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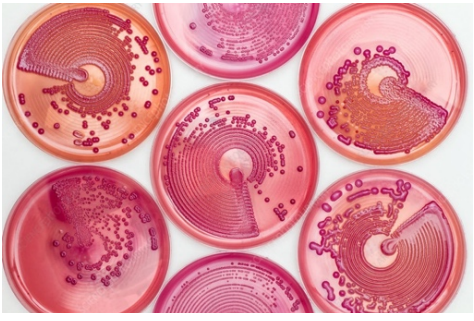
DI PAVIA

Dipartimento di Scienze Clinico, Chirurgiche, Diagnostiche e Pediatriche
Unità di Microbiologia e Microbiologia Clinica.

Multidrug-resistant (MDR) and Extensively Drug-resistant (XDR) Gram-negative bacteria of clinical interest: laboratory diagnosis, clinical management and infection control



Vittoria Mattioni Marchetti

Dottorato di Ricerca in
Genetica, Biologia Molecolare e Cellulare
Ciclo XXXII – A.A. 2016-2019

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To my little, ramshackle and unconventional family I dedicate this thesis

Abbreviations

3GC: cefotaxime and/or ceftriaxone and/or ceftazidime
aac(6′)-Ib-cr: aminoglycoside-(6)-N-acetyltransferase
AMCLI: Italian Society of Clinical Microbiologist
AMR: antimicrobial resistance
ATM: aztreonam
CAT: chloramphenicol acetyl transferase
CAZ: ceftazidime
CC: clonal complex
CDC: Centers for Disease Control and Prevention
cIAI: complicated intra-abdominal infection
CPE: carbapenemase-producing *Enterobacterales*
CRE: carbapenem resistant *Enterobacteriaceae*
CS: conserved segment
CTX: cefotaxime
cUTI: complicated urinary tract infection
DD: double disk synergy test
EARS-Net: European Antimicrobial Resistance Surveillance Network
ECDC: European Centre for Disease Prevention and Control
EDTA: ethylenediaminetetraacetic acid
EEA: European Economic Area
EPIC: Intensive Care Study
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
HAI: health care-associated infections
HGT: horizontal gene transmission
IAI: intra-abdominal infection
ICU: intensive care unit
ICU-HAI: intensive care unit-associated infection
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
MIC: minimum inhibitory concentration
MLST: multilocus sequence typing
MRSA: Methicillin-resistant *Staphylococcus aureus*
MSSA: Methicillin-susceptible *Staphylococcus aureus*
NDM: New Delhi metallo-β-lactamase
OIE: World Organisation for Animal Health
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

PG: polymer peptidoglycan
PMQR: plasmid-mediated quinolone resistance
PPS: Point Prevalence Survey
Rep: replication initiator proteins
ST: sequence type
TZP: piperacillin-tazobactam
UTI: urinary tract infection
VAP: ventilator associated pneumonia
VIM: Verona integron–encoded metallo-β-lactamase
VKM: Norwegian Scientific Committee for Food and Environment
WGS: whole-genome sequencing
WHO: World Health Organization
XDR: extensively-drug resistance

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1. General Introduction

1.1 Antibiotics: from the "Golden Age" to modern Era

The word *antibiotic*, first used in 1941, defines any small molecule made by a microbe that antagonizes the growth of other microbes (Clardy J *et al.* 2009). The discovery of antibiotics, started in 1928 after Penicillin discovery by Alexander Fleming, is justly considered one of the most significant health-related events of modern times, leading to a substantial improvement on infectious diseases treatment (Davis J *et al.* 2010). The following 20 years after Flemming's discovery were considered the 'Golden Age' of antibiotics, even due to the introduction of antimicrobial agents such as Streptomycin and Cephalosporins (Figure 1.1.1).

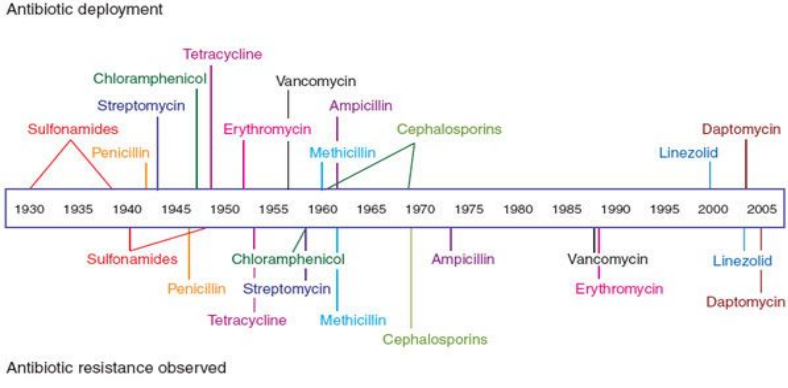

Figure 1.1.1: History of antibiotic discovery and concomitant development of antibiotic resistance.

However, the first cases of antimicrobial resistance were recorded in parallel with the use of antibiotics, leading scientists to look at new ways to enhance existing agents activity and tackle this issue. Methicillin was

introduced in 1959 as the first penicillinase-resistant β-lactam antibiotic, whereas in 1961 ampicillin allowed to reach both a penicillin's spectrum of activity and pharmacokinetics improvement (Gould K 2016). As a strategy against β-lactamases, in 1976 different β-lactamase inhibitors, such as clavulanic acid, were first identified as a by-product of *Streptomyces clavuligerus* cultures. As a number of broad-spectrum antibiotics became available, the incidence of infections caused by drug-resistant bacteria increased due to selection pressure (Gould K 2016).

Despite the use of new strategies, the phenomena of resistance continued to grow, and nowadays specific attention is reverted to the public health issue of resistance in Gram-negative bacteria. Treatment of infections caused by pan-resistant *Acinetobacter* spp., *Enterobacterales* and *Pseudomonas* spp. is proving a challenge to clinicians, particularly in the intensive care unit (ICU) scenario. For this reason, older drugs such as colistin, chloramphenicol and fosfomycin were reconsidered, either alone or in combination with newer agents (Gould K 2016). In 2012, World Health Organization declared the emergency of antimicrobial resistance as one of the top three threats to human health. Antibiotic resistance acquisition occurs mainly through transfer of antibiotic genes between bacteria, particularly encouraged by excessive use/misuse of antibiotics in humans and animals. This leads to clonal dissemination of pathogenic bacteria and emergence of Multidrug-resistant (MDR) bacteria in which the bacterial strains express phenotipical resistance to three or more antibiotic classes at once (Aminov RI *et al.* 2007; Alanis AJ 2005; Kadouri DE *et al.* 2013). The effectiveness of the antibiotic therapies against MDR infections becomes decreased, setting up a global health problem. In particular, the spread of MDR isolates represent a major health issue for patients admitted to ICU wards and affected by Hospital-acquired Infections (HAIs). The ICU-HAI 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 an high frequency of antimicrobial therapy use and risks of cross-transmission between patients and staff (Cornejo-Juarez P *et al.* 2015).
In accordance with ECDC's surveillance report published in 2013 (https://ecdc.europa.eu/sites/portal/files/media/en/publications/Publications/healthcare-associated-infections-antimicrobial-use-PPS.pdf), the occurrence of antimicrobial resistance in Europe is eterogenous, and depends on antimicrobial use, microorganisms and geographic region. From the Figure 1.1.2 it's possible to observe a north to south gradient, with lower antibiotic use percentages in the northern part compared to the southern regions.

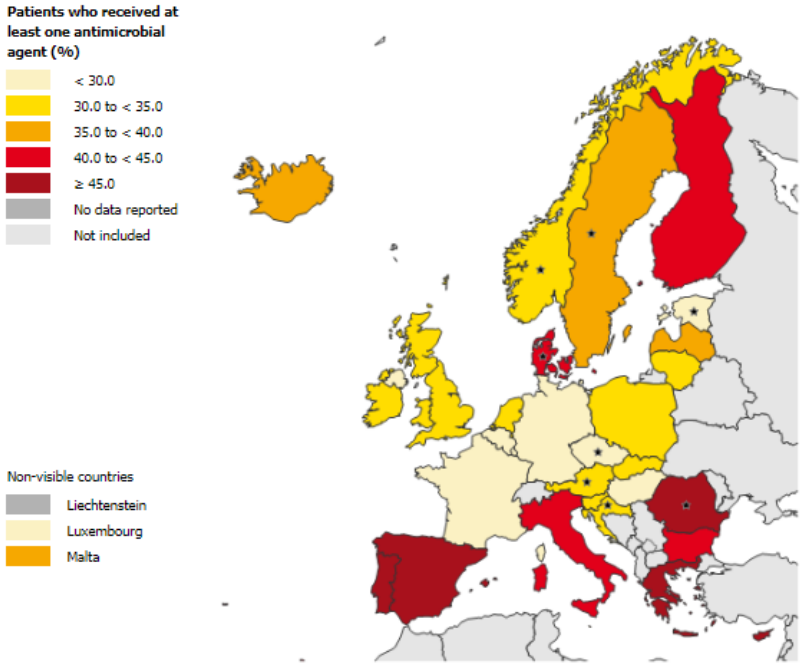

Figure 1.1.2: Prevalence of antimicrobial use in hospitals in acute care hospitals in Europe, ECDC PPS 2011-2012

1.2. "One Health" approach

In accordance with the World Organization for Animal Health (OIE), the **"*One Health*"** term indicates the interconnection between people, animals, plants, and their shared environment. This concept, introduced at the beginning of the 2000s, highlighted the importance of using a collaborative and multisectoral approach, working locally, nationally and globally, in order to achieve optimal global health. This approach was made necessary by the obvious antimicrobial drugs overuse in human and

animal/enviroment setting, and by the conseguent spread of antimicrobial resistance phenomena in human and veterinary medicine (Figure 1.2.1). Medical pioneers of this concept were Rudolf Virchow and William Osler, recognizing the role of a comparative approach to medical investigation (Zinsstag J *et al.* 2012). The link between human and animal medicine was investigated by the veterinarian Calvin Schwabe, who coined the term "One Medicine" and denoted several commonalities in human and animal medicine (Schwabe CW *et al.* 1984).

The majority of antimicrobial compounds is used in both human and animal medicine, including pets, birds, farmed fishes and honey bees (Mcewen SA *et al.* 2002; Van Boeckel TP *et al.* 2015; http://www.fao.org/3/a-i6209e.pdf). Due to their toxicity, few antimicrobial classes are reserved only for humans (e.g., carbapenems) and few others to veterinary use (e.g., flavo-phospholipols) (Mcewen SA *et al.* 2018) (Table 1.2.1). In horticulture, mostly tetracyclines and streptomycin are prescribed for the treatment and prophylaxis of bacterial infections of fruit such as apples and pears (Vidaver AK 2002). In human medicine, the main use of antibiotic drugs is limited to the treatment of clinical infections in individual patients, but prophylactic use can be involved in post-surgery activities and in prevention of diseases such as meningococcal infection (Mcewen SA *et al.* 2018). In veterinary medicine great differences in antimicrobial administrations are noticeable comparing companion animals and food-producing animals; in the former group, drugs are administered to the basis of clinical infection simptoms and in some cases for prophylaxis, such as in post-surgery (Sykes 2013); in the latter, drugs, given through feed or water, are generaly administered to the entire group as prophylaxis (in absence of infection signs). To manage and contain infection events in food-producing factory, antimicrobial drugs are used for metaphylaxis. The term "metaphylaxis" describe therapeutic and/or prophylactic treatment to an entire group with

high risk of infection, usually in the context of mass administration of therapeutic doses (Mcewen SA *et al.* 2002; Van Boeckel TP *et al.* 2015).
In humans medicine, antimicrobial prophylaxis remains uncommon and limited to the management highly diffusible infections such as meningococcal disease. Even in those cases, antimicrobials administration is limited to those with prolonged and close contact (https://assets.publishing.service.gov.uk/government/uploads/system/uploads/attachment_data/file/688835/Public_health_management_of_meningococcal_disease_guidelines.pdf).
Antimicrobial drugs administration for growth promotion purpose represents the most controversial type of treatment. Antibiotics used in such way are administrated to entire groups of animals, usually for prolonged periods of time and at sub-therapeutic doses favoring the selection and spread of resistant bacteria within and between animals, as well as to humans through food or other environmental pathways. The period of exposure is usually greater than two weeks and often is for almost the entire life of an animal (https://www.oie.int/doc/ged/D895.PDF).
Animals are recognized as a relevant reservoir of MDR bacteria and a source of spreading through human contact and food-chain. To minimise a further dissemination of MDR microrganisms in animals and to optimise the terapheutic effects of antimicrobials, several countries, such as Sweden and Australia, wrote guidelines containing principles for good antimicrobial stewardship as follows:

- Antimicrobials should only be administered, dispensed or prescribed when the veterinarian has confirmed that bacterial disease is present, or can reasonably be suspected as the cause of the animal's clinical signs.
- Assessment should be made of the likely target organisms (especially for empirical prescribing).

- Antimicrobial susceptibility testing should be used.
- Antimicrobials should not be used for treatment of self-limiting infections in immunocompetent animals.
- The choice of antimicrobial should be based on consideration of the properties of the drug, the pathogen, the infection site and food safety.
- The antimicrobial with the narrowest spectrum possible should be used to minimise the disruption of normal microflora and limit selection for resistance in bacteria that are not the target of the therapy.
- Antimicrobials should be used for as short a time as needed to successfully treat the patient.
- Topical therapy should be preferred over systemic therapy for treatment of superficial skin infections. Antimicrobial usage is not indicated in many superficial skin infections.
- Off-label use should be avoided when appropriate on-label options are available.
- Use of antimicrobials of high importance that are not registered for veterinary use should be restricted to rare and severe, life-threatening conditions in individual animals. Infection must be confirmed by culture and the antimicrobial therapy must have a reasonable chance of eliminating the infection, and the antimicrobial of high importance should be the most suitable option based on susceptibility testing.
- Antimicrobial therapy should never be used as a substitute for good infection control or optimal medical and surgical practices.
- Perioperative prophylaxis should only be used when necessary. It is not indicated for routine, aseptic surgery of less than 90 minutes duration where no pre-existing inflammation or infection is present, where neither the gastrointestinal or respiratory systems

have been invaded, and aseptic technique is maintained. If required, it should be administered prior to surgery.

- Situations when perioperative antimicrobial therapy is appropriate include: prolonged surgical procedures (> 90 minutes), use of an implant or in-dwelling device, procedures where infection would be catastrophic (e.g. CNS surgery, orthopaedic surgery), when there is an obvious break in asepsis, bowel surgery with a risk of leakage, closed contaminated wounds.
- In these situations, and where surgery is categorised as clean or clean-contaminated, antimicrobial therapy should be discontinued within 24 hours.
- Potential toxicities, inter-current disease (especially kidney or liver disease) and physiological states that could influence drug action should be considered (e.g. pregnant, neonatal and geriatric patients).
- If concurrent administration of different antimicrobials is indicated, the antimicrobials should not have an antagonistic or inhibitory interaction.
- Clients should be educated to ensure compliance and to encourage adoption of good preventive healthcare for their animals (e.g. routine health checks, vaccination, parasite control, exercise and good nutrition)
- Prescription of antimicrobials for a flock or herd must only be provided by a veterinarian with a bona-fide relationship[17] with the individual farm and farm owner.
- A veterinarian prescribing either an in-feed or in-water antimicrobial must have adequate knowledge of the mixing procedures or delivery systems required to ensure homogeneity of distribution of the medication to the animal and provide written instructions on duration of treatment.

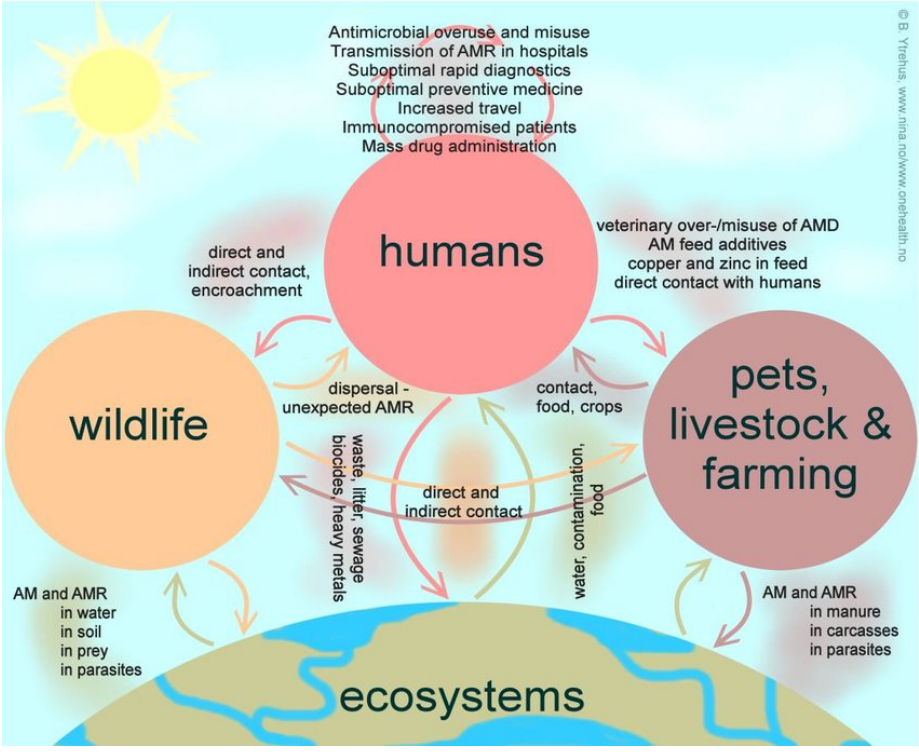

Figure 1.2.1: Representation of the transmission routes of antimicrobial resistance between farm animals, environment, and humans (Report from the Norwegian Scientific Committee for Food and Environment (VKM) 2018)

Table 1.2.1: Classification of antimicrobial classes by importance for human and animal health

Category	Human health (WHO)	Animal health (OIE)
Critically important	Aminoglycoside Ansamycins Carbapenems Cephalosporins (3rd and 4th) Phosphonic acid derivates Glycopeptides Glycylcyclines Lipopeptides Macrolides Monobactams Oxazolidinones Penicillins Polymyxins Quinolones	Aminoglycoside Amphenicols Cephalosporins (3rd and 4th) Macrolides Penicillins Fluoroquinolones Sulphonamides Diaminopyrimidines Tetracyclines
Highly important	Amidinopenicillins Amphenicols Cephalosporins (1st and 2nd) Lincosamides Penicillins Pleuromutilins Pseudomonic acid Streptogamins Sulfonamides Tetracycline	Ansamycins-rifamycins Cephalosporins (1st and 2nd) Ionophores Lincosamides Phosphonic acid Pleuromutilinis Polimyxines First generation quinolones
Important	Aminocyclitols Cyclic polypeptodes Nitrofurantoins Nitroimidazoles	Aminocoumarin Arsenical Bicyclomycin Fusidic acid Orthosomycin Quinoxalines Streptogamins Thiostrepton

1.3 Antibacterial agents

In 1947 Selman Waksman, the discoverer of Streptomycin, published the first definition of antibiotic, defining "as a chemical substance, produced by micro-organisms, which has the capacity to inhibit the growth of and even to destroy bacteria and other micro-organisms" (Kasanah N 2004). Waksman's definition didn't define a class of compound or its natural function, but its application only (Davies J *et al.* 2010). Currently, antibiotics are categorized in bacteriostatic and bactericidal, where the former inhibits bacterial growth, while the latter kills bacteria with a >99.9% efficiency. Considering drug-target interaction, three classes are distinguished: DNA replication inhibitor, protein synthesis inhibitor, and bacterial cell wall turnover inhibitor. Based on mode of action, bacteriostatic drugs can act by inhibiting of ribosome function targeting 30S (tetracycline and aminocyclitol family) and 50S (chloramphenicol and macrolide family) ribosome subunits, while bactericidal antibiotics and quinolones affect DNA replication and repair. Addictionaly, as bacterial wall synthesis inhibitors, β-lactams bind to penicillin binding protein, and glycopeptides interact with peptidoglycan building blocks (Kohanski MA *et al.* 2007). Table 1.3.1 shows a detailed list of the different antibiotics avaible with its relative targets (Wong WR *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	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, sulfapyridine, sulfamethoxazole, sulfadiazine, sulfamerazine	Competitive inhibitor for DHPS 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

The antibiotic resistance can be intrinsic of the species or secondarily "acquired" by the microorganism. Intrinsic resistance refers to the natural presence of genes in bacterial genome that, when expressed, could generate a resistance phenotype, such as intrinsic AmpC β-lactamase expressed by some Gram-negative bacteria and MDR efflux pumps found in many other bacteria. (Table 1.4.1) (Davies J *et al.* 2010). In contrast, acquired resistance manifests itself following mutations in genes targeted by the antibiotic or the transfer of determinant resistant genes through

plasmids, bacteriophages, transposons and/or other mobile genetic materials (Figure 1.4.1).
(http://amrls.cvm.msu.edu/microbiology/molecular-basis-for-antimicrobial-resistance/acquired-resistance/acquisition-of-antimicrobial-resistance-via-horizontal-gene-transfer).

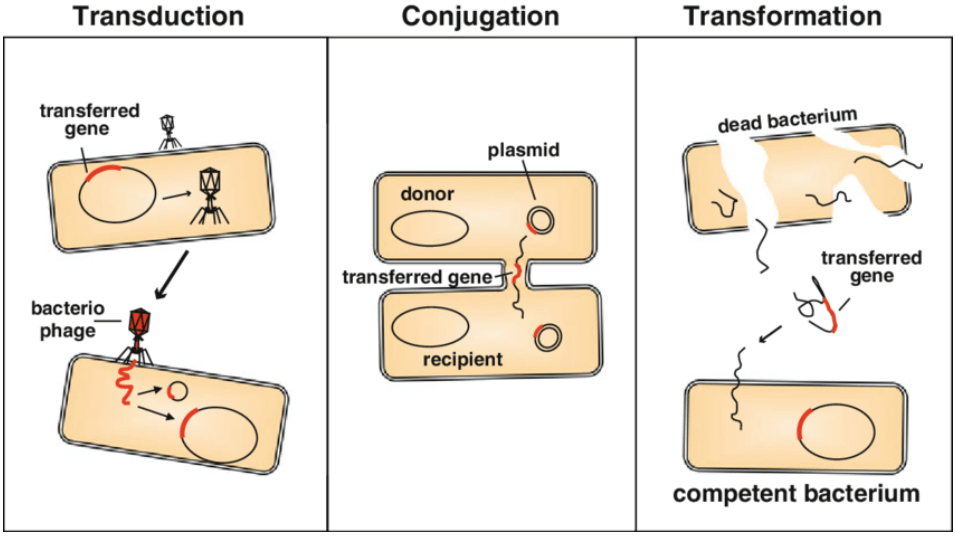

Figure 1.4.1: Mechanisms of antibiotic resistance acquisition

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, caphalosporins, penems, monobactams	Peptidoglycan biosynthesis	Hydrolisis, efflux, altered target
Aminoglycosides	Gentamicin, streptomycin, spectinomycin	Translation	Phosphorylation, acetylation, efflux, altered target
Glycopeptides	Vancomycin, teicoplanin	Peptidoglycan biosynthesis	Reprogramming peptidoglycan biosynthesis
Tetracyclines	Minocycline, tigecycline	Translation	Monooxygenation, efflux, altered target
Macrolides	Erithromycin, azithromicin	Translation	Hydrolisis, glycosylation, phosphorilation, efflux, altered target
Lincosamides	Clindamycin	Translation	Nucleotidylation, efflux, altered target
Streptogramins	Synercid	Translation	C-O lyase, acetylation, 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_1 metabolism	Efflux, altered target

Sulfonamides	Sulfamethoxazole	C_1 metabolism	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

Generally, resistance material can be exchanged by transduction, through which a bacteriophage infectes the bacterial cell, and the inserted DNA harbors resistant genes that will be incorporated into the bacterial DNA (Lerminiaux NA *et al.* 2019). Other ways are by conjugation, via plasmids and conjugative transposons, and by transformation, incorporating chromosomal DNA, plasmids or other DNA pieces from dying organisms into the host chromosome.

Plasmids are extra self-replicating genetic replicons that usually harbor genes able to confer specific property to the host cell. Currently, plasmids exchange is considered one of the most efficient resistant mechanisms implicated in MDR strains dissemination. Plasmids may pass through horizontal gene transfer or conjugation, mostly due to their ability to carry more than one resistant gene and to coexist with other plasmids in the same cell. Moreover, plasmids exchange can occur at both intragenus and intergenus levels, also involving distant bacteria belonging to different genera (Alekshun MN *et al.* 2007).
Nevertheless, horizontal gene transfer is the result of an evolutionary process in which plasmids peculiarities and selection pressure of antibiotic use have played a considerable role. Definitely, antibiotic overuse and disposal placed the bacteria in highly hostile environment and, acting as

selective pressure, favored horizontal gene transfer of resistant genes (Davies J *et al.* 2010).

Transposons are mobile genetic elements that can move from one location to another in the genome (Munoz-Lopez M *et al.* 2010) and can exist on plasmids, or integrate into other transposons or host chromosome. Several plasmid qualities are contained in conjugative transposons and can facilitate the transfer of endogenous plasmids from one organism to another (Alekshun MN *et al.* 2007).
Integrons are "genetic elements that contain a site-specific recombination system able to integrate, express and exchange specific DNA elements, called gene cassettes" (Hall RM *et al.* 1995). Characterized in 1987 by Stokes and Hall, integrons are unusual gene acquisition elements due to their lack of functions for self-mobility. They explicate their mobile activity in association with a variety of transfer and insertion functions (Davies J*et al.* 2010). In particular, the gene cassettes present in integrons can stably integrate into regions of DNA, where they deliver a single exchange containing multiple new genes mostly related to antimicrobial resistance (Alekshun MN *et al.* 2007) (Figure 1.4.2).

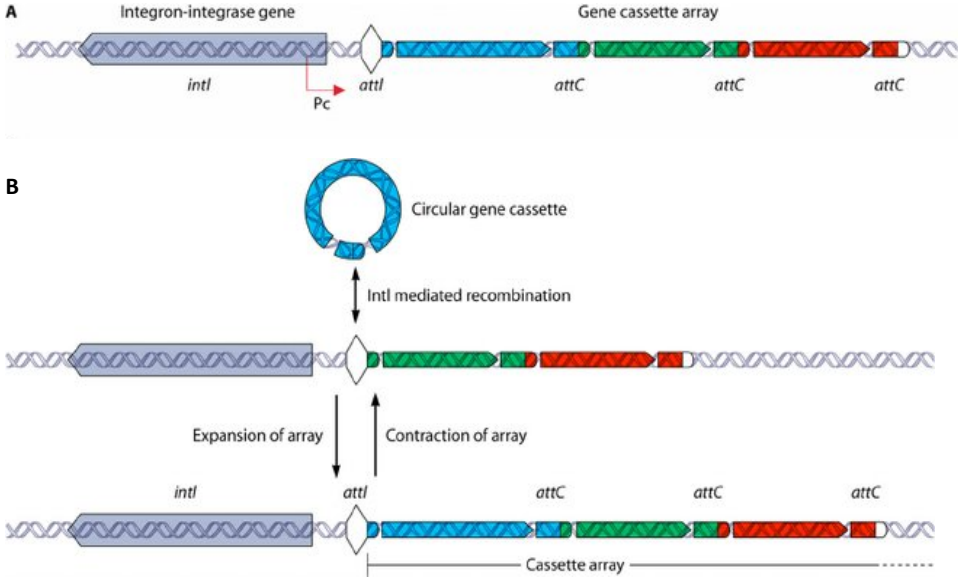

Figure 1.4.2: Integron structure. The basic integron platform consists of the following: intI, a gene for the integron integrase; Pc, an integron-carried promoter; attI, the integron-associated recombination site; and gene cassettes, sequentially inserted into an array via recombination between attI and the cassette associated- recombination sites, attC. A: Gene cassettes normally contain a single open reading frame (ORF) (arrow) expressed from the Pc promoter. In some integrons, Pc lies between intI and attI. B: Acquisition of gene cassettes. Integrons acquire new gene cassettes by recombination between the attC of a circular cassette and the attI site of the integron. Gillings MR 2014).

1.5. β-Lactams: Mechanisms of Action and Resistance

Discovered in 1928 by Alexander Fleming and introduced as clinical treatment in 1940s, β-lactams represent one of the oldest and most used treatment for bacterial infections. They act interfering with the bacterial cell wall synthesis, an essential structure that surround and protect cytoplasmic membrane from osmotic structure. It consists in a matrix structure, the polymer peptidoglycan (PG), obtained by crosslinking glycans chains and

peptides. β-Lactams inhibit PG synthesis, inactivating penicillin-binding proteins (PBPs), that are encoded by bacterial cell and have different roles in the cell wall assembly. Therefore, the antimicrobial activity of β-lactams is expressed in the loss of cell wall integrity, followed by cell lysis (Cho H *et al*. 2014). As peculiar feature, β-lactam drugs have a highly strained and reactive cyclic amide, the β-lactams ring system, on which is based the chemical classification of five relevant groups: penam, cephems, carbapenem and monobactem ring structure.

Penams

Penams are a large group of β-lactams that include penicillin and his derivates. Penams possess a basic bicyclic structure, the 6-aminopenicillanic acid (6-APA), composed of an enclosed dipeptide, obtained by L-cystein and D-valine condensation; this results in the β-lactam ring and in the thiazolidinic ring formation (Long AJ *et al.*, 2005). Due to the reactive nature of the β-lactam ring system, many degradative process alter penams susceptible. In acid environments and room

temperature, the β-lactam ring undergoes reconfiguration steps: protonation of the β-lactam nitrogen, followed by the nucleophilic attack of the remaining lateral chain carbonyl. The intermediate oxazolin ring will originate a new imidazol and form penillic acid (Long AJ *et al.*, 2005). Therefore, these compouds have to be protected from acid enviroment such as in stomach, to be administrated orally. Moreover, many bacteria have developed the ability to synthesize enzymes, known as penicillinases that chemically inactivate β-lactams. Among penicillinases, β-lactamases are the most prevalent, able to disrupt the β-lactam ring and, thus, 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 cause a decrease in the effectiveness of β-lactams, and then result less potent than the parent molecules (Fernandes R *et al.* 2013). Nowadays other compounds are available such as clavulanic acid, tazobactam and sulbactams that bind to the β-lactamases irreversibly, hence inactivating them.

Cephems

Cephems is a sub-group of β-lactam drugs that includes cephalosporins, largely used as anti-infective agents in both human and veterinary medicine since 1970s. Their spread in clinical practice was due to their well tollerance

and their development paralleled that of the penicillins. Several classifications have been proposed: chemical, biological, microbiological, pharmacokinetic and immunological (Fernandes R *et al.* 2013).
Cephalosporins are traditionally divided into first-, second-, third-, fourth- and fifth-generation, in accordance with their antibacterial activity, stability and side-effects (Table 1.5.1) (Fernandes R *et al.* 2013).

Table 1.5.1: Major group of cephalosporins

First-generation	Second-generation	Third-generation	Fourth-generation	Fifth-generation
Cephalothin	Cefamadole	Cefotaxime	Cefepime	Ceftobiprole
Cepharipin	Cefuroxime	Ceftizoxime	Cefpirome	Ceftaroline
Cefazolin	Cefonicid	Ceftriaxone		
Cephalexin[a]	Ceforanid	Ceftazidime		
Cephadrine[a]	Cefoxitin[b]	Cefoperazone		
Cefadroxil[a]	Cefmetazole[b]	Cefixime[a]		
	Cefminox[b]	Ceftibuten[a]		
	Cefotetan[b]	Cefdinir[a]		

[a]Oral cephalosporins. [b]Besides being cephamycins, they are usually included in the microbiological classification as second-generation cephems

First-generation cephalosporins act efficiently against Gram-positive cocci, except methicillin-resistant staphylococci and enterococci, and moderately against some Gram-negative rods, such as *Escherichia coli* and *Klebsiella* spp. Due to their bacterial effect against aerobes but not anaerobes, first-generation cephalosporins are used as alternatives to penicillin in early infection treatment. Among such drugs, cephalexin, cephradine and cefadroxil are absorbed from the gut at different extent and can be used to treat both urinary and respiratory tract infections, whereas other first generation cephalosporins must be injected to obtain adequate levels in blood and tissues (Fernandes R *et al.* 2013). First-generation cephalosporins are unable to penetrate the central nervous system and, thus, not used as drugs of first choice for any nervous system infection (Fung-Tomc JC *et al.* 1995). First-generation cephalosporins own narrowed or limited activity when compared with other-generation broader spectrum cephalosporins.

The second-generation cephalosporins are a heterogeneous group, active against organisms covered by first-generation drugs and additionaly against Gram-negative rods, such as *Proteus* spp., but excluding *Pseudomonas aeruginosa* (Bennett PN *et al.* 2009). Some second-generation cephalosporins, for instance cefaclor and cefuroxime, are orally used to treat sinusitis and otitis caused by *Haemophilus influenzae*, including β-lactamase-producing strains. Moreover cephamycins, like cefoxitin and cefotetan, are clinically considered as second-generation cephlosporins thank to their activity against B*acteroides. fragilis* and, thus, use in mixed anaerobic infections, including peritonitis or pelvic inflammatory disease (Fung-Tomc JC *et al.* 1995).

The main features of third-generation cephalosporins are the enhanced activity against Gram-negative rods (Paladino JA *et al.* 2008) and the decreased activity against Gram-positive cocci (Bennett PN *et al.* 2009). Third-generation drugs, like ceftazidime or cefoperazone, are very useful in the management of hospital-acquired Gram-negative bacteremia thank to their activity against *P. aeruginosa*. Moreover, these drugs combined with aminoglycoside are a good terapheutic options in immunocompromised patients treatment. Additionaly, several third-generation drugs can reach the central nervous system and mantain in the spinal fluid a proper concentrations to treat meningitis caused by Gram-negative rods. Intravenous administration of cefotaxime, ceftriaxone or ceftizoxime is normally the clinical practice for management of Gram-negative bacteria sepsis and meningitis (O'Neill E *et al.* 2006).

The group of fourth-generation cephalosporin consists in extended spectrum agents, such as cefepime and cefpirome, able to bind to PBPs of both Gram-negative and Gram-positive bacteria. This group of cephalosporins is derived from third-generation cephems after adding a C-

3' quaternary ammonium group in position 3 of the cephem nucleus (Garau J *et al.* 1997). This modification gives the properties of penetrating the outer membrane of Gram-negative bacteria rapidly and having poor affinity for β-lactamases located in the periplasmic space (Garau J *et al.* 1997). Cefepime is widely used in treatment of moderate/severe pneumonia, caused by *Streptococcus pneumoniae*, *P. aeruginosa*, *K. pneumoniae*, or *Enterobacter* spp., of uncomplicated/complicated urinary tract infections (including pyelonephritis) caused by *E. coli* or *K. pneumoniae,* and of skin structure infections caused by methicillin-susceptible *S. aureus*.

The small group of fifth-generation cephalosporins includes agents such as ceftobiprole, ceftaroline and ceftolozane. This compouds were developed in laboratory to specifically target against resistant strains of bacteria (Fernandes R *et al.* 2013). Ceftaroline is active against methicillin-resistant *S. aureus* (MRSA) and Gram-positive bacteria, while mantaining the broad-spectrum activity against Gram-negative bacteria. Ceftolozane were developed to treat of infections caused by Gram-negative bacteria, become resistant to conventional antibiotics (Long AJ *et al.* 2014). Ceftolozane inhibits cell wall biosynthesis binding to PBPs and, in association with the β-lactamases inhibitor tazobactam, has a notable activity in treatment against MDR *P. aeruginosa*, urinary tract and complcated inta-abdominal infections.

Carbapenems

Carbapenems are a class of β-lactam antibiotic that derive from thienamycin and have the greatest potency and activity against Gram-positive and Gram-negative bacteria (Papp-Wallace KM *et al.* 2011).
Imipenem, the first drug of this type, posseses good activity against many Gram-negative rods, Gram-positive organisms and anaerobes. For this

reason, it is indicated for infections caused by microorganisms resistant to other drugs. Imipenem penetrates the body tissues and fluids well, including cerebrospinal fluid (Singh GS 2004). Moreover, it is inactivated by dihydropeptidases in renal tubules and, thus, it is administered together with a peptidase inhibitor such as cilastatin (Bennett PN *et al.* 2009).
Meropenem is similar to imipenem in terms of pharmacology and antimicrobial spectrum of activity; however, it is not inactivated by dipeptidases (Bennett PN *et al.* 2009). Meropenem has a broad spectrum of activity against Gram-positive and Gram-negative pathogens.
Ertapenem is was developed to address the short half-life of imipenem and meropenem (Zhanel GG *et al.* 2005) but it posses a more limited activity spectrum if compared with imipenem and meropenem. In particular, ertapenem is weakly active against *P. aeruginosa* and less potent against *A. baumannii* (Papp-Wallace KM *et al.* 2011).

Monobactam ring

Monobactams are synthetic and monocyclic β-lactam compounds active against Gram-negative rods, but not against Gram-positive bacteria or anaerobes (Fernandes R *et al.* 2013). The first monobactam, become avaible in 1986, was aztreonam, primarily used against Gram-negative bacteria infections as those provided by *P. aeruginosa*.

New combination strategies

Currently, there is an urgent need to introduce new therapeutic options to fight the increasing number of MDR/XDR pathogens. However, only few β-lactamase inhibitors and new antibiotics have been introduced to the market over the past thirty years. First-generation β-lactamase inhibitors, as clavulanic acid, sulbactam and tazobactam, are β-lactam derivatives that inactivate class A and some class C serine β-lactamases. Newer β-lactamase inhibitors, such as avibactam and vaborbactam, are based on

non-β-lactam structures and their inhibition activity is extended to KPC. Despite these advances, several class D and virtually all the most important class B β-lactamases are resistant to existing inhibitors.

- **Ceftolozane-tazobactam (Zerbaxa)**
  Zerbaxa was approved by U.S. Food & Drug Administration (FDA) in 2014 for the treatment of complicated intra-abdominal infection (cIAI) and complicated urinary tract infection (cUTI). This combination has a strong activity against ESBL-producer and AmpC-hyperproducing Gram-negative, such as *Enterobacterales* and *P. aeruginosa* (Tehrani KHME *et al.* 2018). However, the synergistic activity is compromised by pathogens expressing highly active carbapenemases and/or MβLs.

- **Ceftazidime-avibactam (Avycaz)**
  Approved by FDA in 2015 for the treatment of cIAI and cUTI, this combination owns high rates of activity against non-MβL carbapenemases, such as KPC- and OXA-48-like. The presence of MβL carbapenemases precludes the use of ceftazidime-avibactam alone. In case of co-presence of MβLs plus other carbapenemase, aztreonam-avibactam rather than ceftazidime-avibactam results more effective therapeutically, further extending its efficacy to CPE harbouring KPC or OXA-48-like carbapenemases. The administration of ceftazidime-avibactam in combination with aztreonam separately may be useful for treating MβL-CPE infections (Chew KL *et al.* 2018).

- **Meropenem-vaborbactam (Vabomere)**
  Approved by FDA in 2017 for the treatment of cUTI and acute pyelonephritis, it owns a potent activity against KPC-producing *Enterobacterales*, but it has no effect on bacterial strains expressing MβL-

or OXA-48 type enzymes. Meropenem-vaborbactam results well tolerated by patients (Tehrani KHME *et al.* 2018).

- **Imipenem-relebactam**
  Relebactam is a novel β-lactamase inhibitor that in combination with imipenem/cilastatin is able to treat patients with antimicrobial-resistant Gram-negative infections. In particular, this combination, approved in 2019 by FDA, is administered for the treatment of cUTI and cIAI bacterial infections where limited or no alternative options are available. Imipenem-relebactam inhibits ESBL- and AmpC-producing strains, and KPC-producing *Enterobacterales.*. Nevertheless, it is ineffective on MβL- or OXA-48-producing bacterial strains (Lob SH *et al.* 2019).

- **Cefiderocol**
  Cefiderocol is an injectable siderophore cephalosporin that inhibits Gram-negative bacterial cell wall synthesis by binding to penicillin binding proteins; the ability to enter the bacterial periplasmic space as a result of siderophore-like property, enhancing stability to β-lactamases, is peculiar to Cefiderocol. Moreover, it is chemically similar to ceftazidime and cefepime, but with high stability to a variety of β-lactamases, including AmpC and ESβLs. Cefiderocol acts against aerobic Gram-negative bacilli and against a variety of Ambler class A, B, C and D β-lactamases. Cefiderocol results also more potent than both ceftazidime-avibactam and meropenem versus *Acinetobacter baumannii*, including meropenem non-susceptible and MDR isolates. Cefiderocol's activity against meropenem-non-susceptible and KPC-producing *Enterobacterales* is comparable or superior to ceftazidime-avibactam, and even more effective than both ceftazidime-avibactam and meropenem against all resistance phenotypes of *P. aeruginosa* (Zhanel GG *et al.* 2019).

