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Detection of carbapenem-resistant *Enterobacterales* in food producing animals and human patients: A "One Health" perspective

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Abstract

In past decades, antimicrobial-resistant (AMR) bacteria became a growing public health concern. Over time, this phenomenon has become a significant problem on a global scale, particularly for those pathogens showing resistance against molecules of primary interest in the medical treatment of bacterial infections in humans, such as carbapenems.

Carbapenems, which belong to the macro-class of β-lactams, are considered by the World Health Organization (WHO) to be “Critically Important Antimicrobials” (CIAs), *i.e.*, drugs used only in extreme cases and as a last line of defense against infections caused by multidrug-resistant Gram-negative microorganisms. The strategies used by bacteria to develop effective resistance to antimicrobials involve many cellular functions, and the main mechanism of resistance to carbapenems is the production of specific enzymes belonging to the β-lactamase class, called carbapenemases. Among these enzymes, those that pose the greatest threat to public health are KPC (*Klebsiella pneumoniae* carbapenemase), NDM (New Delhi metallo-beta-lactamase), VIM (Verona integron-encoded-metallo-β-lactamase), IMP (Imipenemase) and OXA-48 (oxacillinase).

Although the use of carbapenems is banned in veterinary medicine, bacteria resistant to these antimicrobials have been increasingly isolated from food-producing animals in recent years. This phenomenon is probably due to two factors: the selective pressure resulting from the misuse/abuse of antimicrobials, especially other β-lactams, in veterinary medicine, and the contamination from wastewaters, especially those coming from hospitals or highly urbanized environments.

Since humans, animals and the environment are closely interlinked, a “One Health” approach is essential to address the problem. For this reason, this study included samples of both human and animal origin. The samples of human origin consisted of 199 urine samples collected from patients hospitalized at the Parma University-Hospital. The samples of animal origin included 691 rectal swabs collected from cows at slaughter, 317 bovine milk filters and 231 fecal samples collected from slaughtered pigs in Parma province.

Since infections caused by carbapenem-resistant bacteria are mainly associated with healthcare settings and are often caused by bacteria belonging to the *Enterobacterales* family, especially *K. pneumoniae* and *E. coli*, human and animal samples were tested for the presence of carbapenem-resistant *Enterobacterales*. Genotypic methods were used to identify carbapenemases in the isolates that shew signs of resistance to carbapenems in the antimicrobial susceptibility tests (Kirby-Bauer test and Minimal Inhibitory Concentration assay).

A total of 53 *Enterobacterales* strains phenotypically resistant to carbapenems were isolated from the four matrices. Of these, 18 strains (33.96%) were isolated from the human urine samples, 16 (30.19%) from the bovine milk filters, 10 (18.87%) from the bovine rectal swabs and 9 (16.98%) from the pig stools samples. Genes encoding for carbapenemases were found in 7 (13.21%) of these isolates: 2 (3.77%) from human samples, 2 (3.77%) from milk filters and 3 (5.66%) from pig samples. None of the bovine rectal swab isolates were found positive. The genes found in this study belonged to the KPC, VIM and OXA-48-like families.

This project was a collaboration between the Inspection of Food of Animal Origin Unit and the Infectious Diseases Unit of the Department of Veterinary Science and the Nephrology Unit of the Department of Medicine and Surgery of the University of Parma.

1. Introduction

In the Introduction section, an overview on resistance to carbapenems in microorganisms of human and animal origin will be given. The complexity of the subject requires a full description to introduce the study performed during the doctoral period.

1.1. Antimicrobial resistance

Antimicrobial resistance (AMR) is nowadays a global public health crisis that seriously threats our ability to successfully treat bacterial infections. This danger, however, has not been emerged only in recent years. Microbiologists and infectious disease specialists have long recognized this problem, as Sir Alexander Fleming himself, the discoverer of penicillin, drew attention to the threat of resistance from underdosing (Fleming, 1945). Nevertheless, only recently the extent of the gravity of this phenomenon has truly been understood, also due to its ever-increasing diffusion (McEwen and Collignon, 2018). Many infectious agents that could once be successfully treated with several different drug classes have now acquired resistances towards one or more of these. But how did we get from the point where antimicrobials (AMs) were truly "wonder drugs" that could be relied upon to cure a wide range of life-threatening infections, to the point today, where resistance to most AMs is widely prevalent and the supply of new classes of drugs has dwindled to a trickle? The complete answer is not simple, nor, unfortunately, is the solution, however we can say that the overuse and misuse of these drugs in human and veterinary medicine is the main factor that is contributing to the global spread of AMR (O'Neill, 2016; McEwen and Collignon, 2018). In fact, the overuse of AMs forces microorganisms to face a selective pressure that enhances their fitness through the acquisition and expression of resistance genes, which may be later shared with other bacteria via several mechanisms of genetic material exchange, consequently leading to an ever-increasing spread of AMR genes.

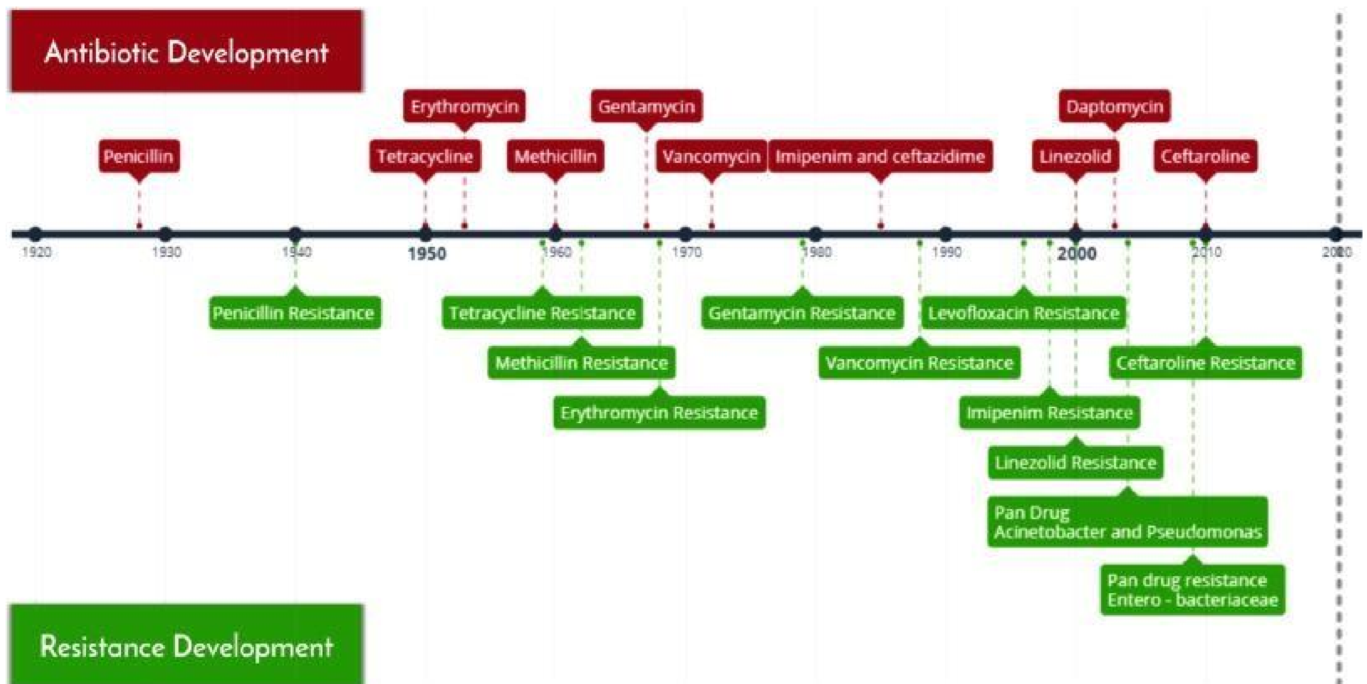


Figure 1: History of antibiotics discovery and antibiotics resistance development (Zainab et al., 2020)

Thus, even though AM overuse and disuse are the main drivers of AMR, there are other important factors that promote the spread of AMR locally and globally (Holmes et al., 2016), such as poor infection control, environmental contamination, and geographical movement of infected humans and animals (Burow and Käsbohrer, 2017). Wherever AMs are improperly used, there are high chances of development for reservoirs of resistance, not only in humans in hospitals and highly anthropized environments, as well as aquaculture and farmed animals, but also in water, soil, wildlife, and other ecological niches, due to pollution by sewage, pharmaceutical industry waste, and manure runoff from farms (Hujibers et al., 2015; McEwen and Collignon, 2018). Bacteria and bacterial genomes can move easily among humans, animals and the environment, and for this reason the adaptations that occur in anyone of these sectors may affect the other two, as well as the actions taken (or not taken) to contain this threat in one of the three sectors can be reflected on the remaining two (O'Neill, 2016).

Therefore, AMR is not a menace that involves only humans or public health, but also an ecological problem, characterized by a complex interaction system, involving diverse microbial populations

affecting the health of humans, animals, and the environment. For these reasons, it makes sense to address the resistance problem by taking this complexity and ecological nature into account using a coordinated, multisectoral approach, such as the One Health approach (So et al., 2015; Robinson et al., 2016), which is defined as “the collaborative effort of multiple health science professions, together with their related disciplines and institutions—working locally, nationally, and globally—to attain optimal health for people, domestic animals, wildlife, plants, and our environment” (One Health Commission, 2018).

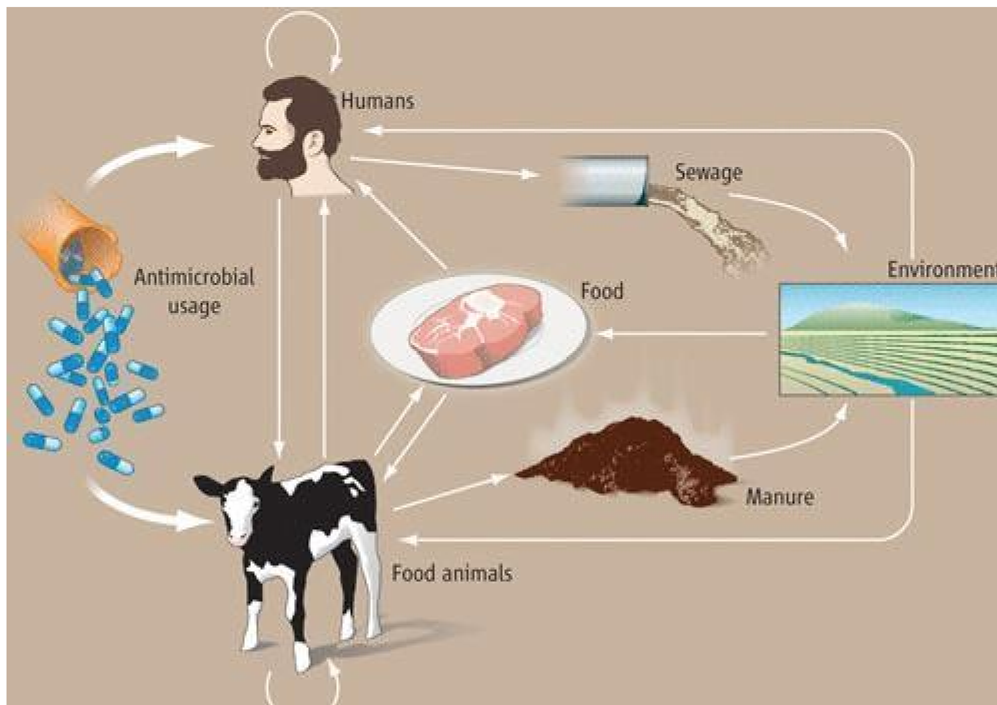


Figure 2: Representation of the routes of transmission of antimicrobial resistance between farm animals, the wider environment, and humans (McEwen and Collignon, 2018).

This includes taking steps not only for prevent the increasingly spreading of this phenomenon, but also to preserve the continued effectiveness of existing AMs by eliminating their inappropriate use. Major concerns in the animal health and agriculture sectors are mass medication of animals with AMs that are critically important for humans, including some β-lactams such as third generation cephalosporins (McEwen and Collignon, 2017).

Farms and aquaculture facilities are ideal environments for the development and dissemination of AMR genes, because AMs were also used for purely preventive and non-therapeutic purposes, which have been banned in EU since 2022 with Regulation 2019/6. (European Commission, 2018). As a consequence, low and sublethal concentrations of antibiotics are consistently present in the gastrointestinal environment of livestock, promoting the selection for AMR genes which are released into the environment, mainly through manure and other animal waste. Several studies in fact show that these genes are found extensively in the wastes of industries associated with animal husbandry (Hille et al., 2017; Ma et al., 2019; Markland et al., 2019). Moreover, due to their use in livestock farming, AMs may residue in manure applied to soil in agriculture practices, and besides this, bacteria potentially carrying AMR genes are shed by animals, contaminating agricultural areas. In this way, bacteria may be run-off from soil and reach surface water, and through these contaminate crops, wild animals and the surrounding environment (Bonardi and Pitino, 2019).

Therefore, environmental pollution plays a key role in the spread of AMR bacteria and genes, which can spread among bacteria by different transfer mechanisms, the most important of which is bacterial conjugation which allows the horizontal transfer of genetic material even between bacteria of different species via transfer of plasmids. The mobile genetic elements (MGE) such as transposons or insertion sequences, and their ability to be carried by a wide range of hosts, made them perfect vectors for AMR spread (Rozwandowicz et al., 2018). Although plasmid conjugation is the main route of horizontal gene transfer (HGT), including AMR genes, this is not the only one. Indeed, HGT can occur by bacteriophage transduction and natural transformation by extracellular DNA, thus allowing genetic material to jump between strains and species (Lerminiaux and Cameron, 2019).

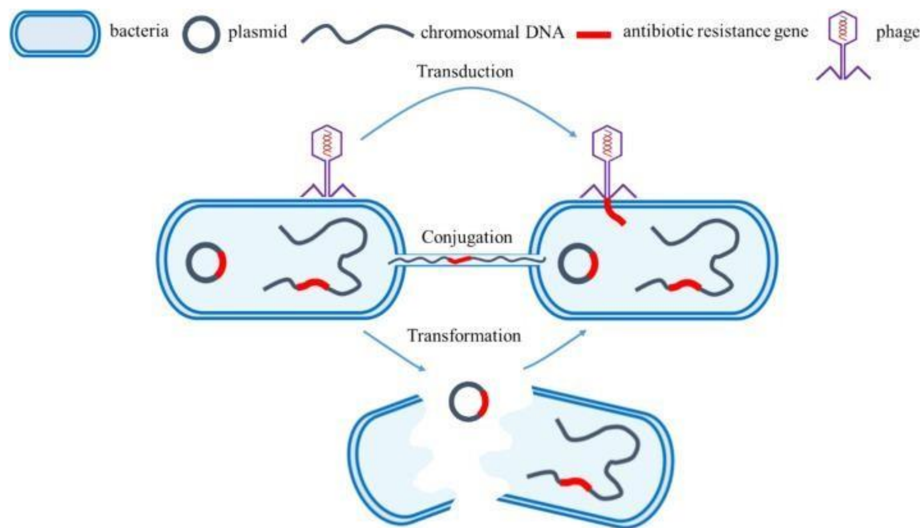


Figure 3: Mechanisms of horizontal gene transfer (Nguyen et al., 2021)

1.2 Classification of antimicrobials

There are several ways to classify antimicrobials (AMs), *i.e*., through their molecular structure, mechanism of action, spectrum of activity and different route of administration (Etebu and Arikekpar, 2016).

These drugs can have two different types of effects on microorganisms:

- Bacteriostatic effect: they block the bacteria growth without killing the microorganism (i.e., quinolones and sulphonamides).
- Bactericidal effect: they cause the death of the bacteria (i.e., β-lactams and tetracyclines).

On the basis of the spectrum of action, antibiotics are classified in broad spectrum (active both against Gram positive and Gram-negative bacteria) and narrow spectrum, that can act efficiently only on certain bacterial species (Gerber et al., 2018)

Very often one or more antibiotics are used synergistically, thus allowing action on different targets and increase in the activity of individual molecules (Cambiotti et al., 2014). However, to set up an effective therapeutic strategy, the molecular structure of the AM and the characteristics of its binding site must be known. Almost all AMs have as primary target the bacterial cell, on

which they cause a wide range of adverse effects with the aim of eliminating the microorganisms (bactericidal effect) or at least blocking their further growth (bacteriostatic effect) (Khan, 2018).

