UNIVERSITA' DEGLI STUDI DI PAVIA
Department of Clinical, Surgical, Diagnostic and Pediatric Sciences

Microbiology and Cinical Microbiology Unit

Director: Prof. L. Pagani

Antimicrobial susceptibilities, drug resistance gene arrays and epidemic potential of *Enterobacteriaceae* and other Gram negative clinical isolates: novel diagnostic and therapeutic strategies

Ibrahim Bitar

Supervised by Prof. Roberta Migliavacca

PhD in
Genetics, Molecular and Cellular Biology
XXIX Cycle – A.A. 2013-2016

UNIVERSITA' DEGLI STUDI DI PAVIA
Deipartment of Clinical, Surgical, Diagnostic and Pediatric Sciences
Microbiology and Cinical Microbiology Unit
Director: Prof. L. Pagani

Antimicrobial susceptibilities, drug resistance gene arrays and epidemic potential of *Enterobacteriaceae* and other Gram negative clinical isolates: novel diagnostic and therapeutic strategies

Ibrahim Bitar

Supervised by Prof. Roberta Migliavacca

PhD in
Genetics, Molecular and Cellular Biology
XXIX Cycle – A.A. 2013-2016

Acknowledgements

This PhD project would not have been possible without the scientific support, but moral one as well, of several people.

I would like to express my special appreciation and thanks to my supervisor Roberta Migliavacca and Professor Laura Pagani for encouraging my research and for allowing me to grow as a research scientist.

My special gratitude goes to my dear friends and colleagues: Melissa, Aurora, Francesca, Roberto, Davide, Betty, Vittoria, Federica and Sofia for their support, believing in my skills they created the utmost motivation to complete this journey.

Many thanks to Dr. Carattoli and Dr. Villa for hosting me for 3 months and helping me finish all my projects

Last but not the least I would also like to thank my family (Aya, Julia, Imad), my parents and sisters for all the support they gave me in all these years.

To Julia and Imad I dedicate this thesis.

Abbreviations

3GC: cefotaxime and/or ceftriaxone and/or ceftazidime
aac(6′)-Ib-cr: aminoglycoside-(6)-N-acetyltransferase
AbGR: DNA homology groups
ABR: antibacterial resistance
AdeABC: *Acinetobacter* drug efflux
AFLP: Amplified Fragment Length Polymorphism Analysis
AMCLI: Italian Society of Clinical Microbiologist
AMR: antimicrobial resistance
ARI-1: *Acinetobacter* Resistant to Imipenem
ATC: anatomical therapeutic chemical
ATM: aztreonam
CA: community-acquired
CA-ESBL: community-acquired ESBL
CAT: chloramphenicol acetyl transferase
CAZ: ceftazidime
CC: clonal complex
CDC: Centers for Disease Control and Prevention
CDT: combination disk test
CHDLs carbapenem-hydrolysing class D β-lactamases
CNS: carbapenem-non susceptibility
CRAb: carbapenem-resistant *A. baumannii*
CRE: carbapenem resistant *Enterobacteriaceae* CS: conserved segment
CTX: cefotaxime
DD: double disk synergy test
DDD: defined daily doses
EARS-Net: European Antimicrobial Resistance Surveillance Network
EARSS: European Antimicrobial Resistance Surveillance System
ECDC: European Centre for Disease Prevention and Control
EDTA: ethylenediaminetetraacetic acid
EEA: European Economic Area
EPIC: Intensive Care Study
ESAC-Net: European Surveillance of Antimicrobial
ESBL extended spectrum β-lactamase
ESBL-KP: ESBL-producing *Klebsiella pneumoniae*
EU: European Union
EUCAST: European Committee on Antimicrobial Susceptibility Testing

FDA: Food and Drug Administration
FEP: cefepime
FMT: fecal microbiota transplantation
HAI: health care-associated infections
HGT: horizontal gene transmission
IC: International clone
ICU: intensive care unit
IEF: isoelettrofocusing
IMP: imipenemase metallo-β-lactamase
Inc: Incompatibility group
intI1: class 1 integrase gene
IS: Insertion Sequence
KPC: *Klebsiella pneumoniae* carbapenemases
LPS: lipopolysaccharides
MBL: metallo β-lactamase
MDR: Multi-Drug-Resistant
MHT: Modified Hodge test
MIC: minimum inhibitory concentration
MLST: multilocus sequence typing
MRSA: Methicillin-resistant *Staphylococcus aureus*
MSSA: Methicillin-susceptible *Staphylococcus aureus*
NAG: N-acetylglucosamine
NAMA: N-acetylmuramic acid
NDM: New Delhi metallo-β-lactamase
NI: nosocomial infection
NICU: Neonatal Intensive Care Unit
ompA: outer-membrane protein A
OMPs: outer membrane porins
Ori: origin of replication
OXA: oxacillinase
PBPs: penicillin-binding proteins
PBRT: PCR-based replicon typing
PFGE: pulse field gel electrophoresis
PMQR: plasmid-mediated quinolone resistance
PPS: Point Prevalence Survey
RAPD: Randomly Amplified Polymorphic DNA
RCR: rolling circle replicating
Rep: replication initiator proteins

Rep-PCR: Repetitive extragenic palindromic
PCR SCCmec: staphylococcal cassette chromosome mec
SG: sequence group
SMAL: S. Matteo/Maugeri Hospitals Acute care and Long term care facilities
ST: sequence type
TZP: piperacillin-tazobactam
UTI: urinary tract infection
VAP: ventilator associated pneumonia
VIM: Verona integron–encoded metallo-β-lactamase
VLBW: very-low-birth-weight
WHO: World Health Organization.

CONTENTS

- List of Tables
- List of Figuers

LIST OF TABLES

LIST OF FIGURES

Chapter 1

GENERAL INTRODUCTION

1.1. Health care-associated infections:

Infections are generally classified into two types: healthcare associated infections (HAI or HCAI) and community acquired infections (CAI). Siegman-Igra *et al.* and Friedman *et al.* introduced the official classification for the first time in 2002. According to Friedman, whose classification is widely used in literature, HAI are infections present upon on set or post 48 hours of the admission of the patient who fulfilled one or more of the following criteria:

- Resided in a long-term care facility or in a nursing home was hospitalized in an acute care hospital for 2 or more days in the previous 3 months
- Attended a hospital or hemodialysis clinic where he/she received intravenous chemotherapy in the previous month
- Received intravenous therapy at home, wound care facility through specialized health care agency, family or friends or even self-administered in the previous month (Cardoso T et al. 2014).

On the other hand, the World Health Organization (WHO) defined the healthcare associated infections (also termed as nosocomial or hospital infections) as infections acquired in the hospital or health care center, not only during the patient's admission, but even after he/she is discharged; taking into account that these infections should not be present upon the patient's admission to the health care center. These can be termed as well as "occupational infections" if the health-care staff is infected while working in the facility (http://www.who.int/en/).

HAIs contribute to a high rate of morbidity and mortality worldwide, leading to 37,000 deaths/year in Europe and 75,000 deaths/year in USA in 2011 only. Moreover, the Centers for Disease Prevention and Control (CDC) and the European Centers for Disease Control and Prevention (ECDC) estimated 25,000 deaths per year in Europe alone due to antibiotic resistant HAIs. In fact, this medical burden expands to an economical level where 9.8 million dollars and 7 billion Euros are spent annually in USA and Europe respectively to fight only five major HAIs (Simoes et al. 2016, Park *et al*. 2016).

WHO lists some factors that contribute to HAI; some of which are related to the patient state itself such as: prolonged hospitalization period, which increases the probability of acquiring an infection, invasive procedures that

may help the pathogens enter the human body, immune-suppression and relative patient's conditions. Other reasons are due to inadequate facility settings and limited resources: inadequate hygienic conditions and environment, poor infrastructure, understaffing, overcrowding and poor knowledge and application of basic infection control measures.

Furthermore, National Healthcare Safety Network with CDC for surveillance has classified HAI sites into 13 types, with 50 infection sites based on biological and clinical criteria. The most common sites are: urinary tract infections (UTI), surgical and soft tissue infections, gastroenteritis, meningitis and respiratory infections. However these sites are in constant rapid change following the rapid increase in HAI cases due to chemotherapy, organ transplant and new-sophisticated invasive techniques (Khan *et al*. 2015).

While protozoans, fungi, viruses and mycobacteria cause HAI, bacteria remain the main causative agent of HAI accounting for 90% of the cases (Bereket *et al*. 2012). In addition to the gram negative and positive bacteria, commensal species are also able to become pathogenic in immunosuppressed individuals (Khan *et al*. 2015) (Table. 1.1.1) (Figure 1.1.1). In fact, CDC in 2014 published the HAI Prevalence Survey, a project tackling the burden of HAI in USA acute care hospitals in 2011 (Table. 1.1.2).

Table 1.1.1: Most common nosocomial pathogens along with infection manifestations and mode of transmission

Nosocomial pathogen	Infection	Mode of transmission
Staphylococcus aureus	Superficial and deep tissues.	Skin and surface contact.
Escherichia coli	UTI, septicemia, pneumonia, neonatal meningitis and peritonitis gastroenteritis.	Skin and surface contact, contaminated food and water.
Vancomycin-resistant enterococci	Blood-borne infections, UTI and wound infections.	Patients with diarrhea; contact with surfaces in the patient's room
Klebsiella pneumoniae	Septicemia, pneumonia, and wound infections.	Contact with: Person to person, respiratory machines, catheters and open wounds
Pseudomonas aeruginosa	UTI, surgical wound infections, pneumonia, cystic fibrosis and bacteremia.	Skin contact with: Breast pumps, incubators, sinks and hand soups.

Clostridium difficile	Colitis.	Person to person and contact with hospital settings surfaces.

Table 1.1.2: Results of HAI Prevalence Survey

HAI Estimates in US Acute Care Hospitals, 2011

Major Site of Infection	**Estimated No.**
Pneumonia	157,500
Gastrointestinal Illness	123,100
Urinary Tract Infections (UTI)	93,300
Primary Bloodstream Infections	71,900
Surgical site infections from any inpatient surgery	157,500
Other types of infections	118,500
Estimated total number of infections in hospitals	**721,800**

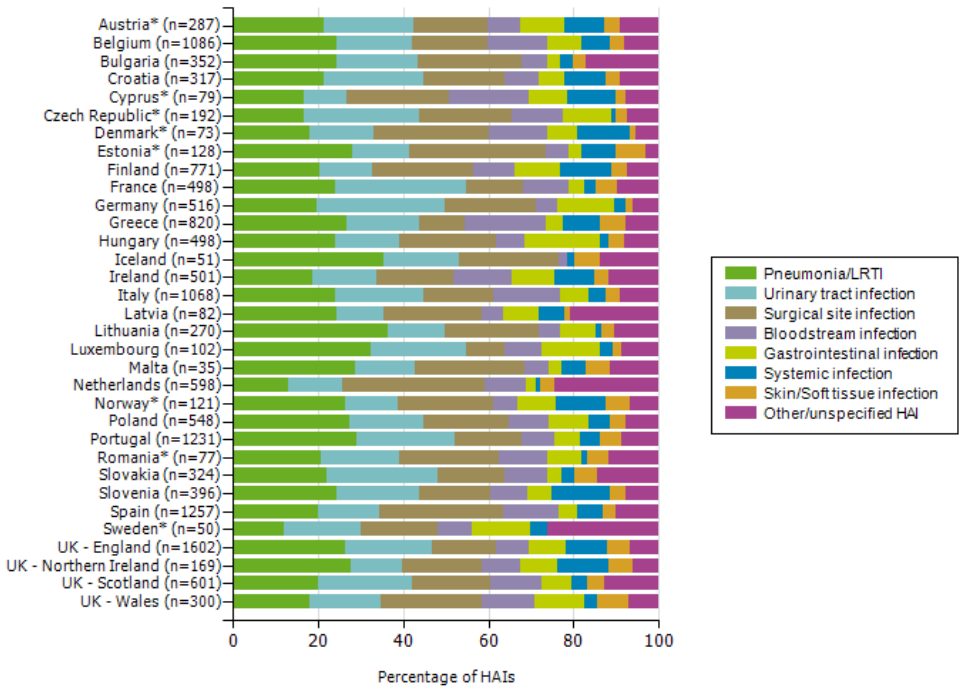

Figure 1.1.1: Distribution of HAI types in acute care hospitals by country (ECDC PPS 2011-2012)

Even though surfaces are usually disinfected using chemical liquid disinfectant in healthcare settings (mostly chlorine derivatives), which are known for their toxicity to humans as well as their slow effect (up to 15 mins) (Bagattini *et al*. 2015), HAI infections are mainly transmitted by contact to surfaces as indicated in Table 1.1.1, with the highest prevalence being at intensive care units (ICU) (Khan M. *et al*. 2015). To be more specific, ECDC coordinated point prevalence survey (PPS) of HAIs and antimicrobial use in acute care hospitals from 2011-2012. Results show that European Economic Area (EEA) had the highest HAIs prevalence for patient admitted to the ICU (Figure 1.1.2).

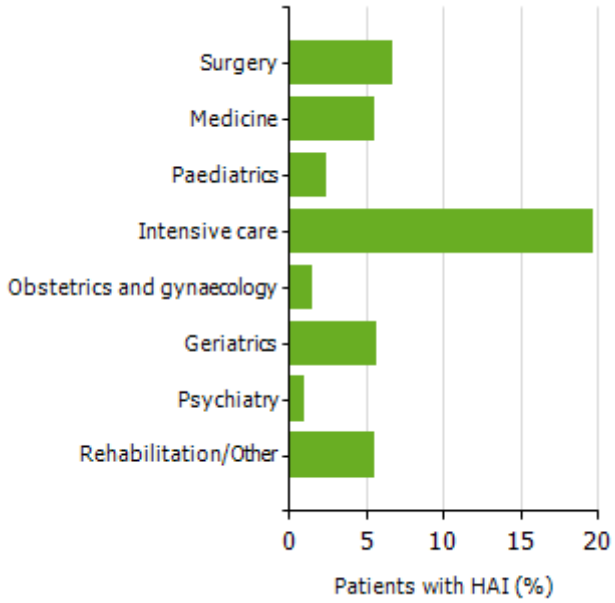

Figure 1.1.2: Prevalence of HAI types in acute care hospitals by specialty in EU/EEA (ECDC PPS 2011-2012)

1.2 Antimicrobial use and antimicrobial resistance

The discovery of antibiotics had a revolutionary impact on the field of medicine, to the extent of being called a turning point in human history for saving countless human lives. However, the excessive usage of these drugs lead to the rapid appearance of resistant bacterial strains (Davies J. and Davies D. 2010).

Penicillin was the first antibiotic to be discovered in 1928 by Alexander Fleming who by noticed that *Penicillium* mould inhibited the growth of bacteria in culture media. However, later on Norman Heatley, Ernst Chain and Howard Florey developed Penicillin as a drug in wartime in England.

In 1941, Florey and Heatley reached the US to seek help in the production of Penicillin. At that time, American companies were focused in collecting microbial metabolic products such as citric acid, which was used to flavor soft drinks and preservation of food's color and flavor in canning process. However as the United States of America entered the Second World War, the War Production Board was instrumental in taking these vat fermentation techniques to industrial pharmaceutical companies, mainly Pfizer. Pfizer was a new company in the market seeking production of Penicillin. By combining efforts of Pfizer and governmental agencies, university research scientists managed to rapidly produce penicillin in mass production amounts. In 1944, production was enough to treat American soldiers. By 1945, enough Penicillin was produced to be released in public market. Penicillin was called the "miracle drug, considering it low side effect relative to therapeutic agents present at that time such as sulfonamides. It managed to cure untreatable diseases with very high efficiency (Landecker 2015).

In 1944, streptomycin was discovered for treating tuberculosis however mutant strains started to arise during the treatment of patients. Moreover, in mid 1950s in Japan, genetically transferable antibiotic were identified that changed the whole picture by introducing the concept of bacterial conjugation. Conjugation is the process of collecting antibiotic resistant genes through which bacteria can share these mobile elements across an entire population. Until the past few years, gene exchange was recognized as a universal property of the bacteria responsible for the bacterial evolution along with resistance dissemination (Davies J. and Davies D. 2010).

The antibiotic discovery timeline started with penicillin in what was called the golden years where most of the known antibiotics were discovered. Then the lean years where the discovery and registration of new antibiotic where lower since scientists were trying to improve the use of antibiotic by regulating the doses and understanding more about the mode of action and biochemical properties to avoid resistance. Moreover, after the thalidomide disaster, the federal drug agency (FDA) of New Drugs led to stricter requirements drug safety, hence slower registration of novel compounds and the disenchantment phase. Because of the failure of enormous investment in

genome-based methods, many companies abandoned drug discovery research (Figure 1.2.1.).

Nevertheless, before antibiotics were discovered, Semmelweis asked people to wash their hands on a regular basis as a way to avoid infection. Today it is strongly recommended to wash hands as a method to prevent transmission (Davies J. and Davies D. 2010).

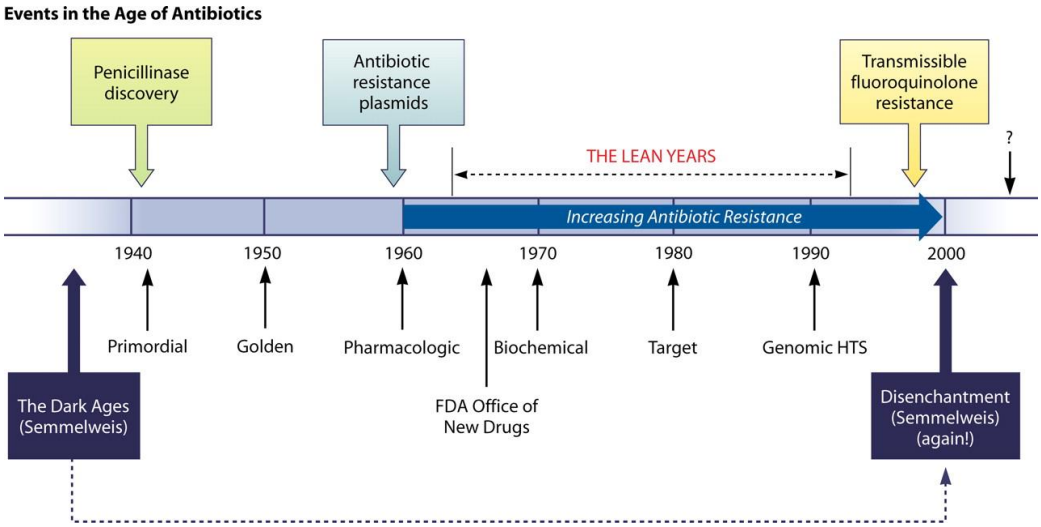

Figure 1.2.1: History of antibiotic discovery and concomitant development of antibiotic resistance

In 2012, WHO declared the evolution of antimicrobial resistance to be among the top three threats to human health. Mortality caused by the antimicrobial resistance matched that of car accidents and gun violence in USA. Moreover, in Europe mortality was estimated to be around 25,000 deaths per year according to ECDC in 2009 (Antonovics J. 2016).

Antibiotic resistance can be acquired mainly through transfer of antibiotic genes between bacteria, or the excessive use/misuse of antibiotics in humans and animals. This leads to clonal dissemination of pathogenic bacteria and emergence of Multi Drug Resistant (MDR) bacteria in which the bacterial strains were resistant to 3 or more antibiotic classes at once, with resistance mechanisms based on altering the target molecules of the used antibiotic (Aminov *et al*. 2007, AlanisA. 2005, Kadouri *et al*. 2013). Consequently, this diminishes the effectiveness of possible therapies to treat such infections, and therefore crates a global health problem. Even though HAIs

and MDR bacterial infections are two different concepts, the ICU patients infected by MDR bacteria had a major health problem considering the critical health situation of these patients. The ICU-HAIS is 5-10 times higher than HAI rates in other general wards due to the complex interaction between the patient's disease, severity, length of hospital stay and invasive procedures. Moreover, the risk of getting MDR infection in ICU is higher due to antimicrobial therapies used in ICU cross-transmission between patients and staff (Cornejo-Juarez *et al*. 2015).

In Europe, the occurrence of antimicrobial resistance varies depending on the antimicrobial use, the microorganisms and the geographic region. The ECDC in the PTT survey however indicates a north to south gradient, with lower resistance percentage in the northern part compared to that of the south (Figure 1.2.2.). This can be partially explained by the reports of antimicrobial consumption to the European Surveillance of Antimicrobial Consumption Network (ESAC-NET) in 2012. In Northern Europe, Netherland recorded a 11.3 defined daily doses (DDD) per 1000 inhabitants per day compared to 31.9 for Greece in the southern part of Europe (Figure 1.2.2).

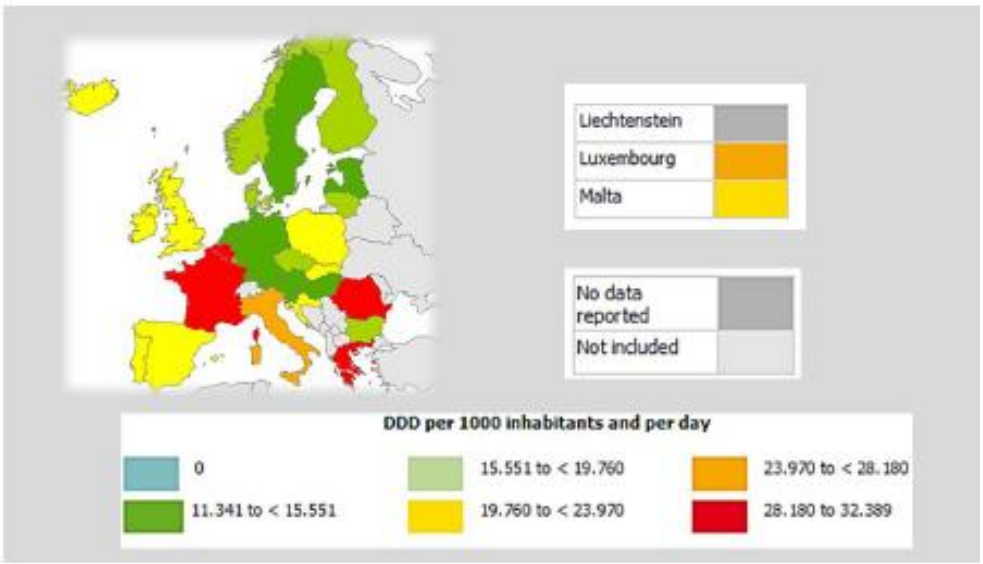

Figure 1.2.2: Geographic distribution of antimicrobial consumption of antibiotics in Europe in 2012

1.3. Antibacterial agents

Selman Waksman, the discoverer of streptomycin and a pioneer in screening soils for the presence of biological compounds, was the first to give the definition of antibiotics. It is simply a description of a use, a laboratory effect, or an activity of a chemical compound. It does not define a class of compound or its natural function, only its application (Davies *et al*. 2010). Accordingly, antibiotics fall into two categories: bacteriostatic and bactericidal, where the former inhibit bacterial growth, while the latter kill bacteria with a >99.9% efficiency. Similarly, the drug-target interaction is generally divided into three classes: DNA replication inhibitor, protein synthesis inhibitor and cell wall turnover inhibitor. Regarding the mode of action, bacteriostatic antibiotics act by inhibiting of ribosome function targeting 30S (tetracycline family and aminocyclitol family) and 50S (macrolide family and chloramphenicol) ribosome subunits, while bactericidal antibiotics and quinolones target DNA replication and repair. On the other hand, β-lactams inhibit cell wall synthesis by binding to penicillin binding protein, and glycopeptides interact with peptidoglycan building blocks (Kohanski *et al*. 2007). Table 1.3.1 shows a detailed list of the different antibiotics available with the available antibiotics and its relative target (Wong *et al*. 2012).

Table 1.3.1: List of antibiotic classes along with the different antibiotics within these categories and its primary targets

Antibiotic Class	Antibiotic Name	Primary Target
Cell Wall Synthesis Inhibitors		
β-lactams (A)	Penicillins cephalosporins [	Penicillin-binding proteins
Glycopeptides	Vancomycin	Peptidoglycan units terminal D-Ala-D-Ala dipeptide
Lipopeptides	Polymixin B	Lipopolysaccharide in the outer membrane
Others	Alafosfalin	Peptidoglycan units terminal D-Ala-D-Ala dipeptide
	Bacitracin	C_{55}-isoprenyl pyrophosphate
	D-cycloserine	D-alanine ligase and alanine racemase
	Fosfomycin	Uridine diphosphate (UDP)-N-acetylglucosamine-3-enolpyruvyltransferase
DNA Synthesis Inhibitors		
Fluoroquinolones	Nalidixic acid, ciprofloxacin, levofloxacin, sparfloxacin, norfloxacin	Topoisomerase II (DNA gyrase), topoisomerase IV
Sulfonamides	Sulfamethazine,	Competitive inhibitor for DHPS

	sulfapyridine, sulfamethoxazole, sulfadiazine, sulfamerazine	involved in folate synthesis
Others	Novobiocin	DNA gyrase
RNA Synthesis Inhibitors		
Rifamycins	Rifampicin, rifabutin, rifaximin	DNA-dependent RNA polymerase
Resistomycins	Resistomycin, resistoflavin	RNA polymerase
Protein Synthesis Inhibitors		
Tetracyclines	Oxytetracycline, doxycycline, tetracycline, demeclocycline, minocycline	30S ribosome (inhibit aminoacyl tRNA binding to ribosome)
Aminoglycosides	Tobramycin, gentamicin, amikacin, streptomycin, spectinomycin	30S ribosome (mistranslation by tRNA mismatching)
Macrolides	Erythromycin, clarithromycin, midecamycin, roxithromycin, spiramycin, azithromycin	50S ribosome (stimulating dissociation of the peptidyl-tRNA molecule from the ribosomes during elongation)
Amphenicols	Chloramphenicol, thiamphenicol, florfenicol	50S ribosome (inhibit elongation step)
Lincosamides	Clindamycin, lincomycin	50S ribosome (stimulate dissociation of the peptidyl-tRNA molecule from the ribosomes during elongation)
Pleuromutilins	Tiamulin	50S ribosome (prevent correct positioning of the cytosine-cytosine-adenine ends of tRNA for peptide transferase)
Others	Thiostrepton	50S ribosome (inhibits messenger RNA-tRNA translocation by the GTPase elongation factor G)
DNA Replication (Intercalators)		
Anthracyclines	Doxorubicin, epirubicin, idarubicin	Intercalate DNA/RNA strand and topoisomerase II
Others	Actinomycin D	Intercalates G-C base pairs and minor groove DNA at the transcription initiation complex
	Mithramycin	Intercalates GC-rich DNA strand
	Tetracenomycin	Intercalates DNA
Anaerobic DNA Inhibitors		
Nitrofurans	Furazolidone, nitrofurantoin	Highly reactive reduced form (by nitrofuran reductase)
Nitro-imidazole	Ornidazole	Damages bacterial DNA
Others		
	Antimycin A	Qi site of cytochrome C reductase
	Bafilomycin	Vacuolar-type H^+-ATPase (inhibits proton transport across membrane)
	Monensin	Membrane ionophore
	Netropsin	DNA replication (binds minor groove

		of AT-rich double stranded DNA)
	Nonactin	Membrane ionophore
	Salinomycin	Membrane ionophore
	Staurosporine	Protein kinase C (prevents ATP binding to the kinase)
	Streptonigrin	DNA and RNA synthesis (DNA and topoisomerase II)
	Tunicamycin	Glycoprotein synthesis (UDP-GlcNAc and Dol-P)
	Valinomycin	Membrane ionophore

1.4. General mechanisms of antimicrobial resistance

Antibiotic resistance can be classified into intrinsic resistance or acquired resistance. Intrinsic resistance mechanisms are naturally occurring due to resistance genes found in the bacterial chromosomes, such as AmpC β-lactamase of gram-negative bacteria and MDR efflux pumps found in many other bacteria. This resistance generates a resistance phenotype (Table 1.4.1.) (Davies J. and Davies D. 2010). In contrast, acquired resistance involves mutations in genes targeted by the antibiotic or the transfer of resistant genes and determinants through plasmids, bacteriophages, transposons and other mobile genetic materials (Figure 1.4.1.).

Table 1.4.1: Modes of action and resistance mechanisms of commonly used antibiotics

Antibiotic class	Example(s)	Target	Mode(s) of resistance
β-Lactams	Penicillins (ampicillin), cephalosporins (cephamycin), penems (meropenem), monobactams (aztreonam)	Peptidoglycan biosynthesis	Hydrolysis, efflux, altered target
Aminoglycosides	Gentamicin, streptomycin, spectinomycin	Translation	Phosphorylation, acetylation, nucleotidylation, efflux, altered target
Glycopeptides	Vancomycin, teicoplanin	Peptidoglycan biosynthesis	Reprogramming peptidoglycan biosynthesis
Tetracyclines	Minocycline, tigecycline	Translation	Monooxygenation, efflux, altered target
Macrolides	Erythromycin, azithromicin	Translation	Hydrolysis, glycosylation, phosphorylation, efflux, altered target
Lincosamides	Clindamycin	Translation	Nucleotidylation, efflux,

			altered target
Streptogramins	Synercid	Translation	C-O lyase (type B streptogramins), acetylation (type A streptogramins), efflux, altered target
Oxazolidinones	Linezolid	Translation	Efflux, altered target
Phenicols	Chloramphenicol	Translation	Acetylation, efflux, altered target
Quinolones	Ciprofloxacin	DNA replication	Acetylation, efflux, altered target
Pyrimidines	Trimethoprim	C_1metabolism	Efflux, altered target
Sulfonamides	Sulfamethoxazole	C_1metabolism	Efflux, altered target
Rifamycins	Rifampin	Transcription	ADP-ribosylation, efflux, altered target
Lipopeptides	Daptomycin	Cell membrane	Altered target
Cationic peptides	Colistin	Cell membrane	Altered target, efflux

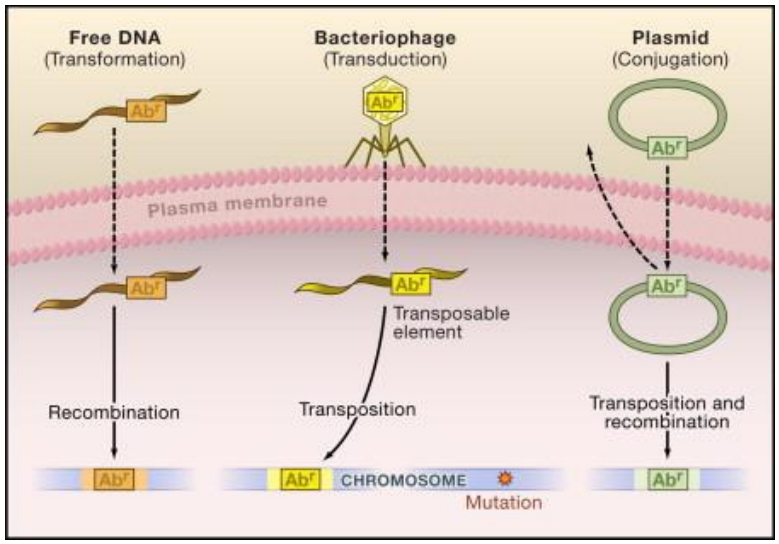

Figure 1.4.1: Acquisition of antibiotic resistance

Generally, exchange of resistance material can be achieved through transduction. A process through which the bacterial cell is infected by a bacteriophage, and the inserted DNA harbors resistant genes that will be later incorporated into the bacterial DNA. Another way is through conjugation, via plasmids and conjugative transposons. The exchange can be achieved as well through transformation by incorporating into the

chromosome chromosomal DNA, plasmids and other DNA pieces from dying organisms.

For instance, plasmids are considered one of the most resistant mechanisms responsible for the dissemination of MDR strains through horizontal gene transfer or conjugation. They replicate independently of the host chromosomes, they can carry more than one resistant gene in most of the cases along with other traits, and they can coexist with other plasmid/plasmids in the same cell. Most importantly, they can be transferred from one genus to the same, or to another distant bacteria belonging to other genera (Alekshun *et al*. 2007). Nevertheless, horizontal gene transfer has occurred through out evolutionary history long before the discovery and the usage of antibiotics. Thus, two sets of independent events, largely differentiated by their time span and the strength of selective pressure, can be distinguished. Therefore, plasmids did exist before the antibiotic era, mostly carrying multi-gene pathways responsible for biodegradation of xenobic molecules, such as phenolic compounds that were common in the days of the industrial revolution. However, the overuse of antibiotics at that time placed the bacteria in highly hostile environment favoring horizontal gene transfer of resistant genes (Davies J. and Davies D. 2010).

Alternatively, transposons are mobile genetic elements than can exist on plasmid, or integrate into other transposons or host chromosome. Conjugative transposons harbor plasmid qualities that can facilitate the transfer of endogenous plasmids from one organism to another (Alekshun *et al.* 2007). In 1987, Stokes and Hall identified and characterized integrons as unusual gene acquisition elements. After the first wave of resistant *Shigella* in Japan in 1950s, these elements were studied for 30 years before the integrin structure was identified. Integrons themselves are not mobile genetic elements; rather, they become so in association with a variety of transfer and insertion functions (Davies J. and Davies D. 2010). They contain a set of genes (gene cassettes). These can stably integrate into regions of DNA, where they deliver a single exchange containing multiple new genes mostly related to antimicrobial resistance (Alekshun *et al.* 2007) (Figure1.4.2.).

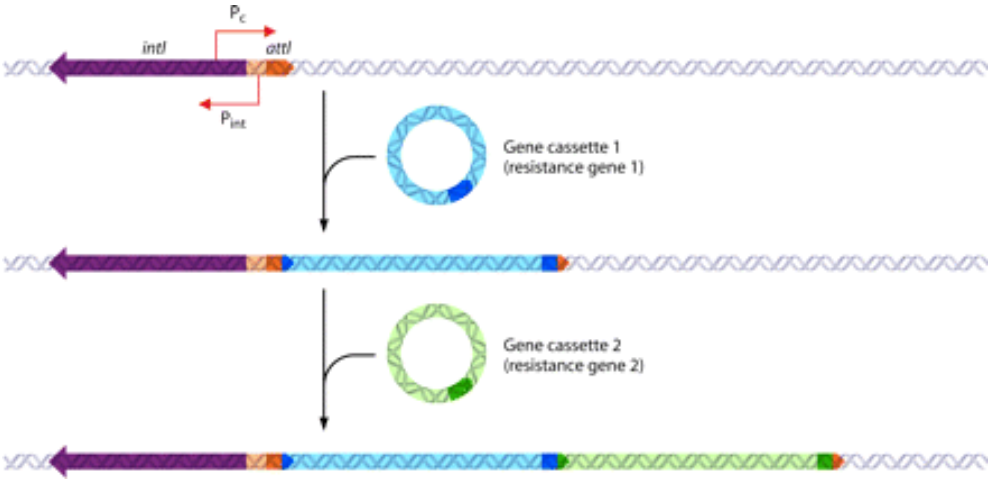

Figure 1.4.2: Integron structure and gene capture mechanism (Davies J. and Davies D. 2010)

1.5. β-Lactams: Mechanisms of Action and Resistance

β-Lactams drugs, one of the oldest antibiotics used in history, are know for its interference with the bacterial cell wall synthesis. The cell wall of the bacteria is an essential polysaccharide structure surrounding the cytoplasmic membrane and protecting it from osmotic rupture. It is build from the polymer peptidoglycan (PG); glycan chains with attached peptides used to crosslink glycans to form a matrix structure. β-Lactams disrupt PG biogenesis by inactivating penicillin-binding proteins (PBPs). The bacterial cell encodes plenty of different PBPs with different molecular weights that have different roles in the assembly of the cell wall. The lethal activity of β-lactams is referred to the loss of the integrity of the cell wall accompanied by cell lysis (Cho *et al*. 2014). One specific feature of the β-lactams antibiotics is the β-lactams ring system, a highly strained and reactive cyclic amide. Based on this, there are five relevant ring systems: penam, penem, carbapenem, cefem and monobactem ring structure.

Penams are a large group of β-lactams including penecillins. They possess a bicyclic structure, composed of an enclosed dipeptide, and formed by the condensation of L-cystein and D-valine resulting in the β-lactam ring. The reactive nature of this ring system makes it susceptible to a variety of degradative processes. In acid environments and room temperature, the β-

lactam ring undergoes a reconfiguration resulting in a malfunction of the antibiotics. This is an important aspect since the drug can not be administered orally, since the compound will be affected by the stomach's acidic environment. Moreover, many bacteria synthesize enzymes, known as penicillinases that will chemically degrade/inactivate β-Lactams. The most prevalent types of penicillinases are the β-Lactamases that attack and disrupt the β-lactam bond and inactivate it. Methicillin was the first synthesized molecule, after that, other molecules were synthesized such as oxacillin and cloxacillin by applying some modification on the biochemical structure. However these modifications resulted in a decrease in the efficiency of β-lactams. Nowadays other compounds are available such as clavulanic acid, tazobactam and sulbactams that bind to the β-Lactamases irreversibly, hence inactivating them.

Since 1970s, cephalosporins were the major representative group of Cephems widely used. They were developed in parallel to penecillins. Numerous classifications had been proposed: chemical, biological, microbiological, pharmacokinetic and immunological. The most widely used classification in microbiology is by default the microbiological classification, which divides cephalosporins into five generations. This classification is based on its antibacterial activity; they differ in their antimicrobial spectrum, β-Lactamase stability, absorption, metabolism, stability and side effects.

- First generation cephalosporins are the choice for treating Gram-positive cocci infections, except for enterococci and methicillin-resistant staphylococci. They have moderate activity against Gram-negative rods namely *Escherichia coli*, *Proteus* and *Klebsiella*. However, none of the first generation drugs penetrates the central nervous system and they can not to be chosen for treatment in case of an infection.
- Second generation cephalosporins are a heterogeneous group, in which all of them are active against organisms covered by the first generation drugs. However, they have an extended coverage against Gram-negative rods including *Klebsiella* and *Proteus* but not against *Pseudomonas aeruginosa*.
- Third generation cephalosporin have a decreased activity against Gram-positive cocci. However, its major advantage on second-generation drugs is that it has enhanced activity against Gram-negative rods such as Pseudomonas aeruginosa. This is very important in management of Gram-negative HAIs. Another important feature of some third generation

cephalosporins is their ability to reach the central nervous system and their ability to treat meningitis caused by Gram-negative rods.

- Fourth generation cephalosporines include only two drugs: Cefepime and Cefpirome. They have an enhanced activity against resistant *Enterobacter* and *Cirobacter* to third generation drugs.

- The fifth generation cephalosporins were developed in the laboratories to tackle resistance, mainly methicillin-resistant *S. aureus* (MRSA). However, this class of drugs is not effective against enterococci bacteria.

Monobactams have a monocyclic β-lactam ring, which is resistant to β-lactamases. They are active against Gram-negative rods but not against Gram-positive bacteria.

Imipenem was the first drug of the Carbapenems. It has good activity against Gram-positive bacteria as well as Gram-negative rods and anaerobes. However, it is administered with peptidase inhibitor since it is inactivated in the renal tubules by dihydropeptidases. Meropenem, a drug similar to imipenem, is used instead since it is not incapacitated in the renal tubule. This class of antibiotics penetrates the body tissues and fluids and can reach the central nervous system. Unfortunately, *Pseudomonas* species rapidly developed resistance to imipenem.

Based on the targets of the β-Lactam drugs, there are four basic mechanisms of resistance (Figure 1.5.1):

- alteration of the antimicrobial target due to loss of affinity;
- reduction of antimicrobial quantities entering the bacterial cell by decreasing permeability through porin mutation or increased exit through efflux pumps;
- presence of enzymatic mechanism that may partially/totally destroys the antibiotic;
- development of alternative metabolic pathway involving precursors.

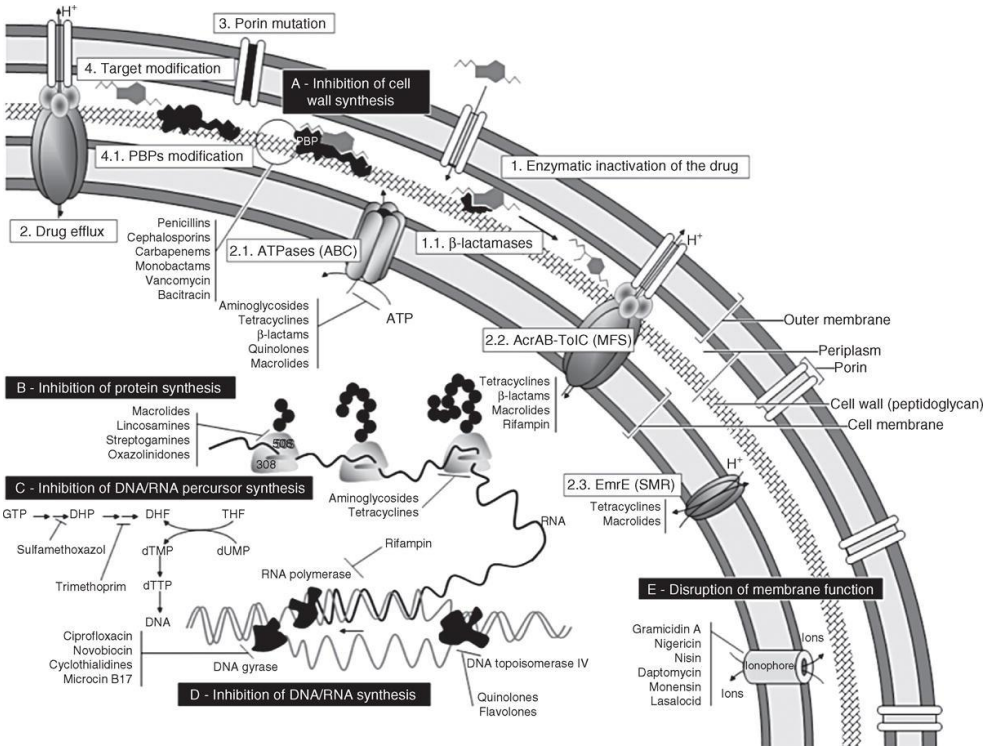

Figure 1.5.1. : Mechanisms of antibiotic action and resistance in Gram negative bacteria

The most important example of target alteration to β-lactams is the case of Methicillin resistance *S. aureus.* MRSA is due to the presence of *mecA* gene. Originally, *S. aureus* has four PBPs, where *mecA* gene encodes for a PBP2' that is different in structure from the wild type PBP2. This altered PBP will not be linked to β-Lactams and thus become insensitive to various β-lactams including methicillin.

Another mechanism of resistance to β-lactams is changing the permeability of the outer membrane. This could be achieved through the presence of efflux proteins or through the alteration/loss of porins. There are two types of efflux pumps: ATP-dependent transporter (ATP-binding cassette ABC), and secondary transporters driven by proton motive force PMF (MDR and toxic compound extrusion MATE family) (Fernandes *at al*. 2013).

