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MULTIDRUG-RESISTANT *ENTEROBACTERALES* IN OMAN: MOLECULAR EPIDEMIOLOGY AND THERAPEUTIC INSIGHTS

Hissa M. Al-Farsi

Karolinska Institutet

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Cover image: A scanning electron microscopic (SEM) of extended-spectrum β-lactamase-producing (ESBL) *Escherichia coli.* The image was created by Alissa Eckert (2013) and obtained from the Public Health Image Library (PHIL) with no copyright restrictions.

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Multidrug-resistant *Enterobacterales* in Oman: Molecular Epidemiology and Therapeutic Insights

THESIS FOR DOCTORAL DEGREE (Ph.D.)

By

Hissa M. Al-Farsi

Principal Supervisor:
Prof. Christian Giske
Karolinska Institutet
Department of Laboratory Medicine
Division of Clinical Microbiology

Co-supervisor(s):
Assoc. Prof. Peter Bergman
Karolinska Institutet
Department of Laboratory Medicine
Division of Clinical Microbiology

Dr. Maarten Coorens
Karolinska University Hospital
Department of clinical Microbiology

Dr. Saleh Al-Azri
Ministry of Health
Central Public Health Laboratories,
Muscat, Oman.

Dr. Amina Al-Jardani
Ministry of Health
Central Public Health Laboratories,
Muscat, Oman.

Opponent:
Dr. Teresa Coque
Ramón y Cajal Institute for Bio Health Research (IRYCIS), Madrid, Spain.
Department of Microbiology
Division of Microbial Biology and Infections

Examination Board:
Assoc. Prof. Åsa Sjöling
Karolinska Institutet
Department of Microbiology, Tumor and Cell Biology. Stockholm, Sweden.

Prof. Mikael Rhen
Karolinska Institutet
Department of Microbiology, Tumor and Cell Biology, Stockholm, Sweden.

Prof. Ann-Beth Jonsson
Stockholm University
Department of Molecular Biosciences, The Wenner-Gren Institute, Stockholm, Sweden.

To the loud peacemakers

paving the way for

the quite peacekeepers

The Red Queen told Alice

“

Now, here, you see, it takes all the running you can do, to keep in the same place”

- Lewis Carroll

From the book Through the Looking Glass (1871)

ABSTRACT

The spread of antibiotic resistance is a concerning issue causing limited treatment options for bacterial infections, particularly with Gram-negative bacteria. Surveillance and epidemiological studies help to determine the magnitude of the problem as well as to establish early measures to slow down the spread of resistance and consequently increase antibiotic lifespan. Currently, there is a visible paucity of published data about resistance from the Arabian Peninsula. In this thesis, we studied a collection of carbapenem non-susceptible *E. coli* (n=35) and *K. pneumoniae* (n=237) isolated in 2015 from various hospitals in Oman. We aimed at identifying resistance mechanisms, mapping the bacterial population structure, investigating bacterial fitness, and studying potential treatment options available to tackle infections caused by such multidrug-resistant strains. These aims were addressed in five papers as discussed below.

NDM and OXA-48 were the only carbapenemases we found in this collection, both among *E. coli* (**Paper I**) and *K. pneumoniae* (**Paper II**). The pattern of resistance among the isolates from Arabian Peninsula mimics the pattern reported from the Indian subcontinent, most likely due to the close socioeconomic interactions between them. Both regions lack KPC enzymes, which are commonly seen in China and the US from strains belong to ST11 and ST258, respectively. Despite ST11 being predominant in this collection, we did not detect KPC. Yet, we detected a high-risk clone of *E. coli*, ST131-H30Rx-CTX-M-15. Additionally, we identified newly emerging clones of *K. pneumoniae* and *E. coli* such as ST231 and ST1193-H64RxC, respectively.

Nearly 10% of the *K. pneumoniae* isolates in our collection were colistin resistant which prompted us to study the mechanisms of colistin resistance (**Paper III**). MgrB-inactivation by insertion elements was seen in 8 isolates while other mutations were seen in other chromosomal genes known to be involved in colistin resistance e.g. *pmrB, phoPQ* and *crrB*. However, we did not detect *mcr* genes. Collectively, the genetic alterations are thought to reduce the net negative charge in bacterial cell wall, hence lowering the binding affinity of colistin. Our data underscores that there is no reduction in the surface charge in colistin-resistant *K. pneumoniae,* due to the MgrB-insertion (**Paper IV**). The genetic alteration might lead to other structural changes in the cell wall such as altering hydrophobicity, which required further investigation. Also, our data shows no difference in the survival rates of colistin resistant and susceptible strains in blood, serum and zebrafish model. Thus, gaining resistance against colistin does not infer a fitness cost in *K. pneumoniae* with MgrB-insertion (**Paper IV**). Additionally, colistin and LL-37 share similar binding mechanism which suggest there might be a cross-resistance between them. Our data supported this hypothesis, but only at high concentrations of LL-37 ($\geq$ 50 mg/L) (**Paper IV**).

Finally, we studied available options to treat infections caused by multidrug-resistant strains. Combining colistin and rifampicin showed good *in vitro* activity against multidrug-resistant strains of *E. coli* (**Paper V**) and *K. pneumoniae* (**Paper III**). To summarise, we conducted comprehensive genomic analysis of *E. coli* and *K. pneumoniae* isolates from Oman to reveal the resistance mechanism, their impact on bacterial cell structural and if there is a fitness cost inferred by the resistance mechanisms. Finally, we studied combination therapy as an available option at hand for tackling infections caused by multidrug-resistant strains.

LIST OF SCIENTIFIC PAPERS

I. **Hissa M. Al-Farsi**, Angela Camporeale, Karolina Ininbergs, Saleh Al-Azri, Zakariya Al-Muharmi, Amina Al-Jardani, Christian G. Giske. Clinical and molecular characteristics of carbapenem non-susceptible *Escherichia coli*: A nationwide survey from Oman. *PLOS ONE* 2020; **15**: e0239924. DOI: 10.1371/journal.pone.0239924

II. **Hissa M. Al-Farsi**[*], Maarten Coorens[*], Isak Sylvin, Saleh Al-Azri, Zakariya Al-Muharmi, Amina Al-Jardani, Christian G. Gisk. Nationwide characterization of carbapenem non-susceptible *Klebsiella pneumoniae* in Oman. Manuscript. [*]*shared first author*

III. **Hissa M. Al-Farsi**, Maarten Coorens, Karolina Ininbergs, Saleh Al-Azri, Zakariya Al-Muharmi, Amina Al-Jardani, Peter Bergman, Christian G. Gisk. Colistin resistance from clinical isolates of *Klebsiella pneumoniae* in Oman: Mechanism of resistance and synergism of colistin with azithromycin and rifampicin. Manuscript.

IV. **Hissa M. Al-Farsi**, Salma Al-Adwani, Sultan Ahmed, Carmen Vogt, Anoop T. Ambikan, Anna Leber, Amina Al-Jardani, Saleh Al-Azri, Zakariya Al-Muharmi, Muhammet S. Toprak, Christian G. Gisk, Peter Bergman. Effects of the antimicrobial peptide LL-37 and innate effector mechanisms in colistin-resistant *Klebsiella pneumoniae* with *mgrB* insertions. *Frontiers in Microbiology* 2019; **10:** 2632. DOI: 10.3389/fmicb.2019.02632.

V. Anna Olsson, Marcus Hong M, **Hissa M. Al-Farsi**, Christian G. Giske, Pernilla Lagerbäck, Thomas Tängdén. Evaluation of the interactions of polymyxin B in combination with aztreonam, minocycline, meropenem and rifampicin against NDM- and OXA-48-like producing *Escherichia coli*. Manuscript.

CONTENTS

LIST OF ABBREVIATIONS

AmpC	Ampicillinase type C
AMPs	Antimicrobial peptides
OXA	Oxacillinase
ArnT	Aminoarabinose glycosyotransferase
BCA	Background corrected absorption
CaMHB	Cation-adjusted media CaMHB
Cas	CRISPR-associated genes
CFU	Colony-forming unit
CMS	Colistinmethate sodium
Col-R	Colistin resistant
Col-S	Colistin susceptible
CP-CRE	Carbapenemase producing-CRE
CRE	Carbapenem-resistant *Enterobacterales*
CRISPR	Cluster regularly interspaced short palindromic repeats
DIW	Deionized water
DR	Direct repeats
EptA	phosphoethanolamine transferase
ESBL	Extended-spectrum β-lactamase
GlcNAc	N-acetylglucosamine
HGT	Horizontal gene transfer
Hi-RC	High-risk clone
HMM	High molecular mass
IM	Inner membrane
IMP	Imipenemase
IS	Insertion elements
Kdo	3-deoxy-D-manno-oct-2-ulosonic acid
KPC	*Klebsiella pneumoniae* carbapenmase
Arn	Aminoarabinose (4-amino-4-deoxy-L-arabinose)
LB	Lysogeny broth
LPS	Lipopolysaccharides
MBL	Metallo-β-lactamase
MCR	Mobile colistin resistant protein
MHB	Mueller Hinton Broth

MIC	Minimum inhibitory concentration
MLST	Multilocus sequence typing
MRSA	Methicillin resistant *S. aureus*
MurNAc	N-acetylmuramic acid
NDM	New Delhi metallo-β-lactamase
OM	Outer membrane
OMP	Outer membrane proteins
PBP	Penicillin binding proteins
PBS	Phosphate buffered saline
PG	Peptidoglycan
RPMI1640	Culture medium developed by Roswell Park Memorial Institute
SEM	Scanning electron microscopy
SNP	Single nucleotide polymorphism
ST	Sequence type
TEM	Transmission electron microscopy
VIM	Verona integron-brone metallo- β-lactamase
VRSA	Vancomycin resistant *S. aureus*
WGS	Whole genome sequencing
WHO	World health organisation

PREFACE

Long time ago, I thought it would be fun to attend a military training, so I did. I joined Nizwa military academic school in Oman. No reasons were required there to yell at someone or treat them like a slave. If the trainer did not like the way you walk, you might end having an extra round (duplication) at the peak of the summer in July where temperature normally goes beyond 40˚C. When we were out for the physical training, the cells were inspected. If they thought, the beds were not tidy, the cell will be turned upside down, the dustbin will be emptied in the sleeping mattress. The number of participants dropped as some could not take it anymore. I left the campus as well but only when the training period was over with ashy skin and a pride being one of those who managed to stand the pressure.

The reason why me and others survived was not only attributed to us as individuals despite that play a role as well since not all participants managed to finish the course. The main factor was the induced pressure was not strong enough to break us but enough to stretch us and turn us resilient. This is a sort of stress with good intention called hormesis. I tried to create such positive stress throughout my PhD study by attending courses and conferences. I know it is a motivation factor to go forward.

This is what we do with bacteria when we start taking an antibiotic yet we do not comply with full treatment course. Bacteria become resilient as much as I did during the military training. Hormetic effect explains what happens during the exposure to increment but low dose of stress. Yet, what does happen if we withdraw the stress? After the military training I reverted back to my old habits, no more waking up at 4:00 am to exercise yet still tidy my bed religiously before I leave home! Why did I carry some of the habits but not others? It depends on the cost I pay to maintain them and the rewards I gain from carrying them.

Generally, we as humans assume bacteria do not anticipate the future as we do but live in the present moment as we fail to do. We assume as such bacteria will revert to be susceptible as they lack the sense of foreseeing the future. A study showed that after 50% decrease in the consumption of certain macrolide in Finland, there was 50% decrease in the prevalence of erythromycin resistance in *Streptococcus pyogenes* between 1991 to 1996[1]. While withdrawal of cotrimoxazole and streptomycin in UK did not affect the prevalence of sulfamethoxazole and streptomycin resistance in *E. coli* [2]. This variation might be attributed to the cost paid by bacteria to keep resistance attributes despite the absence of antibiotic pressure. Much goes with the context of "The red Queen Theory" which was introduced by Van Valen in 1973 that implies organisms must evolve and adapt to new challenges not to extinct. Perhaps bacteria do think about the future, though not as intense as we humans do.

INTRODUCTION

1.1 Multidrug resistance as a public health issue

1.1.1 The pride comes before the fall

Not quite far, 100 years ago, we had no tools to fight back bacterial infections. We look at pregnancy as a safe biological process to bring new lives now. Yet back then, it was a biological process to trade mother's life for infant's life due to many reasons including failure to treat post-partum infections. We as humans, did not have any tools to handle these infections until someone thought outside the box.

Ingaz Philipp Semmelweis proposed the practice of washing hands with chlorinated lime solution in 1847 to tackle the increased incidence of puerperal fever which was the main cause of mortality in obstetrical clinics. He published a book "Etiology, Concept and Prophylaxis of Childbed Fever" and showed results to support his recommendation but was faced with rejection by the medical community then as they felt offended to be asked to wash their hands. He suffered from mental breakdown from stress. In 1865, he passed away from a gangrenous wound, due to infection after being beaten at an age of 47[3]. These challenges from the past continue until the present days. During the COVID-19 pandemic in 2020, following the recommendation of wearing a mask and keeping physical distance, as simple as they sound, were hard to implement. All in all, we see that relying on human behaviour as a tool to fight infectious diseases is quite hard to sustain as such more reliable tools are required such as antibiotics and vaccines.

At the beginning of 1910s, Paul Ehrlich introduced salvarsan as the first antimicrobial agent. It was used to treat syphilis[4]. Followed by Fleming's discovery of penicillin in 1928 and many more antibiotics that gave us quite plenty of tools to fight bacterial infections and save lives. In 1967, a remarkable announcement came from U.S. Surgeon General William Stewart "The time has come to close the book on infectious diseases"[5]. That pride with plenty of antibiotics at hand just faded after 1970s, since we entered a dry innovation season, while bacteria fought back strongly.

1.1.2 The bright past just passed

The first serious battle we had was MRSA (Methicillin resistant *S. aureus*) in the beginning of 1970s against Gram-positive bacteria. We opened our antibiotic closet looking for a solution. Vancomycin, which was first sold in 1954 could be a desperate choice despite the known side effects such as nephrotoxicity (Figure 1). Luckily, a purified form of vancomycin was produced with less toxicity. In 1996, bacteria went ahead again in a second battle known as VRSA (vancomycin resistant *S. aureus*). After this initial nightmare two new antibiotics were introduced; linezolid and daptomycin in 2000 and 2003, respectively (Figure 1). As we humans thought we had won the battle, Gram-negative bacteria pressed the replay button.

Gram-negative bacteria are harder to treat since they have an extra protection layer in their cell wall which is a challenge for drugs to pass. Besides, the transmission of resistance genes between different species of Gram-negative bacteria is frequently high as compared to Gram-positive bacteria. Despite plasmid-encoded penicillinase was first been observed in Staphylococci, there were limited intra-species transmission compared to Gram-negative bacteria[6].

The first clinical impact of resistance in Gram-negative bacteria to β-lactam was in 1974 by penicillinases, when researchers reported ampicillin treatment failure in meningitis patients due to *Haemophilus influenzae* type b producing TEM β-lactamase[7]. The plasmid encoding bla_{TEM} was transmitted to *Neisseria gonorrhoeae*[8]. This resistance mechanism spread widely between different continents due to naval activity between Asia, western Africa and England[9]. The drug development industry came up with a set of 24 novel modified β-lactams with extended-spectrum including β-lactamase inhibitors, third generation cephalosporins, monobactams, and carbapenems in the 1980s[9].

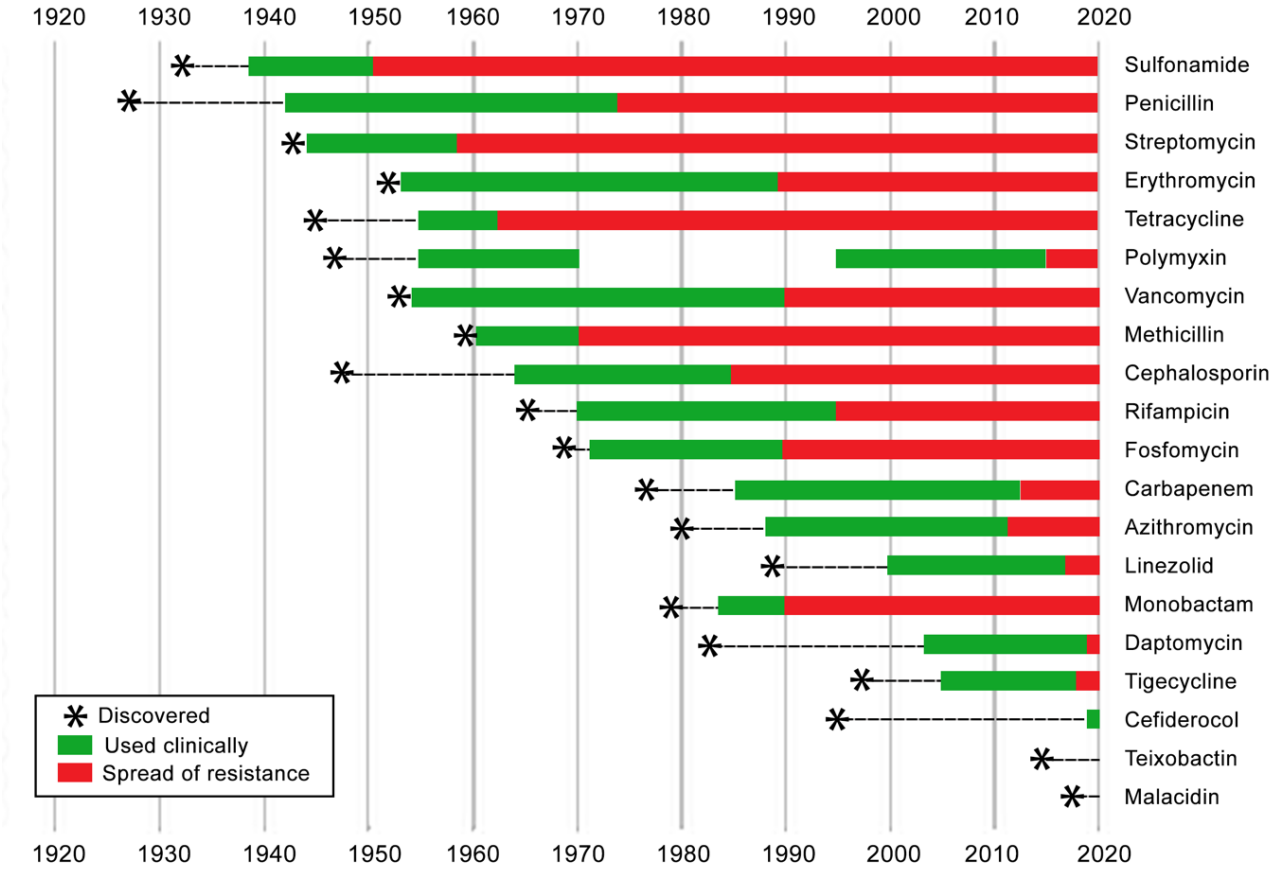


Figure 1. Timeline for key antibiotics. For each antibiotic three dates are represnted; the discovey date, the date when the antibiotic was used clinically and the date when resistance spread. The clinical use of polymyxin was discontinued for concern regarding side effects by 1970s. However, due to lack of treatment options, the drug was re-used again clinically in mid-1990s. Malacidin and teixobactin are not yet approved. Cefiderocol was recently approved in US (2019) and in Europe (2020). Adapted from Gautam and Sommer[5]. Copyright (2014) Sigma Xi, The Scientific Research Honor Society.