Michael Kurilla, the director of the Division of Clinical Innovation at the National Institutes of Health (NIH), comments on the significance of the findings in the context of the antibiotic resistance crisis: "with the specter of antibiotic resistance threatening to turn back healthcare to the pre-antibiotic era, the ability to more judiciously use combinations of existing antibiotics that singly are losing potency is welcome".

Antibiotic	Enterobacteriaceae (e.g. E. coli, Klebsiella spp.)					*Pseudomonas* spp.		*Acinetobacter* spp.
	ESBL	AmpC	KPC	OXA-48	NDM	Efflux	AmpC	
Ceftolozane-tazobactam								
Ceftazidime-avibactam								
Meropenem-vaborbactam								
Imipenem-relebactam								
Aztreonam-avibactam								
Cefiderocol								

Figure 1.5.1: combinations/new antibiotic activity against the bacteria resistant to the current widely used antibiotics (green=active, amber=partial/unreliable activity, red=inactive)

β-Lactams use and abuse result to be the prominent cause of worldwide resistance to β-lactam antibiotics among Gram-negative bacteria, mainly as *Enterobacterales* (Shaikh S *et al.* 2015). The resistance to β-lactams in Gram-negatives is represented by different mechanisms (Figure 1.5.2):

1. reduction of antimicrobial quantities entering the bacterial cell due to the decrease of permeability through porin mutation or increase exit through overexpression of transmembrane efflux pumps;
2. antibiotic target alteration due to loss of affinity;
3. development of alternative metabolic pathway involving precursors;
4. expression of hydrolytic enzymes that partially/totally destroy the antibiotic compounds.

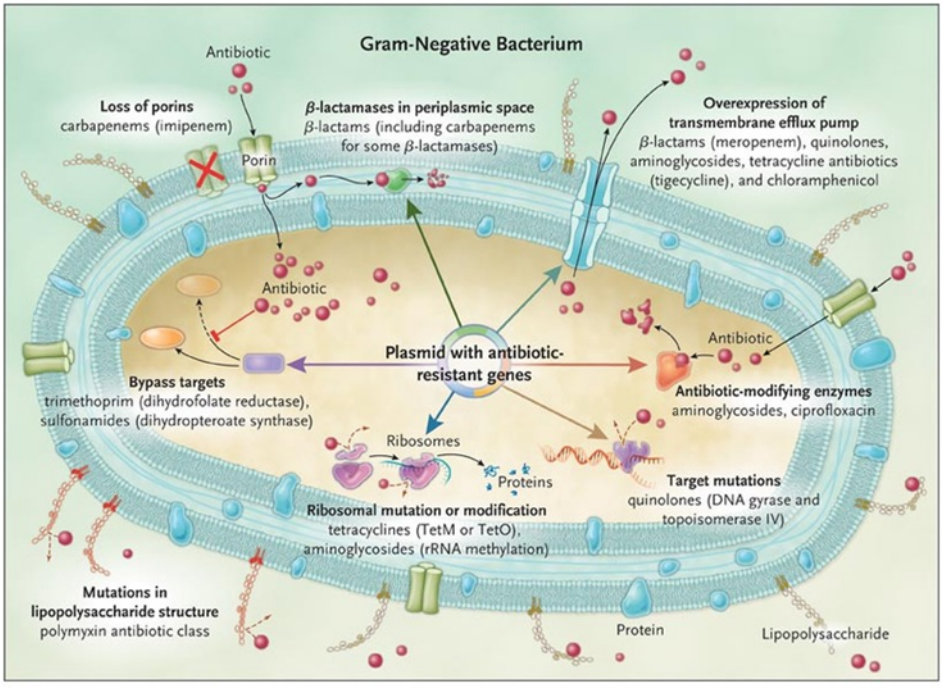

Figure 1.5.2: β-lactamases resistance mechanisms in Gram-negative bacteria (Peleg AY *et al.* 2010)

The production of enzymes such as β-lactamases is one of the most common ways of resistance in Gram-negative bacteria such as *Enterobacterales*. During years several classifications were proposed. The first were given by Ambler in 1980 aminoacids omology, whereas Bush and Jacoby improved a functional classification in 1995.
The current 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 MN *et al.* 2007) (Table 1.5.2). β-lactamase enzymes confer resistance against β-lactams by attack hydrolysis of the β-lactam ring, essential for β-lactams antimicrobial activity. The first plasmid-mediated β-lactamase reported in Gram-negative was TEM-1, isolated in 1965 from a blood culture of a Greek patient named Temoneira (hence the designation TEM). The enzyme, here reported in a *E. coli* strain, spreaded quickly into other bacterial species, due to a plasmid and transposon mediation. Rapid diffusion and 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.*

Table 1.5.2: Ambler classification, substrates, inhibitors and representative enzymes of β-lactamases

Bush-Jacoby-Medeiros-classification	**Ambler classification**	**Distinctive substrate**	**Enzyme(s)**
1	C	Cephalosporins	AmpC
2b	A	Penicillins, early cephalosporins	TEM-1, TEM-2, TEM-13, SHV-1
2be	A	Extended-spectrum Cephalosporins and Aztreonam	TEM-3, SHV-2, PER, VEB, CTX-M-15
2d	D	Cloxacillin	OXA-1, OXA-10
2de	D	Extended-spectrum Cephalosporins	OXA-11, OXA-15
2df	D	Carbapenems	OXA-23, OXA-48
2f	A	Carbapenems	KPC, IMI, SME
3a	B	Carbapenems	MβL

1.6. Extended-Spectrum β-Lactamases (ESβLs)

The persistent exposure of bacterial strains to a multitude of β-lactams has induced dynamic and continuous production and mutation of β-lactamases enztmes in these bacteria, expanding their activity even against the newly developed β-lactam antibiotics. These enzymes are known as extended-spectrum β-lactamases (ESβLs), capable of conferring bacterial resistance to the penicillins, narrow and extended-spectrum cephalosporins and aztreonam by hydrolysis; they are inhibited by β-lactamase inhibitors such as clavulanic acid, sulbactam and tazobactam (Bush K *et al.* 2011). They were discovered in the early 1980s in Europe and since then, the prevalence of ESβLs is increasing worldwide in different variants. The most common ESβLs are SHV-, TEM-, CTX-M and OXA- (Table 1.6.1). Interestingly, SHVs are more prevalent in Europe, TEMs are dominantly present in the USA while the CTX-Ms are being increasingly detected worldwide (Figure 1.6.1) and divided in six lineages (or groups) (Paterson DL *et al.* 2005).

Table 1.6.1: Most common ESβLs

Types	Ambler class	Activity spectrum	Variants	Examples
CTX-M	A	Cefotaxime, cefriaxone	140	CTX-M-1, CTX-M-15
TEM	A	Expanded-spectrum Cephalosporins	238	TEM-3
SHV	A	Broad-spectrum Penicillins, tigecyclin, piperacillin	171	SHV-12
OXA	D	Oxacillin, cloxacillin	438	OXA-10, OXA-17, OXA-18

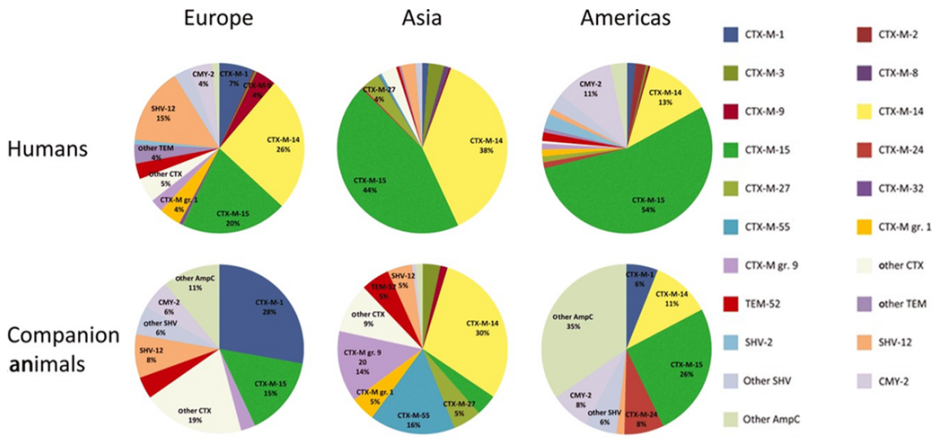

Figure 1.6.1: global epidemiology of the most common ESβLs in human and companion animals.

Class C ESβL includes the chromosomal-encoded AmpC β-lactamases that 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, also known as metallo- β-lactamases or MβL, own a zinc-active site to activate water molecule and catalyze zinc addition to the β-lactam ring. The MβLs are known to major contribute to carbapenem antibiotic resistance worldwide spread. The most common MβL genes are:
- VIM (Verona integron-encoded metallo-β-lactamase) first detected in *P. aeruginosa*, although it is widely disseminated in *Klebsiella* spp, and *E. coli*;

- IMP (active on imipenem) detected in *P. aeruginosa* and in *Enterobacterales* (Watanabe M *et al.* 1991);
- NDM (New Delhi metallo-β-lactamase), recently detected, found in the chromosome of *A. baumannii*, and specially spread in *Enterobacterales* (mostly *Klebsiella* spp. and *E. coli*) (Cornaglia G *et al.* 2011).

1.7. Health care-associated (HAIs) and community-acquired infections (CAIs)

Infections in human medicine are generally classified in healthcare associated infections (HAI) and Community Acquired Infections (CAI). In accordance with the WHO, HAIs are defined as "infections acquired in a hospital or in other health-care facility, not presented/incubated at the time of admission" (http://www.who.int/en/). They also include infections acquired by patients in the hospital or facility but appearing after discharge, and occupational infections among staff (https://www.cdc.gov/hai/surveillance/index.html.). In details, HAIs are infections developed 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).

HAIs contribute to a high rate of morbidity and mortality worldwide. In fact, 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 due to antibiotic resistant HAIs. The WHO Organization has identified many factors that contribute to HAIs, some of which related to the patient characteristics and state such as age, immune-suppression and duration of hospitalization; others depending on inadequate facility settings and limited sources as scarce hygienic conditions, poor infrastructure, understaffing and insufficient application of basic infection control measures. According to the ECDC "Point Prevalence study 2011-2012", the most common HAIs are (Figure 1.7.1):

1. ventilator-associated pneumonia;
2. catheter-associated urinary tract infections;
3. surgical site infections;
4. central line-associated bloodstream infections.

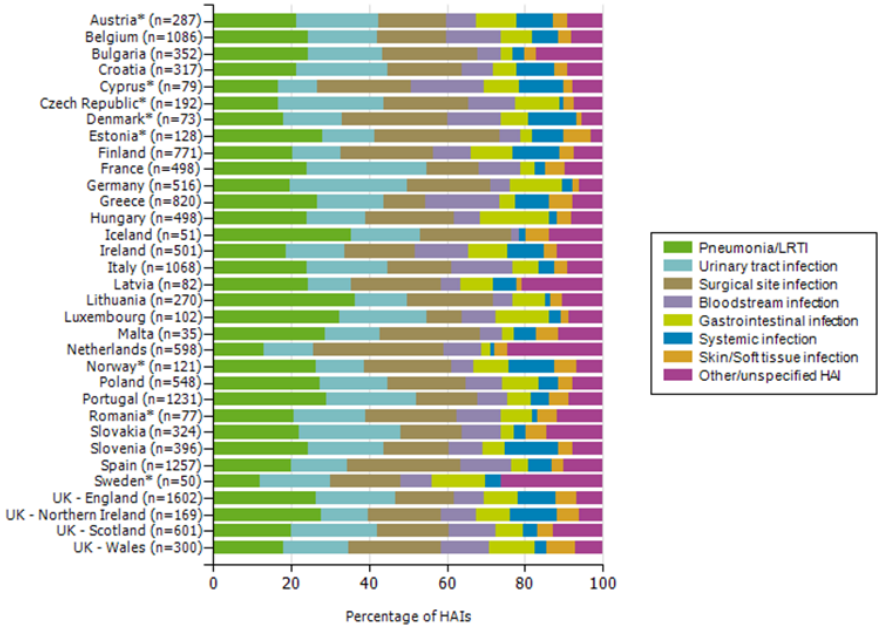

Figure 1.7.1: Distribution of HAI types in acute care hospitals by country (ECDC PPS 2011-2012)

As cause of HAIs protozoans, fungi, viruses and mycobacteria may be involved, bacteria remain the main causative agent of HAI accounting for 90% of the cases (Bereket W *et al.* 2012). In addition to the Gram negative and positive bacteria, commensal species are also able to become pathogenic in immunosuppressed individuals (Table. 1.7.1).

Table 1.7.1: 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
Carbapenem-resistant *Acinetobacter baumannii*	Bacteremia and wound infections	Skin contact, infected wounds, intravascular and urinary catheters
Clostridium difficile	Colitis	Person to person and contact with hospital settings surfaces

On the other hand, community-acquired infections (CAIs) are infections contracted outside of a healthcare setting or infections present on admission. The most commons CAIs are:

- community acquired respiratory infections, commonly involving *Haemophilus influenzae* or *Streptococcus pneumoniae* strains;
- community acquired urinary tract infections, involving ESβL-producing *Enterobacterales*;
- community acquired bloodstream infections, involving methicillin-resistant *Staphylococcus aureus* (MRSA) and ESβL /metallo-β-lactamase-producing *Enterobacteriales* (Laupland KB *et al.* 2014).

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Chapter 2

Interplay among IncA and *bla*KPC carrying plasmids in *Citrobacter freundii*

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2. Abstract

The rapid identification of patients colonized or infected with carbapenemase-producing *Enterobacterales* (CPE) is recognized as a basic tool to control and prevent the global spread of multi-drug resistant (MDR) pathogens. In Italy few studies have reported the isolation of carbapenemase-producing *Citrobacter freundii* strains. The aim of the study was to characterize two isolates of *C. freundii* supected for the carbapenemase-production.

In the period February-March 2016, two *C. freundii* strains with reduced susceptibility to carbapenems were isolated from a rectal swab and an urine specimen of two patients, only one been hospitalized. Species identification and antimicrobial susceptibility testing were carried out by Vitek2 (bioMérieux, France) and Microscan Autoscan4 (Beckman Coulter) semi-automated systems. Carbapenems MIC values were confirmed by E-test. The isolates were analyzed by phenotypic tests, PCR, sequencing and PFGE (XbaI). Conjugation assay, extraction, replicon typing and sequencing of plasmids were also performed.

The isolates were resistant to third generation cephalosporins and carbapenems, being still susceptible to gentamycin, colistin, tigecycline and trimethoprim-sulfamethoxazole. The synergy test gave positive results for the meropenem disk plus boronic acid. PCR and sequencing revealed the presence of two different KPC variants: *bla*KPC-2 gene harbored on an IncN (conjugative) plasmid; and *bla*KPC-3 on an IncX3 plasmid. The KPC-2-producing *C. freundii* isolates presented also a co-resident helper plasmid of IncA/C. The two isolates resulted clonally unrelated by PFGE and belonged to different sequence types.

Our findings show that different clones of KPC *C. freundii* (KPC-Cf) are emerging in Italy. Risk factors for the KPC-Cf carriage of the hospitalized patient could be the gastroenterology prolonged stay under imipenem

therapy due to the rapid identification of KPC-Cf avoid the spread of this unusual type of CPE among hospitalized patients.

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2. Introduction

Citrobacter spp. are a group of anaerobic and facultative Gram-negative bacilli that belong to the *Enterobacterales* family. Citrobacter genus includes 11 species: *C. freundii, C. koseri, C. amalomaticus, C. farmeri, C. youngae, C. braakii, C. werkmanii, C. sedlakii, C. rodentium, C. gillenii, C. murliniae*. For years have been considered as enviromental contaminants and colonizers with low virulence (Liu LH *et al.* 2017). Recently, strains such as *C. freundii* and *C. koseri* are recognized as significant pathogenes reported in infection events involving urinary tract, liver, biliar tract, intestine, respiratory tract, wounds, bloodstreams and meningitis (Pepperell C *et al.* 2002). *Citrobacter* infection can occur as sporadic case or nosocomial dissemination in patient with underlying diseases or immunocompromised status (Samonis G *et al.* 2009; Ferranti M *et al.* 2018); in particular, outbreaks events are reported in intensive unit care wards, resulted in colonization, surgical wound infection and then menigitis (Nada T *et al.* 2004). Among hospitalized patients, *Citrobacter* infection mortality reaches the 6.8%, due in part to ineffective empirical antibiotic therapy.

Citrobacter strains can carry Amp-C ß-lactamases (chromosomal and inducible), broad-spectrum β-lactamases, ESßLs, plasmid-mediated quinolone/aminoglycoside resistance and even carbapenemases such as KPC (Kanamori H *et al.* 2011; Zhang R *et al.* 2008). In cases of multidrug-resistant *C. freundii* infection there is an urgent need to explore new treatment strategies, due to the remarkable toxicity of some sensitive drugs expecially used in newborns treatment (Chen D *et al.* 2019).

2. Review of the literature

2.1. Plasmids and the spread of resistance

Plasmids are considered as a key factor in the evolution and dissemination of antibiotic resistance among the most worrisome clinical pathogens. Plasmids are extra chromosomal, self-replicating genetic mobile elements that exist in a double stranded circular DNA form. They own autonomous replication system and mechanisms that control the copy number and their proper propagation during cell division. Many plasmids are able to kill daughter cells that didn't successfully inherit the plasmid through toxin-antitoxin systems. Additionally, this system furthers plasmid maintenance in the bacterial host cell, even if the plasmid benefits from the bacterial cell and there's an antibiotic selective pressure. Nevertheless, the use of antibiotics acts positively in the propagation and the resistant phenotype of the bacterial population that harbor the relative resistance genes plasmids (Caratolli A 2009).

In 1969, mass outbreaks of dysentery due to antibiotic resistance bacteria occured in Central America leaving behind thousands of dead people. Initially, the bacteria responsible for the outbreak were eradicated using antibiotics, but they re-emerged resistant. The patients resulted infected with resistant bacteria, even though these patients were never administered antibiotics. For these reasons, researchers spent many resources in studying plasmids using molecular tools and they concluded that plasmids 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 H 2015). The main issue with plasmids is

the ability to surpass species and genus barriers due to resistant genes horizontal transfer; plasmid conjugation can occur between bacteria of the same species and an increased rate of plasmid transfer has been observed in heterogeneous communities (Svara F and Rankin DJ 2001).

2.2. Plasmid classification and typing

Plasmids have mobile elements shared between different species and, thus, classification and identification of plasmids resulte problematic.
However, the current classification is based on constant genetic traits regarding the plasmid maintenance and replication control (Carattoli A 2009). These regions are called replicons; they encode functions that activate and control replication (Carattoli A *et al.* 2014).
In 1971, Hedges and Datta proposed the first plasmid classification scheme based on the plasmid incompatibility phenomenom. This event represents the stability of plasmids during conjugation, and is defined as the inability of two co-resident plasmids to be propagated together stably in the same cell in the absence of external selection (Novick RP *et al.* 1976). It is a manifestation of relatedness between plasmids harboring common replication controls.
In 1988, Couturier and colleagues proposed another classification theme based on genetic plasmid typing using Southern blot hybridization and clonal replicons as probes. This approach successfully classified both conjugative and non-conjugative plasmids but resulted low in specificity due to cross hybridization among related replicons (Carattoli A 2009).
Since 2005, has been avaible the current scheme based on replicon typing obtained throught PCR-based replicon-typing (PBRT); it is a multiplex PCR targeting replicons of the major plasmid families found in *Enterobacterales*. Thanks also to the advances in whole genome

sequencing techniques, PBRT is able to detect 25 different replicons, divided in major incompatibility (Inc) groups. However, being a multiplex PCR method, this method cannot cover any 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, phylogroup, etc., could be a good strategy 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 A *et al.* 2014).

The PBRT method is able to detect the major classes of Inc groups in *Enterobacterales*: HI2, HI1, I1α I1ϒ, I2, L, M, K, N, N2, F, FII, FIIS, FIIk, X1, X2, X3, X4, W, Y, P1α, A/C, T, U, and B/O (Figure 2.2.1) (Table 2.2.1). 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 A 2009).

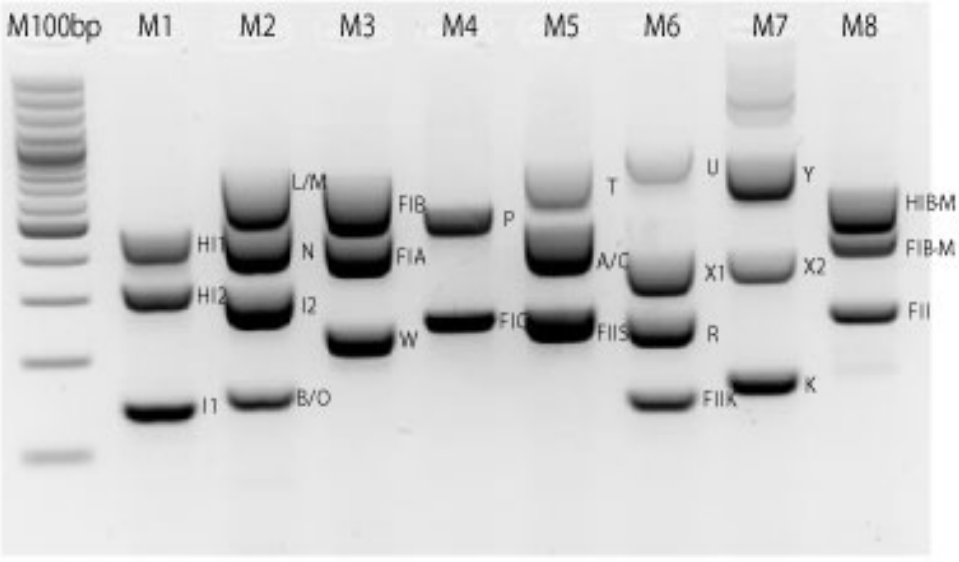

Figure 2.2.1: PBRT kit based on replicons of the major plasmid incompatibility groups in *Enterobacterales* (http://www.diatheva.com)

Table 2.2.1: list of replicons detected by PBRT kit

Replicon	Amplicon bp	Reference plasmid	GenBank	Inc Group
HI1	534	*Salmonella typhi* R27	AF250878	IncHI1
HI2	298-308	*Serratia marcescens* R478	BX664015	IncHI2
HIB-M	570	*K. pneumoniae* pNDM-MAR	JN420336	Not assigned
FIB-M	440	*K. pneumoniae* pNDM-MAR	JN420336	Not assigned
I1α	159	*S. enterica* Typhimurium R64	AP005147	IncI1α
I1ϒ	161	*S. enterica* Typhimurium R64	NC_015965	IncI1ϒ
I2	316	*Escherichia coli* R721	AP002527	IncI2
M	741	*Citrobacter freundii* pCTX-M3	AF550415	IncM
L	854	*K. pneumoniae* pOXA-48	KM406491	IncL
K	190	*Escherichia coli* R387	M93063	IncK
B/O	159	*Escherichia coli* p3521	GU256641	IncB/O
A/C	418	*Aeromonas hydrophila* pRA1	FJ705807	IncA/C
N	514	*Escherichia coli* R46	NC_003292	IncN
N2	177	*Escherichia coli* P271A	JF85549	IncN2
W	242	*Escherichia coli* R721	AP002527	IncW
P1	345	pBS228	BN000925	IncP1-alpha
T	750	*Proteus vulgaris* Rts1	AP004237	IncT

U	843	*Aeromonas hydrophila* pRA3	DQ401103	IncU
R	248	*K. pneumoniae* pK245	DQ449578	Not assigned
X1	370	*Escherichia coli* pOLA52	EU370913	IncX1
X2	376	*Escherichia coli* R6K	M65025	IncX2
X3	284	*K. pneumoniae* pIncX-SHV	JN247852	IncX3
X4	172	*Escherichia coli* pUMNF18_32	CP002895	IncX4
FIA	462	*Escherichia coli* plasmid F	AP001918	IncF
FIB	683	*Escherichia coli* plasmid F	AP001918	IncF
FII	288-292	*Escherichia coli* NR1	DQ364638	IncFII
FIIS	259-260	*S. Enterica* Typhimurium pSLT	AE006471	IncFIIS
FIIk	142-148	*K. pneumoniae* pKPN3	CP000648	IncFIIk
FIB KN	631	*K. pneumoniae* pKPN-IT	JN233704	IncFIIk
FIB KQ	258	*K. pneumoniae* pKpQIL-IT	JN233705	IncFIIk

2.3. Plasmid mediated Carbapenem resistance in *Enterobacterales*

The *bla*KPC gene has been reported mainly in *K. pneumoniae* on several plasmid types, such as IncF, IncI2, IncX, IncA/C, IncR, and ColE1 (Garcia-Fernandez A *et al.* 2012; Chen L *et al.* 2014; Pitout JD *et al.* 2015). IncF with FIIK replicons is the predominant plasmid type, often containing addictional genes responsible for resistance to other antibiotics, including

aminoglycosides, tetracyclines, quinolones, trimethoprim, and sulfonamides (Pitout JD *et al.* 2015). Generally, *bla*KPC genes are inserted in a promiscuous transposon-related structure Tn4401 (10kb), consisting in a transposase gene, a resolvase gene, the *bla*KPC gene, and two insertion sequences, ISKpn6 and ISKpn7 (Figure 2.3.1; Naas T *et al.*, 2008). This transposon is able to jump to numerous conjugative plasmids (Chen L *et al.*, 2014). Tn4401 has five isoforms which differ by deletions (68–255 bp) just upstream of the *bla*KPC gene:

1. deletion of 99 bp;
2. no deletion;
3. deletion of 215 bp;
4. deletion of 68 bp;
5. deletion of 255 bp (Chen D *et al.* 2014).

Tn4401a is frequently associated with the *bla*KPC-3-harboring IncFIIK2 plasmids (Leavitt A *et al.* 2010; Garcia-Fernandez A *et al.* 2012; Chen L *et al.* 2014), whereas Tn4401b and Tn4401d with the IncN and IncFIA plasmids, respectively (Chen L *et al.* 2014). The *bla*KPC-harboring plasmids share as common feature the *tra* operon, encoding the plasmid conjugation machinery proteins, essential for plasmids spread (Chen D *et al.* 2014). This operon may play a key role in the successful dissemination of *bla*KPC-harboring plasmids. The predominant *bla*KPC-harboring plasmids are IncFII plasmids.

In 2006 was entirely sequenced the plasmid pKpQIL, carrying the *bla*KPC-3 in a Tn4401a, and causing a nationwide clonal outbreak in Israel (Leavitt A *et al.* 2010). This plasmid, an IncFIIK2 plasmid, was initially identified in Israel in 2006, then have developed several variants, spread and disseminated to Italy, Poland, the UK, the Czech Republic, the USA and Colombia (Baraniak A *et al.* 2011; Garcia-Fernandez A *et al.* 2012; Hidalgo-Grass C *et al.* 2012; Warburg G *et al.* 2012; Hrabak J *et al.*

2013; Chen L *et al.* 2014). Even non-Tn4401 mobile elements can harbor *bla*KPC genes (Shen P *et al.* 2009; Gomez SA *et al.* 2011) and, based on the insertion sequence upstream of the *bla*KPC gene, these elements are classified in three groups: group I, no insertion (Shen P *et al.* 2009; Liu LH *et al.* 2012; Chen L *et al.* 2014); group II, insertion of truncated *bla*TEM (Gomez SA *et al.* 2011); group III, insertion of Tn5563/IS6100 (Wolter DJ *et al.* 2009). Moreover, non-Tn4401 genetic elements harboring *bla*KPC can contain IS26 transposon (Liu LH *et al.* 2012; Chen L *et al.* 2014).

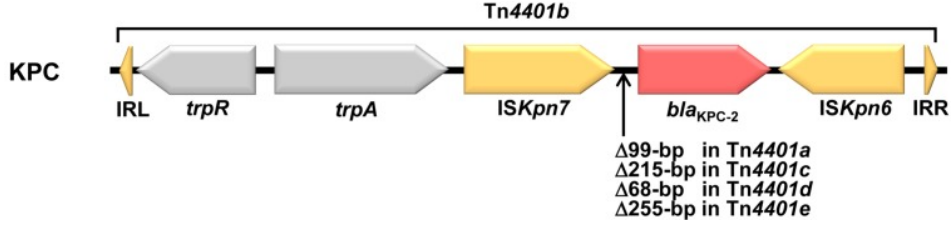

Figure 2.3.1: genomic enviroment of Tn4401-KPC-carrying trasposon

2.4. Plasmid-mediated KPC producing in *C. freundii*

Recently, some studies have investigated the genomic enviroment of MDR *C. freundii*, in order to better characterize the plasmid composition.
Feng *et al.* characterized a 117,288 bp circular molecule, p112298-KPC, with a total of 125 annotated ORFs, an average GC content of 52.25%, and unassigned to any known incompatibility group. p112298-KPC consisted of an 82.3 kb region, encoding plasmid replication (*repA*), stability (*parABM*, *stbB*, *mobC,* etc.) and transfer (*tra*, *trb* and *pil*) functions, and two accessory modules, namely a 1287 bp IS*Ec42* element and a 33.6 kb

MDR region. The deduced replication initiator protein RepA belongs to the IncFII RepA superfamily and matches various RepA proteins from *Citrobacter* and *Enterobacter* (>98% amino acid identity) (Feng J *et al.* 2017). The *bla*KPC platform is composed upstream of a Tn1722, whereas ISKpn27 and ISKpn6 surrounded *bla*KPC-2 upstreamly and downstreamly. Addictionally, five copies of intact or truncated IS26 elements are located at different sites within MDR region (Feng J *et al.* 2017). Tn1722-based transposons are frequently described as *bla*KPC platforms in China (Chen L *et al.* 2014), while *bla*KPC genes in most cases are located in Tn4401 in European and American countries (Bryant KA *et al.* 2013; Chmelnitsky I *et al.* 2014).

Ouyang *et al.* described three novel groups of plasmids carrying *bla*KPC-2: pP10159-3, pHS062105-3 and pECN49-KPC. All of these plasmids contained the core *bla*KPC platform into a truncated Tn3-family transposon Tn1722, called Tn6296. Addictionally, each of the three plasmids carried a single accessory region ΔTn6296, carrying the *bla*KPC-2 gene. Moreover, the three ΔTn6296 elements had several deletions and insertions compare with the prototype Tn6296: 1) ΔTn1722-3′ was lost from all the three ΔTn6296 elements; 2) a 123-bp deletion at the 3′-terminal region of Tn6376 was found in pP10159-3; and 3) a 1754-bp deletion within ΔTn1722-5′ as well as a 73-bp insertion within the variable number tandem repeat (VNTR) region of orf396 was identified in pECN49-KPC (Ouyang J *et al.* 2016*)*.

In 2016, Wu *et al.* reported a whole genome characterization of a *C. freundii* co-producing KPC-2 and NDM-1 enzymes isolated from a Chinese ICU patient. The plasmid (pKPC2_CF65), of 40.9 kb, contained two copies of ISKpn19 flanking *bla*KPC-2 and a Tn*3*-like transposon interrupted by the insertion of IS*Kpn27* were found upstream of *bla*KPC. The upstream genetic context showed similarity with pKPC2_EC14653, a

plasmid from an *E. cloacae* isolate recovered in the same hospital. Downstream of *bla*KPC-2 there was a copy of ISKpn6 and several open reading frames including genes of unknown function (*korC*) and encoding antirestriction protein (*klcA*). Interestingly, this plasmid couldn't be assigned to a known incompatibility group, suggesting a novel type of plasmid (Wu W *et al.* 2016).

In 2017 Villa *et al.* investigated the genomic enviroment of 2 *C. freundii* collected from a tertiary care facility in southern Madrid. The 2 isolates were *bla*KPC-2 positive, inserted in a Tn3-based transposon. The genetic environment of the *bla*KPC-2 gene consisted in Δ*bla*TEM-1, flanked by inverted repeat (IR) sequences, and also harbored a Tn3 transposase gene (tnpA), a Tn3 resolvase gene (tnpR) and four insertion sequences (IS*Kpn6*, IS*Kpn7*, IS*Apu1* and IS*Apu2*). The genetic background of *bla*KPC-2 showed 100% query coverage with overall 99% nucleotide identity to the an *Aeromonas hydrophila* plasmid KPC-producing (KR014106) (Villa J *et al.* 2017).

2. Aim of the Study

Assess by NGS the:

1. analysis of genomic enviroment of two *C. freundii* strains collected in 2015 from two italians un-releated patients;
2. understand the role of *C. freundii* as silent reservoir of relevant resistance genes.

2. Materials and Methods

Bacterial isolates

Two strains of *C. freundii*, designated as AA535 and AA593 were isolated on the February-March 2016 period, from a screening rectal swab of a patient recovered at the "S. Agostino-Estense-Baggiovara" hospital in Modena, Italy, and from an urine sample of a resident in a long term care facility of the same geographical area, respectively. The two patients did not have any apparent epidemiological link.

Identification and susceptibility testing

Identification of the bacterial species was performed using Vitek 2 System (bioMérieux, Marcy l'Etoile, France). Antimicrobial susceptibility was assessed by MicroScan AutoScan-4 (Beckman-Coulter) and confirmed by reference broth microdilution and MICs were interpreted according to EUCAST 2019 breakpoints (http://www.eucast.org).

Genotyping and plasmid content

The two strains were genotyped by XbaI digestion and Pulsed Field Gel Electrophoresis (PFGE; Piazza A *et al.* 2016) and Multilocus Sequence Typing (MLST; Gaibani P *et al.* 2013). MLST profiles obtained were assigned by the PubMLST database (https://pubmlst.org/cfreundii/). Replicon content was detected by PCR-based Replicon Typing (PBRT 2.0 kit DIATHEVA; Carattoli A *et al.* 2005).

Conjugation and trasformation experiments

Conjugation experiments for AA535 and AA593 were performed using streptomycin resistant *E. coli* K12 J62 (pro-, his-, trp-, lac-, Smr) as recipient, selecting transconjugants on 100 µg/ml ampicillin plus 150

μg/ml streptomycin and on 6 μg/ml meropenem plus 150 μg/ml streptomycin. Transconjugants colonies from each experiment were identified as *E. coli* by Matrix-Assisted Laser Desorption Ionization-Time Of Flight mass spectrometry (MALDI-TOF MS) using MALDI Biotyper software (Bruker Daltonics, Bremen, Germany). Plasmids were extracted from donors using PureYield Plasmid Midiprep kit (Promega, USA) and transformed into chemically competent *E. coli* DH5α cells (Invitrogen, USA). Transformants were selected on Luria-Bertani agar plates containing 100 μg/ml ampicillin
(Sigma).

Gene amplification

Presence of the *bla*KPC gene in donors and in transformant cells was detected by PCR using the universal primers:
KPC_FU_1_8 5'-GTGCAGCTCATTCAAGGG-3'
KPC_RU_1_8 5'- GCCAATCAACAAACTGCTG-3'.

NGS and plasmid analysis

Short-read sequence data were obtained from AA535 and AA593 parental strains using Illumina MiSeq next generation sequencer (Illumina Inc., California, USA). *De novo* assembly was performed using the softwares Spades 3.10 and A5 MiSeq (http://bioinf.spbau.ru/spades; Bankevic A *et al.* 2012) using the default spades parameters. Resistance and replicon content were determined by using default threshold parameters in ResFinder (https://cge.cbs.dtu.dk/services/ResFinder/; Zankari E *et al.* 2012) and in PlasmidFinder (https://cge.cbs.dtu.dk/services/PlasmidFinder/; Carattoli A *et al.* 2014). Complete plasmid sequence assembly was obtained by checking pair-end

overlaps and performing PCR-based gap closure, using plasmids from both donors and transconjugants as DNA templates. Plasmid sequences were annotated by BLASTP (https://blast.ncbi.nlm.nih.gov/Blast.cgi?PAGE=Proteins). Assembled genomes were also tested by BLASTN against the plasmid reference sequences, as suggested by PlasmidFinder results.

Nucleotide sequence accession numbers

The nucleotide sequences of the plasmids, pIBAC_IncA, pIBAC_IncN and pIBAC_IncX3_IncA have been deposited in GenBank and the following accession numbers have been assigned respectively; MH594477, MH536949 and MH594478.

2. Results

The studied strains showed resistant profiles to third generation cephalosporins and carbapenems, retaining susceptible to gentamycin, colistin, tigecycline and trimethoprim-sulfamethoxazole. PCR investigation revealed the presence of *bla*KPC gene. The two isolates exhibited different PFGE patterns and the MLST profiles, assigned by the PubMLST database (https://pubmlst.org/cfreundii/), resulted ST19 and ST46 for AA593 and AA535, respectively. After conjugation experiments, ten transconjugants colonies from each experiment were identified. Plasmid content by PBRT 2.0 kit detected A/C and N replicons in all the selected AA535 transconjugants and A/C and X3 replicons in all AA593 transconjugants, respectively. Transformation of AA535 provided two types of transformants, positive to A/C and N replicons, respectively. The *bla*KPC-2 gene was identified in the IncN positive transformants. Transformation of AA593 yielded one type of transformants, positive by PBRT to both A/C and X3 replicons and to the *bla*KPC-3 gene. IncA (previously IncA/C1, renamed by Ambrose SJ *et al.* 2018; Ambrose SJ *et al.* 2018) and IncN plasmid sequences were identified in the AA535 genome in one (144,490 bp) and two (33,378 bp, 15,559 bp) large scaffolds, respectively. The AA535 genome was also positive for Col440 I and Col440I small plasmids (9,532 bp, 1,736 bp). IncA and IncX3 plasmid sequences were identified in five large scaffolds (12,876 bp, 57,440 bp, 45,859 bp, 13,171 bp and 52,783 bp) in the AA593 genome. In addition, IncQ1 was also detected within this genome. The pIBAC_IncA plasmid (MH594477) of AA535 was 145,294 bp in size and carried a class I integron with *aac(6')-Ib-cr5*, *bla*OXA-1, catB3 gene cassettes. It showed 99% nucleotide identity, 90% coverage with pIncAC-KP4898 (KY882285; Esposito EP *et al.* 2017), a *bla*VIM-1-positive plasmid

described in a *K. pneumoniae* from Naples, Italy. The co-presence of pIBAC_IncA and IncN plasmids was identified in most of the AA535 transconjugants by PBRT kit; the presence of *bla*KPC gene was confirmed by PCR in the same strains. An integration of IS3 family element (ISEhe3) was observed between the traW and traU genes, which were not disrupted by this integration. The pIBAC_IncN plasmid (MH536949) of AA535 was 49,804 bp in size, and showed 99% nucleotide similarity, 95% coverage with pKp148, PINH-4900 (KX062091), recovered from a *K. pneumoniae* strain of an urban river in Brazil. The IncN plasmid carried a *bla*KPC-2 on a novel variant of Tn4401 transposon designated by Tn4401i, integrated within the *nuc* gene (Figure 2.1).This transposon had the same genetic environment surrounding the *bla*KPC-2 gene when compared to Tn4401b, except for a deletion of 260 bp upstream *bla*KPC-2 (Figure 2.1). The IncN sequence type was determined as ST15 at the pMLST (https://cge.cbs.dtu.dk/services/pMLST/). ST15 IncN plasmids were described to be efficient shuttles between a variety of species and clones in a study conducted on KPC-producing *Enterobacterales* in Nitanya (Adler A *et al.* 2016).

