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Novel phenotypic and molecular approaches for tackling the problem of microbial drug resistance

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TABLE OF CONTENTS

List of abbreviations

AMR	Antimicrobial resistance
AD	Agar dilution
AST	Antimicrobial susceptibility testing
ATB	Antibiotic
ATG	Antibiotics resistance gene
BLI	β-lactamases inhibitor
BLIC	Combinations β-lactam/β-lactamases inhibitor
BMD	Broth microdilution
BSI	Bloodstream infections
CAMHB	Cation-adjusted Mueller-Hinton broth
CA-MRSA	Community-associated MRSA
CAP	Community-acquired pneumonia
CAZ/AVI	Ceftazidime/avibactam
CC	Clonal complex
CFCD	Cefiderocol
cIAI	Complicated intra-abdominal infections
CPB	Ceftobiprole
CPE	Carbapenemase-resistant *Enterobacterales*
CRAb	Carbapenem resistant *A. baumannii* complex
CRE	Carbapenemase-producing *Enterobacterales*
cUTI	Complicated urinary tract infections
DPA	Dipicolinic acid
DTR	Difficult to treat
EDTA	Ethylenediaminetetraacetic acid
EEA	European Economic Area
EMA	European Medicines Agency
ESBL	Extended-spectrum β-lactamase
EU	European Union
FDA	U.S. Food and Drug Administration
FEP/VNRX	Cefepime/VNRX-5133
FISH	Fluorescent in situ hybridization
HA-MRSA	Hospital-acquired infections caused by MRSA
HAP	Hospital-acquired pneumonia
ID-CAMHB	Iron-depleted cation-adjusted Mueller-Hinton broth
IS	Insertion sequences
KPC-Kp	KPC-producing *Klebsiella pneumoniae*
MBL	Metallo-β-lactamase
MDR	Multi-drug resistant
MEM/VB	Meropenem/vaborbactam
MGE	Mobile genetic element
MHB	Mueller-Hinton broth
MIC	Minimum inhibitory concentration
MRSA	Methicillin-resistant *Staphylococcus aureus*
MSSA	Methicillin-susceptible *Staphylococcus aureus*
NAAT	Nucleic acid amplification techniques
NGS	Next-Generation Sequencing
PBP	Penicillin-binding proteins
PCR	Polymerase chain reaction
PVL	Panton-Valentine Leukocidin
SCC*mec*	Staphylococcal cassette chromosome carrying the *mec* gene
ST	Sequence type
Tn	Transposon
VAP	Ventilator-associated pneumonia
VISA	Vancomycin-intermediate *Staphylococcus aureus*
VRE	Vancomycin-resistant enterococci
WGS	Whole genome sequencing
WHO	World Health Organization

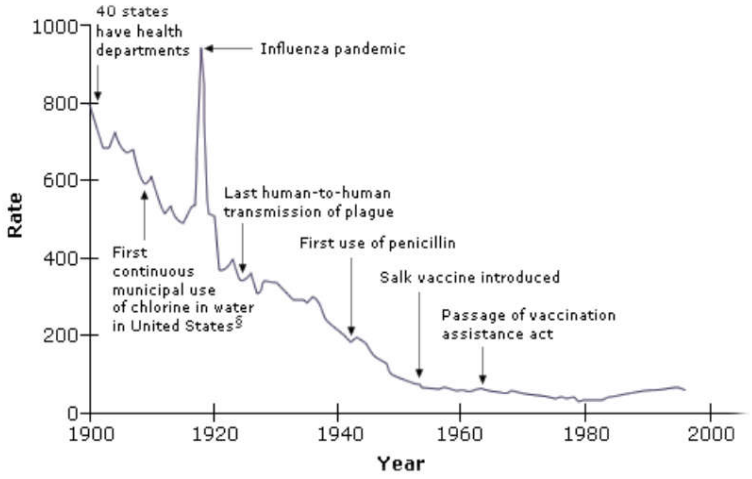
1. AN OVERVIEW ON ANTIMICROBIAL RESISTANCE

"It is not difficult to make microbes resistant to penicillin in the laboratory by exposing them to concentrations not sufficient to kill them, and the same thing has occasionally happened in the body. The time may come when penicillin can be bought by anyone in the shops. Then there is the danger that the ignorant man may easily underdose himself and by exposing his microbes to non-lethal quantities of the drug make them resistant." Alexander Fleming, 1945

Human history has been strongly influenced by infectious diseases. Many ancient civilizations had to face the spread of diseases that could not be controlled or cured, resulting in large demographic declines and in the fall of kingdoms and empires. Just think of how the plague, whose etiological agent is the bacterium *Yersinia pestis*, has inspired works of art and literature over the centuries.

At the begging of the 20th century, life expectancy was just over 40 years in Europe and in the American continent (Riley JC, 2005), while the infant mortality ranged between 15% and 40% in different countries (Wegman M, 2001; Roser and Ritchie, 2019). Most hospitalized patients were assisted with poorly treatments for a disease of infectious origin, with a high prevalence of tuberculosis and other infections related to the respiratory tract, leading to premature death (Bryskier A, 2005, Kenneth *et al,* 2014).

The first major contributions to tackle infection diseases were characterized by the prophylaxis measures, including vaccines, the chlorination of water and antiseptics, proposed by Edward Jenner, Louis Pasteur, Ignaz Semmelweis and Joseph Lister (Figure 1) (Kenneth *et al,* 2014). However, until the diffusion of penicillin in the 1940s, therapies were limited to serotherapy



§ American Water Works Association. Water chlorination principles and practices: AWWA manual M20. Denver, Colorado: American Water Works Association, 1973.

* Per 100,000 population per year.

Figure 1. Crude death rate* for infection diseases in United States between 1900 and 1996. *Adapted from Armstrong GL, Conn LA, Pinner RW. Trends in infectious disease mortality in the United States during the 20th century. JAMA 1999:281; 61–6 (Mort et al, 2013)*

with anatoxins, antiprotozoal vegetable derivates (quinine, emetine), arsenical derivates against syphilis (Salvarsan) (Bryskier A, 2005).

The era of antibacterial agents begins at the end of 19th centuries with pioneering works about two principal research lineages: i) dye substances that led to sulfonamides, the first broad-spectrum antibacterial drugs (Domagk's prontosil); ii) antibiosis, the antagonism of growth between two microorganisms, that culminated in the discovery of penicillin from the mold *Penicillium notatum* by Alexander Fleming in 1928 (Bryskier A, 2005). The Fleming's publication had no clinical repercussions until Howard Florey and Ernst Chain of Oxford University were able to crystallize and purify larger quantity of penicillin between 1941 and 1944 (Mort *et al,* 2013), while the industrial fermentation was the main work of the chairman of Pfizer Co., John Smith (Bryskier, 2005). The advent of anti-infective chemotherapy had a deep social-economic implication by prolonging life expectancy due to a reduction in infant mortality and to the possibility to treat diseases that previously scourged the humanity all around the world such as tuberculosis, leprosy, sexually transmitted diseases and bacterial meningitis (Bryskier A, 2005). After the introduction of treatment for infection diseases with bacterial etiology, antifungal and antiparasitic drugs have been gradually introduced in clinical practice up to the more recently antivirals (Bryan-Marrugo *et al,* 2015). Moreover, the improvement of

the social-economic conditions (higher pro capita income and welfare benefits), as well as the technological progress, has led in some areas of the globe to deploy the surgery and the treatment of oncological, cardiovascular and chronic pathologies as those associated with hospitalization and old age. In this setting, new infection diseases, such as acquired immunodeficiency syndrome (AIDS) or severe acute respiratory syndrome (SARS), as well as non-obligate and/or non-specialist pathogens, defined opportunistic, have emerged (Bryskier A, 2005; Bryan-Marrugo *et al,* 2015).

Unfortunately, microorganisms began to develop resistance against antimicrobial agents, during the first year of antibiotics use (Bryskier A, 2005). Antimicrobial resistance (AMR) has emerged as a worrisome public health issue of the 21st century, liable to affect the prevention and treatment of an ever-increasing range of infections caused by bacteria, fungi, parasites and viruses (Prestinaci *et al,* 2015). The problem of AMR is of concern regarding antibiotic resistance in bacteria with the highest risk of mortality and increased health care cost. (Founou *et al,* 2017). World Health Organization (WHO) enumerated a list of 12 species in respect of which the discovery of some new antibiotics is urgently required, including multidrug-resistant *Mycobacterium tuberculosis,* carbapenem-resistant *Acinetobacter baumannii* and *Pseudomonas aeruginosa*, carbapenem-resistant and/or 3rd generation cephalosporin-resistant *Enterobacteriaceae,* vancomycin-resistant *Enterococcus faecium* and methicillin- vancomycin-resistant *Staphylococcus aureus.* (Tacconelli *et al,* 2018). Extended use of antibiotics, self-medication and exposure to infections in hospitals have selected multi-drug resistant bacteria, answerable for 15.5% of hospital-acquired infections in the world (Mulani *et al,* 2019). Cassini *et al.* estimated that, during 2015, in EU and EEA more than 670.000 infections were caused by antibiotic-resistant bacteria, which led to 33.000 cases of death; 63,5% were healthcare-associated. The highest incidence was observed generally in infants (aged <1 year) and people aged 65 years or older and, between European countries, in Greece and in Italy (Cassini *et al,* 2019).

In 2014, WHO estimated 480 000 new cases of multi-drug resistant tuberculosis, corresponding to 3.6% of new cases and 20.2% of previously treated, with much higher rates in Eastern Europe and Central Asia. (WHO, 2014).

Antimicrobial resistance is a complex phenomenon influenced by many interconnected factors regarding human and animal health, agriculture and environment, so it can't be approach with single direction or sporadic actions. In 2015 WHO promoted the assumption defined "One

Health approach" involving a coordinated action among numerous international and national sectors and actors. This approach provides an integrated view of the interactions between human and veterinary medicine, which entirely includes the antimicrobial resistance chain and the use of antibiotics, in order to increase the appropriate use of antibiotics in both areas (WHO, 2015). The different strategies proposed to tackle AMR can be summed up in two aims: a reduction in the volume of antibiotics currently used to preserve their activities longer and the supply of new antimicrobials active against drug-resistant microbes (WHO, 2015; O'Neil, 2016; OECD, 2019). These aims require measures of antimicrobial stewardship (optimization of antimicrobial drugs use in medicine and veterinary), of infection prevention (enhancement sanitation and hygiene), of diagnostic stewardship (implementation of rapid diagnostic tools) and effective communication and education (increased awareness of AMR between health workers and general population). Moreover, surveillance studies are of fundamental importance to monitor the AMR status, strengthen the knowledge and to examine the effects of measures adopted. These items involve also the allocation of greater resources for the development of new medicines, diagnostics and other interventions.

The next chapters will further describe the bacterial pathogens, their strategies to escape antibiotics, the main cutting-end diagnostic tools and the new alternative therapies.

1.1 ORIGIN AND DISSEMINATION OF ANTIBIOTICS RESISTANCE

Antimicrobial resistance is a natural and ancient phenomenon that evolved in response to the production of antimicrobial molecules during the competition between microorganisms for the same ecologic niches. (Hibbing *et al,* 2010). The production of antimicrobials, which range in their spectrum of activity from strain-specific bacteriocins to more broad-spectrum peptides and antibiotics, could be considered weapons against different species or related strains of the same species, even if may be used for cooperative purposes, such as quorum sensing mechanism (Ghoul and Mitri, 2016). Therefore, it is necessary to discriminate between the intrinsic or chromosomal and the acquired antibiotic resistance. The first was referring to microbes that are constitutively non-susceptible to a certain antibiotic, for example, because of the absence of the target on which the drug acts or because of low drug permeability of its own membrane. The second occurs when chromosomal mutations or genes acquisition confers protection to a previously effective class of molecules (Davies and Davies, 2010). Mutations are spontaneous changes in the bacterium's genetic material that take place at a low frequency. However, if a mutation or pattern of mutations confers an evolutionary advantage in a certain condition, the mutants are selected and their number increases in the population by Darwinian selection (Davies and Davies, 2010).

Genes bestowing a phenotype of antibiotic resistance are largely harbored on mobile genetic elements (MGEs), segments of DNA that mediate the movement of DNA within genomes or between bacterial cells determining intracellular or intercellular mobility, respectively (Frost *et al,* 2005). Among MGEs, insertion sequences (IS) and transposons (Tn) with eventually related resistance genes can interchange their locations in the genome from the chromosome to a plasmid or between plasmids almost randomly, due to the enzymes that they encode. Other elements, such as integrons (In), use site-specific recombination to move resistance genes between defined sites. Moreover, homologous recombination (exchange of sequences between identical or related segments) can be promoted by these types of MGEs which are present in multiple copies in different locations in a genome (Partridge *et al,* 2018). Indeed, resistance genes can accelerate their diffusion between microbes of the same or of different species through horizontal gene transfer mechanisms, including conjugation (transfer of plasmids or integrative conjugative elements [ICE] during a cell-to-cell contact), transduction (bacteriophages-mediated gene transfer), and transformation (uptake and incorporation of extracellular DNA)

(Munita and Arias, 2016). All these interactions between MGEs and spontaneous mutations can lead to accumulating in the same bacterial stem resistant determinants which originated in different species and associated with different types of antibiotics, resulting in the evolution of a multiresistant pathogen (Munita and Arias, 2016; Partridge *et al,* 2018). New resistance determinants, which have generally a fitness cost because their expression can interfere with cellular functions, can resettle permanently in the bacterial population only if there is a positive selection pressure. (Bengtsson-Palme *et al,* 2018). Under stress conditions (like antibiotics), lineages that have increased rates of mutation, recombination and ability for horizontal gene transfer have the selective advantage (Perry *et al,* 2014). In the environment, this selective pressure is exerted by antimicrobials and chemicals produced by other bacteria, fungi or plants on one side, and on the other side derives from anthropic activities such as agriculture and contaminated wastewater. Thousands of tons of antibiotics are reversed in the environment by farm, community and hospital sewages, as well as the direct exposition for the treatment of some plant diseases (e.g. fire blight), altering profoundly how microbes evolve. Some studies support the hypothesis that the environment is the major source and reservoir of resistance with the transfer of antimicrobial resistance genes from environmental to pathogenic bacteria (Prestinaci *et al,* 2015; Surette and Wright, 2017). However, the absence of contact between pathogens and environmental bacteria and the restrict number of mobilization events mitigate the risk of resistance genes transfer into pathogens (Surette and Wright, 2017). An explicative example was the origin and the dissemination worldwide of the extended-spectrum β-lactamase CTX-M from the environmental bacteria of the *Kluyvera* genus (Rodríguez *et al,* 2004). Comparison between sequences of clinical isolates positive for *bla*$_{\text{CTX-M}}$ with sequences in databases highlighted that the different CTX-M clusters derived from different chromosomal *bla*$_{\text{CTX-M}}$ related genes from different species of *Kluyvera*. The capture of these *bla*$_{\text{CTX-M}}$ genes from the environment by highly mobilizable structures, such as insertion sequences (IS*Ecp1* or IS*CR1*) and complex class 1 integrons and transposons, could have been a random event. After incorporation within MGEs, cephalosporins selective force powered mutational events accentuating the diversification of different clusters. Penetration and the later global spread of CTX-M-producing *Enterobacterales* has been associated with few surrounding genetic structures, few plasmids (IncFII, IncN, IncI1, IncL/M, IncK and IncHI2) associated with other resistance determinants (fluoroquinolone or aminoglycoside resistance) and few high-risk bacterial clones (e.g. *Escherichia coli* ST131) (Canton *et al,* 2012). Once new resistance genes

have been acquired by human or animal pathogens, they are easily exchanged and disseminated through bacterial populations in different settings (Surette and Wright, 2017). Animals have a crucial role in the dissemination of infectious diseases such as in the dissemination and development of antibiotic resistance (Surette and Wright, 2017). In fact, CTX-M has been identified in hospitalized patients, in community, in food and companion animals, up to become endemic as colonizers of humans and in the environment in South Asia (Bevan *et al,* 2018). Using a metagenomic shotgun sequencing approach, Munk and colleagues (Munk *et al,* 2018) detected plus of 400 antimicrobial resistance genes, including *bla*$_{\text{CTX-M}}$, *erm, optrA, mcr-1* and *tet,* and across 181 pig and 178 poultry herds in 9 European countries. Zhu and colleagues (Zhu *et al,* 2013) screened Chinese swine farms and observed that resistance genes were three times more numerous in farm soils due to the practice of spreading manure from swine fed a diet containing antibiotics as growth promoters. In the hospital setting, more factors contribute to the emergence and spread of multiresistant nosocomial infections: the intensive and prolonged use of antimicrobial drugs, typically broad-spectrum because of the absence of a complete evidence about the etiologic agents (empiric therapy); the presence of immunocompromised (e.g. patients with cancer or transplant recipients) and fragile elderly patients, invasive surgical procedures and intensive care unit devices, failure to control infections spread from patient to patient the presence of immunocompromised (e.g. patients with cancer or transplant recipients) and fragile elderly patients, invasive surgical procedures and devices of intensive care units, failure to control infections spread from patient to patient (Prestinaci *et al,* 2015). The major part of these factors, together with the length of stay and high levels of inappropriate antimicrobial use, is no exception in nursing homes and long-term care rehabilitation facilities (Morrill *et al,* 2016). An excessive use of antibiotics is also common in community due to self-medication, to prescriptions by general practitioners, even in the absence of appropriate indications as practice of defensive medicine, and to the availability of drugs without prescription of a physician in many low-income countries (Prestinaci *et al,* 2015; Hay *et al,* 2016). A strong correlation between antibiotic consumption and the rate of antibiotic resistance was reported in both humans and food-producing animals in EU/EEA confirming the necessity of the "One Health" approach: a multivariate analyses confirmed that 3rd- and 4th-generation cephalosporins and fluoroquinolones resistance in *E. coli* from humans was associated with corresponding higher antibiotic consumption in humans, whereas resistance to fluoroquinolones in *Salmonella* spp. and *Campylobacter* spp. from humans was related to the consumption of

fluoroquinolones in animals (ECDC/EFSA/EMA, 2017). The huge number of factors interconnected to the emergence and diffusion of resistance to antibiotics are summarized in Figure 2.

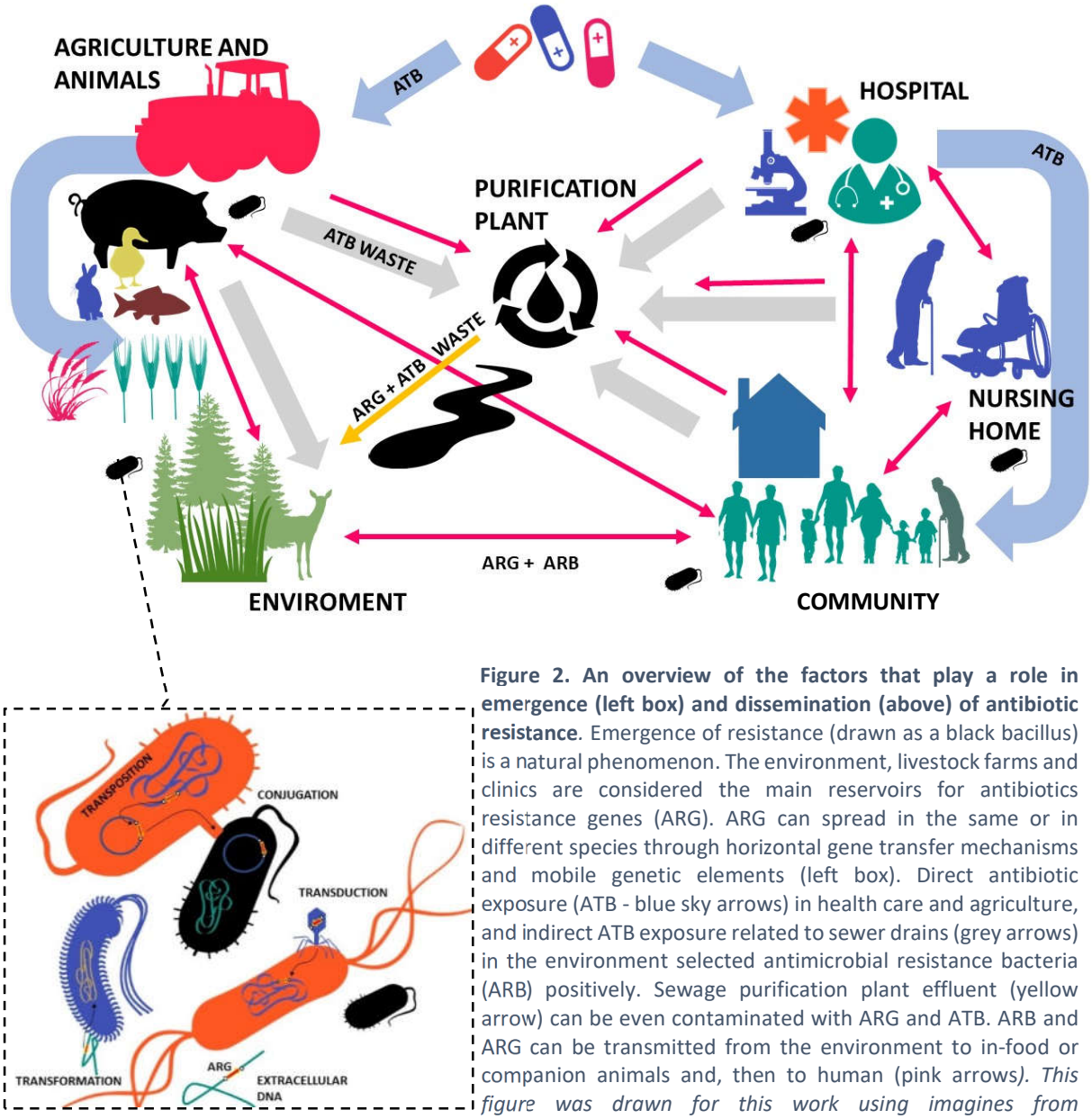


Figure 2. An overview of the factors that play a role in emergence (left box) and dissemination (above) of antibiotic resistance. Emergence of resistance (drawn as a black bacillus) is a natural phenomenon. The environment, livestock farms and clinics are considered the main reservoirs for antibiotics resistance genes (ARG). ARG can spread in the same or in different species through horizontal gene transfer mechanisms and mobile genetic elements (left box). Direct antibiotic exposure (ATB - blue sky arrows) in health care and agriculture, and indirect ATB exposure related to sewer drains (grey arrows) in the environment selected antimicrobial resistance bacteria (ARB) positively. Sewage purification plant effluent (yellow arrow) can be even contaminated with ARG and ATB. ARB and ARG can be transmitted from the environment to in-food or companion animals and, then to human (pink arrows). *This figure was drawn for this work using imagines from https://svgsilh.com under Creative Commons CC0.*

1.2 MECHANISMS OF ANTIBIOTICS RESISTANCE

Bacteria can exhibit an antibiotic-resistance phenotype as a result of biochemical mechanisms that prevent the interaction of antibiotic molecules with their targets. Each biochemical mechanism depends on one or more genetic determinants that can be intrinsically encoded in the chromosome of that species or can be acquired via mutations in chromosomal genes or by horizontal gene transfer. (Antonelli *et al,* 2017; Blair *et al,* 2015) The main molecular targets of antibiotics and the mechanisms to escape from them are described in Table 1.

1.2.1 Altered permeability of the membrane and active efflux

To perform its antibacterial functions, a drug needs to reach its molecular target, which can be sited in periplasmic space or into the bacterial cytosol, with an adequate concentration. The outer membrane, that surrounded a thin peptidoglycan layer in Gram-negative bacteria, blocks the entrance of polar macromolecules inside the cell (e.g. intrinsic resistance of Gram-negative to glycopeptides). Moreover, a reduction of the entrance of the drugs is obtained through the downregulation of the porins-coding genes, protein channels with low-specificity used by small hydrophilic molecules to cross the outer membrane, or through the expression of highly selective porins. This type of permeability alteration contributes to the resistance of many Gram-negative pathogens including *Enterobacterales*, *Pseudomonas* spp. and *Acinetobacter* spp (Blair *et al*, 2015). Examples of this type of mechanism are carbapenems resistance in *P. aeruginosa* mediated by the reduced expression of OmpD2 porins (Satake *et al,* 1991), but also the resistance to a new drug such as ceftazidime/avibactam in *K. pneumoniae* has been associated with the alterations of porins status (OmpK35 and OmpK36) (Nelson *et al,* 2017). Antibiotics can also be actively transported outside the cell via the efflux pumps using ATP or the proton gradient as an energy source. Active efflux systems are involved in the resistance to many antimicrobial agents and are mainly responsible for the intrinsic resistance of Gram-negative bacteria to many antibiotics used in the treatment of Gram-positives. Efflux pumps can be highly specific to a drug and plasmid-encoded (e.g. *tet* genes for tetracyclines resistance, *mef* genes for macrolides resistance, *qepA* and *opxAB* genes for quinolones resistance) or can carry different substrates and chromosome-encoded (multidrug resistance efflux pumps) (Antonelli *et al,* 2017). For example, over-expression of MexA-MexB-OprM system in *P. aeruginosa*

determines β-lactam and fluoroquinolones resistance, while the activation of NorA in *S. aureus* and of AcrAB-TolC systems in *E. coli* results in fluoroquinolones and chloramphenicol resistance, and tetracycline, chloramphenicol, ampicillin, quinolones and rifampin, respectively (Antonelli *et al,* 2017; Blair *et al,* 2015; Okusu *et al,* 1996).

1.2.2 Alterations in antibiotic targets

In this case, the resistance phenotype derives from the modification of the molecular target which reduces its affinity for the antimicrobial agent or from the substitution of the natural target with a new target that doesn't bind to the drug. Changes to the target structure can be caused by mutations in the gene encoding an antibiotic target (e. g. mutations of DNA topoisomerase II for fluoroquinolones or mutations in *rpoB* gene, encoding for the beta-subunit of RNA polymerase, preventing rifampin binding), or by modifications due to specific enzymes (e.g. ribosome methylases encoded by *erm*, *rmt* and *arm* genes). Another strategy to protect the target from antibiotics is that of *qnr* genes described on plasmids in various pathogens. The *qnr* genes encode pentapeptide repeat proteins, which bind topoisomerase IV and DNA gyrase causing the detachment of quinolones. Finally, the acquisition of a homologous target occurs in *S. aureus*, in which methicillin resistance is mediated by the acquisition of the *mec* genes, which encode the β-lactam-insensitive PBPs (Antonelli *et al,* 2017; Blair *et al,* 2015).

1.2.3 Enzymatic Inactivation of the antibiotics

In this case, the resistance phenotype is caused by the production of enzyme enable to modify the molecular structure of drugs avoiding its interaction with the target. This mechanism is frequently involved in β-lactams, aminoglycosides and chloramphenicol resistance. β-lactamases catalyze the hydrolysis of the β-lactam ring which constitutes the crucial component for the interaction with the penicillin-binding proteins (PBPs) involved in the cell wall synthesis. The aminoglycosides can be inactivated by the addition of acyl, phosphate and nucleotide groups mediated by different transferases (ACC, APH, ANT). The acetyltransferases encoded by *cat* genes can inactivate chloramphenicol (Antonelli *et al,* 2017). In Gram-negatives, one of the most prevalent mechanisms of fosfomycin resistance is the production of a glutathione *S*-transferase (FosA) that catalyzes the addition of glutathione to fosfomycin rendering the antibiotic inactive (Ito *et al,* 2017).

Table 1. Characteristics of the main classes of antibiotics.

Classes	Structure	Principal molecules	Type of activity	Molecular target in bacterial cellular	Spectrum of antibacterial activity	Main mechanisms of resistance
β-lactams	Tetratomic β-lactam ring containing an amide bond	Penicillins; cephalosporins; monobactams; carbapenems	Bactericidal	Inhibition of PBPs preventing the peptidoglycan's crosslinks in bacterial cell wall	Gram-positives and Gram-negatives	Antibiotic inactivation enzymes (*bla* genes); reduced affinity of existing PBP components; alteration of permeability and efflux
Glycopeptides	Glycosylated cyclic or polycyclic nonribosomal peptides	Vancomycin; teicoplanin; dalbavancin; telavancin; oritavancin	Bactericidal and bacteriostatic (teicoplanin)	Linkage with D-alanine–D-alanine of N-acetyl-muramic acid preventing the peptidoglycan's crosslinks in the bacterial cell wall.	Gram-positives	Target modification (*van* genes)
Phosphonic antibiotics	L-cis-1,2-epoxypropylphosphonate	Fosfomycin	Bactericidal	Inhibition of bacterial cell wall biogenesis by inactivating UDP-N-acetylglucosamine-3-enolpyruvyltransferase	Gram-positives and Gram-negatives	Antibiotic inactivation enzymes (*fos* genes); alteration of permeability
Ansamycins	Aromatic moiety (naphthalene ring or a naphthoquinone ring) bridged by an aliphatic chain	Rifampicin	Bactericidal	Inhibition of bacterial RNA polymerase	Gram-positives and Gram-negatives	Mutations leading to a change in the structure of the β subunit of RNA polymerase
Aminoglycosides	One or several aminated sugars joined in glycosidic linkages to a dibasic cyclitol	Gentamicin; amikacin; tobramycin	Bactericidal	Inhibition of protein synthesis binding to the 30S ribosomal subunit	Gram-negatives	Alteration of permeability and efflux; target modification; production of aminoglycoside modifying enzymes
Oxazolidinones	Heterocyclic compound containing both nitrogen and oxygen in a 5-membered ring	Linezolid; tedizolid	Bactericidal	Inhibition of protein synthesis binding 50S ribosomal subunit	Gram-positives	Target modification
Tetracyclines	Linear fused tetracyclic nucleus (rings designated A, B, C, and D)	Doxycycline; minocycline; tetracycline; tigecycline	Bactericidal	Inhibition of protein synthesis binding 30S ribosomal subunit	Gram-positives and Gram-negatives	Target modification Alteration of permeability and efflux
Phenicols	Benzene ring	Chloramphenicol; Florfenicol	Bacteriostatic	Inhibition of protein synthesis binding 50S ribosomal subunit	Gram-positives and Gram-negatives	Reduced membrane permeability; target modification (rRNA 50S); antibiotic inactivation enzymes (*cat* genes)
Macrolides	Large macrocyclic lactone	Azithromycin; clarithromycin; erythromycin	Bactericidal	Inhibition of protein synthesis binding 50S ribosomal subunit	Gram-positives and Gram-negatives	Target modification (rRNA 23S)
Lincosamides	Pyrrolidine ring linked to a pyranose moiety	Clindamycin	Bacteriostatic	Inhibition of protein synthesis binding 50S ribosomal subunit	Gram-positives and Gram-negatives	Target modification (rRNA 23S); active efflux; antibiotic inactivation enzymes
Streptogramins	Cyclic peptides	Quinupristin-dalfopristin	Bacteriostatic and bactericidal	Inhibition of protein synthesis binding 50S ribosomal subunit	Gram-positives	Target modification; active efflux; antibiotic inactivation enzymes
Polymyxins	Cationic peptide	Colistin; polymyxin B	Bactericidal	Alteration of bacterial outer membrane	Gram-negatives	Target modification
Lipopeptides	Lipid connected to a peptide	Daptomycin	Bactericidal	Alteration of the cytoplasmatic membrane with rapid depolarization.	Gram-positives	Target modification (yycFGHI operon mutation)
Sulfamides	Aniline derivatized with a sulfonamide group.	Sulfamethoxazole	Bactericidal	Inhibition of DNA synthesis due to the block of folate production (inhibition of dihydropteroate synthase)	Gram-positives and Gram-negatives	Target modification; active efflux
Diamopymidines	Two amine groups on a pyrimidine ring	Trimethoprim	Bacteriostatic	Inhibition of DNA synthesis due to the block of folate production (inhibition of dihydrofolate reductase)	Gram-positives aerobic cocci and some Gram-negatives aerobic bacilli	Target modification; cellular impermeability; loss of binding capacity
Quinolones	Bicyclic ring with a fluorine atom at position C-6 and various substitutions	Nalidixic acid; ciprofloxacin; levofloxacin; moxifloxacin	Bactericidal	Inhibition of DNA gyrase and topoisomerase IV during chromosomal replication and transcription	Gram-positives and Gram-negatives	Plasmid-mediated resistance genes produce a protein able to bind to DNA gyrase; efflux pumps; alterations of DNA gyrase or topoisomerase IV with decreased affinity to quinolones

1.3 β-LACTAMASES

β-lactamases are a large family of enzymes (almost 2800 unique proteins described) with a limited range of molecular structures that can hydrolyze the β-lactam ring (Bush K, 2018). They have been reported since the 1940s (Abraham and Chain, 1940), but phylogenetic analyses have placed their origin 2 billion years ago from environmental sources, as an adaptive response to naturally occurring β-lactams (Hall and Barlow, 2004). β-lactams inhibit the growth of replicating bacteria by acylating an active-site serine in penicillin-binding proteins (PBPs) with an almost irreversible reaction. Similarities in the structural arrangement with an active-site serine, despite low amino acid identities, led to hypothesize that β-lactamases precursors were presumably PBPs with an increased rate of the deacylation step (Bush K, 2018).

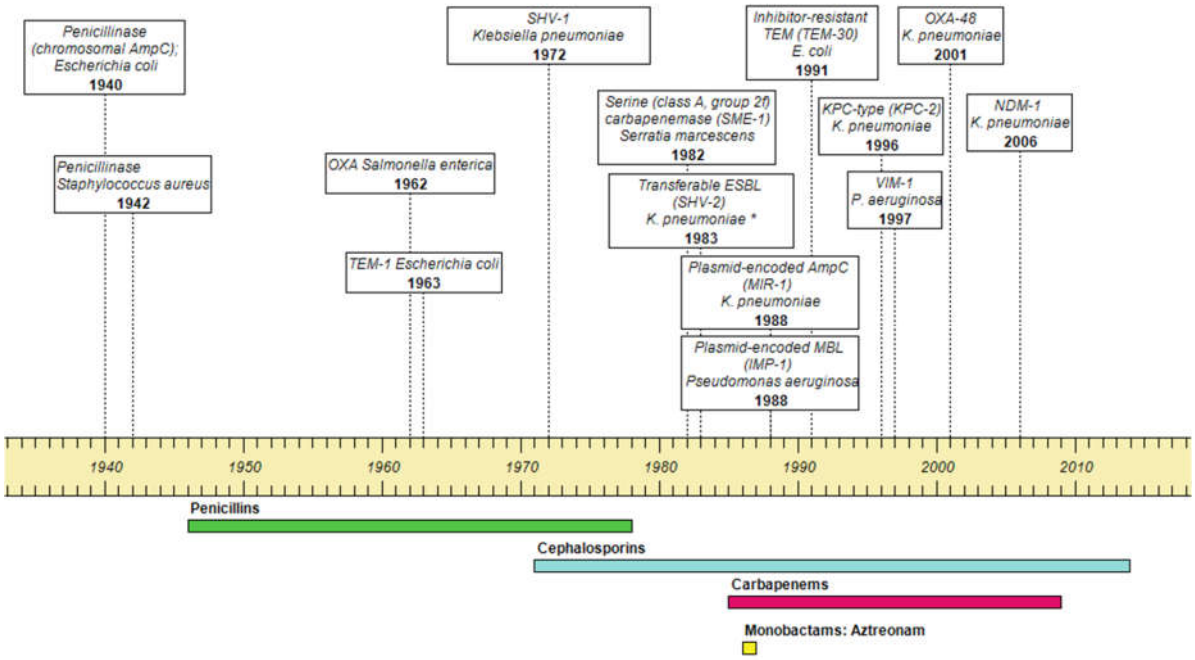


Figure 3. Timeline of the first isolation of a series of β-lactamases with clinical significance. Colored barres indicate the range of time in which FDA has approved new β-lactam drugs for each class. *Date not available, but certainly before 1983. *Drawn for this work using data from Bush and Bradford, 2016; and Bush K, 2018.*

After their first identifications in clinical practice, β-lactamases have shown a remarkable adaptive capacity: the introduction of new molecules in clinical practice has always been followed by the emergence and spread of bacterial strains producing enzymes capable of inactivating them (Figure 3) (Antonelli *et al*, 2017). After the extensive utilization of penicillin to treat streptococcal infections, as collateral damage, *S. aureus* strains quickly developed resistance as a result of penicillinase production (BlaZ) (Medeiros AA. 1997). However, except for BlaZ, β-lactamases

play a marginal role among resistance mechanisms in Gram-positives of clinical relevance. On the other hand, later, in Gram-negative pathogens, β-lactamases became the most important mechanism of resistance to β-lactams, including the recently introduced cephalosporins, monobactams, and carbapenems. The first chromosomal cephalosporinase was described in *E. coli* and, then, known as AmpC, was found in all *P. aeruginosa* isolates and many enterobacteria (Abraham and Chain, 1940; Poole K, 2011). However, it is the mobile β-lactamases, harbored on a transferable genetic element, in Gram-negative bacteria that have created a more insidious threat to β-lactams, because they have been spread horizontally by plasmids with few species barriers for their transmission (Bush K, 2018).

1.3.1 β-lactamases classification

Different types of classification have been proposed to order a huge number of these enzymes. The first large division of β-lactamases is based on the mechanism by which the hydrolysis of the β-lactam ring occurs, either through the formation of an enzymic acyl intermediate at level of the active-site serine (Knott-Hunziker *et al,* 1979) or via the intervention of one or two zinc ions as enzymatic cofactors in the active sites of metallo-β-lactamases (MBLs) (Sabath and Abraham, 1966).

Early schemes classified β-lactamases as penicillinase and cephalosporinases according to the substrates that they were able to inactivate, their inhibitor profiles and other biochemical characteristics of these enzymes (Sawai *et al,* 1968; Richmond and Sykes; 1973). Later, β-lactamases were divided based on their similarities at the level of their nucleotide and amino acid sequences in four molecular classes (A, B, C, and D) (Ambler RP, 1980). The class A, C, and D proteins are serine enzymes, with no significant structural similarities, whereas those of class B, are metalloenzyme (Philippon *et al,* 2015). A recently reviewed nomenclature combines the molecular and biochemical classifications into four main functional groups (1, 2, 2d, 3) and related subgroups (for a total of 17) (Bush and Jacoby, 2010). Enzymes are still differentiated with respect to the relative hydrolysis of the substrates, active inhibitors (clavulanic acid, avibactam and the metal ion chelator EDTA), and there is a correspondence between molecular and functional characteristics (Figure 4) (Bush K, 2018).

The functional group 1, corresponding to molecular Class C, includes AmpC cephalosporinases whose hyperproduction could be selected clinically by the oxyimino-cephalosporins, or expanded-spectrum cephalosporins, mostly chromosomic encoded in many *Enterobacterales* and in all *P.*

aeruginosa isolates (Bush K, 2018). These enzymes are more active on cephalosporins than benzylpenicillin, can hydrolyze 3rd generation cephalosporins and cephamycins, such as cefoxitin, and are usually resistant to inhibition by clavulanic acid, but not by avibactam. In many organisms, including *Citrobacter freundii*, *Enterobacter cloacae* complex, *Serratia marcescens*, *Morganella morganii, Hafnia alvei* and *Pseudomonas aeruginosa*, AmpC expression is low but inducible on exposure to certain β-lactams, such as ampicillin, amoxicillin plus clavulanic acid, narrow-spectrum cephalosporins and cefoxitin. AmpC enzymes can confer carbapenem-resistance (mainly to ertapenem) in strains with decreased permeability or increased efflux. Chromosomic AmpC enzymes are poorly expressed and not inducible in *E. coli*, *A. baumannii* complex, and *Shigella* spp. isolates. Plasmid-encoded AmpC-type enzymes (e.g. CMY, ACT, DHA, FOX, MIR,) on high-copy-number plasmids have been reported but are less frequent as plasmid-mediated ESBL (Jacoby GA 2009; Bush K, 2018).

The groups 2 and 2d are the most numerous and match with the molecular class A and D, respectively. Subgroup 2a includes penicillinases which hydrolyze benzyl-penicillin and some derivatives, mostly chromosomally encoded in Gram-positive cocci (e.g. PC1 from *S. aureus*) (Bush and Jacoby, 2010).

Enzymes belonging to subgroup 2b β-lactamases are many allelic variants of TEM-1 and SHV-1, which hydrolyze penicillins and early cephalosporins, such as cephaloridine and cephalothin, but are strongly inhibited by clavulanic acid, sulbactam and tazobactam. They were identified since the 1970s and in the early 1980s, they had become the most common enzymes among ampicillin-resistant enterobacteria (Bush, 2018). As a consequence of $bla_{\text{TEM-1}}$ and $bla_{\text{SHV-1}}$ point mutations, these respective enzymes have enlarged their substrate spectrum and have been indicated as extended-spectrum β-lactamases (ESBLs). ESBLs (classified in subgroup 2be) have activity against penicillins and first- and second-generation cephalosporins (as subgroup 2b β-lactamases) and in addition hydrolyze one or more oxyimino-β-lactams, such as cefotaxime, ceftazidime, and aztreonam, but not cephamycins and carbapenems. ESBLs are generally susceptible to common β-lactamases inhibitors (e.g. clavulanic acid, sulbactam and tazobactam). CTX-M enzymes also belong to the same subgroup. During the last decade, they have replaced TEM and SHV variants among the most common ESBLs in different epidemiologic settings (Bush K, 2018). Most CTX-M enzymes hydrolyze cefotaxime and cefepime more than ceftazidime. VEB and PER β-lactamases, which showed similar resistance profiles, and SFO, BES, BEL, TLA enzymes are less

common ESBLs (Paterson and Bonomo, 2005). As a result of the extensive use of the combinations β-lactam/β-lactamases inhibitor (BLIC) such as amoxicillin/clavulanic acid and piperacillin/tazobactam, inhibitor-resistant TEM (IRT) and SHV emerged (Bush, 2018). These enzymes (classified in Subgroup 2br) retain a subgroup 2b spectrum of activity (susceptible to cephalothin) but acquired resistance to clavulanic acid. Moreover, subgroup 2ber includes TEM mutants, termed CMT (complex mutant TEM), that exhibit an ESBL profile with relative resistance to clavulanic acid inhibition (Bush and Jacoby, 2010).

Subgroup 2f is formed by the members of the serine class A carbapenemases which can inactivate carbapenems at various levels and are only inhibited by avibactam (Bush, 2018). The class A carbapenemases include GES, KPC, SME, IMI/NMC-A, SHV-38 and SFC-1 enzymes. The genes encoding SME (*Serratia marcescens* enzyme), NMC-A (Non-Metallo enzyme carbapenemase) IMI-1 (Imipenem-hydrolyzing enzyme) and SFC-1 (*Serratia fonticola* carbapenemase-1) are usually located on the chromosome, whereas KPC (*K. pneumoniae* carbapenemase), GES (Guiana extended-spectrum carbapenemase) and IMI-2 enzymes are usually plasmid-borne (Walther-Rasmussen and Høiby, 2007). SME-1, the first identified among class A carbapenemases (1982), has a broad hydrolysis spectrum that includes penicillins, cephalosporins, aztreonam, and carbapenems (Queenan *et al,* 2006). The occurrence of SME such as that of IMI/NMC-A has been sporadic (Walther-Rasmussen and Høiby, 2007). GES-1 is a clinically relevant ESBL, but some GES variants with single point mutations (i.e. GES-2 and GES-5) have acquired the ability to hydrolyze carbapenems (Bontron *et al,* 2015). The *bla*$_{GES}$ genes harbored on transferable plasmids were found in *K. pneumoniae*, *P. aeruginosa* and *A. baumannii* complex (Diene and Rolain, 2014). Among class A β-lactamases, the KPC-type enzymes are the most prevalent in almost any Gram-negative pathogen, although they are predominantly identified in *K. pneumoniae*. After a few years since their discovery in the early 2000s, KPC-producing *K. pneumoniae* had spread worldwide strictly related to high success clonal lineages such as sequence types 11, 15, 101, 258/512 and their derivatives (Bush, 2018; David *et al,* 2019).

OXA-48-*like* enzymes are a family of transferable carbapenemases associated with outbreaks that have been identified from Turkey in 2001 as a class D oxacillinases (Poirel *et al,* 2004). The OXA enzymes are classified in subgroup 2d and are characterized by a hydrolysis rate of cloxacillin and oxacillin greater than 50% of that of benzylpenicillin. However, most of the members of this family are grouped together based on the presence of conserved structural motifs, rather than functional features. They are poorly inhibited by β-lactamase inhibitors (except avibactam), while sodium

chloride acts as an inhibitor (Bush and Jacoby, 2010; Bush, 2018). Some types, such as OXA-1, can inactivate only penicillins, others, such as OXA-11, also are active against expanded-spectrum cephalosporins (subgroup 2de). Subgroup 2df is formed by OXA-type β-lactamases which slowly hydrolyze carbapenems including, in addition to OXA-48 in *Enterobacterales*, chromosomally encoded OXA-51 and plasmid-encoded OXA-23, OXA-24/33/40 in *Acinetobacter baumannii* complex.

Metallo β-lactamases (MBLs) belong to functional group 3 and to molecular class B, which are subdivided into three subclasses (B1, B2, B3) based on amino acid sequence homology (Palzkill, 2013). These enzymes can hydrolyze carbapenems, whereas are inactive on monobactams and are unresponsive to inhibition by clavulanic acid, sulbactam, and tazobactam and, more recently, avibactam. Since their catalysis requires zinc ions as cofactor, the presence of chelators, such as EDTA (ethylenediaminetetraacetic acid) or dipicolinic acid, prevents the action of these enzymes (Palzkill, 2013; Bush K, 2018). There are few species of clinical relevance that have chromosomally encoded MBLs genes (*Aeromonas hydrophila* with CphA and *Stenotrophomonas maltophilia* with L1), while the acquired genes are found in the *Enterobacterales*, in *Pseudomonas aeruginosa*, *Acinetobacter baumannii* complex, and other non-fermentative Gram-negatives (Palzkill, 2013). Initially, chromosomally encoded MBLs were discovered in environmental bacterium *Bacillus cereus* and, for this reason, they have been considered clinically irrelevant enzymes for many decades (Sabath and Abraham, 1966; Bush K, 2018). This situation has changed in the 1990s when plasmid-encoded MBLs (IMP and VIM types) emerged in Japan (Watanabe *et al,* 1991) and in Italy (Lauretti *et al,* 1999). IMP (Imipenemase) and VIM (Verona integron-encoded metallo-β-lactamase) type metallo-β-lactamases have been reported among *Enterobacterales*, in *Pseudomonas* spp. and other non-fermenting Gram-negative bacteria. Outbreaks related to these enzymes have tended to be limited in time and localized to specific geographical regions (Greece, Taiwan, Japan) (Bush K, 2018). In contrast to the other MBLs, NDM, initially found in New Delhi (hence the name), quickly spread worldwide, being the predominant carbapenemase in the Indian subcontinent, but with major outbreaks also reported in the Balkans and the Middle East (Bush, 2018). Twenty-four NDM variants have been identified in various bacterial species responsible for healthcare-associated infections, among which the most prevalent are *K. pneumoniae* (especially ST11, ST14, ST15, or ST147) and *E. coli* (especially ST167, ST410, or ST617) (Wu *et al,* 2019). Others MBLs of clinical interest which were found

only in their region of origin and only sporadically in other countries are GIM (*German imipenemase*), SIM (*Seoul imipenemase*), SPM (*Sao Paulo Metallo β-lactamases*), KHM *(Kyorin Hospital Metallo β-lactamases)*, AIM (*Australian imipenemase*), DIM (*Dutch imipenemase*), SMB (*Serratia metallo-β-lactamase*), TMB (*Tripoli metallo-β-lactamase)* and FIM (*Florence imipenemase*) (Duck *et al,* 2015).