Soon bacteria responded by producing extended-spectrum β-lactamases (ESBLs). The first ESBL in a clinical isolate was reported in 1983 from *Klebsiella ozaenae* in Germany[10]. By the mid-1980s, outbreaks of *Enterobacterales* producing ESBL were reported in France due to TEM derived CTX-1 and CAZ-1 enzymes. As their names imply CTX-1 conferred more resistance to cefotaxime whereas CAZ-1 was more active against ceftazidime[11]. In the early 1990s, CTX-M-type ESBLs emerged and by the mid-1990s they caused large outbreaks of *Salmonella* Typhimurium. More variants of CTX-types were detected including CTX-M-15 which proved to be a game changer regarding the spread of ESBL globally. By the 2000s it became the predominant type of ESBL globally, replacing the TEM- and SHV-derived ESBLs[12]. The access to the drug class carbapenems still kept us quite ahead of the bacteria. However, the frequent use of carbapenems against ESBL-producers predicted carbapenem resistance is the upcoming storm.

Type C ampicillinase (AmpC) enzyme was first discovered by Swedish investigators in 1940 in *E. coli*. As the name implies, it was initially thought to only affect ampicillin[13]. Another report of this enzyme in *Pseudomonas* was published in 1965[14]. Yet, it grabbed more attention in 1976 when it was reported in the UK[15]. Now we know it is capable of not only hydrolysing penicillins but also cephalosporins, cephamycins and monobactams, leaving only carbapenems unaffected. However, AmpC could even hydrolyse carbapenems, if combined with other mechanisms such as porin loss[16]. The threat was magnified when AmpC-type enzymes were detected on plasmids in 1990, as this would enhance its propensity to spread[9].

The first carbapenemase was isolated from *Pseudomonas* in Japan in 1990 and was named impenemase (IMP)[17]. Followed by the detection of Verona integron-borne metallo-β-lactamase (VIM) in Italy from *Pseudomonas* as well in 1997[18]. More notorious types of carbapenemases were detected later such as the *Klebsiella pneumoniae* carbapenemase (KPC), oxacillin-hydrolyzing carbapenemase (OXA-type) and New Delhi metallo-β-lactamase (NDM)[9]. Here, we were literally trapped. We went back to our antibiotic closet and took colistin out despite its limitation in both efficacy and toxicity[19]. As such colistin resistance was foreseen. Multiple outbreaks were reported with colistin and carbapenem resistance *Enterobacterales* (CCRE)[20]. Bacteria were again ahead of humans.

1.1.3 Back to the future

We still lack a realistic estimation of lives lost yearly due to antibiotic resistance as the number is overshadowed by main cause of death. For example, a cancer-patient if expired due to a struggle with infection caused by antibiotic resistant bacteria, often the case will be counted as a death caused by cancer rather than antibiotic resistance. A similar scenario is seen in HIV and transplant patients. Thus, the numbers of death due to antibiotic resistance is far underestimated. Also, many life-saving surgical procedures would cease if we have no effective antibiotics to treat the inevitable infections at surgical sites which adds up in underestimating the impact of resistance in public health. This will not only mask the burden

but will have a crucial impact on allocated resources for intervention. Consequently, the antibiotic resistance issue remains less studied with a big knowledge gap yet to be filled.

A recent report in 2019 estimated the number of deaths attributed to antibiotic resistance only in the US, to be more than 35,000 while the number of infections was estimated to be more than 2.8 million each year[21]. This gives an estimation of less than 0.001% death rate. On the other hand, a study conducted in 2014 estimated the number of death due to antimicrobial drugs resistance, including antibiotics but not limited to them, would be 10 million yearly by 2050[22]. Yet, they estimated the combined deaths caused by road traffic injuries, cancer and diabetes to be around 11 million annually in 2050[22,23]. Bearing in mind diabetes, cancer and road injuries were listed within the top 10 causes of death in 2019 globally[24]. The paper received criticisms regarding uncertainty of assumptions to determine the rate of incidence, as it compiled two sets of data each of them used distinct assumptions and covered particular sources of infections e.g. community versus hospital acquired infections[25]. One advantage of the study was that it considered the impact of resistance on other medical procedures as a secondary effect for the problem. A better approach would be to lay down the estimation from each study separately rather than the risk of combining both, despite the methodological variations used in each. Also, it would be more informative to break the numbers down rather than counting HIV, malaria and TB. We know these diseases already cause high mortality. Yet, researchers in antibiotic resistance are looking for death cases caused by antibiotic resistance specifically. Additionally, listing death caused by primary or secondary attributes separately will give better insight to the magnitude of the problem. However, no doubt the report drove public attention and was a kick-off to think thoroughly about how to estimate the burden of antibiotic resistance and improve data collection as well as analysis in this regard.

Additionally, political barriers and national health policy are other challenges. This has been evident after the discovery of NDM and the reaction from the Indian government to restrict sending biological samples abroad for research purposes. They saw NDM as a stigmatising factor to their country, especially after naming the enzyme by the capital city in India "New Delhi". This impacted tourism which led to economic consequences[26]. Now, it is quite difficult to ship biological samples from India for research purposes and barriers to carry out epidemiological studies are becoming high to pass. Since it is a multifactorial issue with economic and political complications, the nomenclature of new genes could be revisited to minimise provoking logistic barriers for future studies. The awareness NDM brought to this matter was manifested by the neutral name given to the mobile colistin resistance (*mcr*) gene isolated from China in collaboration with some investigators who isolated *bla*$_{\text{NDM}}$ previously[27,28].

Antibiotics are social drugs with respect to their consumption by an individual will impact their effectiveness for others. Unlike the drugs that control high blood pressure or cholesterol level for example, where there is no such impact between individual consumption and long-term efficacy[29]. This creates another challenge related to social and cultural behaviours

towards consumption in different countries. Recent studies linked the increase of living standard in low-income countries to the increase of antibiotic consumption[30,31]. This could be due to the search for a fast fix that is affordable rather than the more time-consuming solution such as building infrastructure for diagnostic facilities which enhance identifying resistant strains prior to antibiotics consumption. Moreover, unregulated policy in private health sectors could play a role, since they go immediately to the last treatment option to satisfy the customer/patient and increase their reputation to cure infections fast andthereby increasing their profits. In less economically fortunate nations the scenario differs, since the majority of people cannot afford the expenses of health services such as visiting clinic and performing diagnostic tests prior to buying the lifesaving antibiotic. Thus, implementing the compulsory regulation for diagnosis and prescription prior to selling antibiotics might prolong antibiotics lifespan but might indirectly cause loss of lives for those who cannot afford paying for such services. Particularly, in the absence of social benefits to protect the vulnerable groups. As we see, despite the global impact of the issue a "one size fits all"-solution is not achievable and a tailored solution for each nation is needed bearing in mind the local socioeconomic structure.

Additionally, the increase of income leads to higher demands for food consumption. Consequently, the use of antibiotics in farming and agriculture are increasing to cover the high demand for proteins[30]. The world health organisation (WHO) introduced the one health concept to tackle this issue. Furthermore, the concept was nourished by other programs such as Joint Programming Initiative on Antimicrobial Resistance (JPIAMR). It implies, the issue of antibiotic resistance requires a collaborative attention from three sectors (human health, animal and environment) as the consumption of antibiotics in any of them will impact the utility and effectiveness of the drug in human health[32]. This expands the concept of antibiotic from a social drug into a universal drug.

As researchers in the field of antibiotic resistance, we acknowledge having a problem that requires intervention. Simultaneously, we acknowledge the multifactorial challenges in estimating the real burden of resistance compared for example to other infectious diseases such as HIV and malaria. I trust that epidemiological studies with ethical permits such as the one presented herein will help mapping the burden of antibiotic resistance. Besides, understanding the evolution of resistance may extend the life of the currently available antibiotics and slow down resistance. Consequently, this would reduce the ultimate cost paid by patients and society.

1.2 Antibiotics: Action and resistance

Antibiotics can be classified into different classes according to their chemical structures or mechanisms of action. Based on their targets, antibiotics could be divided into two main classes. A class of drugs that inhibits intracellular reactions such as proteins or nucleic acids synthesis. Rifampicin and azithromycin belong to this class. The second class includes antibiotics that target bacterial envelope such as β-lactams and polymyxins.

As antibiotics have different targets, bacteria evolve to resist them differently. Some of the resistance mechanisms apply to a wide range of antibiotics while others are tailored for specific drugs based on their chemical structure or the structure of their targets. The physical mechanisms, also known as intrinsic mechanisms, aim to reduce antibiotics uptake through cell surface modification by restricting the uptake (influx) or enhancing the excretion (efflux). This is achieved by porins modification or efflux pumps activation, respectively. The specific resistance mechanisms involve modification of drug target or inactivation of the drug (Figure 2). An example of the former is resistance mechanism against colistin by altering lipid A structure whereas the production of β-lactamase is an example of drug inactivation[5]. The general intrinsic mechanisms are mainly attributed to chromosomal genes while the tailored mechanisms are generally carried in transferable elements such as plasmids. Especially this second type of resistance creates a challenge to control infections as it can be transmitted both vertically and horizontally.

During vertical gene transfer, bacteria inherit their genetic contents from parental cells in the previous generation whereas during horizontal gene transfer (HGT) bacteria could gain genetic material from unrelated bacterial cells or their surroundings. There are three mechanisms of HGT; transformation, transduction and conjugation. They differ in the source of genetic material that bacteria uptake. In transformation bacteria obtain foreign DNA from the environment whereas for the remaining two mechanisms DNA will be obtained from a donor, either a phage or a plasmid by transduction or conjugation, respectively[33]. Conjugation plays a significant role in the spread of resistance genes within and between species. Yet, transduction is more involved in spreading virulence genes, and it is thus far unclear to which extent bacteriophages contribute to spreading resistance genes.

Most of the genes involved in polymyxin (colistin) resistance are chromosomal, which limits their mobilities. In contrast, genes encoding β-lactamases are frequently found in plasmids, which allows for easy horizontal transmission and fast spreading. Both polymyxin and β-lactam antibiotics act by interfering with the biosynthesis of the outer membrane and the cell wall, respectively. Polymyxins compete with and replace divalent cations that usually bring nascent lipid A molecules together physically, while β-lactams chemically inactivate the enzyme responsible for cross-linking nascent peptidoglycans. The following section will describe different components within the bacterial envelope which are used as targets by these drugs as well as how bacteria alter these components to resist them.

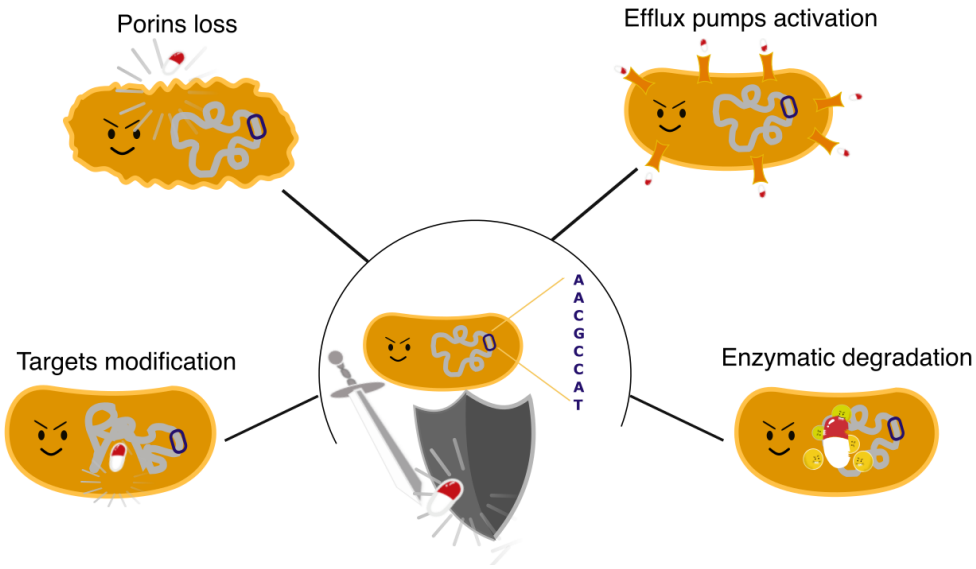


Figure 2. Mechanisms of antibiotic resistnce. Bacteria aquire antibiotic resistance by modifying their DNA. The genetic alteration could cause either a general mechanism of resistance such as porin loss or activation of efflux pump. Alternatively, mutated bacteria could gain more specific mechanisms of resistance, for example the ability to degrade antibiotics enzymatically or modify their targets so the binding would not occur.

The cellular envelope protects bacteria from hostile factors in their environment. In Gram-negative bacteria there are three main layers; the outer membrane (OM), the peptidoglycan (PG) and the cytoplasmic or inner membrane (IM). Among Gram-positive bacteria the envelope has only two layers consisting of a PG and an IM. Generally, Gram-negative bacteria are more resistant to antibiotics, due to the presence of the OM which restricts drug uptake. The bacterial envelope plays a key role in resistance mechanism. Thus, understanding the bacterial envelope is a key factor in slowing down resistance as well as developing new therapeutic drugs to treat infections.

1.2.1 The outer membrane when negativity is a merit

The outer membrane in *Enterobacterales* is composed of phospholipids arranged in a bilayer of inner symmetric leaflets and outer asymmetric leaflets. The outer leaflets are intercalated by lipopolysaccharides (LPS) through Van der Waals forces. In addition, the OM bilayer is embedded with outer membrane enzymes such as PagP and PagL in *E. coli* and *Salmonella enterica*, respectively. These enzymes usually regulate and modify LPS by acylation process[34]. Additionally, OM has outer membrane proteins (OMPs) such as porins which modify cellular permeability (Figure 3).

1.2.1.1 Outer membrane protein: gatekeeper

The outer membrane has β-sheet proteins which are wrapped into cylinders known as outer membrane proteins (OMP). The main function of OMPs is to exchange small molecules across outer membrane. Consequently, they enhance bacterial adaptability to various environments through regulating permeability. Porins are the most abundant OMPs that

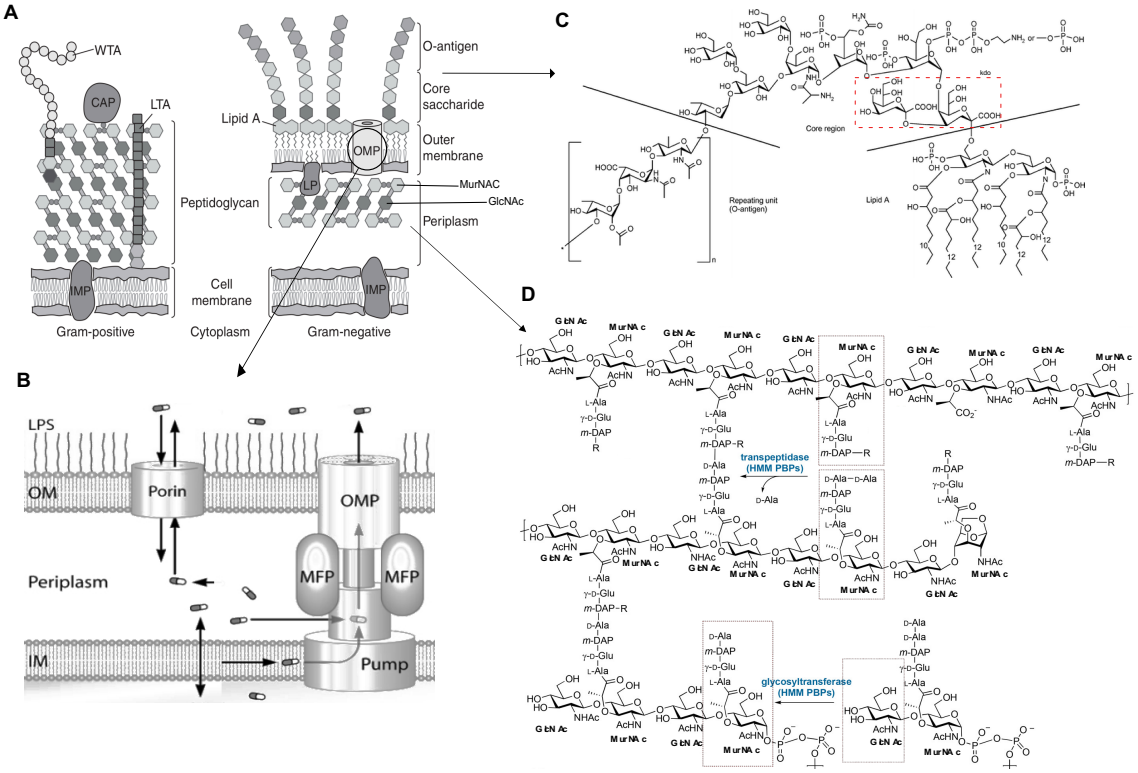


Figure 3. Depiction of cell-envelope components. A| Cell-envelope of Gram-positive and Gram-negative bacteria. Gram-positve bacterium has an exposed yet thick peptidoglycan (PG) layer. Whlist Gram-negative bacterium protects the PG layer by an outer membrane (OM). CAP; covalently attached protein; IMP, integral membrane protein; LP, lipoprotein; LTA, lipoteichoic acid; WTA, wall teichoic acid. Adapted with permission from Silhavy et al.[35] Copyright (2010) Cold Spring Harbor Laboratory Press. **B|** Porin and efflux pump are involved in antibiotic resistance by altering cellular influx and efflux, respectively. Efflux pump consists of three main components; an outer membrane protein (OMP), a membrane fusion proteins (MFP) and a pump as an inner membrane (IM) transporter. LPS; Lipopolysaccharides. Adapted with permission from Li et al.[36] Copyright (2015) American Society for Microbiology. **C|** Chemical structure of LPS components; lipid A, core oligosaccharide and O-antigen. The core oligosaccharide is located in the middle between lipid A and O-antigen. It is attached to lipid A via Kdo (3-deoxy-D-manno-oct-2-ulosonic acid) molecule (framed in red). Adapted with permission from Barkleit et al.[37] Copyright (2008) The Royal Society of Chemistry. **D|** Final steps involved in PG synthesis require two types of high-molecular-mass penicillin-binding proteins (HMM PBP). Glycosyltransferase (HMM PBP) binds to the pre-formed disaccharide units; N-acetylglucosamine (GlcNAc) and N-acetylmuramic acid (MurNAc) to form a linear glycan layer. While transpeptidase (HMM PBP) cross-links glycan linear layers by utilising acyl-D-alanine-D-alanine (D-Ala-D-Ala) in the side chain of a MurNAc unit as a substrate (Figure 5). Thus linking 2 MurNAc units to form a solid mesh-like structure. Adapted with permission from Dik et al.[38] Copyright (2018) American Chemical Society.

affect membrane permeability. Thus, genetic alterations or total loss of porins will enhance bacterial resistance to antibiotics. For example, it is well established that alteration or total loss of specific porins in *E. coli* (OmpF and OmpC) or *K. pneumoniae* (OmpK35 and OmpK36) causes resistance to a wide range of antibiotics including β-lactams[39]. Porins alteration could be achieved by gaining mutations directly into genes encoding porins or the regulatory genes such as *envZ* and *ompR* in *E. coli*[40].

