UNIVERSITA' DEGLI STUDI DI VERONA

DEPARTMENT OF

DIAGNOSTICS AND PUBLIC HEALTH

GRADUATE SCHOOL OF

LIFE AND HEALTH SCIENCE

DOCTORAL PROGRAM IN

APPLIED LIFE AND HEALTH SCIENCE

WITH THE FINANCIAL CONTRIBUTION OF

Cariverona Foundation

Cycle XXIX / Year 2014

TITLE OF THE DOCTORAL THESIS

Evaluation of rapid carbapenemase producers detection method based on MALDI-TOF MS spectra analysis, and molecular and epidemiological characterization of multidrug-resistant *Enterobacteriaceae*

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Evaluation of rapid carbapenemase producers detection method based on MALDI-TOF MS spectra analysis, and molecular and epidemiological characterization of multidrug-resistant Enterobacteriaceae **– Anna Rita Centonze**

PhD Thesis

Verona, 4th July 2017

ISBN

In loving memory of my father and Toni.

In memory of Prof. Roberta Fontana with gratitude and affection.

SOMMARIO

L'emergere ed il rapido diffondersi di batteri resistenti agli antibiotici è stato un importante e crescente problema di salute pubblica a livello mondiale negli ultimi vent'anni.

Attualmente gli Enterobatteri resistenti ai *β*-lattamici, particolarmente quelli resistenti ai carbapenemici, rappresentano uno dei principali motivi di preoccupazione a livello internazionale, poiché la loro resistenza è spesso mediata da plasmidi e quindi facilmente trasmissibile e provocano epidemie nosocomiali con elevato tasso di mortalità.

Prioritario è che i laboratori di microbiologia clinica, soprattutto in ambito ospedaliero, identifichino rapidamente i pazienti colonizzati da ceppi batterici multiresistenti, particolarmente i produttori di carbapenemasi, così da limitarne la diffusione, prevenire episodi epidemici ed indirizzare rapidamente la scelta di una terapia appropriata.

La ricerca si muove, quindi, verso lo sviluppo di nuovi strumenti diagnostici che permettano di individuare rapidamente i ceppi batterici multiresistenti in campioni clinici, riducendo drasticamente il tempo di refertazione.

Obiettivo del nostro studio è stato validare l'utilizzo del sistema MALDI-TOF MS (Matrix Assisted Laser Desorption Ionization Time-of-Flight Mass Spectrometry) come metodo rapido per identificare Enterobatteri produttori di carbapenemasi, confermando la correlazione tra un caratteristico picco MS (11,109Da±8) e la produzione di *Klebsiella pneumoniae* carbapenemasi (KPC).

Sono stati ricercati, inoltre, altri picchi correlati alle carbapenemasi NDM (New Delhi metallo-*β*-lactamase) e VIM (Verona integron–encoded metallo-*β*-lactamase) e si è valutato l'utilizzo del sistema MALDI-TOF MS per individuare rapidamente focolai epidemici.

A questo scopo abbiamo utilizzato campioni clinici selezionati, produttori di KPC e campioni di controllo sensibili ai carbapenemici o resistenti a causa di altri meccanismi o per produzione di altri enzimi (NDM e VIM). La presenza del picco in esame nel 98% dei ceppi produttori di KPC (225/230) e l'assenza dello stesso

nei campioni di controllo, ha confermato la stretta correlazione tra produzione di KPC e presenza del picco di 11,109Da nello spettro MALDI-TOF MS.

Abbiamo, inoltre, verificato la possibilità di utilizzare la ricerca del suddetto picco durante la routine diagnostica, eseguendo l'analisi degli spettri MALDI-TOF MS su 183 campioni isolati durante lo screening MDR. La presenza del picco di 11,109Da negli spettri del 98% dei ceppi (129/132) con test Carba NP positivo, produttori di KPC, conferma la possibilità di utilizzare l'analisi degli spettri MALDI-TOF MS come metodo rapido di screening dei ceppi produttori di carbapenemasi, economico ed adatto ad investigare un elevato numero di campioni. In questo modo si possono riservare i più costosi e complessi metodi molecolari solo ai ceppi che mancano del picco pur essendo positivi ai test fenotipici.

Non abbiamo, invece, individuato altri picchi correlati alla produzione di carbapenemasi diverse dalla KPC (NDM e VIM), analizzando gli spettri di 15 ceppi di Enterobatteri produttori di NDM e 23 ceppi di Enterobatteri e 13 di *P. aeruginosa* produttori di VIM. Questo risultato sembra essere in linea con la molteplicità di plasmidi che portano i geni che codificano per questi enzimi.

Infine l'utilizzo della MALDI-TOF MS per determinare la clonalità di ceppi batterici non è risultato particolarmente promettente, rispetto alla Pulsed-Field Gel Electrophoresis (PFGE) con cui l'abbiamo comparata, utilizzando 44 ceppi di *K. pneumoniae* isolati durante un'epidemia. Il problema potrebbe essere dovuto al software attualmente in uso.

Il sistema MALDI-TOF MS, si conferma, quindi, uno strumento valido per identificare rapidamente ed in modo accurato ceppi batterici produttori di carbapenemasi, il che rappresenta un importante passo avanti nella diagnostica clinica.

ABSTRACT

The occurrence and rapid spreading of multi-resistant bacteria is an important issue of public health, which is increasing worldwide over the last two decades.

β-lactams-resistant *Enterobacteriaceae*, with particular reference to carbapenems, are actually one of the main concerns at international level, as their resistance is often plasmid-mediated, thus rapidly spread and often associated with hospital outbreaks with high rates of mortality.

Clinical microbiology labs, especially in hospital settings, require to quickly identifying patients who carry multi-resistant bacterial strains, especially carbapenemase producers, in order to contain their spreading, preventing epidemic outbreaks and rapidly address proper pharmacological therapy.

Research is moving towards development of new diagnostic tools to speed up identification of multi-resistant strains in clinical specimens, thus drastically shortening the time necessary to obtain analysis reports. Rapid identification of colonized patients, in fact, is the only valid strategy to curb epidemic spreading, mainly when is due to plasmid-mediated resistant determinants.

The objective of this study is to validate the use of Matrix Assisted Laser Desorption Ionization Time-of-Flight Mass Spectrometry (MALDI-TOF MS) for quick identification of carbapenemase-producing Enterobacteria strains, thus confirming that a characteristic MS peak (11,109Da±8) is related to *Klebsiella pneumoniae* carbapenemase (KPC) production.

Moreover, the presence of other peaks related to NDM- (New Delhi metallo-*β*-lactamase) and VIM- (Verona integron–encoded metallo-*β*-lactamase) carbapenemases as well as the evaluation of MALDI-TOF MS as a tool to quickly identify epidemic hotbeds, were investigated.

At this aim, selected clinical samples KPC-producing and control samples carbapenem-susceptible or carbapenem-resistant through other resistance mechanisms or producing hydrolytic enzymes other than KPC (NDM and VIM) were analyzed.

The presence of the 11,109Da peak in 98% (225/230) of the KPC-producing strains compared to the controls, which lacked the peak, confirmed strong correlation between KPC production and the presence of the 11,109Da peak in MALDI-TOF MS spectrum.

The reliability of the method was also verified by searching for this specific peak during routine workflow, analysing the MALDI-TOF MS spectra of 183 patient samples isolated during multidrug-resistant (MDR) screening. The positive correlation between the presence of 11,109Da peak and Carba NP test confirmed the reliability of MALDI-TOF MS analysis as rapid and inexpensive screening method for carbapenemase-producing strains, apt to investigate a high number of samples in KPC-endemic context. In this way, the molecular methods more expensive and difficult to perform during routine workflow, can be used to resolve discrepant strains only.

We did not find, instead, any correlation between other peaks and carbapenemases other than KPC, namely NDM and VIM, through the analysis of the spectra of 15 NDM-producing Enterobacteria strains and 23 Enterobacteria strains and 13 *P. aeruginosa* strains VIM producers. This result is in line with the high number of plasmids harboring the genes codifying for these enzymes.

Finally, the use of MALDI-TOF MS was found not particularly promising as tool to identify clonal relationship between bacterial strains as compared to Pulsed-Field Gel Electrophoresis (PFGE) using 44 *K. pneumoniae* strains isolated during an epidemic outbreak. Drawbacks could be related to the current software.

We therefore confirmed MALDI-TOF MS system is a good tool to quickly and accurate identifying carbapenemase-producing bacterial strains, that represents effective advancement in clinical diagnostic.

CONTENTS

1. Antibiotics

Antibiotics are compounds of natural origin produced by microorganisms, used to treat and prevent bacterial infections. Since their introduction, the occurrence of many illnesses and the number of deaths from infectious diseases has been greatly reduced with a consequent extension of life expectancy.

In nature, the role of molecules with antibacterial activity is not fully understood. Bacteria produce antibacterial compounds that allow survival and provide competitive advantage against other microorganisms. Effectiveness depends on the level of concentration. At high concentration, they have killing effects against bacteria whilst at sub-lethal concentrations they have growth-inhibitory effects. At sub-minimum inhibitory concentrations, however, these small signal molecules intervene in chemical communication, as happen in the quorum sensing [1], they can be modulators for gene transcription [2] or function as pheromones that stimulate bacterial conjugation. [3]

The use of natural substances to fight illnesses was already known two thousand years ago in Egypt, Greece and China. However, it is the discovery of penicillin by Sir Alexander Fleming in 1928 that marks the beginning of the antibiotic era. [4] Chemotherapy takes its roots from the work of Paul Herlich (1854-1915), who advanced the idea of selective toxicity. It becomes a real science in 1935, when Dogmack launched Prontosil, a sulphanilamide derivative for the treatment of streptococcal infections. In the late 30s Chain purified and stabilized penicillin, allowing its clinical use which began in a massive way during the Second World War. A further milestone is represented by Wakeman's 1944 discovery of streptomycin which was later used for tuberculosis treatment. [5] When the industrial production of antibiotics started, it was thought that the battle against infectious diseases was won. However, as early as 1945, Alexander Fleming reported the existence of penicillin-resistant microorganisms and raised the alarm regarding antibiotic overuse. [6] Thereafter, antibiotic resistance quickly became a serious clinical concern.

From the late 1960s through the early 1980s new antibiotics were looked for in order to solve the problem. Tetracyclines, chloramphenicol, cephalosporins, carbapenems were marketed but it was soon realized that the development of resistance is faster than the discovery of new antibiotics. As a result, over a few decades, resistance to all antibiotics that have been developed was observed. [Fig. 1] The idea that synthetic molecules such as fluoroquinolones can be immune to the development of resistance, had failed.

Fig. 1 Developing Antibiotic Resistance: A Timeline of key events based upon early reports of resistance in the literature. (From: CDC-Centers for Disease Control and Prevention) [7]

Based on the huge number of microorganisms and still unknown chemical compounds existing in nature, new antibiotics could be developed by using bioengineering and new genetic methods. [8] However, the processes are not necessarily economically beneficial for pharmaceutical industries and investments in this field are limited. There are very few compounds with new mechanisms of action under development and this represents a particularly evident problem for the treatment of infections caused by multidrug-resistant Gram-negative bacteria. [3] [9] [10] Since the year 2000, five new classes of antibiotics have been marketed: oxazolidinones (Linezolid), lipopetides, pleuromutilins, tiacumicins and diarylquinolines, but they are all active only against Gram-positive bacteria. [11] All other antibacterial compounds have just been variations of the existing ones, and the emergence of resistance does not spare these either.

Antibiotics are classified into different chemical classes and defined according to their target of action: a) cell wall synthesis, b) protein synthesis, c) DNA or RNA synthesis, d) metabolic pathway, e) cell membranes. [Fig. 2]

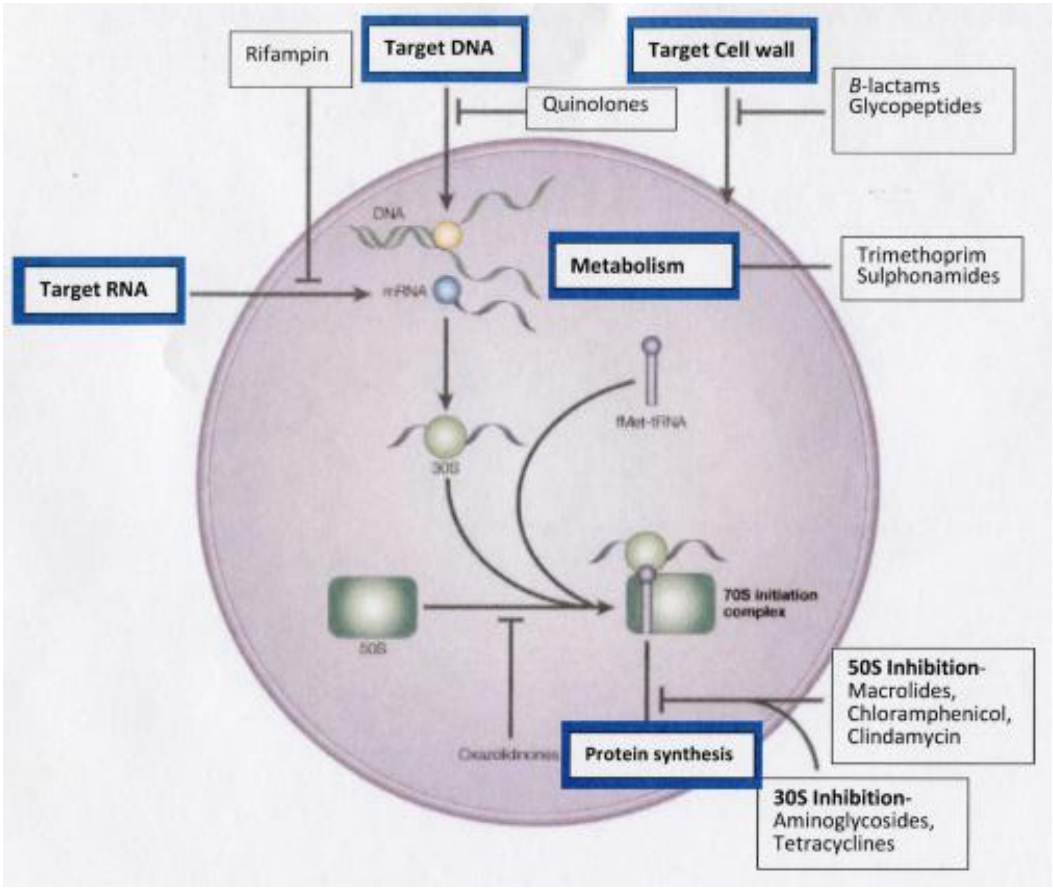


Fig. 2 Mechanisms of action of antibiotics: Inhibition of cell-wall synthesis, DNA and RNA synthesis, protein synthesis and metabolic pathways. (Modified from Coates *et al.* 2002) [12]

They are defined as bactericidal when they cause bacterial death and bacteriostatic if they inhibit cell growth.

a) **Antibiotics that affect cell wall synthesis** are the class of antibiotics most widely used. They have a bactericidal effect since they induce cell death by inhibition of the peptidoglycan biosynthesis, the major constituent of the bacterial cell wall. The structural integrity of the cell envelop is damaged and the microorganism cannot survive.

Cell wall synthesis is inhibited by *β*-lactams (penicillins, cephalosporins, carbapenems, monobactams) and glycopeptides. The *β*-lactams inhibit the transpeptidases enzymes, known as penicillin binding proteins (PBP) preventing the peptidoglycan units cross-links. Glycopeptides prevent transglycosylase and transpeptidase activity binding with peptidoglycan units. [13]

• Penicillins (penicillin, oxacillin, methicillin, amoxicillin, etc.) have a common backbone that is 6-amino-penicillanic acid [Fig. 3] with various acyl radicals. Benzilpenicillin or Penicillin G is a natural compound produced by mould *Penicillium chrysogenum.* Penicillins are active against only a narrow spectrum of Gram-positive bacteria, e.g. *S. pyogenes*, *S. pneumoniae*, Enterococci and sensitive-strains of *S. aureus*, because they fail to penetrate the outer membrane of most Gram-negatives. Semisynthetic drugs such as aminopenicillins were developed later with activity also against some Gram-negative bacteria (*Neisseria*, *Haemophilus*), piperacillin is also active against *Pseudomonas aeruginosa* and methicillin is active against *β*-lactamase-producing strains. [5]

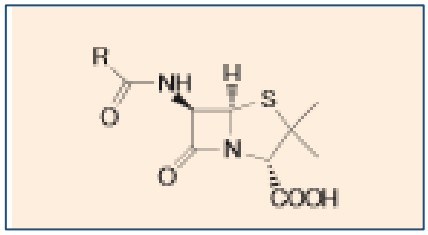


Fig. 3 Penicillins chemical structure.

• Cephalosporins (cephalothin, cefuroxime, cefotaxime, ceftazidime, cefepime, etc.) have the 7-aminocephalosporanic acid as common backbone. [Fig. 4] The first compound isolated from *Cephalosporium acremonium* was

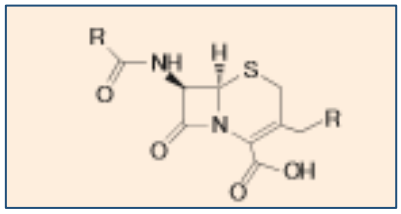


Fig. 4 Cephalosporins chemical structure.

Cephalosporin C. In 1964, the clinical use of cephalothin began. Then other semisynthetic compounds were developed and classified as generations I to V. Compared to penicillins, these compounds have a wider activity against Gram-positive and Gram-negative bacteria. Moreover, IV generation cephalosporins are active also against *P. aeruginosa.* Cephalosporins are the most clinically prescribed class because of their strong activity and high tolerability. [5]

• Carbapenems (imipenem [Fig. 5], ertapenem, doripenem, meropenem) are derivatives of thienamycin, an antibiotic produced by *Streptomyces cattleya*. They are active against a broad spectrum of bacteria, including Gram-positive and Gram-negative, aerobes, anaerobes bacteria and *P. aeruginosa*. This very important group represents the last resort in treatment of infections caused by bacteria resistant to other *β*-lactams and are used in a wide range of severe infections including bloodstream infections, hospital-acquired, complicated urinary and respiratory infections, bone and soft tissue infections, obstetric and gynecologic infections. [5]

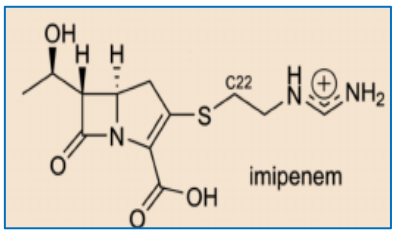


Fig. 5 Imipenem chemical structure.

• Monobactams are monocyclic *β*-lactams isolated from *Chromobacterium violaceum* [Fig. 6]. Only Aztreonam, a synthetic derivative, is approved and marketed. It is active only against Gram-negative and aerobic bacteria. [5]

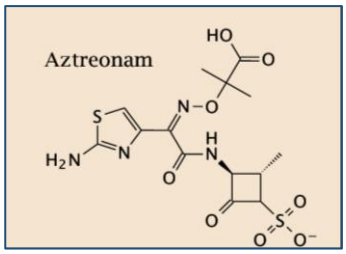


Fig. 6 Aztreonam chemical structure.

• Glycopeptides (vancomycin, teicoplanin) are active against Gram-positive bacteria, aerobe and anaerobes. Vancomycin [Fig. 7] was the first compound of this class to be isolated from *Amycolatopsis orientalis* and patented for clinical use against penicillin-resistant *S. aureus*. Teicoplanin was isolated in 1978 from *Actinoplanes teichomyceticus*, its activity is similar to that of vancomycin. [5]

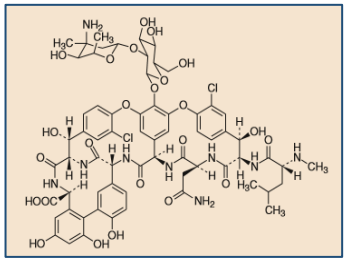


Fig. 7 Vancomycin chemical structure.

b) **Antibiotics that inhibit protein synthesis** interact with ribosomal subunits. They include aminoglycosides (amikacin, gentamicin, kanamycin, etc.) and tetracyclines [Fig. 8] that are able to inhibit ribosome subunit 30S by the alteration of the complex mRNA/aminoacyl-tRNA at the ribosome, causing mismatching and protein mistranslation, or blocking the access of aminoacyl-tRNAs to the ribosome. Protein synthesis inhibitor also include macrolides (azithromycin, clarithromycin, erythromycin, etc.), chloramphenicol, lincosamides (clindamycin) and oxalidinones (Linezolid) [Fig. 9] that interact with 50S subunit blocking initiation of protein translation or translocation of peptidyl-tRNAs. [13]

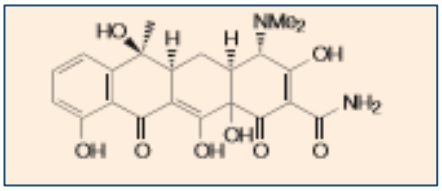


Fig. 8 Tetracycline chemical structure.

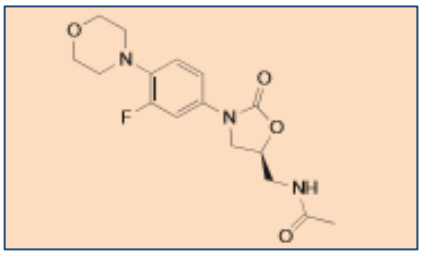


Fig. 9 Linezolid chemical structure.

Most of these compounds, e.g. tetracyclines and aminoglycosides are produced by *Streptomyces* species or related soil bacteria.

• Aminoglycosides are bactericidal compounds active against Gram-negative bacteria and some compounds have additive or synergistic activity with other *β*-lactams also against Gram-positive cocci. [5]

• Tetracyclines, Chloramphenicol, Lincosamides are bacteriostatic, active against Gram-positive and Gram-negative bacteria.

c) **Antibiotics that interfere with the nucleic acid synthesis** are fluoroquinolones (ciprofloxacin [Fig. 10], levofloxacin, moxifloxacin, norfloxacin, etc.) and rifamycins (Rifampin). They are bactericidal with broad antibacterial spectrum.

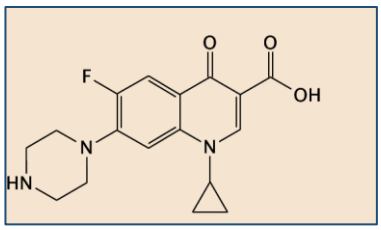


Fig. 10 Ciprofloxacin chemical structure.

• Fluoroquinolones target DNA gyrase and topoisomerase IV, enzymes involved in DNA transcription and replication. They are derivatives of nalidixic

acid introduced in 1960s to treat urinary infections. Their broad spectrum of activity, particularly against Gram-negative bacteria, makes their use very common for treatment of urinary, respiratory, gastrointestinal, and sexually transmitted infections. [13]

• Rifamycins target RNA polymerase which performs RNA synthesis. The first compound was isolated from *Streptomyces mediterranei* in 1950. They are active against many bacterial species and are used in the treatment of tuberculosis. [13]

d) **Antibiotics that interfere with metabolic pathways**: sulfonamides are among the first antibiotics used in the 1930s. Their action interferes with folic acid synthesis. Usually it is used combined with trimethoprim that increases its activity in urinary tract infection, but it is also used in malaria treatment or against *Pneumocystis*.

e) **Antibiotics that act on bacterial cell membranes** have a lower selective toxicity than the previous target and their action can affect eukaryotic cell also.

• Daptomycin, produced by *Streptomyces roseosporus* is able to permeabilize membranes containing phosphatidylglycerol, a lipid plentiful in the bacteria cell membranes but not in human cell membranes. It is active against only Gram-positive bacteria, because it fails to penetrate the outer membrane of Gram-negatives.

• Polymyxin B, produced by Gram-positive bacterium *Bacillus polymyxa*, an antibiotic known for a long time, no longer used because of its toxicity, but now it has reappeared and is being used for the treatment of multi-resistant Gram-negative bacteria, such as *P. aeruginosa*, *Enterobacteriaceae*, *Acinetobacter* and Colistin (Polymyxin E) product of *Bacillus colistinus.* These compounds are able to penetrate into the bacteria cell membranes and disrupt them by interaction with phospholipids. They are also able to interact with lipopolysaccharide by interfering with endotoxin action. [5]

2. Antibiotic resistance

2.1 Causes and effects

After a first enthusiastic period in which everyone thought the fight against infectious diseases was won and when research and production of antibiotic compounds increased, the truth about resistance appeared and it turned out to be a great threat.

Antibiotic resistance is a natural phenomenon. Because antibiotics are natural compounds produced by microorganisms, bacteria encounter them regularly and over millions of years they have adapted to these drugs, making them less effective. [8] Also, it is natural that antibiotic-producing bacteria carry gene encoding for resistance to the antibiotics that they produce and it is hypothesized that many resistance genes, principally encoding for antibiotic hydrolyzing enzymes, for example extended spectrum β-lactamase CTX-M [10], but also target-protecting enzymes, or efflux-pumps, may originate from Actinomycetes. [14] Furthermore, it was observed that often resistance genes are arranged on bacterial chromosome in a cluster with genes for biosynthesis of antibiotics. [15]

We define intrinsic resistance, that of microorganisms which do not have an antibiotic target or of a bacterial cell which is impermeable to antibiotics. That is, species-specific and independent from antibiotic misuse. For example, the cell wall structure of Gram-negative makes them resistant to hydrophobic molecules such as macrolides, that can't pass through outer membrane. [16]

Bacteria can also show an acquired resistance when they become resistant through mutation [17] or acquisition of genetic elements, after exposition to antibiotic. Acquired resistance can be passed by vertical transmission to daughter cells during binary division or by horizontal gene transfer, in this case the transmission is also possible between different species of bacteria. Gene exchange for conjugative transmission is frequent in nature much more than mutations, a very

infrequent event (about 1 per 10^7 to 10^{10} cells), [10] probably used to regulate communication and molecules signalling production. [1]

Resistance genes can encode for various resistance strategies. Resistance to one specific agent corresponds to resistance to a whole class and cross-resistance to other classes with the same action mechanism. If several different mechanisms are present they result in Multidrug-resistance (MDR), or Extensively-drug resistance (XDR) until Pandrug-resistance (PDR) when all antibiotics are ineffective. [18] β-lactams, glycopeptides and fluoroquinolons are the classes most affected by resistance.

The transmission of resistance genes and the spread of antibiotic resistance witnessed an acceleration with the beginning of large scale clinical use of antibiotics and especially with their incorrect use, such as unnecessary prescription; or purchase without prescription; improper dosage and duration; empirical use of wide spectrum drugs; massive use in livestock and the environment. [8] Antibiotic use at sub-inhibitory and sub-therapeutic concentrations, in fact, can kill sensitive bacteria and select resistant strains that then multiply. Moreover, it increases the virulence and the interaction with host immune response. Also, genetic modifications are promoted and changes in the expression of bacterial resistance genes. [3] [17]

Imprudent use of antibacterial products, also sold for hygienic or cleaning purposes and the overuse of antibiotics as growth promoters in livestock increases the problem for the mobilization of resistance genes from environmental and animal's bacteria to commensal and pathogenic strains in humans through water and food. [16] [19] Therefore we use the concept of *resistome*, which is the set of genes that are directly or indirectly involved in antibiotic resistance. [10] [20]

The idea that antibiotic resistance is a new phenomenon, therefore, is not true. Genes encoding resistance to β-lactams, tetracyclines and glycopeptides antibiotics were found in permafrost 30,000 years-old. [20] [21] Resistance is a natural evolution enhanced by misuse of antibacterial, horizontal gene transfer, and increasing human connections around the world. These factors amplify the

phenomenon and increase the global spread of resistant strains, leading to dangerous epidemics for which there is no effective treatment, thus increasing the awareness of the problem. [19]

Development of synthetic molecules does not solve the problem either as their wide and prolonged use leads anyway to the development of resistance even if less quickly at the beginning. This discourages investment in research, development and marketing of new drugs.

Resistant bacteria grow in the presence of antimicrobial substances at concentrations that usually kill or inhibit their growth. Drugs become ineffective and this leads to reduced options for treatment and to longer and more severe illness and an increase in mortality rates. In this way, previously controllable common infections and diseases, again become potential killers, especially in hospitals. Units such as Intensive care (ICU), hematology, neonatology, or transplantation care are exposed to greater risk and have a two-fold mortality rate. [Tab. 1] [22] [23]

The problem is clinical, with high rates of morbidity and mortality that can reach 50-80%, but it also has a high economic impact due to the high costs of prolonged hospitalization, prolonged treatments, the use of more expensive drugs, increase in the need for screening tests and the need for greater investment in new treatments, diagnosis and prevention.

Tab. 1 Mortality attributable to antibiotic-resistant bacteria (From WHO) [24]

Risk of Death is Higher in Patients Infected with Resistant Strains

		Deaths (%)		
	Outcome (number of studies included)	Resistant	Not resistant	RR (95% CI)
***Escherichia coli* resistant to:**				
3rd gen. cephalosporins	Bacterium attributable mortality (n=4)	23.6	12.6	2.02 (1.41 to 2.90)
Fluoroquinolones	Bacterium attributable mortality (n=1)	0	0	
***Klebsiella pneumoniae* resistant to:**				
3rd gen. cephalosporins	Bacterium attributable mortality (n=4)	20	10.1	1.93 (1.13 to 3.31)
Carbapenems	Bacterium attributable mortality (n=1)	27	13.6	1.98 (0.61 to 6.43)
***Staphylococcus aureus* resistant to:**				
Methicillin (MRSA)	Bacterium attributable mortality (n=46)	26.3	16.9	1.64 (1.43 to 1.87)

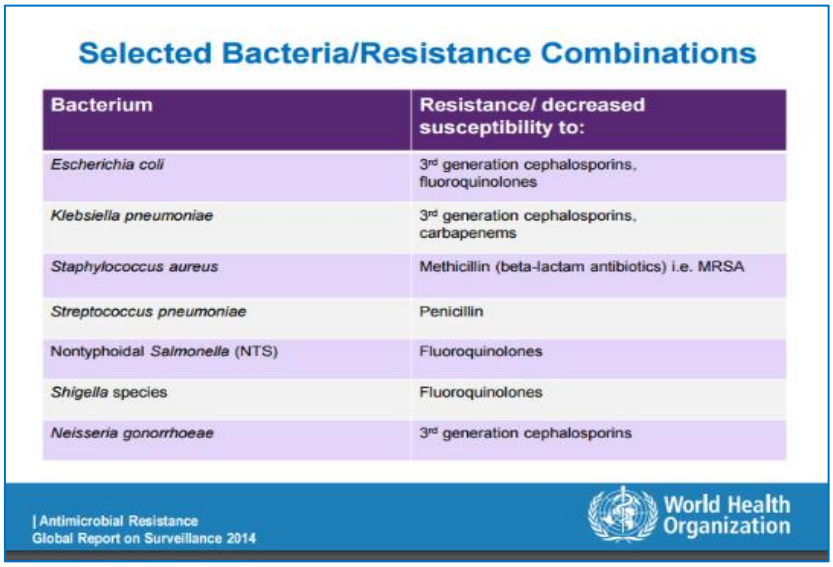
| Antimicrobial Resistance
Global Report on Surveillance 2014

World Health Organization

2.2 The size of the problem

At this point, it is clear that antibiotic resistance is a widespread phenomenon. In 2013, the Centers for Disease Control and Prevention (CDC) declared that the human race is now in the "post-antibiotic era". [7] In 2014, World Health Organization (WHO) recognized that antimicrobial resistance is a global public health crisis, and published a "Global report" to show the situation of antimicrobial resistance in the world, related to tuberculosis, HIV, malaria, influenza and nine selected bacteria. [Tab. 2] [24]

Tab. 2 Combination bacteria/antibiotics focused on WHO Global report 2014 [24]



Selected Bacteria/Resistance Combinations

Bacterium	Resistance/ decreased susceptibility to:
Escherichia coli	3rd generation cephalosporins, fluoroquinolones
Klebsiella pneumoniae	3rd generation cephalosporins, carbapenems
Staphylococcus aureus	Methicillin (beta-lactam antibiotics) i.e. MRSA
Streptococcus pneumoniae	Penicillin
Nontyphoidal *Salmonella* (NTS)	Fluoroquinolones
Shigella species	Fluoroquinolones
Neisseria gonorrhoeae	3rd generation cephalosporins

The real extent of the problem, however, is not known, because not all countries carry out active surveillance on antibiotic-resistant bacteria and routinely surveillance is only for severe infections. Even then data collection is very limited. Of 194 countries, 129 (66%) returned information to WHO for this survey, with the largest gaps in Africa, the Middle East and European countries outside the European Union. [22] [24] [Tab. 3]

Tab. 3 Countries that provide information to WHO on availability of national data on resistance for nine bacteria/antibacterial drug resistance combinations (From Global Report 2014 WHO) [24]

	WHO region						Total
	AFR	AMR/ PAHO[a]	EMR	EUR[a]	SEAR	WPR	
No. of Member States returning information (%)	27/47 (57%)	21/35 (60%)	11/21 (52%)	42/53 (79%)	9/11 (82%)	19/27 (70%)	129/194 (66%)
Returned data set(s)/ no. of Member States (%)	23/47 (49%)	21/35 (60%)	7/21 (32%)	38/53 (74%)	6/11 (55%)	19/27 (70%)	114/194 (59%)
Responded "No national data available"	4	–	4	4	3[b]	0[c]	15
No information obtained for this report	20	14	10	11	2	8	65

AFR, African Region; AMR/PAHO; Region of the Americas/Pan American Health Organization; EMR, Eastern Mediterranean Region; EUR, European Region; SEAR, South-East Asia Region; WPR; Western Pacific Region.

a. To avoid duplicate data collection, ECDC, European Centre for Disease Prevention and Control and AMRO forwarded data already collected in their existing surveillance networks.
b. One country responded there was no national data compilation but still returned data.
c. Two countries responded there was no national data compilation but still returned data.

As for morbidity, disability adjusted life years, mortality, length of hospital stay, cost of care, related to antibiotic-resistant infections, there are only estimates for Europe, USA and the rest of the World published by European Centre for Disease Prevention and Control (ECDC), CDC and WHO. [Fig. 11] [7] [22] [25] [26]

CDC estimate that each year in the USA, about 2 million people acquire infections with antibiotic-resistant bacteria, and 23,000 people die as direct result of these infections. Many others die from conditions complicated by antibiotic resistant infection. [7] ECDC reports that each year, about 25,000 patients die in Europe, from an infection with multidrug-resistant bacteria and these infections count for an extra 2.5 million hospital days. [9]

To obtain information about the costs associated with antibiotic resistant versus antibiotic sensitive pathogens, is very difficult and few economic studies have been carried out. [22] The economic burden of antibiotic resistance was estimated to be at least 55 billion dollars in the USA in 2000 [23] [27] and EUR1.5 billion in Europe in 2007. [25] For the USA, productivity losses are estimated to be 64% of the total estimated 55 billion dollars, whereas for Europe, the estimate is 40% of the total estimated EUR1.5 billion. [7]

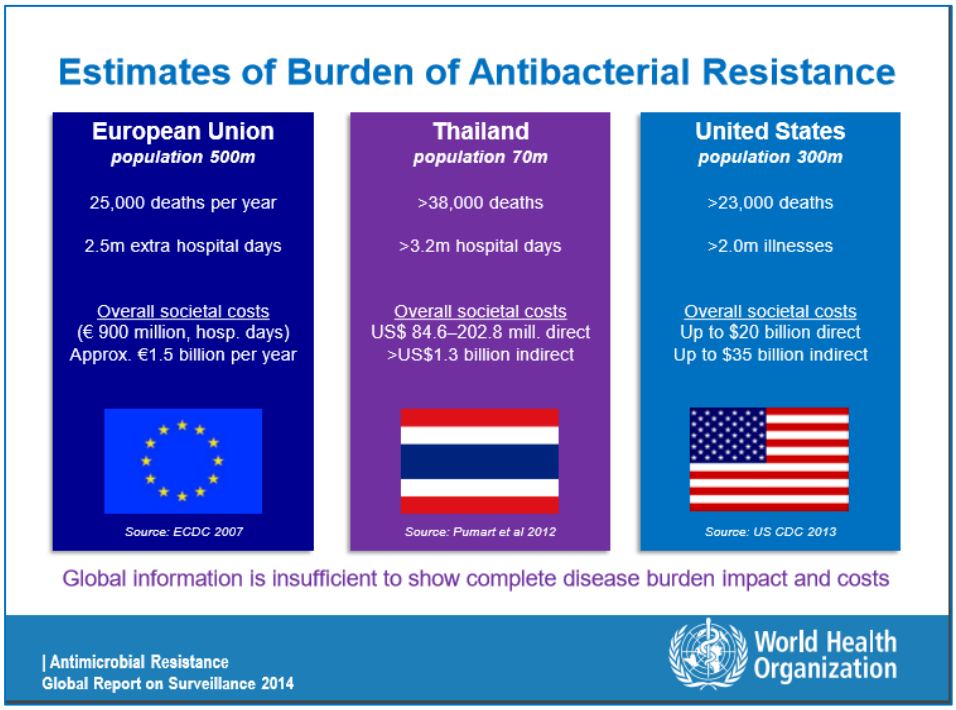


Fig. 11 Estimates of deaths, hospital days and cost for European Union, Thailand and the USA. (From WHO) [24]

The Italian National Health Institute (ISS) published a report on antibiotic resistance surveillance from 2005 to 2008, which shows a stable trend for Gram-positive and an increasing trend for Gram-negative bacteria, especially for fluoroquinolones, aminopenicillins and aminoglycosides in *E. coli* e *K. pneumoniae/oxytoca*, with differences between North and South. [28] [29]

The ECDC report confirms Italy has one of the highest resistance rates in Europe, with an increasing trend in the last years especially for Gram-negative bacteria and high percentages of invasive infections. [25] [26] [28] [29] [Tab. 4] [Tab. 5]

Tab. 4 Antibiotic resistance trend in Italy from 2003 to 2013 (Data ECDC) [26]

Antibiotic resistance from 2003 to 2014

Table 2: Annual percentage (%) of antimicrobial non-susceptible and resistant isolates, 2003–2014

Microorganism by antimicrobial group	2003	2004	2005	2006	2007	2008	2009	2010	2011	2012	2013	2014
Streptococcus pneumoniae												
Penicillin R	5	5	5	<1	4	3	3	5	6	6	9	4
Penicillin RI	13	14	9	7	15	10	6	9	7	12	15	15
Macrolides RI	37	29	31	33	31	26	21	29	27	34	25	29
Staphylococcus aureus												
Oxacillin/meticillin R	39	40	37	38	34	34	37	37	38	35	36	34
Escherichia coli												
Aminopenicilins R	52	53	55	56	58	62	63	64	67	68	66	65
Aminoglycosides R	10	9	11	8	14	14	13	15	18	21	18	19
Fluoroquinolones R	25	28	28	27	32	38	36	39	41	42	42	44
Third-generation cephalosporins R	6	5	8	7	11	16	17	21	20	26	26	29
Carbapenems R	–	–	–	–	<1	<1	<1	<1	<1	<1	<1	<1
Enterococcus faecalis												
Aminopenicilins RI	4	4	4	4	4	13	20	13	11	4	4	10
HL gentamicin R	39	36	38	38	39	47	49	50	50	51	46	55
Vancomycin R	2	2	3	3	2	2	3	2	3	1	1	<1
Enterococcus faecium												
Aminopenicilins RI	80	78	77	86	73	64	60	70	83	87	82	83
HL gentamicin R	44	39	36	48	53	49	52	59	54	62	59	57
Vancomycin R	24	21	19	18	11	6	4	4	4	6	4	8
Klebsiella pneumoniae												
Aminoglycosides R	–	–	8	26	25	28	19	29	35	42	45	49
Fluoroquinolones R	–	–	11	23	27	28	20	39	46	50	54	56
Third-generation cephalosporins R	–	–	20	33	35	39	37	47	46	48	55	56
Carbapenems R	–	–	–	1	1	2	1	15	27	29	34	33
Pseudomonas aeruginosa												
Piperacillin + tazobactam R	–	–	–	23	20	20	24	21	22	30	31	31
Ceftazidime R	–	–	–	20	25	24	16	18	16	26	24	25
Carbapenems R	–	–	–	21	27	33	31	22	21	25	26	25
Aminoglycosides R	–	–	–	32	29	30	29	23	18	30	27	24
Fluoroquinolones R	–	–	–	36	35	36	42	31	26	31	29	28
Acinetobacter spp												
Fluoroquinolones R	–	–	–	–	–	–	–	–	–	86	83	92
Aminoglycosides R	–	–	–	–	–	–	–	–	–	83	83	89
Carbapenems R	–	–	–	–	–	–	–	–	–	82	80	90

Tab. 5 Details about invasive infections caused from antibiotic resistant bacteria (Data ECDC) [25]

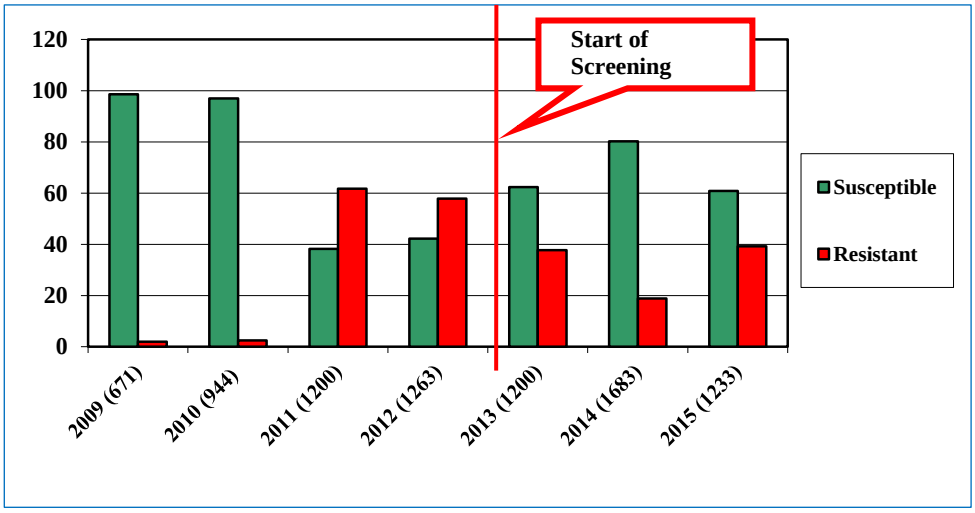
Demographic characteristics

Table 3: Selected details on invasive isolates reported for 2012 and 2013

Characteristic	*S. pneumoniae*		*S. aureus*		*E. coli*		*E. faecalis*		*E. faecium*		*K. pneumoniae*		*P. aeruginosa*	
	% total	% PNSP	% total	% MRSA	% total	% FREC	% total	% VRE	% total	% VRE	% total	% 3GCRKP	% total	% CRPA
Isolate source														
Blood	86	12	100	36	100	42	100	1	100	5	99	52	98	25
CSF	14	24	–	–	<1	33	–	–	–	–	1	81	2	50
Gender														
Male	46	11	48	36	44	47	41	0	48	6	46	50	46	21
Female	36	9	33	37	42	34	22	1	30	3	29	43	27	22
Unknown	18	29	19	32	14	53	37	3	22	6	25	68	27	36
Age (years)														
0–4	2	43	1	25	1	11	1	0	1	0	1	24	1	21
5–19	2	20	1	23	<1	28	0	0	–	–	<1	83	<1	14
20–64	14	14	15	24	12	40	14	1	13	4	15	57	16	34
65 and over	35	12	29	38	33	41	37	2	24	4	30	51	28	21
Unknown	47	14	54	38	54	44	47	1	62	6	53	52	54	26
Hospital department														
ICU	9	11	11	42	6	38	20	2	19	8	21	67	20	36
Internal med.	33	7	39	37	38	41	33	0	32	4	30	46	26	19
Surgery	2	22	15	37	14	45	11	1	18	3	15	52	16	20
Other	55	18	36	32	42	43	36	2	31	5	35	49	38	26

PNSP: penicillin-non-susceptible *S. pneumoniae*; MRSA: meticillin-resistant *S. aureus*; FREC: fluoroquinolone-resistant *E. coli*; VRE: vancomycin-resistant *E. faecalis* or *E. faecium*, 3GCRKP = third-generation cephalosporin-resistant *K. pneumoniae*; CRPA = carbapenem-resistant *P. aeruginosa*.

