UNIVERSITY OF SIENA

Department of Medical Biotechnologies

PhD Program in Medical Biotechnology

Section of Biochemical and Microbiological Sciences

XXVIII Cycle

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Characterization of resistance determinants in clinical and environmental isolates of multi-resistant bacteria

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2016

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1. INTRODUCTION

"Antimicrobial resistance (AMR) is an increasingly serious threat to global public health. AMR develops when a microorganism (bacteria, fungus, virus or parasite) no longer responds to a drug to which it was originally sensitive. This means that standard treatments no longer work; infections are harder or impossible to control; the risk of the spread of infection to others is increased; illness and hospital stays are prolonged, with added economic and social costs; and the risk of death is greater, in some cases, twice that of patients who have infections caused by non-resistant bacteria.

The problem is so serious that it threatens the achievements of modern medicine.
A post-antibiotic era, in which common infections and minor injuries can kill, is a very real possibility for the 21st century."

World Health Organization *Global Report on Surveillance* 2014

The era of antibiotics was born with the observation of the antibacterial properties of a mould, *Penicillum notatum*, made by A. Fleming in 1928; he noticed that in a culture of *Staphylococcus aureus* a green mould was grown and where the bacteria and the mould encountered, the bacterial cells were lysed. Fleming described those results in 1929, suggesting a therapeutic use of the substance he isolated, but he couldn't produce it in order to utilize it as a drug (Fleming, 1929). We had to wait until 1941, when Abram and coworkers were able to produce sufficiently pure penicillin for both external and systemic administration, and Florey and Chain could demonstrate its valuable pharmacological properties (Abraham, 1940). This achievement was the turning point for a revolutionary cure to most of the infective diseases of bacterial origin. The use of antibiotics, despite having represented one of the most important successes of medicine, showed later some complications, among them the most important is antibiotic resistance, because the introduction of new antibiotics was always followed, sooner or later, by the insurgence of mechanisms capable of inhibiting their efficacy. In last years a dramatic increase of the frequency and type of microorganisms that have become resistant to antibiotics have been observed globally. The insurgence of this type of resistance and its capacity of transmission among microorganisms creates serious

therapeutic problems in the near future. According to a recent CDC report, at least two millions infection and 23000 deaths occurs in US each year due to antibiotic resistance (http://www.cdc.gov/drugresistance/threat-report-2013/). Therefore, the research and development of new antibiotic compounds with better activity and capable of overcoming the molecular mechanisms of antibiotic resistance is of paramount importance. It would be also recommended a more careful and appropriate use of the antimicrobial compounds for both men and animals, even if the correlation between the use of antibiotics and the development of new resistances still remains unproven. From this perspective, the epidemiological study of new and emerging determinants of resistance is essential.

1.1 Gram-negative pathogens

Gram-negative bacteria, classified for their inability to retain crystal violet staining, are a very large group of bacteria responsible for many kind of infections including pneumonia, bloodstream infections, wound or surgical site infections, meningitis and urinary tract infections in healthcare settings. Unfortunately, Gram-negative bacteria are often resistant to multiple drugs and are increasingly resistant to most available antibiotics. These bacteria have the intrinsic ability to find new ways to escape therapies and can pass along genetic materials that allow other bacteria to become drug-resistant as well (http://www.cdc.gov/hai/organisms/gram-negative-bacteria.html). The current antibiotic crisis is more serious with Gram-negatives than with Gram-positives (Rossolini, 2014). The most commonly isolated Gram-negatives bacteria cause of infections are *Enterobacteriaceae*, *Pseudomonas aeruginosa* and *Acinetobacter baumanii*, but also other less frequently isolated species can be cause of opportunistic infections, or can be part of the normal human microbita and contribute to the spread of antibiotic resistance.

1.1.1 Enterobacteriaceae

Enterobacteriaceae is a family of bacteria which includes more than 40 genera and hundreds of species of Gram-negative asporigen bacilli, which share an enterobacteric common antigen (Murray, 2015). The genera *Escherichia, Klebsiella, Enterobacter,*

Serratia, and *Citrobacter* (collectively called the coliform bacilli) and *Proteus* include opportunistic pathogens responsible for a wide range of infections. Many of these species are members of the normal intestinal flora and *E. coli* is the most commonly isolated organism in the clinical laboratory. Coliform and *Proteus* bacilli currently cause 29% of nosocomial (hospital-acquired) infections in the United States. In order of decreasing frequency, the major sites of nosocomial infection are the urinary tract, surgical sites, bloodstream, and pneumonias. This group of nosocomial pathogens are responsible for 46% of urinary tract and 24% of surgical site infections, 17% of the bacteremias, and 30% of the pneumonias. All genera except *Klebsiella* are flagellated. Some strains produce capsules. Virulence often depends on the presence of attachment pili (which can be characterized by specific hemagglutination reactions). Sex pili also may be present. The major classes of antigens used in defining strains are H (flagellar), O (somatic), and K (capsular). All enterobacteria can grow in anaerobic conditions, are glucose fermenters and catalase positive and oxidase negative (Murray, 2015). Actually, the global diffusion of Extremely-Drug Resistant (XDR) *Enterobacteriaceae* is one of the most worrying aspects of the ongoing antibiotic resistance crisis (Rossolini, 2015)

1.1.2 Pseudomonas aeruginosa

The opportunistic bacterial pathogen currently known as *Pseudomonas aeruginosa* has received several names throughout its history based on the characteristic blue-green coloration produced during its culture. Sédillot in 1850 was the first to observe that the presence of a transferable agent on surgical wound dressings (Lister, 2009). *P. aeruginosa* is a Gram-negative aerobic non fermenting microorganism that normally lives in moist environment, and it is characterized by small nutritional needs. In humans, the main sites colonized by *P. aeruginosa* are moist areas as perineum, respiratory tract mucosa, and ear (Rossolini, 2005). Even if the frequency of colonization of this microorganism is quite low in healthy humans, this rises in case of hospitalization and repetitive antibiotic therapies with extended spectrum drugs (Pollack, 2000). *P. aeruginosa* is essentially an opportunistic pathogen which requires, for the establishment of the infection, an impairment of the physical barriers (for example skin or mucosa), or the passage through them by means of medical devices as, for example, catheters and assisted respiratory tubes and/or the presence of malfunctions

of the immune system. The derived nosocomial infections affect usually the respiratory tract, with a common evolution in pneumonia, the urinary tract, surgical wounds and burnt skin (Rossolini, 2005). Bacteremia and septic shock are observed in immunocompromised patients and are associated with a high mortality rate (Collin, 2001). Furthermore, *P. aeruginosa* is the most common pathogen in cystic fibrosis (Rossolini, 2005). *P. aeruginosa* in cystic fibrosis affected patients is present in the mucoid phenotype, colonizes the abnormal epithelium of the respiratory tract of those patients and it is responsible for the progression of lung disease (Davies, 2002). Community acquired infections are less frequent; among them we can remember: (i) folliculitis and infections of the auditory canal, generally contracted following contact with infected water; (ii) cheratitis; (iii) osteomyelitis, generally caused by deep wounds; (iv) endocarditis, for intravenous drug users, following the contamination of the injected substances (Rossolini, 2005). The virulence mechanisms of *P. aeruginosa* are complex and only partially known. The colonization of mucosa or other surfaces is mediated by the presence of pili, or the expression of other adhesins, whereas the production of a polysaccharide matrix, which surrounds the cells and links them to each other and the environment, is important for biofilm growth (Drenkard, 2002). Biofilm is a dynamic structure in which the microorganism is protected from the immune system's action and from antibacterial drugs (Hoiby, 2001). *P. aeruginosa* damages the tissue and invades it with secretion products as elastase, alkaline protease, citotoxin, phospholipase C and rhamnolipid (Rossolini, 2005). Moreover, the local and systemic toxicity is linked to the release of endotoxins and the production of the exotoxin A (an extracellular enzyme that inhibits protein synthesis in mammals) and the exoenzyme S (with ADP-rybosylating activity for various GTP binding proteins) (Rossolini, 2005). The production of isoenzymes and virulence factors is controlled by a mechanism of regulation, called quorum-sensing (Bjarnsholt, 2010). This mechanism coordinates the gene expression of virulence factors when the population is large, in order to break the immunologic defenses of the host (Rossolini, 2005).

1.1.3 *Acinetobacter baumanii*

Acinetobacter genus is composed by Gram-negative coccobacillus bacteria, strictly aerobic, oxidase negative non fermentative. *Acinetobacter baumanii* is the most common cause of infections among *Acinetobacter* spp. and is often found with a multi-

drug resistance phenotype (Dent, 2010). Infections caused by *A. baumannii* are associated with significant mortality (attributable mortality rates ranging from 20% to 37%) and an increase in the average length of ICU stay by 15 days (Doan, 2015). *Acinetobacter baumanii* is associated with many nosocomial infections and the most common are pneumonia, urinary tract infections and bacteremia. (Cisneros, 2002). Due to a very common multi-drug resistant profile, infections by *A. baumanii* are among the most difficult to treat (Evans, 2013).

1.1.4 Environmental opportunistic pathogens

Other bacterial species ubiquitarious and most commonly associated with aquatic environment are Aeromonads and *Shewanella spp.* The genus *Aeromonas*, initially considered as an exclusively animal pathogen, is an important disease-causing pathogen of fish and other cold-blooded species, but is also responsible for a variety of infectious complications in both immunocompetent and immunocompromised persons. (Janda, 2010). Most *Aeromonas* spp, with the exception of *Aeromonas caviae*, have a chromosomal inducible metallo-β-lactamase which can give multi-resistance to β-lactams antibiotics, while acquired resistance is uncommon but is increasing in both nosocomial and environment settings (Maravic, 2013, Adler, 2014). *Shewanella xiamenensis* is a species of Gram negative usually found in costal sediments (Huang, 2010), which has been identified as the source of the lineage of OXA-48-like enzymes, which are now globally spreading among *Enterobacteriaceae* (Zong, 2012).

1.2 Gram positive-pathogens

Gram-positive bacteria are characterized by a thick continuous cell wall (20 to 80 nm) composed largely of peptidoglycan. Since the introduction of penicillin into clinical use in the 1940s the treatment of infections caused by Gram-positive bacteria has being revolutionized, especially staphylococci and streptococci. In the subsequent 60 years, many further antibacterial agents have been launched, but bacteria have consistently shown their adaptability by developing resistance to almost every agent with which they have been challenged (Woodford, 2009). Infections caused by multiresistant Gram-positive bacteria represent a major health burden in both community and nosocomial setting (Willems, 2011). Among Gram-positive pathogens, *Staphylococcus aureus*, *Streptococcus pneumoniae* and, more recently, *Enterococcus faecium* and *Enterococcus*

faecalis, each present global resistance challenges, causing significant public health concern and adding to the cost of health care (Woodford, 2009).

1.2.1 *Staphylococcus aureus*

Staphylococcus aureus is a globally diffused human pathogen, often found also asymptomatically on the human body. One third of the general population is colonized by *S. aureus*, and this represents a risk factor for the acquisition of infections mediated by this pathogen. Worldwide, the increasing antibiotic resistance of *S. aureus* complicates treatment of infection and control measures (Kluytmans, 1997). Despite proper antibiotic therapy, staphylococcal infections occur regularly in hospitalized patients and have severe consequences. Due to an increasing number of infections caused by methicillin-resistant *S. aureus* (MRSA) strains, which are most often multiresistant, therapy has become problematic (Rodvold, 2014). Moreover, *S. aureus* carries many virulence factors such as exotoxins, enterotoxins (Aguilar, 2014) and in many cases Phantom-Valentine Leukocidin toxin, which has been associated with leukocyte destruction and necrotizing pneumonia (Vandenesch, 2003). Infection control measures properly applied have lead to a recent decrease in the number of infections mediated by multi-resistant *S. aureus* strains in UK (from 47% in 2002 to 11% in 2014) and Germany (from 21% in 2005 to 11.8% in 2014), but only a slight decrease in Italy (from 44.5% in 2000 to 33.6% in 2014) (Johnson, 2012a, EARS-Net Data 2010-2014).

1.3 Antibiotics

1.3.1 β-lactam antibiotics

β-lactam antibiotics were and are until now the more utilized antibacterial drugs in clinical practice, due to their high efficacy and low toxicity (Giammarellou, 2001).

The targets of these compounds are the *penicillin-binding-proteins* (PBPs), membrane protein of the bacterial cell responsible for the synthesis of the peptidoglycan's cross-links. Peptidoglycan is an essential component of the cellular wall and a peculiar structure of prokaryotes. β-lactam antibiotics interfere with the last step of peptidoglycan synthesis (formation of the cross-links), compromising the bacterial structural integrity. From this follows a growth stop and, in some cases, an osmotic lysis of the cell with a bactericidal effect. For their efficacy, β-lactam antibiotics need microorganisms in active proliferation, whereas they have little or no effect on non-

proliferating bacteria. They are not active on fungi, whose cell walls don't contain peptidoglycan, nor on mycoplasmas, which don't have the cellular wall, neither on protozoa or viruses (Murray, 2015).

Peptidoglycan and bacterial cell wall

The main component of the bacterial wall is peptidoglycan, which is made by two amino sugars acetylated, N-acetyl-glucosamine (GlcNac) and N-acetyl-muramic acid (MurNac) that, bound in a sequence alternated by a β-1,4 glycosidic bond, make the basic structure. The carboxil group of the MurNac is bound to a peptidic portion, which usually, in Gram-negative bacteria, has this sequence: L-alanine (L-Ala), D-glutamic acid (D-Glu), meso-diaminopimelic acid (m-A_2pm), D-alanine (D-Ala) and D-Alanine (D-Ala). The different linear polymers are connected to each other crosswise through peptide bonds that are established, in Gram-negative bacteria, between meso-diaminopimelic acid in third position of a peptide chain, and D-alanine in forth position of a contiguous chain. Biosynthesis and maintenance of the peptidoglycan are catalyzed by a series of enzymes (DD-transpeptidase, transglycosylase, DD-carboxypeptidase and DD-endopeptidase), known as PBPs, present in the bacterial cytoplasmic membrane (Murray, 2015).

Structure of β-lactam antibiotics

β-lactam antibiotics present an analogy of structure with the D-Ala-D-Ala dipeptide, which forms one of the substrates in the formation of the interpeptide bond catalyzed by DD-transpeptidase. Because of this strong similarity, β-lactams interact with the PBPs, creating a very stable covalent complex, and they irreversibly inhibit its activity.

β-lactams constitute a class of antibiotics characterized by the presence in their structure of a tetratomic β-lactam ring containing an amide bond. Some of them are naturally produced by moulds (penicillins, cephalosporins), and bacteria (nocardicins, monobactams, carbapenems), but also semi-synthetic β-lactams exist, obtained with chemical modification of the antibiotic's molecules produced with microorganism fermentation process. Semi-synthetic β-lactams represent most of the molecules available.

B-lactam antibiotics are divided, on a chemical structure basis, in different families (Figure 1):

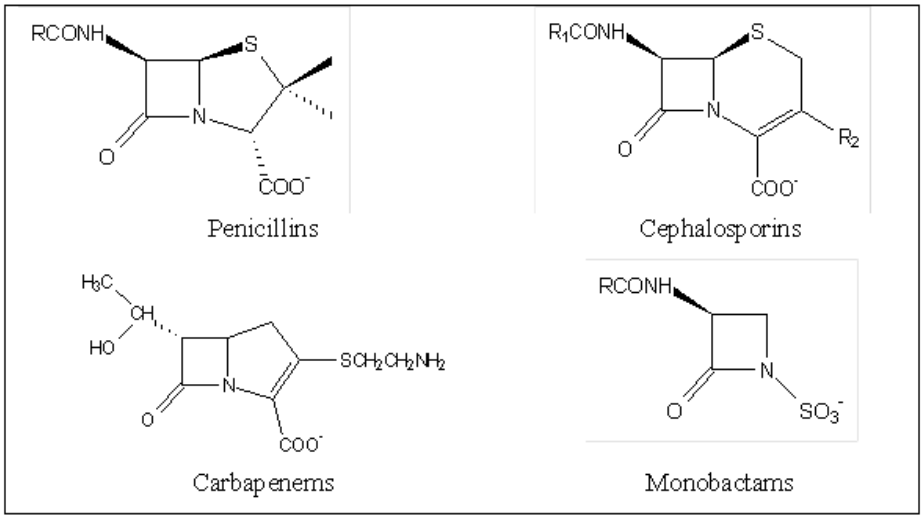


Figure 1. Chemical structure of the main classes of β-lactams antibiotics.

- *Penicillins*

  Natural penicillins are produced from moulds such as *Penicillium chrysogenum.* The basic structure of all penicillins both natural and synthetic or semi-synthetic is the 6-aminopenicillamic acid, constituted by a β-lactam ring melted with a pentatomic thiazolidine ring. Penicillins are globally active on most of the bacterial species of clinical relevance, but mainly on Gram-positive bacteria.

- *Cephalosporins*

  Natural cephalosporins, as cephalosporin C, are produced by the fungus *Cephalosporium acremonium* and by *Streptomyces clavuligerus*. Unfortunately, it is not possible to obtain derivatives through the introduction of precursors during the fermentative process; therefore, the production on a large scale of the fundamental structure, the 7-aminocephalosporanic acid, from which it was possible to proceed with the synthetic way, obtaining a large amount of derivatives, requires a big effort. As a matter of fact most of the cephalosporins utilized in clinic are semi-synthetic derivatives of cephalosporin C, and they are active against many Gram-positives and Gram-negatives infections. Compared with penicillins, cephalosporins have higher resistance to β-lactamase hydrolisis. The group of cephalosporins includes a great variety of compounds classified in "generations" on the basis of their spectrum of action, their intrinsic activity and hydrolysis resistance. These characteristics reflect also the period when they were introduced in commerce (for example ceftazidime is a third generation

cephalosporin, whereas cefepime, a fourth). The fifth generation of cephalosporins (ceftolozane, ceftobiprole, ceftaroline), was designed to combat multi-drug resistant bacteria for which the previous generations are no longer effective (Long, 2014)

- *Carbapenems*

  Carbapenems are β-lactam antibiotics that differ from penicillins because their sulphur atom inside the thiazolidine ring is replaced by a carbon atom. The better-known compounds of this group are imipenem and meropenem, which show a particularly broad spectrum of activity and result resistant to the hydrolytic activity of most part of serin active β-lactamases (molecular classes A, C and D).

- *Monobactams*

  Those compounds show a peculiar structure because their β-lactam ring is not melted with another ring, and for this reason they are called "monocyclic β-lactams". They are produced by environmental bacterial species (ex. *Nocardia uniformis* subsp. *tsuyamanensis*) or they are completely synthetic, as aztreonam, which was introduced in clinical practice in 80's years. They show a good activity against Gram-negatives, a better stability towards β-lactamases and a lower toxicity.

There are some compounds called β-lactamases inactivators provided with a strong inhibitory effect, which interact with β-lactamases making a covalent complex that inhibits the enzyme. For this reason, they are often administered with the classic β-lactam antibiotics to improve their activity (Matagne, 1993). The most used inactivators in clinical practice are clavulanic acid, sulbactam and tazobactam and the recently introduced avibactam (Zhanel, 2013).

1.3.2 Aminoglycosides

Aminoglycosides are natural or semi-synthetic drugs with bactericide action.

They are composed by two or more amino-sugars linked by a glycosidic bond at a hexose nucleus. This structure gives the drug a basic and hydrophilic feature, therefore the diffusion through the cellular membranes occurs with an active transport.

Streptomycin was the first aminoglycoside described, and it was isolated from the actinomyces *Streptomyces griseus* from which it takes the name. Aminoglycosides originate from two different genera: *Streptomyces* from which Streptomycin, Neomycin

and Tobramycin were isolated, and the genus *Micromonospora* from which Gentamycin and Sisomycin were derived.