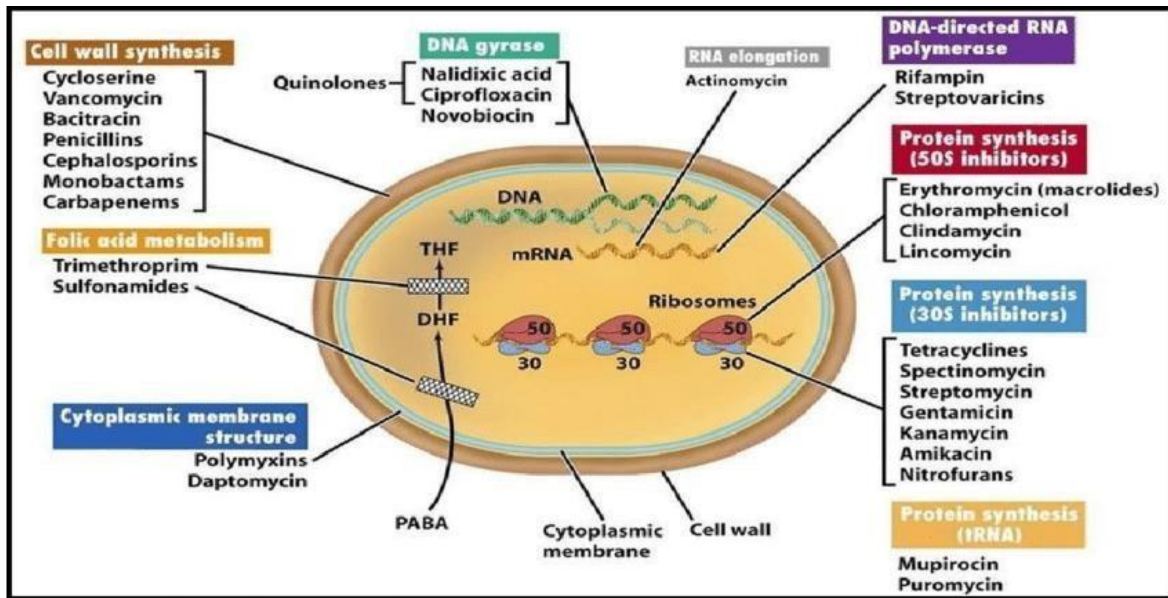


Figure 4: Antimicrobials and their target sites on bacterial cells (Khan, 2018).

A few AM classes, such as carbapenems, are exclusively reserved for humans, while others, such as ionophores, find their use only in veterinary medicine, mainly because their toxicity for humans. This mean that the great majority of AM classes are used in both human and veterinary medicine, including their use in farming practices and aquaculture (Van Boeckel et al., 2015; EFSA and ECDC and EMA, 2015; EMA, 2016; FAO, 2017). In veterinary medicine, there are notable differences in the way AMs are used for companion animals compared to food-producing animals. While in the former their use practices are similar to those in humans, with the administration of drugs made on individual basis and with the primary aim of treat clinical infections, in the latter the management is quite different, with the drugs frequently administrated to entire group of animals (i.e. flock of broilers, pens of pigs) through feed or water, with an approach more prophylaxis-oriented. However, also in food-producing animals AMs are administered for therapeutic use to individual animals affected by clinical infections (i.e., cows with mastitis) (McEwen and Collignon, 2018).

1.3 β-lactams

1.3.1 β-lactams subclasses

Despite having been in use for a long time, β-lactam antibiotics are still one of the most relevant drug classes of antibacterial agents worldwide. The discovery and the market of first β-lactam (Penicillin G) is a symbolic landmark of modern chemotherapy. Since then, several other β-lacta antibiotics have been introduced in therapy, revolutionizing the medical treatment of bacterial infections (Lima et al., 2020). This class is formed by broad spectrum AMs with bactericidal activity based on the interruption of bacterial cell-wall formation, thanks to the covalent binding to essential penicillin-binding proteins (PBPs), which are involved in the terminal steps of peptidoglycan cross-linking in both Gram-negative and Gram-positive bacteria (Bush and Bradford, 2016).

β-lactam antibiotics are structurally-related thanks to their active site, the β-lactam ring that characterizes the molecular structure of these compounds. They are classified in four main groups: penicillins, cephalosporins, carbapenems and monobactams. This classification is based on the chemical nature of the ring fused to the β-lactam pharmacophore unit (Lima et al., 2020).

Penicillins were the first group of β-lactam antibiotic to be used in clinical practice. Some molecules of this class, like amoxicillin, are still widely use nowadays. These drugs are often associated with β-lactamase inhibitors, such as clavulanic acid, to overcome the increasing AMR (Huttner et al., 2020). All penicillins have a fundamental structure formed by a thiazolidine ring linked to a β-lactam ring, which together form the 6-aminopenicillanic acid. Members of the Penicillin group include Penicillin G, Penicillin V, Oxacillin (dicloxacillin), Methicillin, Nafcillin, Ampicillin, Amoxicillin, Carbenicilin, Piperacillin, Azlocillin, Mezlocillin and Ticarcillin (Lima et al., 2020).

Cephalosporins are a group of β-lactam antibiotics similar in structure and in action mechanism to penicillins. These AMs are divided into five generations, based on their spectrum of coverage against Gram-positive and Gram-negative bacteria and their temporal discovery. First-generation cephalosporins like cefazolin, cephalothin, cephapirin, cephradine, cefadroxil, and cephalexin have mostly coverage against Gram-positive bacteria and minimal coverage against some Gram-

negative bacteria (e.g., *Escherichia coli*, *Proteus mirabilis*, and *Klebsiella pneumoniae*) (Bui and Preuss, 2023).

Second-generation cephalosporins like cefuroxime or cefoxitin have less activity against Gram-positive bacteria than first-generation ones, but have increased coverage against *Haemophilus influenza*, *Moraxella catarrhalis*, and *Bacteroides* spp. (Bui and Preuss, 2023).

Third-generation cephalosporins like cefotaxime, ceftazidime, cefdinir, ceftriaxone, cefpodoxime, and cefixime have a more reduced coverage against most Gram-positive bacteria but an increased action against Gram-negative bacteria resistant to first and second generation cephalosporines, like several *Enterobacterales*, *Neisseria* spp., and *H. influenza* (Bui and Preuss, 2023).

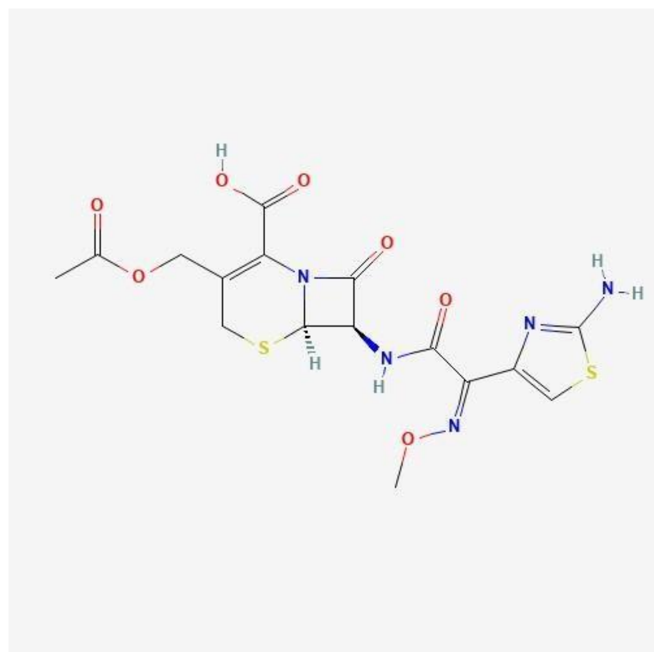


Figure 5: Cefotaxime chemical structure (Pubchem, 2024).

Fourth-generation cephalosporins, like cefepime, have similar coverage as third-generation cephalosporins but with additional coverage against AMR Gram-negative bacteria, in particular they are more effective against β-lactamase producers (Bui and Preuss, 2023).

Fifth-generation cephalosporins include ceftaroline, which is a broad-spectrum AM active against susceptible Gram-positive and Gram-negative microorganisms. However, what makes it unique from the rest of the cephalosporins, is that it has coverage against methicillin-resistant *Staphylococcus aureus*. Ceftaroline can also cover *Listeria monocytogenes* and *Enterococcus faecalis* (Bui and Preuss, 2023).

Monobactams like aztreonam are monocyclic β-lactam AMs active against aerobic enteric bacteria like *Pseudomonas* spp. and, at the time of their introduction, stable to hydrolysis by common β-lactamases due to their peculiar single rather than double ring core structure (Bush and Bradford, 2016). However, the emergence of new β-lactamases like ESBLs and serine carbapenemases has decreased monobactams' efficiency against multidrug-resistant organisms (Wang et al., 2014; Bush and Bradford, 2016).

Carbapenems like biapenem, doripenem, ertapenem, faropenem, imipenem, meropenem and panipenem are the most powerful members of the β-lactam family and occupy a unique position among this class because they are resistant to most β-lactamases produced by Gram-positive and Gram-negative bacteria. In fact, even if carbapenems share a penicillin-like ring with penicillins and cephalosporins, they possess a carbon instead of a sulfone in the fourth position of the thyazolidinic moiety of the β-lactam ring (Bonardi and Pitino, 2019). Carbapenems are very efficient to treat a wide variety of infections such as intra-abdominal infections, skin infections, community-acquired and nosocomial pneumonia, complicated urinary tract infections, meningitis (meropenem only) and febrile pneumonia thanks to their broad-spectrum activity against both Gram-positive and -negative bacteria (Janssen et al., 2015; Bonardi and Pitino, 2019; Lima et al., 2020).

1.3.2 Use of β-lactam antibiotics in veterinary medicine

Members of β-lactam class of drugs constitute one of the most important groups of AM agents used in veterinary medicine and they are the therapy of choice in some well-established practices (Nòbrega and Brocchi, 2014). In companion animals, penicillins like ampicillin and amoxicillin or first and second-generation cephalosporines are used as first-line drugs for the treatment of uncomplicated infections, while the use of third-generation cephalosporins is usually limited to severe infections (Zogg et al., 2018).

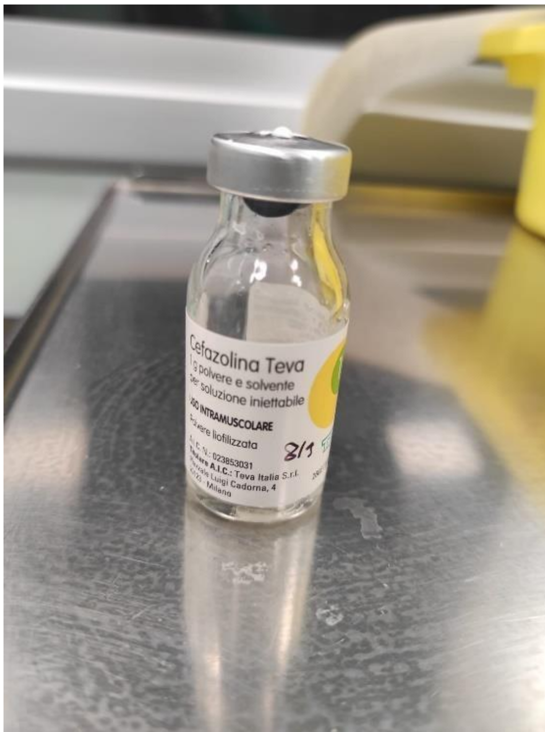


Figure 6: Cefazolin is a β-lactam authorized in veterinary medicine

According to the World Organization for Animal Health (WOAH), β-lactam antibiotics usable in livestock mainly include various first-generation cephalosporins (such as cefalexin and cefazolin), three third-generation cephalosporins (ceftiofur, ceftriaxone and cefoperazone) and one fourth-generation (cefquinome) (WOAH, 2021). Cephalosporins are used in the treatment of septicemias, respiratory infections, and mastitis. In addition to cephalosporins, the use of various

penicillins, including benzylpenicillin, amoxicillin, ampicillin and cloxacillin, is permitted in many species of production animals, mainly for septicemias, respiratory diseases, and urinary tract infections (WHO, 2018a). On the contrary, carbapenems have never been licensed for veterinary treatments worldwide (WHO, 2018b) and their residues in foods of animal origin are not actually allowed (Bonardi and Pitino, 2019).

At last, another sector with a widespread use of AMs, including β-lactam class members, is aquaculture, with amoxicillin and ampicillin being the only two authorized in this practice (WOAH, 2021).

1.3.3 Carbapenems

Carbapenems represent the most powerful and innovative class of β-lactam antibiotics. These AMs are active against a wide range of bacterial species (including *Enterobacterales*, *Acinetobacter* spp. and *Pseudomonas* spp.) due to their broad spectrum. Moreover, they are used to treat infections supported by multidrug-resistant (MDR) bacteria, and are the drugs of choice for the treatment of severe infections by ESBL-producing microorganisms (Partier and Timsit, 2020).

Historically, the first reported carbapenem was thienamycin, a molecule isolated from *Streptomyces catteleya* in 1976, which despite the great potential antimicrobial activity had a very limited use due to its instability in water. Structurally, thienamycin presents the typical β-lactam ring, but with a carbon atom instead of sulfur in position 1 (Kahan et al., 1979). The problem of thienamycin instability was solved years later by adding the N-formimidoyl group to the position 2 of the β-lactam ring, thus creating a new compound, imipenem, today considered the progenitor of carbapenems. Since then, several new molecules have been developed and approved for clinical use, such as ertapenem and meropenem (Kiratisin et al., 2012; Armstrong et al., 2021).

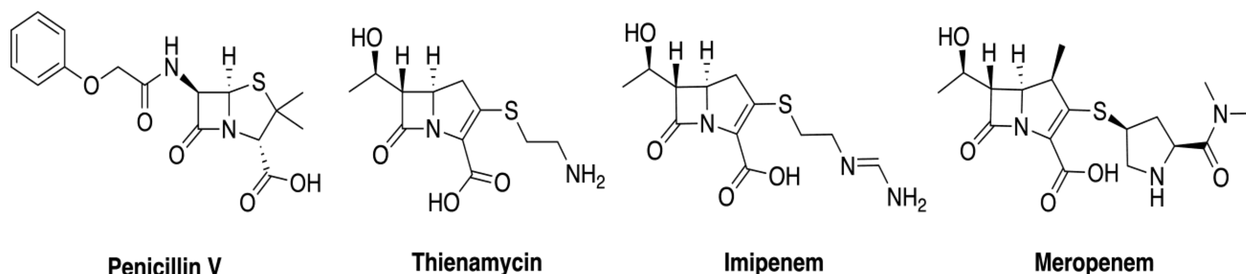


Figure 7: Structure of commonly administered carbapenems in comparison to the β-lactam penicillin V and precursor thienamycin (Armstrong et al., 2021)

Structural differences between carbapenems and other members of the family are the key to their resistance against many β-lactamase enzymes that usually inactivate other drugs. In contrast to penicillins, the 4:5 fused ring system in carbapenems is unsaturated and has no ring sulphur; instead, sulphur is a C2 substituent. The trans configuration of C5–C6 and the C-6 (R)-hydroxyethyl substituent provides better resistance against β-lactamases (Armstrong et al., 2021). In fact, carbapenems tend to have a better stability against these enzymes in comparison to other β-lactam antibiotics, allowing them to be active against β-lactamase-producing bacteria, such as Extended-Spectrum Beta-Lactamase (ESBL) *Enterobacterales*. However, several microorganisms are capable of facing the action of carbapenems through a series of mechanisms, including the production of powerful β-lactamases, called carbapenemases, which can exert hydrolytic activity (El-Gamal et al., 2017)

Carbapenems work by penetrating the bacterial cell wall and binding to PBP enzymes. The resulting effect is lethal as it involves the inactivation of an enzyme autolytic inhibitor within the cell wall resulting in cell death. Specifically, they bind strongly to PBP2 in Gram-negative bacteria, but also bind to PBP1a, PBP1b and PBP3, providing additional killing mechanisms (Sumita and Fukasawa, 1995; Zhanel et al., 2007; El-Gamal et al., 2017).

They have been proven to have a wider action spectrum against bacteria in comparison to the other available β-lactam antibiotics, even in combination with β-lactamase inhibitors such as clavulanic acid. Some carbapenems, such as imipenem, panipenem, and doripenem, exhibited robust activity against Gram-positive bacteria, while others, such as meropenem, ertapenem,

and doripenem proved to be more suitable for the treatment of infections sustained by Gram-negative microorganisms (El-Gamal et al., 2017).

All clinical trials demonstrated that carbapenems have low oral bioavailability and must be administered intravenously because they cannot readily cross the gastrointestinal barrier. In addition, imipenem (if combined with cilastin) and ertapenem can also be administered intramuscularly (El-Gamal et al., 2017)

1.4 β-lactamases

AMs continue to be nowadays the most important resource in the global management of infectious diseases, but the increased occurrence of AMR in pathogens has raised global concern as AMs are steadily losing efficacy in clinical and community settings (Wencewicz, 2019). AMR represents one of the main challenges that public health has to face today, both for the severity of its consequences and its widespread diffusion.