Also in Verona hospital, the number of multi-resistant strains is increasing. During the period 2009-2015 we observed a general increase of resistant strains isolated. For example, carbapenem-resistant *K. pneumoniae* strains isolated in our hospital, were 1.94% in 2009, and arose 61.76% in 2011, dropped to 39.2% in 2015, after the beginning of the screening program in 2013 [Graph. 1]



Graph.1 Meropenem-resistant *K. pneumoniae* strains isolated in Verona hospital between 2009 and 2015

2.3 Action plan

All national and international agencies recognize antibiotic resistance as a complex problem that concerns all nations and requires immediate global measures.

In the last few years many resolutions and recommendations have been proposed and numerous reports have been written. All agree on the need for prudent use of antibiotics, prevention of infections and development of new drugs, and everybody recognize the crucial role of health care workers in preserving the efficacy of antibiotics.

In May 2015, the World Health Assembly adopted a resolution to develop a global action plan [30] to ensure prevention and treatment of infectious diseases using safe and effective drugs. The resolution further recommended that national

and international organizations should take measures to contain the spread of antibiotic-resistant bacteria.

The aims of the global plan are:

1. *To improve awareness and understanding of antimicrobial resistance.*
2. *To strengthen surveillance and research.*
3. *To reduce the incidence of infection.*
4. *To optimize the use of antimicrobial medicines.*
5. *To ensure sustainable investment in countering antimicrobial resistance* [30]

In collaboration with other International Organizations (FAO, OIE), the WHO promote the enhancement of national action plans based on this global plan. The aim being to control antibiotic-resistance with a coordinated approach for an appropriate use of antibiotics in human health, animal health, food and agriculture. It also called for the implementation of a surveillance net and the promotion of economic investment in research for development of diagnostic tools and new antibiotic compounds. [22] In Europe ECDC and national government promote meeting, Technical Advisory Committee (TAC) for the European Antibiotic Awareness Day, report and recommendations. [11] [31] [32] [Fig. 12]

Fig. 12 Action to contain antibiotic resistance. [32]

3. The threat of carbapenem-resistant *Enterobacteriaceae*

In the last two decades, the emergence and spread of carbapenem-resistant *Enterobacteriaceae* (CRE) have become a significant clinical and public health concern.

The CDC assessed hazard levels in infections caused by antibiotic resistant bacteria based on: incidence; 10-year projection of incidence; clinical impact; availability of effective antibiotics; transmissibility; economic impact and barriers to prevention, classifying the biggest threats for the United States as "urgent," "serious," or "concerning". [Fig. 13] [33]

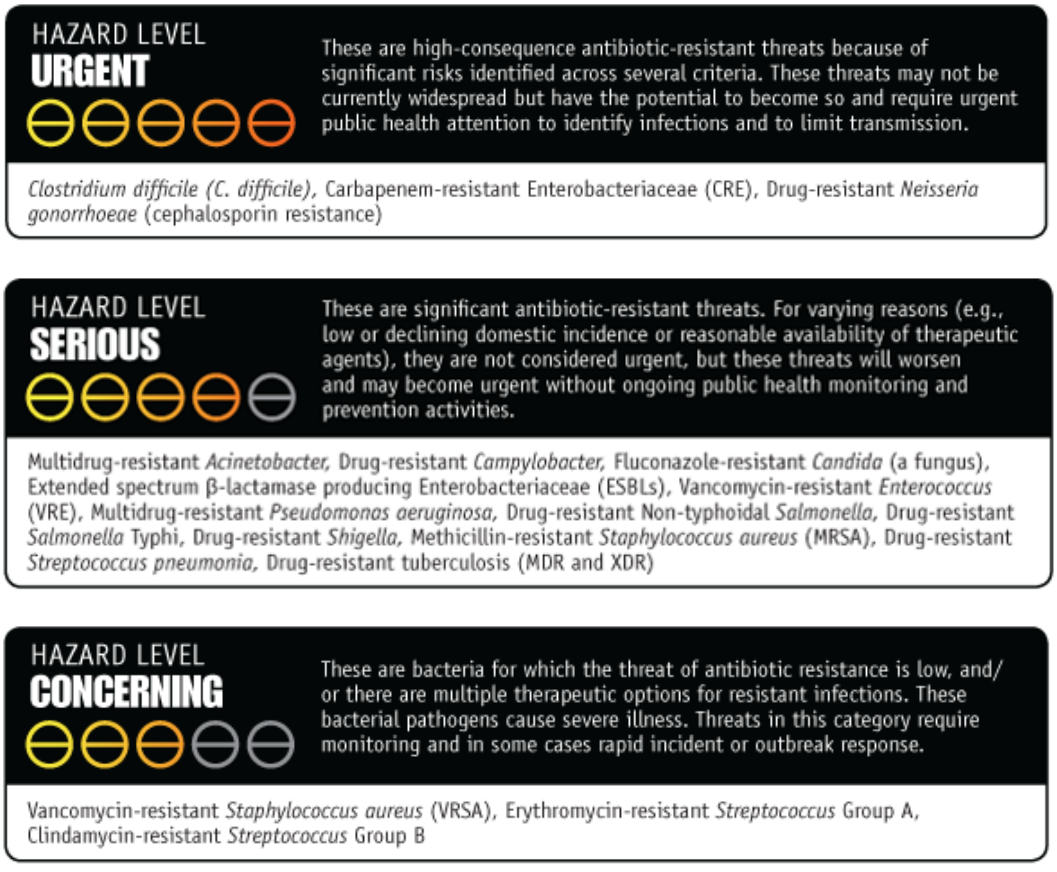


Fig. 13 Hazard level of infections caused by antibiotic-resistant bacteria. (From CDC) [33]

At hazard level "urgent" together with *Clostridium difficile*, and cephalosporin-resistant *Neisseria gonorrhoeae* there are carbapenem-resistant

Enterobacteriaceae, whereas extended spectrum *β*-lactamase (ESBL) producer *Enterobacteriaceae* are at level "serious".

Enterobacteriaceae are Gram-negative bacteria colonizers of the gastrointestinal tract, present also in animals and in the environment. They are also common agents of community-acquired and hospital-acquired infections. *E. coli* for example, is the most frequent urinary tract infection agent, and is also frequently involved in peritonitis, bloodstream infections, neonatal meningitis and food-borne infections. *K. pneumoniae* is causative agent of lower respiratory tract infections, pneumonia, cystitis, pyelonephritis, device associated-infections and bloodstream infections. It is also a major cause of hospital-acquired infections especially in neonatology and in intensive-care units, where there are the most vulnerable patients and the spread is facilitated by the extensive use of antibiotics and extensive use of invasive procedures. [26]

The usual treatment for infections caused by Enterobacteria are fluoroquinolones and cephalosporins especially for urinary tract infections. [5] The extensive use of these antibiotics leads to a high level of resistance and consequently, increased use of carbapenems, with emerging resistance also for these antibiotics.

The strains resistant to carbapenems, are often resistant to nearly all antibiotics and the therapeutic options are limited to combination therapy or older antimicrobial agents such as polymyxins and fosfomycin, although resistance to colistin has also begun to be reported. [26]

The big clinical impact, of carbapenem-resistant infections, with prolonged hospitalization, frequent outbreaks in hospital, high mortality rate [22] [34] [35] and the easiness of transmission, makes us understand why they are classified as an urgent public health threat, not only in the USA, but also globally.

Antibiotic resistance in Enterobacteria, especially *K. pneumoniae* is a public health problem of increasing importance also in Europe. The ECDC report shows that in the years 2010-2014, as *K. pneumoniae* and *E. coli* bacteria resistant to third-generation cephalosporins, ESBL-producing, as strains resistant to cephalosporins,

fluoroquinolones and aminoglycosides have increased significantly in Europe, [26] from 17.7% in 2012 to 18.6% in 2015 for *K. pneumoniae* and from 4.9% in 2012 to 5.3% in 2015 for *E. coli*. [36] Also, carbapenem resistance has increased in *K. pneumoniae* whilst it is still rare and stable in *E. coli*, with percentages of <0.1 % reported by the majority of countries. [26] [36]

There is a large difference in percentage of resistance in European countries, for both *E. coli* and *K. pneumoniae*, with values higher in both southern and eastern Europe. [Fig. 14-16]

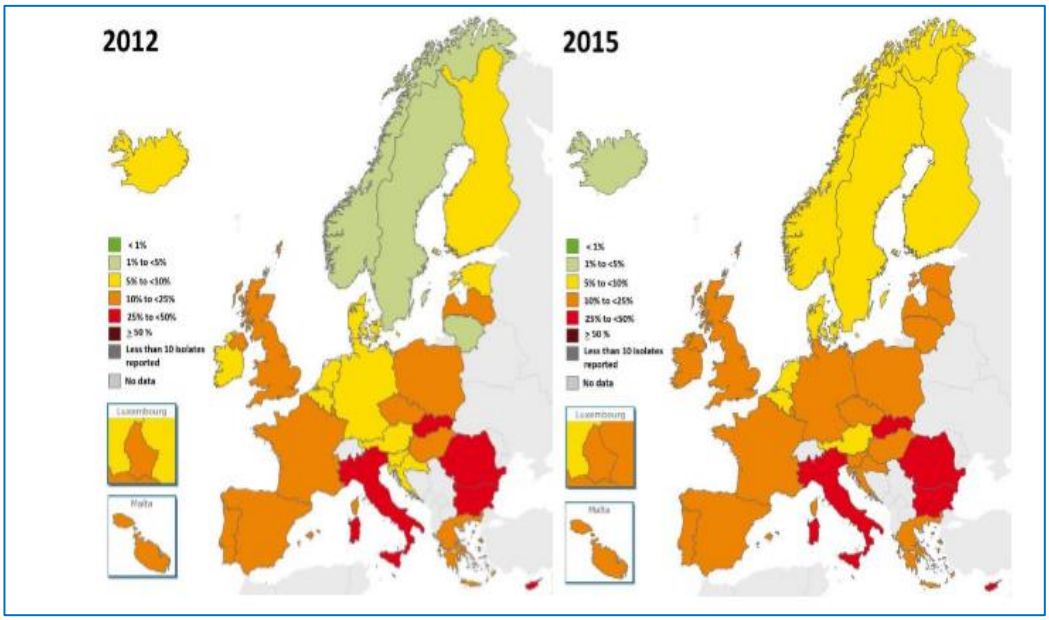


Fig. 14 Percentages of resistance to III generation cephalosporins for invasive isolates of *E. coli* in 2012 and 2015 in European Union (EU)/European Economic Area (EEA). (From ECDC) [36]

Romania, Italy and Greece have the highest percentages of carbapenem-resistant *K. pneumoniae* (31.5%, 32.9% and 62.3% respectively) [Fig. 17] often combined with colistin resistance and resistance to all drugs in use. [26] In Italy the carbapenem resistance has increased from 1.3% in 2006-2008 to 15% in 2010 and 32.9% in 2014. [29]

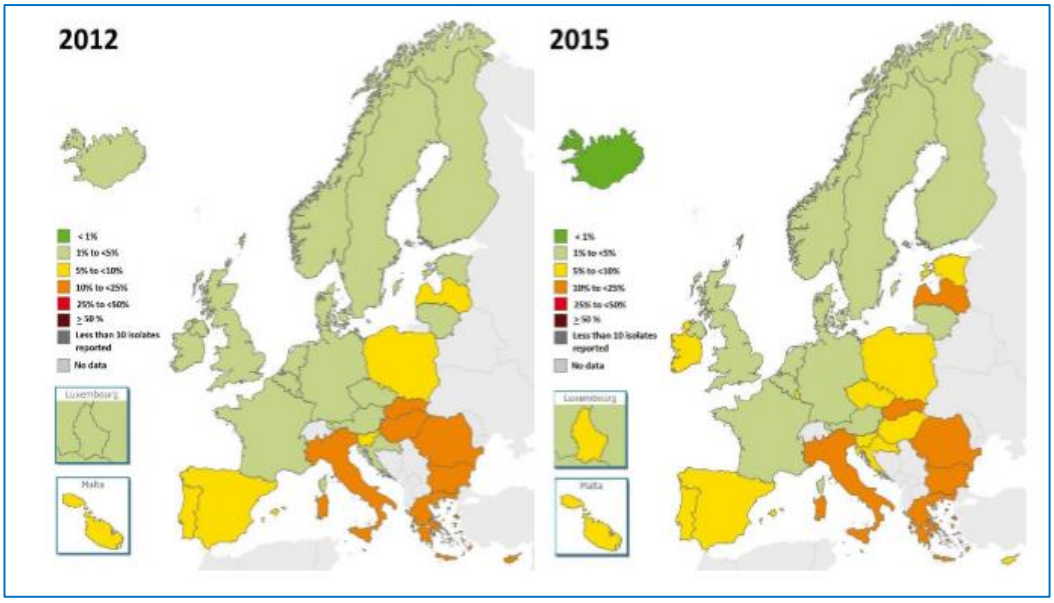


Fig. 15 Percentages of combined resistance to III generation cephalosporins, fluoroquinolones and aminoglycosides for invasive isolates of *E. coli* in 2012 and 2015 in EU/EEA. (From ECDC) [36]

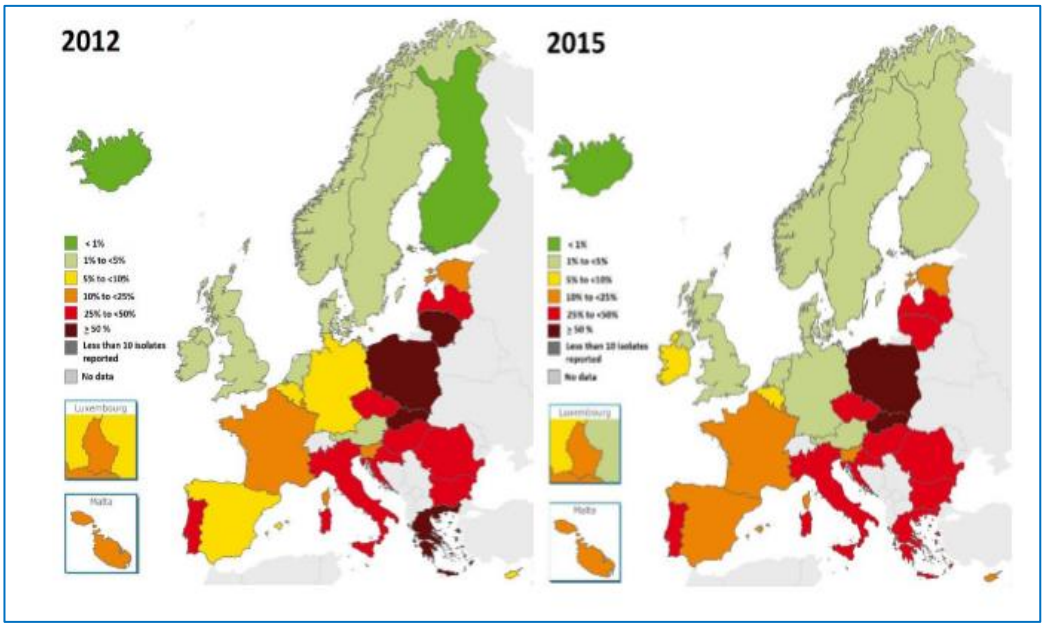


Fig. 16 Percentages of combined resistance to III generation cephalosporins, fluoroquinolones and aminoglycosides for invasive isolates of *K. pneumoniae* in 2012 and 2015 in EU/EEA. (From ECDC) [36]

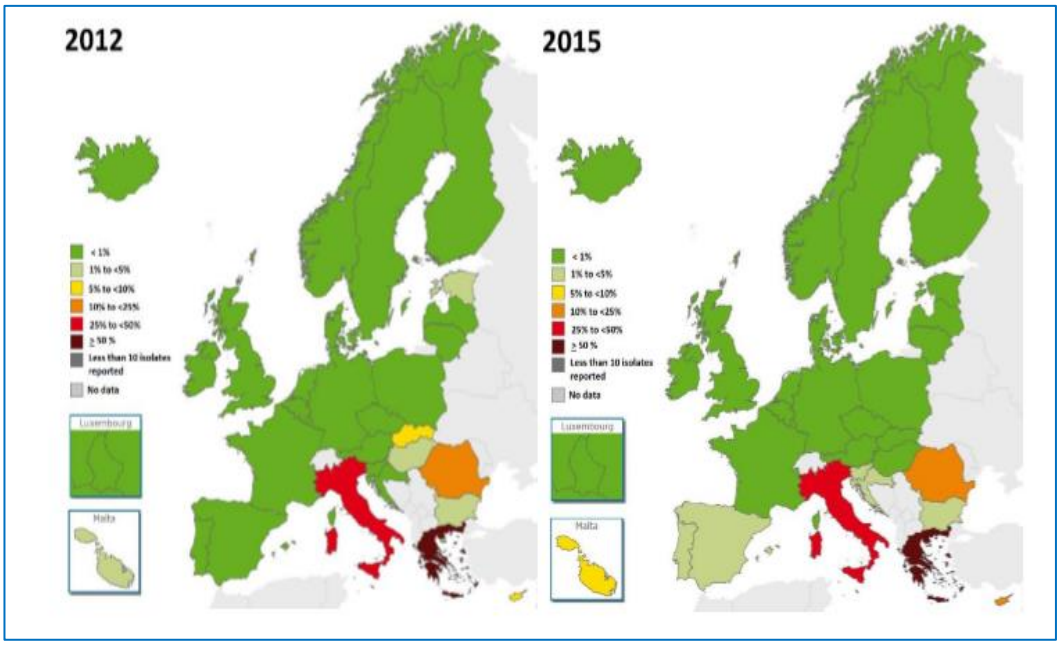


Fig. 17 Percentages of resistance to carbapenems for invasive isolates of *K. pneumoniae* in 2012 and 2015 in EU/EEA. (From ECDC) [36]

These data are cause for great concern, because this resistance is due to the production of hydrolyzing enzymes encoded by genes located on plasmids and therefore easily transferable.

Rapid diagnosis with measures to contain diffusion have become the most important strategies.

4. Mechanisms of resistance

The biochemical basis of resistance mechanisms evolved by bacteria are:

1. Modification of the antibiotic target
2. Alteration of the cell membrane permeability
3. Overexpression of efflux-pumps
4. Degradation of antibiotic by enzymes
5. Overproduction of the target and bypass of the inhibited metabolic pathway [Fig. 18]

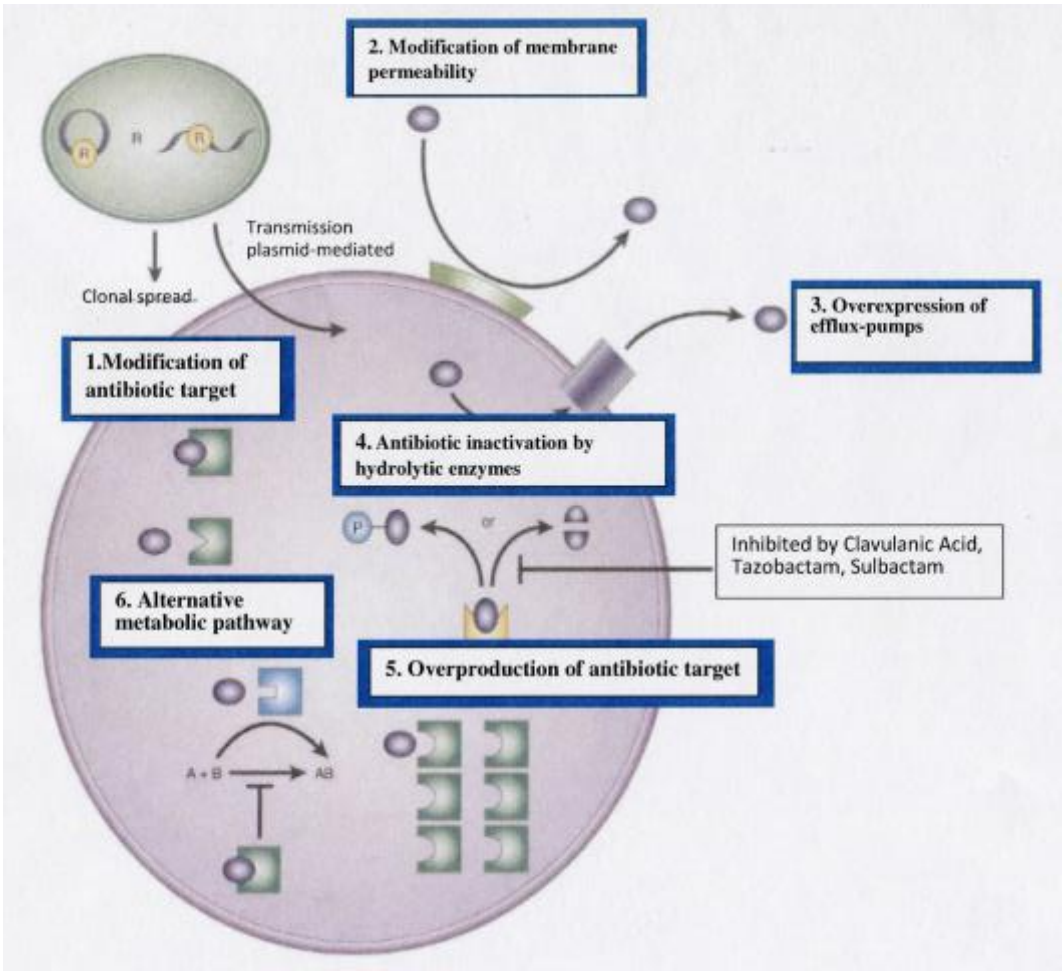


Fig. 18 Mechanisms of resistance to antibiotics evolved by bacteria. 1) Modification of antibiotic target; 2) Modification of membrane permeability; 3) Overexpression of efflux-pumps; 4) Antibiotic inactivation by hydrolytic enzymes; 5) Overproduction of antibiotic target; 6) Alternative metabolic pathway. (Modified from Coates *et al*. 2002) [12]

All mechanisms of resistance are an evolution of bacteria to survive antibiotic action. There are three ways for this evolution: point mutation can occur in a gene encoding for a hydrolyzing enzyme or an antibiotic target. Rearrangements of bacterial genome by integrons, transposons or insertion sequences that result in activation of new resistance genes can occur. Finally, Horizontal Genes Transfer can occur, that is the acquisition of external resistance genes plasmid-mediated, bacteriophages-mediated or by naked DNA sequences. The resistance to carbapenems by hydrolyzing enzymes is an important example of plasmid-mediated resistance. [5]

Plasmids are the main way for resistance-genes to spread. They are small extrachromosomal genetic elements that can auto-replicate and move to another cell belonging to the same species or to different species and genera. They carry much information including resistance-genes, virulence factors or metabolic characteristics. Often plasmids can allocate transposons or other genetic elements transposable. Transposons are also important elements for resistance spreading. [5] Several resistance genes can be linked on the same plasmids and transfer simultaneously with consequent multidrug-resistance.

Resistance through modification of the antibiotic target can occur through the alteration of enzymes targeted by *β*-lactams, the PBPs, thus developing a lower affinity for antibiotics as in *S. aureus* methicillin-resistant. A modification in peptidoglycan reduces affinity for glycopeptides, and an alteration of DNA gyrase causes resistance to fluoroquinolones. Finally, alterations of ribosomal binding sites result in resistance to tetracyclines, macrolides, lincosamides, streptogramins, and aminoglycosides. [5]

Resistance to *β*-lactams, aminoglycosides and carbapenems through alteration of the cell membrane permeability occurs in Gram-negative bacteria when they lose porins. These are proteins that form channels that allow hydrophilic molecules of antibiotics, for example *β*-lactams, to cross the outer membrane. [5]

Overexpression of efflux-pumps is a mechanism that reduces the intracellular accumulation of antibiotics. It is involved in the resistance to tetracyclines in Gram-negative bacteria, to *β*-lactams in *P. aeruginosa* and to

macrolides and streptogramins in streptococci and staphylococci. Moreover, efflux-pumps cause resistance to fluoroquinolones in staphylococci and enteric bacteria. There are four families of these pumps that use H^+ antiporters as energy source: major facilitator superfamily (MFS); resistance nodulation and cell division (RND); staphylococcal multi-resistance (SMR); multidrug and toxic compound extrusion (MATE). ATP-binding cassette (ABC), that bacteria use to eliminate toxic substances and survive in an unfavorable environment, such as enteric tract, use ATP as energy source for active transport. [5]

Antibiotic degradation by enzymes is the most common mechanism confering resistance to *β*-lactams. These enzymes called *β*-lactamases, are excreted extracellularly by Gram-positive or in the periplasmic space by Gram-negative bacteria and break the *β*-lactam ring of antibiotics. They are encoded by *bla* genes, either chromosomal or located on mobile genetic elements, such as plasmids, often linked to other resistance genes in integrons.

β-lactamases are numerous, differentiated by chemical structure and spectrum of activity. For example, penicillinases and cephalosporinases hydrolyze a limited number of antibiotics; extended-spectrum *β*-lactamases (ESBL), inactivate nearly all *β*-lactams including third-generation cephalosporins; carbapenemases can inactivate all or nearly all *β*-lactams. [5] ESBL (TEM, SHV, CTX-M, OXA groups) especially created concern in the ‘90s, when they became very common in Enterobacteria, primarily *E. coli* and *K. pneumoniae*. [37] TEM and SHV are inhibited by clavulanic acid, and CTX-M more so by tazobactam, which are used in combination with antibiotics (for example amoxicillin-clavulanic acid, piperacillin-tazobactam). CTX-M are the most prevalent in all countries. [5] [38]

Ambler classified *β*-lactamases in four classes: A, B, C, D. The classes A, C and D have an active site with a serine residue; class B have a zinc ion at the active site and are called metallo-*β*-lactamases. [Tab. 6]

Tab. 6 Classification of *β*-lactamases by Ambler (From Mandell) [5]

CLASS	ACTIVE SITE	ENZYME TYPE	SUBSTRATES	EXAMPLE
A	Serine	Penicillinases:		
		Broad-spectrum	Benzylpenicillin, aminopenicillins, carboxypenicillins, ureidopenicillins, narrow-spectrum cephalosporins	PC1 in *Staphylococcus aureus* TEM-1, SHV-1 in *Escherichia coli, Klebsiella pneumoniae*, other gram-negative bacteria
		Extended-spectrum (β-lactamase)	Substrates of broad-spectrum plus oxymino-β-lactams (cefotaxime, ceftazidime, ceftriaxone) and aztreonam	In Enterobacteriaceae: TEM-derived, SHV-derived, CTX-M–derived; PER-1, VEB-1, VEB-2, GES-1, GES-2, IBC-2 in *Pseudomonas aeruginosa*
		Carbapenemases	Substrates of extended-spectrum plus cephamycins and carbapenems	KPC-1, KPC-2, KPC-3 in *K. pneumoniae;* NMC/IMI, SME family
B	Metallo-β-lactamases (Zn^{2+})	Carbapenemases	Substrates of extended-spectrum plus cephamycins and carbapenems	NDM-1 in Enterobacteriaceae, IMP, VIM, GIM, SPM, SIM lineages in *P. aeruginosa, Acinetobacter* spp.
C	Serine	Cephalosporinases	Substrates of extended-spectrum plus cephamycins	AmpC-type enzymes in Enterobacteriaceae, *Acinetobacter* spp.
D	Serine	Oxacillinases:		
		Broad-spectrum	Aminopenicillins, ureidopenicillin, cloxacillin, methicillin, oxacillin, and some narrow-spectrum cephalosporins	OXA-family in *P. aeruginosa*
		Extended-spectrum	Substrates of broad-spectrum plus oxymino-β-lactams and monobactams	OXA-derived in *P. aeruginosa*
		Carbapenemases	Substrates of extended-spectrum plus cephamycins and carbapenems	OXA-derived in *Acinetobacter* spp.

AmpC, ampicillin C; CTX-M, cefotaxime-M; GES-1, -2; Guyana extended-spectrum β-lactamase-1, -2; GIM, German imipenemase; IBC-2, integron-born cephalosporinase; IMI, imipenem hydrolyzing; IMP, imipenem; KPC-1, -2, -3, *K. pneumoniae* carbapenemase-1, -2, -3; NDM-1, New Delhi metallo-β-lactamase-1; NMC, not metalloenzyme carbapenamase; OXA, oxacillin; PC1, penicillin 1; PER-1, *Pseudomonas* extended resistance-1; SHV-1, sulfhydryl variable-1; SIM, Seoul imipenemase; SME, *Serratia marcescens* extended-spectrum β-lactamase; SPM, Sao Paulo metallo-β-lactamase; TEM-1, Temoneira-1; VEB-1, -2, Vietnam extended-spectrum β-lactamase-1, -2; VIM, Verona integron-encoded metallo-β-lactamase.

The resistance phenotypes corresponding to ESBL-producing strains can be various, often the resistance is low and consequently, detection can be difficult.

The use of carbapenems to fight ESBL-producing bacteria lead to the appearance of carbapenemase-producing strains that are the major concern currently. They belong to class A (KPC) and class B *β*-lactamases (NDM, VIM, IMP).

Beside *β*-lactamases, there are other enzymes inactivating antibiotics, such as O-adenylyl transferases that modify aminoglycosides or N-acetyltransferases that modify chloramphenicol reducing their affinity for target. [5]

Overproduction of the target and bypass of the inhibited metabolic pathway are the mechanisms that confer resistance to sulphonamides and Trimethoprim. [5]

5. Carbapenemases

Amongst the mechanisms of resistance in Gram-negative bacteria, carbapenemases production is very concerning. Their emergence replaced that of ESBL, the main concern during late '90s. [37] Immediately carbapenemases showed themselves to be even more dangerous for different reasons. Indeed, they hydrolyze all *β*-lactams including carbapenems, that are otherwise the last resort in many infections; also, carbapenemase genes are transferable so their resistance emerges and spreads rapidly; finally, these genes are often associated with other resistance genes leading to multidrug-resistance.

Carbapenemases are numerous, encoded by chromosomal or plasmidic genes. The most relevant are KPC, a serine-*β*-lactamase of class A; NDM, VIM, IMP that are metallo-*β*-lactamase of class B, and OXA-48 belonging to oxacillinases of class D, others are for example, IMI, GES, SME, SPM, GIM, other OXA group. [39] Several carbapenemases can coexist in the same bacterial strain.

The carbapenemase-producing strains are mainly *K. pneumoniae* and *E. coli*, but also other *Enterobacteriaceae* such as *Enterobacter sp*., *Citrobacter sp*., *Proteus sp*. Also, other bacteria families, such as Pseudomonadaceae have been reported producing these enzymes.

5.1 KPC

The first KPC-producing strain (*K. pneumoniae*-carbapenemase) was isolated in North Carolina (USA) in 1996, [40] from here it quickly diffused to the New York area [41] and to the whole of the United States, [42] afterwards to Israel [43] and the rest of the world. [44] [Fig. 19] At the moment, KPC is endemic in many countries outside the USA, including Italy [26] where the first case was reported in 2008, [45] Greece and China. [46]

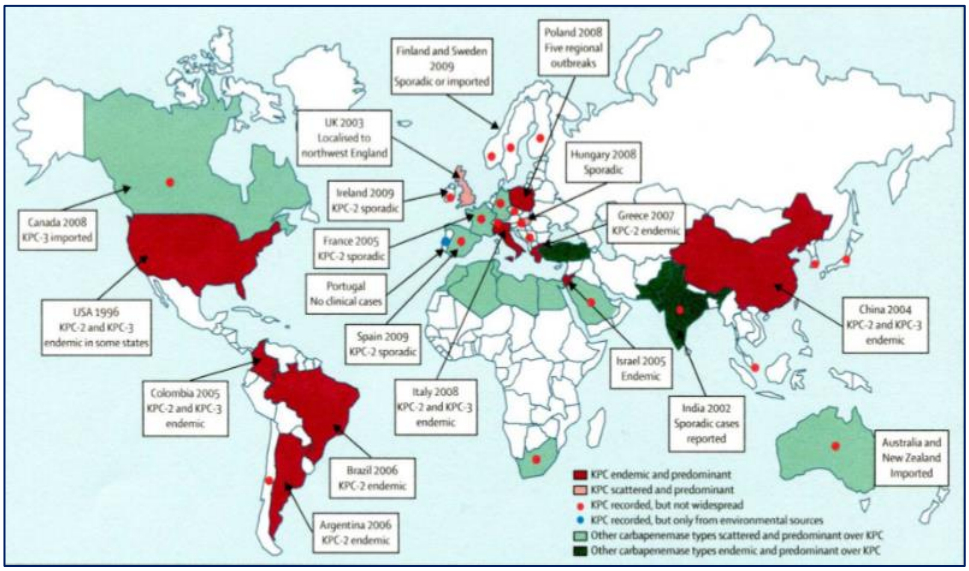


Fig. 19 First isolation and distribution of KPC by countries. [47]

At the moment, 22 variants of this enzyme are known. [48] [49] Among these, we first isolated the variant 19 in 2014 in three *K. pneumoniae* strains. [50]

The most widespread and characterized ones are KPC-2 and KPC-3 that confer very high resistance. Indeed, strains harboring these genes are susceptible only to gentamicin, colistin and tigecycline. Therefore, bloodstream infections caused by KPC-producing strains have high mortality rate. [47] [51]

KPC genes are often associated to transposon Tn4401, [52] [53] [Fig. 20] a Tn3-type transposon capable of inserting itself into plasmids of Gram-negative bacteria that belongs to several incompatibility groups. [54] [55] The plasmid most involved in KPC-3 spreading is pKpQIL of incompatibility group IncFII-like. [53] [56] [Fig. 20] The same plasmid can carry simultaneously more resistance genes, resulting in multidrug-resistance.