The AA593 pIBAC_IncX3_IncA (MH594478) plasmid of 192,802 bp was a fusion of IncA and IncX3 plasmids. The occurrence of such IncA-IncX3 plasmid fusion in the parental strain was ascertained by the results of the transformation experiments here described: a unique type of *E. coli* DH5α transformant, positive to both A/C and X3 replicons and to the *bla*KPC-3 gene, was obtained. The *bla*KPC-3 gene was associated to a Tn4401a transposon flanked by truncated tnpA of IS3000, in a configuration highly similar to the IncX3 plasmid pCfr-145 (KY659388), recently identified in several *C. freundii* in Italy (Figure 2.2) (Venditti C *et al.* 2017). The IncA fused portion of the pIBAC_IncX3_IncA plasmid showed 99% nucleotide identity, 92% coverage with the pIBAC_IncA

plasmid of AA535, carrying the same class I integron with *aac(6')-Ib-cr5*, *bla*OXA-1, *catB3* gene cassettes, but a different IS3 element (ISSen4) integrated within the *traW* and *traU* genes. The fusion of the IncA and IncX3 plasmids in pIBAAC539 could have been mediated by recombination between IS26 elements. The IncX3 plasmid scaffold was interrupted by two directly repeated IS26 elements located next to the *bla*SHV-11, followed by the resistance region of the IncA plasmid. Moreover, hypothetical proteins and an IS903 which were located in proximity of the IS26 in pIBAC_IncA plasmid of AA535 scaffold were lost in the fusion process in pIBAC_IncX3_IncA plasmid of AA593 and a truncated ISLad1, was inserted just before the *stbA* stability gene, probably independently by the fusion process (Figure 2.2).

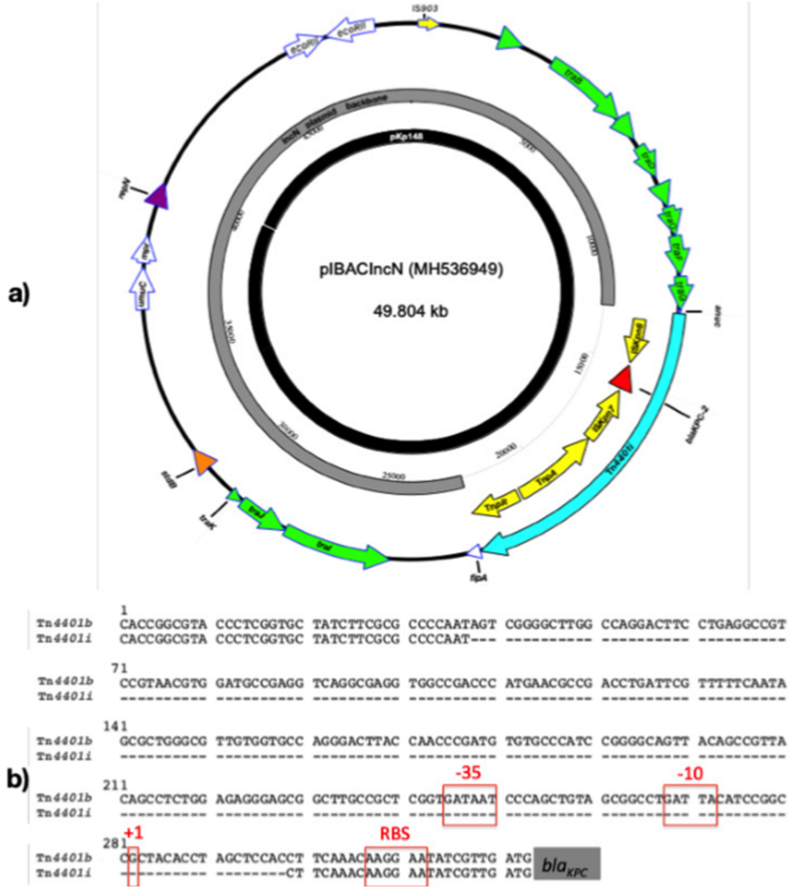

Figure 2.1: a) Circular map of pIBACIncN plasmid compared to pKp148 (black circular box); green arrows represent conjugal transfer system of the plasmid, red arrow represent the *bla*KPC gene, yellow arrows represent mobile elements, white arrows represent hypothetical proteins, purple arrow represent the replication protein repN and orange arrow represent stability protein stdB. Furthermore, the gray circular box represents the plasmid backbone while the transposon Tn4401i is represented in a blue arrow. b) Alignment in the position of interest between the sequence of Tn4401i against the Tn4401b. -35 and -10 represent a putative promoter region, while +1 represent the putative transcription starting point and RBS represent the ribosome-binding sit

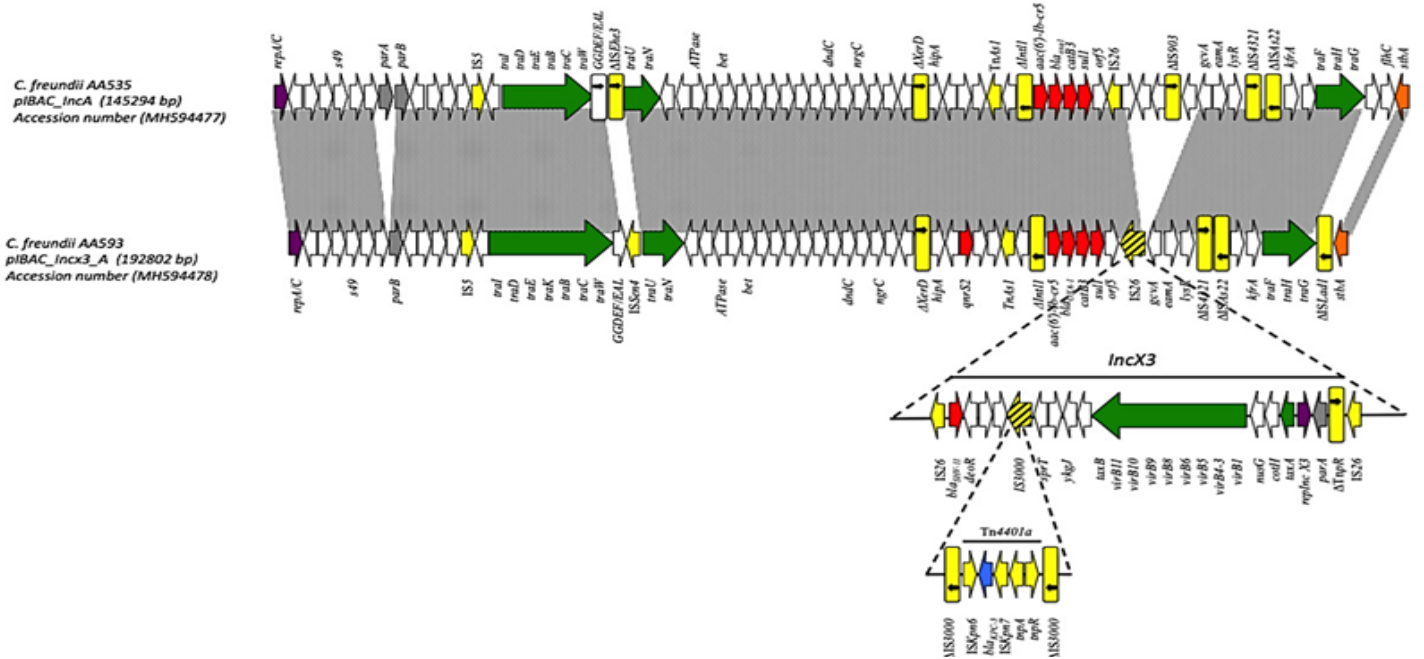

Figure 2.2: Linear map of pIBAC_IncA and pIBAC_IncX3_A; Arrows=direction of transcription of ORFs, rectangles=truncated ORFs. Replicons, partitioning genes, mobile elements, conjugal transfer genes, antibiotic resistance, *bla*KPC-3 and other remaining genes are designated by violet, gray, yellow, green, red, blue and white respectively. Grey shaded area in the upper part shows the similarity of both IncA plasmids while the lower part shows the IncX3 plasmid region of pIBAC_IncX3_A along with its insertion/fusion point, which is shown as black stripped yellow arrows/rectangles in the IncX3 region

2. Discussion

C. freundii, member of the *Enterobacterales* family, is an intestinal tract commensal resident in both humans and animals, causing gastroenteritis and foodborne outbreaks, following the acquisition of virulence factors and resistance determinats (Liu LH *et al.* 2018). In the last fifteen years antibiotic resistant *C. freundii* has increased worldwide, mainly harboring ESβL and plasmid-mediated quinolone resistance determinants (Park YJ *et al.* 2005; Shao Y *et al.* 2011). The increase of MDR *C. freundii* could be resulted from co-selection of genes encoding resistance to multiple antimicrobial classes, thereby leaving few or no options of antimicrobial treatment (Feng J *et al.* 2015; Wu W *et al.* 2016; Xiong J *et al.* 2016).

Citrobacter is recognized to play a unique and worrisome role in bacterial evolution and in human medicine. They are of low virulence and thus can persist in a host population for long periods. These bacteria show broad drug resistance encoded by a diverse array of genetic mechanisms. Addictionally, they can accumulate resistance determinants easly transferable to more virulent organisms. Moreover, they accumulate the means of gene capture and dissemination, i.e., integrons, transposons, and plasmids. In particular, when resistance and virulence determinants are located on the same plasmid or when an inherently virulent organism acquires a resistance plasmid and spreads among hosts, can lead to important outbreaks. Generally, antibiotic-resistant and low-virulence *Citrobacter* species are common colonizers of immunosuppressed patients exposed to multiple antimicrobial agents. It is recognized that *Citrobacter* may contribute to the evolution of bacterial pathogens by acting as persistent sources of resistance genes, complicating surveillance and infection control efforts in hospitalized patients (Pepperell C *et al.* 2002).

In this study, two strains of *C. freundii* producing different KPC enzymes variants were isolated from two different patients without any epidemiological link. *bla*KPC-type harboured in different replicons and plasmids enviroment: *bla*KPC-2-IncN on a newly Tn4401i element and *bla*KPC-3 in a fused IncA-IncX3 plasmids. The evolution of the widely reported Tn4401-*bla*KPC in the new Tn4401i element highlights the hypothesis of *C. freundii* as worrisome and "hot microrganism" for evolution of new and succesfull plasmids carrying persitent resistance genes. Interestingly, Tn4401i was inserted in an IncN/pMLST ST-15-type, recently reported by Adler *et al.* and possessing high conjugation efficiency. In fact, IncN/pMLST ST-15-type seems to facilitate horizontal gene transfer due to his marked and efficient intra- and inter-species conjugation capacity. This strategy has proved to be a winner in the diffusion of KPC enzymes between several *Enterobacterales* clones (Adler A *et al.* 2016). However, the reasons of this ability are still unknown. Addictionally, IncN was associated to a IncA/C plasmid carrying a class I integron with *aac(6')-Ib-cr5*, *bla*OXA-1, catB3 gene cassettes. Despite the two plasmids were physically distinct in the donor and separately transferred in transformation experiments, they conjugated together with the co-resident IncN plasmid carrying *bla*KPC-2. This suggest a possible role of IncA/C in horizontal gene transfer.

On the other hand, *bla*KPC-3 harbored in a hybrid and permanently fused IncA-IncX3 plasmid. IncA/C plasmids are generally large, low copy, plasmids that have been described in the literature for over 40 years but recently intensively studied on the genomic level. They are associated with the emergence of multidrug resistance in enteric pathogens of humans and animals, and possess peculiar modular structure and gene content (Johnson TJ *et al.* 2012).

Interestingly enough, both isolates brought the same resident IncA plasmid. The role of the companion IncA plasmids in these *C. freundii* isolates is not clear. It cannot be predicted if the transfer locus of IncA plasmids was functional or not, while those of the IncX3 and IncN appeared well conserved. IncA could have contributed to stabilize the co-resident IncX3 plasmid in one strain, and used the IncN as a helper plasmid for conjugation in the other. The IncA plasmids could be normal resident of *C. freundii* and may favor adaptation, replication and stability of the plasmids imported from other clinically relevant, carbapenemase-producing *Enterobacterales*, favoring their spread. On the other side, IncA could be symbionts of other co-resident plasmids, using their conjugative properties to spread.

2. Conclusions

In conclusion, this is the first italian report about *C. freundii* KPC-producing strains detected in two peculiar and different plasmid enviroments. Due to *C. freundii* ability of easly accumulating and spreading resistance determinants to more virulence clones, results essential to monitor these strains epidemiology in health-care setting in order to avoid the spread of resistance genes in more virulent strains.

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Chapter 3

Complete nucleotide sequence of plasmids of two *E. coli* strains carrying *bla*NDM-5 and *bla*NDM-5 and *bla*OXA-181 from the same patient

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3.Abstract

In September 2018 two carbapenem-resistant *E. coli* strains were collected at Manerbio Hospital, from a stool sample of a three years-old outpatient affected by diarrhea. The baby, of Indian origin, travelled to India shortly before. Carbapenemase genes detection was performed by GeneXpert (Cepheid); species identification and antimicrobial susceptibilities were obtained by Vitek-2 System and Film-array (bioMérieux) and confirmed by AutoScan4 System (Beckman Coulter); MICs for colistin, tigecycline and fosfomycin were obtained and interpreted according to EUCAST 2018 guidelines. PFGE analysis was accomplished. Whole-Genome Sequencing (WGS) was performed using Illumina MiSeq and PacBio Sequel platforms.Results: Both of the strains, resulted clonally different by PFGE and enterophatogenic, were Multidrug-resistant retaining susceptibility only to colistin, tigecycline and fosfomycin. WGS analysis identified the occurrence of *E. coli* ST2659 and ST405. One strain contained a 130,562 bp multi-replicon plasmid IncFII/IncFIA/IncFIB (pVSI_NDM-5) that carried two main antibiotic resistant islands; ARI-I, 10,030 bp in size, carried genes coding for β-lactams- (*bla*OXA-1, *bla*CTX-M-15), fluoroquinolone- and aminoglycoside- (*aac(6')-lb-cr*) and phenicol- resistance (*catB3*). While ARI-II, 15,326 bp in size, carried genes coding for sulphonamide- (*sul1*), β-lactams- (*bla*NDM-5, *bla*TEM-1B), phenicol- (catB3), trimethoprim-(dfrA17), antiseptic- (qacEΔ1) and aminoglycoside- resistance (aadA5, rmtB). The isolate 5M carried four plasmids: a 153,866 bp multi-replicon plasmid IncFII/IncFIA/IncFIB (pISV_IncFII_NDM-5), a 41,143bp IncI plasmid (pISV_IncI_CMY-42), a 51,480 bp IncX3 plasmid (pISV_IncX3_OXA-181) and an 89,866 bp IncI1 plasmid. pISV_IncFII_NDM-5 carried two main antibiotic resistant islands; ARI-III, 12,220 bp in size, carried genes coding for β-lactams- (*bla*OXA-1), fluoroquinolone- and aminoglycoside- (*aac(6')-lb-cr*),

tetracycline- (*tet(B)*) and phenicol- resistance (*catB3*,*catA1*). ARI-IV, 26,527 bp in size, carried genes coding for macrolide- (*erm(B)*, *mph(A)*), sulphonamide- (*sul1*), β-lactam- (*bla*NDM-5, *bla*TEM-1B), trimethoprim- (*dfrA14*, *dfrA12*), antiseptic- (*qacEΔ1*) and aminoglycoside- resistance (*aadA5*). pISV_IncI_CMY-4 harboured the *bla*CMY-42 gene coding for β-lactams- resistance; while pISV_IncX3_OXA181 harboured genes coding for quinolone- (*qnrS1*) and β-lactams resistance (*bla*OXA-181 In conclusion, the detection of two clonally un-related NDM-5 *E. coli* strains from a pediatric patient with a history of travel to the Far East countries strongly highlight an increasing trend and risk of importation from such areas.

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3. Introduction

Carbapenems are molecules used for the clinical treatment of severe infections caused by MDR *Enterobacterales*, such as *Klebsiella pneumoniae* and *Escherichia coli* (Nordmann P *et al.* 2009). However, over the past 10 years, carbapenemase-producing *Enterobacterales* have been increasingly reported worldwide and nowadays are well known to cause several serious infections resulting in increasing mortality rate, treatment cost, and prolonged hospitalization (Hoang CQ *et al.* 2019). *E. coli* and *K. pneumoniae* are the most common pathogens in *Enterobacterales* family. These carbapenemase-producing bacteria were found in many countries, such as China (Wang J *et al.* 2018), Pakistan (Day KM *et al.* 2013), India (Eshetie S *et al.* 2015), Turkey (Karabay O *et al.* 2016), Brazil (Rossi Gonçalves I *et al.* 2016), Mexico (Torres-González P *et al.* 2015), and Greece (Maltezou HC *et al.* 2009) and mainly in *K. pneumoniae* species.

Carbapenemases are classified according to their dependency on divalent cations for enzyme activation into metallo-carbapenemases (zinc-dependent class B) and non-metallo-carbapenemases (zinc-independent classes A, C, and D; Jeon *et al.* 2015). The class A carbapenemases, such as the *K. pneumoniae* carbapenemase (KPC) enzymes, have been identified worldwide in *K. pneumoniae* (Tangden T and Giske CG 2015). Various class B and D carbapenemases, such as NDM and OXA-48 type, have also been detected in hospital-acquired multi-resistant *K. pneumoniae* (Nordmann P *et al.* 2011), whereas class C carbapenemases (AmpC + efflux pumps) have rarely been reported.

Currently, the spread of carbapenemase-producing *E. coli* in both nosocomial and community setting represents a new and global menance, including NDM- *E. coli* on top of the list of MβL producers in European

countries (Dortet L *et al.* 2014). Moreover, in recent years have been reported infection cases provided by NDM+OXA-48 co-production, firstly in *K. pneumoniae* (Espinal P *et al.* 2019), and then in *E. coli* (Monaco F *et al.* 2018). The main reason of this successful spread could be found in the diffusion of such resistance determinats in higly virulent strains, and in medical tourism, business travel, worldwide diaspora that can play a central role in establishing secondary reservoirs (Nordmann P and Poirel L 2014).

3. Review of the literature

3.1. New Delhi Metallo-β-lactamase

NDM is a Class B β-lactamase, considered as one of the most clinically significant carbapenemases. As MβL, owns a broad-spectrum activity and hydrolyzes all β-lactam antibiotics except for monobactams, and including carbapenems (Jeon JH *et al.* 2015). NDM positive bacteria usually carry a set of genes responsible for a variety of antibiotics such as aminoglycosides, fluoroquinolones, macrolides and sulfonamides.
The first discovery dates back to 2009, with the first report of NDM-1 in a Swedish patient of Indian origin and hospitalized in Orebro, Sweden. The patient was infected by *E. coli* and *K. pneumoniae*, and was previously hospitalized in 2007 New Delhi, India (Yong D *et al.* 2009). Thus far, 17 NDM variants have been assigned (https://card.mcmaster.ca), most of them originated from Asia and share very little identity with other MβLs (Nordmann P and Poirel L 2014). 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 M *et al.* 2014).
The Indian subcontinent, including Pakistan, India and Sri Lanka, has been identified as the main reservoir of NDM-producing *Enterobacterales*. In particular, NDM-producing *K. pneumoniae* results endemic in Indian region (Nordmann P *et al.* 2011). In India, NDM-1 is the most common carbapenemase type detected and accounted for 75.22% of the carbapenemase-producing isolates (Kazi M *et al.* 2015). Sporadic spread has been reported in USA (Doi Y *et al.* 2014), Canada (Lowe CF *et al.*

2013), Spain (Seara N *et al.* 2015), Italy (Espinal P *et al.* 2019), Japan (Nakano R *et al.* 2014) and Australia (Wailan AM *et al.* 2015). Moreover, reports of NDM infections have been also recorded in UK, probably due to the close relationships between India/Pakistan and the UK. Nevertheless, today, reports of NDM producing *Enterobacterales* have been reported worldwide yet becoming new threat to public health (Nordmann P and Poirel L 2014). Other secondary reservoirs of NDM producers have been identified in Balkan states, the Arabian Peninsula and North African countries. The establishing of these secondary reservoirs could be explained by the impact of intercontinental travel as a source of spreading NDM producers (Figure 3.1.1).

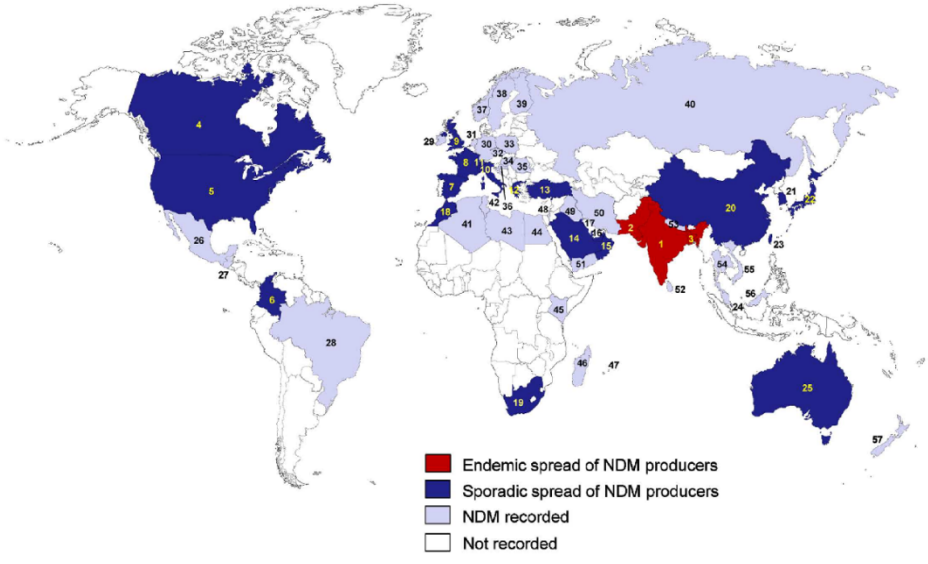

Figure 3.1.1: Geographic distribution of NDM producers (Lee CR *et al.* 2016)

K. pneumoniae and *E. coli* are the main species producing NDM. Very recently, Italy reported an outbreak of NDM-producing CRE, mostly *K. pneumoniae*, affecting seven hospitals in the north-western area of Tuscany, with 350 cases reported between November 2018 and May 2019. This event, with still un-determined source, has led to a national alert due to the rapid spread of such CRE (https://ecdc.europa.eu/sites/portal/files/documents/04-Jun-2019-RRA-Carbapenems%2C%20Enterobacteriaceae-Italy.pdf).

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 been hospitalized for 6 weeks at medical center in Goa, India (Hornsey M *et al.* 2011). The two first NDM-5 producing *E. coli* strains in Italy were reported in 2015 from two un-releated patients came from the same geographical area (Desenzano Area, Northen Italy) (Bitar I *et al.* 2017). NDM-5 differs from NDM-1 for two amino acid substitutions (Val88Leu and Met154Leu), that increase the affinity of resistance to carbapenems and broad-spectrum cephalosporins (Figure 3.1.2) (Yang Q *et al.* 2015).

Figure 3.1.2: Geographic distribution of NDM variants detected worldwide (Sassi A *et al.* 2014)

3.2. Molecular and Genetic environment of NDM

The *bla*NDM gene is mainly inserted in the transposon Tn125 (flanked by two ISAba125 elements) within NDM-producing species of the genus *Acinetobacter*. In NDM-producers *Enterobacterales*, the ISAba125 elements result frequently truncated (Tn125) at various lengths and the Tn125 structure frequently has different IS elements (Figure 3.2.1; Wailan AM *et al.* 2015).

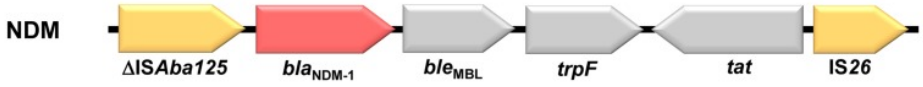

Figure 3.2.1: Structural features of representative genetic environments of *bla*NDM

In *K. pneumoniae bla*NDM genes have been reported on several plasmid types, including IncA/C (Hudson CM *et al.* 2014), IncF (Hishinuma A *et al.* 2013), IncR (Studentova V *et al.* 2015), IncH (Villa L *et al.* 2012), IncL/M (Peirano G *et al.* 2014), and IncX types (Wang X *et al.* 2013). IncA/C type resultes the predominant *bla*NDM-harboring plasmid (Poirel L *et al.* 2011; Pitout *et al.* 2015), frequently carrying also various antibiotic resistance genes including 16S rRNA methylases (*rmtA* and *rmtC*), associated with aminoglycoside resistance; CMY-type β-lactamases, associated with broad-spectrum cephalosporin resistance; and *Qnr*A, associated with quinolone resistance (Pitout JD *et al.* 2015).

3.3. OXA-48 type carbapenemase

OXA-48 is a class D β-lactamase that owns carbapenemase-hydrolysing activity and is not inhibited by clavulanic acid, tazobactam and sulbactam (Poirel L *et al.* 2012). First identified in *K. pneumoniae* from a patient recovered in Istanbul (Turkey) in 2001 (Poirel L *et al.* 2004), nowadays 12 variants of the *bla*OXA-48 gene have been identified: OXA-48, OXA-162, OXA-163, OXA-181, OXA-199, OXA-204, OXA-232, OXA-244, OXA-245, OXA-247, OXA-370 and OXA-405 (Mairi A *et al.* 2018).

Among these, OXA-48, OXA-162, OXA-163, OXA-181, OXA-204 and OXA-232 are the most studied and characterized variants of clinical interest.

OXA-162 differs to OXA-48 variant by a single amino acid substitution and was identified from *K. pneumoniae* isolates in Turkey. Additionally, OXA-162 shares identical hydrolytic activity against β-lactams and carbapenems compared with OXA-48.

The OXA-181 variant, differing from OXA-48 by four amino acid substitutions, has been found associated in *K. pneumoniae* with other carbapenemase genes, such as the *bla*NDM-1, in particular in isolates with an Indian subcontinent link (Poirel L *et al.* 2011; Castanheira M *et al.* 2007; Potron A *et al.* 2011). OXA-181 has been identified in multiple clonally unrelated *K. pneumoniae* isolates in India (Castanheira M *et al.* 2007) and in South Africa (Lowe CF *et al.* 2019). Recently have been reported in literature OXA-181 producing *E. coli* cases in Japan (Tamura S *et al.* 2019), in South Korea (Baek JY *et al.* 2019) and in Italy (Piazza A *et al.* 2018), frequently in association with other carbapenemases such as NDM-1 and NDM-5.

OXA-204 was first identified in 2012 in a *K. pneumoniae* isolate from Tunisia, on a conjugative IncA/C-type plasmid (Potron A *et al.* 2013). OXA-204 exhibits two amino acid substitutions compared with OXA-48, and owns a substrate profile very similar to that of OXA-48.

OXA-232 has been recently identified from *K. pneumoniae* isolates in France, from patients who had been transferred from Mauritius or India (Potron A *et al.* 2013). In 2018, Espinal *et al.* reported the first Italian outbreak provided by *K. pneumoniae* ST16 NDM+OXA-232 co-producer (Espinal P *et al.* 2019). It exhibits five amino acid substitutions compared with OXA-48, and owns a very similar hydrolysis spectrum.

OXA-48 has been found in several *Enterobacterales*, such as *K. pneumoniae* and *E. coli*, due to the high conjugation rate of the pOXA-48a (Potron A *et al.* 2014). Moreover, recently emerged NDM-procuders are *K. oxytoca*, *Enterobacter* spp., *Providencia rettgeri*, *C. freundii*, and *S. marcescens* (Poirel L *et al.* 2012; Berger S *et al.* 2013) (Figure 3.3.1).

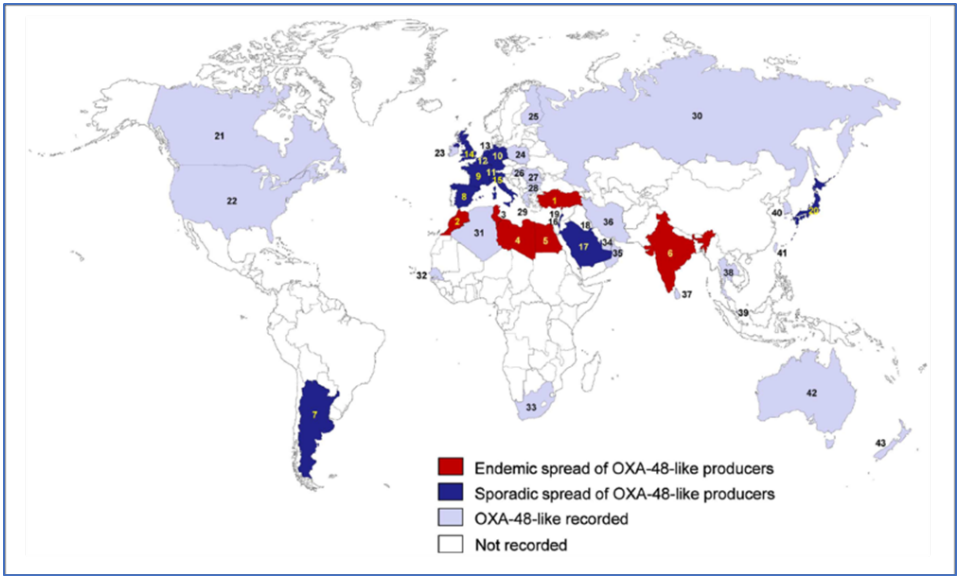

Figure 3.3.1: Epidemiological features of OXA-48-like-producing K. pneumoniae. (1) Turkey; (2) Morocco; (3) Tunisia; (4) Libya; (5) Egypt; (6) India; (7) Argentina; (8) Spain; (9) France; (10) Germany; (11) Switzerland; (12) Belgium; (13) Netherlands; (14) UK; (15) Italy; (16) Israel; (17) Saudi Arabia; (18) Kuwait; (19) Lebanon; (20) Japan; (21) Canada; (22) USA; (23) Ireland; (24) Poland; (25) Finland; (26) Hungary; (27) Romania; (28) Bulgaria; (29) Greece; (30) Russia; (31) Algeria; (32) Senegal; (33) South Africa; (34) United Arab Emirates; (35) Oman; (36) Iran; (37) Sri Lanka; (38) Thailand; (39) Singapore; (40) South Korea; (41) Taiwan; (42) Australia; (43) New Zealand. (Lee CR *et al.* 2016)

3.4. Molecular and genetic context of OXA-48

In *K. pneumoniae* IncL group plasmids, in particular IncL/M type, are mainly responsible for the spread of the *bla*OXA-48 (Pitout JD *et al.* 2015), with a prevalence of 92.5% of the positive isolates in European and North African (Potron A *et al.* 2013). Generally, pOXA-48a plasmids contained *bla*OXA-48, a unique antibiotic resistance gene (Potron A *et al.* 2013). Recently, the *bla*OXA-48-like genes have also been reported on other plasmids and genetic elements, such as IncA/C types (Ma Z *et al.* 2015), IncH types (Wang X *et al.* 2013), and Tn1999 (Poirel L *et al.* 2012). The *bla*OXA-48 gene was originally identified associated with insertion sequence IS1999 in *K. pneumoniae* (Poirel L *et al.* 2004) as part of a composite transposon, named Tn1999. Tn1999 contains two copies of IS1999 bracketing OXA-48 gene (Aubert D *et al.* 2006) (Figure 3.4.1). Thus far, three Tn1999 isoforms have been classified and reported. Tn1999.2 was identified from *K. pneumoniae* isolates from Istanbul, differing by the insertion of IS1R that targeted the region upstream of *bla*OXA-48 and enhanced its expression (Carrer A *et al.* 2010). The third isoform of Tn1999 has been identified in an *E. coli* isolate from Italy, with a second copy of IS1R located downstream of *bla*OXA-48 (Giani T *et al.* 2012). Interestingly, a different genetic environment, laking of IS1999 elements, has been described in particular associated with *bla*OXA-181. Deeply, *bla*OXA-181 is flacked upstream by Insertion sequence ISEcp1, known to be widely responsible for the acqui- sition of *bla*CTX-M and *bla*CMY genes (Poirel L *et al.* 2008; Poirel L *et al.* 2003). In the ISEcp1 transposition process, only one copy mobilizes sequences located at its right-end extremity by recognizing imperfect right inverted repeat sequences (one-ended transposition) (Poirel L *et al.* 2003). In 2011, Potron A *et al.* described the genetic environment of a *bla*OXA-181 inside

a 3139 bp long ISEcp1-made transposon named Tn2013, flanked by a 5 bp-duplicated sequence being the signature of the transposition.

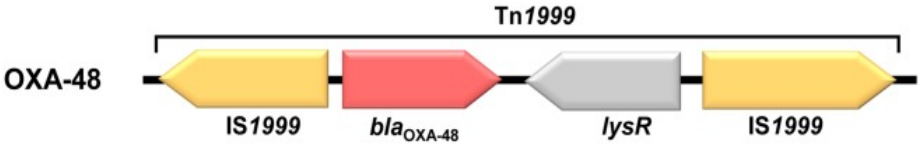

Figure 3.4.1: Structural features of representative genetic environments of *bla*OXA-48

3. Aim of the study

The aim of the study was to describe, for the first time, a *bla*NDM-5 and *bla*OXA-181 co-presence in an *E. coli* clinical strain, in Italy. Moreover, we aim to characterize the plasmids content, comprehensive of the resistance genes array.

3. Materials and Methods

Bacterial Isolation

In 26th September 2018, Manerbio Hospital (Desenzano del Garda, Brescia, Italy) isolated two *E. coli* strains from the stool of a three years old baby of Indian origin. The outpatient was suffering from diarrhea and had a travel history to India shorty before being hospitalized in Italy. The two *E. coli* strains (5M and 5P) resulted positive for NDM production by Xpert Carba-R test (GeneExpert, Cepheid) and were sent to Pavia University and Charles University in Prague for further characterization.

Carbapenemase Production Confirmation and Susceptibility Testing

The strains species identification was confirmed using matrix-assisted laser desorption ionization-time of flight mass spectrometry (MALDI-TOF MS) using MALDI Biotyper software (Brucker Daltonics, Bremen, Germany). Carbapenemase production was tested by MALDI-TOF MS meropenem hydrolysis assay (Rotova V *et al.* 2017). Then, carbapenemase production was screened through metallo-β-lactamase, OXA-48 and KPC using double-disc synergy test with EDTA, phenylboronic acid test and temocillin disc test (Lee CR *et al.* 2003; Doi Y *et al.* 2008; Glupczynski Y *et al.* 2012), respectively. Antimicrobial susceptibility was performed by AutoScan4 System (Beckman Coulter), tigecycline was evaluated using broth microdilution according to EUCAST guidelines (EUCAST 2019).

Plasmid Conjugation

Conjugal transfer of resistance determinants was performed in liquid medium using the *E. coli* A15 strain (Rifr) as recipients and the two strains 5M and 5P as donors respectively. Transconjugants were selected on Muller Hinton agar plates with rifampicin (100 mg/L) and ampicillin (50

mg/L). The presence of *bla*NDM-5 and *bla*OXA-181 was confirmed by polymerase chain reaction (PCR) using the universal primers:
OXA-48 FW 5'-TTGGTGGCATCGATTATCGG-3'
OXA-48 REV 5'- GAGCACTTCTTTTGTGATGGC-3'
NDM FW 5'-GGTTTGGCGATCTGGTTTTC-3'
NDM REV 5'-CGGAATGGCTCATCACGATC-3'

Plasmid size

Plasmid size that carried *bla*NDM-like genes was detected by pulsed-field gel electrophoresis (PFGE) of total DNA digested with S1 nuclease (Promega, Madison, WI, USA; Barton BM *et al.* 1995). Then the DNA was transferred to a BrightStar-Plus positively charged nylon membrane (Applied Biosystems, Foster City, CA) and hybridized with digoxigenin-labeled *bla*NDM-like probe.

Genotyping

The two strains were genotyped by XbaI digestion and Pulsed Field Gel Electrophoresis (PFGE; Piazza A *et al.* 2015).

Whole-genome sequencing (WGS) and Annotation

The genomic DNA of the two *E. coli* strains was extracted using NucleoSpin Microbial DNA kit (Macherey-Nagel, Germany). Sequel I platform (Pacific Biosciences, California, USA) was used for sequencing. Library preparation was done following the microbial multiplexing protocol according to the manufacture instructions for sheared DNA. Shearing was performed using g-tubes (Covaris, USA), and no size selection was done during the library preparations. HGAP4 was used to perform the assemblies of the genomes with minimum seed coverage of 30. ResFInder 3.2 (https://cge.cbs.dtu.dk/services/ResFinder/) (Zankari E

et al. 2012), PlasmidFInder (https://cge.cbs.dtu.dk/services/PlasmidFinder/) (Caratolli A *et al.* 2014), VirulenceFinder 2.0 (https://cge.cbs.dtu.dk/services/VirulenceFinder/) (Joensen KJ *et al.* 2014) ISfinder database (www-is.biotoul.fr/), MLST 2.0 (https://cge.cbs.dtu.dk/services/MLST/)(Larsen MV *et al.* 2012), and CHTyper 1.0 (https://cge.cbs.dtu.dk/services/chtyper/)(Camacho C *et al.* 2009) were utilized to detect resistant genes, plasmid replicon type, virulence genes, mobile elements, Sequence type (ST) and FIMh/FumC type respectively. Open reading frame (ORF) were predicted using RAST 2.0 (Brettin T *et al.* 2015) with default parameters combined with BLASTP/BLASTN. Comparative genome alignments were performed using the Mauve (version 2.3.1). Gene organization and diagrams were sketched using Inkscape 0.92.4 (https://inkscape.org/).

Nucleotide sequence accession numbers

The nucleotide sequences of pISV_IncI_CMY-42, pISV_IncFII_NDM-5 and pVSI_IncFII_NDM-5 plasmids were deposited in the GenBank under the accession numbers MN242251, MN218686 and MN197360 respectively

3. Results

Both strains showed an MDR profile, retaining susceptibility only for colistin (<=1 mg/L), fosfomycin (2 mg/L) and tigecyclin (<=0.25 mg/L). PFGE investigation assigned two different clones, as also obtained by MLST investigation: ST405/ST477 (according to Pasteur and Oxford scheme respectively) for *E. coli* 5P and ST2659/ST759 for *E. coli* 5M (Table 3.1).

Table 3.1: resistance genes content, replicons, ST, and virulence factors of the studied strains

	E. coli 5P	***E. coli 5M***
β-lactamases	*bla*NDM-5, *bla*TEM-1b, *bla*CTX-M-15, *bla*OXA-1	*bla*NDM-5, *bla*TEM-1b, *bla*CMY-42, *bla*OXA-181, *bla*OXA-1
Aminoglycoside	*aadA5, rmtB, aac-(6')Ib-cr*	*aadA2, rmtB, aac-(6')Ib-cr*
Fluoroquinolone	*aac-(6')Ib-cr*	*qnrS1, aac-(6')Ib-cr*
Macrolide	-	*erm(B), mph(A), mdf(A)*
Phenicol	*catB3*	*catA1, catB3*
Sulphonamide	*sul1*	*sul1*
Trimethoprim	*dfrA17*	*dfrA12, dfrA14*
Tetracycline	-	*tet(B)*
Replicons	IncFIA, IncFIB, IncFII	ColKP3, IncFIA, IncFIB, IncFII, IncI, IncI1, IncX3
ST	405/477	2659/759
Virulence factors	*air, eilA, Gad, iha, sta*	*air, eilA, Gad, capU, astA*

The β-lactamase genes were transferable through conjugation in both clinical isolates. Both the transconjugants (*E. coli* A15R5M and *E. coli* A15R5P) exhibited similar susceptibility profiles as the donors, except for aztreonam (in the susceptibility range). Although *E. coli* A15R5M was aztreonam-resistant, the MIC resulted lower (MIC= 4 mg/L).

WGS revealed that 5P belonged to the serotype O102:H6 and FumC37/FimH27. Moreover, it carried virulence genes associated with *air*, *gad*, *iha* and *sat* coding for Enteroagregative immunoglobin reapeat protein, glutamate decarboxylase, adherence protein and secreted autotransporter toxin respectively. The isolate carried a 130,562 bp multi-replicon plasmid IncFII/IncFIA/IncFIB (pVSI_NDM-5). pVSI_NDM_5 plasmid backbone exhibited high similarity scores with the 133,6947 bp plasmid (pM309-NDM5) carried by an *E. coli* strain isolated in Myanmar in 2019 (AP018833.1; Sugawara *et al.* 2019) (99.94% sequence identity, 82% query coverage). The plasmid backbone carried regions responsible for replication (*repA*, *repB*, *repE*), conjugative transfer system (tra, finO genes), maintenance and stability (*parA*, *parB*), toxin-antitoxin (TA) systems (vapC/vapB, ccdA/ccdB, pemI/pemK). Moreover, the plasmid carried two main antibiotic resistance islands (ARI-I and ARI-II); ARI-I, 10,030 bp in size, carried genes coding for β-lactams- (*bla*OXA-1, *bla*CTX-M-15), fluoroquinolone- and aminoglycoside- (*aac(6')-lb-cr*) and phenicol- resistance (*catB3*). ARI-I resembled high similarity scores with plasmids from *K. pneumoniae* and *E. coli* (100% sequency similarity and coverage; USA CP023950.1, Denmark MG462728.1) and even from the chromosome of Enterobacter cloacae in Thailand (CP040827.1). While ARI-II, 15,326 bp in size, carried genes coding for sulphonamide- (*sul1*), β-lactams- (*bla*NDM-5, *bla*TEM-1B), phenicol- (*catB3*), trimethoprim- (*dfrA17*), antiseptic- (qacEΔ1) and aminoglycoside-resistance (*aadA5, rmtB*). ARI-II was a complex transposon that was not

found in the database as whole segment rather fragmented as suspected (Figure 1).

