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DI PAVIA

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Responsabile: Prof. Roberta Migliavacca

Antimicrobial Drug Resistance in *Enterobacterales* & the Impact of Biofilm on *Staphylococcus aureus* Infections Outcomes

Aseel Fayez AbuAlshaar

Dottorato di Ricerca in
Genetica, Biologia Molecolare e Cellulare
Ciclo XXXIV – A.A. 2019-2022

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September 2022
Aseel AbuAlshaar

This thesis is dedicated to the memory of my Father
You will always be remembered
May Your Soul Rest in Peace

Abbreviations

ATCC: American Type Culture Collection
3GC: cefotaxime and/or ceftriaxone and/or ceftazidime
aac(6′)-Ib-cr: aminoglycoside-(6)-N-acetyltransferase
AMC: amoxicillin-clavulanic acid
AMCLI: Italian Society of Clinical Microbiologist
AMP: ampicillin
AMR: antimicrobial resistance
ARMOR: antimicrobial resistance monitoring of ocular microorganisms
ATM: aztreonam
AZITRO: azithromycin
BLAST: Basic local alignment search tool
CAT: chloramphenicol acetyl transferase
CAZ: ceftazidime
CBD: Calgary Biofilm Device
CC: clonal complex
CDC: Centers for Disease Control and Prevention
CEF: cefoxitin
CIP: ciprofloxacin
CLO: chloramphenicol
CLINDA: clindamycin
CLSM: Confocal laser scanning microscopy
CN: gentamicin
CRA: Congo-red agar
CRB: Congo-red broth
CRA-BHI: Congo-red brain heart infusion agar
CRB-TS: Congo-red based on tryptic soy broth
CRE: carbapenem-resistant *Enterobacterales*
CIAI: complicated intra-abdominal infection
CoNS: coagulase-negative Staphylococci
CPE: carbapenemase-producing *Enterobacterales*
CRE: carbapenem resistant *Enterobacterales*
CRKP: carbapenem-resistant *K. pneumoniae*
CS: conserved segment
CTX: cefotaxime
cUTI: complicated urinary tract infection
CVA: crystal violet assay
DAPTO: Daptomycin
DD: double disk synergy test
DHPS: dihydropteroate synthase
DHFR: dihydrofolate reductase
EARS-Net: European Antimicrobial Resistance Surveillance Network
ECDC: European Centre for Disease Prevention and Control
EDTA: ethylenediaminetetraacetic acid

EEA: European Economic Area
EPIC: Intensive Care Study
ERITRO: erythromycin
ESBL extended spectrum β-lactamase
ESBL-KP: ESBL-producing *Klebsiella pneumoniae*
EU: European Union
EUCAST: European Committee on Antimicrobial Susceptibility Testing
FDA: Food and Drug Administration
FEP: cefepime
FOS: Fosfomycin
HCAI: healthcare-associated infections
HGT: horizontal gene transmission
IAI: intra-abdominal infection
IC: immuno-chromatographic
ICU: intensive care unit
IMP: imipenem
IMP: metal-β-lactamase imipenemase
Inc: Incompatibility group
intI1: class 1 integrase gene
IS: Insertion Sequence
KP: *Klebsiella pneumoniae*
KPC: *Klebsiella pneumoniae* carbapenemases
LEV: levofloxacin
LNZ: linezolid
LPS: lipopolysaccharides
MBEC: minimum biofilm eradication concentration
MBL: metallo-β-lactamase
MDR: Multi-Drug-Resistant
MH: Muller-Hinton
MIC: minimum inhibitory concentration
MLST: multilocus sequence typing
MOX: moxifloxacin
MRSA: Methicillin-resistant *Staphylococcus aureus*
MSA: mannitol salt agar
MSSA: Methicillin-susceptible *Staphylococcus aureus*
NDM: New Delhi metal-β-lactamase
NET: netilmicin
OIE: World Organisation for Animal Health
ompA: outer-membrane protein A
OMPs: outer membrane porins
ODc: optical density Cut-off
Ori: origin of replication
OXA: oxacillin
OXA: oxacillinase
PBPs: penicillin-binding proteins
PBRT: PCR-based replicon typing

PBS: phosphate-buffered saline
PEN G: penicillin G
PFGE: pulse field gel electrophoresis
PG: polymer peptidoglycan
PMQR: plasmid-mediated quinolone resistance
Rep: replication initiator proteins
ST: sequence type
SXM: trimethoprim
SYNERCID: quinupristin and dalfopristin
TEICO: Teicoplanin
TET: tetracycline
TMP/SMX: trimethoprim-sulfamethoxazole
TOB: tobramycin
TSB: tryptic soy broth
TZP: piperacillin-tazobactam
UTI: urinary tract infection
VAP: ventilator associated pneumonia
VAN: vancomycin
VIM: Verona integron–encoded metallo-β-lactamase
WGS: whole-genome sequencing
WHO: World Health Organization
XDR: extensively-drug resistance

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Part I: Antimicrobial Drug Resistance in *Enterobacterales*

Chapter 1: General Introduction

I.1.1 Antibiotics: Discovery and Resistance from the Golden Age to the Modern Era

Antibiotics use is not limited to the modern antibiotic era. They have been utilized for at least 2000 years in the form of natural treatments from molds, plant extracts, and honey, among other things [1][2]. Some modern antibiotics may have been available since ancient times, such as tetracyclines, which traces were found in human skeletal [3]. Moreover, red soil was used to treat skin infections due to its richness in culturable antibiotic-producing *Actinomycetes*, with *Streptomyces* spp. being the well-known antibiotic producer genus (*i.e.*, of streptomycin, tetracycline, chloramphenicol, erythromycin, and vancomycin) [3][4][5].

Antibiotic resistance is also an ancient natural phenomenon that coexisted with antibiotic production by other microorganisms in the natural environment long before the antibiotic era [3][6][7]. Several mechanisms of resistance to antibiotics, including to β-lactams, tetracyclines, glycopeptides, aminoglycosides, chloramphenicol, and sulfonamides, were tracked back millions of years ago, along with their mobilization from bacterial chromosomes to plasmids [6][8][9]. One of these mechanisms is the β-lactamases production, that have originated more than a billion years ago, as shown by many structure-based phylogeny studies[3][10][11][12].

The history of antibiotic discovery can be traced back over a century. In 1877, Louis Pasteur unknowingly described the first antibiotic after observing antibiosis when an airborne bacillus inhibited the growth of *Bacillus anthracis*, indicating a bacterial therapeutic potential [13]. In 1930, the antibiotic era started after the introduction of Salvarsan drug, arsphenamine; to treat syphilis by Paul Ehrlich [14]. After the discovery of salvarsan, the sulfonamide Prontosil was introduced to treat tuberculosis by Gerhard Domagk in 1935. In 1940s, salvarsan was replaced with penicillin, the first commercialized antibiotic which was discovered by Alexander Fleming in 1928 [14][3]. The word "*antibiotic"* was introduced in 1941 as 'a compound made by a microorganism to destroy other microbes' by Seman Waksman, the initiator of the 'golden age of antibiotic discovery' (1940s - 1960s) [15]. In that period, thirteen classes of antibiotics were discovered, among which several are currently in clinical use (Fig. I.1.1) [16][17][18]. In the subsequent years after the "Golden Age" of antibiotic discovery, no new structural classes of antibiotics were introduced, only new analogs of known antibiotics were generated to deal with the successive emergence of antibiotic resistance in pathogenic bacteria (Fig.I.1.1) [18].

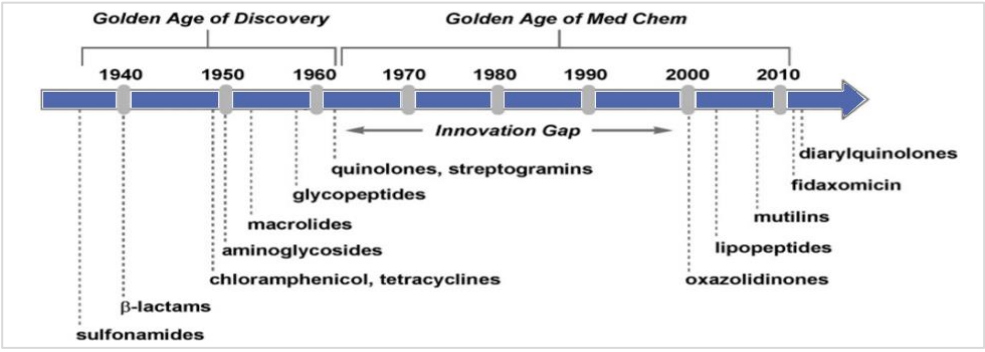

Figure I.1.1: Timeline showing the 'Golden Age' of antibiotic discovery (1940–1960), and the innovation gap (1962-2000) [19].

Like many therapeutic agents, the effectiveness of antibiotics can be compromised by the potential development of tolerance or resistance after first consumption [1].Resistance has been detected nearly to any antibiotics release, as for β-lactams, which efficacy has been eroded shortly after antibiotic deployment (Fig. I.1.2 and Fig. I.1.3) [18].

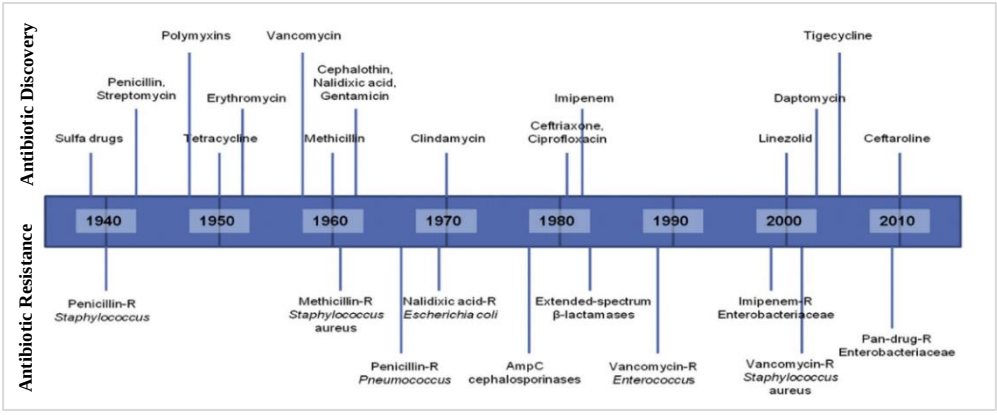

Figure I.1.2: Timeline of β-lactam drug discovery against the development of antibiotic drug resistance in pathogenic bacteria [20]**.**

The slow evolution of antibiotic resistant bacteria before the antibiotic era, cannot be compared to these days trend in terms of selection, diversity, and pace [21]. The high level of resistance, which is more evident these days, is mainly due to the insertion of new resistance genes into existing plasmids, which were not noticed in the pre-antibiotic era [22][23]. Moreover, subsequent cooperative mutations in different genes can be responsible of antibiotic resistance, as in the case of low-level quinolone resistance. To reach high-level fluoroquinolone resistance different mutations in *gyrA* and *grlA* and in regulatory sequences of efflux pumps are required [23].

The excessive and imprudent use of several classes of antibiotics in four main inter-connected sectors: human and animal medicine, zootechny, and agriculture accelerated the development and dissemination of different resistance mechanisms as a consequence of bacterial survival strategy [24][21][25].

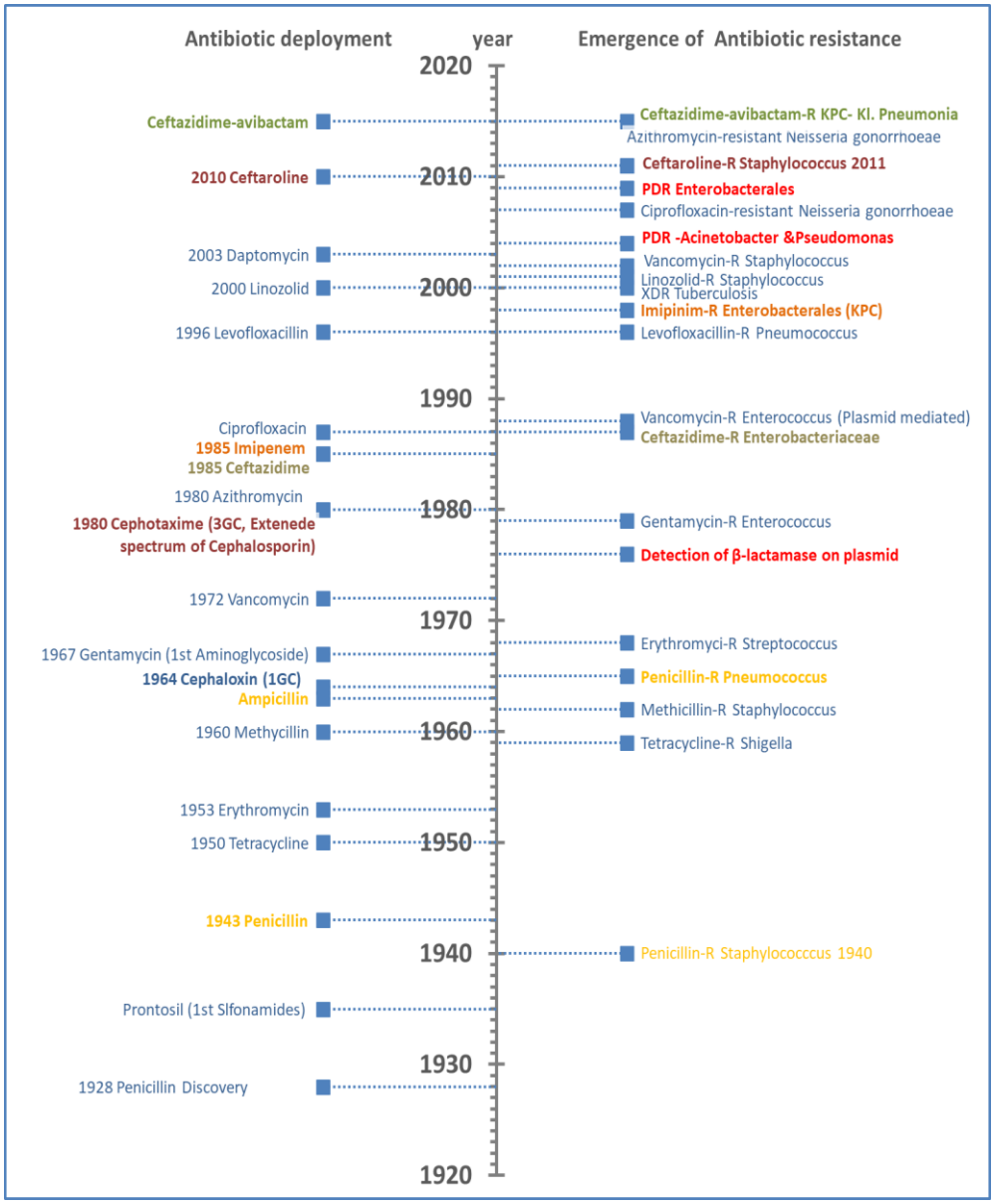

Figure I.1.3: Timeline of Antibiotic introduction against the development of antibiotic drug resistance in pathogenic bacteria, with emphasis on β-lactams [26][27][28][29].

I.1.2 Overview of Antimicrobial Agents

Antibiotics can be classified based on their mode of action into bacteriostatic agents inhibiting bacterial growth and bactericidal agents leading to the death of cells within 24 hours with >99.9% efficacy [30][31]. The Mode of action depends on bacterial targets, which usually involve essential physiological functions and biochemical reactions in bacteria. There are five primary antibiotic targets: the bacterial cell wall, cytoplasmic membrane structure, protein synthesis (i.e., 50S and 30S ribosome units), synthesis of DNA (i.e., DNA gyrase and DNA-directed RNA polymerase), and RNA (i.e., RNA elongation), and folic acid metabolism (Fig. I.1.4) (Table I.1.1) [32].

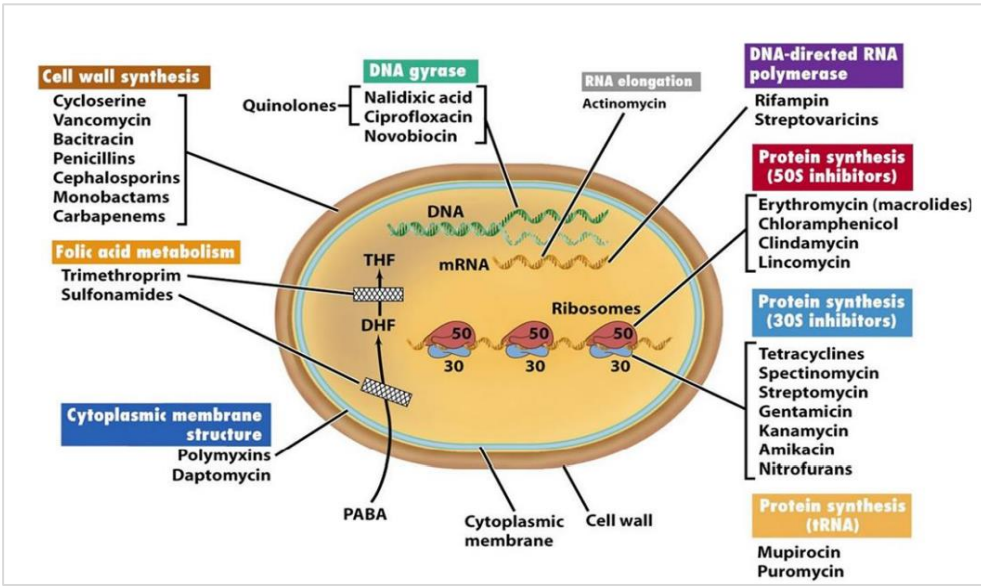

Figure I.1.4: Classes of antibiotics and targets among each class [32].

Antibiotics are characterized by selective toxicity having targets absent and/or different from those present in eukaryotic cells. β-lactam antibiotics, which contain a β-lactam ring, are generally the drugs of choice for treating and preventing many infections due to their bactericidal action, low toxicity, and the ability to be excreted in urine [31]. Penicillins, cephalosporins, monobactam aztreonam, and carbapenems differ in terms of the structural group fused to the β-lactam ring, giving them specific characteristics [31].

Table I.1.1: List of common antibiotics used to treat Gram-negative infections [31][33]

Antibiotic Class	Primary target	Antibiotic Name[a,b]	Spectrum
Cell Wall Synthesis Inhibitors			
β-lactam (Bactericidal)	Penicillin-binding proteins (Transpeptidase)	Penicillins - Natural pinicillins Example: Pinicillin G - Aminopinicillins (Broad spectrum) Examples: **ampicillin**, **Amoxacillin** - Uriedopinicillins (Extended spectrum) Examples: **piperacillin**, cloxacillin	- Natural Pinicillins: active against Gram-positive bacteria and some Gram-negative cocci - Usually combined with β-lactamase inhibitors
		Cephalosporins - 1GC (Narrow spectrum) Examples: Cefazolin, Cephalexin - 2GC (Extended spectrum) Examples: **Cefuroxime** - Cephamycins (Extended spectrum) Examples: Cefotetan, Cefoxitin - 3GC (Broader spectrum) Examples: **Cefotaxime**, **Ceftazidime** - 4GC (Further extended spectrum) Examples: **Cefepime** - 5GC Examples: **Ceftaroline**	- Pinicillinase resistant - Successive generations are more effective against a wider range of Gram-negative bacteria with lower susceptibility to destruction by β-lactamases - 1GC inhibit many Gram-positive bacteria and a few *Enterobacterales* - 2GC have improved coverage of *Enterobacterales* - 5GC have the unique ability to kill MRSA
		- Carbapenems Examples: **Meropenem**, E**rtapenem**, **Imipenem**	- Broadest spectrum of all β-lactam antibiotics.
		- Monobactams The only one: **Aztreonam**	- Exclusively against Gram-negative bacteria, primarily members of the family *Enterobacterales*
β-lactamase inhibitors	β-lactamase preventing enzymatic inactivation of β-lactams	clavulanic acid, sulbactam and tazobactam, avibactam	- When used in combinations with an appropriate β-lactam enhances its spectrum
Outer and cytoplasmic membrane disruptors			
Polypeptides (Bactericidal)	Lipopolysaccharide destruction due to acting as a cationic detergent	**Polymyxin B, Colistin**	- Against Gram-negative cells by damaging cell membranes - Significant toxicity when administered systemically
DNA Synthesis Inhibitors			
Fluoroquinolones (Bactericidal)	Topoisomerase II (DNA gyrase), topoisomerase IV	Nalidixic acid, **ciprofloxacin**, **levofloxacin**, sparfloxacin, norfloxacin	- Wide variety of Gram-positive and Gram-negative bacteria
Sulfonamides (Bacteriostatic)	Competitive inhibitor for DHPS, involved in folate synthesis	Sulfamethazine, sulfapyridine, **sulfamethoxazole**, sulfadiazine, sulfamerazine	- Against a variety of Gram-positive and Gram-negative bacteria
Protein Synthesis Inhibitors			
Tetracyclines (Bacteriostatic)	30S ribosome (inhibit aminoacyl tRNA binding to ribosome)	Oxytetracycline, doxycycline, **tetracycline**, demeclocycline, minocycline	- Some Gram-positive and Gram-negative bacteria Bacteriostatic
Aminoglycosides (Bactericidal)	30S ribosome (mistranslation by tRNA mismatching	boromycin, **gentamicin**, **amikacin**, **tobramycin**, streptomycin, spectinomycin	- Gram-negative aerobic and facultative bacteria
Macrolides (Bacteriostatic)	50S ribosome (stimulating dissociation of the peptidyl-tRNA molecule from the ribosomes during elongation)	Erythromycin, **azithromycin**	- Azithromycin enhanced the Gram-negative spectrum
Amphenicols (Bacteriostatic)	50S ribosome (inhibit elongation step)	**Chloramphenicol**	- Gram-positive bacteria as well as some Mycobacteria

[a]Antibiotics in bold are representative drugs among the class. .[b]1GC, first-generation cephalosporins (Narrow spectrum Cephalosporins); 2GC, second-generation cephalosporins (Extended-spectrum cephalosporins); 3GC, third-generation cephalosporins (Broad spectrum cephalosporins); 4GC, fourth-generation cephalosporins (Extended-spectrum cephalosporins); 5GC, fifth-generation of cephalosporins.

I.1.3 General Mechanisms of Antimicrobial Resistance

Antibiotics act as selectors, promoters, and accelerators of antibiotic resistance [21][34]. In the presence of antibiotics, bacteria with resistance mechanism will be positively selected, whereas the sensitive ones will be cleared from the population [21]. Furthermore, they can act as promoters of resistance by elevating mutational rate, leading to faster acquisition of acquired resistance, and accelerating the evolution of mechanisms of resistance (which can be clearly seen through the recent diversity of β-lactamases in the clinical setting despite being ancient enzymes) [21][34][35].

Essentially, antibiotic resistance is a result of bacterial genetic plasticity in response to high concentrations of antibiotics, enabling the 'survival of the fittest" through mutational adaptations, genetic material acquisition, or upregulation or downregulation of gene expression [36]. The selective pressure of the resistant bacteria allows the spread of resistant clones along with their mechanism of antibiotic resistance.

Antibiotic resistance can be intrinsic (Natural) or acquired (Fig. I.1.5) [36]. Intrinsic resistance refers to the natural presence of genes in the bacterial genomes that have evolved due to exposure to naturally produced antibiotics to overcome their effect in nature. Basically, when these genes are expressed, they can generate a resistance phenotype, such as intrinsic AmpC β-lactamase expressed by some Gram-negative bacteria and multidrug resistance efflux pumps found in many other bacteria [1]. On the other hand, acquired resistance, mainly referred to in clinical settings, occurs in bacterial populations that were initially susceptible to a specific antibiotic [37]. Acquired resistance manifests itself following mutations in genes targeted by the antibiotic, or the transfer of resistance genes through horizontal gene transfer (HGT) of mobile genetic elements, such as plasmids, most probably from intrinsically resistant bacteria from the same or different genera present in the environment [1][37][36].

As a result of these adaptations, bacteria can become resistant to antibiotics through several mechanisms, including (i) changing the permeability in the bacterial cell wall, which restricts antimicrobial access to target sites, (ii) active efflux of the antibiotic from the microbial cell, (iii) enzymatic modification of the antibiotic, (iv) degradation of the antimicrobial agent, (v) acquisition of alternative metabolic pathways to those inhibited by the drug, (vi) modification of antibiotic targets, and (vii) overproduction of the target enzyme (Fig. I.1.5) [38].

Some of these mechanisms are found in intrinsically resistant Gram-positive and Gram-negative bacteria, such as the presence of a thick peptidoglycan layer in Gram-positive and the outer membrane in Gram-negative, which makes the bacteria impermeable to some antibiotics[39]. Another intrinsic resistance mechanism

includes the expression of efflux pump, in addition to the absence of antibiotic targets, making all the bacterial species naturally resistant[39].

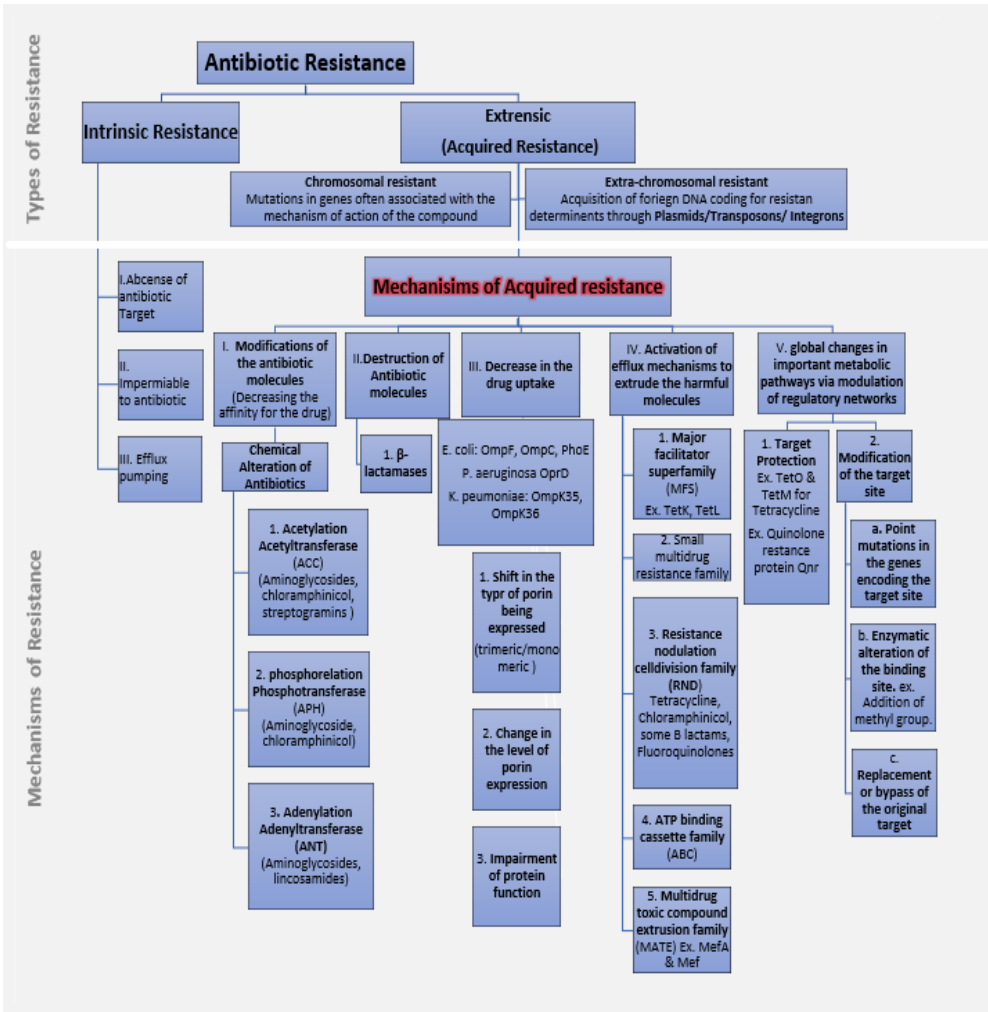

Figure I.1.5: Mechanisms of Antibiotic resistance.

I.1.4 Infectious diseases: Healthcare-associated (HCAIs) and Community-acquired infections (CAIs)

Human infections are usually categorized into healthcare-associated infections (HCAIs), previously called nosocomial infections, and community-acquired infections (CAI). WHO defined HAIs as "infections acquired in a hospital or in other healthcare facilities, not presented/incubated at the time of admission" (http://www.who.int/en/). Haque *et al.*, in their review, define these infections as "acquired infections that occurred and developed in a hospital or other healthcare facility with symptoms that appear 48 hours or more after hospital admission or within 30 days after receiving healthcare" [40]. Usually, infections to be considered hospital-acquired should meet at least one of five criteria, including (i) being a resident of a long-term care facility or a nursing home, (ii) being hospitalized in an acute care hospital for two or more days in the last three months, (iii) being in a hospital or hemodialysis clinic to receive intravenous chemotherapy in the last month, (iv) receiving healthcare at home such as receiving intravenous therapy at home, being in a wound care facility, or (v) being administered or being in contact with somebody that was administered to a healthcare facility in the previous month [41].

According to the CDC and National Healthcare Safety Network, HCAI can be classified into 13 major types containing about 50 specific infection sites with surgical wound and other soft tissue infections, urinary tract infections, respiratory infections, gastroenteritis, and meningitis are the most common HCAI [42]. Moreover, many HCAIs are associated with implants and prostheses, including bloodstream infections, catheter-associated UTIs, and ventilator-associated pneumonia [40].

Many microorganisms are associated with HCAI infections, including protozoans, fungi, viruses, mycobacteria, and bacteria. However, bacteria represent the most prevalent etiological agent accounting for 90 % [43]. The bacteria that are usually associated with HCAI are *Staphylococcus aureus*, enterococci (*Enterococcus faecalis* and *E. faecium),* coagulase-negative staphylococci members, *Streptococcus*spp., *Enterobacter* spp., *Pseudomonas aeruginosa*), *Acinetobacter baumannii*, *Legionella* spp., *Bacillus cereus,* and *Enterobacterales* family members, including *Proteus mirabilis*, *Klebsiella pneumoniae*, *Escherichia coli,* and *Serratia marcescens. Enterococci*, *P. aeruginosa*, *S. aureus*, *K. pneumoniae*, and *E. coli* are the most common etiological agent in HCAI (Table I.1.2) [43][42]. Many Gram-negative bacteria exhibit multidrug-resistant (MDR) phenotypes, particularly the ones isolated from devices showing the highest level of resistance [40]. In hospitals, such organisms can be acquired from (i) having direct contact with other patients or hospital staff, (ii) hospital environment and shared equipment, or (iii) the emergence of resistance to specific drugs in a susceptible isolate that was harbored before patient's admission, after which become epidemiologically significant [44].

Table I.1.2: Most common nosocomial pathogens along with infection manifestations and mode of transmission [45]

Nosocomial pathogen	Infection	Mode of transmission	Virulence factors / antibiotic resistance
***Staphylococcus aureus* (MRSA)**	Bacteremia, pneumonia, cardiovascular infections, Superficial tissue (i.e., Surgical site, ocular infections) and deep tissues (i.e., lower respiratory tract infections)	Skin and surface contact.	Many structural and secreted products play a role in the pathogenesis of various tissues
Escherichia coli	Urinary tract infections (UTI), septicemia, pneumonia, neonatal meningitis, and peritonitis gastroenteritis	Skin and surface contact, contaminated food, and water.	Endotoxin, cytotoxins, capsule, antigenic phase variation, sequestration of growth factors, resistant to serum killing, and antimicrobial resistant
Enterococcus faecalis and E. faecium	Blood-borne infections, urinary tract infections, and wound infections	Patients with diarrhea; contact with surfaces in the patient's room	
Klebsiella pneumoniae	Septicemia, pneumonia, and wound infections	Contact with: Person to person, respiratory machines, catheters, and open wounds	Showing mostly Multidrug resistance phenotype, cytotoxins,
Pseudomonas aeruginosa	UTI, surgical wound infections, pneumonia, cystic fibrosis, and bacteremia	Skin contact with: Breast pumps, incubators, sinks and hand soups.	Pili, enzymes (elastases, proteases, phospholipase C), and (exotoxin A).
Carbapenem resistant *Acinetobacter baumannii*	Bacteremia and wound infections	Skin contact, infected wounds, intravascular and urinary catheters	Showing mostly Multidrug resistance phenotype
Clostridium difficile	Colitis	Person to person and contact with hospital settings surfaces.	

CAIs are infections that develop outside healthcare settings, or infections that appear on admission [46]. The three most prevalent CAIs are: (i) community-acquired respiratory infections, particularly the ones associated with *Haemophilus influenzae* or *Streptococcus pneumoniae* strains, (ii) community-acquired urinary tract infections, particularly the ones associated with ESβL producing *Enterobacterales*; particularly the ones associated with, and (iii) community-acquired bloodstream infections, particularly the ones associated with methicillin-resistant *S. aureus* (MRSA), and ESβL /Metallo-β-lactamase-producing *Enterobacterales* [46].

I.1.5 “One Health” Approach

Antimicrobial resistance (AMR) is considered a global health problem and recognized as One Health challenge because it involves humans, animals (domestic and wild animals), and ecosystems, affecting public health and global economy [47][47]. With the continuous AMR current trends, the number of deaths can reach 10 million annual AMR-associated deaths from a wide range of infections by 2050. Moreover, The World Health Organization (WHO) stated that AMR is one of the top ten global health threats of the 21st century in 2019 [48].

To counteract AMR, it is necessary to apply “One Health” approach, which recognizes that human health is closely connected to the health of animals and the environment due to the intensive contact between humans, domestic and wild animals [47][49].

The concept of One Health is old and can be tracked back for at least two hundred years, where it was coined firstly as One Medicine, then as One World, One Health and finally as One Health [50]. The most frequently used definition is: “One Health is defined as a collaborative, multisectoral, and transdisciplinary approach—working at the local, regional, national, and global levels—with the goal of achieving optimal health outcomes recognizing the interconnection between people, animals, plants, and their shared environment” [50]. This approach considers the health of the human population, animals (domestic animals and wildlife), and the state of the ecosystems are fundamentally linked and affected by each other (Fig. I.1.6) [51].

Antimicrobial overuse and misuse are occurring in three main sectors involving humans, animals, and agriculture, exposing microorganisms in these sectors to antimicrobials and forcing a selection pressure to adapt and enhance their fitness by acquiring, expressing, and sharing resistance genes (Fig. I.1.6) [52].

Most antimicrobial classes are used both in human and animal medicine (domestic and livestock), apart from a few antimicrobial classes, which are reserved exclusively for human use, such as carbapenems, or veterinary use, such as flavophospholipols, and ionophores, due to their toxicity to humans [52]. Antimicrobials are also used in horticulture, such as Tetracyclines, and streptomycin, even for insects (i.e., bees) [52]. For humans and companion animals, antimicrobials are mostly used for treating infections and occasionally for prophylactic use. However, in livestock farms, antimicrobials are intensively used and introduced to animals through feed or water to healthy animals at risk of infections as prophylactic use or to healthy animals in contact with diseased animals as metaphylactic use. Furthermore, antimicrobials can be administered to healthy animals on a regular basis at sub-therapeutic doses to improve growth as they work as growth promoters. Indeed, most antimicrobials are consumed for prophylaxis and metaphylaxis.

Animals are recognized as a relevant reservoir of MDR bacteria and a source of spreading through human contact and food-chain. It is well known that antimicrobial resistant organisms, including MDR, are found in humans, domestic and wild animals, food, plants, and even in natural environments, including water, soil, and air (Fig. I.1.6). Due to the intensive contact between all these parties, AMR can spread rapidly which as observed in many studies. In this context, it is necessary to apply the "One Health" approach to tackle and counteract AMR.

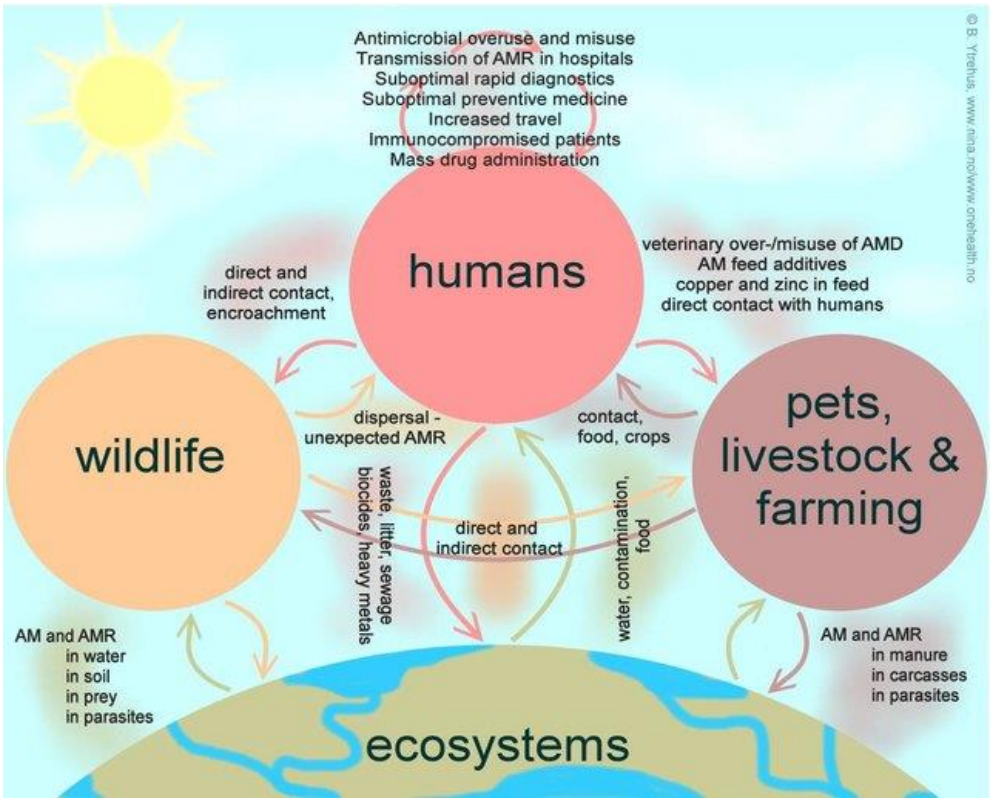

Figure I.1.6: Schematic representations of "One Health" approach showing the drivers of antibiotic resistance that facilitate the spread of AMR genes and resistant pathogens into humans, animals (domestic and wildlife), and ecosystems considering that the health of these three parties are connected and affected by each other [51].

The WHO Plan adopts the "One Health" approach to combat by developing a global plan consisting of five main pillars: 1. Increasing the awareness of antimicrobial resistance through active communication, education, and training 2. improving the knowledge and providing evidence through surveillance and research, 3. Taking safety measures to prevent the spread of antimicrobial resistance, 4. Stopping the overuse of antimicrobials in human and animal medicine, 5. Supporting and increasing investment in research that target new medicines, diagnostic tools, vaccines, and other areas related to antimicrobial resistance [52].

In 2017, WHO issued a list of antibiotic-resistant priority bacterial pathogens categorizing the priority pathogens into three groups, priority 1, critical; priority 2, high; priority 3, medium; (Table I.1.3) [53]. Moreover, the United States centers for disease control and prevention (CDC), in 2019, classified antimicrobial resistant pathogens into three classes, urgent, serious, and concerning threats, considering the level of urgency, severity, morbidity, and mortality that these pathogens cause (Table I.1.4) [26].

Table I.1.3: WHO priority list of antibiotic resistant pathogens published in 2017

Bacterial Pathogen	Antibiotic resistance
Priority 1: CRITICAL	
Acinetobacter baumannii,	carbapenem-resistant
Pseudomonas aeruginosa	carbapenem-resistant
Enterobacterales	carbapenem-resistant, ESBL-producing
Priority 2: HIGH	
Enterococcus faecium	vancomycin-resistant
Staphylococcus aureus	methicillin-resistant, vancomycin-intermediate, and resistant
Helicobacter pylori	clarithromycin-resistant
Campylobacter spp.,	fluoroquinolone-resistant
Salmonellae	fluoroquinolone-resistant
Neisseria gonorrhoeae	cephalosporin-resistant, fluoroquinolone-resistant
Priority 3: MEDIUM	
Streptococcus pneumoniae	penicillin-non-susceptible
Haemophilus influenzae	ampicillin-resistant
Shigella spp.,	fluoroquinolone-resistant

Table I.1.4: CDC list of threats to public health published in 2019

Urgent threats	Serious threats	Concerning threat
carbapenem-resistant *Acinetobacter baumannii*,	Drug-resistant *Campylobacter*	Erythromycin-resistant group A *Streptococcus*
Candida auris	Drug-resistant *Candida*	Clindamycin-resistant Group B *Streptococcus*
Clostridioides difficile	ESBL-producing *Enterobacterales*	Watch list
Carbapenem-resistant *Enterobacterales* (CRE)	Vancomycin-resistant *Enterococci* (VRE)	Azole-resistant *Aspergillus fumigatus*
Drug-resistant *Neisseria gonorrhoeae*	Multidrug-resistant *Pseudomonas aeruginosa*	Drug-resistant *Mycoplasma genitalium*
	Drug-resistant non-typhoidal *Salmonella*	Drug-resistant *Bordetella pertussis*
	Drug-resistant *Salmonella* serotype Typhi	
	Drug-resistant *Shigella*	
	Methicillin-resistant *Staphylococcus aureus* (MRSA)	
	Drug-resistant *Streptococcus pneumoniae*	
	Drug-resistant Tuberculosis	

I.1.6 *Enterobacterales*: *Klebsiella pneumoniae* and *Escherichia coli*

Gram-negative bacteria, specifically *Enterobacterales*, including *E. coli*, *Klebsiella* spp., and *Enterobacter* spp., are common causes of both CAI and HCAI [54]. *Enterobacterales* comprise a modest quantity ($1/10^4$%) of intestinal microbiota colonizing mainly the lower gastrointestinal tract, infrequently female genital tract, and as transient colonizers of the skin. *E. coli* is the most prevalent species of *Enterobacterales* found among the normal flora, followed by *Klebsiella* spp., with *K. pneumoniae* being the most prevalent specie in this genus [55].

Enterobacterales are facultative anaerobic, rod-shaped, non-spore-forming bacteria that can be motile or nonmotile (Fig. I.1.7A) [55]. All *Enterobacterales* are oxidase negative, able to reduce nitrates to nitrites, and ferment glucose and lactose producing 2-5 mm pink colonies after overnight incubation on MacConkey agar. MacConkey agar is a selective/differential media selecting non-fastidious Gram-negative rods due to the presence of crystal violet and bile salts inhibiting the growth of Gram-positive bacteria and the more fastidious Gram-negative organisms, and it is differential due to the presence of lactose and red dye which makes the colony appears pink if the bacteria are lactose fermenters (Fig. I.1.7B). Indole production is one of the biochemical tests that distinguish *E. coli.* Among *Enterobacterales, K. pneumoniae* is the most peculiar specie due to the absence of motility and the presence of a polysaccharide capsule making the colonies shiny and mucoid on agar (Fig. I.1.7C).

Figure I.1.7: Phenotypes of *Enterobacterales*. A. Gram-stained *E. coli*. Magnification, 100×. B. *E. coli* grown on MacConkey agar showing pink colonies. C. *K. pneumoniae* grown on MacConkey agar showing pink shiny mucoid colonies.

Generally, Gram-negative bacteria cause significant morbidity and mortality worldwide [55]. This is mainly due to the presence of an outer membrane making them resistant to a wide range of antibiotics, such as β-lactams, quinolones, and aminoglycosides (Fig. I.1.8). This outer membrane act as a barrier allowing hydrophobic molecules to diffuse through it, while hydrophilic antibiotics, such as β-lactams, need porins to pass through it, while other antibiotics are prohibited completely from passing due to their structure (Fig. I.1.8). Resistance can be exhibited if the outer membrane is altered either by changing the hydrophobic properties or modifying porins' structure through mutations. Cell surface polysaccharides may form a well-defined capsule or an amorphous slime layer (K-antigen). Motile strains have flagella, which extend outside the cell wall (H-antigen).

Almost all *Enterobacterales* members have enterobacterial common antigen, and many have surface pili (fimbriae).

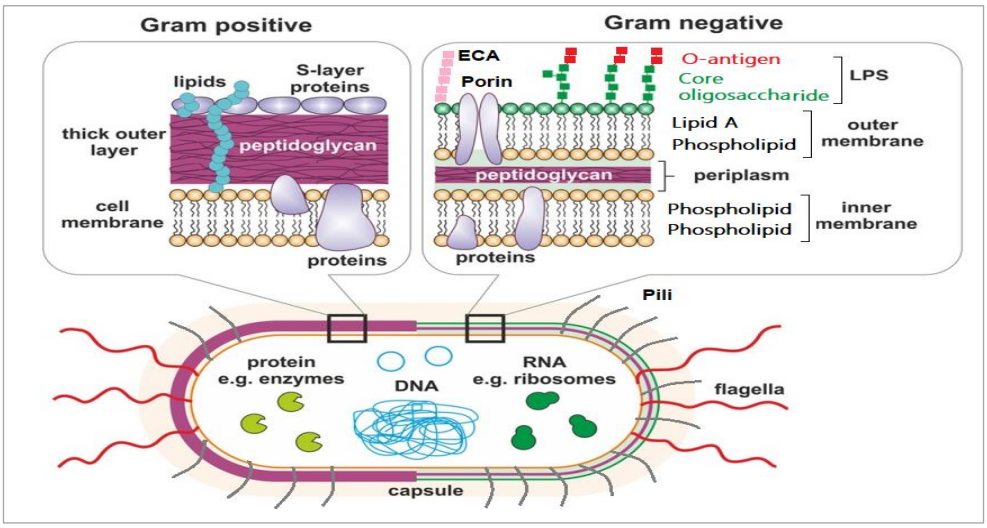

Figure I.1.8: Schematic representation of structure and organization of bacterial cell wall showing Gram-positive and Gram-negative bacteria (Modified after [56]).

Gram-negative bacteria are considered major human pathogens whose continuous increasing antibiotic resistance presents a significant health threat [57]. One of the main opportunistic Gram-negative pathogens is *K. pneumoniae*, which causes many infections such as pneumonia, sepsis, and urinary tract infections. *E. coli* is another opportunistic pathogen that causes infections mainly in the renal system and occasionally int the central nervous system, and some strains of *E. coli* can cause intestinal infections [55][58].

In the context of AMR, *Enterobacterales* spp. are of particular concern due to their ability to develop resistance to antibiotics, making available antibiotics ineffective, including cephalosporins, used as first-line treatment, and carbapenems, used as a last-line antibiotic [59][60]. According to the WHO antimicrobial resistance report, *Enterobacterales*, carbapenem-resistant or ESβL-producers, are classified as a critical group, the highest level, of pathogens that pose the most extreme public health risks worldwide [59]. According to CDC report of antimicrobial-resistant pathogens, carbapenem-resistant *Enterobacterales* (CRE), *i.e*., *Klebsiella* spp., and *E. coli*, are the most urgent resistance threats worldwide [60]. *Klebsiella* spp., and *E. coli* are the most found microorganisms with multi-drug resistance phenotypes with *Klebsiella* spp. currently being the most resistant to antibiotics among all *Enterobacterales* [55].

I.1.7 β-lactams: Overview and Resistance Mechanisms

I.1.7.1 Overview of β-lactams

β-lactams are one of the oldest and most used treatments for bacterial infections worldwide due to their bactericidal action, low toxicity, and the ability to be excreted in urine [31]. β-lactams are grouped based on their chemical structure into penicillins, cephalosporins, carbapenems, and monobactams [61]. All β-lactams have β-lactam ring which is responsible for the bactericidal activity of these agents. β-lactams interfere with transpeptidation reactions by binding to penicillin-binding proteins (PBPs) during the synthesis of peptidoglycan, which is the final step of cell wall synthesis. PBPs are membrane-anchored transpeptidases that catalyze the formation of peptide cross-links. β-Lactams' binding to PBPs blocks the transpeptidation activity affecting cell wall integrity weakening the cell wall, and eventually leading to osmotic lysis [62].

I.1.7.1.1 Penicillins

Penicillin G is the oldest penicillin discovered by Alexander Fleming in 1928 and named after the molds of the genus *Penicillium*. It was introduced into clinical treatment in the 1940s and it is still bactericidal to Gram-positive organisms and a few spirochetes, including *Treponema pallidum,* which causes syphilis [61]. Most Gram-negative bacilli are not affected due to the presence of an outer membrane preventing penicillins from reaching their targets.

Penicillins are still effective in clinical treatment because of three major strategies in drug development [61]. These strategies include (i) developing semisynthetic narrow-spectrum penicillins, such as methicillin, nafcillin, oxacillin, which are susceptible to staphylococcal penicillinase, and effective against methicillin-susceptible *S. aureus* (MSSA);(ii) introducing broad-spectrum penicillins, such as aminopenicillins, ampicillin, and amoxicillin, which have bactericidal activity against some Gram-negative bacteria, but susceptible to staphylococcal penicillinases, and; (iii) the use of penicillins with β-lactamase inhibitors which allow them to resist the hydrolytic action of bacterial β-lactamases.

I.1.7.1.2 Cephalosporins

Cephalosporins structure confers resistance to the hydrolysis by staphylococcal penicillinase and some β-lactamases produced by Gram-negative bacilli [61]. The cephalosporins are divided into first-, second-, third-, fourth-, and fifth- generation based on the chronological sequence of development accompanied with expanding the antimicrobial spectrum through modification of the side chains (Table I.1.5) [61][63]. Typically, higher generation of cephalosporin is associated with a wider spectrum, higher activity (lower MIC) against Gram-negative bacteria, and lower activity (higher MIC) against Gram-positive bacteria [61].

Table I.1.5: Major group of cephalosporins according to their antimicrobial activity [63]

First-generation	Second-generation	Third-generation	Fourth-generation	Fifth-generation
Cephalothin	Cefamadole	Cefotaxime	Cefepime	Ceftobiprole
Cepharipin	Cefuroxime (Oral)	Ceftizoxime	Cefpirome	Ceftaroline
Cefazolin	Cefonicid	Ceftriaxone		Ceftolozane
Cephalexin (Oral)	Ceforanid	Ceftazidime		
Cephadrine (Oral)	Cefoxitin	Cefoperazone		
Cefadroxil (Oral)	Cefmetazole	Cefixime		
	Cefminox	Ceftibuten		
	Cefotetan	Cefdinir		
	Cefaclor (Oral)			

First-generation cephalosporins are effective against Gram-positive organisms except methicillin-resistant staphylococci (MRSA) and *Enterococcus* spp., and relatively active against some Gram-negative bacilli, such as *E. coli* and *Klebsiella* spp. [63]. First-generation cephalosporins can substitute penicillin in early infection treatment. Usually, first-generation cephalosporins must be introduced intravenously to obtain significant concentrations in blood and tissues; however, cephalexin, cephadrine, and cefadroxil are introduced orally to treat both urinary and respiratory tract infections, as they are absorbed in the gastrointestinal tract. First-generation cephalosporins are not usually used for the management of nervous system infections due to their inability to penetrate the central nervous system.

Second-generation cephalosporins have a heterogenous spectrum with activity against organisms covered by first-generation drugs, and aerobic and anaerobic Gram-negative rods, such as *Enterobacterales* spp. and *Proteus* spp., but not *P. aeruginosa* [61]. For example, Cefaclor and cefuroxime are administered orally to treat sinusitis and otitis caused by *Haemophilus influenzae*, including β-lactamase-producing strains [63]. Moreover, cephamycins, such ascefoxitin and cefotetan, can treat Bacteroides fragilis anaerobic infections, including peritonitis or pelvic inflammatory disease.

Third-generation cephalosporins have a wider spectrum of activity against Gram-negative organisms and decreased activity against Gram-positive cocci [61]. Third-generation drugs are less susceptible to β-lactamases than first and second generations, particularly β-lactamases produced by *Klebsiella*, *H. influenzae*, and *E. coli*. Cefoperazone and ceftazidime are usually active against *P. aeruginosa* and useful for managing hospital-acquired Gram-negative bacteremia. Generally, they can be used for the empiric or specific treatment of spontaneous bacterial peritonitis, genitourinary tract infections, bone and joint infections, pneumonia such as *Pseudomonas* pneumonia, skin and soft tissue infections, gram-negative sepsis, streptococcal endocarditis, and central nervous system infections, specifically Gram-negative meningitis, due to their ability to cross the blood-brain barrier [64]. Sometimes, these drugs used in combination with other antibacterial agents such as penicillins, β-lactamase inhibitors, and aminoglycosides. Ceftazidime-avibactam has

been used effectively in treating *Enterobacterales* infections, intra-abdominal and urinary tract infections, sepsis, and pneumonia.

Fourth-generation cephalosporins have improved ability to cross the outer membrane of Gram-negative bacteria with wider activity spectrum against *Enterobacterales* and *P. aeruginosa* and show more resistance to many Gram-negative β-lactamases [61]. These cephalosporins maintained the high affinity of third-generation drugs and the antibacterial activity against *Neisseria* and *H influenzae*. Cefepime is widely used in treating pneumonia, caused by *Streptococcus pneumoniae*, *P. aeruginosa*, *K. pneumoniae*, or *Enterobacter* spp. [64]. Moreover, it is used for uncomplicated/complicated urinary tract infections caused by *E. coli* or *K. pneumoniae*, and meningitis [64].