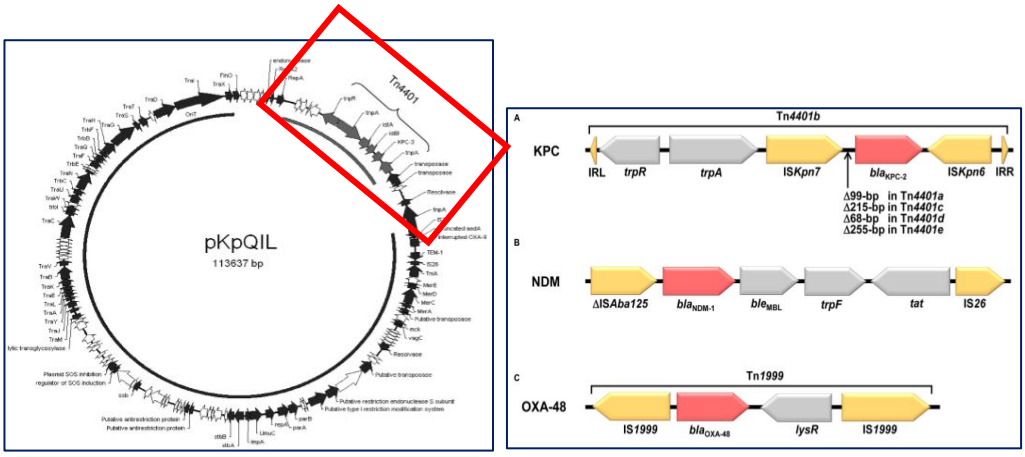


Fig. 20 Complete sequence of pKpQIL plasmid with transoposon Tn4401 containing KPC gene. (On the left) [53] **Representation of gene *bla*$_{KPC}$ on transposon Tn4401 and gene *bla*$_{NDM}$ *and bla*$_{OXA48}$. (On the right)** [48]

In the USA and in Europe, Italy included, carbapenemase-producing *K. pneumoniae* are often clonally related to a specific sequence type (ST), ST258 [52] harboring pKpQIL plasmid. [57] In Italy, the clone ST512 is also common. [58] In Asia, instead, the predominant *K. pneumoniae* clone is ST11 [48] The wide success of clone ST258 is probably due to virulence genes associated with KPC gene in these strains. [48]

5.2 VIM, NDM, OXA-48

VIM (Verona integron–encoded metallo-*β*-lactamase) is a metallo-*β*-lactamase first isolated in *P. aeruginosa* in Verona in 1997, [59] but its' spreading in Europe started in Greece in 2001. [60] [Fig. 21] VIM-producing Enterobacteria have been isolated also in South Africa, Nigeria, Tunisia and Mexico, but they are less common than KPC. [51]

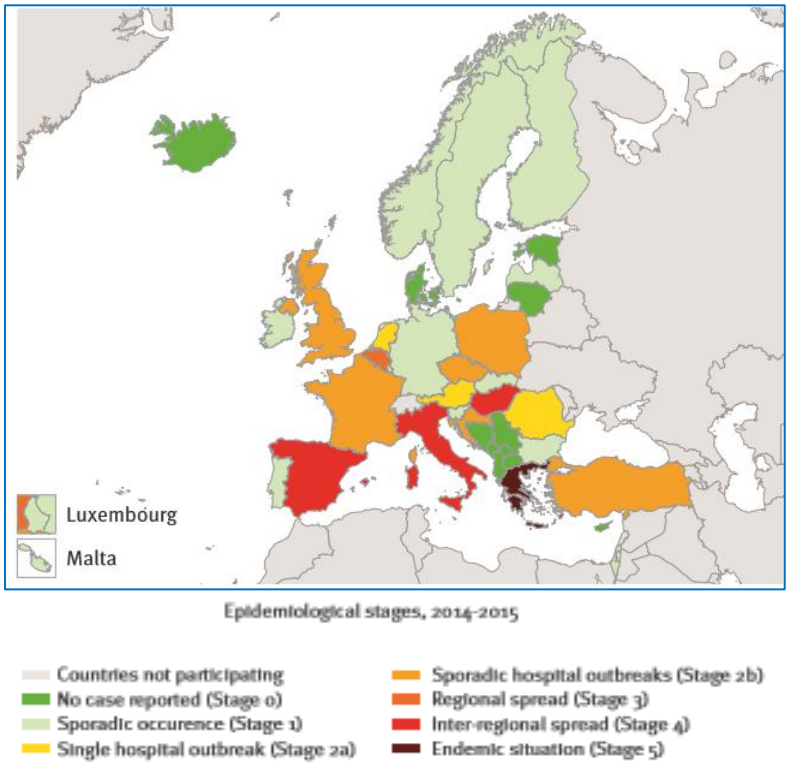


Fig. 21 VIM carbapenemase distribution in Europe (2014-2015). [61]

VIM-encoding gene is located on a mobile cassette in a class 1 chromosomal integron, harbored in *Pseudomonas sp.*, but also in *Enterobacteriaceae*, such as *K. pneumoniae*, *K. oxytoca*, *E. coli*, *E. cloacae*, *Proteus mirabilis*, and it confers a wide resistance at all *β*-lactams except for aztreonam. [59] Clavulanate, sulbactam and tazobactam do not inhibit this enzyme, instead it is inhibited by EDTA, a chelator agent, that cannot have clinical use. Up to now 41 VIM variants in *Enterobacteriaceae* have been detected. [49]

The NDM (New Delhi metallo-*β*-lactamase) carbapenemase was first isolated in 2008 in an Indian patient returning to Sweden from New Delhi. [62] At the moment, it represents the new concern because *K. pneumoniae* and *E. coli* strains producing this enzyme rapidly spread worldwide carrying a very high level of resistance. [48] [Fig. 22] NDM is the most widespread class B carbapenemase. It is endemic in India, where it is the most common carbapenemase, in Pakistan, Bangladesh and the Balkan states, [46] [48] but it was isolated also in many countries worldwide, Italy included. [63]

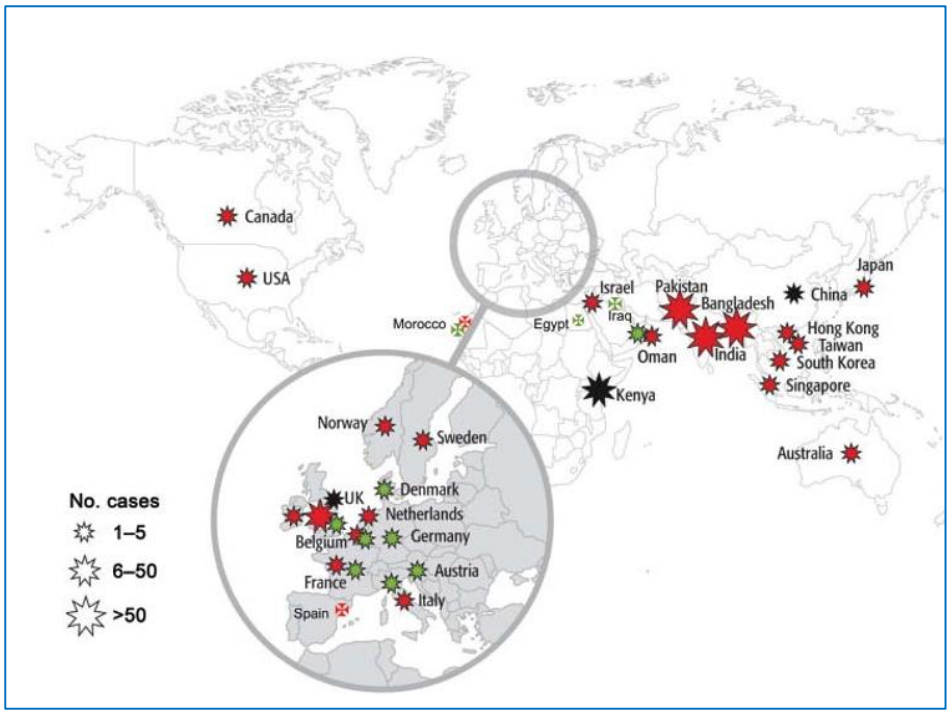


Fig. 22 First isolation and spreading of NDM carbapenemase. [46]

NDM genes [Fig. 20] are allocated on various plasmids, such as IncF, IncR, IncL/M, IncN and IncA/C, that is the predominant, and can spread not only between strains of the same species but also between strains of different species. [64] Often, other resistance genes, such as ESBL genes or genes encoding resistance to aminoglycosides and fluoroquinolones are associated with the NDM gene and *K. pneumoniae* strains carrying them. They belong to various sequence types, such as ST11, ST15, ST16, ST147, ST512, ST972. [48] At the moment we know of 15 NDM variants. [49]

OXA-48 is an oxacillinase that confers resistance to penicillin and reduced susceptibility to carbapenems, but not to third-generation-cephalosporins. First isolated in *K. pneumoniae* in Turkey in 2003 [65] it later spread to Western and Mediterranean Europe (with high prevalence in Spain and France) [46], Northern Africa, the Middle East, India, China, South America. [51] [Fig. 23]

The incidence of OXA-48 producers could be actually, even more elevated, but often they are misdiagnosed because this carbapenemase confers low level resistance. [51]

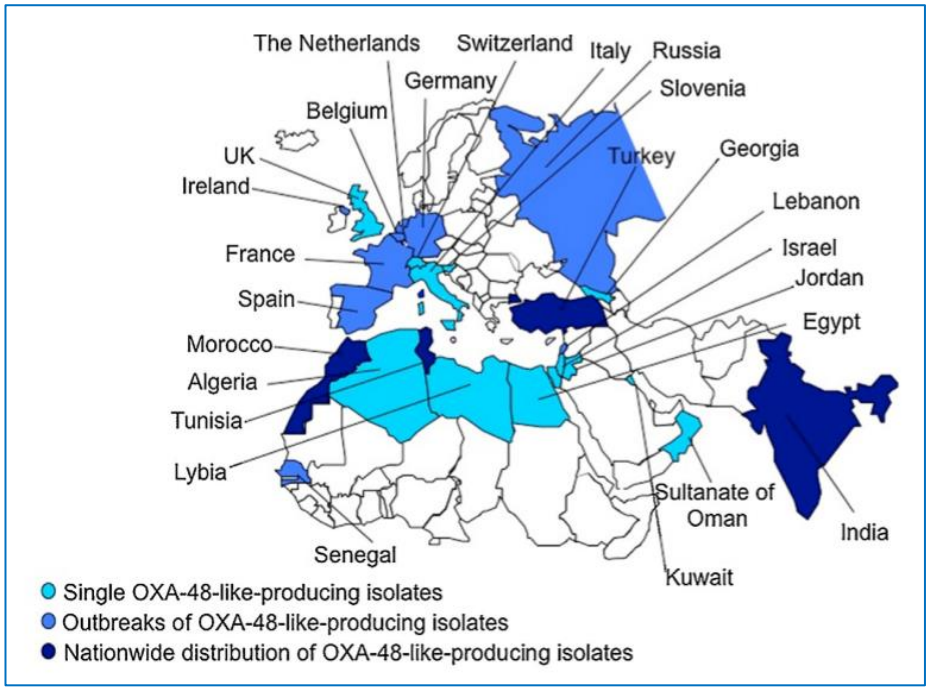


Fig. 23 OXA 48 carbapenemase spreading worldwide. [66]

Different Enterobacteria can harboring OXA-48 gene [Fig. 20], located mainly on highly transferable plasmid of IncL group, associated with an insertion sequence (IS*1999*). [65] The *K. pneumoniae* clones ST11 and ST101 are often associated with this gene. [48] At the moment, 10 variants of this carbapenemase have been detected. [49]

6. Carbapenemase producers detection

The necessity to deal with multidrug-resistance requires multiple and immediate intervention measures, first of all a rapid detection of resistant strains, especially carbapenem-resistant *Enterobacteriaceae* due to their easy and rapid spread and clinical burden.

The role of the laboratory is very important, because in endemic areas there is the need to quickly identify those patients infected by carbapenemase producers in order to provide a proper therapeutic regimen, but also to assess a program for screening and notification of the carriers in a hospital, to prevent nosocomial outbreaks with isolation and contact precautions. For this reason, there is a great need to introduce in all clinical laboratories opportune methods of resistant strains detection.

Right now, there are different diagnostic tools available, but not all of them are reliable actually, due to costs, time consumption, high numbers of sample processing required, as well as laboratory organization. Therefore, some of these tools are implemented only in reference laboratory.

Traditional diagnostics is based on bacterial culture. It uses mostly phenotypic methods because they are easy to perform and are cost contained, but they are time-consuming and not as specific as molecular tests. Phenotypic tests in fact, cannot indicate which enzyme is involved or which other mechanism leads to a resistance, and also, they can miss low level resistance, as in the case of OXA-48 producers.

The new molecular or proteomics diagnostics, instead, can be more rapid and give more information. However, it is often more expensive and not necessarily easy to perform. Another advantage of phenotypic methods versus molecular methods is also the possibility to detect new mechanisms of resistance.

Infection diagnosis can be performed on any clinically significant sample. The screening of carriers is usually performed on rectal swab of patients at risk as for the case of patients admitted to ICU, candidates for transplants, patients from highly endemic countries or from healthcare settings with high prevalence of resistance. A colonized patient in the unit bring to screen all other patient to check and avoid cross-contamination.

In clinical microbiology labs, detection of carbapenemase producers usually takes about 48-72 hours. The first step is overnight culture of the sample, afterwards identification is provided, then susceptibility testing to antibiotics with automated or disc diffusion methods is performed and this requires another 18-24 hours. Reduced inhibition diameters for ertapenem, imipenem and meropenem in disk diffusion assay or Minimum Inhibitory Concentration (MIC) values above the breakpoint can direct towards research for carbapenemase production. Ertapenem is the carbapenem most used for screening, but it is the least specific because other resistance mechanisms also, such as the loss of porins, can affect its MIC value. In 2013, EUCAST (European Committee on Antimicrobial Susceptibility Testing) subcommittee for detection of resistance mechanisms published practical guidelines for clinical laboratories, providing the breakpoints for detection of carbapenemase-producing *Enterobacteriaceae* in infected patients. [Tab. 7] [67]

Tab. 7 EUCAST clinical breakpoints and screening cut-off for carbapenemase-producing *Enterobacteriaceae* detection [67]

Carbapenem	MIC (mg/L)		Disk diffusion zone diameter (mm) with 10 µg disks	
	S/I breakpoint	Screening cut-off	S/I breakpoint	Screening cut-off
Meropenem	≤2	>0.12	≥22	<25
Imipenem	≤2	>1	≥22	<23
Ertapenem	≤0.5	>0.12	≥25	<25

The confirmation of carbapenemase production require further tests, some of them, as Modified Hodge Test or E-test, require another 18-24 hours. However, not only are the results ready only after 24 hours, but Modified Hodge Test has also low sensitivity, principally for NDM producers, as well as low specificity and is therefore not recommended by EUCAST. [67] The MBL E-test can be performed with strips of imipenem and imipenem/EDTA (bioMérieux), for the detection of metallo-β-lactamase producers, but it is little sensitive if MBL producers have low resistance.

It is important, therefore to have access to rapid and sensitive assays and be able to detect and confirm carbapenemase production, also for strains that can possibly produce new carbapenemase, thus providing clinicians with rapid adequate results.

The use of selective chromogenic agar plates, such as ChromID Carba agar (bioMérieux) or ChromID ESBL agar (bioMérieux) combined with a carbapenem disk and the rapid colorimetric assays such Carba NP test, [68] can reduce the turn-around time to less than 48 hours. Carba NP test, indeed is a rapid phenotypic method to identify carbapenemase-producing Enterobacteria strains, it is inexpensive and easy to perform. It is based on hydrolysis of carbapenem (usually imipenem) that causes acidification of the medium detected by phenol red, as pH indicator, which changes from red to yellow. It can be performed directly from a colony that is mixed with lysis solution and phenol red solution with antibiotic, and provides a result within 2 hours maximum.

Molecular techniques based on PCR technology, can be performed in reference laboratories. Numerous tests have been developed, like simplex and multiplex PCR, real-time PCR, microarray, and they are the reference standards for identification of the genes encoding for carbapenemases. They are more rapid than culture-based tests, can give results within 4–6 h; they have high sensitivity and specificity and provide information not only about carbapenemase production, but also about what type of carbapenemase is involved, and also if carbapenemase genes are chromosomal or plasmidic. Techniques such as Multi-Locus Sequence Typing (MLST) and Pulsed-Field Gel Electrophoresis (PFGE) give information

about the emergence and spread of bacterial clones. However, all genotypic techniques are expensive, very time-consuming and require expertise.

In the last few years, Mass-Spectrometry (MS) based methods MALDI-TOF (Matrix Assisted Laser Desorption Ionization Time-of-Flight) [Fig. 24] substituted the traditional methods for bacterial identification in many laboratories, because it is very fast and it has high sensitivity, specificity and low-costs. [69] [70] Numerous studies are being performed to demonstrate the possible use of MALDI-TOF MS also to distinguish resistant from susceptible strains, based on hydrolysis activity of ampicillin, cephalosporins or carbapenems. Indeed, the spectra produced from analysis of strains before and after incubation with antibiotic are different if the strains hydrolyze the drug, with peaks corresponding to antibiotic before incubation and subsequent loss of it or else appearance of a new one which correspond to degradation products. [71] [72] This method is more rapid than the traditional susceptibility test and provide results in 2-4 hours, but it needs further evaluation to optimize the performance and standardize it. Moreover, at the moment there is no available software able to analyze the spectra obtained from this kind of analysis.

Another approach for quick identification of resistant strains by MALDI-TOF MS, is to analyze the spectra trying to detect a pattern in characteristic peaks related to resistance determinants. The published studies about carbapenemase detection, demonstrate the correspondence between KPC production and a peak of 11,109Da in MALDI-TOF MS spectra. [73] [74] [75] This method is faster than hydrolysis, is able to provide a result in a few minutes, does not require any further step other than routine identification analysis, does not add costs and is also very easy to perform. Finally, many studies are being performed to validate the use of MALDI-TOF MS system for typing bacterial strains. [76] [77]

Fig. 24 MALDI-TOF Vitek MS RUO (bioMérieux).

AIM OF THE STUDY

The aim of this study was to evaluate the use of MALDI-TOF MS system to identify antimicrobial resistance mechanisms, and in particular to quickly identify carbapenemase-producing strains.

The study was subdivided in three parts with specific objectives:

1. To confirm the correlation between KPC and a characteristic MALDI-TOF MS spectrum peak, and demonstrate the usefulness of MALDI-TOF MS system to quickly identify KPC-producing Enterobacteria in diagnostics, by the same characteristic peak.

2. To find new peaks correlated with other carbapenemases: VIM and NDM.

3. To evaluate the use of MALDI-TOF MS system to quickly detect hospital outbreaks.

Moreover, the MALDI-TOF MS results were correlated with phenotypic and genetic data to get an epidemiologic and molecular analysis of the diffusion pattern of multidrug-resistant Enterobacteria in Verona area.

1. Bacterial strains

PART I – To validate the use of MALDI-TOF MS system for KPC producers detection.

Group 1. To confirm the correlation between KPC and a characteristic MALDI-TOF MS spectrum peak.

A total of 436 *Enterobacteriaceae* strains (namely 176 KPC-producing; 54 producing carbapenemases other than KPC; 175 ESBL-producing; 4 carbapenem-resistant not producing carbapenemases and 27 susceptible to *β*-lactams) were examined to confirm the correlation between KPC production and MALDI-TOF MS 11,109Da peak. [73] The strains were selected from clinical isolated, based on susceptibility testing results.

Out of 436 total strains, 128 strains were isolated from rectal and pharyngeal swabs from multidrug-resistant (MDR) screening done at the Microbiology Laboratory of Verona Hospital, between April 2013 and January 2014 and stored in glycerol stock vials at -80°C. Identification and antibiotic susceptibility tests were performed beforehand during clinical routine workflow by MALDI-TOF MS system (bioMérieux) and automated system Vitek 2 (bioMérieux).

The strains were namely: 95 KPC-producing *K. pneumoniae*; 4 KPC-producing *E. coli*; 24 ESBL-producing *K. pneumoniae*; 4 *E. coli* with reduced susceptibility to ertapenem due to non-carbapenemase-based mechanisms; 1 VIM-producing *E. coli*.

One hundred and seven strains were isolated in Microbiology Laboratory of "Sacro Cuore" Hospital in Negrar (Verona) from various clinical samples (rectal swabs, urine, respiratory specimens, blood-culture, biological fluids) from August

2014 to March 2015 and stored in glycerol stock vials at -80°C. They had already been identified and tested for antibiotic susceptibility with automated system MicroScan Walk Away 96 Plus (Siemens Healthcare Diagnostics). As confirmatory test for KPC production Modified Hodge Test was used.

The strains were namely: 68 KPC-producing *K. pneumoniae*; 9 KPC-producing *E. coli*; 30 ESBL-producing *K. pneumoniae*.

During the study, we performed for all these strains identification with MALDI-TOF MS system, rapid Carba NP test, [68] MIC evaluation and for carbapenemase-producing strains multiplex and simplex PCR for bla_{IMP}/bla_{VIM}/bla_{KPC}, bla_{NDM}, bla_{OXA-48}, bla_{TEM}/bla_{SHV}/bla_{OXA-1}, $bla_{CTX-M-1}$, $bla_{CTX-M-2}$, $bla_{CTX-M-9}$, $bla_{CTX-M\ 8/25}$ genes; [78] for ESBL-producing strains multiplex PCR for bla_{TEM}/bla_{SHV}/bla_{OXA-1}, $bla_{CTX-M-1}$, $bla_{CTX-M-2}$, $bla_{CTX-M-9}$, $bla_{CTX-M\ 8/25}$ genes. [78] Discrepant strains, that is strains carrying bla_{KPC} gene, without the 11,109Da peak, were retested twice by MALDI-TOF MS, before and after isolation on Mueller-Hinton (MH) plates with Imipenem (2 µg/ml), and bla_{KPC} gene sequencing, plasmid extraction and characterization [56] [79] and PCRs for *p019* and *Tn4401* genes was performed.

As control groups we used collection strains, identified and tested for antibiotic susceptibility during clinic routine workflow and investigated for *bla* genes. They are namely 27 *K. pneumoniae* susceptible to all *β*-lactams; 121 ESBL-producing *E. coli* from Verona; 15 *Enterobacteriaceae* strains from Croatia, namely: 8 *K. pneumoniae*; 2 *E. cloacae*; 2 *C. koseri*; 3 transconjugants that have as recipient *E. coli* J53, all NDM-producing; 37 *Enterobacteriaceae* strains VIM-producing from Croatia, namely: 2 *K. pneumoniae*; 25 *E. cloacae*; 8 *C. freundii*; 2 *K. oxytoca*, and 1 VIM-producing *K. oxytoca* from Verona. For these strains, we performed rapid Carba NP test and the 11,109Da peak detection on MALDI-TOF MS spectra. [Fig. 25]

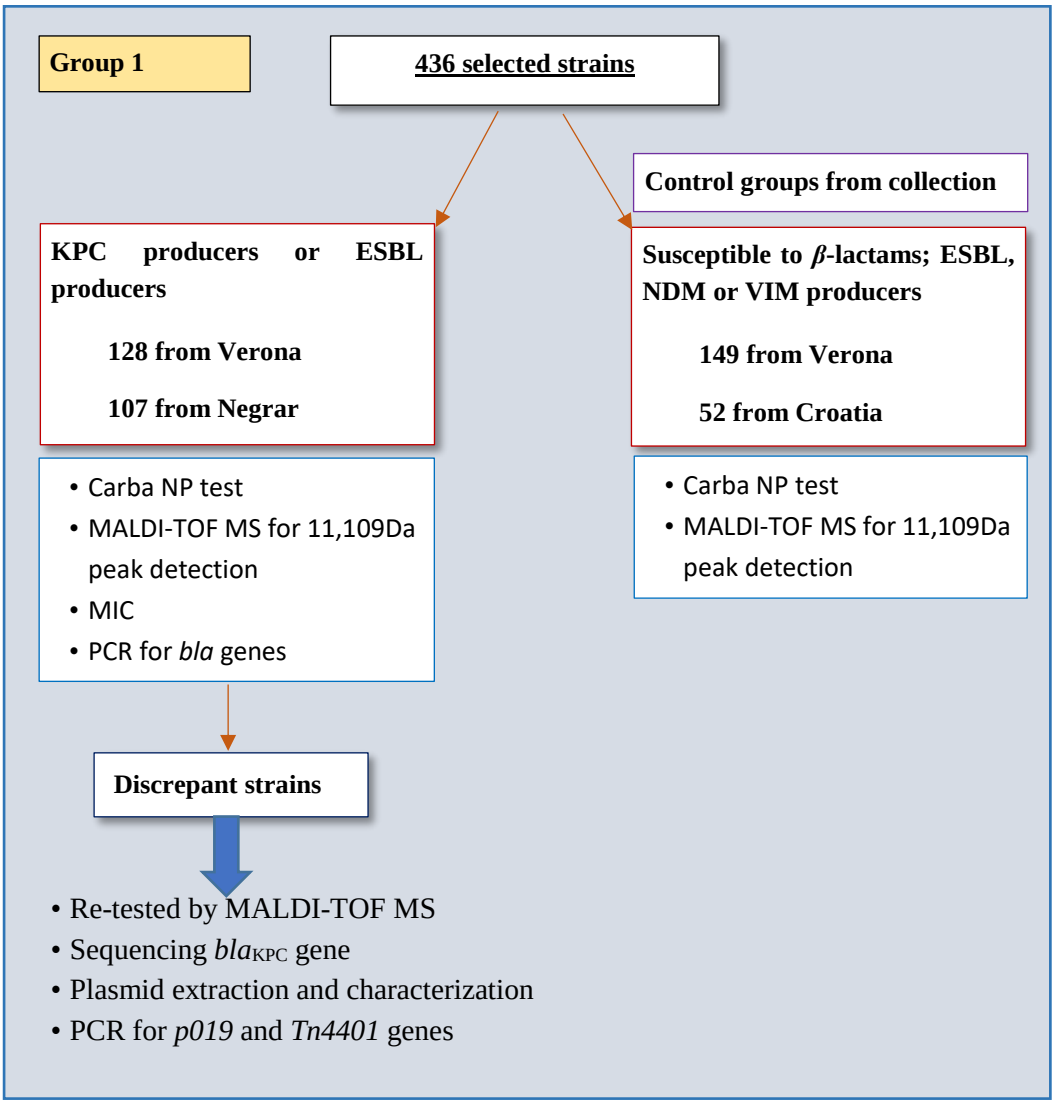


Fig. 25 Workflow for selected strains analyzed to confirm correlation between KPC production and MALDI-TOF MS 11,109Da peak.

<u>Group 2</u>. To demonstrate the usefulness of MALDI-TOF MS system to quickly identify KPC-producing Enterobacteria in diagnostics, by 11,109Da peak.

Between May and July 2016, the MALDI-TOF MS spectra of 183 *Enterobacteriaceae* strains with rapid Carba NP test [68] or ESBL NDP test [80] positive, isolated from rectal and pharyngeal swab during MDR screening at the Microbiology Laboratory of Verona Hospital, were analyzed daily to detect the 11,109Da peak. The strains were namely: 124 *K. pneumoniae*; 8 *E. coli*; 2 *E. aerogenes*; 1 *E. cloacae*, all carbapenemase producers; 47 *K. pneumoniae* and 1 *E. coli* ESBL producers. All strains were isolated on ChomeID ESBL agar (bioMérieux) with Ertapenem (10 µg) disk and identified by MALDI-TOF Vitek MS RUO (bioMérieux). The discrepant strains with positive Carba NP test and without the 11,109Da peak were re-tested twice by MALDI-TOF MS, before and after isolation on MH plates with Imipenem (2 µg/ml), and investigated by multiplex and simplex PCR for bla_{IMP}/bla_{VIM}/bla_{KPC} and bla_{NDM} [78] genes, then if positive for bla_{KPC} gene, we performed sequencing of the gene, plasmid extraction and characterization, [56] [79] and PCR for *p019* and *Tn4401* genes. [Fig.26]

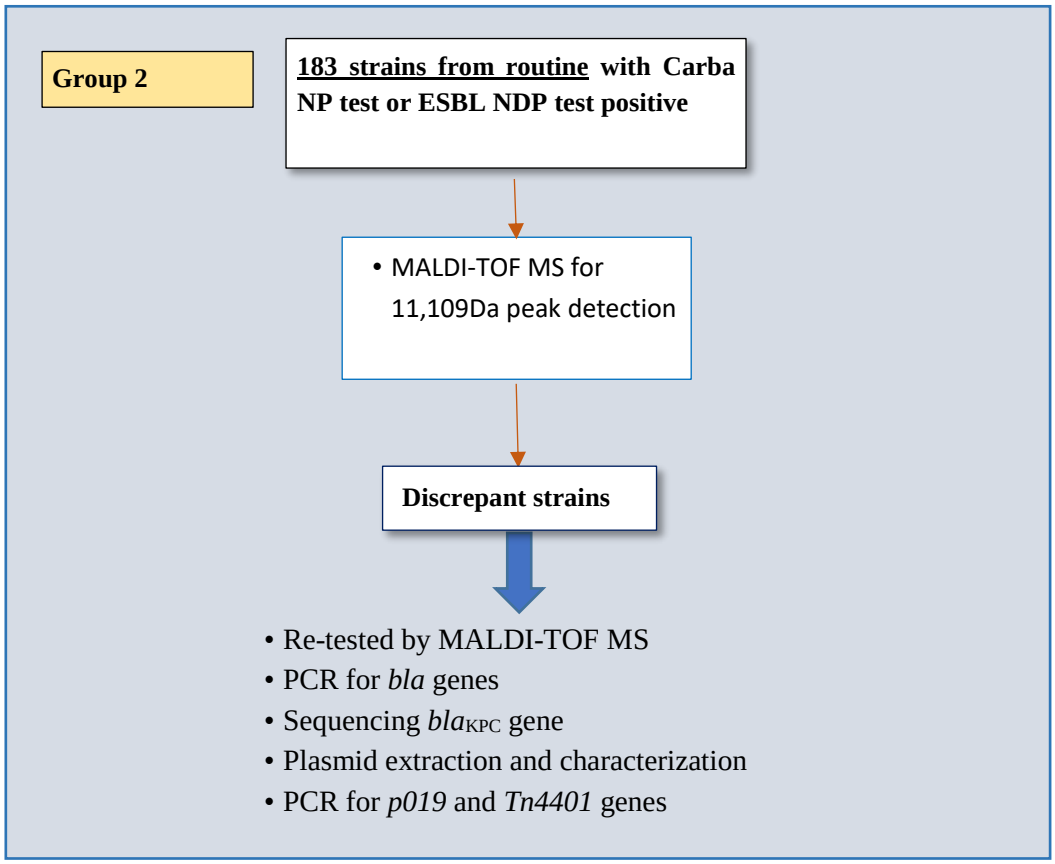


Fig. 26 Workflow to evaluate the application of MALDI-TOF MS 11,109Da peak detection in diagnostic routine.

<u>Group 3</u>. To search NDM-producing *E. coli*.

After finding an NDM-producing *E. coli* strain in the Group 2, we performed a retrospective analysis on 11 *E. coli* strains carbapenem-resistant collected from January to November 2015 and stored at -80°C, to search for other strains producing the same enzyme. All strains were analyzed by MALDI-TOF MS to detect the 11,109Da peak and by multiplex and simplex PCR for bla_{IMP}/bla_{VIM}/bla_{KPC} e bla_{NDM} [78] genes. The discrepant strains, that is strains carrying bla_{KPC} gene, without the 11,109Da peak, were retested by MALDI-TOF MS and we performed sequencing bla_{KPC} gene, plasmid extraction and characterization, [56] [79] and PCR for *p019* and *Tn4401* genes. [Fig. 27]

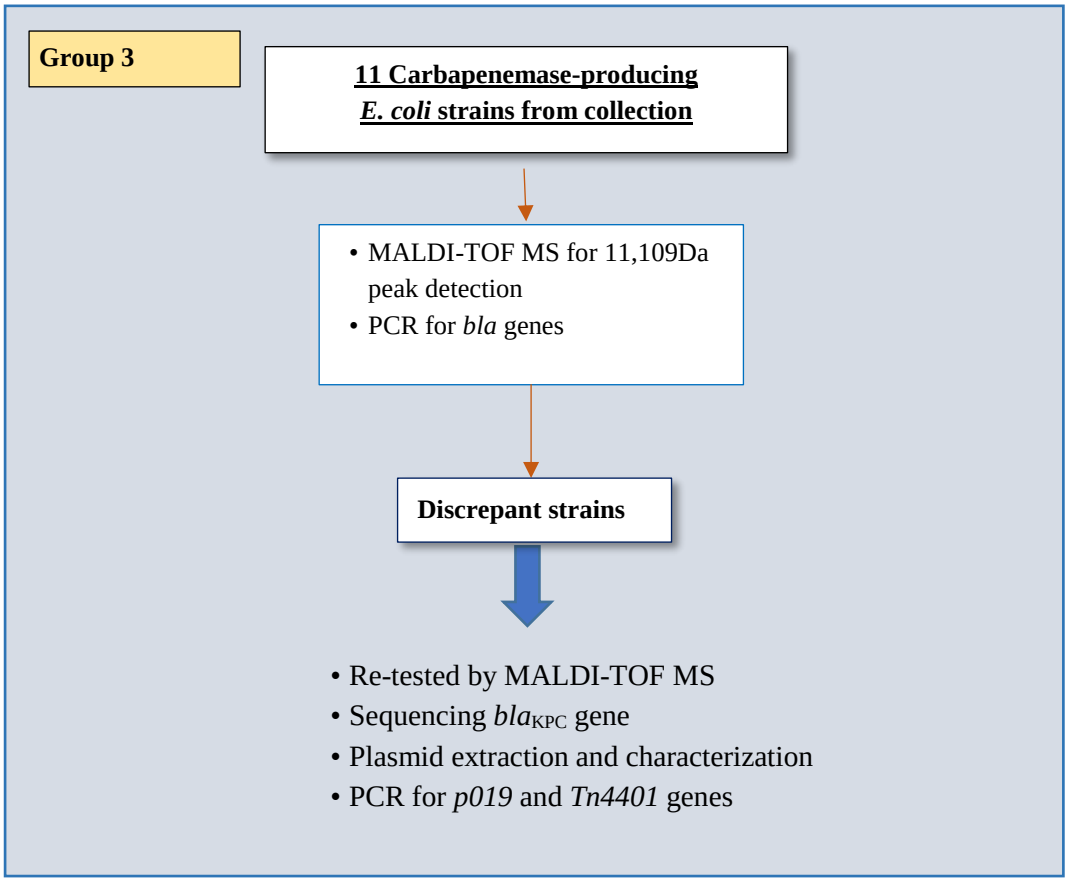


Fig. 27 Workflow to detect NDM-producing *E. coli* strains.

PART II - To find peaks correlated with other carbapenemases: VIM and NDM.

Group 4

To find a specific peak correlated with VIM and NDM enzyme, we analyzed from frozen stock, 57 Enterobacteria strains from Croatia (36 out of 57 were the same of group 1) and 61 *Pseudomonas aeruginosa* strains by collection. After confirmation of carbapenemase production with Carba NP test and PCR for $bla_{IMP}/bla_{VIM}/bla_{KPC}$ e bla_{NDM} [78] genes, we selected 23 and 13 strains respectively, all VIM-producing, positive for Carba NP test, to create a MALDI-TOF MS superspectrum and to search for a specific peak. For 2 strains (1 *C. freundii* and 1 *K. oxytoca*) we performed conjugation with *E. coli* J53 as recipient, to obtain trans-conjugant strain VIM-producing. [Fig. 28]

We created also a superspectrum to search for a NDM specific peak, using the 15 NDM-producing strains of Group 1. [Fig. 25] [Fig. 28] After the first spectra analysis, we characterized the plasmids of the NDM- and VIM-producing strains, and based on the high frequency of appearance, we divided the samples in groups then repeating the spectra analysis to check if, in this way, we would find a common peak, related to one of the plasmids.

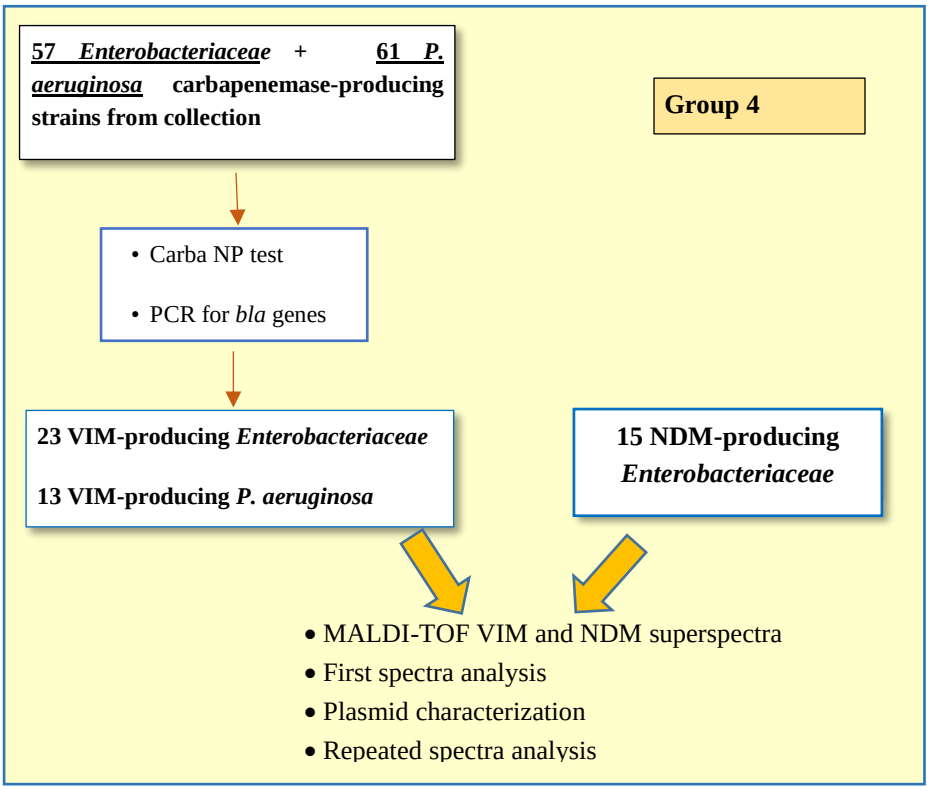


Fig. 28 Workflow to create VIM and NDM superspectra for finding characteristic MALDI-TOF MS peaks correlated with these carbapenemases.

PART III - To evaluate the use of MALDI-TOF MS system to quickly detect hospital outbreaks.

Group 5

Forty-four *K. pneumoniae* strains by frozen collection, isolated during an outbreak onset in our hospital ICU from January to March 2015 and already identified and tested for antibiotic susceptibility during routine clinic workflow. For all strains, we confirmed carbapenemase production with Carba NP test and PCR for bla_{IMP}/bla_{VIM}/bla_{KPC} genes, [78] and we performed MALDI-TOF MS analysis to detect the 11,109Da peak and to find genetic correlation, and PFGE.

The discrepant strains, that is strains which carried bla_{KPC} gene, without the 11,109Da peak, were retested twice by MALDI-TOF MS, before and after using MH plates additioned with Imipenem (2 µg/ml), and we performed sequencing bla_{KPC} gene, plasmid extraction and characterization [56] [79] and PCR for *p019* and *Tn4401* genes. [Fig. 29]

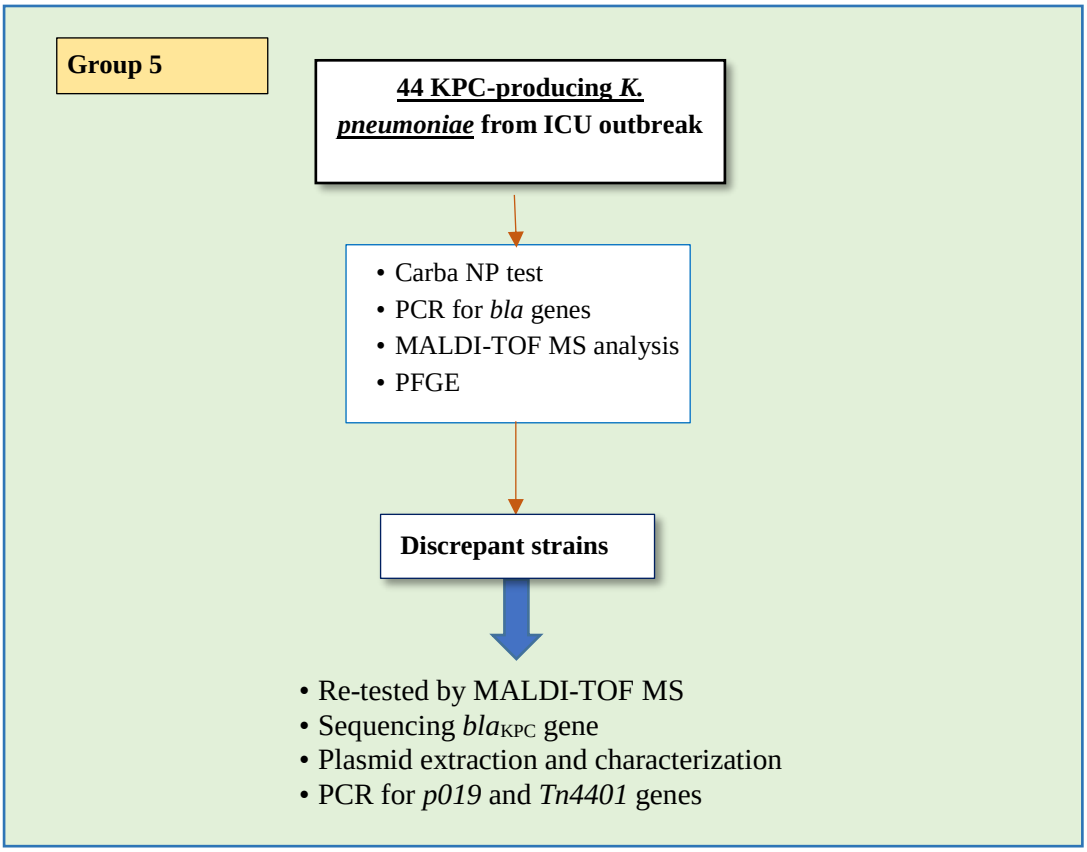


Fig. 29 Workflow to evaluate the use of MALDI-TOF MS to detect clonal correlation between bacterial strains.