Aminoglycosides play their lytic action inhibiting the bacterial protein synthesis irreversibly binding the 30S ribosomal subunit, in particular the 16S rRNA at A site (tRNA acceptor), and altering the integrity of the cellular membrane. Protein synthesis is blocked both in early phase and in extension phase causing a premature interruption of the peptide synthesis and the release of broken proteins. The release and accumulation inside the cell of broken proteins causes the alteration of the cellular membrane permeability. Those protein fragments are included in the membrane structure bringing an increase of the passive permeability of membrane, a release of K+ ions and a further increase of the antibiotic concentration inside the cell. Moreover, the saturation of all the ribosomal bonds with the aminoglycoside, blocking protein synthesis, prevents the synthesis of other ribosomes, causing cell death (Magnet, 2005). Aminoglycosides are the most used chemotherapeutics for the treatment of several infections induced by *Pseudomonas* spp., and other Gram negative microorganisms not only for their efficacy, but also for their positive effects linked to the interaction with other classes of drugs. For example, the combined therapy with β-lactam drugs in cases of lung infection in patients affected by cystic fibrosis, results often effective in limiting the infection (Rossolini, 2005). Even if aminoglycosides are very effective drugs, is important to bear in mind their high toxicity compared to other chemotherapeutics, since they have both oto- and nephro-toxicity (Prayle, 2010). The most common routes of administration, due to their low levels of absorption, are parenteral, intramuscular or intravenous in case of severe infections (Ramirez, 2010).

1.3.3 Quinolones

Quinolones are chemotherapeutic drugs of synthetic origin, which inhibit DNA replication, through the interaction with DNA gyrase and DNA Topoisomerase IV (Topoisomerase II) enzymes, causing bacterial death. DNA topoisomerases II control the topologic state of chromosomal DNA to facilitate replication, recombination and expression through the rupture and rearrangement of DNA helixes. DNA topoisomerase type I is able to cut only one DNA helix, whereas DNA topoisomerase II cuts both the helixes in a reaction coupled with the ATP bond and hydrolysis. DNA gyrase is responsible for the introduction of negative supercoiling in the DNA double helix. The

formation of the complex quinolone-DNA gyrase/DNA topoisomerase is responsible for the inhibition of the DNA replication and the bactericide action of the drug.

The progenitor of this class of drugs is nalidixic acid, which has a limited spectrum of action in enterobacteria and which, for its pharmacokinetic characteristics is exclusively used for the urinary tract infections treatment. Afterwards, new quinolone drugs were developed, fluoroquinolones, that are fluoride derivatives of quinolones, and are characterized by a broader spectrum of action (ex. ciprofloxacin, norfloxacin, levofloxacin, gatifloxacin, moxifloxacin). Fluoroquinolones are used for chemotherapy of infections due both to Gram-positive and Gram-negative microorganisms and act also on *P. aeruginosa* strains, that are intrinsically resistant to I generation quinolones. These molecules show better pharmacokinetic characteristics than the previous, and this allows their use in systemic infections therapies (Figure 2). Unfortunately, up to 39% of fluoroquinolones therapies are unnecessary and up to 27% of the treated patients experience side effects, which could be avoided with a better therapy plan (Werner, 2011).

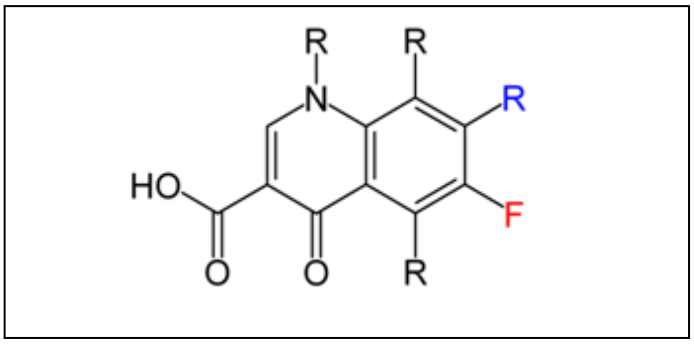


Figure 2. Structure of all quinolone antibiotics. If the phenol ring contains a fluorine atom (in red), the molecule is called fluoroquinolone**.**

1.3.4 Polimixins

Polimixins are a group of cyclic polypeptides naturally produced by *Bacillus polymyxa*, isolated for the first time in 1947 (Biswas, 2012). Polimixin B and E, the most commonly used in clinical practice, have a similar structure with a D-Phe instead of a D-Leu in 6 position for polimixin B and E, respectively (Velkov, *2010*).

The genetic cluster which brings to the production of polimixins is *pmx*, which includes five genes: *pmxA*, *pmxB*, *pmxC*, *pmxD*, *pmxE*. PmxA, PmxB e PmxE are polimixin synthetases, while PmxC e PmxD are membrane transport proteins (Choi, 2009).

Polimixins interact with external membrane polysaccharides and phospholipids causing an increased cell permeability leading to a bactericidal effect (Murray, 2015). They are more effective against Gram-negative bacteria, and recently they have been considered as last-resort antibiotics against multi-resistant bacteria (Rice**,** 2006; Li, 2007). They have important side effects (nephrotoxicity and neurotoxicity) and for this reason their use should be limited to infections by multi-drug resistant Gram-negative pathogens (Falagas, 2005).

1.3.5 Oxazolidinones

The oxazolidinones are a class of antimicrobial agents, which have a unique structure and good activity against Gram-positive pathogenic bacteria. Oxazolidinones contain a 2-oxazolidine (figure 3), and they inhibit protein synthesis by binding at the P site at the ribosomal 50S subunit. They are synthetic antibacterial agents active against multiple-resistant Gram-positive pathogens, including methicillin-resistant *Staphylococcus aureus* (MRSA), penicillin-resistant streptococci, and vancomycin-resistant enterococci (Pandit, 2012). The commercially available oxazolidinones are linezolid (since 2000 in US) and the recently approved tedizolid, which should be more effective with *cfr*-positive (linezolid-resistant) strains (Barber, 2016).

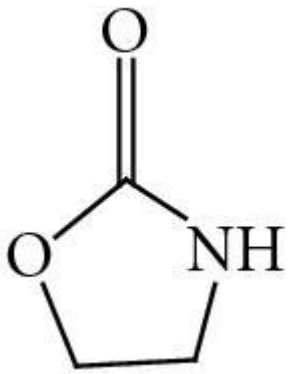


Figure 3: General structure of Oxazolidinones (adapted from Pandit, 2012).

1.4 Mechanisms of antibiotics resistance

The use of antibiotics allowed an effective fight against bacterial infections, but the genetic flexibility of these microorganisms promoted the development of surviving strategies. All bacteria can acquire or develop antibiotic resistance, as a result of chromosomal mutations or genetic material exchange, acquiring and transferring genes that promote antibiotic resistance (Figure 4).

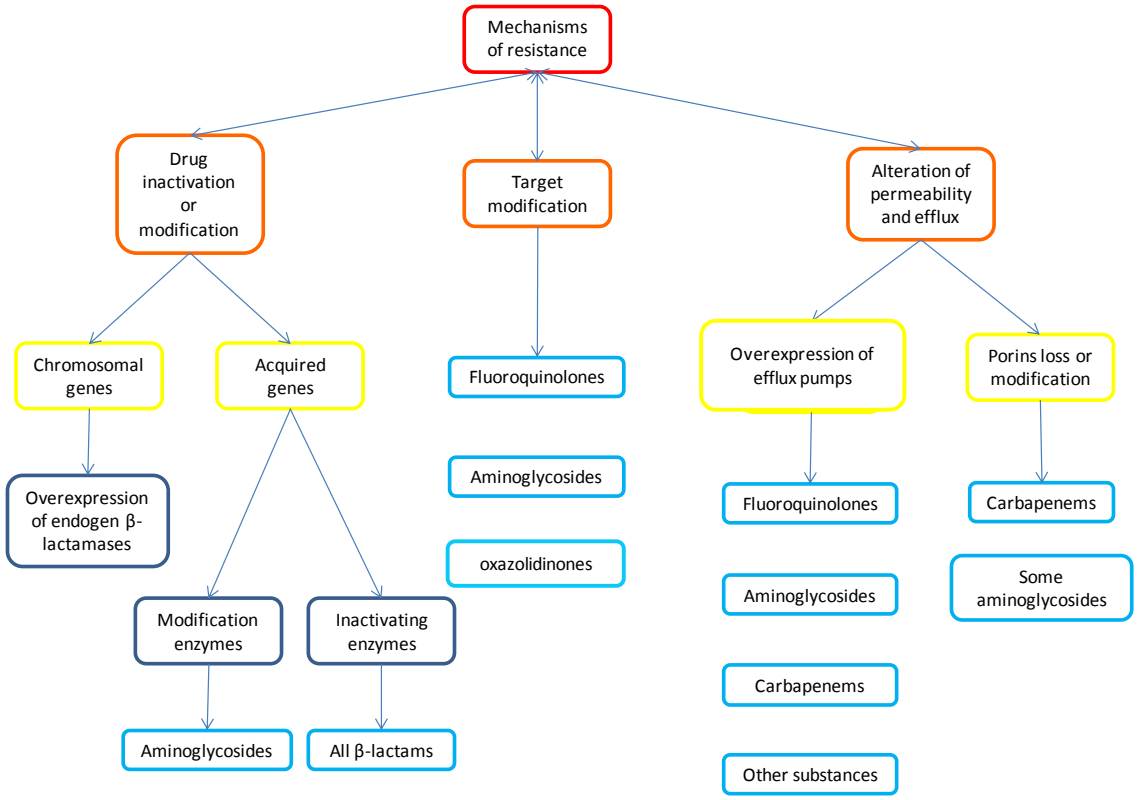


Figure 4. Main mechanisms of antibiotic resistance.

Resistance to an antimicrobial agent can be due to the following mechanisms (Poole, 2011):

- *Altered permeability of membrane*. It is one of the most diffused mechanisms of resistance. The external membrane of Gram-negative bacteria blocks the entrance of big polar molecules inside the cell; on the contrary, little polar molecules, as those of many antibiotics, can penetrate inside the cell through channels called porins. A mutation or porin loss, can slow down or block drug's entrance inside the cell, reducing drug's concentration at the site of action. If the target is intracellular and the drug requires an active transport through the membrane, the mutations or environmental conditions that

block this mechanism of transport can cause resistance. Carbapenems resistance in *P. aeruginosa* is commonly an example of this type of mechanism (when not derived by metallo-β-lactamases).

Active efflux systems are involved in the resistance to many antimicrobial agents for example β-lactams, fluoroquinolones and aminoglycosides. The basal expression of these systems is sufficient itself to assure a little level of protection from antimicrobial drugs, whereas their over-expression, generally due to mutations in regulatory genes, is cause of higher levels of resistance (Davin-Regli, 2008).

- *Alteration of target molecules*. In this case, the resistance is due to a reduced or absent bond between drug and target or to a substitution of the natural target with a new target that doesn't bind to the drug. This mechanism can be caused by mutations of the natural target (mutations of DNA topoisomerase II for fluoroquinolones), modifications of the target (protection of the binding site of the ribosome for macrolides and tetracyclins), and substitution of the natural target with an alternative variant (presence of penicillin-binding-proteins modified for the resistance to meticillin in *S. aureus*) (Pinho, 2001).
- *Modifications of the drug through enzymatic reaction*. The base of this mechanism is the synthesis of enzymes able to inactivate antibiotics. This mechanism is characteristic of the resistance to β-lactam and aminoglycoside antibiotics and was described immediately after the introduction in clinical practice of penicillin, following the isolation of strains of resistant *S. aureus* (Lowy, 2003). β-lactamases are an example of enzymes able to hydrolyze β-lactam antibiotics.

1.5 Mechanisms of resistance to β-lactam antibiotics

Antibiotic resistance is the development of escaping strategies from drug's action made by bacteria. This development is particularly important for β-lactam antibiotics, and their resistance can be the consequence of different mechanisms (Figure 5).

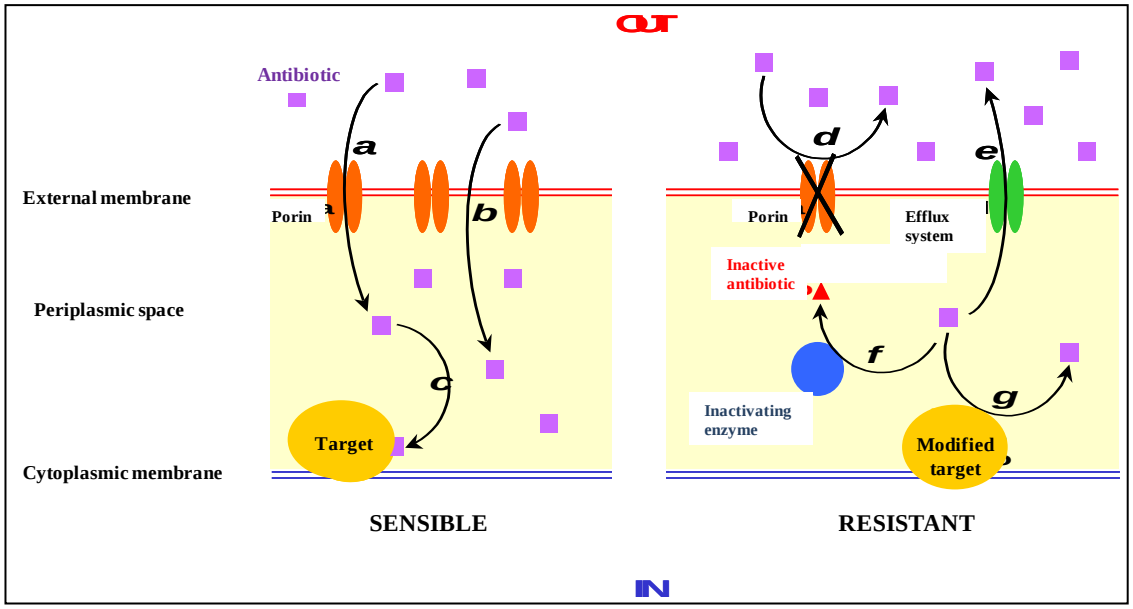


Figure 5. Schematic representation of the bacterial cell, here a Gram-negative, and illustration of the different mechanisms of resistance. The antibiotic can enter the periplasmic space through specific porins (a) or through passive diffusion (b) and inactivate its target (c). Resistance can result also from the modification of the external membrane's permeability through a decrease in the number of porins (d); the production of an efflux system (e), the production of inactivating enzymes (f) or target modification (g).

1.5.1 Target alteration of β-lactams

The target, in this case PBPs, can be altered both on a quality and quantity level in order to counteract antibiotic's action. The simpler mechanism consists in an overproduction of the PBPs, in order to maintain the functionality at sufficient levels in spite of the antibiotic presence. A second mechanism consists in the qualitative modification of the target itself, changed to have lower affinity for the antibiotic. This can be obtained through single or multiples point mutations or genetic material exchange (Ba, 2014).

1.5.2 Alteration of membrane permeability and efflux system over-expression

In Gram-negative bacteria, membrane permeability has a key role on the antibiotic susceptibility level because it offers an additional barrier that the antibiotic must cross to reach the periplasmic space, where its target is localized. The permeability alterations can result in a remarkable reduction of the functional porins due to a reduction of their synthesis or the expression of altered porins, or the over-expression of the efflux system (Poole, 2011). The modification of the bacterial membrane's permeability through a lower expression of porins (i.e. OprD porin down-regulation, a protein involved in β-lactams uptake), or an increase of the efflux pumps expression level was described as a

phenomenon frequently associated with other mechanisms of resistance, among which the production of hydrolytic enzymes or the modification of the drug's target (Poole, 2011). In clinical isolates, the combined action of those mechanisms during an infection can involve a remarkable reduction of sensibility to the antibiotic therapy, and further promote the persistence or development of other resistance mechanisms, with the consequent evolution of a Multi Drug Resistant (MDR) phenotype. For example, the contemporary presence of mutations in regulatory genes of the efflux systems and other resistance mechanisms was frequently described in isolates of *P. aeruginosa* with MDR phenotype (Rossolini, 2005; Davin-Regli, 2008). In *P. aeruginosa* were described four main efflux systems: MexA-MexB-OprM, MexC-MexD-OprJ, MexE-MexF-OprN e MexX-MexY-OprM. They are responsible for the extrusion of all antibiotic classes with the exception of polimixins (Lambert, 2002; Poole, 2004). The genes for those systems are generally not expressed at high levels (Lambert, 2002); however, mutations in regulatory genes, as mexR, which controls the expression of the system MexA-B, are responsible for the over-expression of these efflux systems with a consequent increase of the resistance; the level of resistance is generally variable and depends not only from the gene expression level, but also from the antibiotic and from the type of mutation. In particular, the over-expression of the system MexA-MexB-OprM cause resistance to fluoroquinolones, some disinfectants and all anti-pseudomonas β-lactams exept imipenem; indeed, the antibiotic meropenem is a good substrate for the efflux system, for its chemical structure that is characterized by a hydrophobic lateral chain, whereas the antibiotic imipenem seems to be not an ideal substrate (Poole, 2004; Masuda, 2000; Quale, 2006). In *Enterobacteriaceae* combined outern membrane protein deficiency and inactivating enzymes increases resistance to almost all β-lactam antibiotics including carbapenems (Yang, 2009).

1.5.3 Production of inactivating enzymes

The production of inactivating enzymes able to transform the antibiotic in a biologically inactive compound is one of the most effective resistance mechanism developed by bacteria. The inactivation or modification of antibiotics is due to the presence of enzymes produced by the bacterium itself, present inside the chromosome or acquired through horizontal genetic transfer. This resistance strategy is very important from the clinical point of view as regards β-lactam and aminoglycoside antibiotics. B-lactamases are the most common mechanism of antibiotic resistance of this type.

1.6 β-lactamases

β-lactamases are widely diffused enzymes in the microbial world, mainly among Gram-negative bacteria. In those bacteria they are localized at the periplasmic level, whereas in Gram-positives they are secreted outside the membrane. β-lactamases are able to hydrolyze the amidic bond of β-lactamic ring with the production of an inactive derivative (Frère, 1995). All *P. aeruginosa* and many enterobacteria have an intrinsic chromosomal inducible β-lactamase, AmpC, which is sometimes overexpressed, and confers resistance to ampicillin and most cephems (Poole, 2011).

1.6.1 β-lactamases classification

The first penicillinase was discovered in 1940 (Abraham, 1940), since then, after the introduction of penicillin in clinical use, β-lactamases were subject of several studies. The current identification, which includes more than 1500 enzymes, very different from each other, has made the necessity of a classification. A first large division of β-lactamases was made on a molecular structure basis and their hydrolysis mechanism: they are distinguished in serine active site β-lactamases and metallo-β-lactamases (Jacoby, 2006; Bush, 2010; www.lahey.org/studies).

The hydrolysis of the β-lactam ring occurs with different mechanisms depending on their type, if they are first or second group β-lactamases: (a) formation of a covalent intermediate enzyme-substrate at the serine level, which is in an active site, reconstitution of the enzyme and releasing of a product with an hydrolyzed β-lactam nucleus; (b) intervention of a zinc ion as an enzymatic cofactor in the hydrolysis reaction (Sabath, 1966).

From a structural point of view, β-lactamases were divided in four molecular classes on the basis of their similarities at the level of their amino acid sequence: A, B, C e D (Ambler, 1980). β-lactamases of A, C and D molecular class are characterized by a serine active site (Philippon, 1998) and β-lactamases of class B are placed inside the family of metallo-β-lactamases (Philippon, 1998).

Class A β-lactamases

Enzymes belonging to subgroup 2b β-lactamases are many allelic variants of TEM and SHV, which hydrolyze penicillins and early cephalosporins, such as cephaloridine and

cephalothin, and are strongly inhibited by clavulanic acid and tazobactam. They were identified since the 1970s (G. Jacoby and K. Bush, http://www.lahey.org/Studies/). They are often plasmid mediated and very common among *Enterobacteriaceae* (Bush, 2010).