The isolate 5M belonged to the serotype O50/02:H18 and FumC26/FimH5. Moreover, it carried virulence genes associated with *air*, *gad*, *asta*, *capU* and *eilA* coding for Enteroagregative immunoglobin reapeat protein, glutamate decarboxylase, heat-stable toxin, hexosyltransferase protein and salmonella HilA homologous protein. The isolate carried four plasmids: a 153,866 bp multi-replicon plasmid IncFII/IncFIA/IncFIB (pISV_IncFII_NDM-5) (Figure 1), a 41,143bp IncI plasmid (pISV_IncI_CMY-42) (Figure 2), a 51,480 bp IncX3 plasmid (pISV_IncX3_OXA-181) (Figure 3) and an 89,866 bp IncI1 plasmid. pISV_IncFII_NDM_5 plasmid backbone carried regions responsible for replication (*repA*, *repB*, *repE*), conjugative transfer system (tra, trb, finO genes), maintenance and stability (parA, parB, stb), toxin-antitoxin (TA) systems (ccdA/ccdB, pemI/pemK, doc). Moreover, the plasmid carried two main antibiotic resistant islands (ARI-III and ARI-IV); ARI-III, 12,220 bp in size, carried genes coding for β-lactams- (*bla*OXA-1), fluoroquinolone- and aminoglycoside- (*aac(6')-lb-cr*), tetracycline- (*tet(B)*) and phenicol-l resistance (*catB3,catA1*). ARI-IV, 26,527 bp in size, carried genes coding for macrolide- (*erm(B), mph(A)*), sulphonamide- (sul1), β-lactams- (*bla*NDM-5, *bla*TEM-1B), trimethoprim- (*dfrA14, dfrA12*), antiseptic- (*qacEΔ1*) and aminoglycoside- resistance (*aadA5*). ARI-IV when blasted was found to be in a plasmid isolated in Myanmar pM217_FII (100% sequence similarity, 92% query coverage; AP018147.1). pISV_IncI_CMY-42, that harboured the *bla*CMY-42 gene coding for β-lactam resistance, exhibited high scores when blasted with pCMY-42 (KY463221.1; 99.48% sequence similarity 93% query coverage). While pISV_IncX3_OXA-181, which harboured genes coding for quinolone resistance (qnrS1) and β-lactams

resistance (*bla*OXA-181), showed 100% sequence similarity and coverage to several plasmids found in NCBI database (AP018837.1; Myanmar, MG893567.1; China, CP024806; Denmark, MG570092; Lebanon, KX894452.1; Germany, KX523903; Czech Republic). It is noteworthy that these plasmids as mentioned were all isolated from *E. coli* strains except for the last one from *K. pneumoniae* in Czech Republic.

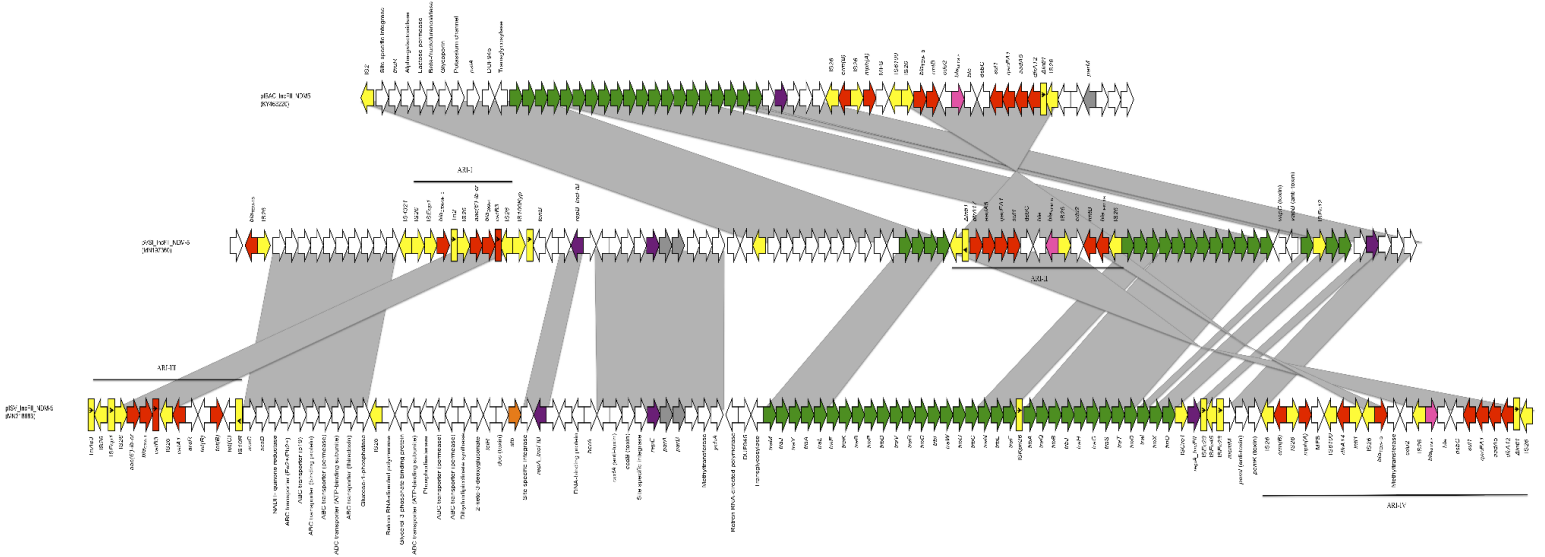

Figure 3.1: Linear map of pIBAC_IncFII_NDM-5, pVSI_IncFII_NDM-5 and pISV_IncFII_NDM-5; Arrows show the direction of transcription of ORFs while rectangles show truncated ORFs. Replicons, partitioning genes, mobile elements, conjugal transfer genes, antibiotic resistance, *bla*NDM-5 and other remaining genes are designated by violet, grey, yellow, green, red, blue and white respectively. Grey shaded area in the upper and lower part shows the similarity of three IncFII plasmids.

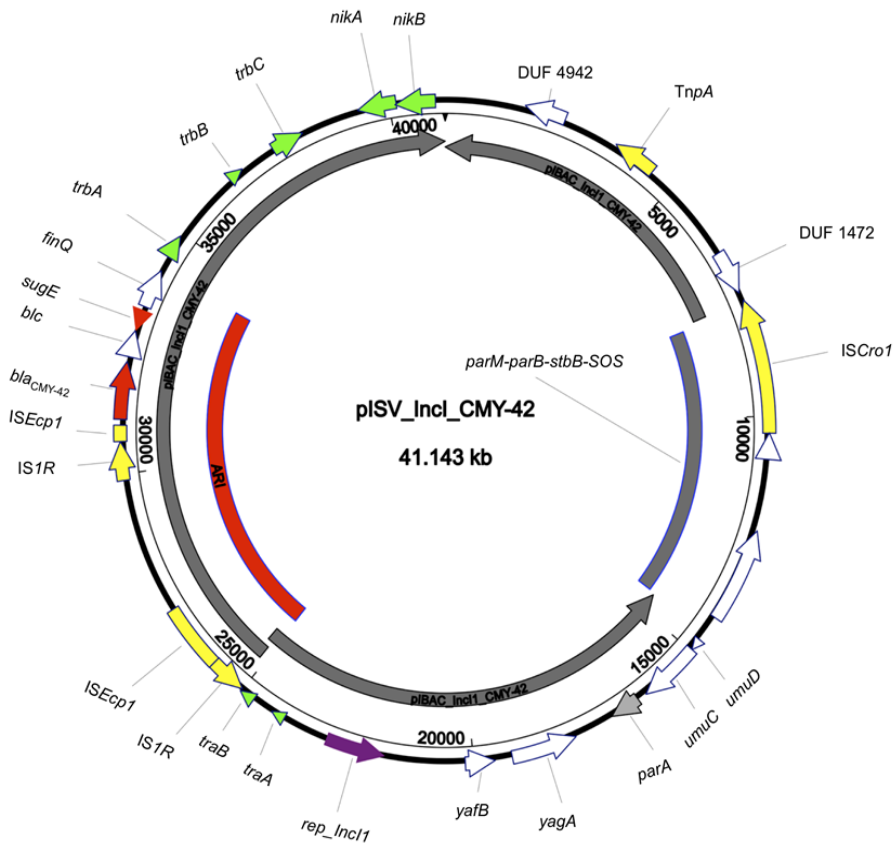

Figure 3.2: circular map of pIVS_IncI_CMY-42 plasmid; green arrows represent conjugal transfer system of the plasmid, red arrow represents the ARI region, yellow arrows represent mobile elements, white arrows represent hypothetical proteins, purple arrow represent the replication protein repI1. Furthermore, the grey circular box represents the plasmid backbone.

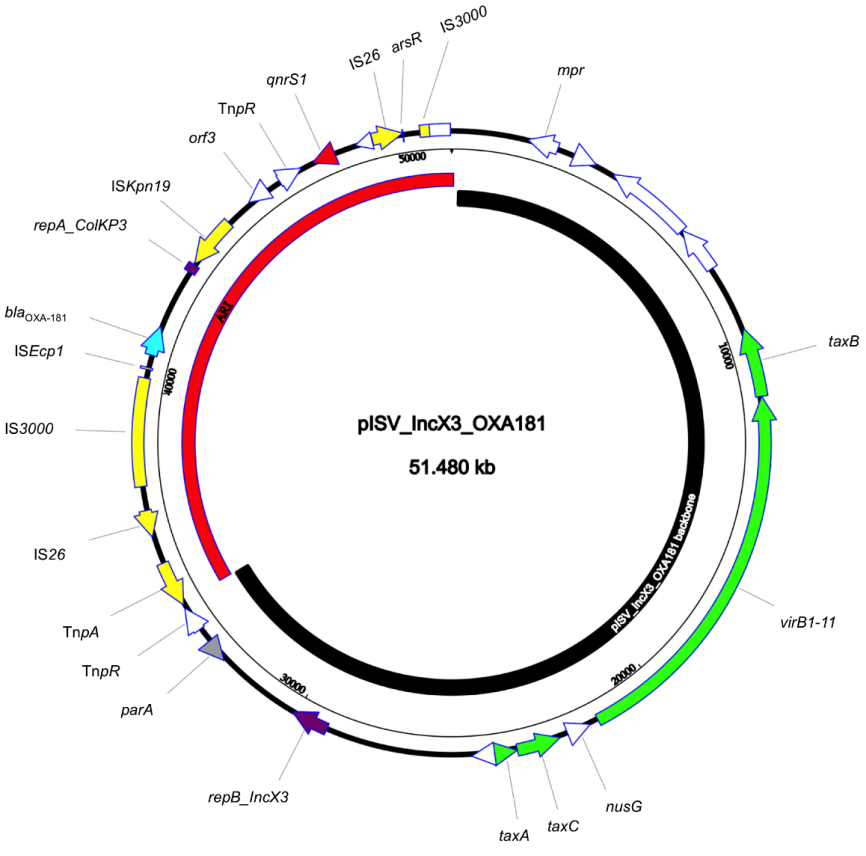

Figure 3.3: circular map of pIVS_IncX3_OXA-181 plasmid; green arrows represent conjugal transfer system of the plasmid, red arrow represents the ARI region, yellow arrows represent mobile elements, white arrows represent hypothetical proteins, purple arrow represent the replication protein repB. Furthermore, the black circular box represents the plasmid backbone.

3. Discussion

In this study we report the isolation of two different *E. coli* isolates from the same patient, one carrying a multi replicon plasmid carrying the *bla*NDM-5 while the other carrying a multi replicon plasmid carrying the *bla*NDM-5 gene, another plasmid carrying *bla*OXA-181 and a plasmid carrying *bla*CMY-42. The emergence of NDM-5 has been reported in Italy (Bitar I *et al.* 2017) and OXA-181 has been also reported in Italy (Piazza A *et al.* 2018).

The comparison of the two plasmids isolated in the study with the first NDM-5 plasmid in Italy shows that the plasmid backbone, as well as the ARI, is different. Even though one of the isolates have the same ST 405, same plasmids incompatibility group IncFII and same hospital isolation, the plasmid backbone (*rep, tra* and stability genes) was strongly different, as well as TA systems.

pVSI_IncFII_NDM-5 shared most of the regions found in pIBAC_IncFII_NDM-5 with more acquired regions (Figure 3.1). Both of the plasmids shared the ARI-II (in opposite direction); even though pIBAC_IncFII_NDM5 had another segment harboring *erm(B)* and *mph(A)* with two IS25 flanking the peripheries in opposite direction, adjacent to the segment resembling ARI-II. Interestingly, pVSI_IncFII_NDM-5 and pISV_IncFII_NDM-5 shared some regions of the backbone but not with pIBAC_IncFII_NDM-5. The ARI, even though shared some regions, yet pISV_IncFII_NDM-5 ARI were larger and carried more resistance genes (except to *bla*CTX-M-15 and *bla*TEM-1B found in pVSI_IncFII_NDM-5 only). The re-arrangement of the plasmid backbone and the excessive presence of insertion sequence in ARI leading to the formation of complex transposons with similar insertion sequences on the flanking regions suggest the possibility of transfer of such regions easily. Nevertheless, this also suggest that the plasmid itself is not conserved and might be rearranged further acquiring more resistant genes as shown comparing the plasmids.

On the other hand, the comparison of pISV_IncI_CMY-42 with the plasmid pIBAC_IncI_CMY-42 isolated with pIBAC_IncFII_NDM5, showed similar plasmid structure except for a small segment that harbored *parM, parB, stbB* and SOS protein, which was replaced by a segment harboring some hypothetical proteins with the insertion sequence IS*Cro (*Figure 2). pISV_IncX3_OXA-181 was the most conserved plasmid among reported IncX3 plasmid harboring *bla*OXA-181. This successful plasmid, as has been reported elsewhere, has conserved plasmid backbone as well as conserved ARI. Moreover, the increasing incidence of this plasmid in different geographic settings confirms the selective pressure and ease of dissemination of this plasmid.

3. Conclusions

In conclusion, the detection in Italy of two clinically important MDR *E. coli* clones (the pandemic ST405/477 and the toxigenic ST2659/ST759) from the same three years old out-patient, represents an unusual and concerning picture, despite the increase of NDM outbreaks reports in Italian area. The presence of genetically different plasmids *bla*NDM-5 positive, suggests that, as reported before, traveling from the Far East countries strongly contributes to the spread in Italy of NDM-type class B carbapenemases (Bitar *et al.* 2017). The high genomic rearrangement-taking place in the plasmid structure could be explained by the selective pressure experienced in different geographical areas settings. Finally, the presence of a *bla*OXA-181 plasmid along with another *bla*NDM-5 harboring plasmid in the ST2659/ST759 *E. coli* 5M, confirms the increasing trend in replicons co-existence, limiting the therapeutic options and increasing the dissemination risk.

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Chapter 4

Deadly puppy infection caused by an MDR *Escherichia coli* O39 *bla*CTX-M-15, *bla*CMY-2, *bla*DHA-1 and *aac(6)-Ib-cr* -positive in a breeding of Central Italy.

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4. Abstract

Antimicrobials consumption in veterinary medicine has led to the spread of MDR zoonotic bacteria, with the companion animals and their living environments involved as emerging reservoirs. While CTX-M-15 and CMY-2 acquired β-lactamases have been widely detected in the bacterial population of companion and breeding animals in European area, DHA-1 enzymes have been rarely reported in veterinary settings. Aim of the study was to screen for ESßL, AmpC and fluoroquinolone/aminoglycoside resistance genes among *E. coli* isolates from a Bulldog puppy in a breeding located in Pesaro area, Central Italy. The *E. coli* strains, of O39 serotype by agglutination, were third and fourth generation cephalosporins, chloramphenicol, aminoglycosides, trimethoprim-sulfamethoxazole and ciprofloxacin resistant, retaining susceptibility to carbapenems, colistin, fosfomycin, levofloxacin (EUCAST clinical breakpoints). PFGE (XbaI) on the five *E. coli* strains revealed the presence of a unique profile. WGS analysis revealed a complex resistome, harbouring *bla*TEM-1b, *bla*CTX-M-15, *bla*OXA-1, *aph(6)-Ib*, *aac(6')Ib-cr*, *aac(3)-Ila*, *aph(6)-Id*, *aadA1*, *qnrB1*, *sul2*, *catA1*, *catB3*, *tetA* and *dfrA14* genes located on an IncHI2/HI2A plasmid, and including other *bla*DHA-1, *qnrB4*, *mph(A)*, *sul1* and *dfrA17* determinants on an IncFII plasmid. Finally, a *bla*CMY-42 determinant was carried on an IncI1 plasmid. The presence of *lpfA*, *iss*, *astA* and *gad* virulence factors was highlighted. This is the first Italian report on a two-weeks old dog invasive infection caused by a MDR *E. coli* O39 *bla*CTX-M-15, *bla*CMY-2, *bla*DHA-1 and *aac(6')-Ib-cr* positive strain. The above MDR *E. coli* clone caused the death of the eight dogs of the same litter, despite amoxicillin-clavulanate and enrofloxacin administration. The water tank-stored used to prepare the milk therapy-based litter meal was suspected as the pathogen reservoir

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4. Introduction

Antimicrobial resistance in veterinary medicine

Considering veterinary medicine, the amount of antibiotics consumed in livestock and companion animals resulted almost double than the one used in humans (Phillips I *et al.* 2004; Aarestrup FM *et al.* 2005) leading to the spread of antimicrobial-resistant bacteria as a complex and serious issue involving even animal's field (Harada K *et al.* 2011; Guardabassi L *et al.* 2004; Chirila F *et al.* 2017). Antimicrobial drugs are extensively used in veterinary for therapy, prophylaxis, metaphylaxis and growth promotion (Marshall BM *et al.* 2011). The most used antimicrobial molecules are penicillins, cephalosporins, polimixins, fluoroquinolones and, in case of *Enterobacterales* severe infections, aminoglycoside class. The continuously inappropriate use of such antibiotics and the close human contact have leaded to an increasing of MDR *Enterobacterales* among animals in European area (Marshall BM *et al.* 2011).

Antimicrobial use in pet animals

The occurrence of MDR microorganisms is a tangible threat in companion animals, mostly due to meticillin-resistant *Staphylococcus pseudointermedius* and ESßL bacteria. Animals at risk include those that have undergone multiple antimicrobial treatments, do not respond to treatment or suffer from multiple infections. The good antimicrobial stewardship in veterinary medicine suggests continuous samplings and testing for resistant bacteria in animals at risk, since the results may affect the treatment and the selection of the antimicrobial drug.

According to the Finnish Food Authority, many diseases in dogs and cats should not be treated with antimicrobial compounds. These include small wounds, minor skin damage and acute gastrointestinal diseases (i.e.

diarrhoea and vomiting), dental illnesses (i.e. gingivitis, periodontitis and acute dental fractures) and clean-contaminated surgical procedures, in which antimicrobials administration should only be used peri-operationally (https://www.ruokavirasto.fi/en/)(Table 4.1).

Table 4.1: Antimicrobials administration in pets

Disease	Causative Microbe	First-line treatment	Alternative treatment	Notes
		Reproductive tract		
Metritis	*E. coli*	Surgical treatment and only perioperative antimicrobial dor prophylaxis. Trimethoprim-sulphonamide admistered orally for a period of time if an animal has systemic signs, in addition to surgical treatment.	If no surgical treatment, an antimicrobial course in addition to a medical evacuation of the uterus: Trimethoprim-sulphonamide Amoxicillin-clavulanic acid	Surgical treatment is most important
Bacterial prostatitis	*E. coli* *Klebsiella* spp.	Trimethoprim-sulphonamide	Fluoroquinolone	Bacterial culture and cytology (urine, prostate fluid, prostate puncture). A long course (4 weeks) anti-testosterone treatment or castration should also been considered. Prostate hyperplasia without a bacterial infection is common and requires no antimicrobial treatment
		Urinary tract		
Acute urinary tract infection with no complications	*E. coli* *Proteus* *Enterococcus* spp. *Staphylococcus* spp. *Streptococcus* spp.	Trimethoprim-sulphonamide Amoxicillin-clavulanic acid	Fluoroquinolone based onthe results of susceptibility tests	Due to the increased resistance, bacterial culture of cystocentesis sample and the determination of drug susceptibility must be performed. Recommended course duration is around 7 days. In the case of recurring urinary tract infections or infections , which do not respond to treatment, solving the underlying cause is essential. In complex urinary tract infections the antimicrobial treatment is for 4 weeks
		Respiratory tract		
Canine infectious respiratory disease complex (CIRD), «kennel cough»	Virus *Bordetella bronchiseptica* *Mycoplasma* spp.	No antimicrobials	Doxycycline Trimethoprim-sulphonamide	Primarilly an acute viral infection; will heal in 7-14 days without treatment if no complications arise
Pneumoniae dogs	*Pasteurella* spp. *E. coli* *Streptococcus* spp. *Bordetella bronchiseptica* *Staphylococcus* spp.	Amoxicillin-clavulanic acid Doxycycline	Fluoroquinolone +aminopenicillin	Except for bordetella, the primary medicine is amoxicillin-clavulanic acid. Severe cases require treatment at a veterinary hospital. Requires a high dose at short intervals (20 mg/kg 3 times a day)
Respiratory tract infection in cats (bronchitis, pneumonia)	*Pasteurella* spp. *Chlamydophila* spp. *Bordetella bronchiseptica* *Mycoplasma* spp.	Doxycycline Amoxicillin-clavulanic acid	Fluoroquinolone +aminopenicillin	Respiratory tract infections in cats are often complex; the possibility of a numerous diseases including ashma, bronchitis or lungworm infections must be taken into consideration

4. Review of the literature

4.1 Overview of *E. coli*

E. coli is one of the most important commensal inhabitants of gastrointestinal tract of warm-blooded animals and humans. As commensal, it lives in the host mutually benefiting and rarely causes diseases. It is one of the most important pathogens causing diseases in humans, mammals and even birds. Currently, its classification is based on serological identification of O (lipopolysaccharide, LPS) and H (flagellar) antigens. Another classification is based on virulence factors and host clinical symptoms proposing the pathogenic types; there are at least seven major pathotypes for enteric *E. coli* and three *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			
EnteroPathogenic *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
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
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

Intestinal pathologies lead to diarrhoea episode due to several *E. coli* pathotypes such as ETEC, EPEC or EHEC *E. coli* (Kaper JB *et al.* 2004). Among EHEC, serotype O157:H7 is reported as an established zoonotic pathogen, mainly in cattle (Manning SD *et al.* 2008; Friesema IH *et al.* 2010). Plausibly, animals can be considered as a reservoir of ExPEC that can potentially cause human diseases (Fairbrother JM and Nadeau E, 2006). Many reports showed similarities among diverse ExPEC strains, common phylogenetic origins of strains from both humans and animals and a genome flexibility, raising concerns about the potential for ExPEC to cause zoonosis (Johnson TJ *et al.* 2007; Brzuszkiewicz E *et al.* 2009). ExPEC can be responsible for UTIs, most of them caused by ExPEC from phylogenetic group B2 (Johnson *et al.* JR 2003; Russo TA and Johnson JR 2000), and bovine mastitis in cattle. Mastitis is an important economic problem causing decreases in milk production. Moreover, mastitis can even lead to the death of the cows (Fairbrother JM and Nadeau E 2010). UTIs are also a relevant cause of mortality in adult swine (Ngeleka M *et al.* 1994) and even companion animals (as dogs and cats) suffer from infections caused by ExPEC (Johnson TJ *et al.* 2008).

Even UPEC isolates are an important cause of extra-intestinal infection in both humans and animals, leading ascending UTI after colonizing the intestine tract (Yamamoto S 2007). UPEC reported from humans and companion animals have showed in some case close phylogenetic relation (Johnson TJ *et al.* 2008) due to the ability of such strains the cross-species barrier and colonizing human host (Johnson JR *et al.* 2009). Interestingly, Johnson *et al.* in 2009 described an UPEC infection affecting pets (five cats and one dog) and four humans living in the same household. The strains caused UTI in the dog and then colonized the four humans living with. In the same household a previous UTI event occurred, caused by a different UPEC strain, and colonizing the same dog and three family members (Johnson TJ *et al.* 2008). These linked reports highlight the evidence not only for human-to-human transmission but also for animal-to-human and human-to-animal transmission of ExPEC, and demonstrates that even pets should be considered as potential reservoirs for the transmission of UPEC to humans.

Poultry has been described as an ExPEC reservoir with zoonotic potential, in particular for the dissemination of APEC strains. APEC are involved in systemic infection, starting as respiratory tract infections and then disseminating to organs. APEC are found in the intestinal microflora of healthy birds and most of the diseases associated with them are secondary to environmental and host predisposing factors (Dho-Moulin M *et al.* 1999).

Relevantly, certain virulence traits appear to be more prevalent in specific groups of human or animal ExPEC strains, assuming a virulence genes/ExPEC pathotypes – host species association (Rodriguez-Siek KE *et al.* 2005; Ewers C *et al.* 2007). However, in many cases were identified common virulence factors apparently not correlated with host specificity. In particular, recent studies have demonstrated that some ExPEC strains

are virulent or capable of infecting both poultry and mammals, further supporting the zoonotic potential of certain APEC strains (Ron EZ 2006). For these reasons, there is mounting evidence that certain ExPEC clonal groups are capable of causing both human and animal infections.

4.2 *E. coli* MDR

Main resistance mechanisms have been found in *E. coli* isolated from veterinary medicine, related to plasmid mediated and/or chromosomal production of enzymes, such as ESßLs, conferring resistance to aminopenicillins, cephalosporins, cephamycins and monobactam, and AmpC β-lactamases, hydrolyzing penicillins and cephalosporins (Marshall BM *et al.* 2014). Recently, plasmids harbouring ESßL/AmpC genes have been founded to carry genes that confer resistance to various classes of antimicrobials, such as fluoroquinolones, aminoglycosides, sulfamethoxazole/trimethoprim. In particular, plasmid-mediated quinolone resistance genes such as *aac(6')-Ib-cr*, *qnrA* and *qnrB* (Zogg AL *et al.* 2018), have been reported largely associated to plasmid-mediated ESßLs/AmpC genes in breeding animals (Wu H *et al.* 2016) and pets (Zogg AL *et al.* 2018), (Liu X *et al.* 2016). These aspects make treatment options for infections caused by ESßL and/or AmpC producing bacteria very limited (Zogg AL *et al.* 2018). Among ESBL family, CTX-M-15 and CTX-M-1 are largely reported in livestock and companion animals in Japan (Maeyama Y *et al.* 2018), China (Li S *et al.* 2017), Canada (Zhang PLC *et al.* 2018), Turkey (Aslantaş O *et al.* 2016), United Kingdom (Timofte D *et al.* 2016) and Poland (Rzewuska M *et al.* 2015). Among the AmpC family, CMY-2 type is most

frequently reported in cattle and retail meats (Pehlivanlar S *et al.* 2015; Yilmaz NO *et al.* 2013) often found in combination with other β-lactamase genes (Aslantaş O *et al.* 2016), whereas DHA genes have rarely been detected in veterinary medicine (Belas A *et al.* 2014), prevalently in *K. pneumoniae* than *E. coli* from companion animals (Hidalgo L *et al.* 2013). Moreover, combinated with ESßL/AmpC genes, *aac(6')-Ib-cr* has been recently detected in companion pets (Zogg AL *et al.* 2018) and breeding animals (Yang F *et al.* 2018; Röderova *et al.* 2017; Jones-Dias D *et al.* 2016).

4. Aim of the study

The aim of this study was to assess the presence of ESβL, AmpC and fluoroquinolone/aminoglycoside resistance genes among *E. coli* from a Bulldog puppy in a dog breeding located in Pesaro area, Central Italy.

4. Materials and Methods

Bacterial isolates

A total of five *E. coli* strains were collected in 8th September 2017, from biopsy specimens of a two-weeks old dog. The Bulldog puppy lived in a dog breeding located in Tavullia (PU) and belonged to an eight-puppy litter. At the beginning of September, the entire litter had haemorrhagic enteritis with renal involvement and cerebral vessels congestion. Puppies were treated for three days with amoxicillin plus clavulanic acid unsuccessfully and then with enrofloxacin before death.

Identification, susceptibility testing and serotyping

Species identification and antimicrobial susceptibility was assessed by MicroScan AutoScan-4 (Beckman-Coulter) and confirmed by reference broth microdilution and MICs were interpreted according to EUCAST 2019 breakpoints (http://www.eucast.org). Serotyping was performed by hot tube agglutination with specific sera.

Genotyping and plasmid content

The two strains, chosen as representative, were genotyped by XbaI digestion and Pulsed Field Gel Electrophoresis (PFGE; Piazza A *et al.* 2015) and Multilocus Sequence Typing (MLST; Gaibani P *et al.* 2013). MLST profiles obtained were assigned by the PubMLST database (https://pubmlst.org/cfreundii/). Replicon content was detected by PCR-based Replicon Typing (PBRT 2.0 kit DIATHEVA; Carattoli A *et al.* 2005).

Conjugation experiments

Conjugation experiments for *E. coli* 2 Liver, *E. coli* 7 Intestine and *E. coli* 8 Intestine were performed using rifampicin resistant *E. coli* K12 J53 (met,

pro-, lac+, Rif^r) streptomycin resistant *E. coli* K12 J62 (pro-, his-, trp-, lac, Sm^r) as recipient, selecting transconjugants on 100 µg/ml rifampicin plus 8 µg/ml cefotaxime and on 100 µg/ml streptomycin plus 8 µg/ml cefotaxime.

Resistance gene investigations

Check-MDR CT103XL (Checkpoint) microarray and/or PCR and sequencing were used for resistance genes investigation. Primers sequences are:

*bla*CTX−M-type FW 5′-ATGTGCAGYACCAGTAARGT-3′
REV 5′-TGGGTRAARTARGTSACCAGA-3′
*bla*CTX−M group-1 FW 5′-GGTTAAAAAATCACTGCGTC−3′
REV 5′- TTGGTGACGATTTTAGCCGC-3
*bla*CMY FW 5’-TGGCCAGAACTGACAGGCAAA-3’
REV 5’-TTTCTCCTGAACGTGGCTGGC-3’
*bla*DHA FW 5’- AACTTTCACAGGTGTGCTGGGT-3’
REV 5’- TCAGCAGATCCGCACGGCTT- 3’
aac(6')-Ib-cr FW 5′-TTGCGATGCTCTATGAGTGG-3′
REV 5′-GCGTGTTCGCTCGAATGCC-3′

NGS and plasmid analysis

Long-reads sequence data were obtained from *E. coli* 2 Liver parental strains using PacBio next generation sequencer (PacBio Sequel System, Pacific Biosense). *De novo* assembly was performed using the softwares SMRT cell portal. Resistance and replicon content were determined by using default threshold parameters in ResFinder (https://cge.cbs.dtu.dk/services/ResFinder/; Zankari E *et al.* 2012) and in PlasmidFinder (https://cge.cbs.dtu.dk/services/PlasmidFinder/; Carattoli

A *et al.* 2014). Plasmid sequences were annotated by RAST. Plasmid images were obtain using SnapGene software (GSL Biotech LLC).

4. Results

All the isolates (n=2 from liver and n=3 from intestine) revealed resistance to ampicillin, piperacillin, third generation cephalosporins, aztreonam, ciprofloxacin, gentamycin and moxifloxacin. The strains showed an identical clonal profile by PFGE. WGS analisys, assessed on a strains chosen as representative, revealed the belonging to serotype O39, ST58/ST186 (according to Oxford and Pasteur scheme respectively) and the presence of FimH32. Concerning the resistance determinats content, the strain contained β-lactams- (*bla*OXA-1, *bla*TEM-1b, *bla*CTX-M-15, *bla*CMY-2, *bla*DHA-1), fluoroquinolone- (*qnrB1, qnrB4, aac(6')-Ib-cr*) and aminoglycoside- (*aac(6')-Ib-cr, aac(3)-Iia, aph(6)-Id, aph(3")-Ib, aadA1*), phenicol- (*catB3, catA1*), sulphonamide- (*sul1, sul2*), trimethoprim- (*dfrA1, dfrA17*) and tetracycline-resistance (*tet(A)*). The PlasmidFinder analisys highlighted the multi-plasmid co-existence: IncFII, IncHI2, IncHI2A, IncI1, IncX1 and IncX4. Moreover, it carried virulence genes associated with *astA*, *gad*, *iss* and *lpfA* coding for heat-stable toxin, glutamate decarboxylase, increased serum survival protein, long polar fimbriae respectively. The β-lactamases and *aac(6')-Ib-cr* genes were transferable through conjugation.

Table 4.1: resistance genes content, replicons, ST and virulence factors of the studied strain

	E. coli 2 Liver
β-lactamases	*bla*OXA-1, *bla*CTX-M-15, *bla*TEM-1B, *bla*CMY-2, *bla*DHA-1
Aminoglycoside	*aac(6')-Ib-cr, aac(3)-Iia, aph(6)-Id, aph(3'')-Ib, aadA1*
Fluoroquinolone	*qnrB1, qnrB4, aac(6')-Ib-cr*
Macrolide	*mph(A), mdf(A)*
Phenicol	*catB3, catA1*
Sulphonamide	*sul1, sul2*
Trimethoprim	*dfrA17, dfrA1*
Tetracycline	*tet(A)*
Replicons	IncFII, IncHI2, IncHI2A, IncI1, IncX1, IncX4
ST	58/186
Virulence factors	*astA, gad, iss, lpfA*

The isolate contained three main plasmids:

a) a 302,597 bp IncHI2α replicon plasmid (pMBC_CTX-M-15). pMBC_CTX-M-15 plasmid backbone exhibited high similarity scores with a 300,375 bp plasmid, carried by a Salmonella typhimurium strain isolated from a Kenyan patient (LN794248.1; Kariuki S *et al.* 2015) (100% sequence identity, 99% query coverage), and with the 309608 bp pEc21617-310 plasmid carried by an *E. coli* strain isolated from human urine specimen in Taiwan (MG878867.1) (99.99% sequence identity, 96% query coverage). In this plasmid harboured *dfrA14, catA1, sul2, aac(6')-Ib-cr, qnrB1, tet(A), bla*OXA-1, *bla*TEM-1b, *bla*CTX-M-15, *aac(3)-Iia*

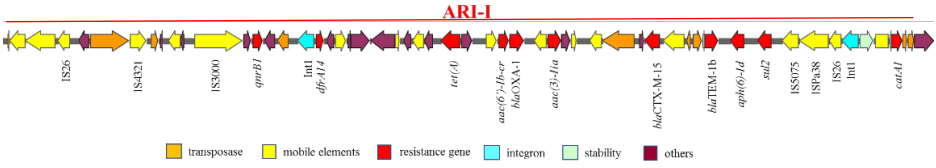
and *aph(6)-Id* resistance genes. Moreover, the plasmid carried one antibiotic resistance islands (ARI-I); ARI-I, 91,179 bp in size, carried genes coding for β-lactams- (*bla*OXA-1, *bla*TEM-1b, *bla*CTX-M-15), fluoroquinolone- (*qnrB1, aac(6')-Ib-cr*) and aminoglycoside- (*aac(6')-lb-cr, aac(3)-Iia, aph(6)-Id*), sulphonamide- (*sul2*) and phenicol- resistance (*catA1*). Moreover, ARI-I is rich in IS element and trasposases, such as IS26 (Figure 4.1a). ARI-I resembled high similarity scores with plasmid pSTm-A54650 from *Salmonella enterica* collected in Sub-Saharan Africa from blood sample (96% coverage and 100% identity; LK056646) and with pCRENT-193_1 from *Enterobacter spp.* collected in South Korea from human wound sample (100% coverage and identity; CP024813.1). The plasmid backbone carried regions responsible for replication (*repA* and *repB*), conjugative transfer system (*tra* and *thr* genes), maintenance and stability (*parA*), arsenic resistance system (*arsH*), tellurium resistance genes (*terA*, *terC*, *terD*) and toxin-antitoxin (TA) system (hipB/higA) (data not showed).

b) An 90,249 bp IncI1 replicon plasmid (pMBC_CMY-2) showing high similarity score with an 87,777 bp plasmid carried by an *E. coli* strain of canine origin isolated in Edinburgh (CP023385.1; 99.98% sequence identity, 96% query coverage) and with a 94,697 bp plasmid carried by *S. enterica* of food origin isolated in China (KX058576.1; 99.80% sequence identity, 98% query coverage). In this plasmid harboured the *bla*CMY-2 determinat only, flanked by an upstream IS1294 and a downstream IS1414 (Figure 4.1b). Additionally, pMBC_CMY-2 backbone carried regions responsible for replication (*repA*) and conjugative transfer system (*tra* and *pil*).

c) An 83,429 bp IncFII replicon plasmid (pMBC_DHA-19) having similarity score with the p133355_SW_C4_Cam-1 plasmid (81,724 bp), carried by *Citrobacter amalonaticus* collected from human stool specimen in Switzerland (CP041363.1; 99.64% sequence identity, 97% query coverage), and with the pUB_DHA-1 plasmid (81,754 bp), existing in an *E. coli* strain from human urine sample (MK048477; 99.55% sequence identity, 97% query coverage). In this plasmid harboured *sul1, qnrB4* and *bla*DHA-1 resistance genes. The plasmid backbone carried regions responsible for replication (*repA*) and conjugative transfer system (*tra* and *trb* genes) (data not showed). Moreover, the plasmid carried ARI-II island (22,425 bp), containing the above resistance genes and flanked by IS26 up- and downstream (Figure 4.1c). ARI-II had high similarity scores with a 184,614 bp plasmid, contained in an *E. coli* strain collected from forest soil in China (CP010239.1; 99.98% sequence identity, 100% query coverage), and with a chromosome region of a *Shighella sonnei* collected in India from human faeces specimen (CP041322.1; 99.98% sequence identity, 100% query coverage).

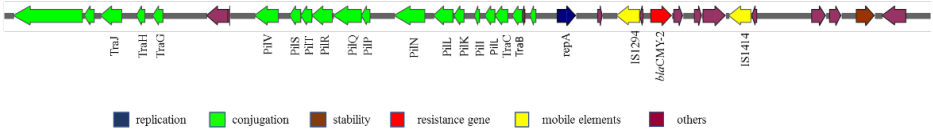
a)

pMBC_CTX-M-15



b)

pMBC_CMY-2



c)

pMBC_DHA-1

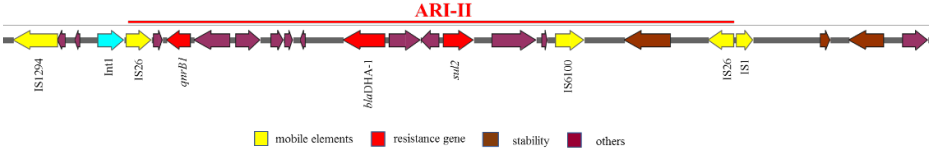


Figure 4.1: linear map of the plasmids; a-b-c) schematic representation of the genetic environment of the three studied plasmids.

4. Discussion and Conclusions

Here, we describe a deadly infection case in a bulldog breeding in Italy. Pets, breeding and food-chain animals are recognized as important reservoir of AMR bacteria. Many papers in literature focus on determinats resistance investigation, such as ESβLs and fluoroquinolone resistance genes, in such animals. However, the co-presence of *bla*CTX-M-15, *bla*CMY-2, *bla*DHA-1 and *aac(6')-Ib-cr* in the same toxigenic strain of animal origin is a concerning event, not yet reported in literature. Moreover, very few cases of *E. coli bla*DHA-1 positive are reported in veterinary medicine (Belas A *et al.* 2014). The genomic environment structure of the three plasmids is well conserved, showing high similarity with worldwide reported plasmids from both human and animal/environment settings. This suggest that the global spread of such plasmids and the human/animal contact play a central role in the diffusion of MDR bacteria.