Control strains

As positive and negative controls for Carba NP test, ESBL NDP test and for PCR for *bla* genes we used well-characterized strains. [Tab. 8]

Tab. 8 Negative and positive controls used for Carba NP test, ESBL NDP test and PCR for *bla* genes

Negative control	*E. coli*	ATCC 25922[81]
KPC positive control	*K. pneumoniae*	KL301 [82]
VIM positive control	*P. aeruginosa*	VR 143/97 [59]
NDM positive control	*K. pneumoniae*	KLZA [83]
OXA-48 positive control	*K. pneumoniae*	KPN2605 [84]
TEM/SHV/OXA-1 positive control	*K. pneumoniae*	KPN2605 [84]
ESBL positive control	*E. coli*	ATCC BAA2326 [81]

2. Carbapenemase-producing strains detection

To isolate carbapenemase-producing strains, the samples were inoculated on selective chromogenic ChromID ESBL agar plates (bioMérieux) with Ertapenem disk (10 µg) and incubated at 37°C overnight. Isolates that showed reduced susceptibility to ertapenem (< 22mm diameter) [85] were further investigated by confirmatory test and MALDI-TOF MS identification, after passage on Mueller-Hinton home-made agar plates. [Fig. 30]

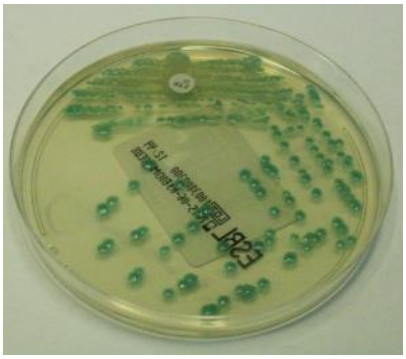


Fig. 30 Green colonies of carbapenemase-producing *K. pneumoniae* on selective ChromID ESBL agar plate (bioMérieux), with ertapenem disk.

3. Identification by MALDI-TOF MS system and spectra analysis to detect the 11,109Da peak

Mass spectrometry has been used from ‘80s to analyze with high accuracy a protein’s molecular weight. [86] Since 1996, this technology has been applied by Matrix Assisted Laser Desorption Ionization Time-of-Flight Mass Spectrometry (MALDI-TOF MS) system, that quickly (~1minute) identify bacterial strains at genus, species and, sometimes, at subspecies level. Two systems of Mass Spectrometry are applied to microbiology: one developed by Bruker Daltonics (Germany) and the other by bioMérieux/Shimadzu (France/Japan). The Bruker system uses the software MALDI Biotyper, whereas the bioMérieux/Shimadzu system uses own software, namely, Shimadzu Launchpad and database SARAMIS^{TM} Premium (Spectral ARchivee And Microbial Identification System) ID-professional (AnagnosTec-bioMérieux). [87] [88]

The MALDI-TOF MS system analyzes ribosomal proteins of the strains spotted in a plastic slide, [Fig. 31] crystallized by a matrix and ionized by a pulsed laser 50Hz ultraviolet frequency, which hits each spot 500 times. The sample is pulsed in a flight tube and the proteins are separated by an electric field according to their molecular weight. The system measures the mass/charge ratio (m/z) and it generates a spectrum with peaks corresponding to molecular masses, with quality representation of relative intensity. The spectrum is automatically acquired and compared with superspectra and reference spectra in the database, to provide identification result of bacterial strains. [69] [89] [90]

This technology is very reliable, rapid and accurate, and it also has low costs. It is very important, however, to guarantee the reproducibility of the results, to always operate in the same conditions, for example to use the same culture conditions, the same plates or extraction method. This is necessary specially to find characteristic peaks and to perform comparisons between strains.

In our study, all strains were identified by MALDI-TOF Vitek MS RUO *Axima@Saramis* (bioMérieux) system, in duplicate, from fresh overnight cultured

colony spotted in a plate with 1 μl of α-cyano-4-hydroxycinnamic acid (α-CHCA) matrix, [Fig. 31] according to manufacturer indications.

For all strains except group 2, all MALDI-TOF MS measurements were performed by the same operator, for Group 2 strains (routine detected strains) also by other operators so as to investigate for potential differences on the results.

Fig. 31 Slides and reagents for MALDI-TOF MS measurements.

Our MALDI-TOF MS spectra were compared with Saramis database (~20,000 bacterial and fungal spectra) for strains identification. According to the manufacturer's criteria, a result was considered valid (accurate identification to the species level) whenever the confidence was ≥90%. The results are showed with different colors according to the confidence of analysis: dark green ≥99% and light green ≥90% if the result is very reliable; yellow ≥85% high probable; white ≥70% not sure, two or more species proposed; red <70%, not acceptable, possible mixture of strains. [Fig. 32]

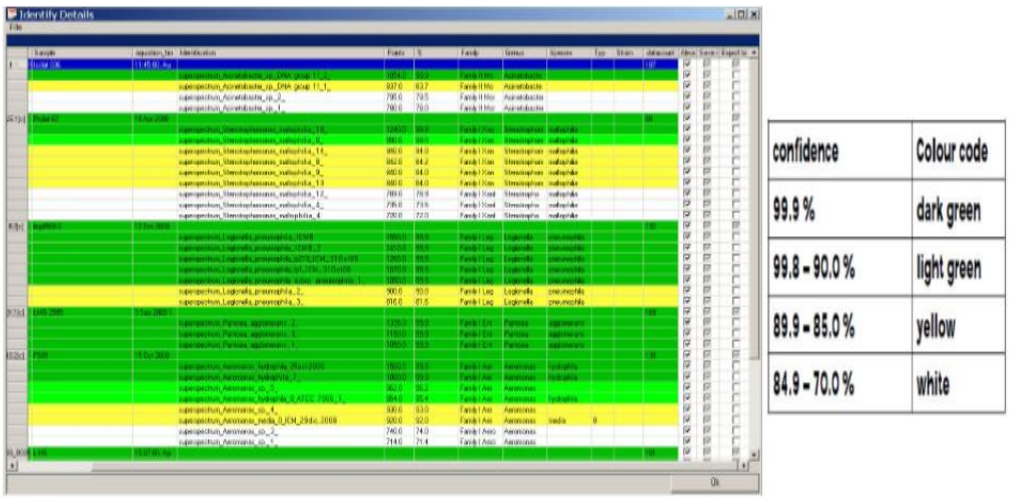


Fig. 32 Color code for MALDI-TOF MS results.

The mass measured by MALDI-TOF MS system using CHCA matrix range from 2,000 to 20,000Da, the parameter settings to detect the 11,109Da peak were: Relative Intensity ≥ 0; Absolute Intensity ≥ 0; Error % 0.08.

Spectra obtained at an initial stage of identification were also used to detect the 11,109Da (± 8Da) peak. They were imported into the database, thus creating a folder for each genus and looking for the peak in question. [Fig. 33]

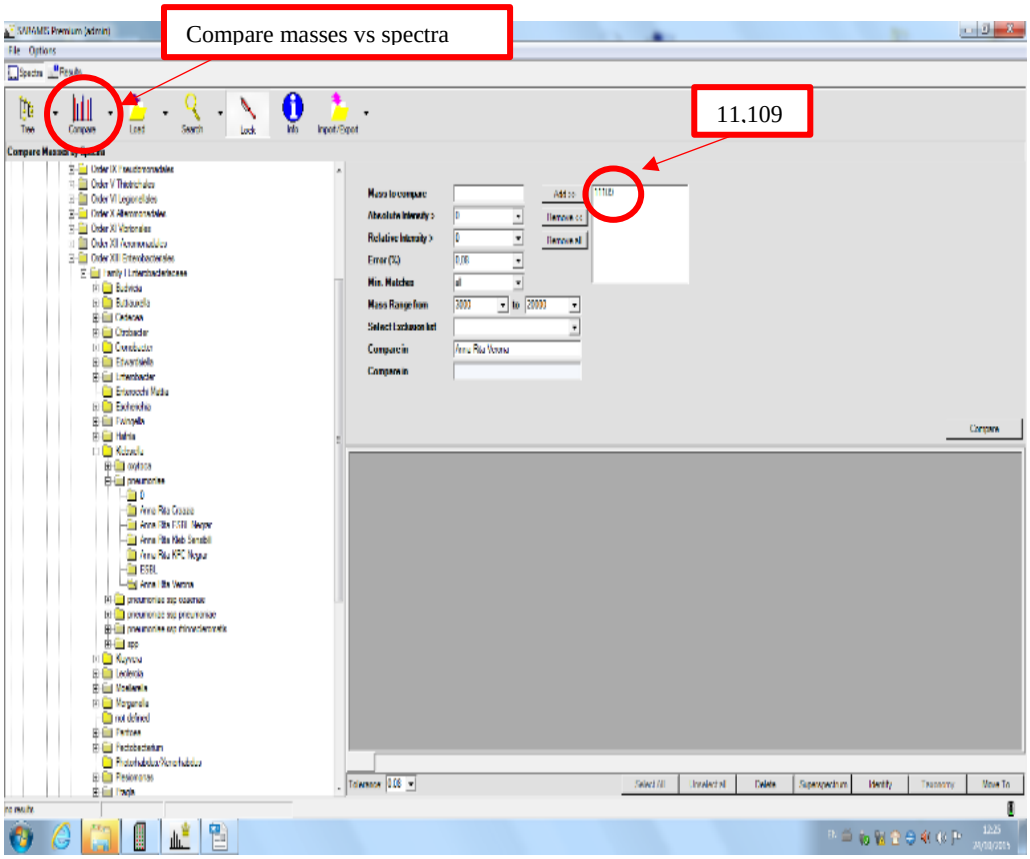


Fig. 33 11,109Da peak detection importing strains spectra in database and comparing mass vs spectra.

4. MALDI-TOF MS superspectra creation

To find a characteristic peak correlated with carbapenemases other than KPC, as NDM and VIM, we used MALDI-TOF MS system to create two specific superspectra. That is a set spectra produced by similar strains, therefore with common peaks, which can use for rapid comparison. [88] To perform this, we made measurements for NDM- and VIM- producing strains and analyzed the spectra by Saramis, then imported our spectra, we created a NDM and a VIM superspectrum. The peaks of these superspectra were used to create an exclusion list to eliminate the proteins most represented and common also to susceptible strains of the database, until step by step, we selected only a few proteins that could be correlated with carbapenemases, to investigate whether the latter could be common to all strains producing the same enzyme. [Fig. 34]

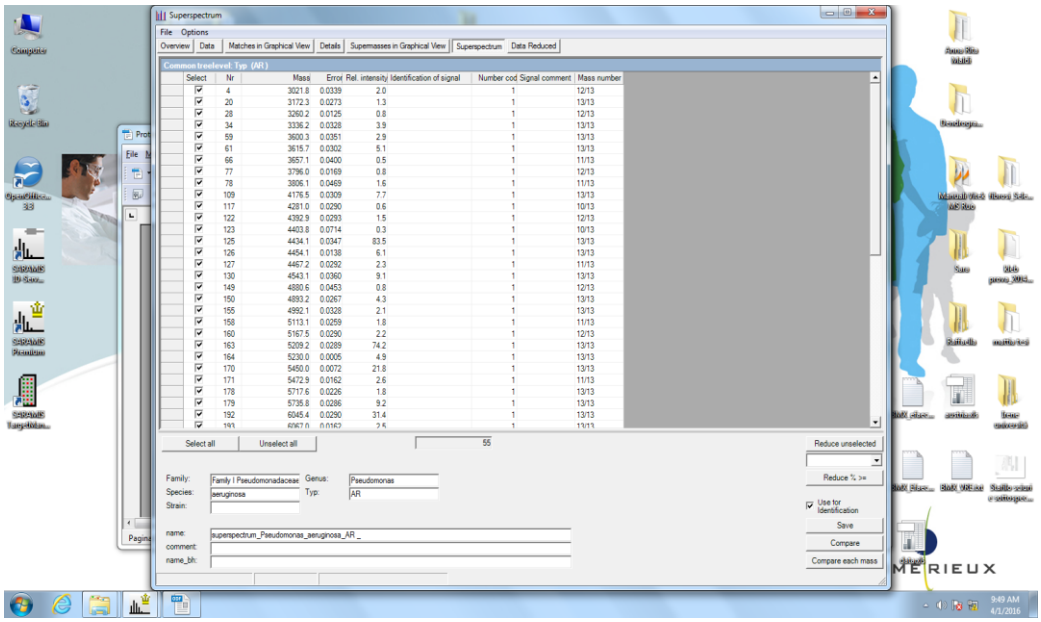


Fig. 34 Creation of superspectrum VIM and selection, step by step, of proteins to find a peak correlated with the enzyme.

5. Antimicrobial Susceptibility Testing

Antimicrobial susceptibility tests were performed with automated systems during clinical routine and by broth microdilution for carbapenems, cephalosporins and aztreonam, in this study. The results were interpreted following the EUCAST breakpoints. [85] [Tab. 9]

Tab. 9 EUCAST clinical breakpoints of cephalosporins, carbapenems and monobactams for *Enterobacteriaceae*

Enterobacteriaceae — **EUCAST Clinical Breakpoint Tables v. 6.0, valid from 2016-01-01**

Cephalosporins[1]	MIC breakpoint (mg/L) S ≤	MIC breakpoint (mg/L) R >	Disk content (µg)	Zone diameter breakpoint (mm) S ≥	Zone diameter breakpoint (mm) R <	Notes
Cefaclor	-	-		-	-	Numbered notes relate to general comments and/or MIC breakpoints. Lettered notes relate to the disk diffusion method.
Cefadroxil (unocomplicated UTI only)	16	16	30	12	12	1. The cephalosporin breakpoints for Enterobacteriaceae will detect all clinically important resistance mechanisms (including ESBL and plasmid mediated AmpC). Some isolates that produce beta-lactamases are susceptible or intermediate to 3rd or 4th generation cephalosporins with these breakpoints and should be reported as tested, *i.e.* the presence or absence of an ESBL does not in itself influence the categorisation of susceptibility. ESBL detection and characterisation are recommended for public health and infection control purposes.
Cefalexin (unocomplicated UTI only)	16	16	30	14	14	
Cefazolin	-	-		-	-	
Cefepime	1	4	30	24	21	2. The cefoxitin ECOFF (8 mg/L) has a high sensitivity but poor specificity for identification of AmpC-producing Enterobacteriaceae as this agent is also affected by permeability alterations and some carbapenemases. Classical non-AmpC producers are wild type, whereas plasmid AmpC producers or chromosomal AmpC hyperproducers are non-wild type.
Cefotaxime	1	2	5	20	17	3. For susceptibility testing purposes, the concentration of tazobactam is fixed at 4 mg/L.
Cefoxitin (screen)[2]	NA	NA	30	19	19	4. Breakpoints are based on high dose therapy (1.5 g x 3), ~~and relate to *E. coli*, *Klebsiella* spp. and *P. mirabilis* only.~~
Cefpodoxime (unocomplicated UTI only)	1	1	10	21	21	
Ceftaroline	0.5	0.5	5	23	23	
Ceftazidime	1	4	10	22	19	
Ceftibuten (UTI only)	1	1	30	23	23	
Ceftobiprole	0.25	0.25	5	23	23	
Ceftolozane-tazobactam	1[3]	1[3]	30-10	23	23	
Ceftriaxone	1	2	30	23	20	
Cefuroxime iv[4], *E. coli*, *Klebsiella* spp. and *P. mirabilis*	8	8	30	18	18	
Cefuroxime oral (unocomplicated UTI only)	8	8	30	18	18	

Carbapenems[1]	MIC breakpoint (mg/L) S ≤	MIC breakpoint (mg/L) R >	Disk content (µg)	Zone diameter breakpoint (mm) S ≥	Zone diameter breakpoint (mm) R <	Notes
Doripenem	1	2	10	24	21	Numbered notes relate to general comments and/or MIC breakpoints. Lettered notes relate to the disk diffusion method.
Ertapenem	0.5	1	10	25	22	1. The carbapenem breakpoints for Enterobacteriaceae will detect all clinically important resistance mechanisms (including the majority of carbapenemases). Some isolates that produce carbapenemase are categorised as susceptible with these breakpoints and should be reported as tested, *i.e.* the presence or absence of a carbapenemase does not in itself influence the categorisation of susceptibility. Carbapenemase detection and characterisation are recommended for public health and infection control purposes.
Imipenem[2]	2	8	10	22	16	
Meropenem	2	8	10	22	16	2. Low-level resistance is common in *Morganella* spp., *Proteus* spp. and *Providencia* spp.

Monobactams	MIC breakpoint (mg/L) S ≤	MIC breakpoint (mg/L) R >	Disk content (µg)	Zone diameter breakpoint (mm) S ≥	Zone diameter breakpoint (mm) R <	Notes
Aztreonam[1]	1	4	30	24	21	Numbered notes relate to general comments and/or MIC breakpoints. Lettered notes relate to the disk diffusion method. 1. The aztreonam breakpoints for Enterobacteriaceae will detect clinically important resistance mechanisms (including ESBL). Some isolates that produce beta-lactamases are susceptible or intermediate to aztreonam with these breakpoints and should be reported as tested, *i.e.* the presence or absence of an ESBL does not in itself influence the categorisation of susceptibility. ESBL detection and characterisation are recommended for public health and infection control purposes.

The antibiotics tested were carbapenems: ertapenem, meropenem, imipenem; cephalosporins: cefotaxime, cefepime, ceftazidime; monobactams: aztreonam. We used 96 well microdilution plates. Each well was filled with 100 µl of Mueller-Hinton (MH) broth, later adding another 100 µl in the first well. Then, for imipenem/cylastatin 10.24 µl from a 10 mg/ml mother solution was pipetted into the first well, and for the other antibiotics 5.12 µl, and made serial dilution, to obtain final 100 µl in each well. Finally, 100 µl of bacterial inoculum in MH broth, with 10^5 cfu/ml was added in each well, obtaining antibiotic concentrations of 128 µg/ml in the first well and 0.06 µg/ml in the last one. The microdilution plates were incubated overnight at 37 ºC. [Fig. 35]

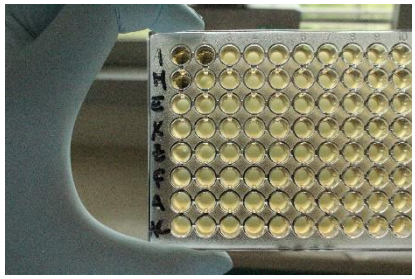


Fig. 35 MIC determination with broth microdilution method.

6. Beta-lactamases detection and characterization

6.1 Colorimetric confirmatory test:

ESBL NDP test and Carba NP test

Rapid phenotypic tests: ESBL NDP test [80] and Carba NP test, [68] were performed for all samples with reduced susceptibility to cephalosporins or to carbapenems, to confirm ESBL or carbapenemases production.

The test was made on samples and on positive and negative controls [Tab. 8] using fresh colony (not more than 24 hours) from overnight culture. For each sample, including both positive and negative controls, two tubes of 1.5 ml were prepared. In all tubes, we distributed 100 µl of Tris-HCl 20 mM lysis buffer (B-PER II, Bacterial Protein Extraction Reagent, Thermo Scientific Pierce) and the colony to be tested, and well mixed. In the first tube of each sample used as test control, we added 100 µl of phenol red solution (solution A). In the second tube of each sample, that is the test tube, we added 100 µl of solution A with antibiotic, at the concentration of 3 mg/mL, prepared just before use: imipenem for Carba NP test, [68] ceftazidime for ESBL NDP test. [80] Solution A was prepared mixing 2 ml of concentrated phenol red solution (0.5% w/v) and 16.6 ml of distilled water, adjusting the pH at 7.8 and adding 180 µl of $ZnSO_4$ (Merck) 10 mM to obtain a final concentration of 0.1 mM.

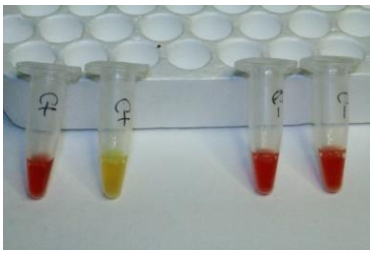


Fig. 36 Rapid Carba NP test home-made. On the left positive result, on the right negative result.

All tubes were incubated at 37°C for max two hours (usually for KPC producers, positive result requires between 2 and 30 min; for MBL producers between 15 min and 1h; for OXA-48-like producers between 20 and 2h). The test was interpreted positive if the color of test tube changed from red to yellow, because carbapenemase or ESBL produced by sample, hydrolyze the antibiotic, causing a pH change evidenced by phenol red (pH indicator) solution becoming yellow. The test was interpreted negative if both tubes were red, and was not interpretable if control test tube or both tubes were yellow. [Fig. 36]

6.2 Molecular detection:

PCRs for carbapenemase and ESBL genes detection

Multiplex and simplex PCRs for carbapenemases genes detection (*bla*$_{IMP}$/*bla*$_{VIM}$/*bla*$_{KPC}$, *bla*$_{NDM}$ and *bla*$_{OXA48}$) [78] were performed for samples of Group 1 carbapenem-resistant and for discrepant samples. PCRs for relevant ESBL genes (*bla*$_{TEM}$/*bla*$_{SHV}$/*bla*$_{OXA-1}$, *bla*$_{CTX-M-1}$, *bla*$_{CTX-M-2}$, *bla*$_{CTX-M-9}$, *bla*$_{CTX-M\ 8/25}$) [78] were performed for ESBL-producing samples of Group 1.

Bacterial DNA was prepared transferring a small amount of sample colony into 1.5 ml tubes with 350 µl of distilled water, boiling at 100°C for 10 minutes and followed by centrifugation at 13,000 rpm for 5 minutes.

We used for each sample 2 µl of supernatant, 20 µl of 5 Prime Hot Master Mix-1000R (5 Prime) (Quanta BioScienses Inc.), a variable concentration of specific-group primers and filled up with distilled water for a total volume of 50 µl PCR reaction. Positive [Tab. 8] and negative control (without DNA template) were included in each amplification process. All primers were synthetized by Eurofins Genomics MWG Operon. [Tab. 10]

The PCR reactions were analyzed by electrophoresis with 0.8% agarose gel at 100 V for 60 min in 1x TAE [40 mM Tris–HCl (pH 8.3), 2 mM acetate and 1 mM EDTA] and stained with 1x Gel Red solution (Biotium).

Multiplex *bla*$_{IMP}$/*bla*$_{VIM}$/*bla*$_{KPC}$ PCR thermal profile was: 30 cycles of denaturation at 94°C for 40 sec; annealing at 55°C for 40 sec; extension at 72°C for 1 min and one final elongation cycle at 72°C for 7 min, obtaining 139bp, 390bp and 538bp product respectively. [78] [Tab. 10]

The *bla*$_{NDM}$ PCR was performed using the thermal profile: 30 cycles at 94°C for 1 min; 55°C for 1 min and 72°C for 1 min, and final elongation cycle at 72°C for 10 min, obtaining 800bp product. [Tab. 10]

The *bla*$_{OXA-48}$ PCR was performed using: 30 cycles at 94°C for 40 sec; 57°C for 40 sec and 72°C for 1 min, and final elongation cycle at 72°C for 5 min resulting in 400bp PCR product. [78] [Tab. 10]

Tab. 10 Primers used for carbapenemase genes screening [78]

Gene amplified	Primer name	Primer sequence 5'⟶3'	Amplicon Size (bp)	Primer concentration (pmol/mL)
***bla*$_{IMP}$**	IMP FW	TTG ACA CTC CAT TTA CDG	139	0.5
	IMP REV	GAT YGA GAA TTA AGC CAC YCT		0.5
***bla*$_{VIM}$**	VIM FW	GAT GGT GTT TGG TCG CAT A	390	0.5
	VIM REV	CGA ATG CGC AGC ACC AG		0.5
***bla*$_{KPC}$**	KPC FW	CAT TCA AGG GCT TTC TTG CTG C	538	0.2
	KPC REV	ACG ACG GCA TAG TCA TAT GC		0.2
***bla*$_{NDM}$**	NDM A	CAC CTC ATG TTT GAA TTC GCC	800	0.5
	NDM B	CTC TGT CAC ATC GAA ATC G		0.5
***bla*$_{OXA-48}$**	OXA-48 FW	TTG GTG GCA TCG ATT ATC GG	400	0.5
	OXA-48 Rev	CAG CAC TTC TTT TGT GAT GCC		0.5

Multiplex *bla*$_{TEM}$/*bla*$_{SHV}$/*bla*$_{OXA-1}$; multiplex *bla*$_{CTX-M-1}$/*bla*$_{CTX-M-2}$/*bla*$_{CTX-M-9}$ groups; *bla*$_{CTX-M-8/25}$ PCR reactions were performed with 30 cycles of denaturation at 94°C for 40 sec; annealing at 60°C for 40 sec; elongation at 72°C for 1 min and a final elongation at 72°C for 10 min. [78] [Tab. 11]

Tab. 11 Primers used for *β*-lactamase genes screening [78]

Gene amplified	Primer name	Primer sequence 5'⟶3'	Amplicon Size (bp)	Primer concentration (pmol/mL)
***bla*$_{TEM-1}$** ***bla*$_{TEM-2}$**	TSO-T FW	CAT TTC CGT GTC GCC CTT ATT C	800	0.4
	TSO-T REV	CGT TCA TCC ATA GTT GCC TGA C		0.4
***bla*$_{SHV-1}$**	TSO-S FW	AGC CGC TTG AGC AAA ATT AAA C	713	0.4
	TSO-S REV	ATC CCG CAG ATA AAT CAC CAC		0.4

***bla*$_{OXA-1}$, *bla*$_{OXA-4}$, *bla*$_{OXA-30}$**	TSO-O FW	GGC ACC AGA TTC AAC TTT CAA C	564	0.4
	TSO-O REV	GAC CCC AAG TTT CCT GTA AGT G		0.4
***bla*$_{CTX-M-1}$, *bla*$_{CTX-M-3}$, *bla*$_{CTX-M-15}$**	CTX-M-1 FW	TTA GGA ART GTG CCG CTG YA	688	0.4
	CTX-M-1-2 REV	CGA TAT CGT TGG TGG TRC CAT		0.2
***bla*$_{CTX-M-2}$**	CTX-M-2 FW	CGT TAA CGG CAC GAT GAC	404	0.2
	CTX-M-1-2 REV	CGA TAT CGT TGG TGG TRC CAT		0.2
***bla*$_{CTX-M-9}$, *bla*$_{CTX-M-14}$**	CTX-M-9 FW	TCA AGC CTG CCG ATC TGG T	561	0.4
	CTX-M-9 REV	TGA TTC TCG CCG CTG AAG		0.4
***bla*$_{CTX-M-8/25}$**	CTX-M-8/25 FW	AAC RCR CAG ACG CTC TAC	326	0.4
	CTXM-8/25 REV	TCG AGC CGG AA8 GTG TYA T		0.4

7. Amplicon Sequencing

For discrepant strains, the PCR products were column-purified by QIAquick® PCR Purification kit (Qiagen) and sent to Eurofins MWG Operon Ebersberg (Germany) for sequencing. Amplicons sequenced were analyzed by NCBI Genebank database on BLAST (basic local alignment search tool) program. [91] For *bla*$_{KPC}$ amplicons sequencing, we used additional primers: KPC FW 5’ TGT CAC TGT ATC GCC GTC TAG 3’; KPC REV 5’ TTA CTG CCC GTT GAC GCC CAA TCC 3’.

8. Plasmid extraction

Plasmid extraction was performed for discrepant strains with QIAPrep® Spin Miniprep Kit (Qiagen, Germany) from an overnight 5ml inoculum in Luria-Bertani (LB) broth. Before and after digestion with *EcoR*I and *BamH*I restriction enzymes (Roche), at 37°C for 1h, the plasmids extracted were loaded in a 0.6% (w/v) agarose gel and visualized after running at 100V for 60 min. The gel was stained with 1x Gel Red solution (Biotium) for 30 min. The presence of bla_{KPC} gene was confirmed by single PCR. [Tab. 10]

9. Plasmid characterization

The characterization of plasmids based on Inc/rep identification was performed for discrepant strains and for 8 KPC-producing strains with 11,109 Da peak as control group.

Five multiplex and 3 simplex PCR-based replicon typing (PBRT) [56] and 4 simplex PCR for IncQ, IncR, IncU, OriColE genes, [79] [92] were performed, using the primers summarized in Tab. 12 and 13.

For each reaction, we used 2 µl of sample, 0.5 µl of each primer, 20 µl of 5 Prime Hot Master Mix-1000R (5 Prime) (Quanta BioScienses Inc.) and filled with distilled water for a total final volume of 50 µl for each reaction.

The thermal profile of all five multiplex PCRs and simplex PCRs, except for F rep and IncQ, was: 30 cycles of 94 ºC for 1 min, 60 ºC for 30 sec, 72 ºC for 1 min and final elongation at 72 ºC for 5 min. Simplex PCR for F rep the annealing was at 52 ºC. [56] For IncQ annealing was at 62°C for 1 min and elongation at 72 ºC for 10 min. [79]

Tab. 12 Primers used for PCR based replicon typing (PBRT) [56]

Replicon amplified	Primer name	Primer sequence 5' ⟶ 3'	Amplicon Size
HI1	HI1FW	GGA GCG ATG GAT TAC TTC AGT AC	471
	HI1 REV	TGC CGT TTC ACC TCG TGA GTA	
HI2	HI2 FW	TTT CTC CTG AGT CAC CTG TTA ACA C	644
	HI2 REV	GGC TCA CTA CCG TTG TCA TCC T	
H1	H1 FW	CGA AAG CCG GAC GGC AGA A	139
	H1 REV	TCG TCG TTC CGC CAA GTT CGT	
X	X FW	AAC CTT AGA GGC TAT TTA AGT TGC TGA T	376
	X REV	TGA GAG TCA ATT TTT ATC TCA TGT TTT AGC	
L/M	L/M FW	GGA TGA AAA CTA TCA GCA TCT GAA G	785
	L/M REV	CTG CAG GGG CGA TTC TTT AGG	
N	N FW	GTC TAA CGA GCT TAC CGA AG	559
	N REV	GTT TCA ACT CTG CCA AGT TC	
FIA	FIA FW	CCA TGC TGG TTC TAG AGA AGG TG	462
	FIA REV	GTA TAT CCT TAC TGG CTT CCG CAG	
FIB	FIB FW	GGA GTT CTG ACA CAC GAT TTT CTG	702
	FIB REV	CTC CCG TCG CTT CAG GGC ATT	
W	W FW	CCT AAG AAC AAC AAA GCC CCC G	242
	W REV	GGT GCC CGG CAT AGA ACC GT	
Y	Y FW	AAT TCA AAC AAC ACT GTG CAG CCT G	765
	Y REV	GCG AGA ATG GAC GAT TAC AAA ACT TT	
P	P FW	CTA TGG CCC TGC AAA CGC GCC AGA AA	534
	P REV	TCA CGC GCC AGG GCG CAG CC	
FIC	FIC FW	GTG AAC TGG CAG ATG AGG AAG G	262
	FIC REV	TTC TCC TCG TCG CCA AAC TAG AT	
A/C	A/C FW	GAG AAC CAA AGA CAA AGA CCT GGA	465
	A/C REV	ACG ACA AAC CTG AAT TGC CTC CTT	
T	T FW	TTG GCC TGT TTG TGC CTA AAC CAT	750
	T REV	CGT TGA TTA CAC TTA GCT TTG GAC	
FIIs	FIIs FW	CTG TCG TAA GCT GAT GGC	270
	FIIs REV	CTC TGC CAC AAA CTT CAG C	
F	FrepB FW	TGA TCG TTT AAG GAA TTT TG	270
	FrepB REV	GAA GAT CAG TCA CAC CAT CC	
K	K/B FW	GCG GTC CGG AAA GCC AGA AAA C	160
	K REV	TCT TTC ACG AGC CCG CCA AA	
B	K/B FW	GCG GTC CGG AAA GCC AGA AAA C	159
	B/O REV	TCT GCG TTC CGC CAA GTT CGA	

Tab. 13 Primers used for Inc group PCRs [79] [92]

Replicon amplified	Primer name	Primer sequence 5' ⟶ 3'	Amplicon size
IncQ repB	IncQ repB 1 FW	TCG TGG TCG CGT TCA AGG TAC G	1160
	IncQ repB 2 REV	CTG TAA GTC GAT GAT CTG GGC GTT	
IncR	IncR FW	TCG CTT CAT TCC TGC TTC AGC	251
	IncR REV	GTG TGC TGT GGT TAT GCC TCA	
IncU	IncU FW	TCA CGA CAC AAG CGC AAG GG	843
	IncU REV	TCA TGG TAC ATC TGG GCG C	
ColE	OriColE FW	GTT CGT GCA TAC AGT CCA	187
	OriColE REV	GGC GAA ACC CGA CAG GAC T	

10. PCR for *p019* and *Tn4401* genes detection

For 8 discrepant strains and 8 KPC-producing strains with the 11,109 Da peak in MALDI-TOF MS spectra, used as control group, we performed also simplex PCRs to detect *p019* and *Tn4401* genes, using the primers summarized in Tab. 14. [Ref. this study]

For each reaction, we used 2 μl of sample, 0.5 μl of each primer, 20 μl of 5 Prime Hot Master Mix-1000R (5 Prime) (Quanta BioScienses Inc.) and filled with distilled water for a total final volume of 50 μl for each reaction. The thermal profiles were for *p019*: 30 cycles of 94 ºC for 1 min, 56 ºC for 1 min, 72 ºC for 1 min and final elongation at 72 ºC for 2 min; for *Tn4401*: 30 cycles of 94 ºC for 1 min, 58 ºC for 1 min, 72 ºC for 1 min and final elongation at 72 ºC for 2 min, obtaining ~300bp and ~400bp PCR product respectively.

Tab. 14 Primers used for *p019* and *Tn4401* genes screening

Gene amplified	Primer name	Primer sequence 5' ⟶ 3'	Amplicon Size (bp)	Primer concentration (pmol/mL)
p019	p019 FW	GCG GTT GAC AAA ACC ATG	~300	0.5
	p019 REV	GCT CAA ACG TCA CTA TGG C		0.5
Tn4401	Tn4401 FW	AAG TCG AGC ATG AAG CGC A	~400	0.5
	Tn4401 REV	TGA CCA CGG ACG ATG CAA T		0.5

11. Conjugation assay

Conjugation assay was performed for 2 strains of VIM-producing Enterobacteria from frozen collection, namely: 1 *C. freundii* and 1 *K. oxytoca* as donors and J53 sodium azide resistant *E. coli* as recipient. [93]

For each strain, 5 ml of LB broth inoculum were incubated at 37 ºC overnight. Afterwards we prepared 3 tubes, one with 500 µl of donor in 5 ml of LB broth, one with 500 µl of recipient in 5 ml of LB broth, the last with 500 µl of both in 5 ml of LB broth, incubated at 37°C for 6 hours. To select trans-conjugant cells, we used LB agar plates supplemented with 100 mg/L sodium-azide and 2 µl/ml imipenem. The growth of trans-conjugant cells was verified by MALDI-TOF MS identification. [94]

12. Strains typing

12.1 Pulsed-field gel electrophoresis (PFGE)

PFGE was performed for *K. pneumoniae* strains isolated during an epidemic onset in ICU, to determine genetic relatedness.

We adopted the Han protocol with some modifications. [95]

For each sample 400 µl of a LB broth inoculum incubated overnight at 37°C, was centrifuged at 8000 rpm for 5 min. The pellet was resuspended in 400 µl TE buffer (50mM Tris-HCl pH 7.5, 50mM EDTA pH 8). Then it was additioned with 20 µl of proteinase K (20 mg/ml) and 400µl 2 % (w/v) low-melting agarose (SeaKem Gold) and pipetted in wells of a reusable plug mold (Bio-Rad). Solidified at room temperature for 10 min and at 4°C for another 10 min, the plugs were transferred in 5 ml of lysis buffer (Tris 50mM, EDTA 50mM at pH 8, 0.5 mg proteinase K/ml; 1 % sarcosine) and incubated at 54 ºC overnight in a water bath. The lysis buffer was removed and the plugs washed two times with 15ml of sterile

ultrapure water and four times with 15 ml of TE buffer (Tris 10mM; EDTA 1mM pH 8.0). The washing steps were performed at room temperature for 2 hours with constant agitation. The plugs were digested with 10 U *Xba*I enzyme (Thermo Scientific) at 37 ºC overnight and placed into the wells of 1% (w/v) PFGE certified agarose gel (Bio-Rad) prepared in 0.5x TBE buffer (Tris 44.5 mM, pH 7.5; boric acid 44.5 mM; EDTA 1 mM at pH 8). The run was accomplished in CHEF MAPPER system (Bio-Rad) in 0.5x TBE buffer with conditions of 6V/cm, 120º angle, initial switch time 6.8 sec and final switch time 63.8 sec for 23 hours at 14 ºC. The gel was stained with 1x GelRed (Biotium) for 30 min. [95] The marker was Pulse Marker™ (Sigma-Aldrich) 50-1,000kb.

The results were analyzed and compared using FPQuest™ Software (Version 4.5, Bio-Rad). Similarity analysis was performed by Dice coefficients (SD) [96] with position tolerance of 1.5%.