Alternatively, the production of enzymes such as β-lactamases is one of the most common ways of resistance. It was first detected before the clinical use of penicillin in soil bacteria. Ambler was the first scientist to propose a classification system in 1980 based on structure. The new classification

divides β-lactamases into A, C, D (Serine residue in the active site) and B (Zinc dependent metalloenzyme in the active site) based on amino acid sequence (Alekshun M and Levy S. 2007). Another type of classification was the functional classification proposed by Bush and collaborators in 1989 and improved in 1995. Ambler β-lactamases classes A, C and D have an active site of serine enzymes, suggesting from the mechanistic point of view: an evolved PBPs. The intermediate catalyst formed attacks the β-lactam ring and inactivates it. TEM-1 was the first plasmid-mediated β-lactamase in Gram negative, isolated in 1960s from single *E. coli* strain obtained from a blood culture of a Greek patient. In fact, it was a plasmid and transposon mediated that quickly spread into other bacterial species. For that reason, extended-spectrum cephalosporins were used to treat these infections. However, the selective pressure applied lead to mutants of TEM genes resistant to extended-spectrum cephalosporins. Another common plasmid mediated β-lactamase found in *K. pneumoniae* and *E. coli* is SHV-1. It is chromosomally encoded in *K. pneumoniae* and usually plasmid mediated in *E. coli.*

Extended-spectrum β-lactamases (ESBLs), by definition, are β-lactamases that hydrolyze the third-generation cephalosporins, penicillins and narrow-spectrum cephalosporins. They were discovered in the early 1980s in Europe and since then, the prevalence of ESBLs are increasing worldwide in different variants. One important variant is the CTX-M (named after its affinity to cefotaxime) that was discovered in 1986 in Germany. CTX-M is the most prevalent of the ESBL nowadays, described in more than 50 different types (Figure 1.5.2.).

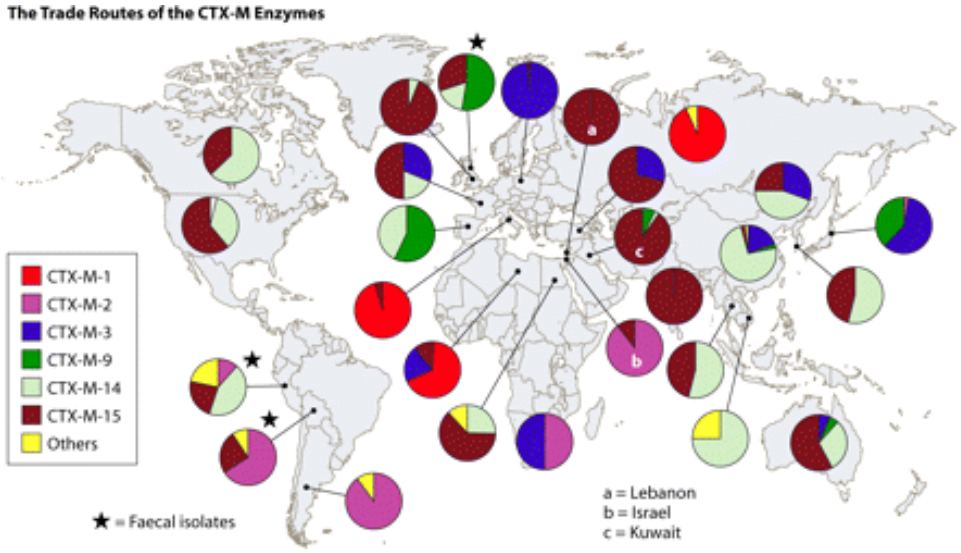

Figure 1.5.2. : Worldwide distribution of different classes of CTX-M β-lactamases (Davies J. and Davies D. 2010)

Class C ESBL, generally the chromosomal-encoded AmpC β-lactamases are widely distributed and generally expressed at low levels in infections. These enzymes are not generally considered to contribute to β-lactams resistance. However in some organisms, such as *Enterobacter cloacae* and *Citrobacter freundii*, it can be induced under certain circumstances when put under β-lactam pressure.

Class B β-lactamases, known as metallo- β-lactamases or MBL, uses zinc in its active site to activate water molecule and catalyze its addition to the β-lactam ring. The MBLs is though to be the major contributor to carbapenem antibiotic resistance. IMP-1 variant was the first discovered in Japan after the extensive use of carbapenem antibiotics.

1.6. Superbugs and Super resistance

The frequency of multi drug resistant (MDR) bacteria is increasing worldwide. The selective pressure of inappropriate use of antibiotic to treat infections is contributing to the spread and enhancement of these resistance traits. New variants for antibiotic resistance such as the New Delhi Metallo-β-lactamase (NDM-1) is considered a public alarm and considered as an extreme drug resistant trait. In 2010, the first " new superbug" was reported in India, a *bla*NDM-1 *Enterobacteriaceae*. Today, infections with NDM-1 and other variants as well from UK, United States, Sweden and many more

developed countries are being reported. This is possible due to the ease of international travel, which will contribute in the dissemination of such infections and the introduction of these strains into other continents (Khan et al. 2016). MDR strains are now associated with some infections, mainly caused by *Pseudomonas aeruginosa*, *Acinetobacter baumannii*, *Escherichia coli*, ESBL *Klebsiella pneumonia*, vancomycin resistant enterococci, methicillin-resistant *Staphylococcus aureus*, vancomycin-resistant MRSA strains, Extensively Drug-Resistant (XDR) *Mycobacterium tuberculosis* and NDM *Enterobacteriaceae* (Khan S. and Khan A. 2016).

1. *References*

Alanis, A. J. (2005). Resistance to antibiotics: are we in the post-antibiotic era?. *Archives of medical research*, *36*(6), 697-705.

Alekshun, M. N., & Levy, S. B. (2007). Molecular mechanisms of antibacterial multidrug resistance. *Cell*, *128*(6), 1037-1050.

Aminov, R. I., & Mackie, R. I. (2007). Evolution and ecology of antibiotic resistance genes. *FEMS microbiology letters*, *271*(2), 147-161.

Antonovics, J. (2016). The value of concept: lessons from the evolution of antibiotic resistance. *Global Policy*, *7*(S1), 97-106.

Bagattini, M., Buonocore, R., Giannouli, M., Mattiacci, D., Bellopede, R., Grimaldi, N., ... & Triassi, M. (2015). Effect of treatment with an overheated dry-saturated steam vapour disinfection system on multidrug and extensively drug-resistant nosocomial pathogens and comparison with sodium hypochlorite activity. *BMC research notes*, *8*(1), 551.

Bereket, W., Hemalatha, K., Getenet, B., Wondwossen, T., Solomon, A., Zeynudin, A., & Kannan, S. (2012). Update on bacterial nosocomial infections. *Eur Rev Med Pharmacol Sci*, *16*(8), 1039-44.

Cardoso, T., Almeida, M., Friedman, N. D., Aragão, I., Costa-Pereira, A., Sarmento, A. E., & Azevedo, L. (2014). Classification of healthcare-associated infection: a systematic review 10 years after the first proposal.*BMC medicine*, *12*(1), 1.

Cho, H., Uehara, T., & Bernhardt, T. G. (2014). Beta-lactam antibiotics induce a lethal malfunctioning of the bacterial cell wall synthesis machinery. *Cell*,*159*(6), 1300-1311.

Cornejo-Juárez, P., Vilar-Compte, D., Pérez-Jiménez, C., Namendys-Silva, S. A., Sandoval-Hernández, S., & Volkow-Fernández, P. (2015). The impact of hospital-acquired infections with multidrug-resistant bacteria in an oncology intensive care unit. *International Journal of Infectious Diseases*, *31*, 31-34.

Davies, J., & Davies, D. (2010). Origins and evolution of antibiotic resistance.*Microbiology and Molecular Biology Reviews*, *74*(3), 417-433.

Fernandes, R., Amador, P., & Prudêncio, C. (2013). β-Lactams: chemical structure, mode of action and mechanisms of resistance. *Reviews in Medical Microbiology*, *24*(1), 7-17.

Kadouri, D. E., To, K., Shanks, R. M., & Doi, Y. (2013). Predatory bacteria: a potential ally against multidrug-resistant Gram-negative pathogens. *PLoS One*, *8*(5), e63397.

Khan, H. A., Ahmad, A., & Mehboob, R. (2015). Nosocomial infections and their control strategies. *Asian Pacific Journal of Tropical Biomedicine*, *5*(7), 509-514.

Khan, M. S., Kundra, P., Cherian, A., Joseph, N. M., & Sistla, S. (2015). Epidemiology of nosocomial infections in an intensive care unit at a tertiary care hospital in India: A retrospective study. *International Journal of Infection Control*, *11*(2).

Khan, E., Irfan, S., Hasan, R., & Nasir, A. (2016). Dissemination and spread of New Delhi Metallo-beta-lactamase-1 Superbugs in hospital settings.*JPMA. The Journal of the Pakistan Medical Association*, *66*(8), 999.

Khan, S. N., & Khan, A. U. (2016). Breaking the Spell: Combating Multidrug Resistant 'Superbugs'. *Frontiers in microbiology*, *7*.

Kohanski, M. A., Dwyer, D. J., Hayete, B., Lawrence, C. A., & Collins, J. J. (2007). A common mechanism of cellular death induced by bactericidal antibiotics. *Cell*, *130*(5), 797-810.

Landecker, H. (2015). Antibiotic resistance and the biology of history. *Body & Society*, 1357034X14561341.

Park, K. S., Huang, C. H., Lee, K., Yoo, Y. E., Castro, C. M., Weissleder, R., & Lee, H. (2016). Rapid identification of health care–associated infections with an integrated fluorescence anisotropy system. *Science Advances*, *2*(5), e1600300.

Simões, A. S., Couto, I., Toscano, C., Gonçalves, E., Póvoa, P., Viveiros, M., & Lapão, L. V. (2016). Prevention and control of antimicrobial resistant healthcare associated infections: the Microbiology Laboratory. .*Frontiers in Microbiology*, *7*, 855.

Wong, W. R., Oliver, A. G., & Linington, R. G. (2012). Development of antibiotic activity profile screening for the classification and discovery of natural product antibiotics. *Chemistry & biology*, *19*(11), 1483-1495.

Chapter 2

Acinetobacter baumannii ST78 Italian Clinical Strains: a hypothesis of MDR to XDR evolution

I. Bitar[1], S. Gaiarsa[2], L. Villa[5] A. Piazza[1], M. Corbella[2], D. Sassera[3], R. Migliavacca[1], E. Scaltriti[4], P. Marone[2], A. Carattoli[5] and L. Pagani[1]

[1]C.S.D.P.S Department, Microbiology and Clinical Microbiology Unit, University of Pavia, Italy
[2] Microbiology and Virology Unit, Foundation IRCCS Policlinico S. Matteo, Pavia
[3]Department of Biology and Biotechnology, University of Pavia, Italy
[4]Istituto Zooprofilattico Sperimentale della Lombardia e dell'Emilia Romagna – Diagnostic Unit of Parma, Italy
[5]Istittuto Superiorie Di Sanita, Rome Italy

Part of this work was presented orally in San Diego, ICAAC/ICC 17-21 September 2015 and Desanzano del Garda, 23-24 October 2015 and as a poster in Rimini, Amicli 19-21 October 2015

2. Abstract

Since antibiotic resistance and long-term persistence in the hospital environment are two factors that most likely contribute to the success of *A. baumannii* as an opportunistic pathogen.

Nine ST78 *A. baumannii* strains (previously identified as "SMAL" by PFGE) obtained during the period of 2002-2012 from 7 Italian hospitals were included in the study. Identification and antimicrobial susceptibilities were determined using Autoscan 4 System. Species identification was confirmed by Vitek MS. PFGE and MLST (Pasteur's scheme) analysis allowed genotyping. NGS was performed using an Illumina MiSeq platform. Genome assembly was performed using MIRA 4 and presence of antibiotic resistance genes were determined using ResFinder.

All the analyzed isolates showed Multi-Drug Resistant (MDR) profiles. The first two strains, isolated in 2002 and 2006, presented the *floR*, *sul2*, *aph(3')-Ic* and *bla*OXA-90 resistance genes, while retaining carbapenem susceptibility. Among isolates obtained from 2009 to 2012, resistance to carbapenems and tetracycline was found, while susceptibility to colistin and tigecycline was maintained. Genomic analysis of this extensively drug resistant (XDR) isolates identified the presence of *bla*OXA-23 and/or *bla*OXA-58.

Our results suggest that, during the study timeframe, isolates belonging to ST78 acquired novel antibiotic resistance genes. Additional phylogenomic analyses and epidemiological screenings are needed to confirm this hypothesis. To our knowledge, this is the first study on the genomic evolution in an epidemic *A. baumannii* genotype (i.e. ST78) beside the three main ICs.

2. Acknowledgments

A particular thanks to:
Dott. D. Sassera and his collegues, for technical support in bioinformatic analysis.
All the IRCCS Institutions for the clinical and epidemiological informations provided together with the *Acinetobacter baumannii* strains.

2. Introduction

Acinetobacter baumannii has emerged in recent years as a leading cause of nosocomial infections, especially in ICUs, becoming a public health problem of major concern in several countries (Zarrilli *et al.* 2013). Of note, cases of community-acquired infections are also increasingly reported (Eveillard *et al*. 2013). The ability to survive in the environment during prolonged periods of time, combined with its innate resistance to desiccation and disinfectants, makes *A. baumannii* difficult to eradicate from the clinical setting.

Extensive use of antimicrobial chemotherapy, particularly carbapenems, has contributed to the emergence of carbapenem-resistant *A. baumannii* (CRAb) that usually exhibit a MDR phenotype (Perez *et al*., 2007). For critically ill patients with MDR infections, therapeutic options are limited and colistin remains the last resource for treatment (Kempf *et al.* 2012). Carbapenem resistance is mostly associated with production of carbapenem-hydrolysing class D β-lactamases, including the acquired OXA-23, OXA-24, OXA-58 and OXA-143, OXA-235 as well as the intrinsic OXA-51 enzyme (Patel *et al*. 2013; Higgins *et al*. 2013). The significant contribution of OXA-type carbapenemases in *A. baumannii* has been emphasized, particularly when *bla*OXA genes are associated with IS*Aba* sequences which provide strong promoters for their expression (Roca *et al*. 2012). Comparative typing of outbreak strains of *A. baumannii* from geographically scattered European hospitals demonstrated the occurrence of three successful clones originally named ―European clones I-III‖ and now renamed as ―International clones I-III‖ (ICs), being distributed worldwide. In addition to these major clones, a wide geographic distribution of some other clones has been reported (Zarrilli *et al*. 2013). MLST analysis conducted on 496 *A. baumannii* strains isolated worldwide identified seventeen clones (considering either clonal complexes, CCs, and STs) distributed also in European countries, with six clones appearing restricted only to Europe (Karah *et al*. 2012).

In Italy, hospital outbreaks caused by CRAb isolates have been repeatedly reported during the last years (Mendes *et al.* 2009; D'Arezzo *et al*. 2011; Mammina *et al*. 2012; Brigante *et al*. 2013). Overall, CRAb Italian isolates were mostly related to the production of OXA-58 carbapenemases and belonged to IC-II, while strains genetically related to IC-I and III were found to be less common (D'Arezzo *et al*. 2011; Migliavacca *et al*. 2013; Mezzatesta *et al*. 2012). Italian CRAb isolates were characterized by:

different STs (e.g., ST1, ST2, ST4, ST20, ST78, ST95, ST109, ST196 and ST197) and sequence groups (SGs, including SG1, SG2, SG5 and SG6). Thus highlighting the presence of international and national clonal *5* lineages in Italy (Carretto *et al*. 2011; Mezzatesta *et al*. 2012; Migliavacca *et al*. 2013). A high proportion of CRAb among bloodstream isolates in Italy has recently been reported by the EARS-Net surveillance system, which has started to monitor *Acinetobacter* resistance in Europe since 2012 (EARS-Net, 2013).

2. Literature review

2.1. *Acinetobacter baumannii* overview

In 1960s, *Acinetobacter* infection was reported for the first time in a hospital setting in ICU. Phenotypic test was unable to determine the species at that time. Yet the presence of *Acinetobacter* in hospital settings allowed the bacteria to be in contact with antibiotics, which applied a selective pressure on the bacteria enabling it to acquire resistant genes.

The genus *Acinetobacter* was first described in 1911, it means non-motile in Greek, and described in 1954 by Brisou and Prevot, yet accepted in 1968. In 1974, the designation was included in *Bergey's Manual of Systematic Bacteriology* and included one species: *Acinetobacter calcoaceticus*. Later in 1986, Bouvet and Girmont observed inconsistencies in phenotypic tests, where a member of this genus had a different metabolic pathway that allowed it to adapt to almost all substrates. Today there are 32 geno-species with *A. baumannii* being the most important since it is frequently isolated in nosocomial infections.

In the period of 1960s-1970s, hospital reports of infection with *Acinetobacter* were recorded in the United States and Europe. Treating patients with β-lactams and sulfonamides easily contained these nosocomial infections. By the end of 1970, *Acinetobacter* was reported to be resistant to sulfonamides, *β*-lactams and aminoglycosides that were considered the treatment of choice in any infection. Moreover it was able to cause outbreaks in hospital settings

In the period of 1980s-1990s, the genetic species was designated and identification of *A. baumannii* was established in 1986. At the same time outbreak analysis studies confirmed that *A. baumannii* was the leading pathogen in nosocomial infections accompanied with increasing antimicrobial resistance. In order to contain these outbreaks, European countries started to search for risk factors and concluded an association between ventilator use and the bacterial strain resistant to more than three antibiotic groups on one hand, and increased mortality on the other hand. These strains were designated as multidrug resistant MDR strains. In 1985, carbapenems emerged and were used to treat such infections, unfortunately resistant strains emerged later in the same year. The enzymes resistant to

such antibiotics were OXA- *β*-lactamases, which were widespread among infectious microorganisms. The increasing number of reports with *A. baumannii* infections led the United States to establish a set of specific features associated with these infections, leading to an important observation; the increased frequency of *A. baumannii* infections is correlated with progressive increase in antibiotic resistance (Figure 2.1.1.).

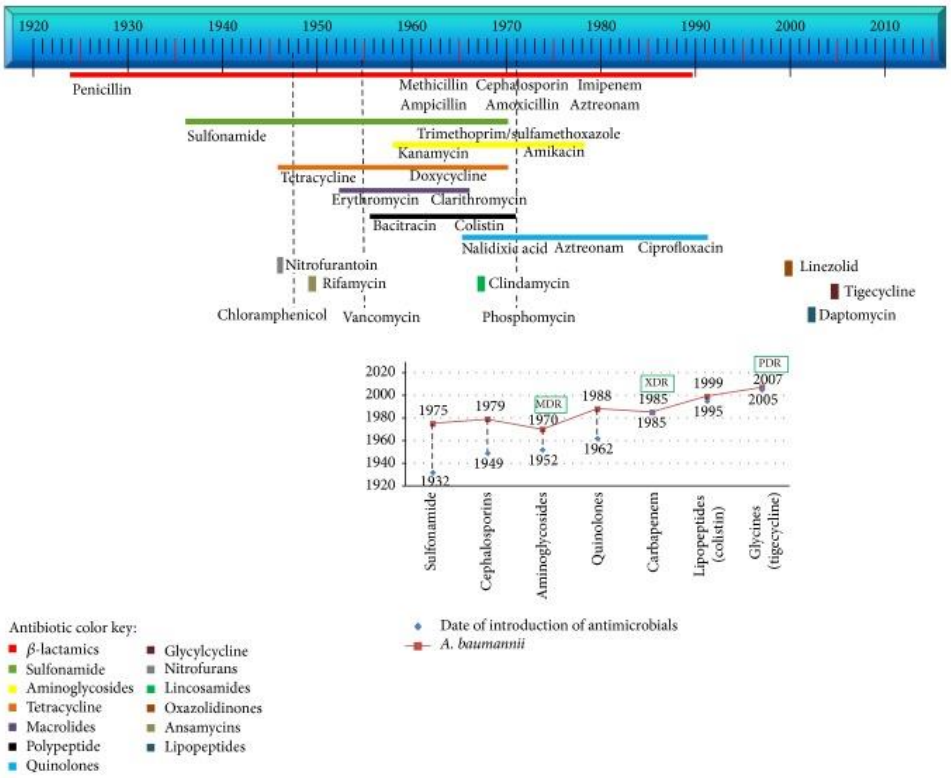

Figure 2.1.1: Top diagram shows dates of introduction of antimicrobial agents, insert graph shows the date of introduction of antimicrobials, date of first report of resistance in *A .baumannii*, emergence of MDR, XDR and pandrug-resistant strains.

The period of 2000-2015 was characterized by a dissemination of carbapenem-resistant *A. baumannii* (CRAB) and the creation of international surveillance networks. In 2001, the first international call for containment was issued by WHO in order to slow down the emergence of bacterial resistance. It was described in "Global Strategy for Containment of Antimicrobial Resistance" introducing bacteria involved in outbreaks associated with high-level resistance leading to public health threat. This group of microorganisms is designated by the acronym ESKAPE (Gonzalez-Villoria A. and Valverde-Garduno V. *et al*. 2016). ESKAPE

bacteria *(Enterococcus faecium, Staphylococcus auerus, Klebsiella pneumoniae, Acinetobacter baumannii, Pseudomonas aeruginosa*, and the *Enterobacter* species) are considered a collection of the most common nosocomial pathogens that escape the effects of clinical antibiotics. Their emergence became of a global concern especially since these pathogens are associated with increasing trends of acquiring antimicrobial resistance (Crooks J. *et al*.2012). The shift from local scale to global scale surveillance was made to contain any endemic infections; this lead to the establishment of European Antimicrobial Resistance Surveillance System (EARSS) in 1998 which was renamed as European Antimicrobial Resistance Surveillance Network (EARS-Net) in 2010 which is controlled by ECDC (Gonzalez-Villoria A. and Valverde-Garduno V. *et al*. 2016) (Figure 2.1.2.).

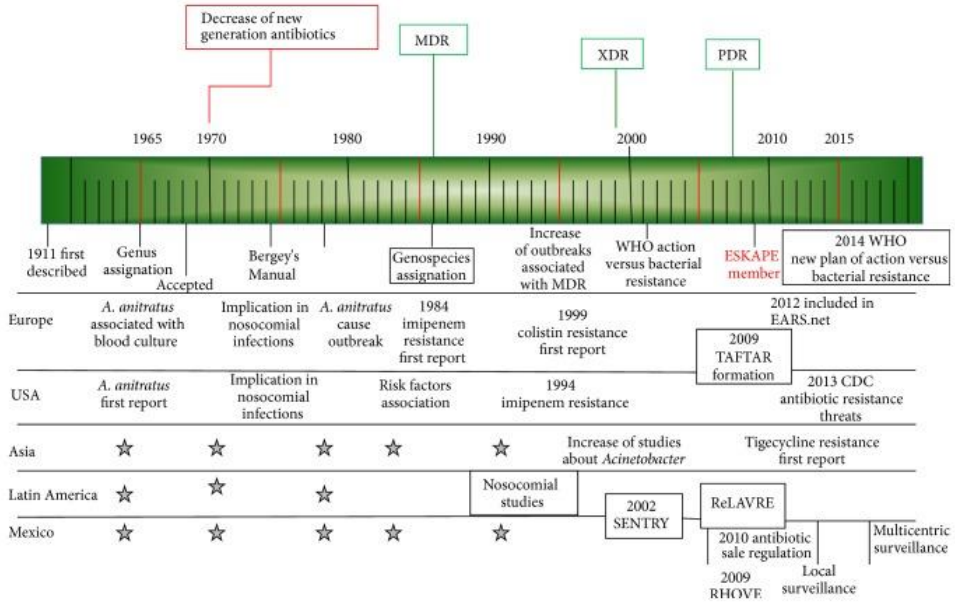

Figure 2.1.2: History of the incorporation of *Acinetobacter baumannii* as one of the successful multidrug-resistant nosocomial pathogens. This has led to the strategic alliance of different countries and continents in monitoring resistant bacteria, and it has resulted in the formulation of a new action plan against bacterial resistance in 2015.

2.2. *A. baumannii* OXA producers

Carbapenem resistance in *A. baumanni* is usually mediated by OXAs and less frequently by MBLs. There are four main OXA subgroups related to *A. baumannii*: the chromosomal OXA-51-like, and the acquired OXA-23-like, OXA-40-like and OXA-58-like. Although OXAs exhibit low activity

against carbapenems and doesn't enable the bacteria to develop resistance, some insertion elements increase the expression of carbapenemase if they are found upstream the OXA gene (Higgins P. *et al*. 2010).

OXA-51-like enzymes consist of 40 sequence-variants contribution to the resistance of carbapenems in a very low level unless associated with the presence of IS*Aba1* upstream of the gene; this insertion sequence will act as a strong transcriptional promoter, which will modulate the expression of the OXA-51-like genes leading to overexpressed levels of carbapenemase. This will lead to the resistance of the corresponding strain to carbapenems (Gordon N. and Wareham D. *et al* 2010).

The first report of *A. baumannii* OXA-58 producer was in in France in 2003. After that, many reports were recorded in different geographic areas (Giordano A. et al. 2007). OXA-58 is a widely spread carbapenem – hydrolyzing class D - *β*-lactamases (CHDLs) reported in *A. baumannii*. It has been reported in Europe, Australia, Argentina, United States and many other Asian countries. OXA-58 also show a low activity against carbapenems unless having an insertion element upstream the gene. *bla*OXA-58 is usually plasmid mediated which explains the wide dissemination. Moreover, another characteristic is the plasmid replicase gene *repAci1* associated with the plasmid carrying the *bla*OXA-58 (Fu Y. *et al*. 2014).

The first report of *A. baumannii* (OXA-23 producer) was in Scotland in 1995. It has also been reported world wide including Italy and Greece with an increased incidents compared to the rest of Europe (Brigante G. *et al* 2012). The first report of carbapenem resistant *A. baumannii* (OXA-23 producer) in Brazil was in 2003. A local outbreak, with eight isolates collected from two hospitals showing to be a part of the same clone. This suggests the possibility of inter-hospital outbreak. In 2009 another outbreak occurred in Rio de Janeiro and later on in other territories in Brazil. Even though the spread of this gene is associated with mobile elements and transposons (such as Tn2006) and plasmids, reports from all around the globe of *bla*OXA-23 encoded chromosomally have been recorded with insertion sequence, IS*Aba1* upstream leading the overexpression of the gene (Chagas T. *et al*. 2014).

2. Aim of the Study

Assess by NGS the:

- Antimicrobial-resistance evolution in a collection of *A. baumannii* strains belonging to ST78 lineage and responsible for outbreaks in Italy since 2002.
- Genomic evolution of this strain in 10 years period.
- Detect genomic characterization associated with this clone.

2. Materials and methods

DNA extraction and sequencing
SMAL strains (as detected in previous publications) were streaked on MacConkey plates at 37°C overnight. One single colony per strain was used for the downstream genomic analyses treated in this work. DNA was extracted with the NucleoSpin® Tissue kit by (MACHERBEY-NAGEL) and sequenced with the Illumina MiSeq technology, using Nextera XT kits for library preparation.

Genome assembly
Reads were assembled with the Mira 4.0 assembler (http://www.chevreux.org/projects_mira.html) using the default settings for Illumina reads and excluding the control for high coverage.

Global database of *Acinetobacter baumannii* genomes
All available *A. baumannii* genomes (April 2016) were downloaded from the NCBI website. All genomes were merged to those sequenced in this work to form the global database of sequenced strains of this species. The Multilocus Sequence Type of all genomes was determined using an in-house script and the Pasteur profiling scheme.

Genomic sequences were aligned to each other using an in-house Perl script and the Mauve software (http://darlinglab.org/mauve/mauve.html) Small Nucleotide Polymorphisms (SNPs) were extracted from regions were all genomes aligned to the others.

Global phylogeny of *A. baumannii*
SNPs were used to investigate the evolution of the species. The software fasttree (http://www.microbesonline.org/fasttree/) was used to build a maximum likelihood phylogeny using the alignment of single nucleotide variants as input.

Fine phylogeny of the SMAL and closely related strains
Eighteen genomes were aligned to the evolutionary closest available complete genome (i.e. AB031, according to the global phylogeny). Each global genomic alignment was performed using the software Mauve and a

set of in-house Perl and Python scripts for output formatting. A global alignment of the 18 genomes of interest was obtained and used to extract SNPs, which are used for phylogeny. The evolutionary analysis was performed using the software RAxML with the ASC_GTRGAMMA evolution model and 100 bootstrap replicates, using the ascertainment bias correction of Lewis.

Recombination analysis

The presence of recombination in the dataset was tested using the software Clonal Frame ML with the EM model on the 18 genomes alignment, excluding all non-SNP positions. Transition on trans version ratio was calculated using the software PhyML; dispersion in parameters among branches was set to 1.0.

***In-silico* Plasmid extraction and characterization**

Assembeled genomes and contigs were uploaded to PlasmidFinder database to detect which contig contains the plasmid replication initiation site (www.cge.cbs.dtu.dk/services/**PlasmidFinder**/), while resistant genes were determined by uploading the contigs to ResFinder database (www.cge.cbs.dtu.dk/services/**ResFinder**/). The process of closing plasmids *In-silico* was done using Bandage (https://rrwick.github.io/**Bandage**/). ORFs and their relative amino acids were detected using Artemis (www.sanger.ac.uk/science/tools/**artemis).** Plasmid annotation was done manually using Sequin and the files were uploaded to Genbank (www.ncbi.nlm.nih.gov/**Sequin)**

Note: the output files of Mira 4 could not be used with Bandage, for that reason, genome was assembled using Spades 3.8, which generate a graph that can be read using Bandage (www.bioinf.spbau.ru/spades).

2.Results

Nine isolates were selected from the strains collected from 2002 till 201). These isolates were selected as representatives of the PFGE clades and based on the antimicrobial susceptibility profiles. All the samples were identified as SMAL isolates based on PFGE (Figure 2.1, Table 2.1).

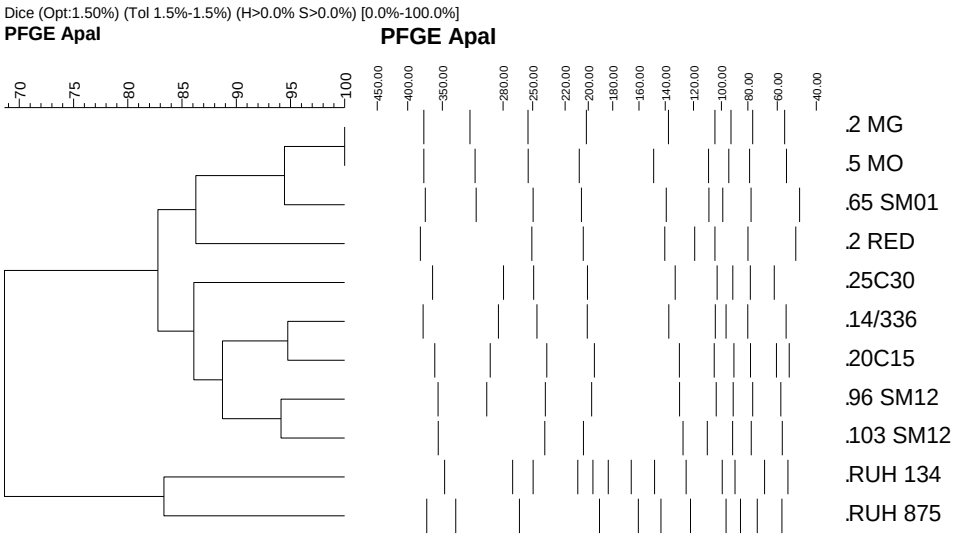

Figure 2.1: PFGE dendogram; the PFGE of the 9 isolates with the 2 outgroups (G1,G2)

Table 2.1: antibiotic profile of the 9 strains selected

Strain	AK	CAZ	CIP	FEP	GM	IMP	MER	TGC	CO
AB2MG	>16	>8	>1	>8	>4	<=2	<=2	<=1	<=2
AB65SM	>16	>8	>1	>8	>4	<=2	<=2	<=1	<=2
AB5MO	>16	>8	>1	>8	>4	>8	>8	<=1	<=2
AB2RED	>16	>8	>1	>8	>4	>8	>8	<=1	<=2
AB14336	>16	>8	>1	>8	>4	>8	>8	<=1	<=2
AB20C15	>16	>8	>1	>8	>4	>8	>8	<=1	<=2
AB25C30	>16	>8	>1	>8	>4	>8	>8	<=1	<=2
AB96SM	>16	>8	>1	>8	>4	<=2	<=2	<=1	<=2
AB103SM	<=8	>8	>1	>8	>4	<=2	<=2	<=1	<=2

The total DNA of the 9 isolates of *Acinetobacter baumannii* was sequenced using the Illumina MiSeq technology. An average of 250 read pairs was obtained per each sample. Reads were assembled using the software Mira and the resulting draft genomes had an average genome size of 4,016,569 and an average N50 of 69,560 nt.

All available genomes of the species *A. baumannii* were retrieved from the NCBI database (n=1043) and joined with those of the isolates presented in this work, to obtain a database of 1052 genomes. The MLST of each genome was determined in silico using the Pasteur classification. Genomes were aligned to each other and core SNPs were called in order to perform a Maximum Likelihood phylogeny. The SMAL isolates were assigned to the ST78 (126 different sequence type was detected) and clustered on the global tree in a single monophylum, together with 9 database genomes of the same sequence type (Table 2.2).

Table 2.2: number of genomes belonging to different STs

	ST 2	**ST 78**	**Others**
Genome #	614	18	420
Percentages	58 %	1.7 %	40.3 %

The ST78 monophylum was further investigated. Genomes were aligned to an evolutionary related complete genome, chosen in light of the global phylogeny (*A. baumannii* Ab031) and core SNPs were called. The resulting alignment of 33,394 core SNPs was used to perform a phylogeny of the ST78 (the tree is reported in Figure 2.2 and 2.3).

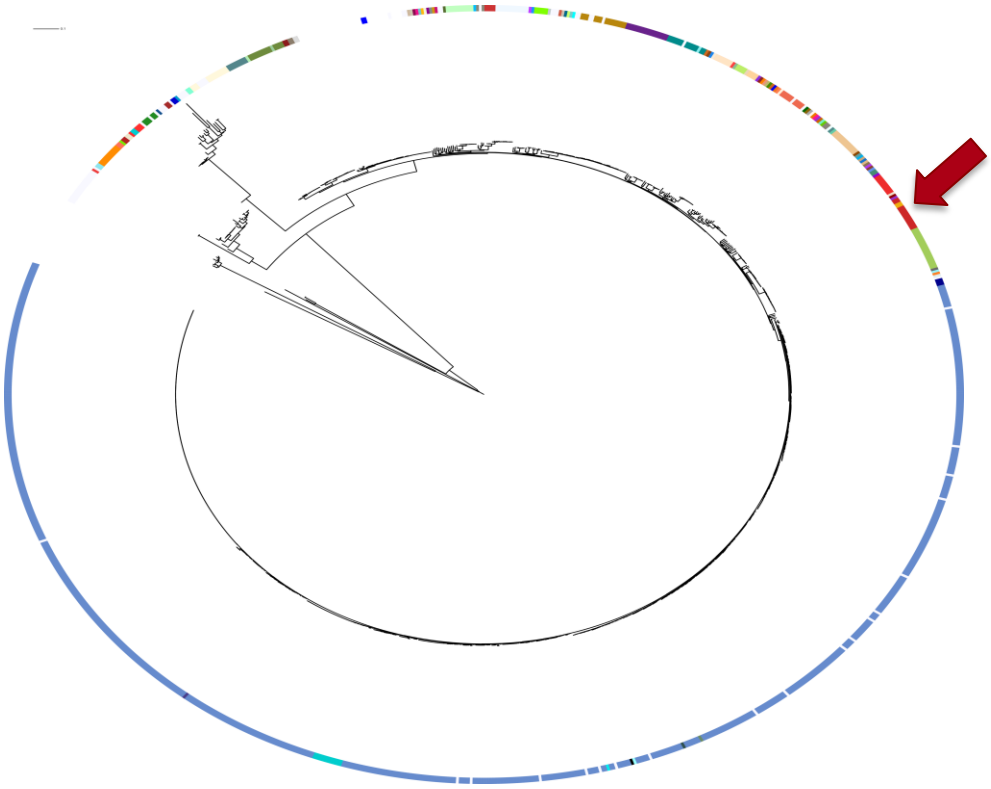

Figure 2.2: circular SNPS phylogenetic tree, of the 1052 genome with the light blue colour corresponding to ST2 and the red arrow indicating ST 78.

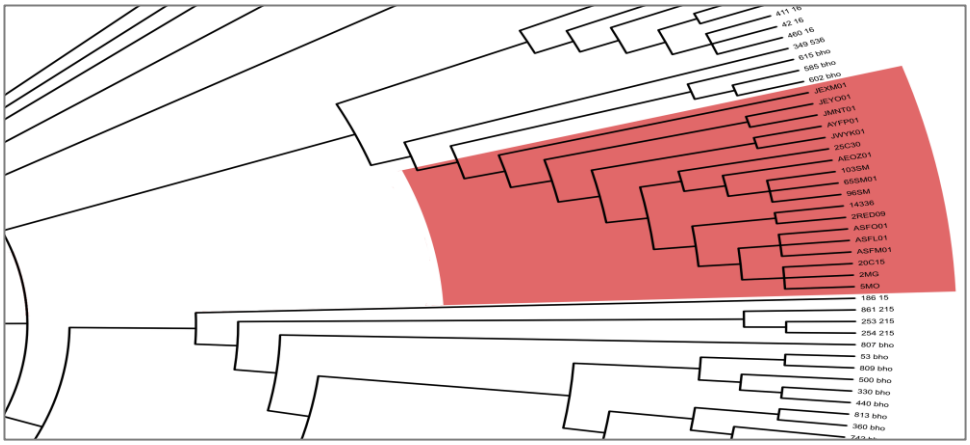

Figure 2.3: ST78 clade including all the 18 genomes.

Taxa were split in two well-supported monophyletic groups and a single evolutionary distant genome (strain AB0025). The nine strains in analysis fit

all in one of the two groups, which contained also four other strains. SMAL isolates were once again clustered in a single monophylum, together with strain 3909. Analysis of available metadata revealed that strain 3909 was isolated in Italy (Zarrilli *et al.* 2011, NCBI reference sequence: NZ_AEOZ00000000). The isolate was requested to the hospital of origin and analyzed with Pulsed Field Gel Electrophoresis. The resulting pattern of migration showed that isolate 3909 belongs to the pulsotype SMAL as well (results not shown). For this reason, from now on, the 10 genomes in this monophylum will be addressed as the SMAL cluster (Figure 2.4).

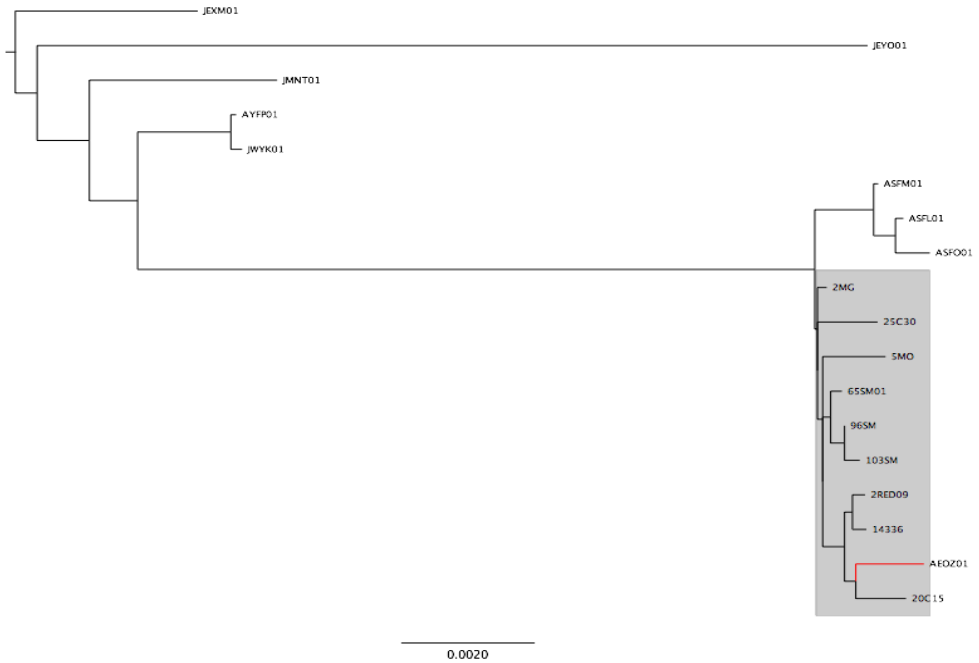

Figure 2.4: SNP based tree (highlighted in grey are the SMAL strains and the red branch corresponds to the strain 3909).

All SMAL strains carried *aph(3')-Ic* gene responsible for aminoglycoside resistance in its genome, and *bla*OXA-9 (OXA-51-like) gene responsible for β-lactams resistance.which is encoded chromosomally. Resistant genes for phenicol, sulphonamide and other genes responsible for beta lactams and aminoglycoside resistance were distributed differently across the strains (Table 2.3). Carbapenems resistant genes were due to the presence of *ISAba* varients upstream of *bla*OXA-90*;* AB25C30, AB96SM and AB103SM. While $bla_{\text{OXA-58}}$ was plasmid mediated in 3 strains; AB2RED (25311 bp plasmid), AB14336 (a 26496 bp plasmid) and AB20C15 (26781 bp

plasmid). On the other hand, *bla*OXA-23 was found on 2 strains, AB20C15 and AB5MO and was chromosomally encoded. Plasmid extracted and annotated showed the OXA-58 environment in the 3 plasmids (Figure 2.5).

