RIASSUNTO

L'organizzazione mondiale della sanità (WHO) ha identificato l'antibiotico-resistenza come uno dei tre problemi più importanti al mondo per la salute dell'uomo. Il naturale succedersi del fenomeno dell'antibiotico-resistenza, viene coercizzato dall'uso distorto degli antibiotici, provocando quindi un'eccessiva pressione evolutiva sui microrganismi. I microrganismi multi-drug resistant (MDR) rappresentano un'emergenza mondiale, soprattutto nell'ambito delle infezioni nosocomiali, in modo particolare, i reparti di terapia intensiva sono quelli più soggetti a questo tipo di problematica. La produzione di β-lattamasi è il meccanismo cardine della resistenza dei gram-negativi nei confronti degli antibiotici β-lattamici; alcune di queste sono codificate da geni cromosomici, altre da geni plasmidici o integrati all'interno di elementi trasponibili, e possono essere costitutive o inducibili. Nel 1985 dopo l'introduzione sul mercato di imipenem, i carbapenemi, nuova classe di beta-lattamici denominati "salva-vita", vennero considerati come farmaci universalmente attivi contro gli Enterobatteri. Questi antibiotici erano capaci di coniugare un'eccezionale attività antibatterica intrinseca con una grande stabilità nei confronti delle β-lattamasi, incluse le ESBLs; divennero così il trattamento d'elezione per le infezioni provocate da ceppi ESBL-produttori. *Klebsiella pneumoniae* carbapenemasi (KPC) è una carbapenemasi a serina codificata dal gene plasmidico bla_{KPC} (Trasposone Tn4401, Tn3-type), appartenente alla classe A di Amber. Il primo isolato *K. pneumoniae* produttore di KPC è stata identificata nel 1996 in North Carolina (USA). Da allora sono state identificate diverse varianti di KPC, in grado di

idrolizzare tutti i β-lattamici, comprese penicilline e cefalosporine. Il trasposone che porta questo gene è un elemento genetico capace di inserirsi all'interno di diversi plasmidi di altri Gram-negativi; questi plasmidi che portano il gene *bla_{KPC}* sono spesso associati a determinanti di resistenza anche per altri antibiotici. La diffusione di *K. pneumoniae* KPC-produttore (Kp-KPC) è associata ad un clone internazionale multi-resistente *K. pneumoniae* ST258 con sensibilità osservata solo a colistina, tigeciclina e gentamicina. Recenti studi hanno descritto la diffusione di diversi cloni di Kp-KPC e che presentano altri pattern di resistenza come quella agli aminoglicosidi, nonché la diffusione sempre più frequente di questo gene tra le diverse specie di Enterobacteriaceae e non. Lo studio condotto in questi tre anni di dottorato in "Ricerca Multidisciplinare Avanzata nei Trapianti" ha avuto come obbiettivo quello di monitorare la diffusione e l'evoluzione dei meccanismi di resistenza di Kp-KPC isolata da pazienti ricoverati nei reparti di Rianimazione e Terapia Intensiva (ICU) degli ospedali Cannizzaro, Policlinico e Vittorio Emanuele di Catania. Lo studio è iniziato con la caratterizzazione di isolati clinici Kp-KPC ST258 colistino-resistenti ed è proseguito con ceppi di

K. pneumoniae ST101 produttori della variante KPC-2 che possiedono un altro gene di resistenza molto importante quale *armA,* che conferisce alti livelli di resistenza agli aminoglucosidi utilizzati spesso nel trattamento di infezioni sostenuti da questi microrganismi MDR. I ceppi sono stati saggiati utilizzando associazioni antibiotiche quali il "doppio carbapenemico" che ha ottenuto un buon risultato sia *in vitro* che *in vivo*. Le prove *in vitro* sono state valutate con le curve di battericidia che, pur rappresentando il "gold standard" nelle metodiche di associazione, sono

lunghe e laboriose. Pertanto un altro step importante del nostro studio è stato quello di mettere appunto una metodica più rapida che permettesse di associare due o più antibiotici in maniera più semplice e rapida quale: Multiple-Combination Bactericidal Test (MCBT) che è stato applicato, fin'ora, solo per *Burkholderia cepacia* e *Pseudomonas aeruginosa* isolati da pazienti con fibrosi cistica.

L'evoluzione della diffusione di KPC ha portato alla identificazione di questo gene in ST e specie diversi. Ciò è dovuto alla trasponibilità del gene *bla*$_{KPC}$ che è stato identificato anche in *E.coli* isolati da pazienti precedentemente colonizzati da Kp-KPC e ricoverati presso il Mediterranean Institute for Transplantation and Advanced Specialized Therapies (ISMETT).

Un altro problema cruciale affrontato nel periodo di dottorato è stata la difficoltà di interpretazione degli antibiogrammi che spesso insorgono quando si uniscono caratteristiche "difficili" legate sia al ceppo in esame (es. mucosità) che all'antibiotico saggiato (diffusibilità in agar di tigeciclina e colistina). Lo studio è stato effettuato su ceppi di Kp-KPC con valori di MIC borderline per tigeciclina e colistina isolati da infezioni gravi in pazienti ricoverati presso la ICU dell'ospedale Cannizzaro.

I risultati ottenuti dimostrano la continua e preoccupante diffusione di Gram negativi MDR per i quali sono necessarie misure atte a contrastare quanto più rapidamente l'insorgere di infezioni da batteri MDR, soprattutto tra pazienti immunocompromessi, quali i trapiantati o i ricoverati presso le ICU. Una buona "arma" per limitare ciò potrebbe essere rappresentata dal controllo delle colonizzazioni nei pazienti a rischio, mediante la "Decontaminazione Selettiva del Tratto Digerente"

al fine di ridurre la trasmissione del gene *bla*$_{KPC}$ e le conseguenti infezioni in pazienti colonizzati e non.

ABSTRACT

The World Health Organization (WHO) has identified antimicrobial resistance as one of the three most important issues in the world for human health. The natural evolution of antibiotic resistance is coerced by misuse of antibiotics, thus causing excessive evolutionary pressure on microorganisms. The microorganisms multi-drug resistant (MDR) represents a 'global emergency, especially in nosocomial infections, in particular, the intensive care units are the most susceptible to this type of problem .The production of β-lactamases is the main mechanism of resistance in gram-negative bacteria against β-lactam antibiotics; some of these are encoded by chromosomal genes, other genes from plasmid or integrated into transposable elements, and can be constitutive or inducible. In 1985, after the introduction of imipenem, carbapenem, new class of beta-lactam antibiotics called "life-saving" drugs were considered to be universally active against Enterobacteriaceae. These antibiotics were able to combine exceptional intrinsic antibacterial activity with high stability against β-lactamases, including ESBLs; thus became the treatment of choice for infections caused by ESBL-producing strains. Klebsiella pneumoniae carbapenemase (KPC) is a serine carbapenemase gene encoded by the plasmid blaKPC (Transposon Tn4401, Tn3-type), belonging to the class A of Amber. The first K. pneumoniae KPC producer, was identified in North Carolina, USA, in 1996 this was resistant to all β-lactams, including carbapenems. Over the years, were identified several variants of KPC, capable of hydrolyzing all β-lactam antibiotics, including penicillins and cephalosporins. The transposon that carries this gene is a genetic element capable of fitting

within different plasmids of other Gram-negative; these plasmids that carry the gene blaKPC are often associated with determinants of resistance to other antibiotics. The spread of KPC-producing K. pneumoniae (KPC-Kp) is associated with an international multi-resistant clone K. pneumoniae ST258 with susceptibility observed only to colistin, tigecycline, and gentamicin. In addition, recent studies have described the spread of different clones of K. pneumoniae carrying the blaKPC and other patterns that have resistance to aminoglycosides like that, as well as the increasingly frequent dissemination of this gene among different species of Enterobacteriaceae and non. The study conducted in these three years of PhD in "Advanced Multidisciplinary Research in Transplantation" has had as objective to monitor the spread and evolution of resistance mechanisms of KPC-Kp isolated from patients hospitalized in the ICU of hospitals Cannizzaro Hospital and Vittorio Emanuele of Catania.

The study has seen the emergence of strains colistin resistant between the ST258, the success of in vivo combination ("double carbapenem"), serious infections by KPC-Kp, whose effectiveness has also been demonstrated in vitro by Time Killing Curves. The latter represents the "gold standard" in the methods of the antibiotic combination, but is very laborious, time for another step of our study was to just put a faster method that would allow to combine two or more antibiotics in a more simple: Multiple-Combination Bactericidal Test (MCBT), so far used only for Burkholderia cepacia and Psedumonas aeruginosa, simple and rapid method that allows combining three antibiotics. The evolution of the spread of KPC has led to the discovery of this gene among different

from ST ST258 clone classic sign of ease passage of intra-species. As in our case the isolation of Klebsiella pneumoniae ST101 that in addition to having just acquired KPC-2 carried a resistance gene armA, which confers high-level resistance to aminoglycosides that many times, in cases of infection with KPC-Kp, prove to be a therapeutic option. The transposability of the blaKPC gene led to another study in collaboration with the Mediterranean Institute for Transplantation and Advanced Specialized Therapies (ISMETT) in which they were isolated 5 KPC-producing Escherichia coli isolated from patients previously colonized with KPC-Kp. Our study showed that there was a "transfer" in vivo between the two species. Another problem faced in these years of PhD has been the difficulty of interpretation of antibiograms, which often arise when you put together the characteristics of "difficult" of the strain in question (eg. mucus) and the molecule in question (agar in diffusibility of Colistin). A study conducted by KPC-Kp isolates from severe infections, patients admitted to the ICU of the hospital Cannizaro, it emerged that must particular attention at borderline results for tigecycline and colistin when using the gradient-test.

All of this highlights the need to fight as soon as the emergence of MDR bacterial infections, especially among immunocompromised patients, such as transplant recipients or admitted to the ICU. A good "weapon" of prevention could be for example the control of colonization of patients at risk by the "Selective Decontamination of the Digestive Tract," by analyzing the feces, a project to which it moves our study.

INTRODUCTION

Klebsiella pneumoniae carbapenemase (KPC)-producing bacteria are a group of emerging highlydrug-resistant Gram-negative bacilli causing infections associated with significant morbidity and mortality. Once confined to outbreaks in the north eastern United States (US), they have spread throughout the US and most of the world. KPCs are an important mechanism of resistance for an increasingly wide range of Gram-negative bacteria and are no longer limited to *K pneumoniae*. KPC-producing bacteria are often misidentified by routine microbiological susceptibility testing and incorrectly reported as sensitive to carbapenems; however, resistance to the carbapenem antibiotic ertapenem is common and a better indicator of the presence of KPCs. Carbapenem antibiotics are generally not effective against KPC-producing organisms. The best therapeutic approach to KPC-producing organisms has yet to be defined; however, common treatments based on *in vitro* susceptibility testing are the polymyxins, tigecycline, and less frequently aminoglycoside antibiotics.

In the 1980s, Gram-negative pathogens appeared to have been beaten by oxyimino-cephalosporins, carbapenems, and fluoroquinolones. Yet these pathogens have fought back, aided by their membrane organization, which promotes the exclusion and efflux of antibiotics, and by a remarkable propensity to recruit, transfer, and modify the expression of resistance genes, including those for extended-spectrum β-lactamases (ESBLs), carbapenemases, aminoglycosideblocking

16S rRNA methylases, and even a quinolone-modifying variant of an aminoglycoside-modifying enzyme.

In 1983, the first report of plasmid-mediated beta-lactamases capable of hydrolyzing extended-spectrum cephalosporins was made. They were named extended-spectrum betalactamases (ESBLs) and they have since been described worldwide. The fact that carbapenems are the treatment of choice for serious infections caused by ESBLs, along with an increasing incidence of fluoroquinolone resistance among *Enterobacteriaceae,* has led to an increased reliance on carbapenems in clinical practice.