1.4 ANTIBIOTIC RESISTANCE IN *ENTEROBACTERALES*

Enterobacteria are a family of Gram-negative aerobic/ facultatively anaerobic, non-spore-forming bacilli which includes 60 genera and over 250 species, many of them are members of the intestinal microbiota, have an environmental ubiquitous distribution and some are of clinical relevance (Adeolu *et al,* 2016). The genera *Salmonella*, *Shigella* and *Yersinia* have evolved specific determinants of pathogenicity that make them capable of causing disease even in normal hosts (Dekker and Frank, 2015) while other species (e.g. *E. coli*, *Klebsiella* spp., *Citrobacter* spp. *Serratia* spp., *Proteus* spp., *Morganella morganii*, *Providencia* spp.) behave as commensals or as opportunistic pathogens, causing extraintestinal infections in conjunction with compromised host defenses or if they acquire particular virulence factors (Guentzel, 1996). Overall enterobacteria are the most frequent causes of healthcare-associated infections and are also responsible for many infections of community origin (intestinal infections, urinary tract infections and sepsis) (ECDC, 2013; Giani *et al,* 2017). A revision of the enterobacteria taxonomy has been recently proposed classifying the various species in an order called *Enterobacterales* in which the families of the *Enterobacteriaceae*, *Erwiniaceae*, *Pectobacteriaceae*, *Yersiniaceae*, *Hafniaceae*, *Morganellaceae* and *Budviciaceae* are included (Adeolu *et al,* 2016). Wild-type *Enterobacterales* are naturally susceptible to many classes of antibiotics but have shown a remarkable ability to acquire determinants of resistance (Iovleva and Doi, 2017). The accumulation of genetic determinants of resistance has led to the progressive emergence of multi-drug resistant strains, which sometimes assume a non-susceptible phenotype to all the different classes of antibiotics (pan-drug resistant strains) (Rossolini *et al,* 2007). Among *Enterobacterales*, *K. pneumoniae* represents the species in which the multidrug issue assumes the most worrisome threat in Europe, in terms of human morbidity and mortality (Cassini *et al,* 2019).

The main acquired mechanisms of resistance against the key antibiotics used to treat MDR *Enterobacterales* infections and their epidemiology are briefly summarized in the next paragraphs.

1.4.1. Trends in resistance to extended-spectrum cephalosporins

Resistance to third-generation cephalosporins has been reported worldwide among *Enterobacterales*, especially in *K. pneumoniae* and *E. coli* with percentages that range widely between countries (e.g. see Figure 4). Currently, the more important enzymes, from a clinical point of view for epidemiologic and therapeutic implications, that mediate resistance to extended-spectrum cephalosporins, are extended-spectrum beta-lactamases (ESBLs), AmpC-type beta-lactamases and carbapenemases (Giani *et al,* 2017).

ESBLs began to spread among enterobacteria from the mid-1980s as a response to the extensive use of third-generation cephalosporins and were initially represented by variants of TEM and SHV, which, since 2000s have been gradually replaced by CTX-Ms, which are the more representative group of ESBLs nowadays (D'Andrea *et al*, 2013).

ESBL-producing *Enterobacterales* are predominantly associated with healthcare infections but in regions with endemic prevalence can become a community-associated risk factor. ESBL-producing *E. coli* isolates are increasingly detected in nursing homes and community-based health-care facilities and have been associated with travel, leading to speculate a potential influx of ESBL-producing microorganisms from the community into the hospital (van der Steen M *et al,* 2015).

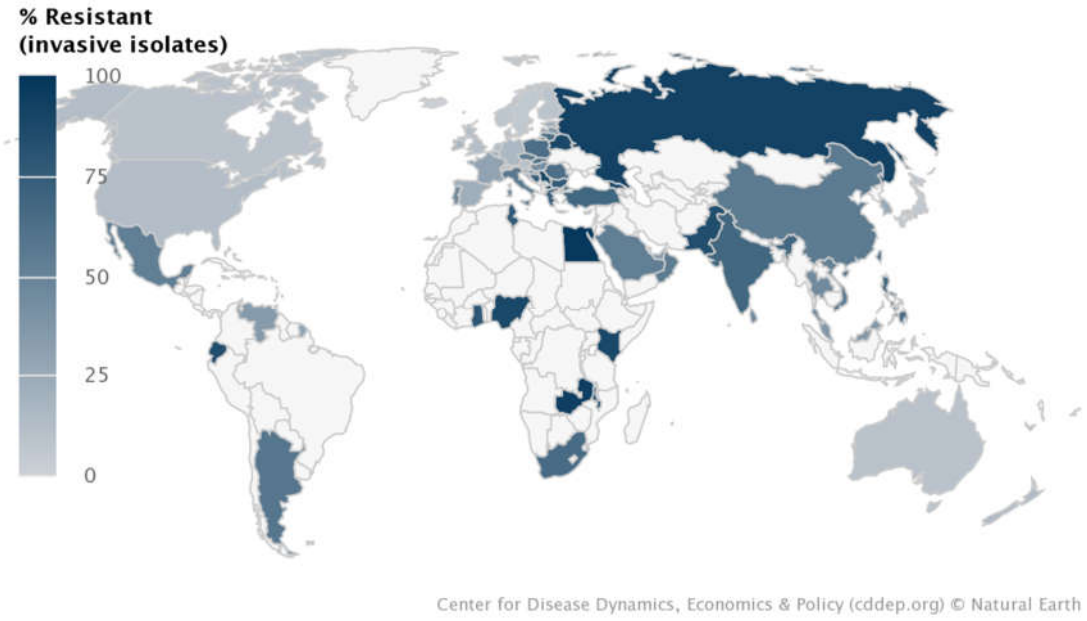


Figure 4. Worldwide distribution of the percentage of invasive *K. pneumoniae* isolates which resulted resistant to 3rd generation cephalosporins. *Adapted from The Center for Disease, Dynamics Economics & Policy. ResistanceMap: Antibiotic resistance. 2019. https://resistancemap.cddep.org/AntibioticResistance.php. Date accessed: October 1, 2019.*

The huge number of CTX-M variants identified can be classified at least in six sublineages or groups (CTX-M-1, CTX-M-2, CTX-M-8, CTX-M-9, CTX-M-25 and KLUC) basing on a difference in aminoacidic sequence ≥10% (D'Andrea *et al*, 2013).

CTX-M-15 (Group 1) has is the most prevalent in most world regions with the exceptions of China, South-East Asia, South Korea, Japan and Spain, where group 9 variants (especially CTX-M-14) are dominant, and South America, where CTX-M-2 is still significant (Bevan *et al,* 2017).

Globally the prevalence of ESBL-producing *Enterobacterales* is increasing over time. A recent review reported upward trends statistically significant for Europe ($R^2 = 0.429$, $P = 0.04$) and in low-outcome countries ($R^2 = 0.814$, $P = 0.0004$) when the rises of rates are considered in community ESBL (Figure 5) (Bevan *et al,* 2017).

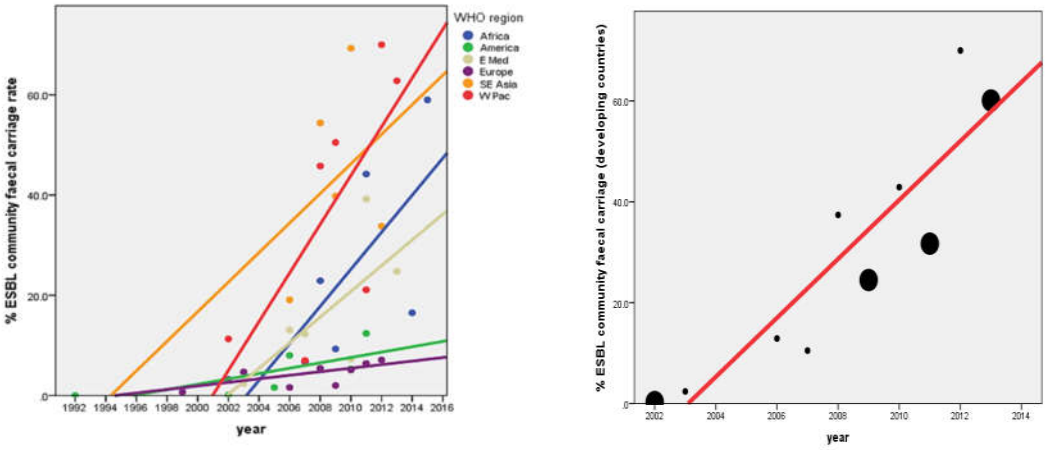


Figure 5. Prevalence rate (%) of community faecal ESBL carriage, according to their geographical distribution (WHO regions) [left] or considering developing countries only (excluding North America and WHO region Europe) [right]. *Adapted from Bevan et al, 2017.*

The reasons of rapid and wide diffusion of $bla_{\text{CTX-M}}$ genes have been attributed to their association with a variety of mobile genetic elements that have mediated an efficient inter-replicon and cell-to-cell dissemination, and with highly successful enterobacterial lineages (e.g. *E. coli* ST131 and ST405, or *K. pneumoniae* CC11 and ST147) (D'Andrea *et al,* 2013).

In Italy, the most recent data from the European Antimicrobial Resistance Surveillance Network (EARS-Net) reported very high percentage of resistance to extended-spectrum cephalosporins among invasive isolates of *E. coli* (29.5%) and *K. pneumoniae* (54,6%), also if compared to the European average of 14.9% and 31.5%, respectively (ECDC, 2018). However, a national

surveillance including *Enterobacterales* isolates from hospital and community infections, collected in 2013, reported that extended-spectrum cephalosporins resistant isolates but susceptible to carbapenems were 23.6% of *P. mirabilis*, 16.1% of *E. coli,* and 9.3% of *K. pneumoniae*, with an higher proportion among isolates from inpatients (20.3%) but a remarkable percentage (11.1%) also among those from outpatients (Giani *et al,* 2017). Moreover, Giani and colleagues found that the proportion of ESBL-producing isolates increased notably in a decade, especially among outpatients and among *E. coli*, and that CTX-M-type enzymes represented the most common type of ESBL in *E. coli* and in *K. pneumoniae,* but not in *P. mirabilis*, where cephalosporins resistance was mediated by ESBLs other than CTX-M-type or AmpC-type β-lactamases, generally of the CMY/LAT/ACT/MIR lineage (Giani *et al,* 2017).

1.4.2 Trends in carbapenems resistance

Over the past decade, carbapenem-resistant *Enterobacterales* (CRE) have had a rapid worldwide spread (Figure 6), which represents an increasing public health issue, with a significant impact on morbidity, mortality, and healthcare-associated costs (Logan and Weinstein, 2017). Among *Enterobacterales,* the resistance to carbapenems is mainly attributable to the production of carbapenemases and, less frequently, to ESBLs or AmpC beta-lactamases in combination with alterations of porin status or efflux systems (Logan and Weinstein, 2017).

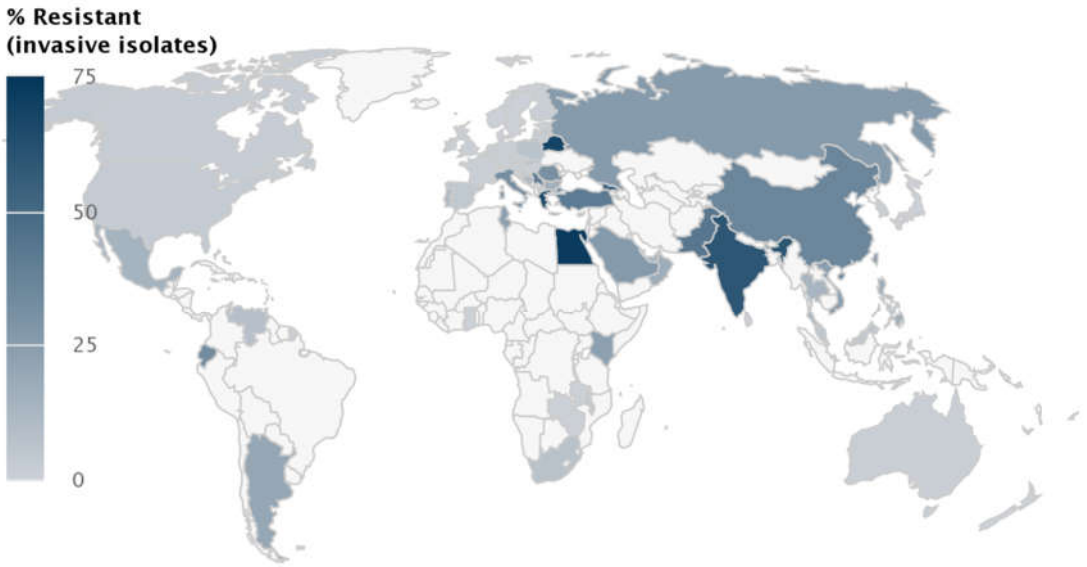


Figure 7. Worldwide distribution of the percentage of invasive carbapenem-resistant *K. pneumoniae* isolates. Adapted from The Center for Disease, Dynamics Economics & Policy. ResistanceMap: Antibiotic resistance. 2019. https://resistancemap.cddep.org/AntibioticResistance.php. Date accessed: October 1, 2019.

The effective spread of carbapenemases within *Enterobacterales* resulted from a continuous interplay between the clonal expansion of successful CPE clonal lineages and horizontal transfer of carbapenemase genes through mobile genetic elements (van Duin and Doi, 2017).

The main carbapenemases in the enterobacteria, especially in *K. pneumoniae*, are the serine β-lactamases of KPC and OXA-48-*like* type and the metallo-β-lactamases (MBLs) of VIM, NDM and IMP type. The Indian subcontinent, United States, Israel, Greece, Italy, Turkey, the Middle East and North Africa are the regions and countries with the highest prevalence of the various CPE (Nordmann and Poirel, 2014).

In Europe, carbapenem-resistant *Klebsiella pneumoniae* is one of the major causes of both hospital- and community-acquired infections with large variability in the national percentages ranging from 0% to 64.7% of invasive isolates (Figure 8) (ECDC, 2019). Most carbapenemase-producing *K. pneumoniae* isolates belong to four clonal lineages (sequence type 11, 15, 101, 258/512) *(David et al, 2019).* On the other hand, carbapenem-resistant *E. coli* isolates have a marginal role in invasive infections with national percentages ranging from 0% to 1.6% and a slightly decreasing trend between 2014 and 2017 (ECDC, 2019). The highest incidence of KPC-producing CPE is found in Greece and Italy which present an endemic situation for KPC, mostly with the KPC-2 and KPC-3 variants (van Duin and Doi, 2017). In a multicenter study from Italy that reported on carbapenem-resistant *Enterobacterales* isolates, during 2013, most (81.9%) were positive for carbapenemase genes, including bla_{KPC} (93.2%), $bla_{\text{OXA-48-like}}$ (4.2%), and $bla_{\text{VIM-type}}$ (2.6%) carbapenemase genes (Giani *et al,* 2017). The impact of KPC in Greece has been similarly dramatic. In a single-center study, the prevalence of KPC producers increased since 2008 and the majority of *K. pneumoniae* isolates carried a bla_{KPC} gene since 2014 (Spyropoulou *et al,* 2016; van Duin and Doi, 2017). However, a retrospective study about the 2015-2018 period in a single ICU highlighted the rise of MBLs-producing isolates, mostly of the VIM-type, after the widespread ceftazidime/avibactam use (Papadimitriou-Olivgeris *et al,* 2019). VIM remains the

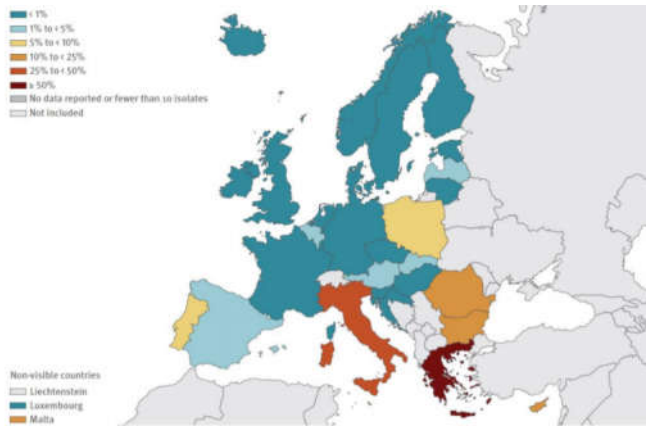


Figure 8. Percentage of carbapenem-resistant *K. pneumoniae* isolates from invasive infections (blood and cerebrospinal fluid) in EU/EEA, 2017. *Adapted from ECDC, 2018.*

predominant MBL in Italy, Spain and Hungary. CRE has a high potential to cause outbreaks in healthcare settings, e.g a large outbreak of NDM-producing CRE was described in Tuscany (Italy) during 2019 (ECDC, 2019). An inter-regional spread of NDM producers was also reported in Romania, Poland and Denmark. In some cases, the isolation of CPE has been linked to contact with healthcare in countries that are endemic for NDM-producers (e.g. India) (Albiger *et al,* 2015; van Duin and Doi, 2017). OXA-48-*like* carbapenemases have an endemic spread in Turkey, where they were described for the first time in 2001, and are quite common in Mediterranean countries, but also Belgium and Romania (Albiger *et al,* 2015; van Duin and Doi, 2017). KPC-producing *Enterobacterales* have been identified in almost every state of the United States but had a marginal role between all infections caused by *Enterobacteriaceae* with an overall estimated incidence of 2.94 CRE cases/100,000 person-year CRE in the 2012–2013 period, including both inpatients and outpatients. Of these, 47% was positive for a carbapenemase of the KPC family (Guh *et al,* 2015; van Duin and Doi, 2017). *Enterobacterales* isolates producing a carbapenemase other than KPC have remained an uncommon etiology of infections and often associated with health care exposures outside the US. For example, sporadic cases or limited outbreaks of *bla*$_{\text{NDM}}$ producing *Enterobacterales* have been reported. VIM-producing and IMP-producing CPE are even less common (van Duin and Doi, 2017). In South and Central America, a multinational observational study (Argentina, Colombia, Ecuador, Guatemala, Mexico, Peru and Venezuela), has revealed that KPC-producing enterobacteria are widespread: 21% of bloodstream infections caused by *Enterobacterales* was positive for a carbapenemase, and among them, 83% were KPC producers, 9% VIM and 8% NDM (Villegas *et al,* 2016). MBL-producing *Enterobacterales* have also been described from Brazil and from Columbia. However, detailed data on the occurrence of CPE in the Americas countries remain scarce.

The widespread of MBLs is of concern in Asia: NDM-positive enterobacteria were prevalent in India, Pakistan, and Bangladesh, not only isolated from patients, but also in public drinking water and wastewater (Walsh *et al,* 2011; van Duin and Doi, 2017). In the SMART study, of the 235 CPE isolates tested from India, 66 (28%) carried at least one carbapenemase gene, with *bla*$_{\text{NDM-1}}$ identified in 50% isolates including 2 isolates carrying also *bla*$_{\text{OXA-48-like}}$ gene (Lascols *et al,* 2011). In a more recent study from Mumbai, out of 111 CPE, 106 were NDM producers, 21 of which were positive also for an additional carbapenemase as well (Kazi *et al,* 2015). In contrast, *bla*$_{\text{KPC}}$ has been infrequently found in CRE from patients in India. On the other hand, KPC and NDM enzymes have also been reported in China, but NDM-producing enterobacteria appear to be

less widespread as compared to India (van Duin and Doi, 2017). For example, in a study from 2011, *bla*$_{KPC-2}$ was present in 71% of 109 ertapenem-resistant *K. pneumoniae* isolates, often in combination with CTX-M type ESBL enzymes (Chen *et al,* 2011; van Duin and Doi, 2017).

In the Middle East, the different carbapenemases had a heterogeneous distribution. At the beginnings of the 2000s, KPC-producing *K. pneumoniae* isolates were endemic and detected nationwide in Israel. However, a successful intervention resulted in a dramatic drop in hospital-acquired CPE cases from 55.5 to 4.8 cases/100,000 patient-days (van Duin and Doi, 2017). NDM and OXA-48-*like* enzymes are the major carbapenemases causing resistance in *Enterobacteriaceae* reported from the Gulf region and Saudi Arabia (Alotaibi F, 2019).

Limited data are available concerning the epidemiology of CPE in Africa. KPC producers have been poorly reported in Tanzania and South Africa, while MBL and OXA-48-*like* enzymes have been reported in various African countries. However, poor data about the current magnitude of the MBL-mediated CPE epidemic in Africa are available. NDM-producing CPE strains were recovered from Kenya, Nigeria, Egypt, Morocco, Algeria, Kenya, Cameroon and Tanzania and South Africa, and VIM-producing CPE from Nigeria and South Africa (van Duin and Doi, 2017). In a recent work of a Tunisian Hospital, 125 CRE isolates have been described: 82.4% was positive for *bla*$_{OXA-48-like}$ genes, followed by *bla*$_{NDM-1}$ (7.8%), *bla*$_{OXA-48-like}$ + *bla*$_{VIM}$ (4.9%), and *bla*$_{OXA-48-like}$ + *bla*$_{NDM-1}$ (4.9%) (Ben Helal *et al,* 2018).

1.4.3 Emergence of colistin resistance

Colistin belongs to the polymyxin family of antibiotics and was previously abandoned in human healthcare due to its nephrotoxicity and neurotoxicity. Colistin has been recently reintroduced as *last-resort* drugs against difficult-to-treat Gram-negative bacteria, including CRE. However, resistance among CRE isolates can develop commonly in hospitals and regions with the increasing use of colistin (ECDC, 2018). During 2012–2013, a global surveillance program showed that overall incidence of colistin-resistant *Enterobacterales* (excluding innate resistant species) was relatively lower and similar between Europe (1.8%), Latin America (1.5%), the Middle East-Africa (1.4%), North America (1.3%) and Asia-Pacific (1.3%) (Bradford *et al,* 2015). However, taking into account each country separately, higher resistance rates were detected in Greece (5.0%), Italy (4.7%) and Romania (3.3%) compared with 2–3% in Spain, The Netherlands, Hungary, Turkey, Brazil, Chile and Thailand and lower of 2% in the remaining participating countries (Bradford *et*

al, 2015; Giamarellou, 2016). The percentage of colistin-resistant isolates among CRE is more worrisome considering that these isolates are also resistant to almost all other antibiotics (ECDC, 2018). In Italy, some national surveillance studies described that 30-40% of carbapenemase-producing *K. pneumoniae* isolates weren't susceptible to colistin (Monaco *et al,* 2014; Giani *et al,* 2017). The main mechanism mediating to colistin resistance in enterobacteria involves modifications of the primary target of colistin which is lipid A moiety of lipopolysaccharide (LPS), through the addition of 4-amino-4-deoxy-l-arabinose and/or phosphoethanolamine molecules: these groups are positively charged and reduced the affinity between the cationic colistin and anionic LPS (Giamarellou H, 2016). These modifications are controlled by chromosomal mutations in the transcriptional regulatory systems (PhoP–PhoQ and PmrA–PmrB), producing the upregulation of the endogenous lipopolysaccharide modification systems, or by the acquisition of mobilized colistin resistance (*mcr*) genes, which encode for exogenous phosphoethanolamine transferases. In *K. pneumoniae*, the alterations leading to the inactivation of the *mgrB* gene, a negative feedback regulator of the PhoP–PhoQ system that controls the LPS modification system, play the main role in colistin resistance (Cannatelli *et al,* 2014). Discovery of the plasmid-encoded *mcr-1* gene in 2015, followed by the detection of other variants, has raised considerable concern in view of their potential of easily transmitting colistin resistance among clinical pathogens (Schwarz and Johnson, 2016). For example, a limited number of clinical isolates has resulted positive for both ESBL or carbapenemase genes and for *mcr-1* (Giamarellou H, 2016). The *mcr* gene was described for the first time in China from animal and human sources (Liu *et al,* 2016), and rapidly was detected worldwide, mostly in *E. coli* and to a lesser extent in *Salmonella*, *K. pneumoniae* and *Shigella sonnei* and from several foods (meat and vegetables), from the environment as well as from infected patients and asymptomatic human carriers including international travelers (Giamarellou H, 2016). The selection and spread of plasmid-mediated colistin resistance has been promoted by the extensive use of polymyxins in livestock as a growth promoter and therapeutic agents, especially for pigs and poultry (Sun *et al,* 2018). Fortunately, the spread of *mcr* genes in clinical settings is limited and regard mostly *E. coli* isolates that retain susceptibility to carbapenems or other antibiotics. However, surveillance studies are fundamental to monitor the evolution of their spread and of their virulence (Poirel *et al,* 2017).

1.5 AMR IN NON-FERMENTING GRAM-NEGATIVE BACTERIA

The rapid spread of carbapenem resistance in Gram-negative pathogens is a worrisome global health threat, not only between *Enterobacterales* (CRE), but also among non-glucose-fermenting Gram-negative bacilli. These organisms, which are intrinsically resistant to multiple antimicrobial classes, can cause difficult-to-treat (DTR) life-threatening infections, especially in patients with remarkable comorbidities, requiring frequently the usage of carbapenems as treatment. In the presence of carbapenem-resistant isolates, the therapeutic options are tragically limited to colistin and few other molecules with lower residual activity (Gniadek *et al,* 2016). Among non-fermentative Gram-negative bacilli, carbapenem-resistant *A. baumannii* complex (CRAb) and ESBL-producing *P. aeruginosa* are included by WHO in the list of pathogens against whom the introduction of new antibiotics is urgent (Tacconelli *et al,* 2018). Both of them are opportunistic pathogens mainly linked with healthcare-acquired pneumonia, central-line-associated bloodstream infections, urinary tract infections and wound infections. Indeed, the main risk factors are advanced age, extensive antibiotic use, presence of indwelling catheters or mechanical ventilation, previous health care exposure, immunocompromising conditions, extended hospital stay, major trauma or burn injuries (ECDC, 2018). Also, *Stenotrophomonas maltophilia,* an emerging opportunistic pathogen, showed DTR phenotype due to L1 and L2 carbapenemases, and an aminoglycoside acetyltransferase, especially in immunocompromised patients or patients under long-duration therapy with broad-spectrum antibiotics (e.g. cystic fibrosis) (Adegoke *et al,* 2017).

1.5.1 *Acinetobacter baumannii* complex

A baumannii complex is a group of species (*A. baumannii*, *Acinetobacter pittii* and *Acinetobacter nosocomialis*) that show intrinsic resistance to several antibiotics, due to their capacity to prevent various molecules from crossing their outer membrane. Consequently, they usually preserve susceptibility only to some antimicrobial families: fluoroquinolones, aminoglycosides, carbapenems, polymyxins and eventually to sulbactam and tigecycline (ECDC, 2018). However, the occurrence of chromosomic mutations or the acquisition of plasmid-encoded genes confers a multidrug phenotype. A recent European surveillance has reported that 43.2% of *A. baumannii* complex isolates from invasive infections were resistant at least to fluoroquinolones, aminoglycosides and carbapenems, with generally higher percentages described from the Baltic countries and from southern and south-eastern Europe (Figure 9) (ECDC, 2018). In Italy, a countrywide cross-sectional survey revealed a high percentage of CRAb, not only between clinical

isolates of *A. baumannii* from inpatients (45.7%) but also among outpatients (22.2%) (Principe *et al,* 2014).

In the United States, carbapenem resistance rates have been described to range from 34% to 62.6% with an increase in the period 2006-2010 (Sievert *et al,* 2013; Gniadek *et al,* 2016). The carbapenem-resistant phenotype is mainly due to the acquisition of genes encoding for class D carbapenemases: in particular, OXA-23, OXA-24/40, OXA-58, and OXA-143. In addition, each *A. baumannii* complex isolate has a $bla_{\text{OXA-51-like}}$ gene, chromosomally encoded, that if it is overexpressed by the upstream insertion of ISA*ba1*, confers carbapenems resistance (Principe *et al,* 2014). MBL enzymes are not the most commonly identified carbapenemases in *A. baumannii* even if IMP-, VIM-, SIM- or NDM-producing isolates has been described (Potron *et al,* 2015).

Figure 9. Percentage of invasive *Acinetobacter* spp. isolates with combined resistance to fluoroquinolones, aminoglycosides and carbapenems. *Adapted from ECDC, 2018.*

Spread of multidrug-resistant *A. baumannii* has been linked with epidemic clones: three out of eight, labeled as international clonal lineages IC1-3, have been predominantly described in Europe and adjacent regions (ECDC, 2016). *A. baumannii* IC2 is by far the most globally spread (USA, Europe, Israel, Asia, Australia and South Africa) in association with nosocomial outbreaks and multidrug resistance, while IC5, IC7 and, to a lesser extent, IC1 are the predominant clones in Latin America (Higgins *et al,* 2010; Levy-Blitchtein *et al,* 2018).

1.5.2 *Pseudomonas aeruginosa*

P. aeruginosa is commonly associated with nosocomial infections in immunocompromised individuals, while it is rarely linked with causing chronic infections in previously healthy patients. *P. aeruginosa* strains are able to cause a wide spectrum including respiratory infections, which in cases of chronic diseases such as cystic fibrosis can widen the lung damage leading to an increased mortality, severe cases of urinary tract infection, bloodstream infections, osteoarticular infections, ear infections, skin infections especially in patients with severe burns (Streeter and Katouli, 2016). The antimicrobial drugs used for the treatment of *P. aeruginosa* infections include some β-lactams

such as piperacillin-tazobactam, ceftazidime, cefepime, ceftolozane-tazobactam, ceftazidime-avibactam and carbapenems, fluoroquinolones, aminoglycosides, and colistin.

P. aeruginosa is not only intrinsically resistant to a wide range of antibiotics but is also inclined to develop resistance to antipseudomonal agents through the selection of mutations in chromosomal genes or by the horizontal acquisition of exogenous resistance genes (Ruiz-Garbajosa and Cantón, 2017). Considering recent data from U.S. nationwide surveillances, roughly 19.3% of *P. aeruginosa* isolates showed a resistant or intermediate phenotype to at least one carbapenem, while approximately 14% of isolates resistant to at least one antipseudomonal agent were MDR (Nguyen *et al,* 2018). In Europe, during 2017, the proportion of *P. aeruginosa* isolates from invasive infections that were non-susceptible to at least one antibiotic class between fluoroquinolones, piperacillin/tazobactam, carbapenems and aminoglycosides were of 20.3%, 18.3%, 17.4% and 13.2%, respectively. A large variability of percentages was present among different countries in EU/EEA with generally higher values of resistance reported in southern and south-eastern Europe (Figure 10) and with a significant decrease between 2014 and 2017 (ECDC, 2018). A multicenter study on *P. aeruginosa* bloodstream and respiratory infections in Italy found that 37.2% of the isolates exhibited an MDR phenotype, 34.7% of all isolates were non-susceptible to meropenem, and 5,1% was positive to produce carbapenemases. Ceftolozane/tazobactam was the most active, followed by amikacin and colistin (Giani *et al,* 2018).

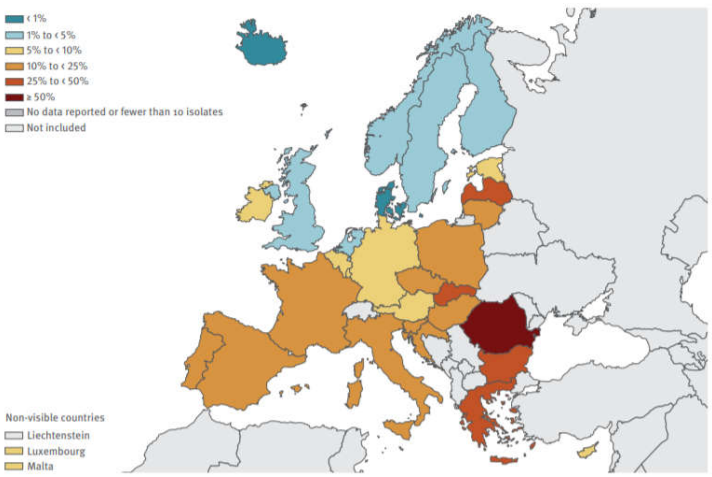


Figure 10. Percentage of P. aeruginosa invasive isolates with resistance to three or more antimicrobial groups among piperacillin/tazobactam, ceftazidime, fluoroquinolones, aminoglycosides and carbapenems. *Adapeted from ECDC, 2018.*

Concerning β-lactams, the main resistance mechanisms include the hyperproduction of the chromosomally encoded AmpC enzymes combined with efflux pump overexpression (MexA-MexB-OprM), the reduction in membrane permeability mediated by the repression or inactivation of the carbapenem porin OprD, penicillin-binding protein mutations and/or the acquisition of plasmid-mediated resistance genes coding for

carbapenemase (Giani *et al,* 2018). The most commonly detected carbapenemases in *P. aeruginosa* are MBLs with VIM and IMP types being the most widely distributed, while class A carbapenemases (mainly KPC) are rarely reported (Ruiz-Garbajosa and Cantón, 2017).

Molecular epidemiology studies have highlighted the existence of high-risk clones that grouped the majority of interhospital-disseminated MDR *P. aeruginosa* isolates: the ST111 and ST235 clones have been described worldwide, while ST175 clone is confined to Europe (Ruiz-Garbajosa and Cantón, 2017).

1.6 ANTIBIOTIC RESISTANCE IN *STAPHYLOCOCCUS AUREUS*

Gram-positives pathogens are one of the most important causes of both community and healthcare-associated infections. Some of them are frequently related to community infections, such as *Streptococcus pneumoniae* and beta-hemolytic streptococci, while others are usually linked to nosocomial infections, for instance, enterococci and coagulase-negative staphylococci (Tong *et al,* 2015). *Staphylococcus aureus* is the most important Gram-positive pathogen in the clinical and epidemiological landscape. It is both a commensal bacterium and a human pathogen. In fact, approximately 20-30% of the human population is colonized with *S. aureus*, without developing any disease *(*Wertheim *et al,* 2005). At the same time, *S. aureus* is the etiological agent of many infections: it is a leading cause of bacteremia, infective endocarditis, osteoarticular, skin and soft tissue, pleuropulmonary, and device-related infections. Besides, the clinical problem is twofold, because of the outbreak and spread of methicillin-resistant strains, which means resistance to β-lactam antibiotics (Tong *et al,* 2015).

1.6.1 Mechanisms of resistance to β-lactams

The main mechanisms of β-lactams resistance in *S. aureus* are the production of a typical serine β-lactamase, BlaZ, which is responsible for the inactivation of penicillin, and the production of PBP2 homologs which are not susceptible to methicillin and oxacillin.

BlaZ β-lactamases, which are strategically located on the outer face of the cytoplasmic membrane, hydrolyze penicillin to protect PBP2s. These β-lactamases are encoded by *blaZ* gene that is carried by the Tn*552-like* transposons, located on plasmids or integrated into the bacterial chromosome (Foster *et al,* 2017).

Resistance to virtually all β-lactam antibiotics in methicillin-resistant *S. aureus* (MRSA) isolates is conferred by the acquisition of a mobile genetic element, the staphylococcal cassette

chromosome (SCC*mec*) carrying the *mecA* gene, or rarely the *mecB* or *mecC* genes, which encode an altered PBP – PBP2a/PBP2′ – with reduced affinity for β-lactam antibiotics. Consequently, cell wall synthesis can continue even in the presence of β-lactam antibiotics (Paterson *et al,* 2014; Becker *et al,* 2018). The expression of *mec* genes is regulated by MecIR regulatory proteins which are homologous to the BlaIR proteins that regulate BlaZ and can also modulate the *mec* genes. Thus, the nature of the expression of PBP2a can vary from strain to strain depending on the presence of functional Mec and Bla regulators (Foster *et al,* 2017).

SCC*mec* elements includes three basic genetic features: i) the *ccr*-gene complex, which is composed of one or two genes for recombinases (*ccrAB* or/and *ccrC)* and of surrounding ORFs; ii) the *mec*-gene complex encoding methicillin resistance determinants, regulatory genes *mecIR,* insertion sequences and surrounding ORFs; iii) the joining region (J region) in which *mec*-gene and *ccr*-gene complex are inserted. The entire SCC element is flanked by inverted repeated regions (IRs). In response to different environments and the pressure of antimicrobial selection, *ccrAB* or/and *ccrC* can mediate the excision and integration of SCCmec to the chromosome of *Staphylococcus* strains (at the 3′ end of the origin of replication *orfX*) and also can mediate the insertion of multiple antibiotic-resistant and heavy metal-resistant genes into SCC*mec* by site-specific recombination. SCC*mec* elements are classi ed into 13 types (I-XIII) and into the corresponding subtypes according to the different kinds of *ccr*-gene complex (8 allotypes), the different order of the elements that are part of the *mec*-gene complex (5 classes), and the disposition of *ccr*-gene and *mec*-gene complexes in the J region (J1, J2, J3) (Liu *et al,* 2016; Baig *et al,* 2018).

1.6.2 Epidemiology of MRSA

The first MRSA isolate was found at the beginning of 1960s shortly after the introduction of methicillin for treatment of penicillin-resistant *S. aureus* and in less than 10 years several sporadic hospital outbreaks related to MRSA occurred in Australia, Western Europe and the USA. Thenceforth until the mid-1990s, hospital-acquired infections caused by MRSA (HA-MRSA) increased continuously, making it an endemic pathogen, while community-associated MRSA (CA-MRSA) strains have gradually been described among groups of patients with no apparent connection to hospitals at the end of the 90s. Surprisingly, CA-MRSA continued to raise with a higher rate than HA-MRSA, which instead presented a steady isolation rate from 1998 to 2008, indicating the transfer of HA-MRSA clones into the community. Furthermore, MRSA has been isolated from many foods, livestock sources and occupational staff linked with colonization, but

also with staphylococcal food poisoning cases, zoonotic pneumonia, endocarditis, and necrotizing fasciitis (Liu *et al,* 2016).

Currently, the prevalence of invasive infections due to MRSA differs widely around the world with higher values in American and Asian countries (Figure 11). In China, a marked decrease in the methicillin resistance rate was seen from 69% in 2005 to 44.6% in 2014 (Hu *et al,* 2016). A significantly decreasing trend was also reported in the US between 2005 to 2016 (Kourtis *et al,* 2019). During 2005-2012 rates of hospital-onset MRSA bloodstream infection decreased by 17.1% annually, but the decline slowed during 2013-2016. Despite reductions in the incidence of MRSA, *S. aureus* infections account for significant mortality in the United States with nearly 20 000 deaths out of 119 000 *S. aureus* bloodstream infections in 2017 (Kourtis *et al,* 2019). In Europe, almost a third of the countries reported significantly decreasing trends during the period 2014 and 2017. However, 9 out of 30 EU/EEA countries, located in southern and southern-east, have reported that 25% or more of *S. aureus* invasive isolates were MRSA compared to a European average of 16.9% (ECDC, 2018).

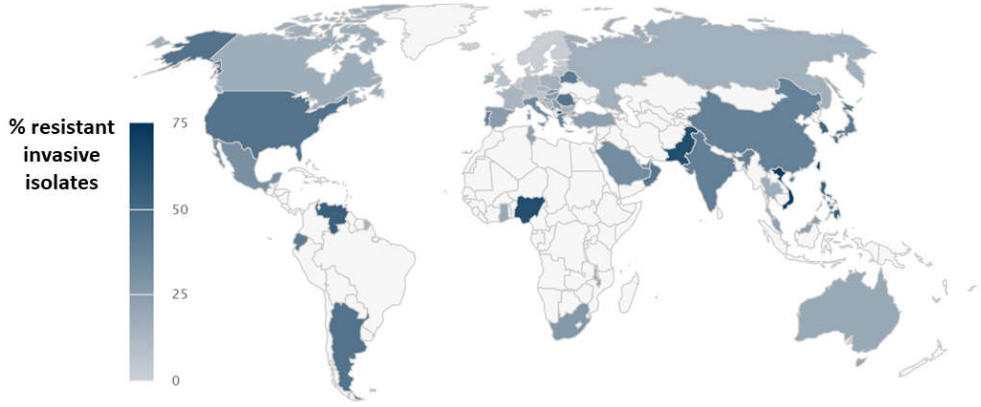


Figure 11. Worldwide distribution of invasive MRSA isolates. *Adapted from The Center for Disease, Dynamics Economics & Policy. ResistanceMap: Antibiotic resistance. 2019. https://resistancemap.cddep.org/AntibioticResistance.php. Date accessed: October 1, 2019.*

The most frequently reported clones are clustering primarily in 2 clonal complexes (CC5 and CC8) which are closely associated genetically. These HA-MRSA clones have evolved several times and have spread worldwide. For example, SNP analysis of CC5 (ST5) validates that this clone evolved multiple times in several countries via the acquisition of the SCC*mec* cassette in a local CC5 (ST5) methicillin-susceptible strain (Lakhundia S and Zhang, 2018). On the other hand, phylogenetic analysis revealed the intercontinental diffusion and hospital transmission of CC8-ST239 isolates

through North America, Europe, Latin America, and Asia. In addition, in the 1990s, the CC8-ST239 subgroup dispersed from Latin America to Europe and from Thailand to China (Lakhundia and Zhang, 2018). In particular, the CC8-ST239 subgroup, CC5 (ST5), and CC22 (ST22) are the most frequently reported CCs in Asian countries (Song *et al,* 2011), while in Africa and in Latin America the CC8 (ST239 subgroup), CC5 (ST5), and CC30 (ST36) lineages predominate (Rodríguez-Noriega *et al,* 2010; Lakhundia and Zhang, 2018). ST239-III and ST5-II are both the major HA-MRSA clones and their presence in the community confirms the exchange in ecological niches of HA-MRSA and CA-MRSA clones. CC30-ST36 is common in the United States and the United Kingdom and CC45 are common in the United States (ST45-II) and Europe (ST45-IV/V) (Lakhundia and Zhang, 2018).

CA-MRSA clones are dispersed in many clonal complexes and many of them are not interrelated suggesting that they evolved in different ways in separate geographical areas (Lakhundia and Zhang, 2018). The most common CA-MRSA clones in North America are USA300 (ST8-IV) and USA400 (ST1-IV) (CDC, 1999). USA300 rapidly superseded USA400, became endemic and has sporadically also been reported in Europe (Kennedy *et al,* 2008; Lakhundia and Zhang, 2018). Moreover, the PVL-producer USA300 virulent clone has rapidly spread in the hospital setting contributed to the problem of HA-MRSA (Seybold *et al,* 2006). Indeed, in recent years the distinction between CA-MRSA and HA-MRSA has somewhat unclear and some CA-MRSA and HA-MRSA clones overlap each other or interchange their epidemiological niches (Lakhundia and Zhang, 2018). In Europe CA-MRSA have remained sporadic compared to those in the United States. ST80 (CC80) is the most principal circulating clone, except for the United Kingdom, where EMRSA-15 (ST22) and EMRSA-16 (ST36) are more frequent of ST80 clone both in hospital and in community. A livestock-associated MRSA, ST398, has been described in Europe and transmitted to humans (Rollason *et al,* 2008; Lakhundia and Zhang, 2018). In the community, ST59 is the dominant CA-MRSA clone in East Asia, followed by ST30, ST239, and ST5: the first two spread from the community to hospitals while these latter are HA-MRSA clones moving in the opposite direction (Song *et al,* 2011).

1.6.3 New challenges: resistance to vancomycin, daptomycin and linezolid

S. aureus was the main Gram-positive pathogen to pose the problem of antibiotic-resistance, because of the outbreak and spread of methicillin-resistant strains, which means resistance to β-lactam antibiotics. MRSA represents the archetype of multi-drug resistant pathogens and its spread

at first in the hospitals and then in the community still is considered an issue in terms of rapid diagnosis and appropriate antimicrobial treatment. Even if in some countries, a decreasing incidence of MRSA had been obtained, thanks to campaigns aiming at preventing and controlling these infections, overall MRSA remains one of the main antibiotic-resistance related pathogens with a high incidence in many countries, including Italy (Viaggi *et al,* 2019). This means that it is necessary to adopt an empirical-therapy based on anti-MRSA drugs (i.e. glycopeptides, daptomycin, oxazolidinones) whenever an invasive infection due to *S. aureus* is suspected (Choo and Chambers, 2016; Viaggi *et al,* 2019). Fortunately, resistance to these antibiotics in MRSA is still sporadic, even if several reports have described the emergence of resistance during therapy (Zhang *et al,* 2015; Liu *et al,* 2011; Gu *et al,* 2013).

Resistance to vancomycin in *S. aureus* (VRSA) can be mediated by the acquisition of plasmid-encoded *van* genes or the occurrence of multiple mutations in chromosomal genes that affect cell wall biosynthesis and homeostasis. The *van* genes encode enzymes able to replace the D-Ala-D-Ala moieties of peptidoglycan precursors with D-Ala-D-lactate groups which have a reduced affinity for vancomycin (Meziane-Cherif *et al,* 2014). However, *van*-positive MRSA isolates have been described rarely and have not established in the hospital setting, probably because *van* genes involve high fitness costs (Foster *et al,* 2017). On the other hand, a common form of treatment failure with vancomycin is due to multiple mutations in chromosomal genes that confer a phenotype known as vancomycin-intermediate *Staphylococcus aureus* (VISA) with a vancomycin MIC of 4–8 mg/L, according to CLSI breakpoint (Foster, 2017; CLSI, 2019). The evolution of VISA requires a multistep process including hetero-VISA (h-VISA) strains which are a subpopulation of cells with a MIC of 4–8 mg/L within the susceptible population (MIC≤2 mg/L) (Foster, 2017). hVISA and VISA strains show alterations in the thickness and structure of the cell wall that prevents vancomycin to reach its target site (Foster, 2017). hVISA and VISA infections are frequently associated with persistent infections, protracted bacteremia, and longer hospitalization. Among MRSA, the prevalence of hVISA and VISA was estimated at 6.05% and 3.01%, respectively, with higher values in Asia than in Europe or America. The most prevalent genotype was SCC*mec* II and the Sequence Type 239 (Zhang *et al,* 2015).