Fifth-generation cephalosporins, including ceftobiprole, ceftaroline, and ceftolozane, were developed in the laboratory to target specific resistant strains [61][64]. Fifth-generation cephalosporins are mainly against Gram-positive bacteria with continued activity against *Enterobacterales*. Ceftolozane binds to the altered penicillin-binding protein (PBP-2A) that confers resistance to other β-lactam antibiotics in MRSA [61]. Using ceftolozane in combination with the β-lactamases inhibitor tazobactam usually shows successful management of infection caused by MDR *P. aeruginosa*, urinary tract and complicated intra-abdominal infections [64].

I.1.7.1.3 Carbapenems

Carbapenems include imipenem, meropenem, ertapenem and doripemen, which have the broadest spectrum of all β-lactam antibiotics due to their easy penetration of Gram-negative and Gram-positive bacterial cells and high level of resistance to β-lactamases [61]. Imipenem, meropenem, and doripemen are active against streptococci, gonococci, including both β-lactamase-positive and -negative strains, *H. influenzae*, and Gram-negative rods. Ertapenem exhibit a similar spectrum to other carbapenems except its inefficacy against *Pseudomonas* spp.. Imipenem is the carbapenem of choice against Gram-positive pathogens, it is administered together with an inhibitor of renal tubular dehydropeptidase-1, cilastatin, due to the ability of renal tubular dehydropeptidase-1 to rapidly hydrolyze imipenem. Other carbapenems do not need the co-administration of cilastatin because they are not significantly degraded by dehydropeptidase-1.

I.1.7.1.4 Monobactams

The monobactam Aztreonam is the first monobactam introduced to clinical medicine with a spectrum restricted to aerobic and facultatively anaerobic Gram-negative bacteria, including *Enterobacterales*, *P aeruginosa*, *Haemophilus* spp., and *Neisseria* spp., with no activity against Gram-positive and anaerobic bacteria [61]. It is worth mentioning that Aztreonam exhibits high resistance to hydrolysis by β-lactamases of *Enterobacterales*.

I.1.7.1.5 β-Lactamase Inhibitors

β-Lactamase Inhibitors, also known as suicide inhibitors, are molecules with almost no antibacterial activity are capable of binding irreversibly to β-lactamase enzymes, making them inactive [61]. They are resistant to staphylococcal penicillinases and broad-spectrum β-lactamases, while more susceptible to cephalosporinases. Classical β-Lactamase Inhibitors include clavulanic acid, sulbactam and tazobactam, which are β-lactam based that inactivate class A and some class C serine β-lactamases. Using these β-Lactamase Inhibitors, in combination with appropriate β-lactams protects the last one from being destructed by many β-lactamases and significantly improves its spectrum. These combinations include amoxicillin/clavulanate, ticarcillin/clavulanate, ampicillin/sulbactam, and piperacillin/tazobactam.

Newer β-lactamase inhibitors include avibactam, vaborbactam, and relebactam based on non-β-lactam structures and their inhibition activity extends to *Klebsiella Pneumoniae* Carbapenemase (KPC) enzymes (Fig. I.1.9) [65]. Avibactam is a synthetic diazabicyclooctane, which inhibits Ambler class A and C and some class D β-lactamases. Vaborbactam is the first boronic acid β-lactamase inhibitor, which exhibits potent inhibition activity against KPC enzymes, and other Ambler class A and C enzymes. Relebactam is a bridged bicyclic urea molecule with a broad spectrum against class A and C β-lactamases. It is worth mentioning that all of these β-lactamase inhibitors with their antibiotic recombination have no activity on several class D and class B β-lactamases.

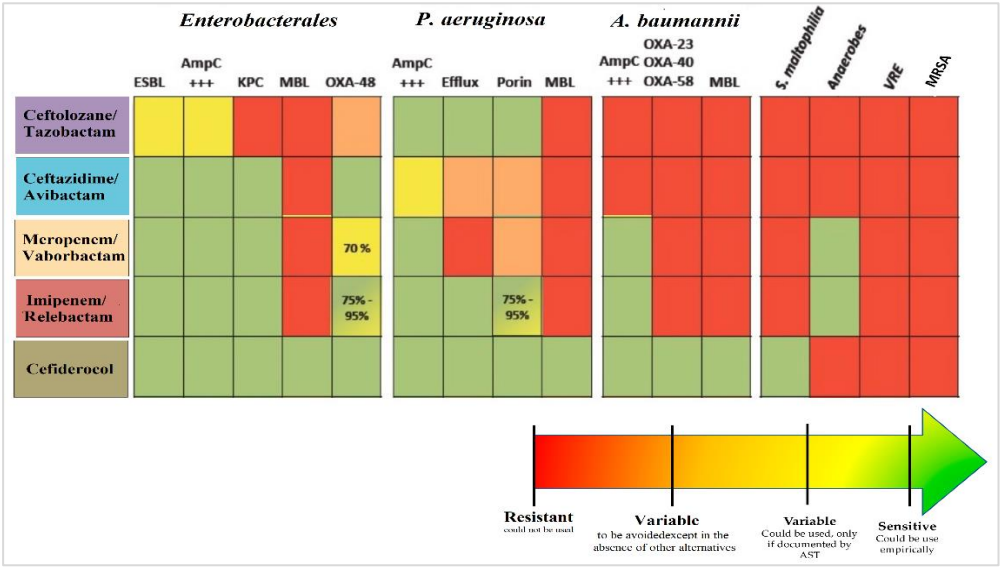

Figure I.1.9: Spectrum of activity of new β-lactamase inhibitors/antibiotics and new antibiotics. AST, Antibiotic susceptibility test; ESBL, Extended-spectrum β-lactamase; AmpC, Cephalosporinase; KPC, *Klebsiella pneumonia* carbapenemase; MBL, metallo-β-lactamase; VRE, vancomycin resistant *Enterococci*; MRSA, Methicillin-resistant *S. aureus* [66].

I.1.7.2 Mechanisms of β-lactams Resistance

The β-lactam antibiotics are a wide class of antibiotics commonly prescribed for treating and preventing many infections due to their bactericidal action, low toxicity and the ability to be excreted in the urine [31]. β-lactams are a wide class of antibiotics, including penicillins, cephalosporins, monobactam aztreonam, and carbapenems. Mechanisms of resistance to β-lactam include (i) synthesis of β-lactamases, (ii) modifications in membrane porins, and (iii) modifications in Penicillin-Binding Proteins, with the first one being the most common resistance mechanism for this class, especially in Gram-negative bacteria including *Enterobacterales* [67].

I.1.7.2.1 β-lactamases

β-lactamase enzymes confer resistance against β-lactams by attacking of the β-lactam ring, essential for β-lactams antimicrobial activity [68]. Ambler classification divides β-lactamases into A, C, D, and B based on amino acid sequence, with the first three being serine proteases having a serine residue in the active site and the last one being metal proteases having zinc in the active site (Fig. I.1.10). β-lactamases of classes A, B, and C have approximately 16% of amino acid identity [68]. In the functional classification of Bush-Jacobi-Medeiros, β-lactamases are classified into class1, class 2, and class 3 depending on β-lactam substrates and the effects of inhibitors (Fig. I.1.10) [12]. Group 1 consists of cephalosporinases classified as class C based on Ambler classification. Group 2 includes β-lactamases other than those in group 1 including classes A and D of Ambler classification. Group 3 consist of metallo-β-lactamases (MBLs) and corresponds to class B, based on Ambler classification [69].

1. Class A

Class A β-lactamases occur in both Gram-positive and Gram-negative bacteria and mostly mediated by plasmids or transposons (Fig. I.1.10). [67]. (i) class A penicillinases belong to the Bush-Jacoby functional subgroup 2a, such as PC1, can hydrolyze a limited spectrum of penicillins. (ii) Class A narrow-spectrum β-lactamases belonging to the Bush-Jacoby functional subgroup 2b, such as TEM-1, TEM-2, and SHV-1, can degrade early cephalosporins and pinicillins [69]. (iii) Class A Extended-spectrum β-lactamases (ESβLs) belong to the Bush-Jacoby functional subgroup 2be such as TEM-3, SHV-2, and CTX-M, can hydrolyze third-generation cephalosporins, such as cefotaxime and ceftazidime, along with penicillins, and they are characterized by their susceptibility to classical β-lactamase inhibitors (clavulanic acid, sulbactam, and tazobactam). ESβLs, which belong to the Bush-Jacoby functional subgroup 2be, originated from 2b. ESβLs are originated from genes of the narrow-spectrum β-lactamases. They are typically encoded by plasmids that can be exchanged between bacterial species. ESβLs variants are classified into nine distinct structural and evolutionary families based on their amino acid sequences such as TEM, SHV, CTX-M, PER, VEB, GES, BES, TLA, and OXA[70]. Class A

carbapenemases belonging to Bush-Jacoby functional subgroup 2f, can hydrolyze all β-lactam antibiotics and are usually characterized by susceptibility to classical β-lactamase inhibitors [69]. Six types of class A carbapenemases have been reported, including KPC (*Klebsiella pneumoniae* carbapenemase), SHV (sulfhydryl variable lactamase), GES (Guiana extended- spectrum β-lactamase), SME (*Serratia marcescens* enzyme), IMI/NMC-A (imipenemase/non-metallocarbapenemase-A), and SFC (*Serratia fonticola* carbapenemase).

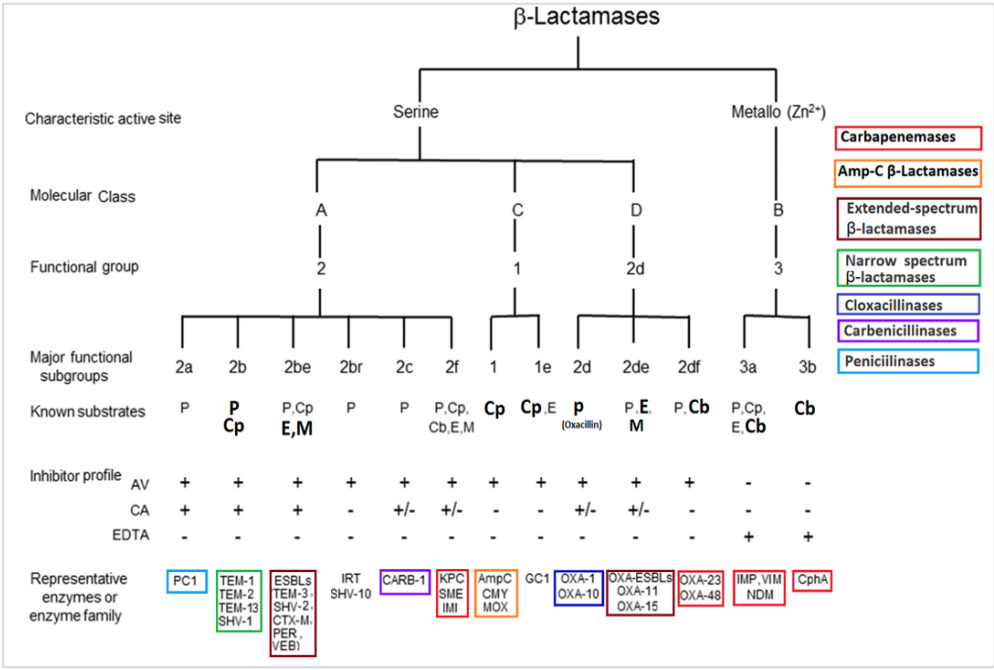

Figure I.1.10: Structural and functional classification of β-lactamases. Ambler method, including Class A, B, C, and D. Functional classification using the Bush-Jacobi-Medeiros method group 1, group 2, and group 3 (Modified after [12]). AV, avibactam; CA, clavulanic acid; Cb, carbapenem; Cp, cephalosporin; E, expanded-spectrum cephalosporin; M, monobactam; P, penicillin.

2. Class C

Class C β-lactamases, which are encoded by *Amp-C* genes, are cephalosporinases hydrolyzing the first four generations of cephalosporins while showing resistance to clavulanic acid but not to cloxacillin and aztreonam, and classified into Bush-Jacoby functional group 1 [69]. Many members of the *Enterobacterales* usually have genes encoding these cephalosporinases on the chromosome.

3. Class D

Class D β-lactamases, known as OXA enzymes, show cloxacillin- and oxacillin-hydrolyzing activity; they are classified into Bush-Jacoby functional group 2d [69]. Most OXA-type β-lactamases do not significantly hydrolyze the extended-spectrum cephalosporins, so they are not considered as ESβLs [71]. However, OXA-11 and OXA-15 extended the degradation ability, that include extended-spectrum cephalosporins, but not carbapenems. They are classified as Bush-Jacoby functional subgroup 2de. Among oxacillinases, there are carbapenem-hydrolyzing oxacillinase, including OXA-23 and OXA-48, belong to functional subgroup 2df. Generally, OXA enzymes of all functional subgroups are codified by resistance determinants on both chromosomes and plasmids.

4. Class B

Class B β-lactamases, metallo β-lactamases (MBL), hydrolyze carbapenems, penicillins, and cephalosporins[69]. They belong to Bush-Jacoby functional group 3. Their enzymatic activity is suppressed by a chelating agent, such as ethylenediaminetetraacetic acid (EDTA), due to the presence of metal in the active. MBLs are classified into three subclasses (B1, B2, B3) according to their amino acid sequence.

I.1.7.2.2 Modifications in Penicillin-Binding Proteins (PBPs)

Mutations in the chromosomal genes encoding the PBPs or the acquisition of supplementary foreign genes encoding new PBPs with lower affinity to β-lactams can render the bacteria resistant to β-lactams [72]. This mechanism of resistance is important in Gram-positive cocci.

I.1.7.2.3 Modifications of membrane proteins

Alterations in porins' structure and their expression reduce the amount of β-lactam antibiotics that can enter the cell. This form of resistance usually leads to multi-drug resistance particularly when the porins are shared by many antibiotics [72]. Resistance to imipenem is an example of drug-specific resistance arising from loss of a carbapenem-specific porin.

I.1.8 Fluoroquinolones: Overview and Resistance Mechanisms

I.1.8.1 Overview of Fluoroquinolones

Fluoroquinolones (FQs) are potent, broad-spectrum antibiotics that exert their bactericidal effect by impairing bacterial DNA synthesis machinery [73]. FQs are one of the most prescribed antimicrobial agents due to their good oral bioavailability, potent activity across a broad spectrum of bacterial species, and favorable pharmacokinetic properties [73]. They are usually used for treating the serious community and hospital-acquired infections, including urinary tract infections, pneumonia, gastroenteritis, and gonococcal infections [73][74]. The backbone of current fluoroquinolones is 1,8-naphthyridine nucleus, quinolone ring [73].

The first quinolone was nalidixic acid, discovered in 1962 and introduced into clinical medicine in 1967 [75]. In 1980s, FQs were introduced by adding a fluorine substituent and a piperazinyl derivative to the quinolone ring. Currently, FQs are divided into four generations, which have enhanced spectrum and high serum concentration levels with successive generations [73]. Second-generation FQs includes ciprofloxacin and ofloxacin, have a wider Gram-negative spectrum but little activity against *S. pneumoniae* than the first generation. Third-generation FQs, including levofloxacin, have improved spectrum for Gram-positive bacteria in comparison to second generations drugs and they are known as respiratory FQs. Fourth-generation FQs, including moxifloxacin, gemifloxacin, and delafloxacin, have a better spectrum for anaerobic bacteria

The bactericidal activity of FQs are associated with direct inhibition of DNA replication by binding to DNA gyrase and DNA topoisomerase IV [73]. DNA gyrase and topoisomerase IV are responsible for relaxing supercoiled DNA produced during DNA replication by the action of helicase by breaking both strands, changing the DNA topology, and promptly re-ligating the broken DNA strands. FQs interfere with this process preventing the re-ligation step and the movement of the DNA replication fork leading to cell death.

I.1.8.2 Mechanisms of Fluoroquinolone Resistance

In Gram-negative bacteria, the mechanisms of FQs resistance in Gram-negative bacteria include target alteration, decreased outer membrane permeability, extrusion by membrane-based efflux pumps, enzymatic modification, and target protection (Fig. I.1.11) [76].

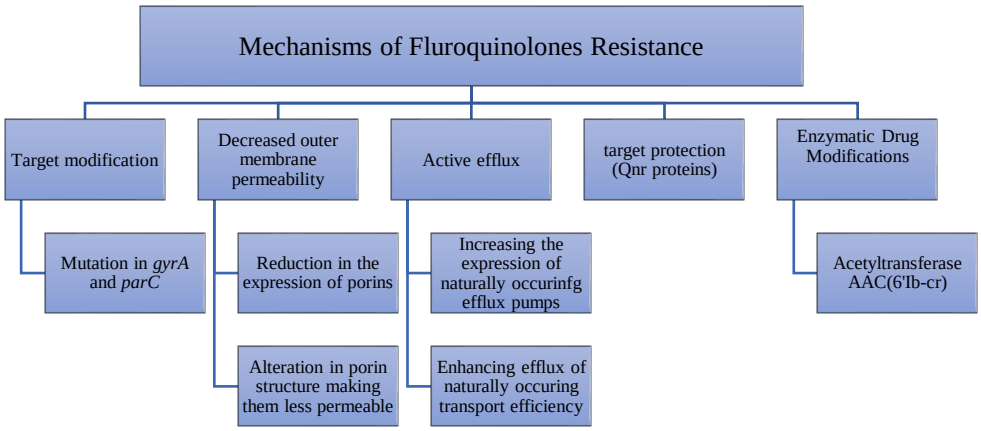

Figure I.1.11: Mechanisms of fluoroquinolones resistance.

I.1.8.2.1 Target-mediated resistance

DNA gyrase, encoded by *gyrA*/*gyrB*, is the main target for FQs in Gram-negative bacteria, while topoisomerase IV, encoded by *parC*/*parE*, is the main target for Gram-positive bacteria [76]. FQs resistance can occur in a stepwise fashion by accumulating deleterious mutations, mainly in *gyrA* and *parC*. Mutations in *gyrB* or *parE* are less frequent and exhibit reduced susceptibility. In Gram-negative bacteria, first-step mutations occur in *gyrA*, which encodes part of the primary target, followed by second-step mutation in *parC*, which encodes part of the secondary target. On the contrary, for Gram-positive bacteria, the first mutations are usually observed in *parC*, followed by a second step mutation in *gyrA*.

I.1.8.2.2 Decreased outer membrane permeability

FQs usually cross the outer membrane through porins such as OmpF, OmpC, and OmpA, while diffusion across the lipid bilayer is dependent on the hydrophobicity of FQ molecules [76]. Mutations in the genes of porins cause a reduction in the expression of porins or alter their structure exhibiting a reduced FQs susceptibility.

The reduction in FQs susceptibility is significantly increased when coupled with an active efflux of FQs.

I.1.8.2.3 Efflux-related resistance

Efflux-related resistance has been detected in almost all clinically important Gram-negative bacteria, including *E. coli*, *P. aeruginosa*, *A. baumanii*, and *M. tuberculosis*, conferring resistance to several classes of antibiotics [76]. Mutations can occur in genes encoding the naturally occurring efflux pump, either in the promoter region, increasing the expression of efflux pumps, or in the coding region, enhancing their transport efficiency. Moreover, plasmid-mediated FQ efflux can also be found in Gram-negative bacteria that harbor *oqxAB*.

I.1.8.2.4 Target protection

Target protection is usually plasmid-mediated and associated with pentapeptide repeat protein, i.e., Qnr, that can bind to topoisomerase- FQ- DNA complex, regenerating topoisomerase activity [76]. QnrB and QnrS are the common ones in *Enterobacterales*.

I.1.8.2.5 Enzymatic Modification of FQs

The *aac(6')-Ib-cr* determinant is a variant of a gene encoding an aminoglycoside acetyltransferase that can reduce the activity of FQs by N-acetylation.

I.1.9 Aminoglycosides: Overview and Resistance Mechanisms

I.1.9.1 Overview of Aminoglycosides

Aminoglycosides (AGs) are potent, broad-spectrum antibiotics that exert their bactericidal effect by impairing bacterial protein synthesis machinery [77]. AGs are used to treat severe hospital-acquired infections due to their rapid bactericidal effect, low levels of resistance compared to other classes of antibiotics, and their ability to act synergistically with other drugs [77][78]. They are used in the empiric and definitive treatment of serious Gram-negative bacterial infections, including endocarditis, ocular, pulmonary, and intestinal infections [79]. AGs are pseudo-polysaccharides with aminocyclitol ring as a backbone connected to amino-sugars giving rise to their polycationic nature [80].

The first AG introduced into clinical use was Streptomycin, which was isolated from *Streptomyces griseus* in 1944, followed by the subsequent introduction of several members, including neomycin in 1949, kanamycin in 1957, gentamicin in 1963, netilmicin in 1967, tobramycin in 1967, and amikacin in 1972 [77]. AGs are mainly active against aerobic, Gram-negative bacteria, particularly potent against members of the *Enterobacterales* family, and act synergistically against certain Gram-positive organisms[77][80]. Indeed, AGs are usually combined with another agent for the empiric management of serious healthcare-associated infections, such as severe sepsis (when MDR is suspected) or in case of a high mortality risk [81]. On the other hand, AGs are inactive against anaerobic bacteria because the uptake of AGs into the cell requires active electron transport [77].

The cationic nature of AGs at physiological pH can lead to their accumulation in the tissue, causing mainly nephrotoxicity, the first main negative effect; irreversible ototoxicity, the second main negative effect; and neuromuscular blockade [81][82]. The toxicity is the nonspecific binding of AGs to the eukaryotic ribosome A-site.

Due to their toxicity, AGs medical use was shifted by introducing β-lactams which were effective and less toxic [78]. However, due to the recent emergence of MDR bacteria, they have started to be used again, but at optimized dosing regimens to improve their safety, and in combination with a variety of other antibacterial classes, such as β-lactams, to synergize the activity enhancing spectrum activity due to increased uptake of AGs [77][78].

The bactericidal activity occurs in a multistep process that starts with the uptake of AGs disrupting membrane integrity and ends with the binding to bacterial ribosomal RNA inhibiting protein synthesis, with the last being the primary mechanism [80][78].

The first step of this multistep process starts with the transport of AGs through the outer membrane by binding electrostatically to the negatively charged components

of the Gram-negative outer membrane, including LPS, replacing essential divalent cations (i.e., magnesium ions, that stabilize neighboring LPS molecules). This replacement disturbs the outer membrane and leads to pore-formation, initiating the uptake and increasing the permeability of AGs [78][83]. The transport across the cytoplasmic membrane requires energy from the electron transport system in an oxygen-dependent manner, which explains the intrinsic resistance of anaerobic bacteria to AGs. After AGs reach the cytosol, they irreversibly bind to the A-site on the 16S rRNA of the 30S subunit of the 70S ribosome, altering its confirmation and disrupting the fidelity of tRNA selection due to codon misreading (errors in proofreading); and blocking translocation of tRNA from A-site to P-site [77][78][83]. Although different classes of aminoglycosides bind to different sites on 16S rRNA, the result of this interaction leads to a misreading of the genetic code and accumulation of abnormal truncated or non-functional proteins in bacteria [77][78].

I.1.9.2 Mechanisms of Aminoglycosides Resistance Mechanisms

The mechanisms of bacterial resistance to AGs include (i) N-acetylation, adenylation, or O-phosphorylation to deactivate AGs, (ii) decreasing intracellular AGs concentration by changing the outer membrane permeability, decreasing inner membrane transport, activating efflux pumps, and drug trapping, (iv) target modification by altering of the 30S ribosomal subunit target by mutation, and the methylation of the AGs-binding site through (Figure I.1.12) [77][78]. The deactivation of AG is the most prevalent mechanism.

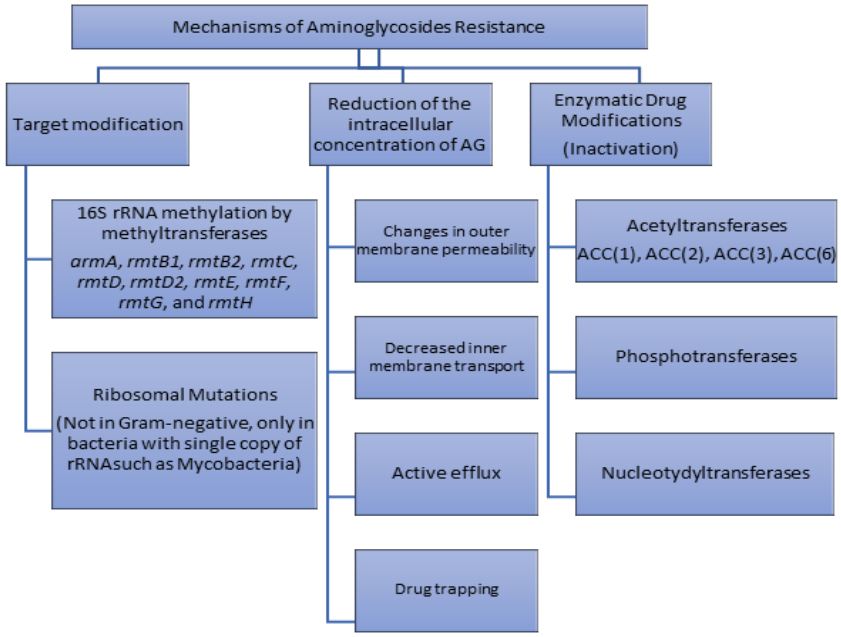

Figure I.1.12: Mechanisms of aminoglycosides resistance.

I.1.9.2.1 Target site modification

Target modification can occur through mutations in the primary target of the antibiotic or through enzymes that modify residues preventing the antibiotic from binding to its target [78]. Usually, bacterial species have different copies of rRNA, making it challenging to have resistance through rRNA modification. This mechanism of resistance can be seen in *Mycobacterium* and *Borrelia* spp. due to posing one copy of the 16S rRNA or a single copy of the entire ribosomal operon. On the other hand, target modification via 16S rRNA methyltransferases (RMTases) is more common in *Enterobacterales*, *A. baumannii* and *P. aeruginosa*. RMTases, including armA, rmtA/B/C/D/E/F/G/H and npmA, confer high-level resistance to all clinically relevant aminoglycosides [84]. RMTases-encoding genes are usually plasmid-mediated, found with other resistance genes, such as ESβLs, carbapenemases, and fluoroquinolone resistance genes.

I.1.9.2.2 Reduction of the intracellular concentration of AGs

Bacteria can reduce the intracellular accumulation of antibiotics necessary for target inhibition, including AGs, by extruding them using energy-dependent efflux systems [78]. Generally, efflux systems confer three levels of antibiotic resistance [85]. Firstly, they can confer intrinsic resistance to low levels of various antibiotics through the constitutive expression of naturally occurring efflux pumps showing a weak increase in MIC values [78][85]. Secondly, they confer acquired resistance by mutations in the promoter or the regulatory genes of the pumps leading to the overexpression or mutations in the coding region, enhancing their activity [85]. Thirdly, they can contribute to transient, non-inheritable phenotypic resistance when bacteria are growing in the presence of an effector of the efflux pump or under growing conditions that trigger their overexpression [78][85]. Efflux systems mainly contribute to a high level of antibiotic resistance when overexpressed or associated with other mechanisms [78].

I.1.9.2.3 Enzymatic drug modification

Enzymatic drug modification is the most prevalent mechanism of AGs resistance faced clinically [78]. The modification of specific amino or hydroxyl groups of the AG molecule reduces AG affinity to bind to the A site of bacterial 16S rRNA, conferring resistance. So far, over 100 enzymes detected, which can be either plasmid-mediated or chromosomally encoded, facilitating the rapid spread of resistance inter- or intra-species. These enzymes include aminoglycoside acetyltransferases (ACC), aminoglycoside phosphotransferases (APH), and aminoglycoside nucleotidyltransferases (ANT) (Fig. I.1.13) [78]. These enzymes show wide differences in their geographical distribution, bacterial species in which these enzymes disseminate, and the specific aminoglycosides they modify [36]. For example, the APH(3) family is frequently found in Gram-positive and Gram-negative

bacteria, altering kanamycin and streptomycin not gentamicin and tobramycin. On the other hand, AAC(6′)-I is mainly found in Gram-negative clinical isolates, including *Enterobacterales*, *P. aeruginosa*, and *A. baumannii* and affects most aminoglycosides.

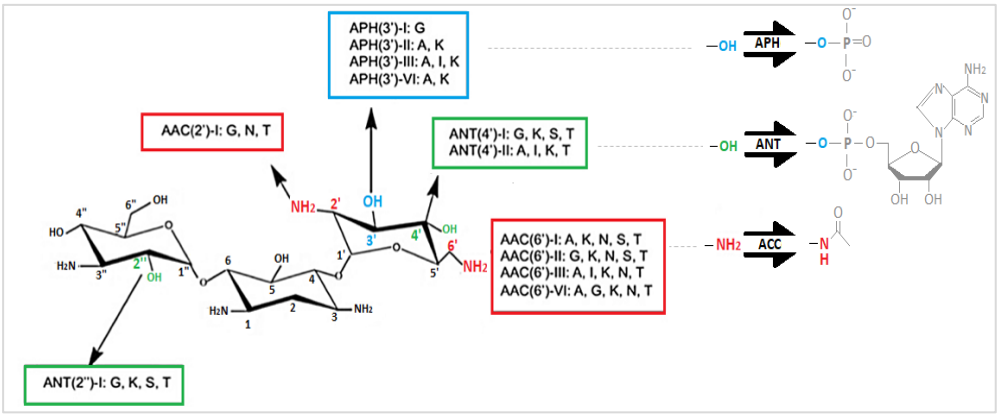

Figure I.1.13: Illustration of aminoglycoside-modifying enzymes and their nomenclature, including phosphotransferase (APH), adenyltransferase (ANT), and acetyltransferase (AAC). The number in parentheses comes after enzyme name and indicates the number of carbon inactivated. A, amikacin; G, gentamicin; I, isepamicin; K, kanamycin; N, netilmicin; S, sisomicin; T, tobramycin (Modified after [36] and [86]).

ACCs are the largest group that catalyzes N-acetylation of amino groups of typical AG molecules [78]. ACC enzymes are categorized into four subclasses depending on amino group position, including AAC(1), ACC(3), ACC(2), and AAC(6), with the last one being the most prevalent AMEs on various mobile elements in diverse bacterial species.

APH, particularly APH(3′), is the second largest group of AG-modifying enzymes after the ACC class of enzymes catalyzing the transfer of phosphoryl group from ATP to hydroxyl groups on AG molecules resulting in poor binding of the drug on its ribosome target [78].

ANTs catalyze the adenylation of the hydroxyl group at different positions 2″, 3″, 4′, 6, and 9 to form the O-adenylated AG with ANT(2″), conferring resistance to multiple AGs due to its ability to adenylate a broad range of substrate molecules [78].

I.1.10 Mobile Genetic Elements

The accumulation and dissemination of ARGs have been facilitated by horizontal gene transfer (HGT), including conjugation (via plasmids and conjugative transposons), and transduction (via bacteriophages), and natural transformation (uptaking extracellular DNA from dying cells), with the first being the main and well-studied mechanism [31][87]. Transduction occurs when a bacteriophage infects the recipient cell with DNA that contains resistance genes, and this DNA is internalized into the genome of the recipient cell. Transformation includes the uptake of extracellular DNA and the incorporation of the DNA into the host chromosome. In conjugation, the donor bacterium transfers DNA, conjugative plasmids or conjugative transposons, to the recipient cell by mating, which can happen at intra- and inter-genus and species level [87].

The most clinically significant antibiotic resistance genes are usually located on different mobile genetic elements (MGEs) that can move intracellularly (between the bacterial chromosome and plasmids), including insertion sequences (ISs), transposons, and gene cassettes, or intercellularly (within the same species or between different species or genera), as conjugative plasmids or integrative conjugative elements which are self-transmissible [88].

I.1.10.1 Plasmids

Plasmids are stable extra self-replicating genetic molecules that usually harbor genes conferring specific properties to the host cell [89]. These replicons are physically distant from the bacterial chromosome except for some plasmids, called episomes, which can insert or integrate into the host cell's chromosome, where their replication is then regulated by the chromosome. Plasmids usually have different combinations of genes associated with virulence, fitness, and antimicrobial resistance, that have a major role in the success of antimicrobial-resistant bacteria [90]. Conjugative plasmids can initiate their own transfer along with other mobilizable plasmids, which are smaller in size and not self-transmissible [89]. Also, they have mechanisms to control their copy-number in the cell and/or replication ability. Additionally, plasmids have a role in the acquisition and dissemination of ARGs to other MGEs in which ARGs are assembled via transposition and recombination mechanisms.

Notably, plasmid exchange is considered one of the most efficient resistant mechanisms implicated in MDR strains dissemination, mainly in Gram-negative bacteria. They are involved in the acquisition of resistance to most antibiotic classes, including β-lactams, aminoglycosides, tetracyclines, chloramphenicol, sulfonamides, trimethoprim, macrolides, polymyxins, and quinolones [89]. This is mostly due to their ability to carry more than one resistant gene, to coexist with other plasmids in the same cell, and their ability to be transferred to others, both intra- and inter-genera [68].

Antimicrobial resistance plasmids can be categorized into a narrow-host-range group, and broad-host-range group based on host range into which plasmids can be transferred and successfully maintained [90]. Narrow-host-range group (their transfer is restricted to the same species or closely related species) commonly belongs to incompatibility group F (IncF), as these plasmids offer a stable environment for antibiotic resistance genes. On the other hand, the broad-host-range group, which can easily be transferred between different species, usually belongs to IncA/C, IncL/M, and IncN [90]. Some plasmids are termed 'Epidemic resistance plasmids' due to their ability to acquire, maintain, and rapidly disseminate their resistance genes. Plasmids belonging to IncF contributed significantly to the rapid dissemination of β-lactamases, such as *bla*CTX-M-15, *bla*KPC-2, and *bla*KPC-3 among *Enterobacterales*[90].

I.1.10.2 Insertion sequences

The smallest transposable mobile elements in bacterial chromosomes and plasmids are insertion sequences (ISs), ranging in size from about 0.7 to 2.5 Kb [89]. IS consists of two inverted repeats (about 10 to 40 bp), which sometimes generate direct target duplications (DRs) when they are integrated into the target DNA. Inverted repeats flank genes necessary for transposition, including transposase that catalyzes the cutting and resealing of the DNA during transposition enabling transposons to cut themselves out of a nucleoid or a plasmid and insert themselves randomly into another location in the same or different DNA molecules within a single cell (Fig. I.1.14A) [88][91]. Usually, IS elements move almost randomly to new locations in the chromosome by themselves; unless there are two closely spaced IS elements randomly flanking a DNA segment that confers a selective advantage, such as antibiotic resistance, they can move as a single unit [88]. However, For some types of IS, a single copy is able to capture and mobilize an adjacent resistance gene [92]. Moreover, ISs can change the gene expression of antibiotic resistance genes by providing an active promoter [89].

I.1.10.3 Transposons

Transposons are large transposable elements capable of jumping from/to different locations in the genome, ranging in size from 2.5 to 21Kb [89]. They usually carry drug resistance genes or other markers besides the functions required for transposition. Bacterial Transposons can be divided into two types, composite and complex. Composite transposons consist of two IS elements flanking a central gene, usually an antibiotic resistance gene (Fig. I.1.14B). The subsequent insertion of the same IS on both sides of a resistance gene allows it to be captured and moved to another DNA molecule (e.g., from the chromosome to a plasmid) as part of a composite transposon. On the other hand, complex transposons contain *tnpA* gene encoding transposase, the *tnpR* gene encoding resolvase, as well as one or more cargo genes which may include resistance gene (Fig. I.1.14C).

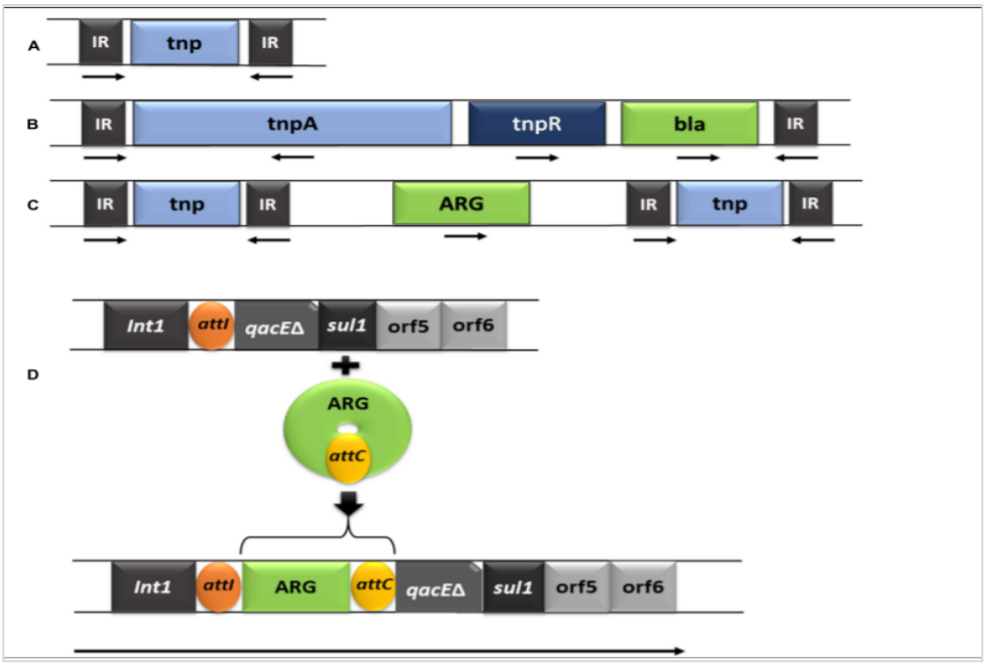

Figure I.1.14: Schematic representation of the predominant MGEs involved in the acquisition and dissemination of ARGs. A. IS element (IR: inverted repeats; *tnp*: transposase gene). B. Tn3 complex transposon (*tnpA*: gene encoding transposase longer than *tnp* in IS, the *tnpR* gene encoding resolvase, *bla*: β-lactamase resistance gene). C. composite transposon with ARG. D. class I integron and gene cassette acquisition (*Int1*: integrase gene; *att1*: recombination site of the integron; *qacEδ*: truncated gene, responsible for quaternary ammonium compounds resistance; *sul1*: sulfonamide resistance gene; orf5/orf6: open reading frames, *attC*: recombination site of the gene cassette) [89].

I.1.10.4 Gene cassettes

A gene cassette is small, non-replicative, and promoter-less mobile element, ranging in size from 0.5 to 1 kb, consisting of a single open reading frame (occasionally two) followed by a short recombination site termed *attC*, allowing it to be captured by larger genetic elements called integrons. Gene cassettes are usually found inserted into an integron, but they can also be found as circularized and free elements (Fig. I.1.14D) [88]. Their gene expression depends on the integron promoter.

I.1.10.5 Integrons

Integrons contain a site-specific recombination system able to integrate, express, and exchange gene cassettes [93]. Integrase, encoded by *intI* gene, allows the integration between *attC* site in gene cassettes and the recombination site (*attI*) in integrons. Many gene cassettes can be captured by the same integrin, forming cassette arrays

whose expression can be directed by integron promoter (Pc), thus conferring multi-resistance (Fig. I.1.14D) [92][89].

There are two types of integrons: sedentary integrons found in chromosomes and mobile resistance integrons found in plasmids [89]. Integrons are divided into several classes (class 1, class 2, and class 3) depending on the amino acid sequence of the IntI enzyme [89]. Class 1 integrons, which are typically associated with plasmids, are the most common classes in clinical isolates. ISCRs are transposable elements that are similar in size to IS elements, are often associated with class 1 integrons, and can mobilize adjacent DNA *via* a rolling-circle mechanism. When ISCR elements are associated with class 1 integrons, they form complex class 1 integrons. The role of these elements in the acquisition and dissemination of ARGs is relevant, especially in Gram-negative bacteria.

I.1.10.6 Integrating and conjugative elements

Integrating and conjugative elements (ICEs) are mobile genetic elements carrying a wide range of genes, including genes that encode the machinery necessary for conjugation, integration, and excision [89]. Moreover, they have genes that confer additional metabolic or colonization abilities, or antibiotics and/or metals resistance. They can spread both vertically to offspring, due to their integration in the bacterial chromosome, and horizontally to recipient cells through conjugation. Several ICEs are capable of autonomous plasmid-like replication, which blurs the line separating it from extrachromosomal plasmids [94].

I.1.11 High Risk Clones: Definition and Characteristics

The rapid dissemination of AMR is primarily due to HGT, mainly through mobile genetic elements, particularly plasmids, and clonal dissemination within different populations, i.e., human and animal, in different environments [95].

MDR bacteria usually have many genetic determinants, mutations, and (or) genes, each contributing to resistance phenotypes. [96]. These genetic determinants can be acquired vertically by chromosomal mutations, which usually occur at multiple loci or horizontally through antibiotic resistance genes [97]. Resistance genes are mainly found on the bacterial accessory genome, which is usually mobile, facilitating the spread of these resistance genes within species and between species.

In the context of AMR, when a bacterium is efficient in maintaining and transferring the resistance determinants vertically to daughter cells or horizontally by acting as a donor to facilitate inter- or intra-species transfer, it is called a 'successful strain' [96]. To evaluate the epidemiological status of such successful MDR strain, it is necessary to know if it is (i) a newly emerged strain, (ii) a previously reported minor strain with resistance as a key driver in its rise to success, or (iii) a successful preexisting strain [96]. This labeling can be investigated by assessing the relatedness of bacterial isolates, i.e., bacterial typing, mainly used when there is strong epidemiological evidence, such as outbreaks in healthcare or community settings [96]. Indeed, isolates that are collected from different specimens, of different patients, at different geographical locations, and possibly at different times can be descendants of a common origin, i.e., belong to the same clone. Isolates of the same clone usually have many similarities at genotypic and phenotypic levels [96]. In other words, bacterial isolates of specific bacterial species that are genotypically indistinguishable are considered a clone, descending from the same recent ancestor [98].

The drivers of intraspecies diversification are mutations, which generally increase intraspecies variation, and gene flow, particularly through HGT, which can increase or decrease intraspecies variation [99]. Acquired DNA by HGT can result in a drastic change in the original character of bacterial species by replacing genetic segments with donor homologs by homologous recombination or by acquiring new genetic material, producing eventually a cluster of increasingly diverse genotypes, i.e., clonal complex) [98][100][99]. These variations are affected by genetic drift through eliminating them and by natural selection through maintaining variations with fitness advantage and eliminating variations with fitness disadvantage. Natural selection is driven by many factors affecting survival and fitness in different niches, such as oxygen, nutrients, antimicrobial compounds, heavy metals, and direct competition or commensalism [99].

Some bacterial clones have an essential role in the global emergence of multidrug resistance because they are able to maintain and propagate antimicrobial resistance

elements [90]. *E. coli* sequence type (ST) 131 and *K. pneumoniae* ST258 are examples of "Eminent or successful" bacterial clones.

The term 'high-risk clones' was coined, in 2011 [101], to describe "highly specialized genetic populations or subpopulations with enhanced ability to colonize, spread and persist in particular niches after having acquired a diversity of adaptative traits that increase their epidemicity and/or pathogenic potential, including antibiotic resistance." Generally, high-risk clones have an enhanced ability to acquire, exhibit, and disseminate antibiotic resistance without their fitness being affected. In fact, they might have some biological factors, e.g., virulence factors, that increase their fitness, counteracting the dogma that states harboring MDR can compromise fitness [90]. High-risk clones with increased fitness and plasmids with antibiotic resistance genes have a major role in the success of MDR bacteria. In *E. coli* ST131 and *K. pneumoniae* ST258, the combination of increased fitness of these clones, due to the presence of virulence factors, along with the presence of epidemic resistance plasmid, i.e., IncF plasmid, harboring *bla*CTX-M-15, and *bla*KPC-2/KPC-3, respectively, allow them to disseminate and become international MDR high-risk clones. Generally, the dissemination of MDR high-risk clones is accelerated by the selective pressures of antibiotics present in healthcare settings and used during food-animal farming [90].

International multidrug-resistant high-risk clones have six characteristics, including (i) worldwide distribution, (ii) posing different antimicrobial resistance determinants, (iii) efficient colonization and persistence in hosts for an extended period of time up to at least six months, (iv) successful transmission among hosts, (v) enhanced pathogenicity and fitness, and (vi) the ability to cause severe and/or recurrent infections [90]. Recently, Lagarde *et al*. revisited the current criteria to adapt them to the "One Health" approach by including the emergence of the pathogen, environmental persistence, and zoonotic transmission, emphasizing the importance of detecting and characterizing high-risk clones in animals [95].

Bacterial typing helps to (i) recognize virulent strains assisting in managing therapeutic options, (ii) determine the source of infections, (iii) determine the epidemiological status of infectious disease, i.e., affirm or exclude outbreaks, and (iv) evaluate the effectiveness of control measures [102].

Bacterial typing has increasingly moved from phenotypic methods, such as serotyping and antibiogram-based typing, to molecular methods, which include amplification-based, fragment-based, sequence-based, and recently genomic-based (Table I.1.6) [44]. The main performance criteria of bacterial genotyping include: (i) stability, which recognizes a clonal relationship between isolates despite genetic differences; (ii) type-ability, generating a result for each isolate tested separately; (iii) discriminatory power, ability to generate distinct information from epidemiologically unrelated isolates, at least at the sub-serotype level; (iv) epidemiologic concordance,

with molecular data produced; and (v) reproducibility, generating the same results when isolates are tested repeatedly in different laboratories [44][102].

Table I.1.6: Performance and convenience criteria of the most commonly used methos for bacterial genotyping [44][103]

Feature	RAPD-PCR	PFGE	MLST	WGS
Typeability[a]	All	All	All	**All**
Repeability[b]	Moderate	Excellent	Excellent	Excellent
Reproducibility[c]	Moderate	Excellent	Excellent	Excellent
Discriminatory power[d]	Good	Excellent	Good to Excellent	**Excellent**
Stability[e]	Moderate	Good	**Good**	**Good**
Ease of interpretation of data generated[f]	Moderate	Moderate	**Good**	**Needs specialist**
Ease of use	Good	Poor	Moderate	**Narrowing down**
High throughput	Yes	No	**Yes**	**yes**
Cost	Low	Moderate	**Moderate**	**expensive**
Time required (days)	**1**	**5**	**>3**	**>2 weeks**

[a]*generate a result for isolates tested separately.*
[b]*generate identical results when isolates are tested repeatedly in the same laboratory.*
[c]*generate identical results when isolates are tested repeatedly in different laboratories.*
[d]*generate distinct results from epidemiologically unrelated isolates, at least at the sub-serotype level.*
[e]*recognize a clonal relationship between isolates despite of genetic differences.*
[f]*Produce clear interpretation.*

Amplification-based methods use non-specific primers for amplifying sequences, after which they are subjected to electrophoresis. They usually include random amplification of polymorphic DNA (RAPD), arbitrarily primed PCR, and variable-number tandem repeat (VNTR) typing. Rep-PCR is the commonest and most validated technique of this group [44].

Fragment-based methods involve the digestion of DNA by one or more restriction enzymes, after which the DNA is subjected to electrophoresis [44]. Pulsed-field gel electrophoresis (PFGE) is the most widely used, particularly for outbreak investigations, due to its high discriminatory power, modest costs, and good reproducibility, despite being time-consuming and the need for special equipment.

Sequence-based typing requires sequencing specific genes to generate allelic profiles by comparing gene sequences to an international database [44]. Typing schemes can use one locus, i.e., single-locus sequence typing (SLST), or multilocus, which is usually based on seven loci, i.e., multilocus sequence typing scheme (MLST), with the second being the most common one. *emm*-typing for *Streptococcus* spp. or *spa*-typing for *S. aureus* are examples of SLST. MLST schemes usually involve seven housekeeping genes that are subject to neutral genetic variation, with MLST schemes for many bacterial species deposited in PubMLST (https://pubmlst.org/). The main advantage of MLST is international standardization facilitating clonal comparisons in different laboratories/countries, while the cost of performing Sanger sequencing is disadvantageous.

Whole-genome sequencing (WGS) is becoming a practical choice allowing for powerful differentiation among isolates in epidemiological studies due to the continuous decrease in sequencing costs. High-resolution typing approaches based on WGS include core genome MLST (cgMLST), whole-genome MLST (wgMLST), and single nucleotide polymorphisms (SNPs) typing, which are arranged ascendingly according to their resolution power. However, appropriate bioinformatic experts and tools are required to manage and interpret sequence data produced [103].

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Chapter 2: ST101/KPC-2 and ST512/KPC-3 *Klebsiella pneumoniae* Outbreak: Multi-antibiotic resistance and multi-clonality in Intensive Care

I.2.1 Abstract

Objective: This study was conducted to characterize isolates of multidrug-resistant and carbapenem-resistant *Klebsiella pneumoniae* (MDR-CRKP), detected during the SARS-CoV-2 pandemic period in the Intensive Care Unit of the Ospedale Civile di Voghera (Lombardia, IT).

Materials and Methods: In the period December 2020 – July 2021, a total of n=33 isolates of CRKP were collected. Identification and antibiograms, initially obtained with BD Phoenix TM M50, were confirmed by Microscan Autoscan-4 System (Beckman Coulter). Antibiograms were interpreted using EUCAST 2021 clinical breakpoints. Production and identification of ESβLs and carbapenemases were evaluated by NG-Test CARBA-5 and/or Microarray Check-Points MDRCT103XL, targeted PCR, and sequencing. The aminoglycosides and fluoroquinolones resistance determinants *aphA6, aadA, armA, aac(6')-Ib-cr, qnrB/S* were investigated by PCR. Clonal relationships were investigated with Pulsed Field Gel Electrophoresis (PFGE) with XbaI restriction enzyme. Representative strains for each PFGE clone were subjected to MultiLocus Sequence Typing (MLST), while plasmid typing was performed with PBRT 2.0 Kit (Diatheva, Fano, Italy).

Results: The 33 characterized MDR-CRKP isolates accounted for 50% (33/66) of the *K. pneumoniae* strains identified during the study period. Most of these CRKPs (66.7%) were obtained from male inpatients, with an average age of 69 years. Biological materials included rectal swabs (12/33), blood (7/33), urine (7/33), bronchoalveolar lavage (6/33) and bronchoaspirate (1/33). Resistance to carbapenems was associated with resistance both to fluoroquinolones (including ciprofloxacin and levofloxacin), and to the aminoglycoside tobramycin. The isolates showed variability in their resistance phenotypes for other aminoglycosides (amikacin 82%, n=27; and gentamycin 27%, n=9), trimethoprim/sulfamethoxazole (67%, n=22), and chloramphenicol (70%, n=23). The 9% (n=3/33) of CRKPs showed a ceftazidime/avibactam resistant phenotype. Thirty-two out of 33 CRKP included in the study were positive for the determinant *bla*KPC-type; the remaining were blaCTX-M-15 gene positives. According to the molecular characterization and typing results, the 33 CRKP isolates were classified into four groups: KPC-2-producing *K. pneumoniae* A by PFGE/ST101 (n=10/33), KPC-3-producing *K. pneumoniae* B by PFGE/ST512 (n=21/33), KPC-3-producing *K. pneumoniae* C by PFGE/ST512 (n=1/33), and CTX-M-15-producing *K. pneumoniae* PFGE D/ST234 (n=1/33). The determinant *bla*KPC-2 was clone A/ST101 related, while the *bla*KPC-

3 was clones B and C of lineage ST512 related. Furthermore, 26/33 strains resulted positive for the *aac(6')-Ib-cr* gene, 9/33 for *armA*, 1/33 for *qnrS*; the *arm*A + *aac(6')-Ib-cr* genes were co-present in one isolate. Moreover, the plasmid content for the clone A/ST101 included FIIK and amplicon with not assigned group, while plasmid content of clone B/ST512 included X3, FIIK, FIB, KQ and FIB KN replicons and for clone C/ST512 FIIK, FIB, and KQ were detected.

Discussion and Conclusions: Preventing CRKP colonization/infection in ICU patients is one of the major aims of modern medicine. Information on risk factors, antibiotic use, and prior hospitalization, together with strict infection control measures and epidemiological-molecular investigations are essential for the containment of MDR difficult to manage strains.

I.2.2 Acknowledgment

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I.2.3 Objectives

The aim of the present study was to investigate carbapenems resistance mechanisms and the molecular epidemiology of 33 clinical CRKP isolates that emerged during the SARS-CoV-2 pandemic period in the Intensive Care Unit of the Civile Hospital of Voghera, in the period December 2020 – July 2021.

I.2.4 Introduction

I.2.4.1 Overview of CRKP

K. pneumoniae is a major opportunistic pathogen responsible for HCAI infections, including respiratory tract, urinary tract, and bloodstream infections [1]. *K. pneumoniae*, a member of the *Enterobacterales* order, is present in the environment and colonizes mainly the lower gastrointestinal tract of humans and animals. *K. pneumaniae* is one of the so named "ESKAPE" pathogens, which exhibit multidrug resistance and virulence through accumulation of AMR genes *via* horizontal gene transfer [2].