In 2001, the first KPC-producing *K pneumoniae* isolate was reported in North Carolina. The enzyme (KPC-1), an Ambler class A beta-lactamase, was not the first carbapenemase to be detected in *K.pneumoniae*, as isolates harboring Ambler class B metallo-beta-lactamases capable of hydrolyzing carbapenems had previously been reported in Japan as early as 1994. However, metallo-beta-lactamases are uncommon in the US and the production of KPC enzymes has become the most prevalent mechanism of carbapenem resistance in the US today.

KPCs are encoded by the gene bla_{KPC}, whose potential for inter-species and geographic dissemination is largely explained by its location within a *Tn3*-type transposon, *Tn4401*. This transposon is a genetic element which is capable of inserting into diverse plasmids of Gram-negative bacteria. Plasmids carrying bla_{KPC} are often also associated with resistance determinants for other antibiotics. Although *K.pneumoniae* remains the most prevalent bacterial species carrying KPCs, the enzyme has been identified in several other Gram-negative bacilli.

A closer look at the molecular epidemiology of KPC-producing bacteria has revealed that a few fit lineages have been responsible for dissemination of the *bla*$_{KPC}$ gene. An examination of all KPC-producing *K pneumoniae* isolates sent to the CDC between the years 1996-2008 from 18 states as well as Israel and India revealed that a single dominant strain, multilocus sequence type 258 (ST258), accounted for nearly 70% of the isolates in the CDC database as well as an isolate from an Israeli outbreak. Although seven variants of the enzyme have been reported (KPC 2-8), most ST258 strains produced KPC-3 and most non-ST258 strains produced KPC-2 (KPC-2 enzyme is genetically identical to KPC-1) **[1, 2]**

EUROPE

In a summary from a meeting on carbapenem-non-susceptible Enterobacteriaceae published in 2010, European countries were classified into a numerical staging system according to the epidemiological situation. This scale includes: 0, no cases reported; 1, sporadic occurrence; 2a, single-hospital outbreaks; 2b, sporadic hospital outbreaks; 3, regional outbreaks; 4, interregional spread; and 5, endemic situation. At the time of that summary, July 2010, two countries each were graded 5 (Greece and Israel) and 4 (Italy and Poland), three were graded 3 (France, Germany, and Hungary), three were graded 2a (Belgium, Spain, and England/Wales), and five were graded 2b (Cyprus, The Netherlands, Norway, Scotland, and Sweden). Other European countries were graded 1 or 0. The situation changed during 2011, as the number of reports from different European countries increased dramatically. According to the EARS-Netsurveillance study (http://ecdc.europa.eu/en/activities/surveillance/EARSNet/database/Pages/database.aspx), antimicrobial resistance in northern European countries is lower than in southern European countries. This is true for ESBL-producing organisms as well as for methicillin-resistant *Staphylococcus aureus*, but only partially true for carbapenemase-producing *E. coli and K. pneumoniae*. Importation of carbapenemases from specific areas in Europe as a consequence of cross-border transfer of patients, travel, medical tourism and refugees might play also an important role in this outburst. This has been clearly demonstrated for carbapenemases of different molecular classes.

ITALY

A different evolution has been observed with *K.pneumoniae* producing KPC-type enzymes. Reported for the first time in late 2008, where the likely source was a medical trainee from Israel, KPC-producing *K.pneumoniae* has since undergone rapid and extensive dissemination in this country, with several reports of hospital outbreaks. The abrupt and remarkable increase in carbapenem resistance rates in *K.pneumoniae* recently reported by the EARSNet surveillance system for Italy (from 1% to 2% during the period 2006–2009 to 15% in 2010,from 2009 to 2012, carbapenem-resistant *K.pneumoniae* diffusion rose from 2.2 % to 19.4%, with a prevalence of KPC enzymes) (http://ecdc.europa.eu/en/ activities/surveillance/EARSNet/database/Pages/database.aspx) appears to be mostly related to the countrywide dissemination of KPC-producing *K. pneumoniae*, as shown by results from a recent countrywide survey. As in Greece, multifocal emergence of colistin resistant isolates of KPC-producing *K.pneumoniae* has been observed, which is a matter of major concern, as colistin is among the few drugs that retain activity against these organisms, and is a cornerstone of antimicrobial chemotherapy for infections caused by these organisms.

In Italy, the first KPC-positive *K.pneumoniae* was isolated in Florence in 2008 from an inpatient with a complicated intraabdominal infection. The isolate had KPC-3 enzyme, with the corresponding gene located on transposon *Tn*4401, which has been described in Israeli ST258 isolates. A second report described two KPC-2- positive *K.pneumoniae* obtained in 2009 from patients admitted to a teaching hospital in Rome. Neither

patient had recently travelled to KPC endemic countries. Active surveillance was done in two hospitals in Padua from 2009 to 2011, and almost 200 cases were identified. The initial epidemiological pattern entailed dissemination of KPC-3-positive *K pneumoniae* ST258 and KPC-2-positive *K pneumoniae* ST14. Subsequently, the former lineage prevailed and spread from ICUs to medical, surgical, and long-term wards. Simultaneously, seven clonally related KPC-3-positive *K.pneumoniae* ST258 isolates were identified from wound cultures of different patients in a surgical ICU in Verona and, more worryingly, horizontal transmission of colistin-resistant KPC-3-positive *K.pneumoniae* was described in different wards of an acute general hospital in Palermo in 2011. KPC-positive *K.pneumoniae* have spread rapidly and extensively in Italy, with a sharp increase reported by the EARS-Net surveillance system71 for bacteraemia isolates, from 1–2% carbapenem resistance in 2006–09 to 30% in 2011, and by the Micronet surveillance network,72 from 2% in 2009 to 19% in 2012. Infection control interventions at the national level are scarce, with only a few reports of local containment. **[3].**

In 2009–2010 in Italy, a few reports outlined the isolation of two KPC variants, i.e. multidrug-resistant (MDR) KPC-2 and KPC-3, belonging to ST258, and susceptible only to colistin, tigecycline and gentamicin. In 2008, the only clone KPC-Kp circulating in Italy was the ST258 while in 2009 different sequence type (both belonging to the same clonal complex or not) KPC-2 and KPC-3 producers were isolated. To date, the clones circulating are the followings: ST101 carrying the bla_{KPC-2} **[4,5,6]; ** ST512 (a single locus variant of ST258) which carries the bla_{KPC-3} **[7];** KPC-3 ST 307 producer **[8]**, and more recently the ST147 producer of both

KPC-2 and KPC-3 and 395 KPC-3 producing **[4,9]**. Possible explanations for this rapid dissemination of *bla_{KPC}* genes reside in their localization on MGE (plasmids) related to some well defined clones, above all ST258, but other minor clone are rising. Plasmids can be found to be of different size, nature and structures.

In most cases, these plasmids are self-transferable at least to *Escherichia coli*. Studies of the genetic structure surrounding *bla_{KPC}* genes have identified a *Tn*3-based transposon, *Tn4401*. This transposon was identified in isolates from different geographical origins, and of different sequence types (ST), in Enterobacteriaceae and in *P.aeruginosa*. In all cases, *Tn4401* was inserted at different loci and on plasmids varying in size and incompatibility group**.**

Aim of the study

The purpose of this study is to monitor and characterize *Enterobacteriaceae* resistant to carbapenems in order to identify the KPC or other mechanisms of resistance. During the period of study, all isolates were characterized to highlight the possible clonal relationship and thus establish the epidemiological profile of the circulation intra / inter-hospitals of these epidemic clones. Plasmid or chromosomal localization of the resistance gene were determined.

The objectives are detailed as follow:

1. To monitor carbapenem-resistant *Enterobacteriacae.*
2. To determine antibiotic activity through combination of different drugs against *Enterobacteriaceae* multi-drug resistant: Tigecycline + Colistin; Tigecycline + Gentamicin; Tigecycline + Fosfomicin; Tigecycline + piperacillin-tazobactam, double Carbapenem with or without Colistin.
3. To characterize different mechanism of resistance to carbapenems.
4. To trace their clonal diffusion by PFGE and MLST.
5. To study genetic elements responsible for this rapid dissemination of resistance.

<u>Material and Methods</u>

<u>Study design:</u> the study began in late 2010 and included all Gram-negative carbapenem-resistant (CR) isolated from serious infections and colonized patients in intensive care units of hospitals Policlinico, Vittorio Emanuele and Cannizzaro of Catania. All strains will be reconfirmed for their phenotypic identification and resistance profile.

<u>PHENOTYPIC METHODS</u>

<u>Isolation and Identification:</u>

After proper treatment of the samples from patients with serious infections microorganisms were isolated on McConkey Agar and identificati systems with Gallery Api 20E (Biomerieux).

Antimicrobial agents and minimum inhibitory concentration (MIC) determination

MIC determinations of the following antibiotics were performed by gradient test (Liofilchem, Roseto degli Abruzzi, Italy): meropenem (MEM); ertapenem (ETP); piperacillin/tazobactam (TZP); amoxicillin/clavulanic acid (AMC); ceftazidime (CAZ); cefotaxime (CTX); cefepime (FEP); amikacin (AK); gentamicin (CN); ciprofloxacin (CIP); trimethoprim/sulfamethoxazole (SXT); colistin (CS) and tigecycline (TG). Susceptibility and resistance categories were assigned according to European Committee on Antimicrobial Susceptibility

Testing (EUCAST) breakpoints 2014. *Escherichia coli* ATCC 25922 was used as the quality control strain.

Phenothypic test for **carbapenemase production**

Phenotypic screening, for the presence of carbapenemases or overexpression of AmpC in combination with porin loss in *K. pneumoniae* strains, was performed by a commercial synergy test (Rosco Diagnostica, Taastrup, Denmark) as following:

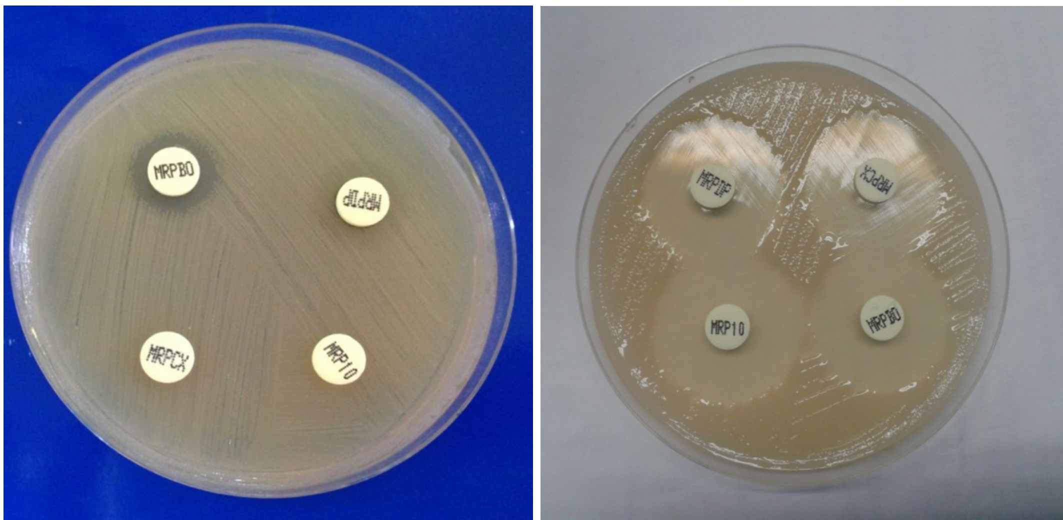
- Meropenem 10μg
- Meropenem 10μg + boronic acid
- Meropenem 10μg + dipicolinic acid

The test strains were adjusted to a McFarland 0.5 standard and inoculated on the surface of a Mueller-Hinton agar plates.