Daptomycin (DAP) can be an alternative for the treatment of MRSA infections caused by vancomycin-reduced susceptibility strains, except for pulmonary infections because of hypersensitivity reactions and myopathies (Duplessis and Crum-Cianflone, 2011; Liu *et al,* 2011). The DAP non-susceptibility in *S. aureus* is the result of mutations in several genes (*mprF,*

dltABCDF, yycG, walKR, vraS) that determine an increase of cell membrane positive charge as well as cell wall perturbations (Sabat *et al,* 2018). Also, in this case, the high fitness cost associated with resistance may explain the reduced diffusion of daptomycin resistance (Rosh *et al,* 2010).

Linezolid, an oxazolidinone class of antibiotics, was approved in 2000 for the treatment of MRSA infections (Watkins *et al,* 2012). Although linezolid-resistant MRSA (LR-MRSA) strains have been rapidly reported since its introduction in clinical practice, linezolid has remained active against more than 98% of staphylococci (Gu *et al,* 2013). In MRSA, the most common mechanisms for oxazolidinone resistance are mediated by chromosomal mutations in genes encoding the 23S rRNA (e.g. G2576T) or L3 and L4 ribosomal proteins, or by the acquisition of the *cfr* gene encoding a ribosomal methyltransferase (Gu *et al,* 2013; Antonelli *et al,* 2016 A). The latter mechanism poses significant challenges to the clinical treatment of these infections because it is transferable and confers also resistance to phenicols, lincosamides, pleuromutilins, streptogramin A and some macrolides (Antonelli *et al,* 2016 A).

Nevertheless, even if finding of MRSA strains highly resistant to glycopeptides, daptomycin, and/or linezolid is sporadic and pharmaceutical industries have recently introduced several new anti-MRSA drugs (glycopeptides and cephalosporins), it is important to strengthen the surveillance of these strains to limit eventually outbreaks and to provide the clinician with useful data for a correct antibiotic *steward-ship* (Viaggi *et al,* 2019).

1.7 ROLE OF RAPID DIAGNOSTICS IN MANAGING MDR BACTERIA

Microbiological diagnostics play a fundamental role in combating resistant pathogens causing difficult-to-treat infectious diseases (Tsalik *et al,* 2017). Nowadays, the treatment of infections related to MDR bacteria is one of the most important challenges for clinicians worldwide, also because resistance may remain unrecognized until the identification of the causative agent and antimicrobial susceptibility testing. It means that the risk of delaying the initiation of effective antibacterial therapy is concrete, with potentially unfavorable consequences in terms of survival (Giacobbe *et al,* 2019).

In this scenario, the desirable features of diagnostic tests are the capacity to rapidly detect or exclude bacterial infection, accurately identify bacterial pathogens, and/or guide the selection of antibacterial agents. The faster, better, and less-expensive versions of existing diagnostics represent clearly advances (Tsalik *et al,* 2017). In this perspective, the reduction of time to response mediated by rapid diagnostic tests will improve on one hand the outcome of patients with MDR bacteria-related infections, and on the other hand will help to avoid misuse of last-resorts antibiotics, according to the antimicrobial stewardship principles. An efficient diagnostic reduces the duration of empirical therapy and allows clinicians to set up a targeted therapy. However, rigorous evidence about the impact on actual rapid tests-driven and not only on turnaround time, and on relevant outcomes in patients with MDR bacteria-related infections is still fragmentary and difficult to interpret (Giacobbe *et al,* 2019).

The standard approach of microbiological diagnostics expects the isolation of bacteria in culture from the clinical samples taken from infection sites. Several selective and chromogenic growth media, useful to identify strains with specific traits from the samples, are now available (Perry JD. 2017). They allow overcoming previous time-consuming methods, that, for example, for the screening of colonization expected an initial stage on selective enrichment broth followed by the subculture of the selected strains (Viau *et al,* 2016). The identification can be achieved by biochemical profile or by MALDI-TOF mass spectrometry. Then, in most cases, it is necessary to assess the susceptibility of bacteria to drugs and to set up, accordingly, a specific therapy. Antimicrobial susceptibility testing (AST) can be performed with the reference broth microdilution (BMD) or, more often, with several commercial systems. Nevertheless, it should be considered that the accuracy of some systems is reduced for certain antibiotics assay (Matuschek *et al,* 2018; Camarlinghi *et al,* 2019).

The conventional approach requires relatively long response times raging usually between 24 and 72 hours from the sample collection, depending on the growth time of microorganisms. (Viaggi *et al,* 2019) Thus, in the case of serious infections as those of bloodstream, it is not possible to wait for the results of the bacterial cultures and of AST before starting the antibiotic therapy which must be necessarily prescribed on an empirical basis and may be subsequently revised (Del Pozo JL, 2019). Moreover, culture-dependent methodologies have a reduced sensitivity compared to molecular methods, especially in cases of previous antimicrobial therapy. For these reasons, diagnostic laboratories flank the conventional methodologies with novel rapid diagnostic methods, which have risen with the aim to improve therapeutic decisions and antimicrobial stewardship (Buehler *et al,* 2016). Innovative diagnostic methods are based on the direct research of DNA/RNA molecules, bacterial proteins or bacterial cells in clinical samples, positive blood cultures or in bacteria isolates.

Molecular diagnostics are based on specific targets of the bacterial genome used for the identification of species and for the definition of the resistance genes content. These technologies are mainly nucleic acid amplification techniques (NAAT) and microarrays, consisting of amplification of specific genetic targets followed by hybridization with oligonucleotide probes or microarray of oligonucleotide probes for target identification (Giacobbe *et al,* 2019). Commercial systems (e.g. Verigene BC-GN – Luminex; FilmArray BCID - BioFire Diagnostics; Unyvero System - Curetis GmbH) based on these technologies are currently available with different syndromic panels, (each one with a narrow spectrum of microorganisms and of resistance mechanisms) for rapid diagnostics of positive blood cultures, but also for lower respiratory tract infections and soft tissue infections (Giacobbe *et al,* 2019; Viaggi *et al,* 2019). Anyway, it is necessary to consider that conventional antibiogram and the molecular one, are not equivalent, since an antibiotic resistance gene, even if present, is not necessarily expressed. Moreover, the molecular antibiogram doesn't provide MIC values for the different antibiotics (Arena *et al,* 2017). Hence the need to integrate conventional and innovative techniques. Despite these limitations, indeed, molecular diagnostic methods are crucial for the rapid identification of MDR pathogens and of its resistance determinants. In the case of positive blood cultures, molecular techniques are able to quickly identify (1-2 hours) the main pathogens responsible for sepsis and the most important types of β-lactamase, leading to adjust the empirical therapy previously set. In addition to the reduction of time to response, the rapid identification of specific resistance mechanisms will be crucial in the near future, because of the specific activity of some novel agents against different

types of resistance mechanisms (Giacobbe *et al,* 2019). Several molecular systems have been developed over the years to detect the microorganisms directly on whole blood in order to obtain results in just a few hours after the blood draw (e.g. T2 Biosystems). However, they have generally shown variable suboptimal sensitivity or suboptimal specificity (Giacobbe *et al,* 2019). Among molecular techniques, fluorescent in situ hybridization (FISH) differs from the other methods mentioned for the use of specific binding of fluorescent probes of nucleic acids to complementary sequences of the bacterial 16S rRNA, with binding observed through a fluorescent microscope (Giacobbe *et al,* 2019). Some molecular diagnostic methods, defined *near-patient,* are suitable to be used in those structures lacking a diagnostic laboratory since they are completely automated and require low expertise to be set up (Viaggi *et al,* 2019). Recently, new immunochromatographic techniques have been introduced to identify within a few minutes and inexpensively the presence of resistance enzymes (some type of carbapenemases, ESBLs and MCR) from bacterial growth or directly from positive blood cultures (Hamprecht *et al,* 2018; Riccobono *et al,* 2018).

Among phenotypic or hybrid molecular/phenotypic methods, novel diagnostic systems include *light scattering technology* (e.g. ALFRED60 – Alifax) which provides rapid automatic AST (3-5 h after positivity of blood cultures) recording turbidity of growing bacteria in liquid media starting from monomicrobial samples or clinical isolates (Giacobbe *et al,* 2019), and *automated time-lapse microscopy* (Accelerate Pheno™ system - Accelerate Diagnostics), which combines FISH with time-lapse microscopy in order to determine both identification (1.5 hours) and rapid phenotypic AST (7 hours) starting from positive blood culture. Indeed, it is an automated system that uses FISH in order to identify the bacteria, and a particular type of imaging of individual bacterial cells to obtain growth curves in presence of different concentrations of antibiotics with the aim to interpolate their MICs (Pancholi *et al,* 2018).

However, the main limitations to the large use of these advanced diagnostic methodologies are the high costs and qualified personnel availability. For this reason, their implementation should be done taken into account the general principles of diagnostic stewardship which deals with the appropriate placement of both conventional and new diagnostic technologies in the laboratory workflow to maximize the impact of diagnostics on clinical outcomes and at the same time ensure maximum efficiency of diagnostic processes (Messacar *et al,* 2017). Finally, it is important to define prioritized access to these diagnostics for specific patients' categories and wards, according to the local epidemiology and the peculiar antimicrobial stewardship needs (Giacobbe *et al,* 2019).

1.8 NOVEL ANTIMICROBIAL DRUGS

One-half of antibiotics used either singly or in combination for the treatment of bacterial infections were discovered in the period 1950-1960 (known as the "Golden Age") and have lost patent protection leading to their overuse and misuse because of their low cost (Davies, 2006).

Over the years, on the one hand, there has been an increase in the spread of resistant bacteria and on the other, since the beginning of the 1980s, a steady decline in the number of new antimicrobial molecules put on the market to reach only two specialties approved by the FDA for the period 2008–2012 (Duval *et al,* 2019). Moreover, at the end of the 1980s, the research focus was on the development of narrow-spectrum antibiotics to tackle what at the end seemed the most worrisome threat, mainly in the United States: the possibility of transfer and diffusion of vancomycin-resistance determinants in MRSA (Duval *et al,* 2019). This clear imbalance has determined that the current *armamentarium* against Gram-positive pathogens, especially against staphylococci, is more abundant than the therapeutic options available for multidrug-resistant Gram-negatives (Duval *et al,* 2019).

The nosocomial spread of carbapenem-resistant *Enterobacterales* and multidrug-resistant isolates of *P. aeruginosa* and *Acinetobacter* spp, with few treatments to combat them and the threat of the development of pan-resistance, has forced clinicians to recover as "*last resort"*" the use of older antibiotics (polymyxins and fosfomycin trometamol), which were abandoned because of their toxicity concerns and/or their tendency to evolve resistance (Wrigh *et al,* 2017).

In this scenario, it is imperative to find new drugs and alternative ways to treat infections. The Centers for Disease Control and Prevention, the WHO, and U.S. and European governments have adopted measures to stimulate pharmaceutical companies to develop new antibiotics including economic incentives, the formation of public-private partnerships, legislative and regulatory changes promoting a streamlined review process ("*fast track*") for new antibacterial agents (Fernandes and Martens, 2017**;** Wrigh *et al,* 2017). These measures have produced the first significant results progress with the approval of several novel agents (i.e., ceftolozane/tazobactam, ceftazidime/avibactam, meropenem/vaborbactam), while others are in late clinical development (i.e., cefiderocol or cefepime/VNRX-5133) (Table 2) (Wrigh *et al,* 2017). The combination of new β-lactams with old β-lactamases inhibitors (BLIs) or vice-versa established antibiotics with new BLIs is the most general approach adopted, followed by the modification of the chemical structure of old antibiotics to circumvents antibacterial resistance mechanisms (e.g. ceftolozane and

cefiderocol among cephalosporins, plazomicin and eravacycline between aminoglycosides and tetracycline, respectively) (Vila *et al,* 2019). Other therapeutic strategies that are currently under trial include phage and enzybiotics therapy, the use of antimicrobial peptides, photodynamic therapy, antibacterial antibodies and nanoparticles as antibacterial agents (Mulani *et al,* 2019; Vila *et al,* 2019).

Table 2. Antimicrobial activity of new antibiotics against Gram-negatives.

Drugs	ESBL-E	CPE	MDR *P. aeruginosa*[c]	MDR *Acinetobacter* spp.	FDA/ EMA approval
Ceftolozane/ tazobactam	Active	Not active	Active, except for carbapenemase-producers	Not active	FDA & EMA
Ceftazidime/ avibactam	Active	Active against Class A, C, and D (OXA-48-*like*) β-lactamases producers	Active against Class A, C and D (OXA-48-like) β-lactamases producers	Not active	FDA & EMA
Aztreonam/ avibactam	Active	Active against Class A, B, C and D (OXA-48-*like*) β-lactamases producers	Active	Not active	Clinical trials
Imipenem/ relebactam	Active	Active against Class A and C β-lactamases producers	Active against Class A and C β-lactamases producers	Not active	FDA
Meropenem/ vaborbactam	Active	Active against Class A and C producers	Not active	Not active	FDA & EMA
Cefepime/ VNRX-5133	Active	Active	Active (except IMP-producers)	No data	Clinical trials
Cefepime/ zidebactam	Active	Active	Active	Active	Clinical trials
Cefiderocol	Active	Active	Active	Active	Clinical trials
Plazomicin[a]	Active	Active	Active	Limited activity	FDA
Eravacycline[b]	Active	Active	Not active	Active	FDA & EMA

ESBL-E: Extended Spectrum β-lactamase-producing *Enterobacterales*; CPE: carbapenemase-producing *Enterobacterales;* MDR: Multi-drug resistant (including carbapenemase-producers); FDA: U.S. Food and Drug Administration; EMA: European Medicines Agency. *References are present in the next paragraphs, except for* [a] *Castanheira et al, 2018 and* [b] *Lee and Burton, 2019.*[c]The antibiotic activity could be affected by the overexpression of efflux systems.

1.8.1 Combination of established β-lactams with new β-lactamase inhibitors

A strategy to overcome the resistance due to the production of β-lactamases is the use of BLIs which are usually molecules without antimicrobial activity but able to inhibit these enzymes and protect β-lactam drugs from the hydrolyzation. The old-generations of BLIs such as tazobactam, clavulanate, and sulbactam, have a molecular structure with a β-lactam ring and are active against class A β-lactamases, excepted for carbapenemases, but are generally less effective against class B, C, and D β-lactamases. They are administered in association with penicillins (ampicillin, amoxicillin or piperacillin) or cephalosporines (ceftolozane) (Drawz and Bonomo, 2010). Significant improvement has been obtained with the development of new β-lactamase inhibitors active against certain extended-spectrum β-lactamases and carbapenemases. According to their molecular structure, novel BLIs can be classified in three main groups: i) the diazabicyclooctane group (i.e., avibactam or relebactam) which inhibits class A, class C, and some class D enzymes, but does not show inhibition of class B MBLs; ii), the boronate BLI group (i.e., vaborbactam and VNRX-5133); iii) the bicyclo-acyl hydrazide group (i.e., zidebactam) (Moya *et al*, 2017; Krajnc *et al,* 2019; Vila *et al,* 2019).

The use of **avibactam** combined with ceftazidime (CAZ-AVI) was approved to treat hospital-acquired pneumonia (HAP), intra-abdominal infections, complicated urinary tract infections, and infections caused by aerobic multidrug-resistant Gram-negative organism with limited treatment options (Shirley, 2018). CAZ-AVI showed a potent *in vitro* activity against *Enterobacterales* producing Ambler class A and C β-lactamases and some Ambler class D enzymes (OXA-48-*like*) and a non-inferiority to the best available therapy in several clinical trials (RECLAIM1-2; RECAPTURE; REPRISE; REPROVE) (Wright *et al,* 2017; Stone *et al,* 2018). Despite its recent introduction, resistance to CAZ-AVI has been reported, either following treatments with CAZ-AVI (Haidar *et al,* 2017 A; Giddins *et al,* 2018; Shields *et al,* 2018) or even in absence of previous exposure to the drug (Humphries *et al,* 2015; Castanheira *et al,* 2017; Gaibani et al 2018). The CAZ-AVI resistance has been most commonly attributed to missense mutations in the KPC enzyme (e.g. D179Y; L169P; T243M; EL165-166; V240G/A; H274Y), which were associated with a decreased activity against carbapenems and other β-lactam antibiotics (Shields *et al,* 2018; Giddins *et al,* 2018; Gaibani et al 2018). Moreover, the combination of multiple mechanisms including nonsense or missense mutations in OmpK35 and OmpK36 porins, increased efflux activity (i.e. mediated by mutations in *ramR* regulator of the *acrAB* efflux system) and increased expression of

KPC or even SHV β-lactamases (i.e., mediated by the presence multiple plasmids carrying *bla*$_{KPC}$ and *bla*$_{SHV-12}$) has been associated with a reduced susceptibility to CAZ-AVI (Castanheira *et al,* 2017; Nelson *et al,* 2017). Avibactam cannot efficiently inhibit MBLs (Ambler class B), and these β-lactamases form one of the most common mechanisms of resistance to CAZ-AVI in *Enterobacterales* (Wright *et al,* 2017). However, the combination of avibactam with aztreonam showed a good activity also against MBL-producing *Enterobacterales,* since MBLs cannot efficiently hydrolyze aztreonam and avibactam can inhibit the other eventually β-lactamases such as AmpC CTX-M and CMY (Vasoo *et al,* 2015; Vila *et al,* 2019). *P. aeruginosa* isolates showed higher aztreonam-avibactam MIC90 values than *Enterobacterales,* while no *in vitro* activity was observed against *A. baumannii* isolates (Wright *et al,* 2017).

Unlike avibactam, **relebactam** has inhibitory activity only against class A and class C β-lactamases, while cannot inhibit class D enzymes. In combination with imipenem/cilastatin (Recarbrio), it has been approved by U.S. FDA (NDA 21281) to treat adults with complicated urinary tract infections (cUTI) and complicated intra-abdominal infections (cIAI), caused by Gram-negative pathogens against whom there are limited treatment options. Several studies reported a good *in vitro* activity against *P. aeruginosa,* MDR isolates included, and carbapenemase-producing *Enterobacterales (*Livermore *et al,* 2013), while this combination resulted in ineffective to restore imipenem susceptibility in *Acinetobacter* spp (Karlowsky *et al,* 2018). Imipenem-relebactam was active also against CAZ-AVI resistant strains positives for mutations in KPC-3. In conclusion, imipenem-relebactam, and CAZ-AVI had overlapping spectra of activity and niches in which each was superior. Such as for CAZ-AVI, the resistance to Recarbrio can be mediated by alterations of porin status (especially OmpK36) (Haidar *et al,* 2017 B).

Vaborbactam derives from boronic acid and inhibits many class A, class C, whereas it does not inhibit class D or class B carbapenemases. This reversible inhibitor was designed to interact with serine β-lactamases and in particular KPC, also in the presence of mutations that affect avibactam activity (Wright *et al,* 2017; Patel *et al,* 2018). When combined with meropenem, vaborbactam at 8 mg/L restores the activity of this carbapenem in KPC-producing *Enterobacterales* (Lomovskaya *et al,* 2017), but has little effect on *A. baumannii* containing OXA-type carbapenemases or *P. aeruginosa* (Wright *et al,* 2017). Meropenem/vaborbactam (Vabomere) has been recently approved by FDA (NDA 209776) and EMA (657766/2018) to treat cUTI, CIAI, lung infections caught in the hospital caused by Gram-negative bacteria when other treatments might not work. Vaborbactam crosses the outer membrane of *K. pneumoniae* using both OmpK35 and OmpK36,

but OmpK36 is the preferred porin, while the multidrug resistance efflux systems don't affect by vaborbactam activity (Lomovskaya *et al,* 2017). Indeed, KPC-producing *K. pneumoniae* isolates with *ompK36* mutations has displayed higher meropenem-vaborbactam MICs than isolates with wild-type *ompK36* (Wilson *et al,* 2019).

VNRX-5133 (VenatoRx Pharmaceuticals, Inc.) is a novel broad-spectrum β-lactamase inhibitor, derived from boronic acid, actually under evaluation able to inhibit the activity of Ambler class A, B, C and D β-lactamases (Krajnc *et al,* 2019) in Gram-negative bacilli, including carbapenem-resistant *Enterobacteriaceae* and *P. aeruginosa* (Bush and Bradford, 2019). The combination of cefepime and VNRX-5133 demonstrates potent *in vitro* activity against CRE clinical isolates, including KPC-variants and MLB-producing CRE that are resistant to CAZ-AVI. Among KPC-producing *K. pneumoniae*, median MICs for both cefepime and cefepime/VNRX-5133 were higher against isolates with OmpK36 porin mutations suggesting that cefepime, and maybe VNRX-5133, relies on intact porin channels for the access to the periplasmic space (Shields *et al,* 2019). VNRX-5133 is currently in phase III clinical trials (https://clinicaltrials.gov/ct2/show/NCT03840148?term=vnrx-5133&rank=2) (Krajnc *et al*, 2019).

Among the inhibitors, **zidebactam** is the only drug that includes Class B carbapenemases and the class D carbapenemases of *Acinetobacter* spp. in its spectrum of activity. Zidebactam is a β-lactamase inhibitor which possesses also intrinsic antibacterial activity. The combination of cefepime plus zidebactam showed a potent activity against isolates producing carbapenemases of Ambler classes A, B, and D, and *P. aeruginosa* isolates with multiple resistance mechanisms including combinations of upregulated efflux, diminished or non-functional OprD porins, and AmpC overproduction (Livermore *et al,* 2017; Thomson *et al,* 2019). Cefepime-zidebactam is currently in phase I clinical trials (https://clinicaltrials.gov/ct2/show/NCT02707107?term=zidebactam&rank=2).

1.8.2 Combination of a new β-lactam with a traditional β-lactamase inhibitor

Ceftolozane/tazobactam is a combination of a novel oxyimino-aminothiazolyl cephalosporin with a suicide β-lactamase inhibitor. The chemical structure of ceftolozane derives from ceftazidime, with the modification of the sidechain at the 3-position of the cephem nucleus, which confers a potent antipseudomonal activity (Zhanel *et al,* 2014). Ceftolozane has a high capacity to cross outer membrane trough porins and high stability in the presence of AmpC β-lactamase, whereas the hydrolysis activity of eventually ESBLs is inhibited by tazobactam. The bactericidal action of

ceftolozane is mediated by the high-affinity interactions with penicillin-binding proteins PBP1b, PBP1c, PBP2 and PBP3(Zhanel *et al,* 2014). Several studies showed that ceftolozane performance was not affected by efflux pump overexpression or by the loss of membrane porins in *P. aeruginosa* (Moya *et al,* 2010; Wright *et al,* 2017). The main mechanisms of resistance are multiple mutations leading to over-expression and structural modifications of AmpC and the production of MBLs and other carbapenemases (Cabot *et al,* 2014; Giani *et al,* 2018).

For these reasons, ceftolozane/tazobactam has demonstrated potent *in vitro* activity against carbapenemase-non-producing MDR *P. aeruginosa* (Giani *et al,* 2018) and ESBLs-producing *Enterobacterales (*Pfaller *et al,* 2017). This combination has been recently approved by the FDA to treat hospital-acquired bacterial pneumonia and ventilator-associated bacterial pneumonia (HAP/VAP) while previously was authorized by both FDA and EMA for the treatment of cUTI and CIAI.

1.8.3. New strategies for vehiculation: hybrid siderophore-cephalosporins

Cefiderocol (Shionogi & Co., Ltd., Japan) is an innovative cephalosporin (being studied in phase III trials) which represents a promising therapeutic option against infections caused by difficult-to-treat Gram-negative pathogens, thanks to an innovative action mechanism: it contains a catechol group that mimics the structure of bacterial siderophores, is therefore actively transported through the outer membrane by iron acquisition system and binds to PBP3 as well as PBP1a, PBP1b, and PBP2 inhibiting peptidoglycan synthesis (Zhanel *et al*, 2019). Moreover, cefiderocol has intrinsic structural stability to hydrolysis by nearly all β-lactamases, included class B metallo-β-lactamases and class D (OXA) β-lactamases (Ito-Horiyama *et al,* 2016; Poirel L *et al,* 2018).

Two clinical trials (phase III) are currently ongoing, one in healthcare-associated pneumonia (HAP, VAP) (NTC030032380), and the other about infections caused by CRE including cUTI, HAP/VAP and sepsis (NTC02714595). Efficacy similar, or even superior, to imipenem-cilastatin for the treatment of cUTI in patients with MDR Gram-negative infections was described in a clinical phase II trial (Portsmouth *et al,* 2018). Cefiderocol showed a strong *in vitro* activity against a CRE and MDR *P. aeruginosa* with MIC values ranged from ≤0.125 to 4 mg/L including for carbapenemase-producers. Cefiderocol was found to be active also against *A. baumannii* producing OXA-type β-lactamases, with MIC values generally higher than *Enterobacterales* isolates and some cases of resistance described. (Ito *et al,* 2016). Moreover, mutations into the *piuA*, *cirA* and *fiu* iron

transporter genes have been associated with an increase in cefiderocol MIC in *P. aeruginosa* and *E. coli* (Zhanel *et al,* 2019).

1.8.4 Cephalosporins anti-MRSA

Ceftaroline and ceftobiprole, often termed "fifth-generation" cephalosporins, are the first broad-spectrum β-lactams with potent bactericidal anti-staphylococcal activity covering also MRSA, VRSA, VISA, and daptomycin-resistant *S. aureus*. The anti-MRSA activity derives from their high affinity not only for the common PBPs but also for PBP2a encoded by *mec* genes and consequently results in cell wall disaggregation and bacterial cell death. However, the structure of these drugs can be hydrolyzed by ESBLs and carbapenemases, though both have some activity against select Gram-negative pathogens (Duplessis and Crum-Cianflone, 2011).

Ceftaroline fosamil (prodrug) has been approved by the FDA and EMA for the treatment of CAP and acute bacterial skin and skin structure infections (ABSSSI). However, it is often used off-label in persistent or recurrent infections because of its broad spectrum of activity, good safety and tolerability profile (Pani *et al,* 2019). Of interest, it has also efficacy against respiratory bacterial pathogens such as *Streptococcus pneumoniae* (including MDR strains), *Haemophilus influenzae*, and *Moraxella catarrhalis* (Duplessis and Crum-Cianflone, 2011). Resistance to ceftaroline is uncommon and usually due to *mec* genes mutations, resulting in changes in the amino acid sequence of PBP2a or to overexpression of PBP4 (Watkins *et al,* 2019).

Ceftobiprole medocaril (prodrug) has been approved in many European countries and in some countries of the North and South America (Canada, Argentina, Peru) and of Middle East (Jordan and Saudi Arabia) for the treatment of HAP (excluding VAP) and CAP (Pfaller *et al,* 2019). Unlike ceftaroline, ceftobiprole also displays a binding profile to PBPs in *P. aeruginosa* comparable to that of cefepime. These characteristics explain also its indication in nosocomial pneumonia which is frequently caused by *P. aeruginosa* (Morosini *et al,* 2019). Ceftobiprole also exhibits *in vitro* bactericidal activity against MRSA strains, with kinetics comparable or superior to those of vancomycin and linezolid, and against *Enterobacterales* with a spectrum of activity similar to ceftazidime and cefepime (Pfaller *et al,* 2019). Finally, ceftobiprole demonstrates a low inclination to evolve resistance among MRSA strains and resistance is probably mediated by multiple mutations in *pbp* genes, especially *mecA* or *pbp4* (Morroni *et al,* 2018).

2. AIM OF THE PROJECT

Over the past decade, infections caused by multi-resistant bacteria have undergone a rapid worldwide spread, which represents a public health problem of major concern, with a significant impact on morbidity, mortality, and healthcare-associated costs.

In this scenario, the possible approaches to fight these infections include the development of new antimicrobial drugs, as well as the optimization of currently available antibiotic therapies by preventive measures, and of diagnostic methods leading to rapid and accurate identification of multi-resistant bacteria.

The general aim of this project was to characterize local and nationwide collections of both Gram-negative and Gram-positive clinical isolates at a phenotypic and genotypic level, evaluating the activity of novel antimicrobial agents and novel molecular and phenotypic diagnostics approaches to tackle antimicrobial resistance. Moreover, advanced genotypic characterization of the most interesting isolates was performed by whole genome sequencing using NGS technology.

In detail, the Ph.D. work focused on two main topics, diagnostic methods and new antimicrobial agents, which have been examined according to the following specific tasks:

1) development of a new phenotypic method for screening isolates carrying acquired colistin-resistance genes;
2) evaluation of different commercial diagnostic systems for antimicrobial susceptibility testing of challenging MDR clinical isolates;
3) evaluation of the *in vitro* activity of novel antibiotics and comparators against bacterial isolates of clinical origin from large multicentric collections;
4) characterization of resistance mechanisms to the novel combination ceftazidime-avibactam.

3. RESULTS AND DISCUSSION

3.1 DEVELOPMENT OF A NEW PHENOTYPIC METHOD FOR SCREENING ISOLATES CARRYING ACQUIRED COLISTIN-RESISTANCE GENES

RELATED PUBLICATION

- **A simple phenotypic method for screening of MCR-1-mediated colistin resistance.** 2018. Coppi M, Cannatelli A, Antonelli A, I. Baccani I, Di Pilato V, Sennati S, Giani T, Rossolini GM. *Clin Microbiol Infect*. 24(2):201.e1-201.e3.

During the last decade, colistin has regained a key role as last-resort antibiotics for the treatment of infections caused by multidrug-resistant and extremely drug-resistant Gram-negative bacterial pathogens (Nation and Li, 2009). Discovery of transferable plasmids encoding phosphoethanolamine transferases of the MCR-type in *Escherichia coli* and other *Enterobacteriaceae* has raised considerable concern for its potential contribution to the dissemination of polymyxin resistance among clinical pathogens (Schwarz and Johnson, 2016). Knowledge of the epidemiology of *mcr* resistance determinants, however, is mainly based on molecular testing. In this work, a novel method (Colistin-MAC test) has been developed for presumptive discrimination of *mcr*-1-positive colistin-resistant *E. coli* strains, which can be implemented by clinical laboratories that are unable to perform molecular tests.

Briefly, the Colistin-MAC test is based on a broth microdilution for colistin MIC testing according to the CLSI guidelines, in the absence or presence of DPA (CLSI, 2018). Since MCR-1 is a metalloenzyme, with zinc ions in the catalytic domain (Hinchliffe *et al,* 2017), the use of zinc chelators in combination with polymyxins can increase colistin susceptibility of MCR-producing strains. In combination with colistin, DPA is used at a fixed final concentration of 900 μg/ml in dimethylsulphoxide (DMSO). Muller-Hinton Broth containing DPA and DMSO at a final concentration of 900 μg/ml and 0.9% (v/v), respectively, was used as growth control (Figure 12). Results are interpreted as positive if in presence of DPA the tested isolate exhibits ≥3-fold MIC decrease compared to the colistin MIC alone (Figure 12).

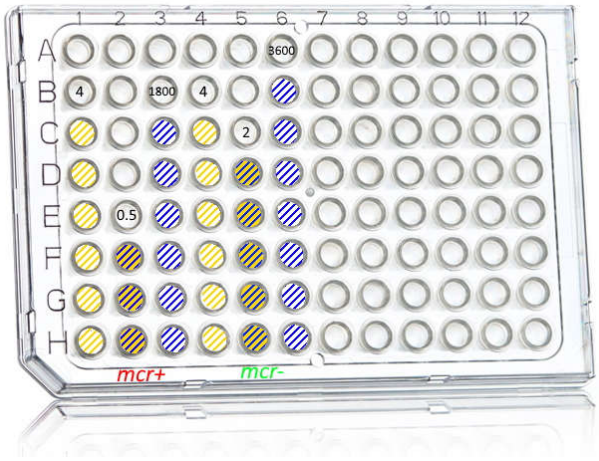


Figure 12. Schematic illustration of Colistin-MAC test for an MCR-positive strain (first three columns) and an MCR-negative strain (second three columns). The diagonal lines in the wells indicate the bacterial growth in presence of colistin alone (yellow), dipicolinic acid alone (blue) and colistin plus and dipicolinic acid (blue and yellow). Numbers indicate the respective MIC values.

The Colistin-MAC test was evaluated with a collection of 74 bacterial strains, including 61 colistin-resistant strains carrying *mcr-1* genes, and 13 colistin-resistant strains in which the absence of *mcr-1* and *mcr-2* determinants had been confirmed by a multiplex Real-time PCR, developed for this study. Most strains (N=65) were *E. coli*, but a few belonged to other enterobacterial species including *Klebsiella pneumoniae* (N=6) *Citrobacter* spp. (N=2) and *Enterobacter cloacae* complex (N=1) (Table 3). In presence of DPA at 900 µg/ml, all the 56 *mcr-1* positive *E. coli* strains exhibited a reduction of colistin MIC of at least an 8-fold dilution (range, 8 – ≥128 fold), and a similar behavior was observed with the *mcr-1*-positive *Enterobacter cloacae* complex strain and with the two *mcr-1*-positive *Citrobacter* strains. On the other hand, colistin MIC was increased, unchanged or at most decreased by a two-fold dilution with the *mcr*-negative *E. coli* and *K. pneumoniae* strains, and with the two *mcr-1*-positive *K. pneumoniae* strains (Table 3). Thus, all the 65 colistin-resistant *E. coli* were properly categorized, whereas the lack of inhibitory effect observed with the *mcr-1*-positive *K. pneumoniae* strains could be due to low permeability of *K. pneumoniae* for DPA and/or to the presence of additional unknown mechanisms of colistin resistance in these strains.

The performance of this test has been recently confirmed by Büdel and colleagues that reported a good accuracy (sensitivity, 94.9%; specificity, 100%), but only when the test was implemented for *E. coli* strains (Büdel *et al,* 2019). Moreover, a variant of this test based on ethylenediaminetetraacetic acid (EDTA) has been described with comparable performance (sensitivity 96.7%; specificities 89.6%) (Esposito *et al,* 2017).

The advantages of using these types of the test include the simple and inexpensive execution for each clinical laboratory, especially in low-income settings, and the possibility to screen large collections of isolates to detect new *mcr*-like genes not yet targeted by the current molecular assays.

Table 3. Bacterial strains tested in this study, and colistin MIC values measured by broth microdilution in the absence or presence of 900 mg/mL DPA (Colistin-MAC test).

Species	Mechanism of Colistin resistance[a]	No. of strains	MIC Colistin (µg/mL) (median value)	MIC Colistin (µg/mL) + DPA 900 µg/ml (median value)	Fold of MIC reduction
Escherichia coli	*mcr-1/ mcr-1*-like	53	4 - >8 (8)	≤0.125 - 1 (≤0.125)	8 - ≥128
	mcr-NEG, n.d.	9	4 - 8 (8)	4 - >8 (8)	≈[b]
Escherichia coli J53AZI^R Transconjugants	*mcr-1*-like	3	4 (4)	≤0.125 - 0.5 (0.25)	8 - ≥32
Klebsiella pneumoniae	*mcr-1*-like*/mcr-1.2*	2	8 - >8	>8	≈[b]
	mcr-NEG PmrB mutant/inactivated *mgrB/*n.d.	4	>8	>8	≈[b]
Citrobacter braakii	*mcr-1*	1	8	0.5	16
Citrobacter freundii complex	*mcr-1*	1	8	≤0.125	≥64
Enterobacter cloacae complex	*mcr-1*	1	>8	≤0.125	≥128

[a] *mcr-1*-like indicates that the gene was amplified with primers for *mcr-1* but was not entirely sequenced; *mcr*-NEG indicates that the strain was negative for *mcr-1*-like and *mcr-2*-like genes as assessed by RT-PCR and end-point PCR. In some cases, WGS data confirmed negativity for all known *mcr*-genes; n.d., indicates that the colistin resistance mechanism remained not determined.

[b] ≈ indicates that colistin MIC, in presence of DPA, was increased, unchanged or at most decreased by a two-fold dilution.

3.2 EVALUATION OF DIFFERENT COMMERCIAL DIAGNOSTIC SYSTEMS FOR ANTIMICROBIAL SUSCEPTIBILITY TESTING

RELATED PUBLICATION

- **Discrepancies in fosfomycin susceptibility testing of KPC-producing *Klebsiella pneumoniae* with various commercial methods.** 2019. Camarlinghi G, Parisio EM, Antonelli A, Nardone M, Coppi M, Giani T, Mattei R, Rossolini GM. *Diagn Microbiol Infect Dis*. 93(1):74-76.
- **Variable performance of different commercial systems for testing carbapenem susceptibility of KPC carbapenemase-producing *Escherichia coli.*** 2019. Clin Microbiol Infect. Antonelli A, Coppi M, Camarlinghi G, Parisio EM, Nardone M, Riccobono E, Giani T, Mattei R, Rossolini GM. 2019. *Clin Microbiol Infect.* pii: S1198-743X(19)30444-6.

3.2.1 Discrepancies in fosfomycin susceptibility testing of KPC-producing *K. pneumoniae* with various commercial methods.

As colistin, also intravenous fosfomycin, in combination with other agents, have recently recovered a key role as last-resort antibiotics for treatment of infections by multidrug-resistant *Enterobacterales*, especially those by KPC-producing *Klebsiella pneumoniae* (Reffert and Smith, 2014). According to EUCAST and CLSI, agar dilution (AD) is the reference method for fosfomycin susceptibility testing (CLSI, 2018; EUCAST, 2019) (Figure 13). However, AD is not suitable for use in routine susceptibility testing and several automated, disk diffusion and gradient diffusion systems are available for fosfomycin susceptibility testing. The aim of this study was to evaluate the performance of Sensititre (ITGNEGF panel, Thermo Fisher Scientific), of Vitek2 (AST 201 card, bioMérieux), of Etest (bioMérieux), of disk diffusion (Bio-Rad Laboratories) and of MIC Test Strip (Liofilchem) to determine the susceptibility to fosfomycin of 78 clinical isolates of KPC-producing *K. pneumoniae* (KPC-KP), compared with reference AD method (AD). Agreement of results obtained with commercial methods in comparison with AD was evaluated according to the International Organization for standardization ISO 20776–2, 2007. Four parameters were used to compare different methods: essential agreement (EA), categorical agreement (CA), major errors (ME), and very major errors (VME). Overall, the EA (range 69.2 -

72.0%) and CA (range 50.0% - 69.2%) for all the commercial methods were in poor agreement with the reference method, with no method being acceptable according to the ISO criteria (EA and CA ≥ 90%). The direct comparison between the agar dilution and each commercial method was reported as scattergram in Figure 13. Sensititre and Etest exhibited high rates of false-susceptible results with a VME of 54.5% and 78.8%, respectively. Therefore, these two methods underestimated MIC values compared to the AD reference method. On the other hand, Vitek2, disk diffusion and MIC Test Strip exhibited high rates of false-resistant results with ME of 75.6%, 84.4% and 76.7%, respectively. Therefore, these three methods overestimated MIC values if compared to the AD (Figure 13). Altogether, these findings indicated that results of susceptibility testing of fosfomycin obtained with the commercial systems evaluated in this study should be considered with caution and laboratory routines should always carry out the AD to test fosfomycin susceptibility, especially for KP-KPC isolates from critical patients.

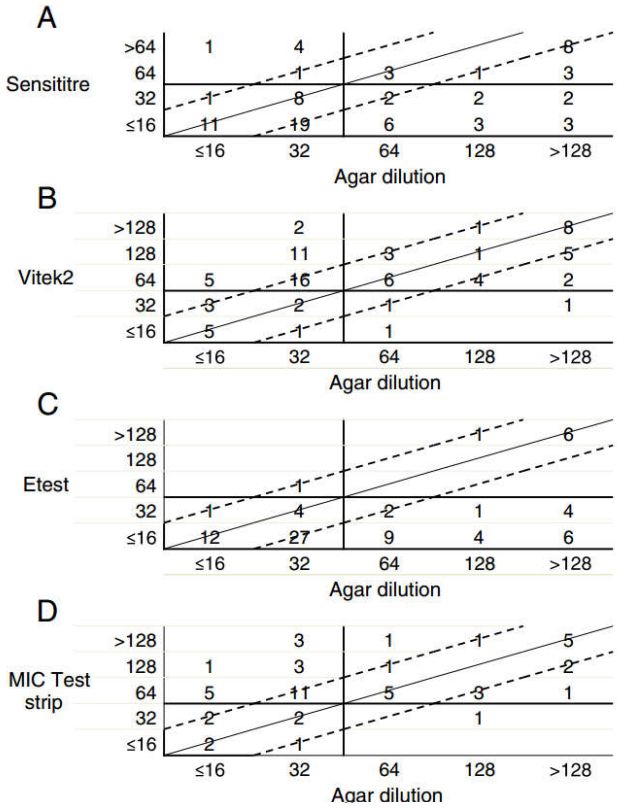


Figure 13. Scattergram of fosfomycin MICs for KPC-KP tested measured by Agar dilution and Sensititre (A), Vitek2 (B), Etest (C), and MIC Test strip (D). MICs were indicated in µg/mL. MIC values between diagonal lines are included in the Essential Agreement. Horizontal and vertical lines indicate the EUCAST breakpoints (susceptible ≤32 µg/mL, resistant N32 µg/mL).

3.2.2 Variable performance of different commercial systems for testing carbapenem susceptibility of KPC carbapenemase-producing *Escherichia coli.*

KPC-producing *K. pneumoniae* is the most prevalent CPE in Italy and some other countries, (Giani *et al,* 2017; Logan and Weinstein, 2017). However, in settings of high endemicity, the emergence of KPC-type carbapenemases has also been reported among *E. coli* (Giani *et al,* 2017; Logan and Weinstein, 2017). Among KPC-producers, *E. coli* usually exhibit lower expression of the carbapenem-resistant phenotype compared to the most diffused *K. pneumoniae*. Therefore, its detection could be sometimes problematic (Landman *et al,* 2011). Moreover, accurate carbapenem MIC measurement is also important for consideration of carbapenem containing regimens (Landman *et al,* 2011).

For this reason, we have investigated, for the first time, the performance of several commercial systems and of disk diffusion for testing carbapenem susceptibility of KPC-producing *E. coli* and found remarkably variable performances of the various methods. Four commercial systems (Vitek2 AST-201 card – bioMérieux; MicroScan Neg MIC Panel Type 44 – Beckman Coulter, Etest – bioMérieux, MIC Test Strip – Liofilchem) was evaluated for antimicrobial susceptibility testing of imipenem, meropenem and ertapenem, whereas the performance of disk diffusion (Bio-Rad Laboratories) and Sensititre (Gram-Negative MIC Plates ITGNEGF – Thermo Scientific™ Sensititre™) were tested only with imipenem and meropenem. All commercial methodologies and the reference broth microdilution (BMD) were performed starting from the same bacterial suspension to favor the comparison between results avoiding inoculum bias. Results were interpreted according to EUCAST clinical breakpoints (v 9.0 2019) and the agreement was determined as established by ISO 20776–2, 2007. A total of 54 non-replicate KPC-producing *E. coli* isolates were collected retrospectively from two different Hospitals located in central Italy (Careggi University Hospital, Florence and San Luca Hospital, Lucca) between 2014 and 2017. The clonality analysis by random amplification of polymorphic DNA (Pacheco *et al,* 1997), showed 44 different profiles, suggesting a marketed clonal diversity.

A comparison of results of reference BMD with those gained with commercial systems is illustrated in Figure 14. For meropenem, the EA ranged from 14.8% to 90.1%, while the CA from 14.8% to 79.6%. Vitek2 exhibited a trend to overcall resistance (ME 50%), while Sensititre, Microscan, Etest, MIC strip and Kirby-Bauer showed a trend to underestimate resistance (VME ranging from 9.1% to 45.5%). For imipenem, the EA ranged from 44.4% to 83%, while the CA from 16.7% to 51.8% with all methods, except for Vitek2, showing an overall trend to underestimate resistance

(VME range 0-66.7%). For ertapenem, the EA of the different commercial methods ranged from 35.2% to 96.3% and the CA from 83.3% to 96.3%. All methods showed an underestimation trend with VME values ranging from 3.7% to 16.7%.

The detection of putative carbapenemase producers based on the ECOFF values (0.125 µg/mL for meropenem and 0.5 µg/mL for imipenem) could be problematic for some isolates using commercial systems: although reference BMD results showed that all the 54 KPC-positive *E. coli* had MIC values ≥the respective ECOFF, the percentage of isolates with a meropenem MIC ≤ECOFF was 5.6% with Etest, 5.6% with MIC strip, 1.9% with Microscan and 1.9% with Sensititre, while imipenem MICs ≤ECOFF resulted in 9.3% of cases with Vitek2, 7.4% with Etest and 9.3% with MIC strip. Moreover, ECOFF values could not be evaluated for meropenem with Vitek2, and for imipenem with Microscan and Sensititre due to the limited MIC range. The range of MICs detectable is limited also for the highest concentrations of antibiotics available: Vitek2 was able to measure for meropenem MIC values up to 8 mg/L, while Sensititre up to 16 mg/L.

This limitation of tested MICs range and the variability of results among the different methods, compared to reference broth microdilution, could significantly impact on the clinical use of carbapenems in case of infections mediated by KPC-producing *E. coli*. Indeed, recent studies indicate that the favorable impact on survival of active drug combinations that include at least one carbapenem is significant only when the meropenem MIC for the CRE-KPC isolate is ≤8 mg/L, but it is not significant when the meropenem MIC exceeds 32 µg/mL (Tumbarello *et al,* 2015). This study suggests that when infections by KPC-producing *E. coli* are suspected, laboratories should always confirm carbapenems MICs with reference BMD, in order to carry out an appropriate antimicrobial therapy.