Table 2.3: SMAL Strains with its resistant genes and the IS*Aba* insertion sequences

Gene / Strain	*aadB*	*floR*	*sul2*	*aph(3')-Ic*	*blaCARB-8*	*blaOXA-90*	*blaOXA-58*	*blaOXA-23*	*ISAba*
AB2MG	X	X	X	X	X	X	-	-	-
AB65SM	-	X	X	X	-	X	-	-	-
AB2RED	-	X	X	X	-	X	X	-	X
AB5MO	-	X	X	X	-	X	-	X	X
AB20C15	-	X	X	X	-	X	X	X	X
AB25C30	X	X	X	X	-	X	-	-	X
AB96SM	-	X	X	X	-	X	-	-	X
AB103SM	-	-	-	X	-	X	-	-	X
AB14336	-	-	-	X	-	X	X	-	X
3909	-	X	X	X	-	X	X	-	X

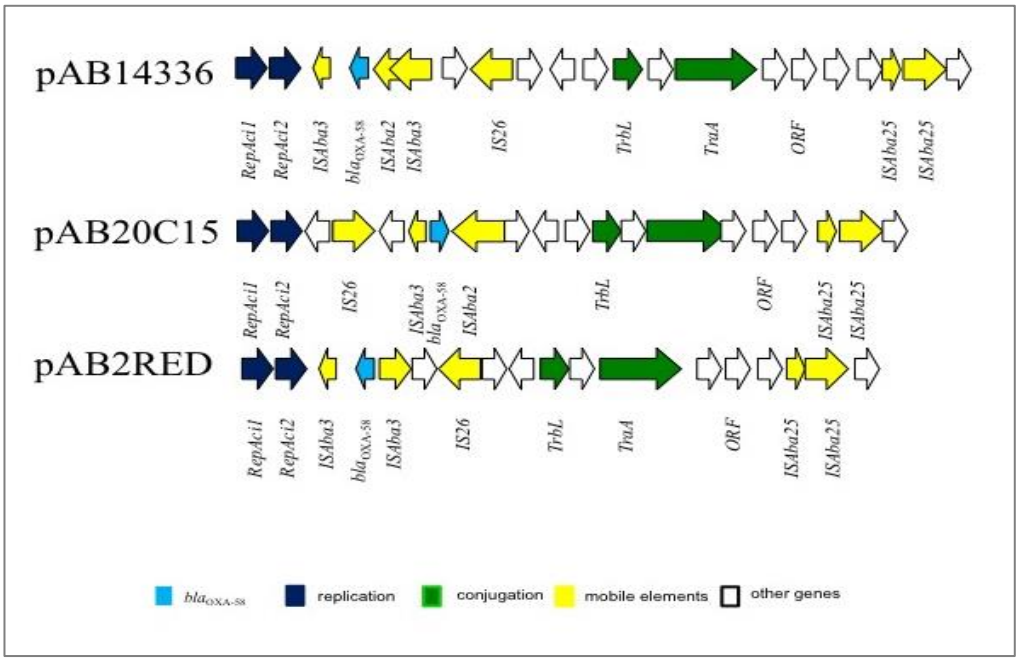

Figure 2.5: Plasmids harboring OXA-58

2. Discussion

A. baumannii is one of the leading pathogen causing nosocomial infections, associated with ventilator-associated pneumonia and surgical and urinary tract infections (Wright M. *et al* 2014). Acquisition of OXA carbapenemase has emerged as one mechanisms contributing to the spread of *Acinetobacter* spp. resistance to a worrisome extent. *A. baumannii* is the source of *bla*OXA-51-like and recently *bla*OXA-23-like. Reports indicated that carbapenem resistance is only expressed in the presence of both b bla_{OXA} genes and insertion sequences such as *ISAba* varients (Boo T. and Crowley B. 2009).

The core genome of the *A. baumannii* causing nosocomial infections is thought to be about 2,200 coding sequences (CDs) responsible for metabolic and general cellular processes while resistance genes are acquired through transposases and plasmids (Imperi F. *et al.* 2011). In our study, recombination event was not detected due to low levels of SNPs density, and this is also confirmed in literature where epidemiological studies show no significant evidence on recombination events within related clones (Wright M. *et al.* 2014).

In this study, a strain of *A. baumanni* belonging to sequence type ST78, also associated with a pulsotype referred as SMAL, was thought to be a strain of Italy that persisted for more than 10 years. The study aims to understand the evolution in terms of genomic characteristics of this strain along with the acquisition of carbapenm resistance. From the NCBI database, nine genomes belonged to ST78 and were included for comparative purposes. After sequencing whole genomes of the nine strains, the genomes were compared to other genomes for strains belonging to sequence type ST78; SMAL genomes clustered together with the strain 3909 that is in fact an Italian strain included in an American study. This confirmed our hypothesis; the strain retained most of its genomic content and managed to spread and circulate until now.

The first two strains included in the study were from the period 2003-2008, AB2MG and AB65SM, were carbapenem susceptible. Even though these strains carry *bla*OXA-90, no *ISAba* sequences were detected upstream which explains the susceptibility. However after 2008, *A. baumannii* strains acquired carbapenem resistance. The first reports of carbapenem resistance was due to the presence of *bla*OXA-58 in

AB2RED and AB14336. While AB20C15 harbored *bla*OXA-58 on a plasmid and *bla*OXA-23 chromosomally, AB5MO had *bla*OXA-23 only in its chromosomes. More over, these strains also carried *bla*OXA-90 with *ISAba1* in the upstream region.

The plasmid analysis showed plasmid backbone similarity in the 3 strains with 2 replication initiation sites *RepAci1* and *RepAci2* and 2 conjugal transfer proteins *TrbL* and *TraA* followed by 2 *ISAba25*. The difference is in the environment of the *bla*OXA-58: the plasmid of AB14336 had a backward alignment of the *ISAba2/ISAba3-bla*OXA-58-*ISAba3* when compared to previously described plasmid p183Eco with 1 IS26 missing (Bertini A. et al. 2007). On the other hand, AB2RED had $bla_{OXA\text{-}58}$ surrounded by 2 *ISAba3* (opposite orientations see figure 2.5). AB20C15 had the *bla*OXA-58 surrounded by *ISAba3* and *ISAba2*. These variations and differences in the insertion sequences introducing the *bla*OXA-58 may partially explain the reason why carbapenem resistance evolved from being encoded by a plasmid mediated *bla*OXA-58 to chromosomally mediated *bla*OXA-23 as reported in China and elsewhere (Wu W. *et al.* 2015).

The strains carrying *bla*OXA-23 (AB20C15 and AB5MO) also carried *ISAba1* upstream of the OXA genes. This resulted in the strong carbapenem resistant profile of the strain. Moreover, strains harboring *bla*OXA-23 with the insertion sequences showed higher level of resistance for carbapenems than those with *bla*OXA-58. Similar results were reported elsewhere, however the exact reason is not yet fully understood; it may be a combination of copy number of insertion sequences and different variants along with other mechanisms such as efflux pumps (Wu W. et al. 2015).

Three strains isolated in 2012-2012, AB96SM, AB25C30 and AB103SM, showed carbapenm resistance however neither of them harbored blaOXA-23 nor *bla*OXA-58. Even though, the *bla*OXA-90 with *ISAba1* in the upstream region was enough to develop carbapenem resistance in this case, reports of other strains in literature having a *bla*OXA-51-like with *ISAba1* did not establish carbapenem resistance (Chen T. et al. 2010). This is still not yet fully understood, yet theories about other resistance mechanisms such as efflux pumps could be involved with the *bla*OXA-51-like-*ISAba1* to express higher level of resistance to carbapenems.

2. Conclusion

In conclusion, this study shows the evolution of *A. baumannii* clone widely spread in Italy. In the period 2002-2012, the SMAL strains retained a sort of genomic stability in terms of recombination events, however resistance to carbapenems evolved greatly from acquiring plasmid encoding OXA-58 to integrating OXA-23 into the genome. The instability and inconsistency of the plasmid contents due to many insertion sequences around the *bla*OXA-58 as shown in three plasmids of same clone may be a reason for the unsuccessful propagation of the plasmid. This may explain the need of the clone to acquire a more stable mechanism such as acquiring *bla*OXA-23 and integrating it in the chromosomal DNA, which makes it more stable and more efficient.

This evolution in the resistance mechanism to carbapenem is reported in Greece and Chine and other countries. More work will be done in detecting other factors that may contribute to the resistance, which will give more insight on the evolutionary process of this clone.

2. References

Boo, T. W., & Crowley, B. (2009). Detection of blaOXA-58 and blaOXA-23-like genes in carbapenem-susceptible *Acinetobacter* clinical isolates: should we be concerned?. *Journal of medical microbiology*, *58*(6), 839-841.

Brigante, G., Migliavacca, R., Bramati, S., Motta, E., Nucleo, E., Manenti, M., ... & Vigano, F. E. (2012). Emergence and spread of a multidrug-resistant Acinetobacter baumannii clone producing both the carbapenemase OXA-23 and the 16S rRNA methylase ArmA. *Journal of medical microbiology*, *61*(5), 653-661.

Carattoli, A., Zankari, E., Garcìa-Fernandez, A., Larsen, M. V., Lund, O., Villa, L., ... & Hasman, H. (2014). PlasmidFinder and pMLST: in silico detection and typing of plasmids. *Antimicrobial Agents and Chemotherapy*, AAC-02412.

Carretto E, Barbarini D, Dijkshoorn L, van der Reijden TJK, Brisse S, Passet V, Farina C, on behalf of the APSI Acinetobacter Study Group. Widespread carbapenem resistant *Acinetobacter baumannii* clones in Italian hospitals revealed by a multicenter study. Infect. Genet. Evol. 2011; 11:1319-1326

Chagas, T. P. G., Carvalho, K. R., de Oliveira Santos, I. C., Carvalho-Assef, A. P. D. A., & Asensi, M. D. (2014). Characterization of carbapenem-resistant *Acinetobacter baumannii* in Brazil (2008–2011): countrywide spread of OXA-23–producing clones (CC15 and CC79). *Diagnostic microbiology and infectious disease*, *79*(4), 468-472.

Chen, T. L., Lee, Y. T., Kuo, S. C., Hsueh, P. R., Chang, F. Y., Siu, L. K., ... & Fung, C. P. (2010). Emergence and distribution of plasmids bearing the blaOXA-51-like gene with an upstream ISAba1 in carbapenem-resistant Acinetobacter baumannii isolates in Taiwan. *Antimicrobial agents and chemotherapy*, *54*(11), 4575-4581.

D'Arezzo S, Principe L, Capone A, Petrosillo N, Petrucca A, Visca P. Changing carbapenemase gene pattern in an epidemic multidrug-resistant *Acinetobacter baumannii* lineage causing multiple outbreaks in central Italy. J. Antimicrob. Chemother. 2011; 66:54-

Eveillard M, Kempf M, Belmonte O, Pailhories H, Joly-Guillou ML. Reservoirs of Acinetobacter baumannii outside the hospital and potential involvement in emerging human community-acquired infections. Int. J. Infect. Dis. 2013; 17:802-805

Fu, Y., Jiang, J., Zhou, H., Jiang, Y., Fu, Y., Yu, Y., & Zhou, J. (2014). Characterization of a novel plasmid type and various genetic contexts of bla OXA-58 in *Acinetobacter* spp. from multiple cities in China. *PloS one*, *9*(1), e84680.

Giordano, A., Varesi, P., Bertini, A., Villa, L., Dionisi, A. M., Venditti, M., ... & Carattoli, A. (2007). Outbreak of *Acinetobacter baumannii* producing the carbapenem-hydrolyzing oxacillinase OXA-58 in Rome, Italy. *Microbial Drug Resistance*, *13*(1), 37-43.

Gonzalez-Villoria, A. M., & Valverde-Garduno, V. (2016). Antibiotic-Resistant *Acinetobacter baumannii* Increasing Success Remains a Challenge as a Nosocomial Pathogen. *Journal of pathogens, 2016*.

Gordon, N. C., & Wareham, D. W. (2010). Multidrug-resistant Acinetobacter baumannii: mechanisms of virulence and resistance. *International journal of antimicrobial agents*, *35*(3), 219-226.

Higgins, P. G., Dammhayn, C., Hackel, M., & Seifert, H. (2010). Global spread of carbapenem-resistant *Acinetobacter baumannii*. *Journal of Antimicrobial Chemotherapy*, *65*(2), 233-238.

Ho, J. Y., Cira, N. J., Crooks, J. A., Baeza, J., & Weibel, D. B. (2012). Rapid identification of ESKAPE bacterial strains using an autonomous microfluidic device. *PloS one*, *7*(7), e41245.

Imperi, F., Antunes, L., Blom, J., Villa, L., Iacono, M., Visca, P., & Carattoli, A. (2011). The genomics of Acinetobacter baumannii: insights into genome plasticity, antimicrobial resistance and pathogenicity. *IUBMB life*, *63*(12), 1068-1074.

Karah N, Sundsfjord A, Towner K, Samuelsen O. Insights into the global molecular epidemiology of carbapenem non-susceptible clones of Acinetobacter baumannii. Drug Resist. Updat. 2012; 15:237-247

Kempf M, Rolain J-M. Emergence of resistance to carbapenems in Acinetobacter baumannii in Europe: clinical impact and therapeutic options. Int. J. Antimicrob. Agents.

Mammina C, Palma DM, Bonura C, Aleo A, Fasciana T, Sodano C, Saporito MA, Verde MS, Calá C, Cracchiolo AN, Tetamo R. Epidemiology and clonality of carbapenem-resistant Acinetobacter baumannii from an intensive care unit in Palermo, Italy. BMC Res Notes. 2012; 5:365

Mendes RE, Spanu T, Deshpande L, Castanheira M, Jones RN, Fadda G. Clonal dissemination of two clusters of *Acinetobacter baumannii* producing OXA-23 or OXA-58 in Rome, Italy. Clin. Microbiol. Infect. 2009; 15:588-592

Mezzatesta ML, D'Andrea MM, Migliavacca R, Giani T, Gona F, Nucleo E, Fugazza G, Pagani L, Rossolini GM, Stefani S.. Epidemiological characterization and distribution of carbapenem-resistant *Acinetobacter baumannii* clinical isolates in Italy. Clin. Microbiol.

Migliavacca R, Espinal P, Principe L, Drago M, Fugazza G, Roca I, Nucleo E, Bracco S, Vila J, Pagani L, Luzzaro F. Characterization of resistance mechanisms and genetic relatedness of carbapenem-resistant *Acinetobacter baumannii* isolated from blood, Italy. Diagn. Microbiol. Infect. Dis. 2013; 75:180-186

Perez F, Hujer AM, Hujer KM, Decker BK, Rather PN, Bonomo RA. Global challenge of multidrug-resistant *Acinetobacter baumannii*. Antimicrob. Agents Chemother.2007;

Roca I, Espinal P, Vila-Farrés X, Vila J. The *Acinetobacter baumannii* oxymoron: commensal hospital dweller turned pan-drug-resistant menace. Front. Microbiol. 2012;

Siguier, P., Pérochon, J., Lestrade, L., Mahillon, J., & Chandler, M. (2006). ISfinder: the reference centre for bacterial insertion sequences. *Nucleic acids research*, *34*(suppl 1), D32-D36.

Wright, M. S., Haft, D. H., Harkins, D. M., Perez, F., Hujer, K. M., Bajaksouzian, S., ... & Adams, M. D. (2014). New insights into dissemination and variation of the health care-associated pathogen *Acinetobacter baumannii* from genomic analysis. *MBio*, *5*(1), e00963-13.

Wu, W., He, Y., Lu, J., Lu, Y., Wu, J., & Liu, Y. (2015). Transition of bla OXA-58-like to bla OXA-23-like in *Acinetobacter baumannii* Clinical Isolates in Southern China: An 8-Year Study.*PloS one*,*10*(9), e0137174.

Zankari, E., Hasman, H., Cosentino, S., Vestergaard, M., Rasmussen, S., Lund, O., . & Larsen, M. V. (2012). Identification of acquired antimicrobial resistance genes.*Journal of Antimicrobial Chemotherapy*,*67*(11), 2640-2644.

Zarrilli R, Casillo R, Di Popolo A, Tripodi MF, Bagattini M, Cuccurullo S, Crivaro V, Ragone E, Mattei A, Galdieri N, Triassi M, Utili R. Molecular epidemiology of a clonal outbreak of multidrug-resistant *Acinetobacter baumannii* in a university hospital in Italy. Clin Microbiol Infect. 2007; 13:481–489

Zarrilli R, Pournaras S, Giannouli M, Tsakris A. Global evolution of multidrug resistant *Acinetobacter baumannii* clonal lineages. Int. J. Antimicrob. 2013; Agents 41:11-19

Chapter 3

Detection of an IncA/C plasmid encodingVIM-4 and CMY-4 -lactamases in *Klebsiella oxytoca* and *Citrobacter koseri* from an inpatient cardiac rehabilitation unit

Mariasofia Caltagirone1, Ibrahim Bitar1, Aurora Piazza1, Melissa Spalla1, Elisabetta Nucleo1, Antonella Navarra2, Roberta Migliavacca1

1Clinical-Surgical, Diagnostic and Pediatric Sciences Department, University of Pavia, Italy;
2I.R.C.C.S. Fondazione Salvatore Maugeri, Pavia, Italy

Caltagirone, M., Bitar, I., Piazza, A., Spalla, M., Nucleo, E., Navarra, A., & Migliavacca, R. (2015). Detection of an IncA/C plasmid encoding VIM-4 and CMY-4 β-lactamases in *Klebsiella oxytoca* and *Citrobacter koseri* from an inpatient cardiac rehabilitation unit. *The new microbiologica*, *38*(3).

3.Abstract

A 62-year-old patient was transferred to the cardiac rehabilitation unit of the I.R.C.C.S. Fondazione S. Maugeri after undergoing heart transplantation at the Acute Care Hospital I.R.C.C.S. S. Matteo of Pavia. On 1 August 2013 and during hospitalization in the rehabilitation unit, *Klebsiella oxytoca* and *Citrobacter koseri* clinical isolates were simultaneously recovered from the patient's preputial swab. Both the *K. oxytoca* and *C. koseri* strains were carbap- enem-resistant by MicroScan System (Beckman Coulter). Carbapenem-resistant *K. pneumoniae* had previously been reported in the same rehabilitation facility.

The aim of the study was to identify the carbapenem resistance mechanisms among the enterobacterial species recovered. Phenotypic screening tests useful to detect the β-lactamases/carbapenemases were performed. Carbapenem MICs were obtained by Etest. AmpC and MBL encoding genes were identified by PCR and sequencing. Conjugation assays and plasmid characterization were performed.

Both of the *K. oxytoca* and *C. koseri* isolates were multi-drug resistant, showing resistance to amoxicillin-clavulanicacid, third generation cephalosporins, ertapenem (K. oxytoca MIC, >32 mg/L; C. koseri MIC, 4 mg/L), imipenem (*K. oxytoca* MIC, 4 mg/L; *C. koseri* MIC, 12 mg/L), thrimethoprim-sulphamethoxazole and gentamicin. Susceptibility was retained to fluoroquinolones, colistin and tigecycline. Molecular characterization confirmed the presence of *bla*CMY-4 and *bla*VIM-4 determinants in a 150 Kb transferable plasmid of IncA/C group.

This case is the first detection in Italy of the *K. oxytoca* and *C. koseri* clinical isolates co-producing theCMY-4 and VIM-4 enzymes.

KEYWORDS: Metallo- -lactamases, Cephalosporinases, Multi drug resistant *Enterobacteriaceae*, Rehabilitation unit.

3.Acknowledgements

I thank Dr. Antonella Navarra and Dr. Silvana Telecco for providing the isolates

3. Introduction

Klebsiella oxytoca and *K. pneumoniae* are opportunistic pathogens increasingly implicated in clusters of community and nosocomial outbreaks, particularly in specific medical units (Watson *et al.* 2005; Migliavacca *et al.* 2013).

The acquisition of an extended-spectrum β-lactamase (ESBL) is the most common mechanism of resistance to broad-spectrum cephalosporins in *K. oxytoca* (Romero *et al.* 2007; Sturm *et al.* 2010), while acquired AmpC cephalosporinases are less frequently detected in this species (Yamasaki *et al.* 2010).

Since AmpC β-lactamase production is frequently accompanied by multi-drug resistance, therapeutic options became limited. In addition, failure to identify AmpC β-lactamase producers may lead to inappropriate antimicrobial treatment and may result in increased mortality (Tsakris *et al.* 2011).

Citrobacter koseri, an environmental Gram-negative bacterium, is occasionally found as a colonizer of the human gastrointestinal tract as part of the normal flora. Although the potential virulence of the species is considered low, it is sporadically implicated in serious nosocomial infections.

The antimicrobial treatment of infection caused by *C. koseri* has been changing, due to several reports of isolates carrying ESBL and other resistance encoding genes (Doran, 1999). Nevertheless, the isolation of *C. koseri* and/or *K. oxytoca* strains showing resistance to carbapenems remains very infrequent in Italy (Giani *et al.* 2013). To date, we have only two reports on the presence of *bla*VIM-1 or *bla*KPC-2 genes in *C. koseri* clinical isolates in the Mediterranean area (Castanheira *et al.* 2009; Mavroidi *et al.* 2011).

Here we report the detection of *K. oxytoca* and *C. koseri* strains co-producing a VIM-4 metallo β-Lactamase (MBL) and an acquired CMY-4 AmpC enzyme from a single patient. The objective of this study was to evaluate the localization of *bla*VIM-4 and *bla*CMY-4 resistance genes and to assess their spreading potential.

3. Literature review

3.1. Plasmids and the spread of resistance.

Plasmids are extra chromosomal, self-replicating genetic mobile elements that exist in a double stranded circular DNA form. They have their own system that will ensure its autonomous replication and the mechanism to control the copy number that will ensure its proper propagation during cell division. Many plasmids possess an addiction system that will kill daughter cells that didn't successfully inherit the plasmid through toxin-antitoxin factors. Additionally, this system promotes plasmid maintenance in the bacterial population whether or not this plasmid is beneficial to the bacterial cell and whether or not there is a selective pressure presented by antibiotic usage. Nevertheless, the use of antibiotics will positively select the propagation and the resistant phenotype of the bacterial population that harbor the plasmid with the relative resistant genes (Caratolli A. 2009).

In 1969, Central America had mass outbreaks of antibiotic resistant bacterial dysentery leaving behind thousands of dead people. The bacteria responsible for the outbreak were thought to be eradicated using antibiotics, however they re-emerged resistant. The patients presented infection with resistant bacteria, even though these patients were never administered antibiotics. For these reasons, alot of research was done on plasmids using molecular tools concluding that plasmids seemed to collect different resistant genes, even against antibiotics no longer in widespread use. This explained the presence of resistance in a patient against some antibiotics regardless whether or not the patient had ever been treated with it (Landecker 2015). The problem associated with plasmids is that the horizontal transfer of resistant genes can surpass the species and genus barriers; plasmid conjugation is not restricted between 2 bacterial cells of the same species, and an increased rate of plasmid transfer has been observed in heterogeneous communities (Svara F. and Rankin D. 2001).

3.2. Plasmid identification and typing

Classification and identification of plasmids are problematic especially with the fact that plasmids have mobile elements shared between different species. However, genetic traits regarding the plasmid maintenance and

replication control are thought to be constant traits from which classification should be based on (Carattoli A. 2009). These regions are called replicons; they encode functions that activate and control replication (Carattoli *et al*. 2014).

In 1971, Hedges and Datta proposed a plasmid classification scheme based on the stability of plasmids during conjugation, a phenomenon called plasmid incompatibility. It is a manifestation of relatedness between plasmids harboring common replication controls. It was primarily defined as the inability of two related plasmids to be propagated stably in the same cell.

In 1988, Couturier an colleagues proposed another classification theme based on genetic plasmid typing using Southern blot hybridization using clonal replicons as probes. This approach was successful in classifying both conjugative and non-conjugative plasmids however providing low specificity due to cross hybridization among related replicons (Carattoli A. 2009).

Since 2005, a PCR-based replicon-typing (PBRT) scheme has been available; it is a multiplex PCR targeting replicons of the major plasmid families found in members of *Enterobacteriaceae*. Initially, it was able to detect 18 major incompatibility (Inc) groups. However, with the advances in whole genome sequencing techniques, PBRT was enhanced and the spectrum of identification was extended to include 25 different replicon. Nevertheless, this method is based on multiplex PCR, thus it needs a lot of lab work and time in order to uncover any new Inc groups, and is not suited for the detection of new Inc groups if the variation is located in the region within the primer binding site. The combination of PBRT and other strain specific characteristics such as multi locus sequence typing MLST, serotype, phylogroup, etc., could be used for comparative analysis of related and unrelated strains as well in an epidemiological study or for the sake of comparing different outbreaks related to plasmid dissemination (Carattoli et al. 2014).

The PBRT method is able to detect the major classes of Inc groups in *Enterobacteriaceae*: HI2, HI1, I1-Y, X, L/M, N, FIA, FIB, FIC, W, Y, P, A/C, T, K, B/O, and can also detect FII, FIII, FIV, and FIV variants (Figure: 3.2.1.). Over than 1000 resistant plasmid have been typed and assigned to a specific plasmid family by PBRT and hybridization/ conjugation methods. Novel plasmid families have been deduced from 800 full-length sequenced

plasmids from *Gammaproteobacteria* (http://www.ncbi.nlm.nih.gov/genome/) (Carattoli 2009).

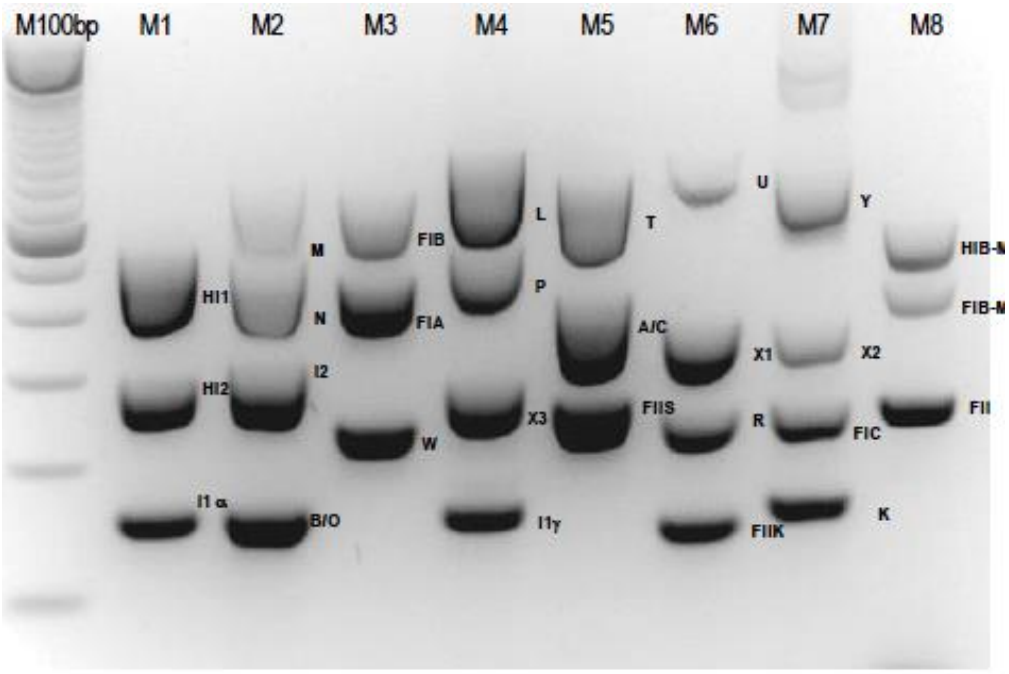

Figure 3.2.1. PBRT kit based on replicons of the major plasmids incompatibility groups in *Enterobacteriaceae* (http://www.diatheva.com)

3.3. Plasmids carrying AmpC β-lactamases in *Enterobacteriaceae*

Enterobacteriaceae β-lactamase genes (*bla*) encodes for extended-spectrum β-lactamase (ESBL) such as CTX-Ms and VEBs, plasmid mediated AmpC (pAmpC) such as CMYs and for carbapenemase such as KPCs, OXA-48 like and NDMs. Pets and food producing animals act as a reservoir of pathogens with these genes, and contributes to its dissemination into human population (Seiffert et al. 2014).

The family of AmpC β-lactamases is generally found to be chromosomal mediated enzymes in *E.coli, Enterobacter* spp., *Citrobacter* spp., M*organella* spp., *Proteus* spp., *P. aeruginosa,* as well as other species. However, plasmid mediated AmpC β-lactamases are increasing worldwide posing a threat on public health due to multidrug resistance and limited therapeutic options in treating such infections. Another factor leading to increased mortality is the failure to identify AmpC β-lactamase produced,

which will lead to inappropriate antibiotic use. Bacteria producing CMY, which is AmpC originated from chromosomal *Citrobacter freundii,* has been linked to specific plasmid families that are found in animal sources and then disseminated to human population (Carattoli A. 2009).

CMY β-lactamases have been reported widely in Gram-negative bacteria in both nosocomial and community infections. The plasmid mediated *cmy-2* being the most frequently isolated in many regions in the world belonging predominantly to IncA/C and to a less extent in IncQ and IncI 1 replicon type (Cejas et al. 2012).

In the past 10 years, IncA/C plasmids have been investigated intensively since they were the main reason of *bla*CMY-2 cephalosporinases gene dissemination in *E. coli* and *Salmonella* spp in United States, Canada and Europe (Carattoli et al. 2012).

IncA/C plasmids were isolated from beef, chicken, turkey and pork from different regions in the United states, showing a common plasmid back bone that is broadly disseminated in the US among resistant zoonotic pathogens. This backbone belonging to the IncA/C (harboring the *cmy-2* gene) was previously identified in *E. coli* and *Salmonella* spp., with high degree of sequence identity with IncA/C plasmids from *S. enterica* and *Yersinia pestis* pIP1202 and fish pathogen *Yersinia ruckeri* YR71. However, the occurrence of IncA/C plasmid was only 1% of E. coli obtained from healthy humans not exposed to antimicrobials and was absent from the fecal flora of healthy birds. This indicated that the occurrence of IncA/C is advantageous in the presence of a positive selective pressure under antibiotic usage. This high distribution of IncA/C, CMY-2 plasmids in animal intestinal tract suggest that the dissemination of these plasmids was initiated from animals treated with antibiotic to human population (Carattoli A. 2009).

3.4. Plasmid mediated Carbapenem resistance in *Enterobacteriaceae*

There are 4 molecular classes of carbapenemases hydrolyzing β-lactams (including carbapenems:

- Chromosomal encoded class A carbapenems (IMI, NMC-A and SME)
- Non chromosomal encoded class A such as KPC in *Enterobacteriaceae*

- GES-type enzymes in E*nterobacteriaceae* and *P. aeruginosa.*
- Class B enzymes, which are most clinically significant, and called the metallo- β-lactamases MBLs. Most prevalent enzymes are the IMP and VIM.

MBLs are known for their resistance to carbapenems, ability to hydrolyze cephalosporins and penicillin yet their lack of ability to hydrolyze azteonam. Chelating substances such as EDTA also inhibits MBLs (Miro et al. 2010).

IMP and Vim have been recorded worldwide carried by different plasmids and integrons (Carattoli A. 2009). The Vim type MBL has been detected worldwide in *P. aeruginosa* and *Acinetobacter* spp., and recently in many species of *Enterobacteriaceae* (Kristof *et al*. 2010).

An increasing number of VIM producing bacteria in *Enterobacteriaceae* has been recorded. Most of them are sporadic and clonally unrelated, although some clonal epidemics have been described. In Greece, *K. pneumoniae* and *E. coli* isolates producing VIM-1 were transmitted through IncN plasmids and through IncW in *Serratia liquefaciens* and *K. oxytoca*. On the other hand, Spain had other plasmid scaffolds harboring the VIM-1 enzyme. For *K. pneumoniae* and *E. coli*, The VIM-1 was carried by IncI1 plasmids, yet with different bla_{VIM-1} genetic environment, while IncH12 plasmids were found in *E. cloacae*. In Italy, plasmids carrying both bla_{VIM-4} and bla_{CMY-4} were identified on IncA/C plasmids in *K. pneumoniae* and *E. cloacae* isolates. These plasmids had similar plasmid scaffolds of that isolated in the U.S.A and U.K yet without carrying the carbapenemase gene. This represents a novel acquisition of carbapenemase genes in IncA/C plasmids.

In the United States, KPC-producing *K. pneumoniae* emerged rapidly, yet PBRT failed to identify replicons of the corresponding plasmids. Only in one case, the plasmid scaffold belonged to IncN group, yet the plasmid replication gene *repA* was slightly different of that of the IncN plasmids (98% similarity). Another KPC-2 producing *K. pneumoniae* in Colombia had a replication origin belonging to ColE-like. This suggests that both IncN-like and ColE-like could be the plasmids responsible for the transmission of *bla*KPC-2 gene.

3. Aim of the study

The aim of the study was to:

- identify the carbapenem resistance mechanisms among the bacterial species recovered;
- evaluate the localization of the resistance genes;
- assess the resistance genes spreading potential.

3. Materials and methods

Case description

On 18 July 2013, a 62-year-old male patient was admitted to the cardiac rehabilitation unit of the I.R.C.C.S. Fondazione S. Maugeri in Pavia (Northern Italy) with a diagnosis of cardiac complications, septic shock, pneumonia and preputial edema. The man had been previously admitted to hospital in February 2013, at the Acute Care Hospital I.R.C.C.S. S. Matteo of Pavia, were he underwent heart transplantation. On 1 August 2013 *Candida glabrata*, *Pseudomonas aeruginosa*, *K. oxytoca* and *C. koseri* were also isolated from both preputial swab and urine samples of the patient. The patient was then treated with colistin in monotherapy (EV 1.000.000 U 4/*die*). After the antibiotic therapy, both the samples resulted negative for the three bacterial species previously identified.

Characterization of the bacterial isolates and of resistance determinants

Species identification and susceptibility testing were carried out using the MicroScan AutoSCAN4 automated-system (Beckman Coulter). Ertapenem (ETP), meropenem (MER) and imipenem (IPM) MICs were determined using Etest (bioMérieux); the results were interpreted according to EUCAST 2014 criteria (The European Committee on Antimicrobial Susceptibility Testing, Version 3.1, 2014). *Escherichia coli* ATCC 25922 and *P. aeruginosa* ATCC 27853 were routinely included during testing for quality assurance. The *K. oxytoca* and *C. koseri* strains were screened and than phenotypically confirmed for carbapenemase production by the Modified Hodge test (MHT) using both ETP and IPM and the KPC/MBL Confirm kit (Rosco Diag- nostic).

Phenotypic ESBL and AmpC detection were performed with both the double disk synergy test (DD) (Jarlier, 1988), using piperacillin-tazobactam (TZP), cefotaxime (CTX), cefepime (FEP), ceftazidime (CAZ) and aztreonam (ATM), and with the ESBL + AmpC Screen kit (Rosco Diagnostic). The β -lactamase preliminary identification was performed by Isoelectricfocusing (IEF), as described elsewhere (Pagani *et al.*, 2002). Crude sonic extracts from *E. coli* harboring TEM-1 (pI, 5.4), SHV-2 (pI,, 7.6) and SHV-12 (pI,8.2) were used as Isoelectric point (pI,) markers.

Conjugal transfer of resistance determinants was performed in liquid medium using the *E. coli* K12 strain J62 (*pro-*, *his-*, *trp-*, *lac-*, *Sm*r) and J53 (*met*, *pro-*,*Rif*r) as recipients. The initial donor/recipient ratio was 0.01. The transconjugants were selected on McConkey agar containing CTX (8 mg/L) plus streptomycin (1000 mg/L) or rifampin (100 mg/L). Species identification and susceptibility testing of the obtained *E. coli* transconjugants were carried out by MicroScan AutoSCAN4 automated system.

Transconjugants MICs against MER, ETP and IPM were determined by Etest (bioMérieux). The presence of *bla*VIM, *bla*IMP and *bla*AmpC genes was assessed by multiplex PCR analysis using the primers and the conditions described elsewhere (Rossolini *et al.*, 2008; Pérez-Pérez *et al.*, 2002; Hujer *et al.*, 2006; Koeleman *et al.*, 2001). PCR products were purified using the kit Quantum Prep PCR Kleen Spin Columns (BioRad) and subjected to double-strand sequencing (Macrogen Inc., Seoul, South Korea). The nucleotide sequences were analyzed according to the BLAST program (http:/www.ncbi.nlm.nih. gov/blast/).

Plasmid DNA was extracted and purified by PureLink™ HiPure Plasmid Filter Purification Kits (Life technologies™). Plasmids were subsequently typed according to their incompatibility group using the PBRT kit-PCR based replicon typing scheme, as described previously (Carattoli *et al.*, 2006). The sizes of the plasmids were estimated using the S1 nuclease PFGE method (Barton *et al.*, 1995).

Table 3.1. Primers used for amplification

Target(s)	Primer	Sequence	Expected amplicon size (bp)
MOX-1, MOX-2, CMY-1, CMY-8 to CMY-11	MOXM-F MOXM-R	5'-GCTGCTCAAGGAGCACAGGAT-3' 5'-CACATTGACATAGGTGTGGTGC-3'	520
LAT-1 to LAT-4, CMY-2 to CMY-7, BIL-1	CITM-F CITM-R	5'-TGGCCAGAACTGACAGGCAAA-3' 5'-TTTCTCCTGAACGTGGCTGGC-3'	462
DHA-1, DHA-2	DHAM-F DHAM-R	5'-AACTTTCACAGGTGTGCTGGGT-3' 5'-CCGTACGCATACTGGCTTTGC-3'	405
ACC	ACCM-F ACCM-R	5'-AACAGCCTCAGCAGCCGGTTA-3' 5'-TTCGCCGCAATCATCCCTAGC-3'	346
MIR-1T ACT-1	EBCM-F EBCM-R	5'-TCGGTAAAGCCGATGTTGCGG-3' 5'-CTTCCACTGCGGCTGCCAGTT-3'	302
FOX-1 to FOX-5b	FOXM-F FOXM-R	5'-AACATGGGGTATCAGGGAGATG-3' 5'-CAAAGCGCGTAACCGGATTGG-3'	190
VIM	VIM-F VIM-R	5'- CAGATTGCCGATGGTGTTTGG-3' 5'-AGGTGGGCCATTCAGCCAGA-3'	523

Detection of class 1 integron was carried out by PCR amplification of an internal fragment of 160-bp within the class 1 integrase gene (intI1) using the primers intIF (5'-CAGTGGACATAAGCCTGTTC-3') and intIR (5'-CCCGAGGCATAGACT GTA-3').

Since the *bla*VIM gene is often found in integrons, primers near the end of this gene were used in combination with primers for the conserved segments of class 1 integron.

Characterization of plasmid segments containing resistance genes was carried out by PCR mapping using the 5‘-CS oligonucleotide, that is specific to the 5‘conserved segment (CS), primers specific for the intI1 and the resistance genes primers that were specific for *bla*VIM and *aadA1* (aminoglycoside resistance gene). PCR primers used in this work for detection and mapping purposes are listed in Table 4.2. The PCR conditions are described elsewhere (Lee YT *et al.* 2008).

Table 3.2. Oligonucleotides for PCR analysis of integron

Primer	**Nucleotide sequence (5’ to 3’)**
5‘CS	5'-GGCATCCAAGCAGCAAG-3‘
3‘CS	5‘-AAGCAGACTTGACCTGA-3‘
Int1-F	5‘-CAGTGGACATAAGCCTGTTC-3‘
Int1-R	5‘-CCCGAGGCATAGACTGTA-3‘
aadA 1-3	5‘-TTATTTGCCGACTACCTTGGTG-3‘
aadA 1-5	5‘-ATGAGGGAAGCGGTGATCG-3‘

3. Results

The antimicrobial susceptibility results by MicroScan System showed that both of *K. oxytoca* and *C. koseri* isolates were characterized by multi-drug resistance, retaining susceptibility only to fluoroquinolones, colistin and tigecycline, and showing resistance to amoxicillin-clavulanic acid (AMC), third generation cephalosporins (3GC), cefoxitin, trimethoprim-sulfamethoxazole and gentamicin (according to the 2014 EUCAST breakpoints).
The *C. koseri* isolate resulted resistant to ETP (MIC, >1 mg/L), IPM (MIC, >8 mg/L) and MER (MIC, >8 mg/L) by MicroScan System, while *K. oxytoca* was only ETP (MIC, >1 mg/L) and MER (MIC, >8 mg/L) resistant, showing an IPM MIC, 8 mg/L, using the same tool (ANNEX 1).
These values were not always coherent with those of the Etest for *K. oxytoca*, ETP MIC being >32 mg/L; IPM MIC, 6 mg/L but MER MIC, 0,75 mg/L, lower than expected. The values for *C. koseri* were consistent only in the case of the ETP MIC >32 mg/L; with IPM MIC, 1 mg/L, and a MER MIC lower than the expected, being equal to 0,5 mg/L.

Synergistic activity between ESBL and tazobactam was shown by DD in the isolates and ESBL + AmpC Screen kit (Rosco Diagnostica) test resulted positive for AmpC enzyme production (Figure 3.1.).

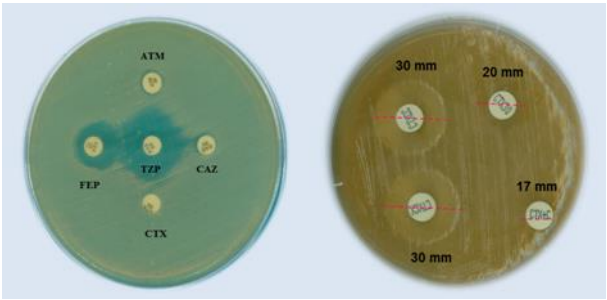

Figure 3.1. Phenotypic AmpC detection: DD and ESBL + Amp-C Rosco tests positive for *C. koseri*, *K. oxytoca* and transconjugants; CTXCX: cefotaxime + cloxacillina; CTXC: cefoxitina + acido clavulanico; CTXCCX: cefotaxime + acido clavulanico + cloxacillina.

MHT carbapenemase screening test showed positive results for both the isolates studied. KPC/MBL Confirm kit and ESBL + AmpC Screen kit tests showed synergistic effect with both dipicolinic and boronic acid; this is

typical of MBL and AmpC producers respectively. The isolates were then further studied for β -lactamase production by biochemical and molecular assays (Figure 3.2).

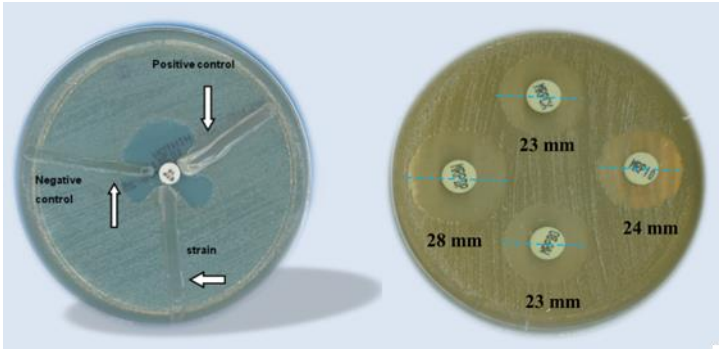

Figure 3.2. Phenotypic carbapenemases detection: MHT and the MBL Rosco tests positive for *C. koseri*, *K. oxytoca* and transconjugants; MRP: meropenem; MRPDP: meropenem + acido dipicolinico; MRPBO: meropenem + acido boronico; MRPCX: meropenem + cloxacillina.

The isolates were than further studied for β-lactamase production by biochemical and molecular assays. Analytical IEF performed using crude enzymatic extracts from the clinical isolates and nitrocefin as chromogenic substrate, showed the presence of an unique β-lactamase band with pI 9.2 corresponded to AmpC cephalosporinase.

Both of the *K. oxytoca* and *C. koseri* donor strains were able to transfer the resistance plasmid to *E. coli* K12 strain J62 (*pro*$^-$, *his*$^-$, *trp*$^-$, *lac*$^-$, *Sm*^{r}) and J53 (*met*, *pro*$^-$, *rif*^{r}) as recipients. Transfer of cefotaxime (CTX) resistance was observed at a frequency of approximately 10^{-3} transconjugants per recipient from both *K. oxytoca* and *C. koseri*.

Compared to the recipient *E. coli* J53 and J62 strains, the transconjugants exhibited a decreased susceptibility to several β-lactams (including carbapenems), trimethoprim-sulphamethoxazole and aminoglycosides. The resistance phenotypes of transconjugants were similar to those of the donors *K. oxytoca* and *C. koseri* for the other antimicrobial agents tested (ANNEX 1).