Additionally, outer membrane vesicles (OMV) are produced by most Gram-negative bacteria. These vesicles are released from the cell envelope. They are simply sacs with high profile of proteins and lipids and have been linked to antimicrobial resistance due to their neutralising capacity. Studies showed mutant *E. coli* that over express OMV can resist polymyxin[39].

Besides the role of the outer membrane in antibiotic resistance, it can enhance bacterial pathogenicity. For example, OmpX in *E. coli* and OmpK17 in *K. pneumoniae* are known to help in adhesion and invasion as well as promoting bacterial resistance to serum by inactivating the complement system[41]. Also, OmpA in *K. pneumoniae* can clear infection and proposed as a potential target for vaccine development[42]. The loss of OmpA in *K. pneumoniae* has been found to reduce capsule expression thereby increase susceptibility to polymyxin[43]. In the other hand, OMPs play an indirect role in active excretion of harmful molecules such as antibiotics as being part of the efflux-pump system.

1.2.1.2 Efflux pump: dealing with gatecrashers

The efflux pump consists of three units; an inner membrane transporter (pump), a periplasmic fusion protein and an outer membrane (OM) channel protein (Figure 3B). The main function of the efflux pump is to expel toxic substances such as antibiotic from passing to bacterial cell. Also, it plays a role in virulence mainly by enhancing colonization through protecting bacteria from antimicrobial compounds present naturally in mucosal surfaces. For example, over expression of the AcrAB-TolC pump is known to cause resistance against β-lactams and polymyxins in *E. coli* and *K. pneumoniae*[40,44,45].

1.2.1.3 Lipopolysaccharides (LPS): polymyxin target

Structurally, LPS is composed of three units; the hydrophobic lipid A, two hydrophilic chains of oligosaccharides and O-antigen. The conserved lipid A anchors LPS in the outer leaflets of outer membrane enhancing its asymmetric structure compared to the symmetric structure of the inner leaflets. The oligosaccharide is located in the middle between lipid A and O-antigen and arranged into two cores; the inner being attached to lipid A and made of 3-deoxy-D-manno-oct-2-ulosonic acid (Kdo), while the outer core being attached to the O-antigen and is made up of variable hydrophilic repeated saccharide units[34,46,47](Figure 3C).

LPS enhances the anionic charge of the outer membrane due to the presence of phosphoester moieties in lipid A as well as carboxylate and phosphate groups within the core oligosaccharide and the O-antigen units[46]. Thus, LPS restricts the entry of big negatively charged molecules. Besides, LPS affects the bacterial ability to respond to the immune system and plays a role in adhesions[47].

Since LPS is an important component with multiple functions, bacteria possess many enzymes to modify its features as an adaptation process to different surrounding[34]. LPS synthesis starts with the Raetz pathway in the inner membrane facing the cytoplasm. It involves a series of reactions with highly conserved enzymes. Eventually, LPS is transported across the inner membrane to the outer membrane. However, adjustment of LPS contents continues even when it reaches its final destination. This is achieved through modification of the variable O-antigens or mainly the conservative lipid A[34,47,48].

Structurally, lipid A is an anionic hydrophobic disaccharide of glucosamine (GlcN) with four to seven acyl chains (fatty acids) and phosphate groups. It is attached to the oligosaccharide by two molecules of Kdo sugars[49] (Figure 3C). Divalent cations (magnesium and calcium) attach to phosphate groups in lipid A and bridge adjacent LPS molecules to add stability[50]. Lipid A exhibits different degrees of acetylation and phosphorylation in different bacteria. The extent of phosphorylation determines the net charge of the membrane. This is affected by different mechanisms like masking phosphate groups from lipid A with positively charged molecules such as phosphoethanolamine (pEtN by EptA) and 4-amino-4-deoxy-L-arabinose (Arn by ArnT) or adding more phosphate groups to increase negativity via LpxT[34]. The net charge is targeted as a binding site by polymyxins and antimicrobial peptides such as LL-37.

1.2.1.4 Polymyxin mechanism of action

Polymyxins are cationic, hydrophobic and lipophilic molecules. The last two properties give polymyxins amphipathic chemophysical properties which is the core of their antibacterial activity[46]. Polymyxin, as positively charged molecule binds to the negatively charged phosphate group in lipid A electrostatically. The binding will consequently, displace the divalent cations (magnesium and calcium) from phosphate groups in lipid A which will disrupt outer membrane stability as these cations bridge LPS. Additionally, polymyxin hydrophobic regions bind to LPS. The detailed mechanism of polymyxin antibacterial activity is still unknown, yet we know electrostatic and hydrophobic interactions are essential steps in polymyxins initial encounter with the bacterial cell[20,46]. As such it was hypothesized that bacteria resist polymyxin by reducing the net negative charge of lipid A[20].

One might wonder, if bacteria aim to reduce the net negative charge, the affinity of divalent cations to bind and bridge lipid A will be lost as well. To what extent these cations are important for the stability of bacterial cell requires more research. Clearly, there is still a gap in our understanding of polymyxin action and resistance mechanisms. Below the known mechanisms of resistance are described.

1.2.1.5 Polymyxin mechanism of resistance

Bacteria develop resistance against polymyxin by decreasing the net negative charge of lipid A. This is achieved by adding positively charged aminoarabinose (Arn) molecules (by ArnT) and phosphoethanolamine (pEtN) molecules (by EptA). Hence lowering the affinity of colistin to lipid A. The modification of LPS is upregulated by the two-component regulatory systems PhoPQ and PmrAB. Upregulation of these systems will increase the expression of genes encoding proteins for the biosynthesis of aminoarabinose and phosphoethanolamine. The two-component systems usually become activate in response to stimuli such as metal concentration or low acidity[34] (Figure 4).

The PmrAB two-component system consists of the *pmrABC* operon that encodes the membrane bound sensor kinase (PmrB), regulatory protein (PmrA) and transferase (PmrC also known as EptA). Low pH or high iron concentration will activate PmrB. PmrB will

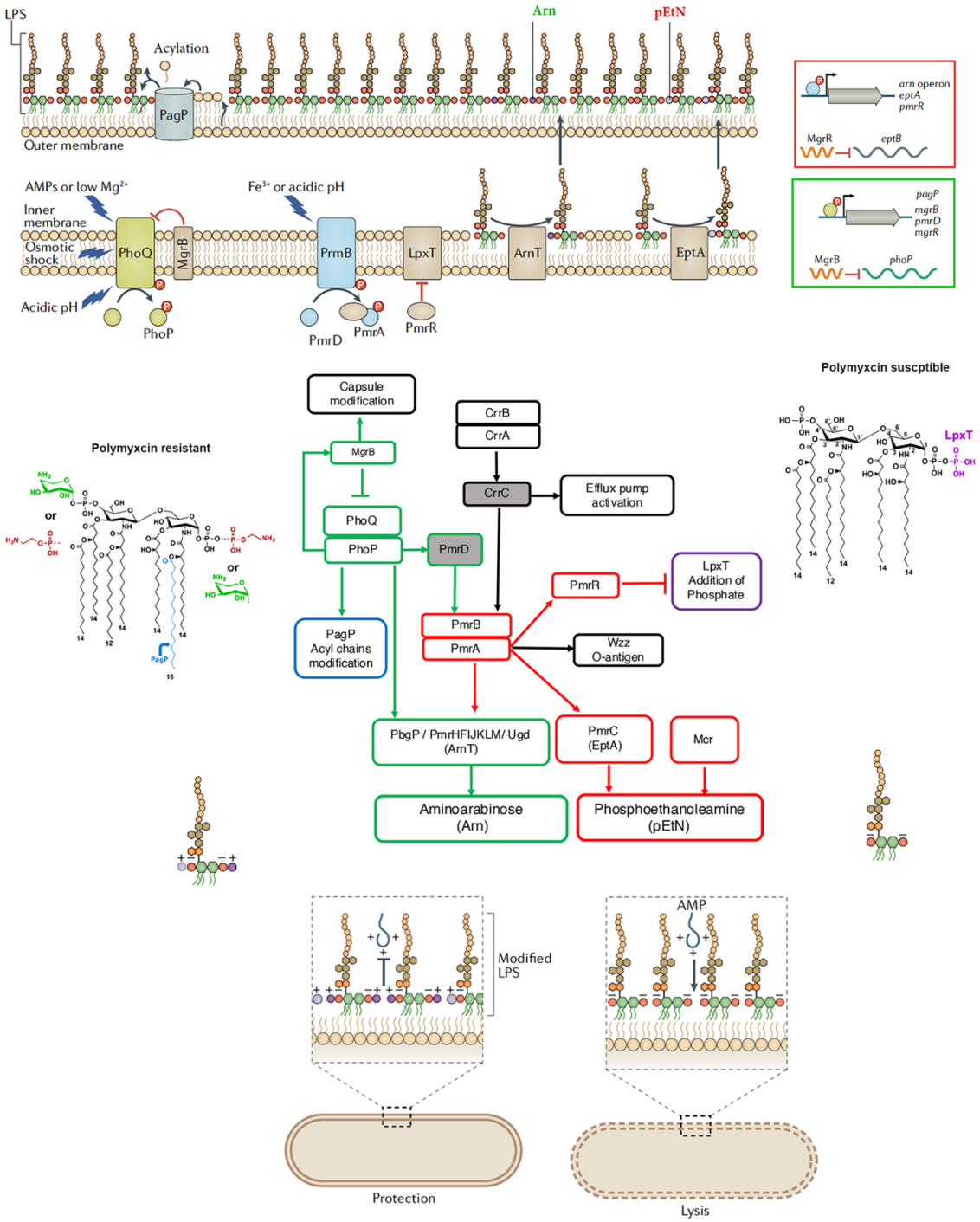


Figure 4. Modification of lipopolysaccharides (LPS). Modification of LPS involves two-component systems; PhoPQ and PmrAB. They work independently or together mediated by PmrD. The activation of PhoPQ or PmrAB eventually reduces the net negative charge in LPS through addition of aminoarabinose (Arn) by ArnT (aminoarabinose transferase) enzyme (pathway indicated by green lines) or phosphoethanolamine (pEtN) by EptA (phosphoethanolamine transferase) enzyme (pathway indicated by red lines), respectively. Also, phosphated PhoP activates PagP, MgrB, PmrD and MgrR. PagP (blue lines) modifies the acyl chain in lipid A by the addition of palmitate chain. MgrB regulates PhoQ negatively. Mutated *mgrB* gene, will allow continuous expression of PhoPQ. Also, mutated *mgrB* could modify capusle structure in *K. pneumoniae*. Whlist PmrAB two-component system activates EptA (PmrC) and PmrR. The latter inactivates LpxT (purple line) to cease the addition of phospahte group, thus lowering the net negative charge. Binding of cationic antimicrobial peptides (AMPs) such as LL-37 to bacterial cell will be reduced as a consequence of LPS modification with reduced net negative charge. In the schematic diagram, mediatores are shaded with gray boxes. The inverted and horizontal "T" shape indicates inhibition. Other mechansims apart from the addition of pEtN (red), Arn (green) or palmitate chain via Pagb (blue) are depicted in black lines. Adapted with permission from Simposon and Trent[34]. Copyright (2019) Springer Nature and Nowicki et al.[51]Copyright (2014) John Wiley and Sons.

phosphorylate and activate PmrA. Activated PmrA could upregulate PbgP (also known as PmrHFIJKLM or ArnBCADTEF) directly which will eventually add aminoarabinose by ArnT. Also, PmrA could activate PmrC (EptA) which will eventually produce phosphoethanolamine (pEtN). Mutated *pmrB* turns on these series of reactions continuously, independent of the external stimuli[40].

Likewise, the PhoPQ system is made up of a membrane bound sensor kinase (PhoQ) and a regulatory protein (PhoP). A stimulus such as low concentration of magnesium or the presence of antimicrobial peptides (AMPs) will activate PhoQ which will phosphorylate PhoP. Activated PhoP will upregulate *pbgP* operon either directly or indirectly by binding to PmrD which will eventually upregulate *pbgP* operon. In either way, activated *pbgP* will activate ArnT. Since PmrD is a mediator between the two-component systems PhoPQ and PmrAB, its activation will activate PmrAB. The later will increase the expression of *pmrC* gene which yields more pEtN. Hence, PhoPQ activation upregulates both ArnT and EptA enzymes. It is worth noting that PhoPQ is downregulated by the *mgrB* gene. Hence mutated *mgrB* will turn the PhoPQ system on and decrease the net negative charge in lipid A which is a common mechanism to resist colistin in *K. pneuomoniae*[52].

Additionally, *K. pneumoniae* has another two-component system known as CrrAB. It encodes a kinase (CrrB), a regulatory protein (CrrA) and a modulator (CrrC). It involves indirectly in modifying LPS by activating PmrAB via CrrC as a mediator between CrrAB and PmrAB. PmrA will activate PmrC and PbgP (Figure 4). Additionally, CrrC could activate a putative efflux pump that cause polymyxin resistance[53]. Still much needs to be revealed about what activates this system. Yet, we know once CrrAB is activated, the production of both aminoarbinose and phosphoethanolamine will increase[40].

Besides the activation of the two-component systems, bacteria have other ways to reduce the negative charge of lipid A. The expression of enzymes in the outer membrane is one of them. Activated PhoP regulates PagP production in *E. coli* which plays a role in modifying acyl chains by adding palmitate chain to maintain asymmetric outer membrane layer. Asymmetric layer protects bacteria from antibiotics entry yet without compensating much by lowering their nutrients uptake[34,54] (Figure 4). Also, LpxT is an inner membrane enzyme responsible for adding phosphate group into lipid A. PmrR inhibits LpxT thereby reducing the net negative charge (Figure 4). Another pathway to add phosphoethanolamine is seen in bacteria that carry a mobile colistin resistance (*mcr*) gene, with a similar effect as the chromosomal mutations mentioned above[27] (Figure 4). Also, mutations in *mgrB* gene seem to modify capsule structure in *K. pneumoniae* and increase resistance to polymyxin as well as affect the expression[55]. Additionally, alterations of O-antigen such the length of the chain can induce polymyxin resistance. In *Salmonella enterica*, PmrA activates *wzz* gene that promotes O-antigen production thereby increases resistance to polymyxin[56](Figure 4).

1.2.1.6 Polymyxin cross-resistance with LL-37

The binding mechanism of colistin through electrostatic interaction with the negatively charged LPS resembles the mechanism used by antimicrobial peptide such as LL-37. LL-37 is part of the innate immune system and has antimicrobial properties against a wide range of microbes including bacteria[57]. It resides in different cell types, most notably in neutrophils[58]. A concern was raised regarding the cross resistance between colistin and LL-37, since they have similar binding mechanism[59,60]. If a bacterial pathogen can resist colistin, it might eventually resist the killing by LL-37 which would lead them to escape host immunity (Figure 4).

1.2.2 Peptidoglycan when less is more: β-lactam target

The bacterial cell wall is made of peptidoglycan (PG) in both Gram-negative and Gram-positive bacteria. The PG layer in Gram-negative is thin (35-40 nm) and hidden beneath an outer membrane compared to the exposed thicker (40-60 nm) PG in Gram-positive bacteria (Fig 3A). Disrupting the construction of PG is the mechanism of action of β-lactam drugs. Hence, penicillin is more effective against Gram-positive bacteria[61].

The building unit of PG is a glycan strand which consists of an alternating saccharide of N-acetylglucosamine (GlcNAc also known as NAG) and N-acetylmuramic acid (MurNAc also known as NAM). The construction of PG is a complex process involving different types of penicillin-binding proteins (PBPs). Two of them are of interest in this context, glycosyltransferase high molecular mass (HMM PBPs) and transpeptidase (HMM PBPs). Glycosyltransferase high molecular mass (HMM PBP) builds linear glycan strand by covalently linking the two saccharides units (GlcNAc and MurNAc) via β-1,4-glycosidic bond. This will result in a linear glycan strand made up of disaccharides. One of them (MurNAc) has a stem made of pentapeptide with acyl-D-Ala-D-Ala end. Acyl-D-Ala-D-Ala is utilised as a substrate by transpeptidase to produce acyl enzyme with transient nature to cross-link two MurNAc units in different linear glycan strands forming eventually a stable mesh structure (Fig 3C-D). In short, glycosyltransferase creates linear glycan strand between GlcNAc and MurNAc, while transpeptidase cross-links two MurNAc units in different glycan strands to form rigid cell wall. β-lactam interferes with the cross-linking process as elaborated below.

1.2.2.1 β-lactamase: when having a ring does matter

β-lactam chemical structure mimics acyl-Ala-D-Ala, as both have a central amide bond. β-lactam will bind and lock transpeptidase (HMM PBPs) and as such interferes with the mesh formation. This will prevent the final crosslinking of the nascent peptidoglycan layer (Figure 5). Thus, structural integrity of the cell wall decreases, which results in osmotic lysis of the bacterial cell[38].