Subgroup 2be comprises the extended spectrum β-lactamases (ESBLs), which are characterized by activity against penicillins and cephalosporins (as subgroup 2b β-lactamases) and in addition hydrolyze one or more oxyimino-β-lactams, such as cefotaxime, ceftazidime, and aztreonam, (usually >10% than benzylpenicillin). The first and largest subset of subgroup 2be was derived by amino acid substitutions in TEM-1, TEM-2, and SHV-1 that broadened their substrate spectrum, but with lower activity for benzylpenicillin and cephaloridine. CTX-M enzymes, actually increasingly found in *Enterobacteriaceae,* belong in ESBL and derive from *Kluyvera* spp. where they are chromosomally encoded. Most CTX-M enzymes hydrolyze cefotaxime more than ceftazidime, and many are active also on cefepime. CTX-M enzymes are inhibited by tazobactam at least an order of magnitude better than by clavulanic acid. Other less common ESBLs unrelated to TEM, SHV, or CTX-M, are BEL-1, BES-1, SFO-1, TLA-1, TLA-2, and members of the PER and VEB enzyme families. Characteristically, subgroup 2be β-lactamases remain sensitive to inhibition by clavulanic acid, and their detection by clinical laboratories is made up with combinatory disks of third and fourth generation cephalosporins with clavulanic acid.

Subgroup 2br enzymes are broad-spectrum β-lactamases that have acquired resistance to clavulanic acid (IC50 $\geq$ 1 μM) and related inhibitors, while retaining a subgroup 2b spectrum of activity. Currently 36 of the 135 functionally characterized TEM enzymes have this property and include enzymes such as TEM-30 and TEM-31 (IRT-2 and IRT-1, respectively), as well as few of the corresponding functionally characterized SHV enzymes (e.g., SHV-10). CTX-M β-lactamase with this characteristic was demonstrated by in vitro mutagenesis and is emerging also in clinical isolates (Ripoll, 2011; Ripoll, 2014; http://www.lahey.org/Studies/).

Subgroup 2ber includes TEM enzymes that combine an extended spectrum with relative resistance to clavulanic acid inhibition. Although all have clavulanic acid IC50s greater than that of TEM-1 (0.08 μM), for some 2ber enzymes the increase in clavulanic acid

resistance is modest. They have also been termed CMT (complex mutant TEM) β-lactamases and include TEM-50 (CMT-1) (Bush, 2010).

Class A carbapenemases include the IMI/NMC, SME, KPC and GES enzymes that confer resistance to carbapenems at various levels, from reduced susceptibility to full resistance. SME, NMC and IMI-1 enzymes are usually chromosomally encoded, whereas KPC, GES and IMI-2 enzymes are usually plasmid-encoded. SME enzymes are usually restricted to *Serratia marcescens*, whereas IMI and NMC enzymes are sporadically detected in *Enterobacter cloacae* and *E. coli* (Rojo-Bezares, 2012). KPC enzymes are found mainly on transferable plasmids, and are highly prevalent, in *K. pneumoniae*, but have also been detected in other *Enterobacteriaceae*, *P. aeruginosa*, and *A. baumannii* (Robledo, 2010). The genes for GES enzymes are found in integrons on transferable plasmids in *P. aeruginosa* and *K. pneumoniae* (Diene, 2014). Recently, two novel class A carbapenemases, FRI-1 in an *Enterobacter cloacae* in France and BKC-1 in a *K. pneumoniae* in Brasil were described (Dortet, 2015; Nicoletti, 2015). The most diffused class A carbapenemase is bla_{KPC}, which has undergone a global dissemination (including Italy) strictly related to high success clones such as CC258, (Tangden, 2015; Giani, 2013).

Class B β-lactamases (Metallo-β-lactamases)

The first metallo-dependent β-lactamase, produced by the environmental bacterium *Bacillus cereus*, was discovered in 1966 (Sabath, 1966). It remained for a long period of time only a biochemical curiosity of no clinical relevance. The discovery in 80's years of clinical isolates of *Stenotrophomonas maltophilia* resistant to carbapenems and producers of metallo-β-lactamases was worrisome. Nevertheless, an important turning point, in early 90's years, was the discovery and the next diffusion of metallo-β-lactamases-coding genes in clinical isolates of *P. aeruginosa* and *Enterobacteriaceae*. The discovery of the potential dissemination of these important resistance determinants in bacterial isolates of remarkable clinical interest, is with no doubts an important issue in the field of microbiology (Bush, 1999; Livermore 2000).

Metallo β-lactamases are able to hydrolyze with high efficiency carbapenems and third and fourth generation cephalosporins, recent and effective antibiotics, whereas are inactive on monobactams (Bush, 1998, 1999). Metallo β-lactamases require, for their function, the presence in their catalytic site of one or two metallic cofactors, one of

them is zinc (Matagne, 1999; Page, 2008). For this reason, metallic ions are inhibited by the presence of chelating agents, as for example EDTA (removes the metallic cofactor), but are not inhibited by the serine β-lactamases inhibitors, as clavulanic acid, sulbactam and tazobactam, for structural and functional reasons (absence of the active serine) (Bush, 1998; Rasmussen, 1997; Payne, 1993).

The presence of metallo-β-lactamases was found in several bacterial species, mainly in Gram-negative microorganisms, both of environmental and clinical origin. Generally, in environmental bacteria the metallo-β-lactamases-coding genes are resident, localized in the chromosome, where the expression of those enzymes can be constitutive or inducible. In most clinical relevant species, instead, the metallo-β-lactamases coding genes were acquired through mobile genetic elements (as for example plasmids, transposons and integrons).

The genes encoding for the different metallo-β-lactamases present a noticeable heterogeneity of sequence, which corresponds to more or less 23% of sequence identity (Queenan 2007, Cornaglia 2011); metallo-β-lactamases of clinical interest actually known are the types VIM, IMP, GIM, SIM, SPM, KHM, AIM, DIM, NDM, SMB, TMB and FIM. For some of the genes that code for those enzymes are known different allelic variants, which generally show a sequence homology of 85% or more (Salabi, 2010; Cornaglia, 2011; Pollini, 2013; Sechiguki, 2008).

Among the different metallo-β-lactamases, *bla*$_{\text{VIM}}$ and *bla*$_{\text{IMP}}$ genes are the most diffused worldwide and in Italy. The *bla*$_{\text{GIM}}$ (*German imipenemase*) and *bla*$_{\text{SIM}}$ (*Seoul imipenemase*) genes were found only in their country of origin or in neighbouring countries (Cornaglia, 2011; Wendel, 2015; Sun 2015). SPM enzyme (*Sao Paulo Metallo β-lactamases*) was described for the first time in Brasil, but recently was found also in Europe (El Salabi, 2012). The *bla*$_{\text{AIM-1}}$ (*Australian imipenemase*) and *bla*$_{\text{DIM-1}}$ (*Dutch imipenemase*) genes were described exclusively in Australia and in Germany in *P. aeruginosa* (Poirel, 2010). Moreover, *bla*$_{\text{KHM-1}}$ gene was identified only in Japan in *Citrobacter freundii* isolates (Sekiguchi, 2008), whereas *bla*$_{\text{NDM}}$ (*New Delhi Metallo β-lactamase*) gene was identified for the first time in India, but was recently found worldwide (Yong, 2009, Cornaglia, 2011, Dortet, 2014). Finally, the *bla*$_{\text{SMB}}$ (*Serratia metallo-β-lactamase*) was found only in Japan (Wachino, 2011), and the TMB (*Tripoli metallo-β-lactamase)* in Libya and Japan (El Salabi, 2012; Kayama, 2014).

IMP and VIM type metallo-β-lactamases represent two of the most important acquired resistance determinants to β-lactamic drugs among *Enterobacteriaceae*, in *Pseudomonas* spp. and in other classes of non fermenting Gram-negative bacteria.

As for other metallo-β-lactamases, IMP and VIM type exhibit a very wide profile of hydrolysis, including cephalosporins of III and IV generation and carbapenems, that generally are not sensible to the action of serine β-lactamases; moreover they don't show sensibility to the common inhibitors of β-lactamases, as tazobactam and clavulanic acid, and in clinical practice other inhibitors for this type of enzymes are not available.

IMP type enzymes were the first to be identified in clinical isolates of *P. aeruginosa*, *Enterobacteriaceae* and other non fermenting Gram-negatives. IMP-1 enzyme was described for the first time in Japan (Watanabe, 1991), but later IMP type enzymes were detected in other eastern countries (Ho, 2002; Zhao, 2011) and also in Europe (Queenan 2007 Hrabak, 2011), Nord America (Borgianni, 2011, Gibb, 2002) and Sud America (Gales, 2003; Santella, 2010), reaching a worldwide distribution (Pfeifer 2010, Cornaglia, 2011).

The molecular characterization of IMP-producing clinical isolates lead to the description of 56 IMP variants diffused worldwide in isolates of Gram-negative non-fermenters and *Enterobacteriaceae* (www.lahey.org/studies).

VIM-type (Verona Integron-encoded Metallo-β-lactamase) enzymes are certainly one of the most heterogeneous subfamilies of the subclass B1. To date 47 bla_{VIM} variants have been recognized by the Lahey Clinic database. Those enzymes were isolated in several bacterial strains of clinical relevance as *Pseudomonas* spp., *Enterobacteriaceae*, *Acinetobacter* spp. and *Achromobacter xylosoxydans*. They were described for the first time in Italy but in the following years they were found throughout all Europe and in many countries worldwide (Livermore, 2012). Nowadays their diffusion is a problem worldwide, in particular in *P. aeruginosa*, whereas actually their diffusion in *Enterobacteriaceae* is lower, except for some cases such as Greece, where the prevalence of isolates that carry VIM-type metallo-β-lactamases is very high; in this country approximately 38% of the clinical isolates of *K. pneumoniae* from blood culture were VIM-1 enzyme producers in 2008 (Livermore, 2009; Vatopoulos, 2008). The only national surveillance program conducted so far on *P. aeruginosa* isolates during the first

half of last decade, reported limited percentages of prevalence of those determinants (1,3%) (Rossolini, 2008). Subsequent studies, conducted at a local level in single epidemiological realities, accounted for an increase in the frequency of those determinants at an alarming rate (Rossolini, 2011).

Class C β-lactamases

Group 1 β-lactamases, molecular class C, are enzymes that are more active on cephalosporins than benzylpenicillins and are encoded on the chromosomes of many *Enterobacteriaceae* and a few other organisms. They have high affinity on aztreonam and are usually resistant to inhibition by clavulanic acid and active on cephamycins, such as cefoxitin. In many organisms, including *Citrobacter freundii, Enterobacter cloacae, Serratia marcescens,* and *Pseudomonas aeruginosa,* AmpC expression is low but inducible on exposure to certain β-lactams, such as amoxicillin, ampicillin, imipenem, and clavulanic acid. In other organisms, the components of induction system are not present (*Acinetobacter baumannii* and *Escherichia coli*). When over-expressed, in a host with reduced β-lactam accumulation (porin loss or mutation), they can provide resistance to carbapenems (usually ertapenem). Plasmid-mediated group 1 enzymes in the CMY, ACT, DHA, FOX, MIR, and other families (known since 1989) are not as frequent as plasmid-mediated ESBL (Jacoby, 2009, Bush, 2010). Nonetheless, in many European countries, included Italy, $bla_{\text{CMY-2-like}}$ genes are higly diffused in *P. mirabilis* (D'Andrea, 2011a).

Class D β-lactamases

Class D β-lactamases, OXA enzymes, were initially distinguished by their ability to hydrolyze cloxacillin or oxacillin at a rate of >50% that for benzylpenicillin and but most members of the OXA family, are currently identified only on the basis of conserved amino acid motifs. Many β-lactamases in this group are inhibited by NaCl and are typically unresponsive to inhibition by clavulanic acid (Bush, 2010). OXA-related enzymes now comprise the second largest family of β-lactamases with around 500 different amino acidic variants (http://lahey.org/studies).

The subgroup 2de is composed by OXA enzymes with an extended spectrum that includes oxyimino-β-lactams but not carbapenems. The majority of 2de enzymes are derived from OXA-10 (1 to 9 amino acid substitutions) or from OXA-48.

Subgroup 2df β-lactamases are OXA enzymes with carbapenem-hydrolyzing activities. They appear frequently in *A. baumannii* and are often chromosomally located, although plasmid-borne OXA-23, and OXA-48 enzymes have been identified in the *Enterobacteriaceae*. OXA-48 producing *Enterobacteriaceae* are actually the most common carbapenemases found in many countries (Glasner, 2013). Class D carbapenemases have usually weak hydrolytic activity for carbapenems, demonstrated by *k*cat values for imipenem and meropenem that are generally $\leq 1\ s^{-1}$ (except OXA-48-like enzymes), with imipenem hydrolyzed faster and more efficiently than meropenem, and *E. coli* trasformants or transconjugants are usually susceptible to carbapenems. These rates compare to much higher *k*cat values for benzylpenicillin or oxacillin (Bush, 2010).

1.6.2 Epidemiology of carbapenemases

In recent years, a rapid increase was detected in the prevalence of carbapenemase-producing *Enterobacteriaceae* in Europe and in Italy. In particular, EARS-NET data show that, in our country from 2009 to 2014, the prevalence of carbapenem resistant *K. pneumoniae* increased from 1.3% to 32.9%. (Figure 6).

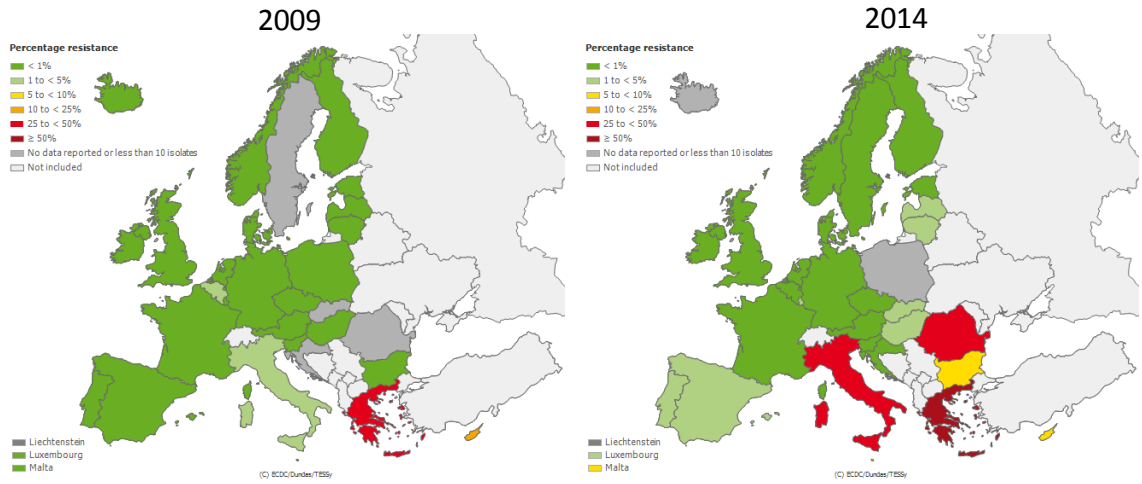


Figure 6. Proportion of Carbapenems Resistant (R) *Klebsiella pneumoniae* Isolates in Participating Countries in 2009 and 2014.
(http://ecdc.europa.eu/en/healthtopics/antimicrobial_resistance/database/Pages/map_reports.aspx#sthash.jPfikoRY.dpuf)

Recent epidemiological studies show en endemic presence of KPC- producing K. pneumoniae in Italy (89.5% of carbapenemase producers), with only a little percentage

of other carbapenemases (VIM 9.2%, and OXA-48 1.3%) (Giani, 2013). Indeed, together with USA and Greece, Italy presents the highest percentage of KPC-producing *K. pneumoniae* world-wide (Figure 7).

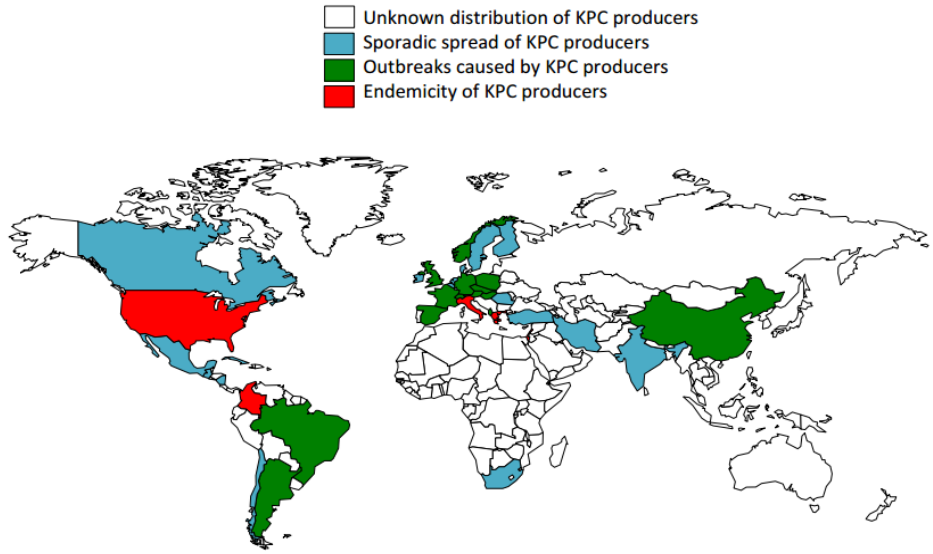


Figure 7. Adapted from Nordmann, 2014. Global distribution of KPC-producers.

1.7 Mechanisms of resistance to oxazolidinones

Oxazolidinones resistance is quite rare among *Sthapylococcus* spp. and enterococci. However, since the introduction of linezolid in 2000, resistance to oxazolidines has emerged. The most common mechanism of resistance described was mutation of the 23S rRNA, and was associated with prolonged treatment with linezolid. But transferable resistance to linezolid was demonstrated by Gram-positive isolates which carried the multi-resistance gene *cfr* (found also in *E. coli*) (Shen, 2013). Cfr enzyme confers resistance also to phenicols, lincosamides, pleuromutilins and streptogramin A. Since the approval of tedizolid by FDA (2014), which is not affected by *cfr* gene, the clinical problem of *cfr*-carrying isolated seemed to be reduced. Unfortunately, in 2015 the gene *optrA* was described in enterococci which reduces the susceptibility to phenicols and oxazolidinones included tedizolid (Wang, 2015). Moreover a new variant of *cfr* gene , named *cfrB* was described in US in *Enterococcus faecium* and *Peptoclostridium difficile* (Deshpande, 2015). Recently, two isolates of *Enterococcus faecium* carrying both *optrA* and *cfr* genes were described in Italy (Brenciani, 2015).

1.8 Mobile genetic elements

All bacteria can develop antibiotic resistance either through the acquisition of resistance genes on mobile genetic elements, or through mutational processes that alter the expression and/or function of chromosomally encoded mechanisms. Mobile genetic elements are segments of DNA that encode enzymes and other proteins that mediate the movement of DNA within genomes or between bacterial cells determining intracellular or intercellular mobility, respectively (Frost, 2005). Both strategies for developing drug resistance can severely limit the therapeutic options for treatment of serious infections (Lister, 2009). Horizontal gene transfer, a common feature in both Gram-negative and Gram-positive bacteria, confers new functions by the acquisition of plasmids, transposons or pathogenicity islands.

1.8.1 Transposons

The simplest of the mobile genetic elements is insertion sequence (IS). These elements just consist of the gene required for element mobility (a transposase) and repetitive sequences, often arranged as inverted repeat at the ends of the element (He, 2015). IS elements can be as short as 1Kb. When these elements contain accessory genes (often antibiotic resistance genes) not involved in element translocation, they are called transposons (Van Hoeck, 2011). Transposons are mobile genetic elements, which are capable of moving from one carrier replicon to another. Transposons usually carry genes for transposition function, or at least a transposase gene (*tnp*A) (Dale, 2004; Grindley, 2002; Mahillon, 1998). Depending on their transposition mechanisms, transposons are subdivided in 4 major classes, but class I and II are the most diffused. Transposons of class I are composite transposons: they consist of 2 copies of an Insertion Sequence (IS) (Figure 8) on either side of a set of resistance genes (Figure 8 A) (Dale, 2004). Their flanking IS might be in inverted orientation or as direct repeats.
Transposons of class II are more complex and are referred to as the Tn*3*-family (Dale, 2004; Grindley, 2002) (Figure 8 B). They usually encode a large transposase (950 to 1020 aa), and transpose a replicative mechanism forming an intermediate called cointegrate (Grindley, 2002). A target DNA molecule containing a Tn*3*-family transposon is immune to further insertions of the same transposon.