AMR towards β-lactam antibiotics can occur by multiple mechanisms, including modification of the target (mutation or expression of alternative PBPs), reduction in cell permeability through downregulation of porins required for β-lactam entry, over-expression of efflux systems and production of modifying or degradative enzymes.

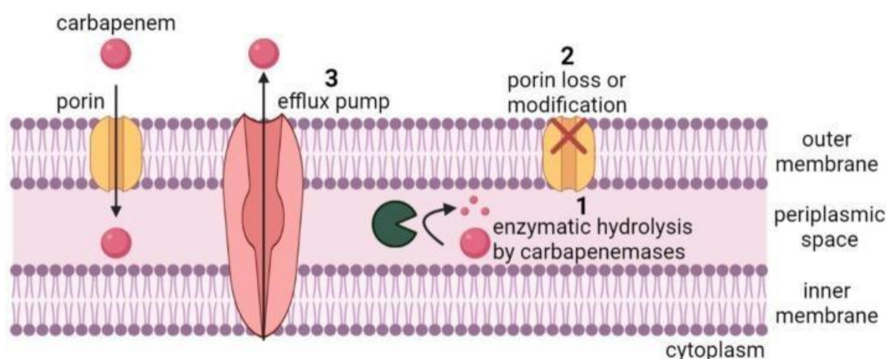


Figure 8: The mechanisms of carbapenem resistance in Enterobacterales (Dixon et al., 2022).

In the case of β-lactam antibiotics, enzyme-mediated resistance arises from the activity of β-lactamases, produced by both Gram-positive and Gram-negative bacteria, that hydrolyze the β-lactam ring. Based on their molecular structure, β-lactamases are divided into four classes according to the Ambler classification system: The active-site serine β-lactamases (classes A, C and D) and the zinc-dependent or metallo-β-lactamases (MBLs; class B) (Ambler, 1980; Tooke et al., 2019). Despite the initial importance of PC1-mediated penicillin resistance in *Staphylococcus aureus*, in Gram-positive bacteria the emergence of strains carrying PBP alterations has largely superseded β-lactamase production as the primary mechanism of β-lactam resistance (Fisher and Mobashery, 2016). On the contrary, in Gram-negative bacteria β-lactamases remain the primary resistance mechanism, although resistance is very often a multifactorial and phenotype driven by combinations of mechanisms that frequently include permeability modifications and/or efflux pump upregulation in addition to β-lactamase production (Tooke et al., 2019). Although all four classes are widely distributed in multiple species of clinically significant and environmental bacteria, within each class a few enzyme families have been successful and have been disseminated widely across the bacteria. Key enzyme families include TEM, SHV, CTX-M and KPC (class A); NDM, IMI and VIM (class B); CMY and ADC (class C). Class D enzymes are termed oxacillinase (OXA); of particular concern are OXA-23, OXA-48 and OXA-181 (Tooke et al., 2019; Bush and Bradford, 2020).

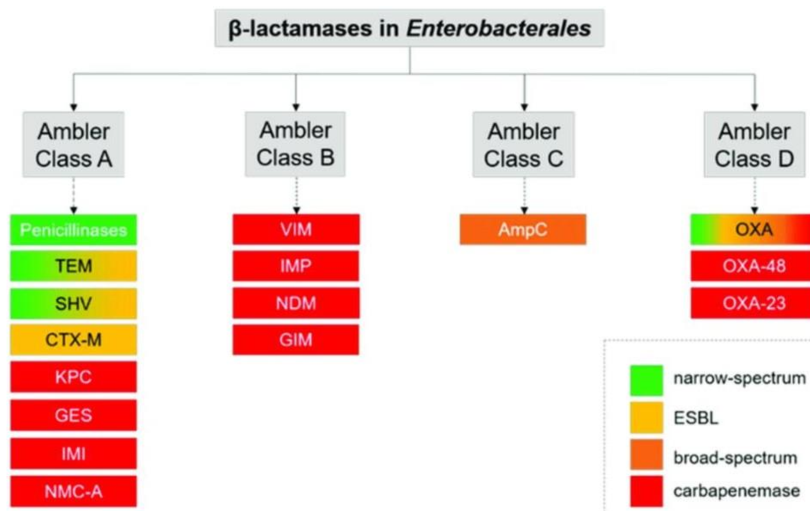


Figure 9: Ambler's classification with examples of main β-lactamases in Enterobacterales (Noster et al., 2021).

These enzymes can be classified not only by their molecular structure, but also towards a functional classification that takes into account subclass and AMs against which they exert their inhibitory action. Enzymes considered of major importance among the β-lactamases are those whose genes are encoded on mobile elements transferable among different bacteria species, mainly via plasmidic conjugation. Major β-lactamase families include plasmid-mediated Extended-Spectrum Beta-Lactamases (ESBLs), cephalosporinases (AmpC) and carbapenemases (Bush and Bradford, 2020).

ESBL-producing bacteria are prevalent worldwide and often correlated with hospital infections, but they have now been evolving as an increasing cause of community-acquired infections. The spread of ESBL bacteria constitutes a major threat for public health, and infections with ESBL-producing microorganisms have been associated with poor outcomes. The majority of ESBLs belong to Ambler class A and include the sulfhydryl reagent variable β-lactamase (SHV), Temoniera β-lactamase (TEM) and cefotaxime-M β-lactamase (CTX-M) types (Karaiskos and Giamarellou, 2020). However, ESBLs group also include several enzymes belonging to Ambler's classes C and D. These molecules are capable to hydrolyze the β-lactam ring of broad-spectrum β-lactam antibiotics, including penicillins, early cephalosporines, monobactams and third generation cephalosporines such as ceftriaxone, ceftazidime, and cefotaxime; additionally, many ESBL-producing strains of *E. coli* and *Klebsiella* spp. have demonstrated co-resistance to other AMs such as fluoroquinolones, aminoglycosides, and tetracyclines. However, they hardly show activity against cephamycin and carbapenems (Kawamura et al., 2017; McDanel et al., 2017).

CTX-Ms are currently the largest group of ESBLs. CTX-M-1 was the first identified enzyme of this family in Germany, France and South America. It originated from *Kluyvera* spp., spread through mobilization from its chromosomal position, and exhibited a "cefotaximase" property, hydrolyzing cefotaxime at high rates but almost sparing ceftazidime (Bevan et al., 2017). Presently, CTX-M-β-lactamases include more than 220 different enzymes, clustered into five subfamilies based on their aminoacid identities that include the CTX-M-1, CTX-M-2, CTX-M-8, CTX-M-9, and CTX-M-25 subfamilies (Naas et al., 2017; Peirano and Pitout, 2019). Enzymes that

originated from subfamilies CTX-M-1 and CTX-M-9 are widely distributed and commonly reported among *Enterobacterales* strains worldwide. Currently, the gene *bla*$_{CTX-M-15}$ (part of the CTX-M-1 subfamily) is the most widely distributed genomic variant in the world, followed by *bla*$_{CTX-M-14}$ (part of the CTX-M-9 subfamily), which is the predominant genotype in several Asian countries (especially China, Japan and South Korea) (Bevan et al., 2017; Peirano and Pitout, 2019; Poirel et al., 2019).

The HGT of *bla*$_{CTX-M}$ genes on conjugative plasmids is fundamental to their evolution and massive global spread while occurring in the human gut, animals and the environment. Plasmids are often carried by *E. coli* or other *Enterobacterales* (Bevan et al., 2017; Robin et al., 2017). Other than their widespread, the most worrying feature of *bla*$_{CTX-M}$ bearing plasmids is their ability to capture additional resistance determinants, including genes encoding for carbapenemases such as OXA-48 and NDM-1 (Partridge et al., 2013; Dautzenberg et al., 2014; Bevan et al., 2017).

Another factor that has contributed to the spread of these genes is the extensive and sometimes indiscriminate use of extended spectrum cephalosporins in farms; third-generation cephalosporins are used for respiratory and systemic infections caused by *Streptococcus suis*, including arthritis or mastitis in suckling and weaned piglets, while the fourth-generation cephalosporin cefquinome is licensed for the therapy of bacterial septicemia in piglets and mastitis-metritis-agalactia syndrome in sows (Randall et al., 2014; Zelendova et al., 2020).

In addition to CTX-M, the other two major ESBL families structurally belonging to Ambler class A are SHV and TEM. In the 1990s, SHV and TEM genes were responsible for nosocomial infections, often associated with *Klebsiella pneumoniae*, and encoded on transferable plasmids (Doi et al., 2017; Peirano and Pitout, 2019). According to a recent study, 243 variants of TEM and 228 variants of SHV have been identified, although not all of these possess the ESBL phenotype. However, unlike CTX-M, ESBLs members of SHV and TEM families are highly related to each other, with variants differing by only few aminoacidic substitutions (Castanheira et al., 2021).

TEM-type β-lactamases are variants of the original plasmid mediated TEM-1, which was found in an *E. coli* isolate from a blood culture of a Greek patient in the early 1960s. The first variant was TEM-2, which differs from TEM-1 for only a single aminoacidic. Prevalence of some of the TEM-

type variants were regional in nature, for example TEM-3 is commonly found in France but rarely seen in the USA, where TEM-10 is the most common variant. On the contrary, some variants, such as TEM-26, were detected in isolates from all the globe (Castanheira et al., 2021).

As the CTX-M-type β-lactamases became the most prevalent ESBL worldwide, TEM-type enzymes became more infrequent. In a recent survey of European isolates, TEM-type ESBLs were detected in less than 1% of ESBL-producing *E. coli* and *Klebsiella pneumoniae* strains (Kazmierczak et al., 2020; Castanheira et al., 2021).

The SHV-type β-lactamases originated as chromosomally encoded enzymes in *K. pneumoniae*. In 1985, the first ESBL described was SHV-2 and was found in a single strain of *Klebsiella ozaenae* isolated in Germany. SHV-2 differed from SHV-1 by a single amino acid. Worldwide, SHV-5 and SHV-12 have been the most common ESBL variants found in *Enterobacterales*; in particular, SHV-type ESBLs are commonly found in clinical *K. pneumoniae* isolates, but to a lesser extent also in other genera of *Enterobacterales* and in *P. aeruginosa* (Castanheira et al., 2021).

β-Lactamases of Ambler's class C are widely distributed on the chromosomes of many Gram-negative species. Many of the most important opportunistic pathogens carry chromosomal genes encoding class C enzymes, typically annotated as AmpC, that under normal conditions are not expressed. The clinical relevance of these enzymes is further enhanced by the dissemination of certain families, such as CMY, FOX and DHA, on mobile genetic elements in both *Enterobacterales* and non-fermenting species such as *P. aeruginosa*. Like ESBLs, AmpC enzymes may exhibit MDR phenotypes, due to co-expression of multiple plasmid-mediated resistance determinants to non-β-lactam antibiotics, such as aminoglycosides. However, although class C β-lactamases have spread widely among *Enterobacterales*, their overall prevalence has remained far lower than that of ESBLs (Meini et al., 2019; Tooke et al., 2019).

Functionally, AmpC enzymes are characterized by an overall greater hydrolyzation capability of cephalosporins (including cephamycin) than penicillin G, hence the term "cephalosporinases" that has been used for these enzymes. In addition, they are resistant to β-lactamase inhibitors, such as clavulanate, sulbactam and tazobactam (Meini et al., 2019). Class C β-lactamases usually have low hydrolyzation rate against fourth generation cephalosporins and do not possess

carbapenemase activity; as a consequence, their involvement in carbapenem resistance is generally due to reduced permeability combined to over-expression of class C enzymes which lead to sequester of carbapenems before they can reach their PBP targets. However, though rare, some studies have reported class C enzymes (CMY-10) capable of imipenem hydrolysis (Goessens et al., 2013; Bonomo et al., 2018; Meini et al., 2019; Tooke et al., 2019).

Although both ESBLs and AmpCs are important threats to public health, nowadays the most worrying danger is represented by carbapenemases, i.e., β-lactamases belonging to different Ambler classes (A, B, D) and encoded by both chromosomal and plasmidic genes. They hydrolyze a broad variety of β-lactam antibiotics, including carbapenems, cephalosporins, penicillins and aztreonam (Bonardi and Pitino, 2019).

1.4.1 Carbapenemases

Carbapenem-resistance is mainly driven by carbapenemases found on mobile genetic elements, such as integrons, insertion sequences, transposons, and mobile plasmids, that can shuttle carbapenemase-expressing genes within and across bacteria of the same or different species (Lugtring and Limbago, 2016; Pedersen et al., 2018; Kopotsa et al., 2019). The ability of plasmids to carry multiple AMR genes and be mobilized across same and different species via conjugation make them very important in the AMR molecular epidemiology (Kopotsa et al., 2019).

Figure 10: Genetic Map of IncA/C2 plasmid encoding a Tn4401 trasposon associated with bla$_{KPC-3}$ carbapenemase (Yao et al., 2021).

The dramatic increase in prevalence and clinical impact of infections caused by carbapenemase-producing (CP) bacteria is a global health concern, especially when *Enterobacterales* such as *K. pneumoniae* or *E. coli* are involved, due to their ability to readily spread and colonize patients in healthcare environments (Bonomo et al., 2018).

Class A carbapenemases can be chromosomally or plasmid encoded. The following enzymes belong to the first group: NmcA (Non-metallo carbapenemase A), SME (*Serratia marcescens* enzyme), IMI-1 (Imipenem-hydrolyzing β-lactamase), SFC-1 (*Serratia fonticola* carbapenemase), BIC-1 (Bicêtre carbapenemase), PenA (penicillinase from *Pseudomonans cepacia*), FPH-1 (from *Francisella philomiragia*) and several members of SHV family. The second group is composed by the following carbapenemases: KPC (*Klebsiella pneumoniae* carbapenemase), GES (Guiana extended spectrum β-lactamase), IMI-2, and FRI-1 (Naas et al., 2017; Bonardi and Pitino, 2019).

KPC enzymes are by far the most clinically significant class A carbapenemases due to their global distribution in invasive bacterial species (in particular *K. pneumoniae*), often responsible for opportunistic infections in healthcare settings (Rapp and Urban, 2012; Tooke et al., 2019). Nowadays, bla_{KPC} positive *Enterobacterales* are widespread in several areas of the globe, such as the United States, Latin America, Southern Europe, Middle East and China (Han et al., 2020). At least 114 variants have been described, with KPC-2 (which is identical to KPC-1) and KPC-3 being the most common. KPC enzymes provide resistance not only against carbapenems, but also to other β-lactam antibiotics (penicillins, cephalosporins, cephamycin and monobactams). However, they are usually inhibited by β-lactamase inhibitors, such as clavulanic acid and tazobactam (weak inhibition) or avibactam and boronic acid (stronger inhibitory effect) (Vasquez-Ponce et al., 2023). The spread of bla_{KPC} primarily occurs via the clonal dissemination of *K. pneumoniae*, in particular of some dominant KPC-producing clones, such as the sequence type (ST) 258, ST512 and ST11 (Bonardi and Pitino 2019). *K. pneumoniae* ST 258 is widespread all over the world, reported in more than 25 countries from four different continents, including several countries where KPC enzymes are considered epidemic; this clone usually possesses plasmids that harbor $bla_{\text{KPC-2}}$ and $bla_{\text{KPC-3}}$ genes and is considered responsible for 77% of the outbreaks in USA and 90% of all infections in Israel (Chen et al., 2014).

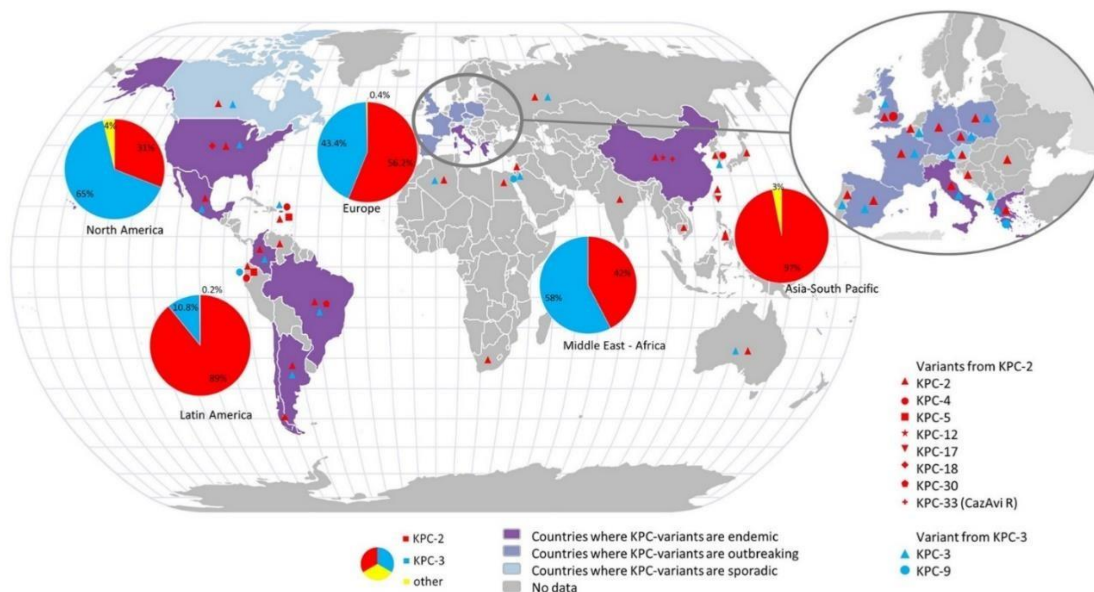


Figure 11: Geographical distribution of bla_{KPC-2} and bla_{KPC-3} and their most frequent circulating variants (Hobson et al., 2020).