12.2 MALDI-TOF MS spectra correlation between strains

With MALDI-TOF MS RUO it is possible to compare different strains to investigate clonal correlation, this is useful to compare strains isolated during an outbreak. For this purpose, we analyzed the spectra of selected strains, then we imported the spectra into the database. The software Saramis analyzed the spectra and compared them, producing a dendrogram based on protein profile. Relative taxonomy and absolute taxonomy can be shown, illustrating the percentages of similarity of masses in different spectra or the coincident mass number. [Fig. 37]

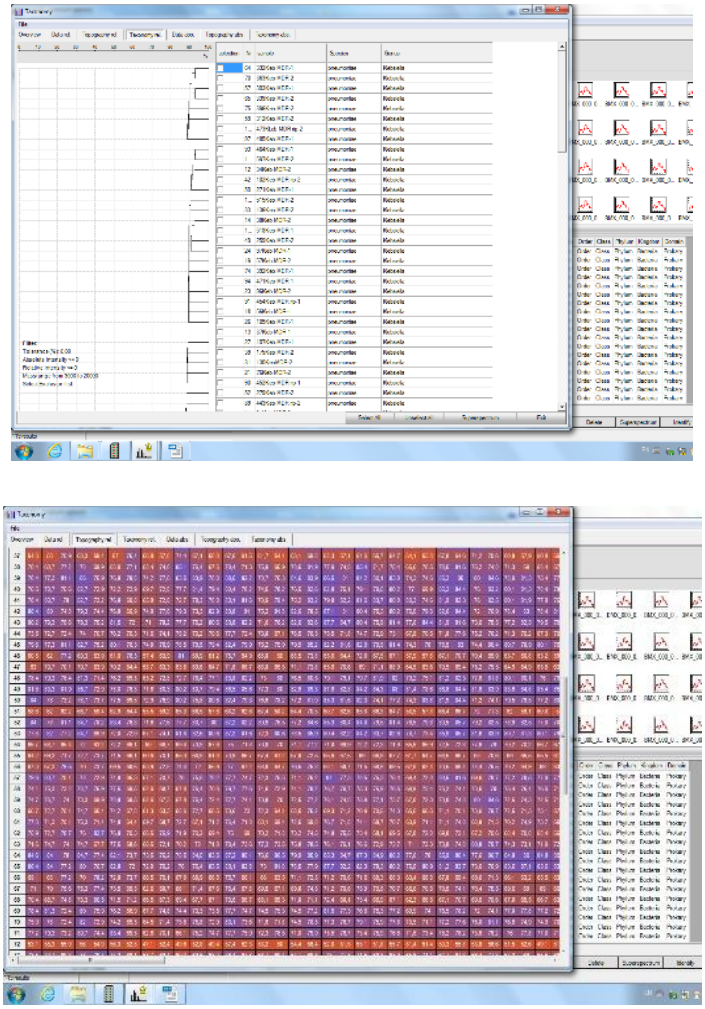


Fig. 37 Relative taxonomy: spectra comparison with dendrogram showing percentage of similarity (above) and masses comparison (below).

PART I

a. MALDI-TOF MS spectra analysis to evaluate the correlation between KPC production and 11,109Da peak

In the first part of the study, we confirmed the association between KPC production in *Enterobacteriaceae* and the presence of 11,109Da peak [73] in MALDI-TOF MS spectra and we evaluated the use of peak detection during clinical routine workflow. For this purpose, we selected two samples groups: the first group was used to confirm the correlation KPC enzyme production/11,109Da peak; the second group was used to confirm the possibility to use the peak search during routine diagnostic work to quickly obtain reliable results about KPC production.

Group 1

A total of 436 *Enterobacteriaceae* strains: 128 from Verona and 107 from Negrar Hospital, isolated during routine diagnostics and 201 as control group from collection, were analyzed.

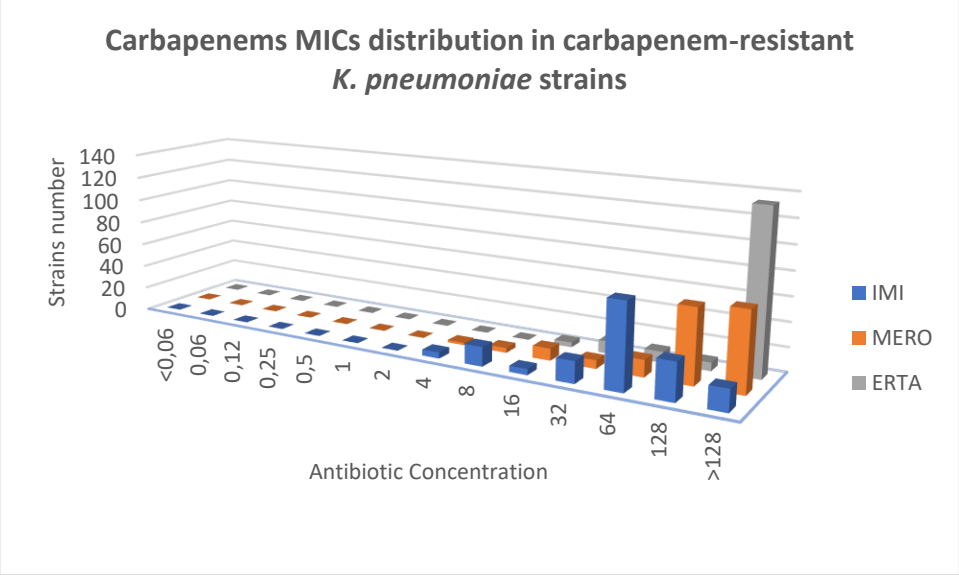
The strains from Verona Hospital were: 100 carbapenem-resistant (95 *K. pneumoniae* and 5 *E. coli*); 4 ertapenem-resistant *E. coli* and 24 cephalosporin-resistant *K. pneumoniae*. The strains from Negrar Hospital were: 77 carbapenem-resistant (68 *K. pneumoniae* and 9 *E. coli*) and 30 cephalosporin-resistant *K. pneumoniae*.

For all these strains, we performed identification and susceptibility testing during routine workflow and during the study we confirmed the pattern of resistance by MIC evaluation and carbapenemase production by rapid Carba NP test. [68] PCRs for *bla* genes were performed to investigate the mechanism of resistance.

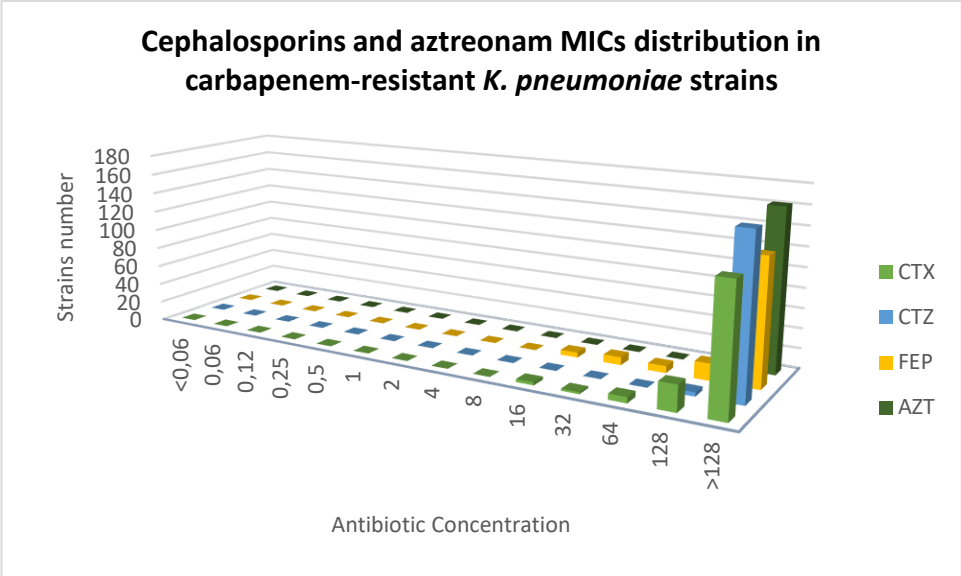
Susceptibility testing by MIC evaluation was performed for carbapenem-resistant *K. pneumoniae* and *E. coli* strains, for ertapenem-resistant *E. coli* strains and for cephalosporin-resistant *K. pneumoniae*.

The carbapenems, cephalosporins and aztreonam MICs distribution in totally 163 carbapenem-resistant *K. pneumoniae* strains are shown in graph. 2 and 3.

Carbapenem MICs ranged from 4 μg/ml to >128 μg/ml for imipenem and meropenem and from 16 μg/ml to >128 μg/ml for ertapenem. Cephalosporins MICs ranged from 16 μg/ml to >128 μg/ml for cefotaxime and cefepime; all strains had ceftazidime MIC ≥128 μg/ml.



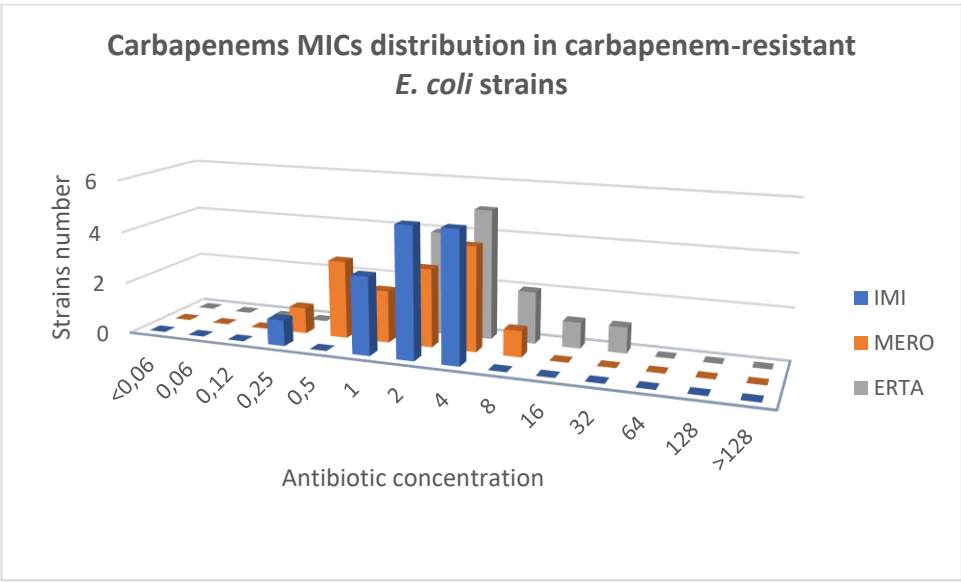
Graph. 2 Carbapenems MICs distribution in totally 163 carbapenem-resistant *K. pneumoniae* strains. (IMI=Imipenem; MERO=Meropenem; ERTA=Ertapenem)



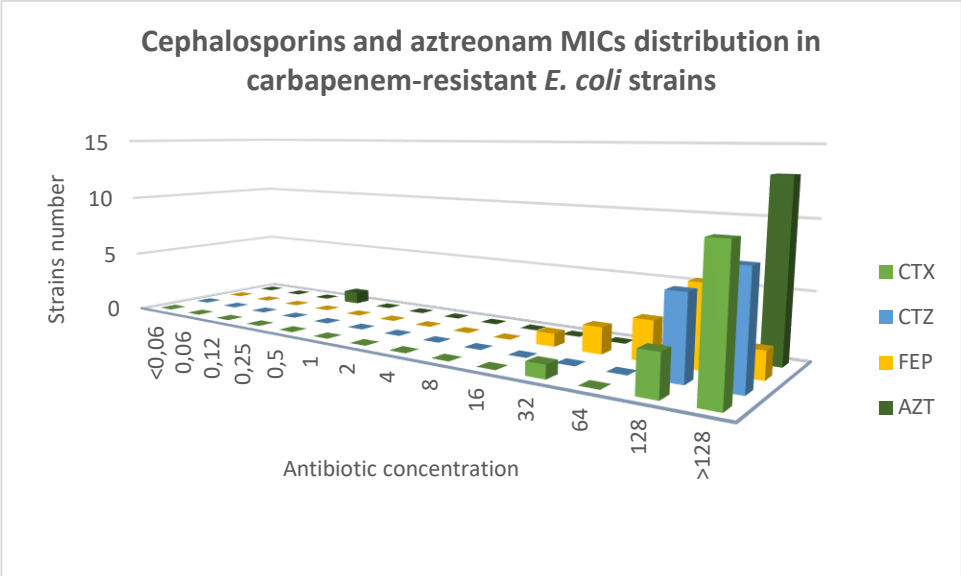
Graph. 3 Cephalosporins and aztreonam MICs distribution in totally 163 carbapenem-resistant *K. pneumoniae* strains. (CTX=Cefotaxime; CTZ=Ceftazidime; FEP=Cefepime; AZT=Aztreonam)

The MICs distribution in totally 14 carbapenem-resistant *E. coli* strains are shown in graph. 4 and 5.

Carbapenem MICs ranged from 0.25 μg/ml to 4 μg/ml for imipenem; from 0.25 μg/ml to 8 μg/ml for meropenem, and from 1 μg/ml to 32 μg/ml for ertapenem. Cefotaxime and cefepime MICs ranged from 16 μg/ml to >128 μg/ml, and all strains had ceftazidime MIC ≥128 μg/ml.



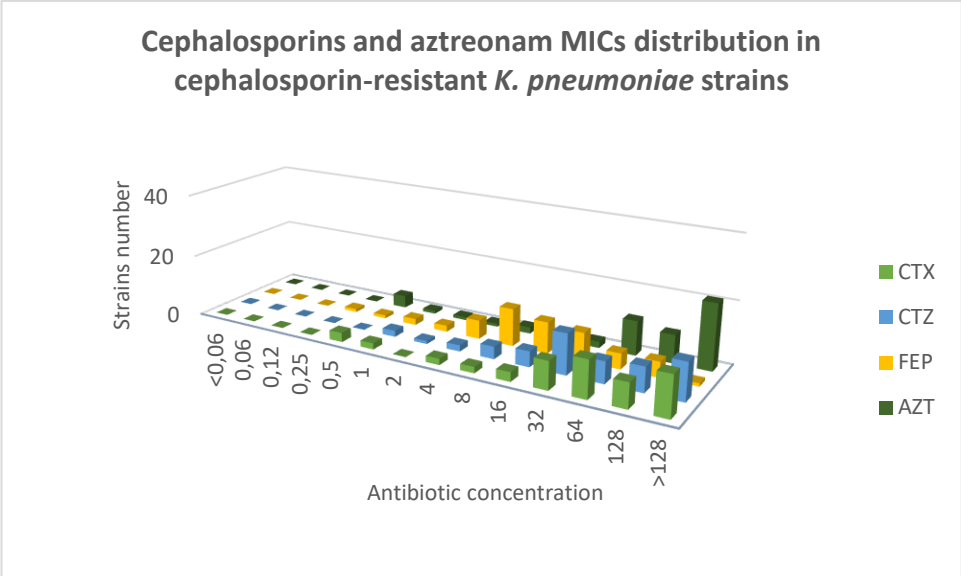
Graph. 4 Carbapenems MICs distribution in 14 carbapenem-resistant *E. coli* strains. (IMI=Imipenem; MERO=Meropenem; ERTA=Ertapenem)



Graph. 5 Cephalosporins and aztreonam MICs distribution in totally 14 carbapenem-resistant *E. coli* strains. (CTX=Cefotaxime; CTZ=Ceftazidime; FEP=Cefepime; AZT=Aztreonam)

Carbapenems MICs in 4 ertapenem-resistant *E. coli* strains had different range: from 0.12 μg/ml and 0.5 μg/ml for imipenem; from <0.06 μg/ml to 8 μg/ml for meropenem, and from 0.5 μg/ml to 64 μg/ml for ertapenem. Cephalosporins MICs ranged from 64 μg/ml to >128 μg/ml for ceftazidime; all strains had MIC ≥128 for cefepime and >128 for cefotaxime and aztreonam.

Cephalosporins MICs distribution in totally 54 cephalosporin-resistant *K. pneumoniae* strains are shown in graph. 6. Cefotaxime MICs ranged from 0.5 μg/ml to >128 μg/ml; ceftazidime from 1 μg/ml to >128 μg/ml, and cefepime from 0.25 μg/ml to >128 μg/ml. MIC range for aztreonam is from 0.5 μg/ml to >128 μg/ml.



Graph. 6 Cephalosporins and aztreonam MICs distribution in totally 54 cephalosporin-resistant *K. pneumoniae* strains. (CTX=Cefotaxime; CTZ=Ceftazidime; FEP=Cefepime; AZT=Aztreonam)

PCR amplification for *bla* genes showed:

Carbapenem-resistant *K. pneumoniae* strains from Verona: 95 (100%) identified as possible KPC producers by positive Carba NP test. By PCR, all carried bla_{KPC} gene, and 1 strain (1.05%) also co-carried a bla_{VIM} gene. All were negative for bla_{IMP}, bla_{NDM}, or bla_{OXA-48}. Two out of 95 strains (2.1%) carried only bla_{KPC} gene; 69 (72.6%) co-carried bla_{KPC} and bla_{TEM}; 1 (1.05%) co-carried bla_{KPC}, bla_{VIM} and bla_{TEM}; 21 (22.1%) bla_{KPC}, bla_{TEM} and $bla_{SHV;}$ 1 (1.05%) co-carried bla_{KPC}, bla_{TEM} and $bla_{CTX-M-1}$; 1 (1.05%) co-carried bla_{KPC} and bla_{SHV}. [Tab.15]

Tab. 15 Results of Carba NP test and PCRs for *bla* genes in 95 carbapenem-resistant *K. pneumoniae* strains from Verona Hospital

Strain	Carba Test	KPC	VIM	IMP	NDM	OXA-48	TEM	SHV	OXA-1	CTX-M-1	CTX-M-2	CTX-M-9	CTX-M-8/25
MDR4	Pos	Pos	Neg	Neg	Neg	Neg	Pos	Neg	Neg	Neg	Neg	Neg	Neg
MDR6	Pos	Pos	Neg	Neg	Neg	Neg	Pos	Neg	Neg	Neg	Neg	Neg	Neg
MDR10	Pos	Pos	Neg	Neg	Neg	Neg	Pos	Neg	Neg	Neg	Neg	Neg	Neg
MDR11	Pos	Pos	Neg	Neg	Neg	Neg	Pos	Neg	Neg	Neg	Neg	Neg	Neg
MDR12	Pos	Pos	Neg	Neg	Neg	Neg	Pos	Neg	Neg	Neg	Neg	Neg	Neg
MDR20	Pos	Pos	Neg	Neg	Neg	Neg	Pos	Neg	Neg	Neg	Neg	Neg	Neg
MDR21	Pos	Pos	Neg	Neg	Neg	Neg	Pos	Neg	Neg	Neg	Neg	Neg	Neg
MDR22	Pos	Pos	Neg	Neg	Neg	Neg	Pos	Neg	Neg	Neg	Neg	Neg	Neg
MDR25	Pos	Pos	Neg	Neg	Neg	Neg	Pos	Neg	Neg	Neg	Neg	Neg	Neg
MDR31	Pos	Pos	Neg	Neg	Neg	Neg	Pos	Neg	Neg	Neg	Neg	Neg	Neg
MDR34	Pos	Pos	Neg	Neg	Neg	Neg	Pos	Neg	Neg	Neg	Neg	Neg	Neg
MDR37	Pos	Pos	Neg	Neg	Neg	Neg	Pos	Neg	Neg	Pos	Neg	Neg	Neg
MDR38	Pos	Pos	Neg	Neg	Neg	Neg	Pos	Neg	Neg	Neg	Neg	Neg	Neg
MDR50	Pos	Pos	Neg	Neg	Neg	Neg	Pos	Neg	Neg	Neg	Neg	Neg	Neg
MDR53	Pos	Pos	Neg	Neg	Neg	Neg	Pos	Neg	Neg	Neg	Neg	Neg	Neg
MDR56	Pos	Pos	Neg	Neg	Neg	Neg	Pos	Neg	Neg	Neg	Neg	Neg	Neg
MDR57	Pos	Pos	Neg	Neg	Neg	Neg	Pos	Neg	Neg	Neg	Neg	Neg	Neg
MDR59	Pos	Pos	Neg	Neg	Neg	Neg	Pos	Neg	Neg	Neg	Neg	Neg	Neg
MDR76	Pos	Pos	Neg	Neg	Neg	Neg	Pos	Neg	Neg	Neg	Neg	Neg	Neg
MDR88	Pos	Pos	Neg	Neg	Neg	Neg	Pos	Neg	Neg	Neg	Neg	Neg	Neg
MDR96	Pos	Pos	Neg	Neg	Neg	Neg	Pos	Neg	Neg	Neg	Neg	Neg	Neg
MDR97	Pos	Pos	Neg	Neg	Neg	Neg	Pos	Neg	Neg	Neg	Neg	Neg	Neg
MDR98	Pos	Pos	Neg	Neg	Neg	Neg	Pos	Neg	Neg	Neg	Neg	Neg	Neg
MDR102	Pos	Pos	Neg	Neg	Neg	Neg	Pos	Neg	Neg	Neg	Neg	Neg	Neg
MDR105	Pos	Pos	Neg	Neg	Neg	Neg	Pos	Neg	Neg	Neg	Neg	Neg	Neg
MDR107	Pos	Pos	Neg	Neg	Neg	Neg	Pos	Neg	Neg	Neg	Neg	Neg	Neg
MDR128	Pos	Pos	Neg	Neg	Neg	Neg	Pos	Neg	Neg	Neg	Neg	Neg	Neg
MDR130	Pos	Pos	Neg	Neg	Neg	Neg	Pos	Neg	Neg	Neg	Neg	Neg	Neg
MDR133	Pos	Pos	Neg	Neg	Neg	Neg	Pos	Neg	Neg	Neg	Neg	Neg	Neg
MDR136	Pos	Pos	Neg	Neg	Neg	Neg	Pos	Neg	Neg	Neg	Neg	Neg	Neg
MDR154	Pos	Pos	Neg	Neg	Neg	Neg	Pos	Neg	Neg	Neg	Neg	Neg	Neg

Strain	Carba Test	KPC	VIM	IMP	NDM	OXA-48	TEM	SHV	OXA-1	CTX-M-1	CTX-M-2	CTX-M-9	CTX-M-8/25
MDR172	Pos	Pos	Neg	Neg	Neg	Neg	Pos	Neg	Neg	Neg	Neg	Neg	Neg
MDR173	Pos	Pos	Neg	Neg	Neg	Neg	Pos	Neg	Neg	Neg	Neg	Neg	Neg
MDR174	Pos	Pos	Neg	Neg	Neg	Neg	Pos	Neg	Neg	Neg	Neg	Neg	Neg
MDR175	Pos	Pos	Neg	Neg	Neg	Neg	Pos	Neg	Neg	Neg	Neg	Neg	Neg
MDR183	Pos	Pos	Neg	Neg	Neg	Neg	Pos	Neg	Neg	Neg	Neg	Neg	Neg
MDR208	Pos	Pos	Neg	Neg	Neg	Neg	Pos	Neg	Neg	Neg	Neg	Neg	Neg
MDR235	Pos	Pos	Neg	Neg	Neg	Neg	Pos	Neg	Neg	Neg	Neg	Neg	Neg
MDR250	Pos	Pos	Neg	Neg	Neg	Neg	Pos	Neg	Neg	Neg	Neg	Neg	Neg
MDR259	Pos	Pos	Neg	Neg	Neg	Neg	Pos	Neg	Neg	Neg	Neg	Neg	Neg
MDR271	Pos	Pos	Neg	Neg	Neg	Neg	Pos	Neg	Neg	Neg	Neg	Neg	Neg
MDR279	Pos	Pos	Neg	Neg	Neg	Neg	Pos	Neg	Neg	Neg	Neg	Neg	Neg
MDR285	Pos	Pos	Neg	Neg	Neg	Neg	Pos	Neg	Neg	Neg	Neg	Neg	Neg
MDR295	Pos	Pos	Neg	Neg	Neg	Neg	Pos	Neg	Neg	Neg	Neg	Neg	Neg
MDR300	Pos	Pos	Neg	Neg	Neg	Neg	Pos	Neg	Neg	Neg	Neg	Neg	Neg
MDR303	Pos	Pos	Neg	Neg	Neg	Neg	Pos	Neg	Neg	Neg	Neg	Neg	Neg
MDR305	Pos	Pos	Neg	Neg	Neg	Neg	Pos	Neg	Neg	Neg	Neg	Neg	Neg
MDR315	Pos	Pos	Neg	Neg	Neg	Neg	Pos	Pos	Neg	Neg	Neg	Neg	Neg
MDR323	Pos	Pos	Neg	Neg	Neg	Neg	Pos	Pos	Neg	Neg	Neg	Neg	Neg
MDR332	Pos	Pos	Neg	Neg	Neg	Neg	Pos	Neg	Neg	Neg	Neg	Neg	Neg
MDR339	Pos	Pos	Neg	Neg	Neg	Neg	Pos	Neg	Neg	Neg	Neg	Neg	Neg
MDR345	Pos	Pos	Neg	Neg	Neg	Neg	Pos	Neg	Neg	Neg	Neg	Neg	Neg
MDR350	Pos	Pos	Neg	Neg	Neg	Neg	Pos	Pos	Neg	Neg	Neg	Neg	Neg
MDR352	Pos	Pos	Neg	Neg	Neg	Neg	Pos	Neg	Neg	Neg	Neg	Neg	Neg
MDR358	Pos	Pos	Neg	Neg	Neg	Neg	Pos	Pos	Neg	Neg	Neg	Neg	Neg
MDR363	Pos	Pos	Neg	Neg	Neg	Neg	Pos	Neg	Neg	Neg	Neg	Neg	Neg
MDR367	Pos	Pos	Neg	Neg	Neg	Neg	Pos	Neg	Neg	Neg	Neg	Neg	Neg
MDR386	Pos	Pos	Neg	Neg	Neg	Neg	Pos	Neg	Neg	Neg	Neg	Neg	Neg
MDR396	Pos	Pos	Neg	Neg	Neg	Neg	Pos	Neg	Neg	Neg	Neg	Neg	Neg
MDR400	Pos	Pos	Neg	Neg	Neg	Neg	Pos	Pos	Neg	Neg	Neg	Neg	Neg
MDR403	Pos	Pos	Neg	Neg	Neg	Neg	Pos	Pos	Neg	Neg	Neg	Neg	Neg
MDR404	Pos	Pos	Neg	Neg	Neg	Neg	Pos	Pos	Neg	Neg	Neg	Neg	Neg

Strain	Carba Test	KPC	VIM	IMP	NDM	OXA-48	TEM	SHV	OXA-1	CTX-M-1	CTX-M-2	CTX-M-9	CTX-M-8/25
MDR409	Pos	Pos	Neg	Neg	Neg	Neg	Pos	Neg	Neg	Neg	Neg	Neg	Neg
MDR420	Pos	Pos	Neg	Neg	Neg	Neg	Pos	Neg	Neg	Neg	Neg	Neg	Neg
MDR427	Pos	Pos	Neg	Neg	Neg	Neg	Pos	Pos	Neg	Neg	Neg	Neg	Neg
MDR428	Pos	Pos	Neg	Neg	Neg	Neg	Pos	Pos	Neg	Neg	Neg	Neg	Neg
MDR430	Pos	Pos	Neg	Neg	Neg	Neg	Pos	Pos	Neg	Neg	Neg	Neg	Neg
MDR436	Pos	Pos	Neg	Neg	Neg	Neg	Pos	Pos	Neg	Neg	Neg	Neg	Neg
MDR443	Pos	Pos	Neg	Neg	Neg	Neg	Pos	Neg	Neg	Neg	Neg	Neg	Neg
MDR452	Pos	Pos	Neg	Neg	Neg	Neg	Pos	Neg	Neg	Neg	Neg	Neg	Neg
MDR454	Pos	Pos	Neg	Neg	Neg	Neg	Pos	Neg	Neg	Neg	Neg	Neg	Neg
MDR463	Pos	Pos	Neg	Neg	Neg	Neg	Neg	Pos	Neg	Neg	Neg	Neg	Neg
MDR464	Pos	Pos	Neg	Neg	Neg	Neg	Pos	Pos	Neg	Neg	Neg	Neg	Neg
MDR471	Pos	Pos	Neg	Neg	Neg	Neg	Pos	Pos	Neg	Neg	Neg	Neg	Neg
MDR473	Pos	Pos	Neg	Neg	Neg	Neg	Pos	Pos	Neg	Neg	Neg	Neg	Neg
MDR480	Pos	Pos	Neg	Neg	Neg	Neg	Pos	Neg	Neg	Neg	Neg	Neg	Neg
MDR485	Pos	Pos	Neg	Neg	Neg	Neg	Pos	Neg	Neg	Neg	Neg	Neg	Neg
MDR488	Pos	Pos	Neg	Neg	Neg	Neg	Pos	Neg	Neg	Neg	Neg	Neg	Neg
MDR494	Pos	Pos	Neg	Neg	Neg	Neg	Pos	Neg	Neg	Neg	Neg	Neg	Neg
MDR506	Pos	Pos	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg
MDR508	Pos	Pos	Neg	Neg	Neg	Neg	Pos	Neg	Neg	Neg	Neg	Neg	Neg
MDR515	Pos	Pos	Neg	Neg	Neg	Neg	Pos	Neg	Neg	Neg	Neg	Neg	Neg
MDR516	Pos	Pos	Pos	Neg	Neg	Neg	Pos	Neg	Neg	Neg	Neg	Neg	Neg
MDR518	Pos	Pos	Neg	Neg	Neg	Neg	Pos	Neg	Neg	Neg	Neg	Neg	Neg
MDR567	Pos	Pos	Neg	Neg	Neg	Neg	Pos	Pos	Neg	Neg	Neg	Neg	Neg
MDR574	Pos	Pos	Neg	Neg	Neg	Neg	Pos	Pos	Neg	Neg	Neg	Neg	Neg
MDR582	Pos	Pos	Neg	Neg	Neg	Neg	Pos	Pos	Neg	Neg	Neg	Neg	Neg
MDR587	Pos	Pos	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg
MDR588	Pos	Pos	Neg	Neg	Neg	Neg	Pos	Neg	Neg	Neg	Neg	Neg	Neg
MDR593	Pos	Pos	Neg	Neg	Neg	Neg	Pos	Pos	Neg	Neg	Neg	Neg	Neg
MDR597	Pos	Pos	Neg	Neg	Neg	Neg	Pos	Pos	Neg	Neg	Neg	Neg	Neg
MDR614	Pos	Pos	Neg	Neg	Neg	Neg	Pos	Pos	Neg	Neg	Neg	Neg	Neg
MDR624	Pos	Pos	Neg	Neg	Neg	Neg	Pos	Pos	Neg	Neg	Neg	Neg	Neg

Pos, positive; Neg, negative

Carbapenem-resistant *E. coli* strains from Verona: 4 out of 5 (80%) identified as possible KPC producers by positive Carba NP test. Four out of 5 (80%) harbored *bla*$_{KPC}$ gene and 1 (20%) *bla*$_{VIM}$. None were positive for *bla*$_{IMP}$, *bla*$_{NDM}$, or *bla*$_{OXA-48}$. Three strains (60%) co-carried *bla*$_{KPC}$ and *bla*$_{TEM}$; 1 (20%) *bla*$_{KPC}$, *bla*$_{TEM}$, *bla*$_{CTX-M-2}$ and *bla*$_{CTX-M-9}$; 1 (20%) *bla*$_{VIM}$ and *bla*$_{TEM}$. [Tab. 16]

Tab. 16 Results of Carba NP test and PCRs for *bla* genes in 5 carbapenem-resistant *E. coli* strains from Verona Hospital

Strain	Carba Test	KPC	VIM	IMP	NDM	OXA-48	TEM	SHV	OXA-1	CTX-M-1	CTX-M-2	CTX-M-9	CTX-M-8/25
MDR219	Neg	Neg	**Pos**	Neg	Neg	Neg	**Pos**	Neg	Neg	Neg	Neg	Neg	Neg
MDR368	**Pos**	**Pos**	Neg	Neg	Neg	Neg	**Pos**	Neg	Neg	Neg	**Pos**	**Pos**	Neg
MDR434	**Pos**	**Pos**	Neg	Neg	Neg	Neg	**Pos**	Neg	Neg	Neg	Neg	Neg	Neg
MDR600	**Pos**	**Pos**	Neg	Neg	Neg	Neg	**Pos**	Neg	Neg	Neg	Neg	Neg	Neg
MDR646	**Pos**	**Pos**	Neg	Neg	Neg	Neg	**Pos**	Neg	Neg	Neg	Neg	Neg	Neg

Pos, positive; Neg, negative

Ertapenem-resistant *E. coli* strains from Verona Hospital: 4 strains all negative for Carba NP Test and positive for ESBL NDP Test. All were negative for carbapenemase genes PCR, but co-carried *bla*$_{OXA-1}$ and *bla*$_{CTX-M-1}$. [Tab. 17]

Tab. 17 Results of Carba NP test and PCRs for *bla* genes in 4 ertapenem-resistant *E. coli* strains from Verona

Strain	Carba Test	KPC	VIM	IMP	NDM	OXA-48	TEM	SHV	OXA-1	CTX-M-1	CTX-M-2	CTX-M-9	CTX-M-8/25
MDR135	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	**Pos**	**Pos**	Neg	Neg	Neg
MDR162	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	**Pos**	**Pos**	Neg	Neg	Neg
MDR220	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	**Pos**	**Pos**	Neg	Neg	Neg
MDR412	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	**Pos**	**Pos**	Neg	Neg	Neg

Pos, positive; Neg, negative

Cephalosporin-resistant *K. pneumoniae* strains from Verona Hospital: 23 out of 24 strains (95.8%) identified as possible ESBL-producers by positive ESBL NDP test; all were negative for Carba NP test. None carried carbapenemase genes. Five out of 24 (20.8%) carried bla_{TEM} gene; 1 (4.2%) bla_{SHV}; 1 (4.2%) bla_{OXA-1}; 3 (12.5%) $bla_{CTX-M-1}$; 1 (4.2%) co-carried bla_{TEM} and $bla_{CTX-M-1;\ 1}$ (4.2%) bla_{SHV} and $bla_{CTX-M-1}$; 5 (20.8%) bla_{TEM}, bla_{OXA-1} and $bla_{CTX-M-1}$; 1 (4.2%) bla_{TEM}, $bla_{CTX-M-1}$, $bla_{CTX-M-2}$ and $bla_{CTX-M-9}$; 1 (4.2%) bla_{TEM}, bla_{SHV}, $bla_{CTX-M-1}$, $bla_{CTX-M-2}$ and $bla_{CTX-M-9}$; 5 (20.8%) carried none of *bla* genes investigated. [Tab. 18]

Tab. 18 Results of Carba NP test, ESBL NDP test and PCRs for *bla* genes in 24 cephalosporin-resistant *K. pneumoniae* strains from Verona Hospital

Strain	Carba Test	ESBL Test	TEM	SHV	OXA-1	CTX-M-1	CTX-M-2	CTX-M-9	CTX-M-8/25
MDR29	Neg	**Pos**	Neg	Neg	Neg	Neg	Neg	Neg	Neg
MDR39	Neg	**Pos**	Neg	Neg	**Pos**	Neg	Neg	Neg	Neg
MDR66	Neg	**Pos**	Neg	Neg	Neg	Neg	Neg	Neg	Neg
MDR108	Neg	**Pos**	Neg	Neg	Neg	**Pos**	Neg	Neg	Neg
MDR121	Neg	**Pos**	**Pos**	Neg	Neg	Neg	Neg	Neg	Neg
MDR137	Neg	**Pos**	Neg	Neg	Neg	**Pos**	Neg	Neg	Neg
MDR255	Neg	**Pos**	**Pos**	Neg	Neg	**Pos**	Neg	Neg	Neg
MDR276	Neg	**Pos**	**Pos**	Neg	**Pos**	**Pos**	Neg	Neg	Neg
MDR277	Neg	**Pos**	**Pos**	Neg	Neg	Neg	Neg	Neg	Neg
MDR293	Neg	**Pos**	**Pos**	Neg	**Pos**	**Pos**	Neg	Neg	Neg
MDR312	Neg	**Pos**	**Pos**	Neg	Neg	**Pos**	**Pos**	**Pos**	Neg
MDR313	Neg	**Pos**	**Pos**	**Pos**	Neg	**Pos**	**Pos**	**Pos**	Neg
MDR329	Neg	**Pos**	**Pos**	Neg	**Pos**	**Pos**	Neg	Neg	Neg
MDR381	Neg	**Pos**	Neg	Neg	Neg	**Pos**	Neg	Neg	Neg
MDR419	Neg	**Pos**	**Pos**	Neg	**Pos**	**Pos**	Neg	Neg	Neg
MDR424	Neg	**Pos**	Neg	Neg	Neg	Neg	Neg	Neg	Neg
MDR433	Neg	**Pos**	Neg	Neg	Neg	Neg	Neg	Neg	Neg
MDR447	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg
MDR478	Neg	**Pos**	**Pos**	Neg	Neg	Neg	Neg	Neg	Neg
MDR496	Neg	**Pos**	**Pos**	Neg	**Pos**	**Pos**	Neg	Neg	Neg
MDR500	Neg	**Pos**	**Pos**	Neg	Neg	Neg	Neg	Neg	Neg
MDR527	Neg	**Pos**	**Pos**	Neg	Neg	Neg	Neg	Neg	Neg
MDR546	Neg	**Pos**	Neg	**Pos**	Neg	Neg	Neg	Neg	Neg
MDR559	Neg	**Pos**	Neg	**Pos**	Neg	**Pos**	Neg	Neg	Neg

Pos, positive; Neg, negative

Carbapenem-resistant *K. pneumoniae* strains from Negrar Hospital: all 68 strains resulted positive at Carba NP test. All carried bla_{KPC} gene, none carried other carbapenemase genes. Two out of 68 strain (2.9%) carried only bla_{KPC} gene; 34 (50%) co-carried bla_{KPC} and bla_{TEM}; 31 (45.6%) bla_{KPC}, bla_{TEM} and bla_{SHV}; 1 (1.5%) co-carried bla_{KPC}, bla_{TEM} and $bla_{CTX\text{-}M\text{-}2}$. [Tab. 19]

Tab. 19 Results of Carba NP test and PCRs for *bla* genes in 68 carbapenem-resistant *K. pneumoniae* strains from Negrar Hospital

Strain	Carba Test	KPC	VIM	IMP	NDM	OXA-48	TEM	SHV	OXA-1	CTX-M-1	CTX-M-2	CTX-M-9	CTX-M-8/25
N5	Pos	Pos	Neg	Neg	Neg	Neg	Pos	Neg	Neg	Neg	Neg	Neg	Neg
N6	Pos	Pos	Neg	Neg	Neg	Neg	Pos	Neg	Neg	Neg	Neg	Neg	Neg
N7	Pos	Pos	Neg	Neg	Neg	Neg	Pos	Pos	Neg	Neg	Neg	Neg	Neg
N15	Pos	Pos	Neg	Neg	Neg	Neg	Pos	Pos	Neg	Neg	Neg	Neg	Neg
N28	Pos	Pos	Neg	Neg	Neg	Neg	Pos	Pos	Neg	Neg	Neg	Neg	Neg
N29	Pos	Pos	Neg	Neg	Neg	Neg	Pos	Pos	Neg	Neg	Neg	Neg	Neg
N45	Pos	Pos	Neg	Neg	Neg	Neg	Pos	Neg	Neg	Neg	Neg	Neg	Neg
N46	Pos	Pos	Neg	Neg	Neg	Neg	Pos	Neg	Neg	Neg	Neg	Neg	Neg
N50	Pos	Pos	Neg	Neg	Neg	Neg	Pos	Neg	Neg	Neg	Neg	Neg	Neg
N54	Pos	Pos	Neg	Neg	Neg	Neg	Pos	Pos	Neg	Neg	Neg	Neg	Neg
N57	Pos	Pos	Neg	Neg	Neg	Neg	Pos	Pos	Neg	Neg	Neg	Neg	Neg
N62	Pos	Pos	Neg	Neg	Neg	Neg	Pos	Pos	Neg	Neg	Neg	Neg	Neg
N63	Pos	Pos	Neg	Neg	Neg	Neg	Pos	Pos	Neg	Neg	Neg	Neg	Neg
N72	Pos	Pos	Neg	Neg	Neg	Neg	Pos	Pos	Neg	Neg	Neg	Neg	Neg
N79	Pos	Pos	Neg	Neg	Neg	Neg	Pos	Pos	Neg	Neg	Neg	Neg	Neg
N87	Pos	Pos	Neg	Neg	Neg	Neg	Pos	Neg	Neg	Neg	Neg	Neg	Neg
N90	Pos	Pos	Neg	Neg	Neg	Neg	Pos	Neg	Neg	Neg	Neg	Neg	Neg
N92	Pos	Pos	Neg	Neg	Neg	Neg	Pos	Pos	Neg	Neg	Neg	Neg	Neg
N98	Pos	Pos	Neg	Neg	Neg	Neg	Pos	Pos	Neg	Neg	Neg	Neg	Neg
N110	Pos	Pos	Neg	Neg	Neg	Neg	Pos	Pos	Neg	Neg	Neg	Neg	Neg
N111	Pos	Pos	Neg	Neg	Neg	Neg	Pos	Neg	Neg	Neg	Neg	Neg	Neg
N114	Pos	Pos	Neg	Neg	Neg	Neg	Pos	Neg	Neg	Neg	Neg	Neg	Neg
N132	Pos	Pos	Neg	Neg	Neg	Neg	Pos	Neg	Neg	Neg	Pos	Neg	Neg