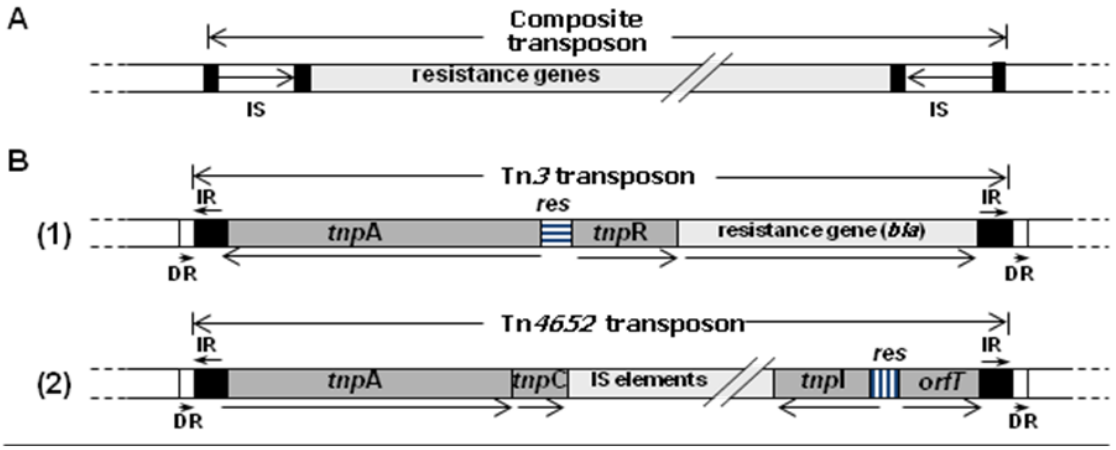


Figure 8. Basic genetic organization of class I and II transposons. (A) Class I transposon or composite transposon (adapted from Dale, 2004). IS are represented by an arrow in a box surrounded by two black boxes. black boxes are IR. (B) Class II transposons or Tn*3*-family transposons (Grindley, 2002). Description of the features is based on (A). Cross-hatched boxes are *res* sites, unfilled boxes are DR. (1) Resolvase-encoding transposon of the Tn*3* subfamily. (2) Integrase-encoding transposons of the Tn*4652* subfamily.

The most widely distributed transposon is the composite transposon Tn21, which is a subgroup of the Tn3 mercury-resistance encoding (mer) transposons (Ng, 2009). Tn21 carries the genes involved in its own transposition (tnp) and a mercury resistance (mer) operon. A subtype of Tn21 is Tn3-like, which is characterized by flanking inverted repeats of about 38 bp and, has two genes: tnpA and tnpR, which encode a transposase and resolvase. The tnpR gene and the res site (where acts the resolvase) are located upstream of tnpA. Trasposition of the transposon Tn21 is carried by the trasposase tnpA. Replicative transposition involves specific recognition and binding to the terminal inverted repeat (IR) elements of Tn21 by tnpA, which then mediates the joining of the donor and recipient replicons. An adjacent putative gene of undefined function, tnpM (351bp) was originally defined as starting at the junction of the tnp region and the integron IRi (left inverted repeat) and terminating with resI (Liebert, 1999). The Tn21 family is widely distributed in both clinical and environmental isolates of Gram-negative bacteria. The transposons mentioned above are not capable of conjugal transfer to other bacteria and they need to be contained within a conjugative element to be mobilized (Van Hoeck, 2011). Most of the characterized examples of the Tn21 family have been found on large conjugative plasmids (Liebert, 1999).

1.8.2 Integrons

Transposon Tn21 and many of its closest relatives carry within them a potentially independent DNA element called integron (Liebert, 1999). Integrons are genetic units, frequently associated with resistance determinants, which include components of a rec-dependent site-specific recombination, capable of catching and mobilizing genes contained in mobile elements, called gene cassettes; moreover, the expression of the cassettes inserted in the integron is made by a common promoter. For this reason they can be considered natural cloning and expression systems (Mazel, 2006, Van Hoeck, 2011). Integrons are not able to move by their selves, but can be associated to transposons and/or plasmids, that promote their transmission intra and inter species and mediate their worldwide diffusion (Mazel, 2006). Integrons are largely diffused in antibiotic resistant Gram-negative bacteria, circulating both in nosocomial and community environment and they are a powerful reservoir of chemoresistance determinants. Furthermore, the possibility of mobilization and transfer of resistance determinants is an important instrument for the creation of new gene combinations, able to bring multi-resistance to antibiotics and disinfectants that could be positively selected in bacterial strains that become immune to the actual pharmacological therapies.

The type of gene coding for integrase, defines the different classes of integrons. Class 1 integrons are the most studied and involved in chemoresistance diffusion in clinical interest strains; moreover they are commonly highly mobilized, being found in plasmids, and conjugation is a major mechanism of resistance genes diffusion between cells, and across genera. However, class 1 resistance integrons can also be found in chromosomes, frequently in Genomic Islands of pathogens like *P. aeruginosa* (Martinez, 2012). Class 1 integrons present two constant regions at the extremities 5' and 3', respectively called 5'CS and 3'CS (Conserved Segment). Each integron has at 5'CS: i) an *intI1*gene, that codes for integrase enzyme, a site-specific recombinase belonging to tyrosine-recombinase family, responsible for the integration and excision of the gene cassettes; ii) an *attI* site, next to the enzyme, that represents the target site of the integrase and is the receptor site for the gene cassettes; iii) a promoter, *Pc*, localized inside *intI1* gene and is necessary for the expression of the cassettes inserted in the variable region (Hall, 1995; Hall, 1994, Recchia, 1997) (Figure 9). 5'CS is a highly conserved region and is essential for the definition of the integron, whereas 3'CS sometimes can be absent. 3'CS of class I integrons usually includes a *sul1* gene that

confers resistance to sulfamidics, a *qacEΔ1* gene that confers a weak resistance to quaternary ammonium compounds (ex. some disinfectants), and one open reading frame (ORF) of unknown function (*orf5*) (Hall, 1994; Paulsen, 1993); in some class 1 integrons the presence of additional ORFs was described, and they can present deletions or can be interrupted by the presence of insertion sequences (IS). Between 5'CS and 3'CS is present a variable region, called *cassette array*, that can have one or more cassettes inserted; those cassettes include each a gene and a specific recombination site, known as *attC* or *59*-base element (59-be), that can be recognized by the integrase and used for their insertion and removal (Figure 9). The dimension of this region is variable and depends on the number of gene cassettes inserted inside it.

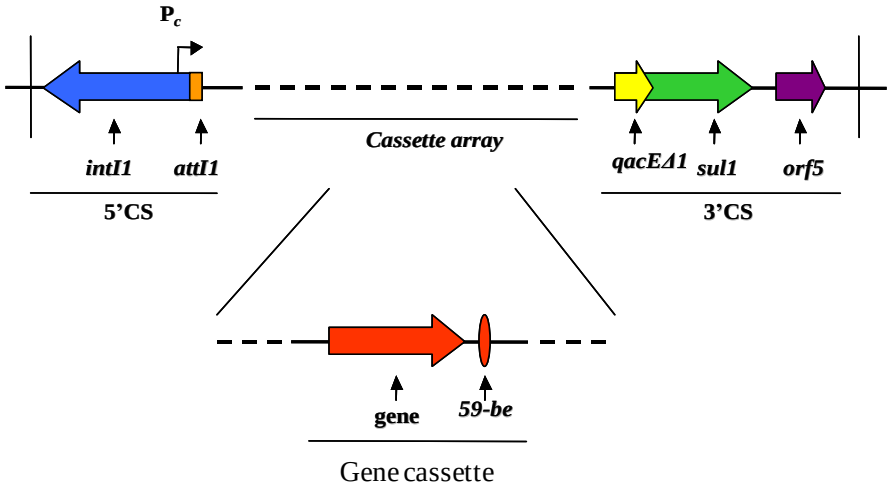


Figure 9. General structure of class 1 integrons. 5'CS extremity is on the left and 3'CS is on the right; on the center the region called cassette array is showed (dotted line) and, in particular, the general structure of a gene cassette, made by the gene and by *59-be*, on the right, pointed by arrows. 5'CS is made by intI1 gene (in the opposite direction of the cassette array), the attI1 site on the right and the P_c promoter (pointed over the gene). 3'CS is made by *qacEΔ1* and *sulI* genes and by *orf5*, placed in the same direction of the gene cassettes and in the opposite direction of *intI1*.

The integron is usually flanked by a *Tn*402-like, which is responsible for its integration in *res* sites found in plasmids or chromosomes (Sajjad, 2011). At least two important steps lead to the generation of the basic class 1 integron backbone: i) the insertion of the integron into a Tn402-like transposon (Figure 10); ii) the generation of the 3′-conserved segment (3′-CS), involving acquisition of *sulI* and loss of part of the *tni* module (Sajjad, 2011).

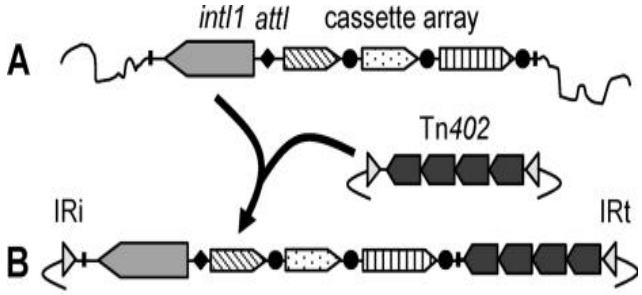


Figure 10. Adapted from Sajjad, 2011. Model for the origin of Tn**402** class 1 integrons. (A and B) A class 1 integron resident in the chromosome of an environmental betaproteobacterium is captured by a *Tn*402 transposon (A) to generate a hybrid structure combining the ability of integrons to sample the environmental gene cassette pool with the mobility of the Tn**402** transposon (B).

1.8.3 Plasmids

Bacteria can bring extrachromosomal genetic elements, called plasmids, defined as a double helix DNA molecule capable of self replication. By definition, plasmids don't bring genes that are essential for the host cell growth. Plasmids have different size, but the majority goes from 1.000 to more than 200.000 base pairs compared to 2 to 6 millions base pairs of the genome. Plasmids have systems that guarantee their self replication, others that regulate their copy number and ensure cell transmission during replication; some plasmid types are present with many copies inside the cell, whereas others can be maintained in a single copy. Furthermore, a single cell can contain different types of plasmids. Plasmids can promote their lateral transfer among very different bacteria, through conjugation process. Plasmid transmissibility through conjugation is controlled by a group of plasmidic genes. In some cases the plasmid can integrate in the host cell chromosome and, in case of wrong plasmid excision, be the mediator of the chromosome DNA transfer from one cell to another.

In 1971, Hedges and Datta proposed a classification on the base of plasmid stability during conjugation, a phenomenon called "plasmid incompatibility". Incompatibility is a manifestation of the correlation between plasmids that share the same replication control mechanisms. Thus, incompatibility is defined as the incapacity of two related plasmids of being steadily spread in the same cellular line; only the compatible plasmids can be found together in transconjugant cells (Carattoli, 2009).

Many plasmids code for additional systems based on toxin-antitoxin factors, able to kill daughter cells that don't inherit the plasmid during cellular division. Those systems promote plasmid maintenance inside the bacterial population, regardless of the selective

pressures, and without giving any apparent benefit to the host bacterium (Blower, 2012).

In any case, most plasmids give positive selective phenotypes through the presence of antibiotic resistance genes. Indeed, in addition to functions involved in replication and transfer plasmids commonly encode resistance to antibiotics. If a resistance gene is on a conjugative or mobilizable plasmid then it has the potential to transfer to new hosts. Sometimes plasmids cannot replicate in the new host or do not replicate well and in these circumstances the plasmid may be lost, but if there is a resistance gene inside a transposon this genetic element can translocate to the bacterial chromosome, and can eventually be maintained also in the absence of the plasmid. Therefore, a plasmid does not necessarily need to be maintained in a particular host in order to contribute to the spread of resistance (Van Hoeck, 2011). Most of the plasmid which replicate in *Enterobacteriacae* have been classified on the basis of their replicon gene (Carattoli, 2005, Johnson, 2012b). Recently Plasmid finder and Plasmid Multi Locus Sequence Typing web tools have been developed for a rapid identification and classification of the most common plasmids directly from whole-genome sequences (http://pubmlst.org/plasmid) (Carattoli, 2014).

1.9 Next Generation Sequencing

A complete turn-around in the field of genetics was the development of Next Generation Sequencing (NGS) techniques that provided a fast and cost-effective method to sequence large amount of DNA sequences within few hours. The first genome was sequenced in 1977 and was bacteriophage ϕX174 (5,386 base pairs), using a technology invented just few years earlier by Fred Sanger and Alan Coulson (Sanger, 1977; Maxam, 1977). Sanger-based sequencing technology has undergone huge improvements in early 1990s with capillary electrophoresis, which separates randomly terminated extension products, by using fluorescently labeled terminators (Kircher, 2010). Sanger sequencing technology, can make up to 384 sequences in parallel, between 600 and 1000-bp in length (Shendure, 2008) with relatively low error rate, long read length and high accuracy, but with a low throughput.

Next (second)-generation sequencing (NGS) technologies were instead able to produce an enormous throughput, in less time and at a substantially lower cost per base (Schuster, 2008). With NGS it was possible to sequence the whole genome of many related organisms (Pareek, 2011; Von Bubnoff, 2008), allowing large-scale comparative an evolutionary studies to be performed, but the analysis and the interpretation of the high volume of data generated are still a limit. The most popular and used NGS technologies and their main characteristics are listed in Table 1.

Next-generation sequencing technologies	
Pyrosequencing (Roche 454)	• Detects pyrophosphate release on addition of a complementary nucleotide to determine the template sequence
	• Lower throughput and subsequently higher sequencing cost per base
	• One of the earlier next-generation technologies, but now being phased out with Roche intending to cease production in 2016
SOLiD sequencing (Life Technologies)	• Sequencing by Oligonucleotide Ligation and Detection (SOLiD) uses a ligation-based approach
	• Less popular than Life Technologies' other platform, the Ion Torrent, and likely to be superseded by newer technologies
Ion semiconductor sequencing (Life Technologies Ion Torrent)	• Uses a sequencing-by-synthesis method, detecting changes in pH due to hydrogen ion release with synthesis of complementary DNA
	• Popular due to lower sequencer cost and speed of sequencing
	• Requires separate emulsion PCR library amplification prior to sequencing (slow and complicated), though automation can be performed using the separate Ion Chef system
	• Higher error rates, particularly homopolymers, than other platforms and poor coverage of extremely AT-rich or GC-rich regions
	• Ion Torrent Personal Genome Machine (PGM) and newer, higher throughput Ion Proton available
Illumina sequencing	• Uses a sequencing-by-synthesis method, detecting release of fluorescent labels from incorporated nucleotides to determine sequence
	• Current market leader with high sequence throughput, with low error rate and low sequencing cost per base
	• Limitations of short read sequences and a longer sequencing run time
	• Several platforms with moderate (MiSeq), moderate-high (NextSeq) and high (HiSeq) throughput
	• TruSeq long read technology recently introduced to produce synthetic reads of 10 kb in length (currently only HiSeq 2000/2500)
Single molecule real-time sequencing (Pacific Biosciences)	• Novel method – observes natural synthesis of unmodified DNA by DNA polymerase, with reads up to 40 kb in length, using nucleotides with fluorescent labels attached to the terminal phosphate (rather than the base)
	• Higher raw error rates, but errors are randomly distributed (vs. ends of reads or homopolymers), and overlapping reads can produce a consensus sequence with high accuracy
	• Has significantly improved *de novo* assembly and bacterial genome completion without needing traditional PCR-based gap closure
	• High setup cost and low throughput have limited implementation, though outsourcing options are available
Emerging technologies	
Nanopore sequencing (Oxford Nanopore)	• Probably the leader of the pack of benchtop sequencing technologies in development
	• Detects characteristic disruptions in a current applied across a protein channel or 'nanopore' as each nucleotide of a strand of DNA is passed through the nanopore
	• Method still being refined, but has the capability of generating long-sequence reads
	• Two portable/affordable benchtop sequencers available – the MinION (disposable USB stick), and the GridION (rack-mountable)

Table 1. Popular next generation sequencing technologies. Adapted from Kwong, 2015.

One of most used technology globally is Illumina sequencing with the highest output of their sequencing platforms and the lowest reagents cost, which with its Miseq instrument sequences up to 2x300 bp reads of DNA. In Illumina Genome Analyzer the starting DNA is converted in a special sequencing library after an enzymatic fragmentation step. The resulting DNA fragments are size selected and ligated to adaptors, which incorporate a sequence complementary to "anchor" oligonucleotides covalently linked to the surface of a flow cell. After annealing to the anchor oligonucleotides, the template DNA molecules are clonally amplified in a modified isothermal PCR reaction termed "bridge PCR" (Metzker, 2010). The bridge PCR step results in the generation of more than fifty million individual clusters containing over one thousand copies of clonally amplified DNA molecules on the surface of the flow cell. Afterwards, the clusters are denatured to provide a single-stranded template, and a sequencing primer oligonucleotide is hybridized to the strand. During each sequencing cycle, the clonally amplified clusters are exposed to DNA polymerase and a mixture of four nucleotides, each labeled with a unique fluorescent label (cyclic reversible terminators). During each sequencing cycle, a single labeled dNTP is added to the nucleic acid chain (Liu, 2012). The nucleotide label serves as a terminator for polymerization and, after each dNTP incorporation, the fluorescent dye is imaged to identify the base; then, the terminator moiety and fluorescent label are cleaved off and removed, and fresh nucleotides and polymerase are added to begin the next sequencing cycle.

The use of NGS technology is particularly important in microbiology due to the huge reduction of costs for clinical isolates characterization. The use of NGS replaces high cost and time consuming techniques like multi-locus sequence typing (MLST), species identification (for the less common species), pulsed-field gel electrophoresis (PFGE) and Real Time PCR. Moreover, NGS can be applied for rapid and cost-effective outbreak analysis with an improved public health response (Gilchrist, 2015).

2. AIMS OF THE PROJECT

Infections caused by multi-resistant bacteria are increasing globally. The high selective pressure (antibiotics) applied at both nosocomial and veterinary level has brought to the selection of multi-resistant bacteria and has favored the diffusion of mobile genetic elements carrying antimicrobial resistance genes. In order to limit the diffusion of these resistance traits there is an urgent need to develop new diagnostic tools capable of a fast and sensitive identification and characterization of multi-resistant bacteria.
During this thesis, the characterization of multi-resistant Gram-negative and Gram-positive pathogens was performed using molecular techniques, phenotypic tests and bioinformatics analysis; moreover, the clonal relationships of the most interesting isolates, together with the genetic context of the relevant resistance genes were analyzed.

In details, the aims of this thesis were:

i) Analysis of the epidemiology of carbapenemase producing *Enterobacteriaceae* and other relevant Gram-negative bacteria isolated from inpatients in Careggi University Hospital and from neighbouring Hospital wastewater;

ii) Characterization of the National epidemiology of carbapenemase, ESBL and AmpC producing isolates of *Enterobacteriaceae* isolated in October 2013;

iii) Characterization of a *cfr* positive, linezolid resistant MRSA strain of *S. aureus* from a cystic fibrosis patient.

3. RESULTS AND DISCUSSION

3.1 Carbapenemase producing *Enterobacteriaceae*

Related publications

1. Antonelli A, Di Palo D M, Galano A, Becciani S, Montagnani C, Pecile P, Galli L, Rossolini G M. 2015. Intestinal carriage of *Shewanella xiamenensis* simulating carriage of OXA-48–producing *Enterobacteriaceae*. *Diagn Microb and Infect Dis.* 82(**1**):1-3.

2. Antonelli A, D'Andrea M M, Vaggelli G, Docquier J-D, Rossolini G M. 2015. OXA-372, a novel carbapenem-hydrolysing class D β-lactamase from a *Citrobacter freundii* isolated from a hospital wastewater plant. *Journal of Antimicrobial Chemotherapy*. 70(**10**):2749-56.

3. Antonelli A, D'Andrea M M, Di Pilato V, Viaggi B, Torricelli F and Rossolini G M. 2015. Characterization of a novel putative Xer-dependent integrative mobile element carrying the $bla_{\text{NMC-A}}$ carbapenemase gene, inserted into the chromosome of members of the *Enterobacter cloacae* complex. *Antimicrob Agents and Chemother*. 59(**10**): 6620-6624.