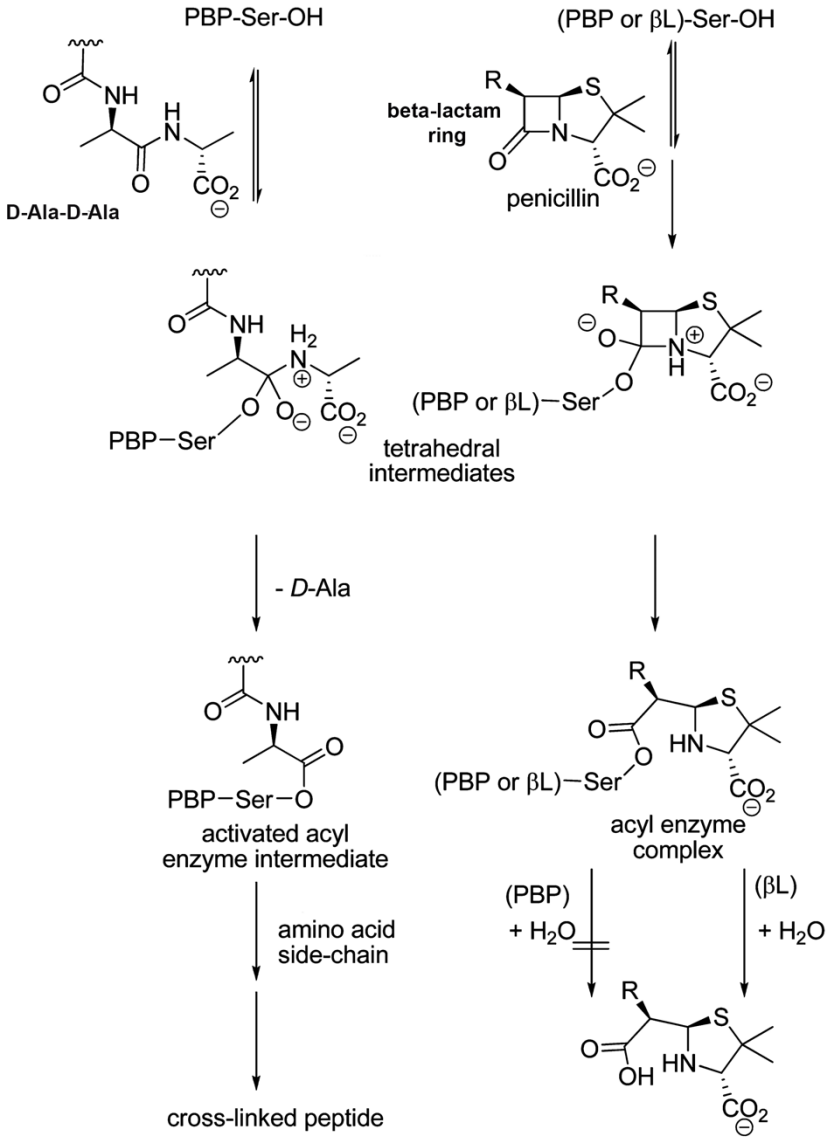


Figure 5. Mechanisms of action and resistance of β-lactam. A| Transpeptidase is a penicillin-binding protein (PBP). It utilises the native D-Ala-D-Ala terminal in MurNAc (N-acetylmuramic acid) saccharide as a substrate to produce acyl enzyme with transient nature. This eventually crosslinks with another MurNAc saccharide in a nascent units of peptidoglycan to form mesh-like structure. **B|** β-lactam, here represented by penicillin, mimics D-Ala-D-Ala of MurNAc molecule as both have amide bond, yet penicillin has the amide in β-lactam ring. PBP will bind to penicillin instead of D-Ala-D-Ala substrate, thus an irreversible complex acyl enzyme will be formed. This will cease the following step of cross-linking and eventually rupture the cell wall. However, in the presence of β-lactamase (βL), the enzyme will hydrolyse β-lactam ring rendering the drug inactive. Adapted with permission from Astrid Zervosen et al.[62]Copyright (2009) American Chemical Society.

1.3 Bacterial fitness: The cost to exist

The most prevalent strain in nature is known as wild-type strain. Such strain fits well in nature as their genetic content is stable. However, under selective pressure they lack the tools to adapt and survive. Under such unfavourable conditions and to avoid extinction, wild-type strain is forced to adapt. In the pre-antibiotic era, the main challenge faced by bacteria is to survive in a host. Thus, the wild-type strains deviated by gaining a set of tools "virulence factors" that enabled them to establish an infection as well as to escape host immunity.

Furthermore, antibiotics added a new challenge that required a new set of tools, "resistance factors". Acquiring these tools will cost wild-type a reduction in their fitness.

Resistant strains can be weakened by altering their structures in a way to bypass antibiotic activity. This can lead to reduced survival strength when competing with wild-type strains in the absence of antibiotic[33]. Therefore, resistant strains usually invade immunocompromised patients with impaired immune systems. They are widely recognized in hospitals causing healthcare-associated infections compared to virulent strains that are usually associated with community acquired infection.

A general model for antibiotic evolution illustrates that wild-type lineages will gradually evolve in three distinct stages under continuous antibiotic pressure (Figure 6A). Initially wild-type lineages will trade their fitness by developing new mutations for survival. At this initial stage, the cost of fitness has raised the hope as if the consumption of antibiotic reduced, the bacteria will revert back to the susceptible phenotype[33]. Additionally, it opens the door to fight bacteria not only by finding new antibiotics but also looking to enhance the immune system. Yet if antibiotic pressure continues, lineages will enter a second stage. There, bacteria would aim to stabilise the newly gained mutant-type by acquiring compensatory mutations to restore fitness and become as competent as the wild-type strains. Another approach to restore fitness is by discarding genes that do not serve the updated genetic structure. When such generation of resistant bacteria emerges, it is unlikely that withdrawal of antibiotic will revert to a susceptible phenotype[33]. What is more concerning is the possible marriage of both virulent and resistant strains. At this stage, bacteria could gain mutations that increase their fitness while maintaining their resistance pattern. For instance, they upgrade advantageous

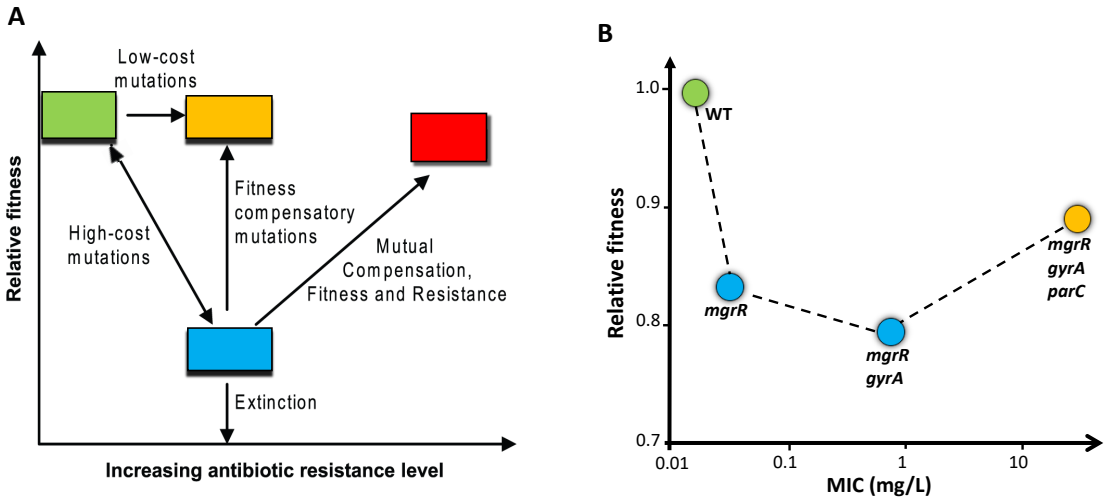


Figure 6. Bacterial fitness. A| General evolutionary model for lineages under antibiotic selection. Adapted from Marcusson et al.(2009)[63]. Copyright via CC BY licence, PloS. **B|** Resistance to fluoroquinolones in *E. coli*. High resistance is achieved when three mutations were acquired and this causes a significant increase in MIC-value from 1 mg/L in double mutations (*mgrR*, *gyrA*) to >32 mg/L in the lineage with triple mutations (*mgrR*, *gyrA, parC*). MIC; minimum inhibatory concentration. Adapted with permission from Anderson and Hughes[33]. Copyright (2010) Springer Nature.

genes to enhance invasiveness or transmission rate. In another word, this particular lineage becomes high risk clone (Hi-RC) such as ST131-H30Rx-CTX-M-15 in *E. coli*.

An example of the model above, is the accumulative mutations seen in fluoroquinolone (FQ)-resistant *E. coli* (Figure 6B). When the resistance occurred due to alteration of *marR* alone or in combination with *gyrA* genes, the cost of the process is high which is evidently seen from the margined elevation of MIC (minimum inhibitory concentration) value around 1 mg/L, which is just enough to survive. However, the addition of a third mutation in *parC* increases both relative fitness and resistance level (MIC> 32 mg/L)[33].

A better understanding of fitness and evolution in antibiotic resistance could be gained by monitoring newly introduced resistance patterns combined with our accumulative knowledge from the previous resistance episodes. For example, by following the evolution of resistance pattern against colistin which emerged recently. Studies on colistin resistant *Acinetobacter baumannii* strains due to loss of lipooligosaccaharide (LOS), showed reduced growth rate and virulence, aligned with the first stage in the model[64]. Additionally, another study showed that overall fitness in LOS-deficient *A. baumannii* could be restored by eliminating two genes only. Both genes are involved in repairing or building the lost LOS which aligns with the second compensation stage[65]. Additionally, colistin and LL-37 have similar binding mechanism so cross-resistance might occur[66]. Thus, LOS-deficient *A. baumannii* might gain the advantage of escaping the killing by immune system free of cost[67]. Combining these features would produce a superbug or Hi-RC. Worth noting, the discovery of the mobile colistin resistance (*mcr*) gene in a plasmid is a major concern but could be still at the initial or the survival stage. This is evident by the low MIC-values in strains with *mcr* compared with those due to chromosomal mutations[68]. Another striking observation about *mcr,* despite it was identified in both *E. coli* and *K. pneumoniae* at the same time, there are more reports of *mcr* in *E. coli* than in *K. pneumoniae*[27]. One might speculate if this could be because *E. coli* is more capable of adapting and lower the fitness cost.

1.4 Enterobacterales

Enterobacterales is a heterogeneous group of Gram-negative bacteria widely distributed in nature. Many of them inhabit the gut of living organisms including humans as part of their normal microbiota. They are rod-shaped, non-sporulated, facultative anaerobes, oxidase negative and catalase positive. They reduce nitrate to nitrite and ferment glucose. Moreover, they can be motile via flagella or non-motile[69]. Within the context of carbapenem-resistant *Enterobacterales* (CRE), two species are particularly clinically important; *E. coli* and *K. pneumoniae*. Infections caused by *E. coli* are mainly linked to community while those caused by *K. pneumoniae* are nosocomial infections. Generally, pathogenic *E. coli* follows the "offense model" by attacking to invade making the most out of its virulence factors from the beginning, whereas *K. pneumoniae* follows a "defense model" by colonizing and only invading at a calculated time[70].

1.4.1 *E. coli* : An old yet turbulent friend

E. coli is one of the most studied organisms worldwide. It inhabits the intestine of healthy human as a commensal strain. However, when *E. coli* affects extra intestinal organs such as the urinary tract or blood it can cause serious health issues, particularly when accompanied by virulence and resistance attributes. There are multiple typing approaches for *E. coli*. The one based on phylogeny divides them into A-F groups, while clinically they could be divided into three subsets: commensal, diarrhoeagenic known as adherent and invasive *E. coli* (AIEC) as well as extraintestinal pathogenic *E. coli* (ExPEC). ExPEC strains belong mainly to B2 and D phylogroups. While commensal strains commonly belong to A, B1, C strains[71]. Treatment of ExPEC and AIEC is increasingly challenging due to the presence of resistance genes against third-generation cephalosporin and carbapenem in strains described as high-risk clones (HiRCs) such as ST131.

1.4.2 *K. pneumoniae:* An opportunist new foe

K. pneumoniae (KP) resides in the environment such as soil and surface water as well as on medical devices. In human, KP colonizes mucosal surfaces such as the intestinal and respiratory tracts where the effects of its colonization are mild yet known to cause opportunistic infections in immunocompromised individuals. Recently, KP gained an advantage by combining virulent and resistant attributes in the absence of treatment options to turn into a resilient pathogen. Thus, KP infections were seen in individuals with competent immune system due to resistance attributes. Also, reports of hypervirulent (HV) strains are increasing and such strains feature virulence factors such as siderophores[72]. Particular clones are capable to spread globally such as ST258 which is linked almost to a specific type of carbapenemases known as KPC.

1.4.3 Carbapenem resistant *Enterobacterales*

Carbapenems are member of the β-lactam antibiotics. They are used to treat infections caused by bacteria resistance to other members of β-lactams including third-generation cephalosporins and monobactams. This is due to their broad spectrum of activity and stability against hydrolysis by AmpC β-lactamases and ESBLs[73]. The high demand of carbapenems in clinical settings, raises carbapenem resistance in Gram-negative bacteria including *Enterobacterales*. Carbapenem-resistant *Enterobacterales* (CRE) arise by two mechanisms; the main one is the production of carbapenemases (CP-CRE), while the second one is the reduction of carbapenem uptake by porin deficiency (non-CP-CPR) particularly when accompanied with another β-lactamases such as ESBLs or AmpCs[74]. Hence the terminology CR and CP is not interchangeable. CRE is a more general term commonly used to describe phenotype when the molecular mechanism is not yet known. Carbapenemases belong to three molecular classes of β-lactamases: Ambler class A, class B and class D (Figure 7).

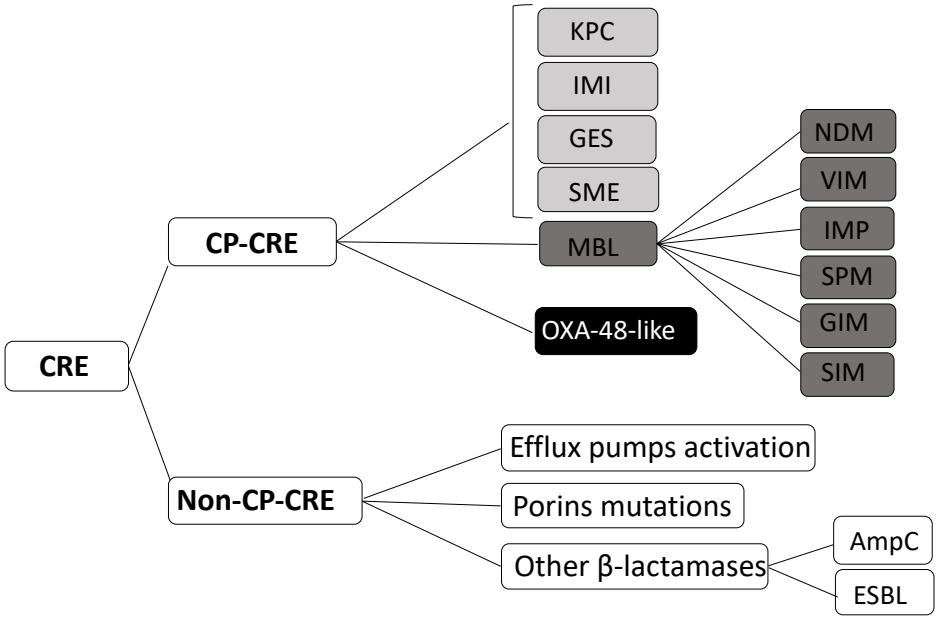


Figure 7. Different mechanisms of carbapenem resistance in *Enterobacterales*. Mechanisms of resistance in carbapenem resistant Enterobacteriaceae (CRE) could be divided into two categories. Those producing carbapenemase enzymes (CP-CRE) and those do not (Non-CP-CRE). CP-CRE could be further divided into 3 classes based on Ambler classification. Ambler class A (Light grey) such as *Klebsiella pneumoniae* carbapenemase (KPC), Imipenemase/non-metallo-β-carbapenemase (IMI), Guiana extended-spectrum β-lactamase (GES) and *Serratia marcescens* enzyme (SME). The second class is Ambler class B (dark grey), also known as metallo-β-lactamase (MBL). Examples of this class are New-Delhi metallo-β-lactamase (NDM), Verona integron-borne metallo-β-lactamase (VIM), Imipenemase/metallo-β-carbapenemase (IMP), Sao Paulo metallo-β-lactamase (SMP), German imipenemase (GIM) and Seoul imipenemase (SIM). The third class is Ambler class D (black), collectively grouped under OXA-48-like family with different variants of oxacillinase (OXA). Despite the second category (Non-CP-CRE) do not produce carbapenemases, they produce other type of β-lactamases such as Type C ampicillinase (AmpC) or Extended-spectrum β-lactamases (ESBLs) which are accompanied by alteration in porins or efflux pumps. Adapted from Beatriz and Perez-Gracia[74]. Copyright (2019) via CC BY license, MDPI.

Class A carbapenemases are capable to hydrolyse a broad variety of β-lactams including monobactams, whereas class B carbapenemases lack the ability to hydrolyse monobactams. Historically class D carbapenemases have been mostly found in *Acinetobacter spp*. However, the OXA-48 group is mainly found in *Enterobacterales*[75]. In the following text, one member from each class will be discussed.

1.4.3.1 *K. pneumoniae* carbapenemases (KPC)

K. pneumoniae carbapenemases (KPC) belongs to class A. It was originally isolated from *K. pneumoniae* but later was found in other *Enterobacterales* including *E. coli*. Still KPCs are commonly isolated from nosocomial infections caused by *K. pneumoniae* compared to other *Enterobacterales*. Interestingly, KPCs are closely associated with specific clone in *K. pneumoniae* known as ST258[76]. Consequently, outbreaks of *K. pneumoniae* with KPCs are commonly found in sites where ST258 predominates such as USA and Israel but not the

Arabian Peninsula or Indian subcontinent for example. Despite the sporadic reported cases from these regions, no outbreaks have been yet reported[77-79].

1.4.3.2 New-Delhi metallo-β-lactamases (NDM)

Class B is unique from other classes by having a metal ion rather than a serine in the active site of the enzyme. The most clinically important member in this class is New-Delhi metallo-β-lactamases (NDM). It is strongly linked to India and the surrounding south Asian countries (Pakistan, Bangladesh). Yet, several NDM-1 producers have been isolated from patients with connection to the Balkan states or the Middle East, suggesting that those areas might be a secondary reservoir of NDM[79,80]. The spread of NDM-1 to the Middle East may be explained by the close social relation and population interaction with the Indian subcontinent for example trade and medical tourism[75].

Unlike KPC, NDM is not linked to certain clones or plasmids. This might explain the global wide spread of this enzyme. Additionally, the wide diversity in plasmids carrying *bla*$_{NDM}$ enables bacteria to carry a high number of resistance genes associated with other carbapenemase genes (*bla*$_{OXA,}$ *bla*$_{VIM}$), *ampC*, aminoglycoside resistance genes (16 rRNA methylases), macrolide resistance genes (esterase), rifampicin modifying enzymes, and sulphamethoxazole resistance genes. Therefore, many NDM producers remain susceptible only to limited treatment options such as tigecycline and colistin[81]. In addition, genetic analysis of the surroundings of the *bla*$_{NDM}$ gene showed an association with *ble*$_{MBL,}$ that encodes bleomycin resistance protein. This coexistence has been suggested to stabilise the *bla*$_{NDM}$ gene[75,82].

1.4.3.3 OXA-48-like carbapenemase (OXA-48-like)

OXA-48-like carbapenemases belong to class D. They are mainly found in north African countries, the Middle East, Turkey and India which are considered as reservoirs for OXA-48 producers[82]. There are many variants of OXA-48-like carbapenemases including OXA-48, OXA-181, OXA-232, OXA-244 and OXA-163 of which OXA-48 is more common[83]. The variants differ by a few amino acid substitutions or deletions. For example, OXA-181 differs from OXA-48 by four amino acids.

Generally, most OXA-48-like carbapenemases hydrolyse penicillin at a high level and carbapenems at a low level, leaving extended spectrum cephalosporin unaffected. Moreover, these enzymes are not inhibited by β-lactamase inhibitors[84]. It is worth noting that, OXA-163 is distinct from other variants by hydrolysing extended-spectrum cephalosporins at a high level and being susceptible to β-lactamase inhibitors, while it very weakly hydrolyses carbapenems[75]. In this aspect, OXA-163 behaves like an ESBL-producer rather than OXA-48-like enzyme, despite it differs from OXA-48 only by a single amino acid variant and a 4-amino-acid deletion[85].

Because of their low level of hydrolysis, OXA-48-like enzymes are the most difficult carbapenemases to identify[84]. So, their true prevalence is most likely underestimated and the attributed mortality rate from infections with OXA-48 producers is unknown[82]. Importantly, OXA-48 producers usually show higher level of resistance to carbapenems when associated with permeability defects and ESBLs[75].