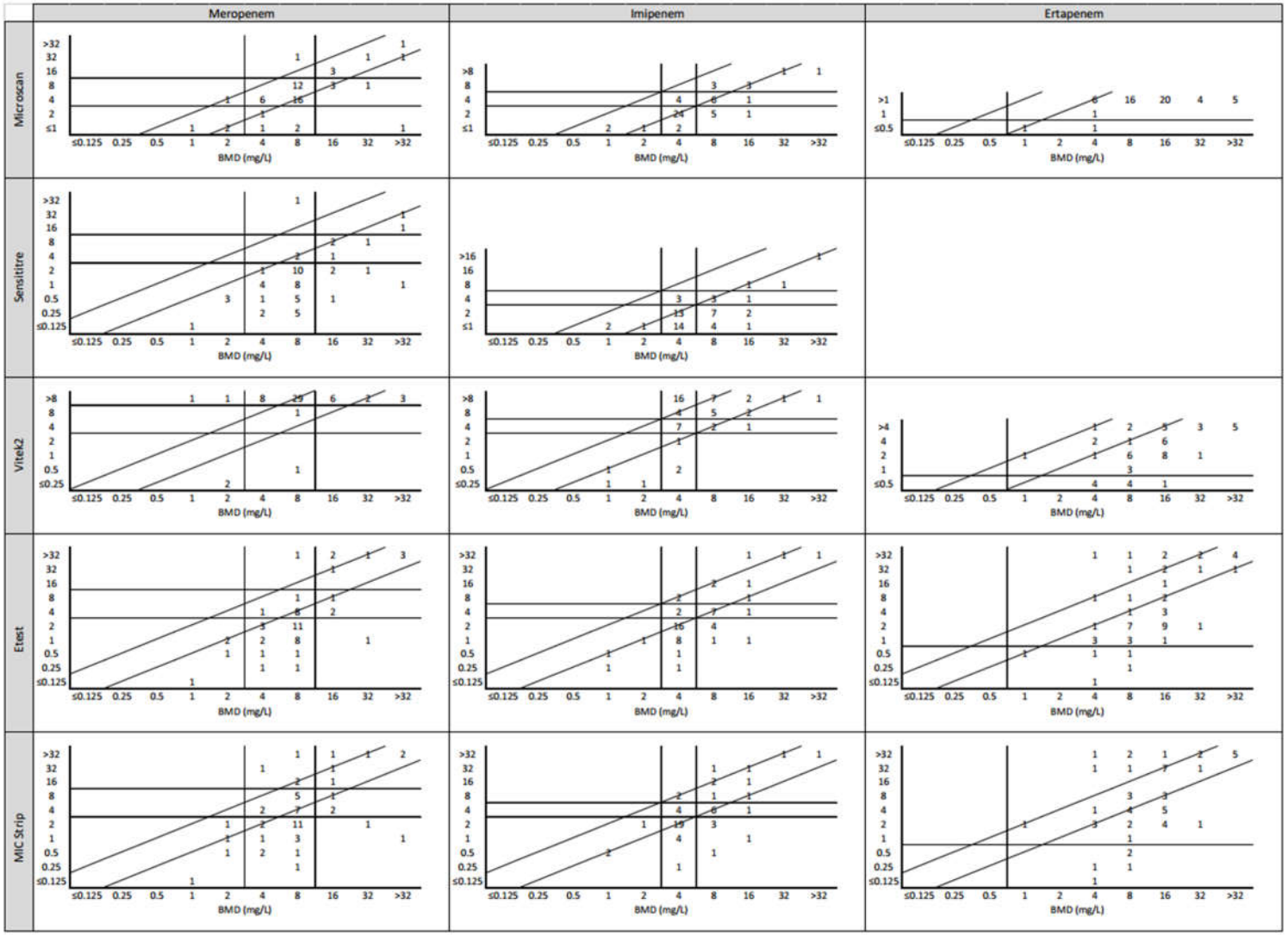


Figure 14. Scattergram of meropenem, imipenem and ertapenem MICs for KPC-producing *E. coli* tested measured by broth microdilution and Microscan, Sensititre, Vitek2, Etest and MIC Test strip. MICs were indicated in mg/L. MIC values between diagonal lines are included in the Essential Agreement. Horizontal and vertical lines indicate the EUCAST breakpoints

3.3 EVALUATION OF THE *IN VITRO* ACTIVITY OF NOVEL ANTIBIOTICS

RELATED PUBLICATION

- ***Staphylococcus aureus* from hospital-acquired pneumonia from an Italian nationwide survey: activity of ceftobiprole and other anti-staphylococcal agents, and molecular epidemiology of methicillin-resistant isolates.** Antonelli A, Giani T, Coppi M, Di Pilato V, Arena F, Colavecchio OL, Conte V, Santerre Henriksen A, Rossolini GM, MRSA-HAP Study Group. *J Antimicrob Chemother.* DKZ371. [Epub ahead of print]

3.3.1 Activity of ceftobiprole and molecular epidemiology of MRSA from HAP in Italy

HAP and CAP are among the most common infections treated in the hospital setting and are associated with high morbidity and mortality rate (Barbier *et al*, 2013; Amin *et al,* 2014). The spread of multidrug-resistant isolates in several settings undermines the possibility of successful empirical antibiotic therapy. Ceftobiprole is a 5th-generation cephalosporin with an expanded-spectrum and potent activity against both Gram-positive and -negative bacteria (Awad *et al,* 2014), which showed potent activity against MRSA causing HAP (Awad *et al,* 2014). The objective of this study was to investigate the *in vitro* activity of ceftobiprole and comparators against a multicentric collection of clinical isolates of *S. aureus* putatively responsible for HAP, proving an update of the currently Italian epidemiology, and the characterization of MRSA involved in such infections by Whole-Genome Sequencing (WGS). A total of 333 consecutive non-replicate isolates were collected by 13 clinical

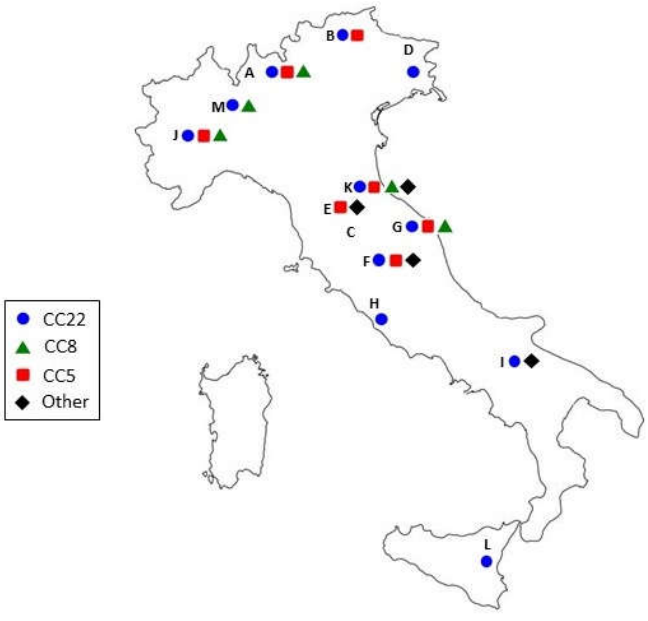


Figure 13. Geographical distribution of the most prevalent MRSA CCs (CC5, CC8 and CC22) among the 13 laboratories participating in the study. *A) Lecco; B) Bolzano; C) Arezzo; D) Udine; E) Florence; F) Perugia; G) Ancona; H) Rome; I) Foggia; J) Turin; K) Cesena; L) Catania; M) Milan.*

microbiology laboratories distributed over the Italian territory (Figure 15), which had been asked to collect up to 25 isolates of all species, putatively responsible of HAP (Jorgensen *et al,* 2015) and all MRSA isolates from HAP during the period January 1 – May 31, 2016. Considering the first 25 strains collected by each center, the most prevalent species in HAP were *P. aeruginosa* (31.5%), followed by *K. pneumoniae* (18.9%), and *S. aureus* (18.6%). Among the *S. aureus,* the proportion of MRSA isolates was 40.3% (25 of 62), ranging from 0% to 100% in the different centers. 41 additional MRSA isolates from putative cases of HAP were also collected, according to the study protocol, yielding a total of 66 MRSA isolates. All isolates identified as *S. aureus* were subjected to MIC testing by reference broth microdilution procedure according to CLSI guidelines (CLSI, 2018), using custom plates (ThermoFisher Scientific, Massachusetts, US), and results were interpreted according to EUCAST breakpoints v 9.0 (http://www.eucast.org/clinical_breakpoints/). All MRSA phenotype was established by cefoxitin broth microdilution and, in case of discrepancy, was confirmed by a cefoxitin screen test using disk-diffusion following EUCAST recommendations. The results were reported in Table 4. All methicillin-susceptible *S. aureus* isolates resulted susceptible to ceftobiprole, teicoplanin, tigecycline, trimethoprim-sulfamethoxazole and oxacillin antibiotics (Table 4). All MRSA isolates were susceptible to vancomycin, teicoplanin, tigecycline and linezolid, whereas ceftobiprole had activity against 95% of these isolates.

Table 4. Susceptibility profiles and MIC_{50} and MIC_{90} of all MSSA and MRSA isolates against ceftobiprole and comparators. MIC values are in mg/L.

MSSA	**n=37**	**CPB**	**ERY**	**LEV**	**LNZ**	**TEC**	**TGC**	**SXT**	**VA**
MIC_{50}		0.5	0.5	0.25	2	0.5	0.25	≤0.06	1
MIC_{90}		0.5	>1	0.5	4	1	0.25	0.5	1
%S		100	76.9 [b]	94.9	100	100	100	100	100
MRSA	**n=66**	**CPB**	**ERY**	**LEV**	**LNZ**	**TEC**	**TGC**	**SXT**	**VA**
MIC_{50}		1	>1	16	2	0,5	0.25	≤0.06	1
MIC_{90}		2[a]	>1	>16	4	1	0.5	0.12	1
%S		95.5	36.4 [b]	12.1	100	100	100	100	100

CPB: ceftobiprole; ERY: erythromycin; LEV: levofloxacin; LNZ: linezolid; TEC: teicoplanin; TGC: tigecycline; SXT: trimethoprim/sulfamethoxazole 1:19; VA: vancomycin. [a] Seven MRSA showed a MIC of ceftobiprole of 2 mg/L, which is within the Areas of Technical Uncertainty according to EUCAST clinical breakpoints; [b] Percentage of isolates susceptible to the standard dosing regimen.

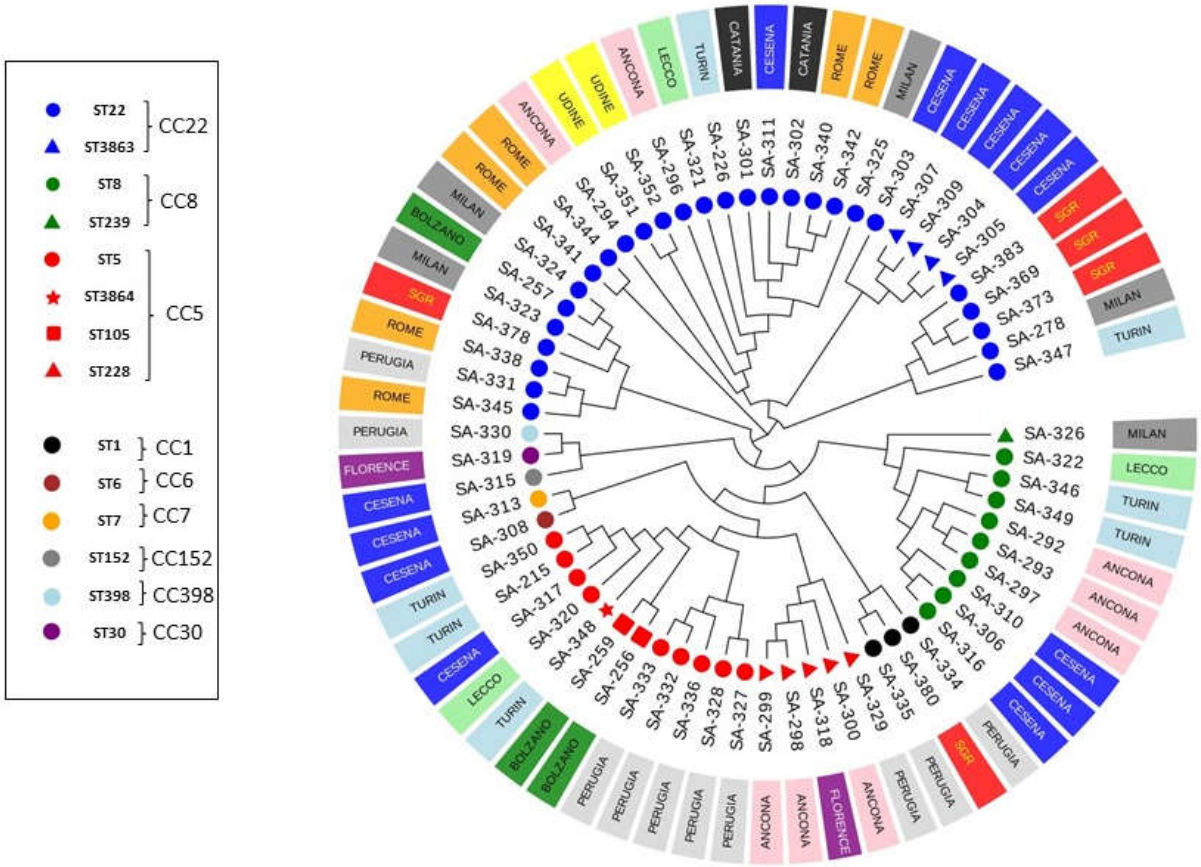


Figure 16. Phylogenetic tree of MRSA isolates. STs, CCs and isolation centres are shown in different colours.

Using a WGS approach with an Illumina MiSeq system (Illumina Inc., San Diego, CA, USA), the draft genomes of all MRSA isolates were determined and further analyzed for clonality and the content of resistance and virulence determinants. Clonality analysis (MLST and SCC*mec*) showed that 47.0% of isolates belonged to clonal complex (CC) 22, including mainly ST22-MRSA-IV but also a novel single locus variant of ST22, labeled as ST3863-MRSA-IV.

CC5 with ST5-MRSA-II, -IV and -VIII, ST105-MRSA-II, ST228-MRSA-I and the novel ST3864-MRSA-II, and CC8 with ST8-MRSA-IV and ST239-MRSA-III represented 25.8% and 15.0% of isolates, respectively. The other lineages were CC1 with three ST1-MRSA-IV isolates and CC6(ST6-MRSA-IV), CC30 (ST30-MRSA-IV), CC152 (ST152-MRSA-V) and CC398 (ST398-MRSA-V) with only a single isolate for each one (Figure 16). This latter sequence type was previously associated with livestock and reported from bloodstream infections and ventilator-associated pneumonia in Italy (Soavi *et al,* 2010; Mammina *et al,* 2010). The isolates belonging to

CC22 were widespread across the Italian territory and highly related to each other, while CC5 and CC8 were apparently more prevalent in central and northern Italy and the variability within CC8 isolates was higher than within CC22 isolates but lower than within CC5. (Figures 15 and 16). The most common clonal complexes of MRSA (i.e. CC22, CC5 and CC8) were overall conserved if they are compared with a previous Italian nationwide surveillance, carried out in 2012 (Campanile *et al,* 2015), but with a substantial decrease in the prevalence of CC5 (ST228) and increase in the prevalence of ST22 as the most remarkable change. The replacement of ST228 with ST22 (firstly discovered in UK where represents the predominant clone since the early 2000s) has already been reported in Italy in a single hospital report, and has also been reported in other countries in recent years, with an interesting differential fitness cost putatively associated with this clonal shift (Baldan *et al,* 2012; Baldan *et al,* 2015).

Analysis of the acquired resistance genes confirmed the presence of *blaZ* gene in most MRSA isolates (81.8%) and of the *mecA* gene in all of them, whereas no *mecC* gene was found in this collection. The main alteration in PBP2a protein was G246E (29.5%), followed by N146K (6.6%), S225R (3.3%) and both G246E and C542G mutations (1.6%). These alterations in PBP2a protein were not associated with a clone, except for $PBP2a_{N146K}$, which was present only in ST228 isolates. This latter mutation has been previously linked to decreased susceptibility to ceftaroline and ceftobiprole (Kelley *et al,* 2015; Morroni *et al,* 2018), and was detected in two of three ceftobiprole-resistant MRSA (ST228-MRSA-I). Moreover, all three isolates exhibited a C197Y substitution in PBP2, previously found in ceftobiprole-resistant isolates. However, both $PBP2a_{N146K}$ and $PBP2_{C197Y}$ variants were also present in ceftobiprole-susceptible isolates of the same clonal lineage, suggesting that these are likely not the only factor contributing to ceftobiprole resistance. No other mutations in PBP2a or PBP1, PBP3, PBP4 (including its promoter region), GdpP, and AcrB, previously associated with ceftobiprole resistance, were detected in these three isolates (Kelley *et al,* 2015; Morroni *et al,* 2018; Chan *et al,* 2015).

Macrolide, lincosamide and streptogramin B resistance genes were relatively less frequent (*ermC* 43.9%, *ermA* 18.2%). Aminoglycoside resistance genes were present in 40.9% of the isolates with *ant(9)-Ia* being the most represented (18.1%), followed by *ant(4')-Ia* (19.7%), *aac(6')-aph(2")* (15.1%), *aph-(3')-III* (12.1%), *ant(6)-Ia (12.1%)*, *aph(3")-Ib* (1.5%) and *aph(6)-Id* (1.5%). In 24.4% of isolates more than one aminoglycoside resistance gene was found. Interestingly, the acquired aminoglycoside resistance genes were very common in CC8 and CC5 isolates, while being nearly absent in those of CC22. Few isolates also had chloramphenicol (*cat*, 12.1%) and

tetracyclines (9.1%) resistance genes.

Among virulence factors, Panton-Valentine Leukocidin (PVL) genes, *LukS* and *LukF,* were detected in two isolates: one isolate belonged to ST152-MRSA-V, a highly divergent clone which has been already associated with PVL toxin in Europe, Oceania and Africa (Ruimy *et al,* 2008), while the second one was a ST8-MRSA-IV. Of interest, the ST152-PVL-carrying strain was also the only strain positive for *edinB* gene, which has been previously associated with an increased risk of translocation of *S. aureus* into the bloodstream during pneumonia in a ST80 MRSA (Courjon *et al,* 2015). Interestingly, 30/66 (45.4%) of the isolates, mostly belonging to CC8 (including the PVL-carrying ST8) and CC5, but none of which belonged to CC22, carried the pore-forming LukED leucocidin to induce dermonecrosis. All MRSA belonging to CC5 and CC8 (and none of CC22) presented the immune escape serine protease encoding *slpA* and *slpB,* with only ST6, ST7 and ST8 being positive also for *slpE* gene. 58/66 (87.9%) of MRSA strains presented also the two immune evasion cluster proteins: the staphylococcal complement inhibitor Scn and the staphylokinase Sak. Among the eight strains not presenting the above described immune evasion genes, five belonged to ST8, and one to ST5, ST1 and ST398, respectively. Overall, this study confirmed that ceftobiprole could be a valid therapeutic option in the case of MRSA from HAP. The exact mechanisms of ceftobiprole resistance have not yet been elucidated, and we can exclude that the alterations in PBP proteins reported above represent the only cause of the resistance phenotype. Moreover, isolates included in this study were collected before the introduction of ceftobiprole among the available clinical treatments; thus, resistance in the cases observed was not mediated by a direct selection pressure. This study, therefore, represents the baseline for further surveillance studies on ceftobiprole activity against MRSA from HAP.

Overall, the complex resistome and virulome of MRSA strains included in this study exhibited a clonal distribution. Of notice, the emerging CC22 strains, which were by far the most prevalent detected in this study, rarely presented aminoglycoside resistance genes and never *lukED* genes denoting a marked difference in acquired resistance and virulence gene content compared to the other most prevalent clones (CC5 and CC8).

3.3.2 Activity of cefiderocol on an Italian multicentric collection of carbapenem-resistant Gram-negatives

Cefiderocol (Shionogi & Co., Ltd., Japan) is an injectable siderophore cephalosporin which represents a promising therapeutic option against infections caused by difficult-to-treat Gram-negative pathogens, thanks to an innovative "Trojan horse" strategy to get inside bacteria and its intrinsic structural stability against a broad spectrum of β-lactamases.

Few data about the activity of cefiderocol against carbapenem-resistant clinical isolates is available, and, in particular, nothing about the Italian epidemiologic setting. For this purpose, the tasks of this work were to evaluate the *in vitro* activity of cefiderocol and of selected comparator antibiotics against two distinct collections consisting of: i) well-characterized, retrospective collected, challenging MDR Gram-negatives producing carbapenemases, extended-spectrum β-lactamases (ESBL) and acquired class C β-lactamases; ii) carbapenem-resistant *Enterobacterales* and Gram-negative non-fermenting (*P. aeruginosa*, *A. baumannii* complex and *Stenotrophomonas maltophilia*) clinical isolates prospectively collected from four Italian hospitals. The antimicrobial susceptibility testing of cefiderocol and comparators was performed using frozen broth microdilution plates (IHMA; Schaumburg, IL, USA) prepared with iron-depleted cation-adjusted Mueller Hinton broth (ID-CAMHB) for cefiderocol and with normal cation-adjusted Mueller-Hinton broth (CAMHB) for reference compounds, according to CLSI guidelines (CLSI, 2019). The use of an iron-depleted medium, which mimics the physiologic free-iron concentrations of patients inducing bacteria to implement an iron transport system, is necessary to correctly determine MICs of cefiderocol. In fact, these latter raise systematically with the rise of iron concentration in the test broth. (Zhanel *et al,* 2019). The agar dilution reference method was used for fosfomycin susceptibility testing (ISO 20776-1). MIC values were interpreted according to EUCAST clinical breakpoints v 9.0, except the cefiderocol results, which were based on CLSI breakpoint: ≤4 and >8 mg/L for susceptible and resistant, respectively (CLSI, 2019).

Retrospective collection. The first part of the study has been carried out with a collection of 42 MDR clinical isolates, previously characterized by the acquired β-lactamase content (Table 5). Cefiderocol showed a good *in vitro* activity (MIC≤4 mg/L) against i) 20 carbapenemase-producing *Enterobacterales* ii) 6 ESBL-producing *Enterobacterales*; iii) one CMY-producing *P. mirabilis*; iv) 6 carbapenemase-producing *P. aeruginosa*; v) 5 carbapenemase-producing *A. baumannii* isolates. Overall cefiderocol exhibited the best inhibitory activity with MIC_{50} and MIC_{90} values

(0.5 mg/L and 4 mg/L, respectively) significantly lower than comparators (Table 6) and susceptibility percentage higher than all other tested antibiotics including colistin, ceftazidime/avibactam and ceftolozane/tazobactam. In particular, cefiderocol resulted also active against a KPC-3-producing *K. pneumoniae* strain resistant to all other antimicrobial agents. Four out of six *A. baumannii* strains were susceptible only to cefiderocol and colistin. All carbapenemase-producing *P. aeruginosa* isolates, that resulted resistant to ceftolozane/tazobactam, cefepime, ceftazidime, ciprofloxacin, gentamicin, had a cefiderocol MIC ≤4 mg/L. On the other hand, a FOX-7-producing *K. pneumoniae*, an NDM-5-producing *E. coli*, an OXA-23-producing *A. baumannii* and a PER-producing *Aeromonas* spp, were non-susceptible to cefiderocol (Table 5).

Table 5. Characteristics of retrospective collected MDR strains (β-lactamases content and number of isolates tested), the cefiderocol MICs range (mg/L) and the percentage of isolates susceptible to Cefiderocol (%S to CFCD).

Species	Resistance mechanisms	CFCD MIC range	% S to CFCD
Enterobacterales **(N=29)**	EBLS/Carbapenemase producers (KPC, OXA-48, VIM, NDM, NMC/A, IMI-2, CTX-M, PER, VEB, TEM-52, TEM-92, CMY, FOX-7)	≤0.03 - >64	93.1
P. aeruginosa **(N=6)**	Carbapenemase producers (VIM-1, VIM-2, IMP-13, GES-5, FIM)	0.12-0.5	100
A. baumanii **complex (N=6)**	Carbapenemase producers (OXA-23, OXA-24, OXA-58, ISAba1-OXA-51)	0.06 - >64	83.3
Aeromonas spp. **(N=1)**	ESBL (PER)	>64	0

Table 6. MIC50, MIC90 (mg/L) and susceptibility profiles (%S; %I; %R) of all retrospective collected MDR strains isolates against cefiderocol and comparators. Species without EUCAST breakpoint or with intrinsic resistance were excluded.

	CFCD	FEP	CAZ	CZA	CT/TZ	MEM	AZT	AMK	GEN	CIP	TGC	CST	FOS
MIC_{50}	0,5	>16	64	2	>64	16	>32	16	>8	>4	1	≤0.5	16
MIC_{90}	4	>16	>64	>64	>64	>64	>32	>64	>8	>4	>4	4	>128
% S	90.5	17.1	11.1	65.7	20.0	31.7	27.8	57.1	39.0	16.7	51.7	86.1	82.9
%I	2.4	5.7	8.3	-	-	12.2	5.6	11.9	2.5	0.0	-	-	-
%R	7.1	77.2	80.6	34.3	80	56.1	66.7	42.9	58.5	83.3	48.3	13.9	17.1

CFDC: cefiderocol; FEP: cefepime; CAZ: ceftazidime; CZA: ceftazidime/avibactam; CT/TZ: ceftolozane/tazobactam; MEM: meropenem; AZT: aztreonam; AMK: amikacin; CIP: ciprofloxacin; GEN: gentamicin; TGC: tigecycline; CST: colistin; FOS: fosfomycin. S%: percentage of susceptible standard dosing isolates; I%: susceptible, increased exposure; R%: resistant.

Multicentric prospective collection. Four hospital laboratories, located in northern (Lecco), central (Florence and Rome), and southern (Potenza) Italy, collected a total of 234 clinical isolates of carbapenem-resistant *Enterobacterales* and Gram-negative non-fermenters (*P. aeruginosa*, *A. baumannii* complex and *Stenotrophomonas maltophilia*). Identification with MALDI-TOF Mass spectrometry yielded 80 *K. pneumoniae*, 58 *A. baumannii*, 54 *P. aeruginosa*, 19 *S. maltophilia*, 11 *E. coli*, 4 *P. mirabilis*, 4 *Enterobacter cloacae* complex, 2 *Klebsiella aerogenes*, 1 *Serratia marcescens*, 1 *Citrobacter* spp. Among the 215 isolates collected excluding *S. maltophilia*, 159 were confirmed as carbapenemase-producers by three multiplex Real-Time PCR (Coppi *et al,* 2017), including 87 KPC-producing *Enterobacterales* (78 *K. pneumoniae*, 7 *E. coli,* 1 *K. aerogenes* and 1 *P. mirabilis*), one VIM-producing *E. cloacae*, one VIM-producing *Citrobacter* spp*,* five VIM-producing *P. aeruginosa,* five GES-producing *P. aeruginosa,* one NDM-producing *K. pneumoniae*, one NDM-producing *E. coli*, one NDM-producing *A. baumannii* and 57 class D carbapenemase-producing *A. baumannii* complex (49 OXA-23-like; 8 OXA-23-like and IS*Aba1*/OXA-51-like co-producers). 12 *Enterobacterales* and 44 *P. aeruginosa* resulted negatives to all the carbapenemases sought. Overall, 80.5% of the isolates were confirmed resistant to meropenem, 83.4% were non-susceptible to one of the cephalosporins tested (cefepime,

ceftazidime, ceftolozane/tazobactam, ceftazidime/avibactam), whereas 62.3% exhibited an MDR phenotype and 16 isolates (15 *A. baumannii* and one *P. aeruginosa*) were resistant to all tested drugs (except cefiderocol).

The results of the antimicrobial susceptibility testing, summarized as MIC range, MIC_{50} and MIC_{90} of all the tested antibiotics, grouped for *Enterobacterales*, *P. aeruginosa*, *A. baumannii* complex and *S. maltophilia,* and the proportion of susceptible isolates were reported in Table 7. The MIC_{50} and MIC_{90} of cefiderocol were lower than those of comparators antibiotics except colistin and tigecycline, which showed slightly lower MIC50 but a higher percentage of resistant isolates between *Enterobacterales.* Moreover*,* as reported in Table 7, non-fermenters*, P. aeruginosa* and *S. maltophilia* especially*,* had lower MIC values of cefiderocol than *Enterobacterales* isolates.

Cefiderocol exhibited percentages of susceptibility higher than those of comparators against both *Enterobacterales* and non-fermentative Gram-negatives (Table 7). In particular, 99% of *Enterobacterales* isolates (N = 102/103) exhibited MIC ≤ 4 mg/L (Table 7), including 98.9% of those positive for bla_{KPC} and 100% of those positive for metallo-β-lactamases production (Table 6). Surprisingly, KPC-producing enterobacteria were more susceptible to cefiderocol than to ceftazidime-avibactam (Table 8).

Excellent *in vitro* activity was also observed with all tested *P. aeruginosa* isolates (S% = 100%; MIC_{50} = 0.5 mg/L and MIC_{90} = 1 mg/L), including GES-producers (resistant to ciprofloxacin and gentamicin) and VIM-producers (resistant to cefepime, ceftazidime/avibactam, ceftazidime, meropenem and ceftolozane/tazobactam) (Table 9). Resistance to ceftolozane, unexpectedly observed in 24,1% of *P. aeruginosa* isolates, did not affect the MICs distribution of cefiderocol (MIC values ≤ 1mg/L) (Table 8).

57 of 58 *A. baumannii* isolates were susceptible to cefiderocol with MIC_{50} and MIC_{90} values of 0.5 mg/L and 2 mg/L, respectively. Cefiderocol retained activity against all the *A. baumannii* isolates that were resistant to all other drugs, or susceptible only to colistin (Table 8).

Cefiderocol was active against all *S. maltophilia* isolates with MIC_{50} and MIC_{90} values of 0.06 mg/L and 1 mg/L, including those resistant to trimethoprim/sulfamethoxazole. The highest MIC value of cefiderocol found was 8 mg/L, corresponding to an intermediate susceptibility, and observed in a KPC-producing *K. pneumoniae* and an NDM-producing *A. baumannii* complex (Table 8).

Discussion. Overall cefiderocol demonstrated a potent in vitro activity with lower MIC values than those of comparators antibiotics against both collections of clinical Gram-negative bacilli isolates, including carbapenemase producers. After testing against MDR-challenging Gram-negative pathogens, cefiderocol showed higher antimicrobial activity (MIC_{90}= 4 mg/L) than the tested comparators (MIC_{90} between >4 and >64 mg/L) against all the isolates producing ESBL and/or carbapenemases. Only colistin presented a similar activity (MIC_{90} 4 mg/L) against these strains, but its use in critically ill patients could cause renal and neurological side effects (Spapen *et al,* 2011). Also, in the multicentric collection cefiderocol had a better performance (MIC_{90} between 1 and 4 mg/L) than comparators, including colistin and tigecycline (Table 3). For the NDM- or VIM-producing isolates, the only antibiotics that retained a strong activity (%S) were fosfomycin (100%), cefiderocol (90%) and colistin (90%). For the enterobacterial isolates, the cefiderocol MIC90 and the percentage of susceptible standard dosing isolates (4 mg/L and 99.0%) were in agreement with those obtained by Hackel *et al* (4 mg/L and 97.0%), by Karlowsk *et al* (4 mg/L and 99.6%), and Jacobs *et al* (4 and 90.5%) (Hackel *et al,* 2018; Karlowsk *et al,* 2018 B; Jacobs *et al,* 2018). Also, MIC90s and percentages of susceptibility (%S), reported in this work for non-fermenters (Table 3), are comparable with those previously described (REF): i) for *P. aeruginosa* ranged from 0.5 to 1 mg/L and from 99.2% to 100%, respectively; ii) for *A. baumannii* complex ranged from 0.5 to 8 mg/L and from 89.7% to 96.1%; iii) for *S. maltophilia* ranged from 0.25 to 1 mg/L and from 99.4% to 100% (Hackel *et al,* 2018; Karlowsk *et al,* 2018 B; Jacobs *et al*. 2018; Falagas *et al,* 2017).

To our knowledge, no other works about cefiderocol susceptibility testing were focused on Italian epidemiology of carbapenem-non-susceptible Gram-negative bacteria and these results could be used as a baseline before treatment for future surveillance studies to monitor the eventual evolution to cefiderocol resistance. Limitations of this study are the restricted number of clinical centers and isolates, of which no clonality assessment was available. However, before this study, no knowledge about the percentage of isolates resistant to cefiderocol was reported. In this study, a total of 6 isolates (four of them from the retrospective collection) exhibited cefiderocol MIC values ≥ 8 mg/L: a FOX-7-producing *K. pneumoniae*, an NDM-5-producing *E. coli*, an OXA-23-producing *A. baumannii*, a PER-2-producing *Aeromonas* spp, a KPC-producing *K. pneumoniae* and an NDM-producing *A. baumannii.* Similarly, Dobias *et al*. reported that 45% of non-susceptible detected isolates were NDM producers and 30% were OXA-23-producing *A. baumannii* (Dobias *et al,* 2017). KPC-producing *K. pneumoniae* isolates with a cefiderocol MIC=8

mg/L has been described by Ito *et al* and Jacobs *et al,* especially associated with KPC-2 enzyme, whereas a PER-producing *Aeromonas* spp and a FOX-7-producing *K. pneumoniae* non-susceptible to cefiderocol have been observed for the first time in this work (Ito *et al,* 2018; Jacobs *et al,* 2018). The full understanding of possible resistance determinants to cefiderocol will be subject to more detailed investigations in the future. However, high cefiderocol MIC values were not associated with any specific β-lactamase class (Table 1 and Table 5) as also previously reported (Kazmierczak *et al,* 2019). Moreover, it is known that deficiencies of outer membrane iron transporter PiuA in *P. aeruginosa* or both CirA and Fiu in *Escherichia coli* were associated with increased cefiderocol MICS (Ito *et al,* 2018).

In the view of the results obtained, cefiderocol is a promising treatment of infection caused in our epidemiological setting by DTR Gram-negative bacteria, especially against those with limited therapeutic option as bacilli harboring metallo-β-lactamases or *A. baumannii* with Class D β-lactamases (no inhibitor combinations actually available), ceftazidime-avibactam resistant or colistin-resistant isolates. Pending the results of the two phase III trials (NCT02714595 and NCT03032380), it will be important to monitor patients who will be treated with cefiderocol for compassionate use to identify the appearance of any resistance.

Table 7. MIC range, MIC_{50}, MIC_{90} (mg/L) and MIC interpretation of cefiderocol and comparators for the prospectively collected isolates.

		CFDC[#]	FEP	CAZ	CZA	CT/TZ	MEM	AZT	AMK	GEN	CIP	TGC	CST*	SXT	FOS
Enterobacterales (N=103)	**MIC range**	0.06-8	≤0.5->16	8->64	0.06-64	0.5->64	≤2->64	2->32	≤4->64	≤0.25->8	≤0.25->4	≤0.25->4	≤0.5->8	NT	≤8->128
	MIC_{50}	2	>16	>64	2	>64	32	>32	16	8	>4	1	≤0.5	NT	16
	MIC_{90}	4	>16	>64	8	>64	>64	>32	>64	>8	>4	4	>8	NT	>128
	S%	99.0	3.88	0.0	91.3	2.9	7.8	0.0	42.7	49.5	8.7	43.7	86.7	NT	73.8
	I%	1.0	4.85	0.0	-	-	20.4	1.0	15.5	3.9	1.0	-	-	NT	-
	R%	0.0	91.26	100.0	8.7	97.1	71.8	99.0	41.8	46.6	90.3	56.3	17.3	NT	26.2
P. aeruginosa (N=54)	**MIC range**	≤0.03-2	1->16	2->64	0.06->64	0.12->64	≤2->64	4->32	≤4->64	≤0.25->8	≤0.25->4	≤0.25->4	≤0.5->8	NT	≤8->128
	MIC_{50}	0.5	16	16	4	1	16	32	8	4	>4	>4	2	NT	64
	MIC_{90}	1	>16	>64	32	64	64	>32	>64	>8	>4	>4	4	NT	>128
	S%	100.0	31.5	38.9	81.5	75.9	5.6	40.7	57.4	55.6	25.9	NI	66.7	NT	88.9
	I%	0.0	-	0.0	-	-	18.5	-	7.4	-	-	NI	-	NT	-
	R%	0.0	68.5	61.1	18.5	24.1	75.9	59.3	35.2	44.4	74.1	NI	33.3	NT	11.1
A. baumannii complex (N=58)	**MIC range**	0.06-8	16->16	64->64	16->64	8->64	32->64	32->32	≤4->64	4->8	4->4	≤0.25->4	≤0.5->8	NT	64->128
	MIC_{50}	0.5	>16	>64	64	16	64	>32	>64	>8	>4	1	1	NT	128
	MIC_{90}	2	>16	>64	>64	>64	>64	>32	>64	>8	>4	2	8	NT	>128
	S%	98.3	NI	NI	NI	NI	0.0	NI	8.6	3.4	0.0	NI	74.1	NT	NI
	I%	1.7	NI	NI	NI	NI	0.0	NI	0.0	-	0.0	NI	-	NT	NI
	R%	0.0	NI	NI	NI	NI	100.0	NI	91.4	96.6	100.0	NI	25.9	NT	NI
S. maltophilia (N=19)	**MIC range**	≤0.03-4	1->16	0.25->64	0.25->64	0.5->64	0.06->64	NT	≤4->64	NT	≤0.25->4	≤0.25->4	≤0.5->8	≤0.25/4.75 ->16/304	32->128
	MIC_{50}	0.06	>16	16	16	8	>64	NT	16	NT	2	≤0.25	>8	2/38	64
	MIC_{90}	1	>16	>64	64	64	>64	NT	>64	NT	>4	1	>8	>16/304	64
	S%	100.0	NI	NI	NI	NI	NI	NT	NI	NT	NI	NI	NI	63.2	NI
	I%	0.0	NI	NI	NI	NI	NI	NT	NI	NT	NI	NI	NI	-	NI
	R%	0.0	NI	NI	NI	NI	NI	NT	NI	NT	NI	NI	NI	36.8	NI

CFDC: cefiderocol; FEP: cefepime; CAZ: ceftazidime; CZA: ceftazidime/avibactam; CT/TZ: ceftolozane/tazobactam; MEM: meropenem; AZT: aztreonam; AMK: amikacin; CIP: ciprofloxacin; GEN: gentamicin; TGC: tigecycline; CST: colistin; SXT: trimethoprim/sulfamethoxazole; FOS: fosfomycin. [#] According to CLSI breakpoint; * *Proteus* spp. and *Serratia* spp. isolates are intrinsically resistant to colistin, so they were excluded from MIC interpretation. S%: percentage of susceptible standard dosing isolates; I%: susceptible, increased exposure; R%: resistant. NI: not interpretable, no EUCAST breakpoints available for this agent. NT: not tested.

Table 8. Distribution of cefiderocol MICs against *Enterobacterales*, *P. aeruginosa*, *A. baumannii* complex isolates from four Italian hospitals. Each taxonomic group of isolates has been sorted based on different resistance patterns.

Family/Species/ resistance pattern (N. of isolates)	Number of isolates (%) with a CFCD MIC (mg/L):								
	≤0.03	**0.06**	**0.12**	**0.25**	**0.5**	**1**	**2**	**4**	**8**
Enterobacterales		2		8	18	21	19	34	1
(N=103)		(1.9)		(7.8)	(17.5)	(20.4)	(18.4)	(33.0)	(1.0)
MEM resistant				6	10	12	13	32	1
(N=74)				(8.1)	(13.5)	(16.2)	(17.6)	(43.2)	(1.4)
CZA resistant					2		3	3	1
(N=9)					(22.2)		(33.3)	(33.3)	(11.1)
All β-lactams tested resistant					1		2	3	1
(N=7)					(14.3)		(28.6)	(42.9)	(14.3)
CZA CST FOS susceptible only					1		1	2	
(N=4)					(25.0)		(25.0)	(50.0)	
P. aeruginosa	1		3	13	27	9	1		
(N=54)	(1.8)		(5.6)	(24.1)	(50.0)	(16.7)	(1.8)		
MEM resistant			2	11	21	6	1		
(N=41)			(4.9)	(26.8)	(51.2)	(14.6)	(2.5)		
CT/TZ resistant			1	1	7	3	1		
(N=13)			(7.7)	(7.7)	(53.8)	(23.1)	(7.7)		
CZA resistant				2	6	2			
(N=10)				(20.0)	(60.0)	(20.0)			
A. baumannii complex		1	1	14	20	15	6		1
(N=58)		(1.7)	(1.7)	(24.1)	(34.5)	(25.9)	(10.4)		(1.7)
CST susceptible only		1	1	9	16	7	4		
(N=38)		(2.6)	(2.6)	(23.7)	(42.1)	(18.4)	(10.5)		
All ATBs tested resistant				4	4	6	1		
(N=15)				(26.7)	(26.7)	(40.0)	(6.7)		
S. maltophilia	3	7		6		1	1	1	
(N=19)	(15.8)	(36.8)		(31.6)		(5.3)	(5.3)	(5.3)	
SXT resistant		3		3		1			
(N=7)		(42.9)		(42.9)		(14.2)			

CFDC: cefiderocol; CZA: ceftazidime/avibactam; CT/TZ: ceftolozane/tazobactam; MEM: meropenem; CST: colistin; FOS: fosfomycin; SXT: trimethoprim/sulfamethoxazole.

Table 9. Percentage of carbapenemases-producing isolates susceptible to cefiderocol and comparators.

Carbapenemase content (number of isolates)	Species (Number of isolates)	CFCD MIC range (mg/L)	Percentage of Susceptible Isolates (S%+I%)											
			CFDC[#]	CT/TZ	GEN	MEM	CAZ	CZA	CST	AZT	AMK	CIP	FEP	TGC	FOS
KPC (N=87)	*E. coli* (N=7) *K. pneumoniae* (N=78) *K. aerogenes* (N=1) *P. mirabilis* (N=1)	0.25-8	100.0	0.0	55.2	19.5	0.0	95.4	84.9	0.0	55.1	5.7	1.1	40.2	73.6
GES (N=5)	*P. aeruginosa* (N=5)	0.5-1	100.0	40.0	0.0	20.0	20.0	80.0	40.0	40.0	20.0	0.0	20.0	NI	60.0
VIM (N=7)	*Citrobacter* spp. (N=1) *Enterobacter cloacae* (N=1) *P. aeruginosa* (N=5)	0.25-4	100.0	0.0	42.9	14.3	0.0	0.0	85.7	42.9	57.1	28.6	0.0	28.6^	100.0
NDM (N=3)	*A. baumannii* (N=1) *E. coli* (N=1) *K. pneumoniae* (N=1)	2-8	100.0	0.0^	0.0	0.0	0.0^	0.0^	100.0	0.0^	33,3	0.0	0.0^	66.7^	66.7^
OXA-23 (N=49)	*A. baumannii* (N=49)	0.06-2	100.0	NI	4.1	0.0	NI	NI	73.5	NI	4.1	0.0	NI	NI	NI
OXA-23 + *IS*AB1/OXA-51 (N=8)	*A. baumannii* (N=8)	0.25-2	100.0	NI	0	0.0	NI	NI	75.0	NI	25.0	0.0	NI	NI	NI

CFDC: cefiderocol; FEP: cefepime; CAZ: ceftazidime; CZA: ceftazidime/avibactam; CT/TZ: ceftolozane/tazobactam; MEM: meropenem; AZT: aztreonam; AMK: amikacin; CIP: ciprofloxacin; GEN: gentamicin; TGC: tigecycline; CST: colistin; FOS: fosfomycin. NI: not interpretable, no EUCAST breakpoints available for this agent.
[#] According to CLSI breakpoint; ^ In this case, *P. aeruginosa* and *A. baumannii* results were interpreted using EUCAST breakpoint for *Enterobacterales*.

3.3.3 Activity of Cefepime/VNRX-5133 against an Italian nationwide collection of *P. aeruginosa* isolates.

P. aeruginosa remains a widespread cause of nosocomial infections and its intrinsic and acquired resistance mechanisms can significantly limit the choices for antimicrobial therapy (Nguyen *et al,* 2018). Carbapenem-resistance in *P. aeruginosa* can be the result of the overexpression of the chromosomal AmpC associated with alterations of the outer membrane permeability and/or the efflux systems or of the acquisition of β-lactamases (Giani *et al,* 2017). In particular, the acquisition of metallo-β-lactamases (MBLs) is of concern because they confer resistance also to recently approved antibiotics, including ceftolozane-tazobactam, ceftazidime-avibactam and meropenem/vaborbactam (Nguyen *et al,* 2018). As well as cefiderocol, even VNRX-5133 is a promising molecule able to inhibit Ambler class B β-lactamases (MBLs) in *P. aeruginosa* (Nguyen *et al,* 2018; Krajnc *et al,* 2019). In this project, the activity of the novel combination cefepime/VNRX-5133 (VenatoRx Pharmaceuticals, Inc., Malvern, USA). and comparator antibiotics was evaluated against a previously characterized nationwide collection of *P. aeruginosa* isolated from cases of bloodstream infections and lower respiratory tract infections (HAP/VAP), in 20 different centers (Figure 17) distributed across Italy. A total number of 48 carbapenemase producers including 32 VIM-type, 12 IMP-type and 4 GES-5 was present in the collection (Giani *et al,* 2017).

Figure 17. Geographical distribution of participating centers across Italy. 1: Milan; 2: Lecco; 3: Torino; 4: San Remo; 5: Bolzano; 6: Modena; 7: Bergamo; 8: Treviso; 9: Udine; 10: Florence; 11: Siena; 12: Perugia; 13: Ancona; 14: Rome; 15: San Giovanni Rotondo; 16: Casarano; 17: Napoli; 18: Cosenza; 19: Catania; 20: Vicenza.

Antimicrobial susceptibility testing of cefepime/VNRX-5133 and comparators, including other innovative combinations (meropenem/vaborbactam, aztreonam/avibactam, ceftazidime/avibactam, ceftolozane/tazobactam), was carried out with broth microdilution method using lyophilized custom plates (Thermofisher Scientific), adding each inhibitor to the respective β-lactam. In fact, the stock solution of each inhibitor was prepared from the respective powders and diluted to obtain

a final concentration of 4 mg/L for avibactam, tazobactam, VNRX-5133, and of 8 mg/L for vaborbactam. Results are presented in the following tables and were interpreted according to EUCAST clinical breakpoints v. 9.0, or in the cases of not-available breakpoints, such as in the case of the combinations aztreonam/avibactam, cefepime/VNRX-5133, and meropenem/vaborbactam, were evaluated using the EUCAST breakpoints for the corresponding β-lactams alone (http://www.eucast.org/clinical_breakpoints/). (Tables 10 and 11).