PCR analysis performed on both donors and transconjugant strains yielded positive results for the co-presence of the *bla*VIM and *bla*CMY determinants in all the isolates. Amplicons sequencing revealed that *K.*

oxytoca, *C. koseri* and *E. coli* transconjugants carried both *bla*VIM-4 and *bla*CMY-4 genes.

Plasmid analysis showed that the above resistance determinants were located in a 150 kb conjugative plasmid belonging to the IncA/C incompatibility group. The IncP and IncN incompatibility groups were also observed in *K. oxytoca* (plasmid 190 Kb; 300 kb) (Figure 3.3, Figure 3.4).

The *K. oxytoca* and *C. kosery* IncA/C multiresistance plasmid was characterized. IntI1 was detected in both isolates analyzed. PCR results showed that the plasmid contained two distinct resistant *loci* carrying the *aadA1* gene and *bla*VIM-4, found as the first gene cassette of a class 1 integron, and CMY-4 β-lactamase gene.

The sizes of the bands obtained (5'-CS and VIM-R primers, 791 bp; Int1-R and VIM-F primers, 526 bp) corresponded to those predicted by joining the published sequences of the of the multiresistance region of pCC416 carrying *bla*VIM-4 (accession no. NG_036464.1).

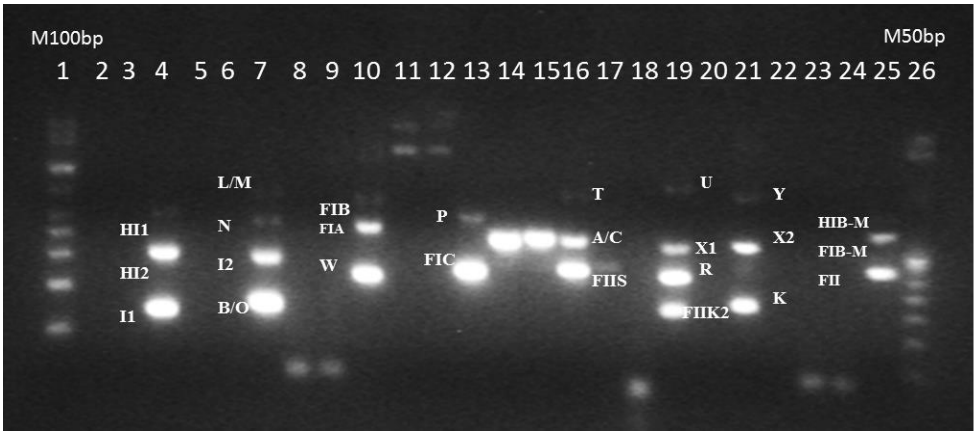

Figure 3.3. *C. kosery* Plasmid Typing using the PBRT kit-PCR based replicon typing scheme

Lane 1: Marker 100 bp; 2: *C. kosery* (Mix1); 3: J62R *C. kosery* (Mix1); 4: C+ (Mix1); 5: *C. kosery* (Mix2); 6: J62R *C. kosery* (Mix2); 7: C+ (Mix2); 8: *C. kosery* (Mix3); 9: J62R *C. kosery* (Mix3); 10: C+ (Mix3); 11: *C. kosery* (Mix4); 12: J62R *C. kosery* (Mix4); 13: C+ (Mix4); 14*: C. kosery* (Mix5); 15: J62R *C. kosery* (Mix5); 16: C+ (Mix5); 17: *C. kosery* (Mix6); 18: J62R *C. kosery* (Mix6); 19: C+ (Mix6); 20: *C. kosery* (Mix7); 21: C+ (Mix7); 22: J62R *C. kosery* (Mix7); 23: *C. kosery* (Mix8); 24: J62R *C. kosery* (Mix8); 25: C+ (Mix8); 26:Marker 100 bp.

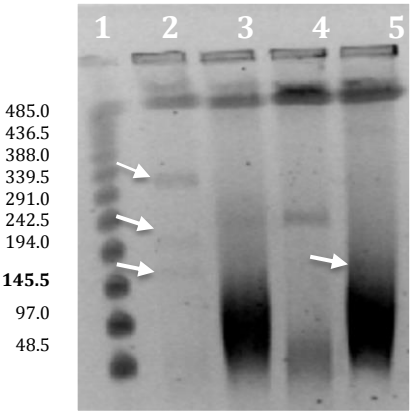

Figura 3.4. S1 nuclease digestion on the pulsed-field electrophoretic mobilit
Lane 1: λ concatamers markers; 2: *K. oxytoca*; 3: *C. kosery*

3. Discussion

To our knowledge this is the first report on the detection of MDR *K. oxytoca* and *C. koseri* clinical isolates co-producing VIM-4 and CMY-4 enzymes, in Italy. Such strains were recovered from the preputial sample of a patient admitted to a Cardiological Rehabilitation ward of "S. Maugeri" hospital of Pavia.

The susceptibility profiles of the studied strains were coherent with a carbapenemase genes expression (MER MIC, >0,5mg/L), and phenotypic tests used for screening/confirmation of carbapenemase production yielded positive results. The co-presence of a CMY-type enzyme was suggested by IEF results (pI 9.2), and confirmed by PCR and sequencing.

The *K. oxytoca* and *C. koseri* MDR strains retained complete susceptibility to colistin. The administration of colistin in monotherapy allowed a positive outcome, with the complete resolution of the infection, even if limited clinical experience suggests satisfactory outcome against carbapenemase-producing *Enterobacteriaceae.* The inferior clinical efficacy of this agent as monotherapy may be associated with suboptimal dosing regimens of drug. The combination treatment with other active agents such as tigecycline, or carbapenems (when the MIC for the infecting organism is ≤4μg/ml) may be considered. *In vitro* data also show synergy between colistin and other agents and support this practice (Kosmidis *et al*. 2012).

The *bla*VIM-4 and *bla*CMY-4 resistance genes were co-transferred to *E. coli* during conjugation.The similarity of the resistance profiles observed in the transconjugants and the donors suggests that all resistance genes were probably harboured in the transferable plasmid (ANNEX 1). This fact highlights the importance of plasmids and other genetic structures that can be mobilized and disseminated among susceptible isolates. Moreover, the high transfer frequency proves the plasmid potential of diffusion and dissemination among susceptible isolates, also of different species. Plasmid analysis showed that the above resistance determinants were located in a 150 kb conjugative plasmid belonging to the IncA/C incompatibility group.

The variability of plasmids mediating antimicrobial resistance in *Enterobacteriaceae* is high. There are plasmid families that are largely prevalent and also plasmids prevalently associated with specific resistance genes. The IncFII, IncA/C, IncL/M and IncI1 plasmids showed the highest

occurrence among typed resistance plasmids. These plasmids can be considered “epidemic”, being detected in different countries, and in bacteria of different origins and sources. The occurrence of these plasmid types seems tightly linked to the selective pressure exerted by antimicrobial use, incrementing their prevalence compared to that observed in bacterial populations that are not preselected for antimicrobial resistance.

Incompatibility group IncA/C plasmids are large, low copy, plasmids that have been described in the literature for over 40 years. However, they have only recently been intensively studied on the genomic level because of their associations with the emergence of multidrug resistance in enteric pathogens of humans and animals. These plasmids are unique among other enterobacterial plasmids in many aspects, including their modular structure and gene content.

Circulation of IncA/C plasmids in Gram-negative pathogens is now common, and these plasmids bring with them the ability to encode resistance to broad arrays of antimicrobial agents (Johnson TJ *et al.* 2012).

IncA/C plasmids carrying both the *bla*VIM-4 and *bla*CMY-4 genes were already identified in Italy in clinical isolates of *K. pneumonia* and *E. cloacae* (Luzzaro F *et al.* 2004). The scaffolds of these plasmids were similar to those of the IncA/C plasmids carrying *bla*CMY-4 or *bla*CMY-2 from *S. enterica* isolated in United States and the United Kingdom, but the carbapenemase gene was not present on these *Salmonella* plasmids and likely represents a novel acquisition for the IncA/C plasmids (Carattoli A 2009).

3. Conclusions

The emergence of carbapenemases in *K. oxytoca* and *C. koseri* poses relevant clinical problems. The coexistence in the same bacterial cell of a plasmid carrying epidemiologically important resistance genes is worrisome, since it could predict the generation and the spread of pan-resistant bacteria and the consequent treatment option limitations that can lead to significant morbidity and mortality (Luzzaro F *et al.* 2004).

This study demonstrates that the problem of MBL-producing pathogens no longer entails Gram-negative non-fermenters alone, but also involves enterobacteria, and confirms the need to continually monitor for the presence of other β-lactamases genes (i.e. AmpC cephalosporinases), that can co-exist in carbapenemase producers. The study highlights the need to monitor the presence and the trends of ESBL and acquired AmpC enzymes among species that can cause infections in immuno-compromised hosts. Surveillance may be of value in the difficult battle against life-threatening bacterial infections.

Plasmids are major vehicles for horizontal spreading of antibiotic resistance genes among bacterial pathogens. Accumulation of various resistance genes on a replicon confers an evolutionary advantage because a single transfer event can result in resistance to multiple antibiotics in the respective host.
The spread of pCC416, a multiresistant plasmid among *K. pneumoniae* and *E. cloacae* clinical strains in Italy has been described recently. The resistance spectrum of pCC416 included virtually all β-lactam antibiotics as well as aminoglycosides. pCC416 represents an efficient vector for the *en bloc* transfer of resistance to most clinically useful antibiotics. In particular, pCC416 carried the MBL gene *bla*VIM-4 and the AmpC-like β-lactamase gene *bla*CMY-4 that were located in two distinct regions. The sequencing data suggest that pCC416 derived from the IncA/C2 CMY plasmid through acquisition of a segment containing a novel *bla*VIM-4 integron (Colinon *et al*. 2007)

Thus it seems to be really interesting to further characterize the plasmid containing *bla*VIM-4 and CMY-4 genes by sequencing approach and do a subsequent comparison with the multiresistant plasmids like pCC416 belonged to the same incompatibility group (IncA/C).

3. References

Barton, B. M., Harding, G. P., & Zuccarelli, A. J. (1995). A general method for detecting and sizing large plasmids. *Analytical biochemistry*, *226*(2), 235-240.

Carattoli, A. (2013). Plasmids and the spread of resistance. *International Journal of Medical Microbiology*, *303*(6), 298-304.

Carattoli, A. (2009). Resistance plasmid families in Enterobacteriaceae.*Antimicrobial agents and chemotherapy*, *53*(6), 2227-2238.

Carattoli, A., Bertini, A., Villa, L., Falbo, V., Hopkins, K. L., & Threlfall, E. J. (2005). Identification of plasmids by PCR-based replicon typing. *Journal of microbiological methods*, *63*(3), 219-228.

Carattoli, A., Miriagou, V., Bertini, A., Loli, A., Colinon, C., Villa, L., ... & Rossolini, G. M. (2006). Replicon typing of plasmids encoding resistance to newer beta-lactams. *Emerg Infect Dis*, *12*(7), 1145-8.

Carattoli, A., Villa, L., Poirel, L., Bonnin, R. A., & Nordmann, P. (2012). Evolution of IncA/C blaCMY-2-carrying plasmids by acquisition of the blaNDM-1 carbapenemase gene. *Antimicrobial agents and chemotherapy*,*56*(2), 783-786.

Castanheira, M., Debbia, E., Marchese, A., & Jones, R. N. (2009). Emergence of a plasmid mediated blaVIM-1 in Citrobacter koseri: report from the SENTRY antimicrobial surveillance program (Italy). *Journal of Chemotherapy*, *21*(1), 98-100.

Cejas, D. A. N. I. E. L. A., Fernandez Canigia, L., Quinteros, M. I. R. T. A., Giovanakis, M., Vay, C., Lascialandare, S., ... & Radice, M. A. R. C. E. L. A. (2012). Plasmid-encoded AmpC (pAmpC) in Enterobacteriaceae: epidemiology of microorganisms and resistance markers. *Rev Argent Microbiol*, *44*(3), 182-186.

Colinon, C., Miriagou, V., Carattoli, A., Luzzaro, F., & Rossolini, G. M. (2007). Characterization of the IncA/C plasmid pCC416 encoding VIM-4 and CMY-4 β-lactamases. *Journal of antimicrobial chemotherapy*, *60*(2), 258-262.

Doran, T. I. (1999). The role of Citrobacter in clinical disease of children: review. *Clinical infectious diseases*, *28*(2), 384-394.

Giani, T., Pini, B., Arena, F., Conte, V., Bracco, S., Migliavacca, R.; AMCLI-CRE Survey Participants, Pantosti, A., Pagani, L., Luzzaro, F., Rossolini, G.M. (2013). Epidemic diffusion of KPC carbapenemase-producing *Klebsiella pneumoniae* in Italy: results of the first countrywide survey, 15 May to 30 June 2011. *Euro Surveill*, 18, 20489.

Hujer, K. M., Hujer, A. M., Hulten, E. A., Bajaksouzian, S., Adams, J. M., Donskey, C. J., ... & Thomson, J. M. (2006). Analysis of antibiotic resistance genes in multidrug-resistant Acinetobacter sp. isolates from military and civilian patients treated at the Walter Reed Army Medical Center.*Antimicrobial agents and chemotherapy*, *50*(12), 4114-4123.

Jacoby, G. A. (2009). AmpC β-lactamases. *Clinical microbiology reviews*,*22*(1), 161-182.

De Blic, J. (1998). Beta-lactamase producers and other bacteria: which ones to take into consideration and when? The viewpoint of the pediatrician.*Presse médicale (Paris, France: 1983)*, *27*, 16.

Johnson, T. J., & Lang, K. S. (2012). IncA/C plasmids: An emerging threat to human and animal health?. *Mobile genetic elements*, *2*(1), 55-58.

Koeleman, J. G., Stoof, J., Van Der Bijl, M. W., Vandenbroucke-Grauls, C. M., & Savelkoul, P. H. (2001). Identification of epidemic strains ofAcinetobacter baumannii by integrase gene PCR. *Journal of clinical microbiology*, *39*(1), 8-13.

Kosmidis, C., Poulakou, G., Markogiannakis, A., & Daikos, G. L. (2012). Treatment options for infections caused by carbapenem-resistant Gram-negative bacteria. *Eur Infect Dis*, *6*(1), 28-34.

Kristóf, K., Tóth, Á., Damjanova, I., Jánvári, L., Konkoly-Thege, M., Kocsis, B., ... & Szabó, D. (2010). Identification of a blaVIM-4 gene in the internationally successful *Klebsiella pneumoniae* ST11 clone and in a Klebsiella oxytoca strain in Hungary. *Journal of Antimicrobial Chemotherapy*,*65*(6), 1303-1305.

Landecker, H. (2015). Antibiotic resistance and the biology of history. *Body & Society*, 1357034X14561341.

Lee, Y. T., Huang, L. Y., Chen, T. L., Siu, L. K., Fung, C. P., Cho, W. L., ... & Liu, C. Y. (2009). Gene cassette arrays, antibiotic susceptibilities, and clinical characteristics of *Acinetobacter baumannii* bacteremic strains harboring class 1 integrons. *Journal of microbiology, immunology, and infection= Wei mian yu gan ran za zhi*, *42*(3), 210-219.

Luzzaro, F., Docquier, J. D., Colinon, C., Endimiani, A., Lombardi, G., Amicosante, G., ... & Toniolo, A. (2004). Emergence in Klebsiella pneumoniae and Enterobacter cloacae clinical isolates of the VIM-4 metallo-β-lactamase encoded by a conjugative plasmid. *Antimicrobial agents and chemotherapy*, *48*(2), 648-650.

Mavroidi, A., Neonakis, I., Liakopoulos, A., Papaioannou, A., Ntala, M., Tryposkiadis, F., ... & Petinaki, E. (2011). Detection of Citrobacter koseri carrying beta-lactamase KPC-2 in a hospitalised patient, Greece, July 2011.*Euro Surveill*, *16*(41), 19990.

Miró, E., Segura, C., Navarro, F., Sorlí, L., Coll, P., Horcajada, J. P., ... & Salvadó, M. (2010). Spread of plasmids containing the blaVIM-1 and blaCTX-M genes and the qnr determinant in *Enterobacter cloacae*, *Klebsiella pneumoniae* and *Klebsiella oxytoca* isolates. *Journal of antimicrobial chemotherapy*, *65*(4), 661-665

Pagani, L., Migliavacca, R., Pallecchi, L., Matti, C., Giacobone, E., Amicosante, G., ... & Rossolini, G. M. (2002). Emerging extended-spectrum β-lactamases in Proteus mirabilis. *Journal of clinical microbiology*, *40*(4), 1549-1552.

Pérez-Pérez, F. J., & Hanson, N. D. (2002). Detection of plasmid-mediated AmpC β-lactamase genes in clinical isolates by using multiplex PCR. *Journal of clinical microbiology*, *40*(6), 2153-2162.

Raimondi, A., Sisto, F., & Nikaido, H. (2001). Mutation in Serratia marcescens AmpC β-lactamase producing high-level resistance to ceftazidime and cefpirome. *Antimicrobial agents and chemotherapy*, *45*(8), 2331-2339.

Romero, E. D. V., Padilla, T. P., Hernández, A. H., Grande, R. P., Vázquez, M. F., García, I. G., ... & Bellido, J. L. M. (2007). Prevalence of clinical isolates of Escherichia coli and Klebsiella spp. producing multiple extended-spectrum β-lactamases. *Diagnostic microbiology and infectious disease*,*59*(4), 433-437.

Rossolini, G. M., Luzzaro, F., Migliavacca, R., Mugnaioli, C., Pini, B., De Luca, F., ... & Toniolo, A. (2008). First countrywide survey of acquired metallo-β-lactamases in gram-negative pathogens in Italy. *Antimicrobial agents and chemotherapy*, *52*(11), 4023-4029.

Seiffert, S. N., Carattoli, A., Tinguely, R., Lupo, A., Perreten, V., & Endimiani, A. (2014). High Prevalence of Extended-Spectrum β-Lactamase, Plasmid-Mediated AmpC, and Carbapenemase Genes in Pet Food. *Antimicrobial agents and chemotherapy*, *58*(10), 6320-6323.

Sturm, P. D., Bochum, E. T., van Mook-Vermulst, S. V., Handgraaf, C., Klaassen, T., & Melchers, W. J. (2010). Prevalence, molecular characterization, and phenotypic confirmation of extended-spectrum beta-lactamases in *Escherichia coli*, *Klebsiella pneumoniae*, and *Klebsiella oxytoca* at the Radboud University Nijmegen Medical Centre in The Netherlands. *Microbial Drug Resistance*, *16*(1), 55-60.

Svara, F., & Rankin, D. J. (2011). The evolution of plasmid-carried antibiotic resistance. *BMC evolutionary biology*, *11*(1), 1.

The European Committee on Antimicrobial Susceptibility Testing. Breakpoint tables for interpretation of MICs and zone diameters. Version 3.1, 2014.

Tsakris, A., Poulou, A., Markou, F., Pitiriga, V., Piperaki, E. T., Kristo, I., & Pournaras, S. (2011). Dissemination of clinical isolates of Klebsiella oxytoca harboring CMY-31, VIM-1, and a New OXY-2-type variant in the community.*Antimicrobial agents and chemotherapy*, *55*(7), 3164-3168.

Watson, J. T., Jones, R. C., Siston, A. M., Fernandez, J. R., Martin, K., Beck, E., ... & Gerber, S. I. (2005). Outbreak of catheter-associated Klebsiella oxytoca and Enterobacter cloacae bloodstream infections in an oncology chemotherapy center. *Archives of internal medicine*, *165*(22), 2639-2643.

Yamasaki, K., Komatsu, M., Abe, N., Fukuda, S., Miyamoto, Y., Higuchi, T., ... & Satoh, K. (2010). Laboratory surveillance for prospective plasmid-mediated AmpC β-lactamases in the Kinki region of Japan. *Journal of clinical microbiology*, *48*(9), 3267-3273.

Zárate, M. S., Gales, A. C., Picao, R. C., Pujol, G. S., Lanza, A., & Smayevsky, J. (2008). Outbreak of OXY-2-producing *Klebsiella oxytoca* in a renal transplant unit. *Journal of clinical microbiology*, *46*(6), 2099-2101.

Chapter 4

ST405 NDM-5 Producing *Escherichia coli* in Northern Italy: The First Two Clinical Cases.

I. Bitar[a], A. Piazza[a], S. Gaiarsa[b,c], L. Villa[d], P. Pedroni[e], E. Oliva[e]., E. Nucleo[a], L. Pagani[a], A. Carattoli[d], R. Migliavacca[a*]

Department of Clinical Surgical Diagnostic and Pediatric Sciences, Microbiology and Clinical Microbiology Unit, University of Pavia, Italy[a]; Microbiology and Virology Unit, Fondazione IRCCS Policlinico San Matteo, Pavia, Italy[b]; Department of Bioscience, University of Milano, Italy[c], Department of Infectious, Parasitic and Immune-Mediated Diseases, Istituto Superiore di
Sanità, Rome, Italy[d]; Clinical Microbiology Laboratory, Clinical Microbiology Laboratory "AO Desenzano del Garda", Brescia, Italy[e]

This work was presented in ICAAC 2015 in San Diego

4. Abstract

ST405 NDM-5-positive *Escherichia coli* strains were isolated from two inpatients at an Italian Hospital in 2014-2015. Bacterial typing was performed and the entire plasmid content was fully sequenced. An IncFII plasmid harboring *bla*NDM-5, *rmtB*, *bla*TEM-1b, *dfrA12*, *aadA2*, *mphA*, *sul1* genes and an IncI1 plasmid, harboring the *bla*CMY-42 gene were co-resident within the ST405 strains. The emergence of a hidden ST405 local reservoir cannot be excluded, even if foreign origin of the MDR ST405 clone was hypothesized.

Keywords: NDM-5; ST405, MDR *Escherichia coli*

4.Acknowledgements

I thank Dr. Oliva and Dr. Pedroni for providing the isolates.

4. Introduction

The spread of carbapenemase producers in *Enterobacteriaceae* represents a global menace, with both nosocomial and community-acquired New Delhi Metallo-beta-lactamase (NDM) *Escherichia coli* now on top of the list of metallo-β-lactamase (MBL) producers in European countries (Dortet et al. 2014). Indeed, the identification of the NDM-producers is no more associated with endemic areas only (e.g. Indian subcontinent, Balkans regions and Middle East); medical tourism, business travel, worldwide diaspora played a central role in establishing secondary reservoirs (Nordman P. and Poirel L. 2014). The early identification of the major pandemic clonal lineages of extraintestinal pathogenic *E. coli* (ExPEC) associated with multi-resistance plasmids is mandatory from both public health and clinical standpoint (Riley L. 2014).

On December 2014 - January 2015, a 61-year-old Italian male, reporting a history of brain hemorrhage and pulmonary embolism, spent a vacation period in Thailand, but was not hospitalized abroad. On 5th March 2015 he was admitted at the Emergency Room of Civil Hospital "La Memoria" of Gavardo (Brescia) due to an acute gallstone pancreatitis. The blood cultures obtained at the time of admission resulted negative for the presence of bacteria and fungi. The patient underwent to surgery for cholecystectomy with biliary drainage and was subjected to empiric therapy with piperacillin-tazobactam (Tazocyn®) for 5 days. Three days after surgery, the patient resulted febrile and a drain blood serum sample resulted positive for the presence of a NDM-positive *E. coli* strain (EcDT-1_GA), detected using the GeneExpert platform with Xpert Carba-R test (Cepheid). The strain displayed high-level resistance to cephalosporins, carbapenems, fluoroquinolones, aminoglycosides, retaining susceptibility only to fosfomycin, colistin and tigecycline by Vitek2 System. A screening rectal swab also identified an intestinal colonization by *E. coli* NDM-positive (EcDT-2_GA). The patient was discharged in good conditions on 31st March 2015.

In late 2015, a 72-year-old Italian female with an history of gallbladder heteroplasia underwent to surgery at the Borgo Roma Hospital of Verona, and a positive rectal carriage of carbapenemase-positive *Klebsiella pneumoniae* and *E. coli* strains was reported at that time. Strains were not available for typing and resistance mechanism investigation. On November 2015, the patient was transferred to the surgical ward and then to the

Palliative Care ward of the Civil Hospital "La Memoria" of Gavardo, where she developed a septicemia due to an *E. coli* MDR strain (EcBA_GA), identified as bla_{NDM} positive by GeneExpert platform. The patient died on 21st December due to gallbladder heteroplasia.

The three clinical NDM-positive *E. coli* strains were sent to the Pavia University for further characterization.

4. Literature review

4.1 Overview of *E.coli*

E.coli is one of the most important commensal inhabitants of the human gastrointestinal tracts of warm-blooded animals and humans. By definition, it commensally lives in the host mutually benefiting and rarely causes diseases. Nevertheless, it is one of the most important pathogen causing diseases in animals and humans. It is mainly classified based on serological identification of O (lipopolysaccharide, LPS) and H (flagellar) antigens. Another classification is based on virulence factors present and host clinical symptoms proposing the pathogenic types; there are at least 7 major pathotypes for enteric *E. coli* and 3 *E. coli* pathotypes as extraintestinal strains (ExPEC) (Table 4.1.1).

Table 4.1.1: *E. coli* pathogenic types

Pathotype (acronym)	Diseases	Symptoms	Virulence factors
Enteric E.coli			
nteroPathogenic *E. coli* (EPEC)	Diarrhoea in children	Watery diarrhoea and vomiting	Bfp, Intimin, LEE
EnteroHaemorrhagic *E. coli*(EHEC)	Haemorrhagic colitis, HUS	Bloody diarrhoea	Shiga toxins, Intimin, Bfp
EnteroToxigenic *E. coli* (ETEC)	Traveler's diarrhoea	Watery diarrhoea and vomiting	Heat-labile and sheat-stable toxins, CFAs
EnteroAggregative *E. coli* (EAEC)	Diarrhoea in children	Diarrhoea with mucus and vomiting	AAFs, cytotoxins
Diffusely Adherent *E. coli* (DAEC)	Acute diarrhoea in children	Watery diarrhoea, recurring UTI	Daa, AIDA
EnteroInvasive *E. coli* (EIEC)	Shigellosis-like	Watery diarrhoea; dysentery	Shiga toxin, hemolysin, Cellular invasion, Ipa
Adherent Invasive *E. coli* (AIEC)	Associated with Crohn disease	Persistent intestinal inflammation	Type 1 fimbriae, Cellular invasion
Extraintestinal E. coli (ExPEC)			
UroPathogenic *E. coli* (UPEC)	Lower UTI and systemic infections	Cystitis, pyelonephritis	Type 1 and P fimbriae; AAFs, hemolysin

Neonatal Meningitis *E. coli*(NMEC)	Neonatal meningitis	Acute meningitis, sepsi	S fimbrie; K1 capsule
Avian Pathogenic *E. coli* (APEC)	Probable source of food-borne disease	-	Type 1 and P fimbriae; K1 capsule

Carbapenem resistance in *E. coli* is a new emerging problem, more importantly in *E.coli* strains since it is mainly found in nosocomial infections. Most of these cases are plasmid- encoded carbapenemases, which facilitate the spread of these infections easily. In Europe the prevalence of these strains appear to follow a north-south distribution, with southern regions having the highest prevalence. This is probably due to different infection control practices and most importantly different antibiotics use. The use of antibiotics in animals led to the emergence of multidrug resistant *E. coli* in animals and food products that is easily transmitted to humans through the consumption of contaminated food.

Moreover, Environment play a vital role in the spread of the antimicrobial resistance, since it serves as a reservoir for a lot of antimicrobial resistant genes from which *E.coli* acquire these genes and share it with potential pathogens in different habitats. Another source of multidrug resistant genes is the hospitals since several studies showed that the presence of antimicrobial resistant genes in hospitals contribute to the spread of these genes through municipal sewage and ventilators.

In the spring of 2011, a novel *E.coli* O10:H4 serotype caused an outbreak in Central Europe, mainly in Germany, infecting 4000 individuals and provoking more than 900 cases of Hemolytic-uremic syndrome (HUS). This strain demonstrated a combination of virulence factors of both EnteroAggregative *E. coli* (EAEC) and EnteroHaemorrhagic *E. coli* (EHEC).

EHEC O157:H7 had been the main pathogen responsible for HUS cases in several European countries. However, the highly virulent EHEC O26:H1 1/H^- is emerging rapidly in Europe. In Italy and Spain, the *E. coli* O25b:ST131 (sequence type 131) is an emerging multi drug resistant ExPEC strain causing a broad spectrum of diseases mainly in UTIs (Allocati *et al*. 2013)

4.2. New Delhi Metallo-beta-lactamase

NDM-1 was the most recent transferable class B β-lactamase discovered. It is able to hydrolyze all beta-lactam antibiotics except for monobactam. In addition, NDM positive bacteria usually carry a set of genes responsible for a variety of antibiotics such as aminoglycosides, fluoroquinolones, macrolides and sulfonamides. The origin of the *bla*NDM-1 is not yet precisely understood, however it is thought to be of bacterial plant origin (chromosomal), such as *Pseudoxanthomonas* and related bacteria that are widely spread in the environment (Berrazeg *et al*. 2014).

In 2009, the first report of NDM-1 was described. A Swedish patient of Indian origin was hospitalized in Orebro in Sweden. The patient was infected by *E. coli* and *K. pneumoniae*, and has been previously hospitalized in 2007 New Delhi, India. And Since the description of NDM-1 in 2009, 8 variants of this enzyme have been published (NDM-1 to NDM-8) and 12 have been assigned (http://www.lahey.org); most of these variants were originated from Asia. Compared to NDM-1, NDM-4, NDM-5 and NDM-7 has increased activity against carbapenems with a systemic association with other resistant determinants against antibiotics; plasmid mediated AmpC cephalosporinase, clavulanic acid-inhibited expanded-spectrum β-lactamases, other types of carbapenemases (OXA-48, VIM and KPC), broad-spectrum resistance to aminoglycosides (16 RNA methylases, quinolones (Qnr), macrolides (esterases), rifampicin, chloramphenicol and to sulphamethoxazole.

Pakistan, India and Sri Lanka have been identified as the main reservoir of NDM-producing *Enterobacteriaceae* not only in humans but also in soil. As previously mentioned, it is likely that the environment is heavily contaminated with NDM producers. It is estimated that the prevalence of carriage to be 5-15% in the Indian subcontinent. However, reports of NDM infections have been recorded in UK, most probably because of the close relationships between India and Pakistan on one hand, and the UK on the other hand. Nevertheless, today, reports of NDM producing *Enterobacteriaceae* have been reported worldwide yet becoming on the top of list of carbapenemase producers in Europe such as France and UK.

The identification of NDM producers is not constricted to the Indian subcontinent, which support the hypothesis of establishment of secondary reservoirs. Balkan states, the Arabian Peninsula and North African countries are considered an important source of NDM producers. This could be

explained by the impact of intercontinental travel as a source of spreading NDM producers and the reason of establishing these secondary reservoirs. On top of this, the persistent of NDM producers in the human gut, will contribute to the human-human transfer leading to autochthonous case in non-endemic areas, as observed in France (Figure 4.2.1)

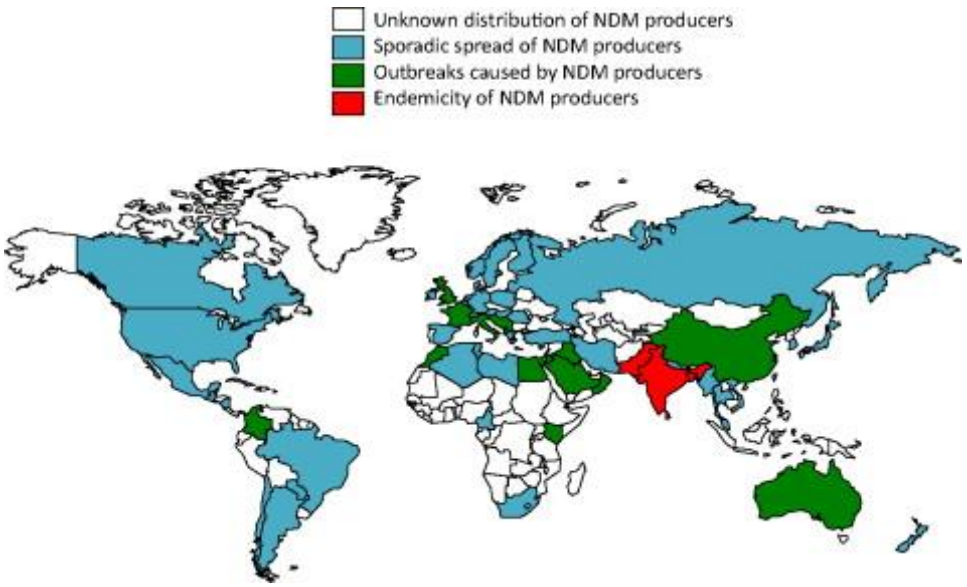

Figure 4.2.1: Geographic distribution of NDM producers

K. pneumoniae and *E. coli* are the main enterobacterial species producing NDM. NDM-producing *E.coli* is of main interest, since it is the main pathogen involved in urinary tract infections, community-acquired infections and diarrhea as well as nosocomial infections. Moreover, diarrhea is considered one of the most important causes of community infection spread, which is difficult to contain and to treat in case of antibiotic resistance (Nordmann P. and Poirel L. 2014).

The first NDM-5 encoding gene was isolated in UK from a patient infected with an *E. coli* (EC045) who had a recent hospitalization in India. NDM-5 is different from NDM-1 in 2 amino acid substitutions (Val88Leu and Met154Leu). These substituations increased the affinity of resistance to carbapenems and broad-spectrum cephalosporins (Figure 4.2.2.) (Yang *et al.* 2014)

Figure 4.2.2: Geographic distribution of NDM variants detected worldwide (Sassi *et al*. 2014).

4. Aim of the study

The aim of th study was to report the transmission of NDM-5 variant to Italy for the first time, and report the dissemenasion of the clone to another patient which might be the start of a hot spot if measures is not taken t contain the spread. Moreover we aim to characterize the plasmid carrying the *bla*NDM and the environment around it and to identify the resistance genes that the plasmid encode.

4. Material and methods

Identification and antibiotic susceptibilities were confirmed using Microscan Autoscan4 System (Beckman Coulter, Italy); MBL production was phenotypically investigated by Mueller-Hinton agar (MHA) (Oxoid, Basingstoke, UK) in the presence of an ertapenem (ETP) and of ETP plus dipicolinic acid disc. The ETP, meropenem (MER) and imipenem (IPM) minimum inhibitory concentrations (MICs) were determined by Etest (bioMérieux, Marcy-l'Étoile, France). Data were interpreted according to EUCAST breakpoint tables v6.0; *E. coli* ATCC 25922 was used as a quality control strain. The three *E. coli* isolates showed a MDR phenotype, being resistant to all β-lactams (ETP, IPM and MER MICs >32 mg/L), aminoglycosides, fluoroquinolones, nitrofurantoin and sulfonamides; retained susceptibility to tigecycline, colistin and fosfomycin. The presence of *bla*NDM, and *bla*CMY, *bla*TEM type β-lactamases was detected by Check-MDR CT103 XL array (Check points Health B.V., Wageningen, The Netherlands).

PCR and sequencing confirmed the identification of the *bla*NDM-5 and *bla*CMY-42 determinants using previously defined PCR protocols (4Nordman P. et al 2011, Perez F. and Hanson N. 2002). Strains were typed by *XbaI*-Pulsed Field Gel Electrophoresis (PFGE, Piazza *et al*. 2016), Multi-locus sequence typing (MLST, http://mlst.warwick.ac.uk), PCR-based Replicon typing (Carattoli *et al*. 2005) and PCR-based phylogroup analysis (Clermont *et al*. 2000). The isolates belonged to phylogenetic group D, ST405 and were indistinguishable by PFGE.

The *bla*NDM-positive plasmids were transferred by conjugation in *E. coli* K12 recipient , selecting transconjugants on McConkey agar containing ETP (1mg/ml) plus streptomycin (1000 mg/ml) or plus rifampin (100 mg/ml). Plasmids were purified by NucleoBondXtraMidiPlus (Macherey-Nagel) and completely sequenced through pair-end 250 bp Illumina MiSEQ system. Scaffolds were generated with Easyfig (Sullivan *et al.* 2011) and Spades 3.8 assemblers and sequence alignments were performed by Mauve (Darling et al. 2004). Scaffolds were screened for resistance genes and replicons using the ResFinder and PlasmidFinder tools, respectively, at the Center for Genomic and Epidemiology (https://cge.cbs.dtu.dk/services/). Scaffolds were assembled in complete plasmid sequences using the pair-end overlapping regions and PCR-based gap closure method.

4. Results and Discussion

Two different conjugative plasmids named pNDM5-IBAC and pCMY-42 were detected within the three isolated ST405 strains. pNDM5-IBAC, was 104.353bp in size, belonged to the IncFII group and harbored *bla*NDM-5, *rmtB*, bla_{TEM-1b}, *dfrA12*, *aadA2*, *mphA*, *sul1* resistance genes, while the IncI1 pCMY-42, 42.792bp in size, was only bla_{CMY-42} positive (Fig. 4.3).

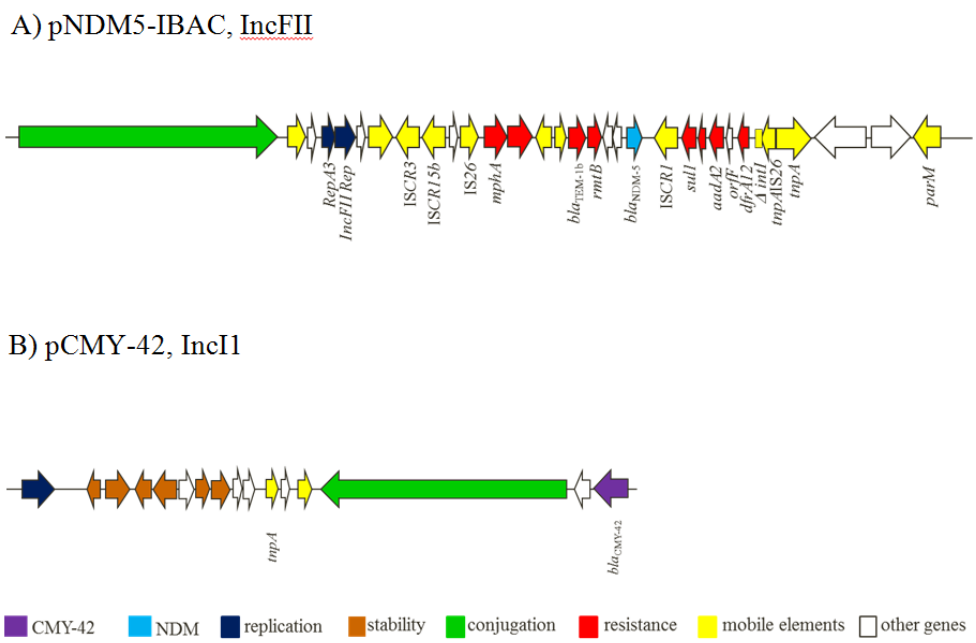

Figure 4.3: Linear map of the plasmids; A) schematic representation of the genetic environment of bla_{NDM-5}. B) Structure of bla_{CMY-42} genetic environment.

Within the IncFII plasmid, the NDM-5 region was bracketed by two IS26 insertion sequences constituting a putative composite transposon, also containing a ISCR1 element and a class I integron with a *intI* gene truncated by the downstream IS26 copy. The *aadA2* and *dfrA12* genes were the integron-borne gene cassettes of this integron (Fig. 4.3). A similar genetic environment of IncFII-NDM-5 plasmid was described in the pHC105 plasmid from Spain (Pitart *et al*. 2015). NDM-1 and -4 have rarely been reported in Italy (Gaihani *et al.* 2001, Coppo *et al.* 2014, D'Andrea *et al.* 2011), but these are the first strains positive for NDM-5.

The presence of the *rmtB* gene in pNDM5-IBAC plasmid justified the high levels of resistance to aminoglycosides observed in the susceptibility profiles of these strains. The co-resident pCMY-42 plasmid likely contributed to the selection of the ST405 clone under piperacillin-tazobactam treatment.

4. Conclusion

NDM-5 *E. coli* have been found in Algeria, India, Spain, UK, Thailand and China but never linked to the ST405 clone (Zhang *et al*. 2016). This clone has been reported both in association with CTX-M-type globally, and with NDM-1 or NDM-4 in Italy. Thanks to infection control measures adopted, further spread of the ST405 clone did not occur at the Gavardo Hospital. Nevertheless, the follow-up of the first patient ascertained the persistence of the EcBA_GA intestinal colonization after six months since the discharge. Long-term NDM-producers persistence in the human gut could contribute to further human-to-human transfer, leading to autochthonous cases in non-endemic areas (Poirel *et al*. 2011, Nordmann *et al*. 2012). Indeed in this case the emergence of a hidden ST405 local reservoir cannot be excluded, even if foreign origin of the MDR ST405 clone was initially hypothesized.

Nucleotide sequence accession number. The complete sequence of pNDM5-IBAC have been deposited at GenBank. The accession number is pending.

4. References.

Allocati, N., Masulli, M., Alexeyev, M. F., & Di Ilio, C. (2013). Escherichia coli in Europe: an overview. *International journal of environmental research and public health*, *10*(12), 6235-6254.

Berrazeg, M., Diene, S., Medjahed, L., Parola, P., Drissi, M., Raoult, D., & Rolain, J. (2014). New Delhi Metallo-beta-lactamase around the world: an eReview using Google Maps. *Euro Surveill*, *19*(20), 20809.

Carattoli A, Bertini A, Villa L, Falbo V, Hopkins KL, Threlfall EJ. 2005. Identification of plasmids by PCR-based replicon typing. J Microbiol Methods 63(3):219-228.

Clermont O, Bonacorsi S, Bingen E. 2000. Rapid and simple determination of the *Escherichia coli* phylogenetic group. Appl Environ Microbiol 66(10):4555-4558.

Coppo E, Del Bono V, Ventura F, Camera M, Orengo G, Viscoli C, Marchese A. 2014. Identification of a New Delhi metallo-β-lactamase-4 (NDM-4)-producing *Escherichia coli* in Italy. BMC Microbiology 14:148.

Dortet L, Poirel L, Nordmann P. 2014. Worldwide dissemination of the NDM-type carbapenemses in Gram-negative bacteria. Biomed Res Int 2014:249856.

D'Andrea MM, Venturelli C, Giani T, Arena F, Conte V, Bresciani P, Rumpianesi F, Pantosti A, Narni F, Rossolini GM. 2011. Persistent carriage and infection by multidrug-resistant *Escherichia coli* ST405 producing NDM-1 carbapenemase: report on the first italian cases. J Clin Microbiol 49:2755-2758.

Gaibani P, Ambretti S, Berlingeri A, Cordovana M, Farruggia P, Panico M, Landini MP, Sambri V. 2011. Outbreak of NDM-1-producing *Enterobacteriaceae* in Northern Italy, July to August 2011. Euro Surveill 16(47):20027.