The source of infection still remains unknown; however: i) the breeding has had a previous history of *E. coli* infections; ii) the puppies' mother, during pregnancy, resulted positive for the presence of *Streptococcus pyogenes* and *Mycoplasma* (treated with ciprofloxacin and cefaclor) but the possible presence of an *E. coli* strain has never been assessed, even after the infection event; iii) in the period of isolation, a strong drought had struck in the Pesaro province. Villages' people used water from a cistern for every-day using, even the owners' breeding that used this water for the preparation of powdered milk to be given to puppies. We suggest the cistern water as a possibly cause of infection, although similar strains were not detected/investigated, and no cases of infection were reported in human population.

In conclusion, this infection event wants to emphasize the impact of human/animal contact in MDR bacteria transmission. According to "One

Health approach", is even essential the continuous monitoring of animal and environment setting for a better comprehension of the "spread routes" of such strains.

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List of original manuscripts

Bitar I., Caltagirone M., Villa L., Mattioni Marchetti V., Nucleo E., Sarti M., Migliavacca R., Carattoli A. Interplay among IncA and blaKPC carrying plasmids in Citrobacter freundii. Antimicrobial Agents and Chemotherapy, 2019.

Abstract

Here we report two KPC-producing *Citrobacter freundii* isolates from unrelated patients. In one case, bla_{KPC-2} was harbored on a novel variant of Tn*4401* transposon of an IncN plasmid, conjugated together with a co-resident IncA plasmid, while in the other one, bla_{KPC-3} was on a Tn*4401a* located on a IncX3-IncA self-conjugative plasmid fusion. The interplay occurring among plasmids carrying bla_{KPC} and the co-resident IncA plasmids offers new information on plasmids co-resident within clinically relevant enterobacteria.

Carbapenem-resistant *Enterobacteriaceae* is emerging as a global threat, especially due to the production of carbapenemase enzymes [1]. The most frequent carbapenemases in clinical isolates of human origin are the class A KPC enzymes, which spread mostly in *Klebsiella pneumoniae*, but also at lower frequency in other *Enterobacteriaceae* such as *Citrobacter freundii* [2,3]. *C. freundii* is considered a low risk pathogen in clinical settings but can act, as in the two examples described here, as a silent reservoir of relevant resistance genes.

Two strains of *C. freundii*, designated as AA535 and AA593 were isolated on the February-March 2016 period, from a screening rectal swab of a patient recovered at the S. Agostino-Estense-Baggiovara hospital in Modena, Italy, and from a urine sample of a resident in a long term care facility of the same geographical area, respectively. The two patients did not have any apparent epidemiological link.

Identification of the bacterial species was performed using Vitek 2 System (bioMérieux, Marcy l'Etoile, France). Antimicrobial susceptibility was assessed by reference broth microdilution and MICs were interpreted according to EUCAST 2019 breakpoints (http://www.eucast.org). The two strains were genotyped by XbaI digestion and Pulsed Field Gel Electrophoresis (PFGE; [4]) and Multilocus Sequence Typing (MLST; [5]). The two isolates exhibited different PFGE patterns (data not shown) and the MLST profiles, assigned by the PubMLST database (https://pubmlst.org/cfreundii/), resulted ST19 and ST46 for AA593 and AA535, respectively. Replicon content was detected by PCR-based Replicon Typing (PBRT 2.0 kit DIATHEVA; [6]). PBRT detected A/C and N replicons in AA535 and A/C and X3 replicons in AA593, respectively.

Conjugation experiments for AA535 and AA593 were performed using streptomycin resistant *E. coli* K12 J62 (*pro*$^-$, *his*$^-$, *trp*$^-$, *lac*$^-$, *Sm*^{R}) as recipient, selecting transconjugants on 100 µg/ml ampicillin plus 150 µg/ml streptomycin and on 6 µg/ml meropenem plus 150 µg/ml streptomycin. Ten transconjugants colonies from each experiment were identified as *E. coli* by

Matrix-Assisted Laser Desorption Ionization-Time Of Flight mass spectrometry (MALDI-TOF MS) using MALDI Biotyper software (Bruker Daltonics, Bremen, Germany). PBRT 2.0 kit detected A/C and N replicons in the all the selected AA535 transconjugants and A/C and X3 replicons in all AA593 transconjugants, respectively.

Plasmids were extracted from donors using PureYield Plasmid Midiprep kit (Promega, USA) and transformed into chemically competent *E. coli* DH5α cells (Invitrogen, USA). Transformants were selected on Luria-Bertani agar plates containing 100 µg/ml ampicillin (Sigma). Transformation of AA535 provided two types of transformants, positive to A/C and N replicons, respectively. Presence of the bla_{KPC} gene was detected by PCR using the universal primers KPC_FU_1_8 5'-GTGCAGCTCATTCAAGGG-3' and KPC_RU_1_8 5'-GCCAATCAACAAACTGCTG-3'. The bla_{KPC-2} gene was identified in the IncN positive transformants. Transformation of AA593 yielded one type of transformants, positive by PBRT to both A/C and X3 replicons and to the bla_{KPC-3} gene.

Short-read sequence data were obtained from AA535 and AA593 parental strains using Illumina MiSeq next generation sequencer (Illumina Inc., California, USA). *De novo* assembly was performed using the softwares Spades 3.10 and A5 MiSeq (http://bioinf.spbau.ru/spades; [7,8]) using the default spades parameters. Resistance and replicon content was determined by using default threshold parameters in ResFinder (https://cge.cbs.dtu.dk/services/ResFinder/; [9]) and in PlasmidFinder (https://cge.cbs.dtu.dk/services/PlasmidFinder/; [10]) respectively. Assembled genomes were also tested by BLASTN against the IncA (FJ705807), IncN (AY046276) and IncX3 (JN247852) plasmid reference sequences, as suggested by PlasmidFinder results. Therefore, IncA (previously IncA/C1, renamed by Ambrose *et al.* 2018; [11]) and IncN plasmid sequences were identified in the AA535 genome in one (144490 bp) and two (33378 bp, 15559 bp) large scaffolds, respectively. The AA535 genome was also positive for Col440 I and Col440I small plasmids (9532 bp, 1736 bp). IncA and IncX3 plasmid

sequences were identified in five large scaffolds (12876 bp, 57440 bp, 45859 bp, 13171 bp and 52783 bp) in the AA593 genome. In addition, IncQ1 was also detected within this genome. Complete plasmid sequence assembly was obtained by checking pair-end overlaps and performing PCR-based gap closure, using plasmids from both donors and transconjugants as DNA templates. Plasmid sequences were annotated by BLASTP (https://blast.ncbi.nlm.nih.gov/Blast.cgi?PAGE=Proteins).

The pIBAC_IncA plasmid (MH594477) of AA535 was 145294 bp in size and carried a class I integron with *aac(6')-Ib-cr5*, bla_{OXA-1}, *catB3* gene cassettes. It showed 99% nucleotide identity, 90% coverage with pIncAC-KP4898 (KY882285; [12]), a bla_{VIM-1}-positive plasmid described in a *K. pneumoniae* from Naples, Italy. The co-presence of pIBAC_IncA and IncN plasmids was identified in most of the AA535 transconjugants by PBRT kit; the presence of bla_{KPC} gene was confirmed by PCR in the same strains. An integration of IS*3* family element (IS*Ehe3*) was observed between the *traW* and *traU* genes, which were not disrupted by this integration.

The pIBAC_IncN plasmid (MH536949) of AA535 was 49804 bp in size, and showed 99% nucleotide similarity, 95% coverage with pKp148, PINH-4900 (KX062091), recovered from a *K. pneumoniae* strain of an urban river in Brazil. The IncN plasmid carried a bla_{KPC-2} on a novel variant of Tn*4401* transposon designated by Tn*4401i*, integrated within the *nuc* gene (Figure 1). This transposon had the same genetic environment surrounding the bla_{KPC-2} gene when compared to Tn*4401b*, except for a deletion of 260 bp upstream bla_{KPC-2} (Figure 1). The IncN sequence type was determined as ST15 at the pMLST (https://cge.cbs.dtu.dk/services/pMLST/). ST15 IncN plasmids were described to be efficient shuttles between a variety of species and clones in a study conducted on KPC-producing *Enterobacteriaceae* in Nitanya [13].

The AA593 pIBAC_IncX3_IncA (MH594478) plasmid of 192802bp was a fusion of IncA and IncX3 plasmids. The occurrence of such IncA-IncX3 plasmid fusion in the parental strain was ascertained by the results of the transformation experiments here described: an unique type of *E.*

coli DH5α transformant, positive to both A/C and X3 replicons and to the bla_{KPC-3} gene, was obtained.

The bla_{KPC-3} gene was associated to a Tn*4401a* transposon flanked by truncated *tnpA* of IS*3000*, in a configuration highly similar to the IncX3 plasmid pCfr-145 (KY659388), recently identified in several *C. freundii* in Italy (Figure 2) [14]. The IncA fused portion of the pIBAC_IncX3_IncA plasmid showed 99% nucleotide identity, 92% coverage with the pIBAC_IncA plasmid of AA535, carrying the same class I integron with *aac(6')-Ib-cr5*, bla_{OXA-1}, *catB3* gene cassettes, but a different IS*3* element (IS*Sen4*) integrated within the *traW* and *traU* genes. The fusion of the IncA and IncX3 plasmids in pIBAAC539 could have been mediated by recombination between IS*26* elements. The IncX3 plasmid scaffold was interrupted by two directly repeated IS*26* elements located next to the bla_{SHV-11}, followed by the resistance region of the IncA plasmid. Moreover, hypothetical proteins and an IS*903* which were located in proximity of the IS*26* in pIBAC_IncA plasmid of AA535 scaffold were lost in the fusion process in pIBAC_IncX3_IncA plasmid of AA593 and a truncated IS*Lad1*, was inserted just before the *stbA* stability gene, probably independently by the fusion process (Figure 2).

In conclusion, both *C. freundii* strains had the same resident IncA plasmid. In AA593, it was permanently fused to IncX3 carrying the bla_{KPC-3}. In the other strain, despite the two plasmids were physically distinct in the donor and separately transferred in transformation experiments, they conjugated together with the co-resident IncN plasmid carrying bla_{KPC-3}. The role of the companion IncA plasmids in these *C. freundii* isolates is not clear. It cannot be predicted if the transfer locus of IncA plasmids was functional or not, while those of the IncX3 and IncN appeared well conserved. IncA could have contributed to stabilize the co-resident IncX3 plasmid in one strain, and used the IncN as a helper plasmid for conjugation in the other. The IncA plasmids could be normal resident of *C. freundii* and may favor adaptation, replication and

stability of the plasmids imported from other clinically relevant, carbapenemase-producing *Enterobacteriaceae*, favoring their spread. On the other side, IncA could be symbionts of other co-resident plasmids, using their conjugative properties to spread.

Nucleotide sequence accession numbers

The nucleotide sequences of the plasmids, pIBAC_IncA, pIBAC_IncN and pIBAC_IncX3_IncA have been deposited in GenBank and the following accession numbers have been assigned respectively; MH594477, MH536949 and MH594478.

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Transparency declarations

We have no conflicts to declare.

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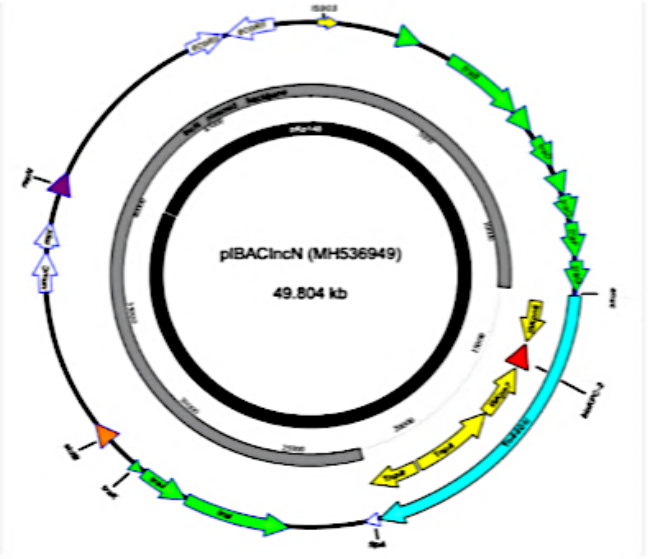
Figure 1: a) Circular map of pIBACIncN plasmid compared to pKp148 (black circular box); green arrows represent conjugal transfer system of the plasmid, red arrow represent the *bla*$_{KPC}$ gene, yellow arrows represent mobile elements, white arrows represent hypothetical proteins, purple arrow represent the replication protein repN and orange arrow represent stability protein stdB. Further more, the gray circular box represents the plasmid backbone while the transposon Tn*4401i* is represented in a blue arrow. b) Alignment in the position of interest between the sequence of Tn*4401i* against the Tn*4401b*. -35 and -10 represent a putative promoter region, while +1 represent the putative transcription starting point and RBS represent the ribosome-binding site.

Figure 2: Linear map of pIBAC_IncA and pIBAC_IncX3_A; Arrows show the direction of transcription of ORFs while rectangles show truncated ORFs. Replicons, partitioning genes,

mobile elements, conjugal transfer genes, antibiotic resistance, bla_{KPC-3} and other remaining genes are designated by violet, gray, yellow, green, red, blue and white respectively. Grey shaded area in the upper part shows the similarity of both IncA plasmids while the lower part shows the IncX3 plasmid region of pIBAC_IncX3_A along with its insertion/fusion point, which is shown as black stripped yellow arrows/rectangles in the IncX3 region.

a)

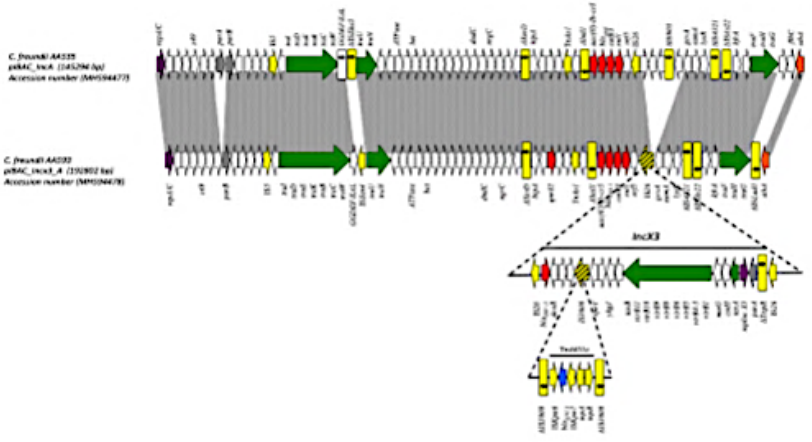


b)

```
Tn4401b   1
          CACCGGCGTA CCCTCGGTGC TATCTTCGCG CCCCAATAGT CGGGGCTTGG CCAGGACTTC CTGAGGCCGT
Tn44011   CACCGGCGTA CCCTCGGTGC TATCTTCGCG CCCCAAT--- ---------- ---------- ----------

Tn4401b   71
          CCGTAACGTG GATGCCGAGG TCAGGCGAGG TGGCCGACCC ATGAACGCCG ACCTGATTCG TTTTTCAATA
Tn44011   ---------- ---------- ---------- ---------- ---------- ---------- ----------

Tn4401b   141
          GCGCTGGGCG TTGTGGTGCC AGGGACTTAC CAACCCGATG TGTGCCCATC CGGGGCAGTT ACAGCCGTTA
Tn44011   ---------- ---------- ---------- ---------- ---------- ---------- ----------
                                                  -35                  -10
Tn4401b   211
          CAGCCTCTGG AGAGGGAGCG GCTTGCCGCT CGGTGATAAT CCCAGCTGTA GCGGCCTAAT TACATCCGGC
Tn44011   ---------- ---------- ---------- ---------- ---------- ---------- ----------
          +1                        RBS
Tn4401b   281
          CCCTACACCT AGCTCCACCT TCAAACAAGG AATATCGTTG ATG blaKPC
Tn44011   ---------- --------CT TCAAACAAGG AATATCGTTG ATG
```



Espinal P., Nucleo E., Caltagirone M., Marchetti V.M., Fernandes M.R., Biscaro V., Rigoli R., Carattoli A., Migliavacca R., Villa L. Genomics of Klebsiella pneumoniae ST16 producing NDM-1, CTX-M-15 and OXA-232. Clin Microbiol Infect. 2018 Nov 22. doi: 10.1016/j.cmi.2018.11.004.

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Research note

Genomics of *Klebsiella pneumoniae* ST16 producing NDM-1, CTX-M-15, and OXA-232

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ABSTRACT

Objectives: Genomic characterization of the internationally spread sequence type (ST) 16 carbapenem-resistant *Klebsiella pneumoniae*.

Methods: The complete genomes of three carbapenem producing ST16 *K. pneumoniae* from Italian patients were analysed by single-nucleotide polymorphism-based phylogeny, core genome multilocus sequence typing, resistance, plasmid, and virulence content and compared with ten genomes of ST16 strains isolated in other countries. Plasmids carrying bla_{NDM-1} or $bla_{OXA-232}$ carbapenemase genes were assembled and sequences were analysed.

Results: The internationally spread ST16 *K. pneumoniae* clone showed variability in terms of distribution of NDM-1 and OXA-232 type carbapenemases. In some ST16 strains, up to six plasmids can be simultaneously present in the same cell, including ColE-like plasmids carrying $bla_{OXA-232}$ and IncF plasmids carrying bla_{NDM-1}. The differences observed in plasmid, resistance, and virulence content and core genome suggested that there is not a unique, highly conserved ST16 clone, but instead different variants of this lineage circulate worldwide.

Conclusions: The ST16 *K. pneumoniae* clone has spread worldwide and may become a high-risk clone.

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Introduction

Klebsiella pneumoniae represents one of the most common opportunistic hospital-associated pathogens and is a major source for multi-antibiotic resistance, including carbapenem resistance, because of the spread of high-risk clones [1]. In *K. pneumoniae* most of the carbapenemase genes have been identified on large, self-conjugative plasmids [1], except $bla_{OXA-232}$, which is frequently encoded by small ColE-like plasmids [2]. Moreover, carriage of different carbapenemase genes in the same strain has been observed, such as the association of bla_{NDM-1} and $bla_{OXA-232}$ in sequence type (ST) 14 [3].

In this study, we report the characterization of ST16 *K. pneumoniae* strains, producing NDM-1, OXA-232 or both, causing an outbreak in the Treviso Hospital area, northern Italy. ST16 has been reported worldwide, showing different antimicrobial resistance profiles. In 2011, *K. pneumoniae* ST16 harbouring CTX-M-15 was the cause of nosocomial infections in Denmark and Sweden [4]. NDM-5-carrying ST16 *K. pneumoniae* was described in Denmark [5], and later the co-presence of NDM-1 and OXA-232 in ST16 *K. pneumoniae* was observed in Italy [6].

Materials and methods

Ethics declaration

This was an observational study on strains selected from anonymized databases. The study did not require the approval of an ethics committee.

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Table 1
Plasmid and resistance content of the fully sequenced ST16 *K. pneumoniae* strains analysed in this study

Strain	KL8	KL11	KL29	FDAARGOS_440	Q517-0029	CNR48	AR_0087	DA33141	UCLAOXA232KP	UCLAOXA232KP
Country	ITALY	ITALY	ITALY	CANADA	THAILAND	FRANCE	NA	SWEDEN	USA	USA
Date	7/3/17	22/3/17	21/9/16	01/2015	04/2017	2012	NA	NA	12/10/14	11/3/15
NCBI GenBank accession numbers	REGZ00000000.1	REGY00000000.1	REHA00000000.1	CP023919 (Chr) CP023922 CP023923 CP023924	CP024038 (Chr) CP024039 CP024040 CP024041 CP024042 CP024043 CP024044	LS399318 (Chr) LT994835 LT994840	CP029738 (Chr) CP029739 CP029740	CP029587 (Chr) CP029588 CP029589	CP012561 (Chr) CP012562 CP012563 CP012564 CP012565 CP012566 CP012567	CP012568 (Chr) CP012569 CP012570 CP012571 CP012572
Acquired resistance genes[a]										
bla_{TEM-1A}		pos	pos	pos	pos					
bla_{TEM-1B}				pos		pos		pos		
$bla_{CTX-M-15}$	pos	pos	pos	pos	pos	pos		pos	pos	
bla_{OXA-9}		pos		pos	pos					
bla_{OXA-1}						pos		pos	pos	
bla_{NDM-1}	pos	pos		pos	pos					
$bla_{OXA-232}$	pos		pos	pos	pos				pos	
bla_{DHA-1}						pos				
bla_{SHV-12}						pos	pos			
aadA1		pos		pos	pos					
aadA2	pos	pos		pos	pos	pos		pos		
aac(6')-Ib		pos		pos	pos					
aac(6')-Ib-cr					pos	pos		pos	pos	
aac(6')-Ilc						pos				
aph(3'')-Ib						pos				
aph(6)-Id						pos				
aph(3')-Ia						pos				
aac(6')-Ib3									pos	pos
sul1	pos	pos		pos	pos	pos		pos	pos	pos
dfrA1									pos	pos
dfrA12	pos	pos		pos	pos	pos		pos		
dfrA19						pos				
catB4						pos		pos	pos	pos
mph(A)		pos		pos		pos				
tet(A)						pos	pos	pos		
tet(B)	pos	pos		pos	pos					
qnrB1			pos	pos			pos			
qnrB4						pos				
erm(B)				pos						
rmtB				pos						
rmtF									pos	pos
mcr-1					pos					
ere(A)						pos				
ARR-2									pos	pos
ARR-3									pos	pos
Plasmids	**Replicons**[b]									
IncF	FII_36	FII_36	FII_36	FII_36	FII_36				FII(pKPX1)	FII(pKPX1)
	FII_22	FII_22	FII_22	FII_22	FII_22					
	FIA_1	FIA_1	FIA_1	FIA_1	FIA_1					
	FIB_20	FIB_20	FIB_20	FIB_20	FIB_20				FIB(pKPHS1)	FIB(pKPHS1)
		FIIK_2	FIIK_2	FIIK_2	FIIK_2	FIIK_5	FIIK_2	FIIK_5	FIIK_5	FIIK_5
		FIB_K (pQil)	FIB_K (pQil)	FIB_K (pQil)	FIB_K (pQil)	FIB_K (pKN3)		FIB_K (pKN3)	FIB_K (pKN3) FIIK_7 (IncR)	FIB_K (pKN3) FIIK_7 (IncR)

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ColE	ColKP3		ColKP3	ColKP3	ColKP3				ColKP3	
	ColE440_2	ColE440_2	ColE440_2		ColE440_2			ColE440_2		
	ColE440_1	ColE440_1	ColE440_1		ColE440_1					
IncHI2						HI2				
IncX					X4	HI2A			X3	

NA, not available; Chr, accession number of the bacterial chromosome, the other accession numbers listed below the chromosome refer to plasmid sequences identified in the same strain and separately submitted in GenBank.
* Acquired resistance genes determined by ResFinder 3.0 (https://cge.cbs.dtu.dk/services/ResFinder/).
† Replicons detected by PlasmidFinder 2.0 (https://cge.cbs.dtu.dk/services/PlasmidFinder/).

Whole genome sequencing

Whole genome sequencing (WGS) was performed using the Illumina MiSeq instrument (Illumina Inc., San Diego, CA, USA). *De novo* assembly was performed by SPADES (Galaxy Version 3.11.1). Plasmid and resistance genes were detected using PlasmidFinder [7] and ResFinder [8], respectively. Replicon alleles were assigned at the plasmid multilocus sequence typing (pMLST) site (https://pubmlst.org/plasmid/).

The core genome MLST on 629 alleles (cgMLST629_S) was identified using the *Klebsiella* Sequence Typing tool (http://bigsdb.pasteur.fr/klebsiella/klebsiella.html) [9,10].

The whole-genome shotgun projects of the KL8, KL11, and KL29 strains have been deposited at DDBJ/EMBL/GenBank under the accession nos. REGZ00000000.1, REGY00000000.1 and REHA00000000.1, respectively).

Plasmid analysis

Purified plasmid DNA was used to transform Library Efficiency DH5α-competent cells (Invitrogen, Carlsbad, CA, USA), selecting the transformants on Luria–Bertani agar plates, containing ampicillin 50 mg/L. Transformants were analysed by plasmid restriction analysis and screened by PCR for the presence of bla_{NDM-1}, $bla_{CTX-M-15}$, and $bla_{OXA-232}$ resistance genes.

Plasmid assembly was obtained mapping ST16 contigs on reference plasmid sequences, checking overlapping paired ends and confirming the assembly by the PCR-based gap closure method. Plasmids were annotated at the RAST server (http://rast.nmpdr.org/).

Phylogenetic analysis

Single-nucleotide polymorphism (SNP) analysis was performed by the Kn35NP (Galaxy version 3.1) software at the ARIES public Galaxy server (https://w3.iss.it/site/aries/) on *K. pneumoniae* genomes from three Italian ST16, ten international ST16, and 22 belonging to other STs (differing from ST16 for one, two, or three alleles, respectively).

Results

Characteristics of the outbreak strains

An outbreak involving 23 infected/colonized patients occurred from 24 February to 29 March 2017 in three different wards of the Treviso Hospital and two long-term care facilities (LTCFs) in the Treviso area. The outbreak clone was identified as ST16 by MLST and pulsed field gel electrophoresis. Retrospectively, nine ST16 carbapenem-resistant strains were isolated in these hospitals before the outbreak from September 2016 to January 2017 and included in the study (Table S1).

The 32 Italian ST16 showed different distribution of two major carbapenemase genes: 14 strains were positive for bla_{NDM-1}, two for $bla_{OXA-232}$, and 16 for both bla_{NDM-1} and $bla_{OXA-232}$ (Table S1). Six carbapenem-resistant strains were also CTX-M-15 extended-spectrum β-lactamase (ESBL) producers.

KL8 (LTCF, March 2017), producing NDM-1, CTX-M-15, and OXA-232, and KL11 (Treviso hospital, March 2017), producing NDM-1 and CTX-M-15, were selected for WGS as prototypic strains of the outbreak clone. KL29 (LTCF, September 2016), producing CTX-M-15 and OXA-232 was also sequenced as prototypes of the strains collected before the outbreak (Table 1).

located in a complex class 1 integron, carrying the *aadA2* and *dfrA12* resistance gene cassettes (Fig. S1). The arginine deaminase (ADI) cluster (*arcA*, *arcB*, *arcC*, and *arcD* genes) was trapped between the two F_{22}:F_{36} replicons [13].

Interestingly, the F_{22}:F_{36}:A_1:B_{20} scaffold was detected in strain KL29, but not the variable region including the bla_{NDM-1} and linked resistance genes (Table 1).

The OXA-232 ColE plasmids

The $bla_{OXA-232}$ gene was located on a 6141-bp plasmid carrying the ColKP3 replicon. This plasmid was obtained by transformation from strains KL8 and KL29 (pKL29-OXA-232; MH523449). It showed the best match (99% nucleotide identity and 100% coverage) with OXA-232 plasmids from QS17-0029 (CP024042), FDAARGOS_440 (CP023924) and the plasmid of the ST14 strain PittNDM1 from USA (CP006802) [3]. All these ColE-like plasmids carried the mobilization system (MobA-D), a replication gene (*repA*), truncated parts of erythromycin esterase (Δ*ereA*) and the transcriptional regulator (Δ*lysR*). Furthermore, all plasmids showed 206 bp upstream of the $bla_{OXA-232}$ corresponding to a vestigial, deleted ΔISEcp*1* insertion sequence.

Location of $bla_{CTX-M-15}$ in ST16 K. pneumoniae

The $bla_{CTX-M-15}$ gene was localized in the chromosome of the three KL8, KL11, and KL29 strains, flanked by the insertion sequence ISEcp*1* and the truncated transposon ΔTn*2*, integrated in the metal-dependent hydrolase tRNA synthetase gene (MH523447). The same structure and integration site was also found in the chromosome of QS17-0029 [11] (between positions 2449972 and 2452365 in CP024038), while it was absent in the chromosome of FDAARGOS_440. However, QS17-0029 (CP024040) and FDAARGOS_440 (CP023922) and strains from Sweden (CP029588) and France (LT994840) carried a plasmid copy of the $bla_{CTX-M-15}$ gene located on IncFIIk.

Discussion

The small number of isolates investigated in this study limited the possibility of describing the emergence and evolution of the outbreak strains within the hospital. However, by comparison with internationally isolated *K. pneumoniae* strains, we demonstrate that ST16 showed considerably divergent genomes, suggesting the existence of different lineages. The most clinically relevant difference lay in the acquisition of multiple plasmids conferring multidrug resistance, including carbapenem resistance. The bla_{NDM-1} and $bla_{OXA-232}$ genes, located on different plasmids, were detected within some strains of the ST16 clone, with up to six plasmids simultaneously resident within the same cell. The versatility to acquire and exchange resistance plasmids suggests that ST16 may become a high-risk clone resulting in important healthcare-associated infections.

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Transparency declaration

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.cmi.2018.11.004.

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SHORT COMMUNICATION

Detection of ST1702 *Escherichia coli* bla_{NDM-5} and bla_{CMY-42} genes positive isolates from a Northern Italian hospital

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SUMMARY

We describe two multi drug-resistant (MDR) carbapenemase-producing *Escherichia coli* clinical isolates from an acute hospital in Milan. Both strains, isolated from a surgical wound sample and a surveillance rectal swab respectively, were positive for a bla_{NDM}-type gene by Xpert Carba-R test. The whole-genome sequence characterization disclosed several resistance determinants: bla_{NDM-5}, bla_{CMY-42}, bla_{TEM-1B}, *rmtB*, *mphA*. The two isolates belonged to phylogenetic group A, sequence type (ST) 1702 and serotype O89:H9. PCR-based replicon typing and conjugation assay demonstrated an IncI1 plasmid localization for both bla_{NDM-5} and bla_{CMY-42} genes. This is the first report of a ST1702 NDM-5 and CMY-42- producing *E. coli* clone in Italy.

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Emergence of New Delhi metallo β-lactamase (NDM)-producing *Enterobacteriaceae* has become a crucial issue of global concern. The bla_{NDM} gene not only confers resistance to most β-lactams, but is often accompanied by several resistance determinants, thus making pathogens multidrug-resistant. NDM-producing bacteria have been implicated in both hospital - and community - acquired infections and have been recovered from several infection sites, including those associated with septicemia, urinary tract infections, and wound infections as well as from companion and livestock animals (Ranjan *et al.*, 2016). The evolution and spread of NDM are rapid, and to date, 21 variants of NDM enzyme have been reported (Naas *et al.*, 2017). bla_{NDM-5} gene was first reported in an *Escherichia coli* strain (EC045) from a patient in the United Kingdom; the protein differed from NDM-1 by only two amino acid substitutions (Val88Leu and Met154Leu) and showed increased resistance to carbapenems and broad-spectrum cephalosporins (Hornsey *et al.*, 2011). Since then, NDM-5-producing strains have also been identified in Algeria, the United States, Australia, China, Denmark, India, Italy, Japan, Poland, Singapore, Spain, the South Korea, Egypt and the Netherlands (Zhu *et al.*, 2016).

Key words:
ST1702, *Escherichia coli*, NDM-5, CMY-42.

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Many reports have indicated a high sequence type (ST) diversity for bla_{NDM-5}-positive *E. coli* and there are several indications of isolates co-harbouring other resistance determinants like the plasmid-borne colistin resistance gene *mcr-1* (Chen *et al.*, 2017; Zhou *et al.*, 2017; Mediavilla *et al.*, 2016) or oxacillinase $bla_{OXA\ 181}$ (Rojas *et al.*, 2017; Gamal *et al.*, 2016). These data, together with the demonstrated horizontal transferability of the bla_{NDM} gene either through plasmids or transposon-related mobile elements, highlighted the importance of continuous epidemiological investigation and surveillance of NDM isolates.

Here we report the detection of two multi drug-resistant (MDR) NDM-5 *E. coli* clinical isolates from the ASST Fatebenefratelli-Sacco hospital (Milan). The first isolate was collected in July 2015 from an Italian man in his 60s with a known history of several hospitalizations. The patient, HIV-positive and suffering from an HCV-related chronic hepatitis, was admitted to the surgical ward for a small intestine partial resection. After surgery, a carbapenemase-producing *E. coli* strain, Ec_S1_3/4, was isolated from a wound sample; it was confirmed as NDM-positive by Xpert Carba-R test (GeneExpert, Cepheid).

The second strain, Ec_S1_6, was isolated in August 2015 from a screening rectal swab of a 76-year-old female patient transferred to the Medicine ward from a long-term care facility. The woman, affected by a urinary tract infection, was empirically treated with a piperacillin/tazobactam, meropenem and fluconazole. The therapy was discontinued based on a reduction of the inflammation indices. The bla_{NDM}-type determinant was detected by Xpert Carba-R test.

The identification of the two *E. coli* strains was obtained by Maldi-TOF MS (BioMèrieux), while the susceptibility profiles were determined by Vitek2 System (BioMèrieux) and in-

Table 1 - *Genetic features of the Ec_S1_3/4 and Ec_S1_6 isolates.*

Carbapenemase/AmpC enzyme	*MLST 1/2**	*Serotype*	*Resistance profile*
NDM-5, CMY-42	1702/2	O89:H19	$bla_{TEM\ 1B}$, *aadA*, *aadA5*, *sul1*, *tet*(A), *tet*(R), *dfr*A17, *dfr*A12, *mph*A, *rmt*B

*MLST 1: seven genes Achtman scheme, MLST 2: eight genes Pasteur scheme.

terpreted according to the EUCAST 2016 breakpoints. Both isolates (Ec_S1_3/4 and Ec_S1_6) resulted MDR, showing a high resistance level to all carbapenems, cephalosporins, fluoroquinolones and trimethoprim-sulfamethoxazole by Vitek2 System. The carbapenem MIC values, determined using the broth microdilution Sensititre system (Termoscientific, Italy), of the two isolates were 8 and ≥16 mg/L for imipenem, ≥16 mg/L for ertapenem and ≥8 mg/L for meropenem.
The genomic DNA was extracted with the QIAamp DNA minikit (Qiagen) following the manufacturer's instructions, and sequenced using Illumina Miseq (2 x 250 paired-end run) after Nextera XT library preparation. Ec_S1_3/4 and Ec_S1_6 reads were assembled using the SPAdes (Nurk et al., 2013) program in 225 and 257 contigs, respectively (accession number ERS2527069 and ERS2527070, available in the project PRJEB27128).
The Enterobase (https://enterobase.warwick.ac.uk/species/ecoli/allele_st_search), ResFinder, and SeroFinder (http://www.genomicepidemiology.org/) databases were used to characterize the STs, the antibiotic resistance mechanisms, and the serotypes of the *E. coli* isolates. The phylogenetic group analysis was also accomplished according to Clermont's scheme (Clermont *et al.*, 2000).
Ec_S1_3/4 and Ec_S1_6 belonged to phylogenetic group A, serotype O89:H9, and ST1702 (*Table 1*). The above ST was reported from an animal sample in China in 2015, and from human specimens in Yemen, Oman (2015) and Ireland in 2016. The strains from Yemen and Oman were NDM-producers but no information was available on the gene variant (http://enterobase.warwick.ac.uk/species/ecoli/search_strains?query=st_search). The presence of bla_{NDM-5} and bla_{CMY-42} gene variants was ascertained in both isolates. Other resistance determinants detected were $bla_{TEM\ 1B}$, *rmtB*, *dfrA17*, *aadA5* and *mphA* (*Table 1*). The two isolates were identified as a single clone according to resistance phenotype results, coupled with the genotyping data. The co-existence of bla_{NDM-5} and bla_{CMY-42} genes in *E. coli* isolates was recently reported in Italy in a ST405 lineage, phylogroup D (Bitar *et al.*, 2017).
In order to assess the transferability of bla_{NDM-5} gene, conjugation assay was performed using the *E. coli* J53 Azide^R as recipient strain at both 25°C and 37°C temperature. Transconjugants were obtained for both strains under the two thermal conditions. The identification and susceptibility profiles were obtained with Microscan Autoscan4 System (Beckman Coulter), and the presence of bla_{NDM-5} and bla_{CMY-42} was confirmed by PCR and sequencing. Plasmid analysis with PBRT kit (Diatheva) was accomplished for both isolates and transconjugants strains. The transconjugants resulted IncI1 replicon positive only, while IncFII and IncI1 were observed in the donor strains. This result differed from the previous Italian data, where a bla_{NDM-5} determinant was detected in an IncFII harbouring plasmid, while the bla_{CMY-42} unique resistance gene was present in an IncI1 plasmid (Bitar *et al.*, 2017).

To the best of our knowledge, this is the first description of ST1702 NDM-5 and CMY-42 co-producing *E. coli* clinical isolates in Northern Italy. In this study, no travel history was ascertained for the two patients and no further NDM-5-producing isolates have since been reported from the hospital. Ec_S1_3/4 and Ec_S1_6 isolates could represent autochthonous strains, with the community setting playing a role as reservoir. However, the patient's several previous hospitalizations may have constituted a risk factor for intra-hospital acquisition of these pathogens.
In summary, our study provides new evidence of persistence and dissemination of MDR NDM-5-producing isolates and the emergence of a new lineage in Italian clinical settings.

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Antimicrobial Resistance and Infection Control

RESEARCH Open Access

Colonization of long-term care facility residents in three Italian Provinces by multidrug-resistant bacteria

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Abstract

Background: Rationale and aims of the study were to compare colonization frequencies with MDR bacteria isolated from LTCF residents in three different Northern Italian regions, to investigate risk factors for colonization and the genotypic characteristics of isolates. The screening included *Enterobacteriaceae* expressing extended-spectrum β-lactamases (ESβLs) and high-level AmpC cephalosporinases, carbapenemase-producing *Enterobacteriaceae*, *Pseudomonas aeruginosa* or *Acinetobacter baumannii*, methicillin-resistant *Staphylococcus aureus* (MRSA) and vancomycin-resistant enterococci (VRE).

Methods: Urine samples and rectal, inguinal, oropharyngeal and nasal swabs were plated on selective agar; resistance genes were sought by PCR and sequencing. Demographic and clinical data were collected.

Results: Among the LTCF residents, 75.0% (78/104), 69.4% (84/121) and 66.1% (76/115) were colonized with at least one of the target organisms in LTCFs located in Milan, Piacenza and Bolzano, respectively. ESβL producers (60.5, 66.1 and 53.0%) were highly predominant, mainly belonging to *Escherichia coli* expressing CTX-M group-1 enzymes. Carbapenemase-producing enterobacteria were found in 7.6, 0.0 and 1.6% of residents; carbapenemase-producing *P. aeruginosa* and *A. baumannii* were also detected. Colonization by MRSA (24.0, 5.7 and 14.8%) and VRE (20.2, 0.8 and 0.8%) was highly variable. Several risk factors for colonization by ESβL-producing *Enterobacteriaceae* and MRSA were found and compared among LTCFs in the three Provinces. Colonization differences among the enrolled LTCFs can be partially explained by variation in risk factors, resident populations and staff/resident ratios, applied hygiene measures and especially the local antibiotic resistance epidemiology.

Conclusions: The widespread diffusion of MDR bacteria in LTCFs within three Italian Provinces confirms that LTCFs are an important reservoir of MDR organisms in Italy and suggests that future efforts should focus on MDR screening, improved implementation of infection control strategies and antibiotic stewardship programs targeting the complex aspects of LTCFs.

Keywords: Long-term care facilities, Multicenter study, ESβL, AmpC, Carbapenemases, MRSA, VRE

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Background

Life expectancy in Italy is rapidly increasing, with present values of 80.1 years for males and 84.7 for females [1]. Due to the ageing population, long-term care facilities (LTCFs), which provide ongoing skilled nursing care to residents and help meet both the medical and non-medical needs of elderly individuals with a chronic illness or disability, play an important role in the Italian healthcare system. Residents in LTCFs have a variety of risk factors for colonization with multidrug-resistant (MDR) bacteria; therefore, these facilities represent reservoirs of: i) *Enterobacteriaceae* expressing extended-spectrum β-lactamases (ESβLs), derepressed/acquired high-level AmpC cephalosporinases or carbapenemases, ii) *Pseudomonas aeruginosa* or *Acinetobacter baumannii* producing carbapenemases and iii) methicillin-resistant *Staphylococcus aureus* (MRSA) and vancomycin-resistant enterococci (VRE) [2–4].