Table 10. MIC_{50}, MIC_{90} (mg/L) and percentage of susceptibility (%S) among collected *P. aeruginosa* isolates (N=934) against cefepime/VNRX-5133 and comparators.

TOT=934	FEP/ VNRX	CAZ	CAZ/ AVI	AZT	AZT/ AVI	FEP	FEP/ TZB	CTZ	MEM	MEM/ VB	PIP/ TZB	AK
MIC50	4	4	2	8	8	4	4	1	1	1	16	4
MIC90	8	64	16	64	32	32	32	4	16	16	>128	16
% S	91.5	68.8	87.2	1.8	86.0	71.8	74.0	91.0	66.5	80.8	59.7	89.4

CAZ: ceftazidime; CAZ/AVI: ceftazidime/avibactam; AZT: aztreonam; AZT/AVI: aztreonam/avibactam; FEP: cefepime; FEP/TZB: cefepime/tazobactam; FEP/VNRX: cefepime/VNRX-5133; CTZ: ceftolozane/tazobactam; MEM: meropenem; MEM/VB: meropenem/vaborbactam; PIP/TZB: piperacillin/tazobactam; AK: amikacin.

Table 11. Distribution of MICs to FEP/VNRX against *P. aeruginosa* are sorted by different phenotypes and/or mechanisms of resistance to ceftolozane/tazobactam (CTZ).

Group of isolates (number)	Number of isolates (%/cumulative %) with an MIC (mg/L) for FEP/VNRX-5133 of:									
	≤0.25	**0.5**	**1**	**2**	**4**	**8**	**16**	**32**	**64**	**>64**
All *P. aeruginosa* (n=934)	2 (0,2/0,2)	10 (1,1/1,3)	83 (8,9/10,2)	259 (27,7/37,9)	260 (27,8/65,7)	241 (25,8/91,5)	52 (5,6/97,1)	7 (0,7/97,9)	5 (0,5/98,4)	15 (1,6/100)
Cefepime resistant (n=263)	-	-	6 (2,3/2,3)	16 (6,1/8,4)	46 (17,5/25,9)	118 (44,9/70,7)	50 (19,0/89,7)	7 (2,7/92,4)	5 (1,9/94,3)	15 (5,7/100)
Sorted by resistance mechanism to CTZ (n=84)	-	-	1 (1,2)	4 (4,8/7)	9 (10,7/16,7)	24 (28,6/45,2)	21 (25/70,2)	5 (6.0/76,2)	5 (6.0/82,1)	15 (17,9/100)
No carbapenemase expression (n=36)	-	-	0 (0/0)	2 (5,6/5,6)	6 (16,7/22,2)	12 (33,3/55,6)	15 (41,7/97,2)	1 (2,8/100)	- (0/100)	- (0/100)
VIM-type (n=32)	-	-	1 (3,1/3,1)	2 (6,3/9,4)	3 (9,4/18,8)	8 (25/43,8)	6 (18,8/62,5)	4 (12,5/75)	4 (12,5/87,5)	4 (12,5/100)
IMP-type (n=12)	-	-	-	-	-	-	-	-	1 (8,3/8,3)	11 (91,7/100)
GES-type (n=4)	-	-	-	-	-	4 (100/100)	- (0/100)	- (0/100)	- (0/100)	- (0/100)

Cefepime/VNRX-5133 and ceftolozane/tazobactam were the most active compounds against 934 clinical isolates of *P. aeruginosa* tested, able to inhibit 90% of isolates at the concentration of 8 mg/L, and 4 mg/L, respectively (Table 10). Considering a putative breakpoint for susceptibility ≤8 mg/L, cefepime/VNRX-5133 exhibited a slightly higher percentage of susceptible isolates (91,5%) than that of ceftolozane/tazobactam (91.0%), amikacin (89,4%), ceftazidime/avibactam (86.7%) and aztreonam/avibactam (86,0%). Cefepime/VNRX-5133 retained activity against the majority of isolates resistant to the first-line agents like cefepime, meropenem, piperacillin/tazobactam and amikacin, specifically against 70.7% (186/263) of cefepime non-susceptible isolates, against 78.99% (297/376) of piperacillin/tazobactam non-susceptible isolates, 64.94% (112/174) of meropenem non-susceptible isolates, 69.8% (44/63) of amikacin non-susceptible isolates and 45.2% (38/84) of ceftolozane/tazobactam resistant isolates (Table 11). Amikacin and aztreonam/avibactam also showed good activity against 65.5% and 60.7% of these latter resistant isolates, while the combination of meropenem with vaborbactam did not substantially improve the meropenem susceptibility (Figure 18).

Among carbapenemase-producers, all GES-5-positive isolates were susceptible to ceftazidime/avibactam, aztreonam/avibactam, and cefepime/VNRX-5133, whereas vaborbactam was inactive. The combination with VNRX substantially improved the activity of cefepime against 48.3% of VIM-producers while no effect was registered against IMP producers (Table 11). Moreover, avibactam led to rescue the activity of aztreonam against 55% of aztreonam-resistant MBLs-producers. For these reasons, the novel combinations cefepime/VNRX-5133 and aztreonam/avibactam could play a central role in the treatment of infections caused by ceftolozane/tazobactam resistant *P. aeruginosa* strains and/or MBLs producers. Against these MDR *P. aeruginosa* isolates current therapeutic options are limited to colistin, amikacin and, in some cases, fosfomycin.

Cefepime/VNRX-5133 demonstrated a potent *in vitro* activity against an Italian collection of *P. aeruginosa* from BSI and HAP/VAP including many isolates resistant to the first line anti-pseudomonal agents. A limitation of this study is that the isolate collection was not very recent (2013–14). Moreover, further investigation is necessary to elucidate the mechanisms of resistance to cefepime/VNRX-5133 and understand if they were enzyme or permeability mediated.

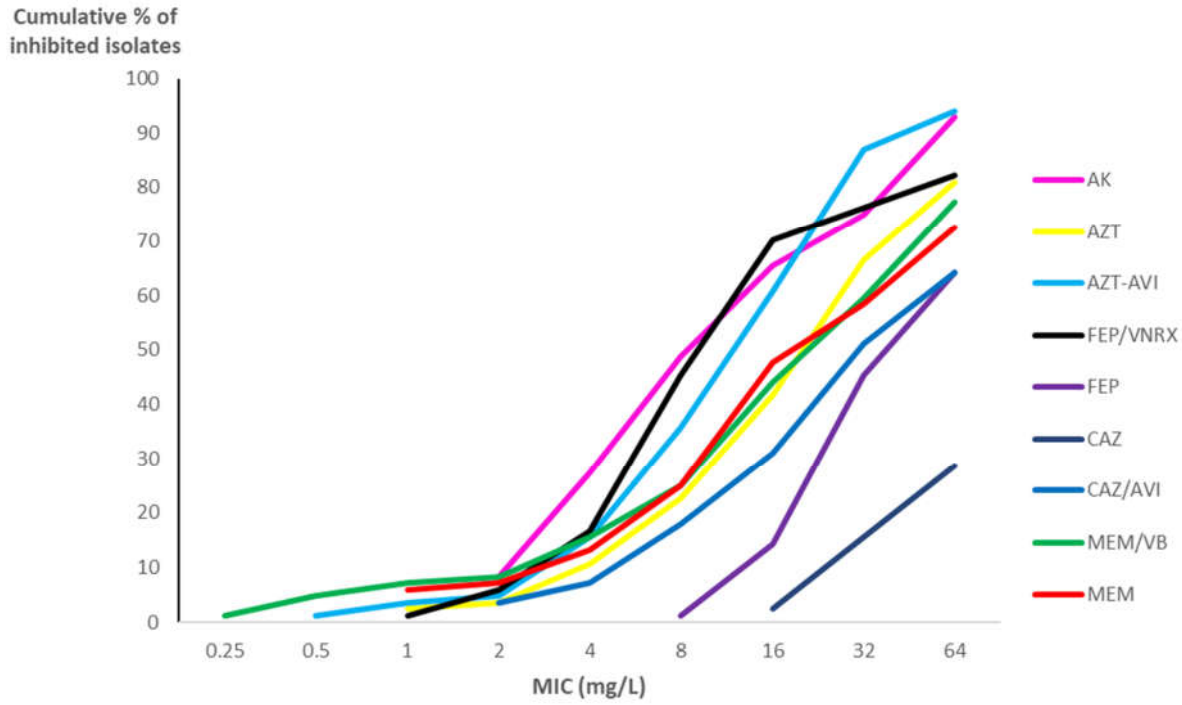


Figure 18. Inhibitory activity of cefepime/VNRX-5133 compared to single β-lactams or to the combination β-lactam/β-lactamase-inhibitors on ceftolozane/tazobactam-resistant *P. aeruginosa* strains (N=84). CAZ: ceftazidime; CAZ/AVI: ceftazidime/avibactam; AZT: aztreonam; AZT-AVI: aztreonam-avibactam; FEP: cefepime; FEP/VNRX: cefepime/VNRX-5133; CT/TZ: ceftolozane/tazobactam; MEM: meropenem; MEM/VB: meropenem/vaborbactam; PIP/TZB: piperacillin/tazobactam.

3.3.4 Activity of ceftazidime/avibactam and meropenem/vaborbactam against a multicentric collection of CRE isolated from BSI.

In this work, a collection of carbapenem-resistant *Enterobacterales* isolated from bloodstream infections from three different Italian centers was investigated to evaluate the *in vitro* activity of two recently introduced β-lactam/β-lactamase-inhibitor combinations: ceftazidime/avibactam (CAZ/AVI) and meropenem/vaborbactam (MEM/VB). The MIC values obtained with these latter antimicrobial agents were compared with those of key antibiotics (meropenem, gentamicin, amikacin, tigecycline, colistin and fosfomycin) for the treatment of invasive CRE infections. The antimicrobial susceptibility testing is part of a major project that aims to evaluate the impact on the outcome of discrepant results between automated and reference methods to determine MIC, providing clinical, microbiological and therapeutic data on CRE BSI.

In this study, 407 consecutive non-duplicate carbapenem-resistant *Enterobacterales* isolates

(imipenem and/or meropenem ≥1 mg/L) from BSI were collected by three Italian centers (Bologna, Turin and Genoa) during the period of 2013 to 2016. The species identification with MALDI-TOF Mass spectrometry yielded 403 *K. pneumoniae* and 4 *Enterobacter cloacae* complex, while the Real-Time PCR screening of the main carbapenemase genes (Antonelli *et al,* 2016 B) revealed the presence of 388 isolates (95.3%) positive for bla_{KPC} , four isolates (1.0%) positive for bla_{VIM}, one isolate (0.3%) positive for $bla_{OXA\text{-}48\text{-}like}$ and 14 isolates (3.4%) negative for all target genes, including also bla_{NDM}. Antimicrobial susceptibility testing was carried out with the broth microdilution method using custom plates (ThermoFisher Scientific) and agar dilution method (for fosfomycin), according to CLSI standards (CLSI, 2018). Results were interpreted according to EUCAST breakpoint v. 9.0 and are reported in Table 12 and Figure 19. MEM/VB and CAZ/AVI exhibited the highest *in vitro* activity with 96.1% and 93.9% of susceptible isolates, respectively. On the other hand, the other antibiotics tested showed percentages of susceptibility that ranged from 9.6% of meropenem to 81.8% of gentamicin. Moreover, the novel combinations presented the lowest MIC_{50} and MIC_{90} values (0.5 and 8 mg/L for MEM/VB; 2 and 8 mg/L for CAZ/AVI) except for tigecycline (1 and 1 mg/L), which, however, had both values out of the susceptibility breakpoint if it was interpreted according to EUCAST clinical breakpoints for *E. coli* (0.5 mg/L). Interestingly, two KPC-producing *K. pneumoniae* isolates resulted susceptible only to CAZ/AVI and MEM/VB.

Resistance to MEM/VB and CAZ/AVI was detected in 16 (3.4%) and 25 (6.1%) strains, among whom 14 (3.4%) isolates are resistant to both combinations. In particular, all VIM-producers (N=4) and 21 KPC-producing *K. pneumoniae* were not-susceptible to CAZ/AVI. Among MEM/VB resistant strains, 14 and two were KPC- and VIM-producing isolates, while two VIM-producers resulted susceptible to MEM/VB because of the concomitant susceptibility to meropenem alone. Looking at the proportion of susceptible isolates, $MIC_{50/90}$ values and the MICs distribution, MEM/VB resulted more active against the collected strains than comparator antibiotics, CAZ/AVI included, and could be considered as a salvage agent in many cases of CAZ/AVI resistant KPC-producing isolates. However, the percentage of KPC-positive isolates resistant to MEM/VB (3.6%) is slightly higher than that described in a recent review (0.7-1%) (Dhillon S, 2019). Interestingly, most resistant strains were collected from the same center, and it will be necessary to investigate the eventual correlation between resistant phenotypes and their clonality. Furthermore, it is important to notice that the collected clinical isolates have never been exposed to these novel combinations during the collection period, and, for this reason, this study can be considered a

baseline for future epidemiologic studies on the efficacy of these antibiotics. The resistance mechanisms to CAZ/AVI and MER/VB in the tested collection are currently under investigation through *whole-genome sequencing* experiments to detect eventual mutations in *bla*$_{KPC}$ gene, alterations of porin status and/or the increase in the *bla*$_{KPC}$ gene copy number.

Table 12. MIC_{50}, MIC_{90} (mg/L) and percentage of susceptibility (%S) among collected CRE isolates (N=407) against ceftazidime/avibactam, meropenem/vaborbactam and comparators.

TOT=407	CAZ/ AVI	MEM	MEM/ VB	AMK	FOT	CST	GNT	TGC
MIC_{50}	2	64	0.5	32	32	1	2	1
MIC_{90}	8	>64	8	32	>128	>8	>8	1
% S	93.9	9.6	96.1	48.2	59.0	66.3	81.8	36.1*

CAZ/AVI: ceftazidime/avibactam; MEM: meropenem; MEM/VB: meropenem/vaborbactam; AMK: amikacin. FOT: fosfomycin; CST: colistin; GNT: gentamycin; TGS: tigecycline. *Tigecycline was interpreted according to EUCAST clinical breakpoints for *E. coli* (breakpoint 0.5 mg/L).

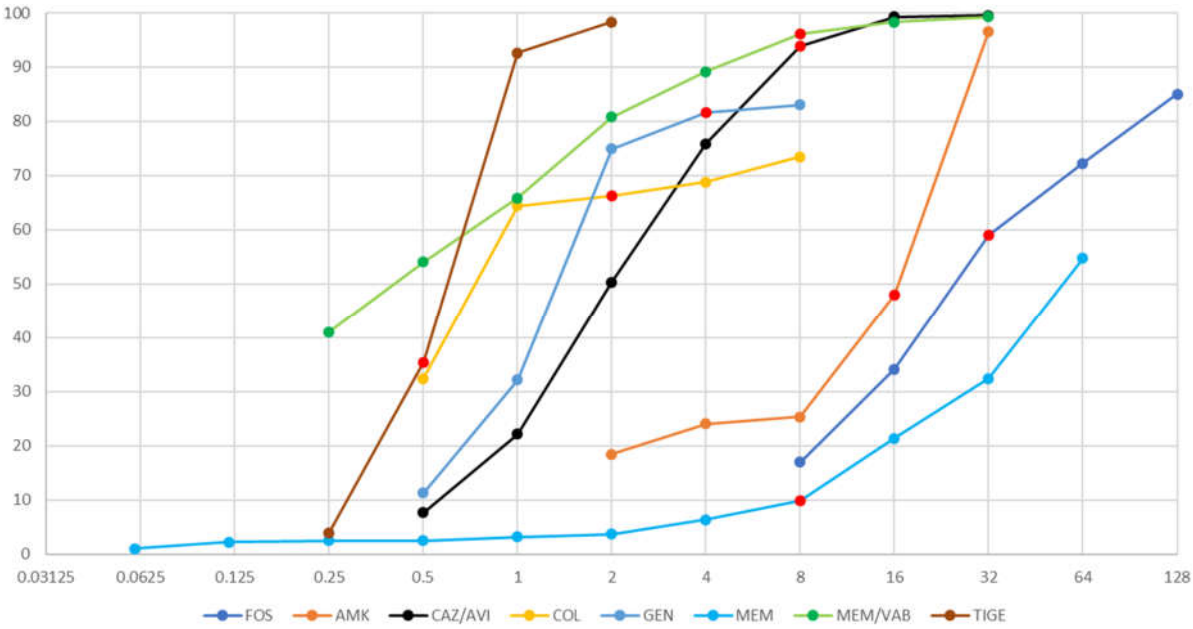


Figure 19. MIC distributions of ceftazidime/avibactam, meropenem/vaborbactam and comparators against all the tested isolates (N=407). Red points indicate the EUCAST breakpoint v. 9.0. CAZ/AVI: ceftazidime/avibactam; MEM: meropenem; MEM/VAB: meropenem/vaborbactam; AMK: amikacin. FOS: fosfomycin; COL: colistin; GEN: gentamycin; TIGE: tigecycline. *Tigecycline was interpreted according to EUCAST clinical breakpoints for *E. coli* (breakpoint 0.5 mg/L).

3.4 CHARACTERIZATION OF RESISTANCE MECHANISMS TO CEFTAZIDIME-AVIBACTAM

RELATED PUBLICATION

- **Ceftazidime/avibactam resistance associated with increased *bla*$_{KPC-3}$ gene dosage mediated by a pKpQIL plasmid derivative carrying two copies of Tn*4401a* in a ST258 *Klebsiella pneumoniae* from a kidney transplant patient.** Coppi M, Di Pilato V, Monaco F, Giani T, Conaldi PG, Rossolini GM. **Submitted (AAC01816-19).*

The urgent need for new antibacterial agents with anti-CRE activity has been partially fulfilled by the recent introduction of novel β-lactam/β-lactamase inhibitor combinations (BLICs), among which ceftazidime-avibactam (CAZ-AVI) was the first to be released for clinical use. Despite its recent introduction, resistance to CAZ-AVI has started to be documented, mainly related to missense mutations in the Ω-loop of KPC. In this work, we have characterized two isolates resistant to CAZ-AVI, although they had never been exposed to it.

During 2017, a 59-years-old female was subjected to renal transplantation, which was complicated by a urinary tract infection caused by a KPC-producing *K. pneumoniae* (isolate KP-14519) which was also resistant to all the tested antibiotics using Vitek-2 (bioMérieux). After 10 days of double carbapenem therapy (meropenem 500 mg/die plus ertapenem 500 mg/die), the patient developed breakthrough bacteremia by a KPC-positive *K. pneumoniae* with the same resistance profile (isolate KP-8788). Both KP-14519 and KP-8788 showed a similar extensively drug-resistant phenotype with ceftazidime-avibactam MIC of 32 and 64 mg/L, respectively, confirmed with reference BMD. The complete genome sequence of both isolates was obtained by whole genome sequencing (Illumina MiSeq and Oxford Nanopore MinION) in order to investigate the mechanisms of resistance to CAZ/AVI. The hybrid assembly of sequencing data generated with both short and long reads sequencing technologies resulted in three complete circular molecules for KP-8788, including the chromosome (5.38 Mb) and two plasmids, named pKPN-IT-8788 (a 271 Kb IncFIIK7-IncFIBk multireplicon) and pIT-8788 (a 102 Kb IncFIBk and ColE multireplicon), respectively. With KP-14519, the hybrid assembly resulted in a draft genome of

5.70 Mb containing both chromosomal and plasmid sequences associated with an IncFIIK7-IncFIBk replicon, and two complete circular molecules represented by two plasmids, named pIT-14519 (an 88 Kb IncFIBk replicon), and ColE-14519 (a 14 Kb ColE replicon). These isolates were representatives of the same sequence type ST258 and differed by a single chromosomal SNP and by the co-integration/rearrangement of two plasmids. Concerning plasmids, plasmid pIT-14519 from KP-14519 was an 88-kb replicon of the IncFIBk lineage, carrying *bla*$_{\text{KPC-3}}$, highly similar (>99% identity over 99% of sequence length) to previously characterized pKpQIL-like plasmids from ST258 KPC-Kp isolated during the 2008-2011 period and including the KPC-2-encoding plasmid pKpQIL-UK from *K. pneumoniae* isolates from various UK centers (Doumith *et al,* 2017), the KPC-3-encoding plasmids pIT-FIPP-1 from the Italian KPC-Kp ST258 index strain FIPP-1 (Papagiannitsis *et al,* 2016), and pKpQIL-LS6 from *K. pneumoniae* LS6 (Villa *et al,* 2013). Interestingly, the major difference between pIT-14519 and pIT-FIPP-1/pKpQIL-LS6 consisted of the presence of an additional copy of Tn*4401a* located within the *tniA* gene present aboard of the plasmid backbone and absence of *bla*$_{\text{TEM-1}}$ (Figure 20). pIT-14519, therefore, carried a double copy of *bla*$_{\text{KPC-3}}$ as compared with previously described pKpQIL-like KPC-encoding plasmids.

Plasmid ColE-14519 from KP-14519 was a 14 kb replicon belonging to the ColE plasmid family, being identical (100% identity over 100% of sequence length) to the ColE-LS6 plasmid from the previously characterized *K. pneumoniae* LS6 (Villa *et al,* 2013). Similarly to the latter plasmid, ColE-14519 carried *aac(6')-Ib* aminoglycoside resistance determinant and a cloacin-like bacteriocin (Figure 20), and was frequently detected among ST258 strains (Bowers *et al,* 2015).

Finally, plasmid pIT-8788 from KP-8788 was an IncFIBk-ColE multireplicon of 102 kb, resulting from the co-integration of pIT-14519 and ColE-14519 likely occurred following IS*26*-mediated homologous recombination. The major differences between pIT-14519 and pIT-8788 consisted of the inversion of the backbone segment laying between the proximal inverted repeats (IR) of Tn*4401a*-1 and Tn*4401a*-2, and in the presence of a *bla*$_{\text{TEM-1}}$ gene in pIT-8788 (Figure 20). Therefore, we hypothesized that the increased gene dosage of *bla*$_{\text{KPC}}$, mediated by the presence of a double copy of Tn*4401a* on a single plasmid, might give rise to a transferable resistance mechanism to ceftazidime-avibactam. To test this hypothesis, the meropenem-hydrolyzing specific activity was determined by a spectrophotometric assay to verify an increased expression of KPC (Lauretti *et al,* 1999), and transfer experiments by conjugation and transformation were carried out with pIT-8788. The amount of carbapenemase activity in crude extracts of KP-14519 and KP-8788 was 2.5-3.3-fold higher than that measured in crude extracts of *K. pneumoniae* FIPP-1 (with a

single copy of *bla*$_{KPC}$). Plasmid pIT-8788 was successfully transferred by electrotransformation into *E. coli* DH10B, while conjugal transfer experiments from either *K. pneumoniae* KP-8788 or *E. coli* DH10B(pIT-8788) were unsuccessful, consistently with the partial deletion of the *tra* locus. Transfer of the KPC-encoding plasmid from KP-8788 to *E. coli* DH10B resulted in an increase of ceftazidime-avibactam (0.25 vs 8 mg/L) and meropenem MIC (≤0.125 vs 32 mg/L), and of the meropenem hydrolysis rate.

At the chromosomal level, investigation of the major porins status in KP-14519 and KP-8788 showed that OmpK35 was nonfunctional due to a frameshift mutation (AA89-STOP), as commonly observed among ST258 members (Bowers et al,2015), while OmpK36 was altered by an original two-amino-acid insertion (Asp135Thr136) within the transmembrane β-strand loop 3 (L3), close to the previously reported duplication (Gly134Asp135) that has been commonly observed in ST258 isolates and was predicted to result in a constricted, partially functional, porin channel (Nelson *et al,* 2017; Agyekum *et al,* 2016). A previous study showed that porin alterations and an increased *bla*$_{KPC-3}$ gene dosage mediated by the presence of a Tn*4401d* transposon on two different plasmids were responsible for decreased susceptibility to CAZ/AVI (Nelson *et al,* 2017).

So, the CAZ/AVI resistance of these isolates was associated with alterations of the major porins OmpK35 (which was inactivated) and OmpK36 (which carried an original two-amino-acid insertion) in combination with overexpression of the KPC-3 enzyme due to an increased gene dosage mediated by the presence of a double copy of Tn*4401a* in the KPC-encoding plasmid. Unlike previously described by Nelson and colleagues (Nelson *et al,* 2017), in this work, the increased *bla*$_{KPC-3}$ gene dosage was mediated by a single plasmid and could, therefore, be transferred *en-bloc* with plasmid transfer resulting in a possible transferable mechanism of CAZ/AVI resistance. Although neither pIT-8788 nor pIT-14159 was self-transferable by conjugation, due to a deletion within the transfer operon, in both plasmids a complete transfer origin was detected, allowing their mobilization in the presence of helper plasmids, such as other IncF-type plasmids which are widely detected among successful clones of *K. pneumoniae* (e.g. ST512) (Papagiannitsis *et al,* 2016; Bowers *et al,* 2015). Moreover, the additional replicon identified within the pIT-8788 backbone (i.e. ColE) could potentially enhance the mobilization capabilities of this element. The presence of plasmids with a double copy of Tn*4401* aboard, as the case of pIT-8788 and pIT-14159, therefore, could represent a hidden threat, since the acquisition of a single element by a new host may directly impact on CAZ/AVI susceptibility following a

single transfer event. This is particularly worrisome in an epidemiological context where *K. pneumoniae* clones carrying alterations of the OmpK35 and/or OmpK36 porins (such as those of clonal group 258, ST11, ST15 and ST395) are frequent (Bowers *et al,* 2015; Chen *et al,* 2014). In fact, these clones may represent a genetic background potentially favoring the emergence of clinical resistance to CAZ/AVI upon transfer of similar plasmids. It should be noted, however, that a lower transferability potential of KPC-encoding plasmids from *K. pneumoniae* strains belonging to the clonal group 258 was previously documented (Papagiannitsis *et al,* 2016). Therefore, additional experiments are required to clarify the transfer behavior of pIT-8788 and pIT-14159 and the role of new duplication in OmpK36.

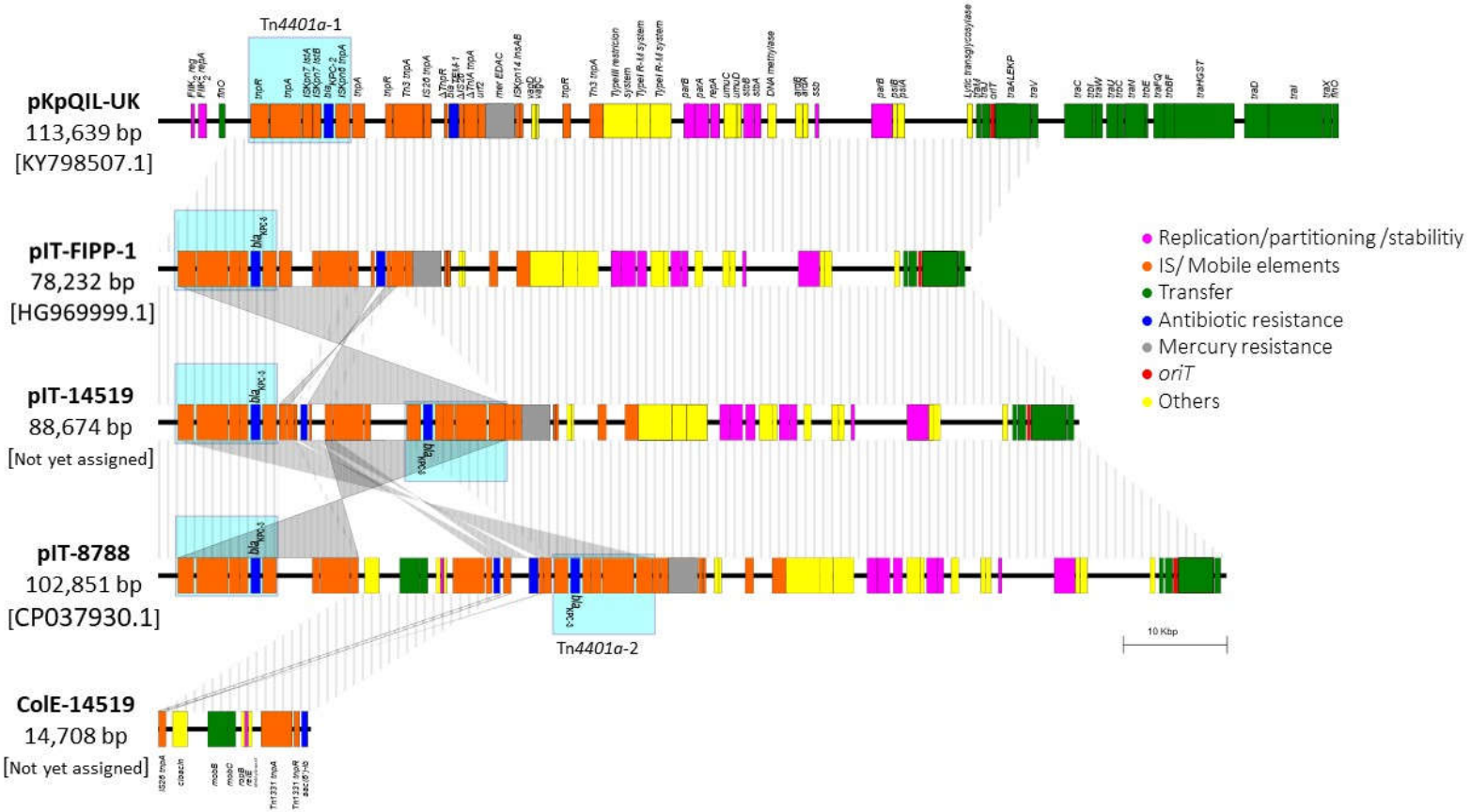


Figure 20. Linear map of two novel KPC-encoding derivative plasmids, pIT-14519 and pIT-8788, which share large regions of homology with plasmids pKpUIL-UK, pIT-FIPP-1 and between them. pIT-8788 originated from the co-integration of pIT-14519 and ColE-14519, a plasmid of ColE family. The straight or ondulate lines between plasmids indicate a sequence identity ≥99%, in the same or in the opposite orientation, respectively. Genes are represented with rectangles colored according to the function, as reported in the legend. Elements belonged to Tn*4401* transposon are grouped with a sky blue rectangles.

4. CONCLUSIONS AND PERSPECTIVES

The health and economic costs of antibacterial resistance have led the mobilization of international and national health-care organizations in order to elaborate action plans to tackle this worrisome health issue. There is a general consensus that the only effective approach against AMR would require multiple strategies, including the optimization of antimicrobial drugs use in medicine and veterinary, the development of new antimicrobial agents, and the improvement of current diagnostic systems. Moreover, multicentric surveillance studies are of paramount importance to monitor the epidemiology of AMR.

In this Ph.D. project, the characterization of local and nationwide collections of Gram-negative and Gram-positive clinical isolates at genotypic and phenotypic level was carried out.

The activity of novel antimicrobial agents (ceftobiprole and cefiderocol) and combinations β-lactam/β-lactamase inhibitors (cefepime/VNRX-5133; meropenem/vaborbactam, ceftazidime/avibactam and aztreonam/avibactam) was evaluated.

In particular, ceftobiprole showed potent activity against both methicillin-susceptible (100%) and methicillin-resistant (95%) *S. aureus* isolates collected from 13 Italian centers. The resistance mechanism to ceftobiprole has not yet been clarified in the 3 resistant MRSA strains detected, even if some mutations in PBP2a, previously associated with ceftobiprole-resistant isolates, have been observed. Moreover, the genotypic characterization of all the 66 MRSA allowed establishing the current image of the Italian epidemiology of MRSA from hospital-acquired pneumonia.

Among antibiotics against Gram-negative pathogens, cefiderocol exhibited the highest activity against both carbapenem-resistant *Enterobacterales* and non-fermentative Gram-negatives. Furthermore, cefiderocol is a promising treatment for infections caused by DTR Gram-negative bacteria, including metallo-β-lactamase-producers and carbapenemase-producing *A. baumannii* for which no β-lactam/inhibitor combinations are currently effective. An NDM-producing *E. coli*, a PER-producing *Aeromonas* spp and an OXA-23-producing *A. baumannii*, which resulted in frankly resistant (MICs >64mg/L), will be subject to more detailed investigations to understand cefiderocol resistance determinants.

Among anti-pseudomonas agents, cefepime/VNRX-5133 exhibited a slightly higher percentage of susceptible isolates (91,5%) against a multicentric collection of 934 *P. aeruginosa* compared to

ceftolozane/tazobactam, which has also been recently introduced. VNRX-5133 restored susceptibility to cefepime in 43.8% of VIM-producers, against whom also aztreonam/avibactam showed potent activity.

Both ceftazidime/avibactam and meropenem/vaborbactam resulted active against a multicentric collection of 407 carbapenem-resistant *Enterobacterales* (mainly KPC-producers) from bloodstream infections. In particular, meropenem/vaborbactam showed efficacy also in some cases of CAZ/AVI resistant KPC-producing isolates.

It will be of interest to further characterize at a genotypic level all the isolates resulted resistant to the novel antibiotics.

The resistance mechanisms to ceftazidime-avibactam in two *Klebsiella pneumoniae* isolates from urine and bloodstream infections, respectively, have been elucidated using a *whole-genome sequencing* approach and gene transfer experiments. The resistance has been associated with an increased $bla_{\text{KPC-3}}$ gene dosage mediated by a new pKpQIL plasmid derivative carrying two copies of Tn*4401a*.

In a scenario of uncertainty due to scarcity of effective therapies against MDR pathogens the availability of accurate and cost-effective methodologies to asses susceptibility to last-resort antibiotics has a crucial role. However, the commercial methods tested in this study showed to be unreliable for testing carbapenems and fosfomycin susceptibility to challenging clinical isolates as KPC-producing *E. coli* and *K. pneumoniae.*

Finally, a new phenotypic method (the colistin-MAC test) has been developed for phenotypic screening of acquired colistin resistance mediated by transferable *mcr-1* resistance determinants, based on colistin MIC reduction in the presence of dipicolinic acid. This inexpensive test will be used to screen large collections of isolates to detect new *mcr*-like genes not yet targeted by the current molecular assays.

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This is the first thesis that I've written in English, I apologize for my poor English. I'll take all the difficulties encountered as an encouragement to do even better in the future.

This emotional paragraph couldn't end without a special thanks to all people that I don't mention here, and maybe don't understand this work, but have the first place in my *macro*-system.

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I conclude with the Vasari's invitation: "*Cerca trova".*

7. ANNEXES

7.1 RELATED PUBLICATIONS

1) **Coppi M**, Cannatelli A, Antonelli A, I. Baccani I, Di Pilato V, Sennati S, Giani T, Rossolini GM. A simple phenotypic method for screening of MCR-1-mediated colistin resistance. Clin Microbiol Infect. 2018. 24(2):201.e1-201.e3.

2) Camarlinghi G, Parisio EM, Antonelli A, Nardone M, **Coppi M**, Giani T, Mattei R, Rossolini GM. Discrepancies in fosfomycin susceptibility testing of KPC-producing *Klebsiella pneumoniae* with various commercial methods. Diagn Microbiol Infect Dis. 2019. 93(1):74-76.

3) Antonelli A, **Coppi M**, Camarlinghi G, Parisio EM, Nardone M, Riccobono E, Giani T, Mattei R, Rossolini GM. Variable performance of different commercial systems for testing carbapenem susceptibility of KPC carbapenemase-producing *Escherichia coli.* Clin Microbiol Infect. 2019. pii: S1198-743X(19)30444-6.

4) Alberto A, Giani T, **Coppi M**, Di Pilato V, Arena F, Colavecchio OL, Conte V, Santerre Henriksen A, Rossolini GM, MRSA-HAP Study Group. *Staphylococcus aureus* from hospital-acquired pneumonia from an Italian nationwide survey: activity of ceftobiprole and other anti-staphylococcal agents, and molecular epidemiology of methicillin-resistant isolates. Journal of Antimicrobial Chemotherapy, dkz371. https://doi.org/10.1093/jac/dkz371

5**) Coppi M***, Di Pilato V, Monaco F, Giani T, Conaldi PG, Rossolini GM.
Ceftazidime/avibactam resistance associated with increased bla_{KPC-3} gene dosage mediated by a pKpQIL plasmid derivative carrying two copies of Tn*4401a* in a ST258 *Klebsiella pneumoniae* from a kidney transplant patient. **Submitted (AAC01816-19).*

7.2 OTHER PUBLICATIONS PROVIDED ONLY FOR INFORMATION

Coppi M, Antonelli A, Giani T, Spanu T, Liotti FM, Fontana C, Mirandola W, Gargiulo R, Barozzi A, Mauri C, Principe L, Rossolini GM. Multicenter evaluation of the RAPIDEC® CARBA NP test for rapid screening of carbapenemase-producing *Enterobacteriaceae* and Gram-negative non fermenters from clinical specimens. *Diagn Microbiol Infect Dis*. 2017. J 88(3):207-213. **This work includes data that was discussed in the master thesis.*

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Research note

A simple phenotypic method for screening of MCR-1-mediated colistin resistance

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ABSTRACT

Objectives: To evaluate a novel method, the colistin-MAC test, for phenotypic screening of acquired colistin resistance mediated by transferable *mcr-1* resistance determinants, based on colistin MIC reduction in the presence of dipicolinic acid (DPA).
Methods: The colistin-MAC test consists in a broth microdilution method, in which colistin MIC is tested in the absence or presence of DPA (900 μg/mL). Overall, 74 colistin-resistant strains of *Enterobacteriaceae* (65 *Escherichia coli* and nine other species), including 61 strains carrying *mcr-1*-like genes and 13 strains negative for *mcr* genes, were evaluated with the colistin-MAC test. The presence of *mcr-1*-like and *mcr-2*-like genes was assessed by real-time PCR and end-point PCR. For 20 strains, whole-genome sequencing data were also available.
Results: A ≥8-fold reduction of colistin MIC in the presence of DPA was observed with 59 *mcr-1*-positive strains, including 53 *E. coli* of clinical origin, three *E. coli* transconjugants carrying MCR-1-encoding plasmids, one *Enterobacter cloacae* complex and two *Citrobacter* spp. Colistin MICs were unchanged, increased or at most reduced by twofold with the 13 *mcr*-negative colistin-resistant strains (nine *E. coli* and four *Klebsiella pneumoniae*), but also with two *mcr-1*-like-positive *K. pneumoniae* strains.
Conclusions: The colistin-MAC test could be a simple phenotypic test for presumptive identification of *mcr-1*-positive strains among isolates of colistin-resistant *E. coli,* based on a ≥8-fold reduction of colistin MIC in the presence of DPA. Evaluation of the test with a larger number of strains, species and *mcr*-type resistance determinants would be of interest. **M. Coppi, Clin Microbiol Infect 2018;24:201.e1–201.e3**

Introduction

Polymyxins have lately regained a key role as last-resort antibiotics for treatment of infections caused by multidrug-resistant and extremely drug-resistant Gram-negative pathogens [1].

In *Enterobacteriaceae,* acquired resistance to polymyxins is mostly mediated by modification of the lipid A target (by addition of 4-amino-4-deoxy-L-arabinose and/or phosphoethanolamine residues) that impair polymyxin binding [2]. Target modification can be dependent on chromosomal mutations up-regulating the endogenous lipopolysaccharide modification systems, or by exogenous phosphoethanolamine transferases encoded by acquired mobilized colistin resistance (*mcr*) genes. Discovery of the latter mechanisms has raised considerable concern in view of their potential for spreading among clinical pathogens [3]. Indeed, after the first description [4], *mcr-1* and variants thereof have been detected worldwide [5], while additional types of *mcr* genes (e.g. *mcr-2*, *mcr-3* and *mcr-4*) have recently been identified [6–8].

Because MCR-1 is a zinc enzyme [9], exposure to chelators could reduce colistin resistance in MCR-1-producing strains. Indeed, ethylenediaminetetraacetic acid (EDTA) was recently reported to potentiate the activity of colistin against MCR-1-producing strains [9].

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Here we describe a novel test, the colistin-MAC test, based on colistin MIC reduction in the presence of dipicolinic acid (DPA), which could be useful for phenotypic screening of *mcr-1*-positive colistin-resistant *Escherichia coli* strains.

Methods

The colistin-MAC test is based on colistin MIC testing, in the absence or presence of DPA, in a broth microdilution (BMD) format according to the Clinical and Laboratory Standards Institute (CLSI) standard [10]. The colistin sulphate (Sigma-Aldrich, St Louis, MO, USA) concentrations tested ranged from 0.125 to 8.0 μg/mL, alone or in combination with DPA at a fixed concentration of 900 μg/mL. DPA was chosen because it was reported to have greater selectivity for zinc ions [11], and it exhibited a better performance than EDTA in phenotypic assays for detection of zinc-β-lactamases [12]. The DPA (Sigma-Aldrich) stock solution was prepared in dimethyl sulphoxide (DMSO) (SERVA, Heidelberg, Germany) at a concentration of 100 mg/mL and stored at −20°C. Cation-adjusted Mueller-Hinton broth (Becton Dickinson, Milan, Italy) was used as the medium for susceptibility testing. Mueller-Hinton broth containing DPA and DMSO at final concentrations of 900 μg/mL and 0.9% (v/v), respectively, was used for growth control. Susceptibility testing was carried out in 96-well microtitre plates (Sarstedt, Nümbrecht, Germany). Results were recorded after 20 hours of incubation at 35 ± 2°C. Susceptibility to colistin was interpreted according to the European Committee on Antimicrobial Susceptibility Testing (EUCAST) clinical breakpoints, version 7.1 (≤ 2 μg/mL) (http://www.eucast.org/). All tests were carried out in duplicate. The two repetitions were considered concordant when a ≥8-fold reduction or a ≤2-fold reduction of colistin MIC in the presence of DPA was observed in both replicates. In case of discordant results, a third replicate was carried out and the modal value was considered.

The colistin-MAC test was evaluated with 74 colistin-resistant strains from our laboratory collection (Supplementary Table S1), including 61 strains carrying *mcr-1*-like genes, in which the genes had been detected by PCR or whole-genome sequencing; and 13 strains in which the absence of *mcr-1*-like and *mcr-2* genes had been confirmed by real-time PCR and end-point PCR (Supplementary Table S2). In nine of these strains, the absence of all known *mcr* genes *mcr-1* to *mcr-4* had also been confirmed by whole-genome sequencing analysis. Most strains were *E. coli* ($n = 65$), and a few belonged to other enterobacterial species including *Klebsiella pneumoniae* ($n = 6$), *Citrobacter braakii* ($n = 1$), *Citrobacter freundii* complex ($n = 1$) and *Enterobacter cloacae* complex ($n = 1$). The tested strains included 70 isolates of clinical origin (blood or urine cultures, or surveillance rectal swabs), one isolate of environmental origin and three laboratory-derived *E. coli* J53 transconjugants harbouring different *mcr-1*-carrying plasmids (Supplementary Table S1). Some of the investigated strains have been previously reported, while others are part of an ongoing study. All *E. coli* strains were confirmed to be clonally unrelated by random amplification of polymorphic DNA PCR (Supplementary Table S1). The *K. pneumoniae* strain carrying *mcr-1*-like gene was ST1 and presented a capsular *wzi-19* allele, while that carrying *mcr-1.2* was ST512 and presented a capsular *wzi-154* allele.

The effect of DPA in increasing colistin susceptibility of *mcr-1*-like-positive strains was also tested in a disc diffusion format [13] using colistin discs (10 μg; Oxoid, Thermo Fisher Scientific, Waltham, MA, USA) added with either 7.5 or 10 μL of the DPA stock solution (i.e. 750 or 1000 μg per disc, respectively) before placing the disc onto the inoculated medium.

Results

The tested strains exhibited a colistin MIC ranging from 4 to >8 μg/mL (Table 1), and all of them grew well in the presence of DPA and DMSO at the concentrations used in the test.

In the presence of DPA at 900 μg/mL, all the 53 *mcr-1*-positive *E. coli* strains exhibited a reduction of colistin MIC of at least an eightfold dilution (range, 8- to ≥128-fold). A similar behavior was also observed with the three *E. coli* J53 transconjugants carrying different *mcr-1* plasmids, with the *mcr-1*-positive *E. cloacae* complex strain and with the two *mcr-1*-positive *Citrobacter* strains (Table 1). Discordant results between the two replicates were only observed with three *mcr-1*-positive *E. coli* strains, which resulted in a fourfold reduction of colistin MIC in one of the two repetitions. In these cases, a third replicate yielded a >8-fold colistin MIC reduction (Supplementary Table S1), and the median value was considered. Colistin MIC was increased, unchanged or at most decreased by a twofold dilution with the *mcr*-negative *E. coli* and *K. pneumoniae* strains, but also with the two *mcr-1*-like positive *K. pneumoniae* strains (Table 1).

In the disc diffusion format, no significant differences were detected in inhibition zones between *mcr-1*-positive and *mcr*-negative colistin-resistant strains (data not shown), probably as a result of the low and variable diffusibility of colistin from discs [14].

Discussion

The colistin-MAC test which we describe here could be a simple method for screening of *mcr-1*-positive strains among colistin-resistant *E. coli,* and possibly also of other species of *Enterobacteriaceae*. In fact, considering as a criterion for *mcr-1* positivity/negativity a ≥8-fold/≤2-fold reduction of colistin MIC in the presence of DPA, the test was able to correctly categorize all the 65 colistin-resistant *mcr-1*-positive *E. coli* (including 62 clinical strains and three laboratory-derived transconjugants), and also a few *mcr-1*-positive strains of other species. The lack of inhibitory effect observed with the *mcr-1*-positive *K. pneumoniae* could be due to a reduced permeability to DPA and/or to the presence of additional unknown mechanisms of colistin resistance in those strains. We encountered only three results falling in the intermediate range (fourfold colistin MIC reduction by DPA), which were confirmed as positive (≥8-fold colistin MIC reduction) in two additional replicates. In case of an intermediate result, we suggest repeating the test and, if the intermediate result is confirmed, reporting the result as indeterminate.