Nordmann P, Couard JP, Sansot D, Poirel L. 2012. Emergence of an autochthonous and community-acquired NDM-1-producing *Klebsiella pneumoniae* in Europe. Clin Infect Dis 54(1):150-1.

Nordmann P, Naas T, Poirel L. 2011. Global Spread of Carbapenemase-producing *Enterobacteriaceae*. Emerging Infectious Diseases 17(10):1791-1798.

Nordmann, P., & Poirel, L. 2014. The difficult-to-control spread of carbapenemase producers among Enterobacteriaceae worldwide. *Clinical Microbiology and Infection*, *20*(9), 821-830.

Piazza A, Caltagirone M, Bitar I, Nucleo E, Spalla M, Fogato E, D'Angelo R, Pagani L, Migliavacca R. 2016. Emergence of *Escherichia coli* Sequence Type 131 (ST131) and ST3948 with KPC-2, KPC-3 and KPC-8 carbapenemases from a Long-Term Care and Rehabilitation Facility (LTCRF) in Northern Italy. Adv Exp Med Biol 901:77-89.

Pérez-Pérez FJ, Hanson ND. 2002. Detection of Plasmid-Mediated AmpC β-Lactamase Genes in Clinical Isolates by Using Multiplex PCR. J Clin Microbiol 40:2153-2162.

Pitart C, Solé M, Roca I, Román A, Moreno A, Vila J, Marco F. 2015. Molecular characterization of *bla*NDM-5 carried on an IncFII plasmid in an *Escherichia coli* isolate from a nontraveler patient in Spain. Antimicrob Agents Chemother 59(1):659-62.

Poirel L, Hervé V, Hombrouck-Alet C, Nordmann P. 2011. Long-term carriage of NDM-1-producing *Escherichia coli*. J Antimicrob Chemother 66(9):2185-6.

Riley LW. 2014. Pandemic lineages of extraintestinal pathogenic *Escherichia coli*. Clin Microbiol Infect 20:380-390.

Sassi, A., Loucif, L., Gupta, S. K., Dekhil, M., Chettibi, H., & Rolain, J. M. (2014). NDM-5 carbapenemase-encoding gene in multidrug-resistant clinical isolates of Escherichia coli from Algeria. *Antimicrobial agents and chemotherapy*, *58*(9), 5606-5608.

Sullivan MJ, Petty NK, Beatson SA. 2011. Easyfig: a genome comparison visualizer. Bioinformatics 27:1009-1010.
Darling ACE, Mau B, Blattner FR, Perna NT. 2004. Mauve: multiple alignment of conserved genomic sequence with rearrangements. Genome Res 14:1394-1403.

Yang, P., Xie, Y., Feng, P., & Zong, Z. (2014). blaNDM-5 carried by an IncX3 plasmid in Escherichia coli sequence type 167. *Antimicrobial agents and chemotherapy*, *58*(12), 7548-7552.

Zhang F, Xie L, Wang X, Han L, Guo X, Ni Y, Qu H, Sun J. 2016. Further Spread of *bla*NDM-5 in *Enterobacteriaceae* via IncX3 Plasmids in Shanghai, China. Front Microbiol 30(7):424.

Chapter 5

Transmission of a *Klebsiella pneumoniae* clone harbouring *bla*CTX-M-15-like genes in an Italian Neonatal Intensive Care Unit

5. Abstract

We report an outbreak of an extended spectrum beta-lactamase *Klebsiella pneumoniae* (ESBL-kp) clones in a Neonatal Intensive Care Unit (NICU) in northern Italy. A total of 118 ESBL-Kp were included in the study. 115 isolates, collected in the outbreak period April-August 2013, were compared with those obtained in August 2012, October 2012 and February 2013. Colonization of patients was assessed by cultures of rectal swabs sampled once a week. Extended spectrum beta-lactamase (ESBL) production was investigated by phenotypic and molecular tests. Plasmid characterization and whole-genome sequencing were performed. Molecular typing was carried out by Pulse Field Gel Electrophoresis (PFGE) and Multilocus Sequence Typing (MLST). Coexistence of ESBL (*bla*SHV-28, *bla*TEM-206, *bla*CTX-M-15, *bla*OXA-1), quinolone and aminoglycoside resistance genes was identified in 19/20 *K. pneumoniae* isolates chosen as representative, that showed an identical banding pattern by PFGE and were attributed to sequence type (ST) 307. Transmission control interventions, including contact precautions and geographic cohorting and restriction of the new admissions were successful in interrupting the outbreak. Nevertheless, the presence of new resistance genes reinforces the necessity for permanent surveillance programmes.

5. Acknowledgements

I thank Dr. Pietro Marone, Dr. Patrizia Cambieri and Dr. Marta Corbella for providing the isolates.

5. Introduction

Gram-negative bacilli are a leading cause of serious infections. Chemotherapeutic options available to clinicians for treatment of invasive Gram-negative infections have been compromised by the rapid emergence of antimicrobial-resistance mechanisms. Of particular concern, is the capacity of Gram-negative bacilli to accumulate co-resistance and cross-resistance mechanisms to commonly used antimicrobial drug classes, which has culminated to the circulation of multidrug-resistant strains (Berezin *et al.* 2014). Newborns admitted to NICU are at a high risk for developing nosocomial infections due to exposure to invasive medical devices (e.g., mechanical ventilator, central venous catheter) and resistant microorganisms (Sohn *et al.* 2001). The organisms responsible for nosocomial infection in NICU are often transmitted by the hands of nurses, physicians, physiotherapists and other hospital personnel. Therefore, it is evident that hand hygiene is the most important procedure in preventing healthcare associated infections.

Multidrug-resistant Gram-negatives (MDRGN), mainly originating from the intestinal tract of hospitalized infants, are reported in NICU to becoming gradually resistant to several commonly used antibacterial drugs. Dissemination of MDRGNs is attributed to several factors: overuse of antimicrobial drugs with a prominent role of third generation cephalosporins, and cross-transmission via caregiver hands, contaminated equipment or inanimate objects. Failure in infection control practices has been proved to allow diffuse horizontal spread. The incidence infections caused by organisms resistant to the β-lactam agents has also increased in recent years and has often been associated to clonal outbreaks (Cipolla *et al*. 2011). *Klebsiella pneumoniae* is one of the most common pathogen of *Enterobacteriaceae* family responsible for nosocomial infections, especially in NICU, showing a clear trend to spread clonally within healthcare institutions and exhibiting a particular ability to cause nosocomial outbreaks (Rodriguez-Bano J. and Pascual A. 2008). Numerous international studies have shown that the majority of clones of *K. pneumoniae*, responsible for outbreaks in neonatal wards, acquired resistance to penicillins, broad-spectrum cephalosporins and monobactams (except for carbapenems and cephamycins) by the production of ESBLs (Mavroidi *et al*. 2014). The European Antimicrobial Resistance Surveillance Network (EARS-Net)'s surveillance system for Italy has shown that the prevalence of 3rd generation cephalosporins resistant *Klebsiella pneumoniae* isolates is of

56,5% in 2014 (http://ecdc.europa.eu/en/activities/surveillance/EARSNet/database/Pages/database.aspx). ESBL-producing isolates are commonly associated with plasmid mediated fluoroquinolone resistance (PMQR) genes in Enterobacteriaceae. Three PMQR mechanisms have been described; these include (i) the Qnr (qnrA, qnrB, qnrS, qnrC and qnrD) proteins, (ii) the aac(6')-Ib-cr enzyme, and, (iii) QepA and OqxAB plasmid-mediated efflux pumps (Lin et al. 2014). Recently in Italian neonatal intensive care units, outbreaks caused by AmpC-producing *Klebsiella pneumoniae* ST14 and ST26 and carbapenemase-producing *K. pneumoniae* ST258 have been described (Nordberg at al. 2013, Arena et al. 2013, Giuffre et al. 2013). Studies reported recently the rapid emergence of ST307 clone in Italy. ST307 was first reported in Netherlands after being isolated from a urine sample in 2008 and later from a human blood culture in the USA. In 2013, this strain was removed as well from a urinary sample and wound culture in Pakistan (Habeeb *et al*. 2013).

In this study, 118 *K. pneumoniae* isolates collected between April and August 2013 from the NICU of the Foundation IRCCS Polyclinic "S. Matteo" hospital, Pavia, were investigated to characterize their molecular and epidemiologic features and to identify the factors that have contributed to their diffusion.

5. Literature review

5.1. Overview of *Klebsiella pneumonia*

In 1882, Friedlander isolated a *Klebsiella pneumpniae* strain for the first time from the lungs of a patient who died after pneumonia. Later in 1882 the bacteria was designated as *Klebsialla pneumonia*. It was described later as a pathogen that colonize the human gastrointestinal tract, skin, nasopharynx, UT and biliary tract.
One of the most impostant virulence factors is the capsular polysaccharides, as well as other factors involoved in adhesion and siderophores. Due to its high pathogenicity, in the pre-antibiotic era, it was considered an important community-acquired pathogen (CA). However, in recent years reports of CA infections became rare. Starting from the 1970s, epidemiology of *K. pneumoniae* changed dramatically where it established a resiviors in hospital environments and becoming one of the leading causes of nosocomial infection.

The infections are associated with efficient colonization as well as resistance to antibiotics that enabled it to spread widley in health-care settings. It is considered the second (after *E. coli*) in nosocomial Gram-negative bacteria as well as UTIs (Vuotto C. *et al*. 2014).

Klebsiella spp. is considered the most frequent pathogen causing multidrug resistance in Gram-negative bacteria, especially after the emergence of ESBLs. By default, patients that require hospital care associated with medical devices and prolonged hospitalization will be at risk of acquiring *Klebsiella* infection especially patients in ICUs (Hendrik *et al*. 2015).
ESBL was first detected in *Klebsiella pneumoniae* in 1983 in Europe and in 1989 in the United States. During 1990s, the most common ESBL types were SHV and TEM, however starting from late 1990s until now, *Klebsiella* acquired new ESBLs such as CTX-M enzymes, which emerged in hospital and community settings and managed to disseminate worldwide (Perano *et al*. 2012).

In 1996, the first carbapenem resistant *K. pneumonia* was isolated; it carried a plasmid-encoded enzyme (KPC) and since then, KPC-producing *K.pneumoniae* has spread worldwide, acquiring more resistant genes to

carbapenems (*bla*NDM, *bla*OXA-48, *bla*VIM and bla_{IMP-1}). More recently, the emergence of colistin resistant strains has been reported. The resistance to antibiotics is not the only aspect that makes *K. pneumoniae* a public threat, also other genetic factors involoved in variable levels of virulence make it more difficult to treat. The most important factors are fimbrial genes, *mrk* and f*im*, which is involved in adhesion to host tissues, genes involved in iron uptake and enterobactin *ent*. Moreover, the capsular production makes it resistant to phagocytosis and other immune responses (Onori *et al.* 2015).

5.2 Epidemiology of CTX-M ESBLs

ESBL are the most important mechanisms for cephalosporin ressitance in Enterobacteriaceae, particularly in *E. coli* and *K. pneumonia*.the first ESBL identified in Klebsialla spp. was SHV-2 in Germany. Now more than 10 families are identified including CTX-M, SHV, TEM, PER, VEB, GES and OXA.

CTX-M enzymes, the plasmid mediated acquired cefotaximases has a huge clinical impact. Most of these enzymes have a strong activity against cefotaxime and ceftriaxone but not against ceftazidime. Howere variants such as CTX-M-15 and CTX-M-16 have an enhanced catalytic activity against ceftazidime. To date, mpore than 109 CTX-M variants have been described both plasmid encoded and chromosomal.

CTX-M ESBLs are high in prevelnce in *K. pneumonia* as well as *E. coli* and *P. mirabilis*. These strains are widespread not only in humans but also in animals and in the environment. CTX-M producing *E. coli* strains have been reported in animals in China, river water in South Korea and in food samples in Tunisia.

Although the incidence of CTX-M variants is geographically different, CTX-M-15 and CTX-M-14 are the most common variants detected worldwide. This is due to the plasmid-mediated horizontal transfer of this gene, which contributed to the spread and increased prevalence.
Moreover, IncF group (FIA, FIB and FII) is the most prevelant in transmitting the CTX-M-15. Along with the CTX-M gene, plasmids usualyy carry other resistant genes to aminoglycoside, tetracycline, sulfonamide and resitant genes agains β-lactams. At least 29 Inc groups have been reported to carry CTX-M genes however IncFII, IncA/C, Inc L/M and IncI1 plasmids

show the highest occurrence among the typed resistance plasmids (Zhao W. and Hu Z. 2013).

5. Aims of the research

Effective epidemiological studies based on typing are indispensible for confirming the genetic relatedness of closely related isolates belonging to the same clonal lineages as well as for screening and controlling the occurrence and spread of epidemic clones.
Therefore, the study aims to:
1) evaluate molecular and epidemiologic characterization of ESBL-Kp strains isolated from the NICU of the Foundation IRCCS Polyclinic ―S. Matteo‖;
2) confirm the genetic relatedness of isolates belonging to the same clonal lineages;
3) identify the factors that contributed to the selection of ESBL producing *K. pneumoniae* in that department.

5. Materials and Methods

Setting

The Polyclinic "S. Matteo" hospital of Pavia is a 1000-bed University Hospital located in Northern Italy. The NICU offers neonatal care activities to critical newborns; it is a regional referral center for neonatal care III level. Approximately 300 newborns are usually admitted annually to S. Matteo NICU, with 23% weighing less than 1500 grams. Those patients present mostly with prematurity, neonatal hypoxia, hypoxic-ischemic encephalopathy. Before the onset of the outbreak, the infection control policy in the NICU did not include routine surveillance cultures for ESBL-Kp. Furthermore, overcrowding, nursing staff shortage, medical staff workload were evident.

Microbiology and molecular epidemiology

Since April 2013, the infection control practices consisted of surveillance rectal swab cultures in all inpatients at admission time and on a weekly basis. The rectal swabs were sent to the Microbiology and Virology Unit where they were directly inoculated on MacConkey agar plates. Identification and antibiotic susceptibility testing were performed using the Phoenix system (Becton Dickinson, Italy) and interpreted according to EUCAST guidelines (http://www.eucast.org.). Outbreak cases were defined by isolation of an ESBL-Kp strain from any culture of neonates in the NICU. Infection was defined by clinical and laboratory criteria and requirement for antimicrobial therapy, while colonization by the absence of relevant symptoms. Before obtaining the clinical information of the neonates, approval was received by the Ethics Committee. Clinical records from the neonates were collected including the following data: date of birth, gender, and number of days in hospital prior to isolation and specimen type. On the basis of resistance phenotype, specimens type/date of collection, neonatal day of born, 20 representative ESBL-KP were selected to perform the phenotypic and molecular characterization. Isolates collected in September 2011, August and October 2012 were also included in the study. Phenotypic ESBL detection were performed with both the double-disc synergy test (DD) (Jarlier at al. 1988) and with ESBL + AmpC Screen kit (Rosco Diagnostica, Taastrup, Denmark). PCR assays were carried out for amplification of genes encoding ESBLs (*bla*CTX-M, *bla*TEM, *bla*OXA) and aac(6')-Ib-cr genes using primers and conditions described in previous studies (Mugnaioli *et al*. 2005) (Table 5.1). PCR products were purified

using the kit Quantum Prep PCR Kleen Spin Columns (BioRad, Alfred Nobel Drive, Hercules, USA) and subjected to double-strand sequencing using amplification primers (Macrogen Inc., Seoul, South Korea). Production of the respective β-lactamases was confirmed by isoelettrofocusing (IEF). IEF was performed in polyacrylamide gels containing ampholines with a pH range of 3.0 to 10.0 (Amersham Biosciences, France). β-lactamase activities were detected with the chromogen nitrocefin (Oxoid, Basingstoke, Hampshire, England). Detection of the activity against CTX substrate of the β-lactamases bands separated by IEF were assayed by a substrate overlaying procedure as reported previously (Pagani et al. 2002). Conjugal transfer of resistance determinants were performed in liquid medium using the *E. coli* K12 strain J62 (pro-, his-, trp-, lac-, SmR) and J53 (met-, pro-, rifR) as recipients. The transconjugants were selected on McConkey agar containing CTX (8 mg/l) plus streptomycin (1000 mg/l) or rifampicin (100 mg/l). Plasmids were typed according to their incompatibility group using the PCR replicon-typing scheme as described previously (Carattoli *et al*. 2006). The sizes of the plasmids were estimated using the S1 nuclease PFGE method (Barton *et al.* 1995). Clonality of the *K. pneumoniae* isolates was investigated by PFGE using *XbaI* restriction enzyme. In addition, MLST was performed and ST was determined using the *K. pneumoniae* MLST database (http://www.pasteur.fr/recherche/genopole/PF8/mlst/Kpneumoniae.html).

Table 5.1. Primers selected and used in PCR and sequencing reactions

Gene	**Primer (sequence)**	**Fragment size (bp)**
blaCTX-M	F- (5‘ATGTGCAGYACCAGTAARGT-3‘) R- (5‘-TGGGTRAARTARGTSACCAGA-3‘)	593
*bla*OXA	F- (5´-TTTTCTGTTGTTTGG GTT TT-3´) R- (5´-TTT CTT GGC TTT TAT GCT TG-3´)	449
*bla*TEM	F -(5‘-ATAAAATTCTTGAAGACGAA-3‘) R-(5‘-ATATGAGTAAGCTTGGTCTGAC-3‘)	960
aac(6‘)-Ib-cr	F- (5‘-TTGCGATGCTCTATGAGTGG-3‘) R- (5‘-GCGTGTTCGCTCGAATGCC-3‘)	482

NGS

Three strains were streaked on MacConkey plates at 37 degrees overnight. One single colony per strain was used for the downstream genomic analyses treated in this work. DNA was extracted with the NucleoSpin® Tissue kit by (MACHERBEY-NAGEL) and sequenced with the Illumina MiSeq technology, using Nextera XT kits for library preparation. Reads were assembled with the Mira 4.0 assembler (http://www.chevreux.org/projects_mira.html) using the default settings for Illumina reads and excluding the control for high coverage.

Assembeled genomes and contigs were uploaded to PlasmidFinder database to detect which contig contains the plasmid replication initiation site (www.cge.cbs.dtu.dk/services/**PlasmidFinder**/), while resistant genes were determined by uploading the contigs to ResFinder database (www.cge.cbs.dtu.dk/services/**ResFinder**/). And the process of closing plasmids *In-silico* was done using Bandage (https://rrwick.github.io/**Bandage**/). ORFs and their relative amino acids were detected using Artemis (www.sanger.ac.uk/science/tools/**artemis).** Plasmid annotation was done manually using Sequin and the files were uploaded to Genbank (www.ncbi.nlm.nih.gov/**Sequin)**

Note: the output files of Mira 4 could not be used with Bandage, for that reason, genome was assembled using Spades 3.8, which generate a graph that can be read using Bandage (www.bioinf.spbau.ru/spades)

5. Results

An outbreak of ESBL-KP was observed during April- August 2013. The onset of the outbreak was recognised on August 2012, when one ESBL-Kp isolate (16781), indicated as the index case, was recovered from the blood sample of one neonate on the 9th day after admission in the NICU, suggesting acquisition of this isolate in this ward. Another case was detected on the 9th of October and more cases were reported during the first six months of 2013. A significant reduction in new cases was observed starting July 2013 after reinforcing the infection control interventions through reduction of overcrowding, supplementary training for all health care workers, geographic cohorting of affected infants and their nursing staff and encouraging daily cleaning of the surfaces by disinfection. In September 2013, the outbreak was successfully controlled with a complete absence of new cases. The epidemic curve of infections/colonization with ESBL-Kp strains in the hospital is shown in Figure 5.1.

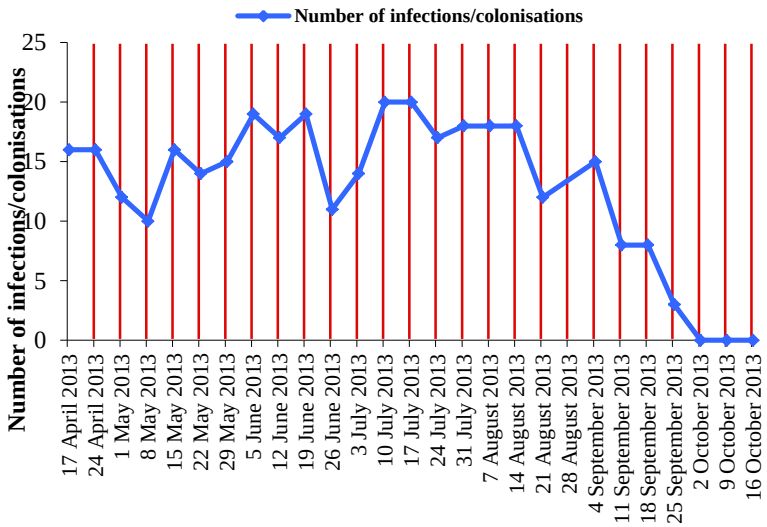

Figure 5.1: Infections/colonisations with beta-lactamase-producing *Klebsiella pneumoniae* strains, NICU of the Foundation IRCCS Polyclinic "S. Matteo" hospital, Pavia, April-August 2013 (n=118)

During the outbreak, 293 children were hospitalized in NICU of which 84 were either colonized (n=66) or infected (n=18) by ESBL-KP. All neonates were born in the maternity unit of the Foundation IRCCS Polyclinic "S.

Matteo" hospital, Pavia, whereas a neonate was admitted to "S. Matteo" hospital from the Civil hospital of Vigevano. Eight of the neonates were delivered by caesarean section. The neonates showed low gestational age (an overall rate of: 32 weeks) and low birth weight (overall rate: 1800 g).

Largely, 118 isolates were obtained from the neonates, of which 93 were from surveillance cultures and 25 from clinical specimens: blood (n=8); lower respiratory tract (n=2); urine (n=9); cerebrospinal fluid specimens (n=1); drainage specimens or swab from other sites (n=5). Isolates of the outbreak clone showed the same MDR pattern (Table 1;ANNEX 1). Phenotypic screening suggested ESBL enzyme production in all 20 representative *Klebsiella pneumoniae* isolates (Figure 5.2). IEF showed four β-lactamase bands common to 19/20 isolates: pI > 8.2, pI 7.5, pI 7.3 and pI of 5.4 generally associated with the expression of CTX-M-type, SHV-type, OXA-type and TEM-type enzyme, respectively (Figure 5.3). One isolate (15332) showed the presence of a unique β-lactamase band with pI 8.2. All 20 isolates presented a band with hydrolytic activity on CTX (1 µg/ml) at pI of 8.2 by overlay assay, related to the production of CTX-M-type enzyme. PCR and sequencing showed that among the 20 ESBL-producing *K. pneumoniae* tested, all carried *bla* CTX-M-15 gene and 19/20 isolates additionally *bla*OXA-1, *bla*TEM-206 and aac(6')-Ib-cr gene.

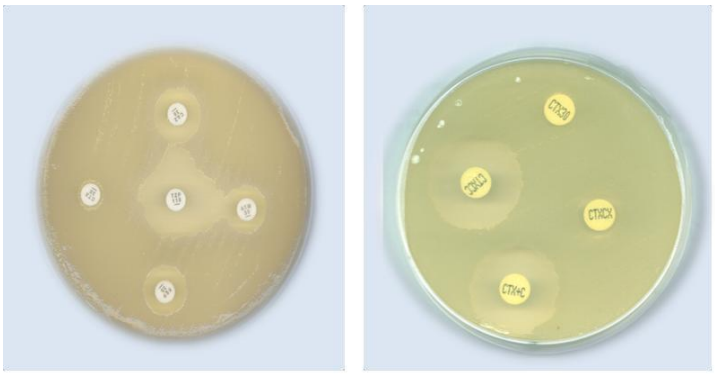

Figure 5.2. Phenotypic detection of ESBL: DD and ESBL + Amp-C Rosco tests

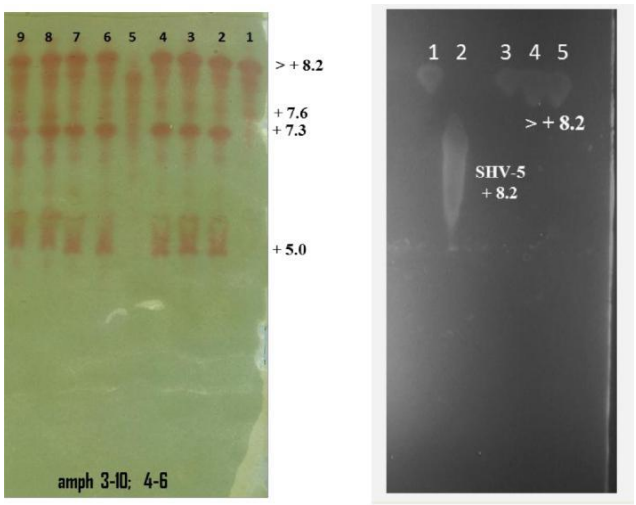

Figure 5.3.. Isoelectric focusing and overlay assay (CTX: 8mg/l)
IEF. LANE 1: Kl 15332; 2: Kl 16781; 3: Kl 16919; 4: J53R Kl 16781; 5: Standard (SHV-5, pI: 8.2); 6: J62R Kl 16781; 7: J53R Kl 16919; 8: J62R Kl 16919; 9: Kl 485.
Overlay assay. LANE 1: Kl 15332; 2: Standard (SHV-5, pI: 8.2); 3: Kl 16781; 4: Kl 16919; 5: J53R Kl 16781.

Plasmid analysis indicated that 19/20 strains had acquired a plasmid of about 200 kb belonged to the IncF incompatibility group. All isolates studied were able to perform conjugation. The transfer of the ESBL determinant was observed at a frequency of approximately 10^{-3} transconjugants per recipient. PFGE was able to classify 19/20 isolates into one PFGE clone, named A, with three main clone A subtypes, A1, A2, and A3, and one strain (15332) in a different clone, named B, according to the criteria described previously (Tenover et al. 1995). MLST attributed 19 out of 20 *K. pneumoniae* strains with the ST307 and one (15332) with the ST14 (Figure 5.4).

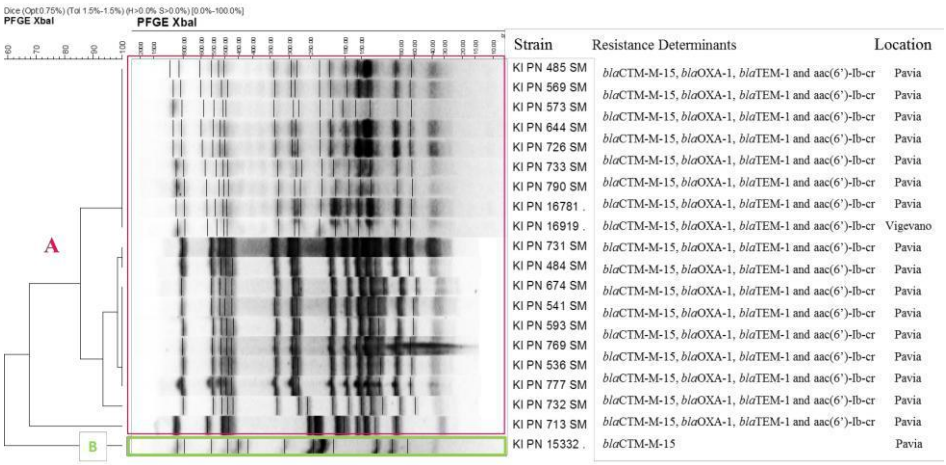

Figure 5.6. Dendrogram of PFGE clusters and genotypic relationships of ESBL-Kp isolates
The 20 isolates identified in this study and analyzed by PFGE are shown. All 19 representative isolates harbored *bla*CTX-M-15/OXA-1/TEM-1 and aac-(6‘)-Ib-cr genes showed the same PFGE fingerprint.

XbaI macrorestriction patterns were analyzed using the Dice coefficient and visualized by unweighted-pair group method, using average linkages with 1.5% tolerance. PFGE groups determined by cluster analysis are named: A (red) and B (green). Resistance determinants and geographic distribution are also included.

The complete genome sequence of three representative strains (732, 733, 16781) confirmed their belonging to ST307 and assigned them to the KN2 capsular type through the presence of the wzi-173 capsular antigen allele. The genome sequencing results confirmed the presence of an IncF multireplicon plasmid pKN3 (194,176 bp) with FIB(k) and FII(k) replicons that carried different resistance genes. Coexistence of ESBL (*bla*SHV-28, *bla*TEM-206, *bla*CTX-M-15, *bla*OXA-1, quinolone and aminoglycoside resistance genes was identified in the isolates, in addition the presence of fosA, tet(A), dfrA14, sul2 resistance genes was observed. Furthermore, it was observed that all ESBL-KP isolates carried the virulence genes encoding the type 3 fimbrial proteins (mrkABDFHIJ) in the chromosome and irp2 gene in the plasmid (Table 2; ANNEX 2).

The plasmid is shared between the 3 strains confirming the dissemination of the same plasmid in the outbreak (plasmid size: 194176 bp) (Figure 5.7.)

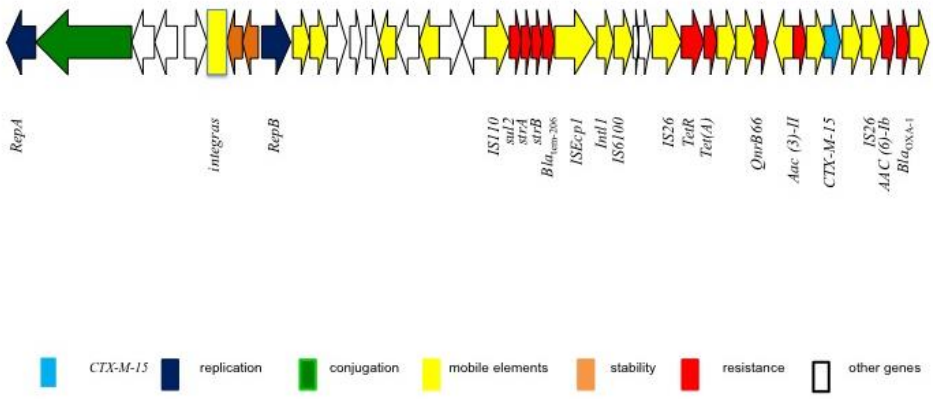

Figure 5.7: Plasmid scheme; CTX-M-15 environment along with other determinants

5. Discussion

The compromised patients admitted to NICU represent a high-risk population for infection with ESBL-KP that are responsible for high rates of morbidity and mortality. This is mainly due to the limited treatment options owing the presence of resistance determinants. ESBL production is the major resistance mechanism to β-lactam antibiotics in *Enterobacteriaceae*. In addition, ESBL producing bacteria are typically associated with multidrug resistance since multiple resistance genes often reside on the same plasmid. Coexistence of ESBL production with resistance to quinolones, aminoglycosides and trimethoprim–sulfamethoxazole has become a global threat for treatment of *K. pneumoniae* infections (Fereshteh et al. 2014). In the present study, we described an outbreak of ESBL-producing *Klebsiella pneumoniae* affecting 84 infants. This occurred during a five-month period in 2013 at the NICU of the Foundation IRCCS Polyclinic "S. Matteo" hospital in Pavia. The outbreak clone has emerged on September 2012 and the strain isolated in August 2012 was identified as the index case. ESBL-Kp were not isolated in the NICU after this period and the outbreak was successfully controlled. A large size cluster was traced over a long period, suggesting the probable role of inadequate hygienic practices by the health care providers. Standard infection control interventions within the NICU were in this case not sufficient to terminate the outbreak. The transmissions were reduced only after the suspension of admission, and cohorting colonized/infected from uncolonized/uninfected newborns into two separate rooms with dedicated staff.

Among the 20 ESBL-KP tested, all carried *bla*CTX-M-15 gene. 19 out of 20 isolates, additionally, carried *bla*OXA-1, *bla*TEM-206 and aac(6')-Ib-cr gene in a pKN3 plasmid belonging to the IncF group. IncF plasmid replicons were most frequently detected in ESBL producing *K. pneumoniae* often associated with *bla*CTX-M-15 gene, *bla*OXA-1 and aac(6')-Ib-cr resistance genes (Carattoli A. 2009). All ESBL-KP isolates showed the presence of *bla*SHV-28, a *bla*SHV-1 mutant detected for the first time in China in 2002. It was subsequently descripted in Italy in clinical *Klebsiella* isolates from dogs and cats (Donati *et al*. 2014). The genome sequencing results confirmed the coexistence of ESBL (*bla*SHV-28, *bla*TEM-206, *bla*CTX-M-15, *bla*OXA-1), quinolone and aminoglycoside resistance genes, in addition to fosA, tet(A), dfrA14, sul2 resistance genes.

To our knowledge, this is the first report of TEM-206 in clinical isolates of *K. pneumoniae* previously found in *E. coli* isolates from farmed chickens in China and in *E. coli* isolates from pig in German (Von Salviati *et al.* 2014). *Klebsiella pneumoniae* strains can produce different virulence factors, such as fimbrial adhesins and siderophores, which are important in the colonization and development of the infection. It was observed that all ESBL-KP isolates carried the virulence genes encoding the type 3 fimbrial proteins (mrkABDFHIJ) in the chromosome and irp2 gene in the plasmid. Ipr2 is a *Yersinia* typical gene involved in the synthesis of siderophore yersiniabactin (Souza Lopes *at al*. 2016). The expression of type 3 fimbriae genes is common to many Gram-negative pathogens that cause catheter-associated urinary tract infection. Type 3 fimbriae belong to the chaperone-usher class of fimbriae and are encoded by five genes (mrkABCDF) arranged in the same transcriptional orientation. Expression of type 3 fimbriae by *K. pneumoniae* facilitates surface adherence, a process strongly activated by the cyclic di-GMP-dependent transcriptional activators. Several studies have clearly demonstrated a role for type 3 fimbriae in biofilm formation. Type 3 fimbriae also mediate various adherence functions such as binding to epithelial cells (from the respiratory and urinary tracts) and extracellular matrix proteins (e.g. collagen V) (Jeremiah *et al*. 2011). One clonal pattern was observed instead among the nineteen ESBL-producing *K. pneumoniae* isolates that coincided with ST307, typing belonging to a clonal complex different from the habitual sequence clone ST258 isolated in Italy. ST307 was first reported from Netherlands by Paltasing et al. and was isolated from a urine sample of a human in 2008. Later on, Farrell *et al.* depicted it in the USA from a human blood culture in 2010 Recently, ST307 bearing KPC-2 has been reported two different Italian hospitals (Richter *et al.* 2012). In more recent surveillance studies from South Italy, multifocal dissemination of KPC-3-producing *K. pneumoniae* clones was observed. This showed the rapid emergence of the KPC-3-Kp ST307 clone, also coproduced the CTX-M-15. Interestingly, the whole genome sequencing of a *Klebsiella pneumoniae* ST307 strain was identified from urine-culture in a tertiary care hospital in South Italy in 2014. It revealed the presence of a pKN3 plasmid with the *bla*CTX-M-15, *bla*OXA-1 and aac(6')-Ib-cr (Villa *et al.* 2016).

Transmission of plasmid carrying these resistant genes will increase the incidence of multidrug resistance. Early detection of these genes will help in prevention and adequate infection control by limiting the spread of these organisms. Strategies to control outbreaks of MDRO colonization/infection in the NICU may include performing hand hygiene, cohorting and isolating

patients, screening healthcare workers and performing admission and periodic surveillance cultures. Hand hygiene is the most important preventive procedure; nevertheless hand hygiene compliance among healthcare workers remains low. Continuous educational strategies can improve hand hygiene and contribute to reducing the incidence of neonatal infections (Cipolla *et al*. 2008).

5. Conclusion

This study reports an ST307 *K. pneumoniae* clone which, carrying resistance and virulence genes imposes significant therapeutic limitations on the treatment of hospital- and community-acquired infections and could compete with the worldwide successful ST258 clone, although larger studies need to determine their epidemiological importance. Nevertheless, the presence of new resistance genes reinforces the necessity for permanent surveillance programs. Local epidemiologic studies, country-, region- or even hospital-specific, are thus need for attending physicians in managing their patients in daily practice, for microbiologists in detecting more accurately the resistance patterns of pathogens circulating within their hospital, and for both in implementing more efficient systems of infection control to prevent the spread of multidrug resistant *K. pneumoniae* infections in Italy, in lines with the other programs adopted in Europe.

5. References

Arena F, Giani T, Becucci E, Conte V, Zanelli G, D'Andrea MM, Buonocore G, Bagnoli F, Zanchi A, Montagnani F, Rossolini GM. Large oligoclonal outbreak due to *Klebsiella pneumoniae* ST14 and ST26 producing the FOX-7 AmpC β-lactamase in a neonatal intensive care unit. J Clin Microbiol. 2013; 51(12):4067-72.

Barton BM, Harding GP, Zuccarelli AJ. A general method for detecting and sizing large plasmids. Anal Biochem. 1995; 226(2):235-40.

Berezin EN, Solórzano F; Latin America Working Group on Bacterial Resistance. Gram-negative infections in pediatric and neonatal intensive care units of Latin America. J Infect Dev Ctries. 2014;8(8):942-53.

Carattoli A. Resistance plasmid families in *Enterobacteriaceae*. Antimicrob Agents Chemother. 2009; 53(6):2227-38.

Carattoli A, Miriagou V, Bertini A, Loli A, Colinon C, Villa L,Whichard JM, Rossolini GM. Replicon typing of plasmids encoding resistance to newer beta-lactams. Emerg Infect Dis 2006; 12(8):1145–8.

Cipolla D, Giuffrè M, Mammina C, Corsello G Prevention of nosocomial infections and surveillance of emerging resistances in NICU. J Matern Fetal Neonatal Med. 2011; 24(1):23-6.

Donati V, Feltrin F, Hendriksen RS, Svendsen CA, Cordaro G, García-Fernández A, Lorenzetti S, Lorenzetti R, Battisti A, Franco A. Extended-spectrum-beta-lactamases, AmpC beta-lactamases and plasmid mediated quinolone resistance in *Klebsiella* spp. from companion animals in Italy. PLoS One. 2014;9(3):e90564.

European Centre for Disease Prevention and Control (ECDC). European Antimicrobial Resistance Surveillance Network (EARS-Net) database. [Accessed: July 2016]. Available from:
http://ecdc.europa.eu/en/activities/surveillance/EARSNet/database/Pages/database.aspx.

Fereshteh R, Fereshteh E, Mohammad MF. Prevalence of Quinolone Resistance Among Extended-Spectrum β -Lactamase Producing Uropathogenic *Klebsiella pneumoniae.* Jundishapur J Microbiol. 2014; 7(6): e10887

Giuffrè M, Bonura C, Geraci DM, Saporito L, Catalano R, Di Noto S, Nociforo F, Corsello G, Mammina C. Successful control of an outbreak of colonization by Klebsiella pneumoniae carbapenemase-producing *K. pneumoniae* sequence type 258 in a neonatal intensive care unit, Italy. J Hosp Infect. 2013;85(3):233-6

Habeeb MA, Haque A, Nematzadeh S, Iversen A, Giske CG. High prevalence of 16S rRNA methylase RmtB among CTX-M extended-spectrum β-lactamase-producing *Klebsiella pneumoniae* from Islamabad, Pakistan. Int J Antimicrob Agents. 2013; 41(6):524-26.

Hendrik, T. C., Voor, A. F., & Vos, M. C. (2015). Clinical and Molecular Epidemiology of Extended-Spectrum Beta-Lactamase-Producing Klebsiella spp.: A Systematic Review and Meta-Analyses. *PloS one*, *10*(10), e0140754.

Jarlier V, Nicolas MH, Fournier G, Philippon A. Extended broad-spectrum _-lactamases conferring transferable resistance to newer β-lactam agents in *Enterobacteriaceae*: hospital prevalence and susceptibility patterns. Rev. Infect. Dis. 1988; 10(4):867–78.

Jeremiah G. Johnson, Caitlin N. Murphy, Jean Sippy, Tylor J. Johnson, and Steven Clegg) Type 3 Fimbriae and Biofilm Formation Are Regulated by the Transcriptional Regulators MrkHI in *Klebsiella pneumoniae* J Bacteriol. 2011; 193(14): 3453–60.

Klebsiella pneumoniae MLST Database. Paris: Institut Pasteur.[Accessed May 2014]. Available from: http://www.pasteur.fr/recherche/genopole/PF8/mlst/Kpneumoniae.html

Lin L, Binting W, Shuai F , Jinnian L , Congming W , Ying W , Xiangchun R , Minghua Z. Prevalence and Characteristics of Extended-Spectrum β-Lactamase and Plasmid-Mediated Fluoroquinolone Resistance Genes in *Escherichia coli* Isolated from Chickens in Anhui Province, China. PLoS One. 2014; 9(8): e104356

Mavroidi A, Liakopoulos A, Gounaris A, Goudesidou M, Gaitana C, Miriagou V, Petinaki E. Successful control of a neonatal outbreak caused mainly by ST20 multidrug- resistant SHV-5-producing *Klebsiella pneumoniae*, Greece. BMC Pediatrics. 2014; 14:105.

Mugnaioli C, Luzzaro F, De Luca F, Brigante G, Amicosante G,and Gian Maria Rossolini GM. Dissemination of CTX-M-Type Extended-Spectrum β-Lactamase Genes to Unusual Hosts Clin Microbiol. 2005; 43(8): 4183–85.

Nordberg V, Quizhpe Peralta A, Galindo T, Turlej-Rogacka A, Iversen A, Giske CG, Navér L. High proportion of intestinal colonization with successful epidemic clones of ESBL-producing *Enterobacteriaceae* in a neonatal intensive care unit in Ecuador. PLoS One. 2013; 8(10):101-7.

Onori, R., Gaiarsa, S., Comandatore, F., Pongolini, S., Brisse, S., Colombo, A., ... & Bandi, C. (2015). Tracking Nosocomial Klebsiella pneumoniae Infections and Outbreaks by Whole-Genome Analysis: Small-Scale Italian Scenario within a Single Hospital. *Journal of clinical microbiology*, *53*(9), 2861-2868.

Pagani L, Migliavacca R, Pallecchi L, Matti C, Giacobone E, Amicosante G, Romero E, Rossolini GM. Emerging extended-spectrum β-lactamases in *Proteus mirabilis.* J Clin Microbiol 2002; 40(4):1549–52.

Peirano, G., Sang, J. H. K., Pitondo-Silva, A., Laupland, K. B., & Pitout, J. D. (2012). Molecular epidemiology of extended-spectrum-β-lactamase-producing Klebsiella pneumoniae over a 10 year period in Calgary, Canada.*Journal of antimicrobial chemotherapy*, dks026.

Richter SN, Frasson I, Franchin E, Bergo C, Lavezzo E, Barzon L, et al. KPC mediated resistance in Klebsiella pneumoniae in two hospitals in Padua, Italy,June 2009–December 2011: massive spreading of a KPC-3-encoding plasmid and involvement of non-intensive care units. Gut Pathog 2012;4:7.

Rodriguez-Bano J, Pascual A. Clinical significance of extended-spectrum beta-lactamases. Expert Rev Anti Infect Ther. 2008; 6(5):671–83.