Disks containing meropenem and chelators were placed on the surface. After incubation overnight at 35°C, an increase of inhibition of ≥ 5 mm with the meropenem in the presence of boronic acid or dipicolinic acid is interpreted as indicating carbapenemase activity (Table 1).

In particular the positive test of meropenem with boronic acid (BOR) suggests the presence of KPC production while the enhancement with dipicolinic acid (DPA) indicates the MBL production (Fig.1).

Fig.1 phenothypic test carbapenemase production



Positive test **Negative test**

		Meropenem + Boronic MR+BO	Meropenem + DPA MR+DP	Meropenem + Cloxacillin MR+CX
AmpC + porin loss	Meropenem 10 µg MRP10	≥ 5mm AND	< 5mm	≥ 5mm
KPC	Meropenem 10 µg MRP10	≥ 5mm	< 5mm	< 5mm
MβL	Meropenem 10 µg MRP10	< 5mm	≥ 5mm	< 5mm

Table 1: Interpretation of the test for synergy

Synergy testing by time-kill assays

Microorganisms was grown on Mueller-Hinton broth for 4 h (log phase of growth) and further diluted in 20 ml of the same medium to yield a concentration of approximately 5×10^5 CFU/ml. Wells containing antibiotic at concentrations corresponding to the MIC and two and four times the MIC were tested for each strain. Aliquots (0.1 ml of broth) were removed from each well and serial dilutions were plated onto Mueller Hinton-agar plates after 0, 2, 4, 8 and 24 h of incubation. Colony counts were performed after 24 h of incubation at 36° C. Bactericidal activity was defined as a ≥ 3 $\log_{10}$ reduction compared with the initial inoculums.

Multiple-Combination Bactericidal Test (MCBT)

MCBTs were carried out in four 96 well, round bottomed microtiter plates (Nunc Inc., Roskilde, Denmark). Antibiotic solutions were prepared for each test from stock solutions stored at -80 °C. The working antibiotic solutions were prepared in Mueller Hinton II Cation Adjusted Broth (MHB II broth; Becton Dickenson Microbiology Systems, Cockeysville, MD) at 10 times the required concentrations. For each MCT, antibiotic working solutions were made fresh on the day of inoculation. One, two, or three antibiotics were added, each in 10μl volumes to the appropriate wells. The necessary volume of MHB II was then added to the wells containing one or two antibiotics so that all the wells had a volume of 30μl before the addition of organism. The organism inoculum consisted of 70 μl of a 100-fold dilution of a 0.5 McFarland turbidity standard prepared from a culture in Tryptone Soya Broth (Oxoid Laboratories, Basingstoke, UK) in the growth phase. This gave a final inoculum concentration in each well of 5×10^5 CFU/ml. Growth and sterility plates (no antibiotics and no organism inoculum, respectively) were run with each MCT procedure as bacteriologic controls. Plates were incubated at 35 °C for 24 h.. The contents of non turbid wells at 24 h were subcultured by streaking 10 μl of suspension onto 5% Columbia sheep blood agar plates (PML Microbiologicals, Mississauga, ON, Canada), which was incubated for 24 h
at 35 °C and examined for 99.9% kill the next day.

The following antibiotics were used in double and triple combinations for MCBT and the antibiotic concentrations were chosen on the basis of peak serum levels after standard intravenous administration: meropenem (120 mg/l) plus ertapenem (70 mg/l) and/or with colistin (20 mg/L); rifampin (6 mg/l) plus tigecycline (0.9 mg/l) and/or with colistin (20 mg/L); and rifampin (6mg/L) plus colistin (20 mg/l).

MOLECULAR METHODS

PCR detection of resistence genes

The strains will be subjected to molecular characterization by previous extraction of genomic DNA according to the protocol **[10]** and subsequent **PCR** to investigate the nature of the genes that give resistance to carbapenems : (KPC, IMP, VIM and OXA). For the amplification of the gene bla_{KPC} were used two pairs of primers **[11, 12]** of this enzyme in fact, there are several variants that differ in few amino acid substitutions. Extended spectrum β-lactamases: (ESBLs; TEM, SHV and CTX-M) aminoglycoside-modifying enzymes: (AAC, APH, AAD and 16S methylase) were performed using previously described primers. To better characterize the localization of KPC-2, which was found as part of the 10 kb *Tn3*-like element *Tn4401*, PCR assays with specific primers for Tn4401 were performed .Published papers have reported that *Tn4401* has been found on *IncN* and *IncFIIk* plasmids (pKpQIL-IT, S9, S12, S15, pKPN101-IT), therefore for the detection of these plasmids we used the following primers:

S9-F, 5′-GCATTGACCTTGGCATCTTC-3′;

S9-R, 5′-GTGATTTACACCAC CACCTCATCA-3′;

S12-F, 5′-CGGACGGTTGATCAGAATCGGATG-3′;

S12-R, 5′ATTGCTGCTGTAGGGGCTGTCATTCT-3′;

S15-F, 5′-GGGGAT CGGTTTTCGCCAGCA-3′;

S15-R, 5′-GCTTTACCGAGGGAGAATGGCTA CTG-3′;

pSLMT-F, 5′-GCATTGACCTTGGCATCTTC-3′;

pSLMT-R, 5′-CTAATAAACTGGTGCTCGGACAGA-3′;

pNYC-F, 5′-GCATCAAACGGAAGCAAAAG-3′;

pNYC-R, 5′-CTTAGCAAATGTGGTGAACG-3′;

pKpQIL-IT-F, 5′-GGTTATTGGGTGAGGTAAGCATTAGGCG-3′;

pKpQIL-IT-R, 5′-GAGTGAGCGAGGAAGCACCAGGG-3′

tnpA up 5’CACCTACACCACGACGAACC 3’

tnpA dw 5’GAAGATGCCAAGGTCAATGC 3’

pLYC up 5’ CTTAGCAAATGTGGTGAACG 3’

tnpA dw 5’ GCGACCGGTCAGTTCCTTCT 3’

designed on the basis of published sequences and specific for each plasmid (GenBank accession numbers FJ223607.1, FJ223605.1, FJ223606.1, HQ589350.1, EU176011.1 and GU595196.1, respectively). **[6]**

Specific primers, using specially designed programs in bioinformatics, can guarantee a correct molecular identification will be made for further research of possible resistance genes. Sequencing of positive PCR products and partial sequencing of the genetic structure were performed using BigDye 3.1 technology (Applied Biosystems, Foster City, CA, USA). Sequence analysis and alignments were performed using SeqManII software (DNAStar, Madison, WI, USA) and compared with sequences deposited in the GenBank database (www.ncbi.nlm.nih.gov)

Molecular typing: PFGE and MLST

PFGE

The strains underwent molecular typing by means of PFGE. The acronym PFGE (Pulsed Field Gel Electroforesis) includes all the techniques of separation of DNA fragments, obtained by treatment with an enzyme restriction, *Klebsiella pneumoniae Xba*I, that use an electric field whose direction with respect to the solid matrix in which migration occurs is periodically varied. Conventional gel and agarose electrophoresis use a static electric field and are able to separate DNA fragments having a maximum size of 20-50 kb. PFGE can separate fragments up to 10 Mb using the large fragments to re-Orientate itself at

each variation of the electric field, the slowness that is proportional to the size of the fragment. In PFGE 24 electrodes are arranged in a hexagon in the electrophoretic chamber so as to generate an electric field with an angle of 120 degrees in all parts of the gel. The fragments were separated using the CHEF DRII system (Contour Clamped Homogeneous Electric Field) (Bio-Rad Laboratories, Hercules, CA). In this way very clear bands are obtained and migration lanes are straight because in all parts of the gel the DNA is under the same conditions. With the PFGE technique "DNA fingerprinting" can be carried out and represents a simple method to compare DNA and includes the fragmentation of the DNA by means of restriction endonuclease and the separation of these fragments to measure the number and size. This obtains a band profile, that looks like a barcode, that can be used as a digital fingerprint to recognise the bacterium. The assignment of cluster membership was based on the criteria of

Tenover **[13]** reported in Table 2.

CATEGORY	NUMBER OF VARIOUS FRAGMENTS	INTERPRETATION EPIDEMIOLOGICAL
indistinguishable	0	The isolates belong to the same cluster
closely related	2-3	The isolates probably belong to the same cluster
possibly related	4-6	The isolates possibly belong to the same cluster
not related	≥ 7	The isolates did not belong to the same cluster

Table: 2 Criteria for the interpretation of the profiles according to Tenover [13].

Multi Locus Sequence Type

MLST is a technique for the typing of multiple loci. The procedure characterizes isolates of bacterial species using the DNA sequences of internal fragments of multiple seven housekeeping genes. Approximately 450-500 bp internal fragments of each gene are used, as these can be accurately sequenced on both strands using an automated DNA sequencer.

The protocol provides for the PCR amplification, sequencing of the PCR products, and reading sequence is compared with the sequences and allelic profiles available on the website of the Institute Pasteur (www.pasteur.fr/mlst). For each housekeeping gene, the different sequences present within a bacterial species are assigned as distinct

alleles and, for each isolate, the alleles at each of the loci define the allelic profile or sequence type (ST).

Plasmids analysis

Plasmid DNA was isolated from the clinical strains using a Qiagen kit (Valencia, CA) according to the manufacturer's instructions. Plasmids were separated on 0.5% agarose gels prepared with 0.04M Tris-acetate-EDTA (pH 8.4) by electrophoresis at 90 V for 18 h at 4°C. The supercoiled plasmid size markers (ranging from 165 kb to 8 kb) used in agarose gel electrophoresis experiments included the BAC-Tracker supercoiled DNA ladder (Epicentre, Madison, WI). DNA-DNA hybridizations were performed as described by Sambrook et al. **[14].** with a Southern transfer of an agarose gel containing total plasmid DNA extracted by the Kieser extraction method **[15].** Southern blot analysis was performed as previously described **[16].**

Southern ibridization was performed with digoxigenin-labeled specific genes fragments. Labeling of the probe and signal detection were carried out by using the ECL nonradioactive labeling and detection kit, according to the manufacturer's instructions (Amersham Biosciences, Orsay, France)

RESULTS AND CONCLUSIONS

Between 19 August and 27 October 2010 in two different hospitals in Catania (University and Vittorio Emanuele hospitals), a hospital outbreak occurred, caused by eight colistin-resistant and carbapenem-resistant *Klebsiella pneumoniae* isolates from eight patients. The first KPC-producing *K.pneumoniae* isolate was obtained from abdominal drainage fluid of a patient aged 84 years admitted to the intensive-care unit (ICU) of the University Hospital and coming from another Sicilian hospital. The rapid spread of *K. pneumoniae* with this MDR resistance pattern in such a short period caused concern, and an investigation was activated to determine the possible source of this outbreak, to establish the increasing risk of transmission, and to start the infection containment procedure.

These isolates were resistant to almost all antibiotics, including colistin (MIC 64 mg/L) and carbapenems. All isolates were susceptible only to tigecycline (MIC 1 mg/l) and gentamicin (MIC 2 mg/L); see Table 3. Analysis of β-lactamase genes by PCR and sequencing revealed the presence of bla_{KPC-3}, bla_{SHV-11}, bla_{TEM-1} and bla_{OXA-9} in all isolates, whereas genes encoding other enzymes (CTX type and VIM type) were not detected. All *K. pneumoniae* isolates, genotyped after *Xba*I digestion by pulsed-field gel electrophoresis, belonged to the same clone, as they were indistinguishable from each other (100% identity) according to the criteria described previously by Tenover et al **[13]**, and with the multilocus sequence typing (MLST) scheme, according to the protocol described on the *K.pneumoniae* MLST website (http:// www.pasteur.fr/recherche/genopole/PF8/mlst/*Kpneumoniae*.html), all

isolates were attributed to sequence type (ST)258. This study reports, for the first time, an outbreak of KPC-3 colistin-resistant *K. pneumoniae* infection in Italy.