Carbapenem-resistant *K. pneumoniae* (CRKP) in healthcare settings is a major health problem worldwide. CRKP infections are associated with high rates of morbidity and mortality, particularly for bloodstream infections, where the mortality rate reached more than 70.0% [3]. The therapeutic options for managing CRKP infections are limited, with tigecycline and colistin as agents of last-resort. It is worth mentioning that tigecycline and colistin are associated with hepatotoxicity and nephrotoxicity, respectively, limiting their use in some patients [4][5].

The rapid increase in the emergence and dissemination of CRKP is mainly due to the presence of carbapenemases harbored on a transmissible plasmid; the overuse of carbapenems in clinical practice exerted a selective pressure on these strains [1]. Indeed, many studies found that *K. pneumoniae* strains of environmental origin are more susceptible to antibiotics than their counterparts in clinical settings, although they are very similar in terms of biochemical patterns, virulence, and pathogenicity. Generally, the spread of CRKP is facilitated in hospitals, which act as reservoirs of CRKP due to intensive antibiotic use and patient-to-patient and patient-to-healthcare worker contact [6]. WHO considers CRE a pathogen of critical priority, and CDC considers them as pathogens of urgent threat [7][8]. CRE infections are endemic in Italy [9].

I.2.4.2 Mechanisms of Resistance and Molecular Epidemiology of CRKP

There are two mechanisms responsible for carbapenems resistance: (i) production of β-lactamases, including ESβLs and AmpC, coupled with reduced concentration of carbapenems in the intermembrane space, and (ii) carbapenemases production [10]. The reduced concentration can be due to the active efflux of the antibiotic molecule or due to structural mutations in genes encoding porins, such as *ompk35* and *ompk36*, which can lead to altered structure or loss of the porins reducing the diffusion of antibiotics into the cell.

Carbapenemases belong to three classes of β-lactamases based on Ambler classification: class A, such as KPC, and GES; D, such as OXA-48; and B, such as NDM, and VIM, with the first two being serine proteases and the last one being MBL which requires zinc for β-lactam hydrolysis (Fig. I.2.1) [10][11]. The first KPC-producing *K. pneumoniae* in Italy was reported in 2008 [12], after which a dramatic increase was observed worldwide. For NDM-1, the first detection was in 2009 in NDM-1-producing *E. coli*, while NDM-1-producing *K. pneumoniae* was reported in 2017 [13].

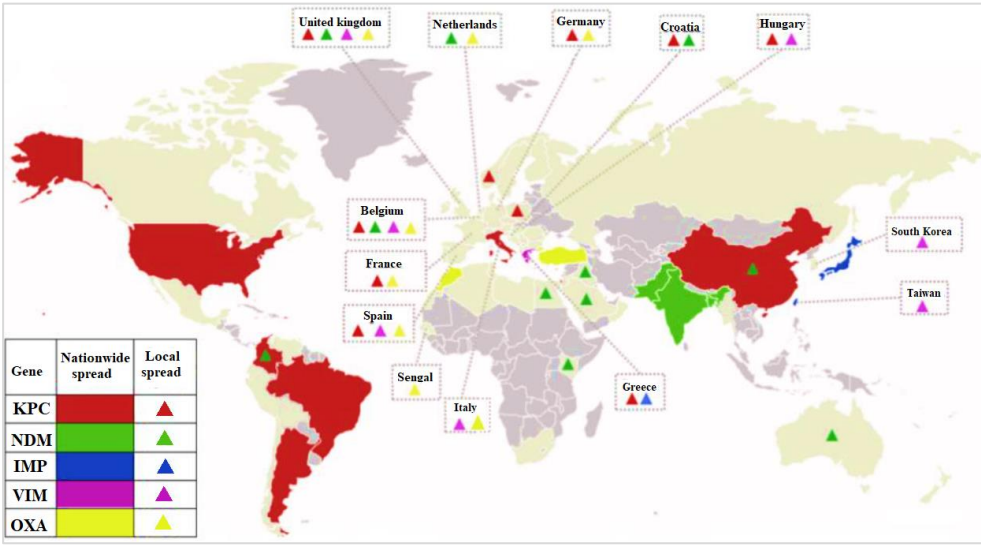


Figure I.2.1: The global distribution of the most prevalent carbapenemases in carbapenem-resistant *Enterobacterales* [11].

Several carbapenemases are locally spread all over Europe, with KPCs being the most prevalent carbapenemases in China, the United States, Italy, and South America; NDMs are prevalent in China, Pakistan, India, and Bangladesh, and widely spread around the world; IMPs are prevalent in Japan and Taiwan, China; VIMs are prevalent in Greece; and OXA-carbapenemases, mainly OXA-48, are prevalent in Turkey, Morocco, and many European countries, such as France, Germany, Netherlands, Italy, the United Kingdom [11].

In Nationwide surveillance covering all regions of Italy, in the period 2014–2017, KPC-producing *Enterobacterales* were responsible for 95.2% of the bloodstream infections, with KPC-3 being the most prevalent [14]. While MBLs, including NDM and VIM enzymes, and OXA-48 were detected in 1.9% and 1.2% of bloodstream infections, respectively. Coproduction of carbapenemases was also observed in 1.3% of *K. pneumoniae* strains.

Class A carbapenemases are serine proteases, including *Klebsiella pneumonia* carbapenemases (KPCs), Guiana extended spectrum (GES), imipenem resistant (IMI), non–metallo-carbapenemase-A (NMC-A), *Serratia marcescens* enzyme

(SME), and *S. fonticola* carbapenemase (SFC), with KPCs as the most prevalent type circulating in *Enterobacterales* [10]. Generally, KPC-producing isolates are MDR bacteria also presenting resistance determinants for fluoroquinolones, aminoglycosides, and trimethoprim-sulfamethoxazole [10]. The worldwide spread of KPC-producing *Enterobacterales* is mainly due to the clonal expansion of *K. pneumoniae* Clonal Group (CG) 258 strains harboring *bla*KPC-2 or *bla*KPC-3 genes in Tn*4401*, a Tn*3*-based transposon, and usually associated with plasmids belonging to IncF incompatibility group [15].

The resistance levels in KPC-producing strains can vary from low to high for carbapenems, reaching MIC value higher than 16 μg/mL, which are associated with increased *bla*KPC gene copy number, deletions directly upstream of the *bla*KPC gene, and/or outer membrane porin losses (OmpK35 and/or OmpK36) [10].

The class D OXA β-lactamases, especially OXA-48 variants, are found in *Enterobacterales*, mainly associated with IncL/M-type plasmids with integration of the *bla*OXA-48 gene as a part of Tn1999 composite transposon [10]. Usually, OXA-48 -producing strains show high penicillins resistance level, and low carbapenems levels resistance.

Among the MBL enzymes, the most represented are IMP, active on imipenem, VIM (Verona integron-encoded MBL), and NDM (New-Delhi MBL). VIM-type and IMP-type MBLs are most commonly class I integrons-supported and associated with transposons or plasmids [10]. The MBLs can hydrolyze all β-lactams, except monobactams; their activity is inhibited by metal-chelating agents, such as ethylenediaminetetraacetic acid (EDTA) [10]. NDM is the most prevalent among this class; unlike class A-KPC, it is mediated by different plasmid incompatibility types, and its dissemination is not associated with a specific dominant clone [10].

In Italy, KPC-KP from invasive infections usually belongs to ST512 and ST258, which was detected for the first time in Italy in 2008. On the other hand, recent epidemiological reports from several countries showed that ST101 and ST307, rarely reported in Italy, are emerging high-risk clones in several countries [16].

I.2.4.3 Antimicrobial Therapy for CRKP Infections

Due to the lack of effective therapy for CRE infections, combined approaches have been recommended to enhance the efficacy of tigecyclines and polymyxins. The combined approaches, mainly tigecycline- and polymyxin-based combinations, showed efficacy in many retrospective studies, clinical studies, and *in vitro* tests, along with reduced mortality rates, particularly with carbapenem-containing combinations [11]. However, these approaches are also threatened by the current resistance mechanisms, such as the upregulation of RND efflux pumps, and already circulating resistance genes for tigecycline, colistin, aminoglycosides, and

fosfomycin. It is worth mentioning that these two antibiotics have suboptimal efficacy and toxicity profiles. For example, tigecycline does not reach the optimal serum level making them ineffective for treating bloodstream infections.

Other combined approaches include novel β-lactamase inhibitors along with β-lactam antibiotics [11]. Avibactam is a novel β-lactamase inhibitor showing efficacy against significant β-lactamases, including KPC, ESβLs, AmpC, and OXA-48, but not MBLs. The combination, ceftazidime/avibactam (CAZ/AVI) showed efficacy against CRKP, in treating complicated urinary tract or intra-abdominal infections. Currently, CAZ-AVI is used empirically as a first-line choice therapy when other treatments fail [9].

Moreover, combining CAZ/AVI with carbapenems showed efficacy in treating pandrug-resistant KP bacteremia [17]. Insufficient data are available about the clinical efficacy and safety of other β-lactamase/β-lactamase inhibitors combinations. These combinations include aztreonam–AVI, imipenem–relebactam, and meropenem–vaborbactam, with the first one showing efficacy against several carbapenemase of class A, B, and D, and the last two showing efficacy against KPC only. These combinations are also affected by resistance mechanisms, including point mutations in *bla*KPC gene, increased expression of KPC enzymes, and mutations in genes encoding porins (such as Ompk35/Ompk36) leading to overexpression or structural changes reducing drug uptake [3][9].

I.2.5 Materials and Methods

1.2.5.1 Bacterial collection

In the period December 2020 – July 2021, a total of 66 non-duplicated clinical *K. pneumoniae* isolates were collected from 46 patients admitted to the ICU of the Civile Hospital of Voghera. Clinical and demographic information was obtained from the microbiological records, including the type of specimen and patient data, such as age, gender, ward hospitalization prior ICU, and CRE screening result at ICU admission. Only carbapenem-resistant isolates were included in this study. CRKP-colonization (CRKP-C) was considered when CRKP was isolated from screening rectal swabs, while CRKP-infection (CRKP-IN), when CRKP was isolated from a clinical specimen.

1.2.5.2 Antimicrobial Susceptibility Testing and Screening for Carbapenemases

The identification at the species level and the antimicrobial susceptibility tests were performed initially using the automated BD PhoenixTMM50 system (BD Diagnostics, Gurgaon, India), and confirmed with Microscan Gram-negative MIC/Combo panels of the semi-automated system MicroScan autoSCAN-4 (Beckman Coulter). Antibiograms were interpreted following EUCAST clinical breakpoint v 12.0. Clinical isolates that were not susceptible to carbapenems (imipenem, meropenem, or ertapenem) were tested for the presence of carbanemases (KPC, VIM, imipenemase [IMP], NDM, oxacillin-hydrolyzing [OXA]-48) using the immuno-chromatographic (IC) assay NG CARBA (NG Biotech, Guipry, France). Antimicrobial susceptibility testing for chloramphenicol (CLO) and tigecycline (TGC) was performed by disk diffusion method using EUCAST guidelines and breakpoints (EUCAST breakpoint tables for interpretation of MICs and zone diameters, version 12, 2022; http://www.eucast.org).

1.2.5.3 Molecular Detection of Resistance genes

Total DNA was extracted from CRKP strains using Wizard Genomic DNA Purification kit (Promega, Madison, WI, USA) or by the boiling method as described previously [1]. Briefly, 3 to 5 colonies of an overnight culture of each isolate were suspended in 500 µL of nuclease-free water and boiled for 10 min, then centrifuged at 13,000 g for 5 min; the DNA-containing supernatant was checked for DNA quality (260/280 and 260/230 ratio) and quantity (ng/µl) with a spectrophometer. Isolates were screened for ESβLs, carbapenemases and AmpC cephalosporinases genes presence using the Check-Points CT 103 XL Check-MDR assay (Wageningen, the Netherlands). Then, PCR assays were performed to assess the presence of resistance

genes including *bla*CTX-M, and *bla*KPC, and resistance genes to quinolones, including *qnrB*, *qnrS*, and *aac(6')-Ib-cr*, and aminoglycosides, including *aadA*, armA. The primer sequences, targeted genes, and amplicon sizes are listed in Table I.2.1. To determine the exact allelic variant of *bla*CTX–M and *bla*KPC, two-directional DNA sequencing was performed. PCR amplicons were purified using a Wizard® SV Gel and PCR Clean-Up System (Promega, Madison, WI, United States). DNA sequencing was performed using the Microsynth services (Microsynth Seqlab, Germany). The alignment between the forward, reverse, and reference DNA sequences was accomplished using ChromasPro software (Technelysium Pty Ltd, South Brisbane, Australia). The allelic variants were identified by sequence alignment with known GenBank sequence using the Basic local alignment search tool (BLAST) (http://blast.ncbi.nlm.nih.gov/Blast.cgi) [18].

Table I.2.1: Primers used in this study for detecting resistance genes

Enzyme/Enzyme family	Target of resistance gene	Resistance gene	Primer Sequences	Annealing temperature (°C)	Amplicon size (bp)	Reference
ESβL	P, Cp, E, M	*bla*CTX-M	FW: 5′-ATGTGCAGYACCAGTAARGT-3′	50	593	[19]
			Rev: 5′-TGGGTRAARTARGTSACCAGA-3′			
Carbapenemases Class A	P, Cp, E, M, Cb	bla KPC	FW: 5′-TGTCACTGTATCCGCGTC-3′	55	1,000	[20]
			Rev: 5′-CTCAGTGCTCTACAGAAAACC-3′			
Aminoacyl transferase	A and Q	*aac(6')Ibcr*	FW:5'-TTGCGATGCTCTATGAGTGGCTA-3'	55	482	[21]
			Rev: 5'-CTCGAATGCCTGGCGTGTTT-3'			
16S methyltransferase rRNA	A	*armA*	FW: 5'-ATTCTGCCTATCCTAATTGG-3'	55	315	[22]
			Rev: 5'-ACCTATACTTTATCGTCGTC-3'			
Aminoglycoside phosphotransferase	A	*aphA6*	FW: 5'-ATGGAATTCCAATATTATTC-3'	49	780	[23]
			Rev:5'-TCAATTCAATTCATCAAGTTTTA-3'			
Aminoglycoside adenylyltransferase	A	*aadA-1*	Fw: 5'- ATGAGGGAAGCGGTGATCG-3'	55	320	[24]
			Rev: 5'- TTATTTGCCGACTACCTTGGTG-3'			
Quinolone resistance protein B	Q	*qnrS*	Fw: 5′-GATCGTGAAAGCCAGAAAGG-3'	55	469	[25]
			Rev: 5′-ACGATGCCTGGTAGTTGTCC-3'			
Quinolone resistance protein B	Q	*qnrB*	Fw: 5′-ACGACATTCGTCAACTGCAA-3'	55	417	[25]
			Rev: TAAATTGGCACCCTGTAGGC-5'			

P: Penicillin, Cp: Cephalosporin, E: Extended-spectrum cephalosporin, M: Monobactam, Cb: Carbapenem, A: Aminoglycosides, Q: Quinolones.

1.2.5.4 Molecular Typing

Pulsed-field gel electrophoresis (PFGE) was performed using the XbaI restriction enzyme, and the obtained genomic fragments were separated on a CHEF-DR II apparatus (Bio-Rad, Milan, Italy) for 26 h at 14°C. Bacteriophage λ concatenamers were used as DNA size markers. DNA restriction patterns of scanned gel pictures

were interpreted following cluster analysis using the Fingerprinting II version 3.0 software (Bio-Rad) using the unweighted pair-group method with arithmetic averages (UPGMA). Only bands larger than 48 Kb were considered for the analysis. The Dice correlation coefficient was used with a 1.0% position tolerance to analyze the similarities of the banding patterns, and a similarity threshold of 90% to define clusters. The restriction patterns of the genomic DNA from the isolates were analyzed and interpreted according to the criteria of Tenover *et al*. [9]. Five *K. pneumoniae* isolates representing the different clones obtained in PFGE were chosen for MultiLocus Sequence Typing (MLST). MLST was performed according to the Pasteur scheme amplifying seven housekeeping genes (*gapA*, *infB, mdh*, *pgi*, *phoE*, *rpoB,* and *tonB*) [10][27]. Alleles and STs were assigned in accordance with the *K. pneumoniae* MLST website (https://pubmlst.org/bigsdb?db=pubmlst_mlst_seqdef).

I.2.6 Results

I.2.6.1 Clinical Characteristics

In the period December 2020 – July 2021, at the Microbiology Laboratory of the Civil Hospital of Voghera, a total of 66 *K. pneumoniae* were isolated from 48 patients admitted to the Intensive Care Unit (ICU). The 50% (n=33/66) of *K. pneumoniae* isolates from different patients were carbapenem resistant. The trend of CRKP detection increased during the study period, starting with the isolation of two isolates in the end of December (6.06%), eight isolates (24.24%) in January 2021, followed by the detection of four (12.12%), five (15.15%) and seven (21.21%) CRKP in February, March, and April 2021, respectively (Fig. I.2.2). In the following three months, the rate of detection decreased to three isolates (9.09%) in May 2021 and two isolates (6.06%) in June 2021 and July 2021 (Fig. I.2.2).

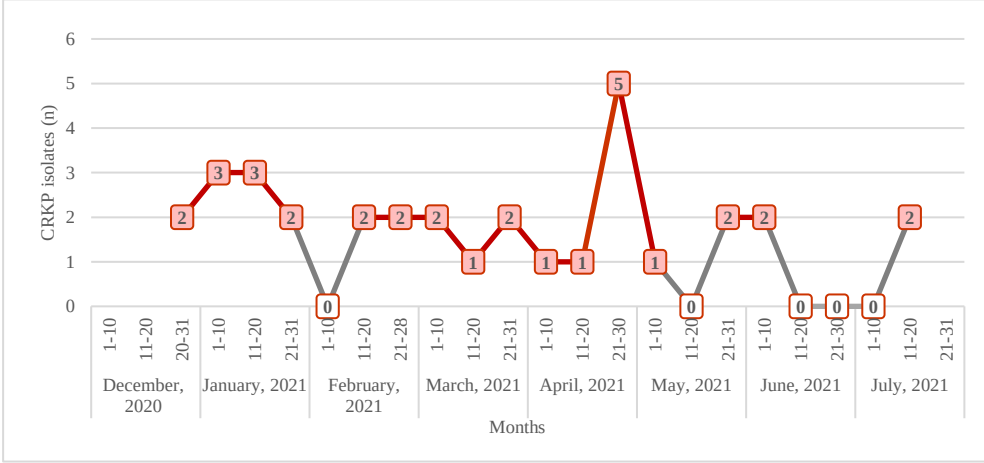


Figure I.2.2: Number and distribution of CRKP cases at Civile Hospital of Voghera (Voghera, Italy) in the period December 2020 to July 2021.

The age of patients colonized or infected by CRKP ranged from 49 to 84 (median=70; mean=68.5;), with 57% of affected patients (n=19/33) being older than 70 years old (Fig. I.2.3A). Among the patients of all age groups, 33.3% (n=11/33) were females and 66.7% (n=22/33) were males. Infected patients with CRKP accounted for 64% (n=21/33), while colonized patients were 36% (n=12/33) (Fig. I.2.3B). In case of infections (n=21), the clinical samples were equally distributed among bloodstream, respiratory, and urine specimens (each n=7; 35%) (Fig. I.2.3B and I.2.3C).

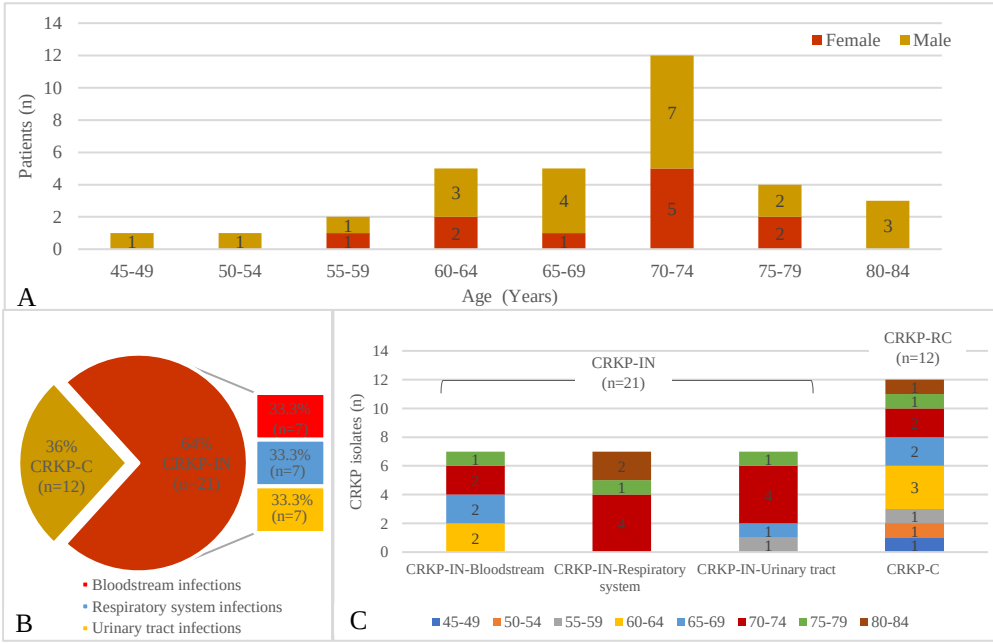


Figure I.2.3: Clinical characteristics of the CRKP infected patients. A. Distribution of CRKP cases according to patients' age and gender. B. Distribution of patients based on colorizations and infections. C. Distributions of CRKP cases according to patients' age and infection/colonization with CRKP.

To track back the source of this outbreak, CRKP cases were divided into three groups based on the ward in which the patients were initially admitted to prior the ICU stay (Fig. I.2.4A). First and second groups included patients from the Emergency Room (ER) (n=10, 30.3%), patients from other departments of the same hospital (n=14, 42.4%), respectively, while the third group included patients from the ER or other departments of other hospitals (n=9, 27.3%). Among the 33 patients, 39% (n=13/33) were CRKP-colonized on admission to the ICU, while the remaining patients acquired CRKP during ICU stay (Fig. I.2.4B). Of the 13 colonized-CRKP patients, 62% (n=8/13) were imported to the ICU from other departments of the same hospital, 23% (n=3/13) were imported from other hospitals, and 15% (n=2/13) were from ER of the Civil Hospital of Voghera (Fig. I.2.4C).

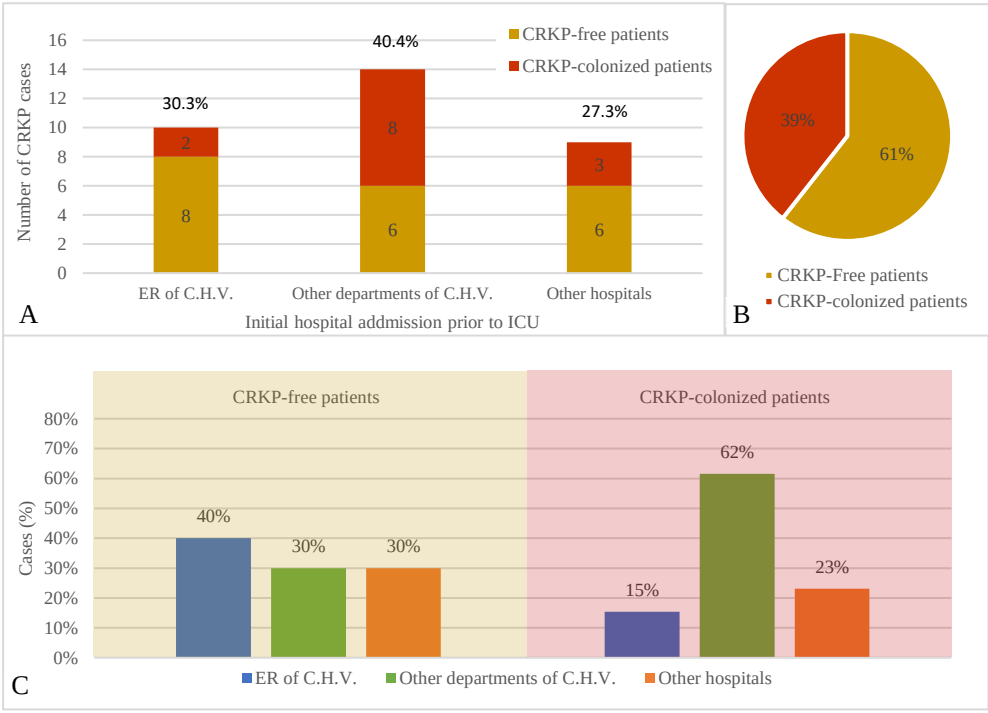


Figure I.2.4: Frequency of CRKP cases before ICU admission. A. Number and distribution of the n=33 CRKP cases according to the patients' initial admission prior ICU admission. B. The incidence of CRKP-colonized and CPRK-free patients on ICU admission. C. The incidence of CRKP-colonized and CRKP-free in ICU patients imported from other department in the Civile Hospital of Voghera and other hospitals.

I.2.6.2 Resistance Profiles of the CRKP Strains

Antimicrobial susceptibility profiles of the 33 CRKP isolates showed resistance to penicillins (100%, n=33), 3GCs (100%, n=33), 4GCs (100%, n=33), amoxicillin/clavulanate (100%, n=33), carbapenems (ertapenem 100%, n=33; meropenem 97%, n=32; and imipenem 97%, n=32), fluoroquinolones (ciprofloxacin 100%, n=33; and levofloxacin 100% , n=33), aminoglycosides (tobramycin 100%, n=33; amikacin 82%, n=27; and gentamycin 27%, n=9), trimethoprim/sulfamethoxazole (67%, n=22), and chloramphenicol (70%, n=23) (Fig. I.2.5). Three isolates (9%) showed resistance to ceftazidime/avibactam. All CRKP isolates exhibited an MDR phenotype being resistant to more than three classes of antibiotics [128]. All isolates with amikacin and tobramycin resistance were gentamicin resistant.

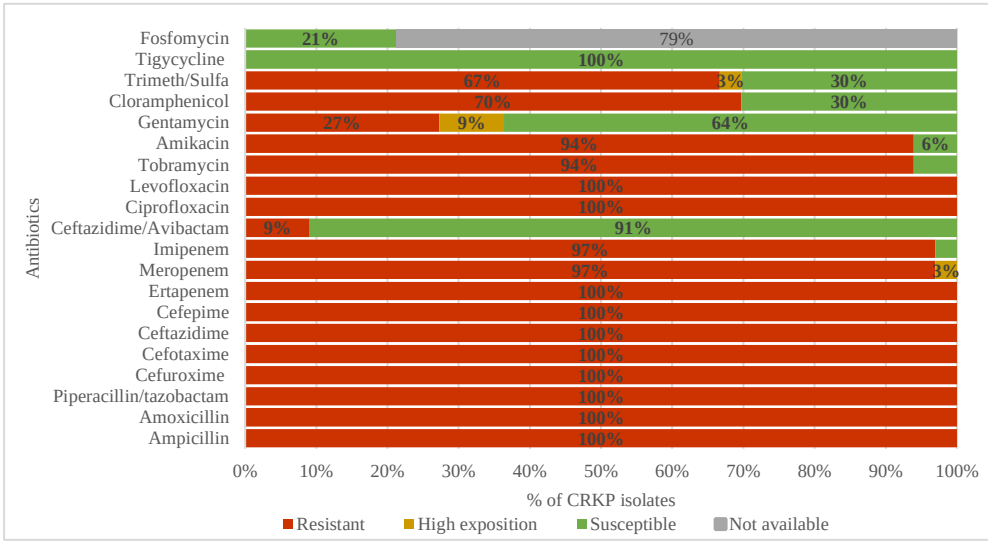


Figure I.2.5: Antimicrobial resistance profiles of CRKP isolates to 20 different antimicrobial drugs.

I.2.6.3 Mechanisms of Resistance in CRKP Isolates

All CRKP isolates were KPC-producers (97%, n=32/33), but one which resulted CTX-M-producer (3%, n=1/3) (Table I.2.2). Among KPC-producing isolates, KPC-3 (68.7%, n=22/32) was the most prevalent enzyme variant, followed by KPC-2 (31.3%, n=10/32). Accordingly, CRKP isolates were divided into two groups, KPC-2-KP and KPC-3-KP. The CTX-M-producing isolate harbored the *bla*-CTX-M-15 gene, thus explaining its low susceptibility profile to carbapenems (resistance to ertapenem, susceptibility with high exposition to meropenem, and susceptibility to imipenem). The three CAZ/AVI-resistant isolates harbored either *bla*KPC-3 (n=2) or or *bla*KPC-2 (n=1). All CRPK isolates were resistant to fluoroquinolones, with *aac(6')-Ib-cr* gene found in 78% (n=26/33) of all the isolates; among those the 12% (n=4) belonged to KPC-2-KP group, while the 66.7% (n=22) belonged to KPC-3-KP group. The CTX-M-15-producing strain harbored the *qnrS* gene. Regarding aminoglycosides resistance, each group of CRKP showed a distinct resistance profile (Fig. I.2.6A). The majority of KPC-2-KP group was resistant to all aminoglycosides (90%, n= 9/10), while the majority of KPC-3-KP group was resistant to amikacin and tobramycin (91%, n=20/22), and susceptible to gentamicin (95%, n=21/22). The CTX-M-15-KP isolate was susceptible to all tested aminoglycosides. Regarding the resistance mechanisms, the majority of KPC-2-KP group harbored *armA* (90%, n=9/10) and to a lesser extent *aac(6')-Ib-cr* (40%, n=4/10) genes(Fig. I.2.6B). On the other hand, all KPC-3-KP isolates harbored *aac(6')-Ib-cr* gene (n=22), explaining resistance to tobramycin and amikacin but not gentamicin; one isolate also harbored *armA* determinant (5%, n=1/22).

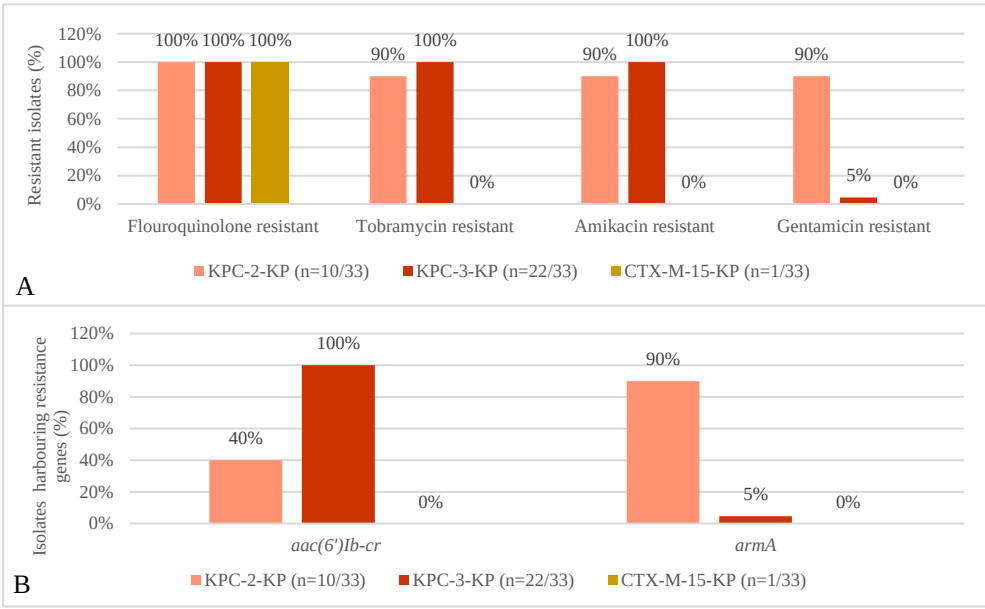


Figure I.2.6: Frequency of fluoroquinolones and aminoglycosides resistance profiles and associated resistance genes. A. Frequency of fluoroquinolones and aminoglycosides resistance in CRKP groups. B. Frequency of fluoroquinolones and aminoglycosides resistance genes in CRKP groups.

Table I.2.2: Summary of the resistance profiles and associated resistance genes for the n=33 CRKP isolates in this study

	Samples names[a,b]	Antimicrobial Resistance profile[b]	Resistance Determinants for		
			β-lactams	Quinolones	Aminoglycoside
1	B.L. 10165	AMP, AMC, PIP/TZP, CTX, FEP, CAZ, CXM, ERT, MER, IMP, CIP, LEV, GEN, TOB, AK	***bla***KPC-2	***aac(6')Ibcr+,*** *qnrB-, qnrS-*	***aac(6')Ibcr+, armA+,*** *aadA-type-, aphA6-*
2	C.G. 10143	AMP, AMC, PIP/TZP, CTX, FEP, CAZ, CXM, ERT, MER, IMP, CIP, LEV, TOB, AK	***bla***KPC-2	***aac(6')Ibcr+,*** *qnrB-, qnrS-*	***aac(6')Ibcr+, armA+,*** *aadA-type-, aphA6-*
3	E.R. 10047	AMP, AMC, PIP/TZP, CTX, FEP, CAZ, CXM, ERT, MER, IMP, CIP, LEV, GEN, TOB, AK	***bla***KPC-2	***aac(6')Ibcr-,*** *qnrB-, qnrS-*	*aac(6')Ibcr-,* ***armA+,*** *aadA-type-, aphA6-*
4	R.G. 10181	AMP, AMC, PIP/TZP, CTX, FEP, CAZ, CXM, ERT, MER, IMP, CIP, LEV, GEN, TOB, AK	***bla***KPC-2	***aac(6')Ibcr+,*** *qnrB-, qnrS-*	***aac(6')Ibcr+, armA+,*** *aadA-type-, aphA6-*
5	S.B. 10045	AMP, AMC, PIP/TZP, CTX, FEP, CAZ, CXM, ERT, MER, IMP, CIP, LEV, GEN, TOB, AK	***bla***KPC-2	***aac(6')Ibcr-,*** *qnrB-, qnrS-*	*aac(6')Ibcr-,* ***armA+,*** *aadA-type-, aphA6-*
6	G.T. 10081	AMP, AMC, PIP/TZP, CTX, FEP, CAZ, CXM, ERT, MER, IMP, CIP, LEV, TOB	***bla***KPC-2	***aac(6')Ibcr-,*** *qnrB-, qnrS-*	*aac(6')Ibcr-, armA-, aadA-type-, aphA6-*
7	S.M.T. 10084	AMP, AMC, PIP/TZP, CTX, FEP, CAZ, CXM, ERT, MER, IMP, CIP, LEV, GEN, TOB, AK	***bla***KPC-2	***aac(6')Ibcr-,*** *qnrB-, qnrS-*	*aac(6')Ibcr-,* ***armA+,*** *aadA-type-, aphA6-*
8	P.M 10213	AMP, AMC, PIP/TZP, CTX, FEP, CAZ, CXM, ERT, MER, IMP, CIP, LEV, GEN, TOB, AK	***bla***KPC-2	***aac(6')Ibcr-,*** *qnrB-, qnrS-*	*aac(6')Ibcr-,* ***armA+,*** *aadA-type-, aphA6-*
9	G.M.S. 10060[b]	AMP, AMC, PIP/TZP, CTX, FEP, CAZ, CXM, ERT, MER, IMP, CIP, LEV, GEN, TOB, AK, **CAZ/AVI**	***bla***KPC-2	***aac(6')Ibcr-,*** *qnrB-, qnrS-*	*aac(6')Ibcr-,* ***armA+,*** *aadA-type-, aphA6-*
10	P.V. 10130	AMP, AMC, PIP/TZP, CTX, FEP, CAZ, CXM, ERT, MER, IMP, CIP, LEV, TOB, SXT, CLO	***bla***KPC-3	***aac(6')Ibcr+,*** *qnrB-, qnrS-*	***aac(6')Ibcr+,*** *armA-, aadA-type-, aphA6-*
11	M.C. 10202[b]	AMP, AMC, PIP/TZP, CTX, FEP, CAZ, CXM, ERT, MER, IMP, CIP, LEV, TOB, AK, CLO, **CAZ/AVI**	***bla***KPC-3	***aac(6')Ibcr+,*** *qnrB-, qnrS-*	***aac(6')Ibcr+,*** *armA-, aadA-type-, aphA6-*
12	M.A. 10182	AMP, AMC, PIP/TZP, CTX, FEP, CAZ, CXM, ERT, MER, IMP, CIP, LEV, GEN, TOB, AK, SXT, CLO	***bla***KPC-3	***aac(6')Ibcr+,*** *qnrB-, qnrS-*	***aac(6')Ibcr+,*** *armA-, aadA-type-, aphA6-*
13	B.P. 10239	AMP, AMC, PIP/TZP, CTX, FEP, CAZ, CXM, ERT, MER (I), CIP, LEV, TOB, SXT, CLO	***bla***CTX-M-15	***aac(6')Ibcr-,*** *qnrB-, qnrS+*	*aac(6')Ibcr-, armA-, aadA-type-, aphA6-*
14	D.R. 10186	AMP, AMC, PIP/TZP, CTX, FEP, CAZ, CXM, ERT, MER (I), CIP, LEV, TOB, SXT, CLO	***bla***KPC-3	***aac(6')Ibcr+,*** *qnrB-, qnrS-*	***aac(6')Ibcr+,*** *armA-, aadA-type-, aphA6-*
15	S.F. 10185	AMP, AMC, PIP/TZP, CTX, FEP, CAZ, CXM, ERT, MER, IMP, CIP, LEV, GEN (I), TOB, SXT, CLO	***bla***KPC-3	***aac(6')Ibcr+,*** *qnrB-, qnrS-*	***aac(6')Ibcr+, armA+,*** *aadA-type-, aphA6-*
16	C.C. 10206	AMP, AMC, PIP/TZP, CTX, FEP, CAZ, CXM, ERT, MER, IMP, CIP, LEV, TOB, AK, SXT, CLO	***bla***KPC-3	***aac(6')Ibcr+,*** *qnrB-, qnrS-*	***aac(6')Ibcr+,*** *armA-, aadA-type-, aphA6-*
17	G.B. 10078	AMP, AMC, PIP/TZP, CTX, FEP, CAZ, CXM, ERT, MER, IMP, CIP, TOB, SXT, CLO	***bla***KPC-3	***aac(6')Ibcr+,*** *qnrB-, qnrS-*	***aac(6')Ibcr+,*** *armA-, aadA-type-, aphA6-*
18	G.V. 10231	AMP, AMC, PIP/TZP, CTX, FEP, CAZ, CXM, ERT, MER, IMP, CIP, LEV, TOB, AK, CLO	***bla***KPC-3	***aac(6')Ibcr+,*** *qnrB-, qnrS-*	***aac(6')Ibcr+,*** *armA-, aadA-type-, aphA6-*
19	Z.A. 10153	AMP, AMC, PIP/TZP, CTX, FEP, CAZ, CXM, ERT, MER, IMP, CIP, LEV, TOB, AK, SXT, CLO	***bla***KPC-3	***aac(6')Ibcr+,*** *qnrB-, qnrS-*	***aac(6')Ibcr+,*** *armA-, aadA-type-, aphA6-*
20	N.P. 10136	AMP, AMC, PIP/TZP, CTX, FEP, CAZ, CXM, ERT, MER, IMP, CIP, LEV, TOB, AK, SXT, CLO	***bla***KPC-3	***aac(6')Ibcr+,*** *qnrB-, qnrS-*	***aac(6')Ibcr+,*** *armA-, aadA-type-, aphA6-*
21	C.R. 10181	AMP, AMC, PIP/TZP, CTX, FEP, CAZ, CXM, ERT, MER, IMP, CIP, LEV, TOB, AK, SXT	***bla***KPC-3	***aac(6')Ibcr+,*** *qnrB-, qnrS-*	***aac(6')Ibcr+,*** *armA-, aadA-type-, aphA6-*
22	S.N. 10248	AMP, AMC, PIP/TZP, CTX, FEP, CAZ, CXM, ERT, MER, IMP, CIP, TOB, SXT, CLO	***bla***KPC-3	***aac(6')Ibcr+,*** *qnrB-, qnrS-*	***aac(6')Ibcr+,*** *armA-, aadA-type-, aphA6-*
23	B.S. 10117	AMP, AMC, PIP/TZP, CTX, FEP, CAZ, CXM, ERT, MER, IMP, CIP, LEV, TOB, AK, SXT, CLO	***bla***KPC-3	***aac(6')Ibcr+,*** *qnrB-, qnrS-*	***aac(6')Ibcr+,*** *armA-, aadA-type-, aphA6-*
24	G.R. 10200	AMP, AMC, PIP/TZP, CTX, FEP, CAZ, CXM, ERT, MER, IMP, CIP, LEV, TOB, AK, SXT, CLO	***bla***KPC-3	***aac(6')Ibcr+,*** *qnrB-, qnrS-*	***aac(6')Ibcr+,*** *armA-, aadA-type-, aphA6-*
25	S.P. 10189	AMP, AMC, PIP/TZP, CTX, FEP, CAZ, CXM, ERT, MER, IMP, CIP, LEV, TOB, AK, SXT, CLO	***bla***KPC-3	***aac(6')Ibcr+,*** *qnrB-, qnrS-*	***aac(6')Ibcr+,*** *armA-, aadA-type-, aphA6-*
26	M.D. 10084	AMP, AMC, PIP/TZP, CTX, FEP, CAZ, CXM, ERT, MER, IMP, CIP, LEV, TOB, AK, SXT, CLO	***bla***KPC-3	***aac(6')Ibcr+,*** *qnrB-, qnrS-*	***aac(6')Ibcr+,*** *armA-, aadA-type-, aphA6-*
27	D.M.C. 10141	AMP, AMC, PIP/TZP, CTX, FEP, CAZ, CXM, ERT, MER, IMP, CIP, LEV, GEN, TOB, AK, CLO	***bla***KPC-2	***aac(6')Ibcr+,*** *qnrB-, qnrS-*	***aac(6')Ibcr+, armA+,*** *aadA-type-, aphA6-*
28	C.L.K.M. 10115	AMP, AMC, PIP/TZP, CTX, FEP, CAZ, CXM, ERT, MER, IMP, CIP, LEV, GEN (I), TOB, AK, SXT (I), CLO	***bla***KPC-3	***aac(6')Ibcr+,*** *qnrB-, qnrS-*	***aac(6')Ibcr+,*** *armA-, aadA-type-, aphA6-*
29	E.T.B. 10061	AMP, AMC, PIP/TZP, CTX, FEP, CAZ, CXM, ERT, MER, IMP, CIP, LEV, TOB, AK, SXT, CLO	***bla***KPC-3	***aac(6')Ibcr+,*** *qnrB-, qnrS-*	***aac(6')Ibcr+,*** *armA-, aadA-type-, aphA6-*
30	D.L.R. 10137	AMP, AMC, PIP/TZP, CTX, FEP, CAZ, CXM, ERT, MER, IMP, CIP, GEN (I), TOB, AK, SXT, CLO	***bla***KPC-3	***aac(6')Ibcr+,*** *qnrB-, qnrS-*	***aac(6')Ibcr+,*** *armA-, aadA-type-, aphA6-*
31	P.F. 10125	AMP, AMC, PIP/TZP, CTX, FEP, CAZ, CXM, ERT, MER, IMP, CIP, LEV, TOB, AK, SXT, CLO	***bla***KPC-3	***aac(6')Ibcr+,*** *qnrB-, qnrS-*	***aac(6')Ibcr+,*** *armA-, aadA-type-, aphA6-*
32	T.F. 10089[b]	AMP, AMC, PIP/TZP, CTX, FEP, CAZ, CXM, ERT, MER, IMP, CIP, LEV, TOB, AK, SXT, CLO, **CAZ/AVI**	***bla***KPC-3	***aac(6')Ibcr+,*** *qnrB-, qnrS-*	***aac(6')Ibcr+,*** *armA-, aadA-type-, aphA6-*
33	P.G. 10306	AMP, AMC, PIP/TZP, CTX, FEP, CAZ, CXM, ERT, MER, IMP, CIP, LEV, TOB, AK, SXT, CLO	***bla***KPC-3	***aac(6')Ibcr+,*** *qnrB-, qnrS-*	***aac(6')Ibcr+,*** *armA-, aadA-type-, aphA6-*

*[a]WGS,subjected for whole-genome sequencing. *Ceftazidime /Avibactam resistant.[b]AMP, ampicillin; AMC, amoxicillin/clavulanic acid; PIP/TZP, piperacillin-tazobactam; CTX, cefotaxime; FEP, cefepime; CAZ, ceftazidime; CXM, cefuroxime; ERT, ertapenem; MEM, meropenem; IMI, imipenem; CIP, ciprofloxacin; LEV, levofloxacin; TOB, tobramycin; AK. Amikacin; GM, gentamicin; SXT, trimethoprim/sulfamethoxazole; I, high exposition.*

I.2.6.4 Molecular Typing

The 33 CRKP isolates, collected from different patients in the period December 2020 to January 2021, were classified into four PFGE pulsotypes, A, B, C, and D. Clone A consisted of all the isolates belonging to KPC-2-group (100%, n=10) (Fig. I.2.7, Table I.2.3). Clone B consisted of all the isolates of KPC-3-KP group (95.5%), collected from different patients in the period of February 2020 to January 2021; only one isolate (4.5%) belonged to clone C and was collected in June 2021. Clone D consisted of the only CTX-M-15-producing *K. pneumoniae* isolate (Fig. I.2.7). MLST analysis was performed for five CRKP representing the different clones based on PFGE results. The two isolates representing clone A belonged to ST101, clone B and C belonged to ST512, and clone D belonged to ST234 (Fig. I.2.7). Moreover, the plasmid content for the clone A/ST101 included plasmids of FIIK and a no group assigned amplicon, while plasmid content of clone B/ST512 included X3, FIIK, FIB, KQ and FIB KN replicons and for clone C/ST512 FIIK, FIB, and KQ were detected.

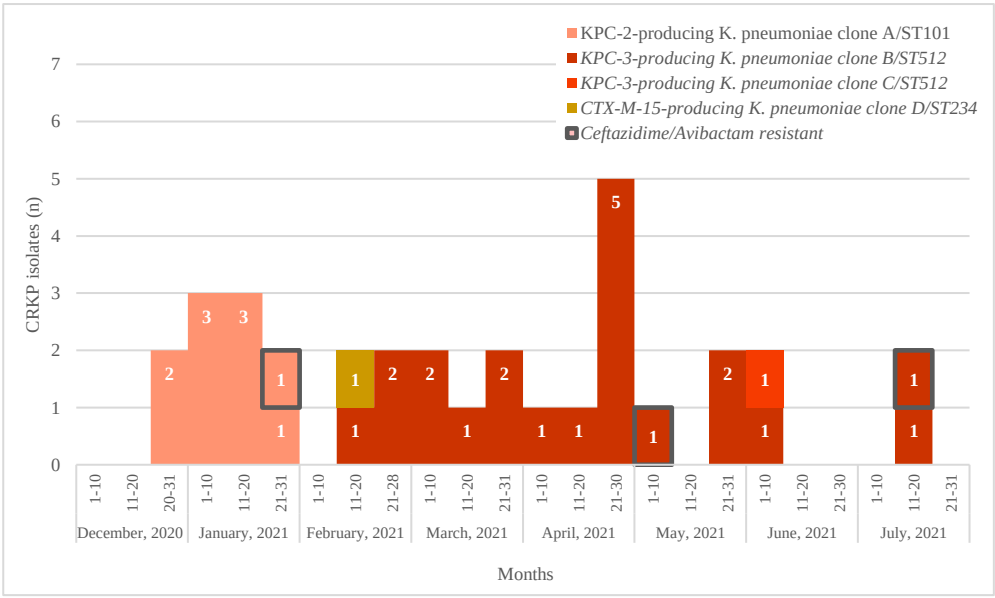
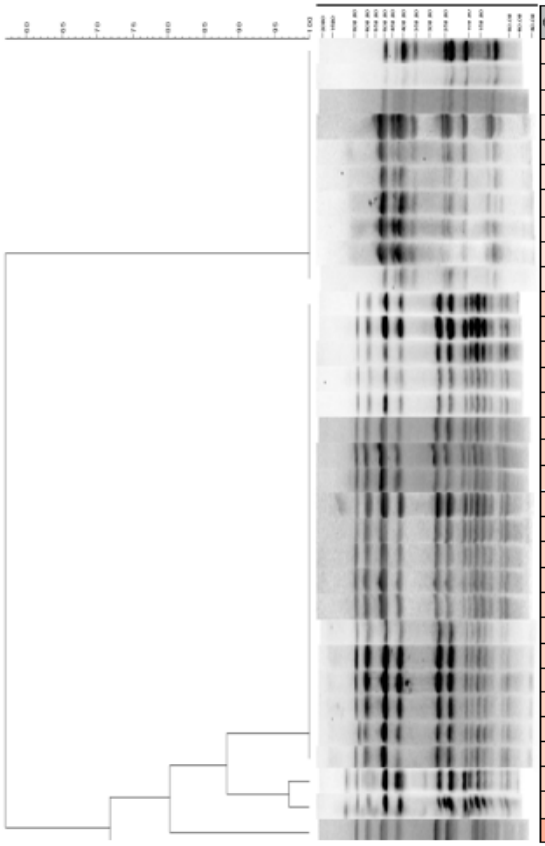


Figure I.2.7: The distribution of the KPC-2-KP, KPC-3-KP and CTX-M-15-KP isolates studied.

Table I.2.3: Cluster analysis of the n=32 KPC-2/3-KP isolates



Clone[a,b]	Isolate code[c]	Date of isolation (Year-month-day)	Specimen type	Resistance genes for			MLST
				β-lactams	Fluoroquinolones	Aminoglycosides	
A	R.G. 10181	2021-01-27	Urine, blood culture, rectal swab	*bla*KPC-2	*aac(6')Ib-cr*	*aac(6')Ib-cr, armA*	NA
A	C.G. 10143[WGS]	2020-12-27	Bronchoalveolar lavage, blood culture, urine, venous catheter	*bla*KPC-2	*aac(6')Ib-cr*	*aac(6')Ib-cr, armA*	NA
A	D.M.C. 10141	2021-01-05	Blood culture, rectal swab	*bla*KPC-2	*aac(6')Ib-cr*	*aac(6')Ib-cr, armA*	101
A	B.L. 10165	2020-12-28	Bronchoalveolar lavage, bronchial aspirate	*bla*KPC-2	*aac(6')Ib-cr*	*aac(6')Ib-cr, armA*	101
A	E.R. 10047	2020-12-29	Rectal swab	*bla*KPC-2	Resistant	*armA*	NA
A	S.B. 10045	2021-01-16	Rectal swab	*bla*KPC-2	Resistant	*armA*	NA
A	G.T. 10081	2021-01-20	Rectal swab, bronchoalveolar lavage, bronchial aspirate	*bla*KPC-2	Resistant	Resistant	NA
A	S.M.T. 10084	2021-01-08	Rectal swab	*bla*KPC-2	Resistant	*armA*	NA
A	P.M 10213	2021-01-08	Rectal swab, bronchoalveolar lavage	*bla*KPC-2	Resistant	*armA*	NA
A	G.M.S. 10060	2021-01-21	Rectal swab	*bla*KPC-2	Resistant	*armA*	NA
B	S.F. 10185	2021-03-10	Blood culture	*bla*KPC-3	*aac(6')Ib-cr*	*aac(6')Ib-cr, armA*	512
B	D.L.R. 10137	2021-05-30	Urine, blood culture	*bla*KPC-3	*aac(6')Ib-cr*	*aac(6')Ib-cr*	NA
B	N.P. 10136	2021-04-08	Blood culture	*bla*KPC-3	*aac(6')Ib-cr*	*aac(6')Ib-cr*	NA
B	P.V.10130 [WGS]	2021-02-18	Urine	*bla*KPC-3	*aac(6')Ib-cr*	*aac(6')Ib-cr*	NA
B	T.F. 10089	2021-07-11	Bronchoalveolar lavage	*bla*KPC-3	*aac(6')Ib-cr*	*aac(6')Ib-cr*	NA
B	G.R.10200 [WGS]	2021-04-26	Blood culture, bronchial aspirate	*bla*KPC-3	*aac(6')Ib-cr*	*aac(6')Ib-cr*	NA
B	G.V. 10231	2021-03-25	Bronchoalveolar lavage	*bla*KPC-3	*aac(6')Ib-cr*	*aac(6')Ib-cr, armA*	NA
B	C.R. 10181	2021-04-20	Bronchoalveolar lavage	*bla*KPC-3	*aac(6')Ib-cr*	*aac(6')Ib-cr*	NA
B	S.N. 10248	2021-04-21	Urine	*bla*KPC-3	*aac(6')Ib-cr*	*aac(6')Ib-cr*	NA
B	D.R. 10186	2021-03-06	Urine	*bla*KPC-3	*aac(6')Ib-cr*	*aac(6')Ib-cr*	NA
B	G.B. 10078	2021-03-18	Urine, bronchoalveolar lavage	*bla*KPC-3	*aac(6')Ib-cr*	*aac(6')Ib-cr, armA*	NA
B	Z.A. 10153 A	2021-02-26	Urine	*bla*KPC-3	*aac(6')Ib-cr*	*aac(6')Ib-cr*	NA
B	S.P. 10189	2021-04-30	Bronchoalveolar lavage, Rectal swab, bronchial aspirate	*bla*KPC-3	*aac(6')Ib-cr*	*aac(6')Ib-cr*	NA
B	M.A. 10182	2021-02-25	Rectal swab, bronchial aspirate, Urine	*bla*KPC-3	*aac(6')Ib-cr*	*aac(6')Ib-cr*	NA
B	C.C. 10206	2021-03-22	Rectal swab	*bla*KPC-3	*aac(6')Ib-cr*	*aac(6')Ib-cr, armA*	NA
B	B.S. 10117	2021-04-26	Rectal swab, urine, bronchial aspirate	*bla*KPC-3	*aac(6')Ib-cr*	*aac(6')Ib-cr*	NA
B	M.D. 10084	2021-04-28	Rectal swab	*bla*KPC-3	*aac(6')Ib-cr*	*aac(6')Ib-cr*	NA
B	E.T.B. 10061	2021-05-22	Rectal swab	*bla*KPC-3	*aac(6')Ib-cr*	*aac(6')Ib-cr*	NA
B	P.G. 10306	2021-07-16	Rectal swab	*bla*KPC-3	*aac(6')Ib-cr*	*aac(6')Ib-cr*	NA
B1	P.F. 10125	2021-06-07	Blood culture, peritoneal fluid, wound and rectal swabs	*bla*KPC-3	*aac(6')Ib-cr*	*aac(6')Ib-cr*	NA
B1	M.C. 10202	2021-05-03	Bronchial aspirate	*bla*KPC-3	*aac(6')Ib-cr*	*aac(6')Ib-cr*	NA
C	C.L. 10115 [WGS]	2021-06-02	Blood culture, other material	*bla*KPC-3	*aac(6')Ib-cr*	*aac(6')Ib-cr*	512

[a]The different clones are shown in different shades and the first cultured isolate of each clone is in red font.
[b]Isolates in red represent the first KPC-2-KP and KPC-3-KP isolates obtained in this outbreak.
[c]WGS: isolates subjected for whole Genome Sequencing.