Strain	Carba Test	KPC	VIM	IMP	NDM	OXA-48	TEM	SHV	OXA-1	CTX-M-1	CTX-M-2	CTX-M-9	CTX-M-8/25
N134	Pos	Pos	Neg	Neg	Neg	Neg	Pos	Neg	Neg	Neg	Neg	Neg	Neg
N135	Pos	Pos	Neg	Neg	Neg	Neg	Pos	Neg	Neg	Neg	Neg	Neg	Neg
N141	Pos	Pos	Neg	Neg	Neg	Neg	Pos	Neg	Neg	Neg	Neg	Neg	Neg
N142	Pos	Pos	Neg	Neg	Neg	Neg	Pos	Neg	Neg	Neg	Neg	Neg	Neg
N143	Pos	Pos	Neg	Neg	Neg	Neg	Pos	Neg	Neg	Neg	Neg	Neg	Neg
N148	Pos	Pos	Neg	Neg	Neg	Neg	Pos	Neg	Neg	Neg	Neg	Neg	Neg
N149	Pos	Pos	Neg	Neg	Neg	Neg	Pos	Pos	Neg	Neg	Neg	Neg	Neg
N156	Pos	Pos	Neg	Neg	Neg	Neg	Pos	Neg	Neg	Neg	Neg	Neg	Neg
N165	Pos	Pos	Neg	Neg	Neg	Neg	Pos	Neg	Neg	Neg	Neg	Neg	Neg
N166	Pos	Pos	Neg	Neg	Neg	Neg	Pos	Neg	Neg	Neg	Neg	Neg	Neg
N180	Pos	Pos	Neg	Neg	Neg	Neg	Pos	Neg	Neg	Neg	Neg	Neg	Neg
N181	Pos	Pos	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg
N187	Pos	Pos	Neg	Neg	Neg	Neg	Pos	Pos	Neg	Neg	Neg	Neg	Neg
N188	Pos	Pos	Neg	Neg	Neg	Neg	Pos	Pos	Neg	Neg	Neg	Neg	Neg
N191	Pos	Pos	Neg	Neg	Neg	Neg	Pos	Neg	Neg	Neg	Neg	Neg	Neg
N205	Pos	Pos	Neg	Neg	Neg	Neg	Pos	Pos	Neg	Neg	Neg	Neg	Neg
N207	Pos	Pos	Neg	Neg	Neg	Neg	Pos	Pos	Neg	Neg	Neg	Neg	Neg
N210	Pos	Pos	Neg	Neg	Neg	Neg	Pos	Neg	Neg	Neg	Neg	Neg	Neg
N217	Pos	Pos	Neg	Neg	Neg	Neg	Pos	Neg	Neg	Neg	Neg	Neg	Neg
N224	Pos	Pos	Neg	Neg	Neg	Neg	Pos	Neg	Neg	Neg	Neg	Neg	Neg
N225	Pos	Pos	Neg	Neg	Neg	Neg	Pos	Neg	Neg	Neg	Neg	Neg	Neg
N227	Pos	Pos	Neg	Neg	Neg	Neg	Pos	Neg	Neg	Neg	Neg	Neg	Neg
N236	Pos	Pos	Neg	Neg	Neg	Neg	Pos	Pos	Neg	Neg	Neg	Neg	Neg
N253	Pos	Pos	Neg	Neg	Neg	Neg	Pos	Pos	Neg	Neg	Neg	Neg	Neg
N258	Pos	Pos	Neg	Neg	Neg	Neg	Pos	Neg	Neg	Neg	Neg	Neg	Neg
N263	Pos	Pos	Neg	Neg	Neg	Neg	Pos	Neg	Neg	Neg	Neg	Neg	Neg
N276	Pos	Pos	Neg	Neg	Neg	Neg	Pos	Neg	Neg	Neg	Neg	Neg	Neg
N282	Pos	Pos	Neg	Neg	Neg	Neg	Pos	Neg	Neg	Neg	Neg	Neg	Neg
N287	Pos	Pos	Neg	Neg	Neg	Neg	Pos	Neg	Neg	Neg	Neg	Neg	Neg
N290	Pos	Pos	Neg	Neg	Neg	Neg	Pos	Pos	Neg	Neg	Neg	Neg	Neg
N291	Pos	Pos	Neg	Neg	Neg	Neg	Pos	Pos	Neg	Neg	Neg	Neg	Neg

Strain	Carba Test	KPC	VIM	IMP	NDM	OXA-48	TEM	SHV	OXA-1	CTX-M-1	CTX-M-2	CTX-M-9	CTX-M-8/25
N292	**Pos**	**Pos**	Neg	Neg	Neg	Neg	**Pos**	**Pos**	Neg	Neg	Neg	Neg	Neg
N293	**Pos**	**Pos**	Neg	Neg	Neg	Neg	**Pos**	**Pos**	Neg	Neg	Neg	Neg	Neg
N298	**Pos**	**Pos**	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg
N326	**Pos**	**Pos**	Neg	Neg	Neg	Neg	**Pos**	Neg	Neg	Neg	Neg	Neg	Neg
N334	**Pos**	**Pos**	Neg	Neg	Neg	Neg	**Pos**	Neg	Neg	Neg	Neg	Neg	Neg
N347	**Pos**	**Pos**	Neg	Neg	Neg	Neg	**Pos**	**Pos**	Neg	Neg	Neg	Neg	Neg
N349	**Pos**	**Pos**	Neg	Neg	Neg	Neg	**Pos**	**Pos**	Neg	Neg	Neg	Neg	Neg
N353	**Pos**	**Pos**	Neg	Neg	Neg	Neg	**Pos**	**Pos**	Neg	Neg	Neg	Neg	Neg
N367	**Pos**	**Pos**	Neg	Neg	Neg	Neg	**Pos**	**Pos**	Neg	Neg	Neg	Neg	Neg
N368	**Pos**	**Pos**	Neg	Neg	Neg	Neg	**Pos**	**Pos**	Neg	Neg	Neg	Neg	Neg
N369	**Pos**	**Pos**	Neg	Neg	Neg	Neg	**Pos**	Neg	Neg	Neg	Neg	Neg	Neg
N378	**Pos**	**Pos**	Neg	Neg	Neg	Neg	**Pos**	**Pos**	Neg	Neg	Neg	Neg	Neg
N379	**Pos**	**Pos**	Neg	Neg	Neg	Neg	**Pos**	**Pos**	Neg	Neg	Neg	Neg	Neg
N383	**Pos**	**Pos**	Neg	Neg	Neg	Neg	**Pos**	Neg	Neg	Neg	Neg	Neg	Neg

Pos, positive; Neg, negative

Carbapenem-resistant *E. coli* strains from Negrar: 8 out of 9 (88.8%) were positive to Carba NP test, 1 gave an unclear result and was considered doubtful. All strains harbored bla_{KPC} gene, none carried other carbapenemase genes. Eight out of 9 strains (88.8%) co-carried $bla_{KPC,}$ bla_{TEM} and $bla_{CTX\text{-}M\text{-}1}$; 1 (11.1%) bla_{KPC}, bla_{TEM}, bla_{SHV}. [Tab. 20]

Tab. 20 Results of Carba NP test and PCRs for *bla* genes in 9 carbapenem-resistant *E. coli* strains from Negrar Hospital

Strain	Carba Test	KPC	VIM	IMP	NDM	OXA-48	TEM	SHV	OXA-1	CTX-M-1	CTX-M-2	CTX-M-9	CTX-M-8/25
N256	**Pos**	**Pos**	Neg	Neg	Neg	Neg	**Pos**	Neg	Neg	**Pos**	Neg	Neg	Neg
N257	**Pos**	**Pos**	Neg	Neg	Neg	Neg	**Pos**	Neg	Neg	**Pos**	Neg	Neg	Neg
N328	**Pos**	**Pos**	Neg	Neg	Neg	Neg	**Pos**	Neg	Neg	**Pos**	Neg	Neg	Neg
N354	Doub	**Pos**	Neg	Neg	Neg	Neg	**Pos**	Neg	Neg	**Pos**	Neg	Neg	Neg
N370	**Pos**	**Pos**	Neg	Neg	Neg	Neg	**Pos**	Neg	Neg	**Pos**	Neg	Neg	Neg
N375	**Pos**	**Pos**	Neg	Neg	Neg	Neg	**Pos**	Neg	Neg	**Pos**	Neg	Neg	Neg
N376	**Pos**	**Pos**	Neg	Neg	Neg	Neg	**Pos**	Neg	Neg	**Pos**	Neg	Neg	Neg
N380	**Pos**	**Pos**	Neg	Neg	Neg	Neg	**Pos**	**Pos**	Neg	Neg	Neg	Neg	Neg
N381	**Pos**	**Pos**	Neg	Neg	Neg	Neg	**Pos**	Neg	Neg	**Pos**	Neg	Neg	Neg

Pos, positive; Neg, negative; Doub, doubtful

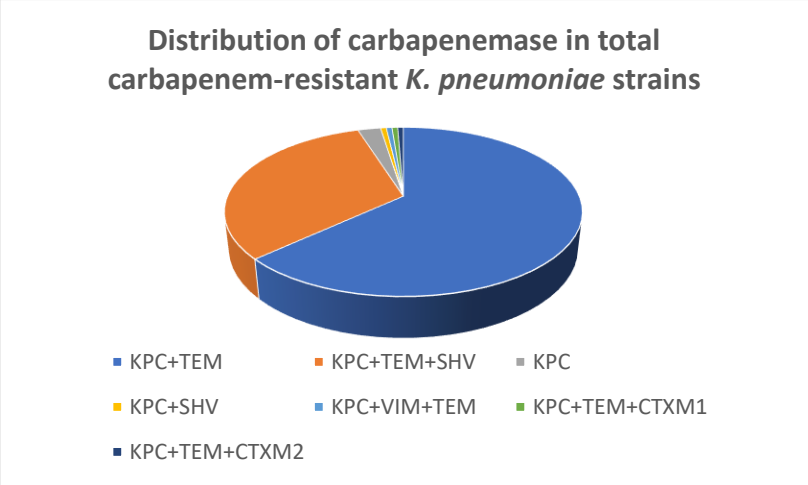
Cephalosporin-resistant *K. pneumoniae* strains from Negrar Hospital: 30 strains, all negative for Carba NP test; 24 out of 30 (80%) were identified as possible ESBL producers by positive ESBL NDP test. By PCR, 3 out of 30 (10%) carried *bla*$_{TEM}$ gene; 3 (10%) *bla*$_{SHV}$; 1 (3.33%) *bla*$_{CTX-M-1}$; 2 out of 30(6.67%) co-carried *bla*$_{TEM}$ and *bla*$_{SHV;}$ 2 (6.67%) *bla*$_{TEM}$, *bla*$_{SHV}$ and *bla*$_{CTX-M-1}$; 6 (20%) co-carried *bla*$_{TEM}$, *bla*$_{OXA-1}$ and *bla*$_{CTX-M-1}$; 3 (10%) *bla*$_{OXA-1}$ and *bla*$_{CTX-M-1}$; 1(3.33%) *bla*$_{SHV}$ and *bla*$_{CTX-M-1}$; 4 (13.3%) *bla*$_{TEM}$, *bla*$_{SHV}$, *bla*$_{OXA-1}$ and *bla*$_{CTX-M-1}$; 5 (16.6%) *bla*$_{SHV}$, *bla*$_{OXA-1}$ and *bla*$_{CTX-M-1}$. [Tab. 21]

Tab. 21 Results of Carba NP test, ESBL NDP test and PCRs for *bla* genes in 24 cephalosporin-resistant *K. pneumoniae* strains from Negrar Hospital

Strain	Carba Test	ESBL Test	TEM	SHV	OXA-1	CTX-M-1	CTX-M-2	CTX-M-9	CTX-M-8/25
N11	Neg	**Pos**	**Pos**	Neg	**Pos**	**Pos**	Neg	Neg	Neg
N16	Neg	**Pos**	Neg	Neg	**Pos**	**Pos**	Neg	Neg	Neg
N39	Neg	**Pos**	**Pos**	**Pos**	**Pos**	**Pos**	Neg	Neg	Neg
N40	Neg	**Pos**	**Pos**	**Pos**	Neg	**Pos**	Neg	Neg	Neg
N42	Neg	**Pos**	**Pos**	**Pos**	**Pos**	**Pos**	Neg	Neg	Neg
N52	Neg	**Pos**	Neg	**Pos**	**Pos**	**Pos**	Neg	Neg	Neg
N88	Neg	**Pos**	**Pos**	**Pos**	**Pos**	**Pos**	Neg	Neg	Neg
N89	Neg	**Pos**	**Pos**	Neg	**Pos**	**Pos**	Neg	Neg	Neg
N95	Neg	**Pos**	**Pos**	Neg	**Pos**	**Pos**	Neg	Neg	Neg
N101	Neg	**Pos**	**Pos**	Neg	**Pos**	**Pos**	Neg	Neg	Neg
N102	Neg	**Pos**	Neg	**Pos**	Neg	**Pos**	Neg	Neg	Neg
N104	Neg	**Pos**	Neg	**Pos**	**Pos**	**Pos**	Neg	Neg	Neg
N113	Neg	Neg	**Pos**	**Pos**	Neg	Neg	Neg	Neg	Neg
N126	Neg	Neg	Neg	**Pos**	Neg	Neg	Neg	Neg	Neg
N138	Neg	**Pos**	**Pos**	**Pos**	Neg	**Pos**	Neg	Neg	Neg
N139	Neg	Neg	**Pos**	Neg	Neg	Neg	Neg	Neg	Neg
N220	Neg	**Pos**	**Pos**	Neg	**Pos**	**Pos**	Neg	Neg	Neg
N237	Neg	Neg	**Pos**	**Pos**	Neg	Neg	Neg	Neg	Neg
N246	Neg	**Pos**	Neg	**Pos**	**Pos**	**Pos**	Neg	Neg	Neg
N289	Neg	**Pos**	Neg	**Pos**	**Pos**	**Pos**	Neg	Neg	Neg
N297	Neg	Neg	Neg	**Pos**	Neg	Neg	Neg	Neg	Neg
N301	Neg	**Pos**	**Pos**	**Pos**	**Pos**	**Pos**	Neg	Neg	Neg
N304	Neg	**Pos**	Neg	**Pos**	**Pos**	**Pos**	Neg	Neg	Neg

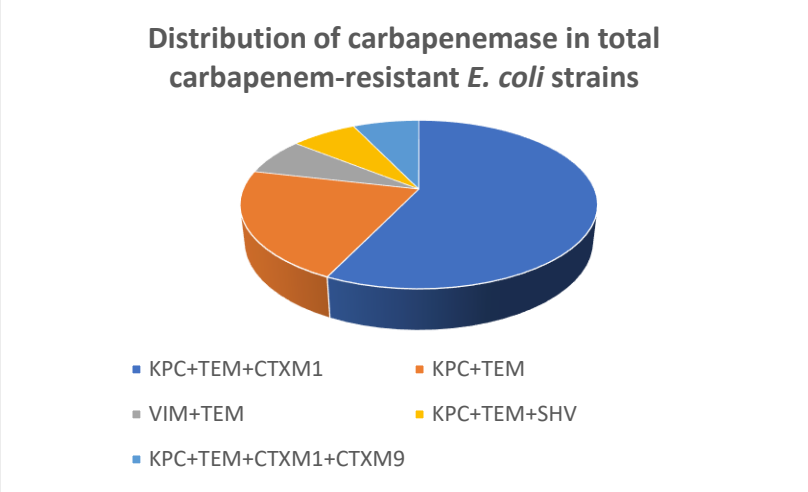
Pos, positive; Neg, negative

The analysis of PCR results for totality carbapenem-resistant *K. pneumoniae* strain showed that all strains harboring bla_{KPC} gene, but the combination bla_{KPC} and bla_{TEM} is prevalent 103 out of 163 (63.2%); 52 out of 163 strains (31.9%) carried $bla_{KPC}/bla_{TEM}/bla_{SHV}$; 4 (2.4%) carried only bla_{KPC}; 1 (0.6%) bla_{KPC}/bla_{SHV}; 1 (0.6%) carried $bla_{KPC}/bla_{VIM}/bla_{TEM}$; 1 (0.6%) $bla_{KPC}/bla_{TEM}/bla_{CTX\text{-}M\text{-}1}$; 1 (0.6%) $bla_{KPC}/bla_{TEM}/bla_{CTX\text{-}M\text{-}2}$. [Graph. 7]



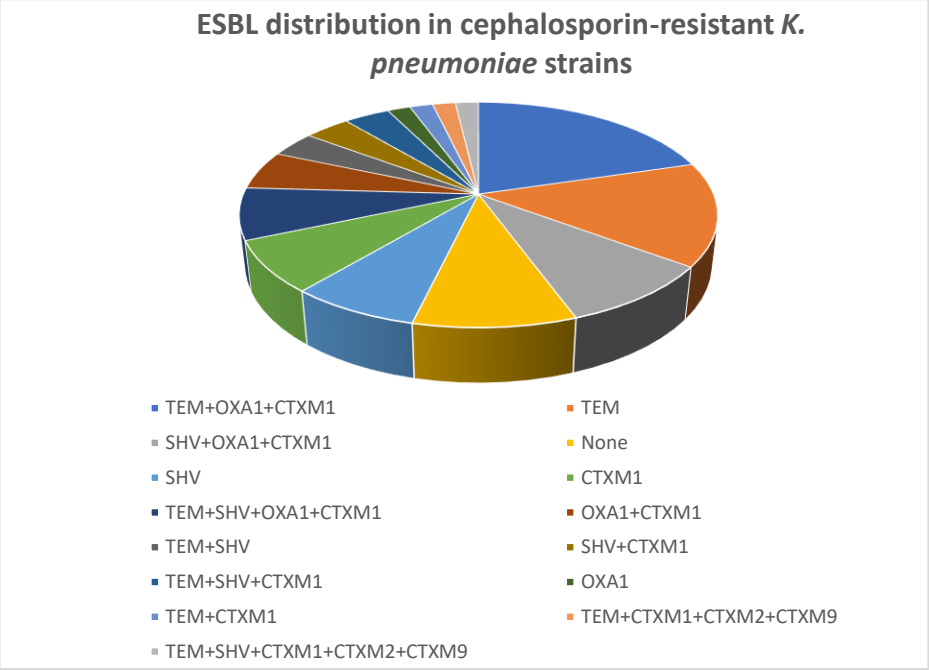
Graph. 7 Distribution of carbapenemase genes in total 163 carbapenem-resistant *K. pneumoniae* strains.

Totally carbapenem-resistant *E. coli* strains showed that prevalent combination is $bla_{KPC}/bla_{TEM}/bla_{CTX\text{-}M\text{-}1}$ carried by 8 out of 14 (57.1%); bla_{KPC} and bla_{TEM} is carried by 3 out of 14 (21.4%); 1 (7.1%) carried bla_{VIM} and bla_{TEM}; 1 (7.1%) $bla_{KPC}/bla_{TEM}/bla_{SHV}$; 1 (7.1%) $bla_{KPC}/bla_{TEM}/bla_{CTX\text{-}M\text{-}1}/bla_{CTX\text{-}M\text{-}9}$. [Graph. 8]



Graph. 8 Distribution of carbapenemase genes in total 14 carbapenem-resistant *E. coli* strains.

Distribution of ESBL genes between totally cephalosporin-resistant *K. pneumoniae* strains is broader. The prevalent combination is $bla_{TEM}/bla_{OXA-1}/bla_{CTXM-1}$ in 11 out 54 strains (20.4%); 8 strains (14.8%) carried bla_{TEM}; 5 (9.2%) co-carried $bla_{SHV}/bla_{OXA-1}/bla_{CTXM-1}$; 4 (7.4%) bla_{SHV}; 4 (7.4%) bla_{CTXM-1}; 4 (7.4%) $bla_{TEM}/bla_{SHV}/bla_{OXA-1}/bla_{CTXM-1}$; 3 (5.5%) bla_{OXA-1}/bla_{CTXM-1}; 2 (3.7%) bla_{TEM}/bla_{SHV}; 2 (3.7%) $bla_{SHV}/bla_{CTX-M-1}$; 2 (3.7%) $bla_{TEM}/bla_{SHV}/bla_{CTX-M-1}$; 1 (1.8%) bla_{OXA-1}; 1 (1.8%) $bla_{TEM}/bla_{CTX-M-1}$; 1 (1.8%) $bla_{TEM}/bla_{CTX-M-1}/bla_{CTX-M-2}/bla_{CTX-M-9}$; 1 (1.8%) $bla_{TEM}/bla_{SHV}/bla_{CTX-M-1}/bla_{CTX-M-2}/bla_{CTX-M-9}$. [Graph. 9]



Graph. 9 Distribution of ESBL genes in total 54 cephalosporin-resistant *K. pneumoniae* strains.

After confirmation of resistance profile, carbapenemase or ESBL-production and detection of *bla* genes carried, we evaluated the correlation between KPC production and 11,109Da (±8Da) peak [Fig. 38] on MALDI-TOF MS spectra.

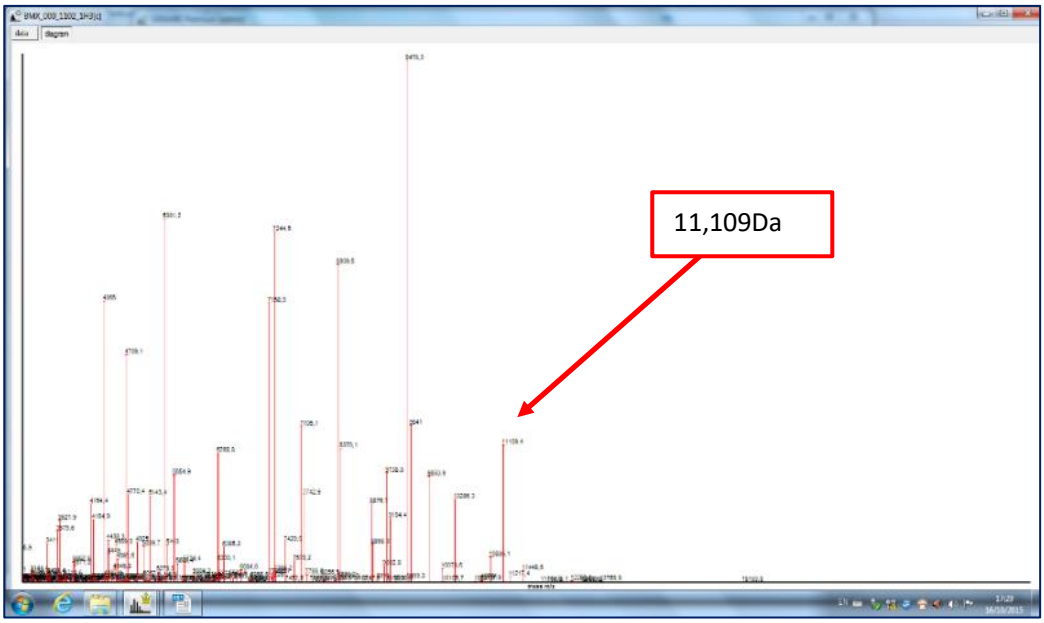


Fig. 38 MALDI-TOF MS spectrum of KPC-producing bacterial strain with the 11,109Da peak.

Spectra of all strains above mentioned, performed in duplicate, were analyzed to detect the 11,109Da peak. The spectra analysis was repeated for discrepant strains (strains resulted positive for *bla*$_{KPC}$, but negative for the 11,109Da peak) after re-isolation. Results of spectra analysis are summarized in Tab. 22 and 23.

Tab. 22 Results of first MALDI-TOF MS spectra analysis and of repeated spectra analysis of discrepant, on KPC-producing and ESBL-producing or Ertapenem-resistant strains distinguished for provenience

		11,109Da peak					
		First MALDI-TOF spectra analysis			After retested discrepant		
		Present	Absent	Tot	Present	Absent	Tot
KPC producers	KPN Verona	94	1	**95**	95	0	**95**
	KPN Negrar	67	1	**68**	67	**1**	**68**
	ECO Verona	3	1	**4**	4	0	**4**
	ECO Negrar	9	0	**9**	9	0	**9**
ESBL producers or Ertapenem-resistant	KPN Verona	0	24	**24**	0	24	**24**
	KPN Negrar	0	30	**30**	0	30	**30**
	ECO Verona	0	4	**4**	0	4	**4**

KPN, *K. pneumoniae*; ECO, *E. coli*

Tab. 23 Results of MALDI-TOF MS spectra analysis on totally KPC-producing and ESBL-producing or Ertapenem-resistant strains, before and after retested discrepant

		11,109Da peak					
		First MALDI-TOF spectra analysis			After retested discrepant		
		Present	Absent	Tot	Present	Absent	Tot
KPC producers	KPN	161	2	**163**	162	1	**163**
	ECO	12	1	**13**	13	0	**13**
ESBL producers or Ertapenem-resistant	KPN	0	54	**54**	0	54	**54**
	ECO	0	4	**4**	0	4	**4**

KPN, *K. pneumoniae*; ECO, *E. coli*

At the first MALDI-TOF MS spectra analysis 161 on 163 (98.7%) KPC-producing *K. pneumoniae* (KPN) strains, and 12 on 13 (92.3%) KPC-producing *E. coli* (ECO) strains were positive for 11,109Da peak.

All 58 cephalosporin-resistant or ertapenem-resistant strains were negative for the presence of the same peak.

On discrepant strains, that are the strains positive for bla_{KPC} by PCR detection and negative at MALDI-TOF MS analysis for 11,109 Da peak, we repeated MALDI-TOF measurements after re-isolation on MH plate. After this, two more strains, namely 1 *K. pneumoniae* and 1 *E. coli*, showed the 11,109Da peak in their spectra, increasing the sensitivity to 99.4% (162/163) and 100% (13/13) respectively. [Tab. 23] Only 1 *K. pneumoniae* out of 176 KPC-producing strains (0.56%), the N181 strain, was confirmed to be discrepant. [Tab. 19] [Tab.22 and 23]

MALDI-TOF MS measurement of N181 strain was repeated in duplicate after 4 passages on MH agar plates containing Imipenem (2 mg/ml) in order to increase expression of carbapenemase. Spectra analysis confirmed anyway the absence of the peak. Multiplex and simplex PCRs for bla_{IMP}/bla_{VIM}/bla_{KPC} and bla_{NDM} [78] were repeated confirming bla_{KPC} presence. The bla_{KPC} PCR product was

purified and sequenced. The sequence analyses proved that the strain harbored a *bla* gene encoding for KPC-3 enzyme. Also for this discrepant strain we performed plasmid extraction and characterization [56] [79] and resulted the strain harbored an OriColE plasmid only. [Tab. 27 and 28]

We performed MALDI-TOF MS analysis in duplicate and spectra analysis also on selected strains control groups: *K. pneumoniae* susceptible to *β*-lactams; ESBL-producing *E. coli*; NDM-producing and VIM-producing *Enterobacteriaceae*. All strains of control groups were negative for bla_{KPC} gene and were negative for 11,109Da peak on MALDI-TOF MS spectra too. [Tab. 24]

Tab. 24 Results of MALDI-TOF MS spectra analysis on selected control groups; ESBL-producing, NDM-producing, VIM-producing and susceptible to *β*-lactams strains

	11,109Da peak		
	Present	**Absent**	**Tot**
ECO ESBL	0	121	121
KPN S	0	27	27
NDM strains	0	15	15
VIM strains	0	39	39

KPN, *K. pneumoniae*; ECO, *E. coli*; S, susceptible

We performed MALDI-TOF MS spectra analysis also on positive and negative control strains [Tab. 8] and only MALDI-TOF spectrum of KPC positive control showed the 11,109Da peak.

Novel variant of KPC enzyme emerging between Group 1 strains

Between the strains of Group 1 we found three *K. pneumoniae* strains (MDR53, MDR136 and MDR345) isolated from screening samples of a patient candidate for liver transplantation and who had undergone selective digestive decontamination. The first strain was isolated before transplantation and the two others after decontamination and transplantation. All three strains were positive for Carba NP test; MICs for meropenem, imipenem and ertapenem were higher than 128 µg/ml. MIC for colistin was 0.5 µg/ml and for tigecycline was 1 µg/ml, all strains were susceptible to gentamycin (MIC 1 µg/ml). The strains carried *bla*$_{KPC}$ gene, and the PCR products were sequenced and analyzed with Blast at the Pub-Med site. [91] The sequence showed a N291T substitution compared to the *bla*$_{KPC-3}$ gene endemic in our hospital. [82] [Fig. 39] The mutated sequence encoded for a novel enzyme that was named KPC-19 at http:/www.lahey.org/studies and accession number of GenBank is KJ775801. [50] [91] These three strains harbored OriColE and IncFII plasmids by using PCR based-replicon typing (PBRT) according to Carattoli *et al*. [56] [79] Also, conjugation with MDR53 as donor and *E. coli* J53 as recipient was performed successfully.

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                CTGGCTGGCTTTTCTGCCACCGCGCTGACCAACCTCGTCGCGGAACCATTCGCTAAACTC 60

                ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
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7170

Query  61       GAACAGGACTTTGGCGGCTCCATCGGTGTGTACGCGATGGATACCGGCTCAGGCGCAACT
120

                ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  7171     GAACAGGACTTTGGCGGCTCCATCGGTGTGTACGCGATGGATACCGGCTCAGGCGCAACT
7230

Query  121      GTAAGTTACCGCGCTGAGGAGCGCTTCCCACTGTGCAGCTCATTCAAGGGCTTTCTTGCT
180

                ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  7231     GTAAGTTACCGCGCTGAGGAGCGCTTCCCACTGTGCAGCTCATTCAAGGGCTTTCTTGCT
7290

Query  181      GCCGCTGTGCTGGCTCGCAGCCAGCAGCAGGCCGGCTTGCTGGACACACCCATCCGTTAC
240

                ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  7291     GCCGCTGTGCTGGCTCGCAGCCAGCAGCAGGCCGGCTTGCTGGACACACCCATCCGTTAC
7350

Query  241      GGCAAAAATGCGCTGGTTCCGTGGTCACCCATCTCGGAAAAATATCTGACAACAGGCATG
300

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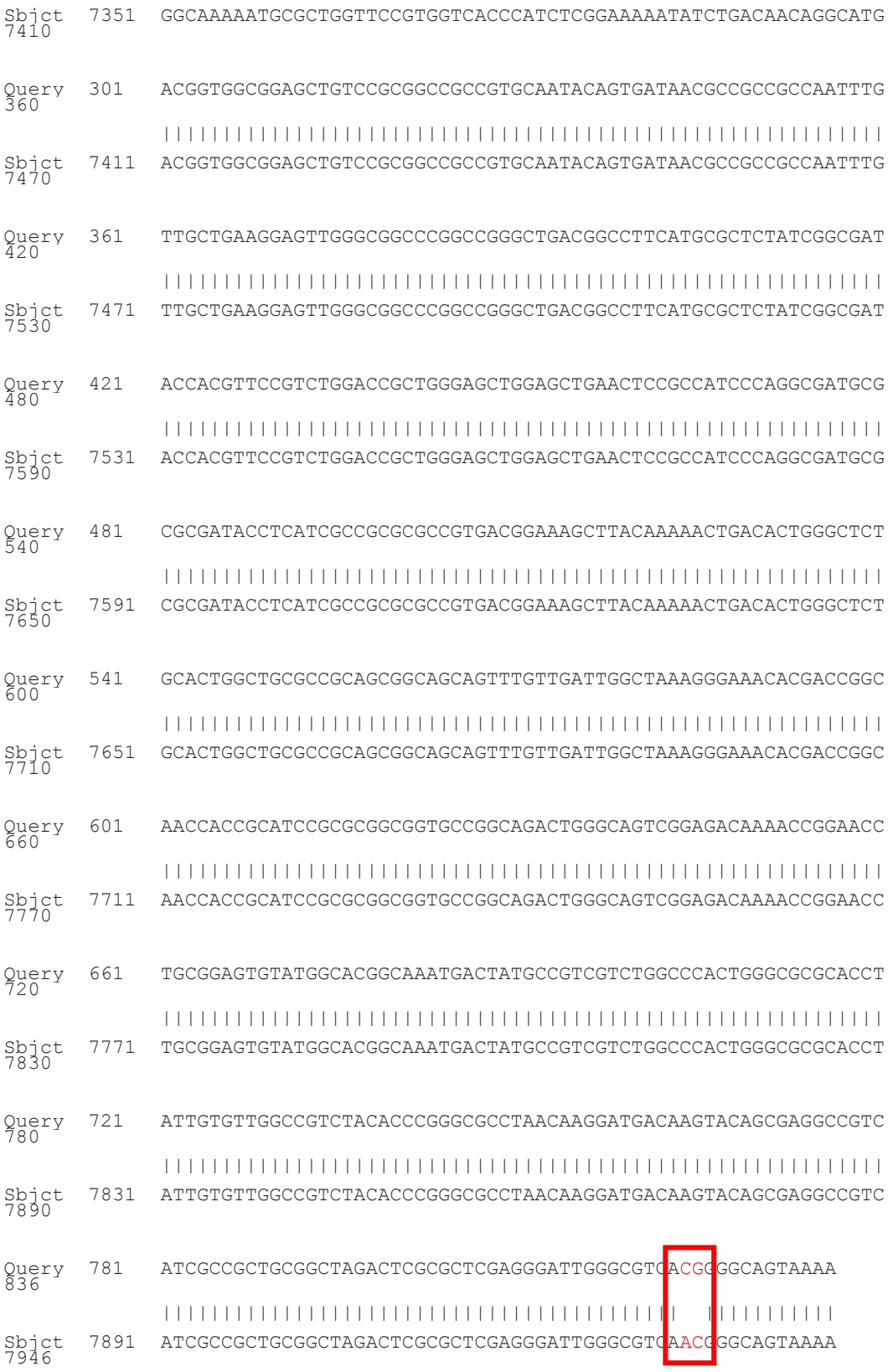

Fig. 39 *bla*$_{KPC}$ sequence of MDR53 *K. pneumoniae* strain compared with KPC-3 sequence, showing N291T substitution. [50]

b. Spectra analysis to evaluate the use in clinical diagnostic workflow of 11,109Da peak detection

Group 2

To evaluate the utility and the feasibility of the 11,109Da peak detection for KPC producers screening in clinical lab, we analyzed daily from May to July 2016 the MALDI-TOF MS spectra of strains investigated for carbapenemase production.

Three technicians working on MDR screening had performed the MALDI-TOF MS identification of bacterial strains isolated during usual routine work and we analyzed their spectra to detect the 11,109Da peak.

Over three months, we analyzed the spectra of 183 strains, namely 171 *K. pneumoniae*, 9 *E. coli*, 2 *Enterobacter aerogenes*, 1 *Enterobacter cloacae*, isolated on ChomeID ESBL (bioMérieux) agar plates with Ertapenem (10 µg) disk and either positive at Carba NP test or positive at ESBL NDP test.

At the first MALDI-TOF MS spectra analysis 121 out of 135 (89,63%) Carba NP positive strains, were positive also for the 11,109Da peak. The same peak was absent in all 48 positive strains for ESBL NDP test only.

We repeated the MALDI-TOF MS identification on discrepant strains after re-isolation on MH plates and 8 more strains positive for Carba NP test showed the 11,109Da peak in their spectrum, increasing the sensitivity to 95,5% (129/135). [Tab. 25]

Tab. 25 Results of MALDI-TOF MS spectra analysis on strains with Carba NP test or ESBL NDP test positive isolated during routinely screening from May to July 2016. On discrepant strains MALDI-TOF measurement was repeated after re-isolation

11,109Da peak

		First MALDI-TOF spectra analysis			**Spectra analysis after repetition of discrepant strains**		
		Present	**Absent**	**Tot**	**Present**	**Absent**	**Tot**
Carba NP test positive	***K. pneumoniae***	114	10	**124**	122	2	**124**
	E. coli	6	2	**8**	6	2	**8**
	E. aerogenes	1	1	**2**	1	1	**2**
	E. cloacae	0	1	**1**	0	1	**1**
ESBL NDP test positive	***K. pneumoniae***	0	47	**47**	0	47	**47**
	E. coli	0	1	**1**	0	1	**1**

On 6 discrepant strains, positive for Carba NP test, but negative for the 11,109Da peak, MALDI-TOF MS identification was repeated in duplicate after 4 passages on MH agar plates additioned with imipenem (2 mg/ml), but spectra analysis confirms absence of the peak.

Multiplex and simplex PCRs for bla_{IMP}/bla_{VIM}/bla_{KPC} and bla_{NDM} [78] were performed on the 6 discrepant strains. Only 3 strains resulted positive for bla_{KPC}, while 3 strains produce other carbapenemases, namely 1 NDM (*E. coli*) and 2 VIM (1 *E. aerogenes*, 1 *E. cloacae*).

These results showed that 132 out of 135 carbapenemase producers, were KPC producers and increased the sensitivity of MALDI-TOF MS detection to 97,7% (129/132). The discrepant strains on this group were only 3 (2.2%), namely: 2 *K. pneumoniae* (87/24U and MDR4597), 1 *E. coli* (MDR4547). [Tab. 27 and 28]

The amplicon of the three positive bla_{KPC} discrepant strains were purified and sequenced. Analysis of the sequences showed the gene encoding for two KPC-19 and 1 KPC-3 enzymes. Plasmid extraction and characterization [56] [79] were performed and proved 1 strain positive for OriColE and IncR, 1 positive for IncFIA and IncFIB, and 1 positive for IncQ. [Tab. 27 and 28]

c. Spectra analysis of collection strains to find NDM-producing *E. coli*

Group 3

After detection of one NDM-producing *E. coli* strain amongst the routine samples, namely: MDR18/12 (Group 2), we performed a retrospective analysis on carbapenemase-producing *E. coli* strains, to detect other NDM producers.