Our isolates were colistin-resistant KPC-3 ST258 *K. pneumoniae*; colistin-resistance is still uncommon among KPC producers, and has been attributed mainly to the modification of lipid A of the outer membrane and the presence of an efflux pump **[17]**

					MIC (mg/L)											
Patients	Date	Hospital	Ward	Specimens	IPM	MEM	DOR	ETP	CEF	CAZ	CTX	TZP	TG	CT	CIP	GM
1	19 August 2010	University	ICU	Abdominal drainage	32	64	64	>128	128	>64	>64	>512	1	16	128	2
2	31 August 2010	University	Surgery	CVC	64	64	64	>128	>128	>64	>64	>512	1	32	128	2
3	10 September 2010	University	ICU	Bloodstream	64	128	64	128	>128	>64	>64	>512	1	16	128	2
4	19 September 2010	University	ICU	Bronchial aspirate	64	64	32	>128	>128	>64	>64	>512	1	32	128	2
5	20 September 2010	University	Internal Medicine	Urine	64	64	64	>128	>128	>64	>64	>512	1	8	256	2
6	23 September 2010	University	Transplant	Sputum	32	64	128	128	128	>64	>64	>512	1	64	128	2
7	26 September 2010	University	Paediatric Haematology	Bloodstream	64	64	256	>128	128	>64	>64	>512	1	32	128	2
8	19 October 2010	VE	Nephrology	Bloodstream	128	512	64	512	512	>512	256	>512	1	8	256	2
3[a]	20 October 2010	University	ICU	Pharyngeal swab	32	64	64	128	128	>64	>64	>512	1	32	128	2
7[a]	26 October 2010	University	Paediatric Haematology	Rectal swab	>512	>512	64	>512	256	>512	256	>512	1	8	128	2
4[a]	27 October 2010	University	ICU	Rectal swab	64	512	64	512	512	>512	256	>512	1	16	128	2

IPM, imipenem; MEM, meropenem; DOR, doripenem; ETP, ertapenem; CEF, cefepime; CAZ, ceftazidime; CTX, cefotaxime; TZP, piperacillin–tazobactam; TG, tigecycline; CT, colistin; CIP, ciprofloxacin; GM, gentamicin; VE, Vittorio Emanuele; CVC, central venous catheter; ICU, intensive-care unit.
[a]Colonization.

Table 3: Clinical characteristics of patients and antibiotic susceptibility of KPC-3-producing *K. pneumoniae*

The most important mechanism involves modifications of the bacterial outer membrane, mainly through the alteration of the LPS moiety. Another mechanism includes the development of an efflux pump/potassium system. Although no enzymatic mechanisms of resistance has been reported so far, strains of *B. polymyxa* are known to produce colistinase .

The modification of the LPS occurs with the addition of 4-amino- 4-deoxy-l-arabinose (LAra4N) to a phosphate group in lipid A. This addition causes an absolute increase in lipid A charge, thus lowering the affinity of positively charged polymyxins. The biosynthesis of LAra4N depends on the genes of polymyxin resistance operon, formerly known as *pmr*, which has been renamed as *arn*. This operon includes pmrHFIJKLM genes. First, UDP-glucose is dehydrogenated to UDP glucuronic acid (UDP-GlcA) by PmrE dehydrogenase. UDP-GlcA is subsequently decarboxylated and transaminated by ArnA(PmrI) and ArnB(PmrH) to UDP-AraN. UDP-Ara4N is formylated by ArnA to UDP-Ara4FN and transferred to the bacterial inner membrane by ArnC(PmrF). UDPAra4FN is deformylated and transferred from the inner to the outer bacterial membrane by mechanisms that are not fully understood, although ArnE(PmrM) and ArnF(PmrL) may have a role in the transportation of UDP-Ara4FN across the bacterial inner membrane. Finally, ArnT(PmrK) tranfers LAra4N to lipid A.

The biosynthesis of LAra4N is mediated by PmrA/PmrB and PhoP/PhoQ two-component regulatory systems (fig.3)

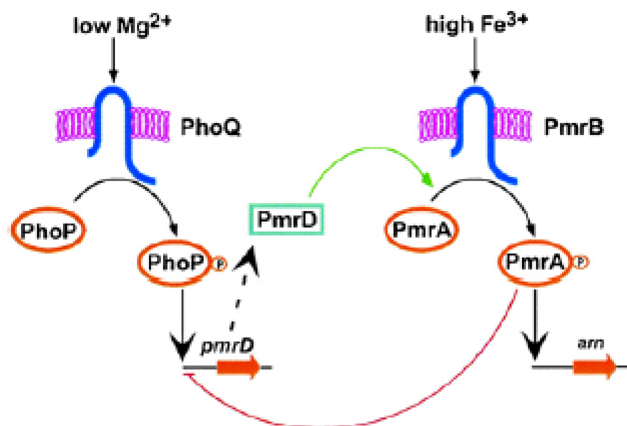


FIG.3 PmrA/PmrB and PhoP/PhoQ two-component regulatory systems

PmrB is a sensor cytoplasmic membrane-bound kinase with a histidine residue in its cytoplasmic domain. It is activated by high concentrations of Fe^{3+} or by low pH. Upon activation, it phosphorylates the aspartate residue of PmrA, which is a regulator protein of *arn* operon. PhoQ is another sensor cytoplasmic membrane-spanning kinase, which is activated by low concentrations of Mg^{2+} or Ca^{2+}. Upon activation, it phosphorylates PhoP regulator, which in turn activates pmrD transcription. PrmD has a protective role by inhibiting PmrA dephosphorylation, thus conferring in the promotion of *arn* transcription. PmrA, in turn, exerts a negative feedback effect by repressing pmrD transcription under conditions that activate PmrA, such us high Fe^{3+} concentrations. These mechanisms, although common in gram-negative bacteria, exhibit interspecies variation. PmrA/PmrB and PhoP/PhoQ systems, as described.

Further modifications of the bacterial outer membrane include the increased production of capsule polysaccharide (CPS) in *K. pneumoniae*. CPS limits the interaction of polymyxins with their target

sites. Thus, up regulation of CPS productions confers to increased polymyxin resistance. Furthermore, increased levels of the outer membrane protein H1 inhibits the action of polymyxins by replacing Mg^{2+} or Ca^{2+} at binding sites on LPS **[18]** . A summary of the resistance mechanisms is presented in table 4.

Resistance mechanism	Pathogen
Modification of lipopolysaccharide moiety of the bacterial outer membrane	*E. coli–S. enterica serotype typhimurium among others*
Efflux pump/potassium system	*Y. pestis*
Increased production of capsule polysaccharide of the bacterial outer membrane	*K. pneumoniae*
Increased levels of the outer membrane protein H1	*P. aeruginosa*
Colistinase production	*B. polymyxa*

Table 4 Summary of polymyxin resistance mechanisms

With rapidly increasing antibiotic-resistance and decline in new antibiotic drug development, the toughest challenge remains to maintain and preserve the efficacy of currently available antibiotics. Therefore, the best rational approach to fight these infections is to 'hit early and hit hard' and kill drug-susceptible bacteria before they become resistant. The preferred approach is to deploy two antibiotics that produce a stronger effect in combination than if either drug were used alone. Various society guidelines, in particular indications, also justify and recommend the use of combination of antimicrobial therapy. Combination therapies have distinct advantage over monotherapy in terms of broad coverage, synergistic effect and prevention of emergence of drug resistance. Recently, treatments based on therapies for severe infections caused by carbapenemase producing *Klebsiella pneumoniae*, with combinations of colistin, tigecycline, meropenem, fosfomycin, and/or aminoglycoside have been suggested. However, the emergence of strains

resistant to almost all of the antibiotics listed above has further complicated the possibility of treating these infections.

In September 2012, a patient admitted to the Neurosurgical Intensive Care Unit of Azienda Policlinico Umberto I in Rome, developed a bacteremia due to *Klebsiella pneumoniae* MDR. A subsequent laboratory

study, in which both microdilution broth (BMD) analysis and an Etest were performed, confirmed that these isolates (4 isolates collected since day 47 of hospitalization from 3 blood cultures and 1 endotracheal aspirate) were resistant to ertapenem, meropenem, imipenem, doripenem, amikacin, colistin, and fosfomycin but evidenced that they were susceptible to tigecycline with both methods, confirming the overestimation of the MIC for this drug if performed with the Vitek2 system (table 5). The same clinical isolates, genotyped by PFGE and MLST, belonged to the same clone and were sequence type (ST) 512. PCR detection showed that all isolates harbored the $bla_{KPC\text{-}3}$ gene. After several inefficient treatments, the patient underwent therapy with ertapenem and doripenem, and this has shown efficacy. The activity of the carbapenem combination was also *in vitro* confirmed by killing curves.

The combination of ertapenem plus doripenem at 1X MIC was strongly synergic after 4 h, achieving 99.9% killing, as was ertapenem plus meropenem, maintaining this behaviour until 24 h. The value for ertapenem alone showed an increase of 1 log after 24 h, while those for doripenem and meropenem alone showed an increase of 3 log (Fig 4).

Our case report on the result obtained *in vitro* and *in vivo* with a KPC-3-producing *K. pneumoniae* seems to corroborate experiments performed by Bulik et al. **[19]**, who recently postulated that the enhanced efficacy of this dual-carbapenem therapy against KPC-2-producing *K. pneumoniae* may be related to the KPC enzyme's preferential affinity for ertapenem. **[7]**

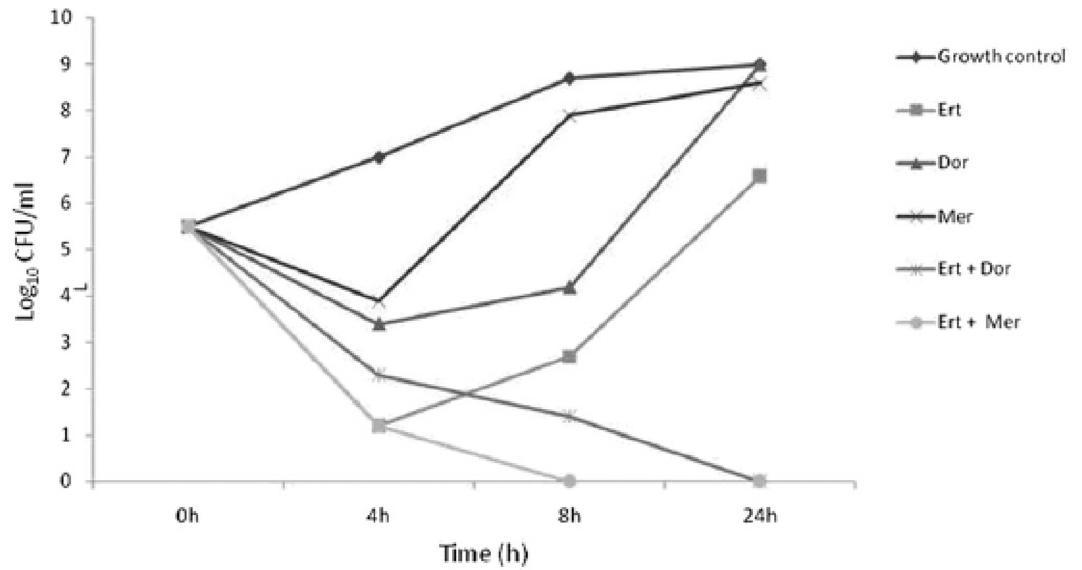


FIG4 Time-kill curves for *K. pneumoniae* with ertapenem (Ert) at 1 X MIC (512 mg/liter), doripenem (Dor) at 1 X MIC (64 mg/liter), meropenem (Mer) at 1 X MIC (64 mg/liter), and the combinations of ertapenem plus doripenem at 1 X MIC and ertapenem plus meropenem at 1 X MIC.

