmission through hand contact ensures their survival in hospital environments and increases the species reservoirs [6]. Beginning in the 1980s, isolates of vancomycin-resistant Enterococcus were demonstrated in Europe, likely arising due to the use of the glycopeptide antibiotic avoparcin in livestock to promote growth. Development in the United States was probably due to increasing use of vancomycin in the clinical setting. Throughout the 1990s and 2000s, multiple epidemics have plagued hospitals due to person-to-person transmission [3]. In contrast, the Middle Eastern countries presented a wide range of enterococcal glycopeptide resistance rates (0.8–75%) [7]. The increasing resistance of VRE in neighboring and other countries poses a serious threat that necessitates using surveillance studies to monitor for such strains especially as this study investigated the first detection of vancomycin-resistant enterococci strains isolated from patients without previous use of vancomycin.

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CONCLUSION AND FUTURE PERSPECTIVES

In this work, we have confirmed that antibiotic resistance has become an international concern worldwide and is a serious public health problem. Prolonged therapy with antibiotics can lead to the development of resistance in a microorganism that initially is sensitive to antibiotics, but later it can adapt gradually and develop resistance to antibiotics [1]. Through this thesis, we have performed several studies that showed the resistance of bacteria to antibiotics of human origin including carbapenems, and colistin in Lebanon. This implies the implementation of emergency measures for the control and use of antibiotics. The overuse of drugs in multiple sectors (human, animal, agriculture) is the main problem. Indeed, microorganisms faced with antimicrobial selection pressure enhance their fitness by acquiring and expressing resistance genes and then share those genes with other bacteria. Thus, antimicrobial use and overuse are important drivers of the resistance phenomenon; the other main drivers are factors that promote the spread of resistant bacteria and their genes locally and globally. These include poor infection control, environmental contamination, and geographical movement of infected humans and animals [2]. Through our work, we reported the emergence of carbapenen-resistant bacteria in humans in Lebanon. Carbapenems are among the drugs of choice for the treatment of nosocomial infections. However, their efficacies are increasingly becoming compromised because of the worldwide emergence of resistant isolate [3]. In the era of severely ill, MDR-colonized patient management, relying on existing guidelines is not enough. A creative multidisciplinary approached based on local epidemiology and surveillance are key factors. In our work, the lack of convincing data regarding the combination therapy and the uncontrollable surge of carbapenem consumption led us to change the standard of care of all XDR-AB infections from colistin-carbapenem combination therapy to colistin mono therapy. We are able to demonstrate that this change in therapeutic pathway of XDR *A. baumanii* (AB) infections dramatically

decreased antibiotic consumption resulting in drop of *A. baumanii* prevalence in sputum culture. In addition, we were able to affirm that the added carbapenem pressure sustained a survival advantage for ST2 XDR- *A. baumannii*. We were succeeded to eliminate the ST2 XDR-*A. baumannii* clone, remarkably reducing *A. baumannii* disease, and the total antimicrobial consumption at our institution was only possible by active surveillance program of antibiotic consumption and resistance profiles as well as trusted collaboration with the ICU and microbiology department.

In this doctoral research, we have also performed studies on colistin resistance. We consider this very important due to the recent use of colistin against multidrug-resistant (MDR) bacteria. We were able to isolate numerous colistin-resistant bacteria from patients that have been treated with colistin. We have reported the prevalence of colistin resistant Enterobactariaceae in Lebanon. This observation further confirms that colistin resistance among Gram-negative bacteria is indeed on increase as demonstrated by the increasing reports of colistin resistance in literature. We showed in this research that mutations in genes influencing bacterial lipid A modifications such as *mgrB* and TCS genes form the major molecular support mediating colistin resistance in Enterobacteriaceae. Thus, surveillance should be implemented urgently because colistin-resistant bacteria begins to spread among Lebanese patients. In Lebanon, the mechanism of colistin resistance at human was described by a single study that reported the detection of three colistin resistant *K. pneumoniae* in a hospital in Beirut; colistin resistance in these latter were due to mutations in the *mgrB, pmrA/B* and *phoQ* genes [4]. On the other hand, two studies were done by Dandachi et al., which described the mechanism of colistin resistance in animals. In these studies, colistin resistance was due to the presence of the plasmid mediated *mcr-1* gene isolated from *E. coli* strain from poultry in Lebanon [5], and from 23 *E. coli* strains isolated from Lebanese swine farms [6] . In fact, the carriage of *mcr-1* in farms could constitute a potential key for the