In contrast to the widespread presence of bla_{OXA} gene in *Acinetobacter spp*. on many different plasmids, the bla_{OXA-48} gene has only been carried in a single plasmid type with the IncL/M replicon, which is called pOXA-48. It has broad host range among *Enterobacterales* and between species[83]. This leads to a remarkable note as the current spread of OXA-48-like is attributed to the spread of only a single plasmid[84].

1.4.4 Polymyxin Resistant *Enterobacterales*

Polymyxins consist of different compounds classified from A to E. Polymyxin B and Polymyxins E (colistin) have gained attention recently as a treatment choice for infections caused by CRE. Despite the fact that they differ by a single amino acid in their structures, they have different pharmacological properties[86]. In Europe and Australia, colistin is used rather than polymyxin B, while in the US both are available. Also, they have a different form of activity[86]. While Polymyxin B is administered directly as an active antibiotic, colistin is administered as an inactive prodrug known as colistin methanesulfonate or colistinmethate sodium (CMS). CMS is an inactive drug and less toxic than colistin sulfate. After administration, it is converted to the active form[86]. Hence, CMS should not be used for diagnostic purposes in lab as an *in vivo* activation is unachievable in such conditions. Despite the differences between polymyxin B and E *in vivo*, both show similar activity *in vitro* as well as similar mechanisms of action and resistance.

Resistance to polymyxin has evolved by genetic alteration for the targeted molecule which is lipid A. Lowering the net negative charge reduces polymyxin affinity. This is achieved with genetic alteration that involves two-component systems PhoPQ and PmrAB and their accompanied regulators. Polymyxins are used at varying extents in clinical settings, but commonly used in farming in some countries which might be responsible for the current increase in resistance. It is worth noting that resistance is more common in *E. coli* than in *K. pneumoniae*. This could be because *E. coli* is more associated with zoonotic spread or could be due to other not yet known factors such as cost of fitness. Also, the transfer of resistance genes is mainly vertical, except for *mcr*. As such we would expect a slow spreading rate. However, the resistance mechanism does not seem to be unique to colistin as the two-component systems are actively used by bacteria in respond to variable external stimuli such as osmotic shock and low acidity. This implies bacteria are familiar to such stimuli and might already be adapted to compensate the cost of fitness.

1.5 Epidemiology of antimicrobial resistance

The current unusual situation we are facing with COVID-19, draws the attention to the importance of epidemiological studies as a tool to estimate the burden of diseases and update the taken measures to limit the spread accordingly. The following discussion will be about the epidemiology of resistance against carbapenem and colistin in *K. pneumoniae* and *E. coli.*

1.5.1 Carbapenem and colistin resistance globally

While Europe and the US have established monitoring programs for surveillance of antibiotic resistance, the rest of the world is still behind. The Global Antimicrobial Resistance Surveillance System (GLASS) was launched in 2015 by the WHO to fill this gap and bridge available data. More countries enrolled in the program since the initial kick-off. Yet, we still lack data from countries that are not enrolled and rely on published reports to map CRE globally.

The general view of carbapenemases from published papers show that KPCs are mainly reported from China, the United States, Italy and South America whereas NDMs are mainly reported from Pakistan, India, Bangladesh, the Middle East and China. IMPs are mostly prevalent in Japan, Taiwan and China, while VIMs are most commonly encountered in Greece. OXA-48 is prevalent in Turkey and Morocco[76].

On the other side, European countries reported various carbapenemase outbreaks which could be tracked with high resolution due to having active infection control programs. According to the European Surveillance Atlas of Infectious Diseases, the national trends for carbapenem resistance in KP increased significantly between 2015-2019 in Slovakia (from 0.9% to 4.6%), Croatia (2.4% to 12%), Bulgaria (3.2% to 27%), Malta (4.5% to 7.8%), Romania (24.7% to 32.3%) Poland (0.5% to 7.7%), Spain (2.2% to 4.4%) and Portugal (3.4 % to 10.9%) whereas a slight decreasing trend was seen in Italy (33.5% to 28.5%) and Greece (61.9% to 58.3%). As expected, resistance to carbapenems was much less common in *E. coli* compared to KP from the same period (2015-2019). The prevalence remained in most countries < 1%. Despite the slight reduction seen in Romania (1.9% to 0.6%) and Greece (1.2% to 1%), which is unlikely to be significant. Likewise, the slight increase in Italy (0.2% to 0.4%) and Spain (0.0% to 1.6%) is most likely insignificant[87]. There were no data regarding colistin resistance in Surveillance Atlas of Infectious Diseases. However, data about antibiotic consumption showed that Greece followed by Italy had the highest consumption of polymyxins between 2005 and 2019. In 2017, the consumption was reduced in Italy, but continued to rise sharply in Greece[88]. Also, published data showed that 43% of *K. pneumoniae* producing carbapenemases (KP-CP) isolates from Italy were resistant to colistin between 2013–2014[89]. In Greece, colistin resistance in KP-CP increased from 0% in 2002 to 26.9% in 2016[90].

The European Centre for Disease Prevention and Control reported three outbreaks of carbapenem resistance in 2019[91]. One of them occurred in north-east Germany and involved

17 patients from different hospitals. It was caused by KP-ST307 producing both NDM and OXA-48 enzymes. The clone was phenotypically resistant to colistin, but no *mcr* gene was detected, suggesting a chromosomal mechanism of resistance[92]. The second outbreak involved 223 isolates in Lithuania, of which 89% were KP and 9% were *E. coli*. Within the KP isolates there was a clonal expansion of KP-ST392 that carried *bla*$_{KPC-2}$ gene in plasmid. The same plasmid was seen in the rest of the KP strains as well as in *E. coli* (n=21). This probably suggests plasmid-mediated spread of CRE in addition to clonal expansion of one single clone (KP-ST392)[93]. The third outbreak was caused by *E. coli*-ST38 producing OXA-244 in different European countries including the UK. The strain has been circulating since 2013 with 116 cases in 2019[93].

Data from the U.S. CDC's 2019 Antibiotic Resistance Threats Report showed that estimated cases of CRE did not increase between 2016 and 2017 as 13,100 cases within hospitalised patients in both years. This is quite good news that reflects the collective efforts to reach this goal[21]. No data regarding specific species were available.

Data from the Arab League showed a predominance of OXA-48 (32.5%) and NDM-1 (46.5%) enzymes in most countries of the region. The prevalence of CRE was ≤ 5% in Saudi Arabia, Lebanon, Morocco, Libya, Tunisia and Mauritania whereas the highest prevalence was seen in Egypt with 40% for CRKP. In the Eastern Mediterranean region, the most commonly produced carbapenemases were NDM-1, OXA-48-like (32.5%) or both (8.9%). KPC enzyme was not notable except in Egypt with only a single report from Jordan and Saudi Arabia. Regarding mobile colistin resistance gene, *mcr-1* was detected in four *E. coli* strains from Saudi Arabia (n=1), Bahrain (n=2), UAE (n=1)[77,78].

1.5.2 Carbapenem and colistin resistance in Oman

Over the past decade, carbapenem resistance in Oman echoes the global situation. It was not common before 2005. All ESBL-producers isolated between 2004 and 2005 from an educational hospital in Muscat (the capital of Oman) were found to be susceptible to carbapenems[94]. When blood culture isolates from a regional hospital between 2004 and 2007 were tested using disk diffusion method, it was found that *K. pneumoniae* (n=4/28), *Enterobacter* spp. (n=2/7) and *E. coli* (n=6/35) isolates were resistant to imipenem[95]. However, no molecular characterization was performed then.

The NDM-1 gene was first detected in two clinical isolates of *K. pneumoniae* recovered from two patients admitted at a hospital in Muscat in 2010. The first patient was Omani yet had received medical treatment for pneumonia in India in 2009, while the second patient had no travel history, this patient was admitted in the same ward after the discharge of the first patient[95]. This was the first description of NDM-1 producers in the Arabian Peninsula and in the Middle East. During the period between 2010 and 2011, carbapenem-resistant *K. pneumoniae*, *E. coli* and *E. cloacae* were identified from three different hospitals. The carbapenemases in these isolates were identified as *bla*$_{NDM-1}$ (n=11/22), *bla*$_{OXA-48}$ (n=5/22) and one isolate had both *bla*$_{OXA-181}$ and *bla*$_{NDM-1}$[96]. Additionally, a study aimed to identify

carbapenemase-producers among clinical isolates at educational hospital in Oman (SQUH) during the period between January 2010 and April 2011 found most of the isolates to be non-MBL producers, though three strains produced NDM-1[97]. Regarding colistin resistance, Sonnevend et al. reported four *E. coli* strains harboring *mcr-1* gene from three neighbouring countries to Oman; the United Arab Emirates (UAE), Saudi Arabia and Bahrain in 2016[98]. In 2017, Mohsin et al. reported the first *mcr-1* from *E. coli* isolates in Oman[99]. In summary, carbapenemases were not common in Oman before 2013, though sporadic cases were reported, no national surveillance with large study samples from different hospitals took place.

1.6 Typing bacterial strains

Tracing back the source of resistant strains helps in controlling the spread and minimising the impact of the problem. Also, it is a tool to evaluate the epidemiological impact of successful clones that require more attention to control. As such the emergence of new high-risk clones will be followed and the evolutionary process will reveal much more about the dynamics and spread of such clones. This will eventually help to narrow down the knowledge gap remains in the field.

There are many approaches for typing bacteria, which depends mainly on bacterial genetic characteristics. For example, capsule typing is more common in KP than in *E. coli* compared to O-serotyping since the variation of capsules is more profound in KP. With current advancement in sequencing approaches, there is a move towards whole genome sequencing (WGS) which opens the door for a wide range of ways to performing *in silico* typing including CRISPR and phages. Here, I discuss CRISPR system as a typing tool in *E. coli.*

1.6.1 CRISPR-Cas system

In 1987, Ishino et al. detected a repetitive stretch of DNA in the chromosome of *E. coli* K-12, downstream of the alkaline phosphatase isozyme *iap* gene[100]. Similar findings were reported from other bacterial species. A distinct feature of these elements was that the repeats were interspaced by noncoding, non-repetitive sequences of similar length now known as spacers. Mojica et al. defined these repetitive elements as short regulatory spaced repeats (SRSR) in 2000[101]. In 2002 Jansen et al. proposed the name cluster regularly interspaced short palindromic repeats (CRISPR)[102]. Additionally, they observed that these repetitive loci were always accompanied by conserved sets of genes encoding nucleic acid processing enzymes named CRISPR-associated genes (Cas). In 2005 three groups independently observed that some of the interspaced sequences were 100% identical to DNA sequences from viruses and plasmids which was a major breakthrough[103-105]. Eventually CRISPR-Cas was considered as an adaptive immune system that protects bacteria from getting invaded by bacteriophages or plasmids carrying similar sequences.

1.6.1.1 Components of CRISPR-Cas system

Generally, the CRISPR-Cas system consists of CRISPR loci and *cas* genes. Each CRISPR locus, also known as CRISPR arrays, motifs or cassettes, have three components; spacers, direct repeats (DR) and a leader (L) (Figure 8). The spacers are greatly variable in their sequences that match part of the DNA sequences in plasmids and phages. The matching sequences in phages or plasmids are called protospacers and assumed to be the origin of the spacers[104].

On the contrary, DR sequences are greatly conserved with the possibility of few modified nucleotides sequences[101]. Kunin et al. classified DR in CRISPR systems for multiple organisms into 12 clusters of which two of them (DR-2 and DR-4) were recognised in *E. coli*[106]. Additionally, each CRISPR locus has one leader, where a leader is an AT-rich sequence with promoters that direct transcription of the adjoining locus.

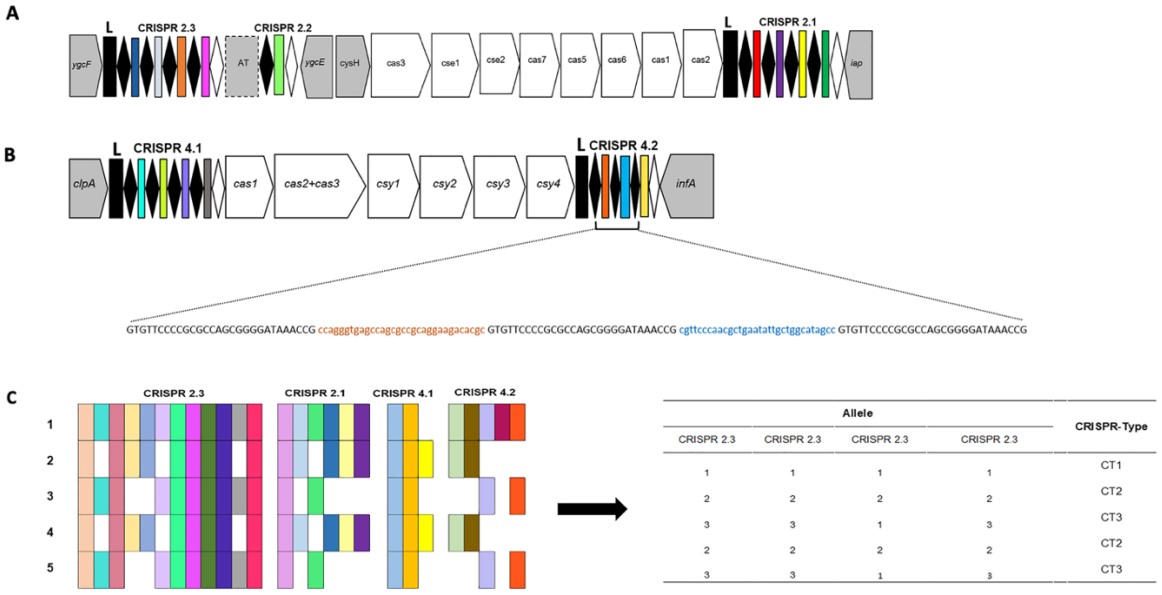


Figure 8. Structure and function of CRISPR-Cas system in *E. coli* as a typing tool. Each CRISPR locus consists of constant repeated sequences called direct repeats (DR; black diamond) and unique sequences embedded between them called spacers (coloured rectangular). In *E. coli*, there are two subtypes of CRISPR-Cas system; I-E and I-F1. **A|** I-E subtype has 2 main CRISPR loci (CRISPR 2.3 and CRISPR 2.1) and one extra locus occurs occasionally (CRISPR 2.2) with leaders (L) at the start of the two main loci. I-E has eight *cas* genes (white boxes). **B|** I-F1 subtype has two CRIPPR loci (CRISPR 4.1 and CRISPR 4.2) with a leader (L) in each locus and six *cas* genes (white boxes). **C|** CRISPR typing is based on extracting spacer sequence information. Unique spacers are represeted with different colored rectangles. An allele is define as a unique combination of spacers in the four CRISPR loci as shown in the table. Adapted with permission from Sharat and Dudley[107]. Copyright (2014) American Societry for Microbiology.

Regarding *cas* genes, there are 8 in I-E subtype(*cas3, cas8e/cse1, cas11/cse2, cas7/cse4, cas5, cas6/cse3, cas1, cas2)* and six in I-F1 subtype (*cas1, cas2-cas3, cas8f1/csy1*, *cas5f1/csy2, cas7f1/csy3, cas6f/csy4*) as per the updated classification done in 2020[108]. Of notice, cas1 and cas2 proteins are present in all CRISPR-Cas systems that are predicted to be active[109].

1.6.1.2 Classification

The CRISPR-Cas system is extremely diverse. The current classification consists of 2 classes, 6 types and 33 subtypes[108]. Each system consists of a set of *cas* genes along with CRISPR arrays. CRISPR-Cas system in *E. coli* belongs to class 1, type I with two subtypes, I-E subtype also known as Ecoli and I-F subtype also known as Ypest. The later was reclassified into I-F1 recently as a defective CRISPR-Cas variant since it lacks *cas3* gene which is required for interference process. Since interference involves recognition and breakdown of the invaded DNA or RNA, this suggests a distinct function other than the adaptive immunity that is already recognised for CRISPR-Cas system[108].

I-E subtype consists of three CRISPR loci; one downstream *iap* gene and two between *ygcE* and *ygcF* genes (Figure 8). The two loci between *ygcE-ygcF* genes were separated by 0.5 kb highly conserved AT rich sequence. Usually only one CRISPR locus is observed between these genes. However, the extra locus following the AT rich sequence might exist occasionally. The less common I-F1 subtype consists of two loci between *clpA* and *infA* genes. *E. coli* CRISPR-Cas system did not gain much attention since its recognition as being static. Thus, not much time was invested on its nomenclature either. While PCR reactions can only amplify DNA sequenced tailored to the designated primers, WGS creates confusion regarding the classification due to the lack of a database that could specifically name the identified CRISPR loci. Previous studies describing *E. coli* CRISPR-Cas system, used three different nomenclatures; Touchon et al., Villasenor et al. and Yin et al.[110-112](Table1). Since Diez-Villasenor et al. classification integrated the type of direct repeat in their nomenclature, here we use their scheme with minor modifications. To keep it simple, we only state the name of the observed arrays and ignore the label indicating the absence of arrays e.g. CRISPR 2.2-3 and CRISPR 4.1-2 (Table 1).

Table 1. Nomenclature of the CRISPR-cas system in *E. coli*[110-112]

	Classification	Touchon et al.	Diez-Villasenor et al.	Yin et al.
I-E	*cysH*-(*cas* genes)-**CRISPR**-*iap*	CRISPR **1**	CRISPR **2.1**	CRISPR **1**
	ygcF-**CRISPR**-(0.5 kb AT rich)-**CRISPR**-*ygcE*	CRISPR **2**-**ND***	CRISPR **2.3**-CRISPR **2.2**	CRISPR-**2a**-CRISPR **2b**
	ygcF-**CRISPR**-(0.5 kb AT rich)-*ygcE*	CRISPR **2**	CRISPR **2.3-2**	CRISPR **2a**
I-F1	*clpA*-**CRISPR**-(*cas* genes)-**CRISPR**-*infA*	CRISPR **3**-CRISPR **4**	CRISPR **4.1**-CRISPR **4.2**	CRISPR **3**-CRISPR **4**
	clpA-**CRISPR**-(*cas* genes)-*infA*	CRISPR **3**	CRISPR **4.1-2**	CRISPR **3**

*ND: Not Determined

1.6.1.3 Function

It is widely accepted that the CRISPR-Cas system plays a role as an active adaptive immune system for bacteria, where a piece of DNA (protospacers) from previous invaders either bacteriophage or plasmid are incorporated within the CRISPR-Cas system in the bacterial genome as spacers. If a bacterium is attacked with a similar bacteriophage or plasmid again, the presence of an active CRISPR system will cleave the foreign DNA. This mechanism consists of three stages, adaptation, expression and interference[109]. However, it is beyond the scope of this thesis as we are more interested in CRISPR as a typing tool and looking for association between CRISPR and bacterial contents of phages and plasmids.