Isolate no. (day of hospitalization)	Specimen	Antibiotic	MIC (mg/liter)[b]		
			Vitek 2	Etest	BMD
1 (48)	Endotracheal aspirate	IPM	≥16	>32	32
		MEM	≥16	>32	64
		ERTA	≥8	>32	256
		DOR	n.t	n.t	64
		AK	≥64	48	32
		COL	≥16	2	32
		FOSFO	≥128	32	64
		TGC	≥8	0.38	0.5
2 (48)	Blood	IPM	≥16	>32	32
		MEM	≥16	>32	64
		ERTA	≥8	>32	512
		DOR	n.t	n.t	32
		AK	≥64	48	32
		COL	≥16	4	16
		FOSFO	≥128	64	128
		TGC	≥8	1	0.5
3 (53)	Blood	IPM	≥16	>32	32
		MEM	≥16	>32	64
		ERTA	≥8	>32	64
		DOR	n.t	n.t	64
		AK	≥64	48	32
		COL	≥16	6	16
		FOSFO	≥128	32	64
		TGC	≥8	0.38	0.5
4 (59)	Blood	IPM	≥16	>32	32
		MEM	≥16	>32	64
		ERTA	≥8	>32	512
		DOR	n.t	n.t	64
		AK	≥64	64	32
		COL	≥16	4	16
		FOSFO	≥128	32	64
		TGC	≥8	0.75	0.5

TABLE 5 Antibiotic susceptibility comparison by Vitek 2, broth microdilution, and Etest methods against 4 *K. pneumoniae* isolates[a]

[a] Abbreviations: BMD, broth microdilution; IPM, imipenem; MEM, meropenem; ERTA, ertapenem; DOR, doripenem; AK, amikacin; COL, colistin; FOSFO, fosfomycin; TGC, tigecycline; n.t, not tested. [b] Data represent 2013 EUCAST breakpoints.

Antibiotic combination *in vitro* and *in vivo* studies are increasing in the literature due to the global spread of MDR Gram-negative bacteria. Different techniques to test synergy are used: among them time-killing (TK) curves are most used increasing a dynamic evolution of the killing activity, even if time-consuming and labor-intensive.

In the 2000 Aaron et al published the results of multiple combination bactericidal antibiotic testing performed in a randomized, double-blind,

controlled clinical trial in patients with acute pulmonary exacerbations of cystic fibrosis who were infected with *Burkolderia cepacia* and *Pseudomonas aeruginosa* isolates **[20];** so I applied this method against thirteen *K.pneumoniae*, using double and triple antibiotic combinations at peak serum level and comparing it with the time-killing method used as a reference [**9].**

All strains were MDR or XDR clones, possessing different mechanisms of carbapenem-resistance including n.1 VIM-1, n.1 OXA-48 and n.11 KPC-producing *K.pneumoniae* strains belonging to STs 258, 512, 101 and 307. The strains were also selected on the basis of colistin-resistance (7 out 13). The single antibiotics and combinations were also evaluated by time-killing curves, at the same concentrations of MCBT, following standard methods **[21,22].**

All strains were resistant to rifampin and susceptible to tigecycline. Eleven strains were resistant to carbapenems with the exception of the OXA-48 producing *K.pneumoniae* showing reduced susceptibility to meropenem and the VIM-1 *K.pneumoniae* was susceptible to carbapenems and colistin.

In time kill analysis, neither tigecycline nor rifampin were cidal against all different MDR *K.pneumoniae* at peak serum concentration. In the various combinations used, the double carbapenem combination demonstrated to be bactericidal 6/13 on TKC and 13/13 times with MCBT: the full bactericidal activity against all strain was obtained only by adding colistin. The same was true for tigecycline + rifampin, cidal only against VIM-1 *K.pneumoniae*: the addition of colistin rendered the combination strongly bactericidal. The most effective double antibiotic

combination against all strains was rifampin + colistin, which was cidal with both methods (table 6).

Methodologically, MCBT is a rapid method requiring 24h to give a results to the clinician an easy of use method, requiring not expensive resources and easily performed in a routine laboratory. In the past decade, this method was used only against *Burkolderia cepacia* and *Pseudomonas aeruginosa* isolated from patients with cystic fibrosis. This is the first study in which MCBT has been applied to Enterobacteriaceae.

Results of our study suggest that: i) MCBT is an easy and realistic methods to study in vitro activity of combination by using standard serum concentration of antibiotics, providing clinicians with in vitro cidal data within 48h to 72h of isolation of the strain; ii) MCBT can be used in place of TKC because these methods showed the same discriminatory power and reproducibility; iii) adding colistin as third antibiotic to a non bactericidal double-drug combination increased the likelihood of bactericidal effects; iv) these combinations, combined with colistin are bactericidal in strains with complex profile of resistance, including the same antibiotics combined. In conclusion, the performance of the MCBT is reliable when colistin has been used in triple combinations.

TABLE 6. Single, double and triple combinations by Time-killing curves and MCBT methods against n.13 *K.pneumoniae*

Strains (n.)	Single antibiotics					Double and triple antibiotics									
	ETP 70 mg/l*	MEM 120 mg/l*	CST 20 mg/l*	TGC 0.9 mg/l*	RIF 6 mg/l*	ETP+MEM (70 mg/l+120 mg/l)		ETP+MEM+CT (70 mg/l+120 mg/l+20 mg/l)		RD+CST (0.9 mg/l+20 mg/l)		TGC+RIF (0.9 mg/l+6 mg/l)		TGC+RIF+CST (0.9 mg/l+6 mg/l+20 mg/l)	
	TKC	TKC	TKC	TKC	TKC	MCBT	TKC	MCBT	TKC	MCBT	TKC	MCBT	TKC	MCBT	TKC
K.pneumoniae KPC-3 ST512 Col R (3)	NB	NB	NB	NB	NB	B	NB	**B**	**B**	B	B	B	NB	**B**	**B**
K.pneumoniae KPC-3 ST307 Col R (2)	B	B	NB	NB	NB	B	B	**B**	**B**	B	B	NB	NB	**B**	**B**
K.pneumoniae KPC-3 ST258 Col R (2)	NB	NB	NB	NB	NB	B	NB	**B**	**B**	B	B	NB	NB	**B**	**B**
K.pneumoniae KPC-3 ST258 Col S (2)	NB	NB	B	NB	NB	B	NB	**B**	**B**	B	B	NB	NB	**B**	**B**
K.pneumoniae KPC-2 ST101 ColS (2)	NB	B	B	NB	NB	B	B	**B**	**B**	B	B	NB	NB	**B**	**B**
K.pneumoniae VIM-1 Col S (1)	B	B	B	NB	NB	B	B	**B**	**B**	B	B	B	B	**B**	**B**
K.pneumoniae OXA-48 Col S (1)	NB	NB	NB	NB	NB	B	B	**B**	**B**	B	B	NB	NB	**B**	**B**

Another worrisome is the spread of clones KPC-Kpn resistant to aminoglycosides, for acquisition of plasmid genes, which can then be disseminated quickly.

One of the most important mechanisms of aminoglycoside resistance is post-transcriptional methylation of 16S rRNA conferred by methylases **[23]**. Among 16S rRNA methylases described thus far, ArmA (aminoglycoside resitance methyltransferase) is a global concern. This methylase was first detected in *Citrobacter freundii* in Poland and later characterized in *Klebsiella pneumoniae* isolated in France in 2003 currently predominates in Europe. ArmA is encoded by the armA gene, which maps to composite transposon *Tn*1548 located on a large (ca. 80-kb to 90-kb) conjugative plasmid that also carries a gene encoding a CTX-M-type extended-spectrum β-lactamase (ESBL). Their presence is responsible of the high-level resistance to a large number of aminoglycosides to clinically useful. The co-existence of 16S rRNA methylases and various ESBLs in Enterobacteriaceae, including KPC-producing pathogens has been reported from time to time. In previous publications, methylase genes and bla_{KPC} were reported to be mostly located on different plasmids. Only Jiang et al. have reported a *K. pneumoniae* plasmid co-carrying bla_{KPC-2} and *armA* to date. This co-existence is quite alarming because additional high-level resistance to aminoglycosides in KPC-producing isolates and their potential spread would strongly limit the therapeutic options **[6].**

Aminoglycosides act by causing translational errors and by inhibiting translocation. Their target sites include ribosomal domains in which the accuracy of the codon-anticodon is assessed In particular, they bind to a highly

conserved motif of 16S RNA, which leads to alterations in the ribosome functions .Substitution or methylation of bases involved in the binding between 16S rRNA and aminoglycosides can lead to a loss of affinity for the antibiotic and to the resistance of the host **[23]**

Recent Italian studies have described the dissemination and the predominance of a KPC-2 variant belonging to ST101. The *armA* gene was found on the same plasmid of the KPC-2 strains previously isolated in Italy3 and China and on different plasmids in isolates from Poland.

In our study we have described five *K. pneumoniae* isolates from five patients in two Italian hospitals (IRCCS Neurolesi, Messina, and Careggi Hospital, Florence) harbouring bla_{KPC-2} and *armA* genes in isolates of ST101 belonging to a clonal complex different from those containing the habitual sequence clone ST258 isolated in Italy. These isolates presented a profile of XDR; two of them were resistant to all classes of antibiotics except tigecycline and colistin and three were resistant to colistin. All strains were also highly resistant to gentamicin, amikacin and kanamycin (MICs between 128 and ≥512 mg/L) in addition to carbapenems (Table 7). Multilocus STs, revealed that all isolates belonged to ST101, an ST already found in other Italian hospitals. Our five ST101 strains also possessed an identical macrorestriction profile by PFGE, performed after *Xba*I digestion, demonstrating the strong epidemic character of this clone. All strains harboured KPC allele 2, TEM-1, APHA1 and ArmA, contributing to the complex phenotype of resistance of these strains. Amplicon sequencing revealed that the bla_{KPC-2} gene was in all cases embedded in a *Tn4401*-like transposon. Published papers have reported that *Tn4401* has been found on *Inc*N and *Inc*FIIk plasmids. In all our

strains amplicon sequence analysis (1071 bp) showed that plasmid sequences matched the pKpQIL-IT plasmid, circulating in Italy and already detected in a strain of *K. pneumoniae* ST258 background. Southern blot experiments on genomic and plasmid DNAs with the *bla*$_{KPC}$, *armA* and pKpQIL-IT probes obtained by PCR fragments were performed. A hybridization signal on the same fragment of 97 kb in all strains was found, suggesting that these genes are located on the same element. In conclusion, our findings suggest that KPC-2- and ArmA producing *K. pneumoniae* strains are emerging in an ST101 background. These clones are extensively resistant, also due to lateral gene transfer, rendering all families of drugs useless and requiring only antibiotic combination.

The horizontal transferability of these elements, together with clonal expansion of these MDR organisms, poses complex challenges to containment programs, and the planning of correct therapies. In fact, infections sustained by MDR organisms harboring *bla*$_{KPC}$ genes are associated with therapeutic failure, and high mortality rates, particularly in high-risk patients **[24, 25]**

Table 7 Clinical characteristics of patients and antibiotic susceptibility of KPC-2 and armA co-producing *K.pneumoniae*

Patients	**Date**	**Place**	**Wards**	**Specimens**		**MIC (mg/L) ***											
					IPM	**ETP**	**MEM**	**LEV**	**CT**	**CEF**	**CAZ**	**CTX**	**TZP**	**TG**	**GM**	**AK**	**KAN**
1	23/11/2011	Messina	Hospitalization	Aspirated Bronchial	4	64	64	32	0.06	512	512	512	512	0,19	512	512	512
2	07/09/2011	Florence	ICU	Throat Swab	128	512	32	16	0.12	64	512	64	256	1	128	128	256
3	11/12/2012	Florence	ICU	Blood	128	512	256	32	64	128	512	512	512	0,25	512	512	512
4	10/11/2011	Florence	ICU	Aspirated Bronchial	128	512	256	16	16	256	512	512	512	0,25	512	512	512
5	13/01/2012	Florence	Oncology	Blood	128	256	32	16	4	64	512	64	256	2	128	128	256

Abbreviations : IPM, imipenem; MEM, meropenem; ETP, ertapenem; CEF, cefepime; CAZ, ceftazidime; CTX, cefotaxime; TZP, piperacillin/tazobactam; TG, tigecycline; CT, colistin; KAN, kanamicin; GM, gentamicin; VE, Vittorio Emanuele; ICU intensive care units.