3.1.1 Surveillance of carbapenemase producing *Enterobacteriaceae*

The worldwide dissemination of carbapenemase-producing *Enterobacteriaceae* (CPE), which usually show an extremely-drug-resistant phenotype, is worrisome (Rossolini, 2015). Unfortunately, the Italian setting is one of the worst in Europe, with an endemic prevalence of CPE, mostly represented by KPC-producing *K. pneumoniae* (Giani, 2013). Careggi University Hospital (Florence) is not an exception to this trend, with a presence since 2008 of CPE (Giani, 2009). In order to limit the diffusion of CPE at nosocomial level, increasing measures of infection control procedures should be applied. For this reason, a protocol for the rapid detection of CPE from surveillance rectal swabs was developed.

During 2015, all rectal swabs were collected using the FECALSWAB™ liquid phase system (Copan, Italy), and the liquid phase was used to inoculate selective chromogenic

medium (Carba Smart, BioMérieux, Marcy l'Etoile, France) for the growth of carbapenem resistant *Enterobacteriaceae*, and incubated at 37°C for 18-22 hours. All the colonies with different morphology were identified with Maldi-Tof (BioMérieux) and the *Enterobacteriaceae* isolated were screened with a homebrew Multiplex Real Time PCR for the detection of bla_{KPC}, bla_{VIM}, bla_{OXA-48}-like and bla_{NDM} genes, which are the most common carbapenemases detected in *Enterobacteriaceae* in Europe (Table 2).

Target	Primer name	Sequence (5'-3')[a]	Reference[b]	Positive control
$bla_{OXA-48-like}$ genes	OXA-48-like-rt-F	GTAGCAAAGGAATGGCAAGAAA	This study	*E. coli* ECBZ-1 (OXA-48), Giani, 2012b
	OXA-48-like-rt-R	GATGCGGGTAAAAATGCTTG	This study	
	OXA-48-like-rt-P	HEX-CTCTGGAATGAGAATAAGCAGCAAGG-BHQ-1	This study	
bla_{KPC} genes	kpc-hind-fwd	GATACCACGTTCCGTCTGG	Hindiyeh,2008	*K. pneumoniae* FIPP-1 (KPC-3), Giani, 2009
	kpc-hind-rev	GCAGGTTCCGGTTTTGTCTC	Hindiyeh, 2008	
	kpc-hind-tq	FAM-AGCGGCAGCAGTTTGTTGATTG-BHQ-1	Hindiyeh, 2008	
bla_{VIM} genes	VIM-rt-fwd	TGGTCTCATTGTCCGTGATG	This study	*K. pneumoniae* VA-416/02 (VIM-4), Luzzaro, 2004
	VIM-rt-rev	CATGAAAGTGCGTGGAGA	This study	
	VIM-rt-tq	ROX-AAGCAAATTGGACTTCCCGTAACGC-BHQ-2	This study	
bla_{NDM} genes	blaNDM1_F	CGCAACACAGCCTGACTTT	Ong JAC 2008	*E. coli* CVB-1 (NDM-1) D'Andrea, 2011b
	blaNDM1_R	TCGATCCCAACGGTGATATT	Ong JAC 2008	
	blaNDM1_P	CY5-CAACTTTGGCCCGCTCAAGGTATTT-BHQ-3	Ong JAC 2008	

[a] The amplification program consisted of 35 two-step cycles of 15 s at 95°Cand 60s at 60°C.

[b] Primer and probe sequences were from different references, while the Multiplex PCR fluorophore-quencher combination is original.

Table 2: Primers and probes used for carbapenemases Real Time PCR.

Simultaneously, all *Enterobacteriaceae* showing resistance to at least one carbapenem from blood culture were also investigated for carbapenemase production and compared to rectal swabs results. All the strains that tested negative for the homebrew Real Time PCR were tested with enzymatic activity assay in order to exclude eventual carbapenemase activity (Lauretti, 1999).

During 2015 a total of consecutive non replicate 387 isolates of *Enterobactericeae* from rectal swabs were screened for carbapenemase production (Table 3A). Most of them

were KPC producing *K. pneumoniae* (72.1%). The other mechanisms detected were bla_{VIM} (12.4%), bla_{OXA-48} (1.8%). Of the samples that tested negative for Real Time PCR an isolate had carbapenemase activity and resulted positive for bla_{IMI-2} (see results of section 3.1.5), while the others showed no enzymatic activity and they were probably grown on Carba Smart plates due to the presence of a class C or ESBL overexpressed and a possible porin loss. Surprisingly, two isolates of *Citrobacter fruendii* co-harbored bla_{KPC} and bla_{VIM} genes.

Of the 48 consecutive non replicate isolates of *Enterobacteriaceae* from blood culture, which showed reduced susceptibility to carbapenems, 41 were KPC-producing *K. pneumoniae* (85.4%), while only four isolates (8.3%) harbored bla_{VIM} gene, with no other carbapenemase detected (Table 3B).

Therefore, in rectal swabs there is a higher variability in the prevalence of the different carbapenemases and species of CPE, reflecting the putative higher potential of invasivity of KPC-producing *K. pneumoniae,* which has usually a clonal diffusion.

A

	Rectal swabs									
	K. pneumoniae	*E. coli*	*E. cloacae complex*	*C. freundii*	*C. koseri*	*C. farmeri*	*S. marcescens*	*E. aerogenes*	*K. oxytoca*	*Total*
KPC	279	13	1	3	-	-	-	-	-	296
VIM	14	11	11	3	1	2	-	-	6	48
OXA-48	4	1	2	-	-	-	-	-	-	7
IMI-2	-	-	1	-	-	-	-	-	-	1
Other	4	26	3	-	-	-	1	3	-	37
Total	301	51	18	4*	1	2	1	3	6	387*

*Two *C. freundii* isolates co-produced VIM and KPC enzymes.

B

	Blood culture				
	K. pneumoniae	*E. coli*	*E. cloacae complex*	*E. aerogenes*	*Total*
KPC	41	-	-	-	41
VIM	1	2	1	-	4
Other	2	-	1	1	4
Total	43*	2	2	1	48*

***** One isolate of *K. pneumoniae* co-produced VIM and KPC enzymes.

Table 3. (A) CPE from rectal swabs in 2015, grown on Carba Smart plate. (B) CPE from blood culture, isolates of *Enterobacteriaceae* were screened for carbapenemase production if they showed reduced susceptibility to at least one carbapenem.

3.1.2 Characterization of OXA-372, a novel class D carbapenemase in a *Citrobacter freundii* isolated from hospital wastewater plant

In September 2013, a screening for CPE in Careggi University Hospital wastewater was carried out. Aliquots of 10 µl of undiluted wastewater were plated on Carba Smart medium (BioMerieux, France) and all the colonies of *Enterobacteriaceae* with different morphology were isolated and screened for the production of carbapenemase. Five different *Enterobacteriaceae* were isolated including two *K. pneumoniae*, one *E. coli*, one *Enterobacter cloacae* and one *C. freundii*. PCR analysis of carbapenemase genes revealed the presence of bla_{KPC} in most CRE isolates (one *K. pneumoniae* and the *E. cloacae* and *E. coli* isolates) and the presence of bla_{VIM} in the other *K. pneumoniae* isolate, while negative PCR results were obtained with the *C. freundii* isolate, named Cfr-FI-07. This strain was the only one grown on both sides of Carba Smart plates (Figure 11A) and resulted negative for production of VIM, KPC, NDM and OXA-48 enzymes, despite being resistant to all β-lactams (except cefepime, for which it had an intermediate susceptibility) including carbapenems and showing imipenem hydrolyzing activity. Moreover, it was neither inhibited by EDTA nor by phenylboronic acid (Figure 11B) suggesting the possible presence of a class D enzyme.

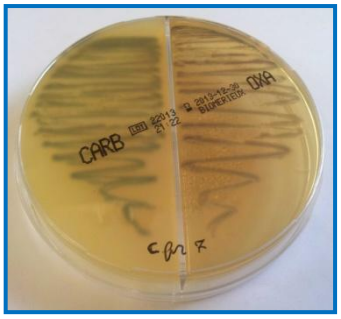
A  B 

Figure 11. (A) *C. freundii* Cfr-FI-07 grown on both sides of Carba Smart plate. (B) Phenotypic test made on *C. freundii* Cfr-FI-07.

Whole Genome Sequencing using an Illumina Miseq (paired end 2x250bp) was performed on *C. freundii* strain (Cfr-FI-07), and annotation with RAST software (Aziz, 2008), followed by bioinformatic analysis showed the presence of four β-lactamases including bla_{OXA-10}, the resident class C enzyme $bla_{CMY-135}$, a novel class C enzyme $bla_{MOX-9,}$ and a novel class D β-lactamase. The new class D β-lactamase, named $bla_{OXA-372,}$ showed only 56% aminoacid identity with the most similar class D (OXA-198; El

Garch, 2011); *bla*$_{OXA-372}$ was susequently cloned in *E. coli* DH5α pLBII and showed imipenemase activity, confirmed also by antimicrobial susceptibility testing. The novel carbapenemase gene was then cloned in pET-9 plasmid *E. coli* BL21 in order to overexpress OXA-372 production, but ion exchange chromatography failed to provide a high quality purification. A second strategy was then applied in order to express the mature part of OXA-372 with glutathione-S-transferase (GST) and obtain an affinity purification with glutathione. The GST was then cleaved with thrombin and antibiotic kinetics were studied on the mature protein (Table 4).

Substrate	**k_{cat} (s^{-1})**			**K_m (μM)**			**k_{cat}/K_m ($\mu M^{-1} \cdot s^{-1}$)**		
	OXA-372	**OXA-48[a]**	**OXA-198[b]**	**OXA-372**	**OXA-48[a]**	**OXA-198[b]**	**OXA-372**	**OXA-48[a]**	**OXA-198[b]**
Benzylpenicillin	40	446	15	110	79	14	2.8	5.6	1.1
Oxacillin	145	130	25	125	95	30	1.2	1.4	0.83
Ampicillin	85	955	37	85	395	216	1.0	2.4	0.17
Ticarcillin	110	45	-[c]	190	55	-	0.58	0.82	-
Temocillin	3	0.3	-	35	45	-	0.086	0.0066	-
Cefalotin	0.17	44	0.19	57	195	12	0.003	0.23	0.016
Cefotaxime	NH[d]	>9	NH	-	>900	-	-	0.01	-
Ceftriaxone	NH	-	-	-	-	-	-	-	-
Ceftazidime	NH	NH	NH	-	-	-	-	-	-
Cefepime	NH	>0.6	NH	-	>550	-	-	0.0011	-
Imipenem	5.8	4.8	0.1	26	13	0.15	0.22	0.37	0.67
Meropenem	0.13	0.07	0.01	0.7[e]	11	0.006	0.52	0.0062	1.7
Ertapenem	0.49	0.13	-	0.25[e]	100	-	0.7	0.0013	-
Aztreonam	NH	NH	NH	-	-	-	-	-	-

Data are the means of three independent measurements. Standard deviations were always within 10% of the mean values. Kinetic parameters for OXA-48 and OXA-198 are also shown for comparison.
[a] Kinetic parameters for all antibiotics except benzylpenicillin (determined in this study), ticarcillin and aztreonam (Poirel, 2004), are from reference (Docquier , 2009).
[b] Kinetic parameters for all antibiotics are from reference (El Garch, 2011).
[c] No data available.
[d] NH, no hydrolysis could be detected with concentrations of enzyme up to 340 nM.
[e] Determined as K_i with benzylpenicillin as the substrate.

Table 4. Kinetic parameters measured for the purified OXA-372 β-lactamase.

OXA-372, despite being highly divergent from other class D enzymes (OXA-198 and OXA-48, 56% and 43% of amino acid identity respectively), shows very similar k_{cat} values for carbapenems if compared to OXA-48. The k*cat* value for imipenem, which is slightly higher than OXA-48, is of particular importance, because denotes OXA-372 as the class D enzyme with the highest catalytic efficiency on imipenem.

S1 nuclease analysis (Barton, 1995), followed by bioinformatic analysis on Cfr-Fi-07 showed that $bla_{OXA-372}$ gene was inserted on a 150 kb IncA/C IncN plasmid and was flanked by a composite transposon, which was mobilized through electrotransformation in *E. coli* DH10B. The gene was carried on a defective transposon of original structure, named Tn*6255*, belonging to the Tn*402*/Tn*5053* lineage, which was in turn inserted into a Tn*3*-family transposon related to Tn*Pa38* (with the addiction of two IS5075-like at the extremities) of original structure, named Tn*6256* (accession no. KP851978), and was flanked by 5-bp direct repeats (Figure 12), revealing a history of insertion by transposition.

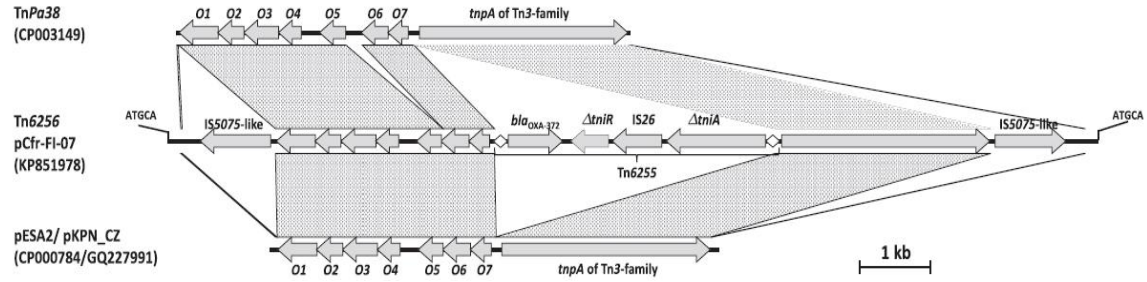


Figure 12. Genetic context of the $bla_{OXA-372}$ gene. Regions showing ≥99% nucleotide sequence identity are connected by dotted areas or lines. Structure of transposon Tn6256 and comparison with homologous transposon TnPa38 (Rau, 2012) and transposons described in plasmids pESA2/pKPN_CZ. The IRi (5′-TGTCGTTTTCAGAAGACGGCTGCAC-3′) and IRt (5′-TGTCGTTTTCAGAAGACGACCGCAC-3′) of Tn6255 are indicated by white diamonds. Direct repeat sequences (5′-ATGCA-3′) flanking Tn6256 are reported. Uncharacterized ORFs are indicated by the letter O followed by a number (O1, DNA-invertase, pin homologue; O2/O3/O4/O6, hypothetical proteins; O5, cupin 2 conserved barrel domain protein; O7, DNA-polymerase).

No clinical isolates of *Enterobacteriaceae* harboring $bla_{OXA-372}$ have been isolated to date, but the presence of the novel carbapenemase on a transferable IncA/C IncN plasmid inside a composite transposon is worrisome, since it could mobilize to other species. The complete characterization of the $bla_{OXA-372}$ harboring-plasmid, is still ongoing.

3.1.3 Intestinal colonization by *Shewanella xiamenensis* misinterpreted as OXA-48 producing *K. pneumoniae*

On July 2013, a 1-year-old child affected by a medulloblastoma, developed a postsurgical central nervous system infection associated with bacteremia caused by a *K. pneumoniae* strain producing the OXA-48 carbapenemase. The infection was successfully treated with intravenous meropenem, amikacin, and tigecycline, plus intrathecal colistin. Since the patient was found to be colonized by the same strain in the intestinal tract, oral amikacin was also given for 20 days to achieve intestinal decolonization. Thereafter, intestinal carriage of OXA-48–producing *K. pneumoniae* was prospectively monitored by culturing rectal swabs onto McConkey agar with a meropenem disk (Giani, 2012a) and onto an indicator medium for CRE (Brilliance CRE, Oxoid, UK) and by testing nucleic acid extracts from the rectal swabs by a Real-Time PCR for the detection of $bla_{\text{OXA-48–like}}$ genes. After a 3-month follow-up period during which 8 consecutive rectal swabs yielded negative results, a rectal swab tested positive for $bla_{\text{OXA-48–like}}$ genes by Real-Time PCR and yielded non–lactose-fermenting bacterial colonies growing closer than 25 mm to the meropenem disk on McConkey agar, while no colonies of carbapenem-resistant *Enterobacteriaceae* were detected. The isolate was a *Shewanella xiamenensis*, which harbored a novel $bla_{\text{OXA-48-like}}$ gene, named $bla_{\text{OXA-416}}$. *S. xiamenensis* is a species, usually found in costal sediments, belonging in *Alteromonadales*, which harbors a chromosomally encoded $bla_{\text{OXA-48-like}}$ and is considered as the environmental source of mobilization of $bla_{\text{OXA-48-like}}$ genes, now identified globally in *Enterobacteriaceae* (Tacao, 2013). The isolation from an intestinal environment of a *S. xiamenensis* is important because $bla_{\text{OXA-48–like}}$ genes could have been mobilized, not only in the environment, but also in the intestinal environment. Moreover, there is a potential for causing “false positivities” when screening rectal swabs for the presence of *K. pneumoniae* or other *Enterobacteriaceae* carrying $bla_{\text{OXA-48–like}}$ genes by molecular testing for surveillance purposes. Nowadays, many systems are being developed for the direct detection of resistance genes from rectal swabs (ex. Check Direct CPE for BD-MAX^{TM}). In these cases, confirmation of positive results by cultural methods would seem advisable if molecular testing is used as a standalone approach.

3.1.4 First detection of *bla*$_{\text{NMC-A}}$ in Italy integrated in *E. ludwigii* chromosome in a novel Xer-dependent integrative mobile element

During the routine screening of CPE from rectal swabs, in August 2014 an *Enterobacter cloacae* complex was isolated on selective medium for CPE from a Japanese tourist inpatient. This strain resulted negative for *bla*$_{\text{KPC}}$, *bla*$_{\text{VIM}}$, *bla*$_{\text{OXA-48}}$ and *bla*$_{\text{NDM}}$ carbapenemase Real Time PCR, while being resistant to carbapenems. This strain showed inhibition by boronic acid, therefore suspecting the presence of a class A carbapenemase. Whole genome sequencing using Illumina Miseq instrument revealed the presence of a *bla*$_{\text{NMC-A.}}$ Species identification as *Enterobacter ludwigii* was made through the analysis of *rpoB, hsp60* and 16S rDNA genes. This is the first description of a NMC-A-producing *E. ludwigii* and the first detection of this resistance determinant in Italy. Genetic context analysis of *bla*$_{\text{NMC-A}}$ showed for the first time that the carbapenemase was inserted in a 29 kb region flanked by XerC/XerD regions, called EludIMEX-1, which putatively were involved in the integration in the chromosome of *E. ludwigii* (Figure 13). Bioinformatic analysis confirmed the presence of an *E. asburiae* MNCRE-14 genome which had a very similar *bla*$_{\text{NMC-A}}$-flanking region (>99% identity), while being different species. Analysis of the sequences of other *bla*$_{\text{IMI-1}}$-producing *E. cloacae* complex showed that despite having many differences inside the flanking regions (Figure 13B), XerC/XerD regions were conserved also in these isolates (accession nos. KR057494.1, and LFDQ01000001).