Class B β-lactamases are also known as metallo-beta-lactamases (MBLs) due to the peculiar structure of their active site, which clearly distinguishes them from serine β-lactamases. In fact, class B enzymes require zinc for their hydrolytic activity and catalysis does not proceed via a covalent intermediate but rather through direct attack of a hydroxide ion that is stabilized by the zinc in the active site (Palzkill, 2012).

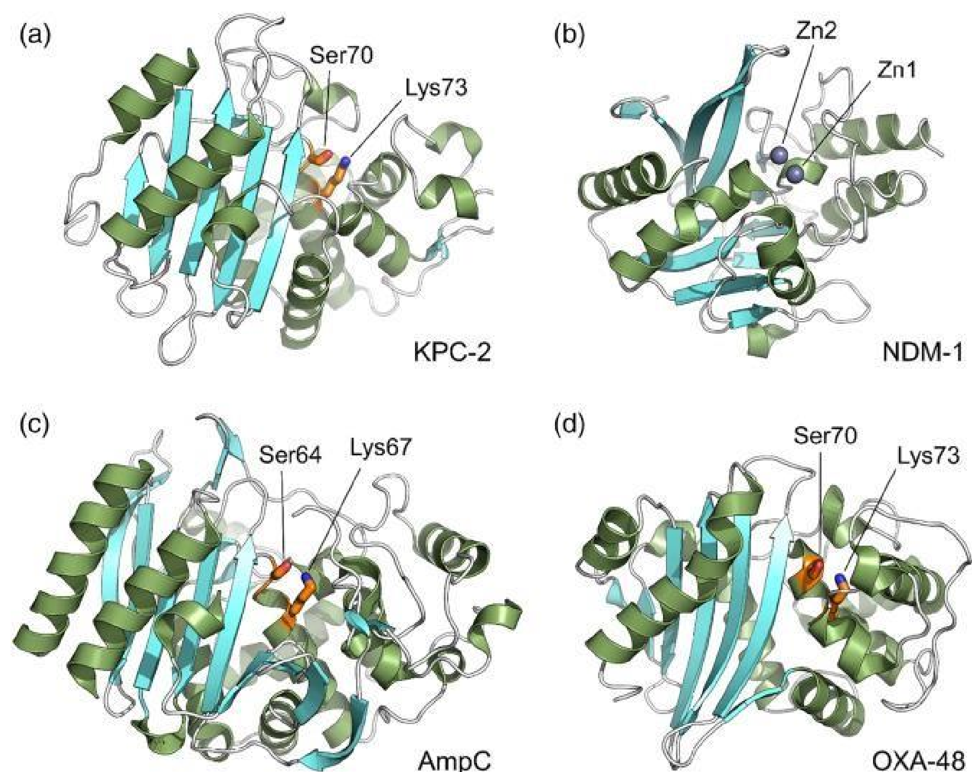


Figure 12: Overall structure of representative β-lactamases from each class (Tooke et al., 2019)

As β-lactamases, the MBLs are notable for their exceptionally broad spectrum of activity, which encompasses penicillins, cephalosporins and carbapenems, although they show little or no activity against monobactams. They are not inhibited by mechanism-based inhibitors such as clavulanate, sulbactam, or tazobactam that are effective against serine-based β-lactamases, but they can be inactivated by metal chelator such as ethylenediaminetetraacetic acid (EDTA) (Palzkill, 2012; Lohans et al., 2017; Tooke et al., 2019).

Based on a molecular criterion, MBLs are divided into three subclasses (B1, B2, and B3) that differ by the active site residues, metal content requirement, and substrate profile. The most clinically

relevant MBLs, such as the NDM, VIM and IMP, belong to the subclass B1, which, differently from B2 and B3, requires two zinc ions to be fully active and has a broad substrate spectrum (Palacios et al., 2020). Genes encoding these enzymes are largely plasmid-borne and can be transferred between different bacterial strains and species, making these genes particularly significant as healthcare threat (Wu et al., 2019).

New Delhi metallo-β-lactamase (NDM) is able to hydrolyze almost all β-lactam antibiotics, including carbapenems (but not monobactams), and its activity cannot be prevented by the clinically available β-lactamase inhibitors (Wu et al., 2019; Ranjan and Thatikonda, 2021).

NDM-1 was first identified in a *K. pneumoniae* strain isolated from a Swedish patient hospitalized in New Delhi, India, in 2008. Since then, NDM-1 has been found in various species of *Enterobacterales*, as well as in *Acinetobacter* spp. and *Pseudomonas* spp. More than 100 NDM variants have been identified in more than 60 species of 11 bacterial families, with several variants having enhanced carbapenemase activity. NDM variants commonly contain between 1 and 5 amino acid substitutions compared to NDM-1 (Wu et al., 2019; WHO, 2023).

K. pneumoniae and *E. coli* are the predominant carriers of bla_{NDM}, with certain sequence types (as ST11, ST14, ST15, or ST147 for *K. pneumoniae* and ST167, ST410, or ST617 for *E. coli*) being the most prevalent. NDM-positive isolates have been identified worldwide, with the highest prevalence in India and China. In particular, NDM-1 is the more common variant not only in these two countries, but also in other areas of the globe (Middle East, Balkans, South America). Otherwise, less common variants have been found in several European countries, as NDM-4 in Italy, and NDM-5 and NDM-7 in France and Denmark. NDM-5 is also commonly found in North Africa (Khan et al., 2017; Wu et al., 2019; Ranjan et al., 2021).

Verona integron encoded metallo-β-lactamase (VIM) is another important MBL spread both in *Enterobacterales* and non-fermenting bacteria. After the initial discovery of bla_{VIM-1} in a clinical isolate of *P. aeruginosa* in Italy during 1997, several variants have been reported in different bacterial species worldwide. The enzymes of this family are divided into two large groups, with the bla_{VIM-1} group detected mainly in *Enterobacterales* and the bla_{VIM-2} group in *Acinetobacter* spp. and *Pseudomonas* spp. (Pena et al., 2014; Govender et al., 2015; Bonardi and Pitino, 2019).

VIM-producing *Enterobacterales* are widespread in all Europe, with Greece being the epicenter of their spread to other European countries, including Spain, Italy and France (Cantòn et al., 2012; Vittecoq et al., 2017)

Since the *bla*VIM genes are often located within class 1 integrons that reside on broad-host range plasmids, they can be easily transferred between bacteria and contribute to the inter-species distribution of VIM-producing genes (Temkin et al., 2014; Bonardi and Pitino, 2019).

The last metallo-β-lactamase of critical importance is imipenemases (IMP), which it is hardly blocked by β-lactamase inhibitors such as clavulanate, sulbactam and tazobactam. IMP-1 is capable to hydrolyze all kinds of β-lactam antibiotics, including penicillins, cephalosporines and carbapenems. Consequently, strains producing IMPs are difficult to control even with β-lactam antibiotics combined with β-lactamase inhibitors, such as the amoxicillin-clavulanic acid combination (Senda et al., 1996; Bonardi and Pitino, 2019; Hu et al., 2021).

IMP-1 was firstly discovered in *Serratia marcescens* in Japan in 1991, while IMP-2 was detected in a clinical isolate of *A. baumannii* in Italy. Actually, at least 96 variants of the bla_{IMP} gene have been described (Shakibaie et al., 2017; Li et al., 2023). These genes can be carried by both *Enterobacterales* and non-fermenting species such as *Acinetobacter* spp. and *Pseudomonas* spp. The bla_{IMP} genes are located in class 1 integrons carried by plasmids and can spread horizontally among different species (Sidjabat and Paterson, 2015; Bonardi and Pitino, 2019).

The class D β-lactamases are also referred to as oxacillinases or OXA β-lactamases due to their capacity of hydrolyzing oxacillin more efficiently than benzylpenicillin (Pitout et al., 2019). These enzymes were first recognized in the 1960s and 1970s and showed hydrolytic activity against penicillins and oxacillin. Later, it was found that some OXA β-lactamases could also inactivate cephalosporins and carbapenems (Evans and Amyes, 2014; Pitout et al., 2019). Currently, more than 1200 members of this family have been reported (Jiang et al., 2023).

Genes encoding for OXAs can be found both on chromosomes and plasmids of several bacterial species, even many chromosomal Class D β-lactamases have been transferred to plasmids, thus becoming a major problem for public health (Antunes and Fisher, 2014).

OXA β-lactamases can have a spectrum of activity that varies greatly from enzyme to enzyme, but as a general principle OXAs are resistant to common β-lactam inhibitors and confer resistance to the amino-, carboxy-, and ureidopenicillins (Antunes and Fisher, 2014; Pitout et al., 2019).

Class D carbapenemases are one of the principal causes of carbapenem resistance among *Enterobacterales* (especially in *K. pneumoniae*) and *A. baumannii*, although their activity against carbapenems is not always as efficient as for other carbapenemases, such as NDM or KPC family members. Surveillance studies have shown that OXA-48-like enzymes are the most common carbapenemases found in *Enterobacterales* in certain regions of the world and are routinely introduced into nonendemic regions, becoming responsible for nosocomial outbreaks. OXA-48, OXA-181, OXA-232, OXA-204, OXA-162, and OXA-244 are, in that order, the most common enzymes identified among the OXA-48-like carbapenemase group (Pitout et al., 2019; Tooke et al., 2019).

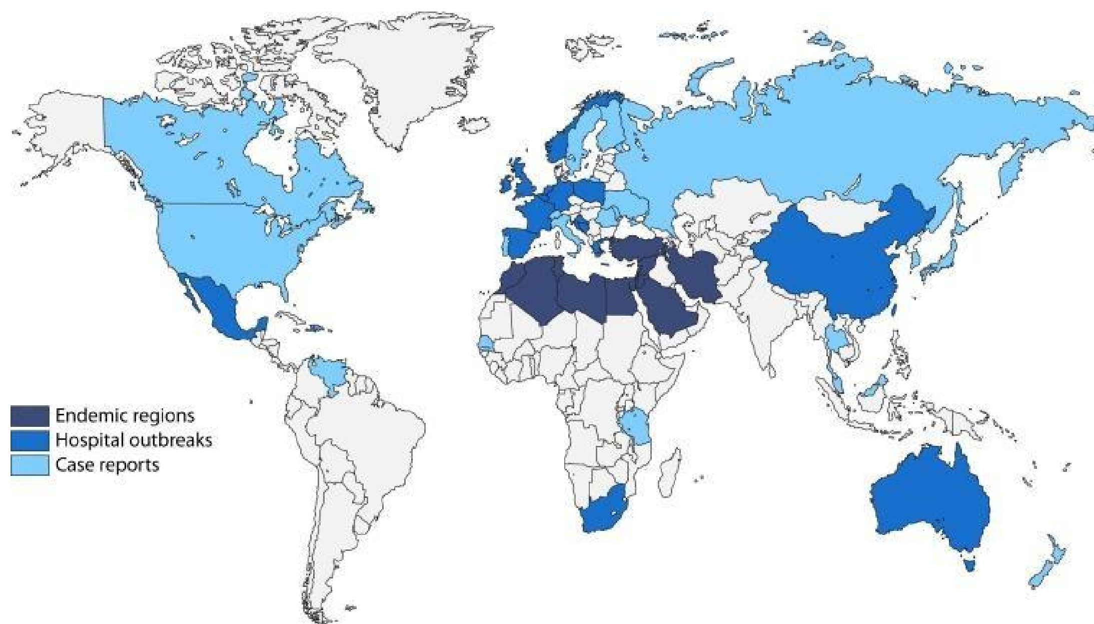


Figure 13: Global distribution of Enterobacterales with bla$_{OXA-48}$-like genes (Pitout et al., 2019).

OXA-23-like enzymes were the first group of carbapenem-resistant class D β-lactamases to be identified. They were first found in an *A. baumannii* isolate in the United Kingdom in 1985. Genes

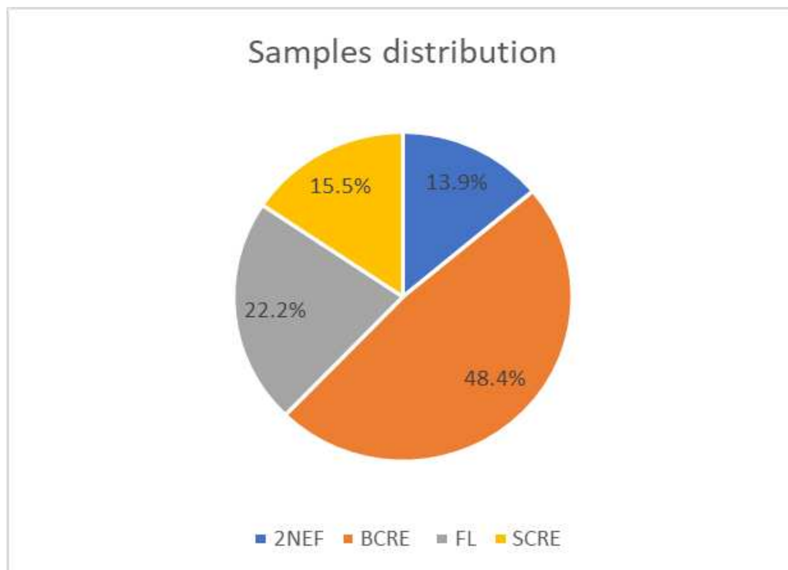
for this group of enzymes are frequently plasmid-borne and have been found in many *Acinetobacter* and *Enterobacterales* species (Evans et al., 2014).

2. Materials and Methods

2.1 Sampling

In this survey, the following samples were analyzed:

- 199 human urine samples collected at the Parma University-Hospital (codified as 2NEF)
- 691 fecal samples collected from cattle at slaughter (codified as BCRE)
- 317 milk filters collected form dairy herds (codified as FL)
- 230 fecal samples collected from pigs at slaughter (codified as SCRE)



Graphic 1: Distribution of the samples among human, bovine, swine, and milk samples

Many of these samples were collected before the PhD period started (November 2020) and the isolates thereof were stored at -80°C, to be further tested.

During the doctoral period, the following samples were collected:

- Human urine samples: From 2NEF 99 to 2 NEF 199 (100 samples)

- Fecal samples from cattle: From BCRE 571 to BCRE 691 (120 samples)
- Milk filters: From FL 181 to FL 317 (136 samples)
- Fecal samples from pigs: From SCRE 89 to SCRE 230 (141 samples)

The 199 urine samples were collected from patients hospitalized at the Renal Intensive Care Unit and the Renal Transplant Unit of the Parma University-Hospital between October 2019 and June 2021. Their collection was authorized by the Rectoral Decree n° 2146/2019.

The 691 bovine fecal samples were collected by using rectal swabs from cattle at slaughter in Parma province between March 2017 and February 2022. The swabs were placed in sterile tubes containing 1ml of Buffered Peptone Water (BPW, Oxoid, Basingstoke, UK). Sampling was carried out on a weekly basis, with an average of 12 swabs taken at each sampling session.

The 230 swine fecal samples were collected from the rectum of pigs at a slaughter plant in the province of Parma between July 2020 and November 2021. Only one pig per batch was tested, so that 230 different batches of animals were sampled. The pigs came from different farms located in four regions of northern Italy, *i.e.*, Lombardy, Emilia-Romagna, Veneto and Piedmont.

Milk filters are gauze used during the milking process to carry out an initial cleaning of milk, given that their mesh retains various types of debris and impurities, such as lumps of faeces and soil, vegetal debris and insects, and, obviously, microorganisms. The 371 milk filters were collected from 150 dairy cattle farms located in 32 different municipalities in the province of Parma between March 2019 and September 2022. The distribution of samples in the different municipalities is shown in Table 1.