Most commonly found in *K. pneumoniae*, KPC enzymes have been identified in a variety of Gram-negative organism, including *Escherichia coli*, *Enterobacter* species, *Pseudomonas aeruginosa* and *Acinetobacter baumannii*. The rapid spread of bla_{KPC} into multiple genera of Gram-negative pathogens, the lack of good treatment regimes and the inability of clinical laboratories to identify all KPC-producing pathogens underscore the need to understand the selective pressures driving the rapid emergence and spread of KPC- producing organism. It is possible that certain antimicrobial drug classes used during empirical therapy, combination therapy or to treat multiple infection within the patient could provide a stimulus to either increase the amount of bla_{KPC} expression or stimulate the mobility of trasposon *Tn4401* carrying bla_{KPC}. **[26]**

In May 2013, in the Mediterranean Institute for Transplantation and Advanced Specialized Therapies (ISMETT), following an active rectal swab surveillance program with chromID® CARBA agar (bio-Mérieux Clinical Diagnostics, France), 5 pairs of KPC-producing *K. Pneumoniae* (KPC Kp) and *E.coli* (KPC Ec) strains were isolated from each of 5 patients (2 who underwent organ transplantation, and 3 who underwent cardiac surgery). At admission, these patients were colonized with KPC Kp, and 2 developed bloodstream infection (BSI) with KPC Kp. In *E. coli*, 3 PFGE clones and 3 different STs were found: clone A was ST131, clone B was ST1672, and clone C was ST394 **[25]**. The two pulsotypes of *K. pneumoniae* (type A in 2 strains,

nd B in 3 strains) coincided with sequence types (STs) (clone A ST512 and clone B ST307) **[27].**In particular, clone ST512 is a single locus variant of ST258, already found in other Italian hospitals **[7]**, while there is no information on the presence of *K. pneumoniae* ST307 in Italy (table 8).

All strains were confirmed to harbor the blaKPC-3 and the blaTEM-1 genes. The only KPC Ec frankly resistant also contained $bla_{CTX\text{-}M\text{-}15}$. The same genes were found in the co-*cultured K. pneumoniae* of the same patient. Plasmid analysis and direct sequencing, revealed that $bla_{KPC\text{-}3}$ genes were in all cases embedded in a *Tn4401a* transposon, and plasmid sequences matched for the presence of the pKpQIL-IT in all strains under study. *E.coli* ST394 was previously not a KPC producer but harbored ESBL genes ($bla_{CTX\text{-}M\text{-}15}$and $bla_{TEM\text{-}1}$); after 2 weeks the same clone was isolated and was a KPC-producer. Among three patients , KPC-3 producing *E.coli* was acquired by cross-transmission during hospitalization in the same ward. All *K. pneumoniae* strains were resistant to all carbapenems with all methods used, while KPC Ec, possessed lower MIC values compared with those observed in *K.pneumoniae*. The KPC-Ec strains, showed reduced susceptibility values to meropenem and imipenem, with the sole exception of one isolate, fully resistant to the three carbapenems with all methods. In the other 4 strains, all susceptible by the Phoenix system, gradient testing revealed a reduced susceptibility to carbapenems, with the exception of ertapenem, which showed a resistant or intermediate level with all methods used. In the light of these findings, this drug was utilized as the marker of resistance (table 9). Our study highlights the simple, and

worrisome, in vivo inter-species transfer of pKpQIL-IT containing the bla_{KPC} gene. This event can be underappreciated and underreported because of the low expression level of this resistance determinant in a genetic background different from *K. pneumoniae*, as already observed **[28]**. Even if little is known about the role that gene expression plays in KPC-mediated resistance, or how the level of expression may affect susceptibility testing, several hypotheses have emerged: i) some studies have reported a possible presence of different isoforms of *Tn4401* with different upstream promoters involved in different degrees of expression **[29]**; ii) the KPC gene can be located in a low number of plasmid copies, as reported in previous studies **[28]**. Further studies will be necessary to fully understand this low level of expression in KPC Ec The ease of KPC in vivo transfer between *K.pneumoniae* and *E.coli* is extremely worrisome, and our study strengthens the importance of infection control measures for rapid detection of KPC in nosocomial pathogens, in order to prevent further dissemination and, in case of infection, for direct targeted therapy **[8]**

Table 8. Typing Characterization of Isolate by PFGE and MLST

Patients	Species	PFGE	ST
426814	*E.coli*	A	131
426814	*K.pneumoniae*	B	512
427835	*E.coli*	B	1672
427835	*K.pneumoniae*	B	512
427862	*E.coli*	B	1672
427862	*K.pneumoniae*	B	512
426010	*E.coli*	B	1672
426010	*K.pneumoniae*	A	307
429422	*E.coli*	C	394
429422	*K.pneumoniae*	A	307

Table9. Susceptibility to Carbapenemsby 3 Methods of KPC-Producing Strains

Patients	Species	Isolation date	MICs of Carbapenems (mg/L)								
			Ertapenem BP (0.5/1)			Meropenem BP (2/8)			Imipenem BP (2/8)		
			Ph	BMD	Gradient test	Ph	BMD	Gradient test	Ph	BMD	Gradient test
426814	*E.coli*	**03/05/2013**	>1	1	0.75	≤ 1	0.5	2	≤ 1	0.5	8
	K.pneumoniae		>1	128	>32	>8	>32	>32	>8	>32	>32
427835	*E.coli*	**10/05/2013**	>1	4	1.5	≤ 1	1	0.75	4	1	3
	K.pneumoniae		>1	128	>32	>8	>32	>32	>8	64	>32
427862	*E.coli*	**15/05/2013**	>1	4	2	≤ 1	4	2	4	1	4
	K.pneumoniae		>1	128	>32	>8	64	>32	>8	64	>32
426010	*E.coli*	**30/04/2013**	>1	1	1.5	≤ 1	2	0.75	4	0.5	3 I
	K.pneumoniae		>1	128	>32	>8	256	>32	>8	256	>32
429422	*E.coli*	**27/04/2013**	<1	0.12	0.25	<1	0.12	0.06	<1	0.12	0.12
	K.pneumoniae		>1	128	>32	>8	512	>32	>8	512	>32
	E.coli	**11/05/2013**	>1	>128	>32	> 8	128	32	>8	64	32
	K.pneumoniae		>1	128	>32	>8	512	>32	>8	512	>32

Abbreviations: Ph: Phoenix; BMD: microdiluition broth; BP: breakpoin

Klebsiella pneumoniae, like other Gram-negative bacteria such as *Acinetobacter baumannii*, in addition to the problem given by the increasingly rapid development of resistance mechanisms and the lack of effective new drugs, another fundamental issue and the difficulty of interpretation methods with simple and fast, such as the gradient test, which not being too laborious turns out to be an excellent support for targeted therapy. The problem concerns essentially two antibiotics, that today' s result to be active against gram-negative MDR, which precisely *Klebsiella pneumoniae*, and *Acinetobacter baumannii*, which are often administered in combination, such as Colistin and Tigecycline, although as mentioned earlier the resistances, following prolonged therapy begin to develop him even for these drugs **[30,31,32].**

The problem arises from the difficulty of interpreting the results in terms of MIC that is born from the joint method used (agar diffusion) and the characteristics of these molecules **[33,34, 35].**

In a study conducted from January 1 to July 31, 2013 were isolated from serious infections in patients admitted to the ICU Cannizzaro Hospital in Catania, n.77 carbapenem-resistant gram-negative bacteria, including 25 *Klebsiella pneumoniae* KPC producers.

All KPC-producing *K. pneumoniae* isolates were MDR: colistin was active against 19 of the 25 strains, and the results regarding the activity of tigecycline showed 32% resistance. Results of the in vitro susceptibility testing, expressed as MIC50and MIC90 (MIC required to inhibit 50% and 90% of the isolates, respectively), are presented in Table 10.

Table 10: Susceptibilities and molecular characteristics of carbapenemase-producing *Klebsiella pneumoniae*

PFGE, pulsed-field gel electrophoresis; ST, sequence type; MIC, minimum inhibitory concentration; MEM, meropenem; ETP, ertapenem; IPM, imipenem; TZP,

Strains (n.)	Speciment (n.)	PFGE pattern	Sequence Type	MIC	Antibiotics (mg/l)																
					MEM	ETP	IPM	TZP	SAM	AMC	ATM	CAZ	CTX	FEP	AMK	GEN	SXT	CIP	RIF	TGC	CST
K.pneumoniae (n.25)	Bronchial aspirate (n.12)	A	258 (n.15)	Range	16->32	2->32	nt	32-256	nt	32-64	32-8	32-8	64-4	16-64	32-64	64-1	8->32	4->32	nt	1-8	0.75-64
				MIC_{50}	16	4	-	64	-	64	16	16	32	16	32	8	16	>32	-	3	1
				MIC_{90}	16	2	-	32	-	64	8	8	4	16	32	1.5	8	4	-	1.5	1
	Urine (n.7)	B	512 (n.6)	Range	4-16	2	nt	32	nt	16-32	8	8	4	8	16-32	1	1-8	2-4	nt	0.75-1	0.5-0.75
				MIC_{50}	8	2	-	32	-	16	8	8	4	8	32	1	1	4	-	1	0.75
				MIC_{90}	8	2	-	32	-	16	8	8	4	8	16	1	8	4	-	1	0.5
	Blood (n.6)	C	147 (n.1)	Range	4	2	nt	32	nt	16	8	4	2	4	2	0.5	0.19	2	nt	0.5	0.19
				MIC_{50}			-		-		-	-	-	-	-	-	-	-	-	-	-
				MIC_{90}			-		-		-	-	-	-	-	-	-	-	-	-	-
		D	395 (n.3)	Range	4	2	nt	32	nt	16	8	8	2-4	8	2-16	1	0.38-1	2	nt	0.75	0.25-0.5
				MIC_{50}	4	2	-	32	-	16	8	8	2	8	2	1	1	2	-	0.75	0.38
				MIC_{90}	4	2	-	32	-	16	8	8	2	8	2	1	0.38	2	-	0.75	0.25

piperacillin/tazobactam; SAM, ampicillin/sulbactam; AMC, amoxi-cillin/clavulanic acid; ATM, aztreonam; CAZ, ceftazidime; CTX, cefotaxime; CEF, cefepime; AMK, amikacin; GEN, gentamicin; SXT, trimethoprim/sulfamethoxazole; CIP, ciprofloxacin; RIF, rifampicin; TGC, tigecycline; CST, colistin; NT, not tested; MIC50/90, MIC required to inhibit 50% and 90% of the isolates, respectively

All strains were tested by gradient test and BMD using both colistin and tigecycline. Of the 25 KPC-producing *K. pneumoniae*, there was only a 60% concordance between the two methods for colistin, whilst for tigecycline the agreement was 88%. The very major error rate was 4% for both antibiotics in *K. pneumoniae*. The agreement data are presented in Table 11.