To promote detailed studies of various microbiological aspects related to LTCFs in Italy, the Association of Italian Clinical Microbiologists (Associazione Microbiologi Clinici Italiani; AMCLI) in 2016 has set up a new working group consisting of Clinical Microbiologists (Gruppo di Lavoro per lo Studio delle Infezioni nelle Residenze Sanitarie Assistite e Strutture assimilabili; *GLISTer*); one of the main objectives of this working group is the study of the distribution and prevalence of MDR organisms in Italian LTCFs and therefore a multicenter point-prevalence survey, including the main MDR bacteria as described above, was performed in 2016 on residents of LTCFs, located in three Northern Italian cities.

Methods

The aim

Rationale and aims of the study were to compare colonization frequencies with MDR bacteria of LTCF residents in three different Northern Italian cities, located in different Italian regions, and to investigate their genotypic characteristics. Moreover, risk factors for colonization were compared between LTCFs and colonization prevalence was correlated with the local epidemiology of invasive MDR isolates.

Facilities, patient characteristics and survey design

In October–November 2016, a multicenter point-prevalence screening study was conducted in four LTCFs concerning i) *Enterobacteriaceae* with ESβLs, carbapenemases or high-level AmpCs, ii) *P. aeruginosa* or *A. baumannii* with carbapenemases, iii) MRSA and VRE. The four facilities, located in the Northern Italian Provinces of Milan ($n = 1$), Piacenza ($n = 2$) and Bolzano ($n = 1$), offer high skilled 24 h nursing care.

Although the overall study was performed over a period of 2 months, the sampling interval in each facility lasted for a maximum of 1 week. All residents of the four LTCFs were eligible to participate, and the study was approved by the Ethics Committees of the three referring hospitals; informed written consent was obtained from the residents or, if they were unable to consent, from their relatives.

Microbiological methods

Sample processing, microbial identification and antibiotic susceptibility testing were carried out in the clinical microbiology laboratories of the referral hospitals. Microbiological methods for the LTCF screening study in Bolzano were previously described [5]. Similar methods were used in the epidemiological studies of Milan and Piacenza LTCFs, with minor modifications.

For the screening of MDR bacteria from LTCF residents in Milan midstream or catheter urine samples were cultured on Oxoid Brilliance™ ESβL plates (Thermo Scientific, UK), applying a 10 μg imipenem (IMP) disc (Oxoid, Thermo Scientific, UK), and on Oxoid Brilliance™ VRE (Thermo Scientific, UK). Inguinal, oropharyngeal and rectal swabs were seeded on Oxoid Brilliance™ ESβL, applying a 10 μg IMP disc, on Oxoid Brilliance™ VRE and on CHROMagar™ MRSA (BD Diagnostics, MD). Nasal swabs were plated on CHROMagar™ MRSA. All plates were incubated at 35 ± 2 °C under aerobic conditions for 24–48 h. Isolate identification and antibiotic susceptibility testing were performed by the BD Phoenix™ System (BD Diagnostics, MD), according to European Committee on Antimicrobial Susceptibility Testing (EUCAST) criteria [6], using PHOENIX NMIC/ID402 for non-urinary Gram-negative bacteria, PHOENIX UNMIC/ID403 for Gram-negative isolates from urine cultures, and PHOENIX PMIC/ID88 for MRSA and VRE. The strains were phenotypically confirmed for β-lactamase production by the ESBL+AMPC Screen Kit and the KPC + MBL Confirm ID Kit (Rosco Diagnostica A/S, Denmark).

Similarly, for screening of MDR bacteria from LTCF residents in Piacenza, midstream or catheter urine samples were seeded on ChromID CPS agar (BioMèrieux, Marcy l'Etoile, France); rectal swabs on ChromID ESBL Agar (BioMèrieux, Marcy l'Etoile, France), on ChromID VRE Agar (BioMèrieux, Marcy l'Etoile, France) and on Mac Conkey agar applying a 10 μg meropenem (MER) disc (Oxoid, Thermo Scientific, UK); nasal swabs on Chapman Agar (Oxoid, Thermo Scientific, UK), on ChromID ESBL and on MacConkey agar applying a 10 μg MER disc; and inguinal swabs on Mannite salt agar (Oxoid, Thermo Scientific, UK). Plates were incubated at 35 ± 2 °C under aerobic conditions for 24–48 h. Isolate identification and antibiotic susceptibility testing were performed using the Vitek 2 System (BioMèrieux, Marcy l'Etoile, France), calibrated against EUCAST criteria [6], with AST-N202 cards (including an ESβL test) for Gram-negative bacteria, AST-P632 cards (with both oxacillin and cefoxitin) for MRSA and AST-P586 cards

for VRE. Identification of β-lactamase types was based on Vitek 2 results and on the synergistic effects obtained by the ESβL+AMPC Screen Kit and the KPC + MBL Confirm ID Kit (Rosco Diagnostica A/S, Denmark). VRE were confirmed by vancomycin and teicoplanin Etest strips (BioMèrieux, Marcy l'Etoile, France).

Molecular characterization of resistance genes

Molecular characterization of all MDR isolates was performed in a common reference laboratory, located at the University of Pavia. Total DNA was extracted by the automated Puro extraction system (DID, Milan, Italy), using the DNA tissue kit, according to manufacturer's instructions. The presence of ESβL and carbapenemase genes was investigated by PCR, targeting bla_{CTX-M}-, bla_{SHV}- bla_{KPC}-, bla_{VIM}-, bla_{IMP}-, bla_{OXA-48}-, bla_{NDM}- and bla_{GES}-type genes, and using published primers and conditions [7–15], summarized in Additional file 1: Table S1. *A. baumannii* isolates were screened for the presence of the following carbapenemase genes: bla_{OXA-23}-like, bla_{OXA-24}-like, bla_{OXA-51}-like and bla_{OXA-58}-like [16–18]. The presence of IS*Aba1* elements adjacent to bla_{OXA-51}-like genes was determined as previously described [19]; AmpC genes were detected by a multiplex PCR [20].

Bacterial isolates collected from the LTCF in Milan were screened for bla_{KPC}-, bla_{VIM}-, bla_{OXA-48}- and bla_{NDM}-type genes by the Cepheid GeneXpert System and confirmed by PCR. Check-MDR CT103 XL array (Check points Health B.V., Wageningen, The Netherlands) has been used to investigate the *bla* gene content of a carbapenem-resistant *P. aeruginosa* strain obtained from an oropharyngeal swab, which tested negative by previous molecular assays.

For gene sequencing, PCR products were purified using the quantum Wizard® SV Gel and PCR Clean-Up System (Promega, Madison, WT, USA) and subjected to double-strand Sanger sequencing. Sequences were analyzed according to the BLAST software [21].

Statistical analysis

A significance level of $p \leq 0.05$ was used. In-house physicians reviewed hospital records and, using a standard questionnaire, recorded demographic and clinical data as follows: patient age, gender, length of stay, Barthel immobility score, coma, comorbidities (dementia, urinary incontinence, diabetes, cancer, vascular diseases, chronic obstructive pulmonary disease, decubitus ulcer), presence of infection, antibiotic treatment in the preceding 3 months and the presence of indwelling medical devices. The significance of differences in risk factors and colonization proportions was calculated using the proportion comparison test. Logistic regression analyses were developed to investigate colonization of at least one site with ESβL producers and MRSA as dependent variables, first as univariate and then as multivariate models, including predictors with $p < 0.05$ in the univariate analysis, comprising the specific LTCF of residence, using stepwise logistic selection. Analysis were performed using the Medcalc® software version 15.11.4 (MedCalc software, Ostend, Belgium).

Results

A variable percentage of LTCF residents, present during the point-prevalence survey in the four LTCFs, agreed to participate: 104/310 (34%) in Milan, 121/326 (37%) in Piacenza (2 LTCFs, with 71/216 and 50/110 participating residents, respectively), and all 115 (100%) residents in the LTCF in Bolzano; no specific LTCF resident selection criteria were used in Milan and Piacenza and resident characteristics of enrolled and not-enrolled residents were similar. The median age of LTCF residents in Milan, Piacenza and Bolzano was 82 years (range: 65–96 years), 86 years (range: 63–102 years) and 77 years (range: 30–94 years) for males, and 90 years (range: 71–102 years), 88 years (69–105 years) and 84 years (24–96 years) for females, respectively. The median length of stay of residents in the LTCFs in Milan, Piacenza and Bolzano was 23 months (range: 1–199 months), 34 months (range: 1–172 months) and 19 months (range: 1–174 months), respectively. Various healthcare staff/resident ratios were found in the LTCFs in Milan (ratio: 0.62; 193/310), Piacenza (ratio: 0.61; 201/326; corresponding to 73/110 and 128/216 in the two enrolled LTCFs, respectively) and Bolzano (ratio: 0.79; 91/115). Demographic and clinical details of the enrolled LTCF residents are summarized in Table 1.

Isolation frequencies and molecular characterization of the antibiotic resistance determinants are shown in Table 2. A high percentage of LTCF residents were colonized with at least one of the target MDR organisms in Milan (75.0%; 78/104), Piacenza (69.4%; 84/121) and Bolzano (66.1%; 76/115); moreover, many residents from Milan (37.5%; 39/104), Piacenza (19.8%; 24/121) and Bolzano (30.4%; 35/115) were colonized with more than one MDR organism.

ESβL-producing *E. coli* expressing bla_{CTX-M}-like genes were highly predominant in Milan (80.4%), Piacenza (97.0%) and Bolzano (80.3%) and CTX-M-type determinants were also identified in *Proteus mirabilis*, *Klebsiella pneumoniae*, *Citrobacter koseri*, *Enterobacter cloacae complex* and *Serratia marcescens*. Most bla_{CTX-M} genes belonged to group-1 (72.4%), followed by group-9 (14.8%) and other groups (12.8%). A bla_{BEL}-like gene was detected in a *P. aeruginosa* strain from the LTCF in Milan.

In total, ten carbapenemase-producing *Enterobacteriaceae* were detected: $n = 7$ KPC-producing *K. pneumoniae* and $n = 1$ VIM-1-producing *E. cloacae*

Table 1 Demographic and clinical details of LTCF residents from three Italian Provinces

	Milan (M), % (n = 104)	Piacenza (P), % (n = 121)	Bolzano (B), % (n = 115)	Significant differences (p-value)
Male sex	30.7	26.4	43.4	M vs. B (0.05); P vs. B (0.006)
Age ≥ 86 years	58.7	60.3	35.6	M vs. B (< 0.001); P vs. B (< 0.001)
Antibiotics in preceding 3 months	24.0	50.4	23.4	M vs. P (< 0.001); P vs. B (< 0.001)
Fluoroquinolones	8.6	7.4	5.2	
Penicillins	2.8	1.6	12.1	M vs. B (0.01); P vs. B (0.001)
Cephalosporins	5.7	24.8	1.7	M vs. P (< 0.001); P vs. B (< 0.001)
Dementia	42.3	79.3	68.7	M vs. P (< 0.001); M vs. B (< 0.001)
Peripheral vascular disease	59.6	47.1	71.3	P vs. B (< 0.001)
Urinary incontinence	74.0	84.3	85.2	M vs. P (0.05); M vs. B (0.04)
Diabetes	19.2	16.5	20.8	
Cancer	8.6	8.2	9.5	
Decubitus ulcer	6.7	5.7	11.3	
Chronic obstructive pulmonary disease	11.5	9.1	18.2	P vs. B (0.04)
Physical disability (Barthel Immobility score of 0)	10.4	41.3	67.8	M vs. P (< 0.001); M vs. B (< 0.001); P vs. B (< 0.001)
Coma	0.0	0.0	17.4	M vs. B (< 0.001); P vs. B (< 0.001)
Any medical device	10.5	23.9	38.7	M vs. P (0.009); M vs. B (< 0.001); P vs. B (0.01)
Percutaneous enteral gastrostomy tube	2.8	11.5	20.8	M vs. P (0.01); M vs. B (< 0.001); P vs. B (0.05)
Tracheostomy tube	0.0	1.6	9.5	M vs. B (0.001); P vs. B (0.007)
Urinary catheter	8.6	6.6	18.2	M vs. B (0.04); P vs. B (0.006)
Nasogastric tube	0.0	9.1	1.7	M vs. P (0.001); P vs. B (0.01)
Length of stay in LTCF < 6 months	17.7	8.2	17.3	M vs. P (0.03); P vs. B (0.03)
Hospital admission in previous 12 months, any department	22.3	15.8	38.2	M vs. B (0.01); M vs. P (< 0.001) P vs. B (< 0.001)
Geriatrics	0.0	1.6	9.5	M vs. B (p = 0.001); P vs. B (p = 0.007)
Medicine	4.8	5.7	6.0	
Orthopedics	3.8	3.3	4.3	
Infection	3.8[a]	5.7[b]	0.8[c]	P vs. B (0.03)

[a]Urinary tract infection - UTI (2), respiratory tract infection - RTI (1), infected prosthesis (1)
[b]RTI (6), UTI (1), skin and soft tissue infection (1)
[c]UTI (1)

complex were isolated from LTCF residents in Milan, and $n = 2$ VIM-1 producers (one *E. coli* and one *Citrobacter amalonaticus*) from residents in Bolzano. Two carbapenemase-positive *P. aeruginosa* were isolated from LTCF residents in Piacenza: in one case a bla_{GES-5} and in the other a bla_{VIM}-like gene were identified. Moreover, two *P. aeruginosa* isolates collected in Milan and Piacenza presented a bla_{GES-1} ESβL. Nine bla_{OXA-23}-positive *A. baumannii* were isolated from two and seven LTCF residents in Milan and Piacenza, respectively.

MRSA strains were most frequently isolated from LTCF residents in Milan and Bolzano, whereas VRE isolates were highly prevalent in Milan ($n = 21$ *Enterococcus faecalis*), but rare in Piacenza ($n = 1$ *E. faecalis*) and Bolzano ($n = 1$ *Enterococcus faecium*).

Colonization of LTCF residents with ESβL-producing enterobacteria and MRSA was associated with several risk factors in univariate and multivariate analysis (Table 3). In multivariate analysis, the LTCF of residence was an independent risk factor for ESβL ($p \leq 0.03$ for all comparisons, except $p = 0.53$ for the comparison of Milan vs. Piacenza) and MRSA ($p \leq 0.02$ for all comparisons) colonization. Risk factors for MRSA colonization were also associated with resident's gender; for the following risk factors significant differences between male ($n = 226$) and female ($n = 114$) residents were found: age > 85 years (M: 34.5%; F: 20.4%; $p < 0.001$), hospitalization within the previous 12 months (M: 35.0%; F: 20.4%; $p = 0.03$), administration of any antibiotic within the previous 3 months (M: 40.3%; F: 29.6%; $p = 0.04$) and coma (M: 10.5%; F: 3.5%; $p = 0.009$).

Table 2 Colonization percentages in residents from LTCFs of three Italian Provinces

	% of LTCF residents colonized with specific resistance phenotype and genotype and significant differences ($p \leq 0.05$)			
	Milan ($n = 104$)	Piacenza ($n = 121$)	Bolzano ($n = 115$)	Significant differences ($p \leq 0.05$)
All resistance groups (MRSA; VRE; ESβL-/AmpC-producing enterobacteria; carbapenemase-producing enterobacteria, *Pseudomonas aeruginosa* and *Acinetobacter baumannii*)	75.0	69.4	66.1	
All ESβL-positive enterobacteria	60.5	66.1	53.0	P vs. B (0.04)
Escherichia coli, ESβL-positive	48.0	55.3	45.2	
*bla*CTX-M-group-1	33.6	41.3	28.7	P vs. B (0.04)
*bla*CTX-M-group-9	6.7	5.7	9.5	
*bla*CTX-M-group, other than 1 or 9	4.8	9.9	0.0	P vs. B (< 0.001)
Proteus mirabilis, ESβL-positive	14.4	9.1	7.0	
*bla*CTX-M-group-1	3.8	4.1	0.0	M vs. B (0.04); P vs. B (0.03)
*bla*CTX-M-group-9	1.9	0.0	0.0	
Klebsiella pneumoniae, ESBL-positive	6.7	5.7	6.1	
*bla*CTX-M-group-1	5.7	4.1	1.7	
*bla*CTX-M-group-9	0.9	0.8	0.0	
*bla*CTX-M-group, other than 1 or 9	0.0	0.0	2.7	
Morganella morganii, ESβL-positive	1.9	1.6	2.6	
Citrobacter koseri, ESβL-positive	0.0	3.3	0.8	
*bla*CTX-M-group other than 1 or 9	0.0	3.3	0.0	
Enterobacter cloacae complex, ESβL-positive	0.9	0.8	0.0	
*bla*CTX-M-group-1	0.0	0.8	0.0	
*bla*CTX-M-group other than 1 or 9	0.9	0.0	0.0	
Serratia marcescens, ESβL-positive	0.0	0.8	0.0	
*bla*CTX-M-group-1, *bla*CTX-M-15-like	0.0	0.8	0.0	
Providencia stuartii	1.9	0.0	0.0	
All high-level AmpC-positive enterobacteria	5.7	3.3	25.2	M vs. B (< 0.001); P vs. B (< 0.001)
Enterobacter cloacae complex, high-level AmpC	0.0	0.8	0.0	
Morganella morganii, high-level AmpC	3.8	0.8	24.3	M vs. B (< 0.001); P vs. B (< 0.001)
*bla*DHA-type	3.8	0.8	8.7	P vs. B (0.004)
Citrobacter freundii, high-level AmpC	0.0	0.8	0.0	
Proteus mirabilis, high-level AmpC	1.9	0.0	0.8	
*bla*CMY-type	0.0	0.0	0.8	
Serratia marcescens, high-level AmpC	0.0	0.8	0.0	
Providencia rustigianii, high-level AmpC	0.9	0.0	0.0	
All carbapenemase-positive enterobacteria	2.6	0.0	1.6	M vs. P (0.002); M vs. B (0.03)
Klebsiella pneumoniae, *bla*KPC-type	6.7	0.0	0.0	M vs. P (0.004); M vs. B (0.05)
Escherichia coli, *bla*VIM-1	0.0	0.0	0.8	
Enterobacter cloacae complex, *bla*VIM-1	0.9	0.0	0.0	
Citrobacter amalonaticus, *bla*VIM-1	0.0	0.0	0.8	
Carbapenemase-positive *Pseudomonas aeruginosa*	0.0	1.6	0.0	
*bla*VIM-type	0.0	0.8	0.0	
*bla*GES-5	0.0	0.8	0.0	
Carbapenemase-positive *Acinetobacter baumannii*	1.9	5.8	0.0	P vs. B (0.009)
*bla*OXA-23-like	1.9	5.8	0.0	P vs. B (0.009)

Table 2 Colonization percentages in residents from LTCFs of three Italian Provinces *(Continued)*

	% of LTCF residents colonized with specific resistance phenotype and genotype and significant differences ($p \leq 0.05$)			
	Milan ($n = 104$)	Piacenza ($n = 121$)	Bolzano ($n = 115$)	Significant differences ($p \leq 0.05$)
MRSA	24.0	5.7	14.8	M vs. P (< 0.001); P vs. B (0.02)
VRE	20.2[a]	0.8[a]	0.8[b]	M vs. P (< 0.001); M vs. B (< 0.001)

Notes: [a]*Enterococcus faecalis*; [b]*Enterococcus faecium*

Discussion

The study evaluated the degree of colonization with drug-resistant bacteria among residents of LTCFs located in three Northern Italian Provinces, finding high colonization of residents in Milan (75.0%), Piacenza (69.4%) and Bolzano (66.1%). Many residents had more than one target organism, underscoring the role of LTCFs as a reservoir for these isolates [2–4].

Colonization of LTCF residents with ESβL-producing enterobacteria was highly prevalent in all the surveyed LTCFs (60.5% in Milan, 66.1% in Piacenza and 53.0% in Bolzano), and group-1 CTX-M-type enzymes were highly predominant, especially in *E. coli* (80–97% of isolates). Notably, about 82% of *K. pneumoniae* and 32% of *P. mirabilis* isolates also harbored a $bla_{\text{CTX-M}}$-type gene. In the same Bolzano LTCF, here screened for ESβL-producing enterobacteria, high colonization percentages, equal to 64.0 and 49.0%, were previously found in 2008 [22] and 2012 [23], respectively; the latter survey also screened a second LTCF in the Province of Bolzano, showing a colonization prevalence of 56.0%. In an Italian study carried out in 2006, a colonization prevalence of 54.0% was found in LTCF residents bearing a urinary catheter [24], while a more recent multicenter study, performed in 2015 and involving 12 Italian LTCFs, reported a mean ESβL colonization of 57.3% (range: 32.8–81.5%) [25]. In all these Italian studies, CTX-M enzymes were the predominantly produced ESβLs. The high ESβL colonization rates of > 50% in Italian LTCF residents are paralleled by high ESβL prevalence in invasive *E. coli* isolates [26]. Generally, ESβL carriage in most European countries is strikingly lower than that found in Italy [4], with exceptions reported from Ireland [27, 28] and Portugal [29].

In our screening study, high-level AmpC-producing *Enterobacteriaceae* were rarely isolated in LTCF residents in Milan and Piacenza, but 24.3% of LTCF residents in Bolzano were colonized by *M. morganii* expressing a high-level DHA-AmpC phenotype; bla_{DHA}-type genes in LTCF isolates have previously been found in a few *E. coli* and *K. pneumoniae* strains from Korea [30], but to our knowledge have not yet been reported in Italian LTCFs.

Carbapenemase-producing enterobacteria were not found in LTCF residents in Piacenza, rarely in Bolzano (1.6%) and more frequently in Milan (7.6%). As found in previous studies of carbapenemase-producing *Enterobacteriaceae* from Bolzano [22, 23, 31], the VIM-1-producing *E. coli* and *C. amalonaticus* isolates from residents in this study were also positive for $bla_{\text{SHV-12}}$. In the present study, all carbapenemase producers from Milan, except an *E. cloacae complex* isolate expressing a $bla_{\text{VIM-1}}$ gene, had KPC-type enzymes; similar results have been reported by other Italian studies in LTCF residents [25, 32, 33]. Carbapenemase-producing enterobacteria, especially KPC-producing *K. pneumoniae*, are epidemically spread in Italy [34] and the emergence of this MDR phenotype in LTCFs is worrying, expanding the reservoir of this health care threat. Nevertheless, as previously summarized [4], carbapenemase-producing *Enterobacteriaceae* are still rare in Italian LTCF residents; the reasons are probably multifactorial, comprising clinical characteristics of the enrolled residents [35] and the low carbapenem selective pressure in LTCFs. On average, only 1.1% of residents enrolled in our screening study received carbapenems within the previous 3 months (data not shown). Nevertheless, a carbapenemase-producing enterobacteria prevalence of 7.6% (mainly KPC-producing *K. pneumoniae*), reported here for the LTCF in Milan, gives rise to concern and has to be addressed by future hygiene and antibiotic stewardship measures.

This study shows the emergence of carbapenemase-producing *P. aeruginosa* in LTCF residents in Piacenza, identifying single isolates with bla_{VIM}-type and $bla_{\text{GES-5}}$ determinants. *P. aeruginosa* expressing bla_{VIM}-type determinants is widely spread in Italy [36], and an outbreak of GES-5-producing *P. aeruginosa* was reported from a LTCF in Japan [37]. Moreover, the ESβL genes $bla_{\text{GES-1}}$ and bla_{PER}-like were found in two and one *P. aeruginosa* isolates, respectively; the latter rarely detected β-lactamase was previously recovered in *P. aeruginosa* strains from Belgium [18]. *A. baumannii* producing OXA-23 carbapenemases have an epidemic diffusion in Italy [38], reflected in the present study by the isolation of this resistance type from LTCF residents in Milan (1.9%) and Piacenza (5.8%).

MRSA colonization prevalence here reported ranged widely in the surveyed LTCFs (5.7, 14.8 and 24.0% in Milan, Piacenza and Bolzano, respectively), similar to other Italian studies [25, 39, 40]. Varying MRSA

Table 3 Resident's risk factors for ESBL and MRSA colonization (cumulative data: Milan, Piacenza, Bolzano)

			Univariate analysis		Multivariate analysis				Univariate analysis		Multivariate analysis	
	ESBL, % (n = 203)	No ESBL, % (n = 137)	OR (CI 95%)	p	OR (CI 95%)	p	MRSA, % (n = 45)	No MRSA, % (n = 295)	OR (CI 95%)	p	OR (CI 95%)	p
Male sex	34.9	31.3	1.17 (0.74–1.86)	0.49			51.1	30.8	*2.34 (1.24–4.42)*	*0.008*	*2.31 (1.16–4.59)*	*0.01*
Age ≥ 86 years	52.7	49.2	1.15 (0.74–1.78)	0.53			39.0	53.0	0.56 (0.29–1.10)	0.09		
Antibiotics in preceding 3 months	39.9	*23.3*	*2.17 (1.34–3.54)*	*0.001*	*1.74 (1.02–2.98)*	*0.04*	37.7	32.5	1.25 (0.65–2.41)	0.48		
Fluoroquinolones	7.8	5.8	1.38 (0.57–3.32)	0.47			*15.5*	*5.7*	*3.01 (1.17–7.73)*	*0.02*	*3.59 (1.26–10.25)*	*0.01*
Penicillins	7.3	2.9	*2.65 (0.86–8.17)*	0.09			11.1	4.7	2.50 (0.85–7.34)	0.09		
Cephalosporins	14.2	6.5	*2.37 (1.08–5.18)*	0.03			4.4	12.2	0.33 (0.07–1.44)	0.14		
Dementia	63.0	66.4	0.86 (0.54–1.36)	0.52			62.2	64.7	0.89 (0.47–1.71)	0.74		
Peripheral vascular disease	67.5	59.1	1.15 (0.74–1.80)	0.52			62.2	61.0	1.05 (0.55–2.00)	0.87		
Urinary incontinence	83.2	78.8	1.33 (0.77–2.31)	0.30			82.2	81.3	1.06 (0.46–2.40)	0.89		
Diabetes	18.7	18.9	0.98 (0.56–1,71)	0.30			26.6	17.6	1.69 (0.82–3.51)	0.15		
Cancer	*11.8*	4.3	*2.92 (1.16–7.36)*	*0.02*	*3.47 (1.32–9.16)*	*0.01*	4.4	9.5	0.44 (0.10–1.93)	0.27		
Decubitus ulcer	9.8	5.1	2.03 (0.83–4.94)	0.12			6.6	8.1	0.80 (0.23–2.79)	0.73		
Chronic obstructive pulmonary disease	11.8	14.6	0.78 (0.41–1.48)	0.45			15.5	12.5	1.28 (0.53–3.08)	0.57		
Physical disability (Barthel immobility score of 0)	*47.7*	*32.3*	*1.91 (1.21–3.02)*	*0.005*	*2.10 (1.15–3.83)*	*0.01*	*37.7*	41.0	0.87 (0.45–1.66)	0.67		
Coma	6.9	4.3	1.61 (0.60–4.31)	0.33			6.6	5.7	1.16 (0.32–4.15)	0.81		
Any medical device	*32.5*	*13.1*	*3.18 (1.79–5.66)*	*< 0.001*	*2.81 (1.44–5.47)*	*0.002*	33.3	23.3	1.63 (0.83–3.21)	0.15		
Percutaneous enteral gastrostomy tube	15.7	6.5	*2.66 (1.22–5.77)*	*0.01*			11.1	12.2	0.89 (0.33–2.42)	0.83		
Tracheostomy tube	4.9	2.1	2.31 (0.67–8.56)	0.21			4.4	3.7	1.20 (0.25–5.60)	0.81		
Urinary catheter	15.7	4.3	*4.08 (1.66–10.06)*	*0.002*			*20.0*	*9.8*	*2.29 (1.00–5.23)*	*0.04*	*2.61 (0.06–6.43)*	*0.03*
Nasogastric tube	5.9	0.7	*8.54 (1.09–66.49)*	*0.04*			4.4	3.7	1.20 (0.25–5.60)	0.81		
Length of stay in LTCF < 6 months	15.6	11.9	1.36 (0.71–2.61)	0.34			16.6	13.7	1.25 (0.51–3.00)	0.61		
Hospital admission in previous 12 months	24.2	27.0	0.87 (0.53–1.43)	0.58			*37.7*	*23.4*	*1.97 (1.02–3.81)*	*0.04*		
Infection	5.4	2.9	1.90 (0.59–6.11)	0.27			8.8	3.7	2.51 (0.76–8.28)	0.12		

ND: not determined; factors included in multivariate analysis are in italics. For multivariate analysis only significant values are shown

colonization prevalence, ranging from close to zero up to levels higher than 37%, has been reported in European studies [4].

Colonization by VRE in the present study was highly variable, ranging from 0.8 to 20.2%. VRE-carriage in European LTCF residents was found to be low, ranging from 0.0–3% [28, 41, 42].

For *Enterobacteriaceae* significant differences in colonization frequencies of LTCF residents were found: i) for CTX-M-type ESβL-producing *E. coli* between Piacenza (highest prevalence) and Bolzano, ii) for high-level AmpC-producing *M. morganii* (highest prevalence in Bolzano), iii) for carbapenemase producers, with highest prevalence in Milan, iv) for carbapenemase-producing *A. baumannii*, showing highest prevalence in Piacenza, and v) for MRSA and VRE, most prevalent in Milan. Therefore, no clear picture of general colonization differences can be deduced from overall colonization prevalence data.

A variety of risk factors for MRSA and ESβL colonization have previously been reported [4]; many of these have also been analyzed in the present survey. Interestingly, male residents carried a more than double risk for MRSA carriage when compared with female residents, probably because of the higher frequencies of other risk factors in males (administration of any antibiotic within the previous 3 months, hospitalization within the previous 12 months and coma), predisposing men rather than women to MRSA acquisition. Moreover, in our study the trend for an inverse correlation ($p = 0.09$) between age > 85 years and MRSA prevalence was associated with a significantly lower percentage of male residents > 85 years, compared to females; similar results have been found by other authors [43]. In the present survey, administration of cephalosporins during the previous 3 months resulted to be an independent risk factor for ESβL colonization; the LTCFs in Piacenza registered the highest consumption of cephalosporins, correlating with highest ESβL prevalence in LTCF residents from Piacenza. Other independent risk factors for ESβL colonization were physical disability, the presence of any invasive medical device and cancer. Whereas no significant differences were found between residents in the three Provinces for cancer as risk factor, physical disability and the presence of any medical device showed highest prevalence in the LTCF in Bolzano; nonetheless, LTCF residents in Bolzano had the lowest ESβL prevalence in the present screening study.

Therefore, further factors may have contributed to the observed differences, comprising staff/resident ratio and practiced hygiene and infection control measures [44]. The LTCF in Bolzano showed the highest staff/resident ratio, and understaffing has been shown to be a risk factor for colonization of LTCF residents by MDR organisms [2]. All of the surveyed LTCFs in the present study follow hygiene, infection prevention and control measures according to guidelines of The Society for Healthcare Epidemiology of America (SHEA) and The Association for Professionals in Infection Control and Epidemiology (APIC) [45]. Nonetheless, the Bolzano LTCF had introduced enforced hygiene measures, according to the World Health Organization guidelines [46], after the 2008 screening study, showing an ESβL colonization prevalence of 64.0% in LTCF residents [22]; colonization frequency decreased significantly to 49.0% ($p = 0.02$) in 2012 [23], arriving at a slightly higher percentage of 53.0% in 2016, but other factors such as changed case mixes and risk factors may also have contributed to this decrease in ESβL prevalence [23].

Significant differences in antibiotic resistance epidemiology of blood culture isolates, used as a proxy for the general local antibiotic resistance epidemiology, were registered, as derived from European Antimicrobial Resistance Surveillance Network (EARS-Net) data for 2016 [26]. Specifically, we found the following antibiotic resistance data referred to the geographic regions of Milan, Piacenza and Bolzano, respectively: *E. coli* third generation cephalosporin-resistant: 22.1% (29/131), 29.4% (71/259) and 17.8% (56/314); *K. pneumoniae* carbapenem-resistant: 29.2% (7/24), 13.5% (10/74) and 6.2% (4/64); *A. baumannii* carbapenem-resistant: 50.0% (1/2,) 100.0% (24/24) and 0.0% (0/2); MRSA: 36.0% (18/50), 49.7% (82/165) and 14.6% (20/137); *E. faecalis* VRE: 0.0% (0/20), 2.4% (2/83) and 0.0% (0/41); *E. faecium* VRE: 10.0% (1/10), 22.2% (6/27) and 8.0% (2/25). This data for blood culture isolates, compared with our LTCF screening data, correlates well for ESβL-producing *E. coli*, carbapenem-resistant *K. pneumoniae* and *A. baumannii*; on the other hand, no correlation for MRSA and VRE can be derived. Patient transfer between acute-care facilities and LTCFs contribute to the diffusion of MDR organisms in both settings; such bi-directional movement of MDR bacteria, related to acute systemic infections, might be more significant for *Enterobacteriaceae* and *A. baumannii* than for MRSA and VRE.

Moreover, the snapshot approach used in this study might lead to the sudden increase in prevalence of a specific resistance phenotype, as shown for high-level AmpC-producing *M. morganii* detected in 2016 from Bolzano LTCF residents [5], which could be a transient phenomenon. Similarly, the high prevalence of VRE in LTCF residents from Milan could be due to a transitory local epidemic event.

Finally, the local circulation of highly transmissible clones, for example ESβL-producing *E. coli*, KPC-producing *K. pneumoniae* and OXA-23-producing *A. baumannii* could contribute to the explanation of the here reported screening results [38, 47].

This study has some limitations. First, it has been done in only four LTCFs, located in three different Provinces

in Northern Italy, and therefore data may not be extrapolated to other Italian LTCFs with differing characteristics. Second, the number of LTCF residents participating in the study was variable, ranging from 34% in Milan up to 100% in Bolzano. Third, we did not use an enrichment step during the laboratory analysis; this limitation is partially compensated by using 4–5 different specimen types for the screening of MDR bacteria. Fourth, different sample types, types of media and laboratory methodologies have been used in the three laboratories processing the samples from the different LTCFs. Fifth, molecular characterization and typing of isolates in the 2016 study was limited, not including pulsed-field gel electrophoresis (PFGE) and sequence typing (ST) of isolates and therefore not permitting the identification of epidemic clusters. Finally, screening of healthcare workers has been done only in one of the enrolled LTCFs [5], but not in the other surveyed facilities. Despite these limitations, the strength of our study is the comparison of colonization prevalence between LTCFs located in three different Provinces, comparing it also with differences in risk factors for colonization and in the local epidemiology of invasive isolates.

Conclusions

We performed a multicenter point-prevalence study in LTCFs located in three different Provinces in Northern Italy and found high colonization prevalence of LTCF residents for MDR organisms, especially ESβL-producing *E. coli*. Variability between the different facilities was noticeable also for other MDR organisms. Differences can be partially explained by i) differences in risk factors for colonization by MDR organisms, ii) changes in resident populations and staff/resident ratios, iii) applied hygiene measures and iv) differences in the local epidemiology of antibiotic resistance of clinical isolates. This widespread diffusion of MDR bacteria in LTCFs of three Italian Provinces confirms that these healthcare facilities are an important reservoir for MDR organisms. Future efforts should focus on screening activities, infection control strategies tailored on the complex aspects of LTCFs and implementation of antibiotic stewardship programs.

Additional file

Additional file 1: Table S1. Oligonucleotides used for PCR and sequencing. (DOCX 17 kb)

Abbreviations

AMCLI: Association of Italian Clinical Microbiologist; APIC: The Association for Professionals in Infection Control and Epidemiology; CDC-M: Cefotaximase-Munich type Extended-Spectrum β-Lactamase; EARS-Net: European Antimicrobial Resistance Surveillance Network; ESβL: Extended-Spectrum β-Lactamase; EUCAST: European Committee on Antimicrobial Susceptibility Testing; GLISTer: Gruppo di Lavoro per lo Studio delle Infezioni nelle Residenze Sanitarie Assistite e Strutture Assimilabili; KPC: *Klebsiella Pneumoniae* Carbapenemase; LTCF: Long-Term Care Facility; MBL: Metallo-β-Lactamase; MDR: Multidrug-Resistant; MRSA: Methicillin-Resistant *Staphylococcus aureus*; PCR: Polymerase Chain Reaction; PFGE: Pulsed-Field Gel Electrophoresis; SHEA: The Society for Healthcare Epidemiology of America; ST: Sequence Typing; VIM: Verona Integron-Encoded Metallo-β-Lactamase; VRE: Vancomycin-Resistant Enterococci

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Availability of data and materials

The datasets generated during and/or analysed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

EN, [1]MC, VMM, RM performed molecular analysis; RDA, [1]MC, EP, AM, FS, GS provided patient's sample data; EF, CR, RA performed and interpreted phenotypic investigations; RA, RM, LP analyzed and interpreted results; RA was a major contributor in writing the manuscript. All authors read and approved the final manuscript.

Ethics approval and consent to participate

The study was approved by the Ethics Committees of the three referring hospitals.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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CASE REPORT

First Report of an ST410 OXA-181 and CTX-M-15 Coproducing *Escherichia coli* Clone in Italy: A Whole-Genome Sequence Characterization

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We investigated an Italian OXA-181-producing *Escherichia coli* clinical isolate (ECS1_14) by whole-genome sequencing. The strain coharbored $bla_{CTX-M-15}$, bla_{CMY-2}, and *qnr*S1 genes; it belonged to ST410(Achtman)/ST692(Pasteur) and phylogroup A. The $bla_{OXA-181}$ gene was harbored on a plasmid highly similar (99% identity) to the pOXA181_EC14828 plasmid, recently reported in China.

Keywords: $bla_{OXA-181}$, IncX3 plasmid, Italy

OXA-181 IS A FOUR amino acid substitutions derivative of OXA-48 carbapenemase, showing similar hydrolytic activity conferring resistance to penicillins and carbapenems. It was first identified in *Enterobacter cloacae* and *Klebsiella pneumoniae* from several locations in India in 2007, and further research indicated that it originated from the Gram-negative waterborne bacterium *Shewanella xiamenensis*.[1] Since the first identification, OXA-181-producing *Enterobacteriaceae* have been reported from several countries, both in the Indian subcontinent (Bangladesh and Sri Lanka) and outside (Canada, Asia, Europe, and Africa).[11,12] The genetic environment of the $bla_{OXA-181}$ gene showed mainly a plasmid localization, including, besides IncX3, also the IncN and IncT plasmids.[7,15] Here we describe the molecular features of the first OXA-181 and CTX-M-15 coproducing *Escherichia coli* clinical isolate in Italy, and its identification as belonging to the phylogenetic group A and ST410 lineage.

An elderly man, previously host at a long-term care facility, was admitted on January 2017 to the emergency room of ASST Fatebenefratelli Sacco hospital (Milan) for a respiratory distress. Owing to his clinical manifestations including cardiovascular decompensation, aortic stenosis, and chronic anemia, he was transferred to the cardiosurgical ward, where rectal carriage of carbapenem-resistant *E. coli* (CREc) was ascertained. The surveillance rectal swab, performed at admission, resulted positive for the presence of an OXA-48-like positive *Escherichia coli* strain (ECS1_14) by Xpert Carba-R test (GeneExpert, Cepheid). The general conditions of the patient worsened and he underwent a cardiac valve surgical replacement. Blood and valve culture resulted negative for the growth of CREc, whereas rectal swabs remained positive. Two weeks after surgery, acute respiratory and chronic renal failures occurred and the patient died.

The ECS1_14 strain showed a multidrug resistant (MDR) profile, being resistant to cephalosporins, carbapenems, fluoroquinolones, and aminoglycosides, retaining susceptibility to fosfomycin, colistin, and tigecycline by Vitek2 System, according to EUCAST guidelines (www.eucast.org/fileadmin/src/media/PDFs/EUCAST_files/Breakpoint_tables/v_7.0_Breakpoint_Tables.pdf). The MIC values for ertapenem (ETP), meropenem (MER), and imipenem (IMP) were determined by Etest, and a carbapenem susceptibility profile coherent with the production of an OXA-48-like enzyme was detected for ECS1_14: high-resistance levels only against

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TABLE 1. GENETIC FEATURES OF THE ECS1_14 ISOLATE

Carbapenemase/ ESBL/AmpC enzyme	*MLST 1/2*	*Serotype*	*fimH subtype*	*Resistance profile*	*MIC (μg/mL) IMP*	*MER*	*ETP*
OXA-181, CTX-M-15, CMY-2	410/692	O8:H9	*fimH24*	bla_{TEM-1B}, bla_{OXA-1}, *qnrS1*, *aadA1*, *aadA5*, *aac(6')Ib-cr*, *aac(3)-IId*, *strA*, *strB*, *mph(A)*, *catA1*, *catB3*, *sul2*, *sul1*, *tet*(B), *dfr*A17	4 (I)	1 (S)	8 (R)

MLST 1, seven genes Achtman scheme; MLST 2, eight genes Pasteur scheme; IMP, imipenem; MER, meropenem; ETP, ertapenem; MLST, multilocus sequence type.