We investigated 11 *E. coli* strains positive for Carba NP test, isolated during MDR screening from January to November 2015. The strains were plated from frozen stock on MH agar plates, MALDI-TOF MS measurements in duplicate and multiplex and simplex PCRs for bla_{IMP}/bla_{VIM}/bla_{KPC} and bla_{NDM} [78] were performed. The spectra analysis showed that in 2 out of 11 strains, the 11,109Da peak was absent; all strains resulted positive for bla_{KPC}. [Tab. 26]

Tab. 26 Carbapenemase-producing *E. coli* strains from collection investigated for bla_{NDM} gene

Strain	Carba Test	11,109DA peak	KPC	VIM	IMP	NDM
MDR1696	**Pos**	11,113	**Pos**	Neg	Neg	Neg
MDR1997	**Pos**	11,110	**Pos**	Neg	Neg	Neg
MDR2114	**Pos**	**Absent**	**Pos**	Neg	Neg	Neg
MDR2225	**Pos**	11,111	**Pos**	Neg	Neg	Neg
MDR2247	**Pos**	11,111	**Pos**	Neg	Neg	Neg
MDR2270	**Pos**	11,107	**Pos**	Neg	Neg	Neg
MDR2367	**Pos**	11,115	**Pos**	Neg	Neg	Neg
MDR2440	**Pos**	11,110	**Pos**	Neg	Neg	Neg
MDR2489	**Pos**	11,107	**Pos**	Neg	Neg	Neg
MDR2664	**Pos**	11,110	**Pos**	Neg	Neg	Neg
MDR2984	**Pos**	**Absent**	**Pos**	Neg	Neg	Neg

Pos, positive; Neg, negative

MALDI-TOF MS identification was repeated for the 2 discrepant strains after streaking on MH plates plus Imipenem (2mg/ml), confirming the strains were discrepant. They were namely: MDR2114 and MDR2984. The *bla*$_{KPC}$ amplicons were purified and sequenced, both resulting for KPC-19. Plasmid extraction and characterization were performed, 1 strain resulting positive for IncN, IncFIB and IncFrepB replicons and the second negative for all Inc/rep investigated. [Tab. 27 and 28]

d. <u>Discrepant strains analysis</u>

Through MALDI-TOF MS spectra analysis of the above mentioned three sample groups, we found 6 discrepant strains, positive for *bla*$_{KPC}$, but negative for the 11,109Da peak. Other 2 discrepant strains were detected in Group 5 after spectra analysis to detect the 11,109Da peak, namely MDR1958 and MDR2025.

Totally, we found 8 discrepant strains out of 362 (2.2%) KPC-producing Enterobacteria strains. Three strains were *E. coli* and 5 were *K. pneumoniae.*

We investigated the correlation between the peak absence and the plasmid profile of the discrepant strains carrying a *bla*$_{KPC}$ gene. On table 27 and 28 the results of *bla*$_{KPC}$ sequencing and plasmid characterization for all discrepant strains are summarized.

The strains differed from each other for plasmid profile, namely: N181, MDR1958 and MDR2025 had positive OriColE; 87/24U IncR and OricolE; MDR4547 FIA and FIB; MDR4597 IncQ; MDR2114 N, FIB and FrepB; MDR2984 not at all. Two out of 8 strains: N181, MDR4547 were harboring KPC-3, the others were harboring KPC-19 enzyme.

Tab. 27 KPC sequencing results and plasmid characterization [79] for discrepant strains: *bla*$_{KPC}$ positive, 11,109Da peak negative

Strain		Sequence	IncQ	IncR	IncU	OriColE
N181	KPN	KPC-3	Neg	Neg	Neg	Pos
87/24 U	KPN	KPC-19	Neg	Pos	Neg	Pos
MDR4547	ECO	KPC -3	Neg	Neg	Neg	Neg
MDR4597	KPN	KPC-19	Pos	Neg	Neg	Neg
MDR2114	ECO	KPC-19	Neg	Neg	Pos	Neg
MDR2984	ECO	KPC-19	Neg	Neg	Neg	Neg
MDR1958	KPN	KPC-19	Neg	Neg	Neg	Pos
MDR2025	KPN	KPC-19	Neg	Neg	Neg	Pos

KPN, *K. pneumoniae*; ECO, *E. coli*; Pos, positive; Neg, negative

Tab. 28 Plasmid characterization [56] for discrepant strains: *bla*$_{KPC}$ positive, 11,109Da peak negative

Strain	HI1	HI2	I1	X	L/M	N	FIA	FIB	W	Y	P	FIC	A/C	T	FIIs	FrepB	K	B
N181	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg
87/24 U	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg
MDR4547	Neg	Neg	Neg	Neg	Neg	Neg	Pos	Pos	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg
MDR4597	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg
MDR2114	Neg	Neg	Neg	Neg	Neg	Pos	Neg	Pos	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Pos	Neg	Neg
MDR2984	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg
MDR1958	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg
MDR2025	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg

Pos, positive; Neg, negative

We performed *bla*$_{KPC}$ sequencing and plasmid characterization also for 8 strains sharing similarities with discrepant ones but also showing the 11,109Da peak on their MALDI-TOF MS spectra, searching for a difference on gene or plasmids. All the strains showing the 11,109Da peak were positive for FIIs replicon, [Tab. 30] whereas the same replicon is negative for all discrepant strain, [Tab. 28] indicating the possibility of a different plasmid harboring *bla*$_{KPC}$ gene in these

strains and confirming the correlation between 11,109Da peak and plasmid, but not between the peak and *bla*$_{KPC}$ gene sequence. [73] [74] One strain (MDR53) harbored KPC-19, all the others had KPC-3. The results are summarized on Tab. 29 and 30.

Tab. 29 KPC sequencing results and plasmid characterization [79] of selected strains: *bla*$_{KPC}$ positive, 11,109Da peak positive

Strain		Sequence	IncQ	IncR	IncU	OriColE
N298	KPN	KPC-3	Neg	Neg	Neg	Pos
MDR53	KPN	KPC-19	Neg	Neg	Neg	Pos
MDR368	ECO	KPC-3	Neg	Neg	Neg	Neg
MDR488	KPN	KPC-3	Neg	Neg	Neg	Pos
MDR600	ECO	KPC-3	Neg	Neg	Neg	Neg
MDR624	KPN	KPC-3	Neg	Neg	Neg	Pos
MDR 1985	KPN	KPC-3	Neg	Neg	Neg	Pos
MDR1696	ECO	KPC-3	Neg	Neg	Neg	Neg

KPN, *K. pneumoniae*; ECO, *E. coli*; Pos, positive; Neg, negative

Tab. 30 Plasmid characterization [56] of selected strain: *bla*$_{KPC}$ positive, 11,109Da peak positive

Strain	HI1	HI2	I1	X	L/M	N	FIA	FIB	W	Y	P	FIC	A/C	T	FIIs	Frep B	K	B
N298	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Pos	Neg	Neg	Neg
MDR53	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Pos	Neg	Neg	Neg
MDR368	Neg	Neg	Neg	Neg	Neg	Pos	Pos	Pos	Neg	Neg	Neg	Neg	Neg	Neg	Pos	Pos	Neg	Neg
MDR488	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Pos	Neg	Neg	Neg
MDR600	Neg	Neg	Neg	Neg	Neg	Neg	Pos	Pos	Neg	Neg	Neg	Neg	Neg	Neg	Pos	Pos	Neg	Neg
MDR624	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Pos	Neg	Neg	Neg
MDR1985	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Pos	Neg	Neg	Neg
MDR2664	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Pos	Neg	Pos	Neg	Neg	Neg	Neg	Pos	Neg	Neg	Neg

Pos, positive; Neg, negative

For the 8 discrepant strains and the 8 control strains, we performed also PCRs for *p019* and *Tn4401* genes, to verify any correlation between p019 protein, transposon Tn4401 and presence of the 11,109Da peak in MALDI-TOF spectra. The results are presented in Tab. 31.

Tab. 31 Results of PCRs for *p019* and *Tn4401* genes performed on 8 discrepant strains (KPC-producing/missing 11,109Da peak) and 8 control strains (KPC-producing/with 11,109Da peak)

Discrepant strains	11,109Da peak	*p019*	*Tn4401*	Control strains	11,109Da peak	*p019*	*Tn4401*
N181	Absent	Neg	Neg	**N298**	Present	Pos	Pos
87/24 U	Absent	Neg	Neg	**MDR53**	Present	Pos	Neg
MDR4547	Absent	Neg	Pos	**MDR368**	Present	Pos	Pos
MDR4597	Absent	Neg	Pos	**MDR488**	Present	Pos	Neg
MDR2114	Absent	Neg	Pos	**MDR600**	Present	Pos	Neg
MDR2984	Absent	Neg	Pos	**MDR624**	Present	Pos	Neg
MDR1958	Absent	Neg	Pos	**MDR 1985**	Present	Pos	Neg
MDR2025	Absent	Neg	Pos	**MDR1696**	Present	Pos	Neg

Pos, positive; Neg, negative

PCR results showed a direct correlation between the 11,109Da peak and *p019* gene. In fact, all discrepant strains, that is the strains having bla_{KPC} gene, but missing the 11,109Da peak in MALDI-TOF spectra, are negative also for *p019* gene. Control strains, instead, that is strains having bla_{KPC} gene, with the 11,109Da peak in MALDI-TOF spectra, are positive also for *p019* gene. The correlation with *Tn4401* gene, however, it is less clear because the gene codifying for transposon was detected in 6/8 of discrepant strains, but only in 2/8 control strains. [Tab. 31]

PART II

Spectra analysis for research of new peaks correlated with other carbapenemases: VIM and NDM

Group 4

After having evaluated the effective correlation between KPC and MALDI-TOF MS 11,109Da peak, we searched for other characteristic peaks correlated with VIM and NDM carbapenemases. For this purpose, we selected bacterial strains VIM and NDM producers and after having performed MALDI-TOF MS identification we analyzed their spectra.

On 57 Enterobacteria strains from Croatia we performed Carba NP Test; MALDI-TOF MS identification; spectra analysis for the 11,109Da peak detection; PCRs for bla_{IMP}/bla_{VIM}/bla_{KPC} and bla_{NDM} genes. [78]

From these 57 strains, we selected 23 strains with Carba NP test positive, negative for the 11,109Da peak and positive for bla_{VIM}. [Tab. 32] The strains with Carba NP test negative were streaked 4 times on MH agar plates plus Imipenem (2mg/ml) and they were eliminated if the test confirmed negative.

The MALDI-TOF MS spectra of these selected strains were used to create a superspectrum to individuate some or at least one protein common to all VIM producers, yet absent in the spectra of other samples not VIM producers. All strains were analyzed in duplicate and we used only spectra with a confidence ≥90% and with a minimum of 170 peaks, the others were repeated, because for this analysis the quality of spectra is extremely important, so as to have high number of peaks and to compare spectra that are similar for peaks number.

We analyzed the spectra with Saramis software, creating an exclusion list to eliminate the proteins most represented (relative intensity from 50% to 80%) and common to other *Enterobacteriaceae* in the database. We produced a first list of 170 proteins that we compared with the whole database to reduce further. Step by step, we reduced the list to 44 proteins, keeping those with a low relative intensity (10%), thus creating a VIM superspectrum. Then, we compared the superspectrum

with the database so as to eliminate the proteins common to *Enterobacteriaceae* susceptible to carbapenems. At the end, we found 7 proteins rare on the database, and performed the comparison mass vs spectra, we verified if one single protein could be correlated with VIM, but all were present also in other bacterial strains not VIM producers.

The same analysis was performed for 13 *P. aeruginosa* strains selected from a collection of 61 strains, all with Carba NP test and *bla*$_{VIM}$ positive, [Tab. 33] but also in this case we found no specific peak correlated with VIM enzyme.

Tab. 32 VIM-producing *Enterobacteriaceae* strains selected to create a superspectrum to search a characteristic peak VIM related

	Strain	Carba Test	11,109Da peak	*bla*$_{VIM}$	*bla*$_{KPC}$	*bla*$_{IMP}$	*bla*$_{NDM}$
1 ZAG	*K pneumoniae*	**Pos**	Absent	**Pos**	Neg	Neg	Neg
2 ZAG	*C freundii*	**Pos**	Absent	**Pos**	Neg	Neg	Neg
3 ZAG	*C freundii*	**Pos**	Absent	**Pos**	Neg	Neg	Neg
7 ZAG	*E cloacae*	**Pos**	Absent	**Pos**	Neg	Neg	Neg
8 ZAG	*E cloacae*	**Pos**	Absent	**Pos**	Neg	Neg	Neg
9 ZAG	*E cloacae*	**Pos**	Absent	**Pos**	Neg	Neg	Neg
10 ZAG	*E cloacae*	**Pos**	Absent	**Pos**	Neg	Neg	Neg
11 ZAG	*E cloacae*	**Pos**	Absent	**Pos**	Neg	Neg	Neg
12 ZAG	*C freundii*	**Pos**	Absent	**Pos**	Neg	Neg	Neg
13 ZAG	*E cloacae*	**Pos**	Absent	**Pos**	Neg	Neg	Neg
26 ZAG	*E cloacae*	**Pos**	Absent	**Pos**	Neg	Neg	Neg
27 ZAG	*K oxytoca*	**Pos**	Absent	**Pos**	Neg	Neg	Neg
28 ZAG	*E cloacae*	**Pos**	Absent	**Pos**	Neg	Neg	Neg
30 ZAG	*E cloacae*	**Pos**	Absent	**Pos**	Neg	Neg	Neg
31 ZAG	*E cloacae*	**Pos**	Absent	**Pos**	Neg	Neg	Neg
32 ZAG	*E cloacae*	**Pos**	Absent	**Pos**	Neg	Neg	Neg
33 ZAG	*E cloacae*	**Pos**	Absent	**Pos**	Neg	Neg	Neg
34 ZAG	*Enterobacter sp.*	**Pos**	Absent	**Pos**	Neg	Neg	Neg
40 ZAG	*C freundii*	**Pos**	Absent	**Pos**	Neg	Neg	Neg
44 ZAG	*C freundii*	**Pos**	Absent	**Pos**	Neg	Neg	Neg
53 ZAG	*K pneumoniae*	**Pos**	Absent	**Pos**	Neg	Neg	Neg
57 ZAG	*K oxytoca*	**Pos**	Absent	**Pos**	Neg	Neg	Neg
61 ZAG	*C freundii*	**Pos**	Absent	**Pos**	Neg	Neg	Neg

Pos, positive; Neg, negative

Tab. 33 VIM-producing *P. aeruginosa* strains selected to create a superspectrum to search a characteristic peak VIM correlated

	Strain	Carba NP Test	*bla*$_{VIM}$	*bla*$_{KPC}$	*bla*$_{IMP}$
PSE BG	*P. aeruginosa*	**Pos**	**Pos**	Neg	Neg
Pae 390	*P. aeruginosa*	**Pos**	**Pos**	Neg	Neg
PSE4	*P. aeruginosa*	**Pos**	**Pos**	Neg	Neg
PSE B	*P. aeruginosa*	**Pos**	**Pos**	Neg	Neg
PSE F	*P. aeruginosa*	**Pos**	**Pos**	Neg	Neg
PSE P	*P. aeruginosa*	**Pos**	**Pos**	Neg	Neg
2523	*P. aeruginosa*	**Pos**	**Pos**	Neg	Neg
2553	*P. aeruginosa*	**Pos**	**Pos**	Neg	Neg
2609	*P. aeruginosa*	**Pos**	**Pos**	Neg	Neg
2622	*P. aeruginosa*	**Pos**	**Pos**	Neg	Neg
2677	*P. aeruginosa*	**Pos**	**Pos**	Neg	Neg
2678	*P. aeruginosa*	**Pos**	**Pos**	Neg	Neg
2682	*P. aeruginosa*	**Pos**	**Pos**	Neg	Neg

Pos, positive; Neg, negative

We performed also a conjugation between 1 *K. oxytoca* and 1 *C. freundii* strains both VIM-producing as donors and *E. coli* J53 as recipient, so as to compare the spectrum of J53 and that of J53 transconjugant, to individuate a possible peak present only on the transconjugant, but the conjugation failed.

In the same way, we searched for a peak NDM correlated, using the spectra of 15 selected NDM-producing Enterobacteria strains from Croatia, positive for Carba NP test and *bla*$_{NDM}$ and negative for 11,109Da peak. [Tab. 34]

Tab. 34 NDM-producing Enterobacteria strains selected to create a superspectrum to search a characteristic peak NDM related

Strain		Carba NP Test	11,109Da peak	*bla*$_{NDM}$
HR40/E	*C. koseri*	**Pos**	Absent	**Pos**
HR85/E	*K. Pneumoniae*	**Pos**	Absent	**Pos**
HR88/E	*K. Pneumoniae*	**Pos**	Absent	**Pos**
HR107/E	*K. Pneumoniae*	**Pos**	Absent	**Pos**
HR112/E	*K. Pneumoniae*	**Pos**	Absent	**Pos**
HR113/E	*K. Pneumoniae*	**Pos**	Absent	**Pos**
HR117/E	*K. Pneumoniae*	**Pos**	Absent	**Pos**
HR324/E	*K. Pneumoniae*	**Pos**	Absent	**Pos**
5 ZAG	*C. freundii*	Doub	Absent	**Pos**
29 ZAG	*K. Pneumoniae*	**Pos**	Absent	**Pos**
60 ZAG	*E. cloacae*	Neg	Absent	**Pos**
66 ZAG	*E. cloacae*	Neg	Absent	**Pos**
H85 transconj	*E. coli*	**Pos**	Absent	**Pos**
H88 transconj	*E. coli*	**Pos**	Absent	**Pos**
H113 transconj	*E. coli*	**Pos**	Absent	**Pos**

Pos, positive; Neg, negative; Doub, doubtful

The spectra of these strains were used to create a NDM superspectrum, but also for NDM we did not find any characteristic peak. Also, comparison of *E. coli* J53 spectrum and transconjugant spectra did not reach a result, not even performing MALDI-TOF analysis for plasmid of strain HR107 harboring *bla*$_{NDM}$ gene. [97]

After the first spectra analysis, we characterized the plasmids of the VIM- and NDM-producing Enterobacteria strains and divided the samples in groups based on the different plasmid type harbored (L/M, A/C, IncR, OriColE), then we repeated the spectra analyses in each group, but also in this way we not found a peak correlated with enzymes. [Tab. 35-36]

Tab. 35 VIM-producing Enterobacteria strains arranged by classes according to their plasmid characterization [56] [79]

1 ZAG	L/M						**7 ZAG**	A/C				
2 ZAG	L/M		IncR			ColE	**8 ZAG**	A/C				
3 ZAG	L/M		IncR			ColE	**9 ZAG**	A/C				
12 ZAG	L/M		IncR			ColE	**10 ZAG**	A/C			IncN	
33 ZAG	L/M						**11 ZAG**	A/C				
40 ZAG	L/M		IncR		IncP	ColE	**13 ZAG**	A/C				
44 ZAG	L/M		IncR			ColE	**26 ZAG**	A/C				
53 ZAG	L/M	A/C		IncN			**28 ZAG**	A/C	IncQ			ColE
61 ZAG	L/M						**30 ZAG**	A/C				
							31 ZAG	A/C				ColE
57 ZAG						ColE	**32 ZAG**	A/C				
							34 ZAG	A/C				

Tab. 36 NDM-producing Enterobacteria strains arranged by classes according to their plasmid characterization [56] [79]

HR40/E	IncR			**HR112/E**	A/C	FIA	ColE		
HR85/E	IncR			**HR117/E**	A/C		ColE		
HR88/E	IncR			**5 ZAG**	A/C				
HR107/E	IncR			**29 ZAG**	A/C			H1	
HR324/E	IncR	ColE	L/M	**60 ZAG**	A/C		ColE		
				66 ZAG	A/C		ColE		
				HR113/E			ColE		

PART III

To evaluate the use of MALDI-TOF MS system to quickly detect hospital outbreaks

Group 5

Fourty-four *K. pneumoniae* strains from a frozen collection, isolated from 23 patients during an outbreak onset in ICU of our hospital, from January to March 2015, were used to evaluate the utility of MALDI-TOF MS system to quickly detect clonal correlation.

For all strains, we performed Carba NP test and PCRs for $bla_{IMP}/bla_{VIM}/bla_{KPC}$ genes, [78] to confirm carbapenemase production. MALDI-TOF MS spectra analysis was used to detect the 11,109Da peak and to find genetic correlation; also, PFGE was performed because it is the Gold Standard for strains typing. [98] [99]

The results of Carba NP test, PCR and MALDI-TOF MS for detection of the 11,109Da peak are summarized in Tab. 37.

Tab. 37 *K. pneumoniae* strains from collection, isolated from 23 patients during an epidemic episode in ICU in 2015

Strain	Carba NP Test	bla_{KPC}	bla_{VIM}	bla_{IMP}	11,109Da peak	Date sample collected	Patient
MDR1689	Pos	Pos	Neg	Neg	11,109	08/01/2015	**1**
MDR1690	Pos	Pos	Neg	Neg	11,111	08/01/2015	**1**
MDR1692	Pos	Pos	Neg	Neg	11,107	08/01/2015	**2**
MDR1693	Pos	Pos	Neg	Neg	11,107	08/01/2015	**2**
MDR1695	Pos	Pos	Neg	Neg	11,110	08/01/2015	**4**
MDR1728	Pos	Pos	Neg	Neg	11,110	15/01/2015	**1**
MDR1730	Pos	Pos	Neg	Neg	11,111	15/01/2015	**1**
MDR1731	Pos	Pos	Neg	Neg	11,109	15/01/2015	**5**
MDR1732	Pos	Pos	Neg	Neg	11,109	15/01/2015	**5**
MDR1741	Pos	Pos	Neg	Neg	11,111	17/01/2015	**6**
MDR1761	Pos	Pos	Neg	Neg	11,109	22/01/2015	**1**
MDR1762	Pos	Pos	Neg	Neg	11,111	22/01/2015	**7**
MDR1764	Pos	Pos	Neg	Neg	11,110	22/01/2015	**7**

MDR1765	Pos	Pos	Neg	Neg	11,110	22/01/2015	**1**
MDR1771	Pos	Pos	Neg	Neg	11,108	24/01/2015	**8**
MDR1794	Pos	Pos	Neg	Neg	11,110	29/01/2015	**1**
MDR1796	Pos	Pos	Neg	Neg	11,107	29/01/2015	**7**
MDR1821	Pos	Pos	Neg	Neg	11,106	03/02/2015	**9**
MDR1822	Pos	Pos	Neg	Neg	11,110	03/02/2015	**9**
MDR1835	Pos	Pos	Neg	Neg	11,110	05/02/2015	**10**
MDR1836	Pos	Pos	Neg	Neg	11,108	05/02/2015	**7**
MDR1868	Pos	Pos	Neg	Neg	11,110	11/02/2015	**11**
MDR1870	Pos	Pos	Neg	Neg	11,109	12/02/2015	**7**
MDR1874	Pos	Pos	Neg	Neg	11,110	12/02/2015	**7**
MDR1914	Pos	Pos	Neg	Neg	11,111	19/02/2015	**12**
MDR1916	Pos	Pos	Neg	Neg	11,108	19/02/2015	**10**
MDR1917	Pos	Pos	Neg	Neg	11,110	19/02/2015	**13**
MDR1919	Pos	Pos	Neg	Neg	11,110	19/02/2015	**14**
MDR1924	Pos	Pos	Neg	Neg	11,112	19/02/2015	**15**
MDR1953	Pos	Pos	Neg	Neg	11,110	27/02/2015	**12**
MDR1955	Pos	Pos	Neg	Neg	11,111	27/02/2015	**1**
MDR1958	Pos	Pos	Neg	Neg	Absent	28/02/2015	**16**
MDR1981	Pos	Pos	Neg	Neg	11,107	05/03/2015	**17**
MDR1985	Pos	Pos	Neg	Neg	11,106	05/03/2015	**18**
MDR2015	Pos	Pos	Neg	Neg	11,114	12/03/2015	**1**
MDR2016	Pos	Pos	Neg	Neg	11,106	12/03/2015	**19**
MDR2018	Pos	Pos	Neg	Neg	11,105	12/03/2015	**20**
MDR2019	Pos	Pos	Neg	Neg	11,112	12/03/2015	**21**
MDR2024	Pos	Pos	Neg	Neg	11,105	13/03/2015	**18**
MDR2025	Pos	Pos	Neg	Neg	Absent	13/03/2015	**16**
MDR2027	Neg	Neg	Neg	Neg	Absent	13/03/2015	**17**
MDR2048	Pos	Pos	Neg	Neg	11,108	19/03/2015	**22**
MDR2081	Pos	Pos	Neg	Neg	11,108	26/03/2015	**23**
MDR2085	Pos	Pos	Neg	Neg	11,107	26/03/2015	**24**

Pos, positive; Neg, negative

Forty-three out of forty-four strains were positive for Carba NP test and positive also for *bla*$_{KPC}$. [Tab. 37]

One strain (number 2027, from patient 17) resulted negative at Carba NP test, PCR for *bla* genes and MALDI-TOF 11,109Da peak detection. [Tab. 37]

After MALDI-TOF analysis, we found 2 discrepant strains (number 1958 and 2025 from patient 16), that is strains that carried *bla*$_{KPC}$ gene, without the 11,109Da peak in their MALDI-TOF spectra. These strains were retested twice by MALDI-TOF MS system, before and after using MH plates additioned with Imipenem (2 µg/ml), and we performed sequencing *bla*$_{KPC}$ gene, plasmid extraction and characterization. [56] [79] [Tab. 27 and 28]

After identification, the MALDI-TOF MS spectra of all 44 strains, including the susceptible ones, were imported in Saramis database to perform the clustering analysis. The software showed the relative taxonomy creating a dendrogram based on mass similarity, with tolerance (%): 0.08; Absolute intensity ≥0; Relative intensity ≥0. [Fig. 40]

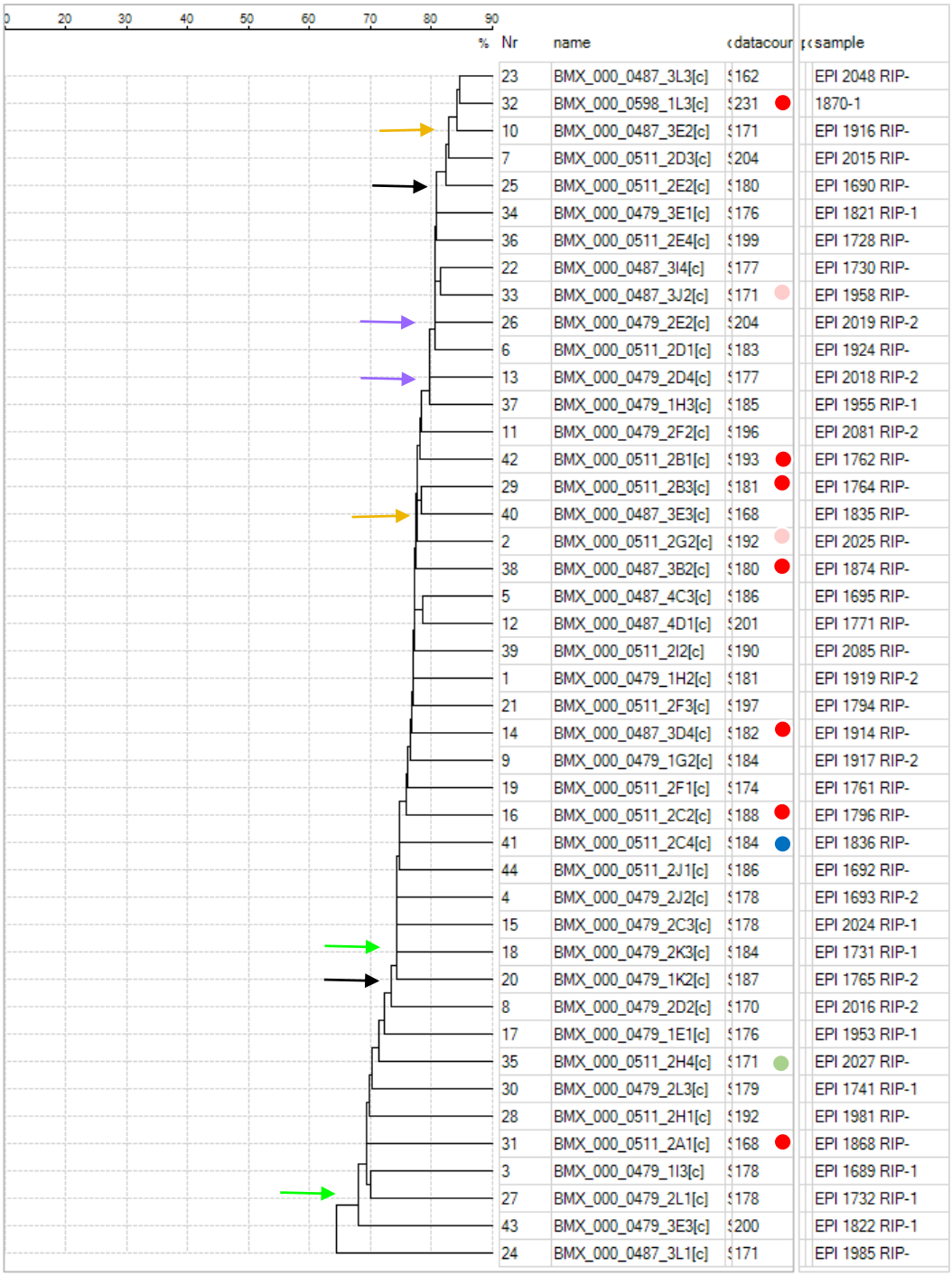

Fig. 40 Dendrogram obtained through MALDI-TOF MS spectra of 44 *K. pneumoniae* strains isolated during an outbreak in our hospital. The strains marked in red and blue are also shown in PFGE fingerprint in Fig. 41 and PFGE dendrogram in Fig. 42. The strains marked in pink and green are also shown in PFGE fingerprint in Fig. 43 and PFGE dendrogram in Fig. 42. The strains marked with an arrow have 100% similarity two by two. They are also shown in Fig. 42-43-44.

For the same strains, PFGE was performed, obtaining a fingerprint after *Xba*I digestion, examples illustrated in Fig. 41, 43 and 44. The results of restriction patterns were analyzed by FPQuest™ Software. Band similarity analysis was performed by Dice coefficients (SD) [96] [98], with position tolerance of 1.5%, on the basis of the unweighted average pair group method (UPGMA), [100] obtaining the dendrogram shown in Fig. 42.

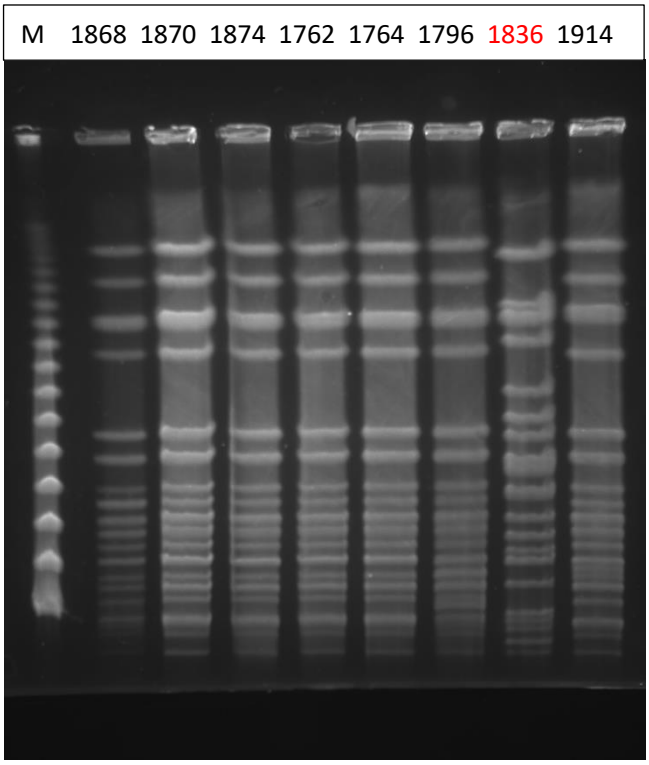

Fig. 41 PFGE fingerprint of 8 *K. pneumoniae* strains out of 44 isolated in ICU during an epidemic onset. Strains 1868 to 1796 and strain 1914 are the same as in Fig. 40 and Fig. 42 where they are marked in red. Strain 1836 is the same as in Fig. 40 and Fig. 42, marked in blue. M=marker.

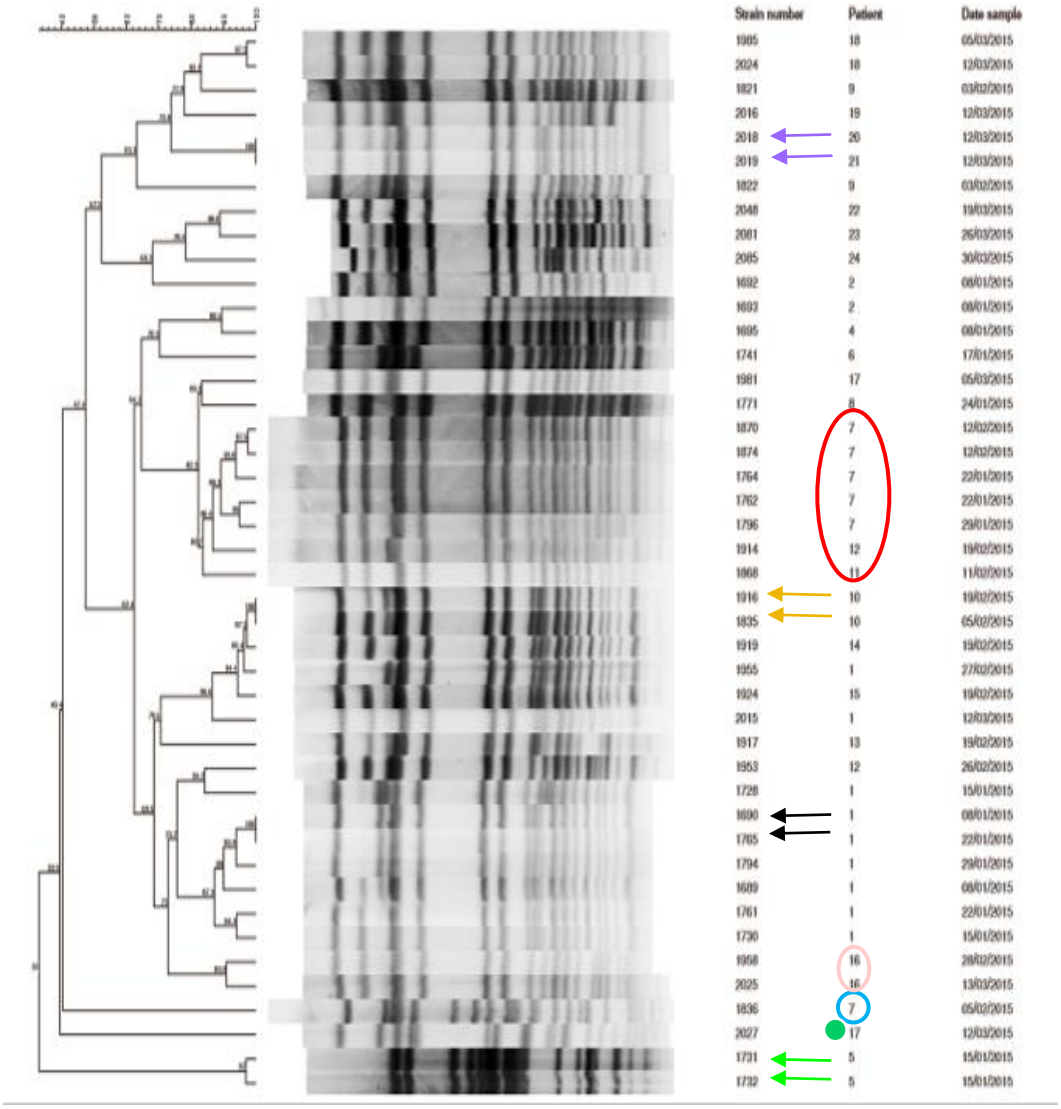

Fig. 42 Results of PFGE on 44 *K. pneumoniae* strains isolated during an outbreak in our hospital, analyzed with FPQuest™ Software. The strains marked in red and in blue are the same as shown in MALDI-TOF dendrogram Fig. 40 and PFGE fingerprint in Fig. 41. The strains marked in pink and in green are the same as shown in Fig. 40 and 43. The strains marked with an arrow have 100% similarity two by two. They are also shown in Fig. 40-43-44.

The fingerprint in Fig. 41 shows 7 strains with similar PFGE pattern (strains from 1868 to 1796, and strain 1914), and strain 1836 with a different PFGE pattern. Strains 1870, 1874, 1764, 1762, 1796 and 1836 were isolated from samples of a same patient (number 7). Instead, strain 1914 was isolated from patient 12 and strain 1868 from patient 11.

The strains 1762 and 1764 were isolated first, on the 22nd of January 2015, strain 1796 on the 29th of January and the others in February 2015, i.e. strain 1836

on the 5th, strains 1870 and 1874, on the 12th and finally, strain 1914 on the 19th. [Tab. 37]

These 8 bacterial strains present dissimilar results regarding MALDI-TOF MS comparison and PFGE.

In MALDI-TOF MS dendrogram, these strains all present a percentual similarity between ~69 and ~85%. None of them show similarity higher than 85%. Strain 1836 clearly different in terms of PFGE fingerprint, has a similarity of about ~75% in MALDI-TOF MS dendrogram. [Fig 40]

The PFGE, instead, shows that strains 1870 and 1874 have percent similarity 97.6%. Strain 1764 has similarity 93.8% with the first two. Strains 1762 and 1796 have 95% similarity, and 89.3% similarity with the previous ones. These five strains were isolated from the same patient. Strain 1914 has 86.8% similarity with the previous ones, and strain 1868 has 83.7% similarity with the previous ones. These last two strains were isolated from patients 12 and 11 respectively. Finally, strain 1836 has 47.4% similarity with all the other strains.

According to PFGE analysis, 5 strains (1868, 1762, 1764, 1796, 1914) are closely related to each other, because they have a percent similarity >81%. Two strains (1870, 1874) are identical, as they have percent similarity >97%. [98] The last strain, instead, with 47.4% similarity is not related to the others. [Fig. 42] [Tab. 38] The results of the MALDI-TOF MS analysis, on the other hand, is not as clear, because the 7 strains (cluster at PFGE) are distributed on the dendrogram, and it would seem they had close relation with other strains but not to each other. Strain 1836, instead, not related to other strains according to PFGE, in MALDI-TOF MS relative taxonomy has ~75-78% similarity with strain 1796, 1761 and others. [Fig. 40] [Tab.38]

Another example of different interpretation of dendrogram results on PFGE and MALDI-TOF MS, are the two strains (1958 and 2025) resulted discrepant at MALDI-TOF MS spectra analysis for the 11,109Da peak detection. Both were isolated from patient 16 with a gap of two weeks between them. [Tab. 37] The PFGE fingerprint [Fig. 43] and the PFGE dendrogram show they are closely related,

with 90.9% similarity, and they differ from the other strains (73% and 68.5% similarity with two cluster). [Fig. 42] Curiously, in MALDI-TOF dendrogram, each of them is related to strains collected from other patients, but not to each other. [Fig. 40]

In Fig. 43 strain 2027 is also shown, the only one resulting negative for carbapenemase production in this group of strains. It was isolated from patient 17 in March 2015, after the isolation of another strain positive for *bla*$_{KPC}$ (1981), one week earlier. According to the PFGE dendrogram, it seems clear that this strain is unrelated to the other strains, as it shows 40.4% similarity with them. [Fig. 42] In MALDI-TOF dendrogram, instead, the strain has over 70% similarity with other strains. [Fig. 40]

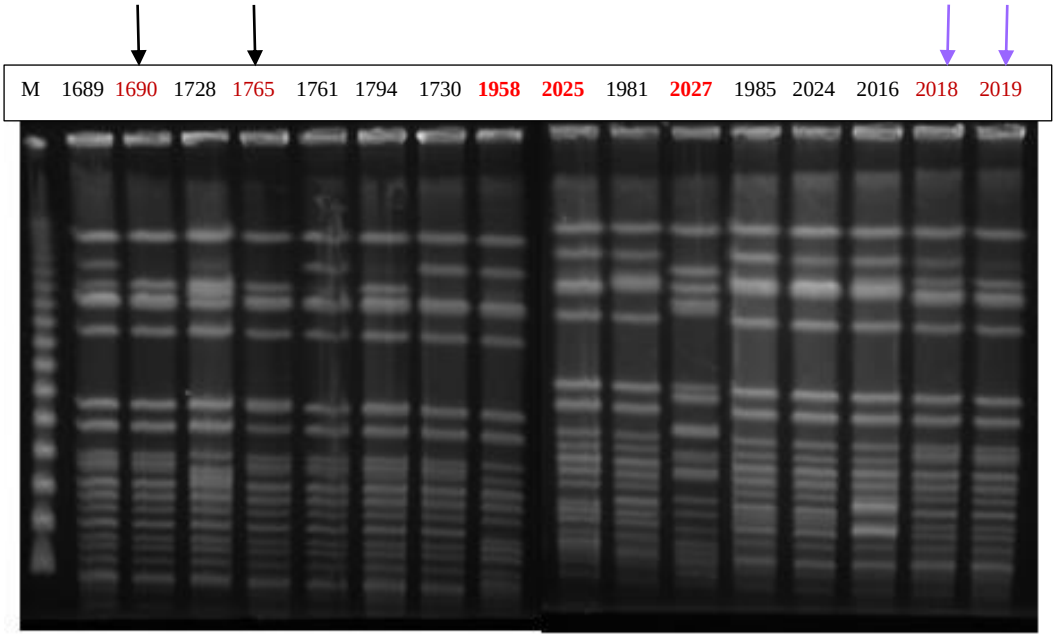

Fig. 43 PFGE fingerprint of 16 *K. pneumoniae* strains out of 44 isolated during an epidemic onset. Strains 1958 and 2025 are discrepant for PCR and the 11,109Da peak detection. In Fig. 40 and 42 are marked in pink. Strain 2027 results negative at Carba NP test, PCR for *bla* genes and 11,109Da peak detection. It is the same as in Fig. 40 and 42 marked in green. At PFGE strains 1690 and 1765 are identical, strains 2018 and 2019 are identical. They are shown also in Fig. 40 and 42. M=marker.