The CRISPR-Cas system was found to be a useful tool for typing bacterial diversity. The resolution of the subtyping depends largely on the spacer's content yielding a higher resolution in strains with diverse spacers. Thus, active CRISPR loci could be a useful tool to trace outbreaks. Such typing system has been used in spoligotyping *M. tuberculosis* and *C. diphtheriae*[113]. In *E. coli*, polymorphisms in CRISPRs of enterohemorrhagic *E. coli* (EHEC) were found to provide a higher resolution typing profile than the established techniques based on *stx* and *eae* polymorphism alone or in combination with O:H serotypes[114]. Likely, Jiang et al. studied shiga toxin-producing *E. coli* (STEC) from various sources. They found a similarity in spacers content and order in strains with identical serogroups[115]. Additionally, Yin et al. found that STEC isolates with same H type would carry same or similar spacers[112]. Also, the virulent strain of B2-phylogroup is known to lack CRISPR-Cas system[110]. Regarding commensal strains, it is questionable if CRISPR is a useful tool for typing or if it plays a role as an active immune system since it was considered static[116]. Also, Mojica et al. suggested that the pathogenicity of prokaryotic population depends largely on bacteriophages and plasmids contents[117]. Thus, targeting these mobile elements by CRISPR might affect bacterial pathogenicity and open the door for a new treatment approach.

On the other side, the correlation between antibiotic resistance and CRISPR-Cas system is not yet conclusive. Studies published on *Enterococcus faecium* published studies showed that absence of CRISPR-Cas system could distinguish antibiotic resistant species with a wide variety of plasmids harbouring resistance genes from susceptible strains. For example, Palmer and Gilmore reported an inverse correlation between CRISPR-Cas and antibiotic resistance[118]. A similar finding was reported by Tremblay et al. as they observed a total absence of CRISPR in seven ampicillin-resistant *E. faecium* isolates[119]. Likewise, Van Schaik et al. studied 10 *E. faecium* isolates from different sources (canine and human) and concluded there was an association between increased antibiotic resistance and the absence of CRISPR-Cas system[120]. However, no such correlation was seen in *E. coli*. A study conducted on *E. coli* strains (n=263) obtained from different sources (humans and animals) with various level of plasmid-encoding antibiotic resistance genes reported no association between the acquisition of plasmids with the presence or activity of CRISPR system[121]. Likewise, Dang et al. compared CRISPR-Cas system in a collection of fecal commensal and uropathogenic *E. coli* (n=162). They did not observe consistent correlation between

CRISPRs and resistance genes yet they observed a correlation between CRISPR activity and pathogenicity between both groups[122].

All in all, active CRISPR-Cas system in *E. coli* is lost to gain virulence factors usually through gaining prophages (transduction). As such CRISPR analysis might help to distinguish pathogenic from commensal *E. coli* strains. However, the gain of resistance genes in *E. coli* which usually transmitted by plasmids (conjugation) does not correlate with CRISPR activity. Such findings suggest CRISPR-Cas in *E. coli* could be particularly active against phages but not plasmids or perhaps plasmids more frequently utilise anti-CRISPR to escape being recognised by CRISPR-Cas system[123]. However, such selective activity of CRISPR or anti-CRISPR between plasmids and bacteriophages is lost not clear[124]. In short, *E. coli* has a distinct CRISPR-Cas system from other microbes where its evolution appears more complex than simple spacer acquisition and its function might be other than immunity system. Many gaps yet remain to be filled in this respect.

1.7 The light at the end of the tunnel

Despite the challenges we face due to antibiotic resistance, we as humans did impressive and work in our collective efforts to make the best out of the current situation and move forwards. We looked outside the antibiotic box for innovative approaches to clear infections. For example, studying ways to optimise or enhance the activity of our own immune system via the intake of vitamin D supplement[125]. Also, developing therapies derived from antimicrobial peptides to treat infections is another approach[126-130].Replacing the notorious drug resistant strains colonising the gut with healthy microbial community by faecal transplant or the intake of probiotics is intensively investigated. Bacteriophages being the natural bacterial enemy, could be utilised to cure infections by making use of CRISPR as a genome editing tool. Most of these approaches are exciting but still under development. Therefore, improving currently available drugs within the antibiotic box seems more practical.

Studies regarding combinational therapy with different classes of antibiotics are actively reported[131,132]. Additionally, a new generation of β-lactamase inhibitors has been introduced to the market recently including imipenem-relebactam (Recarbrio) in 2019[133]. In 2020 cefiderocol was approved for treatment of infections caused by Gram-negative bacteria. It belongs to the cephalosporin family and has a unique entry into the bacterial cell via the iron-transport system. It shows a high spectrum of activity and stability against all β-lactamases including carbapenemases which makes it very interesting as a treatment option when the mechanism of resistance is not yet known[133,134].

However, recycling the available drugs by chemical modifications to manoeuvre around resistance mechanisms is not as efficient as introducing new classes of drugs. Historically, resistance to such modifications has developed rapidly. Therefore, development of completely novel antibiotics is a desirable goal. Malacidin and teixobactin are examples of such novel drugs. although they only have activity against Gram-positive bacteria[135,136]. What is quite interesting about these drugs is the claim that bacteria cannot resist them. The

discovery of drugs with high potential of no resistance would be a game changer that raise our hope for a better future.

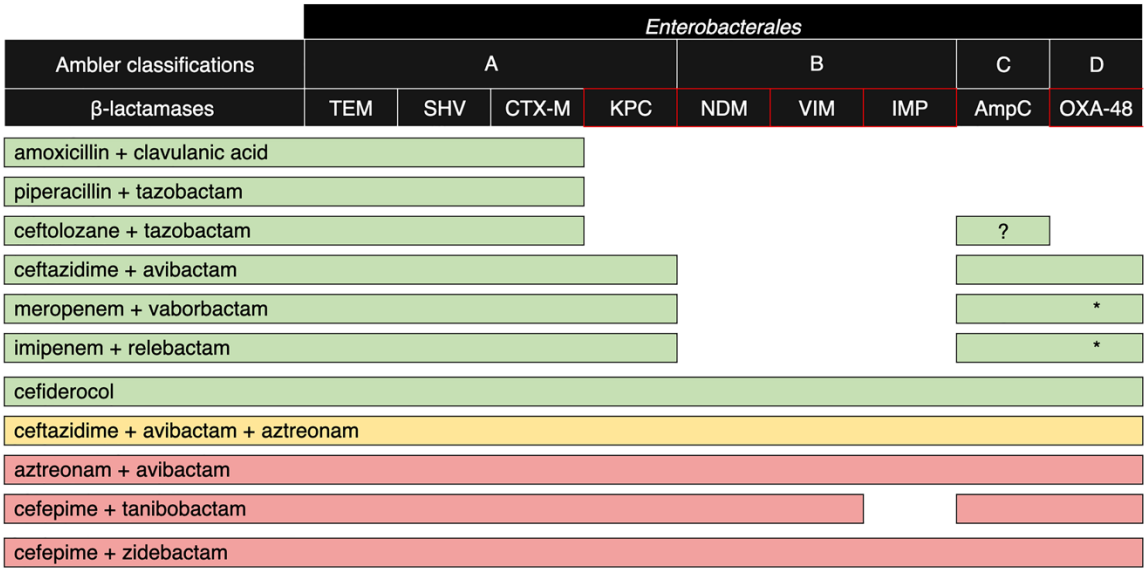


Figure 9: Chemical modification of available β-lactam drugs as a treatment option. The chart represents (green) some of the remarkable recycled β-lactam drugs which were approved by FDA (food and drug adminstration). One combination therapy was used clinically as no other option to treat the patient was available (yellow). Three drugs are under clinical trials with promising high spectrum of activities (pink). ? ; indicates the drug shows variable effect on AmpC. *; varborbactam and relebactam do not inhibit OXA-48-like enzymes. When they are accompnied with meropenem and imipenem, the comination drugs are ferquently active against OXA-48-like family because of the carbapenem component rather than the inhibitory effect. Adapted with permission from half-time examination slides, Copyright (2020), designed by Anna Olsson.

AIMS OF THE THESIS

The thesis is based on three hypotheses (Figure 10) to investigate the following three main aims

I. To investigate the magnitude of the antibiotic resistance in clinical *Enterobacterales* isolates from Oman. Particularly carbapenem-non-susceptible *E. coli* (Paper I) and *K. pneumoniae* (Paper II) as well as colistin resistance (Paper III). This aim is achieved by conducting surveillance studies. Additionally, we aim to uncover the population structure of resistant strains and investigate if they are correlated to the already known international resistant clones.

II. To study the ability of multidrug resistant strains particularly colistin resistant isolates to esacpe innate immune effectors including LL-37 and to examine the putative cross- resistance between colistin and LL-37 (paper III and paper IV).

III. To look into potenential solution to treat infections caused by resistant strains with the current available antibiotics in the market. This is achieved by looking for synergistic effect with antibiotics that already available in the market (paper III and paper V).

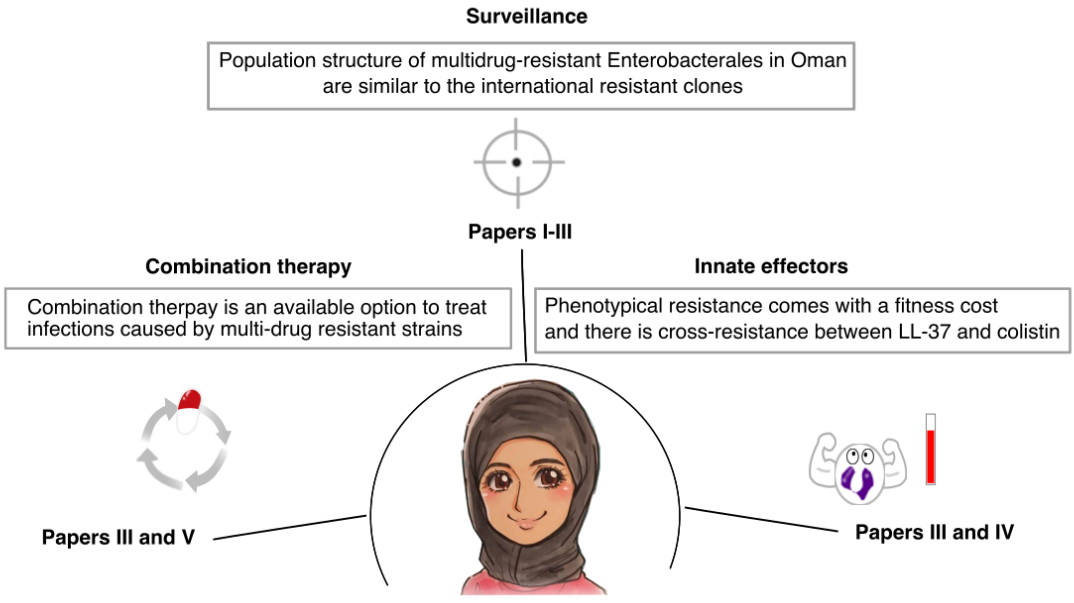


Figure 10. Hypotheses used to address the aims from the research. We aimed to test three hypotheses. First, we hypothesize the resistant strains from Oman belong to some of the major known international clones (Papers I-III). Second, we hypothesize phenotypical resistance comes with a fitness cost. Also, we assume there is cross-resistance between colistin and LL-37 (Paper III-IV). Finally, we hypothesize that combination therapy is an available treatment option for infections caused by resistant strains (Paper III, Paper V).

METHODOLOGICAL CONSIDERATION

In this chapter I will discuss the methodology we used to test the hypotheses framed to achieve the aims. The methodologies we used can be classified into wet or dry lab works. The clinical isolates were obtained from different hospitals in Oman based on one criterion: reduced susceptibility to carbapenem. Such isolates routinely referred to the public health laboratories for further characterization. We used a collection of isolates obtained in 2015 for this study.

3.1 Ethical consideration

Ethical approval for the study was obtained from Ministry of Health in Oman (MD/R&S/32/2015). The approval includes collecting anonymized clinical data, shipping the isolates to Sweden and to carry the research and analysis on them. The isolates were used in all five papers.

Additionally, two ethical permits were granted to carry experiments in paper IV. For the collection of blood samples from healthy volunteers we used initially the permit (dnr 2000-360/00) which was approved by the Regional Ethical Review Board in Stockholm and updated in 2019 by the same committee (dnr 2019-02519). The permit (dnr 19204-2017) to carry zebrafish experiments was approved by the Ethical Review Board, Stockholm, animal research Committee and the Swedish Board of Agriculture.

3.2 Dry lab work

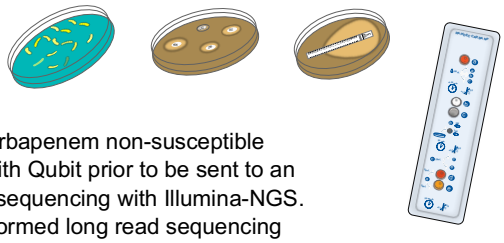
Since most of the analyses were based on data obtained from whole genome sequencing (WGS), the dry lab work constitutes the majority of the work in the study in all papers but to a lower extent in paper IV. The phenotypic test and identification were re-examined at Karolinska Institute. Samples of interest based on their resistance patterns were selected for WGS.

In total there were 35 *E. coli* isolates. All of them were included in Paper I yet 20 isolates were utilized in Paper V. Regarding *K. pneumoniae*, there were 237 isolates. All were included in Paper II. Out of them 25 were colistin resistant isolates which were studied further in-depth in Paper III. In Paper IV, we studied seventeen *K. pneumonia*e isolates of which eight were colistin resistant whereas nine were colistin susceptible as a control group. We followed similar protocol for both species as outlined in the flow chart (Fig 11). In summary, DNA was extracted and quantified prior to sending the sample for Illumina short read sequencing at an external facility (SciLifeLab, Stockholm, Sweden). We checked

1. Around 300 *Klebsiella pneumoniae* and *E. coli* clinical isolates from 12 hospitals in Oman were studied.

2. Species confirmation was performed using MALDI-TOF.

3. We performed phenotypic antimicrobial senstivity tests including disk diffusion, gradient strips (E-test), broth microdilusion and Carba NP.



4. DNA was extracted for carbapenem non-susceptible isolates. Then, quantified with Qubit prior to be sent to an external facility (SciLife) for sequencing with Illumina-NGS. For selected strains we performed long read sequencing via MinION or Sanger sequencing.

5. Reads were assembled using Unicycler or Spades. Several online databases were used for genomic analyses including, MicroSalt, BIGsBD, Kaptive, Kleborate, CARD, CGE, PHASTER, CRISPRcasFINDER and EnteroBase. Also, CLC software was used to screen mutations in genes regulating efflux pumps and porins

6. Snippy was used for SNP-calling after excluding recombination events with Gubbins. A phylogentic tree was infered using FastTree. Graphic design was done using R with addition of packages: tidyverse, tidytree, Biostrings, cluster and ggpubr and grid.

Figure 11. Summary of the workflow related to the analysis based on whole genome sequence. We started the workflow by species identification using MALDI-TOF (matrix assisted laser desorption ionization-time of flight) mass spectrometry followed by confirmation of carbapenem non-sucebtibility prior to sending the strains for short read sequencing. We utalised available analyses tools to screen for virulence and resistance genes. As well as to map the population structure in each species.

the quality of the reads using FASTQC. The reads which passed the quality check were analysed further whereas the reads with low quality were re-sequenced. Regarding *E. coli* strains, reads with accepted quality were used to screen for mutations in genes codify AcrAB-TolC efflux pump and porins or their regulators by CLC program. The generated data were presented in Paper I and Paper V. Furthermore, reads were uploaded in EnteroBase pipeline. The assembled files were downloaded from the database and the gaps were closed by aligning the assembled files back to the original reads via Pilon software. Then, the polished fasta files were uploaded to Center of Genomic Epidemiology, CRISPRcasFINDER and PHASTER databases to screen for resistance, virulence, CRISPR and phage contents. Additionally, phylogenetic trees were constructed in a similar manner to *K. pneumoniae* as described below.

Regarding *K. pneumoniae,* the strains were assembled by Unicycler then the FASTA files were uploaded into BIGSdb to assign new STs. CARD and Kleborate databases were used to screen for resistance and virulence genes, respectively. To understand the population structure, we placed the bacterial genetic background in a phylogenetic tree. This has been achieved by calling the SNPs with Snippy, filtering putative recombination events by Gubbins, reducing the filtered alignment to the core polymorphic sites by SNP-site, building a matrix by SNP-dists and eventually randomized accelerated maximum likelihood (RAxML) tree by RAxML program. *K. pneumoniae* strains used for paper III, were analysed in a similar manner except for phylogenetic tree in which CLC program was used to build a neighbour-joining tree for some strains. Additionally, Sanger sequence or long read sequence with MinION sequencer were performed for selected isolates to increase the genetic resolution obtained by Illumina short read sequencer.

We encountered some challenges in the genomic analyses. These challenges were not unique by any means as they are usually encountered in many labs due to limitation of the available analysis's tools. For example, after filtering recombination sites we noticed unexpected outcome in which isolates with similar classical ST types had high SNP-differences. The recombination events are identified as sites with elevated densities of base substitutions. The drawback with this approach, it cannot distinguish sites with high heterogenicity from those encountered recombination events[137-139]. To overcome this, we masked STs genes prior to filtering out recombination sites. We acknowledge this is not the best approach but the one we could implement with the current tools at hand.

3.3 Wet lab work

Data in Paper IV and to less extend in Paper III involved wet lab works. Here I will discuss some challenges or limitations we encountered with the methodological approaches we have implemented.

3.3.1 Choice of medium

The choice of growth media can affect the outcome of the experiment [140]. Initially we tested different media such as LB with or without RPMI-1640 medium. LB (Lysogeny broth or known to be abbreviated for Luria-Bertani) was developed by Bertani in 1951 to optimize plaque formation[141]. LB media were widely used in molecular microbiology such as plasmid DNA and recombination but discouraged for physiological studies for reasons discussed below.

LB medium has complex sources of carbons. It consists of tryptone as a source of amino acids, yeast extract as a complex source of organic compounds helpful for bacterial growth as well as sodium chloride for osmotic balance and transport. Bacteria during the initial stage of exponential growth will utilize easy to digest compounds as a source of carbons yet when these are depleted then bacteria will switch into more complex sources which will create a pause in bacterial growth[142]. Such slow in growth might alter the size and the structure of cell wall hence interfere with physiological studies. Another disadvantage to utilize LB in physiological studies is the contents of divalent cations such as magnesium and calcium. This has been pointed out by a paper cited three times so far despite the importance of the message it carries[143]. Divalent cations play a role in in the stability of LPS and hence cell wall of Gram-negative bacteria as discussed previously (section 1.2.1.1.3). A third factor is that LB media are sold as premixed capsules contain variable amount of bile salt. Bile salt is toxic to bacterial cell which is usually expelled by activating AcrAB-TolC efflux pump[144,145]. However, once activated the pump will expel other materials along the way such as antibiotic, thus interfering with experiments studying bacterial susceptibility to antibiotics as the case in this thesis. Moreover, bacteria lacking AcrAB-TolC efflux pump will not survive as they will be affected with bile salt toxicity. The content of bile salt in each premixed batch of LB varies. We noticed this as using a fresh batch of LB might change how bacteria behave in the experiment. Particularly since the study of colistin resistance is sensitive to divalent cations and active efflux pump is known to cause resistance to colistin[44]. For these reasons we chose to shift from using LB to MHB (Mueller Hinton Broth).