Municipality code	**Number of filters**	**Percentage**
002PR	16	4.98%
004PR	2	0.62%
007PR	4	1.25%
008PR	2	0.62%
009PR	1	0.31%
010PR	14	4.36%
012PR	13	4.05%
014PR	4	1.25%
015PR	3	0.93%
016PR	5	1.56%
018PR	2	0.62%
019PR	6	1.87%
020PR	6	1.87%
021PR	1	0.31%
023PR	13	4.05%
024PR	1	0.31%
025PR	22	6.86%
026PR	2	0.62%
027PR	36	11.21%
028PR	15	4.67%
032PR	1	0.31%
033PR	3	0.93%
034PR	1	0.31%
035PR	1	0.31%
036PR	4	1.25%
037PR	4	1.25%
038PR	2	0.62%
039PR	11	3.43%
041PR	91	28.38%
042PR	3	0.93%
043PR	3	0.93%
046PR	7	2.18%
Not identified	22	6.85%
Total	321	100.00%

Table 1: Territorial distribution of the milk filters tested in the study period

2.2 Enrichment step

The samples of animal origin were subjected to non-selective enrichment, while the samples collected from the hospitalized patients were subjected to a selective enrichment.

The enrichment step was performed with a 1:10 dilution. In particular, based on the matrixes, the following methods were used:

- Human urine samples: each sample (1 ml) was transferred to a tube containing 9 ml of Trypticase Soy Broth (TSB, Oxoid). A disc containing 10 µg of meropenem was added to the tubes before incubation.
- Bovine rectal swabs: 9 ml of BPW were directly added to the tubes containing the rectal swabs diluted in 1 ml of BPW.
- Milk filters: the filters were weighed in a sterile Stomacher bag with filter to be added with 9 parts of diluent (BPW).
- Pig stool samples: 1 g of faeces was transferred into a sterile tube and added with 9 ml of BPW.

After dilution in BPW, the samples were incubated in at 37°C ± 1°C for 18-24h.

After incubation, the broth cultures were plated onto selective-differential media added with antibiotics to inhibit commensal flora and select carbapenem-resistant bacteria.

2.3 Plating on selective media

Regardless of their matrix of origin, the broth cultures were plated onto selective-differential agar media to obtain well-isolated colonies of bacteria resistant to β-lactam antibiotics. For this purpose, two different media were used: Brilliance CRE agar and McConkey agar.

Brilliance CRE agar (Oxoid): As reported by the manufacturing company, Brilliance CRE is a chromogenic screening medium used for the detection of carbapenem-resistant *Enterobacterales*. This medium allows the presumptive and differential identification of *E. coli*

and KESC group bacteria (consisting of *Enterobacterales* belonging to the genera *Klebsiella*, *Enterobacter*, *Serratia* and *Citrobacter*) thanks to the mix of chromogenic substances. In particular, colonies presumably belonging to the KESC group are colored in blue while the colonies of presumptive *E. coli* are colored in pink. In case of growth of other bacteria resistant to carbapenems, such as *Acinetobacter* spp., their colonies will not interact with the chromogenic mix present in the medium, and therefore will be colorless or will present their natural pigmentation.

Figure 14: Colonies with the typical appearance of KESC group bacteria grown on Brilliance CRE

McConkey agar (Oxoid): selective and differential medium used for the isolation of Gram-negative bacteria. The selective action of this medium is due to crystal violet and bile salts, both capable of inhibiting the growth of Gram-positive bacteria. The differential action, however, is exploited by the neutral red, a pH indicator which allows to distinguish colonies of bacteria capable of fermenting lactose (colored in red) from those which are unable to ferment it (colored in yellow). Since lactose constitutes the only source of carbohydrates, the pH lowering can only be attributed to acidification following its fermentation. Furthermore, a disc containing 5 µg of

cefotaxime (Oxoid) was put into the middle of the McConkey agar plates to favor the growth of bacteria resistant to β-lactam class antibiotics.

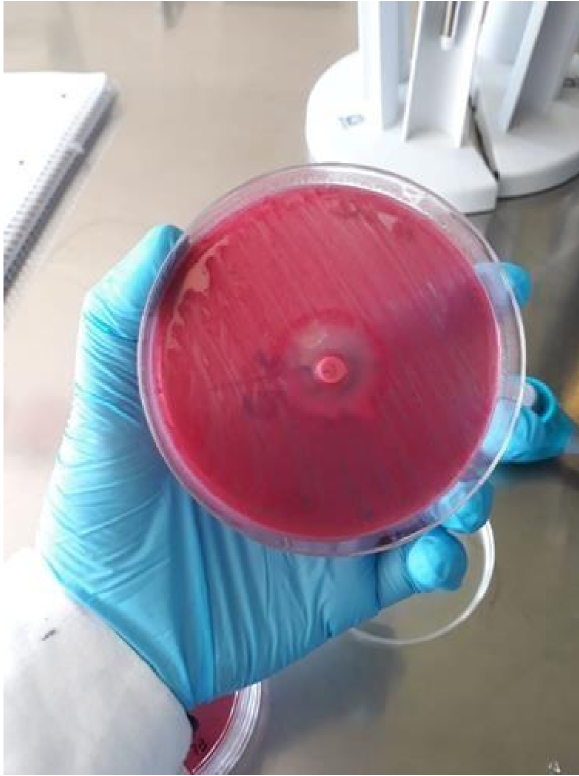


Figure 15: K. pneumoniae resistant to cefotaxime on a McConkey agar plate added with a cefotaxime disc.

The enrichment broths (10 µl) were plated onto Brilliance CRE agar plates to obtain well-isolated colonies by using a sterile loop. In contrast, a sterile swab was used to seed the broths onto McConkey agar plates, before the addition of the cefotaxime disc.

Both media were incubated at 37°C ± 1°C for 18-24 hours. In case of bacterial growth in proximity of the antimicrobial disc, a transplant onto another McConkey agar plate (to incubate at 37°C ± 1°C for 18-24 hours) was often necessary to obtain well isolated colonies.

2.4 Gram staining

Gram staining was the first screening test performed, to ensure that the selected colonies belonged to Gram-negative bacteria. In fact, some Gram-positive bacteria are still able to grow on Brilliance CRE and McConkey agar plates, even though their colonies often have dimensions and morphology that differentiate them from those belonging to the *Enterobacterales.*

2.5 Antimicrobial resistance screening

Screening tests conducted to evaluate AM susceptibility of the selected colonies towards meropenem, cefotaxime and ceftazidime were performed applying the Kirby-Bauer disc diffusion test and the Combination Disk Test.

2.5.1. Kirby-Bauer test

Gram-negative cultures were screened by their phenotypic resistance to β-lactam class antibiotics. For this reason, a Kirby-Bauer test was carried out, to evaluate the resistance against three different drugs, *i.e*., two third-generation cephalosporins (cefotaxime and ceftazidime) and one carbapenem (meropenem). As resistance to carbapenems is often associated with resistance to other classes of β-lactam antibiotics, resistance to meropenem and cephalosporins were tested together. Moreover, the isolates that show resistance to cephalosporins only, and not to carbapenems, can be tested for ESBL-producing activity.

Figure 16: Kirby-Bauer test in a culture showing sensitivity to meropenem and resistance to cefotaxime and ceftazidime

To perform the Kirby-Bauer test, the colony was suspended in 3 ml of TSB (Oxoid) to reach an optical density of 0.5 McFarland degrees, to be plated onto Mueller-Hinton agar (Oxoid), a non-selective medium specifically used in this analysis. Plating was performed by using a sterile swab, in order to distribute the bacterial suspension all over the surface, followed by the addition of the three antimicrobial discs. The plates were incubated at 37°C ± 1°C for 18-24 hours.

The evaluation of bacterial sensitivity to the antibiotics was assessed by the measurement of the inhibition zone diameters around the discs and comparing them with the breakpoint values provided by EUCAST (European Committee on Antimicrobial Susceptibility Testing) (EUCAST, 2023a).

Active Substance	Disk Concentration	Sensitivity (mm)	Resistance (mm)
Cefotaxime (CTX)	5µg	≥20	<17
Ceftazidime (CAZ)	10µg	≥22	<19
Meropenem (MEM)	10µg	≥22	<16

Table 2: Break-point values for cefotaxime, ceftazidime and meropenem according to EUCAST (2023a)

The breakpoint values define the diameter (or the dilution, in the MIC test) of the inhibition zone which corresponds to sensitivity or resistance to specific antibiotics. This range may vary not only in function of the drug considered, but also of the bacterial species under examination. The values used to define the susceptibility category (S-I-R) are called "clinical breakpoints" and are established by EUCAST evaluating clinical, microbiological and pharmacological parameters.

Susceptibility categories are:

- S (Susceptible): values less than or equal to the clinical sensitivity breakpoint. A microorganism is defined as susceptible when there is a high probability of therapeutic success using a standard dosing regimen of the antimicrobial.
- I (Intermediate): intermediate values between the clinical breakpoints of sensitivity and resistance. A microorganism is defined as intermediate when there is a high probability of therapeutic success using an augmented dosing regimen of the antimicrobial.
- R (Resistant): values higher than the clinical sensitivity breakpoint. A microorganism is defined as resistant if there is a high probability of therapeutic failure even with an augmented dosing regimen of the antimicrobial.

However, in addition to clinical breakpoints, EUCAST also introduced the "epidemiological cutoff value" (ECOFF). The ECOFFs distinguish microorganisms without (wild type) and with phenotypically detectable acquired resistance mechanisms (non-wild type) to a specific antimicrobial (EUCAST, 2023b).

The ECOFF values reflect the ability to detect mechanisms of resistance that may become clinically significant in the future. The breakpoints developed for non-wild type strains are distinct from the clinical breakpoints, which are used to highlight the likelihood of a successful antibiotic therapy.

In this study, in addition to the clinical breakpoint, the ECOFF value for meropenem was taken into account both in Kirby-Bauer (< 28 mm) and MIC tests (MIC ≥ 0.125 µg/ml) to detect microorganisms with initial deviations from the wild type strains (EUCAST, 2023b).

2.5.2 Combination Disk Test (CDT)

The Combination Disk Test (CDT) is an additional screening phenotypical test, performed after an isolate had shown to be resistant in the Kirby-Bauer test. The CDT allows to evaluate how bacteria resistant to β-lactam class antibiotics react in presence of β-lactamase inhibitors, *i.e.*, if AM resistance is due to the production of β-lactamases or not.

The phenotypically resistant isolates were subjected to phenotypic confirmation of carbapenemase production by the CDT (KPC, MBL and OXA-48 confirm Kit, ROSCO Diagnostica, Taastrup, Denmark) following the manufacturer's instructions.

The CDT system is composed by tablets containing 10 µg of meropenem alone and in combination with the following inhibitors of β-lactamases:

- Phenylboronic acid (KPC and AmpC inhibitor)
- Cloxacillin (AmpC inhibitor)
- Dipicolinic acid (metallo-β-Lactamase inhibitor)

In addition, the system also provides tablets containing 30 µg temocillin to detect OXA-48-like producing isolates.

To perform the test, the selected colonies were suspended in 3 ml of TSB to reach an optical density of 0.5 McFarland degrees to be plated onto Mueller-Hinton agar using a sterile swab. Using a dispenser, one of each tablet was placed on the plate, ensuring sufficient space between individual tablets to allow proper measurement of the inhibition zones. The plates were incubated at 35±1°C for 18±2 hours. After the incubation, the diameter of the inhibition zones was measured (no zone around a tablet corresponds to a 9 mm inhibition zone).

The results were interpreted by comparing the inhibition zone of Meropenem 10 µg with the inhibition zones of Meropenem 10 µg added with the other molecules. The differences between them should be at least superior to the values shown in Table 3.

Mechanism of Action	Meropenem 10 µg (MRP10)	Meropenem + phenylboronic acid (MRPBO)	Meropenem + dipilcolinic acid (MRPDP)	Meropenem + cloxacillin (MRPCX)	Temocillin 30 µg
AmpC + porin loss		≥ 4 mm	≤ 3 mm	≥ 5 mm	≥ 12 mm
ESBL + porin loss		≤ 3 mm	≤ 3 mm	≤ 3 mm	≥ 12 mm
KPC		≥ 4 mm	≤ 3 mm	≤ 3 mm	Variable
		≥ 4 mm			
MβL		< 4 mm	≥ 5 mm	≤ 3 mm	Variable
OXA-48 and similars		≤ 3 mm	≤ 3 mm	≤ 3 mm	≤ 12 mm
OXA-48 + ESBL		≤ 3 mm	≤ 3 mm	≤ 3 mm	≤ 12 mm

Table 3: Interpretation of the CDT test (Rosco Diagnostica, 2017). https://www.rosco.dk/gfx/pdf/98006-10-15%20-%20Print%20Insert%202017.pdf

In addition to the interpretation table (Table 3), the manufacturer reports also that:

- The microorganism tested does not express AmpC, KPC or MβL activity if all zones of inhibition are < 3 mm.
- OXA-48-producing culture shows negative results with the KPC+MβL Confirm kit, but it is temocillin resistant.

Figure 17: KPC, MBL and OXA-48 confirm Kit. Fisher scientific https://www.fishersci.com/shop/products/kpc-mcb-oxa-48-kit-50-pk/NC1047666

2.6 Species identification

Bacterial species identification was performed by different methods, of which the most common is represented by phenotypic identification through biochemical tests. In recent years, however, also genotypic identification by Polymerase Chain Reaction (PCR) and Whole Genomic Sequencing (WGS) have acquired increasing importance. In this study, traditional phenotypic identification techniques based on a series biochemical tests were used.

Before biochemical testing, the selected colonies were subjected to the oxidase test.

2.6.1 Biochemical identification tests

Species identification was carried out using the API® 20 E system (bioMèrieux, Marcy l’Etoile, France) following the manufacturer’s instructions. This system is formed by a gallery with 20 wells, each one containing a different dehydrated medium able to induce a specific biochemical reaction, which produces color changes in the medium. The identification is obtained starting from the numerical profile based on test positivity or negativity.

Figure 18: API® 20 E system

2.7 Minimum Inhibitory Concentration (MIC) test

The Minimum Inhibitory Concentration (MIC) test measures the lowest concentration of an AM, calculated in µg/ml, capable of inhibiting the growth of the target bacterium (Kowalska-Krochmal and Dudek-Wicher, 2021).

The *Enterobacterales* showing non-susceptibility to meropenem at the screening tests (Kirby-Bauer and CDT) were tested with the MIC assay. The MIC values were provided by the Clinical and Laboratory Standards Institute lines (CLSI) guidelines (CLSI, 2018).

2.7.1 Bacterial inoculum

The bacterial strains were inoculated in sterile Mueller-Hinton Broth (MHB, BD, Sparks, Maryland, USA) and incubated overnight at 37°C ± 1°C. The bacterial suspension was then centrifuged for 20 minutes at 2,000 rpm and 4°C and the pellet was resuspended in phosphate buffer. The turbidity of the bacterial suspension was immediately measured and adjusted by spectrophotometry. At 620 nm, the OD range of 0.08−0.13 was considered to correspond to a bacterial concentration of 10^8 CFU/ml. The obtained suspension was further diluted 1:100 in MHB to obtain a final bacterial concentration of 10^6 CFU/ml and inoculated within 30 minutes.

2.7.2 MIC test

Meropenem trihydrate powder was weighted and diluted in sterile water to prepare a stock solution at the concentration of 2560 µg/ml. This solution was stored in small aliquots at – 80°C until use. The MIC assay was performed in 96-well microtiter plates by incubating meropenem at concentrations ranging from 256 to 0.0005 µg/ml with a final concentration of 5×10^5 CFU/µl of bacterial suspension in a volume of 100 µl. Thereafter, 50 µl of meropenem at each concentration were added in 96-wells microtiter plates, followed by addition of 50 µl of bacterial suspension to each well. The plates were incubated at 37 °C. Three independent experiments, with three replicates each, were performed. After 24 hours of incubation the MIC value was evaluated as the arithmetic average of the lowest concentration of meropenem that completely inhibited the bacterial growth as detected by the unaided eye.

Quality control organisms, such as *E. coli* ATCC 25922 and *S. aureus* ATCC 25923, were tested periodically to validate the accuracy of the procedure.

2.8 Polymerase Chain Reaction (PCR)

The PCR was performed for the bacterial strains belonging to the *Enterobacterales* which showed a MIC value higher than 0.125 µg/ml, i.e., the ECOFF value proposed by EUCAST for meropenem. The multiplex PCR was performed targeting the following gene families: *bla*KPC, *bla*IMP, *bla*NDM, *bla*VIM and *bla*OXA-48-like.

2.8.1. DNA extraction and quantification

The isolated colonies grown on TSA were inoculated in sterile Eppendorf tubes containing 1 ml of distilled water. The bacteria were lysed by heating at 100° C for 10 minutes, and cellular debris were removed by centrifugation at 10,000 rpm for 5 minutes. To perform DNA quantification, 200 µl of supernatant were analyzed with a spectrophotometer. Once quantified, if necessary,

the cultures were diluted with sterile water in order to obtain a final DNA concentration of 25 µg/ml.

2.8.2 Multiplex PCR

Multiplex PCR amplification allows the simultaneous detection of the targeted β-lactamase encoding genes. The reaction was carried out using T-100^{TM} Thermo-Cycler (Bio-Rad, Hercules, CA, USA). The primers used in the reaction are shown in Table 4 (Doyle et al., 2012).