I.2.7 Discussion

Outbreaks of KPC-KP are responsible for a wide range of healthcare-associated infections, particularly in long-term care facilities and ICUs.

In this study, we described an outbreak caused by heteroclonal CRKP strains isolated from patients admitted to the ICU at Voghera Hospital, Lombardy, (Nothern Italy), in the period December 2020 – July 2021. Almost all the CRKP strains were KPC-producers (97%: 31.3% KPC-2, and 88.8% KPC-3), confirming that KPC production is the most prevalent mechanism of carbapenem resistance, as previously reported in other Italian studies [14].

The 32 KPC-KP isolates belonged to three different clones, A/ST101, B/ST512, and C/ST512. Most KPC-KP isolates belonged to ST512 (63.6%) and were all KPC-3-producers; ST512 KPC-3-producers were previously detected in two studies from southern Italy [29][30]. ST512 is a single-locus variant derivative of the ST258, grouped in the Clonal Group CG258 [31]. CG258 is a high-risk lineage responsible for the international spread of CRKP. Clone ST101 included 30.3% of the isolates, which were all KPC-2 producers. Currently, ST101 is recognized globally as one of the main high-risk clones, exhibiting MDR or extensive drug resistance phenotypes [32]. Koster *et. al.* reported that on 574 hospital-associated ST101 *K pneumoniae* isolates the 97% were either ESβL-producers, particularly CTXM-15 followed by CTX-14, or (and) carbapenemase producers, particularly OXA-45, followed by KPC and NDM. This clone has been isolated from specimens of different sources, including environment (natural and human-associated), animals, and humans, both healthy carriers and patients with different infections [32]. According to the same study by Kostrer *et. al*., hospital-associated *K. pneumoniae* ST101 strains can be differentiated from other *K. pneumoniae* ST101 strains by the presence of yersiniabactin, an essential virulence factor, found mainly on mobile genetic elements, and associated with bacterial pathogenesis and invasive infections [32][33]. In Italy, ST101 was associated with either KPC-2 or KPC-3 production and mainly involved with HCAI of increased morbidity and mortality [16][33].

During the outbreak period, one sporadic strain of *K. pneumoniae* was found to be a CTX-M-15 producer belonging to clone D/ST234, exhibiting resistance to ertapenem and reduced susceptibility to meropenem. To our knowledge, this clone has never been reported before in Italy. Ertapenem resistance and low susceptibility to meropenem might be associated with the low ertapenem concentrations in the periplasmic space due to mutations in porin genes, *ompK35* and (or) *ompK36*, *ompK36*, and (or) the active efflux of carbapenems [10][34].

CAZ/AVI resistance phenotype was detected in three isolates, one belonging to KPC-2-producing *K. pneumoniae* clone A/ST101, and two belonging to KPC-3-producing *K. pneumoniae* clone B/ST512. CAZ/AVI resistance in KPC-producing *K.*

pneumoniae strains has been reported in many countries, including Italy [9]. The three isolates had the wildtype *bla*KPC2/3, indicating that the suggested mechanisms for CAZ/AVI resistance phenotypes might be due to the accumulation of multiple resistance mechanisms, including increased expression of KPC2/3, porin alterations due to mutations in *ompk35* and *ompk36*, and increased efflux, which all has been reported in Italy with the first two being the most prevalent [3][35].

I.2.8 Conclusions

After investigating antibiotic resistance mechanisms and the epidemic characteristics of the 33 CRKP isolates from ICU patients, we found out that the epidemiological outbreak was sustained by two different CRKP clones, ST101/KPC-2- and ST512/KPC-3-producing *K. pneumoniae*. Our results clearly highlighted the importance of molecular epidemiology to precisely characterize the clinical impact of such pathogens and investigate the spread dynamics of high-risk clones in our region.

Knowing the epidemic characteristics along with antibiotic resistance mechanisms of MDR strains can give an insight to manage therapeutic options and minimize the selection of resistant bacteria, delaying the emergence of further resistance mechanisms.

Preventing CRKP colonization/infection in ICU patients is one of the major aims of modern medicine. Information on colonization, antibiotic use, prior hospitalization, and epidemiological-molecular investigations, together with strict infection control measures, are essential for the containment of MDR strains that are difficult to manage.

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Chapter 3: Genomic Characterization of an O101:H9-ST167 NDM-5-Producing *Escherichia coli* Strain from a Kitten in Italy

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I.3.1 Abstract

Introduction: New Delhi metallo-β-lactamase (NDM)-producing *Enterobacterales* are rapidly spreading in both clinical and environmental settings. Plasmid-mediated carbapenemase genes are reported as emerging from wild, companion and food animals' biological samples. Aim of the study was the characterization of the NDM-5-producing *Escherichia coli* strain obtained from a cat of a private house, in Italy.

Materials and Methods: The *E. coli* strain 167624/2, collected from a liver sample of a 4-monthold cat who died due to parvovirus haemorrhagic enteritis, was evaluated for antibiotic susceptibility by AUTOSCAN4 semi-automated system (BeckmanCoulter), after which Whole-Genome Sequencing (WGS) was performed on Illumina MiSeq platform and Nanopore technologies to investigate the resistance and virulence genes using Abricate, and the MLST by MLST2.0 tools. The reads quality was evaluated using FastQC, assembled using Shovill. The alignment and the coreSNP calling were performed using the software Purple on the 167624/2 and the 50 closest genomes, retrieved from PATRIC database. The coreSNPs alignments were used to infer phylogeny via RaxML with 100 bootstrap replicates.

Results: The 167624/2 strain exhibited MDR phenotype showing resistance to all β-lactams, flouroquinolones, trimethoprim/sulfamethoxazole, cloramphenicol, and aminoglycosides. Susceptibility was retained only for colistin, amikacin and fosfomycin. WGS analyses showed that the strain belonged to the ST167, clonal complex 10, showing close relatedness to other ST167 *E. coli* isolated from Italy and Switzerland, from both human and animal origin. Various genetic determinants were present, including *bla*NDM-5, *bla*AmpC, *bla*AmpH, *aac(3)-Ila*, *aad*A2, *tet*R, *tet*A, *dfr*A12, *mph*A, *mdf*A, and *sul*1, and mutations in *gyr*A, *par*C and *par*E genes explaining fluoroquinolones resistance. Several virulence factors were found including *fyu*A, *gad*, *iuc*C, *iut*A, *sit*A and *ter*C.

Discussion and Conclusions: The detection of NDM-5 producing *E. coli* belonging to ST167 high-risk clone in a companion animal poses a significant public health problem, highlighting the spreading potential of such antibiotic-resistance genes reservoirs.

I.3.2 Letter to the Editor

The high-risk clone ST167 associated with *bla*NDM-5 resistance determinant is currently recognized to be a source of public health concern worldwide [1][2][3][4], since it has been identified even beyond hospital borders, in companion animals, wastewater, rivers, and wildlife [5][6][7][8]. In this work, we characterized an NDM-5-producing *Escherichia coli* ST167 collected in Italy from a liver sample of a 4-month-old cat who died from parvovirus hemorrhagic enteritis. The *E. coli* strain 167624 was tested for antibiotic susceptibility and sequenced using both Illumina and Nanopore technologies. Bacterial identification and antibiotic susceptibility tests were performed with the semiautomated system MicroScan autoSCAN4 (Beckman Coulter); results were interpreted according to EUCAST guidelines (v10.0-2020, http://www.eucast.org).

The *E. coli* 167624 strain showed a multidrug-resistant (MDR) profile, being resistant to all the antibiotics tested, except for colistin, amikacin, and fosfomycin (Table I.3.1).

Table I.3.1: Antimicrobial susceptibility profile of the ECO167624 strain

Antibiotic	MIC[a] (μg/mL)	Interpretation
AMK	≤8	S
AMP	>8	R
AMC	>8|4	R
AZT	>4	R
FEP	>8	R
CTX	>16	R
CAZ	>8	R
CIP	>1	R
LEV	>1	R
GNT	>4	R
COL	≤2	S
FOS	≤32	S
ERT	>1	R
MER	>8	R
PTZ	>16	R
SXT	>4|76	R
PIP	>16	R
TBR	>4	R

[a]AMK, amikacin; AMP, ampicillin; AMC, amoxicillin/clavulanate; AZT, aztreonam; FEP, cefepime; CTX, cefotaxime; CAZ, ceftazidime; CIP, ciprofloxacin; LEV, levofloxacin; GNT, gentamicin; COL, colistin; FOS, fosfomycin; ERT, ertapenem; MER, meropenem; PTZ, piperacillin-tazobactam; SXT, trimethoprim-sulfamethoxazole; PIP, piperacillin; TBR, tobramycin; S, susceptible; R, resistant. Susceptibility results were interpreted according to the European Committee on Antimicrobial Susceptibility Testing (EUCAST, 2020) criteria.

Genomic DNA was sequenced via both Oxford Nanopore MinION, with library FLO-MIN106 (rapid barcoding kit SQK-RBK004), and Illumina MiSeq platform (Nextera XT library preparation kit, with a 2 250 paired-end run), after extraction with DNeasy blood and tissue kit (Qiagen). A complete hybrid genome was obtained (genome size of 5,141,416 bp, chromosome sequence of 4,849,672 bp) using

Unicycler v0.4.8-beta [9]. A main plasmid, pGA_EcoNDM5 (size of 100,291 bp), harboring the blaNDM-5 gene was detected and annotated (Fig. I.3.1). *In silico* multilocus sequence type (MLST) analysis showed that the strain ECO16724 belonged to the high-risk clone ST167 (MLST Achtman scheme), phylogroup A, and serotype O101:H9. Investigation of the resistance genes content highlighted the copresence of multiple b-lactamase determinants, including the plasmid-borne *bla*NDM-5 and *bla*ble, as well as *bla*AmpH and *bla*AmpC1 on the chromosome. In addition, virulence factors associated with flagellar motility (*Fli*/*Flg* family), fimbriae (*fimF*, *fimG*), and siderophore (*ybtT*, *iucA*) were detected on the chromosome and on the pGA_NDM5 plasmid. Resistance determinants included *bla*NDM-5, *bla*ble, *bla*AmpH, *bla*AmpC1, *gyrA* (S83L, D87N), *parC* (S80 I), *parE* (S458A), *mph(A)*, *tet(A)*, *tet(R)*, *aac(3)-IIa*, *aadA2*, *sul1*, and *dfrA12*. Virulence determinants included *fliN*, *fliM*, *fliL*, *fliJ*, *fliA*, *flgH*, *flgG*, *flgD*, *flgC*, *flgB*, *fimF*, *fimG*, *ybtT*, *iucA*, *cea*, *capU*, *fyuA*, *gad*, *hra*, *irp2*, and aerobactin operon. The pGA_EcoNDM5 belonged to the IncFIA with an identity score of 99.48%.

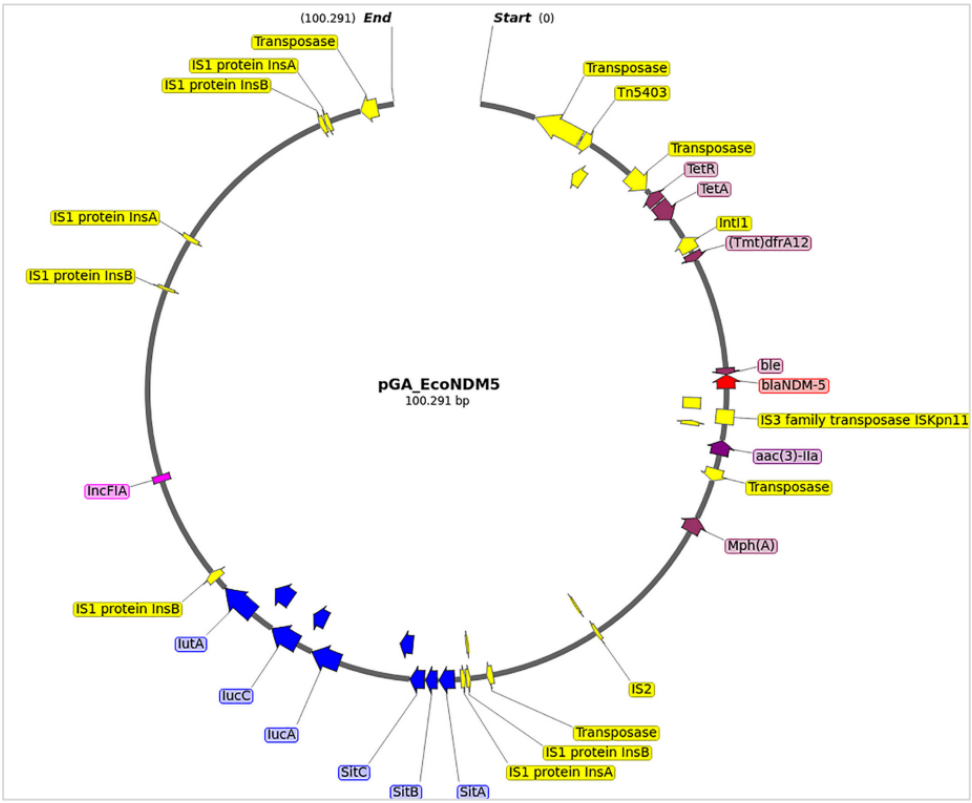

Figure I.3.1: Graphical representation of the pGA_EcoNDM5 plasmid sequence. Colored arrows represent genes or coding regions: red, *bla*NDM-5 gene; purple, antimicrobial resistance genes; yellow, insertion sequences (IS) and transposons; blue, aerobactin operon and virulence genes; fuchsia, incompatibility group.

To place the ECO16724 isolate within the proper taxonomic context, a coreSNP phylogeny was inferred (see supplemental material). The phylogenetic analysis (Fig. I.3.2) showed ECO167624 to be part of a clade including *bla*NDM-5-positive strains: four from human and dog sources in Switzerland (2017 to 2018) and one, LR880734.1, from a dog in Italy (2019). The comparison of the blaNDM-5 genetic environment among the plasmids of the strains within this clade highlighted a high similarity, showing the same NDM-carrying integron. Transmission between animals and humans of ST167 NDM-5-producing *E. coli* has been already demonstrated in a familiar context [7]. Although we were not able to trace the origin of the here-presented ECO167624 strain, a human-animal transmission event could be hypothesized. In Italy, the blaNDM-5 gene is to date associated mainly with human clinical cases [1][2], but our results raise the hypothesis that community could represent a hidden reservoir of NDM-5-producing ST167 high-risk clone. The ability to trace rapidly the source of infection is of relevance in a globalized world, where the boundaries among the different settings (humans, environment, animals) are continuously crossed by bacteria. Hence, the standardization of tools and user-friendly platforms for the genomic surveillance, such as Pathogenwatch and BacWGSTdb 2.0 [10][11], is acquiring an increasingly pivotal role. The increased reports of MDR clones in the hospital, community, and environment surely sound like an alarm bell, suggesting the appropriateness of the "One-Health" approach.

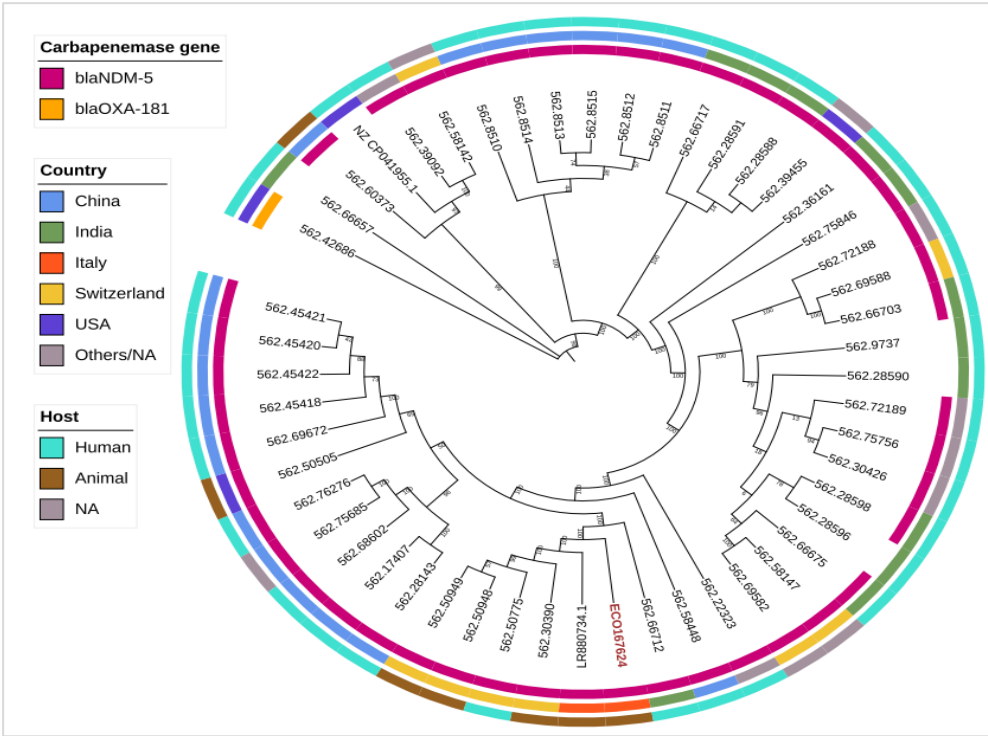

Figure I.3.2: CoreSNP-based phylogeny of the 50 *E. coli* strains closest to ECO167624 retrieved from PATRIC database**.**

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Part II: Impact of Biofilm on *Staphylococcus aureus* Infections Outcomes

Chapter 1: General Introduction

Chapter 2: Evaluation of the Anti-Biofilm Activity of Four Different Antibiotics Against *S. aureus* Strains from Ocular Infections

Chapter 3: Antimicrobial Activity of Benzalkonium Chloride and Zinc Sulphate: Evaluation of the Synergistic Effect in vitro on Ophthalmic Isolates of *Staphylococcus aureus*

Chapter 1: General Introduction

II.1.1 Overview *Staphylococcus aureus*: History, Characteristics, Colonization, and Clinical significance

Staphylococcus aureus is a human pathogen, causing hospital- and community-acquired infections ranging from self-limiting mild infections to life-threatening infections, including pneumonia, bloodstream infections, or endocarditis [1]. Since its discovery in 1880, it remained one of the most important human pathogens due to its unique characteristics, virulence through structural and secreted products, and ability to acquire resistance mechanisms to different classes of antibiotics [1][2].

S. aureus was firstly identified in pus from a surgical abscess in a knee joint by the Scottish surgeon Alexander Ogston in 1880, naming it *Staphylococcus*, (from the Greek staphyle - bunch of grapes - and kokkos - berry). In 1884, The German physician Friedrich Rosenbach named the specie *aureus* (from the Latin word means golden) because of the golden appearance of its colonies on solid media [3]. The Genus Staphylococcus includes more than 50 species divided into two groups based on the ability to coagulase production, coagulase-negative staphylococci (CoNS), and coagulase-positive staphylococci (CoPS), to which the most problematic pathogen within the genus, *S. aureus*, belong.

S. aureus is a facultative anaerobic Gram-positive coccus found in single, pairs, short chains or grape-like clusters able to survive dry conditions and high salt concentrations (up to 10 %) (Fig. II.1.1A) [3][2]. *S. aureus* is differentiated from other staphylococcal species based on gold pigmentation of colonies due to the production of the golden carotenoid pigment, Staphyloxanthin [3]. Mannitol salt agar (MSA) is selective a differential media allowing the selection of *S. aureus* due to being salt tolerant differentiating *S. aureus* from *S. epidermidis*, as the first being mannitol fermenter, shifting the pink color of medium to yellow due to acid production during mannitol fermentation (Fig. II.1.1B). Furthermore, they are catalase-positive, oxidase negative, and able to produce hemolysins showing the typical β-hemolytic phenotype on blood agar (Fig. II.1.1C) [3].

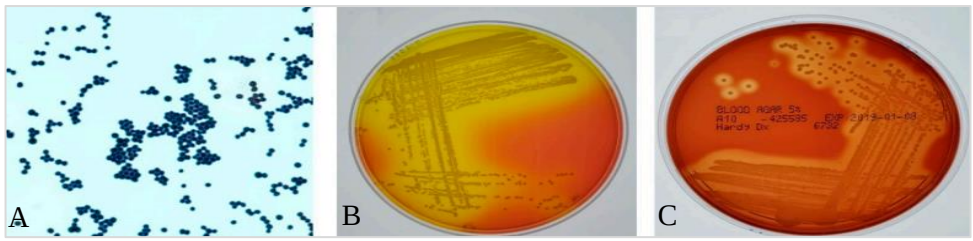

Figure II.1.1: Phenotypes of *S. aureus*. A. Gram stain of *S. aureus*. Magnification, 100×. B. *S. aureus* grown on mannitol salt agar containing 7.5-10% sodium chloride showing the classic yellow halo within the pink agar. C. *S. aureus* grown on 5% sheep blood agar showing β-hemolytic phenotype [4]

S. aureus cell wall is made of thick and rigid layer of peptidoglycan comprising approximately 50% of the cell wall, teichoic acid comprising 40%, and other surface-associated proteins, acting as virulence factors in infection pathogenesis comprising the rest (Fig. II.1.2) [2][3]. Most *staphylococci* produce polysaccharide microcapsules protecting the bacteria from phagocytosis. Capsular serotypes 5 and 8 are associated with 75% of human infections out of the 11 identified serotypes [2][5].

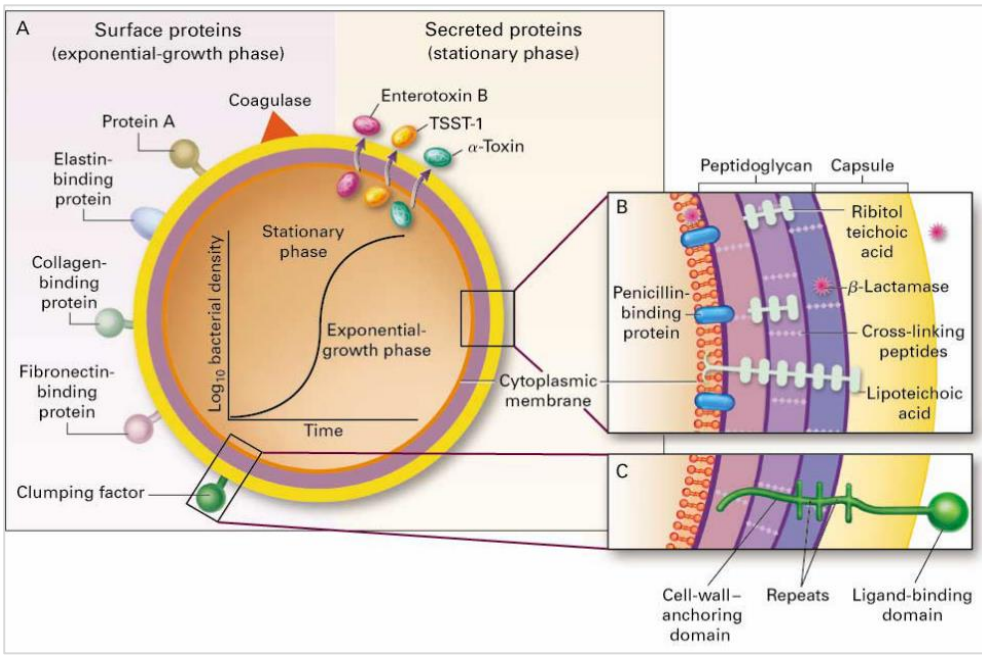

Figure II.1.2: Schematic representation of *S. aureus* cell wall. A. structure and associated virulence factors including surface and secreted proteins. B. Cell wall composition. C. Surface protein clumping factor. TSST-1: toxic shock syndrome toxin [2].

S. aureus is opportunistic pathogen able to colonize different ecological niches, such as skin and mucosa, with anterior nares being the primary ecological niche [6]. The ability of *S. aureus* to colonize different ecological niches of the human body shows its versatility and diversity. In hospital setting, *S. aureus* colonization increases the risk of subsequent infections, particularly infections associated with surgical sites, bloodstream, and lower respiratory system. Approximately 25 - 30% of the human population is colonized with *S. aureus*, either methicillin-susceptible (MSSA) and methicillin-resistant (MRSA) [3]. Based on duration at which a carrier is colonized and the number of positive swabs at different sampling times, carriers can be categorized into two main categories as transient (intermittent carriers) and persistent (long-term carrier) comprising 9-69%, and 9-37% in non-hospitalized populations, respectively [6]. The risk of *S. aureus* colonization increases considerably, reaching 80%, in case of (i) healthcare-related exposure, such as hospital stay, surgery, and antibiotic intake care worker, (ii) general preconditions such as increasing age, being

diabetic, and having immune-compromised system, (iii) occupational exposition (healthcare workers, farmers, veterinarians).

S. aureus is the leading cause of community and healthcare-associated infections causing a wide range of infections from self-limiting mild infections to life threating infections, including bacteremia, infective endocarditis, sepsis, meningitis, osteo-articular, skin and soft tissue infections, pneumonia, surgical site and prosthetic device infections, epidural abscesses, and toxic shock syndrome with being the leading cause in many of them [1]. In a study published in 2022, studying the global burden of antimicrobial resistance in 2019, *S. aureus* was the second contributing to the burden of antimicrobial resistance, with MRSA being responsible for more than 100 000 deaths in 2019 [7]. The clinical significance of this pathogen was highlighted by being listed as one of the "ESKAPE pathogens" to which new antimicrobial development is urgently needed [8]. The antimicrobial resistance in *S. aureus* increases the challenge in treating the infections in both community- and hospital-acquired infections, particularly for MRSA strains [9]. The prevalence of MRSA among clinical isolates varies significantly by geographical location, ranging from single-digit rates in Scandinavian countries to over 50% in the United States and China [10].

MRSA was first identified as a nosocomial pathogen in the United Kingdom in 1961 and community associated pathogen in the USA in 1980s. Infections caused by MRSA strains are generally classified into healthcare-associated MRSA (HA-MRSA) and community-acquired MRSA (CA-MRSA). Moreover, HA-MRSA and CA-MSRA are distinguished according to their origin of infection, resistance pattern, virulence factors, molecular characteristics, and clinical presentation. Both CA-MRSA and HA-MRSA are resistant β-lactams with the first one exhibiting less resistance against non-β-lactam antibiotics while HA-MRSA strains are usually resistant to several classes of non-β-lactam antibiotics [11]. This classification is less applicable now, due to considering CA-MRSA infection are due to HA-MRSA [9][11]. The origin of *S. aureus* infections, particularly invasive infections, is mainly due to asymptomatic colonization that depends on bacterial breach through the epithelial protective layer [10]. In hospital settings, the route of infection may include infected fomites, other infected individuals, and contaminated indwelling medical devices. *S. aureus* may also be acquired from animals, specifically in the livestock industry.

II.1.2 *S. aureus* Virulence Factors

The pathogenesis of *S. aureus* infection is associated with the virulence factors of the bacteria, and the host immune response to the infection [3]. The process of *S. aureus* infections includes five stages (i) colonization, (ii) local infection, (iii) systemic dissemination and/or sepsis, (iv) metastatic infections and (v) toxinosis [12]. These stages are associated with the extensive virulence factors in *S. aureus*, enabling it to evade host natural defenses and be a successful pathogen causing a wide range of human and animal infections.

The virulence of *S. aureus* is multifactorial, associated with a wide range of factors that work together in *S. aureus* pathogenesis, promoting cell adherence and colonization, tissue damage, invading the immune system, causing harmful toxic effects to the host, and finally causing diseases; this in contrast to other bacterial pathogens which rely on only one or a few toxins for pathogenesis [13][14]. The only exception for the multifactorial action of virulence factors is toxic shock syndrome, staphylococcal scalded skin syndrome, and staphylococcal food poisoning, which are caused by toxic shock syndrome toxin, exfoliative toxins A and B, and staphylococcal enterotoxins, respectively [1][14].

Virulence factors of *S. aureus* can be divided into three categories: (i) structural, i.e., cell wall, virulence factors such as peptidoglycan, capsule, protein A, clumping factor (bound coagulase), (ii) enzymes including staphylokinase, DNase, phosphatase, lipase, phospholipase, hyaluronidase, serokinase, and protease. (iii) Extracellular toxins including hemolysin, leucocidin, enterotoxin, TSST, and exfoliative toxin [15].

The expression of cell wall-associated structures generally happens during logarithmic growth (replication), while secreted proteins, such as degradative exoenzymes and toxins, are produced during the stationary phase (Fig. II.1.2A) [16].

Cell adherence to host tissues and extracellular matrix (ECM) is mediated by adhesins, structural surface proteins that allow efficient colonization [17]. Adhesins can be classified to (i) “microbial surface components recognizing matrix molecules” (MSCRAMMs), and “secreted expanded repertoire adhesive molecules” (SERAMS). MSCRAMMs consist of more than 20 members facilitating the binding to host tissues or to other molecules in host, such as fibronectin-binding protein, collagen-binding protein, sialoprotein-binding protein, elastin-binding protein, clumping factors A and B, which binds to fibrinogen, and Staphylococcal Protein A (SpA) (Fig. II.1.2A) [3][10] [17]. SpA is a cell wall anchored protein that can also be released from the cell wall. It has the ability to bind to the conserved region of immunoglobulin IgG, preventing opsonization, and disrupting humoral immune response. It also binds to the Fab region of IgM, acting as a B cell superantigen and causing B cell apoptosis [3][10][18]. SERAMs are a group of five structurally unrelated secreted adhesins, including the fibrinogen-binding protein (FBP) A, the coagulase (Coa), the

extracellular fibrinogen-binding protein (Efb), the ECM-binding protein and the extracellular adherence protein (Eap), which interact with a broad array of host ligands, thereby mediating bacteria adhesion but also interfering with host defense mechanisms which not only), which interact with a broad array of host ligands but also interfering with host defense mechanisms [17]. Another important structural virulence factor is the production of antiphagocytic microcapsule, frequently found in serotypes 5 and 8, allowing the cell to resist phagocytosis and killing polymorphonuclear phagocytes evading the host immune system [18]. Once *S. aureus* adheres to host tissues or abiotic surfaces, it is able to grow and persist by forming biofilms enabling it to persist by evading host defenses and antimicrobials [16].

The secreted products by *S. aureus* consist of numerous degradative exoenzymes and exotoxins [16]. Exoenzymes, such as proteases, lipases, and elastases, enable *S. aureus* to inhibit chemotaxis, invade and destroy host tissues assisting bacterial penetration and metastasizing to other sites (Table II.1.1). In addition, nucleases produced by *S. aureus* can interfere negatively with the antibacterial activity of neutrophils [10]. Toxins can suppress immune responses through manipulating the innate and adaptative immune responses, being able to destroy host cells in infected areas, and degrading inter-cellular junctions, which has an obvious contribution to *S. aureus* proliferation [19].

Table II.1.1: Enzymes considered as *S. aureus* virulence factors and involved in tissue invasion [12][20]

Virulence associated enzyme	Enzymatic action	Effect as virulence factor in host
Catalase	Deactivation of free hydrogen peroxide	Reducing the deleterious effects of hydrogen peroxide which ranging from DNA strand damage to peroxidation of membrane lipids Primarily assist in the attachment to host tissues and host immune evasion
Coagulase	Bound coagulases convert fibrinogen to insoluble fibrin and Cell-free coagulases activated by globulin plasma factor (coagulase-reacting factor) to form staphylothrombin, a thrombin-like factor. catalyzing the conversion of fibrinogen to insoluble fibrin.	make staphylococci clump coats the bacteria with fibrin and makes them resistant to opsonization and phagocytosis
Hyaluronidase	Degrading hyaluronic acid in connective tissue and hydrolyzing the intracellular matrix of acid mucopolysaccharides in tissue	May convert local tissue into nutrients required for bacterial growth May act to spread the organisms to adjacent areas in tissue
Nuclease	Having exonuclease and endonuclease activity	Contributes to evasion of neutrophil extracellular traps May degrade host tissue into nutrients required for bacterial growth
Protease	Degrading human fibronectin, fibrinogen and kininogen Cleves human α1-protease inhibitor, the heavy chain of all human immunoglobulin classes and elastin	May contribute to the ability of *S. aureus* to disseminate and invade tissues
Staphylokinase	Having fibrinolytic effect that converts plasminogen to a serine protease, plasmin More than 67% of *S. aureus* strains express the gene for staphylokinase	Neutralizes the bactericidal effect by forming complex with α-defensin. The bacteria exploit the proteolytic activity of plasmin to degrade components of ECM as well as fibrinogen for dissemination in the host

The main *S. aureus* cytolytic exotoxins can be divided into three major groups: (i) pore-forming toxins, exfoliative toxins, and superantigens (Table II.1.2) [19]. Pore-forming toxins, including hemolysins (Hemolysin-α, Hemolysin-β, δ–hemolysin), and leukotoxins. *S. aureus* expresses superantigen exotoxins, including enterotoxins (A-G) and toxic shock syndrome toxin (TSST-1). The Exfoliative toxins (ETA and ETB) are specific serine proteases, that mediate epidermolysis degrading inter-cellular connections, cell-cell adhesion in the epidermis of the host and modulating immune responses.

Table II.1. 2: Exotoxins considered as *S. aureus* virulence factors [12][19]

Virulence associated enzyme			Enzymatic action	Role in pathogenesis
Pores-forming toxins	Hemolysins	α–toxin	Cytolytic activity on a wide range of human cell types, including epithelial cells, endothelial cells, T cells, monocytes, and macrophage	Hemolytic action causing ce lysis & death
		β-hemolysin (Sphingomyelinase C)	Cytolytic activity on sphingomyelins the most abundant sphingolipid in eukaryotic membrane	
		δ–hemolysin	Neutrophil and monocyte binding	
	Leukocidines		Cytolytic activity on the cells of leukocytic lineage mostly found in community-associated MRSA	Kill leukocytes
Exfoliative toxins A and B (ETA and ETB)			Serine proteases that recognize and hydrolyze desmosomal proteins present in the skin	Inducing skin peeling and blister formation causing scalded skin syndrome (SSSS), necrotizing pneumonia or deep-seated skin infections
Superantigens	Staphylococcal enterotoxins (A-G)		Gastroenteric toxicity; immunomodulation via superantigen activity	Induces toxinosis causing food poisoning
	Toxic shock syndrome toxin (TSST-1)		Toxic for endothelium, direct and cytokine mediated	Causes the rare condition 'toxic shock syndrome' (TSS) characterized by a rapid onset with high fever, rash, vomiting, diarrhea and multiorgan failure

S. aureus can scape host defenses and avoid the antibacterial agents through cellular internalization, host cell invasion; in both phagocytic cells, such as neutrophils and monocytes and non-phagocytic cells, including epithelial and endothelial cells, keratinocytes, and osteoblasts [21]. Moreover, cellular internalization of this pathogen in neutrophils is infectious and believed to contribute to the spread and persistence of *S. aureus* infection while Invasion of non- phagocytic cells contributes to chronicity of *S. aureus* infection. The situation becomes more complicated and persists when *S. aureus* form small-colony variants (SCVs), which can hide in host cells without causing significant host-cell damage which can result in a recurrent infection when these cells revert to their original wild-type phenotype [16]. Of particular concern, the ability of *S. aureus* to form biofilms provide a defense against several clearance mechanism impeding the access of immune cells in addition to exhibiting antibiotic resistance though enabling the infection to persist; this will be discussed later in details.

II.1.3 Antimicrobial Resistance in *S. aureus*

S. aureus has progressively acquired different mechanisms of resistance, allowing it to become resistant to a wide range of antibiotics, including all β-lactams, tetracyclines, aminoglycosides, fluoroquinolones, clindamycin, trimethoprim-sulfamethoxazole- exceptionally vancomycin, daptomycin and linezolid [22]. *S. aureus* is a significant health problem and it is classified as a "high priority" pathogen by the World Health Organization [23].

Emergence of antibiotic resistance by *S. aureus* was introduced in a series of waves, as suggested by, Chamberset *et al*. [24], in which one or a few successful clones was responsible for the initiation of the epidemic waves (Fig. II.1.3). Wave 1 was associated with penicillin-resistant *S. aureus* due to the production of penicillinase in 1940s, shortly after penicillin introduction. Wave 2 was associated with methicillin-resistant *S. aureus,* reported in 1961, due to expressing low-affinity penicillin binding protein, PBP 2a. The expression of PBP2a broadens β-lactam antibiotic resistance to include not only penicillins, but also cephalosporins, and carbapenems, Unlike penicillinase-mediated resistance. Wave 3 started in 1970s, marking the emergence of hospital/healthcare facilities acquired MRSA in hospitals. Wave 4 started in the mid-to-late 1990s, marking the emergence of community associated-MRSA strains. Vancomycin-resistant *S. aureus* (VRSA), firstly reported in 2002, have been isolated exclusively in healthcare settings.

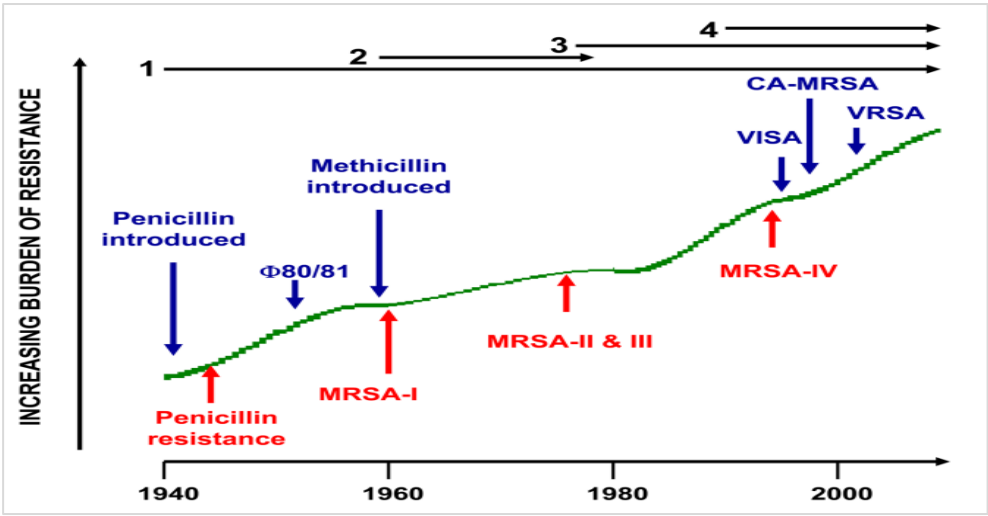

Figure II.1.3: Timeline of the four waves of antibiotic resistance in *S. aureus*, marking the emergence of penicillin-resistant *S. aureus*, MRSA, hospital/healthcare facilities acquired-MRSA in hospitals, and community associated-MRSA strains as well as the emergence of vancomycin-resistant *S. aureus* strain, in healthcare settings [24].

S. aureus, coined as an "evolving pathogen", is well-known for its adaptability and ability to develop resistance to a wide range of antibiotic classes through a variety of different mechanisms, with many mechanisms of resistance remains unclear (Table II.1.3) [25]. Furthermore, MRSA can show a multidrug resistant (MDR) phenotype through developing co-resistance to different classes of antibiotics, including fluoroquinolones, aminoglycosides, macrolides, tetracyclines, and β-lactams, known as MDR MRSA [26].

Table II.1.3: Summary of prevalent antibiotic-resistance mechanisms in *S. aureus* [22][25][27][28]

Antibiotic Class	Mechanism of action	Mechanism of Resistances	Resistance gene
Penicillins	Inhibiting cell wall synthesis (Bactericidal)	Penicillinase	*blaZ*
		Production of PBP2a, PBP2ALGA	*mecA, mecC*
Cephalosporins 1st gen.		PBP2a, PBP2ALGA	*mecA, mecC*
Cephalosporins 2nd gen.		PBP2a, PBP2ALGA	*mecA, mecC*
Cephalosporins 3rd gen.		PBP2a, PBP2ALGA	*mecA, mecC*
Cephalosporins 4th gen.		PBP2a, PBP2ALGA	*mecA, mecC*
β-lactamase inhibitors		PBP2a, PBP2ALGA	*mecA, mecC*
Carbapenems		Development of PBP2a, PBP2ALGA	*mecA, mecC*
Tetracyclines	Inhibiting protein synthesis (Bacteriostatic effect)	Ribosomal protection through dislodging tetracyclines after binding to its target	*tetM, tetO*
		Efflux pumps	*tetK, tetL*
Tigecyclines	Inhibiting protein synthesis (Bacteriostatic effect)	Overexpression of efflux pumps	*mepRAB*
Macrolides, clindamycin, lincosamides, streptogramins	Inhibiting protein synthesis (Bacteriostatic except streptogramins showing bactericidal effect when used together)	Ribosomal methylation of binding sites	ermA
		Efflux pumps	msr*A*, msr*B*, msr*C*, *IsaA, IsaG*
Fluoroquinolones	Inhibit DNA synthesis (Bactericidal effect)	Mutations in topoisomerase IV	grlA *and* grlB
		Mutations in DNA gyrase	gyrA *and* gyrB
		Elevated expression of chromosomally encoded efflux pumps	---
Rifampin		Mutations in RNA polymerase gene	
Ttrimethoprim-sulfamethoxazole		Mutations in DHPS	
		Mutations in DHFR	
Aminoglycosides	Binding to 30SrRNA (Rapid bactericidal)	Aminoglycoside inactivation enzymes	*aac(6')-Ie+aph(2'') ant(4')-Ia*
Daptomycin	Alteration of cell membrane charge (Bactericidal)	Increased L-PG (lysyl- phosphatidylglycerol) synthesis induces electrostatic repulsion of daptomycin complex through an increase of the cell-surface charge	*mprF*
Vancomycin	Inhibiting cell wall synthesis (Bactericidal)	VRSA: modified structure of peptidoglycan precursors from *D-Ala-D-Ala* to *D-Ala-D-Lac*	*vanA*
		VISA: mutations in regulatory genes lead to increased production of peptidoglycan, thicker cell wall, due to trapping *D-Ala-D-Ala* dipeptides on cell surface	*vraSR*
Linezolid	Inhibiting protein synthesis (Bacteriostatic)	Mutations in the 23S rRNA	23S rRNA gene
		Post-transcriptional modifications to the 23S rRNA	*cfr*
		Mutations to the 50S ribosomal L3, L4 and L22	*rplC, rplD* and *rplV*

[a] *PBP, penicillin binding protein; DHPS, dihydropteroate synthase; DHFR, dihydrofolate reductase*

II.1.3.1 Staphylococcal resistance to β-lactams

β-lactams have a bactericidal effect due to their ability to inhibit cell wall synthesis through binding to the transpeptidases, Penicillin Binding Proteins (PBPs); preventing the transpeptidation of the peptidoglycan layer, which is the last step in cell wall biosynthesis [29].

Staphylococcal resistance to penicillin is conferred by two mechanisms. (i) Production of β-lactamase penicillinase encoded by *blaZ*, which inactivates penicillins including penicillin G, V and A, carboxypenicillins and ureidopenicillins by the hydrolysis of its β-lactam ring [30][31][25]. The *blaZ* expression is regulated by two adjacent genes, the anti-repressor *blaR1* and the repressor *blaI* [32]. After exposure to β-lactams, BlaR1, a transmembrane sensor-transducer, cleaves itself and cleaves the repressor protein, *BlaI*, allowing the expression of *blaZ*. (ii) The production of the modified PBP 2a (PBP2a) or PBP2ALGA, a peptidoglycan transpeptidase responsible for crosslinking the peptidoglycans of the bacterial cell wall; the above PBPs are encoded by the *mecA* or *mecC* genes respectively, which are parts of a large mobile genetic element called staphylococcal cassette chromosome mec (*SCCmec*). Modified PBP have a lower affinity to all β-lactams conferring resistance not only to penicillin but also to penicillins, monobactams, cephalosporins and carbapenems [31][25]. PBP2a can take over the transpeptidase function of peptidoglycan biosynthesis in the presence of β-lactam antibiotics inhibiting the function of the four native *S. aureus* PBPs (PBP1, PBP2, PPB3 and PBP4) [29].

II.1.3.2 Staphylococcal resistance to Vancomycin

Vancomycin is a glycopeptide that has a bactericidal effect on Gram-positive bacteria [33]. Vancomycin binds to D-Ala–D-Ala residues of the peptidoglycan precursor in the cytoplasmic membrane to form a stable complex, preventing the use of the precursor for cell wall synthesis.

Few MRSA clones have acquired resistance to vancomycin, the gold standard for the therapy of invasive MRSA infections in hospitalized patients, since 1958 [25]. The mechanism of resistance in VRSA strains is the conjugal transfer of the *vanA* operon from vancomycin-resistant *Enterococcus faecalis* (VRE). *vanA* allows VRSA isolate to synthesize a cell terminal peptide D-Ala-D-Lac instead of the target site for vancomycin D-Ala-D-Ala, resulting in a new cell wall precursor with a decreased affinity to vancomycin, and leading to vancomycin MIC $\geq$ 32 µg-mL [22][25][34]. In contrast, vancomycin intermediate *S. aureus* (VISA) strains show an increased MIC for vancomycin, ranging from 8 to 16 µg-mL, because of the increased thickness of the cell wall [22]. Increased thickness develop, due to mutations in regulatory genes, such as *vraSR*, upregulating enzymes involved in cell wall biosynthesis [22][25]. Moreover, the cell wall of these strains have poorly cross linked additional peptidoglycan, acting as baits to vancomycin, trapping it and clogging the cell wall

[22][34]. Fortunately, strains that have complete resistance to vancomycin (VRSA) are rare, despite the extensive use of vancomycin for treatment of severe MRSA infections. This is thought to be due to the fitness cost associated with the acquisition of *vanA* gene, to the paucity of horizontal gene transfer due to robust *S. aureus* restriction modification systems that prevent foreign DNA uptake, and to the strain-lineage specificity that enable certain strains of *S. aureus* to more readily take up enterococcal plasmids [35].

II.1.3.3 Staphylococcal resistance to Linezolid

Linezolid is an antibiotic of the oxazolidinone class, which has a bacteriostatic effect by inhibiting protein synthesis by binding to the 23S rRNA segment of the 50S ribosomal.[22] Staphylococcal resistance to linezolid is conferred by multiple mechanisms. Firstly, point mutations to the 23S rRNA, conferring significant linezolid resistance through modifying 23S rRNA [22][25]. Secondly, acquisition of chloramphenicol/florfenicol resistance gene (*cfr*), which encodes methyltransferase providing post-transcriptional methylation of the 23S rRNA, conferring multidrug-resistant phenotype due to resistance to multiple antimicrobials including chloramphenicol, lincosamides, oxazolidinones, pleuromutilins, and streptogramin [22][25]. Thirdly, through mutations to the 50S ribosomal proteins L3, L4 and L22, encoded by *rplC*, *rplD* and *rplV*, respectively [22][27].

II.1.3.4 Staphylococcal resistance to Daptomycin

Daptomycin is lipopeptide antibiotic that has a bactericidal effect through altering membrane structure, allowing the leakage of essential ions from the cell [25]. The anionic molecule daptomycin binds to calcium, forming cationic complexes that bind to the negatively charged phosphatidylglycerol on the cell membrane, and lead to the formation of transmembrane cation channels, allowing the influx of potassium ions depolarizing the cell, causing death [22][25]. The most common mechanism of daptomycin resistance is through point mutations in the multi-peptide resistance factor (*mprF*) gene, which encodes a protein that adds a positively charged lysine residue to phosphatidylglycerol increasing cell membrane charge that repels the positively charged daptomycin-calcium complex preventing its binding to the cell-membrane [22]. Moreover, VISA phenotypes, which might be associated with thicker cell wall can lead to decreased daptomycin penetration to reach the cell membrane[22]. Recently, combination of daptomycin with β-lactams has been used in various centers for treatment of persistent bacteremia despite daptomycin treatment. Hypothetically, β-lactams reduces in a way the positive surface charge on the cell wall, facilitating daptomycin adherence [25].

II.1.3.5 Staphylococcal resistance to Tetracyclines

This class of antibiotics have a bacteriostatic effect through inhibiting protein synthesis, as a result of binding to the 30S ribosomal subunit [22][28]. Tetracyclines include different subclasses, including tetracycline, doxycycline, minocycline, and tigecycline. Minocycline is a highly lipophilic molecule with a long half-life, exceptional bioavailability and high tissue penetration used for lung, skin, and soft tissue infections. Minocycline is used in managing central nervous system infections due to its ability to cross blood–brain barrier. Staphylococcal resistance to tetracyclines is mediated either through ribosomal protection proteins or by elongation factor-like proteins, encoded by *tetO* and *tetM*, or through the action of efflux pumps encoded by *tetK* and *tetL* [28]. To overcome these two mechanisms, a modified version of minocycline, tigecycline, was synthesized. Tigecycline has a strong affinity for the ribosomal binding site. On the other hand, its less effective in the penetration of cerebrospinal fluid. Furthermore, tigecycline resistance can also develop through the increased expression of a gene cluster, *mepRAB*, leading to overexpression of multidrug efflux protein [22][36].

II.1.3.6 Staphylococcal resistance to Aminoglycosides

Aminoglycosides belong to class of antibiotics that has a rapid bactericidal effect, due to inducing errors in mRNA translation machinery through binding to the 30S ribosomal subunit [22]. Aminoglycosides increase error rate in amino acids 10-100 folds compared to the average error rate of bacterial ribosome, leading to lethal consequences. In Staphylococci, aminoglycoside resistance is mediated through aminoglycoside acetyltransferases (AAC), aminoglycoside adenylyl-transferases (ANT), and aminoglycoside phosphotransferases (APH). The *aac(6′)-Ie-aph(2″)* resistance gene encodes a bi-functional enzyme displaying AAC(6′) and APH(2″) activity, conferring resistance to gentamicin and concurrent resistance to tobramycin and kanamycin, while the *ant(4′)-Ia* gene encodes for the ANT(4′)-I enzyme, conferring resistance to neomycin, kanamycin, tobramycin, and amikacin [28].

II.1.3.7 Staphylococcal resistance to Fluoroquinolones

Fluoroquinolone antibiotics exert their bactericidal effect by binding to DNA gyrase (bacterial topoisomerase II) and topoisomerase IV, which are responsible for altering the topology of double-stranded DNA within the cell [28][37]. Resistance to fluoroquinolones in clinical isolates of *S. aureus* is achieved by two mechanisms, (i) altering the structure of topoisomerase IV, the primary target; and DNA gyrase, the secondary target, through mutational changes, reducing binding efficiency in both; (ii) upregulation of chromosomally encoded endogenous efflux pumps, including

NorA for extruding the hydrophilic molecules, such as norfloxacin and ciprofloxacin, and NorB and NorC for extruding hydrophobic molecules, such as sparfloxacin and moxifloxacin [37].

II.1.3.8 Staphylococcal resistance to Macrolides, Lincosamide, and Streptogramins

Macrolide, lincosamide, and streptogramin (MLS) antibiotics are inhibitors of bacterial protein synthesis by binding to the 23S rRNA in the 50S ribosomal subunit, with the streptogramin having a bactericidal effect compared to the first two, which have a bacteriostatic effect [22][28]. These antibiotics are effective against Gram-positives, not Gram-negatives, due to low permeability of the outer membrane to these hydrophobic compounds. In staphylococci isolates, resistance to macrolides is mediated through three mechanisms, including (i) alteration of ribosomal binding site by methylation or mutation in the 23S rRNA gene, (ii) active efflux which is prevalent in CoNS isolates, (iii) ribosome protection, and (iv) enzymatic modification of antibiotics, such as macrolide phosphotransferases (*mph*), erythromycin esterases (*er*) which are less prevalent in staphylococci clinical isolates [38]. In *Staphylococci*, cross-resistance can occur showing different MLSB resistant phenotypes depending on the mechanism mediated by different resistance genes (Table II.1.4) [39].