Mueller Hinton Broth was developed by Muller and Hinton to study gonococcus and meningococcus in 1941[146]. It consists of beef extract and casein hydrolysate as sources of amino acids and carbohydrates as well as starch which absorb toxins released by bacteria so they do not interfere with antibiotic activity for example. For this reason and for being nonselective and nondifferential medium in which most bacteria are capable to grow, MHB is recommended to be used for antibiotic susceptibility assays after cations adjustment. Initially the recommendation was to perform broth microdilution (BMD) for cationic antibiotics such as colistin using MHB without cations adjustment as their mechanisms of action relied on competing with calcium and magnesium to bind to bacterial cell wall[147]. However, the latest update recommends using the cation-adjusted media (CaMHB) which we used in our work here.

Another media we have used was RPMI 1640 developed in 1966 at Roswell Park Memorial Institute[148]. It is a growth medium for culture cells. We utilized this media with innate effectors such as blood. Others proposed the use of RPMI1640 with 5% LB in susceptibility assays will improve the estimation of antibacterial activity compared to CaMHB[149]. However, we still have concerns regarding the use of LB and a better approach could be to use RPMI with 5% CaMHB. Yet, we only noticed such significant difference in MIC-value if tested in CaMHB or RPMI with 5% LB in azithromycin.

3.3.2 Bacterial inoculum

Bacterial growth curve can be divided into four stages: lag, exponential, stationery and death phases. During the lag phase bacteria invest time to adjust to the new environment and prepare the precursors to grow and divide. There is no growth or duplication at this phase, as such the growth curve is flat. The next stage bacteria start to grow exponentially. This is an optimum stage to test the effect of intervention in bacteria. Since the nutrient supply is available, the size and the structure of bacterial cell are normal and intact. The changes might only be attributed to the intervention or treatment factor. This phase is followed by a stationary phase in which bacterial rate of growth equals the rate of death, thus presented with a flat line as no net growth. Here, bacteria behave differently as they compete for the limited remaining nutrients and their structure and size might be affected. Also, the medium would contain waste as a by-product of bacterial metabolism. This might be of interest for specific studies like how bacteria behave in starvation. Yet, for intervention studies it is not optimal since many factors might affect the outcome apart from the treatment. Besides, the bacteria initial inoculum determines how fast bacteria could move from one stage to the following one. Exposing bacteria to antibiotic at the lag phase would have a bigger impact in the outcome compared to the exponential phase.

We used different bacterial initial inoculum for different assays (Table 2). We based this on previously published reports. In LL-37-kill assay and blood-kill assay, we used similar initial inoculum of $5x10^7$ cfu/ml. For zebrafish and serum assays we used $4x10^4$ cfu/ml. In experiments related to electron microscopy and zeta potential we used higher inoculum of $1x10^8$ cfu/ml. Whilst we deliberately used low bacterial inoculum for the growth curve experiments ($1x10^3$ cfu/ml) to observe the initial lag phase as starting with a higher inoculum might mask this since bacteria replicate fast. It is worth noting, the initial inoculum might affect the outcome. This is one of the reasons some of our findings do not aligned with the published data. There is no agreement on this in the literature, but the variation is acknowledged to impact the outcome. This is evident by the recommendation to use $5x10^5$ cfu/ml as bacterial starting inoculum for antimicrobial susceptibility assay to control for growth variation. One issue with growth curve experiments is that it does not directly reflect the living bacterial cell, but rather measure the turbidity of the media as an indication of the growth. This could be true until the bacteria enter the stationary phase where bacteria start to die due to lack of nutrients. Yet still the turbidity is high since turbidity is not related to viability.

Table 2: Medium and initial bacterial inoculum used in different assays

Experiments*	Bacterial inoculum cfu/ml (log_{10})	Medium
Time-kill assay		
LL-37	~$5x10^7$ (7.5)	CaMHB
Blood	~$5x10^7$ (7.5)	40% blood diluted in RPMI1640
Serum	~$5x10^4$ (4.5)	20% serum diluted in PBS
Combination assay		
Paper III	~$5x10^7$ (7.5)	5% RPMI1640 diluted in CaMHB with treatment of choice
Paper V	~$1x10^6$ (6)	CaMHB
Zebrafish	~$5x10^4$ (4.5)	E3 medium
Zeta potential	~$1x10^8$ (8)	PBS or deionized water (DIW)
Electron microscopy	~$1x10^8$ (8)	CaMHB
Broth microdilution (BMD)	~$5x10^5$ (5.5)	CaMHB
Growth curve measurement	~$1x10^3$ (3)	CaMHB

CaMHB; cation adjusted Mullar Hinton broth, PBS; phosphate-buffered saline, RPMI1640: culture medium developed by Roswell Park Memorial Institute, CFU; colony forming unit. * If otherwise not indicated, the assays listed were presented in Paper IV.

3.3.3 Time-kill assay

For time-kill experiments, the bacteria were grown exponentially and diluted to the desired concentration prior to the start of the assays (Table 2). Then incubated for two hours with the desired treatment e.g. serum, blood or LL-37. Followed by serial dilution and incubation in blood agar overnight. The next day, we counted the colony-forming unit (CFU-count). Regarding serum-kill assay the practice of using 20 % serum diluted in phosphate buffered saline (PBS) is commonly used and accepted in the literature, despite the rationale behind the practice is not clear. Regarding blood-kill assay we diluted blood in 40% RPMI1640. We optimized the dilution to get a measurable outcome within 2 hours.

3.3.4 Zeta potential

As per the general agreement in literature, colistin resistance arises due to reduced negative charge in the bacterial cell wall. We aimed to measure bacterial charge with zeta potential. Zeta potential will give the whole net charge, we were particularly interested in measuring LPS charge. Despite this, the general trend would remain the same though the magnitude might be affected with other factors such as the ionic content of the medium. Thus, we used two media, PBS and deionized water (DIW) to control for ions effect. Also, we used *Proteus mirabilis* as a control fot machine performance since it is intrinsically resistant to colistin[150]. We studied two genetically close *K. pneumoniae* clinical strains differing in their tolerance to colistin. As a control we used the laboratory reference strain (ATCC25955).

P. mirabilis showed low negative charge compared to the rest of the strains. The data regarding the control showed lower negative charge compared to the colistin resistant strain. The finding we saw was unexpected and surprising. The resistant strains (Col-R) showed higher negativity than the colistin susceptible (Col-S) and the reference strain (ATCC25955). Further work could be done to look in-depth to such striking finding. For example, measuring the contact angle which gives a clue about hydrophobicity of bacterial surface as described previously[151]. This could be relevant since polymyxins utilise electrostatic and hydrophobic interaction to bind with bacterial cell. Another approach could be to extract lipid A for more specific characterization of the surface charges with mass spectrometry as described previously[152]. Additionally, atomic force microscopy (ATM) might help in understanding how polymyxins interact with bacterial cell following similar methodology used for a different goal but could be implemented here[153].

Moreover, measuring zeta potential for other colistin resistant strains might give a clue if this is a trend in the Omani isolates or whether this particular strain is an exception. Another challenge with studies related to colistin resistance is the lack of an international reference strain. The availability of a reference strain aids in relating data from different labs. Currently, only clinical strains were studies. The challenge with clinical strains, their genetic content varies which might affect the findings.

3.3.5 Combination assays

We performed combination assays as presented in Paper III and Paper V. We defined the observed effect as synergistic (reduction of ≥ 2 $\log_{10}$), additive (reduction of ≥ 1 $\log_{10}$) or antagonistic (increase of ≥ 2 $\log_{10}$) by comparing the combination result with the most potent single antibiotic as described previously[154].

In Paper III, we focused on colistin and carbapenem resistant *K. pneumoniae*. We used time-kill assay to test the effect of two antibiotics in combination with polymyxin B (2 mg/L); rifampicin (5 mg/L) and azithromycin (0.25 mg/L). The selection of antibiotic concentrations was based on previously published papers. In Paper V we explored combination therapy for carbapenem-non-susceptible *E. coli* against a wide range of antibiotics with different concentrations; aztreonam (2, 8, 65) mg/L, meropenem (2, 16, 64) mg/L, minocycline (0.5, 4 and 16) mg/L, polymyxin B (0.25, 0.5, 1 and 2) mg/L and rifampicin (1, 8, 32) mg/L. We utilized screening machine based on time-lapse microscopy (oCelloScope). Images were taken at different time interval and bacterial growth was assessed via kinetics algorithms e.g. the background corrected absorption (BCA) and segmented extraction of surface area (SESA)[155]. We considered there is bacterial growth when BCA value is > 8 in combination with a maximum SESA of > 5.8 was noticed. One of the limitations is the inability to distinguish filament from single cell which result in false negative. This is particularly obvious in antibiotic causing filamentation such as aztreonam which was compensated by performing spot assay after 24 hours.

RESULTS AND DISCUSSION

In this chapter, I will discuss the results and the validity of the hypotheses we raised to achieve the aims of this work. Since the work was based on three aims. I will discuss each aim in a specific section.

4.1 Population structure of resistant strains in Oman

We studied population structure and β-lactam resistance mechanisms of *E. coli* strains (n=35) in Paper I. Similarly, we investigated the genetic background of *K. pneumoniae* clinical isolates (n=237) and their mechanisms of resistance against β-lactam (Paper II) and colistin (Paper III).

4.1.1 *E. coli* : Paper I

We observed high clonality reflected by diverse sequence types (ST) in *E. coli* strains. Within 35 strains we found 21 unique STs, 11 distinct H-serotypes, 16 O-serotypes and 22 CRISPR-types. Collectively, we assigned an overall allelic number for each strain by combining these typing schemes. At least there were 6 pairs of closely related strains (Table 3). Furthermore, we studied SNP-differences between strains within these 6 pairs. At least in three pairs, strains were close genetically with SNP-differences ≤ 2 which suggests the occurrence of silent transmission events.

The presence of a diverse CRISPR-type (22 types) was relatively unexpected since the CRISPR-Cas system is considered to be static in strains with similar MLST types in *E. coli*[116]. We have seen three CRISPR-types in ST38 for example (Table 3). The diversity might indicate different genetic background in the studied isolates since the samples were not collected for an outbreak investigation but rather for national surveillance. Also, it implies CRISPR typing could identify outlier strains.

Carbapenemase genes were detected in 22 strains (59%). Isolates produced either NDM (n=13), OXA-48-like (n=7) or both enzymes (n=2). We did not detect any KPC enzyme. This aligns with data published previously that consider the Middle East a reservoir for NDM and OXA-48-like carbapenemases[79,82,156]. Furthermore, three isolates were carbapenem non-susceptible, but we could not detect genes codifying carbapenemase on them. The three isolates were negative by Carba-NP. As Carba-NP has limitation in sensitivity towards OXA-48-like enzymes, one might speculate the isolates possibly could harbour a novel OXA-48-like enzymes which could not be detected in the database[116].

As the three non-carbapenemase producing strains harbored other β-lactamases (ESBL or AmpC), we thought to screen if they mutated to alter cell wall permeability or activate efflux

Table 3: Different typing approaches used to study population structure in *E. coli*

Strain ID	Phylogroup	H-antigen	O-antigen	ST	CRISPR	Strain specific allelic type	SNPs
OM898	A	1702	O89	H9	1	1	0
OM852	A	1702	O89	H9	1	1	
OM1626	A	167	O89	H5	1	3	1
OM855	A	167	O89	H5	1	2	
OM1071	A	167	O89	H9	1	3	
OM979	A	617	NA	H10	2	4	
OM5639	A	617	NA	H10	3	4	
OM693	A	617	O89	H10	4	5	
OM1398	A	540	O9	H30	5	6	
OM211	A	361	O9	H30	6	7	14
OM112	A	361	O9	H30	6	7	
OM1576	A	46	O9	H10	7	8	
OM147	B1	101	O131	H31	8	9	2
OM79	B1	101	O131	H31	8	9	
OM1341	B1	156	O177	H28	9	10	
OM664	B1	448	O160	H8	10	11	
OM561	C	410	NA	H9	11	?	
OM1273	C	410	O8	H9	11	12	
OM1301	C	410	NA	H9	11	?	
OM853	C	652	NA	H9	12	13	
OM1609	F	6870	O45	H6	13	14	
OM78	F	1340	NA	H6	X	15	
OM260	B2	131	O25	H4	X	16	
OM333	B2	73	O6	H1	X	17	
OM1692	B2	127	O6	H31	X	18	
OM1136	B2	1193	O75	H5	X	19	
OM126	D	2914	O166	H15	14	20	
OM1168	D	405	O102	H6	15	21	
OM82	D	405	O102	H6	16	22	7
OM839	D	405	O102	H6	16	22	
OM150	D	2659	O2 /O50	H18	17	23	
OM481	D	38	O86	H18	18	24	
OM1433	D	38	NA	H30	19	25	
OM234	D	38	O7	H18	20	26	10
OM347	D	38	O7	H18	20	26	

NA: Not determined, X: CRISPR-Cas system is absent, ?; Strains similar in known typing approaches but O-serotype since it could not be determined

pumps. One strain had wild-type variants in all screened genes. However, our understanding of the genes involved in regulating porin and efflux pump activities is continuously evolving. Here we screened for mutations in ten genes (*ompCFR*, *envZ, acrR*, *marABR soxSR*) are known to regulate activities of porins and AcrAB-TolC efflux pump. We did not screen for *acrAB*, *tolC* and *ramA* genes known also to affect AcrAB-TolC efflux pump[40]. Recently, a novel AraC-type regulator known as regulator of antibiotic resistance A (RarA) was shown to upregulate AcrAB expression when *E. coli* was exposed to carbapenem[157]. Additionally mutations in other genes known to be involved in the export of proteins in *E. coli* might contribute in carbapenem resistant phenotype e.g. *secDF*[158].

Apart from carbapenem resistance, we did not see colistin resistance in this collection. Furthermore, we detected particular resistant clones known as high-risk clones e.g. ST131-H30Rx/C2, ST410-H24RxC and ST1193-H64RxC. The latter was associated with NDM, to our knowledge for the first time in literature. In short, we confirm the hypothesis that resistant *E. coli* strains in Oman are similar to the international resistant clones and might in fact contribute to the emergence of clones with more resistance attributes such as ST1193-H64RxC.

4.1.2 *K. pneumoniae* : Paper II and Paper III

Paper II presents the population structure and resistance mechanisms of carbapenem-non-susceptible *K. pneumoniae* in Oman based on 218 strains selected from the initial 237 examined isolates. Additionally, in Paper III we studied particularly colistin-resistant *K. pneumoniae* (n=25).

Analysis of genetic background revealed 22 STs of which two were novel (ST4547 and ST4548) and 14 were singletons. The most commonly seen STs were ST231 (32%) and ST11 (31%), followed by ST147 (15%), ST15 (6%) and ST101 (5%). To visualize subclones, classical multidimensional scaling was performed based on SNP-distance matrix. ST11 could be further subtypes into three subclones; one localized in a single hospital (SQH), the second seen was in two hospitals (Khoula and Nizwa) whereas the third one was found in different hospitals. ST101 showed three subclones each one of them was linked to particular hospitals which suggests local transmission events. ST231 was less diverse with no subclones observed, which reflects that one clone was disseminated in the entire country (Figure 12).

Regarding carbapenem resistance, 87% of the isolates harbored carbapenemase genes. We could not detect carbapenemases in 29 isolates, some of them (n=10) were putative MBLs-producers according to the ROSCO phenotypic carbapenemase detection kit and were positive with Carba-NP (n=10). Similar to the pattern seen in *E. coli*, only NDM and OXA-48-like family were detected in *K. pneumoniae*. This also aligns with published molecular data about CRE in Oman[79,159]. While NDM has been observed in different STs, ST231 is almost exclusively linked to OXA-232. This was also reported from other countries in the Arabian Peninsula such as Yemen and UAE as well as India[160-162]. This again links the resistance patterns in the Indian subcontinent and the Arabian Peninsula.

Another common observation between Indian subcontinent and the Arabian Peninsula, is the lack of KPC enzymes. KPC enzymes are usually associated with sequence types within clonal complex (CC258) such as ST258 or ST11. Despite ST11 was one of the major STs in this collection and was known to be associated with KPC epidemic in China, we did not detect KPC in this collection[163-166]. This is an interesting finding and requires further investigation to elucidate the reasons which undoubtedly will reveal much about biological evolution of antibiotic resistance. For example, what factors might contribute to the spread of this particular enzyme apart from the presence of CG258 clone.

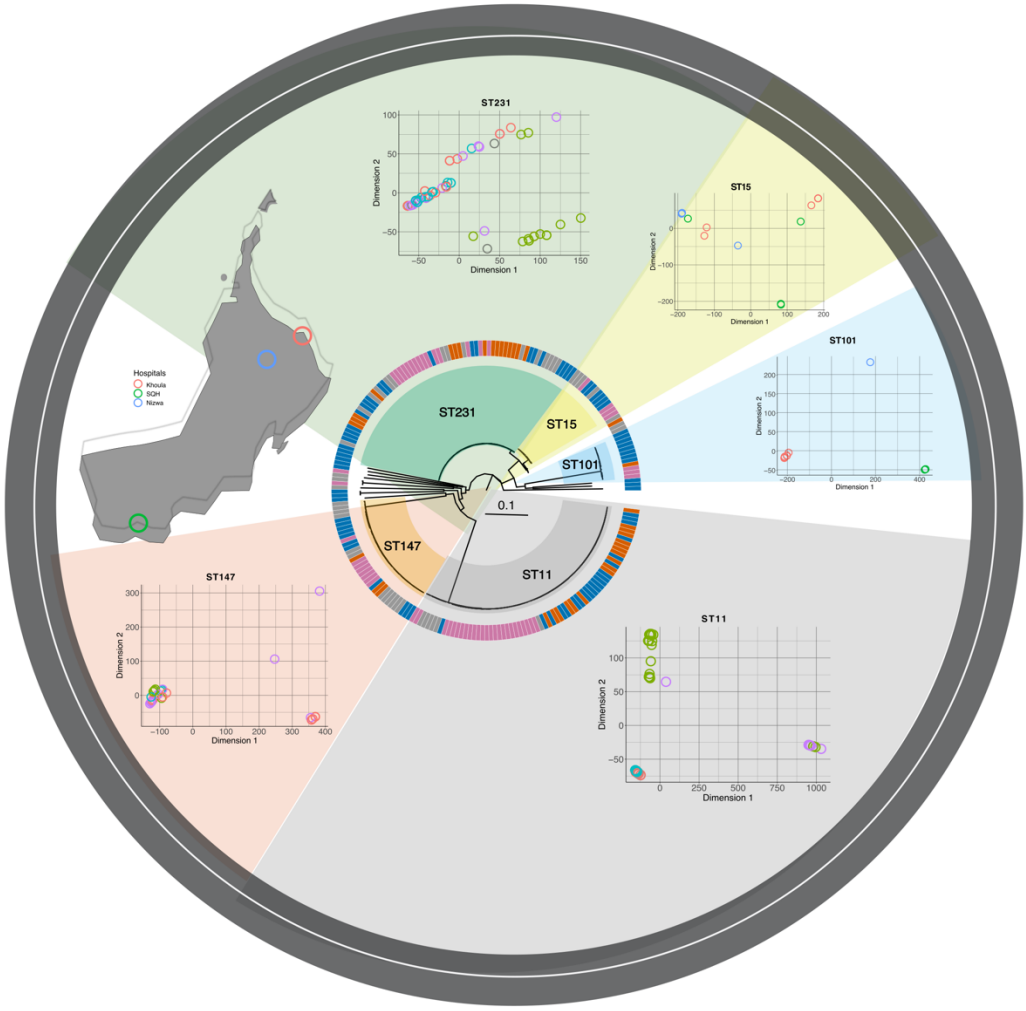


Figure 12. Population structure of carbapenem-non-susceptible K. pneumoniae. Based on SNP-distance matrix and MLST, we could identify five major sequence types (STs); ST231, ST11, ST147, ST15 and ST101 as presented in the inner circle. The coloured ring indicates the hospitals from where these isolates were obtained. Furthermore, based on classical multidimensional scaling, we saw two or three subpopulations in each major STs except ST231. Additionally, we saw low genetic diversity in ST231 which might suggest dissemination of a single clone all over the country. SNP; single nucleotide polymorphism, MLST; multilocus sequence typing.