Sohn AH, Garrett DO, Sinkowitz-Cochran RL, Grohskopf LA, Levine GL, Stover BH, Siegel JD, Jarvis WR; Pediatric Prevention. Network Prevalence of nosocomial infections in neonatal intensive care unit patients: Results from the first national point-prevalence survey study. J Pediatr. 2001;139(6):821-27.

Souza Lopes AC, Rodrigues JF, Cabral AB, da Silva ME, Leal NC, da Silveira VM, de Morais Júnior MA Occurrence and analysis of irp2 virulence gene in isolates of *Klebsiella pneumoniae* and *Enterobacter* spp. from microbiota and hospital and community-acquired infections. Microb Pathog. 2016;96:15-9.

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; 33(9):2233-39

The European Committee on Antimicrobial Susceptibility Testing. Breakpoint tables for interpretation of MICs and zone diameters. 2014; Version 4.0. http://www.eucast.org.

Villa L, Feudi C, Fortini D, Iacono M, Bonura C, Endimiani A, Mammina C, Carattoli A. Complete Genome Sequence of KPC-3- and CTX-M-15-Producing Klebsiella pneumoniae Sequence Type 307.Genome Announc. 2016 ;4(2).

Vuotto, C., Longo, F., Balice, M. P., Donelli, G., & Varaldo, P. E. (2014). Antibiotic resistance related to biofilm formation in Klebsiella pneumoniae.*Pathogens*, *3*(3), 743-758.

Von Salviati C, Friese A, Roschanski N, Laube H, Guerra B, Käsbohrer A, Kreienbrock L, Roesler U. Extended-spectrum beta-lactamases (ESBL)/AmpC beta-lactamases-producing *Escherichia coli* in German fattening pig farms: a longitudinal study. Berl Munch Tierarztl Wochenschr. 2014 Sep-Oct;127(9-10):412-9.

Zhao, W. H., & Hu, Z. Q. (2013). Epidemiology and genetics of CTX-M extended-spectrum β-lactamases in Gram-negative bacteria. *Critical reviews in microbiology*, *39*(1), 79-101.

Chapter 6

Emergence of *Escherichia coli* Sequence Type 131 (ST131) and ST3948 with KPC-2, KPC-3 and KPC-8 carbapenemases from a Long-Term Care and Rehabilitation Facility (LTCRF) in Northern Italy.

Piazza Aurora[1], Caltagirone Mariasofia[1], Bitar Ibrahim[1], Nucleo Elisabetta[1], Spalla Melissa[1], Fogato Elena[2], D'Angelo Roberto[2], Pagani Laura[1], Migliavacca Roberta[1].

[1]Dep. of Clinical Surgical Diagnostic and Pediatric Sciences, Unit of Microbiology and Clinical Microbiology, University of Pavia, Via Brambilla 74, 27100, Pavia, Italy
[2]ASP "Golgi-Redaelli", via Bartolomeo d'Alviano 78, 20146, Milano, Italy

6. Abstract

Aim of the study was to characterize KPC-producing *Escherichia coli* (KPC-Ec) clinical isolates among a Northern Italy Long-Term Care and Rehabilitation Facility (LTCRF) residents. Thirteen consecutive non repeated MDR *E. coli* isolates showing ertapenem Minimum Inhibitory Concentrations (MICs) >0,5 mg/L, collected during the period March 2011 - May 2013 from ASP "Redaelli" inpatients, were investigated. The $bla_{KPC/CTX\text{-}M/SHV/TEM/OXA}$ genes were identified by PCR and sequencing. KPC-Ec isolates underwent phylotyping, Pulsed-Field Gel Electrophoresis (PFGE), multilocus sequence typing (MLST) and repetitive sequence-based PCR (rep-PCR) profiling. Incompatibility groups analysis and conjugation were also performed. Eleven out of 13 isolates, resulted bla_{KPC}-type positive, were consistently resistant to third generation cephalosporins, fluoroquinolones and trimethoprim-sulphametoxazole (84.6%), retaining susceptibility to colistin (EUCAST guidelines). At least n=4/11 of KPC-Ec patients received ≥48 h of meropenem therapy. Sequencing identified 9 $bla_{KPC\text{-}2}$, 1 $bla_{KPC\text{-}3}$ and 1 $bla_{KPC\text{-}8}$ determinants. KPC-Ec plasmids belonged to IncF group (FIIk replicon); conjugation confirmed $bla_{KPC/TEM\text{-}1/OXA\text{-}9}$ genes transferability for 10 KPC-Ec. Although three pulsotypes (A, B, C) were identified, all KPC-Ec belonged to phylogenetic group B2. Clone B (B-B5) caused an outbreak of infection involving nine inpatients at five wards. Rep-PCR showed relatedness for seven representative KPC-Ec isolates. Here we report a LTCRF outbreak caused by an ST131-B2 *E. coli* associated with *bla*KPC-2 and *bla*KPC-8 genes, and the emergence of the new ST3948. Elderly people with co-morbidities are at risk for ST131 colonization. KPC-Ec clones local monitoring appears essential both to avoid their spreading among healthcare settings, and to improve therapeutic choices for LTCRF residents.

Key words: Long-Term Care and Rehabilitation Facility, KPC-positive *E. coli*, Sequence Type 131 (ST131), ST3948

6. Acknowledgements

A particular thanks to:

The Clinical Microbiology Laboratory of the ASP "Golgi-Redaelli" of Milan, and in particular to the Director Dott. R. D'Angelo, for the clinical and epidemiological informations provided together with the *E. coli* strains.

6. Introduction

Carbapenemase-producing *Enterobacteriaceae* (CPE) isolates have been increasingly reported in Europe (Grundmann *et al* 2010, Miriagou et al 2010). The results of the last countrywide cross-sectional survey, carried out during 2011 to investigate the diffusion of Carbapenem-Resistant *Enterobacteriaceae* (CRE) in Italy, showed that CRE were 2% of all collected isolates, *Klebsiella pneumoniae* being the most frequent species (87%). Carbapenemase production was the main mechanism (85%) involved, with bla_{KPC} gene detected mainly in *K. pneumoniae* and only in one *Escherichia coli* CRE (Giani *et al.* 2013). While less prevalent than in *K. pneumoniae*, KPC production in other species of *Enterobacteriaceae* is increasingly reported, presumably as *bla*KPC-carrying plasmids are acquired from *K. pneumoniae* by these non-*Klebsiella* species and then propagate (O'Hara *et al*. 2014). After the first detection of a KPC-positive *E. coli* (KPC-Ec) in Europe, reported in 2008 in France from a patient initially hospitalized in Israel (Petrella *et al.* 2008), sporadic isolation of KPC-Ec strains has been reported in the USA (Urban *et al.* 2008), Israel (Goren *et al*. 2010) and European countries (Naas *et al.* 2011, Morris *et al*. 2011). Moreover, *in vivo* transfer of KPC-2 and KPC-3 from *K. pneumoniae* to *E. coli* of different sequence types (STs) as ST131, ST1672 and ST394 has been recently described in Italy (Richter *et al.* 2011, Gona *et al.* 2014).

E. coli ST131, designated according to the Achtman multilocus sequence typing (MLST) system, was identified in 2008 as a major clone related to the spread of the CTX-M-15 Extended-Spectrum β-lactamase (ESβL) and became the single most prevalent human extra-intestinal *E. coli* strain in many regions, especially among fluoroquinolone- and/or Extended Spectrum-cephalosporin-resistant isolates (Coque *et al*. 2008, Nicolas-Chanoine *et al.* 2014). The increasing detection of ST131 isolates from hospitalized and non-hospitalized individuals and, more recently, from companion (Pomba *et al*. 2014) and foodborne animals (Ghodousi *et al*. 2015, Platell *et al*. 2011), sewage and main rivers of large European cities (Colomer-Lluch *et al.* 2013), highlights the rapid spread and local adaptation to different habitats of this lineage. Worryingly, strains of *E. coli* ST131 resistant to carbapenems have also been reported, further limiting treatment options for this clone (Petrella *et al.* 2008, Urban *et al.* 2008, Goren *et al.* 2010, Naas *et al.* 2011, Morris *et al.* 2011). Sporadic isolations

of KPC-Ec strains have been reported from Ireland (Morris *et al.* 2011), France (Naas *et al.* 2011), Italy (Accogli *et al.* 2014), United States (Kim *et al*. 2012), Taiwan (Ma *et al.* 2013) and China (Cai *et al.* 2014).
Since Long-Term Care and Rehabilitation Facilities (LTCRFs), essential components of healthcare delivery to many patients, have been recently recognized as ‘‘reservoirs of antibiotic resistance’’ (Viau *et al.* 2012) and the spread of carbapenemase-producer *E. coli* strains is related to certain pandemic clones (ST131); aim of the study was: i) to investigate the presence of KPC-Ec in an Italian LTCRF, ii) describe KPC-Ec molecular and epidemiological features.

6. Literature review

6.1 *E. coli* ST 131 overview

In 2008, researchers investigated *E. coli* with *blaCTX-M-15* from different countries including Spain, France, Canada, Portugal, Switzerland, Lebanon, India, Kuwait and South Korea; they performed Multi-Locus Sequence Typing (MLST) only to conclude that all the countries presented *E. coli* strains ST131. Moreover, all multidrug resistant strains belonged to the serotype O25:H4 and belonging to the phylogenetic group B2. This study showed that ST 131 appeared in the community settings in unrelated areas of the globe without any obvious relation between the patients. This suggests that the appearance of ST131 is related to a common contaminated source (water or food) and being introduced into different regions via travelers returning to their relative home country. The prevalence of ST131 among *E. coli* isolates varies depending on the geographic region and host population ranging between 10% to 30% of all *E. coli* isolates.

Like other ExPEC isolates, ST131 causes a variety of extra intestinal infections including pneumonia, UTIs, wound infections…etc. it is strongly associated with community onset infections yet have never been associated with large nosocomial outbreaks in ICUs. This sequence type has a prevelance among the elderly especially among residents of nursing homes and long-term-care facilities. It is also detected in animals, food sources and the environment, however it is in higher frequencies in humans compared to that of the animals suggesting the humans as a primarily reservoir after which it was transmitted to the animals and environment.

The most prevelant lineage of ST131 is named *fimH30* since it contains the H30 variant of the type 1 fimbrial adhesion gene *fimH.* This lineage was the first to be identified among ST131 isolates from different geographic regions. The acquisition of resistant traits against carbapenems is worrisome to the medical community especially with NDM and OXA-48 being the most prevalent among *E. coli* in nosocomial and community infections. Yet, case reports of ST131 with bla_{KPC} from Ireland, France, U.S, Italy, Taiwan and China have been reported and 20 isolates of *E. coli* producing KPC was studied; 60% belonging to ST131 *fimH30* lineage while the bla_{KPC} plasmid belonged to IncF with FII_k replicons. A recent study by the SMART and AstraZeneca global surveillance programs showed that 35% of 116

carbapenemase- producing *E. coli* isolates belonged to ST131, *fimH30* lineage with the presence of *bla*KPC .

Studies showed that the ST 131 is more likely to be transmitted within the same family members and companions (pets), than within patients in hospital environment. This plays an important role in the effective transmission and emergence of this sequence type within the community setting, even though the exact possible reasons and mechanism involved is not yet fully understood (Mathers *et al.* 2015).

6.2 *E.coli* KPC producers

Carbapenem resistance in E. coli is rare, however can be attributed to outer membrane protein deficiency coupled with plasmid-mediated extended-spectrum β-lactamases ESBLs or class C β-lactamases or to carbapenemases. The lateral can be an MBL (VIM, IMP and NDM) or oxacillinases (OXA-48) or class A (mainly KPC) (Naas *et al.* 2011).

KPC producing *K. pneumoniae* emerged decades ago becoming endemic in the United States and in other continents as well. The KPC gene is encoded in a transposon structure on a transferable plasmid; thus enabling it to be transferred to other Gram-negative species (Kim *et al.* 2012). The transmissible element, Tn4401, has been detected in isolates of other E*nterobacteriaceae*, *Acinetobacter* spp., and *Pseudomonas* spp. More importantly, reports of KPC producing *E. coli* have been recorded in the United States (Landman *et al.* 2010).

KPCc hydrolyze all β-lactams, including carbapenems at a sifnificant levels with the exception of cephamycins (Poirel *et al.* 2011). Out of 24 KPC variants, KPC-2 is the mostly disseminated and reported worldwide (Morris *et al.* 2011, http://www.lahey.org/studies). KPC producing E. coli is still not common as that of K. pneumonia. KPC have been associated with epidemic E. coli ST 131 which posses a threat on public health due to its ability to spread in the community and further gain more resistant genes (Chen *et al.* 2014).

6. Aim of the study

Aim of the study was to characterize KPC-producing *Escherichia coli* (KPC-Ec) clinical isolates among a Northern Italy Long-Term Care and Rehabilitation Facility (LTCRF) residents.

6. Materials and methods

Setting.
The Clinical Microbiology Laboratory of the ASP "Golgi-Redaelli" of Milan collects and analyzes biological samples from three different Geriatric Institutes: the "P. Redaelli" of Vimodrone, (n=308 beds), the "C. Golgi" of Abbiategrasso (n=334 beds) and finally the "P. Redaelli" of Milan (n=310 beds).

Bacterial strains.
Thirteen clinical consecutive non-replicate isolates of *E. coli* with ertapenem (ETP) MIC >0,5 mg/L by Phoenix System (Becton Dickinson Diagnostic Systems, Sparks, USA) were collected in the period from March 2011 to May 2013 at the ASP "Golgi-Redaelli", in Milan. All the *E. coli* isolates were obtained from infected elderly inpatients at different long term and rehabilitation Vimodrone wards. Strains were mainly from urine (n=11/13; 84,6%) and in two cases from sputum specimens. Among urine samples, 5/11 were from short-term and the remaining six from indwelling catheters. Demographic, clinical and antibiotic administration data were collected through medical records.

A**ntimicrobial susceptibilities, detection of carbapenemases and other β-lactamases.**
Identification and susceptibility profiles were confirmed using MicroScan4 (Beckman Coulter) NBC46 panels. MICs of imipenem (IPM), meropenem (MER) and ETP were obtained by Etest strips (bioMérieux). Results were interpreted according to EUCAST guidelines (http://eucast.org/clinical_breakpoints). *E. coli* ATCC 25922 was used as a quality control strain. The isolates were screened for their ability to produce carbapenemases by Modified Hodge Test (MHT) and by the confirmatory disk test using ETP and ETP plus aminophenylboronic acid (APBA) or EDTA. Detection of *bla*SHV-, *bla*TEM-, *bla*CTX-M-, *bla*OXA- and *bla*KPC-type genes was performed by PCR as previously described (Tzelepi *et al.* 2003, Giakkoupi *et al.* 2009). PCR amplicons were purified using the kit Quantum Prep PCR Kleen Spin Columns (Bio-Rad) and subjected to direct sequencing. PCR products were sequenced on both strands with an Applied Biosystems sequencer. The nucleotide sequences were analyzed with the BLAST software program (http://blast.ncbi.nlm.nih.gov/Blast.cgi).

Isoelectric focusing.
Production of β-lactamases was confirmed by analytic isoelectric focusing (IEF). IEF of crude cell extracts, visualization of β-lactamase bands by nitrocefin (gels were electrophoresed at 11 to 14 W for 90 min and β-lactamase bands were detected using 0.5 mM nitrocefin). The β-lactamase bands activity was assessed by a substrate overlaying procedure (using 0,5 µg/ml ETP) as previously described (Pagani *et al.* 2002). Crude enzymatic extracts from well-known β-lactamases producers (i.e., TEM-1, TEM-2, TEM-7, TEM-8, TEM-9, TEM-12, SHV-1, SHV-2 and SHV-5) were used as controls.

Conjugation experiments and plasmid characterization.
Conjugative transfer of plasmids was carried out in mixed broth cultures using *E. coli* K12 strain J62 (F^-, *pro*, *his*, *trp*, *lac*, Sm^r) and J53 (F^-, *met*, *pro*, Rif^r) as recipient strains. *E. coli* transconjugants were selected on McConkey agar containing ETP (0,5 mg/L) and streptomycin (1000 mg/L) or rifampin (100 mg/L), respectively. Colonies grown on the selective medium were picked for identification by the MicroScan4 system. Recipients that harbored bla_{KPC}-type genes and exhibited resistance to carbapenems and cephalosporins were defined as transconjugants.
Plasmid DNA of *E. coli* isolates and transconjugants were obtained using Pure Link HiPure Plasmid Midiprep kit (Invitrogen, by life Technologies) and were separated by electrophoresis. Plasmid incompatibility groups were determined in both donor and transconjugant strains by the PCR-based replicon typing (PBRT) method (Carattoli *et al.* 2005) using the commercially available PBRT Kit (Diatheva) according to manufacturer's instructions.

PFGE and rep-PCR analysis.
PFGE was performed using *Xba*I restriction enzyme and fragments were separated on a CHEF-DR II apparatus (Bio-Rad, Milan, Italy) for 22 h at 14°C. Bacteriophage λ concatenamers were used as DNA size markers. DNA restriction patterns of scanned gel pictures were interpreted following cluster analysis with the Fingerprinting II version 3.0 software (Bio-Rad) using the unweighted pair-group method with arithmetic averages (UPGMA). Only bands larger than 48 kb were considered for the analysis. The Dice correlation coefficient was used with a 1.0% position tolerance to analyze the similarities of the banding patterns, and a similarity threshold of 90% to define clusters. The restriction patterns of the genomic DNA from

the isolates were analysed and interpreted according to the criteria of Tenover et al (1995), too.
Repetitive sequence-based PCR (rep-PCR) was performed with the semiautomated Diversilab system (DL) (bioMérieux), according to the manufacturer's instructions. DNA extraction was performed with the UltraClean Microbial DNA isolation kit (Mo Bio Laboratories Inc). Analyses of the PCR amplicons were performed using a 2100 Bioanalyzer (Agilent Technologies). DL fingerprints were analyzed with the DL software 3.4, using the Pearson correlation statistical method to determine clonal relationships. For isolates to be considered indistinguishable in addition to >97% similarity no peak differences should be seen. For similar isolates, in addition to 95-97% similarity one to two peak differences are accepted. In addition to <95% similarity more than two peak variations are considered different. In this study we used the criteria for "indistinguishable" for defining DL profiles (Brolund *et al.* 2010).

MLST and phylogenetic typing.
Multilocus sequence typing (MLST) of representative *E. coli* isolates was performed according to the protocol of Wirth et al. (Wirth *et al.* 2006). Allelic profiling and sequence type (ST) determination were performed using the *E. coli* MLST scheme from the website of the University of Warwick (http://mlst.warwick.ac.uk/mlst/dbs/Ecoli)http://mlst.ucc.ie/mlst/dbs/Ecoli/documents/primersColi_html.
Phylogenetic groups were determined by a two-step triplex PCR described by Clermont et al http://mlst.ucc.ie/mlst/dbs/Ecoli/documents/primersColi_html(2000) using Multiplex PCR kit (Qiagen).

Demographic and clinical data.
Clinical records of patients with KPC-Ec infection or colonization were examined retrospectively. The following data were recorded: age, gender, admission ward, date of admission, previous hospitalization during the last year, site of infection or colonization, antimicrobial administration before or during the infective episode, treatment outcome.
Treatment failure was classified as absence of resolution or worsening of signs and symptoms of infection; not assessable - due to incomplete records of the patient within 72 h of infection

6. Results

A total of 13 consecutive, non-duplicate *E. coli* isolates showing ETP >0,5 mg/L MIC (0.06% of the strains of the same species processed in that period) were identified at Clinical Microbiology Laboratory of ASP "Golgi-Redaelli". Susceptibility results initially obtained by Phoenix System (BD) were then confirmed at the Pavia University by both MicroScan4 System (Beckman Coulter) and Etest (bioMérieux).

The isolates showed a MIC range for ETP of 1 to >32 mg/L (MIC_{50}= 4 mg/L; MIC_{90}= >32 mg/L), MER of 0,064 to >32 mg/L (MIC_{50}= 0,5 mg/L; MIC_{90}= 4 mg/L) and IPM of 0,5 to >32 mg/L (MIC_{50}= 1 mg/L; MIC_{90}= 4 mg/L) (Table 3; ANNEX 3). All the isolates were resistant to piperacillin, amoxicillin/clavulanate, third generation cephalosporins, aztreonam, and ciprofloxacin, 11/13 (84.6%) strains were resistant to trimethoprim-sulphametoxazole (SXT) and 2/13 (15.4%) to amikacin. All isolates retained susceptibility to colistin and tigecyclin.

Although the resistance phenotypes of all the 13 isolates were suggestive of a carbapenemase production, the results of both the MHT and the confirmatory disk test using ETP and ETP+APBA were positive only for 11/13 isolates (84,6%), that underwent molecular typing and were examined for the presence of *bla*KPC-type genes. The remaining two *E. coli* strains resulted negative to both modified Hodge and ETP-EDTA disk combination tests.

Screening for *bla*KPC determinants by PCR yielded an amplification product of the expected size from the same 11/13 isolates. Sequencing of the above 11 *bla*KPC positive isolates, identified the resistance gene as *bla*KPC-2 (n=9) or *bla*KPC-3 (n=1) and *bla*KPC-8 (n=1) (Table 3; ANNEX 3).

Analytical IEF of crude extracts of all the *E. coli* clinical isolates revealed heterogeneous patterns, with multiple β-lactamase bands in 10/13 cases. The presence for 11/13 samples of a pI 6.7 band showing activity on ETP in a bioassay, was consistent with the production of the KPC enzymes. Such a band was accompanied in 10/11 cases by other two bands (pI 7.2 and pI 5.4) the first able to hydrolyse oxacillin, and the other one showing a narrow spectrum of activity by bioassay. Only a pI >8.2 band, showing high level of hydrolytic activity on CTX (1 mg/L) and consistent with the production of

an ESBL of CTX-M-type was present in 2/13 isolates. The BL types detected by PCR in the KPC-Ec included OXA-9 for all isolates and TEM-1 in 10/11 cases. The *bla*CTX-M-type gene presence was confirmed by PCR in the two non-KPC producers; none of the isolates studied was positive for the presence of *bla*SHV-type genes.

The plasmids replicon typing highlighted the presence of two plasmids belonging to the incompatibility group IncF (FIIk replicon and about 200Kb in size) and IncF multireplicon (FII, FIA and FIB), often associated with the *bla*KPC gene dissemination. Conjugation results highlighted the transferability of both plasmids.

All the 11 KPC-Ec strains belonged to the phylogenetic group B2, and showed multiclonal pulsotypes after *XbaI* digestion. PFGE results showed the presence of three KPC-Ec different clones named A, B, and C. Clone B sub-types, named from B to B5 in order of appearance, are shown in Figure 1/A. Clone A appeared once in April 2011; Clone B emerged on June 2012 at Vimodrone LTCRF and persisted until April 2013 causing an intra-hospital outbreak of infection involving nine inpatients at five different wards (V3/1, V4/1, V3/5, VR1, V4/5). The clone C was obtained on May 2013.

Six KPC-Ec isolates, chosen as representative of both KPC variants and PFGE clones, were furthermore investigated by MLST. Two different STs were overall found: the hypervirulent ST131 and the single locus variant in *purA* allele (53-40-47-13-36-**10**-29) ST3948 clone, belonging to the ST131 Clonal complex (Clpx). The ST131 KPC-Ec group was identified in five out of six KPC-Ec chosen as representative. Interestingly, pandemic ST131 included both the PFGE clones A and B, the latter group of KPC-Ec strains harboring both the *bla*KPC-2 or the *bla*KPC-8 gene variants.
The ST3948 clone was associated to the *bla*KPC-3 determinant and to the PFGE unique clone C strain.

Rep-PCR (DL) on seven representative KPC-Ec isolates, belonging to A, B, C PFGE clones, showed genetic relatedness (Figure 1/B). While ST3948 was included in a distinctive clone C by PFGE, ST131strains clustered >97% with ST3948 using the DL tool (Figure 6.1).

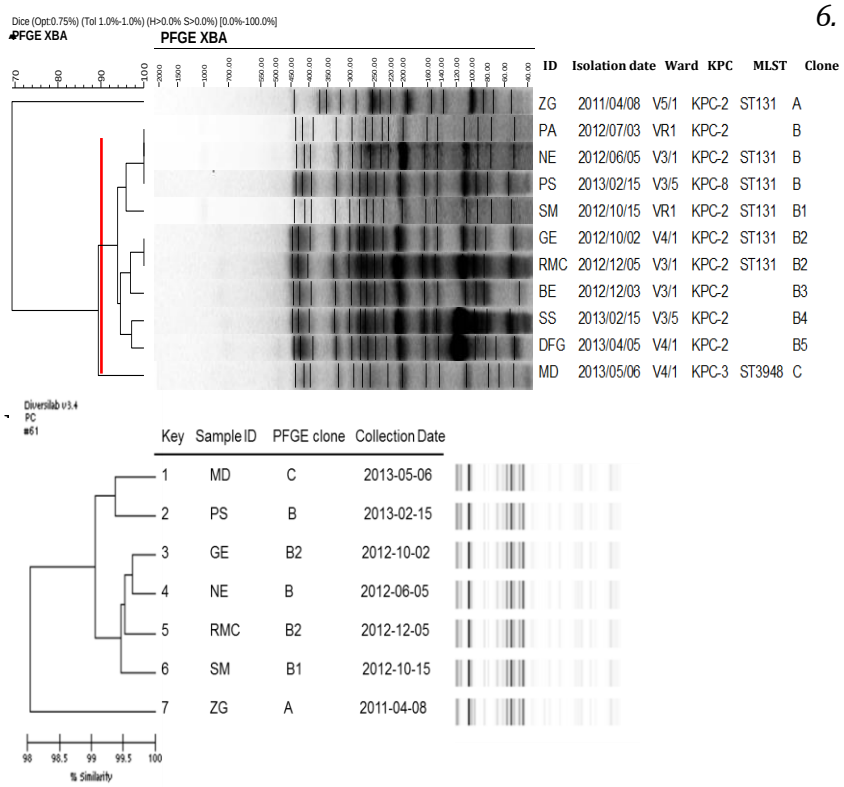

Fig. 6.1:A: UPGMA dendrogram of *Xba*I PFGE profiles of KPC-Ec isolates. B: gel image and dendrogram of rep-PCR DL patterns of KPC-Ec representative strains.

According to clinical records the mean age of the patients was 85 years, with the 54.5% being female. Based on clinical data, at least n=4/11 of KPC-Ec infected patients received ≥48 h of MER therapy in an Acute Care Hospital (ACH) and/or LTCRF located in the area of Milan.

All the patients were affected by several co-morbidities, including diabetes, hypertension, chronic obstructive pulmonary disease. Co-infections were reported for the majority of patients: 5/9 caused by *Clostridium difficile*, 1/9 by *Staphylococcus aureus* and in the remaining cases by Gram-negative bacteria (Table 4; ANNEX 4). It is of note that three out of 11 patients died during hospitalization. Death was attributable or related to KPC-Ec at least in the outbreak index patient NE.

Epidemiological analysis showed that index patient of the KPC-Ec outbreak, was a 89 years old woman (NE), admitted in April 2012 to the V3/1 rehabilitation word of the "P. Redaelli" LTCRF of Vimodrone. The patient was discharged from the S. Raffaele Hospital of Milan just before. After two months of hospitalization, KPC-Ec strain was isolated from the urine of the patient. On admission, patient's physical examination revealed decubitus

ulcers and gastroenteritis symptoms. She was treated with metronidazole 500mg and ciprofloxacin 400mg. Due to the occurrence of UTI-related septic shock symptoms, a combination therapy with MER and amikacin (500mg each) started from May 2012 the 5th. A carbapenem resistant *K. pneumoniae* isolate was obtained from the same patient in May 2012 the 9th. The *K. pneumoniae* strain resulted *bla*KPC-, *bla*TEM-, *bla*OXA-type genes producer, and harboured an IncF (FIIk replicon) plasmid, like the KPC-Ec isolate (data not shown).

Due to NE KPC-Ec isolation (one month later) an 8 days colistin plus IPM therapy was administrated; even so, on June the 20th the rectal swab sampling revealed the intestinal persistence of the same strain.
During the next year, nine additional inpatients from at the same LTCRF, affected by UTIs (n=7) and pneumonia (n=2), resulted positive for the presence of clone B outbreak strains.

The mainly used antibacterial agents are listed in Table 4 (ANNEX 4). A complete or partial response was obtained in eight patients, whereas failure of treatment or relapse was observed in three patients.

6. Discussion

The present study describes the emergence and intra-hospital spread of a clonal strain KPC-Ec in one LTCRF hospital located in Northern Italy and admitting patients from several ACH of the same area. Genetic features and molecular epidemiology were also investigated.

Although KPC-*K. pneumoniae* (KPC-Kp) has become endemic in Italy in many ACH, the presence of KPC-Ec isolates remain limited (Giani *et al.* 2013). The spread of *bla*KPC-type resistance determinants is of particular concern in *E. coli*, being emerging KPC-Ec associated with different STs (Mavroidi *et al.* 2012, Almeida *et al.* 2012, Baraniak *et al.* 2011, Ruiz-Garbajosa *et al.* 2013, Gijon *et al.* 2012). Even more worrisome is the occurrence and dissemination of the MDR ST131 KPC-Ec clones, belonging to the hypervirulent, uropathogenic lineage B2 (Clermont *et al.* 2009), and distinguishable in turn into several pulsotypes (Coque *et al.* 2008, Lau *et al.* 2008, Nicolas-Chanoine *et al.* 2013). In the present study, we report the presence of 11 KPC-Ec producers in an Italian LTCRF.

Interestingly, Etest MIC values for IPM (from 0,5 to 4mg/L) and MER (from 0,5 to >32mg/L) resulted within the susceptible clinical breakpoints for the majority of the carbapenemase producers (n=9/11). Such variability, here observed also in the case of ETP (MIC= 1 - >32mg/L), was already reported for KPC-Ec strains (Accogli *et al.* 2014, Deshpande *et al.* 2006). Moreover, the diversity in carbapenem MICs among KPC-Ec strains of the same clone (B-B5) could be due to differences in plasmid copy numbers (as a result of antibiotic therapy administered) or the presence of sub-populations expressing different resistance levels. Scattered colonies within the Etest inhibition zone were present in few cases, and ETP (instead of MER) resulted the most efficient and sensitive molecule for KPC-Ec producers detection.

A porin loss mechanism coupled with the CTX-M-type enzyme production could justify the ETP MIC values obtained for two non-carbapenemase producers by Microscan4system (1- >1 mg/L) *versus* Etest (4 - 3 mg/L).

Overall, phenotypical tests, IEF, PCR and sequencing revealed heterogeneous β-lactamase patterns: while in the case of the two MDR KPC-Ec negative isolates only a CTX-M-type ESBL was detected, KPC-2,

KPC-3 and KPC-8 MDR strains were also OXA-9 and TEM-1 enzymes producers. Conjugation experiments results together with the plasmid replicon typing analysis (incompatibility group IncF, with FIIk replicon, typical of *K. pneumoniae*) suggested the possible inter-species plasmid exchange between KPC-Kp and KPC-Ec in the retrospectively ascertained (NE) index case patient. Since the clone B strain harbored the 200Kb outbreak conjugative plasmid, the *bla*KPC-8 gene could be due to two points mutations within the *bla*KPC-2 gene sequence (T to G at position 716 leading to a valine-to-glycine substitution, and C to T at 814 position leading to an histidine-to-tyrosine substitution) (data not shown). Nine residents and five wards were involved in the Vimodrone outbreak and the time elapsed between admission and outbreak strain acquisition was in average 38 days.

The molecular and epidemiological results revealed the intra-hospital spread of a ST131-B2 (PFGE clone B) outbreak clone. To note that PFGE clone B-related isolates clustered with two KPC-Ec strains collected on August and October 2012 at Vimercate ACH (our personal data). This confirms how elderly people with many co-morbidities are at greatest risk for ST131 colonization, possibly due to healthcare-associated transmission.

6. Conclusion

Here we describe the emergence in an Italian LTCRF of three KPC-Ec PFGE clones (A, B, C), belonging to ST131 and to the new ST3948 (clone C). Although LTCRFs are well known as reservoirs of other antimicrobial resistant pathogens (Arnoldo *et al.* 2013) and have been implicated as reservoirs of ST131 in Europe (Burke *et al.* 2012, Brisse *et al*. 2012), at our knowledge this study is the first one describing an Italian LTCRF as KPC-Ec ST131 reservoir. DL results clustered all the KPC-Ec isolates in the same profile, thus confirming the higher discriminatory power of PFGE for *E. coli* outbreak studies.

Taken together, our findings highlight the need to continue screening of *E. coli* showing a decreased susceptibility to ETP, and to implement more rigorous infection control measures among LTCRF residents to avoid the spread of difficult to treat ST131 Clpx KPC-Ec in our area. Failure of treatment was observed in three patients, of which two infected by a KPC-Ec strain studied: local monitoring of KPC-Ec clones appears essential to avoid the risk of their spreading among healthcare settings and to improve effective and appropriate therapeutic choices for LTCRF residents.

6. **References**

Accogli M, Giani T, Monaco M et al (2014) Emergence of *Escherichia coli* ST131 sub-clone H30 producing VIM-1 and KPC-3 carbapenemases, Italy. J Antimicrob Chemother 69(8):2293-6. doi: 10.1093/jac/dku132

Almeida AC, de Sá Cavalcanti FL, Vilela MA et al (2012) *Escherichia coli* ST502 and *Klebsiella pneumoniae* ST11 sharing an IncW plasmid harbouring the *bla*(KPC-2) gene in an Intensive Care Unit patient. Int J Antimicrob Agents 40(4):374-6

Arnoldo L, Migliavacca R, Regattin L et al (2013) Prevalence of urinary colonization by extended spectrum-beta-lactamase *Enterobacteriaceae* among catheterized inpatients in Italian long term care facilities. BMC Infect Dis 13:124. doi:10.1186/1471-2334-13-124

Baraniak A, Grabowska A, Izdebski R et al (2011) Molecular characteristics of KPC-producing *Enterobacteriaceae* at the early stage of their dissemination in Poland, 2008-2009. Antimicrob Agents Chemother 55(12):5493-9

Basic Local Alignment Search Tool. http://blast.ncbi.nlm.nih.gov/Blast.cgi

Brisse S, Diancourt L, Laouenan C et al (2012) Phylogenetic distribution of CTX-M- and non-extended-spectrum-beta-lactamase-producing *Escherichia coli* isolates: group B2 isolates, except clone ST131, rarely produce CTX-M enzymes. J Clin Microbiol 50:2974-81

Brolund A, Hæggman S, Edquist PJ et al (2010) The DiversiLab system versus pulsed-field gel electrophoresis: characterisation of extended spectrum β-lactamase producing *Escherichia coli* and *Klebsiella pneumoniae*. J Microbiol Methods 83(2):224-30. doi: 10.1016/j.mimet.2010.09.004

Burke L, Humphreys H, Fitzgerald-Hughes D (2012) The revolving door between hospital and community: extended-spectrum beta-lactamase producing *Escherichia coli* in Dublin. J Hosp Infect 81:192–8

Cai JC, Zhang R, Hu YY et al (2014) Emergence of *Escherichia coli* sequence type 131 isolates producing KPC-2 carbapenemase in China. Antimicrob Agents Chemother 58(2):1146-52. doi: 10.1128/AAC.00912-13

Carattoli A, Bertini A, Villa L et al (2005) Identification of plasmids by PCR-based replicon typing. J Microbiol Methods 63(3):219-28

Chen, L., Hu, H., Chavda, K. D., Zhao, S., Liu, R., Liang, H., ... & Kreiswirth, B. N. (2014). Complete sequence of a KPC-producing IncN multidrug-resistant plasmid from an epidemic *Escherichia coli* sequence type 131 strain in China. *Antimicrobial agents and chemotherapy*, *58*(4), 2422-2425

Clermont O, Bonacorsi S, Bingen E (2000) Rapid and simple determination of the *Escherichia coli* phylogenetic group. Appl Environ Microbiol 66(10):4555–4558 9
Clermont O, Dhanji H, Upton M et al (2009) Rapid detection of the O25b-ST131 clone of *Escherichia coli* encompassing the CTX-M-15-producing strains. J Antimicrob Chemother 64(2):274-7

Colomer-Lluch M, Mora A, Lopez C et al (2013) Detection of quinolone-resistant *Escherichia coli* isolates belonging to clonal groups O25b:H4-B2-ST131 and O25b:H4-D-ST69 in raw sewage and river water in Barcelona, Spain. J Antimicrob Chemother 68:758–765

Coque TM, Novais A, Carattoli A et al (2008) Dissemination of clonally related *Escherichia coli* strains expressing extended-spectrum beta-lactamase CTX-M-15. Emerg Infect Dis 14(2):195-200

Deshpande LM, Rhomberg PR, Sader HS et al (2006) Emergence of serine carbapenemases (KPC and SME) among clinical strains of *Enterobacteriaceae* isolated in the United States Medical Centers: report from the MYSTIC Program (1999-2005). Diagn Microbiol Infect Dis 56(4):367-72

European Committee on Antimicrobial Susceptibility Testing. http://eucast.org/clinical_breakpoints

Ghodousi A, Bonura C, Di Noto AM et al (2015) Extended-Spectrum ß-Lactamase, AmpC-Producing, and Fluoroquinolone-Resistant *Escherichia coli* in Retail Broiler Chicken Meat, Italy. Foodborne Pathog Dis 12(7):619-25. doi: 10.1089/fpd.2015.1936

Giakkoupi P, Pappa O, Polemis M et al (2009) Emerging *Klebsiella pneumoniae* isolates coproducing KPC-2 and VIM-1 carbapenemases. Antimicrob Agents Chemother 53(9):4048-50
Giani T, Pini B, Arena F et al (2013) Epidemic diffusion of KPC carbapenemase-producing *Klebsiella pneumoniae* in Italy: results of the first countrywide survey, 15 May to 30 June 2011. Euro Surveill 18(22)

Gijón D, Curiao T, Baquero F et al (2012) Fecal carriage of carbapenemase-producing *Enterobacteriaceae*: a hidden reservoir in hospitalized and non-hospitalized patients. J Clin Microbiol 50(5):1558-63

Gona F, Barbera F, Pasquariello AC et al (2014) In vivo multiclonal transfer of *bla*(KPC-3) from *Klebsiella pneumoniae* to *Escherichia coli* in surgery patients. Clin Microbiol Infect 20(10):O633-5. doi: 10.1111/1469-0691.12577

Goren MG, Navon-Venezia S, Chmelnitsky I et al (2010) Carbapenem-resistant KPC-2-producing *Escherichia coli* in a Tel Aviv Medical Center, 2005 to 2008. Antimicrob Agents Chemother 54(6):2687-91. doi: 10.1128/AAC.01359-09

Grundmann H, Livermore DM, Giske CG et al (2010) Carbapenem-non-susceptible *Enterobacteriaceae* in Europe: conclusions from a meeting of national experts. Euro Surveill 15(46)

Kim YA, Qureshi ZA, Adams-Haduch JM et al (2012) Features of infections due to *Klebsiella pneumoniae* carbapenemase-producing *Escherichia coli*: emergence of sequence type 131. Clin Infect Dis 55(2):224-31

Landman, D., Urban, C., Bäcker, M., Kelly, P., Shah, N., Babu, E., ... & Quale, J. (2010). Susceptibility profiles, molecular epidemiology, and detection of KPC-producing Escherichia coli isolates from the New York City vicinity.*Journal of clinical microbiology*, *48*(12), 4604-4607.

Lau SH, Kaufmann ME, Livermore DM et al (2008) UK epidemic *Escherichia coli* strains A-E, with CTX-M-15 beta-lactamase, all belong to the international O25:H4-ST131 clone. J Antimicrob Chemother 62(6):1241-4

Ma L, Siu LK, Lin JC et al (2013) Updated molecular epidemiology of carbapenem-non-susceptible *Escherichia coli* in Taiwan: first identification of KPC-2 or NDM-1-producing *E. coli* in Taiwan. BMC Infect Dis 13:599. doi: 10.1186/1471-2334-13-599

Mathers, A. J., Peirano, G., & Pitout, J. D. (2015). The role of epidemic resistance plasmids and international high-risk clones in the spread of multidrug-resistant Enterobacteriaceae. *Clinical microbiology reviews*, *28*(3), 565-591.

Mavroidi A, Miriagou V, Malli Eet al (2012) Emergence of *Escherichia coli* sequence type 410 (ST410) with KPC-2 β-lactamase. Int J Antimicrob Agents 39(3):247-50

Miriagou V, Cornaglia G, Edelstein M et al (2010) Acquired carbapenemases in Gram-negative bacterial pathogens: detection and surveillance issues. Clin Microbiol Infect 16(2):112-22. doi: 10.1111/j.1469-0691.2009.03116.x

Morris D, Boyle F, Ludden C et al (2011) Production of KPC-2 carbapenemase by an *Escherichia coli* clinical isolate belonging to the international ST131 clone. Antimicrob Agents Chemother 55(10):4935-6. doi: 10.1128/AAC.05127-11

Multi Locus Sequence Typing scheme from the website of the University of Warwick. http://mlst.warwick.ac.uk/mlst/dbs/Ecoli

Naas T, Cuzon G, Gaillot O et al (2011) When carbapenem-hydrolyzing beta-lactamase Kpc meets *Escherichia coli* ST131 in France. Antimicrob Agents Chemother 55(10):4933-4. doi: 10.1128/AAC.00719-11

Nicolas-Chanoine MH, Robert J, Vigan M et al (2013) Different factors associated with CTX-M-producing ST131 and non-ST131 *Escherichia coli* clinical isolates. PLoS One 8(9):e72191
Nicolas-Chanoine MH, Bertrand X, Madec JY (2014) *Escherichia coli* ST131, an intriguing clonal group. Clin Microbiol Rev 27(3):543-74. doi: 10.1128/CMR.00125-13

O'Hara JA, Hu F, Ahn C et al (2014) Molecular epidemiology of KPC-producing *Escherichia coli*: occurrence of ST131-fimH30 subclone harboring pKpQIL-like IncFIIk plasmid. Antimicrob Agents Chemother 58(7):4234-7. doi: 10.1128/AAC.02182-13

Pagani L, Migliavacca R, Pallecchi L et al (2002) Emerging extended-spectrum beta-lactamases in *Proteus mirabilis*. J Clin Microbiol 40(4):1549-52

Petrella S, Ziental-Gelus N, Mayer C et al (2008) Genetic and structural insights into the dissemination potential of the extremely broad-spectrum class A beta-lactamase KPC-2 identified in an *Escherichia coli* strain and an *Enterobacter cloacae* strain isolated from the same patient in France. Antimicrob Agents Chemother 52(10):3725-36. doi: 10.1128/AAC.00163-08

Platell JL, Johnson JR, Cobbold RN et al (2011) Multidrug-resistant extraintestinal pathogenic *Escherichia coli* of sequence type ST131 in animals and foods. Vet Microbiol 21;153(1-2):99-108. doi: 10.1016/j.vetmic.2011.05.007

Poirel, L., Barbosa-Vasconcelos, A., Simões, R. R., Da Costa, P. M., Liu, W., & Nordmann, P. (2012). Environmental KPC-producing Escherichia coli isolates in Portugal. *Antimicrobial agents and chemotherapy*, *56*(3), 1662-1663.