Gene	Primers	Amplicon size
bla_{KPC}	F: 5'- TGTCACTGTATCGCCGTC-3' R: 5'-CTCAGTGCTCTACAGAAAACC-3'	900bp
bla_{IMP}	F: 5'-GAAGGCGTTTATGTTCATAC-3' R: 5'-GTACGTTTCAAGAGTGATGC-3'	587bp
bla_{VIM}	F: 5'-GTTTGGTCGCATATCGCAAC-3' R: 5'-AATGCGCAGCACCAGGATAG-3'	389bp
bla_{NDM}	F: 5'-GCAGCTTGTCGGCCATGCGGGC-3' R: 5'-GTCGCGAAGCTGAGCACCGCAT-3'	782bp
$bla_{OXA\text{-}48\text{-}like}$	F: 5'-GCGTGGTTAAGGATGAACAC-3' R: :5'-CATCAAGTTCAACCCAACCG-3'	438bp

Table 4: Primers used and their amplicons sizes (Doyle et al., 2012).

The amplification protocol was described by Doyle et al. (2012). It was carried out using the GoTaq G2 Flexi DNA Polymerase system (Promega italia S.r.l., Milan, Italy). The masterMix was prepared for a final volume of 50 µl containing:

- 10 µL Green GoTaq Flexi Buffer (final concentration 1X)
- 4 µL $MgCl_2$ (final concentration 2 mM)
- 1 µL dNTPs (final concentration 2 mM)
- 0.4 µL GoTaq G2 Flexi DNA Polymerase (final concentration 2U)

The bla_{KPC}, bla_{NDM} and bla_{VIM} primers were added at a final concentration of 0.3 µM, bla_{NDM} at a final concentration of 0.4 µM and bla_{IMP} and $bla_{OXA\text{-}48\text{-}like}$ at a final concentration of 0.5 µM.

At this point, 1 µL of template DNA at the concentration of 25 µg/mL was added. Nuclease Free Water was added to reach the final volume of 50 µl. The PCR protocol is showed in Table 5.

Step	Temperature	Duration	Number of cycles
Initial denaturation	95°C	5 mins	1
Denaturation	95°C	45 secs	35
Annealing	62°C	30 secs	35
Extension	72°C	1 min	35
Final Extension	72°C	8 mins	1
Holding	4°C	-	1

Table 5: PCR protocol (Doyle et al., 2012)

2.8.3 Electrophoresis

The amplicons obtained where subjected to an electrophoretic run in 1.5% agarose gel, with the addition of tris-acetate EDTA (TAE) as buffer and ethidium bromide as base intercalator and stained with SYRB Safe DNA gel stain (Invitrogen, Portland, OR, USA). Once the run was completed, the PCR products were visualized under UV light.

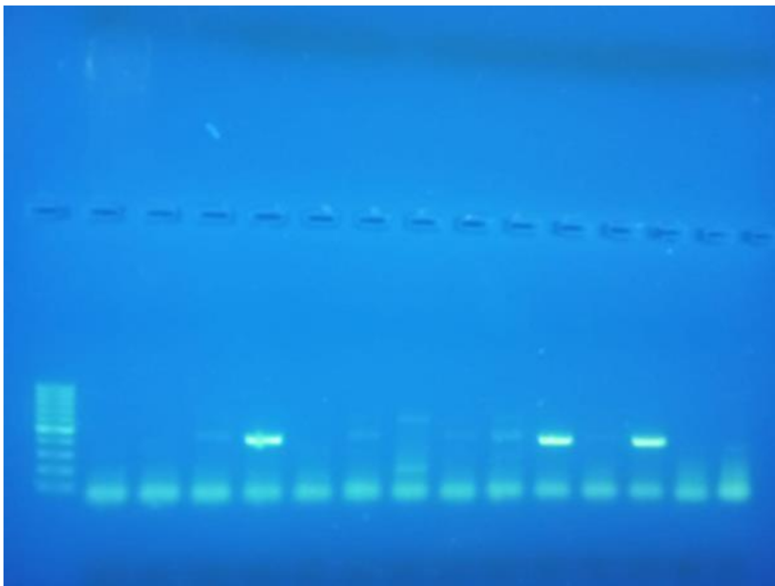


Figure 19: Results of an electrophoretic run seen under UV light. The brighter bands correspond to the bla_{OXA} and bla_{VIM} genes.

2.9 Multiple Drug Resistance (MDR) test

The isolates that harboured the target resistance genes were further tested with for multiple resistance by using the Sensitre^{TM} EUVSEC3 plates (ThermoScientific, East Grinstead, UK) following the manifacturer's instructions. Resistance versus 15 molecules belonging to different AM classes can be tested, including:

- four β-lactams (ampicillin, cefotaxime, ceftazidime and meropenem)
- a sulphonamide (sulfomethoxazole)
- a diaminopyridine (trimethoprim)
- two quinolones (ciprofloxacin, nalidixic acid)
- a macrolide (azithromycin)
- an amphenol (chloramphenicol)
- two tetracycline (tetracycline and tigecycline)
- a polymyxin (colistin)
- two aminoglycosides (gentamicin and amikacin)

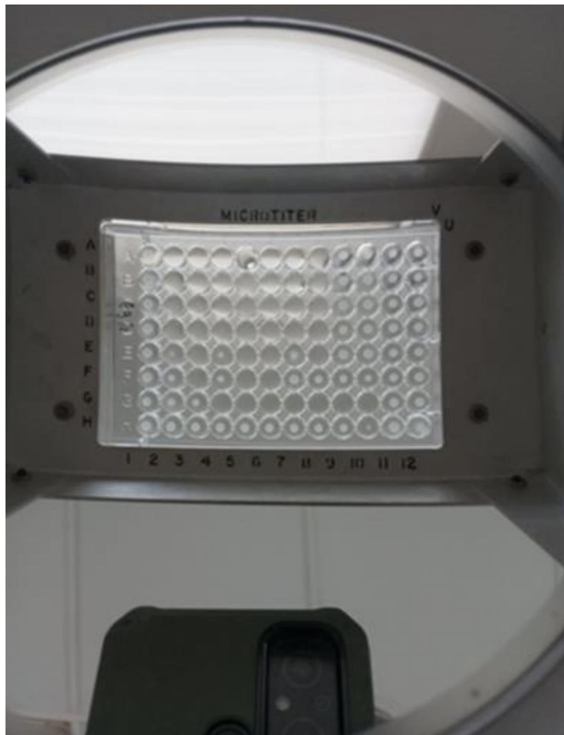


Figure 20: SensitreTM EUVSEC3 plate

2.10 Whole Genome Sequencing (WGS)

The isolates that carried one or more β-lactamase genes were subjected to WGS, which was carried out by the Istituto Zooprofilattico Sperimentale della Lombardia e dell'Emilia-Romagna (IZSLER).

3. Results

3.1 Species identification and AMR: Phenotypical tests

Enterobacterales strains resistant to meropenem by the Kirby-Bauer test and positive to the CDT were detected in 54 (3.76%) out of 1437 samples.

The results of the MIC test and species identification for the isolates detected in the different types of samples will be shown separately in the following subchapters.

3.1.1 Human urine samples (2NEF)

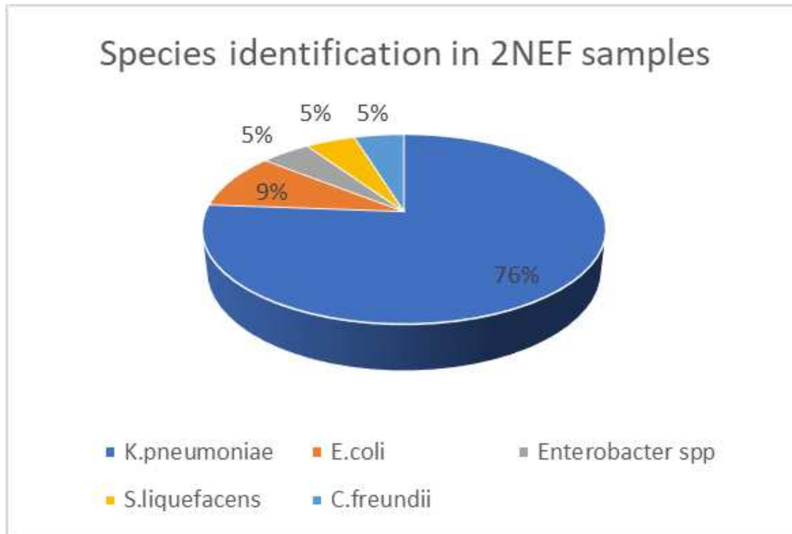
Species identification and MIC test values of the bacteria belonging to the *Enterobacterales* isolated from the human urine samples are shown in Table 6.

Sample code	Species	MIC value
2NEF 15	*E. coli*	0.104 ± 0.036
2NEF 37	*K. pneumoniae*	96.00 ± 45.255
2NEF 43/3	*S. liquefacens*	32.00 ± 0.00
2NEF 43/6	*C. freundii*	96.00 ± 45.255
2NEF 55/1	*K. pneumoniae*	2.021 ± 1.969
2NEF 82	*K. pneumoniae*	32.00 ± 0.00
2NEF 83	*K. pneumoniae*	64.00 ±0.00
2NEF 86/2	*K. pneumoniae*	106.667 ± 36.950
2NEF 99	*Enterobacter* spp.	13.333 ±4.619
2NEF 100	*K. pneumoniae*	21.333 ± 9.238
2NEF 101	*K. pneumoniae*	16.00 ± 0.00
2NEF 120/1	*K. pneumoniae*	512 ± 0.00
2NEF 120/2	*E. coli*	0.073 ± 0.047
2NEF 139	*K. pneumoniae*	32.00 ± 0.00
2NEF 154	*K. pneumoniae*	0.25 ± 0.00
2NEF 155	*K. pneumoniae*	0.292 ± 0.191
2NEF 180/1	*K. pneumoniae*	12.00 ± 4.38
2NEF 188	*K. pneumoniae*	16.00 ± 0.00
2NEF 191/1	*K. pneumoniae*	0.105 ± 0.126
2NEF 191/3	*K. pneumoniae*	10.667 ±4.619
2NEF 195/2	*K. pneumoniae*	256.00 ± 0.00

Table 6: Enterobacterales strains isolated from the human urine samples and meropenem MIC values thereof

Eighteen (9.05%) out of 199 samples were found to be contaminated with *Enterobacterales* showing resistance values for meropenem above the ECOFF and positive to the CDT, for a total of twenty-one isolates. In fact, two different strains of *Enterobacterales* phenotypically resistant to carbapenems and positive to the CDT were isolated from three samples (2NEF 43, 2NEF 120 and 2NEF 191), respectively.

According to the results of the species identification tests, most of these isolates (16; 76.19%) were identified as *Klebsiella pneumoniae*, while 2 isolates (9.52%) were identified as *Escherichia coli*. The remaining three isolates were identified as *Enterobacter* spp., *Serratia liquefacens* and *Citrobacter freundii*.



Graphic 2: Species identification among 2NEF samples tested in the study

Of these 21 isolates, 3 (14.29%) had a MIC value < 0.125 µg/ml and were considered negative despite appearing resistant in the Kirby-Bauer screening test. The remaining 18 isolates (85.71%) had a MIC value above the EUCAST ECOFF value (0.125 µg/ml).

3.1.2 Bovine rectal swabs (BCRE)

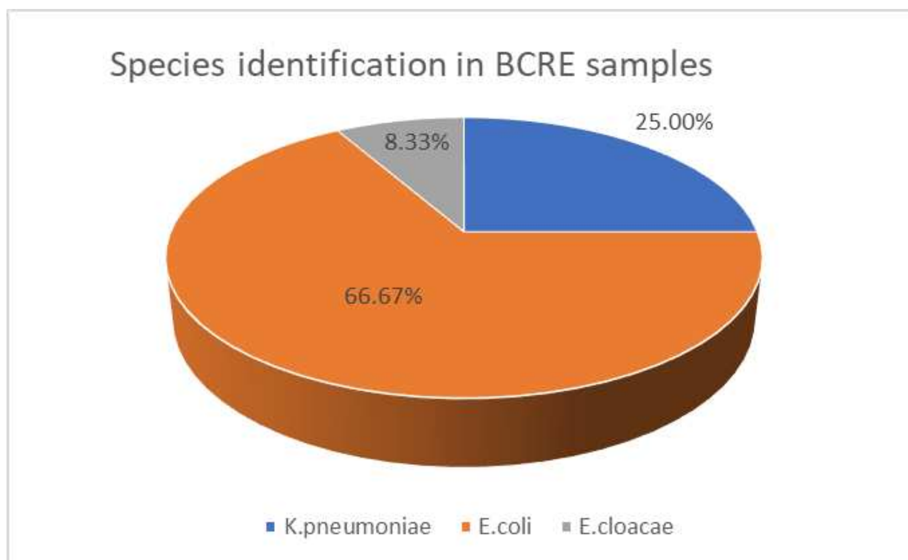
Twelve bovine rectal swabs (1.74%) out of 691 collected at slaughter were positive for bacteria belonging to the *Enterobacterales* family resistant to meropenem according to the Kirby-Bauer test.

Sample code	Species	MIC value
BCRE 142/1	*E. coli*	106.67 ± 36.95
BCRE 148/4	*E. cloacae*	0.167 ± 0.072
BCRE 189/1	*E. coli*	0.027 ± 0.009
BCRE 213	*K. pneumoniae*	>256.00
BCRE 214	*K. pneumoniae*	>256.00
BCRE 232	*E. coli*	0.29 ± 0.19
BCRE 302	*E. coli*	0.25 ± 0.00
BCRE 308	*E. coli*	0.33 ± 0.14
BCRE 314	*E. coli*	0.25 ± 0.00
BCRE 332	*E. coli*	0.016 ± 0.00
BCRE 496	*K. pneumoniae*	4.00 ± 0.00
BCRE 547	*E. coli*	64.00 ± 0.00

Table 7: Enterobacterales strains isolated from the bovine rectal swabs and meropenem MIC values thereof

Contrary to the 2NEF samples, in this matrix the most frequently *Enterobacterales* species was *E. coli* (8 isolates; 66.67%), followed by *K. pneumoniae* (3 isolates; 25%) and *E. cloacae* (1 isolate; 8.33%).

The MIC test was performed for 12 BCRE isolates. Of these, 2 (16.67 %) showed a meropenem MIC value < 0.125 µg/ml, while the remaining 10 (83.33%) showed resistance values higher than the EUCAST ECOFF (> 0.125 µg/ml).



Graphic 3: Species identification among the BCRE samples tested in this study

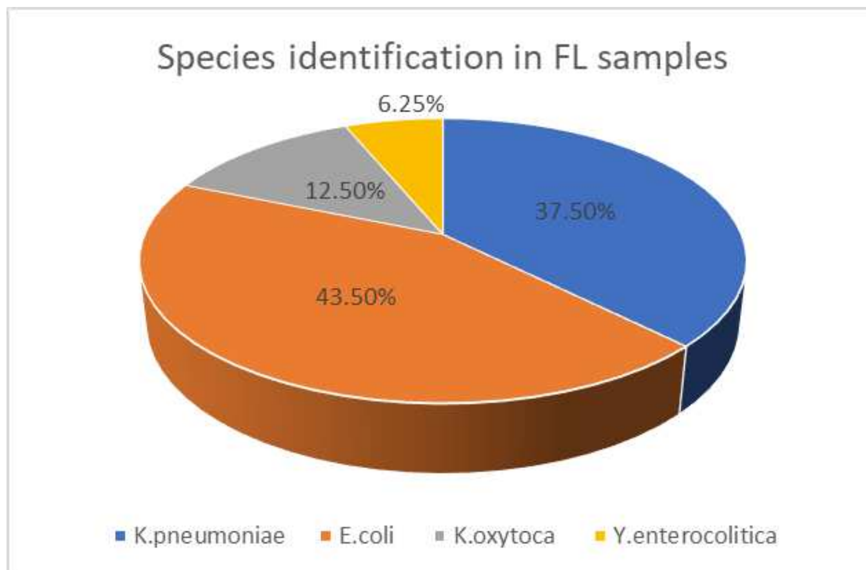
3.1.3 Milk filters (FL)

Fifteen (4.73%) out of 317 milk filters collected from dairy farms were found to be contaminated by *Enterobacterales* resistant to meropenem in the Kirby-Bauer screening test. Interestingly, in one sample (FL 52), two different resistant species were found, i.e., *E. coli* and *Y. enterocolitica*. The isolates detected in the milk filters and their meropenem MIC values are shown in Table 8.