This study was conceived as a preliminary proof-of-concept evaluation of the colistin-MAC test, and as such, it has a number of limitations, including the following: (a) the small sample size of investigated strains; (b) the limited number of investigated species, with a very small number of strains of species other than *E. coli;* (c) the small number of strains with (putative) chromosomal mechanisms of resistance, as negative controls; and (d) the lack of strains carrying *mcr* genes other than *mcr-1.* The performance of this test should be confirmed with a larger collection of strains representative of different species and different colistin resistance mechanisms. The colistin-MAC test has also some inherent limitations: the test apparently does not work with *K. pneumoniae;* and the execution requires the preparation of test-specific BMD panels, which can be labour intensive and could increase costs in case of commercial production. It could be interesting to evaluate the performance of the test carried out with the addition of DPA directly to the wells of a commercial panel.

Nevertheless, the possibility to presumptively detect *E. coli* strains with plasmid-mediated colistin resistance due to

Table 1
Bacterial strains tested in this study, and colistin MIC values measured by broth microdilution in absence or presence of 900 μg/mL DPA (colistin-MAC test)

Species	Mechanism of colistin resistance[a]	No. of strains	MIC colistin (μg/mL) (median)	MIC colistin (μg/mL) + DPA 900 μg/mL (median)	Fold MIC reduction
Escherichia coli	*mcr-1*/ *mcr-1*-like	53	4 to >8 (8)	≤0.125 to 1 (≤0.125)	8 to ≥128
	mcr-NEG, ND	9	4 to 8 (8)	4 to >8 (8)	—[b]
Escherichia coli J53AZI^R transconjugants	*mcr-1*-like	3	4 (4)	≤0.125 to 0.5 (0.25)	8 to ≥32
Klebsiella pneumoniae	*mcr-1*-like/*mcr-1.2*	2	8 to >8	>8	—[b]
	mcr-NEG	4	>8	>8	—[b]
	PmrB mutant/inactivated *mgrB*/ND				
Citrobacter braakii	*mcr-1*	1	8	0.5	16
Citrobacter freundii complex	*mcr-1*	1	8	≤0.125	≥64
Enterobacter cloacae complex	*mcr-1*	1	>8	≤0.125	≥128

For a detailed description of tested strains and results, see Supplementary Table S1.
DPA, dipicolinic acid; ND, colistin resistance mechanism remained not determined.
[a] *mcr-1*-like indicates that the gene was amplified with primers for *mcr-1* but was not entirely sequenced; *mcr*-NEG indicates that the strain was negative for *mcr-1*-like and *mcr-2*-like genes as assessed by real-time PCR and end-point PCR. In some cases, whole-genome sequencing data confirmed negativity for all known *mcr* genes.
[b] Colistin MIC, in presence of DPA, was increased, unchanged or at most decreased by a twofold dilution.

acquisition of *mcr-1* genes (which overall appear to be the most prevalent strains with transferable colistin resistance worldwide) with a test that can be set up in basic laboratories not equipped with facilities for molecular testing could be of remarkable importance for surveillance purposes, especially in low-income settings.

Transparency Declaration

AA reports grants and nonfinancial support from ELITech group outside the submitted work. MC reports nonfinancial support from ELITech group outside the submitted work. TG reports grants from Astra-Zeneca, personal fees from Alifax, personal fees from bio-Merieux, personal fees from Thermo Fisher Scientific and personal fees from Accelerate Diagnostics outside the submitted work. GMR reports grants from bioMérieux, grants from Becton-Dickinson, nonfinancial support from Beckman Coulter, grants and nonfinancial support from Alifax, grants from Check-points, grants, personal fees and nonfinancial support from Accelerate Diagnostics, personal fees from Curetis, grants and personal fees from Cepheid, grants and personal fees from ELITech group, personal fees from Thermo Fisher Scientific, nonfinancial support from Biomedical Service, personal fees from Achaogen, personal fees from Angelini, grants and personal fees from Astra-Zeneca, grants and personal fees from Basilea Pharmaceutica, grants from Biotest, personal fees from Menarini, grants and personal fees from Merck, grants and personal fees from Nordic Pharma, personal fees from Pfizer, grants from Rempex, grants and personal fees from Zambon, grants and personal fees from Angelini ACRAF, grants from Arrow diagnostics, grants from DID Diagnostics, grants from Estor, grants from Liofilchem, grants and nonfinancial support from Novartis and nonfinancial support from Copan outside the submitted work. The other authors report no conflicts of interest relevant to this article.

Appendix A. Supplementary data

Supplementary data related to this article can be found at https://doi.org/10.1016/j.cmi.2017.08.011.

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Antimicrobial Susceptibility Studies

Discrepancies in fosfomycin susceptibility testing of KPC-producing *Klebsiella pneumoniae* with various commercial methods

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ABSTRACT

Fosfomycin susceptibility testing with Sensititre, Vitek2, Etest, Mic Strip Test and disk diffusion methodologies was compared versus reference agar dilution method (AD) with 78 clinical isolates of KPC-producing *Klebsiella pneumoniae*. All methodologies showed a Categorical Agreement and Essential Agreement of ≤69% and ≤72%, respectively, revealing a very low concordance with AD.

Carbapenem-resistant Enterobacteriaceae (CRE) have spread globally in the last decade, and represent one of the most critical challenges to antimicrobial chemotherapy (Nordmann et al., 2011; van Duin and Doi, 2017). Among CRE, *Klebsiella pneumoniae* is the species most affected by carbapenem resistance, and production of carbapenemases of different types (KPC, OXA-48-like, NDM, VIM and IMP) is the most prevalent resistance mechanism in this species (Nordmann et al., 2011; van Duin and Doi, 2017). In Italy, a country where CRE have achieved a high level of endemicity, *K. pneumoniae* strains producing KPC-type carbapenemases (KPC-KP) are by far the predominant CRE type (Giani et al., 2017).

Antibiotic treatment options for carbapenemase-producing *K. pneumoniae* are limited. Fosfomycin, an old antibiotic that inhibits the early stages of peptidoglycan synthesis (Michalopoulos et al., 2011), has long been used for treating uncomplicated urinary tract infections (Keating, 2013) and has recently attracted interest as an anti-CRE agent (Endimiani et al., 2010; Reffert and Smith, 2014). Indeed, recent studies revealed that many CRE strains retain susceptibility to fosfomycin (Kaase et al., 2014), and that fosfomycin has a synergistic effect with carbapenems (meropenem and ertapenem), colistin, aminoglycosides (netilmicin, amikacin) and tigecycline against KPC-KP (Evren et al., 2013; Samonis et al., 2012; Yu et al., 2017).

For these reasons, intravenous fosfomycin, in combination with other agents, is now considered a valuable anti-CRE option (Reffert and Smith, 2014), and the accuracy of susceptibility testing of fosfomycin with CRE strains has become a crucial issue in clinical microbiology laboratories.

According to EUCAST and CLSI, agar dilution (AD) is the reference method for fosfomycin susceptibility testing (CLSI, 2018; EUCAST, 2018). However, AD is not suitable for use in routine susceptibility testing.

The aim of this study was to determine the susceptibility to fosfomycin of a collection of clinical isolates of KPC-KP with agar dilution, and to compare the results with those obtained using automated, disk diffusion and gradient diffusion systems.

A total of 78 non-replicated KPC-KP clinical isolates (44 from rectal-swabs, 21 from urine cultures, 3 from sputum cultures, 7 from blood cultures, and 3 from wound swabs) were collected from 2015 to 2017 at the Clinical Microbiology Laboratory of San Luca Hospital (Lucca, Italy), and were investigated in this study. *Escherichia coli* ATCC 25922 was used as a quality control strain. Identification was carried out by MALDI-ToF (Vitek MS; bioMérieux, Marcy l'Étoile, France). Production of KPC was confirmed in all strains by the KPC K-Set immunochromatographic assay (Coris BioConcept, Gembloux, Belgium).

The same 0.5 McFarland inoculum, prepared from an overnight culture in blood agar plates, was used for fosfomycin testing with

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reference AD (EUCAST, 2018; http://www.eucast.org/clinical_breakpoints/) and the following methods: Sensititre (ITGNEGF panel, with fosfomycin wells added with glucose-6-phosphate (G6P), 25 μg/mL, Thermo Fisher Scientific, Waltham, MA, USA); Vitek2 (AST 201 card, bioMérieux); Etest (bioMérieux), disk diffusion (200 μg fosfomycin/50 μg G6P disks; Bio-Rad Laboratories, Hercules, CA); and MIC Test Strip (performed with 50 of the 78 KPC-KP isolates, randomly selected; Liofilchem, Roseto degli Abruzzi, Italy). All commercial methods were performed as recommended by the manufacturer. The MIC results were interpreted according to the EUCAST clinical breakpoints (susceptible ≤32 μg/mL, resistant >32 μg/mL) (EUCAST, 2018). For disk diffusion, reading of results was performed and interpreted as recommended by EUCAST (2018) for *E. coli*, since no interpretation criteria are present for *K. pneumoniae*.

Agreement of results obtained with different methods in comparison with reference AD was evaluated according to the International Organization for standardization ISO 20776–2, 2007 standard (ISO). Four parameters were used to compare different methods: essential agreement (EA), categorical agreement (CA), major errors (ME), and very major errors (VME). MIC values between doubling dilutions obtained by gradient diffusion methods were rounded up to the nearest doubling dilution. EA was fulfilled when the MIC value obtained with the commercial method was within ±1 doubling dilution compared with that by reference AD. CA was defined as the percentage of isolates classified in the same susceptibility category by the reference AD and the other method. Categorical discrepancies were classified as ME when an isolate was categorized as resistant by one of the methods but susceptible by AD, or VME when an isolate was categorized as susceptible by one of the methods but resistant by reference AD. Acceptable performance was evaluated according to the criteria established by the ISO as follows: ≥90% for EA or CA, ≤ 3% for VME or ME (ISO 20776–2, 2007).

Among the 78 KPC-KP isolates, 45 (57.7%) were susceptible to fosfomycin by AD. Table 1 shows MIC distribution, the percentage of isolates classified as susceptible and resistant to fosfomycin by each testing method, and the values of CA, ME, VME and EA. Overall, the EA and CA for all the commercial methods were in poor agreement with the reference method, with no method being acceptable according to the ISO criteria.

Sensititre and Etest exhibited high rates of VME (54.5% and 78.8%, respectively). Therefore, these two methods are more likely to underestimate MIC values compared to the AD reference method. On the other hand, Vitek2, disk diffusion and MIC Test Strip exhibited high rates of ME (75.6%, 84.4% and 76.7%, respectively). Therefore, these three methods are more likely to overestimate MIC values compared to the AD reference method (Fig. 1).

It should be noted that the reference method for fosfomycin susceptibility testing recommended by ISO 2776–1, 2006 standard is agar dilution since broth microdilution may not give reliable results (ISO 2776–1, 2006).

Altogether, our results indicated that the commercial systems evaluated in this study exhibit a poor correlation with reference AD for fosfomycin susceptibility testing with KPC-KP clinical isolates.

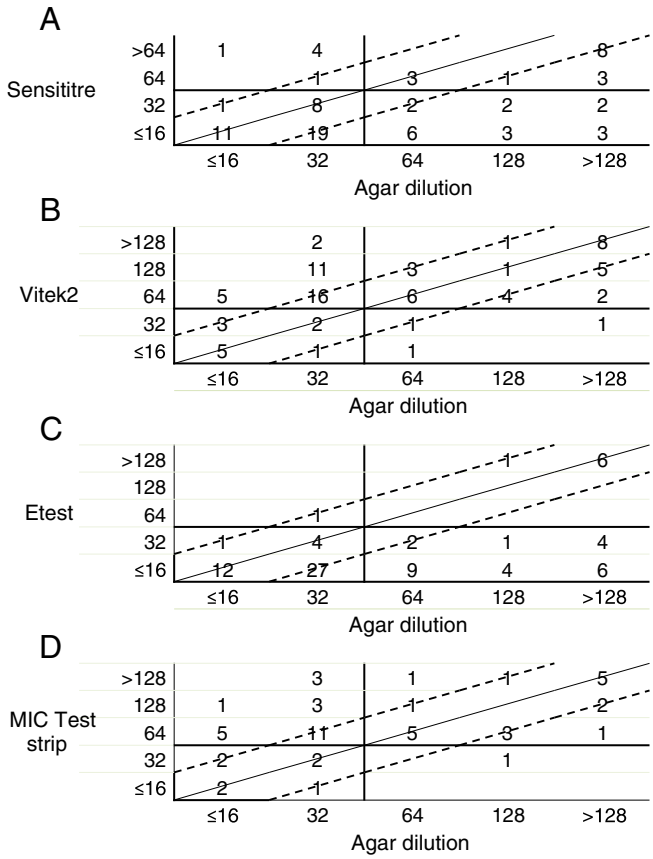

Fig. 1. Scattergram of fosfomycin MICs for KPC-KP tested measured by Agar dilution and Sensititre (A), Vitek2 (B), Etest (C), and MIC Test strip (D). MICs were indicated in μg/mL.

These results are in overall agreement with those of other studies, which have shown that the result of fosfomycin susceptibility testing is dependent on the method used and the microorganisms tested (de Cueto et al., 2006; Perdigão-Neto et al., 2014). Focusing the attention on carbapenemase producing *Enterobacteriaceae* other studies evaluating Etest and disk diffusion showed a poor correlation of these methods with agar diffusion (Kaase et al., 2014; Endimiani et al., 2010; Perdigão-Neto et al., 2014). However, since these studies tested a lower number of isolates, used different reference breakpoints and different methods, the results are not directly comparable.

With disk diffusion and MIC Test Strip, the colonies grown within the inhibition halo (observed with 31.5% and 25% of the strains, respectively) were not taken into account in the MIC reading, as recommended by EUCAST (2018) and by the manufacturer (Liofilchem), respectively. With Etest, the macrocolonies grown within the inhibition halo (observed with 29.7% of the strains) were not taken into account if the number was <5 as recommended by the

Table 1
Results of fosfomycin susceptibility testing with KPC-KP using reference Agar dilution, Sensititre, Vitek2, Etest, disk diffusion and MIC test strip. Seventy-eight KPC-KP strains were evaluated will all methods except for MIC Test Strip (N = 50).

	MIC (μg/mL)					Category (%)		CA (%)	ME	VME	EA
	≤16	32	64	128	>128	S	R				
Agar dilution	13	32	11	6	16	45 (57.7)	33 (42.3)				
Sensititre	42	15	8	13[a]	-	57 (73.1)	21 (26.9)	54 (69.2)	6 (13.3)	18 (54.5)	54 (69.2)
Vitek2	7	7	33	20	11	14 (17.9)	64 (82.1)	41 (52.6)	34 (75.6)	3 (9.1)	56 (71.8)
Etest	58	12	1	-	7	70 (89.7)	8 (10.3)	51 (65.4)	1 (2.2)	26 (78.8)	54 (69.2)
Disk diffusion[b]	-	-	-	-	-	8 (10.3)	70 (89.7)	39 (50)	38 (84.4)	1 (3)	-
MIC Test Strip	3	5	25	7	10	8 (16)	42 (84)	26 (52)	23 (76.7)	1 (5)	36 (72)

[a] Sensititre range ≤ 16- >64 μg/mL.
[b] Zone diameter interpretation breakpoint referred to *E. coli* (EUCAST, 2018).

manufacturer (bioMérieux). The presence of these colonies makes reading difficult, and could impair accuracy of results if reading is performed by unexperienced personnel.

A limitation of this study is that the isolates were collected from a single center and were only KPC-KP. It will be interesting to further investigate the performance of various fosfomycin-testing methods with larger collections of CRE representative of different species and different resistance mechanisms. Another limitation is that all strains were tested only once, and therefore reproducibility of results was not evaluated.

These findings therefore suggest that results of susceptibility testing of fosfomycin obtained with methods other than AD should be considered with caution. Since no results obtained with the investigated methods fell within the ISO parameters, laboratory routines should always carry out the AD to test fosfomycin susceptibility, especially for KP-KPC isolates from critical patients.

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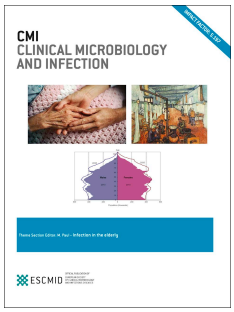

Variable performance of different commercial systems for testing carbapenem susceptibility of KPC carbapenemase-producing *Escherichia coli*

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Variable performance of different commercial systems for testing carbapenem susceptibility of KPC carbapenemase-producing *Escherichia coli*

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Running title: Comparison of methods for carbapenem MIC testing of KPC-producing *E. coli*

Keywords: carbapenemase, MIC, category agreement, essential agreement, discrepancy

Abstract

Objectives

To evaluate different methods for testing carbapenem susceptibility of *Escherichia coli* producing KPC-type carbapenemase.

Methods

Susceptibility to imipenem, meropenem and ertapenem was assayed using the reference broth microdilution method and several commercial methods (Vitek2, MicroScan, Etest, MIC Test Strip) starting from the same bacterial suspension. Susceptibility to imipenem and meropenem was also tested by Sensititre and disk diffusion (Bio-Rad). Results were interpreted according to EUCAST clinical breakpoints. Essential agreement (EA), category agreement (CA) and error rates were calculated as described by the International Organization for Standardization (ISO) guidelines and also considering the new EUCAST definitions. Genotypic diversity of isolates was evaluated with a RAPD profiling protocol.

Results

Of 54 KPC-positive *E. coli* isolates, 5.6%, 7.4% and 0% were susceptible standard dosing regimen (S), 55.6%, 72.2% and 0% susceptible increased exposure (I), and 38.9%, 20.4% and 100.0% resistant (R) to imipenem, meropenem and ertapenem, respectively, using the reference broth microdilution method. CA lower than 90% were observed with all systems for imipenem and meropenem using both the ISO and the modified EUCAST criteria. With ertapenem, CA >90% was observed with all methods except Vitek2. RAPD profiling revealed a remarkable genotypic diversity of the isolates, supporting that results were not biased by an oligoclonal nature of the collection.

Conclusions

Commercial methods can be unreliable for testing susceptibility to carbapenems of KPC-producing *E. coli*. Susceptibility should be confirmed by reference broth microdilution.

INTRODUCTION

The spread of carbapenem-resistant *Enterobacterales* has become a worldwide issue of high priority for healthcare systems [1]. This resistance phenotype is mainly contributed by the diffusion of acquired resistance mechanisms such as class A (i.e. KPC), class B (NDM, VIM and IMP), and class D (OXA-48-like) carbapenemases [2,3]. The epidemiology of carbapenemase-producing *Enterobacterales* (CPE) varies greatly from country to country. In Italy and some other countries, KPC-type carbapenemases are the most prevalent carbapenemases in strains of CPE [3,4]. Although *Klebsiella pneumoniae* is the most affected species, in settings of high endemicity the emergence of KPC-type carbapenemases has also been reported among *Escherichia coli* [4-7]. These findings are a matter of major concern given the primary role of this species as a cause of infections in both hospital and community settings.

Among KPC-producers, *E. coli* usually presents a lower expression of the carbapenem resistant phenotype compared to *K. pneumoniae*. Therefore, detection of KPC-producing *E. coli* could be sometimes problematic [8,9]. Moreover, accurate carbapenem MIC measurement is also important for consideration of carbapenem containing regimens [8].

In this work we investigated the performance of several commercial methods and of disk diffusion for testing carbapenem susceptibility with a collection of KPC-producing *E. coli*, and found remarkably variable performances of the various methods.

MATERIAL AND METHODS

A total of 54 non-replicate KPC-producing *E. coli* isolates were collected from two different Hospitals located in central Italy (Careggi University Hospital, Florence, and San Luca Hospital, Lucca) between 2014 and 2017. Most isolates were from surveillance swabs (n= 42 from rectal swabs and one from vaginal swab), while the remaining were from urinary tract infections (n= 8) or lower respiratory tract infections (n= 3). Species identification was performed by Matrix Assisted Laser Desorption Ionization Time-of-Flight

(Vitek MS; bioMérieux, Marci l'Etoile, France). The presence of KPC-type carbapenemase was confirmed by a Real Time PCR analysis targeting *bla*$_{\text{KPC-type}}$, *bla*$_{\text{OXA-48-like}}$, *bla*$_{\text{VIM-type}}$ and *bla*$_{\text{NDM-type}}$ carbapenemase genes.[10]

Genotypic diversity of the KPC-producing *E. coli* isolates was investigated by random amplification of polymorphic DNA (RAPD) profiling, as described previously [11].

Antimicrobial susceptibility to imipenem, meropenem and ertapenem was assayed using in-house reference broth microdilution method (BMD) [12], and with five commercial systems for antimicrobial susceptibility testing including: Sensititre Gram Negative MIC Plates ITGNEGF (only for imipenem and meropenem; Thermo Scientific™ Sensititre™, MA USA), Vitek2 AST-201 card (bioMèrieux), MicroScan Neg MIC Panel Type 44 (Beckman Coulter CA USA), Etest (bioMèrieux), MIC Test Strip (Liofilchem, Roseto degli Abruzzi, Italy). For testing with each commercial system, the inoculum was prepared starting from the same suspension used for BMD, and all the commercial systems were performed as recommended by the respective Manufacturers. Susceptibility to imipenem and meropenem was also tested by disk diffusion (Bio-Rad Laboratories, CA USA), performed as recommended by EUCAST [12] and starting from the same inoculum.

Results of broth microdilution and disk diffusion methods were interpreted according to EUCAST clinical breakpoints (v 9.0 2019) [14]. Internal colonies for Etest and MIC strip were taken into account as recommended by manufacturer's instructions. *E. coli* ATCC 25922 was used as quality control strain.

Essential agreement (EA), categorical agreement (CA), very major error (VME), major error (ME), and minor error (mE) were evaluated as established by the International Organization for Standardization (ISO) [15]. The CA and error rates for meropenem were also calculated according to the new EUCAST criteria, considering together the categorization susceptible standard dosing regimen (S) and susceptible increased exposure (I) vs resistant (R). For each method, EA was calculated excluding not evaluable MIC values due to truncations in the tested concentration range. Gradient diffusion MIC values between doubling dilutions were rounded up to the nearest doubling dilution. Acceptable performance was evaluated according to the

same ISO criteria as follows: ≥90% for EA or CA, ≤3% for VME or ME, and ≤7% for ME plus mE.

RESULTS

Reference BMD results showed that, of the 54 KPC-positive *E. coli* isolates, 5.6%, 7.4% and 0% were susceptible standard dosing regimen, 55.6%, 72.2% and 0% susceptible increased exposure and 38.9%, 20.4% and 100.0% resistant to imipenem, meropenem and ertapenem, respectively (**Table 1**). In all cases the MIC values determined by BMD were higher than the ECOFF values for each molecule.

The RAPD analysis of KPC-producing *E. coli*, showed 44 different profiles, revealing a marketed clonal diversity. RT-PCR analysis for the most common carbapenemase genes (bla_{KPC}, bla_{OXA-48}, bla_{VIM} and bla_{NDM}) revealed that all isolates were positive only for bla_{KPC}.

Comparison of the results of reference BMD with those obtained with several commercial methods and disk diffusion are shown in **Table 1**.

Overall, for commercial methods returning MIC values and for all molecules, the EA with the reference method was relatively low, with no method being acceptable according to the ISO criteria (with Microscan and ertapenem, the EA could not be evaluated due to the very narrow range of tested concentrations).

Concerning CA and error rates of the various testing methods, calculated according to the ISO criteria for imipenem the CA rates were 16.7-51.8 %, and the VME, ME and mE were 0-66.7%, 0% and 48.1-66.7%, respectively; for meropenem, the CA rates were 14.8-79.6%, and the VME, ME and mE rates were 0-45.5%, 0%-50% and 18.5-75.9%, respectively; for ertapenem, the CA rates were 83.3-96.3%, and the VME and ME rates were 3.7-16.7%, and 0%, respectively. According to EUCAST, the CA rates of the various testing methods were 57.4-75.9% for imipenem, and 27.8-88.9 for meropenem and the VME and ME rates were 14.3-85.7% and 0-60.6% for imipenem and 0-81.8 and 2.3-90.7 for meropenem, respectively (Table 1).

Altogether, with meropenem and imipenem, Vitek2 showed a trend to overcall resistance, while Sensititre, Microscan, Etest, MIC strip and disk diffusion showed a trend to undercall resistance. With ertapenem, all methods showed a trend to undercall resistance (**Table 1** and **Figure 1**).

Concerning ECOFFs, the percentages of KPC-producing isolates reported with a meropenem MIC ≤ECOFF (i. e. ≤0.125 mg/L) were 1.9% with Etest, 1.9% with MIC strip, and 1.9% with Sensititre, while with Vitek2 the ECOFF value was not evaluable (lowest reported MIC value, ≤0.25 mg/L). The percentages of KPC-producing isolates reported with an imipenem MIC ≤ECOFF (i. e. 0.5 mg/L) were 9.3% of cases with Vitek2, 7.4% with Etest and 7.4% with MIC strip. The ECOFF value was on the contrary not evaluable with Microscan and Sensititre (lowest reported MIC value, ≤1 mg/L).

DISCUSSION

To the best of our knowledge, this is the first study comparing different commercial methods and disk diffusion with reference BMD for the assay of carbapenem susceptibility in KPC-producing *E. coli*.

All tested methods demonstrated to be inadequate for the correct evaluation of carbapenems MICs with KPC-producing *E. coli.* In agreement with the results of this study, also a recent EUCAST warning underlined possible carbapenem interpretation problems for carbapenemase-producing *E. coli* and *K. pneumoniae* with a Sensititre panel (http://www.eucast.org/ast_of_bacteria/warnings/).

Using the new EUCAST definition of susceptible categories (S+I), evaluation of the performance of the different systems for meropenem and imipenem showed an overall improvement of CA values, while the rates of ME and VME were increased.

According to the results of this work, the detection of putative carbapenemase producers based on the ECOFF values could also be difficult using commercial systems, due to either the limited MIC range, or the underestimation tendency of some of the methodologies.

Recent studies indicate that the favorable impact on outcomes of CRE-KPC infections by using active drug combinations that include at least one carbapenem is significant only when the meropenem MIC for the

CRE-KPC isolate is ≤8 mg/L, but it is not significant when the meropenem MIC exceeds 32 mg/L [16,17]. For this reason, the use of reliable methods for the evaluation of carbapenem MICs is necessary. Among the studied methods Vitek2 was able to measure for meropenem MIC values up to 8 mg/L, while Sensititre up to 16 mg/L, and the other methods up to 32 mg/L. This limitation of tested MIC range and results variability among the different methods, compared to reference broth microdilution, could significantly impact on the clinical use of carbapenems in case of infections mediated by KPC-producing *E. coli*.

This study has the following limitations: i) the number of isolates tested was limited and more isolates should be included to confirm these results, even if the detection of KPC-producing *E. coli* remains relatively uncommon; ii) *E. coli* isolates producing other types of carbapenemases (e. g. VIM-, OXA-48- and NDM-type) were not investigated, due to the limited number of such isolates available in our collection; iii) the tested isolates were from two centers; however, genotypic profiling of the isolates revealed a substantial diversity of the tested isolates, suggesting that results were not biased by an oligoclonal nature of the collection; iv) when testing disk diffusion, only disks from a single producer were evaluated, and it would be interesting to extend the performance evaluation also to disks from other producers.

In conclusion, our findings reveal substantial discordance in the susceptibility results between the tested methods and BMD, with none satisfying the criteria for acceptable antimicrobial susceptibility testing (AST) performance. This study suggests that, whatever semiautomated AST systems are used for testing KPC-producing *E. coli*, laboratories should always confirm carbapenems MICs with reference BMD in order to carry out an appropriate antimicrobial therapy.

Transparency declaration

AA reports personal fees and nonfinancial support from ELITech group, and nonfinancial support from SymCel and Arrow Diagnostics outside the submitted work. MC reports nonfinancial support from ELITech group, Cardiome and SymCel outside the submitted work. TG reports grants from Venetorx, personal fees from Alifax, personal fees from bioMérieux, personal fees from Thermo Fisher Scientific, personal fees from Accelerate Diagnostics and personal fees from Becton Dickinson outside the submitted work. GMR reports

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Table 1. Comparison of the different antimicrobial susceptibility methods with reference BMD for imipenem, meropenem, ertapenem.

IMIPENEM

Method	S	I	R	TOT	EA (%)[#]	CA (%); *CA (%)**	ME (%); *ME (%)**	VME (%); *VME (%*)*	mE (%)
Broth microdilution	3	30	21	54					
Sensititre	44	7	3	54	21/51 (41.2)	9 (16.7); *36 (66.7)*	0	14 (66.7); *18 (85.7)*	31 (57.4)
Microscan	35	11	8	54	41/50 (82.0)	15 (27.8); *41 (75.9)*	0	6 (28.6); *13 (61.9)*	33 (61.1)
Vitek2	6	10	38	54	22/43 (51.2)	28 (51.8); 31 (57.4)	0; *20 (60.6)*	0; *3 (14.3)*	26 (48.1)
Etest	35	9	8	54	32/52 (61.5)	12 (22.2); 38 (70.4)	0; *2 (6.1)*	6 (28.6); *14 (66.7)*	36 (66.7)
Kirby-Bauer	36	14	4	54	-	12 (22.2); 37 (68.5)	0	8 (38.1); *17 (81.0)*	34 (63)
MIC Strip	32	11	9	54	40/52 (76.9)	16 (29.6); 40 (74.1)	0; *2 (6.1)*	5 (23.8); *12 (57.1)*	33 (61.1)

MEROPENEM

Method	S	I	R	TOT	EA[#]	CA (%); *CA (%)**	ME (%); *ME (%)**	VME (%); *VME (%*)*	mE
Broth microdilution	4	39	11	54					
Sensititre	45	6	3	54	6/54 (11.1)	8 (14.8); 44 (81.5)	0; 1 (2.3)	5 (45.5); *9 (81.8)*	41 (75.9)
Microscan	8	39	7	54	47/53 (88.7)	43 (79.6); 48 (88.9)	0; 1 (2.3)	1 (9.1); *5 (45.5)*	10 (18.5)
Vitek2	3	1	50	54	1/14 (7.1)	14 (25.9); 15 (27.8)	2 (50%); 39 (90.7)	0	38 (70.4)
Etest	34	13	7	54	17/51 (33.3)	20 (37); 48 (88.9)	0; 1 (2.3)	2 (18.2); *5 (45.5)*	32 (59.3)
Kirby-Bauer	39	12	3	54	-	14 (25.9); 44 (81.5)	0; 1 (2.3)	5 (45.5); *9 (81.8)*	35 (64.8)
MIC Strip	27	17	10	54	22/51 (43.1)	24 (44.4); 45 (83.3)	0; 4 (9)	2 (18.2); *5 (45.5)*	28 (51.9)

ERTAPENEM

Method	S	I	R	TOT	EA (%)[#]	CA (%)	ME (%)	VME (%)
Broth microdilution	0	0	54	54				
Microscan	2	0	52	54	NE[§]	52 (96.3)	0	2 (3.7)
Vitek2	9	0	45	54	5/38 (13.2)	45 (83.3)	0	9 (16.7)
Etest	5	0	49	54	11/48 (22.9)	49 (90.7)	0	5 (9.3)
MIC Strip	5	0	49	54	23/47 (48.9)	49 (90.7)	0	5 (9.3)

S: susceptible standard dosing; I: susceptible, increased exposure; R: resistant; TOT: total number; CA: category agreement; ME: major error; VME: very major error; mE: minor error; EA: essential agreement

[#] Due to truncations in the concentration range, the number of tests for EA calculation varied depending of each evaluated method

***** CA, ME and VME for imipenem and meropenem were evaluated also considering S and I as a unique category

[§]NE not evaluable; when evaluable numbers were <10, the value was not calculated.

Figure 1. Scattergram of meropenem, imipenem and ertapenem MICs for KPC-producing *E. coli* tested measured by broth microdilution and Microscan, Sensititre, Vitek2, Etest and MIC Test strip. MICs were indicated in mg/L.

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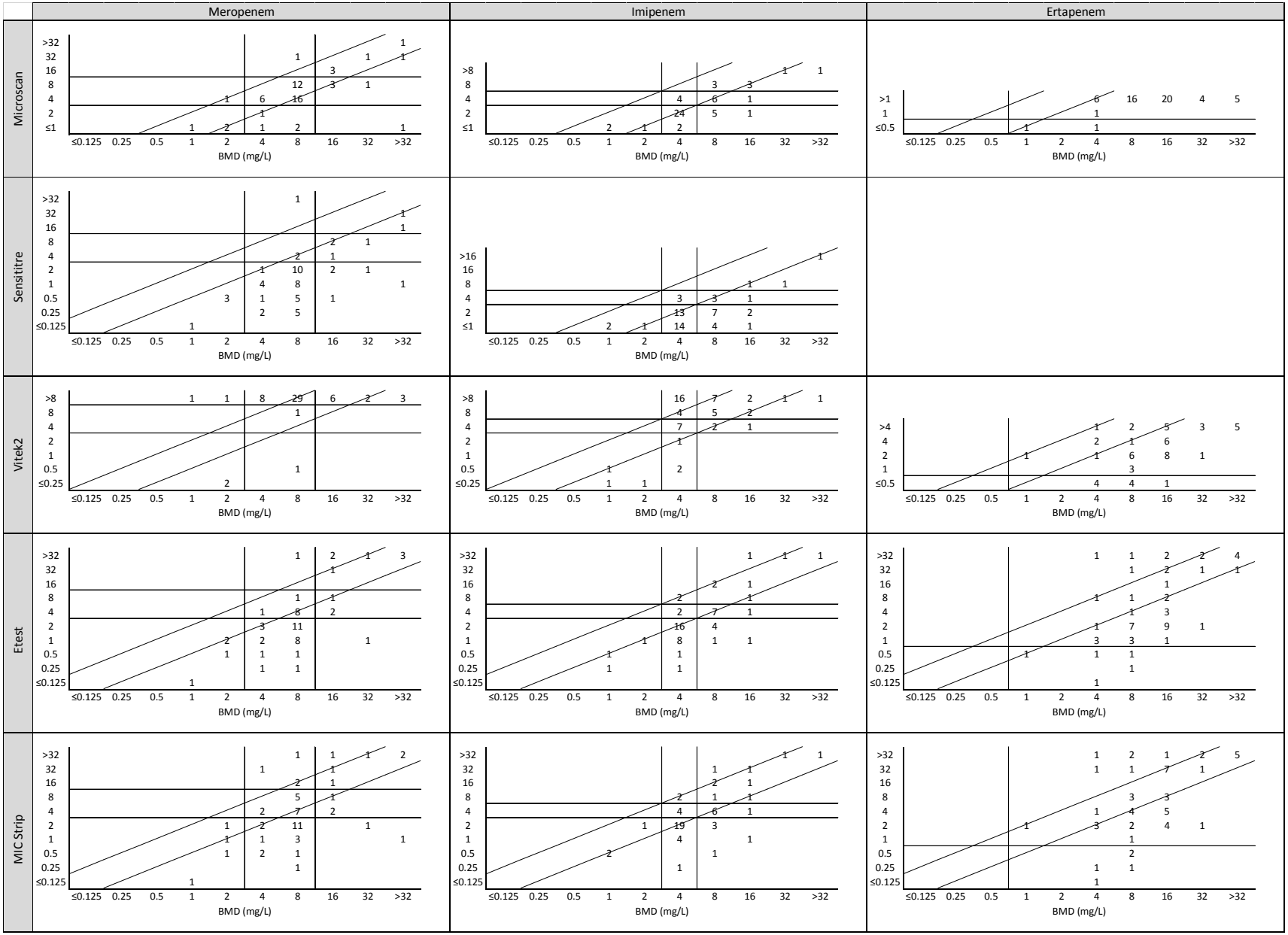

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Journal of Antimicrobial Chemotherapy

Staphylococcus aureus from hospital-acquired pneumonia from an Italian nationwide survey: activity of ceftobiprole and other anti-staphylococcal agents, and molecular epidemiology of methicillin-resistant isolates

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Objectives: To determine the prevalence of *Staphylococcus aureus* from hospital-acquired pneumonia (HAP) in Italy and the susceptibility to ceftobiprole and comparators of MSSA and MRSA isolates. A secondary objective was to characterize the clonality and acquired resistance and virulence genes of MRSA.

Methods: Consecutive non-replicate isolates from HAP were collected from 13 laboratories distributed across Italy, from January to May 2016. Antimicrobial susceptibility testing was performed by broth microdilution, and results were interpreted according to the EUCAST breakpoints. All MRSA isolates were subjected to WGS using an Illumina platform. Clonality and resistance and virulence gene content were investigated with bioinformatics tools.

Results: Among 333 isolates from HAP, *S. aureus* was the third most common pathogen (18.6%). The proportion of MRSA was 40.3%. Susceptibility to ceftobiprole was 100% for MSSA and 95.5% for MRSA. Lower susceptibility rates of 78.4% and 94.6% in MSSA and 36.4% and 12.1% in MRSA isolates were observed for erythromycin and levofloxacin, respectively. The MRSA from HAP mostly belonged to clonal complex (CC) 22 (47.0%), CC5 (25.8%) and CC8 (15.2%), with a minority of other lineages (ST1, ST6, ST7, ST30, ST152 and ST398). Acquired resistance and virulence genes in most cases exhibited a clonal distribution. The three ceftobiprole-resistant isolates exhibited an MIC of 4 mg/L and belonged to ST228-MRSA-I of CC5.

Conclusions: *S. aureus* is an important cause of HAP in Italy. Ceftobiprole exhibited good *in vitro* activity against *S. aureus* isolated from HAP, including MRSA. A trend to replacement of ST228 with ST22 was noticed compared with previous studies.

Introduction

Hospital-acquired pneumonia (HAP) is one of the most common healthcare-associated infections, with a notable burden in terms of morbidity and mortality.[1,2] *Staphylococcus aureus* is the leading Gram-positive pathogen responsible for HAP, although with variable geographical prevalence.[3] In Italy, according to recent ECDC data, *S. aureus* is responsible for 17% of HAP.[4]

Methicillin resistance remains the major resistance issue in *S. aureus*. The global dissemination of MRSA clones inside hospitals has complicated the treatment of staphylococcal HAP, leaving a limited number of treatment options.[5] Ceftobiprole is a fifth-generation cephalosporin with potent anti-staphylococcal activity covering not only MSSA but also MRSA, being the first anti-MRSA β-lactam approved for treatment of HAP (excluding ventilator-associated pneumonia).[6] In previous studies, ceftobiprole showed

excellent activity against MRSA causing HAP,[7] including strains of different resistance phenotypes and clonotypes.[8]

The objective of this study was to investigate the prevalence of *S. aureus* and MRSA among clinical isolates from HAP in Italy, a country where the proportion of MRSA remains among the highest in Europe,[9] and to evaluate the *in vitro* activity of ceftobiprole and other anti-staphylococcal agents against these isolates. The molecular epidemiology, resistome and virulome features of MRSA from HAP were also investigated by a WGS approach.

Methods

Study design

Thirteen clinical microbiology laboratories serving 31 hospitals, including 8 university hospitals distributed over the Italian territory, and covering a catchment population of ~13.3 million people, were involved as a network (referred to as the MRSA-HAP Study Group) during this collaborative study [Figure 1 and Table S1 (available as Supplementary data at *JAC* Online)]. During a 5 month period (1 January to 31 May 2016), all centres were asked to collect up to 25 consecutive non-replicate clinical isolates of any bacterial pathogen putatively responsible for HAP, based on presence with a colony count $\geq 1\times10^4$ cfu/mL or $\geq 1\times10^6$ cfu/mL in bronchoalveolar lavage or bronchial aspirate samples, respectively,[10] from patients with a clinical presentation of pneumonia developed after at least 48 h since hospital admission. The centres were asked to continue the collection of all non-replicate MRSA isolates putatively responsible for HAP until the end of the study period (also exceeding the total number of 25 isolates). Isolates from cystic fibrosis patients were excluded. Identification of the isolates collected by the participating centres was carried out by MALDI-TOF MS according to local protocols. All collected isolates were sent to the central laboratory (Department of Experimental and Clinical Medicine, University of Florence), where confirmation of species identification by MALDI-TOF MS (with Vitek-MS, bioMérieux, Marcy-l'Étoile, France) and further phenotypic and molecular characterization were performed.

Antimicrobial susceptibility testing

All confirmed *S. aureus* isolates were subjected to antimicrobial susceptibility testing. The activity of ceftobiprole and other agents (erythromycin, levofloxacin, linezolid, teicoplanin, tigecycline, trimethoprim/sulfamethoxazole and vancomycin) was tested by broth microdilution[11] using custom-lyophilized plates (ThermoFisher Scientific, Waltham, MA, USA). MIC results were interpreted according to EUCAST clinical breakpoints (version 9.0, 2019).[12] The MRSA phenotype was confirmed by cefoxitin and oxacillin broth microdilution. In the case of discrepancy between MIC results performed by the central laboratory and the MRSA status reported by the collecting centres, a cefoxitin disc screen test, following EUCAST recommendations,[12] was also performed. *S. aureus* ATCC 29213 (MSSA) and *S. aureus* ATCC 33591 (MRSA) were used as quality control strains.

Molecular characterization of MRSA isolates

The genomic DNA of all confirmed MRSA was extracted as follows. A loopful of an overnight culture grown in Mueller–Hinton agar at 35±2°C was suspended in 1 mL of lysis buffer ATL (QIAGEN, Hilden, Germany), corresponding to an OD approximately equivalent to that of a 4 McFarland standard, and boiled for 15 min at 100°C. Afterwards, 0.4 g of glass beads (≤106 μm; Sigma–Aldrich, St Louis, MO, USA) were added and the sample was processed with TissueLyser II (QIAGEN) for 2 min at 30 Hz and then centrifuged for 5 min at 12 000 **g**. The supernatant was processed for DNA extraction with the QIAsymphony DSP Virus/Pathogen Kit with Complex 800 protocol (QIAGEN). The DNA solutions were stored at −20°C until further use.

Genomic DNA was subjected to WGS using an Illumina MiSeq platform (Illumina Inc., San Diego, CA, USA) and a paired-end approach (2×300 bp). *De novo* assembly was performed using SPAdes software version 3.11.1.[13] Contigs obtained with SPAdes were further annotated by the RAST web server.[14] Sequence comparisons were carried out using the BLAST web server at the NCBI web site (http://blast.ncbi.nlm.nih.gov/). Alignment of sequenced genomes and phylogenetic tree construction were done using the CSI phylogeny 1.4 program and the Evolview online software.[15,16] MLST was performed *in silico* with the MLST v.1.8 software.[17] *spa* typing was performed *in silico* with the spaTyper v1.0 software.[18] SCC*mec* typing was performed *in silico* with the SCCmecFinder v1.2 software (https://cge.cbs.dtu.dk/services/SCCmecFinder/) and BLAST analysis. Analysis of resistance genes was performed with the ResFinder 2.1 software.[19] Virulence gene analysis was performed using the VirulenceFinder 1.5 software.[20] Additionally, *speG* and *psm-mec* virulence genes were searched with BLAST analysis.[21,22] Genes putatively involved in ceftobiprole resistance[23–29] were investigated by bioinformatics analysis using the BLAST web server.

Results and discussion

Prevalence of *S. aureus* and other pathogens from HAP

A total of 333 consecutive non-replicate isolates from putative cases of HAP were collected by the 13 participating centres during the study period. The most prevalent species were *Pseudomonas aeruginosa* (*n*=105, 31.5%), *Klebsiella pneumoniae* (*n*=63, 18.9%), *S. aureus* (*n*=62, 18.6%), *Escherichia coli* (*n*=26, 7.8%), *Acinetobacter baumannii* (*n*=21, 6.3%), *Enterobacter cloacae* complex (*n*=12, 3.6%), *Serratia marcescens* (*n*=12, 3.6%), other Gram-negatives (*n*=25, 7.5%) and other Gram-positives (*n*=7, 2.1%) (Table S2).

The prevalence of *S. aureus* from HAP detected in this study was overall similar to that reported by the ECDC,[4] ranging from 0% to 32% in the different centres. The overall proportion of MRSA among the *S. aureus* isolates was 40.3% (25 of 62), ranging from 0% to 100% in the different centres (Table S1).

During the study period, 41 additional MRSA isolates from putative cases of HAP were also collected, according to the study protocol, yielding a total of 66 MRSA isolates.

Antimicrobial susceptibility of the *S. aureus* isolates

All MSSA isolates (*n*=37) were susceptible to ceftobiprole, vancomycin, teicoplanin, tigecycline, linezolid and trimethoprim/sulfamethoxazole. Most of them were also susceptible to levofloxacin, while approximately a fifth were resistant to erythromycin (Table 1).

All MRSA isolates (*n*=66) were susceptible to vancomycin, teicoplanin, tigecycline, linezolid and trimethoprim/sulfamethoxazole. Most of them retained susceptibility to ceftobiprole (95.5%), while lower susceptibility rates to erythromycin (36.4%) and levofloxacin (12.1%) were observed (Table 1). The three ceftobiprole-resistant isolates had an MIC of 4 mg/L (i. e. one dilution above the EUCAST susceptibility breakpoint).

Overall, six isolates, three MSSA and three MRSA, showed a vancomycin MIC of 2 mg/L. All of them were susceptible to teicoplanin and ceftobiprole (data not shown).

Molecular epidemiology of the MRSA isolates

Using a WGS approach, the draft genomes of the 66 MRSA isolates were determined and the sequences were analysed for clonality, and resistome and virulence determinants.