ETP, a reduced susceptibility for IMP, and retained susceptibility to MER (Table 1).

The ECS1_14 genomic DNA was extracted using a QIAamp DNA minikit (Qiagen) following the manufacturer's instructions, and sequenced using Illumina Miseq with a 2 by 250 paired-end run after Nextera XT library preparation. A total of 1,454,081 reads were obtained, and assembled using the SPAdes program giving 409 contigs, of which 194 were >500 bp in length (ERX2145593).

The isolate was *in silico* assigned to phylogenetic group A, sequence type (ST) 410 (http://mlst.warwick.ac.uk/mlst/dbs/Ecoli/), an hyperepidemic clone identified from the three parts of the "one health" approach (humans, animals, and environment),[13] and known to be the founder of the globally disseminated clonal complex 23. In addition, *in silico* multilocus ST with the Pasteur scheme (http://bigsdb.pasteur.fr/ecoli) assigned the isolate to ST692. The ECS1_14 was genotyped using DTU web tools (www.genomicepidemiology.org/): the identified serotype was O8:H9, and the *fimH* subtype was fimH24. The same serotype was identified as peculiar of ST410 clade C *E. coli* strains isolated in Germany from several sources, including companion animals, farm environment, and humans.[2] The complete resistance genes content included, besides the $bla_{OXA-181}$, $bla_{CTX-M-15}$ (a globally distributed extended-spectrum β-lactamase [ESβL] gene), bla_{TEM-1b} (a non-ESβL determinant), bla_{CMY-2} (an AmpC gene), *aac(6')-Ib-cr* (an aminoglycoside acetyl transferase showing low-level resistance to aminoglycoside and fluoroquinolones), *qnrS1* (conferring low-level resistance against fluoroquinolones), *tetB* (a tetracycline resistance gene), and *sul1* (a sulfonamides-resistant determinant) genes (Table 1).

Bioinformatic analyses allowed to identify two assembly contigs highly similar (covering >99% and 100% identity) to the IncX3 pOXA181_EC14828 plasmid. Thus, polymerase chain reaction assays and Sanger sequencing were performed using the pOXA181_EC14828 as template for primers designing, obtaining the complete sequence of the pOXA181_ECS1_14 plasmid (accession number pending). Interestingly, pOXA181_EC14828, first described by Liu *et al.* in China,[6] was recently reported several times across the world (Denmark, the United Kingdom, Czech Republic, Africa, Switzerland, and Germany) from different *Enterobacteriaceae* (*E. coli, K. pneumoniae and K. variicola*) and sources (human, swine, and vegetables).[3,5,6,9,10,14] To note, the IncX3 plasmid is linked to the dissemination of several carbapenem-resistant determinants, including bla_{NDM} and bla_{KPC} types. Interestingly, another ST692(Pasteur) *E. coli* strain, carrying $bla_{OXA-181}$-IncX3 plasmid and with a similar resistance profile, was reported from Burkina Faso in 2016.[5]

The sporadic spread of OXA-48-like-producing *Enterobacteriaceae* has already been reported in Italy, although the prevalence of these carbapenemases remained low[4]; nonetheless the isolation of an MDR *E. coli* isolate belonging to the widely disseminated ST410 and carrying several, transferable, resistance determinants, along with its presence in the community, is of particular concern.

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Disclosure Statement

No competing financial interests exist.

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Occurrence of Extended Spectrum β-Lactamases, KPC-Type, and MCR-1.2-Producing *Enterobacteriaceae* from Wells, River Water, and Wastewater Treatment Plants in Oltrepò Pavese Area, Northern Italy

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To evaluate the water compartment antibiotic-resistance contamination rates, 11 wells, five streams, and four treatment plants located in the Oltrepò Pavese area were screened for the presence of third generation cephalosporins resistant Gram-negative bacteria. *Enterobacteriaceae* were also characterized for the Extended-Spectrum-β-Lactamases (ESBLs), carbapenemases, and *mcr-1* genes presence. From December 2014 to November 2015, 246 water samples were filtered, plated on Plate Count Agar, MacConkey Agar, and MacConkey Agar with cefotaxime. Isolates were species identified using AutoSCAN-4-System and ESBLs, carbapenemases, and colistin resistance determinants were characterized by PCR, sequencing, and microarray. Plasmid conjugative transfer experiments, PCR-based Replicon typing, Pulsed-Field Gel Electrophoresis, Multi-Locus-Sequence-Typing, and *in-silico* plasmid characterization were performed. A total of 132 enterobacteria isolates grew on MacConkey agar with cefotaxime: 82 (62.1%) were obtained from streams, 41 (31.1%) from treatment plants, and 9 (6.8%) from wells. Thirty out of 132 (22.7%) isolates, mainly belonging to *Escherichia coli* ($n = 15$) species, showed a synergic effect with piperacillin-tazobactam. A single ESBL gene of bla_{CTX-M}-type was identified in 19/30 isolates. In further two *E. coli* strains, a $bla_{CTX-M-1}$ gene co-existed with a bla_{SHV}-type ESBL determinant. A bla_{SHV-12} gene was detected in two isolates of *E. coli* ($n = 1$) and *Klebsiella oxytoca* ($n = 1$), while any ESBL determinant was ascertained in seven *Yersinia enterocolitica* strains. A bla_{DHA}-type gene was detected in a cefoxitin resistant *Y. enterocolitica* from a

stream. Interestingly, two *Klebsiella pneumoniae* strains of ST307 and ST258, collected from a well and a wastewater treatment plant, resulted KPC-2, and KPC-3 producers, respectively. Moreover, we report the first detection of *mcr-1.2* ST10 *E. coli* on a conjugative IncX4 plasmid (33.303 bp in size) from a stream of Oltrepò Pavese (Northern Italy). Both ESBLs *E. coli* and ESBLs/carbapenemase-producing *K. pneumoniae* strains showed clonal heterogeneity by Pulsed-Field Gel Electrophoresis and Multi-Locus-Sequence-Typing. During one-year study and taking in account the whole Gram-negative bacterial population, an average percentage of cefotaxime resistance of 69, 32, and 10.3% has been obtained for the wastewater treatment plants, streams, and wells, respectively. These results, of concern for public health, highlight the need to improve hygienic measures to reduce the load of discharged bacteria with emerging resistance mechanisms.

Keywords: water ecosystems, Gram-negative bacteria, carbapenemases, colistin resistance, molecular characterization

INTRODUCTION

Antibiotic resistance, in particular to third generation cephalosporins (3GCs) and carbapenems, threatens healthcare globally. Drug resistance has traditionally been viewed as a clinical problem, but recently natural ecosystems have been recognized as an important reservoir of antibiotic resistance genes (ARGs) (Berglund, 2015).

In aquatic environments the prevalence of antibiotic-resistant bacteria, which may originate from anthropogenic sources such as hospital and municipal effluents, is constantly growing (Baquero et al., 2008; Bouki et al., 2013). The large amounts of antibiotics or their active metabolites released into wastewater with treated urine and feces increase the selective pressure in bacterial populations, allowing the development of antibiotic-resistant microbes' generations (Davies and Davies, 2010).

Furthermore, the surface water could act as resistance hotspots where ARGs disseminate favored by bacteriophages or integrons and new resistant strains are created by horizontal gene transfer (Berglund, 2015; Colombo et al., 2017).

β-lactamase genes have been identified in bacteria isolated from both surface waters and Wastewater Treatment Plants (WWTPs) (Schwartz et al., 2003; Poppe et al., 2006; Bouki et al., 2013). The β-lactamases now include >2,000 naturally occurring amino acid sequences. Some of the clinically most important of these are the Class A penicillinases, the Extended-Spectrum β-Lactamases (ESBLs) (TEM, SHV, VEB, or CTX-M the most spread), the AmpC cephalosporinases (usually CMY, FOX, DHA, ACT, or MOX), and the carbapenem-hydrolyzing enzymes in both the serine (i.e., KPC, IMI, SME, or GES) and metallo-enzyme groups (mainly NDM, VIM, or IMP) (Bonomo, 2017). Because of the versatility of these enzymes to evolve as new β-lactams are used therapeutically, combined approaches to antimicrobial therapy may be required (i.e., carbapenems plus colistin). These genes, frequently located on mobile genetic elements, often coexist with other resistance determinants working on other classes of antibiotics such as aminoglycosides and fluoroquinolones (Marti et al., 2014).

A priority to be currently aware and vigilant of is the worldwide recently emerged plasmid-mediated resistance to colistin, another "last-resort" antibiotic used for the treatment of critical infections caused by MDR Gram-negative pathogens (Olaitan et al., 2016).

The Oltrepò Pavese Plan is located in the province of Pavia with an area of ~1,097 km^2 and a population of 146,579 inhabitants; its name comes to the peculiarity of being south of the river Po, in the full northern Apennines. The Oltrepò Pavese area supports many agricultural and industrial activities causing pollution in the shallower aquifers, for this reason, it represents a good model to evaluate the role of aquatic environment as a potential reservoir for spread and evolution of ARGs and their vectors. The routes by which humans may come in contact with these bacteria include the consumption of crops grown by contaminated sludge used as fertilizer, and/or drinking of water drawn from contaminated ground or surface water. When these resistant bacteria enter humans, they have the opportunity to spread their ARGs to the human microbiome (Wellington et al., 2013).

Only few European studies on surface waters are available; furthermore, due to the lack of local and/or large-scale epidemiological studies, our knowledge on the environmental antibiotic-resistance reservoirs in Italy is still very poor (Perilli et al., 2013; Zanotto et al., 2016).

The purpose of this study was (i) to investigate the occurrence of cefotaxime resistant *Enterobacteriaceae* in Oltrepò Pavese water ecosystem (ii) to determine Gram-negative bacterial counts and percentage of 3GCs resistance, during all the study period, (iii) to characterize the underlying determinants and to assess the clonal features of the most alarming isolates.

MATERIALS AND METHODS

Design Study and Sample Collection

The aim of the present 1-year study was to monitor the Gram-negative Oltrepò Pavese area bacterial counts during

different months per sampling site, and the corresponding 3GCs resistance percentages. Water samples from wells, streams, and WWTPs of the Oltrepò Pavese area were evaluated for the presence of ESBLs/AmpC, carbapenemases, and *mcr-1* genes in *Enterobacteriaceae*.

The sampling sites are located at south of Po river in the Italian Northern Apennines, in the zone between Voghera, Stradella, and Staffora Valley near Varzi.

This area is characterized by the presence of a sandy-gravelly alluvial deposits covering the marine sediments, which are essentially impermeable (Pilla et al., 2007). Local well have a recharging time of 6 months on average and their structure does not provide a complete isolation from the surface water.

Sampling was carried out once a month—during the period December 2014–November 2015. In the study period, a total of 246 water samples were collected. Each of the single stream sampling site (1T–11T) was visited from 9 up to 11 times, while the 11 wells (1P–13P) were sampled from 10 up to 12 times. The four WWTPs included in the study were less frequently inspected (from three to six times), due to the need for a special authorization to get samples.

All the sampled wells are part of the Po Plan Public-Supply System, serving freshwater for human consumption in the area. The outflow of four selected WWTPs, located upstream rivers of the same area, was screened starting from April 2015 only (**Figure 1**).

All water samples were collected in aseptic plastic bottles, stored at 4°C, and analyzed within 24 h after harvesting.

The stream sampling sites have been chosen with different location, upstream, and downstream urban centers, including factories, hospitals, and Long Term Care Facilities (LTCFs).

Filtration Method

A water volume of 100 ml from wells and 1 ml from up/down streams or at the outflow of treatment plants were passed through 0.45 µm-pore size membranes by the filtration method. Filter membranes were placed on Plate Count Agar (PCA), MacConkey Agar (MCA), and selective MCA containing 8 mg/l of CTX (MCA+CTX), to select the potential ESBL/AmpC producers. The bacterial count was estimated after 24 h incubation of the plates at 37°C.

The 3GCs resistance percentage (% R) was calculated by directly comparing the colonies count on the antibiotic plate (MCA+CTX) with the corresponding count on the control plate (MCA) (Watkinson et al., 2007).

Bacterial Identification and Susceptibility Testing

Each colony grown on selective MCA+CTX and distinguishable for the unique morphotype, was identified at species level. Species identification and susceptibility testing were performed using the semi-automated system MicroScan autoSCAN-4 (Beckman Coulter). Colistin (CO) MIC was confirmed by broth microdilution method. Susceptibility results were interpreted according to the EUCAST 2015 (http://www.eucast.org) clinical guidelines. *Escherichia coli* ATCC 25922 was used as control strain.

Phenotypic ESBL detection was performed by the use of chromogenic media ChromArt ESBL (Biolife Italiana S.r.l., Milano, Italy) and the Double-Disc Synergy (DD) test (Jarlier et al., 1988), using piperacillin-tazobactam, CTX, cefepime, ceftazidime, and aztreonam. A phenotype of both cefoxitin and 3GCs resistance without synergistic activity after DD, was regarded as AmpC-production marker. The strains were phenotypically confirmed for the carbapenemases production by the KPC/MBL Confirm kit (Rosco Diagnostic).

Molecular Characterization of Resistance Genes

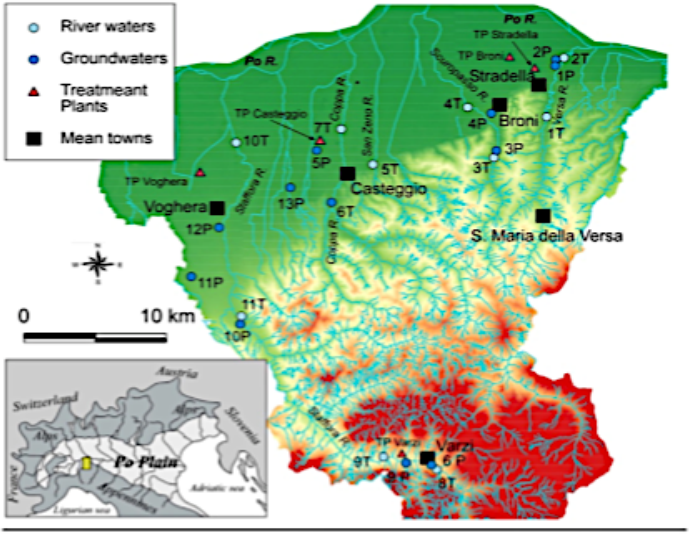
The genomic DNA was extracted using NucleoSpin Tissue (Macherey-Nagel) kit. PCR was used to detect the presence of ESBLs, carbapenemases-, and MCR-1-encoding genes using primers and the annealing temperatures described in **Table 1** (Rasheed et al., 1997; Yigit et al., 2001; Perilli et al., 2002; Pagani et al., 2003; Eckert et al., 2004; Liu et al., 2016). In the case of ESBLs and carbapenemases, the above annealing temperatures were always preceded by an initial 95°C × 5 min denaturation step. The 35 cycling conditions comprised denaturation and extension steps at 94°C × 3 min and 72°C × 1 min respectively; the final extension was at 72°C 5 min. *mcr-1* PCR approach was performed using an initial 94°C × 5 min denaturation step; the 35 cycling conditions comprised the denaturation and extension steps at 94°C and 72°C × 30 s, respectively; the final extension was at 72°C 5 min. PCR products were purified using the quantum PrepPCR Kleen Spin Columns kit (ThermoFisher Scientific) and subjected to double-strand sequencing using the automatic sequencer ABI PRISM 3100 genetic analyzer DNA Sequencer (Applied Biosystems, Foster City, CA, USA) and the BigDye Terminator v1.1 Cycle Sequencing kit (Applied Biosystems, Foster City, CA, USA).

The sequences were analyzed according to the BLAST software program (http://blast.ncbi.nlm.nih.gov/Blast.cgi).

In the case of *Y. enterocolitica* Check-MDR CT103 XL array (Check points Health B.V., Wageningen, The Netherlands) has been used to investigate the *bla* genes content.

Conjugation Transfer Experiments and Plasmid Characterization

Conjugation transfer of resistance determinants was performed in liquid medium using the *E. coli* K12 strain J62 (F^-, *pro*, *his*, *trp*, *luc*, Sm^r) and J53 (F^-, *met*, *pro*, Rif^r) as recipients. To evaluate the transferability of CTX- or CO-resistance, the *E. coli* transconjugants were screened using MCA plates supplemented with streptomycin (1,000 mg/l) or rifampicin (100 mg/l) plus CTX (8 mg/l) or CO (2 mg/l), respectively. Azide-resistant *E. coli* J53Az^R strain was used as a recipient for carbapenem-resistant *Klebsiella pneumoniae* isolates. MCA supplemented with sodium azide (200 mg/l) and ertapenem (0.5 mg/l) was used to select for transconjugants. Conjugation frequency per recipient was expressed by dividing the number of transconjugants by the initial number of recipients.



Sites	Streams	Sites	Wells	Sites	Treatment Plants
1T	Versa upstream	1P	Cascinazza 4	**Broni TP**	Treatment Plant of Broni
2T	Versa Darina downstream	2P	Cascinazza 3	**Stradella TP**	Treatment Plant of Stradella
3T	Scuropasso upstream	3P	Montegrizzo	**Voghera TP**	Treatment Plant of Voghera
4T	Scuropasso downstream	4P	Esegniti 2	**Varzi TP**	Treatment Plant of Varzi
5T	Riale San Zeno	5P	Lottona		
6T	Coppa upstream	6P	Belcredi		
7T	Coppa downstream	9P	Cagnano 2		
8T	Staffora Varzi upstream	10P	Calbiccella		
9T	Staffora Varzi downstream	11P	Bidella		
10T	Staffora Loc. Oriolo	12P	S. Maria Bianca		
11T	Staffora Rivanazzano	13P	Montebello		

FIGURE 1 | Map of the Oltrepò Pavese Plain showing the sampling sites. P, Well; T, Stream; TP, Wastewater Treatment Plant.

Plasmids were typed according to their incompatibility group using the PCR based replicon typing scheme PBRT 2.0 Kit (Diatheva), as described previously (Carattoli, 2009).

Molecular Typing

PFGE was performed on all ESBL-, carbapenemase-, and MCR-positive *E. coli* and *K. pneumoniae* isolates. All the obtained pulsotypes were than compared with the hyper-epidemic clones previously collected in the same area (e.g., 20LOM *E. coli*). Genomic DNA of isolates was analyzed after digestion with *XbaI* restriction enzyme. Fragments were separated on a CHEF DRII system (Bio-Rad, Hercules, CA) at 14°C at 6 V/cm for 20 h with an initial pulse time of 0.5 s and a final pulse time of 30 s. Lambda 48.5 kb concatamers (New England BioLabs, Beverly, MA, USA) were used as molecular size markers.

TABLE 1 | Oligonucleotides used for PCR and sequencing.

Gene	Primer sequences	Ta* (°C)	Fragment size (bp)
$bla_{CTX-M\ type}$*	FW 5'-ATGTGCAGYACCAGTAARGT-3' REV 5'-TGGGTRAARTARGTSACCAGA-3'	50	593
$bla_{CTX-M-1-group}$	FW 5'-CGTTAAAAAATCACTGCGTC-3' REV 5'-TTGGTGACGATTTTAGCCGC-3'	50	1,000
$bla_{CTX-M-9-group}$	FW 5'-ATGGTGACAAAGAGAGTGCA-3' REV 5'-CCCTTCGGCGATGATTCTC-3'	56	835
bla_{SHV} type	FW 5'-GCCCGGGTTATTCTTATTTGTCGC-3' REV 5'-TCTTTCCGATGCCGCCGCCAGTCA-3'	60	900
bla_{TEM} type	FW 5'-ATGAGTATTCAACATTTCCG-3' REV 5'-CTGACAGTTACCAATGCTTA-3'	50	800
bla_{KPC} type	FW 5'-TGTCACTGTATCGCCGTC-3' REV 5'-CTCAGTGCTCTACAGAAAACC-3'	55	1,000
bla_{VIM} type	FW 5'-CAGATTGCCGATGGTGTTGG-3' REV 5'-AGGTGCGCC ATTCAGCCAGA-3'	55	523
bla_{IMP} type	FW 5'-GGAATAGAGTGGCTTAATTCTC-3' REW 5'-GTGATGCGTCYCCAAYTTCACT-3'	50	361
bla_{NDM} type	FW 5'-GCTTTGCGCGATCTGGTTTIC-3' REW 5'-CGGAATGGCTCATCACGATC-3'	52	621
bla_{OXA-48}	FW: 5'-TTGGTGGCATCGATTATCGG-3' REV: 5'-GAGCACTTCTTTTGTGCATGGC-3'	52	743
*mcr-1**	FW: 5'-CGGTCAGTCCGTTTGTTC-3' REV: 5'-CTTGGTCGGTCTGTAGGG-3'	46	309

*Ta, annealing temperature; *primers used only in the PCR reaction.

Dendrograms of strain relatedness were created with Fingerprinting II version 3.0 software (Bio-Rad) using UPGMA. The Dice correlation coefficient was used with a 1.0% position tolerance to analyze the similarities of the banding patterns. The restriction patterns of the genomic DNA from the isolates were analyzed and interpreted according to the criteria described previously (Tenover et al., 1995).

Bacterial strains were cultivated in MCA; one single colony per strain was used for DNA extraction using NucleoSpin Tissue (Macherey-Nagel) kit. MLSTs genes were sequenced using the automatic sequencer ABI PRISM 3100 genetic analyzer DNA Sequencer (Applied Biosystems, Foster City, CA, USA) and the BigDye Terminator v1.1 Cycle Sequencing kit (Applied Biosystems, Foster City, CA, USA).

MLST for *E. coli* was done according to the MLST Databases at University of Warwick (http://mlst.warwick.ac.uk/mlst/dbs/Ecoli) and for *K. pneumoniae* according to the Institute Pasteur MLST (http://bigsdb.pasteur.fr/klebsiella/klebsiella.html). MLST of *Enterobacter cloacae* was done according to the *E. cloacae* MLST website (https://pubmlst.org/ecloacae/) developed by Keith Jolley and sited at the University of Oxford.

Plasmid Characterization

7TE.coli plasmid sequencing was done by Illumina MiSeq technology, using Nextera XT kits for library preparation. Reads were assembled using Spades 3.8 (http://cab.spbu.ru/software/spades/).

The detection of resistance genes and plasmid replication sites have been performed on J53R7TE *coli* using the ResFinder and PlasmidFinder tools in the DTU database (http://www.genomicepidemiology.org/).

Contigs containing plasmid sequences were detected, analyzed, and closed using the software Bandage (https://rrwick.github.io/Bandage/). Open Reading Frames (ORFs) and the relative amino acids were predicted using Artemis (http://www.sanger.ac.uk/science/tools/artemis). The annotation was performed manually using the online blast tool on the ncbi database (https://blast.ncbi.nlm.nih.gov/Blast.cgi?CMD=Web&PAGE_TYPE=BlastHomeNew).

Genbank files were formatted and uploaded using the Sequin software (https://www.ncbi.nlm.nih.gov/Sequin/).

Statistical Analysis

Data were expressed as means (± Standard Error). The difference between means of the bacterial count was tested by the Analysis of Variance (ANOVA). A P value below 0.05 was deemed significant.

RESULTS

Identification of Gram-Negative Bacteria Isolates

A total of 246 water samples was collected; 48% (118/246) from five streams, 43.9% (108/246) were from 11 wells, and 8.1% (20/246) from four WWTPs.

Two hundred and sixty-four non-duplicate microorganisms, one-half (50%; $n = 132$) belonging to enterobacterial species, were overall obtained on MCA+CTX/ChromArt ESBL. The remaining isolates included *Pseudomonas* spp. (19%; $n = 49$), *Acinetobacter* spp. (11%; $n = 29$), *Vibrio fluvialis* (7%; $n = 18$), *Aeromonas hydrophila* (9%; $n = 25$), and other Gram-negative bacteria (4%; $n = 11$).

Bacterial Counts and Percentage of 3GCs Resistance

The number in average of CFU ml^{-1} of bacteria identified after membrane filtration on PCA ranged from zero to 10,000 (data not shown). High bacterial densities were always observed in the case of WWTPs (5,100–10,000 CFU ml^{-1}), while a month to month fluctuating trend have been observed in streams samples, with number in average bacterial counts ranging between 1,023 and 8,272 CFU ml^{-1} (Table 2).

Concerning the wells results, the bacterial detection frequencies varied between 12.9 and 9,005 CFU ml^{-1}; highest densities being found for the three wells named 6P, 9P, and 10P (Table 2).

TABLE 2 | Bacterial counts and percentage of 3GCs resistance for each sampling site.

Site[a]		Avg no. of		
	N[b]	CFU/ml^{-1} (±SE)	% CTX-R[c]	% CTX-R range[d]
1T	11	4,625 ± 1,562	41	0-100
2T	11	4,627.3 ± 1,561	46	0-100
3T	11	3,694.9 ± 1,507	5.3	0-40
4T	11	5,764.3 ± 1,997	68	0-100
5T	11	7,317.3 ± 1,385	49.5	0-100
6T	9	4,500 ± 1,739	42.7	0-100
7T	11	6,272.7 ± 1,159	46	0-100
8T	10	1,023.3 ± 997.4	3.75	0-32.4
9T	11	3,709.1 ± 1,504	25.3	0-100
10T	11	3,696.8 ± 1,507	6.4	0-40
11T	11	2,812.7 ± 1,392	18.8	0-50
1P	12	16.4 ± 6.8	20	0-100
2P	10	2,033.5 ± 1,328	12.9	0-100
3P	10	1,039.5 ± 995	10	0-0.2
4P	10	36 ± 15	10.3	0-77
5P	10	12.9 ± 5.3	10	0-100
6P	10	8,020 ± 1,320	7	0-25
9P	10	9,005 ± 995	13	0-50
10P	11	6,050 ± 1,612	6.6	0-25
11P	10	17.5 ± 10	5	0-50
12P	11	1,003 ± 999	0	0
13P	10	1,028.2 ± 864	18	0-100
TP Varzi	6	10,000	50.8	25-100
TP Broni	3	10,000	100	100
TP Voghera	3	5,100 ± 4,000	75	25-100
TP Stradella	3	6,733.3 ± 3266	52	25-100

% CTX-R, % Cefotaxime resistance; SE, standard error.
[a]T, Well; T, Stream; TP, Wastewater Treatment Plant; [b]Refers to the number of visits to each site; [c]% CTX-R, percentage of 3GCs- resistant Gram-negatives obtained as an average value, in dependence from the overall visits performed at the same sampling site. [d]% CTX-R range, percentage 3GCs-resistant Gram-negatives, obtained comparing the bacterial growth on MCA and MCA+CTX, range 0-100%, depending on the single visit and sampling site.

The percentages of 3GCs-resistant Gram-negatives, obtained comparing the bacterial growth on MCA and MCA+CTX, ranged between 0 and 100%, depending on the single visit and sampling site (**Table 2**).

Statistical Analysis

Seasonal changes of total bacterial count were statistically evaluated. No significant seasonal effects on total vital count were found analyzing samples from wells and WWTPs.

The lowest bacterial count (as average value) was registered from the streams in October (126.4 ± 34.1). A significant increase ($p < 0.05$) in the streams mean bacterial count was observed, starting from the beginning of winter toward the end of spring. If the highest value of the mean bacterial count (10,000 ± 202.5) has been recorded in April, a significant decrease ($p < 0.05$) was observed from the beginning of summer to the end of autumn.

The highest percentage of CTX-resistant Gram-negative microorganisms (65.65 ± 13.1) was recorded in May. A significant decrease ($p < 0.05$) in the same percentage was observed since the beginning of summer toward the end of autumn. The lowest value was found in September (10.2 ± 7.8). The mean values of both total bacterial count and percentage of CTX-resistant Gram-negatives are shown in **Table 3**.

Phenotypic Testing and Antibiotic Resistance Patterns

A total of 132 enterobacteria grew on MCA+CTX: 82 (62.1%) were from streams, 41 (31.1%) from treatment plants, and 9 (6.8%) from wells samples. Only 30/132 (22.7%) *Enterobacteriaceae* showed a synergic effect by DD phenotypic test using 3GCs and piperacillin-tazobactam; 15/30 were *E. coli*, 7/30 *Yersinia enterocolitica*, 5/30 *K. pneumoniae*, 2/30 *Klebsiella oxytoca*, and 1/30 *E. cloacae*. Two (1.5%) out of 132 *Enterobacteriaceae*, were phenotypically confirmed as carbapenemases producers by KPC/MBL kit, while 1/132 (0.8%) was identified as AmpC-producer.

All the collected *Y. enterocolitica* strains showed clinical resistance to 3GCs; the 12.5% were also chloramphenicol and fosfomycin resistant. All the *E. coli* and *Klebsiella* spp. isolates showed resistance to 3GCs; the 13.3% ($n = 2$) of *E. coli* and 22.2% ($n = 2$) of *Klebsiella* spp. resulted, in addition, resistant to the three main carbapenems (ertapenem, meropenem, imipenem). Notably, 60% ($n = 9$) ESBLs-producing *E. coli* isolates showed fluoroquinolones resistance (**Table 4**).

Only 3/15 (20%) of the *E. coli* (7TE. *coli*, 1TPE. *coli* Stradella, 3TPE. *coli* Broni) and 1/7 (14.2%) of the *K. pneumoniae* (9P) out of the 33 ESBLs ($n = 30$)/AmpC ($n = 1$)/carbapenemases ($n = 2$) phenotypically suspected enterobacteria, resulted CO-resistant (MIC > 4 mg/l) by MicroScan-A4 System.

Clinical CO resistance was confirmed only for the strain named 7TE. *coli* (MIC = 8 mg/l) by MIC broth microdilution method.

KPC/MBL confirm kit test showed a positive result for the 9P and 5TP*K. pneumoniae* strains, obtained on June 2015 and July 2015 from a well and a WWTP in the Varzi area, respectively. The boronic acid synergistic effect suggested the production of a KPC-type enzyme.

Detection and Characterization of Resistance Genes

A bla_{CTX-M}-type gene, was identified in a total of 21/33 (63.6%) isolates ($n = 14$ *E. coli*; $n = 5$ *K. pneumoniae*; $n = 1$ *K. oxytoca*; $n = 1$ *E. cloacae*) from all water compartments (**Table 4**).

In four cases (3TE. *coli*; 3TP*K. pneumoniae*; 7T *E. coli*; 6TP *E. coli* Varzi) the bla_{CTX-M}-type gene co-existed with other β-lactamase determinants, as shown in **Table 4**. The most identified bla_{CTX-M} variant was $bla_{CTX-M-1}$ (8/21; 38%), followed by the $bla_{CTX-M-15}$ (5/21; 23.8%), the $bla_{CTX-M-14}$ and $bla_{CTX-M-28}$ (3/21, each; 14.3%, each), and $bla_{CTX-M-136}$ (2/21; 9.5%).

A bla_{SHV-12} gene was present in three isolates, named 11TE. *coli*, 7TE. *coli*, and 6TK. *oxytoca*.

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A bla_{SHV-12} gene was present in three isolates, named 11TE. *coli*, 7TE. *coli*, and 6TK. *oxytoca*.

TABLE 3 | Mean values of total bacterial count and percentage of 3GCs resistance (±SE) from stream samples during different seasons.

	Winter	Spring	Summer	Autumn
Bacterial Count (CFU/ml)	5, 196.485 ± 862	6, 687.636 ± 815.45	3, 137.4 ± 1, 005	2, 231.179 ± 766.8
% CTX Resistance	36.3 ± 6.9	46.3 ± 7.8	12.8 ± 2.95	15.5 ± 6.2

Only 1/8 Chromogenic ESBL-agar selected *Y. enterocolitica* strains (the cefoxitin resistant 11T) harbored a bla_{DHA}-type gene, while the remaining seven resulted negative for any *bla* resistance gene by Check-MDR CT103 XL array.

PCR and sequencing confirmed the presence of the bla_{KPC-2} and bla_{KPC-3} determinants in the 5TP and 9P*K. pneumoniae* strains, respectively. A co-occurrence of *mcr-1.2*, $bla_{CTX-M-1}$, and bla_{SHV-12} genes was detected by *in-silico* plasmid characterization in the 7T*E. coli* collected on November 2015 from Coppa stream (**Table 4**).

Conjugation Experiments Results

Conjugation experiments were performed on all the 25 ESBLs/KPC positive strains: 15 *E. coli*, seven *K. pneumoniae*, two *K. oxytoca*, one *E. cloacae*.

Lateral transfer of resistance genes was observed in 15/25 (60%) isolates: 11 *E. coli*, three *K. pneumoniae*, and one *K. oxytoca*, as shown in **Table 5**. The resistance profiles of donors and transconjugants confirmed lateral transfer of 3GCs, carbapenems (ertapenem), and colistin resistance. PCR analysis confirmed the presence of the resistance genes in all the transconjugants (**Table 5**).

The transfer of CTX resistance was observed at a frequency of $\sim 10^{-3}$ transconjugants per recipient. Compared to the *E. coli* J53 and J62 strains used as recipients, the transconjugants exhibited a decreased susceptibility to 3GCs (data not shown).

While in the case of KPC-3-producing 9P*K. pneumoniae* the conjugation experiment failed, the transfer of the CO resistance trait from MCR-1.2-producing 7T*E. coli* to J53 (met, pro-, rif^r) *E. coli* K12 was possible, at a frequency of $\sim 10^{-2}$ transconjugants per recipient. Susceptibility testing by autoSCAN-4 System revealed a MIC $\geq$ 4 mg/l for the *mcr-1*-harboring J53R7T *E. coli*.

PCR and sequencing analysis on J53R7T *E. coli* yielded positive results for the presence of the *mcr-1.2* gene. Inc group plasmid analysis performed on J53R7T*E. coli* using PBRT 2.0 kit, showed that *mcr-1.2* resistance determinant was located in plasmid belonging to the IncX4 Group. In addition to the IncX4, also IncX3 incompatibility group was observed in the donor 7T *E. coli*.

Molecular Typing

PFGE analysis carried out on all 15 ESBLs-producing *E. coli* showed clonal diversity (**Figure 2**). The 1TE. *coli* strain resulted identical to the 20LOM *E. coli* ST 131 (100% similarity between pulsotypes), previously collected in the same area (**Figure 2B**). Five out of seven ESBLs- and/or carbapenemases- producing *K. pneumoniae* were unique by PFGE (**Figure 3**). Multiple MLST lineages were identified, coherently with the previously obtained PFGE clonal relationships. Among these, were detected the hyperepidemic clones ST131 *E. coli* and ST258 *K. pneumoniae* (**Table 4**). The ST1601 was shared by the two PFGE clonally related strains (but showing different resistance profiles and bla_{CTX-M} variants) collected in May 2015 from Varzi WWTP.

Plasmid Characterization

In-silico analysis of the NGS data of the transconjugant J53R7T*E. coli*, revealed the presence of the CO resistance gene *mcr-1.2* on an IncX4 plasmid. Using Bandage, the plasmid was extracted and closed resulting in a 33,303 bp circular plasmid named pIBMC-MCR1.2 (**Figure 4**).

pIBMC-MCR1.2 showed 99% similarity with the recently reported pMCR1.2-IT plasmid detected in a KPC-3-producing ST512 *K. pneumoniae* clinical isolate collected in Italy (Di Pilato et al., 2016). Other similar IncX4 plasmids carrying *mcr-1* gene were reported in China from a clinical *K. pneumoniae* isolate, in Estonia from a pig sludge *E. coli* isolate, and in South Africa from an *E. coli* clinical isolate (Li et al., 2016; Poirel et al., 2016). In all these plasmids, the *mcr* neighboring regions were highly similar, with the downstream presence of *pap2* gene (encoding for a putative PAP family transmembrane protein), and the absence of the mobilization region IS*Apl1*.

The plasmid pIBMC-MCR1.2 has an average G/C-content of 42%; it comprises 26 ORFs, of which 21 encoding proteins with known functions and five hypothetical proteins. Any additional resistance genes are carried on this plasmid. The plasmid contains a region responsible for mobilization (14.1 kb) and another for replication (1 kb) and maintenance (2.7 kb) (**Figure 4**).

DISCUSSION

The occurrence of antibiotic-resistant bacteria from European aquatic sites is increasingly described (Zarfel et al., 2017).

The dissemination in such settings of ESBLs-producing *Enterobacteriaceae* could be particularly worrisome, due to the ability of nosocomial pathogens to transfer ARGs among different hosts and environments (Machado et al., 2009).

This study describes the occurrence of ESBLs, acquired cephalosporinases and carbapenemases among *Enterobacteriaceae* from surface, ground water and WWTP aquatic compartments of the Oltrepò Pavese Plain, Italy. Moreover, this is the first report on the presence of a CTX-M-1, SHV-12 ST10 *E. coli* strain harboring a *mcr-1.2* gene from an Italian stream. CTX-M-type producing ST10 *E. coli* was already reported from farm animals, healthy, and hospitalized humans (Hansen et al., 2014).

The Oltrepò Pavese area is densely populated (355 inhabitants/km^2 in average), supports many agricultural (as vineyards, orchards), intensive livestock, and industrial activities. Although the study was undertaken in a restricted area, the

TABLE 4 | Molecular characteristics of ESBLs/AmpC/carbapenemase-producing isolates recovered from streams, wells, and WWTPs.