Sample code	Species	MIC value
FL 21	*K. pneumoniae*	2.667 ± 1.155
FL 52/1	*E. coli*	4.00 ± 0.00
FL 52/2	*Y. enterocolitica*	1.667 ± 0.577
FL 88/1	*E. coli*	42.667 ± 18.475
FL 96/2	*E. coli*	4.00 ± 0.00
FL 98/2	*K. pneumoniae*	128.00 ± 0.00
FL 100/2	*K. pneumoniae*	8.00 ± 0.00
FL 111/2	*K. oxytoca*	4.00 ± 0.00
FL 128/2	*K. oxytoca*	6.667 ± 2.309
FL 133/1	*K. pneumoniae*	8.00 ± 0.00
FL 172/1	*K. pneumoniae*	64.00 ± 0.00
FL 211/1	*E. coli*	128.00 ± 0.00
FL 212/1	*K. pneumoniae*	0.583 ± 0.382
FL 224/1	*E. coli*	2.667 ± 1.155
FL 228	*E. coli*	26.667 ± 9.238
FL 230/2	*E. coli*	0.208 ± 0.072

Table 8: Enterobacterales strains isolated from milk filters and meropenem MIC values thereof.

While in the 2NEF and BCRE samples there was a prevalent meropenem-resistant species (*K. pneumoniae* in 2NEF samples and *E. coli* in BCRE samples respectively), in the milk filters the presence of resistant bacteria was more balanced, with 7 isolates (43.75%) identified as *E. coli,* 6 isolates (37.5%) as *K. pneumoniae,* 2 isolates (12.50%) as *K. oxytoca* and 1 isolate (6. 25%) as *Yersinia enterocolitica*. All the tested strains showed MIC values > 0.125 µg/ml.



Graphic 4: Species identification among FL samples tested in this study

3.1.4 Swine fecal samples

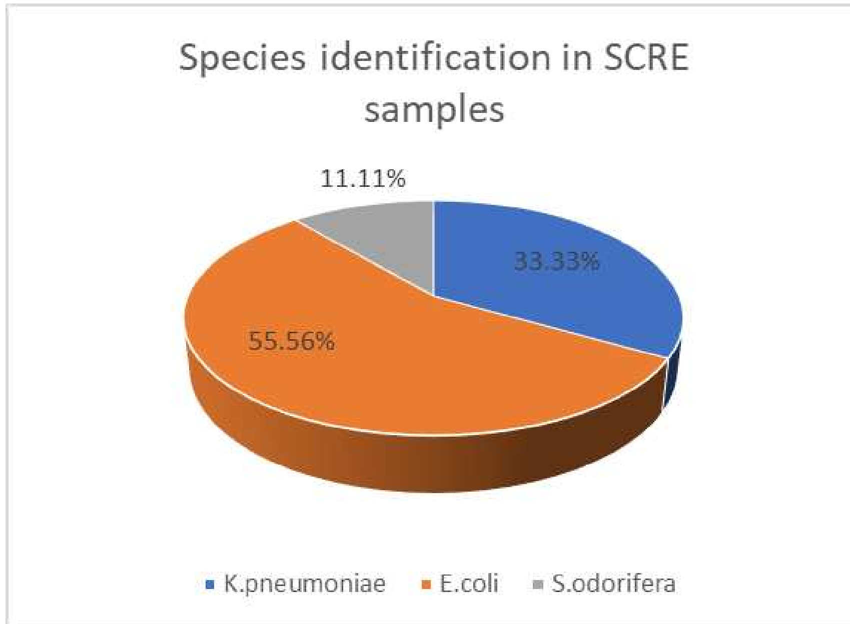
Nine swine fecal samples (4.07%) out of 221 were found to be contaminated with *Enterobacterales* that were resistant to meropenem, according to the Kirby-Bauer screening test.

Sample	Species	MIC value
SCRE 2/4	*E. coli*	6.667 ± 2.309
SCRE 4/2	*K. pneumoniae*	8.00 ± 0.00
SCRE 6/1	*K. pneumoniae*	8.00 ± 0.00
SCRE 7	*E. coli*	4.00 ± 0.00
SCRE 10/3	*K. pneumoniae*	0.50 ± 0.00
SCRE 42/1	*E. coli*	0.583 ± 0.382
SCRE 84/4	*E. coli*	0.25 ± 0.217
SCRE 86/2	*S. odorifera*	128.00 ± 0.00
SCRE 213	*E. coli*	2.667 ± 1.155

Table 9: Enterobacterales strains isolated from pig fecal samples and meropenem MIC values thereof

All the isolates showed a MIC value > 0.125 µg/ml.

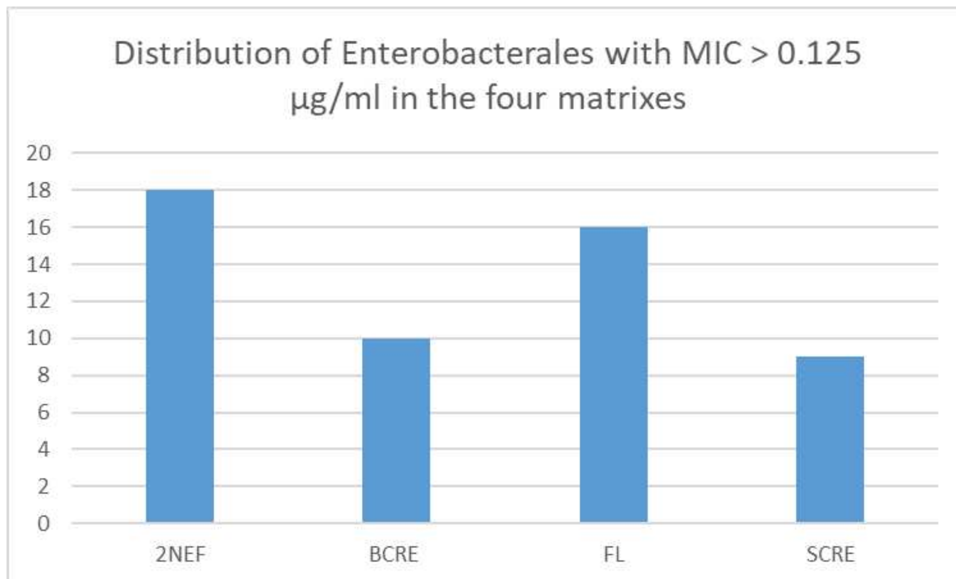
Regarding species identification, 5 (55.56%) strains were identified as *E. coli*, 3 (33.33%) as *K. pneumoniae* and 1 (11.11%) as *Serratia odorifera*.



Graphic 5: Species identification among SCRE samples tested in this study

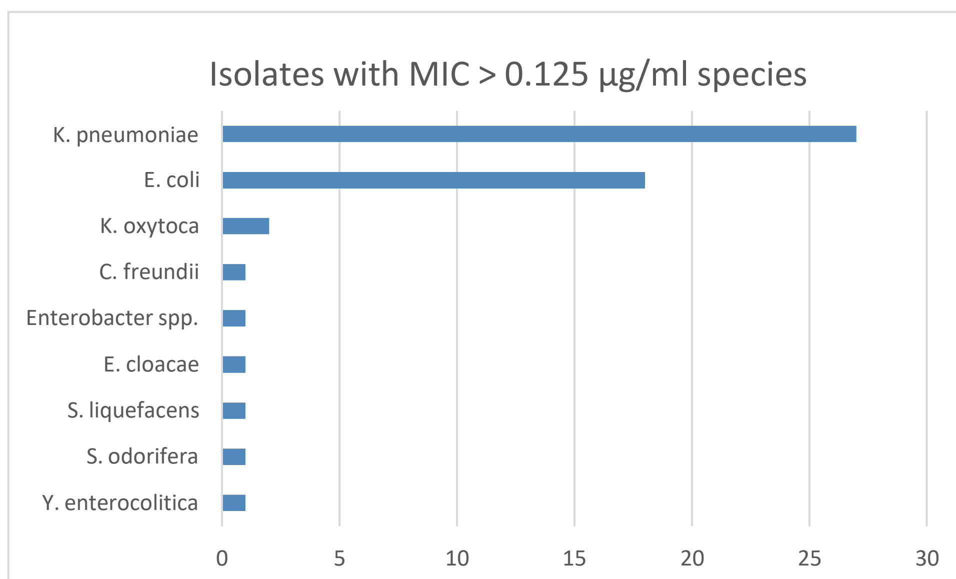
3.1.5 Comparison of results among the four matrices

A total of 53 *Enterobacterales* strains with a meropenem MIC value > 0.125 µg/ml were isolated from 49 samples (3.43%). Of these, 18 strains (33.96%) were isolated from the human urine samples, 16 (30.19%) from the bovine milk filters, 10 (18.87%) from the bovine rectal swabs and 9 (16.98%) from the pig stools samples.



Graphic 6: Distribution of Enterobacterales with MIC for meropenem > 0.125 µg/ml based on source matrices.

The biochemical identification of the isolates showing resistant values higher than the ECOFF revealed that most of them (45; 84.9%) belonged to *E. coli* and *K. pneumoniae* species. In particular, *K. pneumoniae* was the most frequently detected species with a total of 27 (50.94%) isolates, while 18 (33.96%) isolates were identified as *E. coli*. Other less frequently detected species were *K. oxytoca* (2; 3.77%), *S. liquefacens* (1; 1.89%), *C. freundii* (1; 1.89%), *Enterobacter* spp. (1; 1.89%), *E. cloacae* (1; 1.89%), *S. odorifera* (1; 1.89%) and *Y. enterocolitica* (1; 1.89%).



Graphic 7: Biochemical identification of the isolates with MICs above the meropenem ECOFF value

3.2 PCR results

All the isolates which showed a MIC value > 0.125 µg/ml were subjected to a multiplex PCR to identify the presence of the following gene families: *bla*$_{KPC}$, *bla*$_{NDM}$, *bla*$_{IMP}$, *bla*$_{VIM}$ and *bla*$_{OXA-48}$-like.

Of the 53 strains tested, 7 (13.21%) were found positive for the presence of the target genes. They belonged to three matrices only, i.e., human urine samples (2 NEF code; 2 isolates), milk filters (FL code; 2 isolates) and pig fecal samples (SCRE code; 3 isolates). The isolates from bovine rectal swabs were always negative for the carbapenemases-producing genes. The characteristics of the carbapenemases-producing bacteria are shown in Table 10.

Sample code	Species	MIC value (µg/ml)	*bla*$_{KPC}$	*bla*$_{NDM}$	*bla*$_{IMP}$	*bla*$_{VIM}$	*bla*$_{OXA-48}$-like
2NEF 120	*K. pneumoniae*	512	+	-	-	-	+
2NEF 154	*K. pneumoniae*	0.25	-	-	-	-	+
FL 102	*E. coli*	4.00	-	-	-	+	-
FL 172	*K. pneumoniae*	64	+	-	-	-	+
SCRE 84	*E. coli*	0.25 ± 0.217	-	-	-	-	+
SCRE 89	*E. coli*	0.208 ± 0.072	-	-	-	-	+
SCRE 213	*E. coli*	2.667 ± 1.115	-	-	-	-	+

Table 10: PCR results for detection of bla$_{KPC}$, bla$_{NDM}$, bla$_{IMP}$, bla$_{VIM}$ and bla$_{OXA-48}$-like genes

Of these 7 strains, 4 (57.14%) were identified as *E. coli*, while the other 3 (42.86%) were identified as *K. pneumoniae.*

The genes belonging to the *bla*$_{OXA-48}$-like family were the most frequently found, being present in 5 (9.43%) of the 53 strains tested. The *bla*$_{KPC}$ gene was found in 2 (3.77%) strains and the *bla*$_{VIM}$ in the dairy isolate FL 102 (1.89%). None of the isolates carried the *bla*$_{IMP}$ and *bla*$_{NDM}$ genes. Interestingly, the presence of KPC-encoding genes was always associated with the presence of OXA-48-like-encoding genes.

3.3 Multidrug resistance test results

The seven isolates harboring the carbapenemase-producing genes were further tested for phenotypic resistance to other AMs using the Sensititre^{TM} EUVSEC3 plates (Thermofisher). In Table 11 resistance or sensitivity to the 15 antimicrobials is shown.

	AMP	FOT	TAZ	MEM	SMX	TMP	CIP	NAL	AZI	CHL	TGC	TET	COL	GEN	AMI
2NEF 120	R	R	R	R	R	R	R	R	S	S	S	R	S	R	S
2NEF 154	R	R	R	R	R	R	R	S	S	S	R	R	S	R	S
FL 102	R	R	R	R	R	R	R	S	S	R	S	R	S	S	S
FL 172	R	R	R	R	R	R	R	R	S	S	S	R	S	R	S
SCRE 84	R	S	S	R	R	R	R	R	S	R	S	R	S	S	S
SCRE 89	R	S	S	R	R	R	S	S	S	R	S	R	R	S	S
SCRE 213	R	S	S	R	R	R	S	S	S	R	S	R	R	S	S

Table 11: Results of the MIC test performed with Sensititre™ EUVSEC3 plates.

Legend: AMP= ampicillin; FOT= cefotaxime; TAZ= ceftazidime; MEM= meropenem; SMX= sulfamethoxazole; TMP= trimethoprim; CIP= ciprofloxacin; NAL= nalidixic acid; AZI= azithromycin; CHL= chloramphenicol; TGC= tigecycline; TET= tetracycline; COL= colistin; GEN= gentamicin; AMI= amikacin

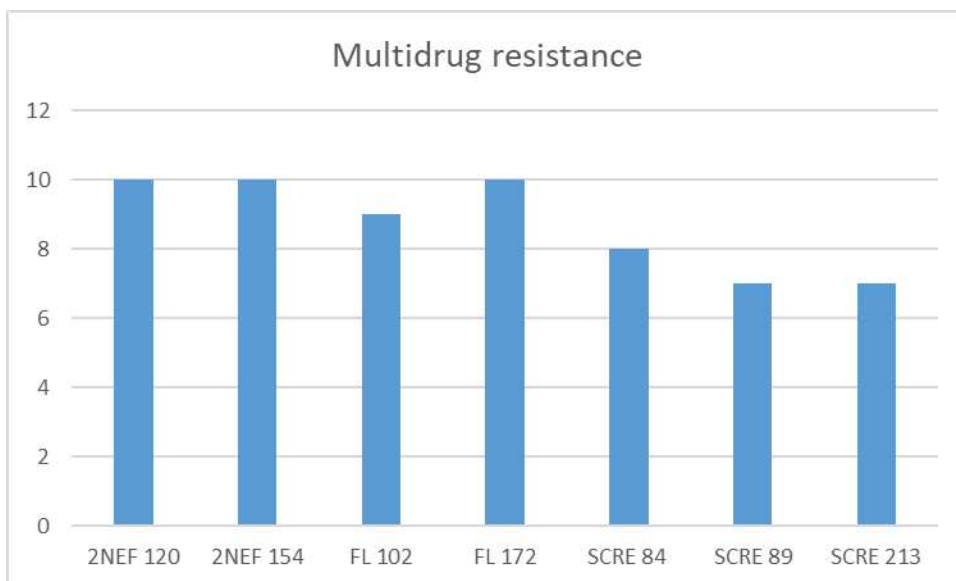
Results interpretation: S= sensitive; R= resistant

All isolates were found to be multidrug resistant (MDR), being resistant to at least 3 different classes of AMs. To evaluate sensitivity or resistance to the different antimicrobials, the breakpoint values provided by EUCAST were used as a reference (EUCAST, 2023).

All meropenem-resistant strains were also resistant to AMP, SMX, TMP and TET, whereas none of the strains showed resistance to AZI and AMI.

Interestingly, resistance to third-generation cephalosporins (FOT and TAZ) was detected in the human and bovine isolates, but not in those of porcine origin.

All isolates were resistant to a minimum of seven up to a maximum of ten AMs, as observed in 2NEF 120, 2NEF 154 (human urine samples; *K. pneumoniae*) and FL 172 isolates (milk filter; *K. pneumoniae*).



Graphic 8: Number of AMs to which the strains were resistant according to the Sensititre™ EUVSEC3 test

3.4 Whole genome sequencing (WGS) results

Four isolates were selected to be tested by WGS by the Istituto Zooprofilattico Sperimentale della Lombardia e dell'Emilia-Romagna (IZSLER) (Table 12).