Pomba C, López-Cerero L, Bellido M et al (2014) Within-lineage variability of ST131 *Escherichia coli* isolates from humans and companion animals in the south of Europe. J Antimicrob Chemother 69(1):271-3. doi: 10.1093/jac/dkt343

Richter SN, Frasson I, Bergo C et al (2011) Transfer of KPC-2 Carbapenemase from *Klebsiella pneumoniae* to *Escherichia coli* in a patient: first case in Europe. J Clin Microbiol 49(5):2040-2. doi: 10.1128/JCM.00133-11

Ruiz-Garbajosa P, Curiao T, Tato M et al (2013) Multiclonal dispersal of KPC genes following the emergence of non-ST258 KPC-producing *Klebsiella pneumoniae* clones in Madrid, Spain. J Antimicrob Chemother 68(11):2487-92
Tenover FC, Arbeit RD, Goering RV et al (1995) Interpreting chromosomal DNA restriction patterns produced by pulsed-field gel electrophoresis: criteria for bacterial strain typing. J Clin Microbiol 33(9):2233-9

Tzelepi E, Magana Ch, Platsouka E et al (2003) Extended-spectrum beta-lactamase types in *Klebsiella pneumoniae* and *Escherichia coli* in two Greek hospitals. Int J Antimicrob Agents 21(3):285-8

Urban C, Bradford PA, Tuckman M et al (2008) Carbapenem-resistant *Escherichia coli* harboring *Klebsiella pneumoniae* carbapenemase beta-lactamases associated with long-term care facilities. Clin Infect Dis 46(11):e127-30. doi: 10.1086/588048
Viau RA, Hujer AM, Marshall SH et al (2012) "Silent" dissemination of *Klebsiella pneumoniae* isolates bearing *K. pneumoniae* carbapenemase in a long-term care facility for children and young adults in Northeast Ohio. Clin Infect Dis 54(9):1314-21. doi: 10.1093/cid/cis036

Wirth T, Falush D, Lan R et al (2006) Sex and virulence in *Escherichia coli*: an evolutionary perspective. Mol Microbiol 60(5):1136-51

ANNEX 1. Table 1: Characteristics of patients and antibiotic susceptibility of ESBL-producing *Klebsiella pneumoniae* , NICU of the Foundation IRCCS Polyclinic "S. Matteo" hospital, Pavia, April-August 2013 (n=20)

Date of birth	Sex	Admission NICU	No. of days prior to isolation	Clinical sample	Isolate(s)	Minimum inhibitory concentration (mg/L)										
						TZP	CAZ	CTX	FEP	ATM	IPM	MEM	ETP	SXT	GN	CIP	AK
11/09/11	M	11/09/11	5	BS	15332	≤ 4/4	8	>4	>8	>16	≤1	0.064	≤0.25	≤1/19	>4	≤0.125	≤4
23/08/12	M	23/08/12	9	BS	16781	> 16/4	>8	>4	>8	>16	≤1	0.064	≤0.25	>4/76	>4	>1	≤4
06/10/12	M	06/10/12	3	BS	16919	= 16/4	>8	>4	>8	>16	≤1	0.064	≤0.25	>4/76	>4	>1	≤4
15/02/13	M	15/02/13	5	BS	485	= 16/4	>8	>4	>8	>16	≤1	0.064	≤0.25	>4/76	>4	>1	≤4
09/03/13	M	09/03/13	38	RS	573	> 16/4	>8	>4	>8	>16	≤1	0.064	≤0.25	>4/76	>4	>1	≤4
09/03/13	M	09/03/13	7	BS	569	> 16/4	>8	>4	>8	>16	≤1	0.064	≤0.25	>4/76	>4	>1	≤4
06/05/13	F	08/05/13	7	RS	644	> 16/4	8	>4	>8	>16	≤1	0.064	≤0.25	>4/76	>4	>1	≤4
10/07/13	F	10/07/13	7	RS	726	> 16/4	8	>4	>8	>16	≤1	0.064	≤0.25	>4/76	>4	>1	≤4
10/07/13	F	10/07/13	8	BS	733	> 16/4	8	>4	>8	>16	≤1	0.064	≤0.25	>4/76	>4	>1	≤4
10/07/13	F	29/08/13	0	CFS	790	> 16/4	8	>4	>8	>16	≤1	0.064	≤0.25	>4/76	>4	>1	≤4
05/02/13	M	05/02/13		CW	484	> 16/4	8	>4	>8	>16	≤1	0.064	≤0.25	>4/76	>4	>1	≤4
15/03/13	F	15/03/13		NA	536	> 16/4	8	>4	>8	>16	≤1	0.064	≤0.25	>4/76	>4	>1	≤4
22/01/13	F	22/01/13		BL	541	> 16/4	8	>4	>8	>16	≤1	0.064	≤0.25	>4/76	>4	>1	≤4
03/04/13	M	03/04/13		U	593	> 16/4	8	>4	>8	>16	≤1	0.064	≤0.25	>4/76	>4	>1	≤4
25/05/13	M	25/05/13		RW	674	> 16/4	8	>4	>8	>16	≤1	0.064	≤0.25	>4/76	>4	>1	≤4
30/06/13	M	30/06/13		BS	732	> 16/4	8	>4	>8	>16	≤1	0.064	≤0.25	>4/76	>4	>1	≤4
28/06/13	M	28/06/13		BS	713	> 16/4	8	>4	>8	>16	≤1	0.064	≤0.25	>4/76	>4	>1	≤4
06/07/13	F	17/07/13		RW	731	> 16/4	8	>4	>8	>16	≤1	0.064	≤0.25	>4/76	>4	>1	≤4
05/08/13	M	05/08/13		CW	769	> 16/4	8	>4	>8	>16	≤1	0.064	≤0.25	>4/76	>4	>1	≤4
11/08/13	M	11/08/13		RW	777	> 16/4	8	>4	>8	>16	≤1	0.064	≤0.25	>4/76	>4	>1	≤4

AK: amikacin; BL: bronchoalveolar; BS: bloodstream; CAZ: ceftazidime; CEF: cefepime; CIP:ciprofloxacin; CTX: cefotaxime; CSF: cerebrospinal fluid; CW: corneal swab; ETP: ertapenem; GM: gentamicin; IPM: imipenem; MEM: meropenem; NA: nasopharyngeal aspirate; RS: rectal swab; **SXT**: ….; TZP: piperacillin–tazobactam; U: urine;

ANNEX 2. Table 2. Summary of whole-genome sequencing results

Gene	**Gene type**	**Phenotype**	**Location**
aac(6')Ib-cr	resistance	Fluoroquinolone and aminoglycoside resistance	plasmid
aac(3)-IIa	resistance	Aminoglycoside resistance	plasmid
*bla*SHV-28	resistance	Beta-lactam resistance	chromosome
*bla*TEM-206	resistance	Beta-lactam resistance	plasmid
*bla*CTX-M-15	resistance	Beta-lactam resistance	plasmid
blaOXA-1	resistance	Beta-lactam resistance	plasmid
dfrA14	resistance	Trimethoprim resistance	plasmid
fosA	resistance	Fosfomycin resistance	chromosome
irp2	resistance	Synthesis of siderophore	
oqxA	resistance	Quinolone resistance	chromosome
oqxB	resistance	Quinolone resistance	chromosome
QnrB66	resistance	Quinolone resistance	plasmid
strA	resistance	Aminoglycoside resistance	plasmid
strB	resistance	Aminoglycoside resistance	plasmid
sul2	resistance	Sulphonamide resistance	plasmid
tet(A)	resistance	Tetracycline resistance	plasmid
mrkA(12)	virulence	MrkA fimbrial protein, major subunit	chromosome
mrkB (2)	virulence	MrkB fimbrial protein, chaperone	chromosome
mrkD (8)	virulence	MrkD fimbrial protein, adhesin	chromosome
mrkF (4)	virulence	MrkF fimbrial protein, minor subunit	chromosome
mrkH (2)	virulence	MrkH fimbrial protein, cyclic di-GMP-dependent transcriptional activator	chromosome
mrkI (4)	virulence	MrkI fimbrial protein, cyclic di-GMP-dependent transcriptional activator	chromosome
mrkJ (2)	virulence gene	MrkJ fimbrial protein, cyclic di-GMP-dependent transcriptional activator	chromosome

ANNEX 3. Table 1. Characteristics of the 13 *E. coli* isolates considered in the study.

		MicroScan4 MIC, mg/L			Etest MIC, mg/L			Beta-Lactamase (BL) content		Molecular typing			
ID	Collection date (yyyy/mm/dd)	(Susceptibility category)			(Susceptibility category)								
		IPM	MER	ETP	IPM	MER	ETP	Carbapenemase	BL	PFGE	MLST	DL	Phylogenetic group
VR	2011-03-09	<=1 (S)	<=1 (S)	1 (I)	0,25 (S)	0,064 (S)	4 (R)	-	CTX-M Gr 1	-			D
ZG	2011-04-08	4 (I)	8 (I)	>1 (R)	1 (S)	>32 (R)	8 (R)	KPC-2	OXA-9	A	131	A	B2
RA	2011-09-30	<=1 (S)	<=1 (S)	>1 (R)	0,75 (S)	0,125 (S)	3 (R)	-	CTX-M Gr. 2	-			B2
NE	2012-06-20	8 (I)	8 (I)	>1 (R)	2 (S)	0,5 (S)	1,5 (R)	KPC-2	TEM-1; OXA-9	B	131	A	B2
PA	2012-07-03	<=1 (S)	<=1 (S)	>1 (R)	0,5 (S)	0,5 (S)	1,5 (R)	KPC-2	TEM-1; OXA-9	B			B2
GE	2012-10-02	>8 (R)	8 (I)	>1 (R)	1 (S)	1 (S)	>32 (R)	KPC-2	TEM-1; OXA-9	B2	131	A	B2
SM	2012-10-15	>8 (R)	>8 (R)	>1 (R)	1 (S)	0,38 (S)	4 (R)	KPC-2	TEM-1; OXA-9	B1	131	A	B2
BE	2012-12-03	4 (I)	8 (I)	>1 (R)	0,5 (S)	1 (S)	2 (R)	KPC-2	TEM-1; OXA-9	B3			B2
RMC	2012-12-05	<=1 (S)	<=1 (S)	2 (R)	1 (S)	0,5 (S)	2 (R)	KPC-2	TEM-1; OXA-9	B2	131	A	B2
SS	2013-02-15	<=1 (S)	<=1 (S)	>1 (R)	4 (I)	1,5 (S)	24 (R)	KPC-2	TEM-1; OXA-9	B4			B2
PS	2013-02-15	<=1 (S)	<=1 (S)	>1 (R)	1 (S)	0,25 (S)	1 (I)	KPC-8	TEM-1; OXA-9	B	131	A	B2
DFG	2013-04-05	<=1 (S)	<=1 (S)	>1 (R)	4 (I)	4 (I)	>32 (R)	KPC-2	TEM-1; OXA-9	B5			B2

S: susceptible; I: intermediate; R: resistant; IPM: imipenem; MER: meropenem; ETP: ertapenem; PFGE: pulsed-field gel electrophoresis; MLST: multilocus sequence typing; DL: Diversilab

ANNEX 4. Table 4. Clinical and epidemiological data of KPC-Ec infected patients.

Patient		Admission ward	Admission date (yyyy/mm/dd)	Previous hospitalization	Previous exposure to CB	Reason for admission	Sample	KPC-Ec isolation date (yyyy/mm/dd)	Other bacteria	Therapy	
ID	Age/Sex									Agent	Duration (days)
ZG	77/F	V3/1	2011/02/26	Yes (Policlinico, MI)	NA	Ictus, COPD, diabetes, decubitus, AH, pneumonia	Urine (IC)	2011/04/08	-	SAM MER	10 5
*NE	89/F	V3/1	2012/04/02	Yes (SRH, MI)	Yes (MER)	Decubitus ulcer, gastroenteritis, AH, UTI	Urine (IC)	2012/06/05	*K. pneumoniae* *P. aeruginosa*	MDZ CIP AK CO IPM VA AMC	10 10 8 8 8 8 10
*PA	92/F	VR1 V4/1	2012/05/09	Yes (SRH, MI)	No	Femur fracture, AH, decubitus, pneumonia	Urine (IC)	2012/07/03	*C. difficile*	LEV VA CAZ Cefuroxim MER CRO	10 11 7 10 10 5
GE	85/M	V4/1	2012/09/12	Yes (Melegnano, MI)	No	COPD, hydrocephalous, diabetes, UTI	Urine (STC)	2012/10/02	-	TZP CO	NA 7
SM	85/M	VR1 V4/5	2012/09/19	Yes (SRH, MI)	Yes (MER)	COPD, pneumonia, UTI, cerebral haemorrhage	Urine (IC)	2012/10/15	*P. mirabilis* *K. pneumoniae*	CRO TZP IPM LEV FOS CIP	5 5 5 8 10 7
BE	87/F	V3/1	NA	Yes (G. Pini, MI)	No	Pelvis fracture, COPD, AH	Urine (STC)	2012/12/03	*K. pneumoniae*	CIP FOS NFR	10 3 10
RMC	84/F	V3/1	2012/11/16	Yes (SRH, MI, Melegnano, MI)		Cerebral hematoma, pneumonia	Urine (IC)	2012/12/05	*C. difficile*	TZP VA	5 4
SS	87/F	V3/5	2013/02/11	Yes (SRH, MI)	No	Femur fracture, AH , diabetes, UTI	Urine (STC)	2013/02/15	*P. mirabilis* *C. difficile*	NFR AK AMC MER	3 6 10 10
PS	85/M	V3/5	2013/01/09	Yes (Melegnano, MI SRH, MI)	No	Femur fracture, COPD, AH, diabetes, pneumonia	Urine (STC)	2013/02/15	*C. difficile*	LEV Metronidazole CAZ NFR VA	5 3 3 3 15
*DFG	82/M	V4/1	2012/11/29	No	Yes (MER)	COPD, diabetes, AH	Sputum	2013/04/05	*C. difficile*	Metronidazole CAZ AK VA	3 12 15 35
MD	84/M	V4/1	2013/02/27	Yes (Cernusco, MI, SRH, MI)	Yes (MER)	Femur fracture, COPD, pneumonia	Sputum	2013/05/06	*S. aureus*	CAZ Zetamicin TZP	15 15 7

CB: carbapenems; SRH: S. Raffaele Hospital; COPD: Chronic Obstructive Pulmonary Disease; AH: Aortic Hypertension; IC: Indwelling Catheter; STC: Short-Term Catheter MDZ: metronidazole; CIP: ciprofloxacin; CAZ: ceftazidime; MER: meropenem; AK: amikacin; CO: colistin; IPM: imipenem; VA: vancomycin; CRO: ceftriaxone; FOS: fosfomycin; SAM, ampicillin–sulbactam AMC: amoxicillin-clavulanate; NFR: nitrofurantoin; NA: Not Available. *: patients with an adverse outcome.

List of original manuscripts

Caltagirone, M., Bitar, I., Piazza, A., Spalla, M., Nucleo, E., Navarra, A., & Migliavacca, R. (2015). Detection of an IncA/C plasmid encoding VIM-4 and CMY-4 β-lactamases in Klebsiella oxytoca and Citrobacter koseri from an inpatient cardiac rehabilitation unit. *The new microbiologica*, *38*(3).

New Microbiologica, 38, 387-392, 2015

Detection of an IncA/C plasmid encoding VIM-4 and CMY-4 β-lactamases in *Klebsiella oxytoca* and *Citrobacter koseri* from an inpatient cardiac rehabilitation unit

Mariasofia Caltagirone[1], Ibrahim Bitar[2], Aurora Piazza[1], Melissa Spalla[1], Elisabetta Nucleo[1], Antonella Navarra[1], Roberta Migliavacca[1]

[1]Clinical-Surgical, Diagnostic and Pediatric Sciences Department, University of Pavia, Italy; [2]I.R.C.C.S. Fondazione Salvatore Maugeri, Pavia, Italy

SUMMARY

A 62-year-old patient was transferred to the cardiac rehabilitation unit of the I.R.C.C.S. Fondazione S. Maugeri after undergoing a heart transplantation at the Acute Care Hospital I.R.C.C.S. S. Matteo of Pavia. On 1 August 2013 and during hospitalization in the rehabilitation unit, *Klebsiella oxytoca* and *Citrobacter koseri* clinical isolates were simultaneously recovered from the patient's preputial swab. Both the *K. oxytoca* and *C. koseri* strains were carbapenem-resistant by MicroScan System (Beckman Coulter). Carbapenem-resistant *K. pneumoniae* had previously been reported in the same rehabilitation facility.

The aim of the study was to identify the carbapenem resistance mechanisms among the enterobacterial species recovered. Phenotypic screening tests useful to detect the β-lactamases\carbapenemases were performed. Carbapenem MICs were obtained by Etest. AmpC and MBL encoding genes were identified by PCR and sequencing. Conjugation assays and plasmid characterization were performed.

Both of the *K. oxytoca* and *C. koseri* isolates were multi-drug resistant, showing resistance to amoxicillin-clavulanic acid, three generation cephalosporins, ertapenem (*K. oxytoca* MIC, >32 mg/L; *C. koseri* MIC, 4 mg/L), imipenem (*K. oxytoca* MIC, 4 mg/L; *C. koseri* MIC, 12 mg/L), thrimethoprim-sulphamethoxazole and gentamicin. Susceptibility was retained to fluoroquinolones, colistin and tigecycline. Molecular characterization confirmed the co-presence of bla_{CMY-4} and bla_{VIM-4} determinants in a 150 Kb transferable plasmid of IncA/C group.

This case is the first detection in Italy of the *K. oxytoca* and *C. koseri* clinical isolates co-producing the CMY-4 and VIM-4 enzymes.

KEY WORDS: Metallo-β-lactamases, Cephalosporinases, Multi-drug resistant *Enterobacteriaceae*, Rehabilitation unit.

Received January 29, 2015 — *Accepted* May 21, 2015

INTRODUCTION

Klebsiella oxytoca and *K. pneumoniae* are opportunistic pathogens increasingly implicated in clusters of community and nosocomial outbreaks, particularly in specific medical units (Watson *et al.*, 2005; Migliavacca *et al.*, 2013).

The acquisition of an extended-spectrum β-lactamase (ESβL) is the most common mechanism of resistance to broad-spectrum cephalosporins in *K. oxytoca* (Romero *et al.*, 2007; Sturm *et al.*, 2010), while acquired AmpC cephalosporinases are less frequently detected in this species (Yamasaki *et al.*, 2010). Since AmpC β-lactamase production is frequently accompanied by multi-drug resistance, therapeutic options became limited. In addition, failure to identify AmpC β-lactamase producers may lead to inappropriate antimicrobial treatment and may result in increased mortality (Tsakris *et al.*, 2011). *Citrobacter koseri*, an environmental Gram-neg-

Corresponding author
Dott.ssa Roberta Migliavacca
Via Brambilla, 74 - 27100 Pavia, Italy
E-mail: r.miglia@unipv.it

ative bacterium, is occasionally found as a coloniser of the human gastrointestinal tract as part of the normal flora.
Although the potential virulence of the species is considered low, it is sporadically implicated in serious nosocomial infections.
The antimicrobial treatment of infection caused by *C. koseri* has been changing, due to several reports of isolates carrying ESβL and other resistance encoding genes (Doran, 1999). Anyway, the isolation of *C. koseri* and/or *K. oxytoca* strains showing resistance to carbapenems remains very infrequent in Italy (Giani *et al.*, 2013). To date, we have only two reports on the presence of bla_{VIM-1} or bla_{KPC-2} genes in *C. koseri* clinical isolates in the Mediterranean area (Castanheira *et al.*, 2009; Mavroidi *et al.*, 2011).
Here we report the detection of *K. oxytoca* and *C. koseri* strains co-producing a VIM-4 metallo-β lactamase (MBL) and an acquired CMY-4 Amp-C enzyme from a single patient. The objective of this study was to evaluate the localization of bla_{VIM-4} and bla_{CMY-4} resistance genes and to assess their spreading potential.

MATERIALS AND METHODS

On 18 July 2013, a 62-year-old male patient was admitted to the cardiac rehabilitation unit of the I.R.C.C.S. Fondazione S. Maugeri in Pavia (Northern Italy) with a diagnosis of cardiac complications, septic shock, pneumonia and preputial edema.
The man had been previously admitted to hospital in February 2013, at the Acute Care Hospital I.R.C.C.S. S. Matteo of Pavia, were he underwent a heart transplantation.
On 1 August 2013 *Candida glabrata*, *Pseudomonas aeruginosa*, *K. oxytoca* and *C. koseri* were also isolated from both preputial swab and urine samples of the patient. The patient was then treated with colistin in monotherapy (EV 1.000.000 U 4/die). After the antibiotic therapy, both the samples resulted negative for the three bacterial species previously identified.
Species identification and susceptibility testing were carried out using the MicroScan AutoSCAN4 automated-system (Beckman Coulter).
Ertapenem (ETP), meropenem (MER) and imipenem (IPM) MICs were determined by Etest (bioMérieux); the results were interpreted according to EUCAST 2014 criteria (The European Committee on Antimicrobial Susceptibility Testing, Version 3.1, 2014). *Escherichia coli* ATCC 25922 and *P. aeruginosa* ATCC 27853 were routinely included during testing for quality assurance.
The *K. oxytoca* and *C. koseri* strains were screened and than phenotypically confirmed for carbapenemase production by the Modified Hodge test (MHT) - using both ETP and IPM - and the KPC/MBL Confirm kit (Rosco Diagnostic).
Phenotypic ESBL and AmpC detection were performed with both the double disk synergy test (DD) (Jarlier, 1988), using piperacillin-tazobactam (TZP), cefotaxime (CTX), cefepime (FEP), ceftazidime (CAZ) and aztreonam (ATM), and with the ESBL + AmpC Screen kit (Rosco Diagnostic). The β-lactamase preliminary identification was performed by Isoelectricfocusing (IEF), as described elsewhere (Pagani *et al.*, 2002).
Crude sonic extracts from *E. coli* harbouring TEM-1 (pI, 5.4), SHV-2 (pI,, 7.6) and SHV-12 (pI,8.2) were used as Isoelectric point (pI,) markers.
Conjugal transfer of resistance determinants was performed in liquid medium using the *E. coli* K12 strain J62 (*pro*⁻, *his*⁻, *trp*⁻, *lac*⁻, *Sm*ʳ) and J53 (*met*⁻, *pro*⁻, *Rif*ʳ) as recipients. The initial donor/recipient ratio was 0.01.
The transconjugants were selected on McConkey agar containing CTX (8 mg/L) plus streptomycin (1000 mg/L) or rifampin (100 mg/L). Species identification and susceptibility testing of the obtained *E. coli* transconjugants were carried out by MicroScan AutoSCAN4 automated-system.
Transconjugants MICs against MER, ETP and IPM were determined by Etest (bioMérieux).
The presence of bla_{VIM}, bla_{IMP} and bla_{AmpC} genes was assessed by multiplex PCR analysis using the primers and the conditions described elsewhere (Rossolini *et al.*, 2008; Pérez-Pérez *et al.*, 2002; Hujer *et al.*, 2006; Koeleman *et al.*, 2001). PCR products were purified using the kit Quantum Prep PCR Kleen Spin Columns (BioRad) and subjected to double-strand sequencing (Macrogen Inc., Seoul, South Korea). The nucleotide sequences were analyzed according to

the BLAST program (http:/www.ncbi.nlm.nih.gov/blast/).
Plasmid DNA was extracted and purified by PureLink™ HiPure Plasmid Filter Purification Kits (Life technologies™).
Plasmids were subsequently typed according to their incompatibility group using the PBRT kit-PCR based replicon typing scheme, as described previously (Caratioli *et al.*, 2006).
The sizes of the plasmids were estimated using the S1 nuclease PFGE method (Barton *et al.*, 1995).

RESULTS

The antimicrobial susceptibility results by MicroScan System showed that both of *K. oxytoca* and *C. koseri* isolates were characterized by multi-drug resistance, retaining susceptibility only to fluoroquinolones, colistin and tigecycline, and showing resistance to amoxicillin-clavulanic acid (AMC), third generation cephalosporins (3GC), cefoxitin, trimethoprim-sulfamethoxazole and gentamicin (according to the 2014 EUCAST breakpoints).
The *C. koseri* isolate resulted resistant to ETP (MIC, >1 mg/L), IPM (MIC, >8 mg/L) and MER (MIC, >8 mg/L) by MicroScan System, while *K. oxytoca* was only ETP (MIC, >1 mg/L) and MER (MIC, >8 mg/L) resistant, showing a IPM MIC, 8 mg/L, using the same tool.
These values were not always coherent with those of the Etest for *K. oxytoca*, ETP MIC being >32 mg/L; IPM MIC, 6 mg/L but MER MIC, 0,75 mg/L, lower than expected. The values for *C. koseri* were consistent only in the case of the ETP MIC >32 mg/L; with IPM MIC, 1 mg/L,

TABLE 1 - *In vitro activity of selected antimicrobial agents tested against Citrobacter koseri, Klebsiella oxytoca and their transconjugants.*

Antimicrobial Agents	**MIC mg/L*				
	Citrobacter koseri	*J53/J62R C. koseri*	*Klebsiella oxytoca*	*J53/J62R K. oxytoca*	*E. coli J53/J62*
Amikacin	≤8	≤8	16	≤8	≤8
Amoxicillin/clav	>8/4	>8/4	>8/4	>8/4	≤2/1
Ampicillin	>8	>8	>8	>8	≤2
Cefepime	4	4	8	4	≤1
Cefotaxime	>16	>16	>16	16	≤1
Cefpodoxime	>1	>1	>1	>1	≤1
Ceftazidime	>8	>8	>8	>8	≤1
Ciprofloxacin	≤0.5	≤0.5	≤0.5	≤0.5	≤0.5
Cloramphenicol	>8	>8	>8	>8	≤8
Colistin	≤2	≤2	≤2	≤2	≤2
Ertapenem	>1	>1	>1	1	≤0.5
Fosfomicin	≤32	≤32	≤32	≤32	≤32
Gentamicin	>4	>4	>4	>4	≤2
Imipenem	>8	≤2	8	≤2	≤2
Levofloxacin	≤1	≤1	≤1	≤1	≤1
Meropenem	>8	≤2	>8	≤2	≤2
Moxifloxacin	≤0.5	≤0.5	≤0.5	≤0.5	≤0.5
Piperacillin/tazobactam	>16	>16	>16	16	≤4
Piperacillin	-	>16	>16	16	≤4
Tetracyclin	>8	>8	>8	>8	≤4
Tigecycline	≤1	≤1	≤1	≤1	≤1
Tobramycin	>4	>4	>4	>4	≤2
Trimethoprim/sulfamethoxazole	>4/76	>4/76	>4/76	>4/76	≤2/38

MIC: Minimum Inhibitory Concentration; *According to European Committee on Antimicrobial Susceptibility Testing (EUCAST 2014) criteria; MIC values were determined using NBC46 card, AutoScan4 System (Beckman Coulter).

and a MER MIC lower than the expected, being equal to 0,5 mg/L.
MHT carbapenemase screening test showed positive results for both the isolates studied.
KPC/MBL Confirm kit and ESBL + AmpC Screen kit tests showed synergistic effect with both dipicolinic and boronic acid; this is typical of MBL and AmpC producers respectively.
The isolates were then further studied for β-lactamase production by biochemical and molecular assays.
Analytical IEF performed using crude enzymatic extracts from the clinical isolates and nitrocefin as chromogenic substrate, showed the presence of a unique β-lactamase band with pI 9.2.
Both of the *K. oxytoca* and *C. koseri* donor strains were able to transfer the resistance plasmid to *E. coli* K12 strain J62 (*pro⁻, his⁻, trp⁻, lac⁻, Sm^r*) and J53 (*met, pro-, Rif^r*) as recipients.
Transfer of CTX resistance was observed at a frequency of approximately 10^{-3} transconjugants per recipient from both *K. oxytoca* and *C. koseri*.
Compared to the recipient *E. coli* J53 and J62 strains, the transconjugants exhibited a decreased susceptibility to several β-lactams (including carbapenems), trimethoprim-sulphamethoxazole and aminoglycosides. The resistance phenotypes of transconjugants were similar to those of the donors *K. oxytoca* and *C. koseri* for the other antimicrobial agents tested (Table 1).
PCR analysis performed on both donors and transconjugant strains yielded positive results for the co-presence of the bla_{VIM} and bla_{CMY} determinants in all the isolates. Amplicons sequencing revealed that *K. oxytoca*, *C. koseri* and *E. coli* transconjugants carried both bla_{VIM-4} and bla_{CMY-4} genes.
Plasmid analysis showed that the above resistance determinants were located in a 150 kb conjugative plasmid belonging to the IncA/C incompatibility group. The IncP and IncN incompatibility groups were also observed in *K. oxytoca*.
The IncA/C multi-resistance plasmid, from the *E. coli* transconjugant, was characterized. PCR results showed that the plasmid contained two distinct resistant *loci* carrying the VIM-4 and CMY-4 β-lactamase genes, with bla_{VIM-4} found as the first gene cassette of a class 1 integron.

DISCUSSION

Although VIM-type carbapenemases have already been described as widely spread in *K. pneumoniae* in Italian rehabilitation hospitals (Nucleo E. *et al.*, 2013), to our knowledge this is the first report on the detection of MDR *K. oxytoca* and *C. koseri* clinical isolates co-producing VIM-4 and CMY-4 enzymes in Italy. The above clinical strains were both recovered from the preputial sample of a patient admitted to the cardiac rehabilitation unit of I.R.C.C.S. Fondazione S. Maugeri of Pavia.
The susceptibility profiles of the studied strains were coherent with carbapenemase production (MER MIC ≥0,5 mg/L), and phenotypic tests used for screening/confirmation of carbapenemase production yielded positive results. The co-presence of a CMY-type enzyme was suggested by IEF results (pI 9.2) and confirmed by PCR and sequencing.
CMY-4 enzyme differs from CMY-2 by one substitution (Arg for Trp at position 221) and from CMY-3 by two substitutions (Glu for Gly at position 42 and Ser for Asn at position 363). The deduced amino acid sequence is 98-99% identical to CMY-3 and to those of the plasmid-mediated AmpC-type β-lactamases originated from *C. freundii*.
The *K. oxytoca* and *C. koseri* MDR strains retained complete susceptibility to colistin. The administration of colistin monotherapy led to a positive outcome, with a complete resolution of the infection.
The bla_{VIM-4} and bla_{CMY-4} resistance genes were co-transferred to *E. coli* during conjugation. The high transfer frequency highlights the plasmid potential of diffusion and dissemination among susceptible isolates, also of different species.
The variability of plasmids mediating antimicrobial resistance in *Enterobacteriaceae* is high. There are plasmid families that are largely prevalent and also plasmids prevalently associated with specific resistance genes. The IncFII, IncA/C, IncL/M and IncI1 plasmids showed the highest occurrence among typed resistance plasmids. These plasmids can be considered "epidemic", being detected in different countries, and in bacteria of different origins and sources. The occurrence of these plasmid types

seems closely linked to the selective pressure exerted by antimicrobial use, incrementing their prevalence compared to that observed in bacterial populations that are not preselected for antimicrobial resistance.

Incompatibility group IncA/C plasmids are large, low copy, plasmids that have been described in the literature for over 40 years. However, they have only recently been intensively studied at the genomic level because of their association with the emergence of multi-drug resistance in enteric pathogens of humans and animals. These plasmids are unique among other enterobacterial plasmids in many aspects, including their modular structure and gene content.

Circulation of IncA/C plasmids in Gram-negative pathogens is now common, and these plasmids bring with them the ability to encode resistance to broad arrays of antimicrobial agents (Johnson *et al.*, 2012).

IncA/C plasmids carrying both the bla_{VIM-1} and bla_{CMY-4} genes were already identified in Italy in clinical isolates of *K. pneumoniae* and *E. cloacae* (Luzzaro *et al.*, 2004). The scaffolds of these plasmids were similar to those of the IncA/C plasmids carrying bla_{CMY-4} or bla_{CMY-2} from *Salmonella enterica* isolated in United States and the United Kingdom, but the carbapenemase gene was not present on these *Salmonella* plasmids and likely represents a novel acquisition for the IncA/C plasmids (Carattoli, 2009).

The emergence of carbapenemases in *K. oxytoca* and *C. koseri* poses major clinical problems. The coexistence in the same bacterial cell of a plasmid carrying epidemiologically important emerging resistance genes is worrisome, since it could predict the generation and the spread of pan-resistant bacteria and the consequent treatment option limitations that can lead to significant morbidity and mortality (Luzzaro *et al.*, 2004).

This report demonstrates that the problem of MBL-producing pathogens no longer entails Gram-negative non-fermenters alone but also involves enterobacteria. Moreover, this case study confirms the need for continuous monitoring for other β-lactamases genes (i.e. AmpC cephalosporinases) that can co-exist in carbapenemase producers, and underline the importance of an active surveillance on the trends of ESBL and acquired AmpC enzymes among species which can cause infections in immuno-compromised hosts (Zhrate et al., 2008). Surveillance may be of value in the difficult battle against life-threatening bacterial infections.

ACKNOWLEDGEMENTS:

The authors thank Dr Silvana Telecco for providing the isolates.

Funding:

This work was supported by internal funds from the University of Pavia. MC and IB are PhD students.

REFERENCES

Barton B.M., Harding G.P., Zuccarelli A.J. (1995). A general method for detecting and sizing large plasmids. *Anal. Biochem.* **226**, 235-240.

Carattoli A. (2009). Resistance plasmid families in *Enterobacteriaceae*. *Antimicrob. Agents Chemother.* **53**, 2227-2238.

Carattoli A., Miriagou V., Bertini A., Loli A., Colinon C., Villa L., Whichard J.M., Rossolini G.M. (2006). Replicon typing of plasmids encoding resistance to newer beta-lactams. *Emerg. Infect. Dis.* **12**, 1145-1148.

Castanheira M., Deshpande L., Marchiese A., Jones R.N. (2009). Emergence of a plasmid mediated bla_{VIM-1} in *Citrobacter koseri*: report from the Sentry Antimicrobial Surveillance Program (Italy). *J. Chemother.* **21**, 98-100.

Doran T.I. (1999). The role of *Citrobacter* in clinical disease of children: Review. *Clin. Infect. Dis.* **28**, 389-394.

Giani T., Pini B., Arena F., Conte V., Bracco S., Migliavacca R.; AMCLI-CRE Survey Participants, Pantosti A., Pagani L., Luzzaro F., Rossolini G.M. (2013). Epidemic diffusion of KPC carbapenemase-producing *Klebsiella pneumoniae* in Italy: results of the first countrywide survey, 15 May to 30 June 2011. Euro Surveill. 30; 18(22):pii=20489. Available online: http://www.eurosurveillance.org/ViewArticle.aspx?ArticleId=20489

Huber K.M., Huber A.M., Hutton E.A., Bajaksouzian S., Adams J.M., Donskey C.J., Ecker D.J., Massire C., Eshoo M.W., Sampath R., Thompson J.M., Rather P.N., Craft D.W., Fisher J.T., Ewell A.J., Jacobs M.R., Patterson D.L., Bonomo R.A. (2006). Analysis of antibiotic resistance genes in multi-drug resistant *Acinetobacter* spp. isolates from military and civilian patients treated at the Walter Reed Army Medical Center. *Antimicrob. Agents Chemother.* **50**, 4114-4123.

Jarlier V. (1988). Beta-lactamase producers and other bacteria: which ones to take into consideration and when? The concept of beta-lactamase inhibitor. *Presse Med.* **27**, 17-18.

Johnson T.J., Lang K.S. (2012). IncA/C plasmids: an emerging threat to human and animal health? *Mob. Genet. Elements.* **2**, 55-58.

Koeleman J.G., Stoof J., Van Der Bijl M.W., Vandenbroucke-Grauls C.M., Savelkoul P.H. (2001). Identification of epidemic strains of *Acinetobacter baumannii* by integrase gene PCR. *J. Clin. Microbiol.* **39**, 8-13.

Luzzaro F., Docquier J-D, Colinon C., Endimiani A., Lombardi G., Amicosante G., Rossolini G.M., Toniolo A. (2004). Emergence in *Klebsiella pneumoniae* and *Enterobacter cloacae* clinical isolates of the VIM-4 Metallo-ß-Lactamase Encoded by a Conjugative Plasmid. *Antimicrob. Agents Chemother.* **48**, 648-650.

Mavroidi A., Neonakis I., Liakopoulos A., Papaioannou A., Ntala M., Tryposkiadis F., Miriagou V., Petinaki E. (2011). Detection of *Citrobacter koseri* carrying beta-lactamase KPC-2 in a hospitalised patient, Greece. Euro Surveill. Available online: http://www.eurosurveillance.org/ViewArticle.aspx?ArticleId=19990

Migliavacca R., Nucleo E., Asticcioli S., Casari E., Bracco S., Sironi M.C. (2013). Multifocal diffusion of a KPC-3 producing ST512 *K. pneumoniae* clone in Northern Italy. *New Microbiol.* **36**, 279-282.

Nucleo E., Spalla M., Piazza A., Caltagirone M.S., Asticcioli S., Debiaggi M., Matti C., Daturi R., Navarra A., Labonia M., Migliavacca R. (2013). Emergence of a VIM-1 MBL and CTX-M-15 ESBL-producing *Klebsiella pneumoniae* clone from acute and rehabilitation hospitals in Italy. *New Microbiol.* **36**, 109-110.

Pagani L., Migliavacca R., Pallecchi L., Matti C., Giacobone E., Amicosante G., Romero E., Rossolini G.M. (2002). Emerging extended-spectrum beta-lactamases in *Proteus mirabilis*. *J. Clin. Microbiol.* **40**, 1549-1552.

Pérez-Pérez F.J., Hanson N.D. (2002). Detection of Plasmid-Mediated AmpC β-Lactamase Genes in Clinical Isolates by Using Multiplex PCR. *J. Clin. Microbiol.* **40**, 2153-2162.

Romero E.D., Padilla T.P., Hernández A.H., Grande R.P., Vázquez M.F., García I.G., García-Rodríguez J.A., Muñoz Bellido J.L. (2007). Prevalence of clinical isolates of *Escherichia coli* and *Klebsiella* spp. producing multiple extended-spectrum-Beta-lactamases. *Diagn. Microbiol. Infect. Dis.* **59**, 433-437.

Rossolini G.M., Luzzaro F., Migliavacca R., Mugnaioli C., Pini, B., De Luca, F., Perilli M., Pollini S., Spalla M., Amicosante G., Toniolo A., Pagani L. (2008). First Countrywide Survey of Acquired Metallo-β-Lactamases in Gram-Negative Pathogens in Italy. *Antimicrob. Agents Chemother.* **52**, 4023-4029.

Sturm P.D., Bochum E.T., van Mook-Vermulst S.V., Handgraaf C., Klaassen T., Melchers W.J. (2010). Prevalence, molecular characterization, and phenotypic confirmation of extended-spectrum-ß-lactamases in *Escherichia coli*, *Klebsiella pneumoniae*, and *Klebsiella oxytoca* at the Radboud University Nijmegen Medical Centre in The Netherlands. *Microb. Drug Resist.* **16**, 55-60.

The European Committee on Antimicrobial Susceptibility Testing. Breakpoint tables for interpretation of MICs and zone diameters. Version 3.1, 2014.

Tsakris A., Poulou A., Markou F., Pitiriga V., Piperaki E-T, Kristo I., Pournaras S. (2011). Dissemination of Clinical Isolates of *Klebsiella oxytoca* harboring CMY-31, VIM-1, and a New OXY-2-Type variant in the community. *Antimicrob. Agents Chemother.* **55**, 3164-3168.

Watson J.T., Jones R.C., Siston A.M., Fernandez J.R., Martin K., Beck E., Sokalski S., Jensen B.J., Arduino M.J., Srinivasan A., Gerber S.I. (2005). Outbreak of catheter-associated *Klebsiella oxytoca* and *Enterobacter cloacae* bloodstream infections in an oncology chemotherapy center. *Arch. Intern. Med.* **165**, 2639-2643.

Yamasaki K., Komatsu M., Abe N., Fukuda S., Miyamoto Y., Higuchi T., Ono T., Nishio H., Sueyoshi N., Kida K., Satoh K., Toyokawa M., Nishi I., Sakamoto M., Akagi M, Nakai I., Kofuku T., Orita T., Wada Y., Imamoto T., Kinoshita S., Miyamoto K., Hirai I., Yamamoto Y. (2010). Laboratory surveillance for prospective plasmid mediated AmpC B-lactamases in the Kinki region of Japan. *J. Clin. Microbiol.* **48**, 3267-3273.

Zárate M.S., Gales A.C., Picão R.C., Pujol G.S., Lanza A., Smayevsky J. (2008). Outbreak of OXY-2-producing *Klebsiella oxytoca* in a renal transplant unit. *J. Clin. Microbiol.* **46**, 2099-2101.

Caltagirone, M., Bitar, I., Piazza, A., Spalla, M., Nucleo, E., Navarra, A., & Migliavacca, R. (2015). Detection of an IncA/C plasmid encoding VIM-4 and CMY-4 β-lactamases in Klebsiella oxytoca and Citrobacter koseri from an inpatient cardiac rehabilitation unit. *The new microbiologica*, *38*(3).

Adv Exp Med Biol - Advances in Microbiology, Infectious Diseases and Public Health (2016) 901: 77–89
DOI 10.1007/5584_2015_5017

Published online: 26 January 2016

Emergence of *Escherichia coli* Sequence Type 131 (ST131) and ST3948 with KPC-2, KPC-3 and KPC-8 carbapenemases from a Long-Term Care and Rehabilitation Facility (LTCRF) in Northern Italy

Aurora Piazza, Mariasofia Caltagirone, Ibrahim Bitar, Elisabetta Nucleo, Melissa Spalla, Elena Fogato, Roberto D'Angelo, Laura Pagani, and Roberta Migliavacca

Abstract

Aim of the study was to characterize KPC-producing *Escherichia coli* (KPC-Ec) clinical isolates among a Northern Italy Long-Term Care and Rehabilitation Facility (LTCRF) residents. Thirteen consecutive non repeated MDR *E. coli* isolates showing ertapenem Minimum Inhibitory Concentrations (MICs) >0.5 mg/L, collected during the period March 2011 – May 2013 from ASP "Redaelli" inpatients, were investigated. The $bla_{KPC/CTX\text{-}M/SHV/TEM/OXA}$ genes were identified by PCR and sequencing. KPC-Ec isolates underwent phylotyping, Pulsed-Field Gel Electrophoresis (PFGE), multilocus sequence typing (MLST) and repetitive sequence-based PCR (rep-PCR) profiling. Incompatibility groups analysis and conjugation were also performed. Eleven out of 13 isolates, resulted bla_{KPC}-type positive, were consistently resistant to third generation cephalosporins, fluoroquinolones and trimethoprim-sulphametoxazole (84.6 %), retaining susceptibility to colistin (EUCAST guidelines). At least n = 4/11 of KPC-Ec patients received ≥48 h of meropenem therapy. Sequencing identified 9 $bla_{KPC\text{-}2}$, 1 $bla_{KPC\text{-}3}$ and 1 $bla_{KPC\text{-}8}$ determinants. KPC-Ec plasmids belonged to IncF group (FIIk replicon); conjugation confirmed $bla_{KPC/TEM\text{-}1/OXA\text{-}9}$ genes transferability

A. Piazza, M. Caltagirone, I. Bitar, E. Nucleo, M. Spalla, L. Pagani, and R. Migliavacca (✉)
Laboratory of Microbiology and Clinical Microbiology, Department of Clinical Surgical Diagnostic and Pediatric Sciences, University of Pavia, Via Brambilla 74, 27100 Pavia, Italy
e-mail: r.migli@unipv.it

E. Fogato and R. D'Angelo
ASP "Golgi-Redaelli", via Bartolomeo d'Alviano 78, 20146 Milan, Italy

for 10 KPC-Ec. Although three pulsotypes (A, B, C) were identified, all KPC-Ec belonged to phylogenetic group B2. Clone B (B-B5) caused an outbreak of infection involving nine inpatients at five wards. Rep-PCR showed relatedness for seven representative KPC-Ec isolates. Here we report a LTCRF outbreak caused by a ST131-B2 *E. coli* associated with bla_{KPC-2} and bla_{KPC-3} genes, and the emergence of the new ST3948. Elderly people with co-morbidities are at risk for ST131 colonization. KPC-Ec clones local monitoring appears essential both to avoid their spreading among healthcare settings, and to improve therapeutic choices for LTCRF residents.