Table11: Minimum inibitory concentration (in mg/L) of tigecycline and colistin for *Klebsiella pneumoniae* by gradient test and broth microdiluition (BMD)

ST	Isolate	Colistin		Tigecycline	
		Gradient test	BMD	Gradient test	BMD
258	1	1	0.25	0.5	0.5
	2	0.75	0.5	1[a]	4
	3	64	256	2	2
	11	24	>32	1	2
	4	1	0.25	3	4
	5	0.38	0.5	4	4
	6	0.75	0.25	1	1
	7	0.75	1	0.75	2
	8	0.75	0.5	3	4
	9	1	2	1	2
	19	0.5	0.5	2	4
	21	2[a]	64	6	8
	22	0.19	0.5	3	4
	23	0.5	1	0.75	1
	24	0.5	1	0.75	0.5
512	10	0.75	1	1.5	1
	12	1	0.5	3	4
	13	4	64	1	0.25
	14	1	1	2	4
	15	0.25	0.5	4	2
	16	1	0.5	8	4
395	17	8	>256	0.75	1
	18	16	>256	1.5	1
	20	16	>256	1	1
147	25	0.5	0.25	1.5	2
Antibiotic susceptibility testing and agreement with BMD					
%S	72	68	44	36	
%I	4	4	24	24	
%R	24	28	32	40	
Agreement BMD (%)	60	NA	88	NA	
Very major error (%)	4	NA	4	NA	

ST, sequence type; S, susceptible; I, intermediate; R, resistant; NA, not applicable.

[a] Very major error.

All *K. pneumoniae* resistant to meropenem and ertapenem were positive by carbapenemase phenotypic test, and PCR detection showed that all strains harboured the bla_{KPC-3} gene and no MBL(bla_{IMP} and bla_{VIM}), bla_{NDM-1} and bla_{OXA-48} genes were detected. Epidemiological investigation by PFGE identified four pulso-types among all of the KPC-producing *K. pneumoniae* (A, B, C and D). MLST of these isolates identified four distinct STs: pulsotype A strains belonged to ST258 and pulsotype B was categorised as ST512 detected in most isolates. Pulsotypes C and D were also identified in a few strains, as ST147 and ST395, respectively .

The resistance rates for colistin in KPC-producing *K. pneumoniae* isolates, tested by the gradient test and BMD methods, did not show significant differences; however, the agreement was only 60%with a 4% very major error rate because in some strains the MIC values were very different (i.e. strain no. 17 in Table 3: 8 mg/L by gradient test and >256 mg/L by BMD). This feature is probably due to the characteristics of the strain and the low diffusibility of colistin in agar owing to its high molecular weight, resulting in an underestimation of resistance **[36,37].**

This is an issue when the MIC determined by gradient test is intermediate because it hides the resistant phenotype.

This study also described 25 *K. pneumoniae* isolates harbouring the bla_{KPC-3} gene in isolates of four different clones, of which two were more widespread (A and B), belonging to the same clonal complex (ST258 and ST512) already found in other Italian hospitals. Analysis of antimicrobial susceptibility patterns showed that *K.pneumoniae* isolates with PFGE type A ST258 all had a multiresistant antibiotype characterised by higher MICs to most antimicrobial agents compared with B, C and D

pulsotypes. The other two clones (ST147 and ST395) belonged to a different clonal complex and were identified for the first time in Italy carrying KPC-3. OXA-48-producing ST395 was previously isolated in the Netherlands whilst ST147 was spread in Canada and Hungary carrying the *bla*$_{CTX\text{-}M\text{-}15}$ and *bla*$_{OXA\text{-}48}$ genes, respectively **[38,39,40]**

To date, this is the first study to report the spread of KPC-3-producing *K. pneumoniae* ST147 and ST395 in Italy. The capacity for rapid evolution of resistance determinants in these nosocomial pathogens and the difficulty of interpretation of molecules that represent the last therapeutic option against super-bugs led to the conclusion that a rapid and reliable method for colistin susceptibility testing of *K. pneumoniae* is required. **[8]**

DISCUSSION

KPC-producing *K.pneumoniae* strains are a growing problem in the healthcare setting because KPC reside on transmissible plasmids, and the KPC-Kp harboring these enzymes are multidrug-resistant **[9]**. Due to the unavailability of effective antibiotics, they are generally associated with serious outcomes and high case-fatality rates in critically ill patients. These patients are especially prone to colonization and infection by MDR strains because of their severe underlying conditions coupled with selective pressure due to the extensive use of antibiotics. KPC-Kp MDR has been of particular concern, jumping from sporadic reports to approximately 20% of blood systemic infections isolates in Italy **[41]**. These infections have limited therapeutic options and a striking associated mortality, above all in selected patients **[42]**. Few papers were published in the literature considering the possible use of gut decontamination in the treatment of these critically ill patients as an infection-prevention measure and has been associated with a reduction in morbidity as well as a reduction in overall mortality rate. Results are controversial: some of them indicating that the use of mixed antibiotics were not effective for decontamination often associated with resistance development **[43]** others published results in favor of these procedures **[44,45]**. All paper published until now have used standard phenotypic protocols to detect MDR *K.pneumoniae*, some of them have confirmed their KPC results with PCR **[46, 47]**

Gut colonization represents the main source for KPC-producing *K. pneumoniae* epidemic dissemination. Furthermore, gut colonization

seems to be associated with a substantial risk (around 10%) of developing subsequent KPC-Kp infection and may be a contraindication for some surgical procedures, organ transplantation and other major medical interventions. Therefore the future studies could investigate the changes of faecal microflora after the use of gut decontamination in patients infected/colonized with KPC-Kp *K. pneumoniae*.

Gut decontamination for KPC-Kp colonization could be of interest as a complementary approach for removing patient from isolation, reducing transmission, and preventing subsequent infectious episodes in already colonized patients.

The use of synergetic control procedures in combination with molecular characterization of isolates should represent a priority for the containment of outbreaks of these alert MDR organisms.

REFERENCE

1.**Arnold RS**, **Thom KA**, **Sharma S**, **Phillips M**, **Kristie Johnson J**, **Morgan DJ** *Emergence of Klebsiella pneumoniae carbapenemase-producing bacteria***.** South Med J. 2011 Jan; 104(1):40-5. doi: 10.1097/SMJ.0b013e3181fd7d5a

2. **David M. Livermore***: Current Epidemiology and Growing Resistance of Gram-Negative Pathogens*. Korean J Intern Med. 2012 Jun;27(2):128-42. doi: 10.3904/kjim.2012.27.2.128. Epub 2012 May 31.

3. **Munoz-Price LS, Poirel L, Bonomo RA, Schwaber MJ, Daikos GL, Cormican M, Cornaglia G, Garau J, Gniadkowski M, Hayden MK, Kumarasamy K, Livermore DM, Maya JJ, Nordmann P, Patel JB, PatersonDL, Pitout J, Villegas MV, Wang H, Woodford N, Quinn JP**: *Clinical epidemiology of the global expansion of Klebsiella pneumoniae carbapenemases* Lancet Infect Dis. 2013 Sep;13(9):785-96. doi: 10.1016/S1473-3099(13)70190-7.

4. **Mammina C, Bonura C, Aleo A, Fasciana T, Brunelli T, Pesavento G, Degl'Innocenti R, Nastasi A**. *Sequence type 101 (ST101) as the predominant carbapenem-non-susceptible Klebsiella pneumoniae clone*

in an acute general hospital in Italy. Int J Antimicrob Agents. 2012 Jun;39(6):543-5. doi: 10.1016/j.ijantimicag.2012.02.012. Epub 2012 Apr 23.

5. Frasson I, Lavezzo E, Franchin E, Toppo S, Barzon L, Cavallaro A, Richter SN, Palù G. *Antimicrobial treatment and containment measures for an extremely drug-resistant Klebsiella pneumoniae ST101 isolate carrying pKPN101-IT, a novel fully sequenced blaKPC-2 plasmid*. J Clin Microbiol. 2012 Nov;50(11):3768-72. Doi : 10.1128/JCM.01892-12. Epub 2012 Sep 12.

6. Maria Lina Mezzatesta, Floriana Gona, Carla Caio, Chiara Adembri, Pia Dell'utri, Maria Santagati and Stefania Stefani: *Emergence of an extensively drug resistant ArmA- and KPC-2-producing ST101 Klebsiella pneumoniae clone in Italy*. J Antimicrob Chemother. 2013 Aug;68(8):1932-4. Doi: 10.1093/jac/dkt116. Epub 2013 May 10.

7. Giancarlo Ceccarelli, Marco Falcone, Alessandra Giordano, Maria Lina Mezzatesta, Carla Caio,Stefania Stefani, Mario Venditti: *Successful Ertapenem-Doripenem Combination Treatment of Bacteremic Ventilator-Associated Pneumonia Due to Colistin-Resistant KPC-Producing Klebsiella pneumonia.* Antimicrob Agents

Chemother. 2013 Jun;57(6):2900-1. doi: 10.1128/AAC.00188-13. Epub 2013 Apr 9.

8. F. Gona, F. Barbera, A.C. Pasquariello, P. Grossi, B. Gridelli, M.L. Mezzatesta, C.Caio, S.Stefani and P.G.Conaldi. *In vivo multiclonal transfer of blaKPC-3 from Klebsiella pneumoniae to Escherichia coli in surgery patients.* Clin Microbiol Infect. 2014 Jan 30. doi: 10.1111/1469-0691.12577. [Epub ahead of print]

9. Maria Lina Mezzatesta, Carla Caio, Floriana Gona, Roberta Cormaci, Iasmine Salerno, Tiziana Zingali, Carmelo Denaro, Mauro Gennaro, Cristiana Quattrone, Stefania Stefani: *Carbapenem and multidrug resistance in Gram-negative bacteria in a single centre in Italy: Considerations on in vitro assay of active drugs.* Int J Antimicrob Agents. 2014 Aug; 44(2):112-6. doi: 10.1016/j.ijantimicag.2014.04.014. Epub 2014 Jun 2.

10. Pitcher DG, Sanders NA, Owen RJ. *Rapid extraction of bacterial genomic DNA with guanidium thiocyanate.* Lett Appl Microbiol 1989.

11. David Goldfarb,Sarah-Beth Harvey, Kelsi Jessamine ,Peter Jessamine, Baldwin Toye and Marc Desjardins *Detection of Plasmid-Mediated KPC-Producing Klebsiella pneumoniae in Ottawa, Canada: Evidence of Intraospital Trasmission***.** J Clin Microbiol. 2009 Jun; 47(6):1920-2. doi: 10.1128/JCM.00098-09. Epub 2009 Apr 8.

12. Tommaso Giani, Marco Maria D'Andrea, Patrizia Pecile, Luisa Borgianni, Pierluigi NIcoletti, Francesco Tonelli, Alessandro Bartoloni, Gian Maria Rossolini. *Emergence in Italy of Klebsiella pneumoniae Sequence Type 258 Producing KPC-3 Carbapenemase* J Clin Microbiol. 2009 Nov;47(11):3793-4. doi: 10.1128/JCM.01773-09. Epub 2009 Sep 16

13. Tenover FC, Arbeit RD, Goering RV, Mickelsen PA, Murray BE, Persing DH, Swaminathan B.. *Interpreting chromosomal DNA restriction patterns produced by pulsed-field gel electrophoresis criteria for bacterial strain typing***.** J Clin Microbiol. 1995 Sep;33(9):2233-9

14. Sambrook, J., Fritsch, E. F. & Maniatis, T.. *Bacterial media, antibiotics and bacterial strains. In Molecular Cloning: a Laboratory Manual, 2nd***.** Cold Spring Harbor, NY: Cold Spring Harbor Laboratory 1989.

15. Kieser T. *Factors affecting the isolation of CCC DNA from Streptomyces lividans and Escherichia coli.* Plasmid. 1984

16. Ørjan Samuelsen1, Umaer Naseer, Sta° le Tofteland, Dag Harald Skutlaberg, Annette Onken, Reidar Hjetland, Arnfinn Sundsfjord, and Christian G. Giske: *Emergence of clonally related Klebsiella pneumoniae isolates of sequence type 258 producing plasmid-mediated KPC carbapenemase in Norway and Sweden* J Antimicrob Chemother. 2009 Apr;63(4):654-8. doi: 10.1093/jac/dkp018. Epub 2009 Feb 13.