The prevalent mechanism of MLS resistance is ribosomal methylation of the binding site of the antibiotics, mediated by erythromycin ribosomal methylase (*erm*) genes, which can be can be expressed constitutively or erythromycin induction [22][38]. Constitutively resistant MLSB strains (cMLSB) are typically resistant to all macrolides, lincosamides, streptogramin B, while strains with inducible MLSB (iMLSB) resistance phenotype are usually resistant to 14- and 15-membered macrolides [22].

Treating iMLSB infections with clindamycin can select constitutive *erm* mutants, leading to treatment failure, although these strains are sensitive to clindamycin in susceptibility testing [22]. To avoid eliminating clindamycin as a therapeutic option for clindamycin-susceptible staphylococcal isolates, it is necessary to conduct, “D-test”, which can efficiently show iMLSB resistance. D-test involves growing *Staphylococcus* spp. isolates on an agar plate with erythromycin and clindamycin diffusion disk. Susceptibility for both antibiotics is suggested in the absence of bacterial growth, while cMLSB resistance phenotype is suggested in presence of growth around both disks, and iMLSB resistance phenotype, where clindamycin should not be used, is suggested when there is growth around the erythromycin disk that extends to the clindamycin zone forming a straight line [22].

The second significant mechanism of resistance in staphylococci is the upregulation of active efflux pumps, including (i) ATP-dependent efflux pump (ABC), encoded by msr genes, *msr*A, *msr*B, *msr*C, conferring resistance to macrolides and streptogramin B in *Staphylococcus* spp. [38] (ii) active efflux ABC transporter-like

transmembrane protein encoded by *lsa* genes *lsa*A and *lsa*C; conferring resistance to lincosamides and streptogramin type A [38]. Resistance to lincosamides and streptogramin B (LSb) phenotypes is mediated by enzymatic inactivation of the antibiotic. Lincosamide nucleotidyl transferases encoded by *lnu* genes, *lnu*A and *lnu*B genes, are considered the most critical enzymes that modify antibiotics are

Table II.1.4: Resistance phenotypes of Staphylococci to Macrolides-Lincosamides-Streptogramins B [38][39]

Resistance phenotype	Mechanism of resistance	Affected Antibiotics	Genes
Constitutive MLSB phenotype	Ribosome methylation	All macrolides, lincosamides streptogramin B	*ermA, ermC*
Inducible MLSB phenotype	Ribosome methylation in the presence of erythromycin	14-membered macrolides 15-membered macrolides Clindamycin in the presence of erythromycin	*ermA, ermC*
MSB-phenotype	Efflux pumping ATP-dependent efflux pump (ABC)	14-membered macrolides 15-membered macrolides streptogramin B in the presence of inducer	*msrA,* msr*B,* msrC
M-phenotype	Enzymatic inactivation of antibiotics by phosphotransferases	macrolides	*mphC*
PLSA-phenotype	active efflux pumping	lincosamides, pleuromutilins and streptogramins A	*vgaA, vgaC, vgaE, IsaA, IsaG, lsaE*
L-phenotype	Enzymatic inactivation of antibiotics by nucleotidyl transferases	lincomycin	*lnuA,* lnu*B*
SB-phenotype	Enzymatic inactivation of antibiotics by lyases	streptogramin B	*vgbA, vgbB*

II.1.4 Biofilm Overview: Definition, Formation, Regulation, Structure, and Composition

II.1.4.1 Definition of Biofilm

The life cycle of bacteria consists of two alternating phases of growth, a unicellular phase involving planktonic cells, and multicellular involving sessile cells, with the first phase allowing bacterial dispersion and colonization of new environments in contrast to the second one allowing sessile cells to live in a synchronized manner that favors their proliferation [40].

Most bacteria produce biofilm as a part of their life cycle, allowing the cells to survive in hostile environments, to colonize new niches, particularly when implicated in infectious diseases [41]. In the context of infectious diseases, bacteria face various environmental challenges, such as shear forces generated by bodily fluids, host immune responses, and shifts in nutrients, forcing the bacteria to switch to the more favorable phenotype for the host environment, which often ends in biofilm development [42].

The term "biofilm", suggested by Bill Costerton in 1978, refers to highly organized communities of microorganisms made up of one or more species surrounded by self-synthesized extracellular matrix (ECM) that allows their reversible attachment to biotic or abiotic surfaces and their adherence to each other exhibiting with attached cells exhibiting a different phenotype with respect to growth rate and gene expression [40][43][44]. It is assumed that Microbial biofilm can be monomicrobial, consisting of a single or polymicrobial, consisting of multiple species, with the last one more likely to happen in nature, including human bodies and other environments [44].

II.1.4.2 Biofilm Formation

S. aureus can produce complex and multilayered biofilms, as a part of their life cycle life, in four chronologically ordered stages based on its progression (Fig. II.1.4), (i) reversible aggregation of planktonic cells on biotic or abiotic surface followed by irreversible adhesion, initially mediated by van der Waals, electrostatic forces and hydrophobic interactions followed by irreversible attachment of through bacterial adhesins[45], (ii) cell proliferation to form a monolayer which can be accompanied with early dispersal "exodus" to reduce biomass and restructure biofilm, (iii) biofilm maturation which is initiated with the production of ECM composed of extracellular polymeric substances (EPS) followed by the accumulation of bacteria as sticky aggregations called "microcolonies", also known as towers or mushroom-like structures, after the formation exopolysaccharide matrix, and (iv) bacterial dispersion from the microbial community into the surrounding environment which is mediated by enzymes degrading biofilm matrix, physical forces, as well as quorum sensing system [46][47][48].

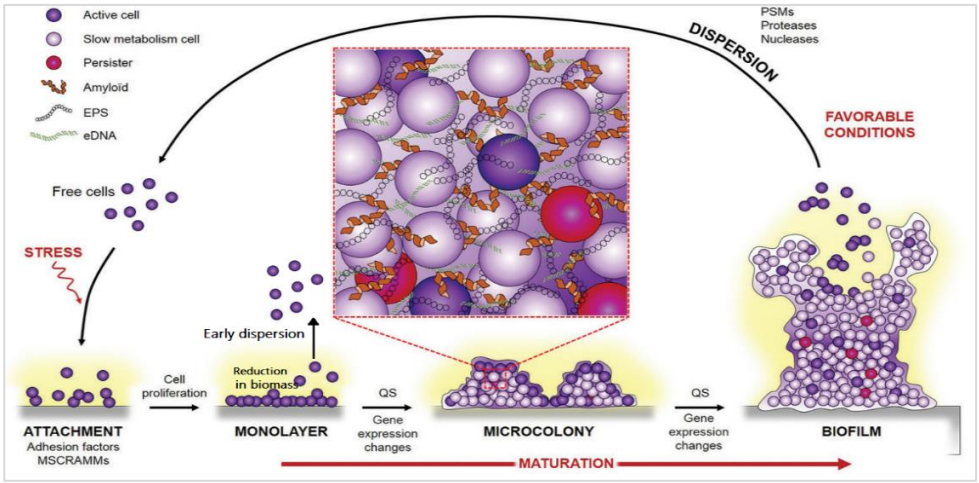

Figure II.1.4: Representation of the stages of biofilm development in *S. aureus*, including (i) attachment; (ii) proliferation and early dispersion; (iii) maturation forming extracellular matrix and having microcolonies; and (iv) detachment (modified after [48]).

II.1.4.3 Regulation of Biofilm Synthesis

S. aureus controls the expression of extracellular virulence factors, such as cell attachment and dispersion during biofilm formation, through cell-to-cell signaling mechanisms called "quorum-sensing", relying on the synthesis of autoinducing signals enabling bacteria to collectively modify behavior in response to fluctuations in cell density and species composition of the surrounding microbial community [48]. Quorum-sensing allows bacteria to evaluate bacterial population density and express specific genes at a high cell density. As the size of the bacterial population increases, the concentration of the autoinducing signal increases. Upon reaching a significant concentration of the signal, they interact with a transcriptional regulator, allowing the activation or the repression of target genes to coordinate the behavior of bacterial cells at high density.

S. aureus biofilms are mainly regulated by two quorum-sensing systems, accessory gene regulator (*agr*) and staphylococcal accessory regulator (*sarA*), working in a complementary and opposing manner where agr negatively regulates the adhesion genes responsible for biofilm formation leading to biofilm dispersal, while *sarA* induces bacterial colonization [49][50]. The *agr* locus consists of four genes, namely *agrD*, *agrB*, *agrC*, *agrA*, organized as operon encoding autoinducing peptides (AgrD), which are extracellular signal post-translationally modified and exported via AgrB, that activates auto-phosphorylation of the histidine kinase AgrC, which phosphorylate the DNA-binding response regulator AgrA to activate it [48]. agr expression leads to upregulation of a number of virulence factors to evade the host immune response and supports bacterial dispersion in a nuclease, protease and surfactant-dependent manner [48][51]. On the other hand, SarA induces attachment

and early biofilm formation, possibly by repressing nucleolytic and proteolytic extracellular enzymes [49]. Moreover, SarA upregulates the transcription of *icaADBC* gene cluster and its suppressor *icaR*, which are responsible for polysaccharide intercellular adhesin (PIA) production and preventing biofilm over formation, respectively [52]. Therefore, *sarA*- and *agr*- quorum systems can be considered as molecular switches responsible in which *sarA*-quorum systems mainly responsible for bacterial colonization, while *agr*-quorum systems responsible for the shifting from adhesion phenotypes by downregulating genes controlling the expression of adhesions and pili and upregulating factors crucial to the bacterial survival in a community [50][42].

II.1.4.4 Structure and Composition of Biofilm

Bacterial biofilms consist of bacteria (only 5–25%), and ECM (comprising 75–95%) [44]. ECM plays a critical structural and functional role essential for giving biofilm its properties. Although the composition of EPSs is variable, depending mainly on bacterial species and host conditions, it mainly consist of biomolecules such as exopolysaccharides, nucleic acids (eDNA and eRNA), and proteins, lipids, and other biomolecules [53].

The Components of ECM promote microbial adhesion bringing cells into proximity allowing intercellular interactions, provides stable chemical microenvironments essential for biofilm lifestyle. Moreover, ECM enhances biofilm tolerance to antimicrobials and host immune cells and products [53].

S. aureus biofilm has two distinct components, water (approximately 97%), and the organic matter (3%) including microcolonies, and EPS (extracellular polymeric substances) which constate the majority (about 50 to 90%) of the total organic matter of a biofilm. EPS include extracellular DNA (eDNA), proteins, and polysaccharides (Fig. II.1.5) [54]. The abundant component of EPS is the polysaccharide intercellular adhesin (PIA), also known as poly-β(1-6)-N-acetylglucosamine (PNAG) [54]. PIA/PNAG are cationic in nature and play a significant role in colonization and maintaining the structural integrity of staphylococcal biofilms, biofilm formation, immune evasion, and resistance to antimicrobials and phagocytosis [54].

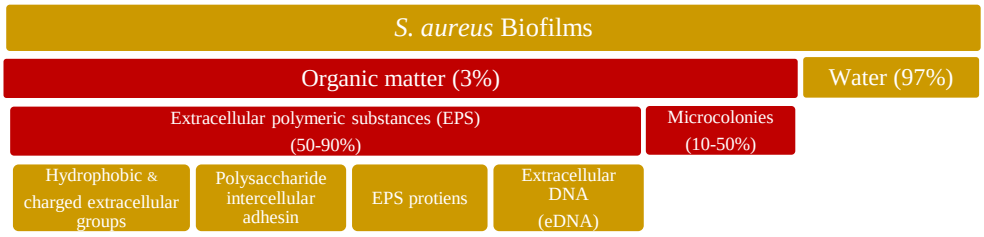

Figure II.1.5: Components of *S. aureus* biofilms and their respective percentages.

PIA/PNAD is encoded by the ica operon-encoded enzymes, consist of *icaR* (regulatory) and *icaADBC* (biosynthetic) genes – are responsible for the synthesis, export, and modification of PIA [55]. *icaA* gene product is a transmembrane protein with homology to *N*-acetyl-glucosaminyl transferases involved in PIA synthesis, requiring the *icaD* gene product to reach its optimal activity exhibiting the full phenotypic expression of the capsular polysaccharide [55].

The expression of the *icaADBC* operon products is regulated by *tcaR* (transcriptional regulator of the teicoplanin-associated locus) and *icaR*, that are able to downregulate PIA synthesis inhibiting biofilm formation [51]. The expression of *icaR*, in turn, is upregulated and downregulated by proteins Spx (global regulator of stress response genes) and Rbf (protein regulator of biofilm formation), respectively. Moreover, *Ica* production is also regulated by a series of environmental factors, such as oxygen, glucose, ethanol, osmolarity, temperature and antibiotics, such as tetracycline. For example, anaerobic conditions induce the production of SrrAB (staphylococcal respiratory response regulator), which promotes the expression of the *icaADBC* gene cluster, leading to PIA production and biofilm formation.

ica-dependent biofilm mechanisms are found in most *S. aureus* isolates, however, there are *S. aureus* isolates able to form *ica*-independent biofilms, such as *bap*-dependent biofilms, in which SarA, responsible for *bap* upregulation during biofilm formation.

The second component of matrix composition is cell wall-anchored proteins (CWP). In *S. aureus* biofilms, PIA-dependent and PIA-independent, EPS contain a range of CWPs implicated in the attachment and the development of the matrix. Their expression depends on growth phase and growth condition [54]. Generally, *S. aureus* can express up to 24 CWP, posing N-terminal secretory signal sequence, and C-terminal sorting signal [56]. The N- terminus direct the protein in the cytoplasm to secretory machinery in the cytoplasmic, while carboxyl terminus facilitates the covalent anchorage of the secreted protein to cell wall peptidoglycan. Different CWPs are categorized into seven families including: (i) MSCRAMM, (ii) G5-E Repeat Domains, (iii) Three-Helical Bundle, (iv) The NEAT Motif Family, (v) 3.5 The Legume Lectin Domain, (vi) Fibronectin Binding by Tandem β-Zipper, and (vii) Nucleotidase Motif. CWA proteins contribute biofilm formation by facilitating the adhesion to EPS, to host surface, and adjacent cells. Similarly, amyloid fibers maintain the stability of biofilm through keeping *S. aureus* cells anchored.

The third important element of EPS is eDNA, which is a polyanionic molecule, released from lysed cells, are involved in biofilm development and the formation of the tower mushrooms shapes [56]. eDNA contributes to irreversible attachment, horizontal gene transfer of mobile resistance determinants, and host immune system evasion [57].

In addition to three previous components, EPS have negatively charged groups, including carboxyl groups, phosphates, sulfates, glutamic acid and aspartic acid, and positively charged ones, including amino sugars [57].

The formation of the extracellular polymeric matrix during biofilm maturation leads to the establishment of microenvironments (i.e., gradients of oxygen, nutrients, signaling compounds, chemicals, and bacterial waste), providing different confined habitats, divided into 3 different zones: (i) outer layer zone where oxygen and substrate available; (ii) an intermediate zone where substrate is available but oxygen is depleted zone in which cells depend heavily on fermentation; (iii) and a substrate- and oxygen-depleted zones consisting of metabolically dormant cells near the adhesion surface (Fig. II.1.6) [42][58].

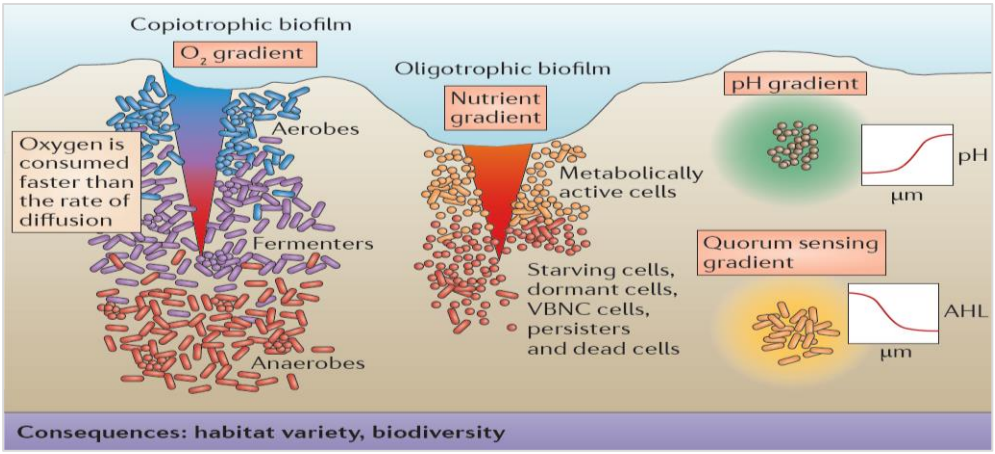

Figure II.1.6: Schematic representation of the association between microenvironments of biofilm and cell heterogeneity in terms of metabolism and growth rate [58].

In the upper layers, nutrient and oxygen consumption by organisms in the upper layer lead to the starvation of organisms in the lower layers leading the bacteria in these layers to adopt slow growth states, found in dormant cells, or leading to cell death. This is accomplished by altering gene expressions affecting cell density within a biofilm, their growth rates and protein production causing cells in mature biofilm to be phenotypically and metabolically distinct from the planktonic form [58]. The alteration in gene expression profile lead to heterogeneous cell population with four distinct metabolic states, which are usually biofilm-location dependent, including (i) metabolically active cells growing aerobically, (ii) fermentatively, (iii) dormant (including very slow growing cells and persisters), or (iii) dead (Fig. II.1.6) [44][51][59]. In the context of biofilm, The first two types of cells are mainly responsible for matrix production, while persisters are non-dividing cells exhibiting antibiotics tolerance by being not affected with antibiotic concentrations that are usually lethal to the planktonic form, rendering antibiotic ineffective and leading to persisting infections [42].

II.1.4.5 Antimicrobial Tolerance and Resistance of Microbial Biofilms

Antibiotic resistance is further complicated with bacterial biofilm production, making microbial cells highly tolerant/ resistant to antimicrobial drugs and thus difficult to eradicate with standard antimicrobial therapy [44]. Biofilm-related infections are more difficult or impossible to eradicate with the empiric antibiotic therapy, usually effective against the bacterium in a planktonic state [60]. Biofilms are considered a major contributor to chronic and recurrent infections [61]. According to the available information from the National Institute of Health, up to 80 % of the clinical infections seen in humans have a biofilm origin, subsequently translated into increased morbidity and mortality, increased hospital stay, and the additional economic cost associated with the care and treatment of recalcitrant microbial infections for prolonged periods of time [44]. *Staphylococcal* bacteria are recognized as the most frequent cause of biofilm-associated infections [62]. Many studies showed that biofilm cells display phenotypic drug tolerance tolerating up 100-1000 times higher concentrations of antibiotics thank planktonic counterparts [63]. Growing in a biofilm provides a defense against host clearance mechanisms impeding the access of certain types of immune defenses, such as macrophage. At the same time enhances bacterial ability to survive lethal concentrations of antibiotics in the planktonic form, exhibiting antimicrobial resistance through genetically-encoded mechanisms of antibiotic resistance (irreversible, genetic, heritable phenotype that is acquired either by mutation or by gene exchange and that remains even when cells in the biofilm are dispersed) or (and) through exhibiting antimicrobial tolerance (a reversible, transient, and nonheritable phenotype depending on the physiological state of biofilm cell populations as well as the ability of biofilm to prevent drug diffusion and activity [60][44]. Tolerance in biofilms is a result of ECM, through the entrapment or inactivation of antimicrobials, and as a result of the slow growth in biofilms [44].

Antibiotic tolerance/resistance can be achieved through different mechanisms, including (i) Low level of cell division, and reduced working as chelating metabolic rates, (ii) the presence of persister cells, (iii) ECM as chemical composition and the architecture of the ECM acting as a physical barrier and its component capable of chelating and enzymatic degradation, (iv) acquired resistance by horizontal gene transfer, (v) upregulation of MDR Efflux Pumps (Fig. II.1.7) [44][58].

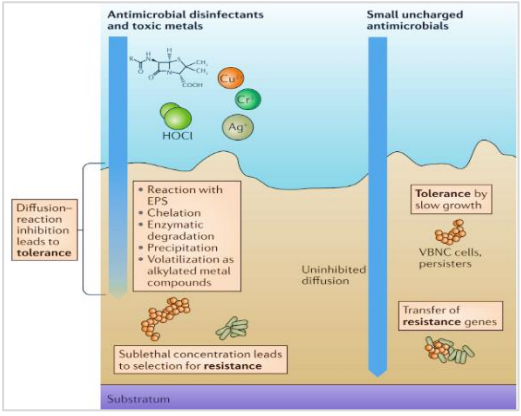

Figure II.1.7: Mechanisms of antimicrobial tolerance and resistance in biofilms [58].

Low levels of cell division, and reduced metabolic rates are exhibited by typical microbial biofilm showing population heterogeneity in terms of metabolism and growth rate ranging from metabolically active dividing cells to metabolically slow nondividing cells, near dormant but live cells that persist [58]. Antibiotics usually target cellular functions in actively dividing cells rendering metabolically inactive or slow-growing cells less affected, particularly persister cells that are highly antibiotic tolerant [60][44][58].

ECM and its components are acting as a molecular sieve, allowing selective permeability of some antibiotics [60]. At the same time, ECM can reduce antibiotics effect as they diffuse in the biofilm in a form known as "diffusion–reaction inhibition", which can involve chelation by complex formation, enzymatic degradation of antimicrobials due to the numerous anionic and cationic molecules, including exopolysaccharides, minerals, proteins, and extracellular DNA that has the ability to bind to different antibiotics preventing the antibiotics from binding and acting on cells [44][58]. Moreover, Diffusion–reaction inhibition can cause a reduction in the concentration to sublethal levels that induce the selection for antimicrobial resistance in biofilm cells [58].

Acquired resistance by horizontal gene transfer (cell to cell, not parent cell to daughter cell) in biofilm can take place through transformation, conjugation, and transduction, allowing the uptake of resistance genes. Beneficial genetic traits to the survival and fitness, such as antimicrobial drug resistance determinants, ability to utilize alternate nutrient sources, and ability to metabolize toxic chemicals, will be maintained by natural selection and eventually become the dominant strain [44].In transformation, eDNA from lysed cells is transported across the cellular membrane by actively growing bacterial cells in a physiological state called "competence", after which the material is incorporated into their own genome by genetic recombination [44].

For conjugation, the biofilm growth condition is ideal for genetic transfer due to providing proximity and stable undisturbed environment and proximity between neighboring cells to allow the direct physical contact between the donor and the recipient facilitated by F pilus encoded by the F plasmid in the donor cell [44]. In fact, a study of *S. aureus* showed the ability *S. aureus* biofilm embedded-cells were able to transfer conjugative plasmid, which was not observed in planktonic cells [58].

Efflux pumps are highly expressed when the cells are in environments of limited nutrients and oxygen or sub-minimum inhibitory concentration (sub-MIC) of different antibiotics, both of which are criteria of biofilm environment [60].

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Chapter 2: Evaluation of the Anti-biofilm Activity of Four Different Antibiotics against *Staphylococcus aureus* Strains from Ocular Infections

II.2.1 Abstract

Introduction: We investigated the biofilm forming ability of n=45 *Staphylococcus aureus* (14/45 MRSA; 31/45 MSSA) of the period 2017-2021 and involved in ocular infections, in Lombardy region- Italy, after retrospectively analyzing the etiology and resistance patterns of bacterial species involved in ocular infections in the period 2015-2020. We evaluated MICs/MBCs and Minimal Biofilm Eradication Concentration (MBEC) and the ability of levofloxacin (LEV), chloramphenicol (CLO), netilmicin (NET), and tobramycin (TOB) in avoiding cells adhesion and biofilm eradication at sub-inhibitory MIC (sub-MICs), MIC and over MBC values.

Materials and Methods: A total of n=463 microbiological reports were analysed retrospectively, collected in the period 2015-2020 from three Acute Care Hospitals located in Lombardy region (n=408 from IRCCS "Policlinico S. Matteo" of Pavia, n=42 from "A. Manzoni" of Lecco, and n=13 from "L. Sacco" of Milan". MICs/MBCs of levofloxacin (LEV), chloramphenicol (CLO), Tobramycin (TOB), and netilmicin (NET) were assessed for available cultures of *S. aureus* isolates (n=45) by broth microdilution assay (EUCAST breakpoints). The ability of biofilm production was qualitatively and quantitatively assessed by Congo-red Agar/broth and crystal violet assay, respectively, together with screening of icaA/D genes (*S. aureus* ATCC 25923 as positive control). Cell adherence was assessed in the presence of sub-MICs/MIC/MBC values of the antibiotics tested above for six MSSA strong biofilm producing isolates selected as not clonally related by RAPD, n=5 MSSA and the ATCC *S. aureus* 25923 as control, using crystal violet assay. Calgary biofilm device was used to determine MBEC/MICs of the antibiotics tested above for biofilm and biofilm-detached cells of the six MSSA isolates.

Results: *S. aureus* (34.1%, n=158/463) resulted the most prevalent pathogen in ocular infections (with an increasing trend during the study period), followed by *Pseudomonas aeruginosa* (12.5%, n=58/463). 43/45 (95.6%) were CLO susceptible (93.5% MSSA vs 100% MRSA); 33/45 (73.3%) LEV susceptible (100% MSSA vs 14.3% MRSA); 28/45 (62.2%) resulted wild-type (71% MSSA vs 42.9% MRSA); 32/45 (71.1%) TOB susceptible (100% MSSA vs 14.3% MRSA). All the *S. aureus* strains resulted strong biofilm producers. The 6 MSSA tested isolates tested showed almost similar MIC and MBC values for LEV (0.125-0.25mg/L and 0.125-0.5mg/L), CLO (8mg/L and ≥32mg/L), and TOB and NET (0.125-0.25mg/L and 0.125-0.25mg/L). CLO, LEV, NET and TOB resulted effective in preventing bacterial adhesion if used at concentrations equal to or higher than the MIC of the sensitive strains. On the contrary, A significant enhancement in cell adherence was observed at sub-MIC values equal to 0.03mg/L of LEV in three isolates, 0.5 and 1mg/L of CLO in one and two isolates, respectively, and 0.06 and 0.125mg/L of NET in three and

two isolates, respectively. MSSA Cells of newly formed biofilms (24h) of susceptible strains were more resistant than their planktonic counterparts to LEV (MBEC>512-1024xMIC), CLO (MBEC=64xMIC for ophthalmic isolates and for the reference strain MBEC=512xMIC), TOB (MBEC>128-256xMIC) and NET (MBEC>512-1024 xMIC). Biofilm-detached cells were more resistant than their planktonic counterparts to LEV (≤2xMIC), CLO (equal to 4xMIC), TOB and NET (equal to 16xMIC).

Discussion and Conclusions: CLO/LEV/NET/TOB resulted effective in preventing bacterial adhesion and biofilm formation when used at concentrations equal to or higher than the MICs of the sensitive strains. Based on the dosages commonly reachable for CLO/LEV (with only one exception)/NET/TOB ophthalmic use, the results showed a clear efficacy in the eradication of newly formed biofilms of sensitive *S. aureus* strains. The efficacy of each drug against biofilm forming *S. aureus* strains is however subjected to the local epidemiology in terms of susceptible strains presence.

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II.2.3 Introduction

II.2.3.1 *S. aureus* Ocular Infections

Bacteria are the major contributor of a variety of ocular infections worldwide, particularly Gram-positive bacteria causing a range of ocular Infections [1][2]. These infections can include anatomical structure that surround the eye (such as conjunctivitis, blepharitis, panniculitis, dacryocystitis, orbital and periorbital cellulitis), of the surface of the eye (keratitis), or within the globe of the eye (endophthalmitis and uveitis/retinitis) [3]. Among gram positive bacteria, *S. aureus*, Coagulase-negative Staphylococci (CoNS), *Streptococcus pneumoniae*, and *Streptococcus pyogenes* represent the predominant isolated species from ocular infections worldwide [2][4]. For Gram-negative, *Pseudomonas aeruginosa*, *Klebsiella pneumoniae*, *Escherichia coli*, and *Haemophilus influenzae* are the main bacteria involved in ocular infections [2].

Isolates of the genus *Staphylococcus* have been associated with all ocular infections, particularly conjunctivitis, blepharitis, endophthalmitis, keratitis, in addition to dacryocystitis and orbital cellulitis.

S. aureus is the leading cause of eye infections such as, conjunctivitis dacryocystitis, sight threatening microbial keratitis, cellulitis, corneal ulcers, blepharitis, and endophthalmitis [4][5]. *S. aureus* is a well-known pathogen, whose wide range of intrinsic virulence factors allow the onset of mild to serious infections, with an overall mortality of 20–40% [6][7]. *S. aureus* can colonize ocular surface structures increasing the risk of subsequent infections, particularly infections associated with surgical sites [8]. Approximately 9-37% of non-hospitalized people are asymptomatic carriers, and the risk of *S. aureus* colonization increases considerably, reaching 80%, with healthcare-related exposure [8]. Despite of the several defense mechanisms and actions present in the ocular site, *S. aureus* can infect eyelids, lacrimal sac, conjunctiva, cornea, the intraocular fluids (vitreous and aqueous) causing *Blepharitis*, dacryocystitis, conjugtivitis, keratitis, and endophthalmitis, respectively [9].

Usually *Blepharitis*, dacryocystitis, conjunctivitis are not sight-threatening unless the cornea becomes involved and commonly encountered among the general population [10]. On the other hand, keratitis and endophthalmitis can cause significant damage resulting in scarring reducing visual acuity and tissues damage critical to vision, especially the retina, due to the combination of immune response and the action of bacterial toxins.

II.2.3.2 Virulence Factors associated with *S. aureus* Ocular Infections

The eye is one of the most complex sensory organs of the human body and the integrity of its parts is essential for proper sight [9]. The eye has some defense mechanisms to protect against bacterial infection avoiding the negative impact of infecting organisms including, (i) tears components (lysozyme, immunoglobulins, lactoferrin, lipocalin, β-lysin, etc.), (ii) blinking the eyelids also spreading the tears across the ocular surface acting as a barrier to the microbial-colonization of the ocular surface, (iii) the action of innate immunity involving corneal epithelial cells, corneal nerves, keratocytes, polymorphonuclear cells, neutrophils, eosinophils, macrophages, NK cells, Langerhans cells, etc.), (iv) molecular elements (components of complement, interferons, interleukins, etc.), and (v) being impermeable to most environmental agents [11][12]. However, *S. aureus* can evade these protection mechanisms thanks to its virulence factors, which can cause serious infections such as conjunctivitis or dacryocystitis, or sight-threating infections such as corneal ulcers, endophthalmitis, or orbital cellulitis [11]. Depending on the site of ocular infection, *S. aureus* can express virulence factors critical for bacterial survival and spread; such factors include pore forming cytolytic toxins, and biofilm [11].

Staphylococcal cytolytic toxins pore-forming toxins (PFTs) toxins can cause significant damage to ocular structure. These toxins include: (i) hemolysin alpha (Hla; α-toxin), (ii) Hemolysin-β (Sphingomyelinase C), (iii) leukotoxin including, Gamma-Hemolysin (Hlg, γ-toxin), and Panton-Valentine Leucocidin (PVL), Leukotoxin ED, and Leukotoxin AB/GH, and phenol soluble modulins (PSM) [13][14].

α-toxin/α-hemolysin is one of the most important toxins in the pathogenesis of staphylococcal infections. It has the ability to cause β-hemolysis of red blood cells, as the name implies [11]. It is expressed by 95–100% of *S. aureus* isolates from various sites causing different infections central nervous system infections, endocarditis, endophthalmitis, keratitis, mastitis, pneumonia, sepsis, and skin and soft tissue infections [11]. It is responsible for corneal epithelial ulceration in keratitis, due to its ability to cause death of cells at high concentration, and tissue damage at sublethal concentrations through activating ADAM10 metallo-protease which cleaves E-cadherin adherents' junctions and results in disruption of tissue barriers. It has the ability to lyse neutrophils, platelets, monocytes, T cells, pneumocytes, keratinocytes, endothelium and endothelial cells [14][9]. The expression of α-toxin is strongly regulated by the quorum-sensing systems *agr* (accessory gene regulator), *sarA* (staphylococcal accessory gene regulator), and *sae* (staphylococcal accessory protein effector) [11].

Hemolysin-β, a neutral sphingomyelinase, is responsible for α-hemolysis on blood agar plates and able to hydrolyze the plasma membrane lipid sphingomyelin, the most abundant sphingolipid in eukaryotic membrane [11]. β-hemolysin shows cytotoxicity towards human keratinocytes, polymorphonuclear leukocytes, monocytes, and T-lymphocytes and inhibits interleukin-8 (IL-8) expression by endothelial cells [14].

Leukotoxins, including PVL, LukED, LukAB/GH, and γ-Hemolysin, are bi-component pore-forming toxins consisting of two different protein components that assemble to form pores lysing leukocytes with the exception of LukED and γ-Hemolysin which can also lyse red blood cells [14]. PVL toxin is less prevalent than α-toxin/α-hemolysin mostly found in CA-MRSA strains targeting neutrophils, monocytes, macrophages, natural killer cells, dendritic cells, and T lymphocytes [11]. It has a synergistic interaction with other *S. aureus* toxins such as β-toxin, δ-toxin, γ-toxin, LukED, and PSMα3 synergize with PVL to amplify IL-1β release to trigger inflammation.

PSMs peptides, of which δ-toxin is a member, have multiple roles in *S. aureus* pathogenesis, such as facilitating biofilm dissemination, cytolytic activity, and proinflammatory activity [11]. Specifically, PSMs peptides form an α-helix amphipathic structure allowing it to attach to the cytoplasmic membrane in a non-specific way leading to membrane disintegration [14].

The ability of *S. aureus* to grow on ocular surfaces is associated with their ability to form biofilm which give the cells advantageous characteristics including antimicrobial tolerance/ resistance, and establishing chronic infections [12][15].

Growing in a biofilm provide a defense against several clearance mechanism impeding the access of certain types of immune defenses, such as macrophage, in addition to exhibiting resistance to antibiotic treatment. The last can be achieved through (i) genetically-encoded mechanisms of antibiotic resistance which is usually inherited and (ii) a reversible phenotype conferring drug tolerance which is transient and nonheritable phenotype depends on the physiological state of biofilm cell populations as well as the ability of biofilm to prevent drug diffusion and activity [16]. Antibiotic tolerance can be achieved through different mechanisms [16]. Firstly, biofilm can act as physical barrier due to the presence of hydrated matrix of extracellular polymeric substances (EPS) which contain numerous anionic and cationic molecules including exopolysaccharides, minerals, proteins, and extracellular DNA that can bind to different antibiotics. Secondly, biofilm has metabolically inactive, slow growing cells and "persister cells" which are less affected by antibiotics because such molecules usually target cellular functions required in actively replicating cell. Thirdly, biofilms facilitate the emergence and dissemination of antibiotic-resistance genes through horizontal gene transfer (HGT) due to the polymicrobial nature of biofilms and proximity between species as well as natural transformation, due to the highly hydrated matrix. Fourthly, biofilm matrices and EPS that sequester antibiotics may confer cross-species shelter such as increased tolerance to vancomycin when *S. aureus* cells embedded within *Candida* spp. biofilm. Fifthly, sub-MIC antibiotic can induce biofilm formation, such as sub-MIC azithromycin that can induce *S. aureus* biofilm development and sub-MIC erythromycin, tetracycline, and which can induce the intercellular adhesion gene cluster (*ica*) expression in *S. epidermidis*.

II.2.3.3 Treatment of Ocular Infections

Ophthalmic infections, if left untreated, can lead to visual impairments and blindness due to the damage of ocular structures caused by toxins and degradative enzymes released by the bacteria, in addition to the effect of immune-driven inflammation around the site of infection (i.e. damage resulting from the heavy influx of inflammatory cells into the posterior segment of the eyeball) [1][2].

From a clinical point of view, many factors play a role in choosing the therapeutic approaches to *S. aureus* infections including type of infection, patient age, clinical manifestation of the disease, co-morbidity, antibacterial susceptibility of infecting organism and hospitalization [3]. Various drugs as single agent and drug combinations have been used to treat *S. aureus* infection. Generally, management of MRSA infections is more difficult compared to that of MSSA [3].

Treatment for most bacterial ocular infections is primarily empiric, to avoid treatment delays associated with the required time to obtain culture, and get susceptibility results, and/or to avoid the costs of culturing, though effective management requires the knowledge of the specific microorganism etiology [1][4][5]. Empiric treatment involves broad-spectrum antibiotics, which are effective against the most common bacteria associated with these ocular infections [5]. Broad spectrum antibiotics can be bactericidal (killing the bacteria) or bacteriostatic (inhibiting bacterial growth and relying on the host defense mechanisms to clear and eradicate the infective organism) [5][6]. Bactericidal antibiotics like penicillins, cephalosporins, aminoglycosides, and fluoroquinolones are used for severe infections, acting fast enough to prevent sight-threatening sequelae which may occur due to the release of bacterial toxins and degradative enzymes which can damage tissue and impair function of ocular tissues. Bacteriostatic drugs like tetracyclines, macrolides, chloramphenicol, and sulfonamides are instead used for the cases of less severe infections or to obtain a specific benefit, such as tetracycline in the treatment of ocular rosacea, or to overcome an allergy problem [6].

The most commonly used antibiotics in the treatment of ocular infections against MRSA and methicillin-resistant *S. epidermidis* (MRSE) are netilmicin (NET), tobramycin (TOB), levofloxacin (LEV), chloramphenicol (CLO) and vancomycin [7].

The third-generation aminoglycoside netilmicin (NET), is one of the most effective against both MSSA and MRSA. Moreover, it is able to overcome TOB resistance derived from the action of enzymes modifying the drug such as the bi-functional N-acetyltransferase enzyme combined with O-phosphotransferases enzyme [ACC(60)-APH(200)] in *Staphylococci* [7]. Since NET is primarily used as a topical agent, it may inhibit the emergence, spreading and persistence of antibiotic-resistant bacteria. Chloramphenicol and its fluorinated derivative florfenicol represent highly potent inhibitors of bacterial protein biosynthesis showing broad spectrum activity with a bacteriostatic effect [8].

Chloramphenicol is an old that had been partially abandoned due to its ability to cause fatal aplastic anemia after its systemic administration. Now, it is experiencing its renaissance because it is widely used in the treatment and prevention of superficial eye infections due to maintaining its susceptibility due to the low resistance rate, its ability to interfere with bacterial adhesion before biofilm formation, its ability to penetrate biofilm matrix reducing biofilm biomass and viability. It could therefore represent a means of combating biofilm-related infections and improving patient outcomes.

The resistance of ocular pathogens to topical antimicrobial agents is a worldwide problem, narrowing treatment choices for the management of ocular infections even for the management of mild and more common ocular infections [9][5]. Antibiotic resistance of ocular pathogens is mainly influenced by the characteristics of the pathogen, the overuse of broad spectrum antibiotics for systemic infections and of topical molecules on external ocular surfaces, in addition to short-term and frequent exposure [5][10][11]. Overuse of antibiotic-prescribing practices, including the widespread use of broad-spectrum systemic antibiotics is exacerbated by inadequate compliance to full treatment duration, leading to a global increase in resistance among both Gram-positive and Gram-negative bacteria to some old and new generations antimicrobials used to treat ophthalmic infections [9][5]. Many Antibiotic Resistance Monitoring in Ocular micRoorganisms (ARMOR) surveillance studies have shown that the resistance rates were high in *Staphylococcus* spp., particularly CoNS and MRSA [9][4][11][12][13].

According to an Italian 30-year retrospective study of bacterial ocular infections published in 2021, CLO demonstrated to be the most effective antimicrobial toward bacterial ocular infections, particularly those caused by Gram-positive bacteria, followed by tetracycline, ampicillin, and aminoglycosides [9]. The aminoglycoside netilmicin showed the highest *in-vitro* activity toward *S. aureus* isolates followed by moxifloxacin, CLO, LEV, and amikacin. CoNs isolates were highly susceptible to moxifloxacin followed by NET, CLO, amikacin, and LEV. Furthermore, resistance rate was lower for NET and gentamicin, than or TOB, likewise resistance rate for the newer fluoroquinolones, levofloxacin, and moxifloxacin, in comparison to old fluoroquinolones.

Among broad spectrum antibiotics, CLO and its fluorinated derivative florfenicol represent highly potent inhibitors of bacterial protein biosynthesis [8].

In order to have effective antibiotic action against bacterial pathogens, a concentration above the minimum inhibitory concentration (MIC) value should be achieved in successive doses [15]. However, during antibiotic therapy or in natural conditions, bacteria may be found in presence of sub-inhibitory concentrations (sub-MICs) of antibiotics [14]. Many studies have shown that sub-MICs conditions can act as signal molecules altering their physicochemical characteristics and inducing the expression of bacterial virulence genes [15]. Particularly, the MDR-MRSA can frequently be exposed to subinhibitory concentrations of antibiotics, which leads to

gene transfer, biofilm formation, and virulence gene expression [16]. In *S. aureus*, five main features of bacterial virulence can be changed upon exposure to sub-MIC levels of antibiotics as mentioned by Chen *et al.* in 2021; these criteria can be however strain and antibiotic dependent [14]. The effects of sub-MIC concentrations on *S. aureus* include: (i) bacterial cell deformation which can stimulate abnormal host immune responses and induce the cell to release toxins; these deformations include cell morphology deformation, cell wall component changes, and cell wall breakdown. (ii) controlling the expression levels of *S. aureus* virulence factors (such as a-toxin, PVL, SpA, PSM, and enterotoxins) to alter pathogenesis; (iii) regulating strain-specified adhesion and invasion capabilities to affect bacterial colonization and diffusion; (iv) modifying *S. aureus* biofilm formation; and (v) influencing bacterial SCV formation to achieve persistent infection and recurrence.

II.2.4 Objectives

1. To provide a retrospective study on the ophthalmic isolates to assess bacterial etiology of external *ocular*/periocular *infections* and antibiotic susceptibility patterns in a five-years period (2015-2020) assisting the empiric management of ocular infections.

2. To evaluate the *in vitro* antimicrobial susceptibility of n=45 *S. aureus* strains isolated from any type of ocular infection.

3. To obtain the "real" MIC values for LEV, CLO, TOB, and NET, commonly used in treating ocular infections, using broth microdilution method according to EUCAST 2020 guidelines, breakpoints, and ECOFF to identify non-wild type isolates with possible resistance mechanisms and to determine the MIC value for some antibiotics that are not usually included in automated testing panels usually used for routine susceptibility testing.

4. To assess the ability of ophthalmic *S. aureus* isolates to produce biofilm using different methods.

5. To assess the effect of sub-inhibitory and over-MBC concentrations of the four antibiotics tested above on cell adherence, the first step in biofilm formation, of ophthalmic MSSA isolates.

6. To assess the ability of the four antibiotics tested above to eradicate biofilm produced by ophthalmic MSSA isolates and their ability to inhibit the growth of biofilm-detached MSSA cells using Calgary method.

II.2.5 Materials and Methods

II.2.5.1 Collected Data

During the five-year period 2015 - 2020, microbiologic records of n= 463

bacterial isolates were collected from three medical structures located in Lombardia, Italy. A total of n=412 were from IRCCS Policlinico San Matteo (Pavia), n=40 was from Manzoni Hospital of Lecco, and n=13 from "Luigi Sacco" University Hospital (Milan). The data collected included i-ii) bacterial species identification/susceptibility patterns (when performed), iii) ward from which the bacterial isolates were obtained, iv) specimen type used to detect the pathogen/diagnose the presence of an ocular infection. The species identification and antimicrobial susceptibilities of n=112 *S. aureus* from the above different hospitals, were reviewed and analyzed at the Microbiology and Clinical Microbiology Unit of the Dept. Clinical, Surgical, Diagnostic and Pediatric Sciences - UniPV.

II.2.5.2 *S. aureus* Strains and Culture Conditions

A total of 45 *S. aureus* isolates have been obtained from the three medical structures IRCCS Policlinico San Matteo N= 40, "Luigi Sacco" University Hospital n=2, and Manzoni Hospital of Lecco n=3; in the period of 2017 through 2021. The *S. aureus* isolates were collected from the ocular site of in- or out-patients with superficial/intraocular infections, or from in-patients need to be checked for colonization before proceeding with ophthalmic procedure. Frozen stock cultures of all strains were stored in Luria-Bertani broth plus 40% glycerol at -80°C. Prior experiments, cells were sub-cultured from stock on mannitol salt agar (MSA) at 37 ^{o}C for 24 hours.

II.2.5.3 Species Identification, Antimicrobial Susceptibility Testing, and *mecA* Detection

All Gram-positive ophthalmological isolates were identified at species level and tested for antimicrobial susceptibility using MicroScan dried Gram-positive BP Combo Panel Type 33 (Beckman Coulter, CA, USA) and analyzed through the semi-automated system MicroScan autoSCAN-4 (Beckman Coulter, CA, USA) following the manufacturer instructions. Clinical categorization of the isolates as susceptible (S), susceptible high-exposure (I) or resistant (R) was performed according to European Committee on Antimicrobial Susceptibility Testing (EUCAST) breakpoints (http://www.eucast.org). The antimicrobial susceptibilities of these Gram-positive bacteria were determined using the following antibiotics: ampicillin(AMP), amoxicillin-clavulanic acid (AMC), azithromycin (AZITRO), ciprofloxacin (CIP), clindamycin (CLINDA), Daptomycin (DAPTO), erythromycin (ERITRO), Fosfomycin (FOS), cefoxitin (CEF), gentamicin (CN), imipenem (IMP), levofloxacin (LEVO), linezolid (LNZ), moxifloxacin (MOX), oxacillin (OXA),

penicillin G (PEN G), quinupristin and dalfopristin (SYNERCID), tetracycline (TET), Teicoplanin (TEICO), trimethoprim/sulfamethoxazole (SXT), and vancomycin (VAN).

Based on EUCAST guidelines, cefoxitin is used as a marker for the detection of methicillin *resistance* phenotype predicting resistance to all cephalosporins, cephems, and other β-lactams (such as ampicillin-sulbactam, amoxicillin-clavulanic acid, ticarcillin-clavulanic acid, piperacillin-tazobactam, and the carbapenems) regardless of the *in vitro* test results obtained with indicating agents except for the anti-MRSA cephalosporins. Therefore, *S. aureus* isolates which showed MIC values >8 mg/L for cefoxitin were considered methicillin-resistant phenotypically. All MRSA isolates were screened for the presence of *mecA* gene by PCR using the primers *mecA* forward (5′- GTGAAGATATACCAAGTGATT-3′) and *mecA* reverse (5′-ATGCGCTATAGATTGAAAGGAT-3′) amplifying 147 bp fragment of the *mecA* gene using 30 PCR cycles of denaturation at 94 °C for 30 seconds, annealing at 55°C for 90 seconds, followed by extension at 72 °C for 90 seconds [17].

Antimicrobial categorization of *S. aureus* as MDR was based on the guidelines recommended by the joint initiative of the European Centre for Disease Prevention and Control (ECDC) and the Centres for Disease Control and Prevention (CDC) [18]. According to these guidelines, the isolates exhibiting non-susceptibility (including both high-exposure susceptibility and resistance) to at least one agent in at least three antimicrobial categories considering MRSA isolates MDR organism by virtue of being an MRSA.

II.2.5.4 Determination of Real MIC values for LEV, CLO, TOB, and NET

Minimum Inhibitory Concentrations (MICs) were determined in duplicate for all *S. aureus* isolates (n=45, 31 MSSA and 14 MSSR) using microdilution technique according to EUCAST guidelines in Muller-Hinton (MH) broth [19]. For the preparation of antibiotics LEV, TOB, and NET were dissolved in pure sterile water, while CLO was dissolved in ethanol. Twofold microbroth serial dilutions in concentrations ranging from 0.03 mg/L to 16 mg/L of LEV, 0.25 µg/m to 128 mg/L of chloramphenicol, 0.06 mg/L to 32 mg/L of TOB or NET with final inoculum of 5 × 10^5 CFU/mL were dispensed in each well of the 96-well culture plate. After incubation for 24 hours at 35°C, 30 µl of 0.015% resazurin were added to all wells, and further incubated for 2-4 hours for the observation of blue to pink color change indicating bacterial growth [20]. Two wells in each row of the 96-well plate served as growth control well with no antibiotics added, and another served as sterility control, without bacteria in the same media nor antibiotics. The American Type Culture Collection (ATCC) 25923 *S. aureus* was included in each batch as quality control. MIC was determined from visual reading before and after adding resazurin considering the MIC is the lowest concentration able to inhibit microbial growth. To check inoculum density for each strain, 2.5 µL from the growth control well was spread immediately after inoculation with 50 µl of sterile distilled water on standard Plate Count Agar plates. Plate count was made by counting the Colony Forming Units

(CFUs) after incubation for 24 hours at 35 °C. The interpretation of susceptibility/resistance patterns was done as suggested by EUCAST for topical agents using systemic breakpoints (for antibiotics administered systemically/topically) or ECOFFs (for antibiotics administered topically) with the last one used to categorize isolates as wild type (susceptible) or non-wild type (reduced susceptibility/resistant) demonstrate reduced susceptibility [21].

The MBC values were determined for the 5 MSSA isolates, that were extensively investigated in the following experiments, by plating 10 µL from the well that correspond to the MIC value and two higher concentrations than the MIC value. The MBC values was determined after incubation for 24 hours at 37 ◦C, with MBC being the lowest concentration of the substance at which no colonies formed.

II.2.5.5 Phenotypic Characterization of Biofilm Production using Congo Red

The phenotypic identification of biofilm-production was performed *in vitro* using Congo Red Agar (CRA) [22]. CRA plates were prepared by dissolving 37 g of Brain Heart Infusion agar (Biolife Italiana S.r.l., Milano, Italy) and 50 g of sucrose (BIO-RAD, California, USA) in 800 ml distilled water autoclaved. After autoclaving, the agar cooled down to 55 °C and Congo Red stain (Sigma Aldrich, India) added to the agar after it was separately prepared and autoclaved as a concentrated aqueous solution of 0.8 g dissolved in 200 ml distilled water. CRA was inoculated with fresh isolates of *S. aureus* strains adjusted to 0.5 McFarland standard turbidity. The plates were subsequently incubated aerobically for 24 hours at 37 °C and overnight at room temperature. *S. aureus* ATCC 25923 was used as a positive control for the CRA test. Biofilm-producing strains appeared as black colonies dry crystalline consistency on the CRA while non-biofilm-producers appeared red-colored with occasional darkening at the center of the colonies. For Trysptic soy-Congo-red broth (CRB;), it was based on tryptic soy broth (TSB) (Biolife Italiana S.r.l., Milano, Italy) which was supplemented with 3.6% sucrose (Kanto Chemical Co., Inc., Tokyo, Japan) and 0.08% Congo red dye (Sigma Aldrich, India)[23]. *S. aureus* isolates that turned the broth black or reddish black were considered slime producers, while strains that turned the broth yellow were considered non-biofilm producers.

II.2.5.6 Quantification of Biofilm Formation by Crystal Violet Assay (CVA)

The biofilm forming capacity of each strain was determined using crystal violet (CV) assay as described previously [24]. The *S. aureus* strain ATCC 25923, a strong biofilm former, was used as a positive control. Overnight cultures of staphylococci were diluted 1:200 in BHI. Aliquots (200 µl) of the diluted bacterial suspensions were added to 96-well flat-bottom sterile polystyrene microplates (Costar; Corning, New York, NY) and incubated statically for 24 h at 37°C. Biofilms formed on the plates were gently washed twice with phosphate-buffered saline (PBS) (137 mM NaCl, 2.7 mM KCl, 4.3 mM Na2HPO4 [pH 7.4]) to remove planktonic and loosely adhering bacteria. Adherent cells were fixed with 96% ethanol for 10 min then ethanol was removed and left to dry for 15 minutes, then stained with 0.1% crystal violet for 15

min, and—after several washings—the wells were air dried. For a quantitative estimation of biofilm density, bound crystal violet was solubilized with 10% glacial acetic acid, and the absorbance of the solubilized dye was read at 595 nm in a microplate reader (model 680; Bio-Rad Laboratories, Inc., Hercules, CA). All experiments were done in triplicates. Mean absorbance values of each sample were calculated and compared with the mean values of controls. The cut-offs proposed by Stepanović et al. were used to classify the level of biofilm production [25] Cut-off OD (ODc) is defined as three standard deviations above the mean OD of the negative control. Strains were interpreted as follows.