Keywords
Long-Term Care and Rehabilitation Facility • KPC-positive *E. coli* • Sequence Type 131 (ST131) • ST3948

1 Introduction

Carbapenemase-producing *Enterobacteriaceae* (CPE) isolates have been increasingly reported in Europe (Grundmann et al. 2010; Miriagou et al. 2010). The results of the last countrywide cross-sectional survey, carried out during 2011 to investigate the diffusion of Carbapenem-Resistant *Enterobacteriaceae* (CRE) in Italy, showed that CRE were 2 % of all collected isolates, *Klebsiella pneumoniae* being the most frequent species (87 %). Carbapenemase production was the main mechanism (85 %) involved, with bla_{KPC} gene detected mainly in *K. pneumoniae* and only in one *Escherichia coli* CRE (Giani et al. 2013). While less prevalent than in *K. pneumoniae*, KPC production in other species of *Enterobacteriaceae* is increasingly reported, presumably as bla_{KPC}-carrying plasmids are acquired from *K. pneumoniae* by these non-*Klebsiella* species and then propagate (O'Hara et al. 2014). After the first detection of a KPC-positive *E. coli* (KPC-Ec) in Europe, reported in 2008 in France from a patient initially hospitalized in Israel (Petrella et al. 2008), sporadic isolation of KPC-Ec strains has been reported in the USA (Urban et al. 2008), Israel (Goren et al. 2010) and European countries (Naas et al. 2011; Morris et al. 2011). Moreover, *in vivo* transfer of KPC-2 and KPC-3 from *K. pneumoniae* to *E. coli* of different Sequence Types (STs) as ST131, ST1672 and ST394 has been recently described in Italy (Richter et al. 2011; Gona et al. 2014).

E. coli ST131, designated according to the Achtman multilocus sequence typing (MLST) system, was identified in 2008 as a major clone related to the spread of the CTX-M-15 Extended-Spectrum β-lactamase (ESβL) and became the single most prevalent human extra-intestinal *E. coli* strain in many regions, especially among fluoroquinolone- and/or Extended Spectrum-cephalosporin-resistant isolates (Coque et al. 2008; Nicolas-Chanoine et al. 2014). The increasing detection of ST131 isolates from hospitalized and non-hospitalized individuals and, more recently, from companion (Pomba et al. 2014) and foodborne animals (Ghodousi et al. 2015; Platell et al. 2011), sewage and main rivers of large European cities (Colomer-Lluch et al. 2013), highlights the rapid spread and local adaptation to different habitats of this lineage. Worryingly, strains of *E. coli* ST131 resistant to carbapenems have also been reported, further limiting treatment options for this clone (Petrella et al. 2008; Urban et al. 2008; Goren et al. 2010; Naas et al. 2011; Morris et al. 2011). Sporadic isolations of KPC-Ec strains have been reported from Ireland (Morris et al. 2011), France (Naas et al. 2011),

Italy (Accogli et al. 2014), United States (Kim et al. 2012), Taiwan (Ma et al. 2013) and China (Cai et al. 2014).

Since Long-Term Care and Rehabilitation Facilities (LTCRFs), essential components of healthcare delivery to many patients, have been recently recognized as "reservoirs of antibiotic resistance" (Viau et al. 2012) and the spread of carbapenemase-producer *E. coli* strains is related to certain pandemic clones (ST131); aim of the study was: (i) to investigate the presence of KPC-Ec in an Italian LTCRF, (ii) describe KPC-Ec molecular and epidemiological features.

2 Materials and Methods

2.1 Setting

The Clinical Microbiology Laboratory of the ASP "Golgi-Redaelli" of Milan collects and analyzes biological samples from three different Geriatric Institutes: the "P. Redaelli" of Vimodrone, (n = 308 beds), the "C. Golgi" of Abbiategrasso (n = 334 beds) and finally the "P. Redaelli" of Milan (n = 310 beds).

2.2 Bacterial Strains

Thirteen clinical consecutive non-replicate isolates of *E. coli* with ertapenem (ETP) MIC >0.5 mg/L by Phoenix System (Becton Dickinson Diagnostic Systems, Sparks, USA) were collected in the period from March 2011 to May 2013 at the ASP "Golgi-Redaelli", in Milan. All the *E. coli* isolates were obtained from infected elderly inpatients at different long term and rehabilitation Vimodrone wards. Strains were mainly from urine (n = 11/13; 84.6 %) and in two cases from sputum specimens. Among urine samples, 5/11 were from short-term and the remaining six from indwelling catheters. Demographic, clinical and antibiotic administration data were collected through medical records.

2.3 Antimicrobial Susceptibilities, Detection of Carbapenemases and Other β-Lactamases

Identification and susceptibility profiles were confirmed using MicroScan4 (Beckman Coulter) NBC46 panels. MICs of imipenem (IPM), meropenem (MER) and ETP were obtained by Etest strips (bioMérieux). Results were interpreted according to EUCAST guidelines (http://eucast.org/clinical_breakpoints). *E. coli* ATCC 25922 was used as a quality control strain. The isolates were screened for their ability to produce carbapenemases by Modified Hodge Test (MHT) and by the confirmatory disk test using ETP and ETP plus aminophenylboronic acid (APBA) or EDTA. Detection of bla_{SHV}-, bla_{TEM}-, $bla_{CTX\text{-}M}$-, bla_{OXA}- and bla_{KPC}-type genes was performed by PCR as previously described (Tzelepi et al. 2003; Giakkoupi et al. 2009). PCR amplicons were purified using the kit Quantum Prep PCR Kleen Spin Columns (Bio-Rad) and subjected to direct sequencing. PCR products were sequenced on both strands with an Applied Biosystems sequencer. The nucleotide sequences were analyzed with the BLAST software program (http://blast.ncbi.nlm.nih.gov/Blast.cgi).

2.4 Isoelectric Focusing

Production of β-lactamases was confirmed by analytic isoelectric focusing (IEF). IEF of crude cell extracts allowed the visualization of β-lactamase bands by nitrocefin (gels were electrophoresed at 11–14 W for 90 min and β-lactamase bands were detected using 0.5 mM nitrocefin). The β-lactamase bands activity was assessed by a substrate overlaying procedure (using 0.5 μg/ml ETP) as previously described (Pagani et al. 2002). Crude enzymatic extracts from well-known β-lactamase producers (i.e., TEM-1, TEM-2, TEM-7, TEM-8, TEM-9, TEM-12, SHV-1, SHV-2 and SHV-5) were used as controls.

2.5 Conjugation Experiments and Plasmid Characterization

Conjugative transfer of plasmids was carried out in mixed broth cultures using *E. coli* K12 strain J62 (F^-, *pro*, *his*, *trp*, *lac*, Sm^s) and J53 (F^-, *met*, *pro*, Rif^r) as recipient strains. *E. coli* transconjugants were selected on McConkey agar containing ETP (0.5 mg/L) and streptomycin (1000 mg/L) or rifampin (100 mg/L), respectively. Colonies grown on the selective medium were picked for identification by the MicroScan4 System. Recipients that harbored bla_{KPC}-type genes and exhibited resistance to carbapenems and cephalosporins were defined as transconjugants.

Plasmid DNA of *E. coli* isolates and transconjugants were obtained using Pure Link HiPure Plasmid Midiprep kit (Invitrogen, by Life Technologies) and were separated by electrophoresis. Plasmid incompatibility groups were determined in both donor and transconjugant strains by the PCR-based replicon typing (PBRT) method (Carattoli et al. 2005) using the commercially available PBRT Kit (Diatheva) according to manufacturer's instructions.

2.6 PFGE and Rep-PCR Analysis

PFGE was performed using *Xba*I restriction enzyme and fragments were separated on a CHEF-DR II apparatus (Bio-Rad, Milan, Italy) for 22 h at 14 °C. Bacteriophage λ concatenamers were used as DNA size markers. DNA restriction patterns of scanned gel pictures were interpreted following cluster analysis with the Fingerprinting II version 3.0 software (Bio-Rad) using the unweighted pair-group method with arithmetic averages (UPGMA). Only bands larger than 48 kb were considered for the analysis. The Dice correlation coefficient was used with a 1.0 % position tolerance to analyze the similarities of the banding patterns, and a similarity threshold of 90 % to define clusters. The restriction patterns of the genomic DNA from the isolates were analysed and interpreted according to the criteria of Tenover et al. (1995), too.

Repetitive sequence-based PCR (rep-PCR) was performed with the semiautomated Diversilab system (DL) (bioMérieux), according to the manufacturer's instructions. DNA extraction was performed with the UltraClean Microbial DNA isolation kit (Mo Bio Laboratories Inc). Analyses of the PCR amplicons were performed using a 2100 Bioanalyzer (Agilent Technologies). DL fingerprints were analyzed with the DL software 3.4, using the Pearson correlation statistical method to determine clonal relationships. For isolates to be considered indistinguishable in addition to >97 % similarity no peak differences should be seen. For similar isolates, in addition to 95–97 % similarity one to two peak differences are accepted. In addition to <95 % similarity more than two peak variations are considered different. In this study we used the criteria for "indistinguishable" for defining DL profiles (Brolund et al. 2010).

2.7 MLST and Phylogenetic Typing

MLST of representative *E. coli* isolates was performed according to the protocol of Wirth et al. (2006). Allelic profiling and sequence type (ST) determination were performed using the *E. coli* MLST scheme from the website of the University of Warwick (http://mlst.warwick.ac.uk/mlst/dbs/Ecoli).

Phylogenetic groups were determined by a two-step triplex PCR described by Clermont et al. (2000) using Multiplex PCR kit (Qiagen).

2.8 Demographic and Clinical Data

Clinical records of patients with KPC-Ec infection or colonization were examined retrospectively. The following data were recorded: age, gender, admission ward, date of admission, previous hospitalization during the last year, site of infection or colonization, antimicrobial administration before or during the infective episode, treatment outcome.

Treatment failure was classified as absence of resolution or worsening of signs and symptoms

of infection; not assessable – due to incomplete records of the patient within 72 h of infection.

3 Results

3.1 Antimicrobial Susceptibilities and bla_{KPC} Genes Detection

A total of 13 consecutive, non-duplicate *E. coli* isolates showing ETP >0.5 mg/L MIC (0.06 % of the strains of the same species processed in that period) were identified at Clinical Microbiology Laboratory of ASP "Golgi-Redaelli". Susceptibility results initially obtained by Phoenix System (BD) were then confirmed at the Pavia University by both MicroScan4 System (Beckman Coulter) and Etest (bioMérieux).

The isolates showed a MIC range for ETP of 1 to >32 mg/L (MIC_{50} = 4 mg/L; MIC_{90}= >32 mg/L.), MER of 0.064 to >32 mg/L (MIC_{50} = 0.5 mg/L; MIC_{90} = 4 mg/L.) and IPM of 0.5 to >32 mg/L (MIC_{50} = 1 mg/L; MIC_{90} = 4 mg/L.) (Table 1). All the isolates were resistant to piperacillin, amoxicillin/clavulanate, third generation cephalosporins, aztreonam, and ciprofloxacin; 11/13 (84.6 %) strains were resistant to trimethoprim-sulphametoxazole (SXT) and 2/13 (15.4 %) to amikacin. All isolates retained susceptibility to colistin and tigecyclin.

Although the resistance phenotypes of all the 13 isolates were suggestive of a carbapenemase production, the results of both the MHT and the confirmatory disk test using ETP and ETP + APBA were positive only for 11/13 isolates (84,6 %), that underwent molecular typing and were examined for the presence of bla_{KPC}-type genes. The remaining two *E. coli* strains resulted negative to both modified Hodge and ETP-EDTA disk combination tests.

Screening for bla_{KPC} determinants by PCR yielded an amplification product of the expected size from the same 11/13 isolates. Sequencing of the above 11 bla_{KPC} positive isolates, identified the resistance gene as bla_{KPC-2} (n = 9) or bla_{KPC-3} (n = 1) and bla_{KPC-8} (n = 1) (Table 1).

Analytical IEF of crude extracts of all the *E. coli* clinical isolates revealed heterogeneous patterns, with multiple β-lactamase bands in 10/13 cases. The presence for 11/13 samples of a pI 6.7 band showing activity on ETP in a bioassay, was consistent with the production of the KPC enzymes. Such a band was accompanied in 10/11 cases by other two bands (pI 7.2 and pI 5.4) the first able to hydrolyse oxacillin, and the other one showing a narrow spectrum of activity by bioassay. Only a pI >8.2 band, showing high level of hydrolytic activity on CTX (1 mg/L) and consistent with the production of an ESBL of CTX-M-type was present in 2/13 isolates. The BL types detected by PCR in the KPC-Ec included OXA-9 for all isolates and TEM-1 in 10/11 cases. The bla_{CTX-M}-type gene presence was confirmed by PCR in the two non-KPC producers; none of the isolates studied was positive for the presence of bla_{SHV}-type genes.

3.2 Molecular Characterization and Typing

The plasmids replicon typing highlighted the presence of two plasmids belonging to the incompatibility group IncF (FIIk replicon and about 200 Kb in size) and IncF multireplicon (FII, FIA and FIB), often associated with the bla_{KPC} gene dissemination. Conjugation results highlighted the transferability of both plasmids.

All the 11 KPC-Ec strains belonged to the phylogenetic group B2, and showed multiclonal pulsotypes after *XbaI* digestion. PFGE results showed the presence of three KPC-Ec different clones named A, B, and C. Clone B sub-types, named from B to B5 in order of appearance, are shown in Fig. 1a. Clone A appeared once in April 2011; Clone B emerged on June 2012 at Vimodrone LTCRF and persisted until April 2013 causing an intra-hospital outbreak of infection involving nine inpatients at five different wards (V3/1, V4/1, V3/5, VR1, V4/5). The clone C was obtained on May 2013.

Six KPC-Ec isolates, chosen as representative of both KPC variants and PFGE clones, were furthermore investigated by MLST. Two different STs were overall found: the hypervirulent ST131 and the single locus variant in *purA* allele

Table 1 Characteristics of the 13 *E. coli* isolates considered in the study

ID	Collection date (yyyy/mm/dd)	MicroScan4 MIC, mg/L			Etest MIC, mg/L			Beta-Lactamase (BL) content		Molecular typing			
		(Susceptibility category)			(Susceptibility category)								
		IPM	MER	ETP	IPM	MER	ETP	Carbapenemase	BL	PFGE	MLST	DL	Phylogenetic group
VR	2011-03-09	<=1 (S)	<=1 (S)	1 (I)	0.25 (S)	0.064 (S)	4 (R)	–	CTX-M Gr. 1	–			D
ZG	2011-04-08	4 (I)	8 (I)	>1 (R)	1 (S)	>32 (R)	8 (R)	KPC-2	OXA-9	A	131	A	B2
RA	2011-09-30	<=1 (S)	<=1 (S)	>1 (R)	0.75 (S)	0.125 (S)	3 (R)	–	CTX-M Gr. 2	–			B2
NE	2012-06-20	8 (I)	8 (I)	>1 (R)	2 (S)	0.5 (S)	1.5 (R)	KPC-2	TEM-1; OXA-9	B	131	A	B2
PA	2012-07-03	<=1 (S)	<=1 (S)	>1 (R)	0.5 (S)	0.5 (S)	1.5 (R)	KPC-2	TEM-1; OXA-9	B			B2
GE	2012-10-02	>8 (R)	8 (I)	>1 (R)	1 (S)	1 (S)	>32 (R)	KPC-2	TEM-1; OXA-9	B2	131	A	B2
SM	2012-10-15	>8 (R)	>8 (R)	>1 (R)	1 (S)	0.38 (S)	4 (R)	KPC-2	TEM-1; OXA-9	B1	131	A	B2
BE	2012-12-03	4 (I)	8 (I)	>1 (R)	0.5 (S)	1 (S)	2 (R)	KPC-2	TEM-1; OXA-9	B5			B2
RMC	2012-12-05	<=1 (S)	<=1 (S)	2 (R)	1 (S)	0.5 (S)	2 (R)	KPC-2	TEM-1; OXA-9	B2	131	A	B2
SS	2013-02-15	<=1 (S)	<=1 (S)	>1 (R)	4 (I)	1.5 (S)	24 (R)	KPC-2	TEM-1; OXA-9	B4			B2
PS	2013-02-15	<=1 (S)	<=1 (S)	>1 (R)	1 (S)	0.25 (S)	1 (I)	KPC-8	TEM-1; OXA-9	B	131	A	B2
DFG	2013-04-05	<=1 (S)	<=1 (S)	>1 (R)	4 (I)	4 (I)	>32 (R)	KPC-2	TEM-1; OXA-9	B5			B2
MD	2013-05-06	32 (R)	32 (R)	32 (R)	2 (S)	1 (S)	>32 (R)	KPC-3	TEM-1; OXA-9	C	3948	A	B2

S susceptible, *I* intermediate, *R* resistant, *IPM* imipenem, *MER* meropenem, *ETP* ertapenem, *PFGE* pulsed field gel electrophoresis, *MLST* multilocus sequence typing, *DL* DiversiLab

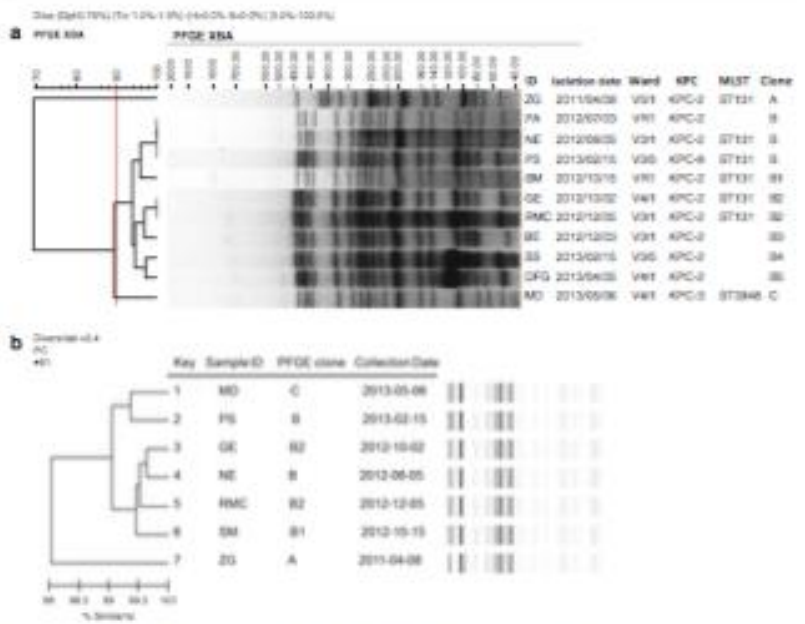

Fig. 1 (**a**) UPGMA dendrogram of *Xba*I PFGE profiles of KPC-Ec isolates. (**b**) Gel image and dendrogram of rep-PCR DL patterns of KPC-Ec representative strains

(53-40-47-13-36-**10**-29) ST3948 clone, belonging to the ST131 Clonal complex (Clpx). The ST131 KPC-Ec group was identified in five out of six KPC-Ec chosen as representative. Interestingly, pandemic ST131 included both the PFGE clones A and B, the latter group of KPC-Ec strains harboring both the bla_{KPC-2} or the bla_{KPC-3} gene variants.

The ST3948 clone was associated to the bla_{KPC-3} determinant and to the PFGE unique clone C strain.

3.3 DL and PFGE

Rep-PCR (DL) on seven representative KPC-Ec isolates, belonging to A, B, C PFGE clones, showed genetic relatedness (Fig. 1b). While ST3948 was included in a distinctive clone C by PFGE, ST131 strains clustered >97 % with ST3948 using the DL tool.

3.4 Demographic and Clinical Data of KPC-Ec Patients

According to clinical records, the mean age of the patients was 85 years, with the 54.5 % being female. Based on clinical data, at least n = 4/11 of KPC-Ec infected patients received ≥48 h of MER therapy in an Acute Care Hospital (ACH) and/or LTCRF located in the area of Milan.

All the patients were affected by several co-morbidities, including diabetes, hypertension, chronic obstructive pulmonary disease. Co-infections were reported for the majority of patients: 5/9 caused by *Clostridium difficile*, 1/9 by *Staphylococcus aureus* and in the remaining

cases by Gram-negative bacteria (Table 2). It is of note that three out of 11 patients died during hospitalization. Death was attributable or related to KPC-Ec at least in the outbreak index patient NE.

Epidemiological analysis showed that index patient of the KPC-Ec outbreak, was a 89 years old woman (NE), admitted in April 2012 to the V3/I rehabilitation ward of the "P. Redaelli" LTCRF of Vimodrone. The patient was discharged from the S. Raffaele Hospital of Milan just before. After 2 months of hospitalization, KPC-Ec strain was isolated from the urine of the patient. On admission, patient's physical examination revealed decubitus ulcers and gastroenteritis symptoms. She was treated with metronidazole 500 mg and ciprofloxacin 400 mg. Due to the occurrence of UTI-related septic shock symptoms, a combination therapy with MER and amikacin (500 mg each) started from May 2012 the 5th. A carbapenem resistant *K. pneumoniae* isolate was obtained from the same patient in May 2012 the 9th. The *K. pneumoniae* strain resulted bla_{KPC}-, bla_{TEM}-, bla_{OXA}-type genes producer, and harboured an IncF (FIIk replicon) plasmid, like the KPC-Ec isolate (data not shown).

Due to NE KPC-Ec isolation (one month later) an 8 days colistin plus IPM therapy was administrated; even so, on June the 20th the rectal swab sampling revealed the intestinal persistence of the same strain.

During the next year, eight additional inpatients at the same LTCRF, affected by UTIs (n = 7) and pneumonia (n = 1), resulted positive for the presence of clone B outbreak strain.

The mainly used antibacterial agents are listed in Table 2.

A complete or partial response was obtained in eight patients, whereas failure of treatment or relapse was observed in three patients.

4 Discussion

The present study describes the emergence and intra-hospital spread of a clonal strain KPC-Ec in one LTCRF hospital located in Northern Italy and admitting patients from several ACH of the same area. Genetic features and molecular epidemiology were also investigated.

Although KPC-*K. pneumoniae* (KPC-Kp) has become endemic in Italy in many ACH, the presence of KPC-Ec isolates remain limited (Giani et al. 2013). The spread of bla_{KPC}-type resistance determinants is of particular concern in *E. coli*, being emerging KPC-Ec associated with different STs (Mavroidi et al. 2012; Almeida et al. 2012; Baraniak et al. 2011; Ruiz-Garbajosa et al. 2013; Gijón et al. 2012). Even more worrisome is the occurrence and dissemination of the MDR ST131 KPC-Ec clones, belonging to the hypervirulent, uropathogenic lineage B2 (Clermont et al. 2009), and distinguishable in turn into several pulsotypes (Coque et al. 2008; Lau et al. 2008; Nicolas-Chanoine et al. 2013).

In the present study, we report the presence of 11 KPC-Ec producers in an Italian LTCRF.

Interestingly, Etest MIC values for IPM (from 0.5 to 4 mg/L) and MER (from 0.5 to >32 mg/L) resulted within the susceptible clinical breakpoints for the majority of the carbapenemase producers (n = 9/11). Such variability, here observed also in the case of ETP (MIC = 1 – >32 mg/L), was already reported for KPC-Ec strains (Accogli et al. 2014; Deshpande et al. 2006). Moreover, the diversity in carbapenem MICs among KPC-Ec strains of the same clone (B-B5) could be due to differences in plasmid copy numbers (as a result of antibiotic therapy administered) or the presence of sub-populations expressing different resistance levels. Scattered colonies within the Etest inhibition zone were present in few cases, and ETP (instead of MER) resulted the most efficient and sensitive molecule for KPC-Ec producers detection.

A porin loss mechanism coupled with the CTX-M-type enzyme production could justify the ETP MIC values obtained for two non-carbapenemase producers by Microscan4 System (1–>1 mg/L) *versus* Etest (4–3 mg/L).

Overall, phenotypical tests, IEF, PCR and sequencing revealed heterogeneous β-lactamase patterns: while in the case of the two MDR

Table 2 Clinical and epidemiological data of KPC-Ec infected patients

Patient											Therapy	
ID	Age/Sex	Admission ward	Admission date (yyyy/mm/dd)	Previous hospitalisation	Previous exposure to CB	Reason for admission	Sample	KPC-Ec isolation date (yyyy/mm/dd)	Other bacteria	Agent	Duration (days)
ZG	77/F	V3/1	2011/02/26	Yes (Policlinico, MI)	NA	Ictus, COPD, diabetes, decubitus, AH, pneumonia	Urine (IC)	2011/04/08	–	SAM	10
										MER	5
*NE	89/F	V3/1	2012/04/02	Yes (SRH, MI)	Yes (MER)	Decubitus ulcer, gastroenteritis, AH, UTI	Urine (IC)	2012/06/05	*K. pneumoniae*	MDZ	10
									P. aeruginosa	CIP	10
										AK	8
										CO	8
										IPM	8
										VA	8
										AMC	10
*PA	92/F	V8/1	2012/05/09	Yes (SRH, MI)	No	Femur fracture, AH, decubitus, pneumonia	Urine (IC)	2012/07/03	*C. difficile*	LEV	10
		V4/1								VA	11
										CAZ	7
										Ceftriaxim	10
										MER	10
										CRO	5
GE	85/M	V4/1	2012/09/12	Yes (Melegnano, MI)	No	COPD, hydrocephalus, diabetes, UTI	Urine (STC)	2012/10/02	–	TZP	NA
										CO	7
SM	85/M	V8/1	2012/09/19	Yes (SRH, MI)	Yes (MER)	COPD, pneumonia, UTI, cerebral haemorrhage	Urine (IC)	2012/10/15	*P. mirabilis*	CRO	5
		V4/5							*K. pneumoniae*	TZP	5
										IPM	5
										LEV	8
										FOS	10
										CIP	7
BE	87/F	V3/1	NA	Yes (G. Pini, MI)	No	Pelvis fracture, COPD, AH	Urine (STC)	2012/12/03	*K. pneumoniae*	CIP	10
										FOS	3
										NIT	10

(continued)

Table 2 (continued)

Patient												Therapy	
ID	Age/Sex	Admission ward	Admission date (yyyy/mm/dd)	Previous hospitalisation	Previous exposure to CB	Reason for admission	Sample	KPC-Ec isolation date (yyyy/mm/dd)	Other bacteria	Agent	Duration (days)
RMC	84/F	V3/1	2012/11/06	Yes (SRH, MI, Melegnano, MI)		Cerebral hematoma, pneumonia	Urine (IC)	2012/12/05	*C. difficile*	TZP	3
										VA	4
SS	87/F	V3/5	2013/02/11	Yes (SRH, MI)	No	Femur fracture, AH, diabetes, UTI	Urine (STC)	2013/02/15	*P. mirabilis*	NFR	3
									C. difficile	AK	6
										AMC	10
										MFR	10
PS	85/M	V3/5	2013/01/09	Yes (Melegnano, MI SRH, MI)	No	Femur fracture, COPD, AH, diabetes, pneumonia	Urine (STC)	2013/02/15	*C. difficile*	LEV	5
										Metronidazole	3
										CAZ	3
										NFR	3
										VA	15
*TBG	82/M	V4/1	2012/11/29	No	Yes (MER)	COPD, diabetes, AH	Sputum	2013/04/05	*C. difficile*	Metronidazole	3
										CAZ	12
										AK	15
										VA	35
MD	84/M	V4/1	2013/02/27	Yes (Cernusco, MI, SRH, MI)	Yes (MER)	Femur fracture, COPD, pneumonia	Sputum	2013/05/06	*S. aureus*	CAZ	15
										Zeftazidim	15
										TZP	7

CB carbapenems, *SRH* S. Raffaele Hospital, *COPD* chronic obstructive pulmonary disease, *AH* aortic hypotension, *IC* indwelling catheter, *STC* short-term catheter, *MEM* meropenem, *CIP* ciprofloxacin, *CAZ* ceftazidime, *MER* meropenem, *AK* amikacin, *CO* colistin, *IPM* imipenem, *VA* vancomycin, *OBO* cefuroxime, *FOS* fosfomycin, *SAM* ampicillin–sulbactam, *AMC* amoxicillin–clavulanate, *NFR* nitrofurantoin, *NA* not available

*Patients with an adverse outcome

KPC-Ec negative isolates only a CTX-M-type ESBL was detected, KPC-2, KPC-3 and KPC-8 MDR strains were also OXA-9 and TEM-1 enzymes producers. Conjugation experiments results together with the plasmid replicon typing analysis (incompatibility group IncF, with FIIk replicon, typical of *K. pneumoniae*) suggested the possible inter-species plasmid exchange between KPC-Kp and KPC-Ec in the retrospectively ascertained (NE) index case patient. Since the clone B strain harbored the 200Kb outbreak conjugative plasmid, the bla_{KPC-8} gene could be due to two points mutations within the bla_{KPC-2} gene sequence (T to G at position 716 leading to a valine-to-glycine substitution, and C to T at 814 position leading to an histidine-to-tyrosine substitution) (data not shown). Nine residents and five wards were involved in the Vimodrone outbreak and the time elapsed between admission and outbreak strain acquisition was in average 38 days.

The molecular and epidemiological results revealed the intra-hospital spread of a ST131-B2 (PFGE clone B) outbreak clone. To note that PFGE clone B-related isolates clustered with two KPC-Ec strains collected on August and October 2012 at Vimercate ACH (our personal data). This confirms how elderly people with many co-morbidities are at greatest risk for ST131 colonization, possibly due to healthcare-associated transmission.

Here we describe the emergence in an Italian LTCRF of three KPC-Ec PFGE clones (A, B, C), belonging to ST131 and to the new ST3948 (clone C). Although LTCRFs are well known as reservoirs of other antimicrobial resistant pathogens (Arnoldo et al. 2013) and have been implicated as reservoirs of ST131 in Europe (Burke et al. 2012; Brisse et al. 2012), at our knowledge this study is the first one describing an Italian LTCRF as KPC-Ec ST131 reservoir. DL results clustered all the KPC-Ec isolates in the same profile, thus confirming the higher discriminatory power of PFGE for *E. coli* outbreak studies.

Taken together, our findings highlight the need to continue screening of *E. coli* showing a decreased susceptibility to ETP, and to implement more rigorous infection control measures among LTCRF residents to avoid the spread of difficult-to-treat ST131 Clpx KPC-Ec in our area. Failure of treatment was observed in three patients, of which two infected by an KPC-Ec strain studied: local monitoring of KPC-Ec clones appears essential to avoid the risk of their spreading among healthcare settings and to improve effective and appropriate therapeutic choices for LTCRF residents.

References

Accogli M, Giani T, Monaco M et al (2014) Emergence of *Escherichia coli* ST131 sub-clone H30 producing VIM-1 and KPC-3 carbapenemases, Italy. J Antimicrob Chemother 69(8):2293–2296. doi:10.1093/jac/dku132

Almeida AC, de Sá Cavalcanti FL, Vilela MA et al (2012) *Escherichia coli* ST502 and *Klebsiella pneumoniae* ST11 sharing an IncW plasmid harbouring the *bla* (KPC-2) gene in an Intensive Care Unit patient. Int J Antimicrob Agents 40(4):374–376

Arnoldo L, Migliavacca R, Regattin L et al (2013) Prevalence of urinary colonization by extended spectrum-beta-lactamase *Enterobacteriaceae* among catheterized inpatients in Italian long term care facilities. BMC Infect Dis 13:124. doi:10.1186/1471-2334-13-124

Baraniak A, Grabowska A, Izdebski R et al (2011) Molecular characteristics of KPC-producing *Enterobacteriaceae* at the early stage of their dissemination in Poland, 2008–2009. Antimicrob Agents Chemother 55(12):5493–5499

Basic Local Alignment Search Tool. http://blast.ncbi.nlm.nih.gov/Blast.cgi

Brisse S, Diancourt L, Laouenan C et al (2012) Phylogenetic distribution of CTX-M- and non-extended-spectrum-beta-lactamase-producing *Escherichia coli* isolates: group B2 isolates, except clone ST131, rarely produce CTX-M enzymes. J Clin Microbiol 50:2974–2981

Brolund A, Haggman S, Edquist PJ et al (2010) The DiversiLab system versus pulsed-field gel electrophoresis: characterisation of extended spectrum β-lactamase producing *Escherichia coli* and *Klebsiella pneumoniae*. J Microbiol Methods 83(2):224–230. doi:10.1016/j.mimet.2010.09.004

Burke L, Humphreys H, Fitzgerald-Hughes D (2012) The revolving door between hospital and community: extended-spectrum beta-lactamase producing *Escherichia coli* in Dublin. J Hosp Infect 81:192–198

Cai JC, Zhang R, Hu YY et al (2014) Emergence of *Escherichia coli* sequence type 131 isolates producing KPC-2 carbapenemase in China. Antimicrob Agents

Chemother 58(2):1146–1152. doi:10.1128/AAC.00912-13

Carattoli A, Bertini A, Villa L et al (2005) Identification of plasmids by PCR-based replicon typing. J Microbiol Methods 63(3):219–228

Clermont O, Bonacorsi S, Bingen E (2000) Rapid and simple determination of the *Escherichia coli* phylogenetic group. Appl Environ Microbiol 66(10):4555–4558

Clermont O, Dhanji H, Upton M et al (2009) Rapid detection of the O25b-ST131 clone of *Escherichia coli* encompassing the CTX-M-15-producing strains. J Antimicrob Chemother 64(2):274–277

Colomer-Lluch M, Mora A, Lopez C et al (2013) Detection of quinolone-resistant *Escherichia coli* isolates belonging to clonal groups O25b:H4-B2-ST131 and O25b:H4-D-ST69 in raw sewage and river water in Barcelona, Spain. J Antimicrob Chemother 68:758–765

Coque TM, Novais A, Carattoli A et al (2008) Dissemination of clonally related *Escherichia coli* strains expressing extended-spectrum beta-lactamase CTX-M-15. Emerg Infect Dis 14(2):195–200

Deshpande LM, Rhomberg PR, Sader HS et al (2006) Emergence of serine carbapenemases (KPC and SME) among clinical strains of *Enterobacteriaceae* isolated in the United States Medical Centers: report from the MYSTIC Program (1999–2005). Diagn Microbiol Infect Dis 56(4):367–372

European Committee on Antimicrobial Susceptibility Testing. http://eucast.org/clinical_breakpoints

Ghodousi A, Bonura C, Di Noto AM et al (2015) Extended-spectrum ß-lactamase, AmpC-producing, and fluoroquinolone-resistant *Escherichia coli* in retail broiler chicken meat, Italy. Foodborne Pathog Dis 12(7):619–625. doi:10.1089/fpd.2015.1936

Giakkoupi P, Pappa O, Polemis M et al (2009) Emerging *Klebsiella pneumoniae* isolates coproducing KPC-2 and VIM-1 carbapenemases. Antimicrob Agents Chemother 53(9):4048–4050

Giani T, Pini B, Arena F et al (2013) Epidemic diffusion of KPC carbapenemase-producing *Klebsiella pneumoniae* in Italy: results of the first countrywide survey, 15 May to 30 June 2011. Euro Surveill 18(22)

Gijón D, Curiao T, Baquero F et al (2012) Fecal carriage of carbapenemase-producing *Enterobacteriaceae*: a hidden reservoir in hospitalized and non-hospitalized patients. J Clin Microbiol 50(5):1558–1563

Gona F, Barbera F, Pasquariello AC et al (2014) In vivo multiclonal transfer of *bla*(KPC-3) from *Klebsiella pneumoniae* to *Escherichia coli* in surgery patients. Clin Microbiol Infect 20(10):O633–O635. doi:10.1111/1469-0691.12577

Goren MG, Navon-Venezia S, Chmelnitsky I et al (2010) Carbapenem-resistant KPC-2-producing *Escherichia coli* in a Tel Aviv Medical Center, 2005 to 2008. Antimicrob Agents Chemother 54(6):2687–2691. doi:10.1128/AAC.01359-09

Grundmann H, Livermore DM, Giske CG et al (2010) Carbapenem-non-susceptible *Enterobacteriaceae* in Europe: conclusions from a meeting of national experts. Euro Surveill 15(46)

Kim YA, Qureshi ZA, Adams-Haduch JM et al (2012) Features of infections due to *Klebsiella pneumoniae* carbapenemase-producing *Escherichia coli*: emergence of sequence type 131. Clin Infect Dis 55(2):224–231

Lau SH, Kaufmann ME, Livermore DM et al (2008) UK epidemic *Escherichia coli* strains A-E, with CTX-M-15 beta-lactamase, all belong to the international O25:H4-ST131 clone. J Antimicrob Chemother 62(6):1241–1244

Ma L, Siu LK, Lin JC et al (2013) Updated molecular epidemiology of carbapenem-non-susceptible *Escherichia coli* in Taiwan: first identification of KPC-2 or NDM-1-producing *E. coli* in Taiwan. BMC Infect Dis 13:599. doi: 10.1186/1471-2334-13-599

Mavroidi A, Miriagou V, Malli E et al (2012) Emergence of *Escherichia coli* sequence type 410 (ST410) with KPC-2 β-lactamase. Int J Antimicrob Agents 39(3):247–250

Miriagou V, Cornaglia G, Edelstein M et al (2010) Acquired carbapenemases in Gram-negative bacterial pathogens: detection and surveillance issues. Clin Microbiol Infect 16(2):112–122. doi:10.1111/j.1469-0691.2009.03116.x

Morris D, Boyle F, Ludden C et al (2011) Production of KPC-2 carbapenemase by an *Escherichia coli* clinical isolate belonging to the international ST131 clone. Antimicrob Agents Chemother 55(10):4935–4936. doi:10.1128/AAC.05127-11

Multi Locus Sequence Typing scheme from the website of the University of Warwick. http://mlst.warwick.ac.uk/mlst/dbs/Ecoli

Naas T, Cuzon G, Guillot O et al (2011) When carbapenem-hydrolyzing beta-lactamase Kpc meets *Escherichia coli* ST131 in France. Antimicrob Agents Chemother 55(10):4933–4934. doi:10.1128/AAC.00719-11

Nicolas-Chanoine MH, Robert J, Vigan M et al (2013) Different factors associated with CTX-M-producing ST131 and non-ST131 *Escherichia coli* clinical isolates. PLoS One 8(9), e72191

Nicolas-Chanoine MH, Bertrand X, Madec JY (2014) *Escherichia coli* ST131, an intriguing clonal group. Clin Microbiol Rev 27(3):543–574. doi:10.1128/CMR.00125-13

O'Hara JA, Hu F, Ahn C et al (2014) Molecular epidemiology of KPC-producing *Escherichia coli*: occurrence of ST131-fimH30 subclone harboring pKpQIL-like IncFIIk plasmid. Antimicrob Agents Chemother 58(7):4234–4237. doi:10.1128/AAC.02182-13

Pagani L, Migliavacca R, Pallecchi L et al (2002) Emerging extended-spectrum beta-lactamases in *Proteus mirabilis*. J Clin Microbiol 40(4):1549–1552

Petrella S, Ziental-Gelus N, Mayer C et al (2008) Genetic and structural insights into the dissemination potential of the extremely broad-spectrum class A beta-lactamase KPC-2 identified in an *Escherichia coli* strain and an *Enterobacter cloacae* strain isolated from the same patient in France. Antimicrob Agents Chemother 52(10):3725–3736. doi:10.1128/AAC.00063-08

Platell JL, Johnson JR, Cobbold RN et al (2011) Multidrug-resistant extraintestinal pathogenic *Escherichia coli* of sequence type ST131 in animals and foods. Vet Microbiol 153(1–2):99–108. doi: 10.1016/j.vetmic.2011.05.007

Pomba C, López-Cerero L, Bellido M et al (2014) Within-lineage variability of ST131 *Escherichia coli* isolates from humans and companion animals in the south of Europe. J Antimicrob Chemother 69(1):271–273. doi:10.1093/jac/dkt343

Richter SN, Frasson I, Bergo C et al (2011) Transfer of KPC-2 Carbapenemase from *Klebsiella pneumoniae* to *Escherichia coli* in a patient: first case in Europe. J Clin Microbiol 49(5):2040–2042. doi:10.1128/JCM.00133-11

Ruiz-Garbajosa P, Curiao T, Tato M et al (2013) Multiclonal dispersal of KPC genes following the emergence of non-ST258 KPC-producing *Klebsiella pneumoniae* clones in Madrid, Spain. J Antimicrob Chemother 68(11):2487–2492

Tenover FC, Arbeit RD, Goering RV et al (1995) Interpreting chromosomal DNA restriction patterns produced by pulsed-field gel electrophoresis: criteria for bacterial strain typing. J Clin Microbiol 33 (9):2233–2239

Tzelepi E, Magana C, Platsouka E et al (2003) Extended-spectrum beta-lactamase types in *Klebsiella pneumoniae* and *Escherichia coli* in two Greek hospitals. Int J Antimicrob Agents 21(3):285–288

Urban C, Bradford PA, Tuckman M et al (2008) Carbapenem-resistant *Escherichia coli* harboring *Klebsiella pneumoniae* carbapenemase beta-lactamases associated with long-term care facilities. Clin Infect Dis 46(11):e127–e130. doi:10.1086/588048

Viau RA, Hujer AM, Marshall SH et al (2012) "Silent" dissemination of *Klebsiella pneumoniae* isolates bearing *K. pneumoniae* carbapenemase in a long-term care facility for children and young adults in Northeast Ohio. Clin Infect Dis 54(9):1314–1321. doi:10.1093/cid/cis056

Wirth T, Falush D, Lan R et al (2006) Sex and virulence in *Escherichia coli*: an evolutionary perspective. Mol Microbiol 60(5):1136–1151