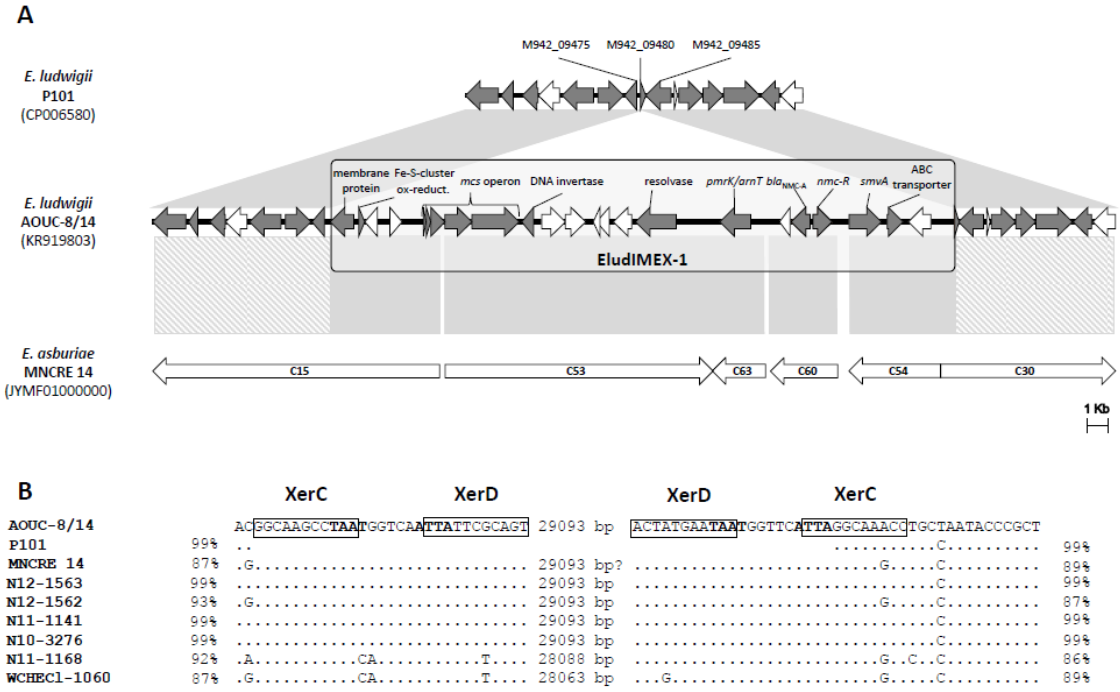


Figure 13. Panel A. Comparison between the genomic region of *E. ludwigii* AOUC-8/14 carrying the *bla*$_{NMC-A}$ gene and the corresponding chromosomal region of *E. ludwigii* P101 (region 1872587 - 1888317 of GenBank entry CP006580), revealing the chromosomal insertion of the EludIMEX-1 element in AOUC-8/14. Comparisons with the corresponding regions of the EcWSU1 and EN-119 *E. ludwigii* strains yielded identical results and are not shown. ORFs are indicated by arrows, showing direction of transcription. Proteins of unknown function are filled in white. Regions connected by grey areas exhibit >98% nucleotide sequence identity. Comparison with the draft genome of *E. asburiae* MNCRE14 (accession no. JYMF01000000) is shown in the lower part of this panel. In this case, contigs from WGS of MNCRE14 (indicated by C and contig number) were aligned and ordered according to homology with the corresponding parts of the AOUC-8/14 genome. The regions connected by striped areas exhibit <91% nucleotide sequence identity.
Panel B. Nucleotide sequences at the junctions of EludIMEX-1 with the *E. ludwigii* AOUC-8/14 chromosome, revealed by alignment with the corresponding *E. ludwigii* P101 chromosomal region. The imperfect repeats homologous to the XerC/XerD binding sites are boxed, the conserved consensus is boldfaced (Krupovic,2011), and the size of the intervening DNA segment (in bp) is indicated. A comparison is also shown with the presumptive *bla*$_{NMC-A}$ carrying element of strain MNCRE14, with the elements associated with *bla*$_{NMC-A}$ from ECC strains N12-1563 (KR057496.1), N12-1562 (KR057495.1), N11-1141 (KR057493.1), N10-3276 (KR057492.1), and with the elements associated with *bla*IMI from ECC strain N11-1168 (KR057494.1) and *E. cloacae* WCHECl-1060 (LFDQ01000001). Percentages at the left and right sides of the sequences represent the nucleotide identity with the corresponding region of AOUC-8/14 (for a 10-kb region, except for N12-1563,N12-1562, N11-1141, N10-3276, and N11-1168, which have shorter flanking regions).

In conclusion, XerC/XerD could be the putative recombination mechanism of integration not only for *bla*$_{NMC-A}$, but also for *bla*$_{IMI-1}$ genes inside the chromosome of *E. cloacae* complex isolates.

3.1.5 First detection of *bla*$_{IMI-2}$ in Italy in *E. ludwigii* on a transferable plasmid

On July 2015, an *Enterobacter cloacae* complex, grown on Carba Smart plate, was isolated from a surveillance rectal swab of an italian inpatient of Careggi University Hospital. Phenotypic test showed decreased susceptibility to imipenem and inhibition by phenylboronic acid. Carbapenemase activity was confirmed by spectrophotometric assay and the strain was subjected to WGS with Illumina Miseq (paired end 2x300 bp). WGS analysis showed the presence of a *bla*$_{IMI-2}$ within an original genetic context with common features with that described in *E. coli* in 2012 (Rojo-Bezares, 2012). Species identification as *Enterobacter ludwigii* (the same as the NMC-A-producing AOUC-8/14) was made by *rpoB, hsp60* (Hoffmann, 2003; Paauw, 2008), 16S r-DNA and GGDC software analysis (Auch, 2010). Conjugation experiments in *E. coli* J53Azi^{R} were not successful, while electrotransformation experiments on *E. coli* DH10B demonstrated transferability of an IncFII plasmid harboring *bla*$_{IMI-2}$ gene. S1 nuclease digestion showed a plasmid size around 170 kb. Antimicrobial susceptibility testing on *Enterobacter ludwigii* revealed a resistance profile to penicillins, carbapenems, and fosfomycin (derived by the presence of *fosA* gene in the chromosome), while only β-lactams resistance was transferable to *E. coli* DH10B with plasmid pEL-IMI2 (Table 5). Further studies in order to characterize the genetic context of *bla*$_{IMI-2,}$ and the mobilization event, which could have integrated *bla*$_{IMI-2}$ on this trasferable plasmid are still ongoing.

	MIC (mg/L)		
	E. ludwigii	*E. coli*	
Antibiotic	AOUC-07/15	DH10B pEL-IMI2	DH10B
Piperacillin	64	64	2
Piperacillin/tazobactam[a]	16	16	2
Amoxicillin	>64	>64	4
Amoxicillin/clavulanic acid[b]	>64	>64	4
Ceftazidime	0.5	0.5	≤0.25
Cefotaxime	0.5	≤0.25	≤0.25
Cefepime	≤0.25	≤0.25	≤0.25
Aztreonam	16	16	≤0.25
Imipenem	>32	>32	≤0.25
Meropenem	>32	32	≤0.25
Ertapenem	>32	16	≤0.25
Amikacin	1	1	1
Gentamicin	0.5	≤0.25	0.5
Tobramycin	≤0.25	≤0.25	0.5
Ciprofloxacin	≤0.25	≤0.25	≤0.25
Colistin	0.5	≤0.25	≤0.25
Trimethoprim/sulphamethoxazole[c]	≤0.25	≤0.25	≤0.25
Fosfomycin[d]	64	≤0.5	≤0.5

[a]MIC of piperacillin with tazobactam at 4 mg/L fixed concentration;
[b]MIC of amoxicillin with clavulanic acid , at 2 mg/L fixed concentration;
[c] MIC of trimethoprim/sulfamethoxazole, at 1:19 proportion;
[d] MIC of fosfomycin was obtained with agar microdilution method;

Table 5. Antimicrobial susceptibilities of IMI-2 producing *E. ludwigii* AOUC-07/15.

3.2 Carbapenemase producing Gram-negative bacteria, non-*Enterobacteriaceae*

Related publications

1. **Pollini S, Antonelli A, Venturelli C, Maradei S, Veggetti A, Bracco S, Rumpianesi F, Luzzaro F Rossolini G M.** 2012. Acquisition of plasmid-borne $bla_{\text{IMP-19}}$ gene by a VIM-1-positive *Pseudomonas aeruginosa* of the Sequence Type 235 epidemic lineage. *Journal of Antimicrobial Chemotherapy* . 68(**3**):722-4. *****

   *****This paper has been published before the PhD period

2. **Antonelli A, D'Andrea M M, Montagnani C, Bartolesi A M, Di Pilato V, Fiorini P, Torricelli F, Galli L, Rossolini G M.** Newborn bacteraemia caused by an *Aeromonas caviae* producing the VIM-1 and SHV-12 β-lactamases,

encoded by a transferable plasmid. *Journal of Antimicrobial Chemotherapy* 2015 Sep 25; Epub ahead of print.

3.2.1 MO-11c33: characterization of a *P. aeruginosa* isolate harbouring the resistance determinants *bla*$_{\text{VIM-1}}$ and *bla*$_{\text{IMP-19}}$

The strain of *P. aeruginosa* MO-11c33 was isolated in June 2011 from the urine of an elderly (81 y/o) female inpatient in Modena University hospital. The patient, resident in a long-term care facility and affected by senile dementia requiring a permanent urinary catheter and nasogastric tube, had been admitted with diagnosis of sepsis and treated with meropenem for 10 days before isolation of MO-11c33 from urine. Clinical records reported two additional hospital admissions for septic episodes during the previous two months, successfully treated with meropenem. MO-11c33 was considered a colonizer, and no specific anti-pseudomonas treatment was given following its isolation.
Susceptibility testing revealed that MO-11c33 exhibited a MDR phenotype, including all anti-pseudomonas β-lactams, while it retained susceptibility to amikacin and colistin.

MO-11c33 belonged in ST 235 (Curran, 2004), the deduced founder of CC 235, a high-risk clone (HiRiC) lineage (Woodford, 2011) that has been involved in the dissemination of the *bla*$_{\text{VIM-1}}$ MBL determinant in Italy and elsewhere (Woodford, 2011, Giske, 2006, Rossolini, 2008). The *bla*$_{\text{VIM-1}}$ gene was found to be carried by an In70.2 integron platform identical to that of the VIM-1-producing *P. aeruginosa* index strain (Riccio, 2005) and of most other VIM-1 producing isolates circulating in Italy (Rossolini, 2008). The *bla*$_{\text{IMP-19}}$ gene was found to be carried by an In804 integron platform (Figure 14) identical to that previously detected in an *Achromobacter xylosoxidans* isolate from Japan (Yamamoto, 2012). Apart from the integron associated conserved sequences, no information were available on the surroundings of the different *bla*$_{\text{IMP-19}}$-harbouring integrons (Figure 14) (Neuwirth, 2007; Yamamoto, 2011; Yamamoto, 2012); the further analysis, with specific *bla*$_{\text{IMP-19}}$ and external primers, of the genetic context of In804 from MO-11c33 revealed the presence of a copy of ISPa7, which has been frequently reported as associated to MBL genes in *P. aeruginosa*, upstream of the integrase gene and remnants of Tn402-like transposition modules (*tniB* gene), that are often linked to class1 integrons as well, at the 3' end (Figure 14). The sequence of In804 with its genetic context was submitted to GenBank (JX421697).

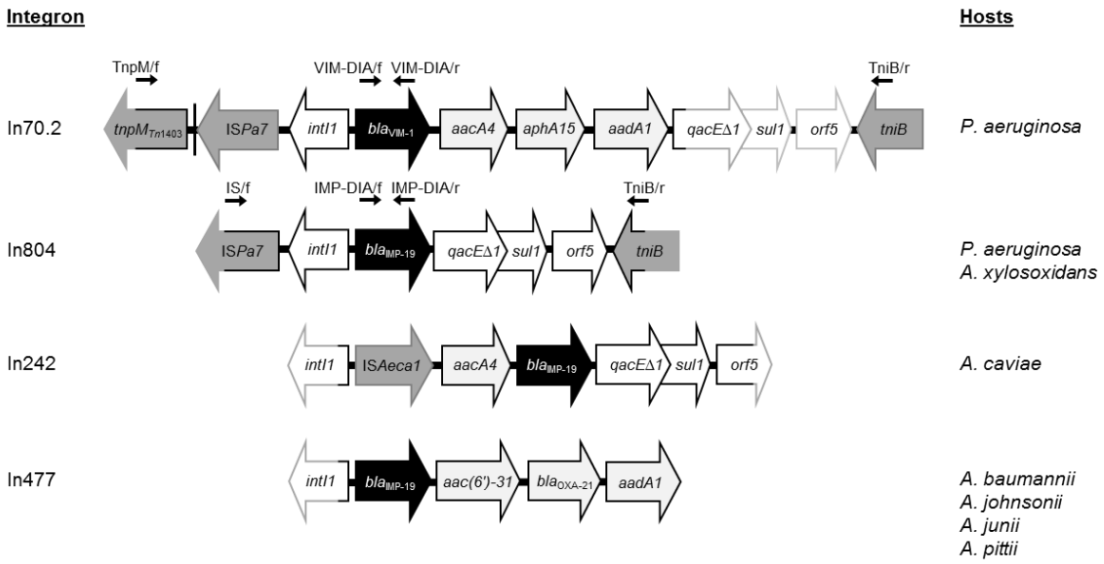


Figure 14. Structure of the variable regions and genetic context of In70.2 and In804 class 1 integrons from *P. aeruginosa* MO-11c33. The previously described In242 and In477 integrons harbouring *bla*$_{\text{IMP-19}}$ are also shown. The gene cassettes are indicated by arrows, filled with different patterns: black, cassettes encoding MBLs; light grey, cassettes encoding other resistance enzymes (e. g. for aminoglycosides and phenicols); white, *intI1* integrase gene and 3'-conserved segments flanking the cassette array; dark grey, transposition modules and insertion sequences. 25 IRi is indicated by a vertical line. Black outlines indicate fully sequenced regions while gray ones indicate regions that were only partially sequenced or deduced by PCR mapping. The positions of primers TnpM/f (5'-CATGCCTCAGTCTGACAGTG-3'), IS/f (5'-AGGATTTCTTCGCCTTTGAA-3'), and TniB/r (5'-CGACGATTGCTGCTCACTG-3'), used for PCR mapping in combination with primers VIM-DIA/f and /r and IMP-DIA/f and /r are also shown.

Moreover, the genetic localization of the In804 was investigated through S1 nuclease followed by hybridization experiments with probes for the specific detection of 16S ribosomal RNA and of the *bla*$_{\text{VIM-1}}$ and *bla*$_{\text{IMP-19}}$ genes. The result showed the chromosomal location of the gene *bla*$_{\text{VIM-1}}$ in the strain MO-11c33 while the *bla*$_{\text{IMP-19}}$ gene resulted to be located on a ca. 120 kb plasmid (Figure 15).

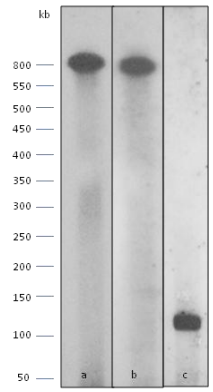


Figure 15: PFGE of an S1 nuclease digestion of the MO-11c33 strain. The figure shows the hybridization with 16S probe of MO-11c33 (a), the hybridization with VIM probe of MO-11c33 (b) and the hybridization with IMP probe of MO-11c33 (c). The hybridization exhibits the chromosomal location of the gene *bla*VIM-1 in MO-11c33, and the plasmid location of the gene Imp-19 in MO-11c33.

Different attempts to transfer the *bla*$_{IMP-19}$-carrying plasmid were made, but it couldn't be transferred either by transformation or conjugation. Different commonly used bacterial hosts were utilized for transformation (*E. coli* DH5α, MC1061 and DH10B strains; *P. aeruginosa* PAO1 strain), including restriction/modification system mutants, and conjugation (*E. coli* MKD135 and *P. aeruginosa* 10145/3 strains) experiments. As the *bla*$_{IMP-19}$-carrying plasmid couldn't be mobilized, its contribution to resistance, in terms of carriage of additional resistance genes, couldn't be assessed as well as the contribution of the *bla*$_{IMP-19}$ gene to the β-lactams resistance profile in MO-11c33. Nevertheless, the isolation of this determinant in an epidemic and very successful clonal lineage represents a matter of concern and suggests a potential role of this CC in the spreading of this resistance gene. This is the first detection of the *bla*$_{IMP-19}$ gene in Italy and the second description of this resistance determinant in Europe since 2007. Moreover, this isolate was found to co-produce the VIM-1 MBL. This peculiarity makes the isolate MO-11c33 the first double-MBL-carrying *P. aeruginosa* detected world-wide. *Bla*$_{IMP-}$19 is a *bla*$_{IMP}$-type allelic variant that was originally detected in *Enterobacter cloacae* and *Pseudomonas aeruginosa* from Japan (GenBank/EMBL accession no. AB201264 and AB184976) and subsequently, in an *Aeromonas caviae* isolated in France (Neuwirth, 2007). More recently, it has been reported in *Achromobacter xylosoxydans* and *Acinetobacter* spp. isolates from Japan (Yamamoto, 2011; Yamamoto 2012), and in an *Acinetobacter baumannii* isolate from Iran (GenBank/EMBL accession no. JQ766528).

3.2.2 Newborn bacteraemia caused by an *Aeromonas caviae* producing the VIM-1 and SHV-12 β-lactamases, encoded by a transferable plasmid

A. caviae AOUC-AA14 was isolated in autumn 2014 from the blood cultures of a 1-day-old newborn transferred to the Anna Meyer Children's University Hospital (Florence, Italy) from another hospital due to prematurely ruptured membranes and meconium aspiration. On admission, blood was drawn for culture during umbilical vein catheter insertion, and empirical antibiotic treatment with ampicillin/sulbactam (100 mg/kg/day) plus gentamicin (4 mg/kg/day) was started. An *A. caviae* was isolated from blood culture. Results of the preliminary anti-biogram, carried out by disc diffusion using 0.075 mL of the blood culture medium directly inoculated on to Mueller–Hinton agar plates, were suggestive of resistance to ESCs, intermediate susceptibility to

meropenem and gentamicin, and production of MBL activity based on synergism between meropenem and EDTA. Strains of this species are normally resistant to penicillins, and narrow-spectrum cephalosporins due to the production of chromosomally encoded β-lactamases of class C (AmpC type) and class D (OXA type), but susceptible to expanded-spectrum cephalosporins (ESCs) and carbapenems (Janda, 2010; Rossolini, 1995; Walsh, 1997). Due to its unusual resistance phenotype AOUC-AA14 strain was subjected to WGS analysis using an Illumina MiSeq platform (Illumina Inc.) with a paired-end approach (2×250 bp). Sequence comparisons confirmed identification as *A. caviae* by using the Multi-Locus Sequence Analysis scheme REF and revealed the presence of a complex resistome, including the resident AmpC type (accession no. KGY70007) and OXA type (accession no. KT355710) β-lactamase genes, two acquired β-lactamase genes (*bla*$_{\text{VIM-1}}$ and *bla*$_{\text{SHV-12}}$) and resistance determinants for aminoglycosides [*aac(6′)-Ib′* and *aadA1b*], chloramphenicol (catB2), sulphonamides (*sul1*), trimethoprim (*dfrA14*) and macrolides [*mph(A)*]. The *bla*$_{\text{VIM-1}}$ gene was associated with a novel class 1 integron platform, named In*1164*, carrying the *bla*$_{\text{VIM-1}}$, *aac*(6′)*-Ib'* , *aadA1b* and *catB2* gene cassettes, followed by a 3′ conserved segment composed of *qacED1*, *sul1* and *orf5* genes (accession no. KR869764) (Figure 16).

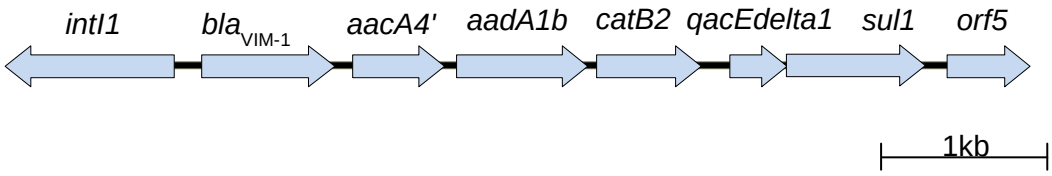


Figure 16. Integron In*1164* containing *bla*$_{\text{VIM-1}}$ gene of *A. caviae* AOUC-AA14.