OD ≤ ODc	non-adherent
ODc ≤ OD ≤ 2 x ODc	weakly adherent
2 x ODc ≤ OD ≤ 4 x ODc	moderately adherent
4 x ODc ≤ OD	strongly adherent

II.2.5.7 Detection of *icaA* and *icaD* responsible for PIA Synthesis

DNA was extracted by boiling method [26]. For the detection of *ica*A, primers ICAAF (ACACTTGCTGGCGCAGTCAA) and ICAAR (TGTTGGATGTTGGTTCCAGA) were used to amplify a 188 bp fragment.[27] Similarly for amplifying *ica*D, primers ICADF (ATGGTCAAGCCCAGACAGAG) and ICADR (CGTGTTTTCAACATTTAATGCAA) were used for a 198 bp fragment [28]. A 25 µL reaction volume consisted of 2.5 mM MgCl2, 200 mM of each nucleotide, 1 mM of each primer, 1.25 U of Taq polymerase and 100 ng of template DNA. Forty-five cycles of amplification, each consisting of denaturation at 95 °C for 30s, annealing at 56 for *icaA* primers and 62 °C for *icaD* primers for 30 s and elongation at 72 °C for 30 s, along with a final extension at 72 °C for 5 min were performed in a thermocycler (BioRad, USA). The presence and size of the amplified products were confirmed by electrophoresis on 2% agarose gel. The *S. aureus* strain ATCC 25923, a strong biofilm former, was used as a positive control.

II.2.5.8 In Vitro cell Adherence Assay in 96-Well Microtiter Plates

Biofilm formation was assessed in the presence of sub-MIC, MIC and MBC values of chloramphenicol, levofloxacin, netilmicin and tobramycin using broth microdilution according to EUCAST guidelines followed by CV assay, as described above for the two assays. Five MSSA strains were selected based on their AST profiles preferring strains susceptible to the four antibiotics and their ability to produce biofilm after confirming that they are not clonally related using random amplified polymorphic DNA (RAPD) -PCR using RAPD-PCR kit (Amersham biosciences UK limited, England).

The *S. aureus* strain ATCC 25923 and the five selected MSSA isolates were incubated overnight in MH broth at 37ºC. The selected MSSA strains (10^5 CFU/mL each) were inoculated in 96-well flat-bottom sterile polystyrene microplates (Costar; Corning, New York, NY) containing 250 µl of twofold serial antibiotic dilutions and

incubated for 24 hours at 37°C. Chloramphenicol, levofloxacin, Netilmicin, and tobramycin concentrations ranged from 0.25-128 mg/L, 0.03-16 mg/L, and 0.06-32 mg/L for the last two, respectively. After incubation, CV assay was performed as mentioned above. Two wells in each row of the 96-well plate served as growth control well with no antibiotics added, and another served as sterility control, without bacteria in the same media nor antibiotics. Three technical replicates in each experiment were performed for all the antibiotics with *S. aureus* strain ATCC 25923 serving as a quality control in every batch. (Cell adherence in the presence or absence of antibiotics) were analyzed by calculating p-values based on two-way ANOVA using GraphPad Prisim version 9.4.0. Statistical significance-based differences in $OD_{595,}$ was calculated using Two-way ANOVA with Dunnett's multiple comparison post-test comparing values with growth control values (Antimicrobial concentration = 0) after normalization against blank wells (Sterility control) for each strain tested. Red/black asterisks correspond to significant increase/reduction in cell adherence, respectively. (*P < 0.05; **P< 0.01; ***P< 0.001; ****P<0.0001).

II.2.5.9 In Vitro Biofilm Inhibition assay in 96-Well Microtiter Plates

The biofilm inhibitory activity of LEV, CLO, TOB, and NET compound was tested on *S. aureus* ATCC 25923 and on one MSSA isolate, chosen based on cell adherence results at sub-MICs concentration [29]. The bacterial cells were cultured in TSB + 1% glucose O/N at 37 °C and diluted to 10^7–10^8 CFU/mL; then, 100 µL of culture was pipetted into the microtiter plate either in the absence or presence of different concentrations of LEV, CLO, TOB, and NET ranged from 0.25-128 mg/L, 0.03-16 mg/L, and 0.06-32 mg/L for the last two, respectively. After 2 h of incubation, the supernatant (containing non adherent cells) was removed and 100 µL of fresh sterile medium (containing the same concentration of antibiotics tested) was added to each well and incubated for 20 h at 37 ◦C. Biofilm biomass was quantified by staining with crystal violet and absorbance measurements at OD 595 nm. Results were expressed as the ratio between biofilm absorbance and the OD 600 absorbance of the corresponding after the incubation and before the crystal violet treatment.

II.2.5.10 Biofilm evaluation at sub-MICS by Confocal Laser Scanning Microscopy (CLSM)

CLSM was used to determine the three-dimensional architecture, thickness and morphology of biofilms formed at sub-MICs as described before [30]. Briefly, bacteria were cultured O/N in TSB + 1% glucose and diluted to an OD600 = 0.05 (about 1×10^7 CFU/mL) in the same medium. Bacterial suspensions (400 µL) of the *S. aureus* ATCC 25923 and of the MSSA number 15 were incubated into the "µ-Slide 4 Well chambered coverslip ibiTreat" Ibidi for two hours at 37ºC, in the absence or in the presence of LEV (0.125 mg/L), CLO (0.5 mg/L), and NET (0.25 mg/L) for, for TOB (1 mg/L) sample 15 was replaced with sample 4 because of its resistance to TOB. The concentrations of antibiotics were selected based on the results of biofilm inhibitory assay mentioned above choosing the concentration that was able to inhibit the formation of biofilm. After two hours of incubation, the medium was removed,

the wells were washed once in PBS to remove nonadherent cells, and fresh medium containing the same concentration of antibiotic was added. After an overnight incubation, the medium was removed, and biofilms were washed twice with PBS and stained with Syto 9 (Invitrogen) (5 µM). A 63× oil immersion objective and a Leica DMi8 with 500- to 530-nm (green fluorescence representing Syto 9) emission filters were used to take five snapshots randomly at different positions in the confocal field of each chamber. The Z-slices were obtained every 0.3 microns. For visualization and processing of biofilm images, ImageJ was used. The thickness, biomass, roughness coefficient, and biofilm distribution were measured using the COMSTAT 2 software. All confocal scanning laser microscopy experiments were performed two independent experiments, and standard deviations were measured [31].

II.2.5.11 Determination of the Minimum Biofilm Eradication Concentration (MBEC)

The efficacy of the antibiotics LEV, CLO, TOB, and NET against biofilm was assessed for 5 isolates, which were strong producers and susceptible to all antibiotics, using MBEC High-throughput (HTP) assay (Innovotech Inc., AB, Canada) using Calgary biofilm device (CBD) to grow mature *S. aureus* biofilms, according to manufacturer instructions, as in vitro model for antimicrobial susceptibility testing of *S. aureus* in biofilm.[32] In brief, second sub-cultures of *S. aureus* isolates grown MH agar were used to inoculate MBEC assay plates after adjusting to a cell density of 10^6 CFU/ml and incubated overnight at 37ºC on a rocking platform. Inoculum confirmation was preparing through serial dilution of 10^{-1} to 10^{-7} of the inoculum using the 1:100 diluted bacterial suspension and then transferring 20 µL to each of the eight rows of the 96-well plate. 20 µL was removed from each well and spot-plated onto the MH agar. After incubation, the established biofilms on peg lid were rinsed in saline solution for 10 seconds to disrupt the planktonic cells and biofilm growth check was performed using 3 specified pegs removed from the lid using flame-sterilized pliers and each were placed in a new 96-well plate with surfactant supplemented Mueller-Hinton broth. The plate was sonicated for 30 min to recover the biomass. The biofilm cell density was confirmed by serial dilution and spot plating. After removing biofilm growth control pegs, the peg lid was transferred to antimicrobial challenge plate and incubated overnight at 37ºC. The antimicrobial challenge plate has ten twofold serial dilutions of levofloxacin, chloramphenicol, tobramycin, or netilmicin ranging from 0.25-128 mg/L, 8-4096 mg/L, 0.06-32 mg/L, and 0.25-128 mg/L, respectively. After incubation the lids containing biofilms removed from the antimicrobial challenge plate, placed in recovery plate containing 200 µl of surfactant supplemented MU broth which was then left to rest for 30 minutes at room temperature followed with sonication at high for other 30 minutes. After sonication, 100 µl were used for the determination log10 reduction according to manufacturer instruction. 100 µl of MH broth were added to the other 100 µl in each well of the recovery plate and incubated overnight at 37 °C to determine the MBEC value via observing turbidity. This test was carried out in quadruplicate. The MBEC value is the minimum concentration of antibiotic at which the bacteria of the biofilm fail to regrow [32].

II.2.6 Results

II.2.6.1 Five-year Retrospective Analysis of Bacterial Species Involved in Ocular Infections

II.2.6.1.1 Identification and Evaluation of Antimicrobial Susceptibility of Ocular Bacterial Isolates

During the five-year period, 2015 – 2020, microbiological records for a total of 463 isolates were collected including n=408 isolates from IRCCS Policlinico San Matteo (Pavia), n=42 isolates from Manzoni Hospital (Lecco), and n=13 isolates from "Luigi Sacco" University Hospital (Milano). The 463 isolates belonged to 48 different bacterial species, 22 species being Gram-positive (45.8 %) and 29 species Gram-negative (54.2%).

Among the 463 isolates, 49.5% were Gram-negative bacteria (n=229/463) and 50.5% were Gram-positive bacteria (n=234/463). Among all the 463 isolates, *S. aureus* (34.1%, n=158/463) was the most common species followed by *Pseudomonas aeruginosa* (12.5%, n=58/463) (Fig. II.2.1A).

Among Gram-positive bacteria, *S. aureus* (67.5%, n=158/234) was the most prevalent bacterial specie, while *P. aeruginosa* (25.3%, n=58/229) was the most prevalent isolate among Gram-negative, followed by *Haemophilus influenzae* (19.7%, n=45/229) and *Escherichia coli* (12.7%, 29/229). Among *Staphylococcus* spp., *S. aureus* was the most prevalent followed by *S. epidermidis* (Fig. II.2.1B). Based on the data available for 129 *S. aureus* isolates, an increasing trend in *S. aureus* detection was observed in the period 2015 to 2020; for both MRSA and MSSA, particularly in 2019 (Fig. II.2.1C)

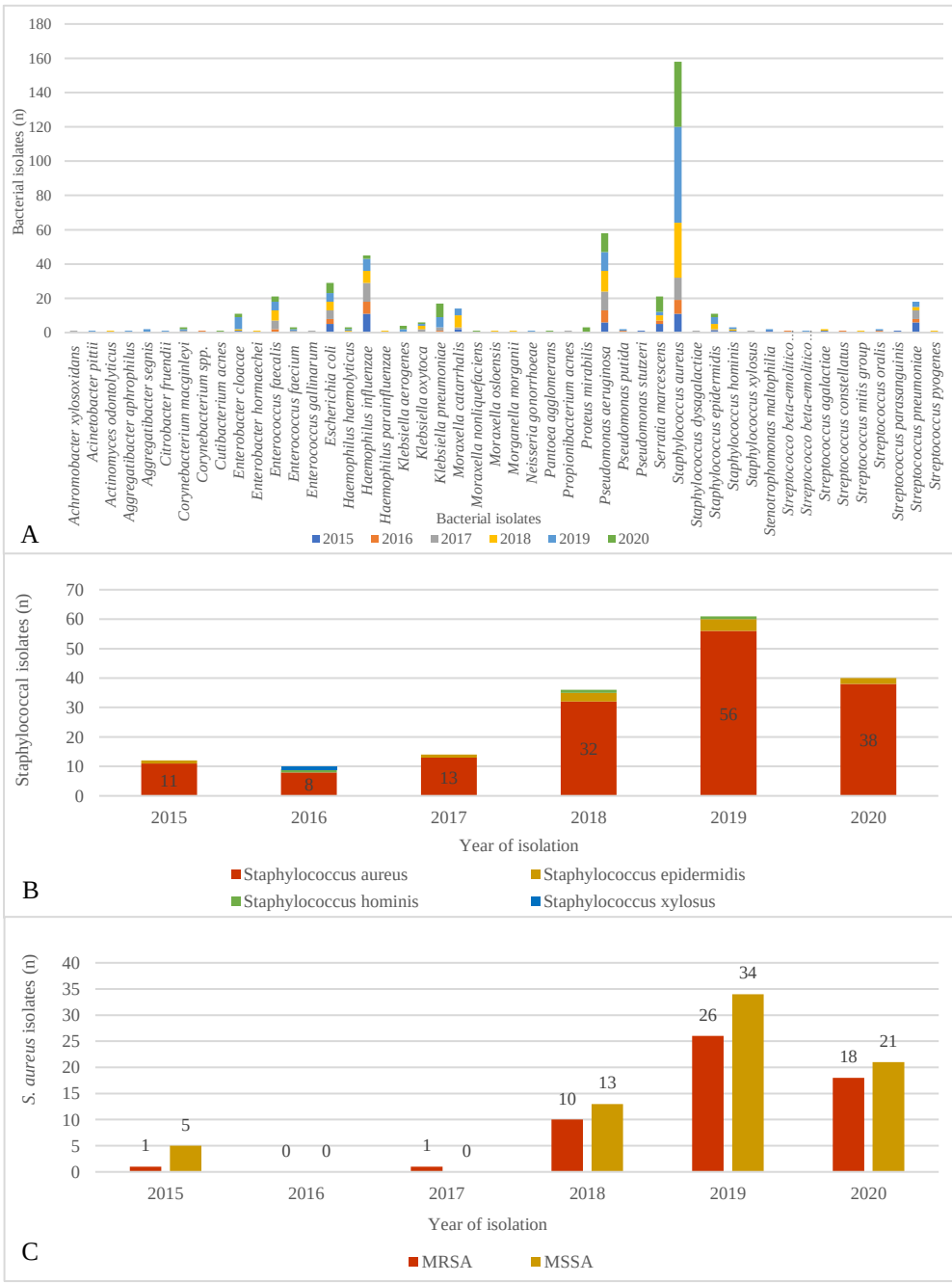


Figure II.2.1: Summary of bacterial species identified in the period 2015-2020. A. Number of isolates and bacterial species identified in the three Italian Hospitals involved in the retrospective epidemiological study, B. Number of isolates of different S*taphylococcus* spp. identified in the retrospective epidemiological study period. C. Number of MRSA and MSSA identified in this the study, from three hospitals.

S. aureus isolates (n=123), collected at the three medical structures, were mainly from conjunctival swabs (94.5% MRSA, 85.3% MSSA), cornea (4% MRSA, 15% MSSA) and aqueous humor (2% MRSA, 0%MSSA) (Fig. II.2.2A). Both MRSA and MSSA isolates were significantly more common among patients with healthcare exposure, inpatients, and less common in outpatients. Most of the *S. aureus* isolates collected at S. Matteo Hospital were from pediatric/neonatal units, (85% MRSA, 57% MSSA) (Fig II.2.2B).

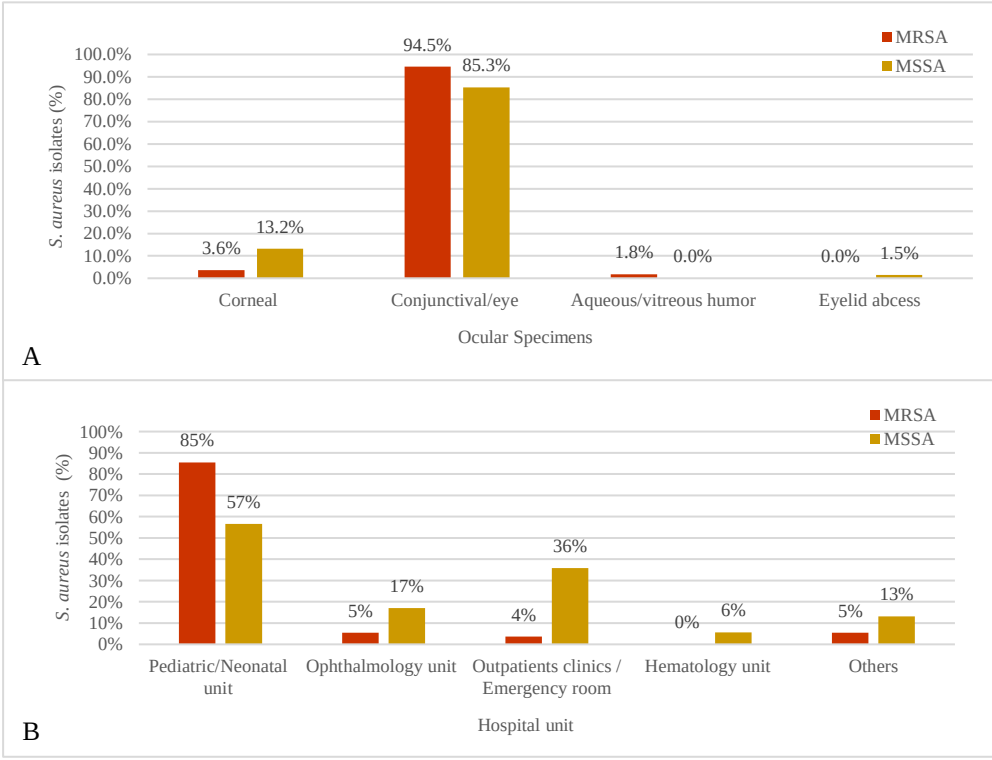


Figure II.2.2: Types of specimens and hospital units from which n=123 *S. aureus* isolates were collected in the study. A. Distribution of *S. aureus* specimens obtained from the three medical structures. B. Distribution of *S. aureus* isolates among the hospital units of the three medical centers. Outpatient clinics includes ophthalmology and infectious disease clinics. Others include eye bank, medical clinic, dermatology day hospital.

Antimicrobial susceptibility profiles were available for 122 *S. aureus* (54 MRSA, 68 MSSA) isolates collected in the period of 2018 to 2020 from the three Hospitals. Almost all MRSA isolates were significantly more resistant than MSSA to several antibiotics, including ciprofloxacin (96.3%; 0%, respectively), erythromycin (59%; 19%), moxifloxacin (96%; 0%) and TOB (96%; 3%) (Fig. II.2.3A, II.2.3B). Almost all the isolates have shown susceptibility to daptomycin, linezolid, and fusidic acid. (Fig. II.2.3A, II.2.3B).

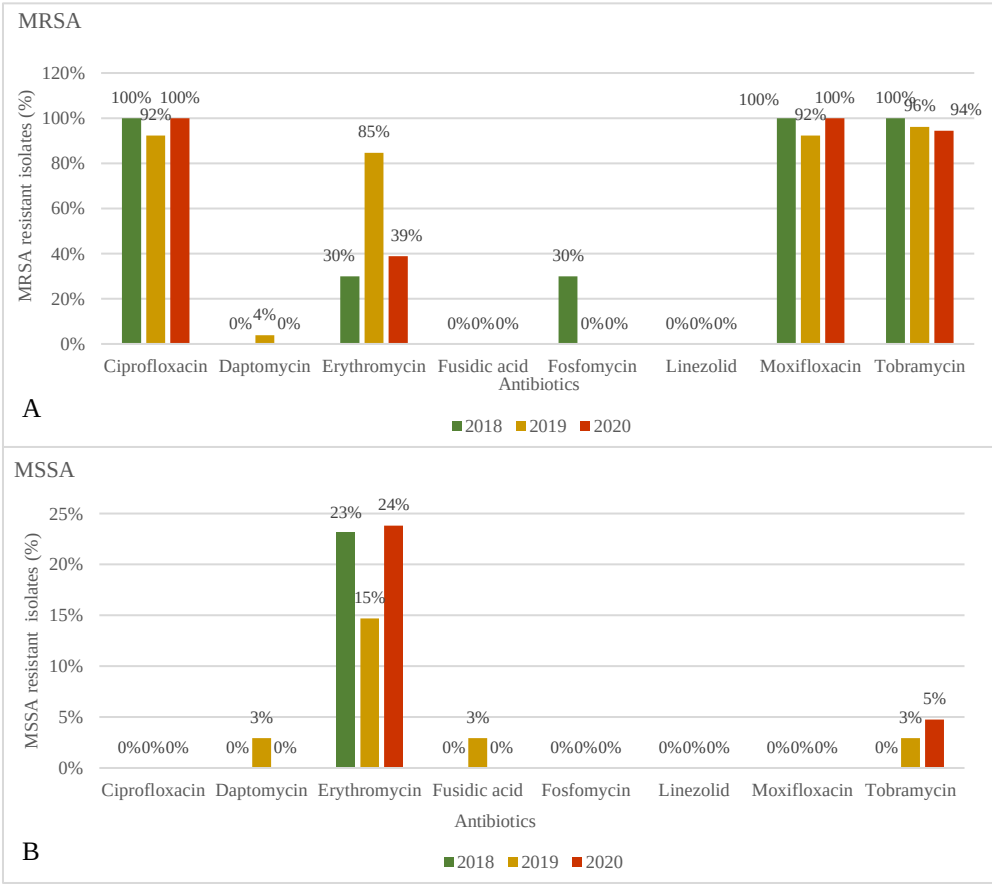


Figure II.2.3: Antimicrobial resistance patterns of *S. aureus* isolates**.** A. Antimicrobial resistance patterns of the n=54 MRSA isolates. B. Antimicrobial resistance patterns of the n=68 MSSA isolates.

The 408 isolated collected from IRCCS Policlinico San Matteo of Pavia belonged to 43 different species. *S. aureus* (37%) was the most isolated species, followed by *Pseudomonas aeruginosa* (13%), *Haemophilus parainfluenzae* (7%), and *Haemophilus haemolyticus* (7%) (Fig. II.2.4A). Among the 117 *S. aureus* isolates, 89.7% (n=105/117) were from conjunctival swabs, the remaining being identified from corneal swabs (9.4%, n=11/117), and aqueous humor specimens (0.8%, n=1/117) for suspected keratitis and endophthalmitis, respectively (Fig. II.2.4B). *S. aureus* isolates were collected from inpatients at different units, including pediatric/neonatal units (85% MRSA vs 45% MSSA), ophthalmological unit (5% MRSA vs 10% MSSA) and hematology unit (0% MRSA vs 5% MSSA), and outpatients (4% MRSA vs 29% MSSA) at ophthalmological, infectious disease outpatient clinics, and emergency room (Fig. II.2.4C).

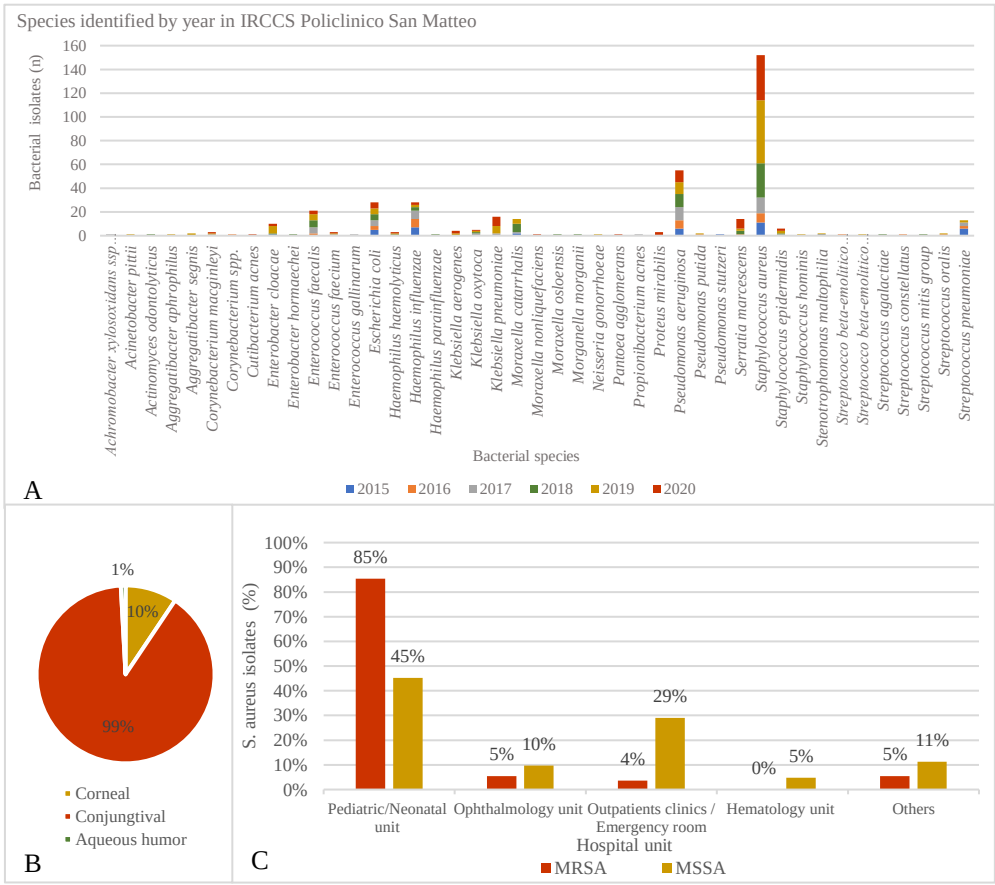


Figure II.2.4: Bacterial species identified during the period 2015-2020 in the IRCCS "Policlinico San Matteo". B. Distribution of the specimen type of *S. aureus* isolates obtained from IRCCS Policlinico San Matteo. B. Distribution of hospital units from which the n=117 *S. aureus* (55 MRSA isolates vs 62 MSSA) isolates have been collected in IRCCS Policlinico San Matteo.

The 42 isolates collected from Manzoni Hospital of Lecco belonged to 12 different species (Fig. II.2.5A). Among these, the majority was represented by Gram-negatives (78.6%, n=33/42) belonged to *H. influenzae* representing (40%, n=) of isolated species and *Serratia marcescens* representing 17% of isolated species, while *Streptococcus pneumoniae* (12%) and *S. aureus* (7%) were among the most isolated Gram-positive bacteria, isolated mainly in 2018 and 2019 from both hospital and community settings. The three *S. aureus* isolates from Lecco were all MSSA, obtained from conjunctival swabs, and collected from paediatric unit (67.7%, n=2/3) and the emergency unit (33.3%, n=1/3).

The 13 isolates collected from "Luigi Sacco" University Hospital belonged to 6 different species, 4 species were of *Staphylococcus* including *S. epidermidis* (38%), *S. aureus* (23%), *S. hominis* (15%), and *S. xylosus* (8%), the other 2 belonged to *Streptococcus* spp. including *S. dysagalactiae* (8%), and *S. parasanguinis* (8%) (Fig. II.2.5B). The *Staphylococcus* spp. isolates were all MSSA, obtained from different specimens, including conjunctival swabs (45.5%, n=5/11), aqueous humour (36.4%, n=4/11), and eye abscess samples (9.1%, n=1/11). The three *S. aureus* isolates were recovered from two patients of the ophthalmological unit (67.7%, n=2/3) and one outpatient at Emergency Room (33.3%, n=1/3).

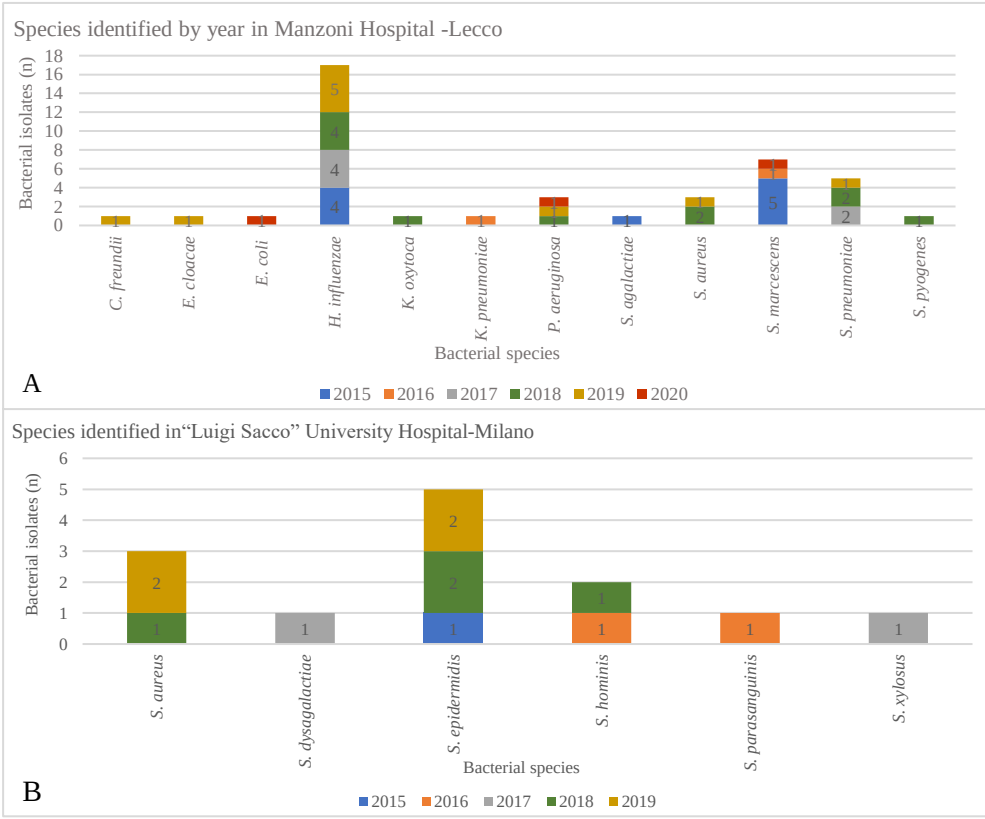


Figure II.2.5: Bacterial species identified during the period 2015-2020 in the A. "Manzoni Hospital" of Lecco, and B. "Luigi Sacco" University Hospital.

II.2.6.2 Real MIC Values and Anti-biofilm Activity of Four Different Antibiotics Against *S. aureus* Strains from Ocular Infections

II.2.6.2.1 Clinical Characteristics and Antimicrobial Susceptibility of Ocular *S. aureus* Isolates

A total of n=45 *S. aureus* ophthalmological isolates, obtained from the above three medical centers, were subjected to further *in vitro* investigations. The strains included both MSSA (69%, n=31/45) and MRSA (31%, n=14/45) (Fig. II.2.6). These *S. aureus* ophthalmological isolates were collected from superficial or intraocular infections, among which 82% of the isolates (n=37/45) were identified in conjunctival samples for suspected conjunctivitis, while 16% of the isolates (n=7/45) were identified in corneal samples, and 2% of the isolates (n=1/45) were identified in aqueous humor samples for suspected keratitis and endophthalmitis, respectively (Fig. II.2.7).

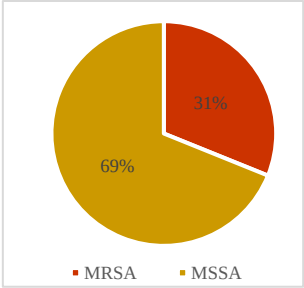


Figure II.2.6: The **d**istribution of MRSA vs MSSA among the n= 45 *S. aureus* isolates.

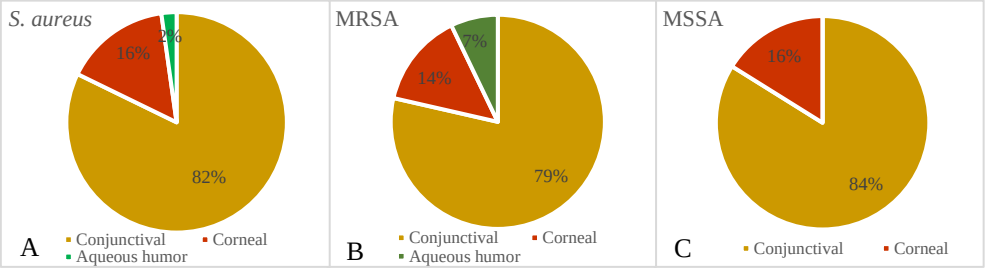


Figure II.2.7: Distribution of specimen type for the n=45 *S. aureus* isolates. A. the 45 *S. aureus*, B. 14 MRSA, and C. 31 MSSA isolates.

The n=45 *S. aureus* isolates were from outpatients in emergency room and other clinics (0% MRSA, 45% MSSA) and from inpatients in various hospital units including neonatal unit (57.1%, MRSA, 22.7% MSSA), ophthalmological unit (28.6%MRSA, 45.5%MSSA), hematology unit (0% MRSA, 13.64% MSSA), dermatology unit (0% MRSA, 4.6% MSSA), infectious diseases unit (7.1% MRSA, 4.6% MSSA), and medical clinic (7.14% MRSA, 4.6% MSSA) (Fig. II.2.8).

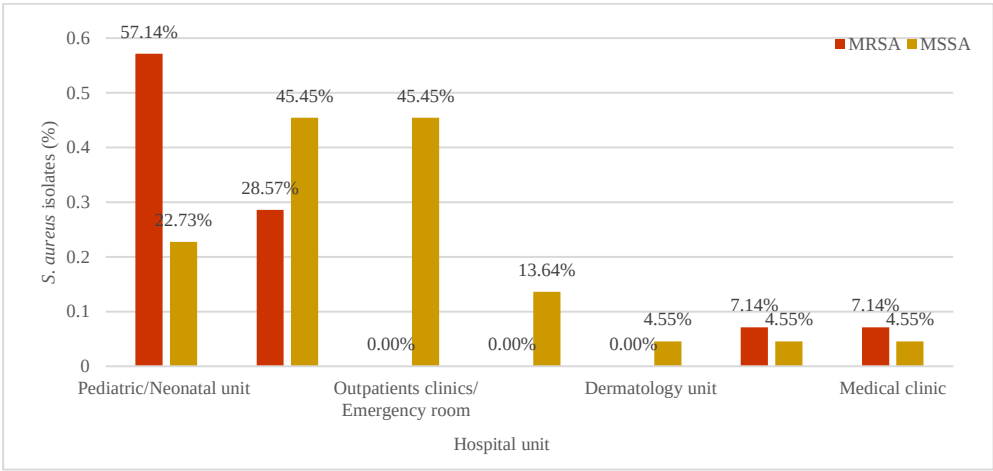


Figure II.2.8: Distribution of hospital units from which *S. aureus* isolates, n=14 MRSA and the n=31 MSSA, were collected.

All the MRSA isolates had *mecA* gene and were considered MDR organisms, while 35% of MSSA isolates (n=11/31) exhibited mild MDR phenotype due to being resistant to at least three categories of antibiotic resistance [18].

Overall, drug resistance pattern between MRSA and MSSA isolates was found to be similar for some antibiotics and highly variable for others (Table II.2.1) (Fig. II.2.9A, II.2.9B) All MRSA isolates (n=14) exhibited resistance (86%, n=12/14) or susceptible with high exposition (14%, n=2/14) to ciprofloxacin and levofloxacin, while all MSSA (n=31) isolates exhibited Susceptibility with high exposure (I) except one showed resistance to both antibiotics. For moxifloxacin, all MRSA isolates were found resistant except one, while all MSSA isolates were found to be susceptible except one which was resistant.

Regarding aminoglycoside and macrolide resistance, MRSA isolates were significantly more resistant than MSSA to gentamicin (58% vs 9%), erythromycin (64% vs. 29%) but less for azithromycin (64% vs 55%). Clindamycin resistance – positive inducibility test - was found in 28.9 % (n=13/45) of *S. aureus* isolates including 50% (n=7/14) MRSA and 19.4% (n=6/31) MSSA isolates, with no isolates showing a macrolide-lincosamide-streptogramin$_B$ (MLS_B) resistance phenotype. all *S. aureus* isolates of both groups were susceptible to streptogramin B, quinupristin and dalfopristin, linezolid, tetracycline, trimethoprim/sulfamethoxazole, daptomycin, teicoplanin, fosomycin, and vancomycin (Table II.2.1).

Table II.2.1: Antibiotic Susceptibility patterns of the n=45 *S. aureus* isolates studied

Antibiotics	MRSA			MSSA (%)		
	R (%)	I (%)	S (%)	R (%)	I (%)	S (%)
Penicillin G	100%	0%	0%	71%	0%	29%
Ampicillin	100%	0%	0%	84%	0%	16%
Amoxicillin-clavulanic acid	100%	0%	0%	10%	0%	90%
Oxacillin	100%	0%	0%	0%	0%	100%
Imipenem	100%	0%	0%	23%	0%	77%
Cefoxitin	100%	0%	0%	0%	0%	100%
Ciprofloxacin	86%	14%	0%	3%	97%	0%
Levofloxacin	86%	14%	0%	3%	97%	0%
Moxifloxacin	93%	0%	7%	3%	0%	97%
Gentamicin	64%	0%	36%	10%	0%	90%
Erythromycin	64%	0%	36%	29%	0%	71%
Azithromycin	64%	0%	36%	55%	0%	45%
Clindamycin	64%	7%	29%	29%	26%	45%
Tetracycline	7%	0%	93%	0%	0%	100%
Daptomycin	0%	0%	100%	0%	0%	100%
Fosfomycin	0%	0%	100%	0%	0%	100%
Vancomycin	0%	0%	100%	0%	0%	100%
Teicoplanin	0%	0%	100%	0%	0%	100%
Lilinezolid	0%	0%	100%	0%	0%	100%
Trimethoprim/sulfamethoxazole	0%	0%	100%	0%	0%	100%
Quinupristin and Dalfopristin	0%	0%	100%	0%	0%	100%

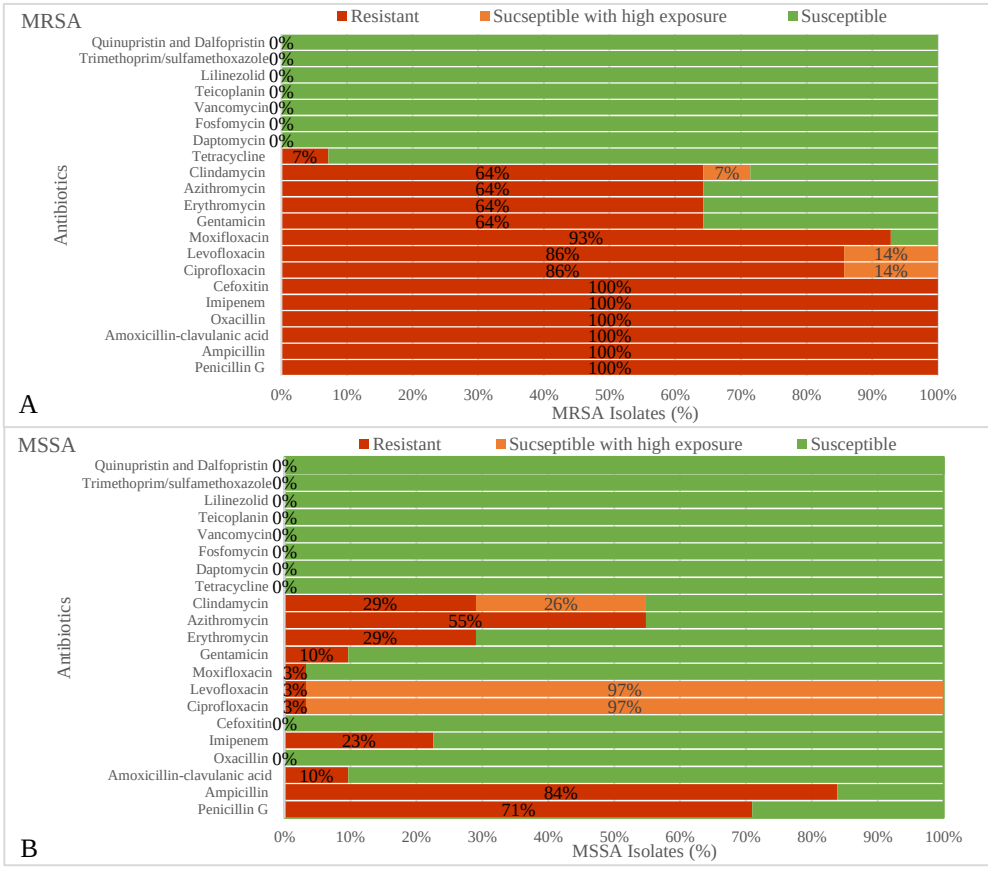
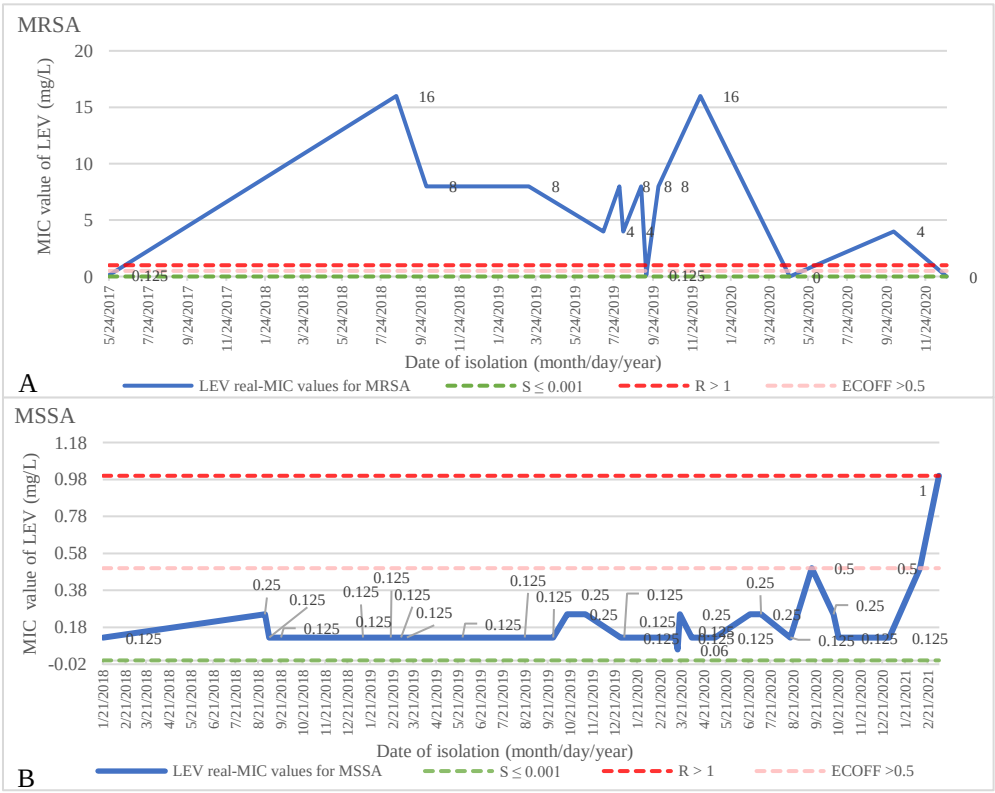


Figure II.2.9: Antimicrobial susceptibility patterns of the n=45 *S. aureus* isolates. A. The n=14 MRSA isolates, and B. The n=31 MSSA isolates.

II.2.6.2.2 Real MIC values for LEV, CLO, TOB, and NET

The MICs obtained by broth-microdilution for LEV, CLO, TOB, and NET, four molecules of therapeutic interest for the treatment of ocular infections, showed a bacterial trend of growing resistance due to the presence of non-wildtype or resistant strains.

The "real MIC values" for LEV obtained by broth dilution method, confirmed the Autoscan4 Semi-automated system susceptibility results, on LEV concentrations of 0.001, 1 mg/L, and 2 mg/L. Levofloxacin susceptibility profiles according to EUCAST clinical breakpoints are MIC ≤ 0.001 for susceptibility, MIC 0.001 ≥ 1 for Susceptibility with increased exposure, and MIC >1 mg/L for Resistance. All but two MRSA strains were LEV Resistant; susceptibility with increased exposure was present for one MRSA strain collected in 2017 and another in 2020 (Fig. II.2.10A, Table II.2.2). All MSSA isolates resulted Susceptible with increased exposure to levofloxacin. However, three MSSA strains showed a higher MIC in comparison with the other previously identified n=28 MSSA isolates; one isolate, collected in December 2020, had an MIC value of 0.5 mg/L and two other, collected in February and March 2021, showed MIC values of 0.5 and 1 mg/L, respectively (Fig II.2.10B, Table II.2.3).



(Figure II.2.10 continues next page)

Concentrations of LEV (mg/L)	*S. aureus* (n=45) (%)	MSSA (n=31) (%)	MRSA (n=14) (%)
0.03	0	0	0
0.06	1 (2%)	1 (3%)	0
0.125	22 (49%)	20 (56%)	2 (14%)
0.25	7 (16%)	7 (23%)	0
0.5	2 (4%)	2 (6%)	0
1	1 (2%)	1 (3%)	0
2	0	0	0
4	3 (7%)	0	3 (21%)
8	5 (11%)	0	5 (36%)
16	2 (4%)	0	2 (14%)
>16	2 (4%)	0	2 (14%)

Figure II.2.10: Real-MIC values of LEV for *S. aureus* isolates. A. MRSA isolates. B. MSSA isolates. Dashed lines indicate EUCAST clinical breakpoints of LEV between the susceptibility categories. R, resistant when MIC is >1; I, susceptible with increased exposure when MIC is 1> and ≥0.001 mg/L; S, susceptible when MIC is ≤ 0.001 mg/L.

Table II.2.2: Real-MIC values of LEV, CLO, TOB, and NET for MRSA isolates (Resistant in red, susceptible with increased exposure in yellow, and susceptible in green)

Date of isolation (month/day/year)	LEV[a] real-MIC value (mg/L)	CLO[b] real-MIC value (mg/L)	TOB[c] real-MIC value (mg/L)	NET[d] real-MIC value (mg/L)
05/24/2017	0.125	8	>32	2
08/17/2018	16	8	>32	8
10/03/2018	8	8	0.25	0.5
03/13/2019	8	8	0.125	0.25
07/08/2019	4	8	0.25	0.25
08/02/2019	8	8	32	0.25
08/08/2019	4	8	16	2
09/05/2019	8	8	>32	1
09/13/2019	0.125	8	0.5	2
10/02/2019	8	8	>32	0.25
12/07/2019	16	8	32	8
04/25/2020	>16	8	0.125	0.25
10/05/2020	4	8	>32	4
12/27/2020	>16	8	0.25	0.25

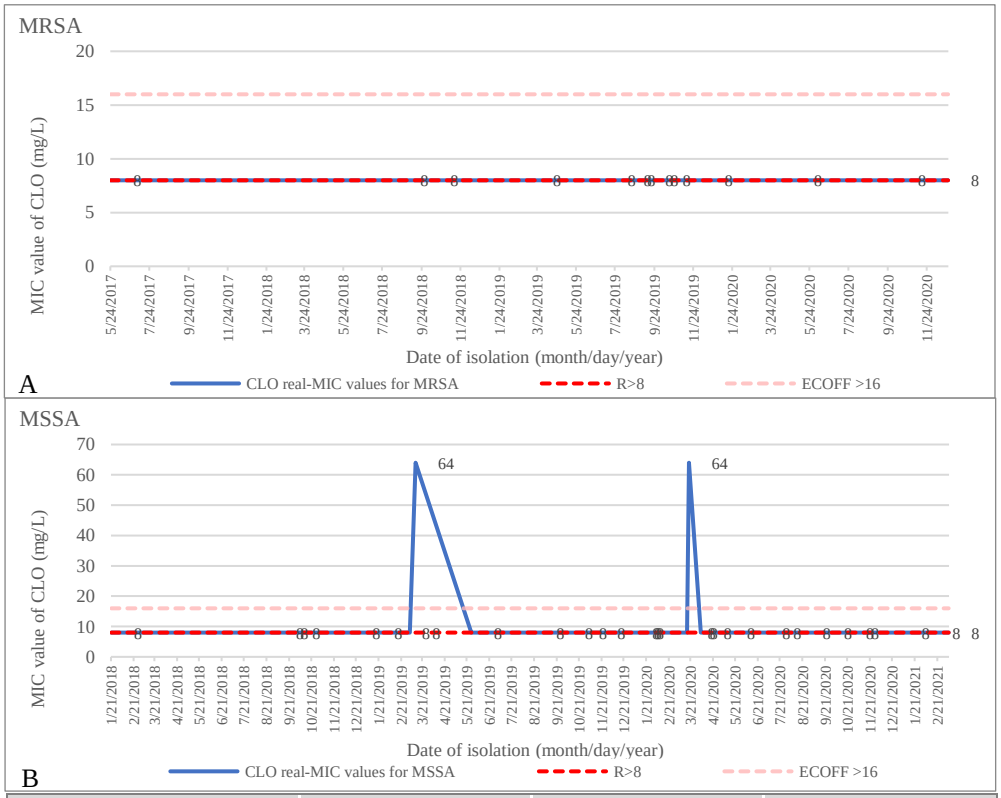
[a]*Eucast LEV clinical breakpoint values: S ≤ 0.001 mg/L, 0.001> I ≥1, R > 1 mg/L. ECOFF: Wild type ≤ 0.5 mg/L.*
[b]*Eucast CLO clinical breakpoint: R>8 mg/L. ECOFF: Wild type ≤ 16 mg/L.*
[c]*Eucast TOB clinical breakpoint: R>2 mg/L. ECOFF: Wild type ≤ 2 mg/L.*
[d]*Eucast NET clinical breakpoint value is insufficient evidence (IE) that S. aureus is a good target for therapy with this drug.*

Table II.2.3: Real-MIC values of LEV, CLO, TOB, and NET for MSSA isolates (Resistant in red, susceptible with increased exposure in yellow, and susceptible in green)

Date of isolation (month/day/year)	LEV[a] real-MIC value (mg/L)	CLO[b] real-MIC value (mg/L)	TOB[c] real-MIC value (mg/L)	NET[d] real-MIC value (mg/L)
01/21/2018	0.125	8	0.25	0.25
08/30/2018	0.25	8	0.25	0.25
09/05/2018	0.125	8	0.125	0.5
09/21/2018	0.125	8	32	2
12/12/2018	0.125	8	0.5	0.25
01/11/2019	0.125	8	0.25	0.125
02/18/2019	0.125	8	0.25	0.25
03/04/2019	0.125	8	0.125	0.125
03/12/2019	0.125	64	0.25	0.25
05/27/2019	0.125	8	0.125	0.125
08/20/2019	0.125	8	8	0.125
09/28/2019	0.125	8	0.5	0.25
10/17/2019	0.25	8	8	4
11/11/2019	0.25	8	0.125	0.25
12/29/2019	0.125	8	0.125	0.25
12/31/2019	0.125	8	0.125	0.25
01/03/2020	0.125	8	4	2
03/13/2020	0.125	8	0.25	0.5
03/16/2020	0.06	8	0.25	0.25
03/19/2020	0.25	64	0.125	0.25
04/04/2020	0.125	8	0.25	0.25
05/06/2020	0.125	8	0.125	0.25
06/23/2020	0.25	8	0.25	0.25
07/08/2020	0.25	8	1	0.5
08/17/2020	0.125	8	0.125	0.25
09/15/2020	0.5	8	0.125	0.125
10/15/2020	0.25	8	0.25	0.25
10/22/2020	0.125	8	0.25	0.125
12/30/2020	0.125	8	0.125	4
02/10/2021	0.5	8	0.25	4
03/08/2021	1	8	8	4

[a]*Eucast LEV clinical breakpoint values: S ≤ 0.001 mg/L, 0.001> 1 ≥1, R > 1 mg/L. ECOFF: Wild type ≤ 0.5 mg/L.*
[b]*Eucast CLO clinical breakpoint: R>8 mg/L. ECOFF: Wild type ≤ 16 mg/L.*
[c]*Eucast TOB clinical breakpoint: R>2 mg/L. ECOFF: Wild type ≤ 2 mg/L.*
[d]*Eucast NET clinical breakpoint value is insufficient evidence (IE) that S. aureus is a good target for therapy with this drug.*

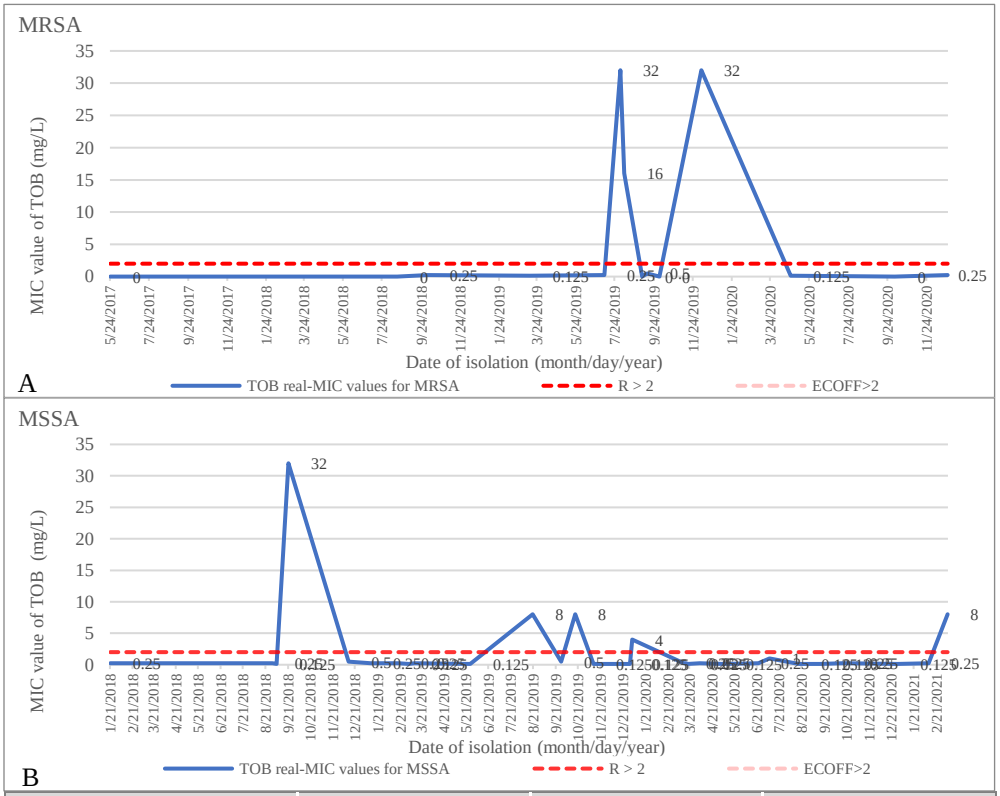
All MRSA isolates (n=14) were susceptible to CLO, having an MIC value of 8 mg/L (Fig. II.2.11A, Table II.2.2). Similarly, all except two susceptible MSSA isolates (n=29/31) showed susceptibility to CLO (Fig. II.2.11B, Table II.2.3).