Also, according to PFGE fingerprint there are 6 strains that have 100% similarity with each other: strains 2018 and 2019 (patient number 20 and 21); strains 1916 and 1835 (both patient 10); and finally strains 1690 and 1765 (both patient 1). [Fig. 42-43-44]

Also in this case, there is no accordance between PFGE and MALDI-TOF results. Only the strains 2018 and 2019, in fact, have about 80% of similarity, the others are not closely related in the MALDI-TOF taxonomy. [Fig. 40]

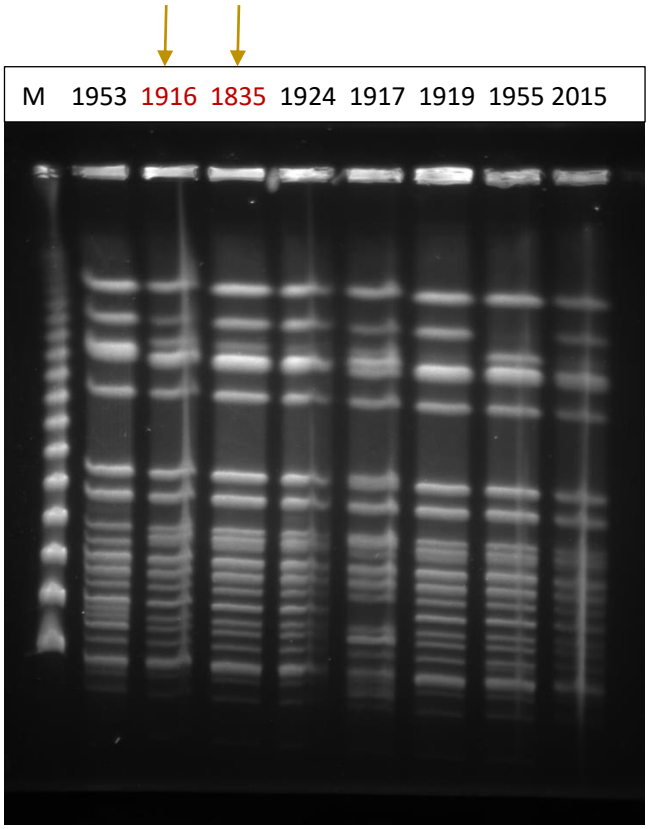

Fig. 44 PFGE fingerprint of 8 *K. pneumoniae* strains out of 44 isolated during an epidemic onset. Strains 1916 and 1835 are identical. They are shown also in Fig. 40 and 42. M=marker.

Finally, according to PFGE fingerprint, 2 strains isolated from pharyngeal and rectal swab from patient number 5 (strain 1731 and 1732) result almost identical to each other (97% similarity), but not related with the other strains (33% similarity). [Fig. 42] [Fig. 45] The MALDI-TOF MS dendrogram does not show the same result. Strain 1732 has about 70% similarity with strain 1689, but it is not closely related to strain 1731, which in turn has ~75% similarity with strain 1765. [Fig. 40]

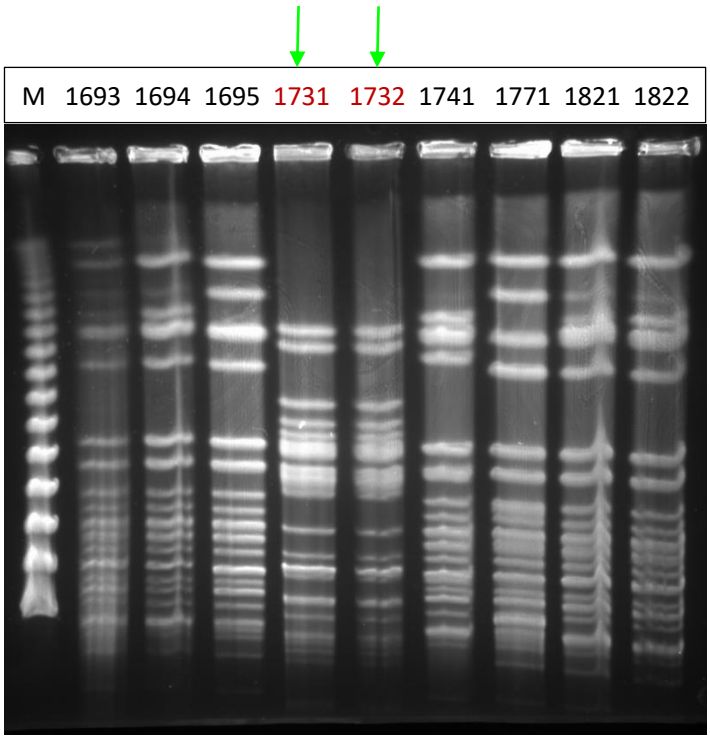

Fig. 45 PFGE fingerprint of 8 *K. pneumoniae* strains out of 44 isolated during an epidemic onset. Strains 1731 and 1732 isolated from pharyngeal and rectal swab from patient 5 are identical and differ from all the other strains. They are shown also in Fig. 40 and 42. M=marker.

Tab. 36 synthetizes the results of PFGE and MALDI-TOF MS relative taxonomy of the strains already presented in Fig. 40 to Fig. 45.

Tab. 38 Results of the comparison between PFGE and MALDI-TOF MS relative taxonomy, concerning the strains already presented in Tab. 41 to Tab. 46. Cut-off for similarity between strains was 81% for type (closely related) and 97% for subtype (identical) [98]

Strain	Patient	PFGE Percent similarity	MALDI-TOF MS Percent similarity
1870	7	97.6% / 93.8% / 89.3% / 86.8% / 83.7%	Relationship not clear Similarity between 69% and 85%
1874	7		
1764	7		
1762	7	95%	
1796	7		
1914	12		
1868	11		
1836	7	47.4% Not related with the 7 strains above	
1958	16	90.9%	Related with other strains. Not closely related to each other
2025	16		
2027	17	Not related with any other 51.6% only with one strain	~ 71% similarity with other strains
2018	20	100%	~ 80%
2019	21		
1916	10	100%	Relationship not clear
1835	10		
1690	1	100%	Relationship not clear
1765	1		
1731	5	97% / 33% with other strain	Not closely related each other. Related with other strains
1732	5		

After observing the difference between PFGE and MALDI-TOF MS relative taxonomy, we have decided to verify whether this diversity was due to the

number of our samples. Therefore, we selected the spectra of the strains which at PFGE fingerprint clearly formed a cluster, then revaluating the MALDI-TOF MS relative taxonomy in each group. [Fig. 46] [Fig. 47]

For example, the 7 strains mentioned above (1868, 1870, 1874, 1762, 1764, 1796 and 1914) as well as strains 1771 and 1981, which resulted a cluster at PFGE, if evaluated separately for MALDI-TOF MS relative taxonomy, they show different percentages of similarity, ranging from ~68% to ~78%, but still different from those of PFGE. [Fig. 46]

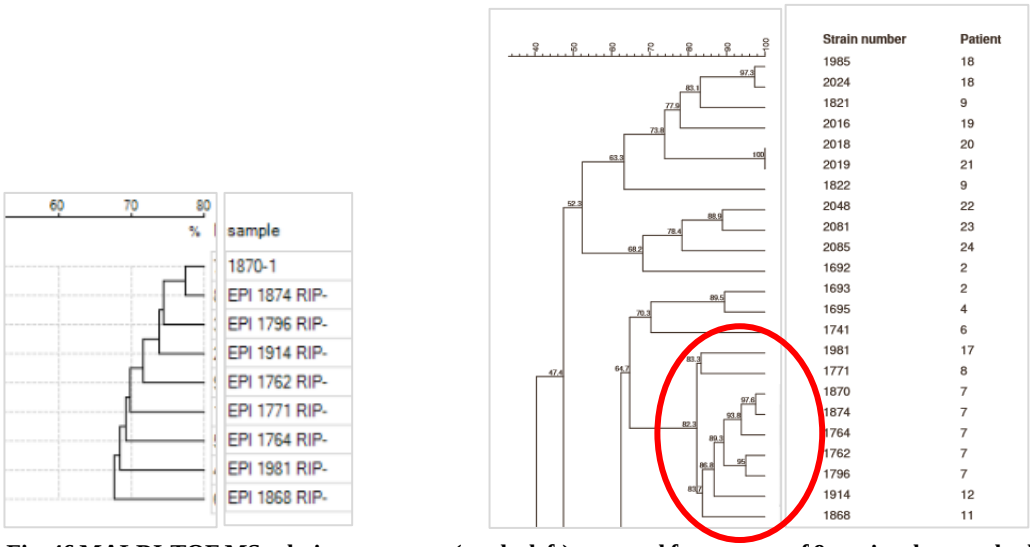

Fig. 46 MALDI-TOF MS relative taxonomy (on the left) repeated for a group of 9 strains that resulted a cluster at PFGE (strains marked on the right).

We also repeated the evaluation of the relative taxonomy at MALDI-TOF MS for 17 strains, including some already presented in Tab. 38 (namely strain 1958, 2025, 1916, 1835, 1690, 1765). Again, strains with 100% similarity in groups of two by two at PFGE (strains 1916 and 1835; strains 1690 and 1765) were not closely related at MALDI-TOF MS evaluation. Also strains 2025 and 1958 were identical to each other, but different from the other ones according to PFGE fingerprint, yet they do not have the same similarity at MALDI-TOF MS evaluation (<80% similarity).

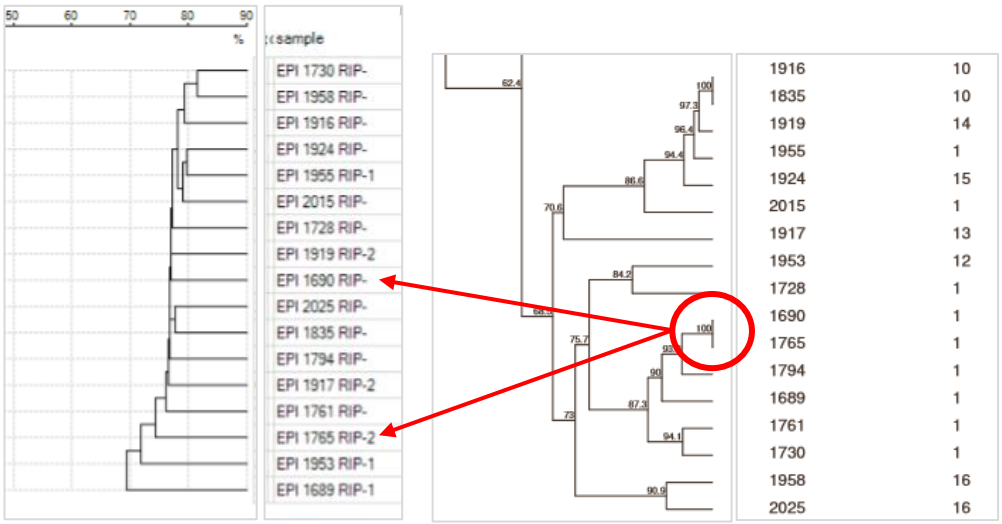

Fig. 47 MALDI-TOF MS relative taxonomy (on the left) repeated for a group of 17 strains distributed in 4 cluster at PFGE (on the right). The strains marked (1690 and 1765) are identical according to PFGE (100% similarity) but not even closely related for MALDI-TOF MS evaluation.

Finally, also revaluating these 17 strains with the 4 strains already presented in Tab. 37 (namely strain 1836, 2027, 1731 and 1732), resulting clearly not related with the others at PFGE, the difference between PFGE and MALDI-TOF MS evaluation remains confirmed. [Fig. 48]

Fig. 48 Repeated MALDI-TOF MS relative taxonomy (on the left) of a group of 17 strains (previously presented in Fig. 47) forming four clusters according to PFGE, with 4 strains resulting clearly not related (on the right). The strains marked (1731 and 1732) are identical according to PFGE (97% similarity) but not closely related at MALDI-TOF MS evaluation. Strains 2027 and 1836 are not related with anyone at PFGE, but at MALDI-TOF MS relative taxonomy they presented ~72% and ~75% similarity with other strains.

Strains 1731 and 1732, in fact, which have 97% similarity at PFGE, on MALDI-TOF MS relative taxonomy are not even closely related (strain 1732 has 70% similarity with strain 1689 and <70% with a group of strains including strain 1731). Strain 2027 clearly not related to any other at PFGE (40.4% similarity), at MALDI-TOF MS has ~72% similarity with strain 1953 and other ones. Strain 1836 which at PFGE has 40.4% similarity with other strains, at MALDI-TOF MS relative taxonomy it has ~75% similarity with strain 1761 and some more. [Fig. 48]

DISCUSSION AND CONCLUSION

Antibiotic resistance is a widespread phenomenon, recognized by International Health Organizations as a new global public health crisis. [7] [22] Carbapenem-resistant *Enterobacteriaceae* especially represent a major concern mostly in hospital settings, since they spread very quickly. The problem is clinical, with severe illness, frequent epidemic outbreaks in hospitals and rate of mortality that reach 50-80%, but economic also, for prolonged hospitalization and treatments, for the use of expensive drugs and increasing demand of screening tests.

ECDC estimate that each year, about 25,000 patients die in Europe, from an infection with multidrug-resistant bacteria. These infections count for an extra 2.5 million hospital days, with an economic burden estimated to be at least EUR1.5 billion in Europe in 2007. [9] [25]

In Italy, resistance percentages have had an increasing trend during the last years, among the highest in Europe, especially for Gram-negative bacteria. For example, percentage of isolates of carbapenem-resistant *K. pneumoniae* arose from 1% in 2006 to 33% in 2014. [26] [29] [Tab. 4]

In Verona, too, we observed a general increase of resistant strains isolated in our hospital. For example, carbapenem-resistant *K. pneumoniae* strains isolated in our clinical lab, were 1.94% in 2009, and arose to 61.76% in 2011. However, after the beginning of the screening program in 2013, we observed a slight decrease (39.2% in 2015). [Graph. 1] Screening, thus, proves itself to be an important strategy to reduce resistance rate, because it helps contain the transmission of resistant-strains in those cases in which resistance is plasmid-mediated, and therefore easily spread.

At the moment, available diagnostic assays are countless, ranging from traditional culture-based methods to more recent genetic and proteomic ones. They are highly sensitive and specific, but often time-consuming, some are very

expensive and hard to perform and require specific instruments and expertise. Thus, they are not feasible in clinical laboratories, but only in reference laboratories.

As a consequence, it is a priority for researchers to provide new diagnostic tools for clinical labs, more suited for their needs so that they can respond to an increasing number of requests from clinicians, giving them accurate results which also save time and money.

The main necessity is to quickly identify carbapenemase-producing bacterial strains in infected and colonized patients, in order to efficiently contain the spreading and assess proper therapy. Carbapenem-resistant *Enterobacteriaceae*, in fact, have been classified by CDC at hazard level "urgent", as they cause hospital-acquired infections, which leave very limited therapeutic options and consequently bring high mortality rates. [7]

The routine screening for multidrug-resistant strains (MDR) in our clinical laboratory, performed on rectal swabs, is based on traditional overnight culture. The time required to report was reduced from the 72 hours needed with traditional identification, susceptibility tests and confirmatory tests, to 24 hours only, with the use of selective chromogenic agar plates (ChomID ESBL agar) with ertapenem disk, rapid phenotypic Carba NP test [68] (results in 2 hours) and MALDI-TOF identification (results in a few minutes). Also, costs were reduced (about EUR5 per screening including the costs for detection of several types of multi-resistant strains, not only of carbapenemase-producers). Molecular technics such as Real-Time PCR could reduce the detection time even further starting directly from samples, but their costs are not always compatible with the high number of samples tested in a hospital clinical laboratory (about EUR30-50 for each single screening).

Mass-Spectrometry-based method MALDI-TOF, represents an innovative system for bacteriology, is currently used in diagnostics to identify bacterial species, but could be implemented as a new tool to quickly detect resistant strains with high sensitivity, and with a significant decrease in time and costs. This would be especially useful for laboratories, like our clinical Microbiology lab, that

provides a service for a medium/big hospital, with high and increasing number of MDR screening requests (about 12,000 tested samples last year).

Numerous studies have demonstrated the possible use of MALDI-TOF MS to recognize resistant and susceptible strains. At the moment, most of the published data concerns the detection of carbapenemase activity measuring antibiotic hydrolysis. [71] [101] Few studies instead, were performed in order to use MALDI-TOF MS spectra acquired during the ordinary identification to detect resistance determinants, based on the presence/absence of a specific peak. [73] [74] [102] [103] There are, moreover, very few data about clinical application and utility [74] [102] and all studies were performed only by one of two MALDI-TOF systems applied to microbiology, namely that patented by Bruker Daltonics, whereas we have employed the other system for microbiology patented by bioMérieux, which makes use of the same technology, but with a different software (Shimadzu Launchpad) and different database (SARAMIS^{TM} Premium (Spectral ARchivee And Microbial Identification System) ID-professional (AnagnosTec-bioMérieux)). In addition, the use of MALDI-TOF for typing, compared with PFGE, was also evaluated from many research groups, but again these studies were performed using Bruker Daltonics system. [76] [77]

The initial aim of this study, started in January 2014, was to look for a peak in MALDI-TOF spectra, which could be used as marker for rapid detection of carbapenemase production, especially for KPC. During our research, we found a study based on 18 outbreak isolates published by Lau and coll., [73] who had already identified a MALDI-TOF peak (11,109Da) corresponding to pKpQIL plasmid, harboring KPC gene, and proposed it as marker for this plasmid and consequently for KPC detection. [73] Therefore, we have continued our experiments to confirm our theory and the published study on a wider range of samples, and verify if it would also apply to our specific context, using a different MALDI-TOF system.

Our purpose was indeed, to evaluate the ability of MALDI-TOF MS system used in our clinical laboratory to detect antimicrobial resistance mechanisms and to validate the use of this peak detection to quickly spot carbapenemase-producing *Enterobacteriaceae*, through the spectra already acquired during species identification, in order to improve the analytical capability of our lab.

The main goal was reached through three specific objectives that represent the three sections of this work, performed in close collaboration with our clinical Microbiology laboratory.

Part I - To confirm the correlation between KPC production and a characteristic MALDI-TOF MS spectrum with 11,109Da peak detected in 2014 by Lau and coll. [73] We also wanted to demonstrate the usefulness of 11,109Da peak detection in diagnostic workflow for rapid screening of KPC-producing Enterobacteria.

Part II - To eventually detect new characteristic MALDI-TOF MS peaks for carbapenemase other than KPC: NDM and VIM.

Part III - To evaluate the use of MALDI-TOF MS system during hospital outbreaks to identify bacterial strains clonally related.

The study included *Enterobacteriaceae* strains isolated in clinical samples collected during MDR screening in Microbiology Laboratory of Verona Hospital and Negrar Hospital (Verona). We identified the bacterial strains with MALDI-TOF MS system, evaluated the antibiotic susceptibility with systems in use in our laboratories and detected and confirmed carbapenemase production. We correlated also the results of MALDI-TOF MS measurements with phenotypic and genetic data to obtain an epidemiologic and molecular analysis of the diffusion pattern of multidrug-resistant Enterobacteria in Verona geographic area.

In the first part of the study, we confirmed that 11,109Da peak in MALDI-TOF MS spectra is correlated with KPC production and later, we demonstrated how to use this correlation in ordinary diagnostic workflow.

In a first step, 436 Enterobacteria strains (Group 1) were used to investigate the correlation between the presence of the 11,109Da peak in MALDI-TOF MS spectra and KPC-production. We acquired and analyzed the MALDI-TOF MS spectra of 176 well-characterized KPC-producing strains (*K. pneumoniae* and *E. coli*) and 58 well-characterized ESBL-producing and carbapenem-resistant, but not carbapenemase producer strains (*K. pneumoniae* and *E. coli*). As control group, we analyzed the spectra of 27 *β*-lactam-susceptible *K. pneumoniae,* 121 ESBL-producing E. *coli,* 15 NDM-producing and 39 VIM-producing Enterobacteria strains from collection. [Fig. 25] The 11,109Da (±8) peak was present in 99.4% (175/176) of the spectra of KPC-producing strains. In all 260 strains negative for KPC the peak was absent. Only one strain, therefore considered discrepant, was positive for KPC production, confirmed by *bla* genes PCR, without 11,109Da peak in MALDI-TOF MS spectra. [Tab. 23 and 24]

Afterwards, we also analyzed two other groups of strains by collection: 11 *E. coli* all KPC producers (Group 3) [Fig. 27] and 44 *K. pneumoniae* (43 KPC producers and 1 negative for carbapenemase production) (Group 5). [Fig. 29] By MALDI-TOF spectra analysis, we found 2 discrepant strains in each group. [Tab. 27 and 28] Therefore, totally the 11,109Da peak by spectra analysis was observed in 97.8% (225/230) of KPC-producing strains. On the other hand, none of 261 strains carbapenem-susceptible, carbapenemase-producing other than KPC (VIM or NDM) or carbapenem-resistant but not carbapenemase producers, as well ESBL-producing strains, had the same peak.

Thus, we demonstrated there is indeed a strong correlation between MALDI-TOF MS 11,109Da peak and KPC production, confirmed by PCR. Sensitivity in fact, is 97.8% and specificity 100%, with Positive Predictive Value 100% and Negative Predictive Value 98%. These results were encouraging and lead us into evaluating a possible applicability of spectra analysis to routine screening.

In the second step, thus, we demonstrated that the analysis of MALDI-TOF MS spectra to detect the 11,109Da peak can be used not only in research laboratory, but also for MDR rapid screening in clinical laboratory, and demonstrate many practical advantage of this method for diagnostic routine.

The reliability of the method was verified by searching for this specific peak during routine workflow in our clinical microbiology lab. Rectal and at times pharyngeal swabs were processed by three different technicians according to protocols in use. From May to July 2016, we examined daily, the MALDI-TOF MS spectra obtained during bacterial species identification. We limited the investigation to strains identified as possible carbapenem-resistant, by isolation on ESBL ChromID agar plate, showing an inhibition zone diameter with ertapenem disk <22 mm and with positive Carba NP test. The same way, we investigated as control group some strains isolated in the same context, showing zone diameter for ertapenem disk >22 mm, with negative Carba NP test and positive ESBL NDP test. (Group 2) [Fig. 26]

Totally, we analyzed 183 spectra, 135 of strains with positive Carba NP test (124 *K. pneumoniae*, 8 *E. coli*, 3 *Enterobacter sp*.) and 48 strains with negative Carba NP test and positive ESBL NDP test. (47 *K. pneumoniae*, 1 *E. coli*). It results that 129 out of 135 MALDI-TOF MS spectra of the strains with Carba test positive had also the 11,109Da peak. None of the spectra of the 48 strains with Carba NP test negative had the 11,109Da peak. [Tab. 25] Spectra analysis for detection of the 11,109Da peak, in this group of strains had 95.5% sensitivity (129/135) and 100% specificity (48/48).

Analysing the discrepant strains, however, we found 3 strains producing carbapenemases other than KPC, namely 1 *E. coli* NDM producer, and 1 *E. aerogenes* and 1 *E. cloacae*, VIM producers. We concluded, therefore, that the real discrepants were 3 only, namely 2 *K. pneumoniae* and 1 *E. coli*, and so sensitivity arose to 97.7% (129/132) and specificity confirmed 100% (51/51). Thus, Positive Predictive Value (PPV) was 100% and Negative Predictive Value (NPV) is 94.4%.

Analysis of discordant strains was performed to clarify the absence of the peak.

Totally, discrepant strains were 8/362 KPC-producing strains, namely 3 *E. coli* and 5 *K. pneumoniae*. All were confirmed KPC producers based on PCR for bla_{KPC} and confirmed expressing the gene, based on Carba NP test positive, yet

without the 11,109Da peak in MALDI-TOF MS spectra. We looked for any common characteristic amongst the strains that could possibly justify the discordance, to try to understand if it could interfere with the usage proposed.

By sequencing the *bla*$_{KPC}$ gene we concluded that 2 were KPC-3 and the others were KPC-19. The resistance plasmids harboring the KPC gene were investigated through PBRT protocol of Carattoli *et al.* [56] [79] and showed scaffolds that resulted different. Three strains harbored plasmid replicon OriColE; 1 carried IncR and OriColE; 1 carried FIA and FIB; 1 IncQ; 1 IncN, FIB and FrepB; 1 strain none at all. [Tab. 27 and 28]

As control group, we selected 8 not discrepant strains, namely 3 *E. coli* and 5 *K. pneumoniae*. All these strains were KPC producers and had the 11,109Da peak in their MALDI-TOF spectra. We performed *bla*$_{KPC}$ sequencing and plasmid typing for these strains too. Only one strain resulted harboring KPC-19, the others carried all KPC-3. [Tab. 29 and 30] These strains showed different replicons, according plasmid characterization, [56] [79] and the element that appeared common and yet different from discordant strains was the presence of FIIs replicon in these, but not in the discrepant ones. [Tab. 28 and 30] The FIIs rep is the replicon most frequently present in plasmid pKpQIL, carrying transposon Tn4401, very common in KPC-producing strains.

Finally, for all 8 discrepant strains and 8 control strains we performed PCRs to detect *p019* and *Tn4401* genes. The results were that only control strains (positive for 11,109Da peak and IncFII plasmid) contained *p019* gene. The distribution of *Tn4401* gene, instead, is not the same in discrepant and control group (6 positive and 2 positive respectively). [Tab. 31]

Our results, thus, when analysing both selected strains and random strains during routine diagnostic practice, are in line with published data, [73] [74] and mainly they confirmed our hypothesis that use of MALDI-TOF spectra analysis for detection of the 11,109Da peak could be relevant to our routine screening. Indeed, we analyzed a consistent number of strains, and we only found 2% false negative

(8/362) and none false positive. Since the 8 discrepant strains did not show the peak in the spectrum analysis, but were positive to Carba NP test, we propose to use the two methods in combination, because we find them complementary, so as not to miss other possible discrepant strains.

The correlation between the presence of 11,109Da peak and positive results by Carba NP test confirmed the reliability of MALDI-TOF MS analysis as rapid screening method of KPC-producing bacterial strains.

Epidemiology of our geographic area, with over 90% of carbapenemase which are KPC, [Graph. 7] is favourable to implement this analysis. The utility of the 11,109Da peak detection, moreover, is not only direct to find KPC-producing strains during the routine identification, but also to find strains producing other types of carbapenemases, for example NDM or VIM which are also increasing in frequency, or new carbapenemases. The presence of the 11,109Da peak combined with a positive Carba NP test confirms KPC production. On the other hand, the absence of the peak combined with positive Carba NP test can lead to perform PCR for *bla* genes and to search for carbapenemase other than KPC. In fact, the 3 strains above mentioned, were detected and characterized as NDM and VIM producers exactly through spectra analysis, since the Carba NP test provide information only about carbapenemase production, but not about the type of carbapenemase involved. This is important not only to provide epidemiologic information, but mostly, to detect strains with high level of resistance and transmissibility like NDM producers, the new threat emerging worldwide. Also, detailed information about the specific type of carbapenemase is important when a therapy effective against only some specific types of carbapenemase producers is required, like with Ceftazidime-Avibactam which is not active against MBL producer strains. [104]

The peak of 11,109Da was detected in 2014 by Lau and coll. [73] who were investigating 18 KPC-producing *K. pneumoniae* strains from an outbreak. They proved the peak to be present only in KPC-producing strains and which they

supposed to correspond to a product of *bla*$_{KPC}$ gene, adjacent to Tn4401, carried on plasmid pKpQIL. [73]

Later, the same group proved the 11,109da peak to be corresponding to a protein named p019 not to KPC. [74] The gene codifying for this protein, however is associated with some type of plasmid harboring KPC gene too. In fact, they detected the 11,109Da peak in spectra of carbapenemase-resistant strains, positive for KPC and for p019. The presence or the absence of the peak is therefore related with the presence or absence of the gene of p019 protein and therefore with the transposon and with the type of plasmid harbored KPC gene, not directly with KPC. [74] In over 90% of the KPC producer strains, *bla*$_{KPC}$ and *p019* genes are associated, carried simultaneously on pKpQIL plasmid.

In 2016, Gaibani and coll., demonstrated that the *p019* gene was associated with transposon Tn4401a isoform [105] as part of an Insertion Sequence present only in plasmid harboring KPC gene. [106] Probably, in discrepant strains, different mobile elements carry *bla*$_{KPC}$ gene, without harboring *p019* gene.

Then, these studies indicate a strong association between the 11,109Da peak (corresponding to p019 protein) and KPC production, reporting a small percentage of discrepant strains.

Our results too, confirmed that the presence of the 11,109Da peak is correlated with KPC, depending on the type of plasmid harboring *bla*$_{KPC}$ gene. In our analysis, the 11,109Da peak is present in strains carrying the *p019* gene in IncFII type plasmid, and absent in strains not carrying the *p019* gene and harboring other plasmids. [Tab. 31] That it is explained with epidemiological data. In our region, that is, the prevalent KPC sequence type is ST258, spread worldwide. [82] This clonal strain harbors different plasmids, the most frequent being pKpQIL, which carries the transposon Tn4401 and belonging to Inc group FII. [52] [57] These strains seem to be the same carrying the *p019* gene and therefore, those detectable as KPC producers through the 11,109Da peak. The percentage of strains harboring other mobile genetic elements different from pKpQIL plasmid, carrying *bla*$_{KPC}$ gene, but not *p019* gene and therefore not detectable through the 11,109Da peak

appear to be very few. In our strains, however, the *Tn4401* gene shows no strong correlation with the *p019* gene and 11,109Da peak, because it is observed in both groups of strains (discrepant and control strains), and this requires further evaluation.

The peak searching is a screening method which is sensitive, specific, it provides immediate useful information to clinicians, allowing them to adopt prompt containing measures for carriers, because it is very rapid, simple to perform, does not require much expertise, nor extra steps on top of ordinary identification by MALDI-TOF MS system. Moreover, the costs are much lower compared to other diagnostic tests, like for example molecular methods, which are also time-consuming, more difficult to perform and their use could be then restricted for discrepant strains only.

Other advantages of this sort of analysis are the followings: the MALDI-TOF MS measurements can be performed directly from colony, by more than one operator and they can be used for screening of a large number of samples. Peak detection can be performed also retrospectively, on spectra already acquired for identification, whenever there is a need to confirm a diagnostic suspect or on spectra of strains isolated from samples not send for screening and therefore following longer diagnostic procedures, rapidly identifying the potential presence of KPC producers.

To ensure reliable results, it is extremely important to acquire high quality spectra (confidence ≥90%). Therefore, it is necessary to guarantee for calibration of the instrument and for standardization of the pre-analytical phase, culturing the bacterial strains on the same type of plate, under the same conditions.

It would be desirable and very useful, however, to have the chance to rely on a software able to automatically spot the presence or absence of the 11,109Da peak.

Part II – In the second part of this study, we tried to find new characteristic MALDI-TOF MS peaks corresponding to carbapenemase other than KPC: NDM and VIM. Isolation of strains producing these enzymes, in fact, is arising in our region, too.

For this purpose, we used well-characterized strains from collection, namely 15 NDM-producing *Enterobacteriaceae* and 36 VIM-producing strains (23 *Enterobacteriaceae* and 13 *P. aeruginosa*). [Fig. 28] After acquiring their MALDI-TOF MS spectra, we used them to create two superspectra, one for NDM producers and the other for VIM producers. Through the superspectra we wanted to select a few peaks present only in spectra of resistant-strains, excluding the masses common to susceptible strains. In a second step, we tried to find a peak common to all resistant strains NDM-producing and VIM-producing respectively, but we did not find any specific peak.

Afterwards, we performed plasmid characterization only for Enterobacteria strains, so as to classify the strains by frequency of plasmids, because we supposed that the peak searched could be common not to all strains, but to strains harboring a certain plasmid. In the NDM group we observed that the most represented plasmids are A/C (6/15), OriColE (6/15) and IncR (5/15); in VIM group, they are A/C (12/23), L/M (9/23), OriColE (8/23) and IncR (5/23). [Tab. 34 and 35]

Then, we divided the strains in groups based on plasmid and repeated manually the spectra analysis, but also this way we found no common peak.

This result can be explained with a greater variability in plasmid harboring bla_{NDM} and bla_{VIM}, compared to plasmid carrying bla_{KPC}. It also could indicate that there is not such a thing as a single protein being associated with a detectable peak in MALDI-TOF spectra corresponding to NDM and VIM producer strains.

Since we could not find any new peak yet, we can adopt the same 11,109Da peak detection in order to have an indication about strains producing carbapenemase other than KPC, as explained above, if a positive Carba NP test does not correspond to presence of the peak.

Part III – In the third and last part of our study, we investigated the use of MALDI-TOF MS system to type bacterial strains during hospital outbreaks. In literature, most of the studies were performed with MALDI-TOF MS system of Bruker Daltonics producer, as far as we know no study was performed with the MALDI-TOF MS system bioMérieux in use in our laboratory.

To perform this part of the study, we used a group of 44 *K. pneumoniae* strains isolated from clinical samples during an outbreak in ICU from January to March 2015. [Tab. 36]

For all strains, we confirmed the identification by MALDI-TOF MS system and carbapenemase production by PCR for bla_{KPC} gene. Then, we acquired the spectra to perform an analysis and generate a dendrogram. This step is rapid and performed automatically, obtaining relative or absolute taxonomy. We chose to use relative taxonomy that generates a dendrogram based on percentage similarity, [Fig. 40] while the absolute taxonomy shows the coincident masses number.

As control, we performed the PFGE on the same strains, analyzed the pattern of DNA by FPQuest™ Software, and thus obtained a dendrogram. [Fig. 42]

Finally, we compared the results of MALDI-TOF MS spectra analysis and PFGE.

Our results were clearly different according to the two system. The dendrogram generated by PFGE was easier to read than the dendrogram of MALDI-TOF MS. The strains correlated, included in a cluster in PFGE dendrogram, were early detectable, unlike in MALDI-TOF MS taxonomy. Moreover, we obtained discrepant results, because, for example, strains identical in PFGE analysis (similarity 97% or 100%) seems lightly related in MALDI-TOF MS taxonomy (similarity ~70%) and strains totally unrelated for PFGE analysis (similarity 40%) show the same percentage of similarity (~70%) as related strains. [Fig. 40 and following] Also, our results, are different from most published data, showing

comparable results with PFGE and propose the use of MALDI-TOF MS to type bacterial strains. [76] [77]

In our study, the difference between the two systems seems very important. We repeated the analysis also on separate groups of strains according to the clusters generated by DNA patterns analysis, but also this way the difference was still confirmed.

It is necessary to clarify if the difference in results is intrinsic to the systems (since PFGE compares DNA fragments and MALDI-TOF MS compares masses) or if it is a problem based on different cut-off and/or the software used by MALDI-TOF MS system.

PFGE is the Gold Standard for bacterial strains typing, especially during outbreaks. [99] [107] [108] It is based on DNA fragments analysis, obtained through Enzyme of Restriction, separated by pulsed-electrophoresis and shown as band. The profile analysis can be performed visually or automated and there are guidelines for interpretation. [109] For dendrogram interpretation there is percentage similarity cut-point. [98] The PFGE is very useful, but it has the disadvantages that it is very time-consuming, is not easy to perform and it needs equipment and expertise. The use of MALDI-TOF MS spectra analysis to typing bacterial strains could be very useful in terms of time-saving, costs containment and easiness of performance. Our results, however, were not very promising.

Our study confirmed that PFGE is an assay with higher performances. The evaluation offered through MALDI-TOF MS to clustering with the software currently in use seems little understandable and hard to interpret.

Extremely important for this analysis is the high quality of the spectra (confidence ≥90%) and the homogeneous consistency concerning similar number of peaks (>170) so as to guarantee a correct comparison among the spectra, and allow for reliable results.

In conclusion, in this study we confirmed the strong correlation between KPC production and the presence of the 11,109Da peak in MALDI-TOF MS spectra in a large number of samples. We also demonstrated that it is very easy to perform spectra analysis to detect the 11,109Da peak and it would be very convenient to introduce it in diagnostic routine workflow.

This system is suitable for a context like our region, where KPC is endemic and represents over 90% of the carbapenemases detected and can be used to search for other carbapenemases, too, since at this moment we have not found any other characteristic peak specific to NDM and VIM production.

And yet, it is evident this method cannot substitute susceptibility test. MALDI-TOF MS spectra analysis combined with Carba NP test can provide very quickly, adjunctive information to clinicians, but if performed without further results, it is not sufficient to diagnose carbapenemase production on its own.

Since we observed the correlation between *p019* gene and 11,109Da peak, in a small number of strains (8 control strains), [Tab. 31] in the future, we are going to further investigate other discrepant strains and hopefully confirm the presence or absence of *p019* and *Tn4401* genes related with the 11,109Da peak in MALDI-TOF MS spectrum on a larger number of strains.

Finally, at this moment, the use of MALDI-TOF MS system (bioMérieux) to rapidly typing epidemic strains is not viable yet, and cannot substitute the Gold Standard PFGE. It needs more investigations to this purpose, and it would be useful to improve the software for spectra analysis and dendrogram generation.

Acknowledgements

I thank Prof. A. Mazzariol and all staff of Microbiology Laboratory especially TLB Anna Oliani, Mirta Bragantini, Elena Lucchini, Anna Bertoncelli and Marco Aldegheri for their technical support. Dr. Zeno Bisoffi, Dr. Francesca Perandin, Dr. Chiara Savio, Dr. Pierantonio Orza of Sacro Cuore Don Calabria Hospital Laboratory (Negrar) for providing the strains. Dr. Teresa Zannino (bioMérieux Specialist) for her precious technical support. Mrs. Paola Menini for administrative and friendly support. Dr. Antonella Sangalli and Dr. Pamela Lorenzi for the use of Uvitec Gel Documentation System. Mr. Michele Governo for his informatic support. My dear friends Cinzia and Craig for revising the English version and my dear friends Laura, Antonella and Maurizio for their friendship and moral support.

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