Regarding colistin resistant strains (n=25), most belonged to ST231 (n=9) and ST11 (n=6) as they were the major STs in this collection. The mechanism of resistance could be attributed to genetic alteration in genes that regulate the two-component systems in *K. pneumoniae* such as PhoPQ , PmrAB and CrrAB. Mutations in *pmrB* gene were the commonest (n=19). To the contrary, only wild-type *pmrA* gene was detected in this collection. We did not either detect *mcr* gene in this collection.

Another common colistin resistance mechanism is the presence of mutations in the *mgrB,* gene which encodes a negative regulator of PhoPQ system. We observed a frame shift at the 9th codon (Ser9fs) of *mgrB* in three strains. Apart from mutations in *mgrB*, the gene could be inactivated by insertion elements (ISs) which was detected in 8 isolates via three different insertion elements (ISs). Particularly, IS*Kpn*14 as the commonest as it was seen in 6 isolates. In four of them IS*Kpn*14 had identical insertion site as it disturbed *mgrB* at nucleotide 116 by same direct repeat that suggest a close relation between these strains. Clinically, three of them (OM300, OM290, OM536) were obtained from the same hospital (Nizwa) whereas the fourth isolate (OM124) was obtained from a patient transferred from Nizwa hospital to Khoula hospital which suggests a root of transmission across the hospitals. Genetically, the strains belonged to ST11 and were clustered into two groups apart from each other by 18 SNPs. It is highly likely, the strains had common ancestor and presenting a cross hospital transmission.

Apart from resistance genes to survive antibiotic treatment, the initial ability of *K. pneumoniae* to cause infections is determined to a large extend by its content of virulence factors. For example, the presence of K2 capsule type, *rmpA/rmpA2* and specific siderophores molecules (yersiniabactin and aerobactin) are associated with virulence in *K. pneumoniae*[167,168]. Here, K2 capsule was detected in four carbapenemase producering strains. Also, yersiniabactin and aerobactin were detected in 73% and 26% of the strains, respectively. Additionally, an ST2096 colistin resistant and OXA-232 producer strain (OM5488) harbored *rmpA/rmpA2*, aerobactin (*iuc1*) and yersiniabactin *(ybt*-14*).* Such clone was isolated recently from India and reported to cause an ICU (intensive care unit) outbreak in Saudi Arabia[169,170]. Clones with both resistance and virulence attributes are concerning since they have the ability not only to establish infection in immunocompromised individuals but also in healthy individuals.

All in all, we confirm the hypothesis that population structure of resistant strains in Oman is similar to the international resistant clones such as ST131 in *E. coli* and ST11 in *K. pneumoniae*. However, we noticed newly emerging clones such as ST1193 in *E. coli* with carbapenemase gene. Also, a high prevalence of ST231 in *K. pneumoniae* was seen, which is not commonly observed in other regions. The most concerning is the presence of highly resistant and virulent clones such as ST2096. More about virulence is discussed in the following section.

4.2 Interaction of innate effectors with colistin resistant strains: Paper IV

In Paper IV we aimed at investigating if there was a cross-resistance between colistin and LL-37. We examined this by time-kill assay and growth curve experiment. Additionally, we studied structural and intracellular changes induced by LL-37 against polymyxin resistant strain via electron microscopy. We looked into the genetic changes that confer resistance to polymyxin and its implication such as reduction of the net negative charge in outer membrane. Finally, we investigated bacterial fitness of the colistin resistant strains and how the innate effectors interact with them as well as how their survival in a zebrafish model.

4.2.1 Interaction of colistin resistant strain with LL-37

LL-37 is a component of the innate immune system and has a wide antimicrobial activity. Both colistin and LL-37 share similar binding mechanism to bacterial cell wall. As such the emergence of resistance against colistin might affect how bacteria interact with LL-37. We investigated if resistance to colistin induces high tolerance to LL-37. Additionally, we looked into intracellular and extracellular changes in bacterial cell when treated with LL-37 using electron microscopy.

4.2.1.1 Cross-resistance with LL-37

Here, we studied how LL-37 interacts with two genetically similar strains yet differ in their susceptibility to colistin. OM124 gained resistance by inactivation of *mgrB* gene with insertion element whereas the susceptible strain (OM322) had an intact *mgrB* gene. From the designed time-kill and growth curve experiments, we saw a cross-resistance between LL-37 and colistin but only at a concentration higher than 50 mg/L (or 11.25 μM). Furthermore, similar finding was seen when more isolates (n=8) with *mgrB* insertion were included compared to colistin susceptible strains (n=9). However, only strains with *mgrB*-insertion were studied. The cross-resistance might be specific to this particular resistance mechanism.

Published data were inconclusive regarding the cross-resistance hypothesis. In *K. pneumoniae,* Kadar et al., reported high tolerance to different antimicrobial peptides including lactoferrin and lysozyme[67].However, Dobias et al. showed no difference in the interaction with LL-37 in colistin resistant *K. pneumoniae* stains due to different mechanisms including *mgrB*-insertion. Nevertheless, our data supported their finding as they examined LL-37 at concentrations ≤ 20 mg/L (or ≤ 4.5 μM)[171]. We have not seen cross-resistance at LL-37 concentration < 50 mg/L (or 11.25 μM). Regarding colistin resistance in *Acinetobacter baumannii* , Moffatt et al. reported a colistin resistant isolate due to an LPS-deficient mutation in the *lpxA* gene (deletion of a single base at nucleotide 90), which was more susceptible to LL-37 at concentrations ≥ 5.5 mg/L (or ≥ 1.25 μM) but no difference was observed below this concentration[172]. Yet, Garcia-Quintanilla showed no difference at a concentration of 3.13 mg/L (or 0.7 μM) in a collection of resistant strains due to different mechanisms including *lpxA* mutation (I205N)[173], which is aligned with the previous finding by Moffatt et at. despite the particular mutation in *lpxA* varied between the studied strains. The finding of decreased

susceptibility to LL-37 in colistin resistant strain ≥ 5.5 mg/L by Moffatt et al. was contradicted by Napier et al. as they showed cross-resistance at a concentration of 6.25 mg/L (or 1.4 μM), though the mechanism of resistance was not mentioned[60]. Such discrepancies might be attributed to using variable methodology e.g. protocols regarding growth medium, incubation time, initial bacterial inoculum, LL-37 concentration and mechanism of resistance in the studied strains.

The impact of cross-resistance on individuals depends on their health status. In a healthy individuals the concentration of LL-37 is estimated to be below 2 mg/L in serum and plasma[174]. Yet in individuals with autoimmune diseases such as psoriasis, LL-37 level in plasma could reach as high as 1,366 mg/L[175].Thus, the ability of LL-37 to clear infection caused by colistin resistant strains is significantly reduced compared to infection caused by colistin susceptible strain. Also, neutrophil stores high concentration of LL-37 estimated to be 640 mg/L, which is released during inflammation to clear infection[174]. This implies the activity of LL-37 released from neutrophil to clear infection caused by colistin resistant strain is lower than that of colistin susceptible strains even in healthy individuals. However, the concern of using antimicrobial peptides as a therapeutic approach could compromise natural immunity might be overestimated[59,176]. Yet, evaluating cross-resistance to host AMPs in new therapeutic AMPs is highly desirable[126-130].

In summary, our data support there is cross-resistance between colistin and LL-37 but only at high concentration of ≥ 50 mg/L (or ≥ 11.25 μM). This aligns with our knowledge that LL-37 has antimicrobial activity against Gram-positive bacteria which lack LPS and are not commonly treated with colistin[177]. Most likely the negativity of LPS in Gram-negative bacteria enhances LL-37 binding to bacterial cell but is not the only means of interaction. Next, we thought to complement this concentration dependence finding by further looking into the variation of cellular changes induced by different concentrations of LL-37 on bacterial cell as discussed in the following section.

4..2.1.2 Extracellular and intracellular impact of LL-37 on bacterial cell

Gram-negative bacteria undertake modification in the structure of their cell walls to gain resistance against colistin. Such changes might also interfere with how they interact with other molecules, including LL-37, compared to wild-type. In addition, the intracellular changes induced by LL-37 to eventually kill bacteria might be concentration dependence. To that end, we studied morphological changes induced by different concentrations of LL-37 (50-200 mg/L) at different exposure time (0.5 and 2 h) via transmission electron microscopy (TEM) and scanning electron microscopy (SEM). Two genetically similar isolates differing in their susceptibility to colistin were included in this study.

Intracellular changes were inferred from TEM images. Bacterial exposure of 100 mg/L induced morphological changes within 30 minutes of treatment. We observed clustering of DNA and ribosomes in both resistant and susceptible strains. Despite the resistant strain was less affected by LL-37 at concentration above 50 mg/L in CFU-counts that was performed in

parallel with TEM experiment. Worth noting, the isolates lost their capsules after exposure to LL-37 compared to the control untreated strains. Interestingly, using atomic force microscopy (ATM), Formosa et al. showed that the capsule of polymyxin resistant *K. pneumoniae* due to *mgrB* inactivation was tightly bound to bacterial cell wall when exposed to polymyxin as well as it became harder as the concentration of polymyxin increased, while the strain with intact *mgrB* lost their capsule[55]. This implies *mgrB* alteration has an impact in capsule structure. Although we did not notice variation between the strains as they did. Also, since capsule has anionic charge whereas LL-37 has cationic charge, the capsule could neutralise the killing effect of the peptide via electrostatic interaction and thereby reduce the amount of LL-37 that can reach bacterial cell wall as shown previously[178].

Changes in the surface structure were inferred from SEM images. Exposure to LL-37 (100 mg/L) for 30 minutes turned both strains to appear more swollen compared to control untreated strains. This effect was more profound at higher concentration (200 mg/L) and exposure time (2 h).

In summary, exposure to LL-37 had profound impact on both strains with no obvious differences between them. These data and the CFU-counts which were performed in parallel with TEM and SEM experiments, indicate resistance to colistin might affect the quantity but not the quality of changes induced into bacterial cell. As far as LL-37 manages to enter the cell, the morphological effects were neutral to resistance pattern. This also emphasizes in the ability of LL-37 to bind to resistant strain despite the changes induced by the genetic variation causing colistin resistance. Thus, we wonder if resistance mechanism against colistin indeed reduces the net negative charge or lead to other changes beside or apart from that.

4.2.2 The *mgrB*-insertion did not impact surface charge

It is commonly accepted that colistin resistance arises due to reduction of the net negative charge in LPS which is induced by genetic modification in genes regulating lipid A synthesis in Gram-negative bacteria. The hypothesis is supported by finding that showed reduced negativity in species known to be intrinsically resistant to colistin such as *Proteus mirabilis*[150]. Genomic analysis of the studied strains revealed the cause of colistin resistance to be due to *mgrB*-insertion by IS*Kpn*14. We investigated if such genetic alteration might impact the surface charge via measuring zeta potential in two different media PBS and deionised water.

Unexpectedly, the data showed no significant changes in the negativity of the studied strains ($P=0.54$ in DIW and $P \geq 0.99$ in PBS). Similar finding was reported by Ayerbe-Algaba et al. as they studied colistin resistant strain due to *mgrB*-insertion as well[179]. However, our data contradict with what was reported by Velkov et al. They reported a reduction on the surface charge of *K. pneumoniae* that gained colistin resistance phenotype *in vitro*[180]. It could be that different mechanisms of resistance result in different structural or chemical changes in *K. pneumoniae.*

Colistin mechanism of action relies on two initial steps; the binding of the cationic drug to the anionic lipid A via electrostatic interaction and the insertion of the drug fatty acid tail or head group into the membrane via hydrophobic interaction. Consequently, resistance to colistin involves modification of lipid A structure mainly via the two-component systems PhoPQ and PmrAB. The alteration leads eventually to either reduction in the net negative charges which is achieved by different approaches e.g. addition of positively charged molecules (phospohethanolamine or aminoarabinose) or blocking LpxT to cease the addition of phosphate groups. Another modification could not affect the surface charge but the hydrophobic interaction. This could be achieved by different mechanisms including addition of palmitate via PagP or acyl chain substitution by secondary acyl-transferases (*lpxL* and *lpxM*). *K. pneumoniae* encodes two LpxL homologoues LpxL1 and LpxL2. They are expressed at normal growth condition and both compete to add, at the same position, a fatty acyl chain in lipid A either a lauroyl or myristoyl group, respectively[34]. Lipid A in *K. pneumoniae* differs from the *E. coli* only by the presence of myristoyl residue[181].

As the strains here showed no variation in the net negative charge, we speculate that bacteria could modify the hydrophobicity trait. This could be achieved by secondary acylation in *K. pneumoniae*. Published data showed an increase in sensitivity to antimicrobial peptides and colistin in *lpxM*-mutated (*msbB*/*waaN*) *K. pneumoniae* strain. The effect could be reverted when the mutation was complemented *in vitro*[181]. Additionally, *Bordetella bronchiseptica* is highly resistant to antimicrobial peptides compared to *Bordetella pertussis* which has been attributed to ArnT[182]. MgrB-insertion is known to induce ArnT indirectly by activating the two-component system PhoPQ. Thus, we hypothesize, *mgrB* truncation will indirectly activate ArnT that will promote acylation through yet unrecognised pathway. However, the genetic alteration might induce changes other components of LPS such as the O-antigen.

Another distinctive genetic difference between the two studied isolates was the O-antigen. The resistant strain had O3b while the susceptible strain had OL104. Both are considered subgroups of O3 serotype. They varied only in *wbdD* gene[183]. Besides, we noticed that the susceptible strain lacked *manB* gene compared to the resistant strain, which is involved in monosaccharide synthesis[183]. This might lead to variation in the length of O-chain between the two strains. Additionally, Campos et al. showed that O-polysaccharides conferred resistance against polymyxin in *K. pneumoniae* in the absence of the capule[184]. Since both strains lost their capsules when treated with LL-37 as evident from TEM experiment, LL-37 would probably interact with O-antigen directly. Also, Benemann et al. reported the presence of O-specific sugar side chains conferred resistance to antimicrobial peptides in *B. bronchiseptica*[185].

In summary, no reduction in surface negative charge due to *mgrB*-insertion was observed yet the two strains differed in O-antigen. We hypothesize resistance mechanism in the studied strain of *K. pneumoniae* could be similar to what is seen in *B. bronchiseptica* rather than in *P. mirabilis.*

4.2.3 Pathogenicity and virulence of colistin resistant strains

We investigated if gaining colistin resistance implies fitness cost by exposing the strains to blood and serum following by CFU-counts. We noticed no difference in fitness and survival rate in both strains. Moreover, we studied the strains survival in zebrafish. Similarly, we did not see significant difference in their survival. We conclude *mgrB* truncation in *K. pneumoniae* do not affect bacterial fitness.

In short, we rejected the hypothesis that there is fitness cost which bacteria pay to gain resistance trait as no variation seen in both colistin resistance and colistin susceptible isolates. To the contrary, bacteria gain tolerance to LL-37 free of cost.

4.3 Impact of combination therapy onto resistant strains

We studied options to treat infections caused by resistant strains. In paper III for *K. pneumoniae* and in Paper V for *E. coli.* We achieved this by setting a time-kill assays with the combination of the drugs and looked into CFU-count.

4.3.1 Combination therapy in *E. coli*: Paper V

In this paper we examined the impact of polymyxin B in combination with aztreonam, meropenem, minocycline and rifampicin against 20 carbapenemase producing *E. coli* strains. We found synergistic effect between polymyxin B with minocycline as well as between polymyxin B with rifampicin against 11 or 9 isolates, respectively. Interestingly, we saw a correlation between the presence of *tet*(B) gene and wildtype *soxR* gene and the outcome of polymyxin and minocycline combination. Besides, aztreonam or meropenem with polymyxin B showed synergistic effect in 2 out of 20 strains. We could not see any synergistic effect between meropenem or aztreonam with polymyxin.

4.3.2 Combination therapy in *K. pneumoniae* : Paper III

We looked into the activity of colistin with rifampicin or azithromycin as a treatment option for colistin and carbapenem resistant *K. pneumoniae* strains (n=25). We saw synergistic effect ($\geq$ 1 $\log_{10}$ reduction) with colistin and rifampicin while we saw an additive effect with azithromycin ($\geq$ 1 $\log_{10}$ reduction). Both azithromycin and rifampicin antibiotics target intracellular components of bacterial cell while colistin target cell wall. Colistin will expose bacterial cell to the other antibiotics and enhance their activities.

In summary, rifampicin in combination with polymyxin was the best combination *in vitro* versus both *K. pneumoniae* and *E. coli* resistant strains.

CONCLUSION

The main findings from this thesis could be summarised as below:

I. Oman as being part of the Arabian Peninsula has high prevalence of NDM and OXA-48 carbapenemases mirroring the antibiotic pattern seen in the Indian subcontinent. We identified international high-risk clones including ST131 in *E. coli* and ST11 in *K. pneumoniae* as well as the potential of newly emerging high-risk clones such as ST1193-H64RxC in *E. coli* and ST231 in *K. pneumoniae.*

II. Colistin resistance did not imply cost of fitness or redcuction in bacterial pathogencity at least in *K. pneumoniae* with *mgrB*-insertion. To the contrary, the resistant bacteria tolerate high concentrations of LL-37 (≥ 50 mg/L or ≥ 11.25 μM). Thus, having the advantage to escape one of the innate immunity free of cost.

III. There is no reduction in the surface charge of colsitin resistant *K. pneumoniae* with *mgrB*-insertion. Suggesting the genetic alteration in *mgrB* gene might interfer with other chemical or structural changes e.g. O-antigen or hydrophobicity.

IV. Rifampicin combined with polymyxin E (colistin) showed good *in vitro* activity against multi-drug resistant strains of *K. pneumoniae* and *E. coli.*

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