Concentrations of CLO (mg/L)	*S. aureus* (n=45) (%)	MSSA (n=31) (%)	MRSA (n=14) (%)
0.125	0	0	0
0.5	0	0	0
1	0	0	0
2	0	0	0
4	0	0	0
8	43 (96%)	29 (94%)	14 (100%)
16	0	0	0
32	0	0	0
64	2 (4%)	2 (6%)	0
128	0	0	0

Figure II.2.11: Real-MIC values of CLO for *S. aureus* isolates. A. MRSA isolates. B. MSSA isolates. Dashed lines indicate EUCAST clinical breakpoints for CLO between the susceptibility categories. R, resistant when MIC is >8; and S, susceptible when MIC is ≤ 8 mg/L.

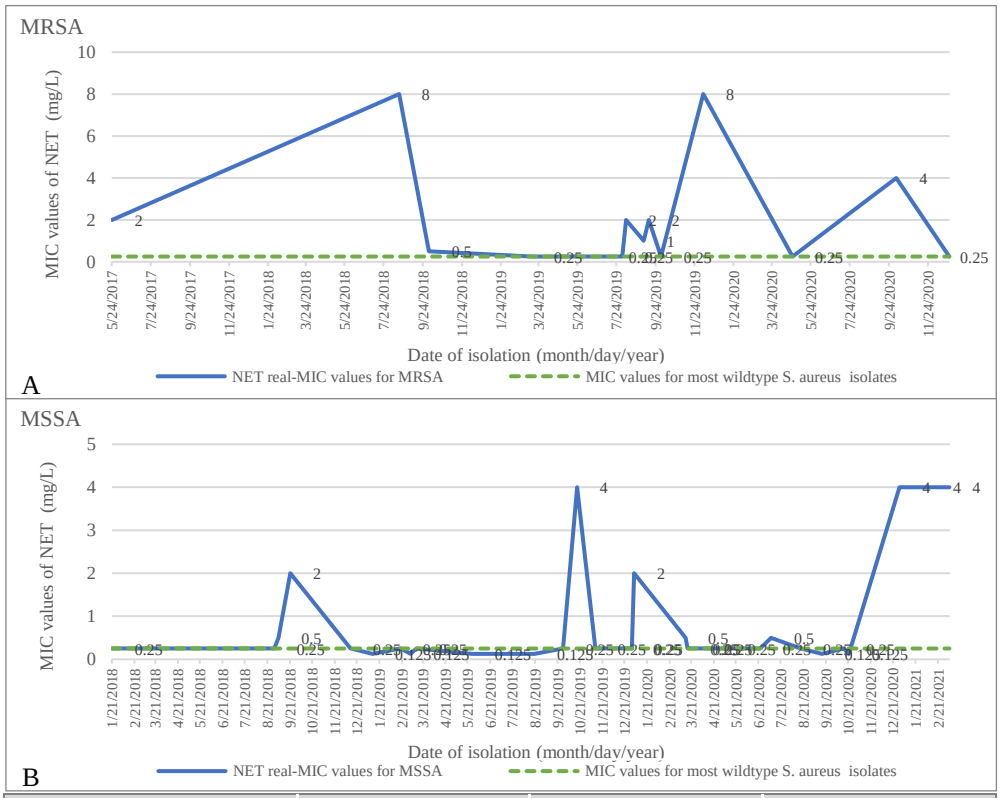
More than half of of MRSA isolates (57.1%, n=8/14) were TOB resistant, all having an MIC value of 32 mg/L or higher except one isolate having MIC=16 mg/L (Fig. II.2.12A, Table II.2.2). On the other hand, 16.1% (n=5/31) of MSSA isolates showed resistance with MICs lower than the ones observed for MRSA isolates (Fig. II.2.12B, Table II.2.3).



Concentrations of TOB (mg/L)	*S. aureus* (n=45) (%)	MSSA (n=31) (%)	MRSA (n=14) (%)
0.06	0	0	0
0.125	13 (29%)	11 (35%)	2 (14%)
0.25	15 (33%)	12 (39%)	3 (21%)
0.5	3 (7%)	2 (6%)	1 (7%)
1	1 (2%)	1 (3%)	0
2	0	0	0
4	1 (2%)	1 (3%)	0
8	3 (7%)	3 (10%)	0
16	1 (2%)	0	1 (7%)
32	3 (7%)	1 (3%)	2 (14%)
> 32	5 (11%)	0	5 (36%)

Figure II.2.12: Real-MIC values of TOB for *S. aureus* isolates. A. MRSA isolates. B. MSSA isolates. Dashed lines indicate EUCAST clinical breakpoints of TOB between the susceptibility categories. R, resistant when MIC is >2; and S, susceptible when MIC is ≤ 2 mg/L.

For netilmicin, 57.1% (n=8/14) of MRSA isolates had MIC values ranging from 0.5 to 8 mg/L above the expected MIC value for wildtypes, 0.25 mg/L (Fig. II.2.13A, Table II.2.2). On the other hand, 29% (n=9/31) of MSSA isolates had MIC values ranging from 0.5 to 4 mg/L above the expected MIC value for wildtypes (Fig. II.2.13B, Table II.2. 3). .



Concentrations of NET (mg/L)	*S. aureus* (n=45) (%)	MSSA (n=31) (%)	MRSA (n=14) (%)
0.06	0	0	0
0.125	6 (13%)	6 (19%)	0
0.25	22 (49%)	16 (52%)	6 (43%)
0.5	4 (9%)	3 (10%)	1 (7%)
1	1 (2%)	0	1 (7%)
2	5 (11%)	2 (6%)	3 (21%)
4	5 (11%)	4 (13%)	1 (7%)
8	2 (4%)	0	2 (14%)
16	0	0	0
32	0	0	0

Figure II.2.13: Real-MIC values of NET for *S. aureus* isolates. A. MRSA isolates. B. MSSA isolates. Dashed line indicates the MIC value for most wild type *S. aureus* strains according to EUCAST ECOFF distribution.

II.2.6.2.3 Phenotypic Characterization and *icaA/D* gene Detection of Biofilm Production

In this study biofilm production was detected qualitatively using CRA and CRB assays, and quantitatively using crystal violet assay (CVA). CVA, all MRSA and MSSA isolates resulted strong biofilm producers matching the results obtained by the less commonly used qualitative method, CRB-TS (Fig. II.2.14, Fig. II.2.15, table II.2.4, table II.2.5). Using the CRA-BHI, 79% (n=13) of MRSA isolates and 55% (n=17/31) of MSSA isolates produced characteristic black colonies indicating their ability of biofilm production (Fig. II.2.14). For genotypic identification of polysaccharidic biofilm, the isolates were screened for the presence of *icaA* and *icaD* genes. 79% (n=11/14) of MRSA isolates and 87% (n=27/31) of MSSA isolates were *icaA/D* positive. However, 21% (n=3/14) of MRSA isolates and 9.7% (n=3/31) of MSSA isolates did not amplify *icaA* or *icaD* genes with one MSSA isolate was negative for both genes, but they showed characteristics of exopolysaccharide production in vitro through both CRA and CRB (Table II.2.4, and table II.2.5).

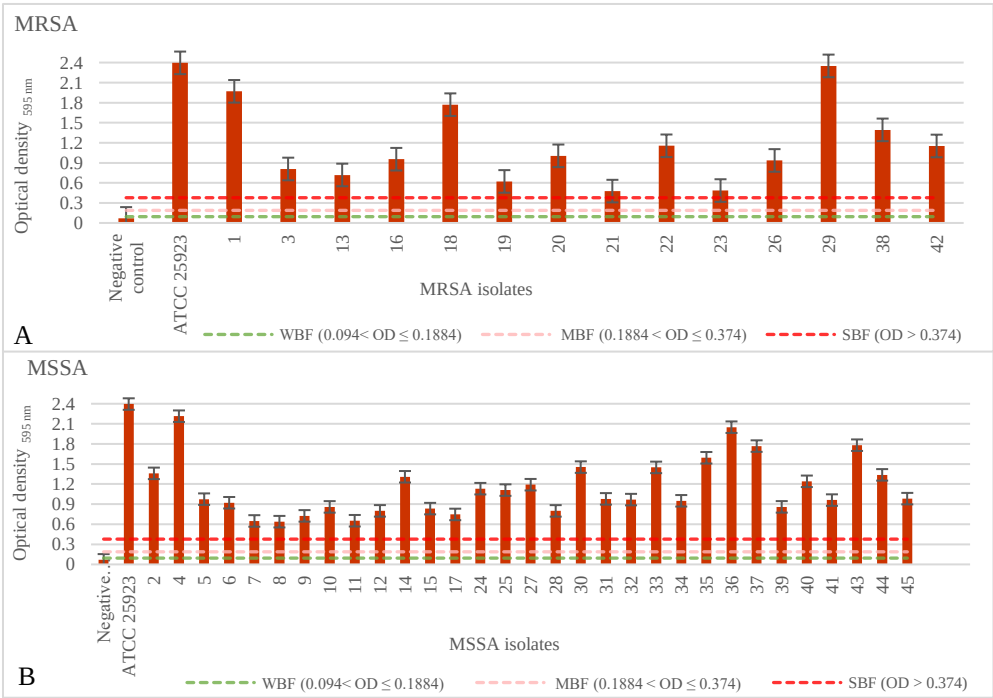


Figure II.2.14: Biofilm phenotypic characterization and classification by crystal violet assay (CVA) of *S.aureus* isolates, A. MRSA. B. MSSA isolates. Dashed lines correspond to cut-off values for the classification of biofilm producing isolates, NBF, non-biofilm producers with OD ≤ 0.0942; WBF, weak biofilm producers with 0.0942 < OD ≤ 0.1884; MBF, moderate biofilm producers with (0.1884 < OD ≤ 0.3778); SBF, strong biofilm producers with OD > 0.3778.

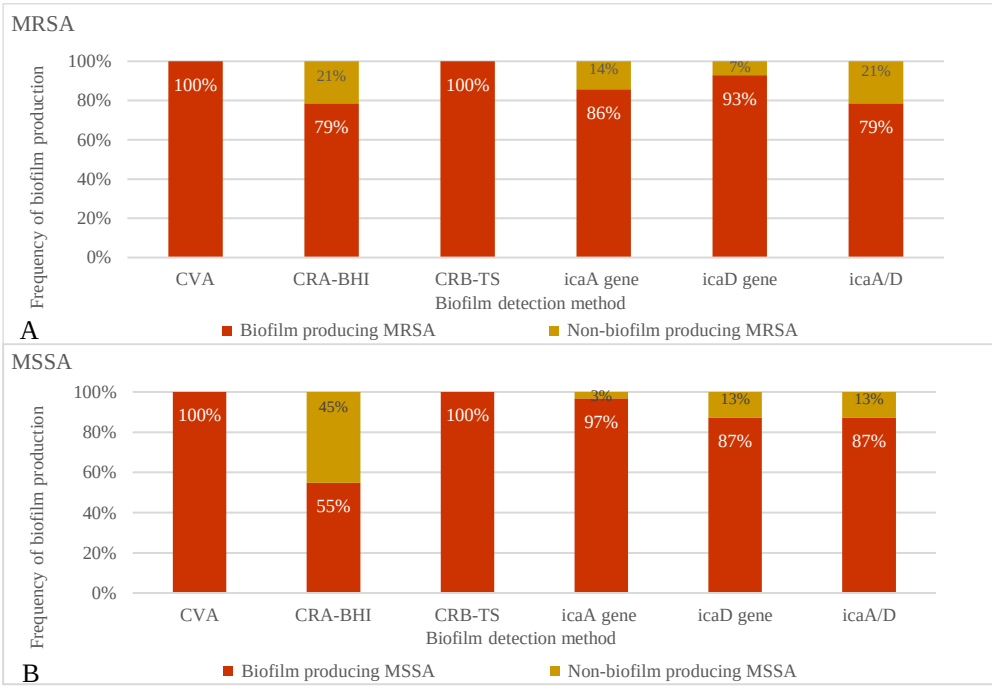


Figure II.2.15: Biofilm phenotypic characterization of *S.aureus* isolates by crystal violet assay (CVA), Congo-red based on brain heart infusion agar (CRA-BHI), and Congo-red based on tryptic soy broth (CRB-TS), and genotypic identification of polysaccharidic biofilm producing genes *icaA* and *icaD* genes in A. MRSA isolates, and B. MSSA isolates.

Table II.2.4: Biofilm characterization of MRSA isolates

MRSA Isolates[a]	Congo-Red agar	Congo-Red broth	Optical density obtained from crystal violet assay (nm)	Classification of biofilm production[b] (Strong, moderate, weak)	*icaA*	*icaD*
ATCC 25923	Positive	Positive	2.39	Strong	***icaA*⁺**	***icaD*⁺**
1	Positive	Positive	1.97	Strong	***icaA*⁺**	***icaD*⁺**
3	Positive	Positive	0.81	Strong	***icaA*⁺**	*icaD*⁻
13	Positive	Positive	0.72	Strong	***icaA*⁺**	***icaD*⁺**
16	Positive	Positive	0.95	Strong	***icaA*⁺**	***icaD*⁺**
18	Positive	Positive	1.77	Strong	***icaA*⁺**	***icaD*⁺**
19	Positive	Positive	0.62	Strong	IcaA-	***icaD*⁺**
20	Negative	Positive	1	Strong	***icaA*⁺**	***icaD*⁺**
21	Positive	Positive	0.48	Strong	IcaA-	***icaD*⁺**
22	Negative	Positive	1.15	Strong	***icaA*⁺**	***icaD*⁺**
23	Positive	Positive	0.48	Strong	***icaA*⁺**	***icaD*⁺**
26	Positive	Positive	0.93	Strong	***icaA*⁺**	***icaD*⁺**
29	Negative	Positive	2.35	Strong	***icaA*⁺**	***icaD*⁺**
38	Positive	Positive	1.39	Strong	***icaA*⁺**	***icaD*⁺**
42	Positive	Positive	1.15	Strong	***icaA*⁺**	***icaD*⁺**

[a]Isolates in red were chosen to be further investigated.
[b]NBF, non-biofilm producers with OD ≤ 0.0942; WBF, weak biofilm producers with 0.0942 < OD ≤ 0.1884; MBF, moderate biofilm producers with (0.1884 < OD ≤ 0.3778); SBF, strong biofilm producers with OD > 0.377.

Table II.2.5: Biofilm characterization of *MSSA* isolates

MSSA Isolates[a]	Congo-Red agar	Congo-Red broth	Optical density obtained from crystal violet assay (nm)	Classification of biofilm production[b] (Strong, moderate, weak)	*icaA*	*icaD*
ATCC 25923	Positive	Positive	2.39	Strong	***icaA*** $^{+}$	***icaD***$^{+}$
2	Negative	Positive	1.36	Strong	***icaA***$^{+}$	***icaD***$^{+}$
4	Negative	Positive	2.21	Strong	***icaA***$^{+}$	***icaD***$^{+}$
5	Positive	Positive	0.97	Strong	***icaA*** $^{+}$	***icaD***$^{+}$
6	Negative	Positive	0.92	Strong	***icaA*** $^{+}$	***icaD***$^{+}$
7	Negative	Positive	0.65	Strong	***icaA*** $^{+}$	***icaD***$^{+}$
8	Negative	Positive	0.64	Strong	***icaA***$^{+}$	***icaD***$^{+}$
9	Negative	Positive	0.72	Strong	***icaA*** $^{+}$	***icaD***$^{+}$
10	Negative	Positive	0.86	Strong	***icaA*** $^{+}$	*IcaD-*
11	Positive	Positive	0.65	Strong	***icaA*** $^{+}$	***icaD***$^{+}$
12	Positive	Positive	0.8	Strong	***icaA*** $^{+}$	***icaD***$^{+}$
14	Negative	Positive	1.31	Strong	***icaA*** $^{+}$	***icaD***$^{+}$
15	Positive	Positive	0.84	Strong	*icaA*$^{-}$	*icaD*$^{-}$
17	Negative	Positive	0.75	Strong	***icaA*** $^{+}$	***icaD***$^{+}$
24	Negative	Positive	1.13	Strong	***icaA*** $^{+}$	***icaD***$^{+}$
25	Negative	Positive	1.11	Strong	***icaA*** $^{+}$	*IcaD-*
27	positive	Positive	1.19	Strong	***icaA*** $^{+}$	***icaD***$^{+}$
28	positive	Positive	0.8	Strong	***icaA*** $^{+}$	***icaD***$^{+}$
30	Positive	Positive	1.45	Strong	***icaA***$^{+}$	***icaD***$^{+}$
31	Positive	Positive	0.98	Strong	***icaA*** $^{+}$	***icaD***$^{+}$
32	Positive	Positive	0.97	Strong	***icaA*** $^{+}$	***icaD***$^{+}$
33	Positive	Positive	1.4	Strong	***icaA*** $^{+}$	*IcaD-*
34	Positive	Positive	0.95	Strong	***icaA*** $^{+}$	***icaD***$^{+}$
35	Negative	Positive	1.59	Strong	***icaA*** $^{+}$	***icaD***$^{+}$
36	Positive	Positive	2.05	Strong	***icaA***$^{+}$	***icaD***$^{+}$
37	Positive	Positive	1.77	Strong	***icaA*** $^{+}$	***icaD***$^{+}$
39	Negative	Positive	0.86	Strong	***icaA*** $^{+}$	***icaD***$^{+}$
40	Positive	Positive	1.24	Strong	***icaA*** $^{+}$	***icaD***$^{+}$
41	Positive	Positive	0.96	Strong	***icaA*** $^{+}$	***icaD***$^{+}$
43	Positive	Positive	1.78	Strong	***icaA*** $^{+}$	***icaD***$^{+}$
44	Negative	Positive	1.34	Strong	***icaA*** $^{+}$	***icaD***$^{+}$
45	Positive	Positive	0.98	Strong	***icaA*** $^{+}$	***icaD***$^{+}$

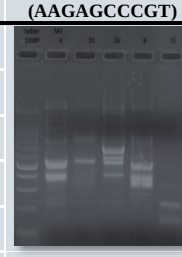
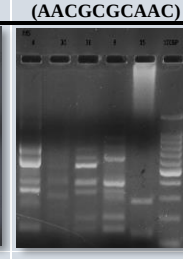
[a] *Isolates in red were chosen to be further investigated.*

[b] *NBF, non-biofilm producers with OD ≤ 0.0942; WBF, weak biofilm producers with 0.0942 < OD ≤ 0.1884; MBF, moderate biofilm producers with (0.1884 < OD ≤ 0.3778); SBF, strong biofilm producers with OD > 0.377.*

II.2.6.2.4 The Effect of Sub-inhibitory Concentrations of Antibiotic on Cell Adherence

We evaluated the effect of the CLO, LEV, TOB, and NET antibiotics presence on *S. aureus* cell adherence and ability to form biofilm. Six non-clonally related (Table II.2.6) *S. aureus* strong biofilm producing strains, including five clinical MSSA strains, and the ATCC 25923, were cultured in the presence of different concentrations, sub-MICs and over the MBC values; of the four different antibiotics, for 24h. The selected MSSA strains were all susceptible to the four antibiotics studied but one was TOB resistant.

Table II.2.6: Clonal typing of the five MSSA strains chosen for antibiotic-driven biofilm formation/eradication ability experiments

Strains[a]	**Date of isolation (d/m/year)**	**Hospital**[b]	**Ward**	**Specimen type**	Clones RAPD Typing	**RAPD primers used for typing**	
						Primer 4 (AAGAGCCCGT)	**Primer 5 (AACGCGCAAC)**
MSSA 4	17/08/2020	San Matteo (PV)	External	corneal	E		
MSSA 8	04/04/2020	San Matteo (PV)	Hematology	Conjunctival	D		
MSSA 15	17/10/2019	San Matteo (PV)	External	Conjunctival	C		
MSSA 30	21/01/2018	Manzoni, (LC)	Pediatrics	Conjunctival	A		
MSSA 36	30/08/2018	San Matteo (PV)	Pediatrics	Conjunctival	B		
***S. aureus* ATCC 25923**	-	-	-	-	-	-	-

Biofilm inhibition by LEV, CLO, TOB, and NET was tested using three different methods: (i) Cell adherence using EUCAST guidelines for determining the MIC values followed with crystal violet assay for biofilm quantification, this was tested for the 5 MSSA isolates (ii) biofilm inhibitory assay, (iii) CLSM.

Cell adherence was evaluated in MH-broth for the above strains at defined antibiotic concentrations, and as control condition, without antibiotic. Cell adherence was significantly enhanced at sub-MIC concentrations of CLO, LEV, and NET, but not TOB (Fig. II.2.16). The presence of 0.03 mg/L of LEV, a concentration 4.16 times lower than the MIC for all the studied strains, significantly promoted the biofilm formation in 3 isolates (50%), in comparison with the LEV-free condition (Fig. II.2.16A). On the contrary, the presence of a concentration of LEV at least equal to 0.125 mg/L, the MIC of all the strains (n=6) ensured a complete cell adherence inhibition preventing biofilm formation (Fig. II.2.16A). In particular, the presence 0.5 mg/L and 1 mg/L of CLO, a concentration 16- and 8-times lower than the MIC for all strains studied, significantly promoted biofilm formation in one (16.7%) and two isolates, respectively, in comparison with the antibiotic-free condition (Fig. II.2.16B). On the contrary, the presence of a concentration of CLO at least equal to 8 mg/L, the MIC obtained for all the strains, ensured a complete inhibition of cell adherence preventing biofilm formation (Fig. II.2.16B).

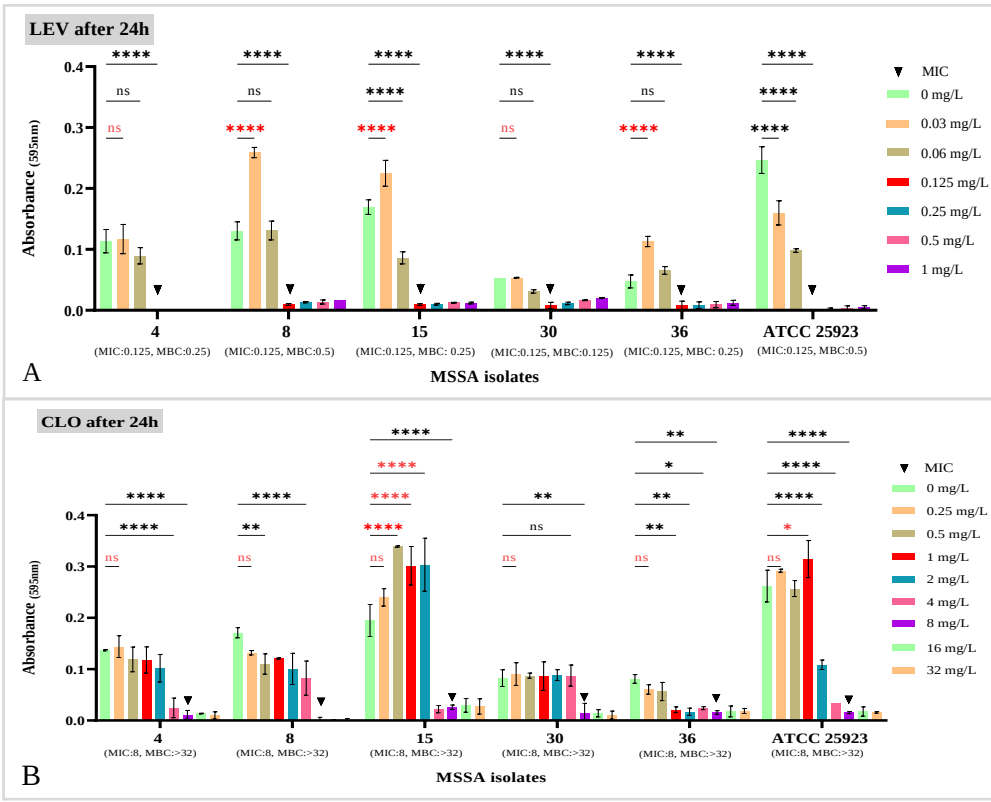


Figure II.2.16: The effect of subinhibitory concentrations of A. LEV, and B. CLO on cell adherence of 5 clinical ophthalmic MSSA isolates and *S. aureus* ATCC 25923. (X-axis represents two-fold serially diluted concentrations of the antibiotic tested showing the results for each MSSA strain tested independently). Results are expressed as means ± SD (n=3 replicates). Statistical significance was calculated using Two-way ANOVA with Dunnett's multiple comparison post-test. Red/black asterisks correspond to significant increase/reduction in cell adherence, respectively. (*P < 0.05; **P< 0.01; ***P< 0.001; ****P<0.0001).

For TOB, All the strains did not show a significant increase in the cell adherence growing at sub-MIC TOB conditions (Fig. II.2.17A). On the contrary, at 0.06 mg/L, a concentration 1- or 2-times lower the MIC value in three isolates (n=3/5, 60%) and 2 isolates (n=2/5, 40%), respectively, significantly reduced cell adherence. However, the presence of a concentration of TOB at least equal to the MIC value, 0.125 in 0.25 mg/L in three isolates (n=3/5, 60%) and 2 isolates (n=2/5, 40%), respectively, ensured a complete cell adherence inhibition preventing biofilm formation (Fig. II.2.17A). The presence of 0.06 mg/L of NET, a concentration one- and two-times lower than the MIC for 2 (n=2/6, 33.3%) and 4 (n=4/6, 66.7%), respectively, promoted cell adherence in 3 isolates ophthalmic isolates (n=3/5, 60%) significantly, and in the other 2 ophthalmic isolates (2/5, 40%) there was an increase but not significant in comparison with the NET-free condition (Fig. II.2.17B). However, the presence of a

concentration of NET at least equal to 0.25 mg/L ensured a complete inhibition of cell adherence (Fig. II.2.17B).

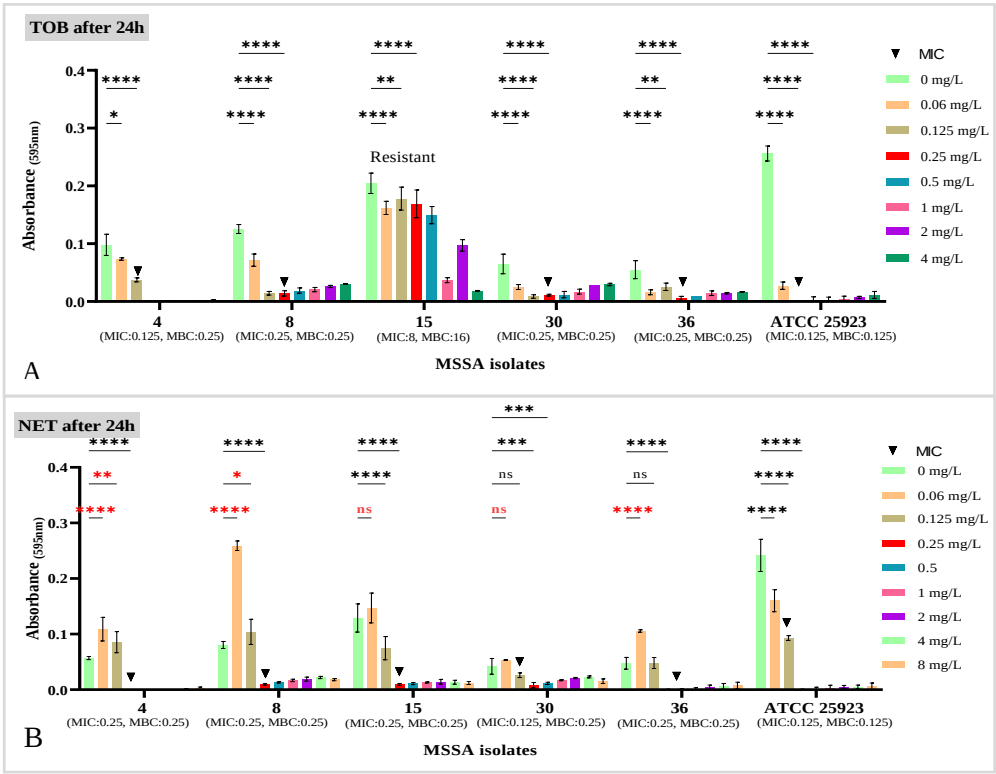


Figure II.2.17: The effect of subinhibitory concentrations of A. TOB and B. NET on cell adherence of 5 clinical ophthalmic MSSA isolates and *S. aureus* ATCC 25923. (X-axis represents two-fold serially diluted concentrations of the antibiotic tested showing the results for each MSSA strain tested independently). Results are expressed as means ± SD (n=3 replicates). Statistical significance was calculated using Two-way ANOVA with Dunnett's multiple comparison post-test. Red/black asterisks correspond to significant increase/reduction in cell adherence, respectively. (*P < 0.05; **P< 0.01; ***P< 0.001; ****P<0.0001).

To determine biofilm inhibitory activity of LEV, CLO, TOB, and NET against *S. aureus*, the 96-well microplate crystal violet staining assay was carried out for *S. aureus* ATCC 25923 and one MSSA isolate, sample number 15 for all antibiotics except TOB sample number 4 was used instead. We observed a significant decrease in biofilm formation at 0.125 mg/L (MIC) of LEV, 0.5 mg/L of CLO (1/16MIC), 1 mg/L of TOB (MIC), and 0.25 mg/L of NET (MIC).

To better characterize the effect of LEV, CLO, TOB, and NET on *S. aureus* biofilm morphology, a confocal laser scanning microscopy (CLSM) analysis with Syto9staining was performed. *The* biofilms of *S. aureus* ATCC 25923 and one

ophthalmic MSSA isolate (S15 for all antibiotics except for TOB S4) were grown as static cultures in two parallel chambered cover glasses, in the absence or presence of 0.125 mg/L (=MIC) of LEV, 0.5 mg/L of CLO (1/16 MIC), 0.25 mg/L of NET (=MIC), and 0.25 mg/L of TOB (4MIC), at 37 °C for 20 hours. Biofilm properties, including biofilm thickness, and roughness coefficient, were analyzed using COMSTAT 2 for MSSA strains tested.

In the absence of the antibiotics, medium biofilm thickness of *S. aureus* ATCC 25923, MSSA S15, and MSSA S4 was 12.3 µm, 8.451 µm, and 17.8 µm, respectively.

In the presence of 0.125 mg/L (=MIC) of LEV, biofilm of *S. aureus* ATCC 25923 was completely altered showing a significant reduction in biofilm thickness (3.15 µm), and a significant decrease in roughness coefficient due to the absence of biofilm (Fig. II.2.17A/B, Fig. II.2.18A/B). For MSSA S15, the biofilm was not significantly altered, but LEV was able to alter biofilm organization. Indeed, there was a slight increase of biofilm thickness (10.652 µm), and roughness coefficient decreased indicating that the antibiotic can affect only partially biofilm structure (Fig. II.2.17A/B, Fig. II.2.18A/B).

In the presence of 0.5 mg/L (1/16MIC) of CLO, biofilm of *S. aureus* ATCC 25923 was considerably altered showing a significant reduction in biofilm thickness (5.67 µm), and a significant increase in roughness coefficient indicating biofilm heterogeneity and less compactness (Fig. II.2.17A/C, Fig. II.2. 18A/B). The same treatment of MSSA S15 biofilm showed a decrease in biofilm thickness (6.345 µm), however, there was an no significant change in roughness coefficient indicating that the antibiotic acts only partially on biofilm, but it is not able to alter biofilm structure (Fig. II.2.17A/C, Fig. II.2.18A/B).

In the presence of 0.25 mg/L (=MIC) of NET, biofilm of *S. aureus* ATCC 25923 was slightly altered with almost intact structure showing a slight significant reduction of biofilm thickness (9.89 µm), and almost no change in roughness coefficient, as the biofilm is still structured (Fig. II.2.17A/D, Fig. II.2. 18A/B). For MSSA S15, biofilm was not significantly altered only exhibiting structure heterogeneity showing a slight reduction in biofilm thickness (7.97 µm) and a significant increase in roughness coefficient indicating that the NET was able to alter the organization of the biofilm (Fig. II.2.17A/D, Fig. II.2.18A/B).

In the presence of 1 mg/L (4MIC) of TOB, biofilm of *S. aureus* ATCC 25923 was considerably altered showing a great significant reduction in biofilm thickness (1.1 µm), as well as a significant increased roughness coefficient indicating a less compact and a more heterogenous biofilm (Fig. II.2.17A/E, Fig. II.2.18A/B). For MSSA S15, biofilm was significantly altered exhibiting a reduction in biofilm thickness (8.68 µm) and a significant increase in roughness coefficient indicating that the TOB was able to alter the organization of the biofilm (Fig. II.2.17A/E, Fig. II.2.18A/B).

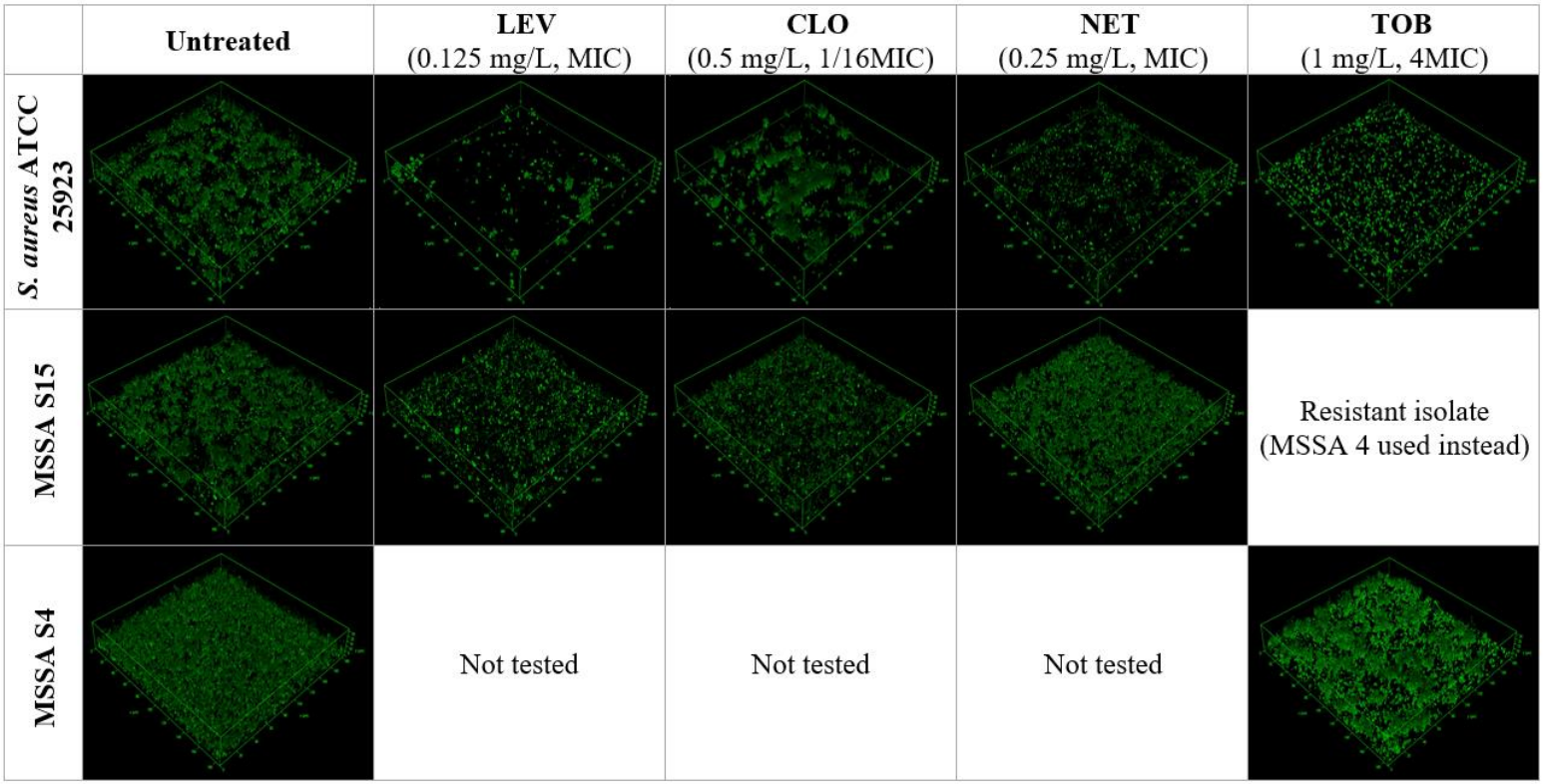


Figure II.2.18: 3D Confocal Laser Scanning Microscopic (CLSM) reconstructed image of *S. aureus* ATCC 25923 and ophthalmic MSSA biofilms grown in a chambered slide. A. no antibiotic (Control), B. 0.125 mg/L (MIC) of levofloxacin (LEV), C. 0.5 mg/L (1/16MIC) of chloramphenicol (CLO), D. 0.25 mg/L (MIC) of netilmicin (NET), or E. 1 mg/L (4MIC) of tobramycin (TOB). Eighty planes at equal distances along the Z-axis of the biofilm were imaged by CLSM. These 2D images were stacked to reconstruct the 3D biofilm image

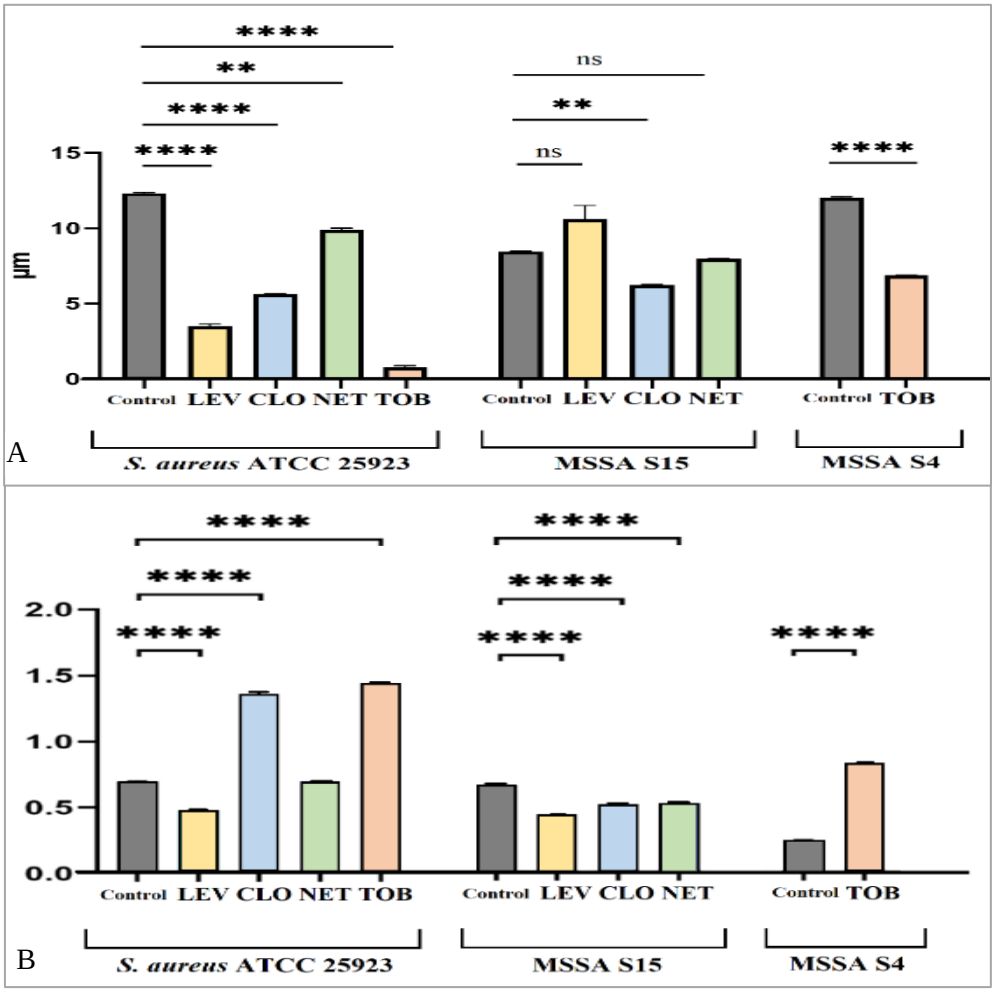


Figure II.2.19: Analysis of biofilm properties of *S. aureus* ATCC 23925 and ophthalmic MSSA isolates by COMSTAT 2. A. Measures of average biofilm thickness. B. Measures of average roughness coefficient. Biofilms was cultured in the absence of antibiotic (Control), in the presence of 0.125 mg/L (MIC) of levofloxacin (LEV), 0.5 mg/L (1/16MIC) of chloramphenicol (CLO), 0.25 mg/L (MIC) of netilmicin (NET), or 1 mg/L (4MIC) of tobramycin (TOB). Measures of average thickness Data are the mean ± SD of the results from two independent experiments. Black asterisks indicate significant reduction ** $p < 0.01$*** $p < 0.001$, **** $p < 0.0001$ (One-way ANOVA test).

II.2.6.2.5 Biofilm Eradication Efficacy of Antibiotics

To assess biofilm eradication potential, MBEC assay system using *Calgary biofilm* device was used to grow mature *S. aureus* biofilms which they were treated with 16 (4× MIC) and 32 (8× MIC) μg/mL of the compound for 24 h. MBEC of LEV, CLO, TOB, and NET was determined for the same five not clonally related MSSA isolates tested above, which were all strong biofilm producers. All the antibiotics tested were bactericidal except CLO showed a bacteriostatic effect with MBC being more than fourfold higher than MIC (Table II.2.7) [33]. Generally, the 6 MSSA isolates within the biofilm and MSSA biofilm-detached cells were much more resistant to all the antibiotics tested with MBEC and MIC extremely higher than the MIC for planktonic counterparts (Table II.2.7).

For LEV, the majority of MSSA isolates (n=4/6, 66.7%) in biofilm were at least 1024 times more resistant than their planktonic counterparts, the rest isolates (n=2/6, 33.3%) were at least 512-times more resistant than their planktonic counterparts (Table II.2.7). For MSSA biofilm-detached cells, the MIC values of LEV were lower or equal to lower or two times higher than the MIC value for planktonic cells in 2 (33.3%), and 4 (66.6%) of MSSA isolates tested, respectively.

For CLO, the majority of MSSA isolates (n=5/6, 83.3%) in biofilm were 64 times more resistant than their planktonic counterparts with one isolate (n=1/6, 16.7%) was 512-times more resistant than their planktonic counterparts (Table II.2.7). The MIC values of CLO for biofilm-detached cells were equal, 2 times or 4 times higher than the MIC values in 2 (33.3%), 3 (50%), and 1 (16.7%) of MSSA isolates tested, respectively.

For TOB, all MSSA isolates tested (n=5) had an MBEC equal or at least 128-times more resistant than their planktonic counterparts (Table II.2.7). The MIC values of TOB were more variable for biofilm-detached cells of the 5 MSSA isolates ranging from equal, 2-times, 8-times to 16-times higher than the MIC value of their planktonic counterparts in 1 (20%), 2 (40%), 1 (20%), and 1 (20%) of MSSA isolates tested, respectively.

For NET, all MSSA isolates tested (n=6) had an MBEC higher than 512-times more resistant than their planktonic counterparts except one showing an MBEC value of 4-times higher than the MIC of their planktonic counterparts (Table II.2.7). The MIC values of NET were more variable for biofilm-detached cells of the 5 MSSA isolates ranging from lower or equal to 16-times higher than the MIC value of their planktonic counterparts.

Comparing the MBEC of the different antibiotics with the MIC values of planktonic cells, chloramphenicol showed stronger impact on biofilm reduction than the rest.

Table II.2.7: MIC and MBEC values of LEV, CLO, TOB, and NET against the five MSSA isolates

c	Levofloxacin					
	Planktonic Cells		MIC for biofilm-detached cells (mg/L)	MBEC (mg/L)	MIC for biofilm detached cells vs MIC for planktonic cells	MBEC vs MIC for planktonic cells
	MIC (mg/L)	MBC (mg/L)				
ATCC 25923	0.125	0.5	≤0.25	>128 (256)	**≤2 MIC**	**>1024 MIC**
4	0.125	0.25	≤0.25	>128	**≤2 MIC**	**>1024 MIC**
8	0.125	0.5	≤0.25	>128 (256)	**≤2 MIC**	**>1024 MIC**
15	0.25	0.125	≤0.25	>128	**≤1 MIC**	**>512 MIC**
30	0.125	0.125	≤0.25	>128	**≤2 MIC**	**>1024 MIC**
36	0.25	0.25	≤0.25	>128	**≤1 MIC**	**>512 MIC**
	Chloramphenicol					
	Planktonic Cells		MIC for biofilm-detached cells (mg/L)	MBEC (mg/L)	MIC for biofilm detached cells vs MIC for planktonic cells	MBEC vs MIC for planktonic cells
	MIC (mg/L)	MBC (mg/L)				
ATCC 25923	8	>32	32	4096	**4 MIC**	**512 MIC**
4	8	>32	8	512	**1 MIC**	**64 MIC**
8	8	>32	16	512	**2 MIC**	**64 MIC**
15	8	>32	8	512	**1 MIC**	**64 MIC**
30	8	>32	16	512	**2 MIC**	**64 MIC**
36	8	>32	16	512	**2 MIC**	**64 MIC**
	Tobramycin					
	Planktonic Cells		MIC for biofilm-detached cells (mg/L)	MBEC (mg/L)	MIC for biofilm detached cells vs MIC for planktonic cells	MBEC vs MIC for planktonic cells
	MIC (mg/L)	MBC (mg/L)				
ATCC 25923	0.125	0.125	2	>32	**16 MIC**	**>256 MIC**
4	0.125	0.25	1	32	**8 MIC**	**256 MIC**
8	0.25	0.25	0.5	32	**2 MIC**	**128 MIC**
15	8	16	Resistant	Not tested	**Not tested**	**Not tested**
30	0.25	0.25	0.25	>32	**1 MIC**	**>128 MIC**
36	0.25	0.25	0.5	>32	**2 MIC**	**>128 MIC**
	Netilmicin					
	Planktonic Cells		MIC for biofilm-detached cells (mg/L)	MBEC (mg/L)	MIC for biofilm detached cells vs MIC for planktonic cells	MBEC vs MIC for planktonic cells
	MIC (mg/L)	MBC (mg/L)				
ATCC 25923	0.125	0.125	0.5	>128	**4 MIC**	**>1024 MIC**
4	0.25	0.25	1	>128	**4 MIC**	**>512 MIC**
8	0.25	0.25	1	>128	**4 MIC**	**>512 MIC**
15	0.25	0.25	4	>128	**16 MIC**	**>512 MIC**
30	0.125	0.25	≤0,25	1	**≤ 2 MIC**	**4 MIC**
36	0.25	0.25	≤0,25	>128	**≤ 1 MIC**	**>512 MIC**

II.2.7 Discussion

In our 5-year-old multicenter retrospective study, which included a total of n=463 ocular isolates in Lombardia area (Northern Italy), Gram-positive and Gram-negative bacteria contributed almost equally as etiological agents of ocular infections, with *S. aureus* (34.1%, n=158/463) being the most prevalent among all the species of the two categories, and *P. aeruginosa* (12.5%, n=58/463) the first of Gram-negatives and second most prevalent among all the species. These findings were in agreement with other surveillance studies in Italy [2][34], United states [35][4], China [36], India [37], and turkey [38]. *Staphylococcus* spp., particularly *S. aureus*, was reported as the leading cause of conjunctivitis, followed by keratitis and less frequently endophthalmitis [1].

The 44.7% of *S. aureus* strains (n = 112) isolated in the three-year period 2018-2020 were found to be methicillin resistant (MRSA). High percentages of resistance to ciprofloxacin, moxifloxacin, TOB (92% -100%) and to erythromycin (30-85%) were detected in MRSA. However, the 15% -24% of the MSSA strains of the same period showed clinical resistance to erythromycin on the bases of EUCAST breakpoints.

Afterward, we tested four different antibiotics that are commonly used for ocular infections, LEV, CLO, TOB, and NET. Out of the 45 vital *S. aureus* strains available for broth-microdilution MIC evaluation, 14.3%, 100%, and 14.3% of MRSA resulted susceptible to LEV, CLO, and TOB, respectively. For NET, 42.9% of MRSA were wildtype. On the other hand, MSSA were more susceptible to the antibiotics tested with 100%, 93.5%, and 100% were susceptible to LEV, CLO, and TOB. For NET, 42.9% of MRSA were wildtype. Out of the above *S. aureus* strains, wild-type phenotype represents 42.9% and 71% of MRSA and MSSA, respectively. On average, 73.3%, 95.6%, and 71.1% of the studied *S. aureus* strains were susceptible to LEV, CLO, and TOB. The 62.2% of the *S. aureus* resulted wildtype for NET. These results, clearly highlight the importance to perform a screening for methicillin resistance before antibiotic therapy with LEV, TOB, and NET is established. The antibacterial activity of CLO toward Gram-positive bacteria was also observed in 30-year study of Italy published in 2021 [2]. In comparison to fluoroquinolones, CLO rarely induce bacterial resistance and have a limited rate of resistance [39]. Moreover, a CLO anti-biofilm activity was hypothesized, due to the ability to penetrate biofilm and reducing its mass. Therefore, CLO seems appropriate to be used as topical eye formulation in the empiric management of ocular infections. However, the observations regarding fluoroquinolones of the later study were not consistent with our results which demonstrated the high percentages of resistance against the members of fluoroquinolones.

All the *S. aureus* strains analyzed, MRSA and MSSA, resulted strong biofilm producers by CV assay, the ideal method and the gold standard where the basic dye CV is binds to negatively charged surface molecules and polysaccharides in both the extracellular matrix and cytoplasm [40][41]. Moreover, there was a high correlation

between the results of CV assay and *icaA/icaD* genotype in this study with most of *S. aureus* isolates had *icaA* (93.3%), *icaD* (88.9%), or *icaA*/*icaD* (82.2%). These results were consistent with a previous report of 2017 [40]. The results of CR-TSB broth, as a qualitative test, were correlated with CV assay results with a sensitivity of 100% considering the CV assay the gold standard. These results were consistent with Jung-Su Lee et al who suggested CR-TSB as a useful tool for detection/screening biofilm-forming *Staphylococcus* spp. allowing the identification of 100% of the *icaA*/*D*-positive strains [23]. On the other hand, Congo red agar method, the most wildly used method for biofilm detections, was found less correlated that CRB with a sensitivity of 55%. In this study, we noticed that there are some biofilm-producing isolates, detected through CR-TSB, were negative for *icaA*, *icaD*, or *icaA/D* negative, suggesting the presence of genes other than *ica* genes might be involved in the formation of polysaccharide biofilm in these strains.

Since *S. aureus* frequently forms intractable biofilms during chronic infections that are often associated with antibiotic treatment failure[42], we tested the effect of CLO, LEV, NET and TOB on cell adherence/biofilm inhibition, and eradication.

Biofilm inhibition by LEV, CLO, TOB, and NET was tested using three different methods. (i) Cell adherence using EUCAST guidelines for determining the MIC values followed with crystal violet assay for cell adherence quantification, tested for the 5 MSSA isolates (ii) biofilm inhibitory assay, (iii) CLSM.

These antibiotics resulted effective in preventing bacterial adhesion and biofilm formation if used at concentrations equal to or higher than the MIC of the susceptible strains. However, the presence of sub-inhibitory concentrations of LEV (1/4MIC) and CLO (1/16 MIC and 1/8 MIC) enhanced cell adherence in 50% and 33.3% of MSSA isolates tested, respectively. The findings regard LEV were consistent with a previous study published in 2016 [43], where cell adherence of MRSA isolates was enhanced upon exposure to ½ MIC value of LEV and was accompanied with an increase up to more than 5-fold in the expression level of adhesion-associated genes (*fnbA*, *fnbB*, *clfA*, *clfB*, and *icaD*). The enhancement of cell adherence was also in the presence of ¼ MIC and ½ MIC in 50% and 33.3% of MSSA isolates tested, respectively. In a study of 2003 [44], sub-MIC of NET was able to slightly increase the hydrophobic interactions which plays a role in bacterial adhesion to biotic and abiotic surfaces.

S. aureus biofilms were visualized by confocal laser scanning microscopy, showing significant differences in the biofilm thickness when the cells were treated cells compared to the untreated control for all the antibiotic tested. The biofilms were thinner, less structured, and less uniform in the presence of antibiotics: actually, antibiotics treatments led to the formation of more heterogeneous biofilms that were unable to cover the entire abiotic surface. Since the presence of biofilm-growing bacteria is often associated with antibiotic treatment failure, our results highlight the ability of these antibiotics particularly CLO to disrupt *S. aureus* biofilm

establishment and, consequently, increase the effectiveness of the recognized therapies.