introduction of this gene into the community as well as to the clinical settings in Lebanon by horizontal transfer from animals to humans. Due to the spread of carbapenemase producers in hospitals in Lebanon, it is expected that once *mcr-1* would be introduced, this latter will be selected by the frequent use of colistin. In Lebanon, the detection of colistin resistance is still new; many clinical microbiology laboratories still depend on the antibiotic disk method to determine resistance or susceptibility toward colistin. As the disk diffusion method is not reliable for antibiotic susceptibility testing against colistin, the *mcr-1* positive stains could diffuse silently in the clinical settings, resulting in an epidemic in Lebanon. Therefore, surveillance studies targeting colistin resistance in hospitals are warranted in Lebanon. Regarding this situation, we need to find solutions to fight and stop the dissemination and increasing of colistin resistance. As of today, few antibiotics remain active against infections caused by colistin and polymyxin B. Moreover, the fight against these bacteria can be through prevention, which consists, to understand their transmission mechanisms, and to find these resistance determinants. In addition, it is so important to introduce supplementary tests in clinical laboratories for the detection of colistin-resistant strains isolated from infected patients including those without a previous history of colistin usage.

Moreover, we reported the emergence of vancomycin resistant *E. faecium* in Lebanon. Vancomycin resistant isolates were isolated from asymptomatic patients and without previous treatment with vancomycin. As we do not have any idea about the history of those patients and why they carried the vancomycin resistant *E. faecium*, one questions remained. Do we have in Lebanon the same problem that was encountered in Europe in the mid-1990s when they used the avoparcin (a vancomycin analogue not used in humans) as a feed additive in livestock to promote growth in animals, and consequently, we have the dissemination of vancomycin

resistant Enterococci (VRE) in humans? [7]. On the other hand, in the United States, studies showed that the development of vancomycin resistant Enterococci (VRE) in humans was probably due to increasing use of vancomycin in the clinical setting, which is not the case at the hospital where this study was done [8]. This emergence in Lebanon is worrisome and reemphasizes the importance of implementing surveillance programs and different research studies must be done in order to avoid the spread of such bacteria in human.

At the end, some points should be implemented in the near future to further investigate this resistance; we should look for new resistance genes from soil, water, environment, and animals because they represent very large reservoirs of resistance genes never explored before. With the continuous rise of bacterial resistance, new approaches are needed to fight infections caused by MDR bacteria especially for carbapenem and colistin-resistant ones. Due to the lack of development of new antibiotics in the pharmaceutical companies, the re-introduction of old antibiotics can constitute attractive alternative therapeutic options, in addition to the introduction of new molecules to treat MDR bacteria as it is one of the purpose of our group . Moreover, with the creation of new bioinformatic tools, specifically ARG-ANNOT, which can detect existing antibiotic resistance genes in bacterial genomes, real-time sequencing will become a reference method for detecting resistance genes. In addition, we believe that it is necessary to set up quality training for health professionals and strengthen the supervision of the prescription of anti-infection in human. Another important point is the strengthening of surveillance of black markets and self-medication and medication by non-professionals. Finally in Lebanon, it is necessary to implement surveillance programs in order to avoid the spread of MDR bacteria, implement an infection control measures in healthcare settings, control antibiotic resistance dissemination, develop rapid diagnostic tests such as (LBJMR medium, Malditof, E-test, broth microdilution,

carba NP test, PCR, RT-PCR), promote research on antibacterial resistance prevention and surveillance, and make the whole genome sequencing to guide therapeutic intervention. In conclusion, our thesis work contributed to a better knowledge of the epidemiology and the risk factors for the acquisition of multidrug-resistant bacteria in human in Lebanon, and clearly demonstrate that surveillance programs and healthcare policies should be implemented in this country in a “one health” perspective.

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POSTERS, PRESENTATION AND SCIENTIFIC JOURNEY

- **Investigation of multidrug-resistant ST2 *Acinetobacter baumannii* isolated from Saint Georges hospital in Lebanon.** Tania Nawfal Dagher, Selma Chabou, Charbel Al-Bayssari, Antar Nadine, Seydina. M. Diene, Eid Azar, and Jean-Marc Rolain. In Les Journées de l'Infectiopôle, Marseille, France 2017.
- **Successful control and elimination of XDR *A. baumanii* ST-2 at a tertiary care center ICU by changing standard of care: colistin monotherapy carbapenem sparing regimen.** Tania Nawfal Dagher, Eid Azar, Jean-Marc Rolain, Amanda Chamieh, Tala Ballouz, Claude Afif, George Juvelekian, and Sani Hleis. In ESCMID conference, Madrid, Spain, April 2018.
- **Emergence of colistin resistant gram-negative bacteria in clinical isolates at Saint Georges hospital in Lebanon**. Tania Nawfal Dagher, Eid Azar, Selma Chabou, Linda Hadjadj, Seydina. M. Diene, and Jean-Marc Rolain. In ASM conference, Atlanta, June 2018.

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