MLST (ST)	Isolate	Date of isolation (Month, Year)	Origin[a] (Sample)	Resistance[b]	Resistance determinant	Inc/Replicon[c]
993	6P *E. coli*	June 2015	6P	AMC, AMP, CTX, ERT	CTX-M-1	IncP (P1, incL (L), IncF (FIA)
N/a	7T *E. cloacae*	March 2015	7T	AMC, AMP, CAZ, PIP, CTX	CTX-M-14	IncF (FIA), IncI (I1)
131	1T *E. coli*	January 2015	1T	AMP, CAZ, CTX, FEP, CIP, PIP, LEV, MOX, NOR, TOB, TMB	CTX-M-1	IncF (FIB), IncFII (FII)
94	3T *E. coli*	January 2015	3T	AMC, AMP, CAZ, CTX, FEP, PIP, ERT, MEM, TZP, CIP, CL, MOX, NOR	CTX-M-28, TEM-1	IncP (P), IncF (FIB)
6151	11T *E. coli*	May 2015	11T	AMC, AMP, CAZ, CTX, FEP, PIP, TZP	BHV-1/2	IncN (N), Inc B/O (B/O), IncF (FIB)
5717	17P *E. coli*	June 2015	7P Brovi	AMC, AMP, CTX, CAZ, FEP, PIP, CIP, LEV, MOX, NOR, TOB, CL, FOS	CTX-M-28	IncH2 (H2), Inc B/O (B/O), IncF (FIA, FIB), IncA/C (A/C), IncFII (FII)
5590	2TP *E. coli*	July 2015	TP Brovi	AMP, FEP, CTX, CAZ, PIP, CIP, CL, MOX, NOR, LEV,	CTX-M-14	IncX1 (X1)
7519	5TP *E. coli*	October 2015	TP Brovi	AMP, FEP, CTX, CAZ, PIPCIP, CO, MOX, NOR, LEV, TMB, TOB	CTX-M-130	IncH2 (H2), IncM (M)
6080	1TP *E. coli*	June 2015	TP Bralelle	AMP, CTX, FEP CIP, CO, GM, MOX, NOR, LEV, TMB, TOB	CTX-M-1	IncF (FIA, FIB)
3132	1TP *E. coli*	May 2015	TP Verzi	AMP, CTX, FEP, PIP, TMB	CTX-M-15	IncF (FIA), IncF (T)
399	2TP *E. coli*	May 2015	TP Verzi	AMP, CTX, CIP, LEV, MOX, NOR, PI, TMB	CTX-M-15	IncF (FIA)
7228	3TP *E. coli*	May 2015	TP Verzi	AMP, CTX, CAZ, FEP, PIP	CTX-M-130	IncN (N), IncF (FIA, FIB)
2432	4TP *E. coli*	June 2015	TP Verzi	AMP, CTX, FEP, PIP CIP, MOX, NOR, LEV	CTX-M-1	IncM (M), IncN (N), HIBM (NAst, FIBM (NAst)
2208	5TP *E. coli*	June 2015	TP Verzi	CIP, MOX, NOR, LEV	CTX-M-14	IncL (L), IncF (FIA,FIB)
362	6TP *E. coli*	July 2015	TP Verzi	AMP, CTX, CAZ, PIP, CIP, MOX, NOR, LEV, GM, TMB	CTX-M-1, BHV-6	IncP (P), IncF (FIA,FIB)
10	7T *E. coli*	November 2015	7T	AMC, AMP, CAZ, PIP, TMB, CO	CTX-M-1, BHV-1/2, MOR-1/2	IncM (M), IncM3 (M3)
N/a	6T *K. oxytoca*	April 2015	6T	AMC, AMP, CAZ, PIP, CTX	BHV-1/2	IncH2 (H2), IncN (N), Inc F (FIA)
N/a	9T *K. oxytoca*	December 2014	9T	PIP, AMP, CTX, FEP, FOS	CTX-M-1	IncN (N), R (NAst)
258	9P *K. pneumoniae*	June 2015	9P	AMC, AMP, CAZ, CTX, PIP, CO	KPC-3	IncFII K (FIK, FIB, K2)
466	2T *K. pneumoniae*	January 2015	2T	AMC, AMP, CAZ, CTX, FEP, PIP, CL, GM	CTX-M-1	IncN (N)
1296	1TP *K. pneumoniae*	May 2015	TP Verzi	AMP, CTX, FEP, PIP	CTX-M-15	IncF (FIA), IncF (T)
1601	2TP *K. pneumoniae*	May 2015	TP Verzi	AMC, AMP, CAZ, FEP, PIP, CL	CTX-M-28	IncH1 (H1), Inc B/O (B/O), IncF (FIA)
194	3TP *K. pneumoniae*	May 2015	TP Verzi	AMP, CAZ, CTX, FEP, PIP, TMB	CTX-M-15, TEM-1	IncM (M), IncN (N), IncB/O (B/O), IncF (FIK)
1601	4TP *K. pneumoniae*	May 2015	TP Verzi	AMP, CTX, CAZ, FEP, PIP, CL, CIP, MOX, NOR, LEV	CTX-M-15	IncH1 (H1), Inc. (L)
307	5TP *K. pneumoniae*	July 2015	TP Verzi	AMC, AMP, CTX, CAZ, FEP, MEM, ERT, TZP, CIP, NOR, MOX, FOS, GM, TMB, CL	TEM-1, KPC-2	IncFIK, FIK, FIB RN, FIB K2)
N/a	1T *Y. enterocolitica*	June 2015	1T	AMC, AMP, CTX, FOS	Other mechanism	N/a
N/a	2T *Y. enterocolitica*	March 2015	2T	AMC, AMP, CTX, PIP	Other mechanism	N/a
N/a	3T *Y. enterocolitica*	May 2015	3T	AMC, AMP, CTX, PIP	Other mechanism	N/a
N/a	6T *Y. enterocolitica*	January 2015	6T	AMC, AMP, CTX, CAZ, CL	Other mechanism	N/a
N/a	6T *Y. enterocolitica*	February 2015	6T	AMC, AMP, CTX, CAZ	Other mechanism	N/a
N/a	6T *Y. enterocolitica*	March 2015	6T	AMC, AMP, CTX, CAZ	Other mechanism	N/a
N/a	11T *Y. enterocolitica*	May 2015	11T	AMC, AMP, CTX, PIP	Other mechanism	N/a
N/a	11T *Y. enterocolitica*	June 2015	11T	AMC, AMP, CTX, PIP	DHA-type	N/a

[a] W, well; T, stream; TP, Treatment Plant; [b] AMC, amoxicillin/clavulanic acid; AMP, ampicillin; CAZ, ceftazidime; CIP, ciprofloxacin; CL, chloramphenicol; CO, colistin; CTX, cefotaxime; ERT, ertapenem; FEP, cefepime; FOS, fosfomycin; GM, gentamicin; LEV, levofloxacin; MEM, meropenem; MOX, moxifloxacin; NOR, norfloxacin; PIP, piperacillin; TZP, piperacillin-tazobactam; TOB, tobramycin; TMB, trimethoprim/sulfamethoxazol; [c] Inc, incompatibility group; *N/A, not assigned; *n/t, not tested.

TABLE 5 | Phenotypical and molecular characteristics of 3GCs-resistant transconjugants; comparison with *E. coli* and *Klebsiella* spp. donors resistance and β-lactamases.

Isolates	Resistance[a]	Enzyme	Inc (Replicon)[b]
1T *E. coli*	**AMP, CAZ, CTX, FEP, CIP, PIP, LEV, MOXI, NOR**, TOB, **TMS**.	**CTX-M-1**	IncF (FIB)
3T *E. coli*	AMC, **AMP**, CAZ, **CTX, FEP, PIP**, ERT, MEM, TZP, **CIP, CL, MOXI, NOR**.	**CTX-M-28, TEM-1**	IncF (F), IncI (FIB)
7T *E. coli*	AMC, AMP, CAZ, PIP, TMS, **CO**.	CTX-M-1, **MCR-1.2**, SHV-12	IncX4 (X4)
11T *E. coli*	**AMC**, AMP, CAZ, **CTX**, FEP, **PIP**, TZP.	**SHV-12**	IncF (FIB)
1TP *E. coli* Ebroni	AMC, **AMP, CTX**, CAZ, **FEP, PIP, CIP, LEV, MOXI, NOR**, TOB, **CL**, FOS.	**CTX-M-28**	IncI B2 (FIB), IncF (FIA, FIB)
2TP *E. coli* Ebroni	**AMP**, CAZ, **CTX, FEP, PIP**, CIP, **LEV**, MOXI, NOR, CL.	**CTX-M-14**	IncX1(X1)
1TP *E. coli* Vaiti	**AMP, CTX, FEP, PIP**, TMS.	**CTX-M-15**	IncI (I)
2TP *E. coli* Vaiti	**AMP, CTX, CIP, LEV, MOXI, NOR**, PI, TMS.	**CTX-M-15**	IncF (FIA)
3TP *E. coli* Vaiti	**AMP, CTX**, CAZ, FEP, PIP.	**CTX-M-15**, TEM-1	IncF (FIA, FIB)
6TP *E. coli* Vaiti	**AMP, CTX, CAZ, PIP, CIP, LEV, MOXI, NOR, TOB, TMS**.	**CTX-M-1, SHV-5**	IncF (FIA, FIB)
11P *E. coli* Stradella	**AMP, CTX, FEP**, CIP, CO,GM, MOXI, NOR, LEV, **TMS**, TOB.	**CTX-M-1**	IncF (FIA, FIB)
1TP *K. pneumoniae* Vaiti	**AMP, CTX, FEP, PIP**.	**CTX-M-15**	IncF (FIA)
3TP *K. pneumoniae* Vaiti	**AMP**, CAZ, **CTX**, FEP, PIP, TMS.	**CTX-M-28**	IncN (N)
5TP *K. pneumoniae* Vaiti	**AMC, AMP, CTX**, CAZ, FEP, MEM, **ERT, TZP**, CIP, NOR, MOXI, FOS, CL, GM, TMS.	**TEM-1, KPC-2**	IncI Bk (FIK, FIBK2)
9T *K. oxytoca*	**AMP, CTX, FEP**, FOS, **PIP**.	**CTX-M-1**	IncN (N), R (NAC)

[a]*AMC, amoxicillin/clavulanic acid; AMP, ampicillin; CAZ, ceftazidime; CIP, ciprofloxacin; CL, chloramphenicol; CO, colistin; CTX, cefotaxime; ERT, ertapenem; FEP, cefepime; FOS, fosfomycin; GM, gentamicin; LEV, levofloxacin; MEM, meropenem; MOXI, moxifloxacin; NOR, norfloxacin; PIP, piperacillin; TZP, piperacillin-tazobactam; TOB, tobramycin; TMS, trimethoprim/sulfamethoxazole;* [b]*Inc, incompatibility group;* [c]*NA, not assigned.*
The antibiotic resistance and the determinants transferred in the transconjugants are underlined and provided in bold.

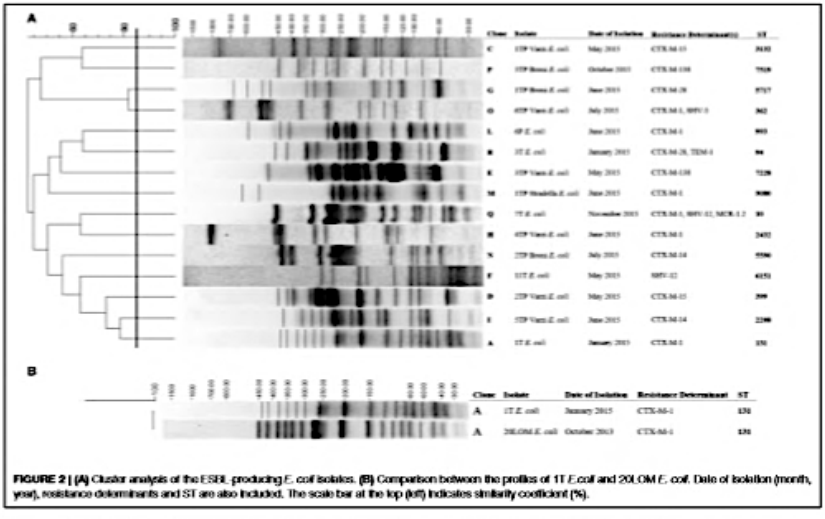

FIGURE 2 | (A) Cluster analysis of the ESBL-producing *E. coli* isolates. **(B)** Comparison between the profiles of 1T *E.coli* and 20LOM *E. coli*. Date of isolation (month, year), resistance determinants and ST are also included. The scale bar at the top (left) indicates similarity coefficient (%).

microbiological results here presented can be regarded as representative, since this hydrogeological site is typical of many other Po plain zones.

During one-year period, a total of 33 CTX-M-, SHV-, DHA-, KPC-type producing *Enterobacteriaceae* were identified from 11 wells, five streams, and four WWTPs. A β-lactamase production

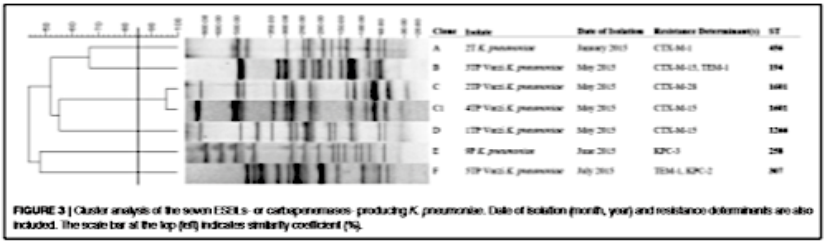

FIGURE 3 | Cluster analysis of the seven ESBLs- or carbapenemases- producing *K. pneumoniae*. Date of isolation (month, year) and resistance determinants are also included. The scale bar at the top (left) indicates similarity coefficient (%).

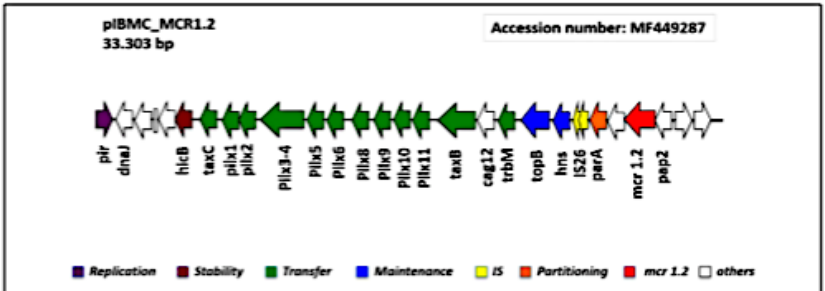

FIGURE 4 | Linear map of the pIBMC_MCR1.2 plasmid, showing the genetic structures surrounding *mcr 1.2* gene.

mechanism was not detected in a large amount of the selected 3GCs resistant enterobacterial species ($n = 99/132$; 75%).

High levels of bacterial contamination and CTX-resistance rates were constantly observed in WWTPs, while seasonal changes—with highest values in spring—were recorded from stream samples. Moreover, fluctuations occurred depending on the sampling sites location, up- or downstream the rivers. The above trend, that well highlights how the local anthropogenic activities directly affect the water quality, was observed also in a recent research from Guadeloupe (Guyomard-Rabenirina et al., 2017).

Surprisingly, high bacterial counts were detected sampling 2/11 wells located nearby the heavily contaminated Varzi WWTP; these data confirm the huge pollution risks existing in this short term (about 60 days) ground water recharge area.

The CTX-M-type ESBLs resulted the most spread 3GCs hydrolyzing enzymes among the collected isolates. The overall β-lactamases detected were mainly of CTX-M-type, followed by SHV-type, TEM-1, and KPC-type.

The high (60%) fluoroquinolones non-susceptibility level detected in 3GCs resistant *E. coli* represents a particular concern, as this antibiotic class is frequently employed in treatment of urinary tract infections.

Fifteen out of 33 (45.4%) β-lactamases-producing *Enterobacteriacae* were collected from WWTPs.

Although in wells 3GCs-resistance rates resulted very low, a ST258 KPC-3-producing 9P*K. pneumoniae* strain has been found in Varzi area. To our knowledge, only one description of an environmental strain ST512 KPC-3-producing *K. pneumoniae* was previously reported in Central Italy from a WWTP (Perilli et al., 2013). In 2011, the European Antimicrobial Resistance Surveillance Network (EARS-Net) described an ongoing worrisome increasing trend of carbapenemase-producing *K. pneumoniae* from invasive samples in Italian Intensive Care Units. Moreover, recent National data show that KPC-producing *K. pneumoniae* Clonal Complex 258 is also frequently found in patients of geriatric or medicine wards (Cristina et al., 2016).

Seven out of eight *Y. enterocolitica*, although initially suspected as ESBLs-positive using phenotypic tests, resulted *bla* genes negative by microarray. The detection of *Y. enterocolitica* showing CTX and cefepime clinical resistance is not common in our country. Thereby, as the intrinsic AmpC production is not

enough to explain this phenotype, other mechanisms as porin loss and/or over-expression of efflux systems could be responsible in the resistance detected in our isolates (Frazão et al., 2017).

The plasmid-mediated *mcr-1* gene has recently been reported from food animals, vegetables, asymptomatic carriers, and hospitalized patients in China (Liu et al., 2016). Since then, it has been across five continents (Wang et al., 2017). Regarding the environmental compartment, *mcr-1* was reported in Malaysa (Zurfuh et al., 2016), Switzerland river (Li et al., 2016), and Spain sewage water (Ovejero et al., 2017). In the present study, a *mcr-1.2* variant was detected in the 7TE. *coli* on a conjugative IncX4 plasmid named pIBMC-MCR1.2. Other similar IncX4 plasmids carrying *mcr-1* determinat were reported in China, Estonia, South Africa, and very recently Italy, in different *Enterobacteriaceae* from both clinical and animal origin (Di Pilato et al., 2016; Li et al., 2016; Poirel et al., 2016). In the Italian strain, in particular, the *mcr1.2* gene is carried on a plasmid showing 99% similarity with the here reported pIBMC-MCR1.2.

The spreading potential of *mcr-1*-harboring plasmids in MDR microorganisms poses significant challenges for clinical treatment and infection control strategies, as when β-lactams, aminoglycosides or quinolones are ineffective, colistin serve as the final alternative. The advent of transmissible colistin resistance indicates that the evolution of *Enterobacteriaceae* from extensively to pan-drug resistant is inevitable (Biswas et al., 2012).

The occurrence of ESBLs-, DHA-, KPCs-, MCR-1.2-environmental *Enterobacteriaceae* highlights the importance to improve surveillance and remediation actions on surface and ground waters of Oltrepò Pavese area. In this regard, and out of the aims of this report, we observed clonal relatedness between the 1TE. *coli* and the 20LOM.*coli* strain isolated in October 2013 from a Long Term and Rehabilitation Facility located near the Versa stream (Figure 2B). Appropriate measures urgently need to be enforced in order to reduce the anthropogenic burden of antibiotic resistance. Improvement of water status is of major concern: new strategies for the treatment of wastewaters, i.e., the use of sand filters or more-stringent chlorine disinfection, need to be taken into consideration to prevent resistant bacteria from being released into the aquatic environment.

The current results support the hypothesis that environmental water might represent an important hidden resistance reservoir.

NUCLEOTIDE SEQUENCE ACCESSION NUMBER

The complete sequence of the pIBMC_MCR1.2 plasmid was deposited into GenBank under the accession number MF449287.

AUTHOR CONTRIBUTIONS

MC and EN played an important role in interpreting the results and in writing the manuscript. MS and GP helped to acquired data. FZ, SP, FN, VM, AP, IB, and MD carried out experimental work. LP and RM supervised the experiments and revised the final manuscript, which was approved by all authors.

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FULL PAPER

Colonization of residents and staff of an Italian long-term care facility and an adjacent acute care hospital geriatric unit by multidrug-resistant bacteria

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SUMMARY

In 2016, we undertook a point prevalence screening study for *Enterobacteriaceae* with extended-spectrum β-lactamases (ESBLs), high-level AmpC cephalosporinases and carbapenemases, and also methicillin-resistant *Staphylococcus aureus* (MRSA) and vancomycin resistant enterococci (VRE) in a long-term care facility (LTCF) and the associated acute care hospital geriatric unit in Bolzano, Northern Italy.
Urine samples and rectal, inguinal, oropharyngeal and nasal swabs were plated on selective agars. Demographic data were collected. ESBL and carbapenemase genes were sought by PCR.
We found the following colonization percentages with multidrug-resistant (MDR) bacteria in 2016 in LTCF residents: all MDR organisms, 66.1%; ESBL producers, 53.0%; carbapenemase-producers, 1.7%; MRSA, 14.8%; VRE, 0.8%. Colonization by all MDR bacteria was 19.4% for LTCF staff and 26.0% for geriatric unit patients. PCR showed that 80.3% of *Escherichia coli* isolates from LTCF residents, all *E. coli* isolates from LTCF staff, 62.5% and 100% of *Klebsiella pneumoniae* from LTCF residents and geriatric unit patients, respectively, had a bla_{CTX-M}-type gene. All carbapenemase-producing *Enterobacteriaceae* harboured a bla_{VIM}-type gene.
To conclude, the ongoing widespread diffusion of MDR bacteria in the LTCF suggests that efforts should be strengthened on MDR screening, implementation of infection control strategies and antibiotic stewardship programs targeting the unique aspects of LTCFs.

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INTRODUCTION

Long-term care facilities (LTCF) play an important role in contemporary healthcare systems due to an ageing population in industrialized countries, such as Italy where 22% of the population were over 65 years in 2015 (http://www.istat.it/). LTCFs provide ongoing skilled nursing care to residents in need of assistance with activities of daily living and help meet both the medical and non-medical needs predominantly of elderly people with a chronic illness or disability. Residents in these facilities have a variety of risk factors for colonization with multidrug-resistant (MDR) organisms.
Therefore these facilities represent reservoirs of *Enterobacteriaceae* expressing extended-spectrum β-lactamases (ESBLs), derepressed or acquired AmpC cephalosporinases, carbapenemases, methicillin-resistant *Staphylococcus aureus* (MRSA) and vancomycin-resistant enterococci (VRE) (Moro *et al.*, 2013; Cassone *et al.*, 2015; Aschbacher *et al.*, 2016). In 2008 and again in 2012 we undertook a point-prevalence survey for bacteria with these resistance phenotypes among residents and staff of a LTCF in Bolzano, Northern Italy, and among geriatric unit patients in the associated acute care hospital (March *et al.*, 2010; March *et al.*, 2014). The rationale for the repetition of the point prevalence screening study in 2016 in the same LTCF and hospital acute care unit was to determine the long-term trend in colonization prevalence with MDR bacteria of residents and staff, compared with geriatric unit patients of the associated acute care hospital.

Key words:
Long-term care facility, AmpC, ESBL, Carbapenemase, MRSA, VRE.

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MATERIALS AND METHODS

Facilities, resident and patient characteristics, survey design
In October 2016 we repeated a point prevalence study for MDR bacteria, first carried out in 2008 (March *et al.*, 2010) and then repeated in 2012 (March *et al.*, 2014), in a 120-bed LTCF that manages residents with different levels of independence, basal disease, comorbidity and functional status. All residents and staff were eligible to participate, as were patients in the 50 bed geriatric unit of the acute care hospital. The study was approved by the Ethics Committee of Bolzano Hospital.

Microbiological and molecular methods

Microbiological methods were similar to those used in the 2008 and 2012 studies (March *et al.*, 2010; March *et al.*, 2014), with minor modifications. Midstream or catheter urine samples, rectal, inguinal and oropharyngeal swabs from all participants were spread on Brilliance™ ESBL Agar (Oxoid Microbiology Products, Thermo Scientific, UK), applying a 10 µg imipenem disc (BIO-RAD, Marnes-la-Coquette, France), and on Liofilchem® Chromatic VRE (Liofilchem, Italy) agar plates, applying a 5 µg vancomycin disc (Becton Dickinson, USA); nasal swabs and the above mentioned samples except urine were also spread on BBL™ CHROMagar® MRSA II (Becton Dickinson, USA).

Isolates were identified by matrix-assisted laser desorption-ionization-time of flight (MALDI-TOF) Vitek MS mass-spectrometry (bioMérieux, France).

Antibiotic susceptibility testing was performed using the Vitek 2 System, calibrated against European Committee on Antimicrobial Susceptibility Testing criteria (www.EUCAST.org), with AST-N202 cards (including an ESBL test) for Gram-negative bacteria, AST-P632 cards (with both oxacillin and cefoxitin) for MRSA and AST-P586 cards for VRE.

Identification of β-lactamase types was based on Vitek 2 results, Etests (meropenem +/- EDTA for MBL in enterobacteria and imipenem +/- EDTA for MBLs in *Pseudomonas aeruginosa*, cefotaxime +/- clavulanate, ceftazidime +/- clavulanate and cefepime +/- clavulanate for ESBLs, bioMérieux) and the ESBL+AMPC Screen Kit and KPC+MBL Confirm ID Kit (ROSCO DIAGNOSTICA A/S, Denmark). Identification of bla_{CTX-M}, bla_{SHV}, bla_{TEM} and bla_{VIM}-type genes was accomplished by PCR as previously described (Tzelepi *et al.*, 2003; Giakkoupi *et al.*, 2009).

Statistical analysis

The significance of differences in risk factors and colonization proportions was calculated by using the Chi-squared test or the proportion comparison test with Medcalc® version 15.11.4 (MedCalc software, Ostend, Belgium).

RESULTS

All 115 residents of the LTCF present in October 2016 participated in the point-prevalence study; 67 of the 91 (73%) LTCF staff also agreed to be enrolled, mainly nurses and physicians. The median age of LTCF residents was 83 years (range 24-96 years) and the percentage of women was 56%. All 50 acute care geriatric unit patients, with a median age of 85 years (range 74-98 years), joined the study; none was ordinarily a resident of the LTCF.

Table 1 shows demographic and clinical characteristics of LTCF residents and geriatric unit patients involved in the 2016 screening study. The median length of stay of residents in LTCF was 19 months (range <1-172 months).

As shown in *Table 2*, 66.1% of LTCF residents in 2016 were colonized by at least one resistant organism, as were 19.4% of LTCF staff members and 26.0% of geriatric unit patients; 63.4% of residents were colonized by extended-spectrum cephalosporin-resistant (ESCR) *Enterobacteriaceae*, predominantly ESBL-producers (53.0% of residents colonized, 7.8% by more than one species) and high-level AmpC-cephalosporinase producers (25.2% of residents colonized). Interestingly, high-level AmpC-producing *Morganella morganii* isolates from LTCF residents were highly prevalent (24.3%) in the 2016 screening study. Only two LTCF residents (1.7%) were colonized by carbapenemase-producing *Enterobacteriaceae*.

Table 1 - *LTCF resident and geriatric unit patient characteristics in the 2016 screening study.*

	LTCF residents colonized (%) Number of residents –115	*Geriatric unit patients colonized (%) Number of patients –50*
Male sex	43.4	48.0
Age ≥ 86 years	35.6	48.0
Antibiotics in last 3 months	23.4	58.0
Fluoroquinolones	5.2	14.0
Penicillins	12.1	38.0
Cephalosporins	1.7	6.0
Dementia	68.7	62.0
Peripheral vascular disease	71.3	98.0
Incontinence	85.2	76.0
Diabetes	20.8	22.0
Cancer	9.5	20.0
Decubitus ulcer	11.3	0.0
Chronic obstructive pulmonary disease	18.2	22.0
Physical disability (Barthel immobility score of 0)	67.8	
Apallic state (coma)	17.4	0.0
Any medical device	38.2	24.0
Percutaneous enteral gastrostomy tube	20.8	0.0
Tracheostomy tube	9.5	0.0
Urinary catheter	18.2	24.0
Nasogastric tube	1.7	0.0

Table 2 - *Percentages of colonization in residents and staff from the LTCF and geriatric unit patients.*

	LTCF residents colonized (%)	*LTCF staff colonized (%)*	*Geriatric unit patients colonized (%)*	*p-value for differences between LTCF residents and geriatric unit patients*
	Number of residents = 115	*Number of staff members = 67*	*Number of patients = 50*	
All resistance groups (MRSA, VRE and enterobacteria ESBL, AmpC, MBL)	66,1%	19,4%	26,0%	<0.001
All enterobacteria, ESBL-positive	53,0%	11,9%	12,0%	<0.001
Escherichia coli, ESBL-positive	45,2%	11,9%	8,0%	<0.001
Proteus mirabilis, ESBL-positive	7,0%	0,0%	0,0%	0.05
Klebsiella pneumoniae, ESBL-positive	6,1%	0,0%	4,0%	0.58
Morganella morganii, ESBL-positive	2,6%	0,0%	0,0%	0.25
Citrobacter koseri, ESBL-positive	0,8%	0,0%	0,0%	0.52
Citrobacter freundii, ESBL-positive	0,0%	0,0%	2.0%	0.19
Alle enterobacteria, high-level AmpC	25,2%	0,0%	8,0%	0.01
Enterobacter cloacae, high-level AmpC	0,0%	0,0%	4,0%	0.03
Morganella morganii, high-level AmpC	24,3%	0,0%	0,0%	<0.001
Citrobacter freundii, high-level AmpC	0,0%	0,0%	4,0%	0.03
Proteus mirabilis, high-level AmpC	0,8%	0,0%	2,0%	0.51
All enterobacteria, MBL-positive	1,7%	0,0%	4,0%	0.37
MRSA	14,8%	7,4%	6,0%	0.11
VRE	0,8%	0,0%	0,0%	0.52

PCR testing showed that 80.3% of *Escherichia coli* isolates from LTCF residents had bla_{CTX-M}-type genes, 15.1% harbored bla_{SHV}-type genes and 83% had a bla_{TEM}-type determinant. The bla_{VIM}-type gene was present in one *E. coli* isolate, together with a bla_{TEM}-type determinant (corresponding to 1.9% of isolates from LTCF residents). Moreover, all *E. coli* isolates from LTCF staff had bla_{CTX-M}-type genes. Four out of five *E. coli* isolates collected from geriatric unit patients had a bla_{CTX-M}-type gene; in the remaining *E. coli* strain, a bla_{VIM}-type gene was detected. By PCR testing 62.5% of *Klebsiella pneumoniae* isolates from LTCF residents and all *K. pneumoniae* from geriatric unit patients had bla_{CTX-M}-type genes; one of the latter isolates, besides the bla_{CTX-M}-type gene, also harboured bla_{VIM}. Finally, no bla_{CTX-M}-type determinant was detected in the 31 *M. morganii*, nine *Proteus mirabilis*, five *Citrobacter spp.* and three *Enterobacter spp.* The single isolate of *Citrobacter amalonaticus* from a LTCF resident and the unique *Enterobacter aerogenes* strain from a geriatric unit patient (who was also colonized by the bla_{VIM} producing *K. pneumoniae*) harboured a bla_{VIM}-type gene together with a bla_{SHV-12} determinant.

MRSA colonization prevalence was 14.8% in LTCF residents, 7.4% in LTCF staff and 6.0% in geriatric unit patients. Colonization of residents with VRE was rare (0.8%). No LTCF staff member was colonized with *Pseudomonas aeruginosa*, whereas 75.6% of LTCF residents (rectal – 62%, inguinal – 35%; urine – 20%; oropharyngeal – 20%) and 32% of geriatric unit patients were colonized; none of these isolates was a MBL producer. No *Acinetobacter baumannii* grew on the Brilliance ESBL Agar (an OXA-23 carbapenemase producing strain was used as positive control).

Enterobacteriaceae producing ESBLs, derepressed or acquired AmpCs or MBLs in LTCF residents were most often recovered from rectal swabs (90%) and MRSA from oropharyngeal (88%) specimens (*Table 3*). The best screening strategies to detect colonized residents required both rectal and inguinal samples for *Enterobacteriaceae* and oropharyngeal, combined with nasal or rectal samples for MRSA.

Table 3 - *Percentages of residents to be positive for methicillin-resistant Staphylococcus aureus (MRSA) or Enterobacteriaceae producing extended-spectrum β-lactamase (ESBL), derepressed or acquired AmpC-cephalosporinases or metallo-β-lactamases (MBLs) with various specimen type combinations.*

	MRSA (%)	*ESBL, AmpC or MBL producing Enterobacteriaceae (%)*
Rectal	17	90
Inguinal	23	79
Oropharyngeal	88	15
Nasal	47	
Urina		52
Rectal+inguinal	29	100
Rectal+oropharyngeal	94	90
Rectal+nasal	53	
Rectal+urine		98
Inguinal+oropharyngeal	88	84
Inguinal+nasal	53	
Inguinal+urine		89
Oropharyngeal+nasal	94	
Oropharyngeal+urine		58

DISCUSSION

In 2016, we repeated a screening study, first undertaken in 2008 (March et al., 2010) and then repeated in 2012 (March *et al.*, 2014), for MDR *Enterobacteriaceae*, MRSA and VRE in a LTCF and the associated acute care hospital geriatric ward. Comparing data from the 2016 screening study with data from the two previous studies, 66.1% of LTCF residents in 2016 were colonized by at least one resistant organism, compared with 74.8% in 2008 (p=0.15) and 53.8% in 2012 (p=0.06); 19.4% of LTCF staff members in 2016, compared with 27.5% in 2008 (p=0.26) and 10.5% in 2012 (p=0.17) were also colonized, as were 26.0% of geriatric unit patients in 2016, compared with 22.2% in 2008 (p=0.66) and 22.7% in 2012 (p=0.71).

Differences in LTCF resident colonization prevalence with ESBL producing *Enterobacteriaceae* between 2016 (53.0%) and 2008 (64.0%) or 2012 (49.0%) were not statistically significant (p-values of 0.09 and 0.55, respectively), as were not significant differences for LTCF staff or geriatric unit patients.

On the other hand, LTCF resident colonization prevalence for high-level AmpC-producing *Enterobacteriaceae* was significantly higher (p<0.001) in 2016 (25.2%) compared with 2008 (4.5%) and 2012 (3.8%); this difference was caused by a significant increase (p<0.001) of high-level AmpC-producing *Morganella morganii* in 2016 (24.3%), according to phenotypic tests, compared with 2008 (0.0%) and 2012 (1.9%). Only two LTCF residents (1.7%) were colonized by carbapenemase-producing *Enterobacteriaceae* in 2016.

Colonization prevalence of LTCF residents with MRSA was significantly lower in 2016 (14.8%) compared with 2008 (38.7%; p<0.001), but it was similar in 2012 (13.2%; p=0.73). A striking feature is the colonization of 19.4% of LTCF staff with resistant bacteria in 2016 (ESBL: 11.9%; MRSA: 7.4%), compared with 27.5% in 2008 (ESBL: 14.5%; MRSA: 14.8%) and 10.5% in 2012 (ESBL: 5.2%; MRSA: 7.0%). This carriage probably reflects resident-to-staff transmission facilitated by the low functional and cognitive status and the generally low mobility score of LTCF residents, requiring continuous nursing care for daily living activities, with many occasions for horizontal transmission of MDR organisms between residents and health care workers.

Differences in colonization prevalence between LTCF residents and geriatric unit patients are highly significant (p<0.001) for ESBL and high-level AmpC-producing *Enterobacteriaceae*. Similarly to colonization rates in LTCF residents, percentages of acute care hospital's inpatient MRSA isolates (2008: 21.8%; 2012: 18.9%; 2016: 20.3%) or of ESCR *E. coli* isolates (2008: 10.9%; 2012: 12.8%; 2016: 10.0%) did not change significantly (p=0.64 and p=1.00, respectively). On the other hand, ESCR isolates of *K. pneumoniae* increased significantly (2008: 12.2%; 2012: 16.7%; 2016: 29.2%; p<0.001), but the increase in this resistance phenotype in the acute care hospital was not paralleled by increasing colonization prevalence of the same phenotype in LTCF residents (data for 2008 and 2012: March *et al.*, 2014; data for 2016: Aschbacher R, unpublished data).

In 2016, in our LTCF, 63.4% and 14.8% of residents were colonized by extended-spectrum cephalosporin-resistant enterobacteria and MRSA, respectively. Slightly lower colonization rates of 56.0% and 12.0%, respectively, were found in 2012 in a second LTCF in the same Bolzano health district (March *et al.*, 2014). In 2006, a mean colonization percentage of 54% for residents bearing a urinary catheter in 23 Italian LTCFs (Arnoldo *et al.*, 2013) was reported, whereas in 2015 in four Italian cities on average 57.3% of residents were colonized by ESBL-producing enterobacteria and 17.2% by MRSA (Giufrè *et al.*, 2017). Nasal MRSA carriage of 7.8% and 19.3%, respectively, was found in 2006 in residents of two Italian LTCFs (Brugnaro *et al.*, 2009) and in 2005 in residents of one LTCF (Monaco *et al.*, 2009), respectively; the last study found a MRSA prevalence for staff of 5.8%. In various screening studies in other European countries, a high variability of colonization frequencies for ESBL-producing *Enterobacteriaceae* and MRSA, ranging from close to 0% up to levels higher than 50% were found; variability is high especially among countries but also among different LTCFs within single countries (Aschbacher *et al.*, 2016). As shown for ESBL-producing *E. coli* isolates from LTCF residents and staff from the two previous screening studies in the same LTCF (March *et al.*, 2010; March *et al.*, 2014), a high predominance of CTX-M-type enzymes was also found in 2016; isolates from the two previous screening studies were mainly *E. coli* ST131 expressing CTX-M-15-like enzymes. VRE colonization in our LTCF in 2016 was low (0.8% of residents), similarly to 2008 (2.7%) and to other European studies in LTCFs (March *et al.*, 2010, Aschbacher *et al.*, 2016).

In 2016, in our LTCF, colonization of residents by carbapenemase-producing enterobacteria was low (1.7%) and no staff member was colonized. A similarly low, mean colonization rate (1.0%) was found in LTCF residents of various Italian Provinces in 2015 (Giufrè *et al.*, 2017). The two carbapenemase producers in our study expressed VIM-type enzymes, predominant in the Bolzano healthcare district (Carattoli *et al.*, 2010; Aschbacher *et al.*, 2011; Aschbacher *et al.*, 2013). Other authors found VIM-type carbapenemases (Accogli *et al.*, 2014; Giufrè *et al.*, 2017), *K. pneumoniae* carbapenemases (KPCs) (Del Franco *et al.*, 2015; Piazza *et al.*, 2016; Giufrè *et al.*, 2017) and New Delhi metallo-β-lactamases (NDMs) (Gaibani *et al.*, 2011) in screening or clinical isolates from Italian LTCFs. The low colonization prevalence of carbapenemase-producing *Enterobacteriaceae* in Italian LTCF residents is surprising because Italy has one of the highest prevalence rates of this resistance phenotype in blood isolates from Europe (Annual report EARS-Net, 2015).

Much lower colonization rates with MDR bacteria in the screening studies were found in the Bolzano geriatric unit of the acute care hospital compared with LTCF residents. The carbapenemase-producing *Enterobacteriaceae* isolates (1 *E. coli*, 1 *K. pneumoniae*, 1 *E. aerogenes*) from geriatric unit patients harboured bla_{VIM}-type genes, similar to previous studies (March *et al.*, 2010; March *et al.*, 2014). No significant differences in colonization frequencies were found for geriatric unit patients between the screening studies in 2008, 2012 and 2016, though significant differences were found for percentages of male patients (2008: 28.9%, 2012: 27.3%, 2016: 48.0%) and dementia patients (2008: 33.3%, 2012: 31.8%, 2016: 62.0%), significantly higher in 2016 compared with 2008 (p=0.05 and p=0.05, respectively) and 2012 (p=0.04 and p=0.003, respectively).

Usage of an oropharyngeal swab for screening of LTCF

residents for MRSA and a rectal swab for screening of enterobacteria-producing ESBLs, high-level AmpCs or carbapenemases, gave recovery rates of 88% and 90%, respectively (compared with the combination of all four specimen types). Recovery rates for MRSA from oropharyngeal swabs in 2016 are in contrast with the two previous screening studies in 2008 and 2012 in the same LTCF where recovery rates of only 50% and 72%, respectively, were obtained (March *et al.*, 2010; March *et al.*, 2014). Moreover, using nasal swabs alone we would have obtained MRSA recovery rates of only 45%, 52% and 47% in 2008, 2012 and 2016, respectively. In many Italian and other European colonization studies, the only sample type for MRSA screening was nasal swabs, leading to possible significant underestimation of real colonization frequencies. On the other hand, using only rectal swabs for ESBL screening, as done in most of the colonization studies, still permits high recovery rates of 77-96% (Aschbacher *et al.*, 2016).

Few risk factors for colonization with MDR bacteria in LTCF residents or geriatric unit patients changed significantly from 2008 (March *et al.*, 2010) or 2012 (March *et al.*, 2014) to 2016. The number of coma residents in the LTCF was lower in 2012 (10 residents) compared with 2008 and 2016 (20 residents each), the percentage of catheterized residents was also lower in 2012 (24.5%) compared with 2008 (48.6%; p<0.001) and 2016 (38.2%; p=0.03), whereas the percentage of dementia residents in 2012 (85.8%) was significantly higher compared with 2008 (66.7%; p=0.002) and 2016 (68.7%; p=0.002). Chronic obstructive pulmonary disease increased significantly in 2016 (18.2%) compared with 2008 (6.3%; p=0.006). The lower number of coma residents and catheterized residents and the higher percentage of dementia residents in 2012 compared with 2008 and 2016 could be a partial explanation for the lower colonization rates by MDR bacteria in 2012 compared with 2008 (p=0.002) and for the marginally lower rates compared with 2016 (p=0.06). Nevertheless, strengthened hygiene measures introduced after the 2008 screening, according to World Health Organization guidelines (WHO guidelines on hand hygiene, 2009), were maintained throughout the period 2009-16. Therefore, similar colonization rates in 2016, compared with the previous studies, confirm that control of colonization by MDR organisms in LTCFs is challenging (Smith *et al.*, 2008). Nevertheless, the snapshot approach (samples were collected only once in 2008, 2012 and 2016, respectively, in a short period of time) might lead to the sudden increase in prevalence of specific resistance phenotypes, as shown for high-level AmpC-producing *M. morganii* in 2016 which could be a transient phenomenon, and the choice of a point prevalence study might therefore explain the discrepancy in the conclusions of the two studies (preventive measures seemed to be effective in 2012 but not in 2016).

This study has some limitations. First, it was carried out in a single LTCF, not allowing for extrapolation of data to other LTCFs in other Italian cities. Second, molecular characterization of isolates in the 2016 study was limited. Despite these limitations, the strength of our study is the 100% participation of LTCF residents, the comparison with 100% of geriatric unit patients present in the associated acute care hospital unit and the inclusion of a high percentage of LTCF staff in the screening survey. Moreover, the repetition of the screening in four-year intervals (2008, 2012 and 2016) allows evaluation of a long-term trend in colonization frequencies.

The ongoing widespread diffusion of MDR bacteria in LTCFs in the Bolzano healthcare area confirms that LTCFs are a potentially important reservoir for MDR organisms and suggests that future efforts should focus on MDR screening, improved implementation of infection control strategies and antibiotic stewardship programs targeting the unique aspects of LTCFs. To promote further studies of various microbiological aspects related to LTCFs, the Association of Italian Clinical Microbiologists (Associazione Microbiologi Clinici Italiani; AMCLI) in 2016 has set up a new working group consisting of Clinical Microbiologists (Gruppo di Lavoro per lo Studio delle Infezioni nelle Residenze Sanitarie Assistite e Strutture assimilabili; GLISTer); the present study was supervised by this working group.

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Conflict of interest

All authors: nothing to declare.

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