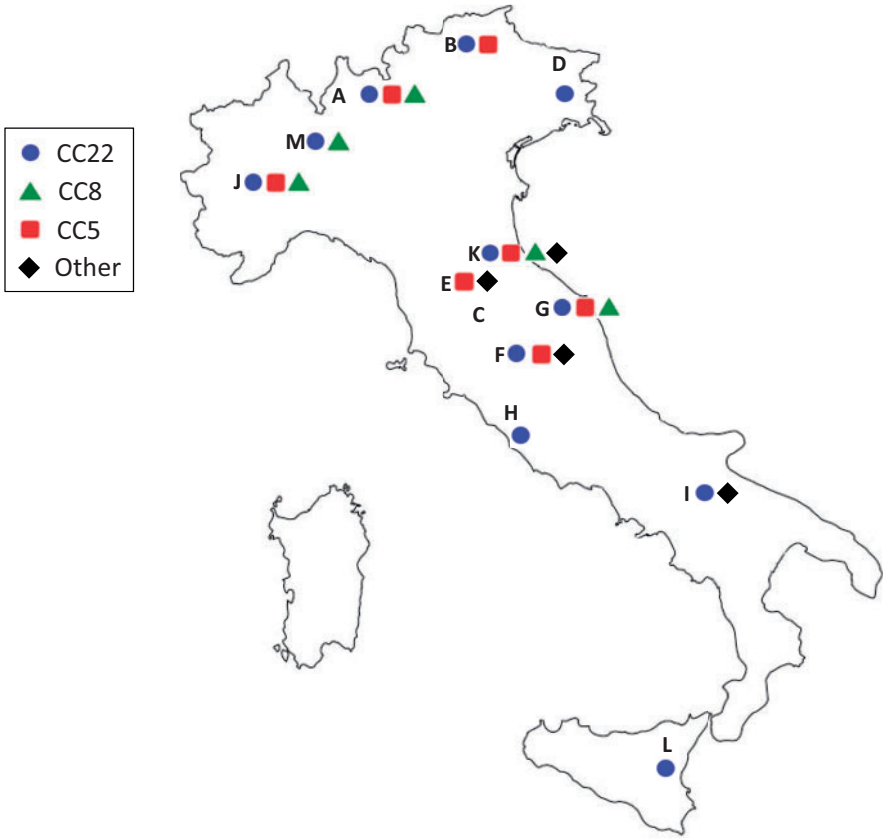

Figure 1. Geographical distribution of the 13 laboratories participating in the study. Centre codes are as follows: A, Lecco; B, Bolzano; C, Arezzo; D, Udine; E, Florence; F, Perugia; G, Ancona; H, Rome; I, Foggia; J, Turin; K, Cesena; L, Catania; and M, Milan. Geographical distribution of the most prevalent MRSA CCs (CC5, CC8 and CC22) is also shown. This figure appears in colour in the online version of *JAC* and in black and white in the print version of *JAC*.

Table 1. Susceptibility of MSSA and MRSA isolates to ceftobiprole and comparators

	CBP	ERY	LVX	LZD	TEC	TGC	SXT	VAN
MSSA, *n*=37								
MIC_{50} (mg/L)	0.5	0.5	0.25	2	0.5	0.25	≤0.06	1
MIC_{90} (mg/L)	0.5	>1	0.5	4	1	0.25	0.5	1
percentage susceptible	100	78.4[a]	94.6	100	100	100	100	100
MRSA, *n*=66								
MIC_{50} (mg/L)	1	>1	16	2	0, 5	0.25	≤0.06	1
MIC_{90} (mg/L)	2	>1	>16	4	1	0.5	0.12	1
percentage susceptible	95.5	36.4[a]	12.1	100	100	100	100	100

CBP, ceftobiprole; ERY, erythromycin; LVX, levofloxacin; LZD, linezolid; TEC, teicoplanin; TGC, tigecycline; SXT, trimethoprim/sulfamethoxazole (1:19); VAN, vancomycin.
[a]Percentage of isolates susceptible to the standard dosing regimen.

In silico MLST and SCC*mec* typing revealed that most isolates (47.0%) belonged to clonal complex (CC) 22, being mostly represented by ST22-MRSA-IV but also by ST3863-MRSA-IV (a novel single locus variant of ST22). One isolate, belonging to ST22-MRSA-IV, also presented a novel *spa*-type allele. The other most prevalent lineages were CC5 (25.8%), with ST5-MRSA-II, -IV and -VIII, ST105-MRSA-II, ST228-MRSA-I and ST3864-MRSA-II (a novel single locus variant of ST5), and CC8 (15.2%), with ST8-MRSA-IV and ST239-MRSA-III. Interestingly, three ST8-MRSA-IV isolates additionally presented *ccrA4/B4* genes, typical of SCC*mec*-VI elements. The presence of both *ccrA2/B2* and *ccrA4/B4* genes was previously described in an MRSA, in CoNS and in *Staphylococcus pseudintermedius*.[30–32] There were also three isolates of ST1-MRSA-IV and singletons of ST30-MRSA-IV, ST152-MRSA-V, ST6-MRSA-IV and ST398-MRSA-V (Table 2 and Figure 2). Notably, the livestock-associated MRSA belonging to ST398 have already been isolated from bloodstream infections and ventilator-associated pneumonia in Italy.[33,34]

The isolates belonging to CC22 were highly related to each other (SNP variation=4–247, mean=104, median=89), while those of CC5 were more divergent (SNP variation=11–519, mean=354, median=462). Within CC8 isolates, variability was lower than within CC5 but higher than within CC22 isolates (SNP variation=0–514, mean=239, median=223), with the ST239 isolates as outliers (SNP variation: 4346–4457) (Figure 2). Concerning geographical distribution, CC22 was found to be widespread across the Italian territory, while CC5 and CC8 were apparently more prevalent in central and northern Italy (Figure 1).

Table 2. Clonality and acquired resistance and virulence gene analysis of MRSA isolates

					Acquired resistance genes (% of strains)					Virulence genes (% of strains)				
CC	ST	SCC*mec*	*spa* type	*n*	β-LACT	Ags	MLS	other resistance genes	exoenzymes	host immune genes	haemolysin	enterotoxins	leucocidins	other virulence genes
1	1	IV	t127	3	*blaZ*, *mecA*	*ant(6)-Ia*, *aph(3′)-III*	*erm*(C)	*tet*(K)	*slpA*, *slpB*	*scn* (66%), *sak* (66%)	*hlb*, *hlgA*, *hlgB*, *hlgC*	*seh*	*lukD*, *lukE*	
5	5	II (75%), VIII (25%)	t242	4	*blaZ* (75%), *mecA*	*aadD* (50%), *ant(9)-Ia*	*erm*(A) (75%), *erm*(C) (25%)		*slpA*, *slpB*	*scn* (75%), *sak* (75%)	*hlb*, *hlgA*, *hlgB*, *hlgC*	*ser* (75%), *sej* (75%), *sed* (75%), *seo*, *sem*, *sei*, *seu*, *sen*, *seg*	*lukD*, *lukE*	*psm-mec*, *speG* (25%)
		IV	t002	5	*blaZ* (20%), *mecA*	*aac(6′)-aph(2″)* (40%)	*erm*(C) (20%)	*cat* (40%)	*slpA*, *slpB*	*scn*, *sak*	*hlb*, *hlgA*, *hlgB*, *hlgC*	*ser* (25%), *sej* (25%), *sed* (25%), *seo*, *sem*, *sei*, *seu*, *sen*, *seg*, *sea/sep* (40%)	*lukD*, *lukE*	
	105	II	t002	2	*blaZ*, *mecA*	*ant(4′)-Ia* (50%), *ant(9)-Ia*	*erm*(A)		*slpA*, *slpB*	*scn*, *sak*	*hlb*, *hlgA*, *hlgB*, *hlgC*	*ser*, *sej*, *sed*, *seo*, *sem*, *sei*, *seu*, *sen*, *seg*	*lukD*, *lukE*	*psm-mec*
	3864	II	t002	1	*blaZ*, *mecA*	*ant(4′)-Ia*, *ant(9)-Ia*	*erm*(A)		*slpA*, *slpB*	*scn*, *sak*	*hlb*, *hlgA*, *hlgB*, *hlgC*	*ser*, *sej*, *sed*, *seo*, *sem*, *sei*, *seu*, *sen*, *seg*	*lukD*, *lukE*	*psm-mec*
	228	I	t001	2	*mecA*	*aac(6′)-aph(2″)*, *ant(6)-Ia* (50%), *aph(3′)-III*, *ant(9)-Ia*	*erm*(A)		*slpA*, *slpB*	*scn*, *sak*	*hlb* (50%), *hlgA*, *hlgB*, *hlgC*	*seo*, *sem* (50%), *sei* (50%), *seu* (50%), *sen* (50%), *seg* (50%), *sea/sep*	*lukD*, *lukE*	
		I	t288	1	*mecA*	*aac(6′)-aph(2″)*, *ant(6)-Ia*, *aph(3′)-III*, *ant(9)-Ia*	*erm*(A)		*slpA*, *slpB*	*scn*, *sak*	*hlb*, *hlgA*, *hlgB*, *hlgC*	*seo*, *sem*, *sei*, *seu*, *sen*, *seg*, *sea/sep*	*lukD*, *lukE*	
		I	t12898	2	*blaZ*, *mecA*	*aac(6′)-aph(2″)*, *ant(6)-Ia*, *aph(3′)-III*, *ant(9)-Ia*	*erm*(A)		*slpA*, *slpB*	*scn*, *sak*	*hlb*, *hlgA*, *hlgB*, *hlgC*	*seo*, *sem* (50%), *sei* (50%), *seu* (50%), *sen* (50%), *seg* (50%), *sea/sep*	*lukD* (50%), *lukE* (50%)	
6	6	IV	t304	1	*blaZ*, *mecA*				*slpA*, *slpB*, *slpE*	*scn*, *sak*	*hlb*, *hlgA*, *hlgB*, *hlgC*	*sea/sep*	*lukD*, *lukE*	
7	7	IV	new	1	*blaZ*, *mecA*				*slpA*, *slpB*, *slpE*	*scn*, *sak*	*hlb*, *hlgA*, *hlgB*, *hlgC*	*sea/sep*	*lukD*, *lukE*	
8	8	IV[a]	t008	6	*blaZ* (50%), *mecA*	*ant(4′)-Ia* (83.3%), *aac(6′)-aph(2″)* (16.7%)	*erm*(C) (50%)	*cat* (33.3%)	*slpA*, *slpB*, *slpE*	*scn* (50%), *sak* (50%)	*hlb*, *hlgA*, *hlgB*, *hlgC*	*ser* (67%), *sej* (67%), *sed* (67%), *sea/sep* (50%)	*lukD*, *lukE*	*speG* (33%)
		IV[a]	t024	1	*blaZ*, *mecA*	*ant(4′)-Ia*			*slpA*, *slpB*, *slpE*	*scn*, *sak*	*hlb*, *hlgA*, *hlgB*, *hlgC*	*ser*, *sej*, *sed*, *sea/sep*	*lukD*, *lukE*	*speG*
		IV	t211	1	*blaZ*, *mecA*				*slpA*, *slpB*, *slpE*	*scn*, *sak*	*hlb*, *hlgA*, *hlgB*, *hlgC*	*sek*, *seq*	*lukD*, *lukE*, *lukS-PV*, *lukF-PV*	
		IV	t622	1	*blaZ*, *mecA*				*slpA*, *slpB*, *slpE*		*hlb*, *hlgA*, *hlgB*, *hlgC*	*ser*, *sej*, *sed*	*lukD*, *lukE*	
	239	III	t459	1	*blaZ*, *mecA*	*aac(6′)-aph(2″)*, *ant(9)-Ia*	*erm*(A)	*tet*(M)	*slpA*, *slpB*	*scn*, *sak*	*hlb*, *hlgA*, *hlgB*, *hlgC*	*sek*, *seq*, *sea/sep*	*lukD*, *lukE*	*psm-mec*
22	22	IV	t022	5	*blaZ*, *mecA*		*erm*(C) (40%)	*cat* (20%)		*scn* (80%), *sak* (80%)	*hlb*, *hlgA*, *hlgB*, *hlgC*	*seo*, *sem*, *sei*, *seu*, *sen*, *seg*, *sel*, sec (60%)		
		IV	t025	1	*blaZ*, *mecA*		*erm*(C)			*scn*, *sak*	*hlb*, *hlgA*, *hlgB*, *hlgC*	*seo*, *sem*, *sei*, *seu*, *sen*, *seg*		

	IV	t032	7	*blaZ, mecA*	*ant(4′)-Ia* (14.3%)	*erm*(C) (57.1%)		*scn, sak*	*hlb, hlgA, hlgB, hlgC*	*sed* (14.3%), *seo, sem, sei, seu, sen, seg, sel* (86%), sec (86%)		
	IV	t515	1	*blaZ, mecA*		*erm*(C)		*scn, sak*	*hlb, hlgA, hlgB, hlgC*	*seo, sem, sei, seu, sen, seg*		
	IV	t790	1	*blaZ, mecA*		*erm*(C)		*scn, sak*	*hlb, hlgA, hlgB, hlgC*	*seo, sem, sei, seu, sen, seg, sel, sec*		
	IV	t1297	2	*blaZ, mecA*		*erm*(C)		*scn, sak*	*hlb, hlgA, hlgB, hlgC*	*seo, sem, sei, seu, sen, seg, sel, sec*		
	IV	t1328	2	*blaZ, mecA*		*erm*(C) (50%)		*scn, sak*	*hlb, hlgA, hlgB, hlgC*	*seo, sem, sei, seu, sen, seg, sel, sec*		
	IV	t5605	1	*blaZ, mecA*		*erm*(C)		*scn, sak*	*hlb, hlgA, hlgB, hlgC*	*seo, sem, sei, seu, sen, seg*		
	IV	t5713	1	*blaZ, mecA*		*erm*(C)		*scn, sak*	*hlb, hlgA, hlgB, hlgC*	*seo, sem, sei, seu, sen, seg*		
	IV	t8530	1	*blaZ, mecA*		*erm*(C)		*scn, sak*	*hlb, hlgA, hlgB, hlgC*	*seo, sem, sei, seu, sen, seg*		
	IV	t12754	1	*blaZ, mecA*			*cat*	*scn, sak*	*hlb, hlgA, hlgB, hlgC*	*seo, sem, sei, seu, sen, seg, sel, sec*		
	IV	t17659	1	*blaZ, mecA*		*erm*(C)		*scn, sak*	*hlb, hlgA, hlgB, hlgC*	*seo, sem, sei, seu, sen, seg*		
	IV	t17661	1	*blaZ, mecA*		*erm*(C)		*scn, sak*	*hlb, hlgA, hlgB, hlgC*	*seo, sem, sei, seu, sen, seg, sel, sec*		
	IV	t17662	1	*blaZ, mecA*				*scn, sak*	*hlb, hlgA, hlgB, hlgC*	*seo, sem, sei, seu, sen, seg*		
	IV	new	1	*blaZ, mecA*		*erm*(C)		*scn, sak*	*hlb, hlgA, hlgB, hlgC*	*seo, sem, sei, seu, sen, seg, sel, sec*		
3863	IV	t032	4	*blaZ, mecA*		*erm*(C) (50%)		*scn, sak*	*hlb, hlgA, hlgB, hlgC*	*seo, sem, sei, seu, sen, seg*		
30	IV	t975	1	*blaZ, mecA*			*slpE*	*scn, sak*	*hlb, hlgA, hlgB, hlgC*	*seo, sem, sei, seu, sen, seg*		
152	V	t355	1	*blaZ, mecA*	*aac(6′)-aph(2″)*	*erm*(C)	*cat*	*scn, sak*	*hlgA, hlgB*		*lukS-PV, lukF-PV*	*edinB*
398	V	t899	1	*blaZ, mecA*			*tet*(M)		*hlb, hlgA, hlgB, hlgC*			

β-LACT, β-lactam antibiotics; Ags, aminoglycosides; MLS, macrolides, lincosamides and streptogramins.
When SCC*mec* and resistance and virulence genes were not present in all isolates of the same *spa* type, the prevalence is indicated in parentheses.
[a]In three isolates (two t008 and one t024) additionally to SCC*mec*-IV, the presence of *ccrA4/B4* genes was detected.

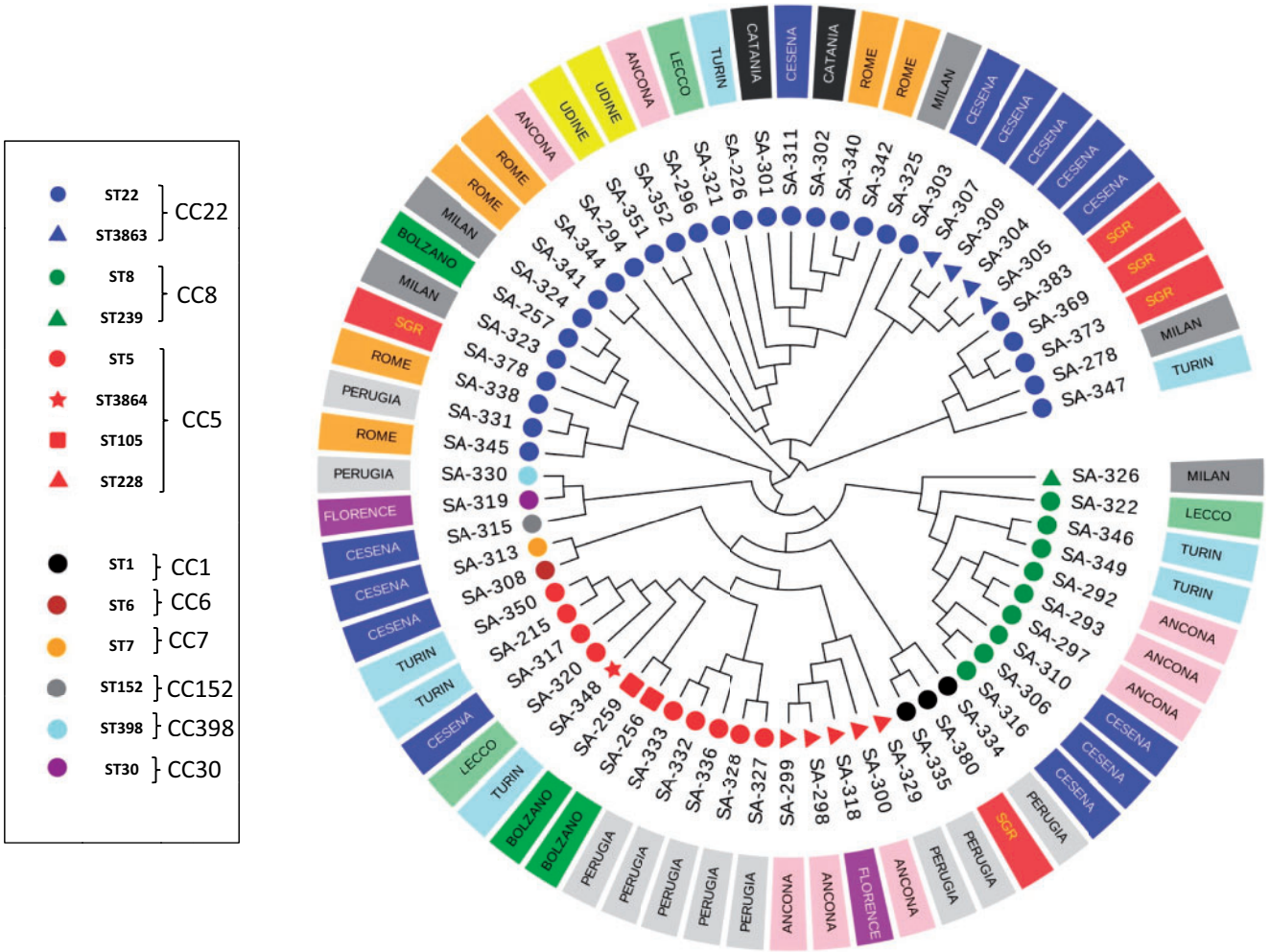

Figure 2. Phylogenetic tree of MRSA isolates. STs, CCs and isolation centres are shown in different colours. This figure appears in colour in the online version of *JAC* and in black and white in the print version of *JAC*.

Compared with a previous Italian nationwide surveillance, carried out in 2012,[35] the most common lineages of MRSA (i.e. CC22, CC5 and CC8) were overall conserved, but with different proportions. ST22-MRSA-IV (E-MRSA15-like) was remarkably increased (from 16.7% to 40.9%), while the CC5 strains were decreased (the ST228-MRSA-I/Italian clone from 33.3% to 7.6% and ST5-MRSA-II/USA100-like from 14.7% to 4.5%) and CC8 ST8-MRSA-IV/USA500-like was also slightly decreased (from 17.6% to 13.6%).[35]

Resistome analysis in the MRSA isolates

Analysis of the acquired resistome of the MRSA isolates confirmed the presence of the *mecA* gene in all of them. An archetypal *mecA* (accession no. NC_007793.1) was present in 59% of the isolates, while *mecA* alleles encoding PBP2a variants were detected in the remaining isolates (PBP2a$_{G246E}$, 29.5%; PBP2a$_{N146K}$, 6.6%; PBP2a$_{S225R}$, 3.3%; PBP2a$_{G246E/C542G}$, 1.6%). The allelic variants of PBP2a were not associated with a particular clone, except for PBP2a$_{N146K}$, which was only present in ST228 isolates.

Consistent with a previous survey on the Italian epidemiology of MRSA in clinical settings, no *mecC* gene was found in this collection,[35] while the β-lactamase gene *blaZ* was present in most isolates (89.4%).

The three ceftobiprole-resistant MRSA belonged to ST228-MRSA-I: two presented the same *spa* type (t001), while the third was of a different *spa* type (t12898). Concerning their possible ceftobiprole resistance determinants, one of these isolates had a C197Y substitution in PBP2, while the others had both an N146K substitution in PBP2a and a C197Y substitution in PBP2. Both amino acid substitutions were previously associated with decreased susceptibility to ceftobiprole, within the same ST.[26] However, each of these amino acid substitutions was also present in ceftobiprole-susceptible isolates of the same clonal lineage (Table S3). None of the other mutations in PBP2a or PBP1, PBP3, PBP4 (including its promoter region), GdpP and AcrB, previously associated with ceftobiprole resistance,[24–30] was detected in these three isolates.

Aminoglycoside resistance genes were present in almost 40% of the isolates, with *ant(9)-Ia* being the most represented, followed by *ant(4′)-Ia*, *aac(6′)-aph(2″)*, *aph-(3′)-III* and *ant(6)-Ia*. Interestingly, the acquired aminoglycoside resistance genes were very common in CC8 and CC5 isolates, while being nearly absent in

those of CC22. In 22.7% of isolates, multiple aminoglycoside resistance genes were found (Table 2).

Macrolide, lincosamide and streptogramin B (MLS_B) resistance genes [*erm*(C) or *erm*(A)] were overall less prevalent. Chloramphenicol (*cat*) and tetracycline [*tet*(M) and *tet*(K)] resistance genes also exhibited a low prevalence (Table 2). No acquired resistance genes were found for fusidic acid and trimethoprim.

In most cases, there was a clear association between certain clonal lineages and the acquired resistance gene content. The few exceptions regarded the sporadic presence of aminoglycoside resistance genes in single isolates belonging to CC8 and CC22 (Table 2).

Virulence genes in MRSA isolates

Leucocidins

Panton–Valentine leucocidin (PVL) genes *lukS* and *lukF* were detected in only two MRSA isolates. One was an ST152-MRSA-V, a highly divergent clone which has been already associated with PVL toxin in Europe, Oceania and Africa,[36] and was also the only isolate positive for the *edinB* gene, which was previously associated with an increased risk of translocation of *S. aureus* into the bloodstream during pneumonia in an ST80 MRSA.[37] The other was an ST8-MRSA-IV but lacking the arginine catabolic mobile element (ACME) typical of USA300 isolates.[21]

On the other hand, 30 of 66 (45.5%) MRSA isolates carried the pore-forming LukED leucocidin genes. These mostly belonged to CC8 (including the PVL-carrying ST8) and CC5, while none was of ST22. The LukED leucocidins have been demonstrated to induce dermonecrosis, but are less leucotoxic compared with PVL[38] and have already been associated with both community-acquired and hospital-acquired MRSA.[39]

Enterotoxins

All MRSA, except two ST8 and the singletons ST152 and ST398, carried acquired staphylococcal enterotoxin (SE) genes. As for the other virulence genes, the SE genes also mostly exhibited a clonally related distribution. All ST1 isolates carried the food poisoning *seh* gene. The related ST6 and ST7 isolates carried the *sea* gene. All CC22 isolates carried the *seo*, *sem*, *sei*, *seu*, *sen* and *seg* genes, with 54.8% of them also carrying the *sec* gene and one of them the *sed* gene. Of CC8 isolates, 60% carried the *ser*, *sej* and *sed* genes. All ST228 isolates carried the *seo* and *sea* genes, with 60% of them also carrying the *sem*, *sei*, *seu*, *sen* and *seg* genes. All ST5 isolates carried the *seo*, *sem*, *sei*, *seu*, *sen* and *seg* genes, with 44.4% of them also carrying the *ser* gene and two the *sea* gene (Table 2). In previous studies, enterotoxin prevalence was also found to be clonally related.[40] However, no previous data are available from Italy about the prevalence of these genes among MRSA of clinical origin.

Exoenzymes, immune evasion proteins and haemolysins

All the MRSA belonging to CC5 and CC8 (and none of CC22) carried the immune escape serine protease-encoding *slpA* and *slpB* genes, while only the ST6, ST7 and ST8 isolates were also positive for the *slpE* gene (Table 2). Most (58/66 87.9%) MRSA isolates carried genes encoding the two immune evasion cluster proteins: the staphylococcal complement inhibitor Scn and the staphylokinase Sak.[41] Among the nine isolates lacking those genes, four belonged to ST8 and the remainder to ST5, ST1, ST22 and ST398 (Table 2). Almost all MRSA isolates carried the β-haemolysin *hlb* and γ-haemolysin *hlgA*, *hlgB* and *hlgC* genes (except the ST152 isolate which lacked *hlgC* gene). All MRSA-II and -III isolates (8/66, 12.1%) presented the virulence *psm-mec* gene, concordantly with previous literature (Table 2).[42]

Polyamine resistance

The *speG* polyamine tolerance gene was present in three PVL-negative ST8-MRSA-IV (the same also presenting the additional *ccrA4/B4* genes) and in one PVL-negative ST5-MRSA-II isolate. This important polyamine resistance gene, which potentiates the ability of *S. aureus* to colonize and infect,[43] has been associated mainly with ST8-MRSA-IV,[21] but only in Japan has it previously been associated with ST5-MRSA-II isolates.[43] None of the MRSA isolates, even those positive for *speG*, carried the ACME.

Conclusions

Summarizing, this study provided an updated picture of the Italian epidemiology of *S. aureus* from HAP, with both phenotypic and genotypic characterization of the MRSA isolates. To the best of our knowledge, this is the first study including a nationwide survey on *S. aureus* isolated from HAP, including phenotypic tests and characterization of clonality and acquired resistance and virulence genes of MRSA isolates. A limitation of this laboratory-based surveillance study was that information about demographic data, clinical parameters, ward of hospitalization and antimicrobial treatment was not available to the laboratory.

Compared with a previous nationwide survey, carried out in 2012, this study revealed some changes in the molecular epidemiology of the circulating MRSA, with a substantial decrease in the prevalence of ST228 and increase in the prevalence of ST22 as the most remarkable change. However, it should be noted that this collection was not entirely comparable to the previous one due to differences in the sample source (in the previous collection, bloodstream and skin and soft tissue infections were also present) and in the number of participating laboratories (52 versus 13 in the current study).[35] The replacement of ST228 with ST22 (first discovered in the UK, where it has been the predominant clone since the early 2000s)[44,45] has already been reported in Italy in a single hospital report[46] and has also been reported in other countries in recent years. This clonal shift was putatively associated with an increased resistance of ST22 to host and environment stressors compared with ST228.[47]

Overall, ceftobiprole was active against all MSSA and most MRSA from HAP, and results of this study confirmed that ceftobiprole could be a valid therapeutic option in the case of MRSA HAP. Other studies previously reported the presence of ceftobiprole-resistant MRSA strains in Italy,[23,26,48–50] with ST228 isolates previously associated with this phenotype.[23,26,48] In this study, we detected three ceftobiprole-resistant MRSA isolates of ST228 from two centres, with MIC values of 4 mg/L (i.e. just above the resistance breakpoint). Although the PBP2a from these isolates exhibited allelic variations that were previously associated with ceftobiprole resistance, we also detected these variants in ceftobiprole-susceptible isolates. Therefore, the exact mechanisms of ceftobiprole resistance could not be elucidated.

It should be noted that ceftobiprole was approved for HAP treatment in Italy in October 2014 but it was introduced in the formularies of many Italian hospitals only in the second half of 2016 (i.e. after completion of this study). In particular, in both Ancona University Hospital and Perugia University Hospital, where ceftobiprole-resistant isolates were detected, ceftobiprole was introduced after their isolation, thus suggesting that resistance in these cases was not mediated by selection of ceftobiprole-resistant strains following treatment with the drug. This study therefore represents the baseline for further surveillance studies on ceftobiprole activity against MRSA from HAP.

Overall, the complex resistome and virulome of MRSA strains included in this study exhibited a clonal distribution. Of note, the emerging CC22 strains, which were by far the most prevalent detected in this study, rarely presented aminoglycoside resistance genes and never the *lukED* genes, denoting a marked difference in acquired resistance and virulence gene content compared with the other most prevalent clones (CC5 and CC8).

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Supplementary data

Tables S1 to S3 are available as Supplementary data at *JAC* Online.

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Ceftazidime/avibactam resistance associated with increased *bla*$_{KPC-3}$ gene dosage mediated by a pKpQIL plasmid derivative carrying two copies of Tn*4401a* in a ST258 *Klebsiella pneumoniae* from a kidney transplant patient

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Running Title: CZA resistance due to two copies of KPC on a plasmid

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Abstract

This work reports on the characterization of two ceftazidime-avibactam resistant KPC-producing *K. pneumoniae* isolates from a patient never treated with this antibiotic. KP-14519 and KP-8788 were isolated from a urinary tract and a bloodstream infection, respectively, of a kidney transplant patient in 2017, and showed a similar extensively drug-resistant phenotype with ceftazidime-avibactam MIC of 32 and 64 mg/L, respectively. The complete genome sequence of both isolates was obtained by WGS (Illumina MiSeq and Oxford Nanopore MinION). A high genetic relatedness was demonstrated by WGS for both isolates that: i) belonged to ST258; ii) had nonfunctional outer membrane porins due to a missense mutation in OmpK35 (AA89-STOP) and a novel mutation (Asp135Thr136 insertion) in OmpK36; iii) carried an IncFIB plasmid of original structure harboring two copies of bla_{KPC-3}. Transfer of the KPC-encoding plasmid from KP-8788 to *E. coli* DH10B resulted in an increase of ceftazidime-avibactam (0.25 vs 8 mg/L) and meropenem MIC (≤0.125 vs 32 mg/L), and of the meropenem hydrolysis rate. Although ceftazidime-avibactam resistance mechanisms to date documented has largely been attributed to nonsynonymous mutations occurring in the KPC-3 enzyme, alteration of the major outer membrane porin status couple with an increased expression of bla_{KPC} could severely impact ceftazidime-avibactam susceptibility. The presence of a single plasmid mediating the increased gene dosage of bla_{KPC} might give rise to a transferable resistance mechanism to ceftazidime-avibactam.

Introduction

Over the past decade, carbapenem-resistant Enterobacterales (CRE) have undergone ~~a~~ rapid and global dissemination worldwide, becoming a public health problem of major concern [1].

Carbapenemase production represents the most important resistance mechanism to carbapenems among CRE and depends on the horizontal acquisition of genes coding for different types of enzymes including those of the KPC, NDM, VIM and OXA-48-like lineages [2].

In Italy, the remarkable spread of CRE observed during the past decade has mostly been driven by the dissemination of high-risk clones of KPC-producing *Klebsiella pneumoniae* (KPC-Kp), which are characterized by difficult-to-treat resistance (DTR) phenotypes which leave very few treatment options [3].

The urgent need for new antibacterial agents with anti-CRE activity has been partially fulfilled by the recent introduction of novel beta-lactam/beta-lactamase inhibitor combinations (BLICs), among which ceftazidime-avibactam (CAZ-AVI) was the first to be released for clinical use [4]. Avibactam is a non-βlactam β-lactamase inhibitor of the diazabicyclooctane family with activity vs. class A, C and some class D β-lactamases, but not against class B metallo-β-lactamases [4]. Approved indications for CAZ-AVI use include hospital-acquired pneumonia, intra-abdominal infections, complicated urinary tract infections, and infections caused by aerobic multidrug-resistant Gram-negative organism with limited treatment options [4].

Despite its recent introduction, resistance to CAZ-AVI has been reported, either following treatments with CAZ-AVI [5-8] or even in absence of previous exposure to the drug [9-12]. The CZA resistance has been most commonly attributed to missense mutations in the KPC enzyme (e.g. D179Y; L169P; T243M; EL165-166; V240G/A; H274Y), which were associated with a decreased activity against carbapenems and other β-lactam antibiotics [4, 5, 7, 11-14]. Moreover, the combination of multiple mechanisms including nonsense or missense mutations in OmpK35 and

OmpK36 porins, increased efflux activity (i.e. mediated by mutations in *ramR* regulator of the *acrAB* efflux system) and increased expression of KPC or even SHV β-lactamases (i.e mediated by the presence multiple plasmids carrying bla_{KPC} and bla_{SHV-12}) has been associated with ~~a~~ reduced susceptibility to CZA [10; 15; 16].

Here we report on the characterization of CZA resistant KPC-producing *K. pneumoniae*, isolated in the absence of previous exposure to the drug, which carried a novel missense mutation in OmpK36 and overexpressed the KPC-3 enzyme due to gene amplification within the same pKpQIL-derivative plasmid. The latter resistance mechanism could be transferred by plasmid transfer, representing a novel type of potentially transferable CZA resistance mechanism among *Enterobacterales*.

Methods

Bacterial isolates and *in vitro* susceptibility testing

K. pneumoniae KP-8788 and KP-14519 were isolated in 2017 respectively from the blood culture and from urine culture of a patient who had received a kidney transplant. Bacterial identification was carried out using MALDI-TOF mass spectrometry (bioMérieux, Marcy l'Etoile, France) and confirmed by WGS data. Confirmatory antimicrobial susceptibility testing was carried out with broth microdilution using lyophilized custom plates (MICRONAUT-S MHK; MERLIN Diagnostika GmbH, Berlin, German) and interpreted according to the EUCAST clinical breakpoints (EUCAST breakpoint tables version 9.0, 2019, www.eucast.org/clinical_breakpoints/). Broad range meropenem (MEM; 0.125-4096 mg/L), ceftazidime (CAZ; 0.125-4096 mg/L), ceftazidime-avibactam (CAZ-AVI; 0.0625-128 mg/L with avibactam at the fixed concentration of 4 mg/L)

susceptibility testing was performed using reference broth microdilution according to CLSI guidelines [17] and antibiotic powders obtained from Sigma-Aldrich (Saint Louis, Missouri, USA).

Whole-genome sequencing and bioinformatics

Total bacterial DNA was isolated from KP-8788 and KP-14519 using the phenol-chloroform method [18] and subjected to Whole-Genome Sequencing (WGS) on an Illumina MiSeq platform (Illumina® San Diego, CA, USA), as previously described [19]. WGS was also performed by the Oxford Nanopore MinION system (Oxford Nanopore Technologies, Oxford, UK) to exploit the long-reads sequencing technology. Raw data from Illumina and MinION were *de novo* assembled using the SPAdes Genome Assembler v. 3.11 [20] and Unicycler v. 0.4.6 [21]. Genome and plasmid annotation were performed by RAST (rast.nmpdr.org/rast.cgi) and by the NCBI Prokaryotic Genome Annotation Pipeline (PGAP) (https://www.ncbi.nlm.nih.gov/genome/annotation_prok/). Comparative genome alignments were performed using Mauve 2.3.1 (http://darlinglab.org/mauve/mauve.html) and the BLASTn and BLASTp algorithms (http://blast.ncbi.nlm.nih.gov/). Annotation of IS elements was performed using the ISFinder database (www-is.biotoul.fr/). *In silico* identification of antimicrobial resistance genes, bacterial sequence type (ST), plasmid replicons and single nucleotide polymorphisms (SNP) on core genome were carried out using dedicated tools available at the Center for Genomic Epidemiology (http://www.genomicepidemiology.org/). Physical maps were generated using Easyfig software [22].

Gene transfer experiments

Electrotransformation experiments were carried out using competent *E. coli* DH10B (Invitrogen; Waltham, Massachusetts, USA) and *K. pneumoniae* KP-8788 total DNA. Transformants were selected on Mueller-Hinton agar (MHA) supplemented with 8 mg/L ceftazidime. Conjugal transfer experiments were carried out as previously described, using both *E. coli* MKD-135 and *E. coli* J53

(Sodium Azide resistant) as recipients [23]. MHA containing ceftazidime (8 mg/L) and rifampin (250 mg/L) or Sodium Azide (100 mg/L) were used to select MKD-135 and J53 transconjugants, respectively. Transfer of bla_{KPC} gene was always confirmed by Real-Time PCR [24]

Assay of carbapenemase activity.

Meropenem-hydrolyzing specific activity was determined by a spectrophotometric assay on cell crude extracts as previously described [25]. *E. coli* DH10B(pIT-FIPP-1) and *E. coli* DH10B(pIT-01C022), two DH10B transformants with KPC-encoding plasmids highly similar to pIT-8788 and to the archetypal KPC-encoding plasmid pKpQIL [26], respectively, were included for comparison in assays of carbapenemase activity.

Sequence accession number(s)

The genome sequences of *K. pneumoniae* KP-8788, plasmid pIT-8788 and *K. pneumoniae* KP-14519 have been registered under the BioProject number PRJNA526630 and were deposited in GenBank under accession number CP037928, CP037930 and [*not yet available*] respectively.

Results

Case Description

A 59-years-old female affected by end-stage renal disease (ESRD) secondary to polycystic kidney disease was subjected to renal transplantation. For the transplantation procedure the patient received antibiotic prophylaxis with cefazolin (500 mg i.v. every 12 hours) for 3 days. The early post-transplantation course was complicated by a urinary tract infection (UTI) caused by a carbapenem-resistant *K. pneumoniae* (isolate KP-14519) which was also resistant to amoxicillin/clavulanic acid, piperacillin/tazobactam, cefepime, cefotaxime, ceftazidime, ciprofloxacin, colistin, fosfomycin, amikacin, gentamicin, tigecycline, and trimethoprim/sulfamethoxazole (tested using Vitek-2, bioMérieux and interpreted according the

EUCAST v.7.1 breakpoints;) . The isolate was confirmed to be a KPC producer by a phenotypic test (Rosco Diagnostica KPC, MBL and OXA-48 Confirm Kit; Taastrup, Denmark). The patient was treated with meropenem (500 mg i.v. every 6 hours) in combination with ertapenem (500 mg per day) for 10 days. After 10 days since treatment suspension, the patient developed a breakthrough bacteremia by a KPC-positive *K. pneumoniae* with the same resistance profile (isolate KP-8788), suggesting the occurrence of a secondary bacteremia of urinary origin (a persistent UTI was also documented at the same time). Double carbapenem therapy was restarted and, after 7 days, the dosage was increased (meropenem 1000 mg i.v. every 6 hours, in combination with ertapenem 1000 mg per day). However, both bacteremia and UTI persisted, and bilateral nephrectomy was carried out, which was complicated by a surgical site infection caused by the KPC-positive *K. pneumoniae* and a vancomycin-susceptible *E. faecium*. Antibiotic treatment with double carbapenem was continued for one month and initially associated with tigecycline (50 mg twice daily for 15 days) and then with teicoplanin (600 mg/day for 15 days) until recovery.

Characterization of *K. pneumoniae* KP-14519 and KP-8788

The *K. pneumoniae* isolates from the post-transplantation UTI (KP-14519) and from the subsequent bacteremia (KP-8788) were available for further characterization.

Antimicrobial susceptibility testing using reference broth microdilution confirmed resistance to carbapenems and other β-lactams, fluoroquinolones, gentamicin, trimethoprim-sulfamethoxazole and colistin, and showed that both isolates were also resistant to CZA despite the patient had never been treated with this drug. On the other hand, the isolates were susceptible, increased exposure to amikacin, while tigecycline MICs were 0.5-1 mg/L (Table 1).

To investigate the resistance mechanisms, the strains were characterized by a WGS approach. The hybrid assembly of sequencing data generated with both short and long reads sequencing technologies resulted in three complete circular molecules for KP-8788, including the chromosome

(5.38 Mb) and two plasmids, named pKPN-IT-8788 (a 271 Kb IncFII$_{K7}$-IncFIB$_k$ multireplicon) and pIT-8788 (a 102 Kb IncFIB$_k$ and ColE multireplicon), respectively. With KP-14519, the hybrid assembly resulted in a draft genome of 5.70 Mb containing both chromosomal and plasmid sequences associated with an IncFII$_{K7}$-IncFIB$_k$ replicon, and two complete circular molecules represented by two plasmids, named pIT-14519 (an 88 Kb IncFIB$_k$ replicon), and ColE-14519 (a 14 Kb ColE replicon).

In silico analysis of the KP-8788 and KP-14519 genomes confirmed identification as *K. pneumoniae sensu stricto* and revealed that both isolates belonged to the clade II lineage of ST258 [27]. Alignment of KP-14519 short reads against the chromosome of KP-8788 revealed that the two isolates differed only by an SNP in the *rseB* gene, causing a missense mutation (Lys56Glu) in the sigma-E factor regulatory protein.

At the chromosomal level, investigation of the major porins status showed that OmpK35 was nonfunctional due to a frameshift mutation (AA89-STOP), as commonly observed among ST258 members [28], while OmpK36 was altered by an original two-amino-acid insertion (Asp135Thr136) within the transmembrane β-strand loop 3 (L3), close to the previously reported duplication (Gly134Asp135) that has been commonly observed in ST258 isolates and was predicted to result in a constricted, partially functional, porin channel [16; 29]. Moreover, the isolates also carried mutations leading to amino acid substitutions in the topoisomerase IV ParC subunit (Ser80Ile) and in the DNA gyrase GryA subunit (Ser83Ile), known to be associated with fluoroquinolone resistance, and a nonfuctional *mgrB* gene due to a novel deletion (Δ110/119), similar to a previously described alteration associated with colistin resistance [30].

Concerning plasmids, both isolates carried the same IncFII$_{K7}$-IncFIB$_k$ replicon of 271 kb, although in KP-14519 it could only be identified in a draft assembly status because long-read sequencing did not yield a complete circular molecule for the IncFII $_{K7}$-IncFIB$_k$ replicon. This replicon was highly

similar (99% identity over 81% of sequence length) to pKPN-LS6 (GenBank accession no. JX442974), an IncFII$_{K1}$ plasmid from KPC-3-positive ST258 *K. pneumoniae* LS6 isolated in Italy in 2011 [31], and to pKPN-a68 (GenBank accession no. CP009777.1), an IncFII$_{K1}$ plasmid from KPC-3-positive ST258 *K. pneumoniae* KPNIH32 isolated in the U.S.A. in 2013 [32]. Sequence analysis of pKPN-IT-8788 revealed the presence of genes associated with resistance to aminoglycosides (*aph(3')-Ia, aadA2, aac(3)-IIa, aac(6')-Ib3*), beta-lactams (*bla*$_{OXA-1}$), chloramphenicol (*catA1, catB3*), sulphonamides (*sul1*), trimethoprim (*dfrA12, dfrA14*) and fluoroquinolones (*qnrB1*).

Plasmid pIT-14519 from KP-14519 was an 88-kb replicon of the IncFIB$_k$ lineage, carrying *bla*$_{KPC-3}$, highly similar (>99% identity over 99% of sequence length) to previously characterized pKpQIL-like plasmids from ST258 KPC-Kp isolated during the 2008-2011 period and including: the KPC-2-encoding plasmid pKpQIL-UK from *K. pneumoniae* isolates from various UK centres [33], the KPC-3-encoding plasmids pIT-FIPP-1, from the Italian KPC-Kp ST258 index strain FIPP-1 [26], and pKpQIL-LS6, from *K. pneumoniae* LS6 [31].

Plasmids pIT-FIPP-1 and pKpQIL-LS6 were almost identical IncFIB$_k$-type replicons and, as pIT-14159, carried *bla*$_{KPC-3}$ embedded in a Tn*4401a* transposon inserted at the same backbone position and were deletion derivatives of the archetypal IncFII$_{K2}$ KPC-encoding plasmid pKpQIL (GenBank accession no. NC_014016) [34], where portions of the FII$_{K2}$ replicon and part of the *tra* locus were lacking (Figure 1). The major difference between pIT-14519 and pIT-FIPP-1/pKpQIL-LS6 consisted in the presence of an additional copy of Tn*4401a* (named Tn*4401a*-2), located within the Δ*tniA* gene present aboard of the plasmid backbone and absence of *bla*$_{TEM-1}$ (Figure 1). pIT-14519, therefore, carried a double copy of *bla*$_{KPC-3}$ as compared with previously described pKpQIL-like KPC-encoding plasmids.

Plasmid ColE-14519 from KP-14519 was a 14 kb replicon belonging to the ColE plasmid family, being identical (100% identity over 100% of sequence length) to the ColE-LS6 plasmid from the

previously characterized *K. pneumoniae* LS6 [31]. Similarly to the latter plasmid, ColE-14519 carried an *aac(6')-Ib* aminoglycoside resistance determinant and a cloacin-like bacteriocin (Figure 1), and was frequently detected among ST258 strains [28].

Finally, plasmid pIT-8788 from KP-8788 was an IncFIB$_K$-ColE multireplicon of 102 kb, resulting from the co-integration of pIT-14519 and ColE-14519 likely occurred following an IS*26*-mediated homologous recombination (Figure 1). The major differences between pIT-14519 and pIT-8788 consisted in the inversion of the backbone segment laying between the proximal IRs of Tn*4401a*-1 and Tn*4401a*-2, and in the presence of a *bla*$_{TEM-1}$ β-lactamase gene in pIT-8788 (Figure 1).

Mechanisms of CZA resistance in *K. pneumoniae* KP-14519 and KP-8788

Loss of porins function and increased gene dosage of *bla*$_{KPC}$, mediated by the co-presence of two different plasmids carrying a single copy of Tn*4401*, have previously been associated with decreased susceptibility to CZA [16]. Therefore, we hypothesized that the combination of these two mechanisms was also responsible for the CZA resistance phenotype observed in these two *K. pneumoniae* isolates.

Indeed, the amount of carbapenemase activity in crude extracts of KP-14519 and KP-8788 was 2.5-3.3-fold higher than that measured in crude extracts of *K. pneumoniae* FIPP-1, an ST258 strain carrying a similar KPC-3-encoding plasmid but with a single Tn*4401* copy (Figure 1 and Table 2).