17. M. L. Mezzatesta, F. Gona, C. Caio, V. Petrolito, D. Sciortino, A. Sciacca, C. Santangelo and S. Stefani: *Outbreak of KPC-3-producing, and colistin-resistant, Klebsiella pneumonia infections in two Sicilian hospitals.* Clin Microbiol Infect. 2011 Sep;17(9):1444-7. doi: 10.1111/j.1469-0691.2011.03572.x. Epub 2011 Jun 10..

18. Matthew E. Falagas, Petros I. Rafailidis, Dimitrios K. Matthaiou: *Resistance to polymyxins: Mechanisms, frequency and treatment options*. Drug Resist Updat. 2010 Aug-Oct;13(4-5):132-8. doi: 10.1016/j.drup.2010.05.002. Epub 2010 Jun 17..

19. **Bulik CC, Nicolau DP.** *Double-carbapenem therapy for carbapenemase- producing Klebsiella pneumoniae.* Antimicrob Agents Chemother. 2011 Jun;55(6):3002-4. doi: 10.1128/AAC.01420-10. Epub 2011 Mar 21

20. Aaron SD1, Ferris W, Henry DA, Speert DP, Macdonald NE. *Multiple combination bactericidal antibiotic testing for patients with cystic fibrosis infected with Burkholderia cepacia***.** Am J Respir Crit Care Med. 2000 Apr;161(4 Pt 1):1206-12.

21. Mezzatesta ML, Trovato G, Gona F, Nicolosi VM, Nicolosi D, Carattoli A, Fadda G, Nicoletti G, Stefani S. *In vitro activity of tigecycline and comparators against carbapenem-susceptible and resistant Acinetobacter baumannii clinical isolates in Italy*. Ann Clin Microbiol Antimicrob. 2008 Feb 8;7:4. doi: 10.1186/1476-0711-7-4.

22 Pournaras S, Vrioni G, Neou E, Dendrinos J, Dimitroulia E, Poulou A, Tsakris A*Activity of tigecycline alone and in combination with colistin and meropenem against Klebsiella pneumoniae carbapenemase (KPC)-producing Enterobacteriaceae strains by time-kill assay.* Int J Antimicrob Agents. 2011 Mar;37(3):244-7. doi: 10.1016/j.ijantimicag.2010.10.031. Epub 2011 Jan 13

23. Galimand M, Courvalin P, Lambert T. *Plasmid-mediated high-level resistance to aminoglycosides in Enterobacteriaceae due to 16S rRNA methylation*. Antimicrob Agents Chemother. 2003 Aug;47(8):2565-71.

24. Kalpoe JS, Sonnenberg E, Factor SH, del Rio Martin J, Schiano T, Patel G, Huprikar S. *Mortality associated with carbapenem-resistant Klebsiella pneumoniae infections in liver transplant recipients.* Liver Transpl. 2012 Apr;18(4):468-74. doi: 10.1002/lt.23374.

25. Bergamasco MD, Barroso Barbosa M, de Oliveira Garcia D, Cipullo R, Moreira JC, Baia C, Barbosa V, Abboud CS. *Infection with Klebsiella pneumoniae carbapenemase (KPC)- producing K. pneumoniae in solid organ transplantation*. Transpl Infect Dis. 2012 Apr;14(2):198-205. doi: 10.1111/j.1399-3062.2011.00688.x. Epub 2011 Oct 28..

26. Roth AL, Lister PD, Hanson ND. *Effect of drug treatment options on the mobility and expression of blaKPC*. J Antimicrob Chemother. 2013 Dec;68(12):2779-85. doi: 10.1093/jac/dkt280. Epub 2013 Jul 16

27. Wirth, T., Falush, D., Lan, R., Colles, F., Mensa, P., Wieler, L.H., Karch, H., Reeves, P. R.,Maiden, M. C., Ochman, H., and Achtman M. *Sex and virulence in Escherichia coli: an evolutionary perspective***.** Mol Microbiol. 2006 Jun; 60(5):1136-51.

28. Amanda L. Roth, Philip M. Kurpiel, Philip D. Lister, and Nancy D. Hanson *blaKPC RNA Expression Correlates with Two Transcriptional Start Sites but Not Always with Gene Copy Number in Four Genera of Gram-Negative Pathogens Antimicrob Agents Chemother. 2011 Aug;55(8):3936-8. doi: 10.1128/AAC.01509-10. Epub 2011 May 16.*

29. Naas T, Cuzon G, Truong HV, Nordmann P. *Role of ISKpn7 and deletions in blaKPC gene expression.* Antimicrob Agents Chemother. 2012 Sep;56(9):4753-9. doi: 10.1128/AAC.00334-12. Epub 2012 Jun 25.

30. Betts JW, Phee LM, Hornsey M, Woodford N, Wareham DW. *In vitro and in vivo activities of tigecycline-colistin combination therapies against carbapenem-resistant Enterobacteriaceae*. Antimicrob Agents Chemother. 2014 Jun;58(6):3541-6. doi: 10.1128/AAC.02449-14. Epub 2014 Mar 31

31. Nielsen LE, Snesrud EC, Onmus-Leone F, Kwak YI, Avilés R, Steele ED, Sutter DE, Waterman PE, Lesho EP. *IS5 Element Integration, a Novel Mechanism for Rapid In Vivo Emergence of Tigecycline Non susceptibility in Klebsiella pneumoniae.*; Antimicrob Agents Chemother. 2014 Oct;58(10):6151-6. doi: 10.1128/AAC.03053-14. Epub 2014 Aug 4.

32. Poirel L, Jayol A, Bontron S, Villegas MV, Ozdamar M, Türkoglu S, Nordmann P. *The mgrB gene as a key target for acquired resistance to colistin in Klebsiella pneumoniae.* J Antimicrob Chemother. 2014 Sep 3. pii: dku323. [Epub ahead of print]

33. Gales AC, Reis AO, Jones RN. *Contemporary assessment of antimicrobial susceptibility testing methods for polymyxin B and colistin: review of available interpretative criteria and quality control guidelines*. J Clin Microbiol. 2001 Jan;39(1):183-90.

34. Ronald N. Jones, Tamara R. Anderegg, Jana M. Swenson, and The Quality Control Working Group: *Quality Control Guidelines for Testing Gram-Negative Control Strains with Polymyxin B and Colistin (Polymyxin E) by Standardized Methods*. Journal of Clinical Microbiology 2005.

35. **Leal Castro AL, Buitrago Gutierrez G, Ovalle V, Cortes JA, Alvarez CA; Colombian Tigecycline Susceptibility Surveillance Group**. *Comparing in vitro activity of tigecycline by using the disk diffusion test, the manual microdilution method, and the VITEK 2 automated system.* Rev Argent Microbiol. 2010 Jul-Sep; 42(3):208-11.

36. Pelletier MR, Casella LG, Jones JW, Adams MD, Zurawski DV, Hazlett KR, Doi Y, Ernst RK. *Unique structural modifications are present in the lipopolysaccharide from colistin-resistant strains of Acinetobacter baumannii*. Antimicrob Agents Chemother. 2013 Oct;57(10):4831-40. doi: 10.1128/AAC.00865-13. Epub 2013 Jul 22.

37. Galani I1, Kontopidou F, Souli M, Rekatsina PD, Koratzanis E, Deliolanis J, Giamarellou H.. *Colistin susceptibility testing by Etest and disk diffusion methods*. Int J Antimicrob Agents. 2008 May;31(5):434-9. doi: 10.1016/j.ijantimicag.2008.01.011. Epub 2008 Mar 6.

38. Potron A, Kalpoe J, Poirel L, Nordmann P. *European dissemination of asingle OXA-48-producing Klebsiella pneumoniae clone*. Clin Microbiol Infect. 2011 Dec; 17(12):E24-6. doi: 10.1111/j.1469-0691.2011.03669.x. Epub 2011 Oct 6.

39. Yang J, Ye L, Guo L, Zhao Q, Chen R, Luo Y, Chen Y, Tian S, Zhao J, Shen D, Han L. *A nosocomial outbreakof KPC-2-producing Klebsiella pneumoniae in a Chinese hospital: dissemina-tion of ST11 and emergence of ST37, ST392 and ST395*. Clin Microbiol Infect. 2013 Nov;19 (11):E509-15. doi: 10.1111/1469-0691.12275. Epub 2013 Jul 10.

40. Damjanova I, Tóth A, Pászti J, Hajbel-Vékony G, Jakab M, Berta J, Milch H, Füzi M*Expansionand countrywide dissemination of ST11, ST15 and ST147 ciprofloxacin-resistant CTX-M-15-type-betalactamase-producing Klebsiella pneumoniae epidemic clones in Hungary in 2005—the new 'MRSAs'?* J Antimicrob Chemother. 2008 Nov;62 (5):978-85. doi: 10.1093/jac/dkn287. Epub 2008 Jul 30.

41. Giani T, Pini B, Arena F, Conte V, Bracco S, Migliavacca R; AMCLI-CRE Survey Participants, Pantosti A, Pagani L, Luzzaro F, Rossolini GM. *Epidemic diffusion of KPC carbapenemase-producing K.pneumoniae in Italy.* Euro Surveill. 2013 May 30;18(22). pii: 20489.

42. Mouloudi E, Protonotariou E, Zagorianou A, Iosifidis E, Karapanagiotou A, Giasnetsova T, Tsioka A, Roilides E, Sofianou D, Gritsi-Gerogianni N. *Bloodstream infections caused by metallo-ß-lactamase/K.pneumoniae Carbapenemase-producing K. pneumoniae among intensive care unit patients in Greece.* Infect Control Hosp Epidemiol. 2010 Dec; 31(12):1250-6. doi: 10.1086/657135. Epub 2010 Oct 25.

43. Lübbert C, Faucheux S, Becker-Rux D, Laudi S, Dürrbeck A, Busch T, Gastmeier P, Eckmanns T, Rodloff AC, Kaisers UX. *Rapid emergence of secondary resistance to gentamicin and colistin following selective digestive decontamination in patients with KPC-2-producing K. pneumonia.* Int J Antimicrob Agents. 2013 Dec; 42(6):565-70. doi: 10.1016/j.ijantimicag.2013.08.008. Epub 2013 Sep 19.

44. Oostdijk EA, Smits L, de Smet AM, Leverstein-van Hall MA, Kesecioglu J, Bonten MJ. *Colistin resistance in gram-negative bacteria during prophylactic topical colistin use in intensive care units*. Intensive Care Med. 2013 Apr ;39(4):653-60. doi: 10.1007/s00134-012-2761-3. Epub 2012 Dec 1.

45. Tascini 1, Sbrana F, Flammini S, Tagliaferri E, Arena F, Leonildi A, Ciullo I, Amadori F, Di Paolo A, Ripoli A, Lewis R, Rossolini GM, Menichetti F; GENGUT Study Group. *Oral gentamicin gut decontamination for prevention of KPC-producing K. pneumoniae infections*. Antimicrob Agents Chemother. 2014; 58(4):1972-6. doi: 10.1128/AAC.02283-13. Epub 2014 Jan 13.

46 Landman D, Salvani JK, Bratu S, Quale J. *Evaluation of techniques for detection of carbapenem-resistant K. pneumoniae in stool surveillance cultures*. J Clin Microbiol. 2005 Nov;43(11):5639-41.

47. Samra Z, Bahar J, Madar-Shapiro L, Aziz N, Israel S, Bishara J. *Evaluation of CHROMagar KPC for rapid detection of carbapenem-resistant Enterobacteriaceae*. J Clin Microbiol. 2008 Sep;46(9):3110-1. doi: 10.1128/JCM.00249-08. Epub 2008 Jul 16.