The *dfrA14* gene was associated with another integron platform, linked with *mph(A)* in a genetic context identical to that described in plasmid pRSB225 (accession no. JX127248.1). WGS data also revealed the presence of an IncA/C-type plasmid replicon. S1 nuclease analysis revealed that the MBL gene was carried on an approximate 150 kb plasmid, named pAOUC-AA14. The plasmid could be transferred by conjugation to *Escherichia coli* J53 aziR at high frequency (1.4×10^{-3} transconjugants/recipient cell). PCR analysis of transconjugants confirmed the presence of the *bla*$_{\text{VIM-1}}$, *bla*$_{\text{SHV-12}}$ and *dfrA14* genes, and that pAOUC-AA14 belonged to the IncA/C type. The resistance

profile conferred to *E. coli* upon plasmid acquisition was consistent with plasmid carriage of In1164, bla_{SHV-12} and *dfrA14* (Table 6).

	MIC, mg/L		
Antibiotic	AOUC-AA14	J53 azi^R p(AOUC-AA14)	J53 azi^R
ampicillin	>256	>256	4
piperacillin	>256	>256	2
piperacillin/tazobactam[c]	>256	>256	2
amoxicillin/clavulanate[d]	64	>256	8
cefotaxime	>32	32	0.06
cefepime	>32	>32	0.06
ceftazidime	>32	>32	0.25
aztreonam	>32	>32	0.12
amikacin	0.5	2	1
gentamicin	1	1	0.25
tobramycin	8	8	0.25
imipenem	1	8	0.25
meropenem	0.25	8	0.03
doripenem	0.25	8	0.03
colistin	0.25	0.25	0.06
chloramphenicol	>32	>32	16
ciprofloxacin	≤0.015	≤0.015	≤0.015
streptomycin	>32	>32	4
trimethoprim	>32	>32	0.5
trimethoprim/sulfamethoxazole[f]	>32/608	>32/608	0.06/1.14

[a] NA, not applicable
[b] -, not determined
[c] MIC of piperacillin with tazobactam at 4 mg/L fixed concentration
[d] MIC of amoxicillin/clavulanic acid at 1:2 proportion
[e] PBA, phenylboronic acid
[f] MIC of trimethoprim/sulfamethoxazole, at 1:19 proportion

Table 6. Antimicrobial susceptibility of *A. caviae* AOUC-AA14, and of the *E. coli* J53 aziR (pAOUC-AA14) transconjugant and of the *E. coli* J53 aziR recipient strain.

This study confirmed the circulation of *A. caviae* strains with acquired VIM-type MBLs, and indicated that beyond intestinal colonization similar strains can also cause invasive infections. In previously reported *A. caviae* strains, bla_{VIM} determinants were apparently not transferable by conjugation (except in one case, but at very low frequency) (Adler, 2014). The high conjugation frequency showed by p(AOUC-AA14) is worrisome since it could spread all its resistance genes to other species in both nosocomial and community setting.

3.3 Evolution of the β-lactamases epidemiology in *Enterobacteriaceae*: ESBLs, AmpCs and carbapenemases from an Italian nationwide surveillance

Related publication

Antonelli A, Caltagirone M, Mauri C, Nicchi J, Giani T, Arena F, Nucleo E, Bracco S, Luzzaro F, Pagani L, Rossolini G M. 2015. Molecular characterization of ESBLs, AmpCs and carbapenemases among *Enterobacteriaceae* from a recent Italian nationwide survey. *25th European Congress of Clinical Microbiology and Infectious Diseases*. April 2015 Copenhagen. O143, oral communication.

In Italy, concerning carbapenemases, ESBL and AmpC resistance determinants, previous surveillance studies, carried out in 1999 and in 2003, have documented a countrywide diffusion of *Enterobacteriaceae* producing ESBLs, initially mostly represented by TEM- and SHV-type ESBL variants (Spanu, 2002), and subsequently largely replaced by CTX-M-type ESBLs (Luzzaro, 2006), but no more recent studies are available. In 2013, a nationwide surveillance study on CPE, isolated in 2011, has documented that the problem of carbapenems resistance is mostly sustained by *K. pneumoniae* producing KPC-type enzymes (Giani, 2013).

The Committee for antimicrobial agents (CoSA) and the Association of Italian Clinical Microbiologists (AMCLI) promoted a nationwide Italian survey aimed at investigating the prevalence and nature of ESBL, ACBLs and carbapenemase determinants among isolates of *E. coli*, *K. pneumoniae* and *Proteus mirabilis* of clinical origin. This study reports the results of that survey, that confirms the endemic presence of KPC-producing *K. pneumoniae* and new insights of ESBL and class C β-lactamases (ACBL) types and diffusions.

Design of the study. Fourteen hospitals from 13 Italian cities participated in the study. During the survey period, (1^{st} – 15^{th} October, 2013) all referring microbiology laboratories were asked to collect all consecutive non replicate clinical isolates of *Escherichia coli*, *Klebsiella pneumoniae* and *Proteus mirabilis*, from any site of

infection, with a minimum inhibitory concentration (MIC) for extended-spectrum cephalosporins (ESC) (cefotaxime, and/or ceftriaxone, and/or ceftazidime and/or cefepime), and/or ertapenem of >1 mg/L**.** The isolates fulfilling these criteria were sent to reference laboratories for confirmation of species identification and phenotypic and molecular characterization of the resistance mechanisms.

Bacterial identification, antimicrobial susceptibility testing (AST) and phenotypic characterization of resistance mechanisms. At satellite laboratories susceptibility testing was carried out by Phoenix or Vitek2 automated systems. Identification of collected isolates was confirmed at the reference laboratory of Lecco Hospital by MALDI-TOF (Vitek MS, bioMérieux). Susceptibilities to carbapenems (including ertapenem, imipenem, and meropenem), amikacin, gentamicin, ciprofloxacin and trimethoprim/sulphametoxazole were determined by disc-diffusion on Mueller-Hinton, and interpreted according to the current EUCAST breakpoints (http://www.eucast.org/clinical_breakpoints/). Basing on results from AST, all collected isolates were categorized in two groups: i) isolates that showed an MIC >1 to at least one of the tested cephalosporins but <=1 to all tested carbapenems (ESCR-carbaS) or ii) isolates that showed an MIC >1 to at least one of the tested cephalosporins and to at least one of the tested carbapenems (ESCR-carbaNS). Confirmatory test for ESBL production was performed using double disk test method (testing cefotaxime, ceftazidime, cefepime and aztreonam) on all isolates. Isolates that tested positive to ESBL confirmatory test, were categorized as ESBL producers (ESBL-P).

Molecular characterization of β-lactamase determinants. ESCR-carbaS and ESCR-carbaNS isolates were analyzed by a homebrew multiplex Real Time PCR (mRT) able to detect all $bla_{\text{CTX-M}}$ genes and to distinguish among different $bla_{\text{CTX-M-type}}$ variants of groups 1, 2, 9, 8/25 (mRT-1) (Table 7).

Target	Primer name	Sequence (5'-3')[a]	Reference[b]	Positive control
bla$_{CTX-M}$ group 1 genes	CTX-M-group-1_FW	AAAAATCACTGCGCCAGTTC	Woodford, 2006	*E. coli* V460a (CTX-M-15), unpublished
	CTX-M-group-1_REV	AGCTTATTCATCGCCACGTT	Woodford, 2006	
	CTX-M-group1-P	HEX-TGGCGACGGCAACCGTCACGCTGTT-BHQ-1	This study	
bla$_{CTX-M}$ group 2 genes	CTX-M-group-2_FW	CGACGCTACCCCTGCTATT	Woodford, 2006	*E. coli* C277a (CTX-M-2), unpublished
	CTX-M-group-2_REV	CCAGCGTCAGATTTTTCAGG	Woodford, 2006	
	CTX-M-group2-P	FAM-TATTGAGCGTGGGCTCGGTTCTGTCCAG-BHQ-1	This study	
bla$_{CTX-M}$ group 8/25 genes	CTX-M-group-8/25_Fwd	CGATACCACCACGCCATTAG	This study	*E. coli* M26a (CTX-M-8), unpublished
	CTX-M-group-8/25_REV	AACCCACGATGTGGGTAGC	Woodford, 2006	
	CTX-M-group8/25-P	CY5-CCTGAATGCTGGCAGCGCCGGTG-BHQ-3	This study	
bla$_{CTX-M}$ group 9 genes	CTX-M-group-9_FW	CAAAGAGAGTGCAACGGATG	Woodford, 2006	*E. coli* V404a (CTX-M-14), Riccobono, 2014
	CTX-M-group-9_REV	ATTGGAAAGCGTTCATCACC	Woodford, 2006	
	CTX-M-group9-P	ROX-CGTGCATTCCGCTGCTGCTGGGCA-BHQ-2	This study	
All *bla*$_{CTX-M}$ genes	U-CTX-M-Fwd	ATYRAYACMGCVGATAAYWCGCA	This study	
	U-CTX-M-Rev	CSGCAATSGGRTTRTAGTTAAC	This study	
	U-CTX-M-P	CY5.5-ATGTGCAGYACCAGTAARGTKATGGC-BHQ-3	This study	
PhHV (internal control)[c]	PhHV-267s	GGG CGA ATC ACA GAT TGA ATC	Van Doornum, 2003	PhHV DNA cloned in pGEM-T-easy *E. coli* DH5α
	PhHV-337as	GCG GTT CCA AAC GTA CCA A	Van Doornum, 2003	
	PhHV-305tq	Cy5.5 -TTTTATGTGTCCGCCACCATCTGGATC-BHQ-3	Van Doornum, 2003	

[a] The amplification program consisted of 35 two-step cycles of 15 s at 95°Cand 60s at 60°C.

[b] Primer and probe sequences were from different references, while the Multiplex PCR fluorophore-quencher combination is original.

[c]PhHV internal control was added with *bla*$_{KPC}$, *bla*$_{OXA-48}$, *bla*$_{VIM}$ and *bla*$_{NDM}$ multiplex Real Time PCR (Table 2).

Table 7. Primers and probes used for the detection of the different *bla*$_{CTX-M}$ groups and PhHV internal amplification control.

ESCR-carbaNS isolates were further analyzed by mRT-2, an homebrew mRT targeting *bla*$_{KPC-type}$, *bla*$_{VIM-type}$, *bla*$_{OXA-48-type}$ and *bla*$_{NDM-type}$ genes (Table 2). The isolates that tested negative for mRT-1 and mRT-2 were analysed by a multiplex-PCR targeting genes encoding ACBLs as described previously (D'Andrea, 2006). For *E. coli* specific primers designed to distinguish between acquired and chromosomal AmpC-type were used (D'Andrea, 2006).

MIC microdilution assays on carbapenemase producers and spectrophotometric assay for carbapenemase activity detection. ESCR-carbaNS *K. pneumoniae* that

tested positive for mRT-2 (CARB-P) were subjected to MIC testing using broth microdilution method and custom lyophilized plates (TREK Diagnostic Systems, Cleveland, OH) following CLSI guidelines. Results were interpreted according to EUCAST breakpoints.

For ESCR-carbaNS that tested negative for mRT-2, carbapenemase production was further investigated by measuring the imipenem-hydrolyzing specific activity in bacterial crude extracts as described previously (Lauretti, 1999), using a Cary 100 UV-Vis (Varian, Walnut Creek, CA) spectrophotometer.

Prevalence of *Enterobacteriaceae* with resistance mechanisms to ESC and/or carbapenems. During the study period, a total of 3,895 consecutive non-replicate clinical isolates of the family *Enterobacteriaceae* (1,864 from inpatients, and 2,031 from outpatients) were isolated in the 14 centers participating to the survey. Of them, 3,324 belonged to the target enterobacterial species, including 2,352 (70.7%) *E. coli*, 697 (21%) *K. pneumoniae*, and 275 (8.3%) *P. mirabilis*. Overall, 652 isolates (19.6%) were confirmed to belong to the target species and to exhibit an ESCR-carbaS (N=508) or an ESCR-carbaNS (N=144) phenotype. Of these, 65.6% were from inpatients- and 34.4% were from outpatients. ESCR-carbaS phenotypes were contributed by all three species with higher proportions among isolates from inpatients, more evident for *E. coli* and *K. pneumoniae*. ESCR-carbaNS phenotypes were mostly contributed by *K. pneumoniae* with higher proportion among inpatients but a remarkable proportion (7.7%) also among outpatients. A detailed distribution of the resistance phenotype by species and type of patients is reported in Table 8.

Species	Isolates from inpatients				Isolates from outpatients				All isolates			
	Total	ESCR(%)	ESCR-CarbaS (%)	ESCR-CarbaNS (%)	Total	ESCR/CR (%)	ESCR-CarbaS (%)	ESCR-CarbaNS (%)	Total	ESCR/CR (%)	ESCR-CarbaS (%)	ESCR-CarbaNS (%)
Escherichia coli	920	230 (25.0)	219 (23.8)	11 (1.2)	1432	162 (11.3)	159 (11.1)	3 (0.2)	2352	392 (16.7)	378 (16.1)	14 (0.6)
Klebsiella pneumoniae	437	159 (36.4)	49 (11.2)	110 (25.1)	260	36 (13.8)	16 (6.2)	20 (7.7)	697	195 (28.0)	65 (9.3)	130 (18.7)
Proteus mirabilis	152	39 (25.7)	39 (25.7)	0 (0.0)	123	26 (21.1)	26 (21.1)	0 (0.0)	275	65 (23.6)	65 (23.6)	0 (0.0)
Total target species	1509	428 (28.4)	309 (20.3)	121 (8.0)	1815	224 (12.3)	201 (11.1)	23 (1.3)	3324	652 (19.6)	508 (15.3)	144 (4.3)

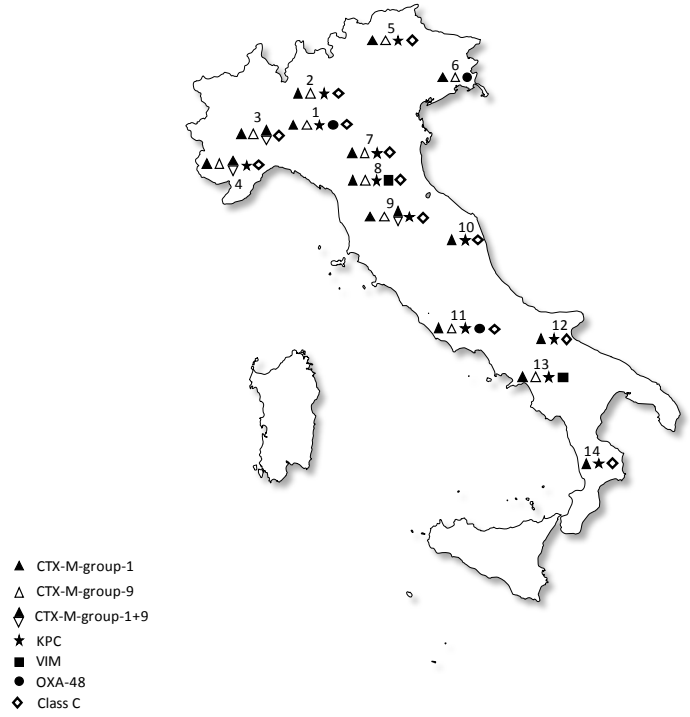
Table 8. Proportions of ESCR-CarbaS and ESCR-CarbaNS *Enterobacteriaceae*

β-lactamase phenotypes and genotypes of the ESCR/CR isolates. Of the ESCR-CarbaS isolates, 460 (90.5%) were ESBL-positive, and 384 of them carried a CTX-M

ESBL gene (Figure 17 and Table 9). The proportion of *bla*$_{CTX-M}$ determinants was 92.2% among ESBL-positive *E. coli* and 85% among ESBL-positive *K. pneumoniae*, while no such determinants were detected among ESBL-positive isolates of *P. mirabilis*. Most CTX-M enzymes belonged to group 1 (81.6%), while a minority belonged to group 9 (15.9%), and a small proportion were positive for both CTX-M groups (2.5%) (Table 9). CTX-M-producing isolates were detected from all centers (Figure 17). The majority of the ESCR-CarbaS isolates that were ESBL-negative produced an acquired AmpC-type β-lactamase (n=38, 79%) (Fig. 17) which, in most cases (N=36), belonged to the CMY/LAT/ACT/MIR lineage (Table 9). Most of the AmpC-producing isolates (65.8%) were *P. mirabilis* (Fig. 17). Concerning the ESCR-Carba NS isolates, most (82%) were carbapenemase producers, including KPC (93.2%), OXA-48 (4.2%), and VIM (2.6%). The majority of carbapenemase producers were contributed by *K. pneumoniae*, while only five *E. coli* produced a carbapenemase (two producing OXA-48, two KPC-type and one a VIM-type carbapenemase). Interestingly, seven KPC-positive *K. pneumoniae* and the majority of the OXA-48-producing isolates also co-harbored a *bla*$_{CTX-M}$ determinant (four out of five, with three CTX-M group1 and one CTX-M group 9) (Table 9). The 26 ESCR-CarbaNS (18% of the overall ESCR-carbaNS) isolates that tested negative for carbapenemase genes, were confirmed to be carbapenemase non-producers by spectrophotometric assay. Most of these isolates carried *bla*$_{CTX-M}$ genes (65.3%) while a minority was positive for an acquired ampC-type gene (*bla*$_{DHA}$ or *bla*$_{CMY}$). A detailed distribution of the resistance determinants in the Italian territory is reported in Figure 17 and the distribution by species is reported in Table 9.

		E. coli (%)	*K. pneumoniae* (%)	*P. mirabilis* (%)	Total (%)
	Total	2352	697	275	3324
	Resistant*	392 (16,7)	195 (28,0)	65 (23,6)	652 (19,6)
ESCR-carbaS	Total	378 (96,4)	65 (33,3)	65 (100,0)	508 (77,9)
ESBL-P	CTX-M-1	264 (69,8)	50 (76,9)	0 (0,0)	314 (61,8)
	CTX-M-9	61 (16,1)	0 (0,0)	0 (0,0)	61 (12,0)
	CTX-M-1+9	8 (2,1)	1 (1,5)	0 (0,0)	9 (1,8)
	Other ESBL	28 (7.4)	9 (13.8)	39 (60.0)	76 (15.0)
ampC-P	CMY/ACT/MIR	12 (3,2)	0 (0,0)	24 (36,9)	36 (7,1)
	DHA	0 (0,0)	0 (0,0)	1 (1,5)	1 (0,2)
Other	Other R mechanism	5 (1.3)	5 (7,7)	1 (1,5)	87 (17,1)
ESCR-carbaNS	Total	14 (3,6)	130 (66,7)	0 (0,0)	144 (22,1)
CARB-P	KPC	2 (14,3)	108 (83,2)	0 (0,0)	110 (76,4)
	VIM	1 (7,1)	2 (1,5)	0 (0,0)	3 (2,1)
	OXA-48	2 (14,3)	3 (2,3)	0 (0,0)	5 (3,5)
ESBL-P	CTX-M-1	4 (28,6)	9 (6,9)	0 (0,0)	13 (9,0)
	CTX-M-9	3 (21,4)	0	0 (0,0)	3 (2,1)
	CTX-M-1+9	0 (0,0)	1 (0,8)	0 (0,0)	1 (0,7)
ampC-P	CMY/ACT/MIR	2 (14,3)	0 (0,0)	0 (0,0)	2 (1,4)
	DHA	0 (0,0)	2 (1,5)	0 (0,0)	2 (1,4)
other	Other R mechanism	0 (0,0)	5 (3,8)	0 (0,0)	5 (3,5)
Total	Total	392	195	65	652
CARB-P	KPC	2 (0,5)	108 (55,4)	0 (0,0)	110 (16,9)
	VIM	1 (0,3)	2(1,0)	0 (0,0)	3 (0,5)
	OXA-48	2 (0,5)	3 (1,5)	0 (0,0)	5 (0,8)
ESBL-P	CTX-M-1	268 (68,4)	59 (30,3)	0 (0,0)	327 (50,2)
	CTX-M-9	64 (16,3)	0 (0,0)	0 (0,0)	64 (9,8)
	CTX-M-1+9	8 (2,0)	2 (1,0)	0 (0,0)	10 (1,5)
AmpC-P	CMY/ACT/MIR	14 (3,6)	0	24 (36,9)	38 (5,8)
	DHA	0 (0,0)	2 (1,0)	1 (1,5)	3 (0,5)
Other	Other R mechanism	33 (8,4)	19 (9,7)	40 (61,5)	92 (14,1)

Table 9. Resistance determinants detected in this study.