In this study, we also measured MBEC values of newly formed biofilms, 24h biofilms, using Calgary biofilm device, an innovative approach that allows biofilms to grow on suspended pegs before exposing them to antibiotics. MSSA cells of newly formed biofilms (24h) of susceptible strains were more resistant than their planktonic counterparts to LEV (MBEC>512-1024MIC), CLO (MBEC=64MIC for ophthalmic isolates and for Reference strain Mic=512MIC), TOB (MBEC>128-256) and NET (MBEC>512-1024 MIC). In consistence with many other studies that showed that biofilm cells display phenotypic drug tolerance tolerating up 100-1000 times higher concentrations of antibiotics than planktonic counterparts [45]. This is mainly due to several factors among which some would explain the increased MBEC values for the newly formed biofilms (24h). Firstly, biofilm extracellular matrix and its components can act as a physical barrier chelating agents preventing antimicrobials from reaching bacterial cells. Secondly, the slow growth and low metabolic rate of biofilm cells make them tolerant because most antibiotics are effective against rapidly dividing. Moreover, some antibiotics, such as fluoroquinolones, β-lactams, and aminoglycosides, are not active in anerobic conditions making them ineffective in killing the biofilm cells in deeper layers. Thirdly, biofilm cells can upregulate the expression of efflux pump. So far, there is no standardized procedure for MBEC determination and the results are incomparable due to the technical difficulties and the variability of the parameters in the procedure available, Calgary assay [46][47]. The MBEC values can considerably be affected with many experimental parameters including biofilm growth age, antibiotic concentration and treatment duration, and growth media [47]. In a Brazilian study of 2021 [48], *S. aureus* strains (n=15), isolated from chronic rhinosinusitis, LEV was effective for both planktonic cells (MIC= 1mg/L) as well as for their biofilm counterparts (MBEC= 1 mg/L) using modified Calgary assay. In a Brazilian study of 2017 [49], *S. aureus* isolates (n=10), isolated from catheters, MBEC value (MBEC=750mg/L) of CLO were 120 higher than MIC of planktonic counterparts (MIC=6.25mg/L) using broth microdilution assay.

Biofilm-detached cells were more resistant than their planktonic counterparts. In a study of 2019 [50], Khelissa et.al. noticed that resistance behavior of *S. aureus* biofilm-detached cells is associated with lower membrane fluidity through increasing fatty acid composition, particularly long-chain fatty acids, which can pack together to make a rigid membrane bilayer compared to planktonic cells.

It is worth mentioning that biofilm formation *in vitro* may not certainly reflect the presence of biofilm *in vivo* due to the complexity of *in vivo* conditions [47]. Moreover, the clinical Relevance of One-day MBEC assays may overestimate the required concentrations to kill organisms in biofilm because MBEC values can decrease with increased time of exposure for longer than 24 hours [47][33]. Nonetheless, One-day MBEC assays may also underestimate the required concentration because 72 h biofilms can be more difficult to be eradicated than 24 h

biofilms regardless of the antibiotic being time- or concentration- dependent, as shown by Chen et. al, [47]. This might be solved by conducting studies that reflect the association between biofilm resistance and clinical outcomes in biofilm-related infections. However, the possibility of preventing biofilm formation using one of the four antibiotics tested empirically, is a direct consequence of the proportion of susceptible strains, as assessed by local epidemiology.

Experimentally, CLO, LEV (with one exception), NET and TOB are effective considering the currently available ophthalmic formulations, in the eradication of newly formed biofilms (24h) of sensitive strains (Table II.2.8). However, Future studies are needed to evaluate whether the MBEC values achieved *in vitro*, i.e., Calgary assay, correlates with the MBEC values, achieved clinically to obtain good therapeutic outcomes considering antimicrobial exposure time.

Table II.2.8: The efficacy of commercially available ophthalmic formulas of LEV, CLO, TOB, and NET

Antibiotic	Antibiotic Formula	Concentration of antibiotic (mg/ml)	Concentration of antibiotic (µg/drop)	Number of applications/ dose[a]	Amount required to eradicate biofilm (mg)	Number of applications to eradicate biofilm	Efficacy
Levofloxacin	0.5% in eye wash	5	250	1-2 drops 8-11times daily	128/ 256/ 512/2048	1/ 2/ 28 drops	effective with one exception
Chloramphenicol	0.5% in eye wash	5	250	2 drops 8 times daily	512	3 drops	effective
	1% in ointment	3	150	Small amount 8 times daily	128	-	effective
Netilmicin	0.3% in eye wash	3	150	1-2 drops/3 times daily	128	1 drop	effective
Tobramycin	0.3% in eye wash	3	150	drops	32	1 drop	effective

[a]*based on updated Summary of Product Characteristics (SmPC) in the electronic medicines compendium (emc) contains up to date, easily accessible information about medicines licensed for use in the UK.* https://www.medicines.org.uk/emc/.

There are some limitations in this study limitations. Firstly, the absence of clinical breakpoints for topical agents, and phenotype interpretation using systemic breakpoints/ECOFFs (for agents administered topically) as EUCAST suggested which may underestimate the activity of antibiotics in topical formulas because of the high concentrations that can be achieved at the site of application [51][21][52]. Secondly, the absence of breakpoints for biofilm-cells makes it difficult to predict the efficacy of antibiotics, i.e., therapeutic success, in biofilm-associated infection, in contrast to planktonic bacteria for which breakpoints are well-established enabling the prediction of therapeutic success in planktonic-associated infections. Moreover, MBEC values after one-day of exposure to antimicrobials might overestimate the concentration of antibiotics needed to eradicate biofilm when it can be lower with longer-time of antimicrobial exposure which can be achieved clinically from local delivery of antibiotics at specific concentrations lower than or equal to the MBEC values. The MBEC values was achieved by growing monomicrobial biofilms may not reflect the *in vivo* status of biofilms which is more are likely to be polymicrobial according to metagenomic approaches decreasing antimicrobial susceptibility [53][54]. Melphine et.al. showed that *S. aureus* cells embedded within *Candida*

biofilm had an increased tolerance to vancomycin through mediating cross-species tolerance by sequestering vancomycin in Candida matrix [55]. Moreover, biofilms were formed under favorable conditions, which is not the case *in vivo*, avoiding stressors such as unfavorable pH, O_2 tension, osmolality, nutrient availability, or host defenses (antibody and cellular) which has a negative effect microorganism's survival within the biofilm [33].

II.2.8 Conclusions

This study provided a 5- year local surveillance of etiology and susceptibility patterns of ophthalmic isolates, assisting in providing the appropriate empiric management of ocular infections; this with the limitation that spectrum of ocular pathogens and susceptibility patterns can change over time and differ according to geographical area. *S. aureus* resulted the most prevalent etiological agent across the 5 years of the study. CLO/LEV/NET/TOB resulted effective in preventing bacterial adhesion, and the first step of biofilm formation, when used at concentrations equal to or higher than the MICs of the susceptible strains. Based on the dosages commonly reachable in topical ophthalmic formulations for CLO/LEV (with only one exception)/NET/TOB, the results showed a clear efficacy in the eradication of newly formed biofilms (24h) of sensitive *S. aureus* strains. However, the efficacy of each drug against biofilm forming *S. aureus* strains is subjected to the local epidemiology in terms of susceptible strains presence. Therefore, continued surveillance of antibiotic resistance is recommended to guide therapy choices, particularly when instituting empiric therapy for ocular infections.

II.2.9 References

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Chapter 3: Antimicrobial activity of Benzalkonium Chloride and Zinc Sulphate: evaluation of the synergistic effect *in vitro* on ophthalmic isolates of *Staphylococcus aureus*

II.3.1 Abstract

Introduction: No studies have been conducted on the susceptibility of ophthalmic clinical isolates to benzalkonium chloride (BAC) and zinc sulfate, which are commonly used in ophthalmic solutions as antimicrobial preservatives. The objective of this study was to evaluate the *in vitro* antimicrobial inhibitory and bactericidal effect of BAC and zinc sulfate both singly and in combination, against clinical isolates of *S. aureus,* including MSSA and MRSA responsible for ocular infections, as well as *Pseudomonas aeruginosa* and *Acinetobacter baumannii*.

Materials and Methods: The inhibitory effect of zinc sulfate was determined against n=11 clinical isolates, including n=5 isolates of MSSA, n=5 MRSA, one *P. aeruginosa,* and three reference strains, including *S. aureus* ATCC 25923, *P. aeruginosa ATCC 27853*, and *A. baumannii ATCC 19606*. The inhibitory and bactericidal effects were evaluated *in vitro* using broth microdilution and agar dilution for BAC and zinc sulfate, respectively. The combined effect of the two molecules was evaluated using checkerboard broth microdilution assay against n=6 MSSA isolates, including n=5 ophthalmic clinical MSSA isolates and the reference strain *S. aureus* ATCC 25923.

Results: MIC and MBC of BAC and zinc sulfate were higher for Gram-negative isolates tested: the ophthalmic clinical *P. aeruginosa* isolate, and the two reference strains *P. aeruginosa ATCC 27853*, and *A. baumannii ATCC 19606.* The findings indicate that BAC has an inhibitory effect on the clinical isolates of *S. aureus* at concentrations of BAC ranging from 0.5-1.0 mg/L for MSSA and 1.0-2.0 mg/L for MRSA, by broth microdilution method. The same results were obtained for zinc sulfate at concentrations ranging from 0.6-0.8 mg/ml for MSSA and 1-2mg/ml for MRSA. The combined effect of the two molecules was of "indifference" both for the n=5 MSSA isolates and for the *S. aureus* ATCC25923 isolate.

Discussion and Conclusions: Based on our findings, BAC have high antimicrobial efficacy against *S. aureus* isolates, both MSSA and MRSA, while higher concentrations of this compound is required to be effective against Gram-negative isolates. For zinc sulfate, higher concentrations are required to inhibit the growth of *S. aureus* (at least 1.6 mg/ml), *A. baumannii,* (MIC >2mg/ml), and *P. aeruginosa* (MIC >2mg/ml). An additive action between the two compounds was finally observed, but not enough to show synergistic effect when used in combination.

II.3.2 Introduction

II.3.2.1 Overview of BAC and Zinc Sulfate

The quaternary ammonium compound benzalkonium chloride (BAC) and zinc sulfate (Zn) are commonly found in ophthalmic solutions.

BAC is commonly used as a preservative due to its antimicrobial activity in wide range of applications ranging from domestic to agricultural, cosmetics, and clinical fields (Fig. II.3.1) [1]. The wide use of these compounds in different products, usually at low concentrations, is expected to impose selective pressure inducing the selection of bacteria with decreased susceptibility to such compounds, which can represent a clinical problem if the bacterial population is also resistant to antimicrobial agents by means of cross-resistance or co-resistance [1] [2].

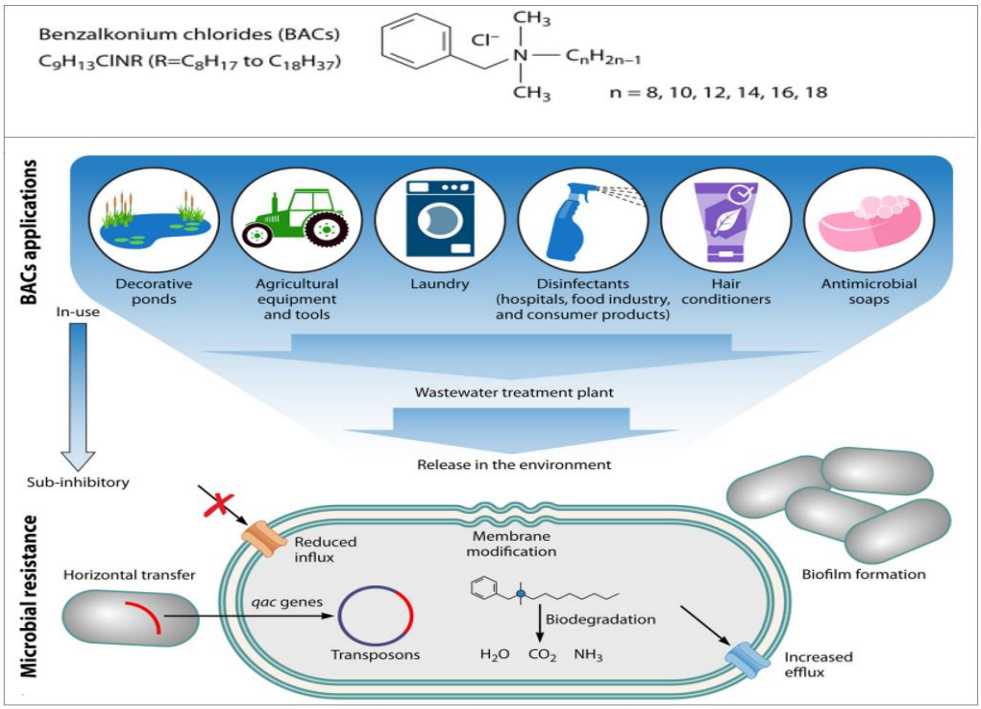


Figure II.3.1: A. Structure and formula of Benzalkonium Chloride (BAC). B. BACs' applications and the associated resistance mechanisms for BACs [1].

The use of BAC as antiseptic is threatened by antimicrobial resistance or tolerance; and its toxicity can be furthermore associated with adverse effect [1]. Resistance to BAC has been observed in different species, such as *S. aureus*, *P. aeruginosa*, *Escherichia coli* and *Listeria monocytogenes.*

Another compound commonly used in ophthalmic formulations is zinc sulfate. Generally, the trace element Zinc (Zn) is essential for the life of living organisms and involved in a wide range of physiological functions, controlling metabolism and growth [3]. In bacterial cells, the optimal level of Zn^{2+} ions ranges from 10^{-7} to 10^{-5}M with concentrations above 10^{-4}M showing cytotoxicity, through interfering with bacterial homoeostasis of Zn^{2+} ions and enhancing cell membrane permeability [3][4]. Moreover, and also in agreement with the antimicrobial activity displayed against fungal microorganisms, Zn-containing compounds serve as antimicrobial preservatives in cosmetic and pharmaceutical products [4]. Moreover, Zn-containing compounds serve as antimicrobial preservatives in cosmetic and pharmaceutical products. The most common derivative of zinc is zinc sulfate (ZnSO4.7H2O).

II.3.2.2 Antimicrobial activity of BAC and zinc sulphate for ophthalmic infections

Few studies have been conducted to evaluate the antimicrobial activity against bacterial isolates of clinical relevance, with none done for isolates obtained from ocular site.

Topical ophthalmic treatments represent 90% of the marked ophthalmic formulations in ocular drug delivery, including *eye drops*, ointments and hydrogels, emulsions, and contact lenses, with the first one being the most commonly used formulation [5].

Due to the need of maintaining the sterility of multidose ophthalmic formulations, antimicrobial preservative is incorporated into ophthalmic formulations according to pharmacopeia international standards to prevent ocular infections from a contaminated multidose ophthalmic solution [6]. The most used preservative is BAC, included in about 70% ophthalmic solutions, at different concentrations ranging from 0.004% to 0.02%[7][8].

The cationic amphipathic nature of BAC makes it a cationic surfactant able to adhere to porous surfaces particularly to negatively charged surfaces, showing antimicrobial activity against many common pathogens through denaturing proteins and disrupting cytoplasmatic membranes [8][9]. The antimicrobial activity of BAC is shown against both Gram-positive and Gram-negative bacteria due to having a negatively charged outermost surfaces associated with teichoic acid and polysaccharide elements of Gram-positive bacteria, the lipopolysaccharide of Gram-negative bacteria and the cytoplasmic membrane itself [9]. Moreover, the cationic amphipathic allows BAC to stabilize poor water-soluble drugs, and act as penetration enhancer, excipient; of active compounds through ocular membranes [5][8]. As noticed for all medications, exposure to high doses or prolonged treatments with formulations containing BAC are associated with adverse events on ocular surfaces and deeper ocular structures particularly in the case of preexisting ocular surface problems [8][10]. It was shown to cause the superficial cell loss observed in the cornea of rabbits with an ophthalmological solution containing 0.02% BAC [11]. Moreover, at the cellular level, it can cause cytotoxic damage to conjunctival and corneal epithelial cells, and

at the DNA level it was able to cause small but significant genotoxic effects *in vitro* in both plant and mammalian cells at concentrations of 1 mg/liter [7][12]. Therefore, it is necessary to monitor the antimicrobial activity of BAC to balance the antimicrobial efficiency against ocular pathogens and the toxicity of BAC-containing formulations.

Generally, the wide use of quaternary ammonium compounds, such as BAC, is not restricted to ophthalmic formulations, but also involves many other formulations and daily/regularly used products, such as disinfectants, preservatives, pest control agents, and others [2]. This wide use of these compounds in different products, usually at low concentrations, is expected to impose selective pressure inducing the selection of bacteria with decreased susceptibility to these compounds, which can be a problem of clinical relevance if the bacteria is also resistant to antimicrobial agents by means of cross-resistance or co-resistance. There are some reports indicating the presence of MRSA isolates with reduced susceptibility to biocides, such BAC [1] [13].

Few studies have been conducted to evaluate the antimicrobial activity of BAC against bacterial isolates of clinical relevance, with none done for isolates obtained from ocular sites.

In a 2018 study [13], BAC was tested for antimicrobial activity against 182 *S. aureus* isolates, including MSSA and MRSA, exhibiting MIC_{90} of $\leq$2 mg/L and 4mg/L for isolates from human and animal origin, respectively. In another 2012 study [9], BAC activity was evaluated against six important foodborne pathogens including three Gram-positive (*S. aureus*, *L. monocytogenes* and *Bacillus cereus*) and three Gram-negative bacteria (*Salmonella typhimurium*, *E. coli* and *P. aeruginosa*) exhibiting higher antibacterial efficacy on Gram-positive than on Gram-negative bacteria (except *B. cereus* because of its ability to spore formation). MIC and MBC values of BAC for *S. aureus* were 40mg/L and 45 mg/L, respectively, while *P. aeruginosa* showed MIC and MBC of 60 mg/L and 80 mg/L, respectively.

Another compound commonly used in ophthalmic formulations is zinc sulfate. This compound is used in concentrations ranging from 0.2 to 0.25% [14], as an antiseptic and astringent, useful for drying fluids, shrinking exposed tissues, relieving, and treating bacterial infection [15]. It is also used for treating superficial ocular infections, such as conjunctivitis [16].

Generally, zinc is an essential trace element necessary for the normal function of all living systems at structural and regulatory levels, furthermore it has a role in maintaining normal ocular structures and function partially [17][18]. The optimal concentration of zinc in bacteria typically between 10^{-7}M and 10^{-5}M depending on the bacteria [19]. Long time ago, it was established that excessive zinc concentrations has significant toxicity to bacteria inhibiting it is growth at concentration above $\sim 10^{-4}$ M [19]. Unfortunately, the literature about antimicrobial inhibitory effect on clinical bacterial isolates of zinc, particularly zinc sulfate, is scarce with none

considering the effect on ophthalmic isolates. Some experimental studies evaluated the efficacy of zinc sulfate on enteric pathogens, including S*almonella, enteropathogenic E. coli, Shigellae* and *Vibrio cholerae* [20], nosocomial pathogens, including *P. aeruginosa* and *A. baumannii* [21], clinical multidrug resistant (MDR) isolates, including *S. aureus*, *Staphylococcus epidermidis*, *Proteus* spp., *E. coli*, *P. aeruginosa*, *Klebsiella* spp., *Enterobacter* spp. [22], and the oral pathogen *Streptococcus mutans*.

Few studies showed that BAC has a synergistic effect when combined with other molecules. Synergistic interactions had been demonstrated in combination with other biosides like chlorocresol, against reference bacterial species of *A. baumannii, P. aeruginosa, S. aureus*, and *Enterococcus faecalis* [23]. Moreover, synergistic interaction had been observed when combined with other antibiotics, such as gatifloxacin and moxifloxacin against MRSA, and with MIC values reduction of 2-500 fold [24]. However, in the same study, no synergic activity was observed for BAC combined with ciprofloxacin and levofloxacin.

In 1995 study [25], synergistic bactericidal activity was observed when Zinc sulphate was combined with BAC against Gram-negative bacteria, including *E. coli* and *P. aeruginosa*, and the Gram-positive *S. aureus*. Another study published in 2008, highlighted the synergic bactericidal and antibiofilm activity of metal ions as copper, and quaternary ammonium compounds, specifically benzalkonium chloride, against *P. aeruginosa* [26]. In the latter study, synergy between zinc sulfate and BAC against the tested bacterial species, including *S. aureus*, was not observed.

Based on our knowledge, this combination is found solely in one ophthalmic formulation, ZINCOMETIL (Théa Group, France), with 0.02% zinc sulfate and 0.01% BAC. According to ZINCOMETIL Summary of Product Characteristics (SmPC), it is used as a disinfectant solution for ophthalmic use in adults with a dose of 1-2 drops introduced into the conjunctival sac of the affected eye(s) 2-4 times a day. This product contains two active substances, zinc sulfate and BAC. Zinc sulfate, at the concentration (0.02%, 200 mg/L), has antiseptic partly due to the ability of the zinc ions to precipitate proteins. It also has astringent properties at the ocular level clearing mucus from the outer surface of the eye. On the other hand, BAC, at the concentration of use (0.01%, 100 mg/L), is widely used in ophthalmic preparations for the antiseptic action widely tested and reported in the literature.

The wide use of BAC and zinc in many products in different formulations at different concentrations could result in the selection of bacteria with decreased susceptibility to these compounds, which can be a problem of clinical relevance if the bacteria are also resistant to antimicrobial agents by means of cross-resistance or co-resistance. There are some reports indicating the presence of MRSA isolates with reduced susceptibility to biocides, such BAC and metals, as Zinc [13]. Thus, balancing is required taking into consideration the toxicity of these compounds when used

separately or combined [27]. From this perspective, it is necessary to establish standardized methodology, thus far not available, to monitor biocide resistance.

To date, there are no studies conducted to evaluate the bacterial inhibitory effect of BAC and zinc sulfate on ocular pathogens when used individually or combined, despite the need to correctly establish the antimicrobial activity and toxicity of these compounds.

II.3.3 Objectives

1. To determine the antimicrobial inhibitory effect of zinc sulfate and BAC against the ophthalmic clinical isolates of *S. aureus* and *P. aeruginosa*, leading cause of ocular infections among Gram-positive and Gram-negative bacteria, respectively. We also included reference strains belong to *S. aureus*, *P. aeruginosa* and *A. baumannii*m, with the last being included due to its ability to cause an aggressive ocular infection.

2. To evaluate the synergistic interaction of BAC and zinc sulfate in combination against MSSA ophthalmic isolates.

II.3.4 Materials and Methods

II.3.4.1 Strains and Culture Conditions

Strains evaluated in this study included three reference stains from the American type of culture collection (ATCC), including *S. aureus* ATCC 25923, *P. aeruginos*a ATCC 27854, and *A. baumanii* ATCC 19606, and n=11 ophthalmic clinical isolates, including n=5 MSSA, n=5 MRSA, and n=1 *P. aeruginosa.*

The ophthalmic isolates have been obtained from IRCCS Policlinico San Matteo Hospital (n=4 MSSA and n=5 MRSA), and Manzoni Hospital of Lecco, (n=1 MSSA and n=1 *P. aeruginosa*), in the period 2017-2020. *S. aureus* strains were routinely cultured on mannitol salt agar (MSA), while *P. aeruginosa* and *A. baumannii* strains were cultured on MacConkey agar. Frozen stock cultures of all strains were stored in Luria-Bertani broth plus 40% glycerol at -80°C. Prior experiments, cells were sub-cultured from stock on a suitable medium at 37 °C for 18-24 hours.

II.3.4.2 Species Identification, Antimicrobial Susceptibility Testing, and mecA Detection

Ophthalmic isolates were identified at species level and subjected to automated antimicrobial susceptibility testing using MicroScan dried Gram-positive BP Combo Panel Type 33 (Beckman Coulter, CA, USA) for Gram positive, and Microscan Gram-negative MIC/Combo panels for Gram-negative isolate. The panels were analyzed through the semi-automated system MicroScan autoSCAN-4 (Beckman Coulter, CA, USA) following the manufacturer instructions. Clinical categorization of the isolates as susceptible (S), susceptible high-exposure (I) or resistant (R) was performed according to European Committee on Antimicrobial Susceptibility Testing (EUCAST) breakpoints (http://www.eucast.org). Based on EUCAST guidelines, cefoxitin is used as a marker for the detection of methicillin resistance phenotype predicting resistance to all cephalosporins, cephems, and other β-lactams (such as ampicillin-sulbactam, amoxicillin-clavulanic acid, ticarcillin-clavulanic acid, piperacillin-tazobactam, and the carbapenems) regardless of the in vitro test results obtained with indicating agents except for the anti-MRSA cephalosporins. Therefore, *S. aureus* isolates which showed MIC values >8 mg/L for cefoxitin were considered methicillin-resistant phenotypically. All MRSA isolates were screened for the presence of *mecA* gene by PCR using the primers *mecA* forward (5′-GTGAAGATATACCAAGTGATT-3′) and *mecA* reverse (5′-ATGCGCTATAGATTGAAAGGAT-3′) amplifying 147 bp fragment of the *mecA* gene using 30 PCR cycles of denaturation at 94 °C for 30 seconds, annealing at 55°C for 90 seconds, followed by extension at 72 °C for 90 seconds [28].

II.3.4.3 Measurement of Antibacterial Activity of BAC and Zinc Sulfate

The antimicrobial activity of BAC was evaluated by broth dilution method according to EUCAST guidelines in triplicates for both the n=11 clinical ophthalmic isolates and the n=3 reference strains. Before proceeding with broth microdilution, MSSA and MRSA strains were tested for clonal relatedness using random amplified polymorphic DNA (RAPD) -PCR using RAPD-PCR kit (Amersham biosciences UK limited, England).

To determine the MIC and the MBC values of BAC, 250 µl of twofold serial dilutions of BAC (alkyldimethylbenzyl ammonium chloride, PHR1371-5ml, Sigmaldrich, USA) was prepared in Muller Hinton broth (MHB) and dispensed in 96-well U-bottom polystyrene microtiter plates (CELLSTAR-greiner bio-one, USA) with concentrations ranging from 0.06 mg/L to 32 mg/L for testing *S. aureus* isolates, and 8 to 512 mg/L for testing Gram-negative isolates. A volume of 5µl of overnight bacterial broth cultures, adjusted to final inoculum of 5×10^5 CFU/mL, was dispensed in each well of the 96-well culture plate, and incubated for 24 hours at 35°C. Two wells in each row of the 96-well plate served as growth control well with no antibiotics added, and another as sterility control, without bacteria in the same media nor antibiotics. The American Type Culture Collection (ATCC) ATCC 25923 *S. aureus* was used as a quality control in each batch for testing MSSA and MRSA. MIC was defined as the lowest concentration able to inhibit visible growth. To check inoculum density for each strain tested, 2.5 µL from the growth-control well was spread immediately after inoculation with 50 µl of sterile distilled water on standard Plate Count Agar plates. Plate count was made by counting the Colony Forming Units (CFUs) after incubation for 24 hours at 35 °C. The MBC values were determined for the *S. aureus* isolates by plating 10 µL from the well that correspond to the MIC value and two higher concentrations than the MIC value on muller Hinton agar (MHA). The MBC values was determined after incubation for 24 hours at 37 ◦C, with MBC being the lowest concentration of the substance at which no colonies formed.

The antimicrobial activity of zinc sulfate was evaluated by agar dilution method according to EUCAST guidelines in triplicates for the same strains tested above. A stock solution of zinc sulfate with a concentration of 20 mg/ml was prepared by dissolving 0.2 g of ZnSO4 (ZnSO4.H2O, 221376-100G, Sigmaldrich, USA) in 10 ml of distilled water and sterilized by filtration, using a Millipore filter (Millipore Co., Bedford, Mass., USA). The agar media with various concentrations of zinc sulfate was prepared by adding volumes of zinc sulfate stock solution (20 mg/ml) ranging from 0.2 to 2.0 ml into tubes containing 20 ml melted (50°C) Mueller-Hinton agar to obtain active zinc in ZnSO4. H2O. The concentrations of zinc sulfate in agar include 200, 400, 600, 800, 1000, 1200, 1400, 1800, and 2000 mg/L. The zinc sulfate supplemented MH was poured into a sterile Petri plate and set at room temperature to solidify. 1 µl and 2 µl of the 0.5 McFarland adjusted overnight bacterial cultures in MH broth was used to inoculate ZSA. The broth cultures were also inoculated onto plain (no Zinc Sulfate) MHA as a control. Plates was incubated for 18–20 h at 37°C.

The MIC values was recorded as the lowest concentration of Zinc Sulfate that completely inhibited bacterial growth, disregarding a single colony or faint haze caused by the inoculum.

II.3.4.4 In Vitro Evaluation Synergistic/Antagonistic Activity of BAC and Zinc Sulfate

The antibacterial activity of BAC and zinc sulfate in combination were evaluated using checkerboard broth microdilution assay against ophthalmic clinical MSSA isolates. Two-fold serial concentrations combinations of BAC (0.0075 to 8 mg/L) and zinc (0.175 to 11.2 mg/ml) were arrayed in a 96-well U-bottom microtiter plate and resuspended in MH. Every well of the checkerboard plate was inoculated with 90 µl of 5 × 105 CFU/mL bacterial solution and incubated for 24 hours at 35°C with at a final volume of 200 µl. At the end of the incubation period, the MICs were recorded for BAC, Zinc sulfate, and BAC/Zinc sulfate in combination after visual examination of the wells. The BAC and zinc sulfate MICs were determined as the lowest concentrations that showed no visible growth, turbidity. For each isolate the test was performed in triplicates. At high concentrations of BAC and zinc sulfate, precipitation occurred leading to the turbidity of the wells before incubating the plates for 24h. to avoid the confusion with result interpretation after incubation, 20 µl of the suspected wells were streaked on MHA to confirm the result of no growth. The turbidity can be due to metal precipitation which can be a result of metal complexation by an ingredient of the growth media used to grow the microorganism, or the change in pH, likely due to the acidic, neutral or slightly basic conditions [29]. The observed MIC values were used to calculate the fractional inhibitory concentration (FIC) of each compound. The FIC value was calculated for each compound by dividing the MIC value of the compound in combination with the MIC value of the compound alone. Then, the FIC values of BAC and zinc sulfate were added to find the FIC index (FICI). FICI values were compared to threshold values whereby synergism between two compounds is regarded at an FICI of equal or lower than 0.5 , antagonism at an FICI of equal or higher than 4.0, and no interaction at an FICI between 0.5 and 4.0 (Fig. II.3.2) [30].

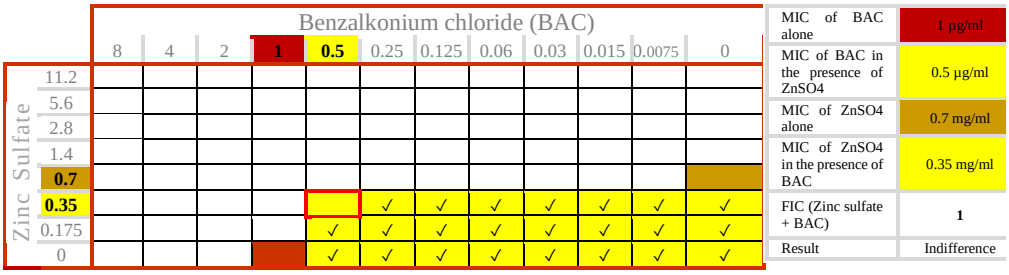


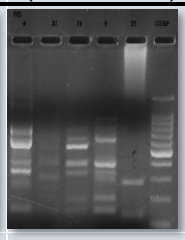
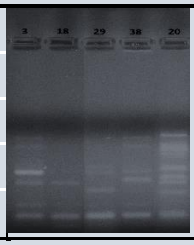
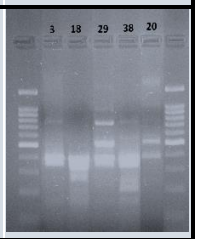
Figure II.3.2: Representative image of BAC and zinc sulfate checkerboard 96-well plate and results' interpretation. ✓ represents growth on the well. The yellow zone represents the bacterial growth, and the white zone represents the inhibition of bacterial growth.

II.3.5 Results

II.3.5.1 Susceptibility of clinical isolates to BAC and zinc sulfate

MIC and MBC values of BAC and zinc sulphate were obtained using microbroth dilution and agar dilution, respectively, against n=11 *S. aureus* isolates, including n=5 MSSA, n=5 MRSA, and *S. aureus* ATCC 25923, and n=3 Gram negative isolates, including one ophthalmic *P. aeruginosa* isolate, *P. aeruginosa ATCC 27853*, and *A. baumannii ATCC 19606* (Table II.3.1).

Table II.3.1: Characteristics and clonal typing of the 14 bacterial isolates

Strains[a]	Date of isolation (d/m/year)	Hospital[b]	Ward	Specimen type	Clones by RAPD Typing	RAPD primers used for typing	
						Primer 4 (AAGAGCCCGT)	Primer 5 (AACGCGCAAC)
MSSA 4	17/08/2020	San Matteo (PV)	External	corneal	E		
MSSA 8	04/04/2020	San Matteo (PV)	Hematology	Conjunctival	D		
MSSA 15	17/10/2019	San Matteo (PV)	External	Conjunctival	C		
MSSA 30	21/01/2018	Manzoni, (LC)	Pediatrics	Conjunctival	A		
MSSA 36	30/08/2018	San Matteo (PV)	Pediatrics	Conjunctival	B		
***S. aureus* ATCC 25923**	-	-	-	-	-	-	-
MRSA 3	10/05/2020	San Matteo (PV)	Pediatrics	Conjunctival	E		
MRSA 18	13/09/2019	San Matteo (PV)	Ophthalmic	Corneal	D		
MRSA 20	08/08/2019	San Matteo (PV)	Pediatrics	Conjunctival	C		
MRSA 29	17/08/2018	San Matteo (PV)	Ophthalmic	Corneal	B		
MRSA 38	24/05/2017	San Matteo (PV)	Pediatrics	Conjunctival	A		
PSA 380/20	04/09/2020	Manzoni, (LC)	Pediatrics	Conjunctival	-	-	-
PSA ATCC 27854	-	-	-	-	-	-	-
AB ATCC 19606	-	-	-	-	-	-	-

[a]*PSA, pseudomonas aeruginosa; AB, Acinetobacter baumannii.*
[b]*PV, Pavia; LC, Lecco*

For BAC, no significant differences have been observed between the MIC values obtained for ophthalmic MSSA, showing MICs of 0.5 mg/L to 1 mg/L-, and the one obtained for MRSA isolates, exhibiting MICs ranging from 1mg/L to 2mg/L. The above results confirm the absence of acquired resistance mechanisms for BAC, as ATCC 25923 *S. aureus* and wild-type isolates have a MIC ≤2 [2] (Fig. II.3.2A, table II.3.2, table II.3.3). On the other hand, the ophthalmic *P. aeruginosa* isolate showed MIC=128 mg/L, a very high concentration in comparison to the MIC values obtained both for ophthalmic *S. aureus* isolates and the MICs for *P. aeruginos*a ATCC 27854 (with BAC MIC=32mg/L) and *A. baumannii* ATCC 19606 (BAC MIC=16mg/L) (Table II.3.2, table II.3.3). The BAC MBC values against ophthalmic clinical *S.*

aureus isolates were found to be 2-8, and 2-4-fold higher than the MIC values of MSSA and MRSA, respectively. The BAC MBC value of the ATCC 25923 *S. aureus* was two-fold higher than MIC value for the same strain.

Table II.3.2: Susceptibility of clinical isolates to BAC and zinc sulfate (Reference strains shaded in green)

Strains[a]	Date of isolation (d/m/year)	MIC of BAC (mg/L)	MBC of BAC[b] (mg/L)	MIC of Zinc sulphate (mg/ml)
MSSA 4	17/08/2020	1	4	0.800
MSSA 8	04/04/2020	0.5	4	0.800
MSSA 15	17/10/2019	0.5	4	0.600
MSSA 30	21/01/2018	1	4	0.800
MSSA 36	30/08/2018	1	4	0.600
***S. aureus* ATCC 25923**	-	**2**	**4**	**1.400**
MRSA 3	10/05/2020	**2**	**4**	**1.600**
MRSA 18	13/09/2019	1	4	1.000
MRSA 20	08/08/2019	2	8	1.200
MRSA 29	17/08/2018	1	4	1.400
MRSA 38	24/05/2017	1	4	1.000
PSA 380/20	04/09/2020	128	Not tested	> 2.000
PSA ATCC 27854	-	32	Not tested	> 2.000
AB ATCC 19606	-	16	Not tested	> 2.000

[a]*PSA, pseudomonas aeruginosa; AB, Acinetobacter baumanii.*
[b]*not tested due to high MIC value.*

Table II.3.3: Benzalkonium chloride (BAC) susceptibility in ophthalmic clinical isolates and the reference strains of *S. aureus*, *P. aeruginosa*, and *A. baumannii*

Concentrations of BAC (mg/L)	*S. aureus* (n=11) (%)	MSSA (n=6) (%)	MRSA (n=5) (%)	*P. aeruginosa* (n=2) (%)	*A. baumannii* (n=1) (%)
0.06	0 (0%)	0 (0%)	0 (0%)	Not tested	Not tested
0.125	0 (0%)	0 (0%)	0 (0%)	Not tested	Not tested
0.25	0 (0%)	0 (0%)	0 (0%)	Not tested	Not tested
0.5	2 (18.2%)	2 (33.3%)	0 (0%)	Not tested	Not tested
1	6 (54.5%)	3 (50%)	3 (60%)	Not tested	Not tested
2	3 (27.3%)	1 (16.7%)	2 (40%)	Not tested	Not tested
4	0 (0%)	0 (0%)	0 (0%)	Not tested	Not tested
8	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)
16	0 (0%)	0 (0%)	0 (0%)	0 (0%)	1 (100%)
32	Not tested	Not tested	Not tested	1 (50%)	0 (0%)
64	Not tested	Not tested	Not tested	0 (0%)	0 (0%)
128	Not tested	Not tested	Not tested	1 (50%)	0 (0%)
256	Not tested	Not tested	Not tested	0 (0%)	0 (0%)
512	Not tested	Not tested	Not tested	0 (0%)	0 (0%)

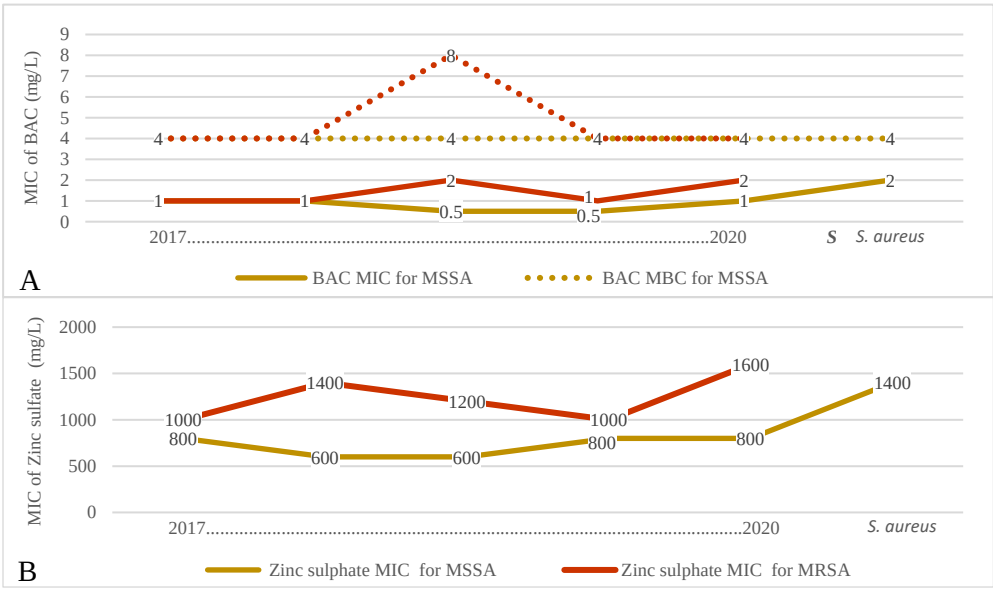


Figure II.3.3: A. Distribution of MIC and MBC values of BAC against MSSA and MRSA taking into consideration the year of strain detection. B. Distribution of MIC values of zinc sulfate against MSSA and MRSA taking into consideration the year of the year of strain detection.

The n=5 MSSA isolates were inhibited by zinc sulfate at MIC values ranging from 0.6 mg/ml to 0.8 mg/ml, while the n=5 MRSA isolates were inhibited at higher concentrations, ranging from 1 mg/ml to 1.6 mg/ml. The ATCC 25923 *S. aureus* growth was inhibited at a zinc sulfate concentration of 1.4 mg/ml. The tested Gram-negative isolates, including one ophthalmic *P. aeruginosa* isolate, the ATCC 27854 P. aeruginosa, and the ATCC 19606 *A. baumannii*, were inhibited at concentration higher than 2 mg/ml (Fig. II.3.2B, table II.3.2, table II.3.4).

Table II.3.4: Zinc sulfate susceptibility in ophthalmic clinical isolates and the reference strains of *S. aureus*, *P. aeruginosa*, and *A. baumannii*

Concentrations of Zinc sulfate (mg/ml)	*S. aureus* (n=11) (%)	MSSA (n=6) (%)	MRSA (n=5) (%)	*P. aeruginosa* (n=2) (%)	*A. baumannii* (n=1) (%)
0.2	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)
0.4	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)
0.6	2 (18.2%)	2 (33.3%)	0 (0%)	0 (0%)	0 (0%)
0.8	3 (27.3%)	3 (50%)	0 (0%)	0 (0%)	0 (0%)
1	2 (18.2%)	0 (0%)	2 (40%)	0 (0%)	0 (0%)
1.2	2 (18.2%)	1 (16.7%)	1 (20%)	0 (0%)	0 (0%)
1.4	1 (9.1%)	0 (0%)	1 (20%)	0 (0%)	0 (0%)
1.6	1 (9.1%)	0 (0%)	1 (20%)	0 (0%)	0 (0%)
1.8	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)
2	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)
>2	0 (0%)	0 (0%)	0 (0%)	2 (100%)	1 (100%)

II.3.5.2 In Vitro Evaluation Synergistic/Antagonistic Activity of BAC and Zinc sulfate

BAC was tested for the synergistic effect when combined with zinc sulfate using the checkerboard assay and against the five MSSA strains yet evaluated for the MIC of the individual compounds. The results of the checkerboard assay for the n=5 MSSA, demonstrated various degree of additivity (4 > FICI > 0.5) between BAC and zinc sulfate, with the FICI ranging from 0.75 to 1.5 (Table II.3.5). Based on such FICI, no synergism (FICI < 0.5) nor antagonism (FICI > 4) can be detected analyzing the MIC values obtained, for each tested isolate, in absence and in presence of the two compounds combinations.

Table II.3.5: Checkerboard results for the 5 ophthalmic MSSA isolates

	BAC[a]		Zinc sulfate MIC[b] (mg/ml)	Checkerboard assay						
	MIC (mg/L)	MBC (mg/L)			MIC of BAC in the presence of ZnSO4 (mg/L)	MIC of BAC (mg/L)	MIC of zinc sulfate in the presence of BAC (mg/ml)	MIC of zinc sulfate (mg/ml)	FICI[c] (Zinc sulfate + BAC)	Result
ATCC 25923	2	4	1.4	Replicate 1	0.25	1	0.35	0.7	**0.75**	Indifference
				Replicate 2	0.5	2	0.175	0.7	**0.5**	Indifference
				Replicate 3	0.5	2	0.35	0.7	**0.75**	Indifference
MSSA 4	1	4	0.8	Replicate 1	1	1	0.35	0.7	**1.5**	Indifference
				Replicate 2	1	1	0.175	0.35	**1.5**	Indifference
				Replicate 3	1	2	0.175	0.35	**1**	Indifference
MSSA 8	0.5	4	0.8	Replicate 1	0.5	1	0.175	0.35	**1**	Indifference
				Replicate 2	0.5	1	0.175	0.35	**1**	Indifference
				Replicate 3	0.5	1	0.175	0.35	**1**	Indifference
MSSA 15	0.5	4	0.6	Replicate 1	0.5	1	0.175	0.35	**1**	Indifference
				Replicate 2	0.5	1	0.175	0.35	**1**	Indifference
				Replicate 3	0.5	1	0.175	0.35	**1**	Indifference
MSSA 30	1	4	0.8	Replicate 1	0.5	1	0.175	0.35	**1**	Indifference
				Replicate 2	0.5	1	0.175	0.35	**1**	Indifference
				Replicate 3	0.5	1	0.175	0.7	**0.7**	Indifference
MSSA 36	1	4	0.6	Replicate 1	0.125	2	0.175	0.35	**0.5625**	Indifference
				Replicate 2	0.25	2	0.175	0.35	**0.625**	Indifference
				Replicate 3	0.25	0.5	0.175	0.35	**1**	Indifference

[a]MIC and MBC values using broth microdilution assay
[b]MIC values based on agar dilution assay
[c]Fractional inhibitory concentration index (FICI) = $\frac{\text{MIC of BAC alone}}{\text{MIC of BAC in combination}} + \frac{\text{MIC of Zinc sulfate alone}}{\text{MIC of zinc sulfate in combination}}$

II.3.6 Discussion

BAC and zinc sulfate are two compounds commonly used in many ophthalmic formulations as antimicrobial preservatives. We conducted this pilot study to evaluate the efficacy of these compounds in inhibiting the growth of ophthalmic pathogens at varying concentration of BAC (0.06 32 mg/L for Gram positive bacteria, and 8.0-512 mg/L for Gram-negative bacteria) and zinc sulfate (0.2-2.0 mg/ml); this taking into consideration the most provided concentrations in ophthalmic solutions among which the concentrations of BAC and zinc sulfate usually range from 0.004 to 0.02% [8] and 0.2 to 0.25% [14], respectively, which are equivalent to 40 to 200 mg/L of BAC and 2 to 2.5 mg/ml of zinc sulfate.

In the present study, BAC showed stronger antibacterial activity against ophthalmic MSSA, with MIC= 0.5-1 mg/L and MRSA isolates, showing MIC=1-2 mg/L, than against the ophthalmic *P. aeruginosa* isolate, MIC=128 mg/L. These results were in accordance with the results of previously published reports.

In a 2012 study [9], the antimicrobial efficacy of BAC was evaluated against six important foodborne pathogens including three Gram-positive (*S. aureus*, *L. monocytogenes* and *Bacillus cereus*) and three Gram-negative bacteria (*Salmonella typhimurium*, *E. coli* and *P. aeruginosa*) exhibiting higher antibacterial efficacy on Gram-positive bacteria (except *Bacillus cereus* because of its ability to spore formation): MIC and MBC values of BAC for *S. aureus* were 40 mg/L and 45 mg/L, respectively, while *P. aeruginosa* had an MIC and MBC equal to 60mg/L and 80 mg/L, respectively. Moreover, similar results with comparable MIC values were also observed in a 2018 report [13], when BAC was tested against 182 *S. aureus* isolates, including MSSA and MRSA, exhibiting MIC_{90} of ≤ 2 from human. In Gram-negative bacteria, resistance mechanisms are more complicated since these organisms have an inner and an outer membrane with the ability of this bacteria to alter the hydrophobic properties and structure changing permeability of the latter one, in addition to their efficiency in acquiring and spreading antibiotic resistance gene [9][31].

The MIC value of BAC for the clinical ophthalmic *P. aeruginosa* isolate (MIC=128 mg/ml) was three-times higher than the MIC value of the reference strain *P. aeruginosa* ATCC 27854, while the reference strain *A. baumannii* ATCC 19606 had the lowest MIC value among the gram-negative strains tested. This difference might be due to the continuous exposure to BAC taking into considerations eye infections are usually treated empirically.

For zinc sulfate, the same pattern was observed, with *S. aureus* strains more susceptible to the compound (MIC: 0.6 -1.6 mg/ml) than the Gram-negative isolates tested (MIC >2 mg/ml). Among *S. aureus* isolates, MSSA were more susceptible to zinc sulfate (MIC ranged from 0.6mg/ml to 0.8 mg/ml for ophthalmic MSSA isolates, with *S. aureus* ATCC 23925 MIC = 1.4 mg/ml) than MRSA isolates (MIC= 1mg/ml-1.6 mg/ml). Interestingly, the highest MIC value was obtained for a recently

identified MRSA isolate, indicating the possibility of an increasing trend of resistance.

The efficacy of zinc sulfate against *S. aureus* were concordant with the results of a 2020 study conducted on MDR pathogens, with *S. aureus* being one of the pathogens tested (the study used agar diffusion assay while in our study we used agar dilution) [22]. In a study of 2020 [21], most *P. aeruginosa* (n=60) and *A. baumannii*,(n=60), were inhibited at a concentration of 1 mg/ml and 0.5 mg/ml, respectively. In our study, the three Gram-negative isolates tested (the ophthalmic *P. aeruginosa* and the two reference strains of *P. aeruginosa* and *A. baumannii*) showed MIC > 2 mg/ml.

The results of checkerboard assay showed that BAC and zinc sulfate in combination were able to reduce the MIC value for BAC, but not enough to show synergistic activity through calculating FICI, which resulted in additive interaction.

Comparing our findings with the concentrations of the active ingredients in ZINCOMETIL, the quantity of BAC 0.01% (100 mg/L) present in the formula, resulted able to inhibit the growth of *S. aureus* isolates (MIC=2 mg/L), and *A. baumannii* (MIC=16 mg/L) but not effective against the clinical strain of *P. aeruginosa* (MIC=128 mg/L). The quantity of zinc sulfate 0.02% (200 mg/L) present in the formula, although too poor in inhibiting the growth of both the ophthalmic isolates and the reference strains tested (*S. aureus* -MIC= 1.6 mg/ml-, *A. baumannii* -MIC >2mg/ml-, and *P. aeruginosa* -MIC >2mg/ml-), it was responsible (albeit at limited extent) for the augmented inhibitory activity of the associated compound BAC observed *in vitro*, towards MSSA isolates. Even if a synergistic effect has not been demonstrated (FICI: “indifference”), an additive action in terms of a MICs values decrease for each MSSA tested in presence of the second compound (BAC or Zn) compared with a single use, was observed.

II.3.7 Conclusions

Based on our findings, BAC have antimicrobial high efficacy against *S. aureus i*solates, both MSSA and MRSA, while higher concentrations of this compound are required to be effective against Gram-negative isolates. For zinc sulfate, concentrations at least of 1.6 mg/ml for *S. aureus*, and higher than 2mg/ml for *A. baumannii*, and *P. aeruginosa* are required to obtain an effective growth inhibition. n additive action between the two compounds was finally observed, but not enough to show synergistic effect when used in combination.

Further studies are recommended to confirm the results obtained, including larger number of ophthalmic isolates of the species studied and including a wider range of species ophthalmic infections associated.

II.3.8 References

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1. Piazza Aurora, **Aseel AbuAlshaar**, Antonietta Grosso, Francesca Piscopiello, Spalla Melissa, Michela Sturini, Federica Maraschi, Pilla Giorgio, Migliavacca Roberta.**Quality assessment of surface and groundwaters in Lombardy, Italy: detection and characterization of ESBLs-producing *Enterobacterales***. Selected for FLASH COMMUNICATION. Società Italiana Microbiologia 50° congresso, 18-21 settembre 2022, Napoli.

2. **Aseel AbuAlshaar**, Aurora Piazza, Francesca Piscopiello, Marta Corbella, Patrizia Cambieri, Carola Mauri, Alessandra Consonni, Sara Rimoldi, Roberta Migliavacca. **Evaluation of the anti-biofilm activity of four different antibiotics against *Staphylococcus aureus* strains from ocular infections.** Società Italiana Microbiologia 50° congresso, 18-21 settembre 2022, Napoli. Selected for ORAL PRESENTATION

3. **Abualsha'ar Aseel**, Mercato Alessandra, Piazza Aurora, Nucleo Elisabetta, Marta Corbella, Patrizia Cambieri, Carola Mauri, Francesco Luzzaro, Sara Rimoldi, Migliavacca Roberta. **Antibiotic-resistance in ocular pathogens: a six-year trend, Italy.** ECCMID congress; 2022 April.

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5. **Abu-Alsha'ar Aseel**, Gherard Batisti Biffignandi, Aurora Piazza, Paola Prati, Alessandra Mercato, Federica Marchesini, Giuseppina Andreoli, Melissa Spalla, Davide Sassera, Roberta Migliavacca. **Detection and Characterization of ST167 NDM-5-producing *Escherichia coli* strain from a cat, in Italy.** ECCMID congress; 2021 July.

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7. **Abu-Alsha'ar Aseel**, Marchesini Federica, Mercato Alessandra, Mancinelli Marika, Mattioni Marchetti Vittoria, Elena Fogato, Lattanzi Fabio, Nucleo Elisabetta, Megliavacca Roberta. ***Escherichia coli* KPC-2/3, OXA-181, and VIM-1 producers from an Italian Long-term care facility (LTCF).** ECCMID on-line congress 2020.