Interestingly, in this case, the increased *bla*$_{KPC-3}$ gene dosage was mediated by the presence of a double copy of Tn*4401* on a single plasmid, a condition thus far original and which could be potentially associated with transferability of the resistance mechanism to CAZ-AVI. To test such hypothesis, transfer experiments by conjugation and transformation were carried out with pIT-8788. Plasmid pIT-8788 was successfully transferred by electrotransformation into *E. coli* DH10B, while conjugal transfer experiments from either *K. pneumoniae* KP-8788 or *E. coli* DH10B(pIT-8788) were unsuccessful, consistently with the partial deletion of the *tra* locus. Of note, the MIC of

CAZ-AVI, ceftazidime and meropenem of *E. coli* DH10B(pIT-8788) were significantly higher than those of *E. coli* DH10B transformed with two plasmids of the same lineage but carrying a single copy of Tn*4401*, namely pIT-FIPP-1 [26], a plasmid highly related to pIT-8788 but with a single copy of bla_{KPC-3} embedded in Tn*4401a*, and pIT-01C22 [26], a plasmid highly related to the archetypal and widespread KPC-encoding pKpQIL plasmid (Table 2). These findings were consistent with an increased production of carbapenemase activity by DH10B(pIT-8788) compared with DH10B(pIT-FIPP-1) and DH10B(pIT-01C22), and confirmed the role of the increased bla_{KPC-3} gene dosage effect, mediated by a double copy of Tn*4401* present on the same plasmid, in decreasing susceptibility to CAZ-AVI, and also to ceftazidime and meropenem (Table 2).

Discussion

In this work we report on the characterization of ST258 KPC-3-producing *K. pneumoniae* isolates highly resistant to CZA from a kidney transplant patient who had never been treated with this antibiotic (that was not yet available for clinical use at the time of the infection). Due to the apparent pandrug-resistant phenotype of the isolates returned by susceptibility testing with a popular semiautomated system (Vitek-2), the infection was treated with a double carbapenem regimen which, however, was unsuccessful until combined with source control by removal of the infected kidneys and additional tigecycline treatment. Pre-transplantion carbapenem-resistant *K. pneumoniae* infection or colonization was reported as a risk factor associated with increased mortality and with antimicrobial treatment failure, but not with graft failure [35]. In endemic areas, early detection of colonizing CRE is critical for the management of immunocompromised hosts and, in case of infection, for the rapid introduction of targeted therapy [36], that in case of KPC-producers includes the combination of β-lactams and the new inibitors avibactam [4].

253 However, the two isolates from UTI and bacteremia, described in this work, resulted highly
254 resistant to CZA (32-64 mg/L).
255 The CZA resistance of these isolates, which were actually representatives of the same strain and
256 differed by a chromosomal SNP and the co-integration\rearrangement of two plasmids, was
257 apparently related with alterations of the major porins OmpK35 (which was inactivated) and
258 OmpK36 (which carried an original two-amino-acid insertion within the L3 loop, constituting the
259 porin channel eyelet) in combination with overexpression of the KPC-3 enzyme due to an
260 increased gene dosage mediated by the presence of a double copy of Tn*4401a* in the KPC-
261 encoding plasmid. Indeed, a previous study showed that similar porin alterations and an increased
262 bla_{KPC-3} gene dosage mediated by the presence of a Tn*4401d* transposon on two different plasmids
263 were responsible for decreased susceptibility to CZA [16]. In this case, however, the increased
264 bla_{KPC-3} gene dosage was mediated by a single plasmid and could therefore be transferred *en-bloc*
265 with plasmid transfer resulting in a possible transferable mechanism of CZA resistance. Although
266 neither pIT-8788 nor pIT-14159 were self-transferable by conjugation, due to a deletion within the
267 transfer operon, in both plasmids a complete transfer origin was detected, allowing their
268 mobilization in the presence of helper plasmids, such as other IncF-type plasmids which are widely
269 detected among successful clones of *K. pneumoniae* (e.g. ST512) [26; 28]. Moreover, the
270 additional replicon identified within the pIT-8788 backbone (i.e. ColE) could potentially enhance
271 the mobilization capabilities of this element.
272 The presence of plasmids with a double copy of Tn*4401* aboard, as the case of pIT-8788 and pIT-
273 14159, therefore, could represent a hidden threat, since the acquisition of a single element by a
274 new host may directly impact on CZA susceptibility following a single transfer event. This is
275 particularly worrisome in an epidemiological context where *K. pneumoniae* clones carrying
276 alterations of the OmpK35 and/or OmpK36 porins (such as those of clonal group 258, ST11, ST15

277 and ST395) are frequent [28; 37-39]. In fact, these clones may represent a genetic background
278 potentially favoring the emergence of clinical resistance to CZA upon transfer of similar plasmids.
279 It should be noted, however, that a lower transferability potential of KPC-encoding plasmids from
280 *K. pneumoniae* strains belonging to the clonal group 258 was previously documented [26], and
281 additional experiments are therefore required to clarify the transfer behavior of pIT-8788 and pIT-
282 14159.

283

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289

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Antibiotic	MIC (mg/L) (category)			
	***K. pneumoniae* KP-14519**	***K. pneumoniae* KP-8788**	***E. coli* DH10B (pIT-8788)**	***E. coli* DH10B**
Ceftazidime	>32(R)	>32(R)	>32(R)	0.5 (S)
Ceftazidime-Avibactam	>8/4(R)	>8/4(R)	4/4(S)	0.25 (S)
Cefepime	>8(R)	>8 (R)	>8 (R)	≤1 (S)
Imipenem	>8(R)	>8 (R)	>8 (R)	≤1 (S)
Meropenem	>16(R)	>16 (R)	>16 (R)	≤0.125(S)
Piperacillin-tazobactam	>128/4	>128/4 (R)	>128/4 (R)	≤1/4 (S)
Ciprofloxacin	>8(R)	>8 (R)	≤0.0625 (S)	≤0.0625 (S)
Levofloxacin	>8(R)	>8 (R)	≤0.125 (S)	≤0.125 (S)
Amikacin	16(I)	16 (I)	16 (I)	≤4 (S)
Gentamicin	32(R)	>32(R)	1 (S)	1 (S)
Tigecycline	1(*)	0.5(*)	≤0.125 (S)	≤0.125 (S)
Tobramycin	8 (R)	16 (R)	8 (R)	0.5 (S)
Trimethoprim-sulfamethoxazole	>8/152 (R)	>8/152 (R)	≤1/19 (S)	≤1/19 (S)
Colistin	>8(R)	>8 (R)	≤1 (S)	≤1 (S)

Table 1. MICs and their interpretation for *K. pneumoniae* KP-14519 associated with UTI, *K. pneumoniae* KP-8788 associated with BSI, the transformant strain *E. coli* DH10B (pIT-8788) and *E. coli* DH10B. S: susceptible standard dosing isolates; I: susceptible, increased exposure; R: resistant. *No EUCAST breakpoint (V9.0) for *K. pneumoniae* are available, but they should be categorized as susceptible considering EUCAST breakpoint V.7.0 in use when the bacteria were isolated.

Strain	ST	*bla*$_{KPC-3}$ copies per plasmid	*bla*$_{KPC}$-carrying plasmid replicon(s)	MIC (mg/L)			MEM Specific activity	Porin status		Reference
				MEM	CAZ	CAA		OmpK35	OmpK36	
***K. pneumoniae* KP-14519**	258	2	FIB	1024	4096	32	77±2	AA89-STOP	Asp135 Thr136	This study
***K. pneumoniae* KP-8788**	258	2	FIB-ColE	2048	>4096	64	101±20	AA89-STOP	Asp135 Thr136	This study
***K. pneumoniae* FIPP-1**	258	1	FIB	512	1024	8	30.5±0.3	AA122-STOP	Disrupted by IS*5-like*	[26]
***E. coli* DH10B(pIT-8788)**	-	2	FIB	32	4096	8	114±18	-	-	This study
***E. coli* DH10B(pIT-FIPP-1)**	-	1	FIB	4	128	1	43±2	-	-	[26]
***E. coli* DH10B(pIT-1C022)**	-	1	FII$_{K2}$-FIB	1	128	0.5	31±3	-	-	[26]
***E. coli* DH10B**	-	-	-	≤0.125	0.5	0.25	-	-	-	-

Table 2. Comparison between meropenem (MEM), ceftazidime (CAZ), ceftazidime-avibactam (CAZ-AVI; 4 mg/L) MICs and MEM-hydrolysing activity measurement (nmol/min/mg of protein extract) against: I) *K. pneumoniae* FIPP-1 harbouring a plasmid (pIT-FIPP-1) similar to the one detected in *K. pneumoniae* KP-15159 (pIT-14519) and in *K. pneumoniae* KP-8788 (pIT-8788), and showing a similar porins' genetic background; II) *E. coli* DH10B carrying pIT-8788 or comparator pKpQIL-like plasmids (i.e. pIT-FIPP-1 or pIT-01C022).

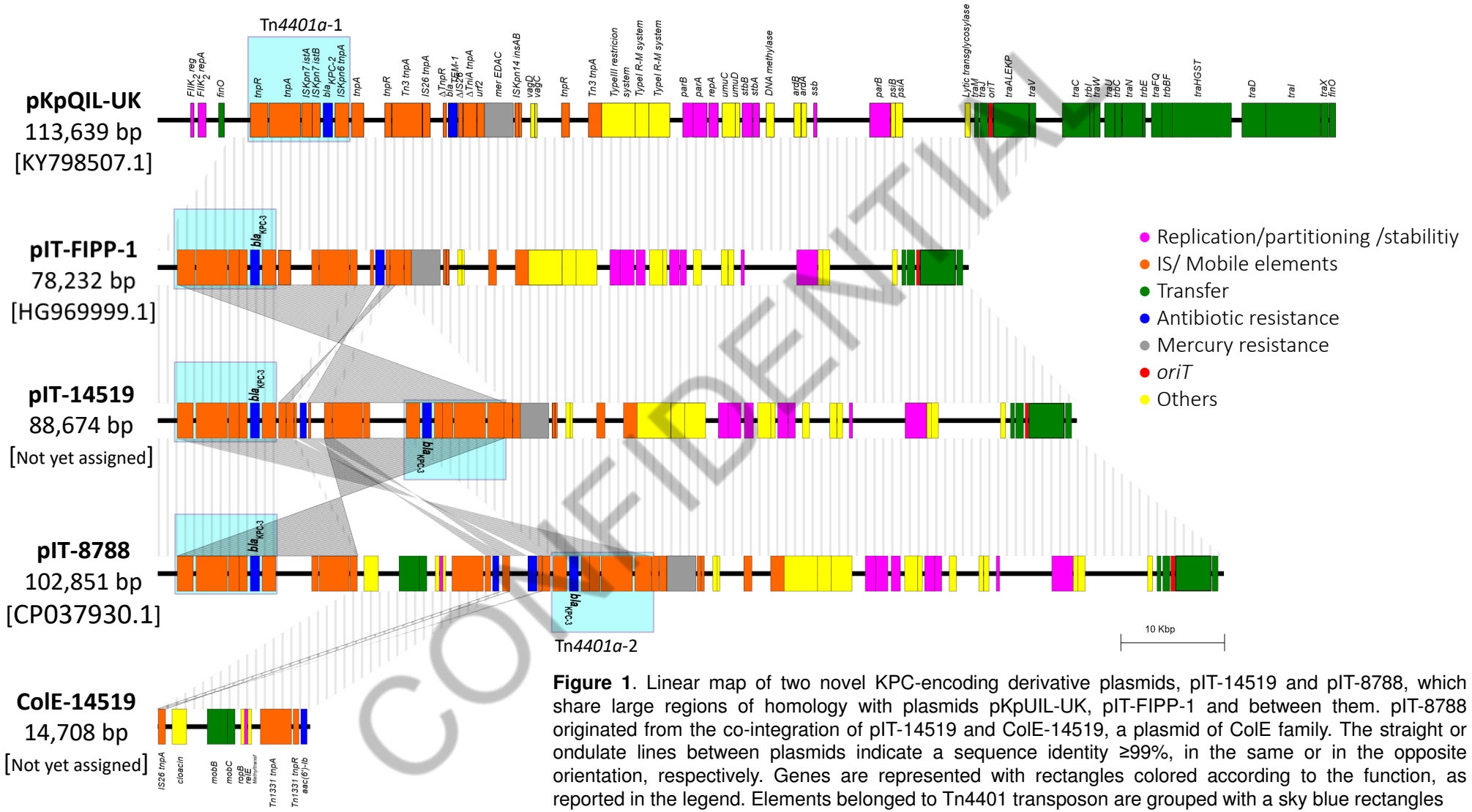

Figure 1. Linear map of two novel KPC-encoding derivative plasmids, pIT-14519 and pIT-8788, which share large regions of homology with plasmids pKpUIL-UK, pIT-FIPP-1 and between them. pIT-8788 originated from the co-integration of pIT-14519 and ColE-14519, a plasmid of ColE family. The straight or ondulate lines between plasmids indicate a sequence identity ≥99%, in the same or in the opposite orientation, respectively. Genes are represented with rectangles colored according to the function, as reported in the legend. Elements belonged to Tn4401 transposon are grouped with a sky blue rectangles

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Bacteriology

Multicenter evaluation of the RAPIDEC® CARBA NP test for rapid screening of carbapenemase-producing *Enterobacteriaceae* and Gram-negative nonfermenters from clinical specimens

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ABSTRACT

The rapid diagnosis of carbapenemase-producing (CP) bacteria is essential for the management of therapy and infection control. In this study, RAPIDEC® CARBA NP (RCNP) was evaluated for the rapid screening of CP *Enterobacteriaceae, Acinetobacter baumannii* complex, and *Pseudomonas aeruginosa* from clinical specimens collected at five Italian hospitals. Firstly, each site tested 20 well-characterized strains in a blinded fashion. Secondly, each center prospectively tested 25 isolates from blood cultures processed with a rapid workflow (6 h after subculture) and 25 isolates from other specimens processed after an overnight culture. The presence of carbapenemases was confirmed by multiplex real-timePCRs targeting carbapenemase genes. RCNP presented an overall sensitivity, specificity, positive predictive value, and negative predictive value of 70%, 94%, 82%, and 89%, respectively, with a higher performance in detection of CP *Enterobacteriaceae* and a poorer performance in detection of CP *A. baumannii* complex. With isolates from blood cultures, RCNP could significantly reduce the time required for identification of CP *Enterobacteriaceae* (less than 9 h since the positivization of blood cultures).

1. Introduction

Antibiotic resistance is an issue of growing importance for public health, and involves a large variety of pathogenic bacteria responsible for healthcare-associated and community-acquired infections (Tang et al., 2014). Carbapenems are considered among the last resort antibiotics for treatment of resistant Gram-negatives (Papp-Wallace et al., 2011), but carbapenem-resistant strains of *Enterobacteriaceae* and Gram-negative nonfermenters are now spreading worldwide (Ruppé et al., 2015). The main mechanisms of resistance to carbapenems in Gram-negative pathogens are represented by the production of carbapenemases, reduction of outer membrane permeability mediated by the loss of porin function, and up-regulation of efflux systems (Papp-Wallace et al., 2011). The spread of carbapenemase-producing (CP) strains of Gram-negative bacteria (GNB), including *Enterobacteriaceae*, *Pseudomonas aeruginosa* and *Acinetobacter* spp., is of notable concern since these strains often carry additional resistance determinants and exhibit complex multidrug-resistant (MDR) phenotypes. Moreover, carbapenemase genes are usually associated with mobile genetic elements and their expression can be associated with higher-level carbapenem resistance (Kaye and Pogue, 2015; Rossolini et al., 2014; Ruppé et al., 2015).

Therefore, rapid identification of CP-GNB is important to implement infection control strategies that limit their spread in hospitals, and to the selection of appropriate antimicrobial therapy (Miriagou et al., 2010). Several approaches can be used for rapid identification of CP-GNB, including phenotypic and genotypic methods (Osei Sekyere et al., 2015). Among the phenotypic methods, the RAPIDEC® CARBA NP test (bioMérieux, Marcy l'Etoile, France) is a commercial test for rapid screening of CP-GNB developed basing on the original CARBA NP colorimetric method (Nordmann et al., 2012). The RAPIDEC® CARBA NP test is easy to use and provides results in 2 h, while being cheaper than

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molecular assays and able to detect also unknown carbapenemase genes. This test, based on the colorimetric detection of hydrolysis of imipenem using phenol red as indicator, has been previously validated or compared with other tests in several studies in which it was retrospectively applied on a collection of isolates previously characterized for the presence of carbapenemases (Aktaş et al., 2016; Dortet et al., 2015; Garg et al., 2015; Hombach et al., 2015; Kabir et al., 2016; Lifshitz et al., 2016; Österblad et al., 2016; Poirel and Nordmann, 2015), or prospectively applied on *Enterobacteriaceae* isolates (Noël et al., 2017).

In this work, we carried out a multicenter evaluation of the RAPIDEC® CARBA NP test, including a proficiency test with well-characterized strains, followed by further testing for the detection of CP-GNB among bacterial isolates prospectively collected from various clinical specimens. Moreover, a fast-track workflow for the detection of CP-GNB using the RAPIDEC® CARBA NP from blood cultures was implemented.

2. Materials and methods

2.1. Participating centers

Five laboratories associated with hospitals located in northern (Lecco and Modena) and central (Florence and Rome) Italy, representative of different Italian Regions, were involved in the study carried out from April to September 2015.

2.2. Proficiency test

A collection of 20 well-characterized strains, previously confirmed as CP (n = 14) or carbapenem-resistant but carbapenemase-non-producers (CNP, n = 4) or carbapenem-susceptible (n = 2) (Table 1), was provided to each participating center in a blinded fashion. Each strain was cultured for 18–24 h on blood agar and then tested with the RAPIDEC® CARBA NP test according to the Manufacturer's instructions.

2.3. Test on clinical isolates

A total of 250 (50 per participating center) consecutive, non-replicate clinical isolates of *Enterobacteriaceae* and Gram-negative nonfermenters (*P. aeruginosa* and *A. baumannii* complex) were tested with RAPIDEC® CARBA NP. Of them, 125 isolates (25 per participating center) were from blood cultures processed with a fast-track workflow, and 125 isolates (25 per participating center) were from other clinical specimens (surveillance specimens were not included). Positive blood cultures from BACTEC™ (Becton Dickinson, Franklin Lakes, NJ, USA) or BacT/ALERT® (bioMérieux) systems were evaluated with Gram staining and plated onto blood agar plates (bioMérieux). The fast-track workflow foresaw that after 6 h of incubation (35 ± 2 °C, 5% CO_2), bacterial isolates were identified by MALDI-TOF with the VITEK® MS system (bioMérieux) and, if they belonged to the target species, they were included in the study and tested with the RAPIDEC® CARBA NP test. Since the laboratories did not process positive blood cultures on a 24/7 schedule, only the blood cultures that became positive during the night or in the morning (until 12 a.m.) were processed with the fast-track workflow, by the staff in charge of the afternoon shift. Blood cultures yielding Gram-positive bacteria or mixed Gram-positive/Gram-negative bacteria and/or yeasts at Gram staining were excluded (Fig. 1). Urine samples were cultured on chromID® CPS® Elite medium (bioMérieux) for 18–24 h, while other materials were cultured on blood agar (bioMérieux) for 18–24 h. Bacterial isolates were identified by MALDI-TOF with the VITEK® MS system (bioMérieux) and, if they belonged to the target species, they were included in the study and tested with the RAPIDEC® CARBA NP (Fig. 1).

2.4. RAPIDEC® CARBA NP test

The RAPIDEC® CARBA NP test was performed according to the Manufacturer's instructions, as follows. In case of isolated colonies from 18 to 24 hour-old cultures, several colonies were deposited in the dedicated well. For the 6-hour bacterial growth from blood cultures, the bacterial growth was transferred directly to the well of RAPIDEC® CARBA NP, until the indicated turbidity was reached. Samples presenting an insufficient bacterial growth were excluded. Strips were incubated at 35 ± 2 °C for up to 120 minutes, and inspected at 30, 60 and 120 minutes. Results were interpreted by comparing the test well and the control well colors. A test was considered positive when a change of color of the well (from red to red-orange, orange or yellow) was observed.

2.5. Antimicrobial susceptibility testing

Antimicrobial susceptibility testing (AST) was performed using reference broth microdilution according to CLSI guidelines (CLSI, 2015)

Table 1
Gram-negative strains selected for the evaluation of RAPIDEC® CARBA NP proficiency with the test results obtained in each site.

Strain	Species	Principal Acquired β-lactamase	Reference	MIC Meropenem (μg/mL)	RAPIDEC® results (center)					
					Expected	1	2	3	4	5
6–419	*Escherichia coli*	No-one	–	0.5	–	–	–	–	–	–
23–1786	*Enterobacter ludwigii*	NMC-A	Antonelli et al. (2015c)	32	+	+	+	+	+	+
7–556	*K. pneumoniae*	NDM-1	–	32	+	+	+	+	+	+
22–1706	*E. coli*	NDM-5	–	>32	+	+	+	+	+	+
7728	*P. aeruginosa*	IMP-13	–	4	+	+	–	+*	+	+
ATCC 25922	*E. coli*	no-one	–	0.5	–	–	–	–	–	–
47–3752	*E. cloacae* complex	IMI-2	–	>32	+	+	+	+	+*	+
CVB-1	*E. coli*	NDM-1	D'Andrea et al. (2011)	32	+	+	+	+	+	+
ECBZ-1	*E. coli*	OXA-48	Giani et al. (2012)	1	+	+	+	+	+*	+
FIPP-1	*K. pneumoniae*	KPC-3	Giani et al. (2009)	>32	+	+	+	+	+	+
VA-417/02	*E. cloacae* complex	VIM-4	Luzzaro et al. (2004)	32	+	+	+	+	+	+
FI-14/157	*P. aeruginosa*	FIM-1	Pollini et al. (2013)	>32	+	+	–	+	+	+
Cfr-FI-07	*C. freundii*	OXA-372	Antonelli et al. (2015b)	16	+	+	+	+	–	+
45A02	*K. pneumoniae*	FOX-7 + porin deficiency	Arena et al. (2013)	4	–	–	–	–	–	–
NV132	*A. baumannii* complex	OXA-58	–	8	+	+*	–	+	+*	–
8–27	*K. pneumoniae*	CTX-M-1-like + OMP deficient	–	2	–	–	–	–	–	–
10–52	*K. pneumoniae*	CTX-M-1-like + OMP deficient	–	2	–	–	–	–	–	–
VA-416/02	*K. pneumoniae*	VIM-4	Luzzaro et al. (2004)	32	+	+	+	+	+	+
GW1	*P. aeruginosa*	GES-2	Poirel et al. (2001)	16	–	–	–	–	–	–
PIEcl	*E. cloacae* complex	VIM-1	–	>32	+	+	+	+	+	+

* Borderline positive: there was correctly a change of color in the test well, but it was not clear as described in manufacturer's instructions.

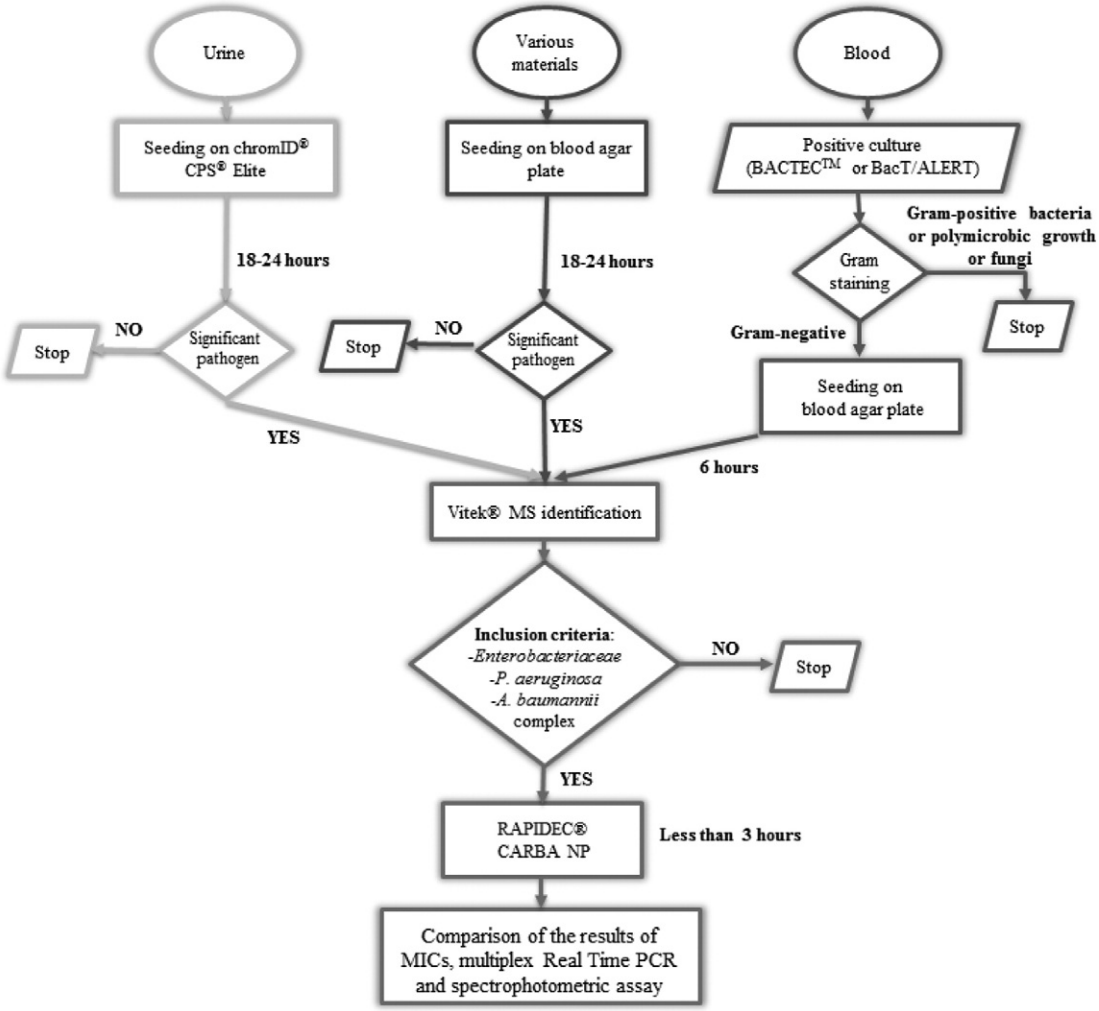

Fig. 1. Workflow for the detection of carbapenemase producers in different clinical isolates. Significant pathogen indicates the presence of a suspect pathogen in a significant count (if applicable for the specimen type), present as a pure culture or as a mixed population with commensals derived from sampling the nonsterile site, considered to be clinically significant and subjected to further identification, AST and reporting.

and results were interpreted according to EUCAST criteria v 6.0 (http://www.eucast.org/clinical_breakpoints/).

2.6. Molecular detection of carbapenemase genes

After performing the RAPIDEC® CARBA NP, each isolate was processed with three homebrew multiplex Real-Time-PCR mixes for the detection of the main carbapenemase genes, including bla_{KPC}, bla_{VIM}, bla_{NDM}, $bla_{OXA\text{-}48\text{-}like}$ genes in *Enterobacteriaceae*, bla_{IMP}, bla_{VIM}, $bla_{FIM\text{-}1}$, bla_{GES} genes in *P. aeruginosa*, and $bla_{OXA\text{-}23\text{-}like}$, $bla_{OXA\text{-}24\text{-}like}$, $bla_{OXA\text{-}58\text{-}like}$, IS*Aba1* + $bla_{OXA\text{-}51\text{-}like}$ genes for *A. baumannii* complex. Primers and probes used in every reaction, and reaction conditions are described in Table 2. An internal control, consisting of phocine herpesvirus DNA (PhHV), and primers and probe targeting PhHV, was added in each reaction mix as a positive amplification control (Van Doornum et al., 2003).

2.7. Spectrophotometric assay

All meropenem non-susceptible isolates which tested negative with molecular assays for the detection of carbapenemase genes were further investigated by a spectrophotometric assay with crude extracts, using imipenem as substrate, as previously described (Lauretti et al., 1999), for detection of carbapenemase activity to exclude the presence of carbapenemase types not included in the molecular assay.

3. Results

3.1. Proficiency test with RAPIDEC® CARBA NP

Considering that RAPIDEC® CARBA NP is based on a colorimetric method and results are assigned by visual inspection, a proficiency test was initially performed with a collection of 20 well-characterized strains provided to each participating center in a blinded fashion, to evaluate the reproducibility of interpretation of results obtained at different centers.

All the CNP (n = 4) and the susceptible (n = 2) strains were correctly identified as carbapenemase-negative with the RAPIDEC® CARBA NP test by all laboratories, while four of the 14 CP strains were not reported as carbapenemase-positive by one or more laboratories. In particular, the OXA-58-positive *A. baumannii* complex strain was not detected as CP by two laboratories, while the FIM-1-positive and the IMP-13-positive *P. aeruginosa* strains, and the OXA-372-positive *Citrobacter freundii* strain, were not detected as CP by one laboratory each (Table 1).

The overall good results of the proficiency test (specificity for detection of CP, 100%; sensitivity for detection of CP, 93%) prompted us to proceed with the analysis of isolates from clinical samples in each center.

Table 2
Primers and probes used in this study for three different multiplex Real Time PCR.

Investigated bacteria	Target	Primer name	Sequence (5′-3′)	Reference	Positive control	Concentration of use in reaction mix (nM)
		OXA-48-like-rt-F	GTAGCAAAGGAATGGCAAGAAA	Antonelli et al. (2015a)		500
		OXA-48-like-rt-R	GATGCGGGTAAAAATGCTTG	Antonelli et al. (2015a)		500
	$bla_{OXA-48-like}$ genes	OXA-48-like-rt-P	HEX-CTCTGGAATGAGAATAAGCAGCAAGG-BHQ-1	Antonelli et al. (2015a)	*E. coli* ECBZ-1 (OXA-48) (Giani et al., 2012)	125
		KPC-rt-F	GATACCACGTTCCGTCTGG	Hindiyeh et al. (2008)		500
		KPC-rt-R	GCAGGTTCCGGTTTTGTCTC	Hindiyeh et al. (2008)		500
	bla_{KPC} genes	KPC-rt-P	FAM-AGCGGCAGCAGTTTGTTGATTG-BHQ-1	Hindiyeh et al. (2008)	*K. pneumoniae* FIPP-1 (KPC-3) (Giani et al., 2009)	125
		VIM-rt-fwd	TGGTCTCATTGTCCGTGATG	Antonelli et al. (2016)		500
		VIM-rt-rev	CATGAAAGTGCGTGGAGA	Antonelli et al. (2016)	*K. pneumoniae* VA-416/02	500
	bla_{VIM} genes [b]	VIM-rt-tq	ROX-AAGCAAATTGGACTTCCCGTAACGC-BHQ-2	Antonelli et al. (2016)	(VIM-4), (Luzzaro et al., 2004)	125
		*bla*NDM1_F	CGCAACACAGCCTGACTTT	Ong et al. (2011)		500
		*bla*NDM1_R	TCGATCCCAACGGTGATATT	Ong et al. (2011)	*E. coli* CVB-1	500
	bla_{NDM} genes	*bla*NDM1_P	CY5-CAACTTTGGCCCGCTCAAGGTATTT-BHQ-3	Ong et al. (2011)	(NDM-1) (D'Andrea et al., 2011)	125
		FIM-rt-F	CGCCTTAACACCCGTCGTGA	This study		500
	bla_{FIM-1} gene	FIM-rt-R	GTCTCCTTTTTCAACGATTAGCC	This study	*P. aeruginosa* FI-14/157	500
		FIM-rt-P	HEX- CTGGCGTACAAGCGGCTCAACCCAA- BHQ-1	This study	(FIM-1) (Pollini et al., 2013)	125
	bla_{GES} genes	GES-rt-F	AGAATTGACTCAGGCACCGAG	This study	*P. aeruginosa* GW1 (GES-2)	500
		GES-rt-R	GTTAGTAGCCCCATTGTCGC	This study	(Poirel et al., 2001)	500
		GES-rt-P	CY5- GAACCGTCATGTGTCCCGATGCTAG-BHQ-3	This study		125
		IMP-rt-F	GANGCYTAYHTRATWGAYACTCCA	This study		2000
		IMP-rt-R	GRRATDGAYYGAGARTTAAGCCA	This study	*P. aeruginosa* FI-5/7728	2000
P. aeruginosa [c]	bla_{IMP} genes	IMP-rt-P	FAM- ATTCCNSCYGHRCTRTCRCYATGRAAATG-BHQ-1	This study	(IMP-13), unpublished	250
		oxa-23-like-rt-F	GATTGTTCAAGGACATAATCAGGTG	This study	*A. baumannii* Ab13 (OXA-23) (Corvec et al., 2007	500
		oxa-23-like-rt-R	GGTTCTCCAATCCGATCAGGG	This study		500
	$bla_{OXA-23-like}$ genes	oxa-23-like-rt-P	FAM- AGGCTGGCACATATTCTGTATTTGCGG-BHQ-3	This study		125
		oxa-24-like-rt-F	CTTCCTATHYTCAGCATTTCTATTCTAG	This study	*A. baumannii* VA-566/00 (OXA-40/24), (D'Andrea et al., 2009)	1000
		oxa-24-like-rt-R	ATCTTAAATGTTGAYGCAGGGAC	This study		*1000*
	$bla_{OXA-24-like}$ genes	oxa-24-like-rt-P	HEX- GCTATTTTGATGAAGCTCAAACACARGGT-BHQ-1	This study		250
		oxa-58-like-rt-F	AAAGCATGGGACAAAGATTTTAC	This study		500
		oxa-58-like-rt-R	CAAACTTTACTTCTTGTATAGGTGT	This study	*A. baumannii NV132* (OXA-58), unpublished	500
	$bla_{OXA-58-like}$ genes	oxa-58-like-rt-P	ROX- CAGTGCCTGTATATCAAGAATTGGCAC-BHQ-2	This study		125
		ISABA1-oxa-51-rt-F	ATAATCACAAGCATGATGAGCG	This study		500
		ISABA1-oxa-51-rt-R	GTGARCAGGCTGAAATARRAATAG	This study		500
A. baumannii complex [c]	IS*Aba1* + $bla_{OXA-51-like}$ genes	ISABA1-oxa-51-rt-P	CY5-ATGAACATTAAAGCACTCTTACTTATAACAAG-BHQ-3	This study	*A. baumannii 696/03* (OXA-58), unpublished	125
		PhHV-267 s	GGG CGA ATC ACA GAT TGA ATC	Van Doornum et al. (2003)		500
		PhHV-337as	GCG GTT CCA AAC GTA CCA A	Van Doornum et al. (2003)		500
Everyone	PhHV (internal control)	PhHV-305tq	Cy5.5 -TTTTATGTGTCCGCCACCATCTGGATC-BHQ-3	Van Doornum et al. (2003)	PhHV DNA cloned in pGEM-T-easy *E. coli* DH5α	125

[a] The amplification program consisted of 35 two-step cycles of 15 s at 95 °C and 60 s at 60 °C.
[b] bla_{VIM} genes were also target of the multiplex Real Time PCR used for *P. aeruginosa* isolates.
[c] The amplification program consisted of 35 three-step cycles of 15 s at 95 °C, 30 s at 50 °C and 30 s at 60 °C.

3.2. Performance of the RAPIDEC® CARBA NP test on isolates from clinical specimens other than blood cultures

Among 125 isolates from various materials (mainly urine and respiratory specimens), 31 were confirmed as CP by molecular methods. These isolates included 10 KPC-producing *K. pneumoniae*, one OXA-48-producing *K. pneumoniae*, one KPC- and VIM-coproducing *K. pneumoniae*, and 19 class D carbapenemase-producing *A. baumannii* complex (n = 16 OXA-23-like, n = 2 OXA-24-like, n = 1 OXA-51-like overexpressed by an IS*Aba1* inserted upstream). Of these, the RAPIDEC® CARBA NP test correctly detected the 12 CP *Enterobacteriaceae*, but failed to identify 11 of the 19 CP *A. baumannii* complex (including 9 positive for a $bla_{\text{OXA-23-like}}$ gene, one positive for a $bla_{\text{OXA-24-like}}$ gene, and one carrying a $bla_{\text{OXA-51-like}}$ gene preceded by an IS*Aba1* insertion sequence). Moreover, the RAPIDEC® CARBA NP test was positive with one *K. pneumoniae* and five *P. aeruginosa* for which the molecular tests and the spectrophotometric assay had not identified carbapenemases (Table 3). Consequently, overall sensitivity, specificity, positive predictive values (PPV), and negative predictive values (NPP) were found to be 65%, 94%, 77%, and 89%, respectively. If *A. baumannii* complex strains were not included in the study, the sensitivity, specificity, PPV and NPP of RAPIDEC® CARBA NP would be 100%, 93%, 67%, 100%, respectively. Considering only the 61 *Enterobacteriaceae* isolates, the test yielded an even better performance (100%, 98%, 92%, 100%, respectively) (Table 4).

Table 4
Performance of RAPIDEC® CARBA NP test with clinical isolates.

Sources	Species/Family	Sensitivity %	Specificity %	PPV%	NPV%
All	**All (N = 247)**	**70**	**94**	**82**	**89**
	Excluding *A. baumannii* complex (N = 208)	100	94	78	100
	Enterobacteriaceae (N = 142)	100	98	94	100
Blood cultures	**All (N = 122)**	**75**	**95**	**87**	**90**
	Excluding *A. baumannii* complex (N = 107)	100	95	86	100
	Enterobacteriaceae (N = 81)	100	98	95	100
Other materials	**All (N = 125)**	**65**	**94**	**77**	**89**
	Excluding *A. baumannii* complex (N = 101)	100	93	67	100
Enterobacteriaceae	(N = 61)	100	98	92	100

3.3. Performance of the RAPIDEC® CARBA NP test with positive blood cultures in a fast-track workflow

Of the 125 isolates from blood cultures tested with RAPIDEC® CARBA NP, 122 gave interpretable results, while three isolates (one *P. aeruginosa*, one *A. baumannii* complex and one *Enterobacter cloacae*) yielded insufficient growth to perform the test at 6 h after subculture. Among the 122 evaluable isolates, 36 were confirmed as CP by molecular methods, including 18 KPC-producing *K. pneumoniae*, two OXA-48-producing *K. pneumoniae*, one VIM-producing *K. pneumoniae*, three VIM-producing *P. aeruginosa* and 12 class D carbapenemase-producing *A. baumannii* complex (n = 10, OXA-23-like; n = 1, OXA-24-like; n = 1, OXA-23-like and OXA-24-like co-producer). Of them, RAPIDEC® CARBA NP assay correctly identified 27 isolates as CP, but missed nine *A. baumannii* complex carrying $bla_{\text{OXA-23-like}}$ and/or $bla_{\text{OXA-24-like}}$ genes (Table 3). RAPIDEC® CARBA NP correctly categorized as CNP 82 out of 86 isolates from blood cultures, while false-positive results were obtained with one *K. pneumoniae* and three *P. aeruginosa* isolates for which no carbapenemase genes nor carbapenemase activity were detected using Real-Time-PCRs and spectrophotometric activity, respectively. According to these results, the RAPIDEC® CARBA NP showed a sensitivity of 75%, a specificity of 95%, a PPV of 87%, and a NPV of 90%. However, excluding

Table 3
Comparison of the results obtained from RAPIDEC® CARBA NP test with the molecular detection of carbapenemase genes. Discrepant RAPIDEC® CARBA NP results are underlined.

Origin of Isolates	Species	No. of isolates	MIC MEM (μg/ml)	Carbapenemase genes	RAPIDEC® Result
various materials	*Enterobacteriaceae*	10	16 to >128	bla_{KPC}	+
		1	32	$bla_{\text{OXA-48-like}}$	+
		1	16	$bla_{\text{KPC}} + bla_{\text{VIM}}$	+
		48	≤0.03–4	No one	−
	P. aeruginosa	1	0.25	No one	+
		4	16 to >128	No one	+
		35	≤0.03–64	No one	−
		35	≤0.03–64	No one	−
	A. baumannii complex	1	2	No one	+
		7	64 to >128	$bla_{\text{OXA-23-like}}$	+
		9	8–128	$bla_{\text{OXA-23-like}}$	−
		1	64	$bla_{\text{OXA-24-like}}$	+
		1	2	$bla_{\text{OXA-24-like}}$	−
		1	2	IS*Aba1* + $bla_{\text{OXA-51-like}}$	−
		5	0.25–8	No one	−
blood cultures	*Enterobacteriaceae*	18	8 to >128	bla_{KPC}	+
		2	1–4	$bla_{\text{OXA-48-like}}$	+
		1	4	bla_{VIM}	+
		59	≤0.03–2	No one	−
	P. aeruginosa	1	≤0.03	No one	+
		1	≤0.03	No one	[a]
		3	8 to >128	bla_{VIM}	+
		1	>128	bla_{VIM}	[a]
		1	>128	No one	+
		20	0.12 to >128	No one	−
		2	0.12; 0.5	No one	+
		1	128	$bla_{\text{OXA-23-like}}$ and $bla_{\text{OXA-24-like}}$	−
		7	16 to >128	$bla_{\text{OXA-23-like}}$	−
		3	16–64	$bla_{\text{OXA-23-like}}$	+
		1	>128	$bla_{\text{OXA-24-like}}$	−
		1	>128	$bla_{\text{OXA-23-like}}$	[a]
	A. baumannii complex	3	0.25	No one	−

[a] insufficient biomass 6 h after subculture.

the *A. baumannii* complex isolates, sensitivity, specificity, PPV and NPV were 100%, 95%, 86%, 100%, respectively, and these percentages further increased when considering only the 81 *Enterobacteriaceae* isolates (100%, 98%, 95%, 100%, respectively) (Table 4).

3.4. Overall performance of the RAPIDEC® CARBA NP test with clinical isolates

Overall, considering all types of clinical specimens, the RAPIDEC® CARBA NP test yielded sensitivity, specificity, PPV and NPP of 70%, 94%, 82%, and 89%, respectively. The best results were observed with *Enterobacteriaceae*, as described in Table 4.

No significant differences were observed among the performance of the RAPIDEC® CARBA NP test carried out at the five centers.

4. Discussion

The global spread of CP-GNB represents a major public health challenge. Clinical Microbiology laboratories are increasingly asked for rapid detection of CP strains for infection control and antimicrobial stewardship purposes.

The RAPIDEC® CARBA NP test is a simple phenotypic test that does not require any specific technical skills or expensive equipment (Garg et al., 2015; Poirel and Nordmann, 2015), which allows rapid detection of CP strains in a timeframe comparable to that of molecular tests (i.e. 1–2 h) but at a substantially lower cost.

In this multicenter work, involving five different Italian laboratories, we evaluated the performance of RAPIDEC® CARBA NP in a proficiency test carried out by each laboratory with a collection of 20 well-characterized carbapenem-resistant strains representative of different Gram-negative species and resistance mechanisms, and then in a field test with 50 clinical isolates from each laboratory.

Results revealed an overall high specificity of RAPIDEC® CARBA NP for detection of CP strains, similar to that previously reported by Poirel and Nordmann (2015) (96%), Garg et al. (2015) (96.2%) and Kabir et al. (2016) (98.5%). The false-positive results, observed with *K. pneumoniae* and *P. aeruginosa* isolates, and reported also by other Authors (Lifshitz et al., 2016; Österblad et al., 2016; Poirel and Nordmann, 2015), could be attributable to a reduced stability of the imipenem substrate used in the test toward strains producing enzymes that are not true carbapenemases but have some weak carbapenemase activity (e.g. AmpCs, CMY, CTX-M-type producers), and possibly also to the use of the inoculum recommended in the Manufacturer's instructions. Indeed, Dortet et al. (2015) identified a critical impact of the bacterial inoculum in the performance of the test, and recommended the use of a much higher inoculum to avoid false positive results.

On the other hand, the test frequently failed to detect *A. baumannii* complex strains producing class D β-lactamases, a problem reported also by other authors (Kabir et al., 2016; Poirel and Nordmann, 2015) and probably due to the overall weak carbapenemase activity of class D carbapenemases (Queenan and Bush, 2007). A lower overall sensitivity (70%) was detected in this work compared to previously reported studies by Garg et al. (2015) (92.6%), Kabir et al. (2016) (97.8%), and Poirel and Nordmann (2015) (96%). This difference could be partially ascribed to the higher percentage of class D producing *A. baumannii* complex (12.5%) tested in our study (Garg et al. (2015) 0%, Kabir et al. (2016) 4.7%, and Poirel and Nordmann (2015) 8.5%). Also Noël et al. (2017) showed that RAPIDEC® CARBA NP performed poorly for the detection of class D carbapenemase-producing *Acinetobacter* spp. isolates (sensitivity 36.4%, specificity 75%), leading us to conclude that this test should not be used with *A. baumannii* complex isolates in its present format. A much higher bacterial inoculum, compared to the Manufacturer's instructions, could increase the sensitivity of the tests (Lifshitz et al., 2016). Indeed, Dortet et al. (2015) recommended to perform the test using a standardized inoculum (a full 10 μl loop), which is critical for test reliability.

A limitation of the prospective evaluation of RAPIDEC® CARBA NP carried out in this work is represented by the relatively low number of isolates producing some types of carbapenemases (e.g. OXA-48 or VIM), and of carbapenemase-producing *P. aeruginosa* isolates, which reflected the local epidemiology of infections.

Considering *Enterobacteriaceae* isolates only, the values of sensitivity and specificity were high and comparable with other works (Dortet et al., 2015; Hombach et al., 2015; Lifshitz et al., 2016), showing that RAPIDEC® CARBA NP test could be useful also for detecting CP *Enterobacteriaceae* from positive blood cultures processed with a fast-workflow approach. With this approach, RAPIDEC® CARBA NP can be used to reduce the time required for identification of CP *Enterobacteriaceae* to less than 9 h since positivization of blood cultures (from at least 24 to 48 h of routine methods) (Morgenthaler and Kostrzewa, 2015). The rapid identification of CP Gram-negatives from blood cultures can be of remarkable importance to antimicrobial stewardship (Barlam et al., 2016). It should be noted, however, that the possibility of using a fast workflow is dependent on the laboratory schedule. For instance, in the laboratories participating in this work, which do not process positive blood cultures on a 24/7 schedule, the fast workflow could only be performed with blood cultures that were found to be positive or became positive in the morning. When the RAPIDEC® CARBA NP is used with positive blood cultures in the rapid workflow, another limitation could be represented by an insufficient bacterial growth for inoculum at 6 h. However, in our experience, this occurred only with a small number of cases (3 of 122, 2.4%).

A limitation of the RAPIDEC® CARBA NP test is that it cannot discriminate the type of carbapenemase produced. This information is important for antimicrobial stewardship since the new antibiotics active against CP-GNB that are entering clinical practice (e.g. ceftazidime-avibactam) may not cover all types of CP strains. In this perspective, the RAPIDEC® CARBA NP could have a role as a screening test for excluding carbapenemase production and selecting the most suitable candidates for characterization of the carbapenemase type by molecular platforms or immunoenzymatic assays (Banerjee et al., 2015; Meunier et al., 2016; Raich and Powell, 2015).

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