1-Milano; 2-Lecco; 3-Novara; 4-Sanremo; 5-Bolzano; 6-Udine; 7-Modena Bg; 8-Modena Pc; 9-Firenze; 10-Ancona; 11-Roma;12-San Giovanni Rotondo ;13-Avellino; 14-Cosenza

Figure 17. Nationwide distribution of the different β-lactamases detected in this study.

Proportion of ESCR-carbaS and ESCR-Carba NS among inpatient and outpatient. The overall proportion of ESCR-CarbaS isolates were approximately two times higher in those from inpatients than in isolates from outpatients (20.1% vs. 11%) while ESCRCarba NS were 8 times higher among inpatients than outpatients (8% vs. 1.2%) (data not shown).

Susceptibility assays. Results of antimicrobial susceptibilities showed that most of *E. coli* were resistant to of ciprofloxacin and trimethoprim/sulfamethoxazole (87.5% and 56%) and showed a non-susceptibility rates to gentamicin of 36.9%. No *E. coli* was resistant to imipenem and/or meropenem (only few with intermediate values), and only 3.6% were non-susceptible to ertapenem. Broth microdilution assays on the 113 Carba-P *K. pneumoniae* revealed that 38.3% were resistant to colistin, and 5.6% to tigecyclin (Table 10).

		Susceptible (%)												
		Amikacin	Gentamicin	Ciproflox acin	Ceftazidime	Cefotaxime	Cefepime	Imipenem	Meropenem	Ertapenem	Aztreonam	Trim/Sulf	Colistin*	Tigecyclin*
E. coli	ESBL	94,8	61,7	9,8	10,6	0,8	14,9	100,0	100,0	98,1	6,3	43,8	-	-
	Carbapenemase-P	80,0	60,0	20,0	20,0	0,0	20,0	80,0	60,0	0,0	20,0	40,0	100,0	100,0
	Others	100,0	89,5	52,6	0,0	0,0	94,7	100,0	100,0	89,5	5,3	42,1	-	-
	Total	94,9	63,0	12,0	10,2	0,8	18,9	99,7	99,5	96,4	6,4	43,6	-	-
K. pneumoniae	ESBL	91,8	39,7	21,9	4,1	6,8	6,8	100,0	100,0	82,2	8,2	26,0	-	-
	Carbapenemase-P	16,8	48,7	0,9	0,0	0,0	0,0	3,5	3,5	0,0	0,0	25,7	61,7	94,4
	Others	66,7	88,9	44,4	0,0	66,7	77,8	100,0	88,9	55,6	77,8	55,6	-	-
	Total	47,2	47,2	10,8	1,5	5,6	6,2	44,1	43,6	33,3	6,7	27,2	-	-
P. mirabilis	ESBL	92,3	0,0	30,8	43,6	7,7	61,5	92,3	100,0	100,0	87,2	25,6	-	-
	Carbapenemase-P	-	-	-	-	-	-	-	-	-	-	-	-	-
	others	96,2	57,7	7,7	0,0	0,0	88,5	84,6	100,0	100,0	100,0	7,7	-	-
	Total	93,8	23,1	21,5	26,2	4,6	72,3	89,2	100,0	100,0	92,3	18,5	-	-

*susceptibilities to Colistin and Tygecyclin were determined by reference broth microdilution on Carbapenemase producers

Table 10. Percentage of antimicrobial susceptibility retained by the studied isolates.

Results of this cross-sectional survey define the current epidemiology of ESCR *Enterobacteriaceae* among inpatient and outpatient in Italy. In addition, compared to previous surveys (Luzzaro, 2006; Giani, 2013), this study provides an updated picture of prevalence, distribution, types of produced enzymes and susceptibility profiles of ESBL, carbapenemase and ampC producing enterobacteria. Overall, the design of the study was similar to those adopted in previous nationwide surveys but considering the magnitude of the problem, the sampling time was limited to a 15-days period and the survey was focused on *E. coli*, *K. pneumoniae* and *P. mirabilis*.

In this survey, the overall prevalence of ESCR Enterobacteria was 19.6%, 28.4% among inpatients and 12.3% among outpatients. Thus, compared to the 2003 survey, data show a high increase with proportion of resistant isolates that are three times higher in 2013 than in 2003 confirming a still expanding phenomenon that afflicts both hospital and community settings. In 2013 the proportion of ESCR isolates in community is higher than the proportion of ESBL-producing isolates in hospital detected in 2003 (12.3% vs. 7.4%).

In *E. coli*, ESCR account for 16.7% of isolates (25% for inpatients and 11.3% for outpatients) and was almost entirely due to ESBL production, with CTX-M-type enzymes present in the vast majority of resistant isolates (85%). CarbaNS was rare in *E. coli* (1.2% of all ESC *E. coli*) and was associated in 5/14 cases (35.7%) with carbapenemase production.

In *P. mirabilis* ESCR (accounted for 23.6% of isolates of which 25.7% from inpatients and 12.3% from outpatients) may be due to either ESBL or ACBL production (60% and 40% respectively), with ESBLs other than CTX-M-type and ACBLs mostly of the CMY/LAT lineage (96%). No CarbaNS *P. mirabilis* were detected.

In *K. pneumoniae* ESC-R was present in 28% of isolates (36.4% for inpatients and 13.8% for outpatients). ESC phenotype was due in part to the presence of an ESBL gene (35.8%) but in the majority of cases (58%) is associated to a carbapenemase gene (mostly of $bla_{\text{KPC-type}}$) that only in few isolates (9,7%) was superimposed to ESBL production. Carbapenem resistance is a problem that affected almost *K. pneumoniae* (90% of CarbaNS are *K. pneumoniae*).

The overall proportion of ESBL producing isolates compared to the 2003 survey, was higher (11.8% vs. 6.4%). As in 2003 *E. coli* was the most prevalent ESBL-producing species, while prevalence of ESBL positive *P. mirabilis* (the second most common species among ESBL producers in 2003) decreased (14.2% vs 22.3%) and ESBL positive *K. pneumoniae* increased (10.6% vs. 8.6%).

Concerning proportion among inpatient and outpatient, among outpatients the proportion of ESBL producers is increasing (10.6% vs 3.5% in 2006), in details proportion is higher in 2013 among *E. coli* (1.9% vs 10.9%) and *K. pneumoniae* (2.6% vs 7.7%) while comparable among *P. mirabilis* (14.4% vs 12.2%). The results of this study show that CTX-M prevalence is increasing (19.7% vs. 83.4%) among ESBLs producers and are nowadays the most common ESBLs (while in 2003 were TEM-type ESBLs). In particular, CTX-M-group 1 represent the most widespread ESBLs in *E. coli* and *K. pneumoniae*. CTX-M group 9 producers (proportion among CTX-M producers, 18.5%), not detected in 2003 survey, are increasing. (Mugnaioli, 2006; Luzzaro, 2006).

In this study, for the first time, the presence of acquired ampC-type genes was investigated. AmpC production was responsible for ESCR phenotype in few cases in *E. coli* and *K. pneumoniae* (3.6% and 1%, respectively) while was frequent among ESCR *P. mirabilis* (38.4%). To the best of our knowledge this is the first report of a DHA-1 producing *P. mirabilis* in Italy.

The proportion of CarbaNS isolates compared to 2011 survey showed an increase both in *E. coli* (0.06% vs. 0.6%) and in *K. pneumoniae* (11.9% vs. 18.7%). Most of CarbaNS isolates was KPC producers and KPC-KP continues to represent the major responsible of the CPE endemicity in Italy, while other species and carbapenemases remain in the

backstage. It is notable, among carbapenemase producers, the little increase of OXA-48-producers (4.2% vs 1.3%) and the decrease of VIM-type producers (2.5% vs 9.2%) compared to 2011 survey (Giani,2013) even if these differences are calculated basing on a limited number of isolates.

Interestingly the proportion of KPC-KP among outpatients has doubled in this study compared to 2011 survey (2.2% vs 4.6% $p<0.05$). Even if all except one KPC-KP were obtained from urine, which was by far the most common specimen, this represents an alarming phenomenon that underlines the increasing diffusion in the community of such resistant determinant, mirroring what has been described for CTX-M enzymes, and probably reflecting the colonization ability showed by KPC-KP that continues outside the hospital settings over the patient discharge (Giani, 2013).

3.4 Linezolid-resistant *cfr*-positive methicillin-resistant *Staphylococcus aureus* from a cystic fibrosis patient, first Italian case

Infections caused by methicillin-resistant *Staphyloccoccus aureus* (MRSA) remain a serious healthcare problem worldwide (Stryjewski, 2014). Linezolid represents one of the most important treatment options for MRSA infections (Kullar, 2015; Liu, 2011).

Linezolid-resistant MRSA (LR-MRSA) have been reported since the early 2000s (Tsiodras, 2001), but their prevalence has remained overall low (Gu, 2013; Cafini, 2015). In MRSA, linezolid resistance can be mediated by mutations in 23S rRNA genes or in the L3, L4 ribosomal proteins, or by acquisition of the *cfr* gene, encoding a 23S rRNA methylase (Munita, 2015). The latter mechanism is particularly worrisome since it is horizontally transferable and confers multiresistance to several anti-ribosomal drugs including phenicols, lincosamides, oxazolidinones, pleuromutilins, streprogramins A, and 16 membered-ring macrolides (Long, 2006).

The first LR-MRSA carrying the *cfr* gene was detected in the U.S.A. in 2005, from a cystic fibrosis (CF) patient (Locke, 2014). Thus far, *cfr*-positive LR-MRSA have been reported from several countries, as sporadic cases or even responsible of hospital outbreaks (Cai, 2015). Most recently, *cfr*-positive LR-MRSA isolates have been reported from CF patients in Spain (Caballero, 2015).

This study reports the first detection of a *cfr*-positive LR-MRSA in Italy, isolated from a CF patient.

S. aureus AOUC-0915 was isolated in September 2015 from the respiratory secretions of a 22-year-old CF patient. The patient had moved from USA to Italy in 2009, and had undergone several hospitalizations in Italy since 2009. During 2015 the patient had experienced exhacerbations of respiratory infections by MRSA (susceptible to linezolid) and *Burkholderia cepacia* complex (*Burkholderia multivorans*, genomovar II), and also an episode of sepsis caused by *B. multivorans*, and had received several antibiotics including Vancomycin, Trimethoprim-sulfamethoxazole, Ceftazidime, Meropenem, Daptomycin, Amikacin, Tetracycline and Minocycline. While at home, the patient had been given Trimethoprim-sulfametoxazole, Minocycline and Azythromycin to control MRSA. In July 2015 the patient reported to have taken Linezolid by self-medication, independent of medical prescription. To date, the patient is in good health and has not been re-hospitalized.

S. aureus AOUC-0915 was identified by MALDI-TOF (Vitek-MS, bioMérieux). Susceptibility testing, carried out by reference broth microdilution, revealed resistance to oxacillin (MIC, >2 mg/L), linezolid (MIC, 32 mg/L), clindamycin (MIC, >1 mg/L), erythromycin (MIC, >4 mg/L), levofloxacin (MIC, 16 mg/L), and susceptibility to ceftaroline (MIC, 0.5 mg/L), ceftobiprole (MIC, 1mg/L), vancomycin (MIC, 2 mg/L), daptomycin (MIC, 0.5 mg/L), rifampin (MIC, ≤0.06 mg/L), and fusidic acid (MIC, 1 mg/L).

To investigate the mechanisms of resistance and clonal lineage of AOUC-0915, the strain was subjected to whole genome sequencing (WGS) analysis using an Illumina MiSeq platform (Illumina Inc.) with a paired-end approach (2x300 bp). De novo assembly of WGS data was performed using the ABySS software (http://www.bcgsc.ca/platform/bioinfo/software/abyss). Genome analysis revealed that AOUC-0915 was ST5, spa-type T306 (CC5), and scc*Mec* II. A previous study in China found *cfr* gene in a S. aureus ST5 scc*Mec* II, but the linezolid resistance gene was plasmid located and presented a different genetic context (Cai, 2015).

Resistome analysis, using the Res finder program (Zankari, 2012), revealed the presence of a complex array of acquired resistance genes including *cfr*, *fexB*, *erm*(A), *erm*(B), *erm*(C), *mecA*, *tet*(M), *aad* and *spc* . WGS data also revealed the presence of a mutation in the L3 ribosomal protein gene leading to a G152D substitution, which has been associated with linezolid resistance (Klitgaard, 2015). No other mutations were detected in other ribosomal protein genes or in the rRNA genes. The *cfr* gene was flanked by two

IS*Enfa5* insertion sequences in a 5093 bp-context similar to that described in *Staphylococcus suis* S10 plasmid pStrcfr (Wang, 2013, accession no. KC844836).

Genome analysis revealed that AOUC-0915 was ST5 and spa-type T306 (CC5). AOUC-0915 was also positive for Panton-Valentine leukocidin (PVL) genes. S1 nuclease analysis followed by hybridization with a specific probe revealed a chromosomal location of *cfr* gene. Further studies are necessary in order to investigate the putative transposition event that brought to the integration of *cfr* gene in the chromosome.

5. CONCLUSIONS AND PERSPECTIVES

The increasing spread of multi-resistant bacteria in nosocomial setting and also in the community has raised the necessity of a prompt increase of surveillance and infection control measures. Unfortunately, Italian epidemiology is characterized by high levels of resistance in nosocomial setting for both Gram-positive and Gram-negative pathogens. In this study, the resistance determinants in clinical and environmental isolates of multi-resistant bacteria were characterized. For the mechanisms of particular relevance, the prevalence, the genetic context and the chromosomal or plasmidic location of resistance genes were analyzed.

Most of this PhD study was focused on the detection of emerging resistance determinants in both Gram positive and Gram-negative bacteria, with particular attention on carbapenemase producing *Enterobacteriaceae*.

The rapid increase nationwide of CPE (Giani, 2013), underlined the necessity of an active strategy of surveillance on CPE and the development of methods for the rapid screening of presence of the most common carbapenemases. Simultaneously, the constant monitoring of the emerging carbapenemases, which could be actually very rare or underestimated, but that could represent a risk for the future, is not less important.

In this PhD study, two novel Real Time PCR were validated for the detection of bla_{KPC}, $bla_{OXA\text{-}48\text{-}like}$, bla_{VIM}, bla_{NDM} carbapenemases and $bla_{CTX\text{-}M}$ (group 1, 2, 9, 8/25) genes, in order to create a rapid and cost-effective method for the screening of CPE and ESBL producers. Both local and national epidemiology of CPE was assessed. Data on the prevalence of ESBL and in particular of $bla_{CTX\text{-}M}$ genes among ESBL producers was lacking in Italy since 2006. No study of the national class C prevalence was made before.

Moreover in this study $bla_{OXA\text{-}372}$, a novel class D carbapenemase, found in a *Citrobacter freundii* from a hospital wastewater plant, was characterized. This finding is particularly important because this carbapenemase, highly divergent from any other previously described enzyme, is harbored in a transferable plasmid and presents an activity spectrum similar to the highly diffused OXA-48. Despite OXA-372-producing *Enterobacteriaceae* has never been found in clinical isolates to date, its finding in hospital wastewater is worrisome because it could promote its diffusion in the

environment. Further studies on the structure of the transferable *bla*$_{\text{OXA-372}}$-harboring plasmid will be interesting.

Moreover, in this study, for the first time in Italy,were identified *bla*$_{\text{NMC-A}}$ and *bla*$_{\text{IMI-2}}$ carbapenemases in two unrelated isolates of *E. ludwigii* both from surveillance rectal swabs. These rare carbapenemases are uncommon worldwide and rarely caused outbreaks, but their identification and recognition is important in order to limit their diffusion in Italian territory. The mechanism of integration in the chromosome of *Enterobacter cloacae* complex of *bla*$_{\text{NMC-A}}$ and *bla*$_{\text{IMI-1}}$ has been explained for the first time. On the other hand, *bla*$_{\text{IMI-2}}$ was found in an *E. ludwigii* on a transferable plasmid and this mobile genetic emelent could promote its dissemination. A rapid screening method should be developed in order to identify at low cost and in a short time these emerging carbapenemases.

As regards Gram-negative non enterobacteria pathogens, in this thesis the first case of VIM-1-producing bloodstream infection by *Aeromonas caviae* was described and also the first infection by double-MBL (IMP-19 and VIM-1) producing *P. aeruginosa*. All the results presented in this thesis give an update epidemiology of β-lactamase producers present in the Italian territory, with particular attention on the Careggi University Hospital epidemiology of CPE. Further studies for the investigation of the clonal relationship between the carbapenemase, ESBL and Ampc producers should be performed.

Finally, an MRSA isolate of *S. aureus* from a cystic fibrosis patient, isolated in September 2015, showing resistance to linezolid, was found positive for *cfr* gene. The diffusion of linezolid resistance mechanisms in MRSA isolates of *S. aureus* in Italy, is worrisome. Other linezolid resistance mechanisms (ribosomal protein mutations, *optrA* gene) in Gram-positive pathogens will be investigated (among *Staphylococcus* spp. and *Enterococcus* spp.) in order to determine the epidemiology of linezolid resistant isolates. Increasing infection control measures should be applied in order to limit the diffusion of this resistance determinant in our territory, and surveillance studies on the colonization by linezolid-resistant Gram-positive isolates should be made in the future.

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6. ACKNOWLEDGEMENTS

Prof. Gian Maria Rossolini.

All the researchers, co-workers, PhD students, and students of the Microbiology Laboratory in Florence and Siena.

My parents and family members.

My girlfriend Valeria Pellegrini.

7. ANNEXES

7.1 RELATED PUBLICATIONS

1. **Antonelli A, D'Andrea M M, Montagnani C, Bartolesi A M, Di Pilato V, Fiorini P, Torricelli F, Galli L, Rossolini G M.** 2016. Newborn bacteraemia caused by an *Aeromonas caviae* producing the VIM-1 and SHV-12 β-lactamases, encoded by a transferable plasmid. *Journal of Antimicrobial Chemotherapy*. 71(**1**):272-4.

2. **Antonelli A, D'Andrea M M, Di Pilato V, Viaggi B, Torricelli F and Rossolini G M.** 2015. Characterization of a novel putative Xer-dependent integrative mobile element carrying the *bla*$_{\text{NMC-A}}$ carbapenemase gene, inserted into the chromosome of members of the *Enterobacter cloacae* complex. *Antimicrobial Agents and Chemotherapy*. 59(**10**):6620-4.

3. **Antonelli A, D'Andrea M M, Vaggelli G, Docquier J-D, Rossolini G M.** 2015. OXA-372, a novel carbapenem-hydrolysing class D β-lactamase from a *Citrobacter freundii* isolated from a hospital wastewater plant. *Journal of Antimicrobial Chemotherapy*. 70(**10**):2749-56.

4. **Antonelli A, Di Palo D M, Galano A, Becciani S, Montagnani C, Pecile P, Galli L, Rossolini G M.** 2015. Intestinal carriage of *Shewanella xiamenensis* simulating carriage of OXA-48–producing *Enterobacteriaceae*. *Diagnostic Microbiology and Infectious Diseases.* Feb 2015; 82(**1**):1-3.

5. **Pollini S, Antonelli A, Venturelli C, Maradei S, Veggetti A, Bracco S, Rumpianesi F, Luzzaro F Rossolini G M.** 2013. Acquisition of plasmid-borne *bla*$_{\text{IMP-19}}$ gene by a VIM-1-positive *Pseudomonas aeruginosa* of the Sequence Type 235 epidemic lineage. *Journal of Antimicrobial Chemotherapy*. 68(**3**):722-4.*

*****This paper has been published before the PhD period

6.2 PUBLICATIONS PROVIDED ONLY FOR INFORMATION

1. **Meini S, Laureano R, Fani L, Tascini C, Galano A, Antonelli A, Rossolini G M.** 2015 . Breakthrough *Lactobacillus rhamnosus* GG bacteremia associated with probiotic use in an adult patient with severe active ulcerative colitis: case report and review of the literature. *Infection.* 43(**6**):777-81.

2. **Dani C, Coviello C, Corsini I, Arena F, Antonelli A, Rossolini GM.** 2015. *Lactobacillus* Sepsis and Probiotic Therapy in Newborns: Two New Cases and Literature Review. *American Journal of Perinatology Reports.* Epub.