Identification Code	Source	Species
2NEF 120	Human	*K. pneumoniae*
2NEF 154	Human	*K. pneumoniae*
FL 172	Cattle	*K. pneumoniae*
SCRE 213	Pig	*E. coli*

Table 12: Subset of isolates selected for WGS

The results of the WGS are shown in Table 13

Source	Sample code	Species	MIC (µg/ml)	MLST	*bla* genes	Additional resistance genes
Human urine	2NEF 120	*K. pneumoniae*	>256	307	KPC-3, OXA-1, OXA-9, SHV-106, CTX-M-15, TEM-1B	aac(3)-lla, aac(6')-lb-cr, aph(3'')-lb, aph(6)-la, dfrA14, fosA6, oqxA, oqxB, qnrB1, sul2, tet(A)
Human urine	2NEF 154	*K.pneumoniae*	0.25	902	OXA-1, SHV-187, CTX-M-15, TEM-1B	aac(3)-lla, aac(6')-lb-cr, aph(3'')-lb, aph(6)-la, dfrA14, fosA, oqxA, oqxB, qnrB1, sul2, tet(A)
Milk filter	FL 172	*K.pneumoniae*	64	307	KPC-3, OXA-9, CTX-M-15, SHV-106	aac(3')-IIa, aph(3'')-Ib, aph(6)-Id, dfrA14, fosA6, oqxA, oqxB, qnrB1, sul2, tet(A)
Pig faeces	SCRE 213	*E. coli*	2.667	189	OXA-181	aadA1, aadA2, cmlA1, floR, qnrS1, sul2, tet(M), tet(A), dfrA12

Table 13: WGS results of four selected isolates

By WGS, ESBL genes belonging to the bla_{SHV}, $bla_{CTX\text{-}M}$ bla_{TEM} and bla_{OXA} families were found in both 2NEF isolates. In addition, carbapenemase-encoding genes were detected in 2NEF 154 ($bla_{OXA\text{-}1}$) and in 2NEF 120 ($bla_{OXA\text{-}1,}$ $bla_{OXA\text{-}9}$ and $bla_{KPC\text{-}3)}$. The FL 172 isolate carried the ESBL genes $bla_{CTX\text{-}M\text{-}15}$ and $bla_{SHV\text{-}106}$ and the carbapenemase-encoding genes $bla_{KPC\text{-}3}$ and $bla_{OXA\text{-}9}$. SCRE 213 carried the gene coding for $bla_{OXA\text{-}181}$ β-lactamases, in absence of any ESBL-encoding genes.

Moreover, a minimum of 9 to a maximum of 11 other antimicrobial resistance genes were found, conferring resistance to aminoglycosides (*aac(3)-lla, aac(6')-lb-cr, aph(3'')-lb, aph(6)-la, aadA1, aadA2*), fluorquinolones (*oqxA, oqxB, qnrB1, qnrS1*), trimethoprim (*dfrA14, dfrA12*), phosphonic acids (*fosA6, fosA*), sulphonamides (*sul2*), tetracyclines (*tetA, tetM*) and chloramphenicol (*cmlA1*).

4. Discussion

4.1 The One Health Perspective

Antimicrobial resistance is one of the most serious threats to public health today. Despite the great benefits of these drugs, their prolonged and often inappropriate use has led to the continuous emergence of resistance phenomena. AMs can almost be defined as a double-edged weapon. On the one hand, they are essential for treating many infections and saving lives. On the other hand, their overuse leads to increasing resistance, which reduces the efficacy of the compounds used and makes it increasingly difficult to treat infections with a given drug.

In addition, the extensive use of AMs in animal husbandry needs to be considered. The use of AMs in animal husbandry has several benefits, including improved animal health, increased production and, in some cases, reduced presence of microorganisms, including pathogens, in derived foods. However, the use of these drugs in livestock has also been shown to contribute to an increase bacterial resistance to AMs commonly used in both veterinary and human medicine (McEwen and Collignon al., 2018).

Given the interconnectedness of human health, animal health and the environment, it is essential that the problem of AMR is addressed from a One Health perspective. As this study also shows, the presence of bacteria resistant even to strategically important antibiotics such as carbapenems is no longer limited to hospital settings (Bonardi and Pitino, 2019; Jelic et al., 2019).

4.2 Carbapenemase-producing *Enterobacterales* in food-producing animals

The emergence of carbapenem-resistant bacteria in livestock is therefore a growing concern. However, little is known about the spread of these microorganisms in food-producing animals, seafood, aquaculture, wildlife and their environment, or the associated health risks for humans (Ramìrez-Castillo et al., 2023).

The occurrence of carbapenemase-producing (CP) *Enterobacterales* in food-producing animals has been reported in several countries. The first detection of these bacteria in livestock dates

back to 2011, when a VIM-1 producing *E. coli* was isolated from a pig farm in Germany (Fischer et al., 2012). Although this strain was phenotypically classified as sensitive/intermediate to various carbapenems (ertapenem, imipenem and meropenem), the isolate was found to harbor the *bla*$_{VIM-1}$ gene by PCR. This *E. coli* was the first CP microorganism isolated in Europe, but its sequence type (ST88) was previously identified in chickens, cattle and humans (Fischer et al., 2012). Retrospectively, this strain was found to be widespread in fattening pig farms in Germany. Since then, CP *Enterobacterales* have been found in various food-producing animals worldwide, including dairy cattle (He et al., 2017), poultry (EFSA and ECDC, 2018) and fishery products (Roschanski et al., 2017).

Many studies have shown that genes encoding for the expression of carbapenemases are widespread in other healthcare-associated pathogens, such as *Acinetobacter* spp. and *Pseudomonas* spp. (Bonardi et al., 2023; Tenover et al., 2022; Massik et al., 2023; Shields et al., 2023). Nevertheless, the main goal of the study was specifically focused on CP *Enterobacterales* not only because carbapenem-resistant *Enterobacterales* (especially *E. coli* and *K. pneumoniae*) are a major global threat for public health (Kaye et al., 2023), but also because the most common analytical methods are specifically developed for *Enterobacterales* detection, as the CDT and the multiplex PCR protocol (Doyle et al., 2012; ROSCO Diagnostica, 2017). In addition, many pathogenic species for food producing animals belong to the *Enterobacterales* family (Cheng et al., 2020, Köck et al., 2021, Edesoukey et al., 2022).

In this study, *Enterobacterales* carrying genes for production of carbapenemases were isolated from bovine, porcine and human sources. In particular, two strains were isolated from human urine samples collected from hospitalized patients (2NEF 120 and 2NEF 154), two from milk filters (FL 102 and FL 172) and three from pig faeces (SCRE 84, SCRE 89 and SCRE 213). None of the strains isolated from bovine faeces (BCRE) were found to harbor the carbapenemase-producing genes.

4.2.1 Bovine rectal samples (BCRE)

Failure to isolate bacteria carrying carbapenemase-producing genes from bovine rectal swabs should not make us less vigilant. In fact, although no genes for carbapenemase production were detected by PCR, 10 *Enterobacterales* strains isolated from the bovine rectal swabs had MIC values above the epidemiological cut-off (ECOFF) set by EUCAST.

These values can be attributed mainly to three factors:

- The presence of intrinsic resistance factors (*i.e.,* increased expression of membrane porins);
- Expression of carbapenemases other than those targeted in our study (*i.e*., GES, IMI-1 and many others);
- Overexpression of ESBL genes (*i.e*., CTX-M, SHV or TEM).

In addition to the production of carbapenemases, resistance to carbapenems can also result from the production of ESBL and/or AmpC β-lactamase enzymes associated with alterations in outer membrane porins (Hamzaoui et al., 2018; Ramìrez-Castillo et al., 2023).

As already described, only *Enterobacterales* were considered in this study, mostly because carbapenem-resistant *Enterobacterales* (especially *E. coli* and *K. pneumoniae*) are a major global threat for public health (Kaye et al., 2023) but also because *E. coli* and *K. pneumoniae* may be responsible for infections in bovines (Cheng et al., 2020; Quiroga et al., 2022). Other microorganisms, not tested in our study, could be carriers of genes encoding for the expression of carbapenemases, such as carbapenemase-producing *Acinetobacter* spp. and *Pseudomonas* spp., which are healthcare-associated pathogens and are considered as major threats to public health (Tenover et al., 2022; Massik et al., 2023; Shields et al., 2023) and have been previously isolated in cattle in several studies (Webb et al., 2016; Klotz et al., 2017; Alam et al., 2022; Gharieb et al., 2022).

4.2.2. Milk filter samples (FL)

Of the 16 strains isolated from milk filters and subjected to multiplex PCR, only two (FL 102 and FL 172) were found to carry the target genes for carbapenemases production.

FL 102 was an *E. coli* strain harboring a *bla*$_{VIM}$ β-lactamase and phenotypically resistant to sulphonamides, trimethoprim, quinolones, chloramphenicol and tetracyclines by the MIC test. Although it is not the first time that a VIM-producing strain has been isolated from cattle (Edesoukey et al., 2022), *E. coli* and other *Enterobacterales* carrying *bla*$_{VIM}$ genes are most frequently isolated from porcine-derived matrices (Irrgang et al., 2019; Roschanski et al., 2019; Pauly et al., 2020).

However, enteropathogenic *E. coli* (EPEC) strains carrying *bla*$_{VIM}$ and other AM resistance genes have recently been isolated from dairy cattle in Egypt. This is certainly a public health concern, as EPEC strains are a major cause of diarrhoeagenic diseases in humans and cattle worldwide (Edesoukey et al., 2022).

FL 172 was a *K. pneumoniae* strain carrying genes for the production of several β-lactamases. *K. pneumoniae* is the most common *Klebsiella* species infecting animals and is a causative agent of mastitis in dairy cattle (Cheng et al., 2020). Multiplex PCR and WGS results revealed the presence of the *bla*$_{KPC-3}$ gene in FL 172. To the best of our knowledge, this was the first report of a KPC-3-producing *K. pneumoniae* ST307 strain detected in a bovine dairy farm in Italy (Bonardi et al., 2023). In addition, this strain carried other β-lactam resistance genes encoding for a class D carbapenemase (OXA-9) and ESBLs (CTX-M-15 and SHV-106), together with determinants of resistance to other AMs (aminoglycosides, fluorquinolones, phosphonic acids, tetracyclines, sulfonamides and trimethoprim). This strain shared several characteristics with a human clinical KPC-3 encoding ST307 *K. pneumoniae* strain (2NEF 120), as well as the high-risk clone KPC-3 *K. pneumoniae* ST 307 distributed worldwide (Fuentes-Castillo et al., 2021; Bonardi et al., 2023).

According to WGS, FL 172 and 2NEF 120 were not clonal strains, despite their similarities and geographical proximity of their isolation area. However, finding a MDR strain similar to a clinical isolate in a dairy cattle farm is certainly worrying, because it reflects carbapenem-resistance bacteria spreading outside the hospital setting.

As the use of carbapenems is prohibited in food-producing animals, some suggestions could be made from the literature to justify the occurrence of KPC-3-producing *K. pneumoniae* in livestock. A report on KPC-2 producing *K. pneumoniae* ST258 isolated from a river water sample in Croatia in 2019 showed that these microorganisms were associated with the presence of a hospital near the river, consistent with a previous report of *K. pneumoniae* in that hospital. When compared, the strains isolated from the hospital and the river showed a close genetic relationship, including the presence of the bla_{KPC-2} gene in the non-conjugative plasmid IncFII. This finding also suggests the role of the aquatic environment in the spread of clinical isolates out of the hospital settings and contamination of the environment (Brkic et al., 2017; Jelic et al., 2019).

As well known, the prevalence of AMR bacteria originating from anthropogenic sources, such as hospital and municipal wastewaters, is increasing globally in aquatic environments (Bouki et al., 2013; Bonardi et al., 2023) thus contributing to the spread to the animal compartment. The AMR contamination rate of the water compartment was also investigated in Italy, where *K. pneumoniae* ST307 and ST258 isolates detected in a well and a wastewater treatment plant (WWTP) resulted to be KPC-2 and KPC-3 producers, respectively. In addition, different CTX-M-, SHV- and DHA-type positive *Enterobacterales* were identified from wells, streams and WWPTs in our country (Caltagirone et al., 2017).

Other *Enterobacterales* strains carrying genes encoding for carbapenemases have been reported in hospital wastewater and WWPTs worldwide. *E. coli* carrying NDM-type carbapenemase encoding genes and *K. pneumoniae* carrying IMP-type carbapenemase encoding genes were found in wastewater in Japan (Urase et al., 2022), while several MDR *E. coli* strains harboring bla_{KPC-2} and five different NDM variants were isolated from hospitals and WWPTs in China (Wang et al., 2023).

However, the use of antimicrobials, whose resistance genes are located on mobile genetic elements (MGEs) together with carbapenemase-encoding genes, can enhance the co-selection of MDR phenotypes, including carbapenem-resistance, even in absence of carbapenem treatments (De Oliveira et al., 2020). Through genetic mutations and the acquisition of MGEs, microorganisms have developed resistance mechanisms against a wide range of antimicrobial

classes, including molecules that are considered as the last line of defense against MDR infections, such as carbapenems, polymyxins and glycopeptides (Giddins et al., 2017; Herc et al., 2017; De Oliveira et al., 2020).

4.2.3 Swine fecal samples (SCRE)

Among the strains detected in pig faeces, 3 *E. coli* isolates (SCRE 84, SCRE 89 and SCRE 213) harboured genes encoding for carbapenemases. By PCR, $bla_{\text{OXA-48}}$-like genes were detected in all the porcine isolates.

As well known, class D carbapenemases are the main cause of carbapenem-resistance in *Enterobacterales* isolated from human infections in many countries (Pitout et al., 2019) and their detection in food-producing animals is becoming more and more frequent. OXA-48-like producing *E. coli* have been isolated from pigs and cattle in Italy (Carfora et al., 2022) and from pigs in Germany (Irrgang et al., 2020). The wide distribution of OXA genes is also highlighted by this study, where genes belonging to this family were found in six (85.71%) of the strains tested by multiplex PCR.

The three porcine isolates showed resistance to other AMs when tested with the Sensititre^{TM} EUVSEC3 plates and were therefore classified as MDR strains. In addition, SCRE 89 and SCRE 213 isolates were resistant to colistin. This co-resistance is certainly very worrying, as colistin, like carbapenems, is considered a last resort treatment against MDR bacteria in human medicine (El-Sayed Ahmed et al., 2020) and is part of the WHO list of Critical Importance Antimicrobials (CIA) (WHO, 2019). However, bacteria with these resistance proprieties have been already reported in pigs. For example, two different *E. coli* strains carrying $bla_{\text{OXA-181}}$ and the colistin resistance determinant *mcr-1* were isolated from pigs in Italy in 2017 (Pulss et al., 2017).

To investigate the genomic profile of our porcine isolates resistant to colistin and meropenem, one of them (SCRE 213) was sequenced, even though the PCR assay did not find any carbapenemase-producing genes. By WGS, *mcr-1* or other colistin resistance genes were not found, but SCRE 213 carried the $bla_{\text{OXA-181}}$ gene. OXA-181 is, after OXA-48, the second most

frequently identified carbapenemase among the OXA-48-like group (Pitout et al., 2019) and its presence in *E. coli* strains isolated from pigs in Italy and Europe has already been highlighted (Pulss et al., 2017; Carfora et al., 2022). Interestingly, while all the other isolates sequenced in this study harbored multiple genes encoding for β-lactamases, including ESBLs, the porcine SCRE 213 carried only $bla_{OXA\text{-}181}$. Although carbapenem-resistant MDR isolates usually carry multiple genes encoding for β-lactamases (both carbapenemases and ESBLs), *Enterobacterales* carrying only one carbapenemase-encoding gene and no other β-lactamase encoding genes have been already reported (Khalid et al., 2023).

4.3 Carbapenemase-producing *Enterobacterales* in human patients

Most (83.33%) of the meropenem-resistant strains isolated from human patients proved to be *K. pneumoniae.* These data are consistent with the trend observed in recent years, when *K. pneumoniae* has become a leading cause of nosocomial infections in several areas of the world. *K. pneumoniae* infections are often healthcare-associated, usually in patients with a compromised immune system or underline medical disorders (ECDC, 2020; Di Pilato et al., 2021).

In the present study, two *K. pneumoniae* (11.11%) strains isolated from human patients (2NEF 120 and 2NEF 154) tested positive for the presence of carbapenemases-producing genes. Both these isolates were subjected to WGS, which revealed the presence of several genes encoding for both carbapenemases and ESBLs. In particular, both isolates harboured $bla_{KPC\text{-}3}$ and $bla_{OXA\text{-}1}$ genes, with 2NEF 120 carrying also $bla_{OXA\text{-}9}$ gene.

Over the past decade, carbapenem-resistant *K. pneumoniae* has emerged and spread worldwide as a major health threat, causing infections with high mortality rates and becoming endemic in several countries across Europe (Monaco et al., 2014; Grundmann et al., 2017; Di Pilato et al., 2021). In Italy, the proportion of carbapenem-resistant *K. pneumoniae* among invasive isolates increased dramatically from 1% in 2009 to 15% in 2010 and to 34% in 2016, with a slight decrease (27%) in 2018 (Di Pilato et al., 2021). According to the last report on antimicrobial resistance surveillance in Europe, 26.7% of the invasive *K. pneumoniae* isolates in Italy were resistant to carbapenems (ECDC and WHO, 2023).

In the early stages, the epidemic strains observed in Italy were found to be mostly associated with the spread of high-risk clones of sequence type (ST) ST512 and ST258, capable of producing KPC carbapenemases (Conte et al., 2016; Wyres et al., 2020; Arcari et al., 2023). Subsequent epidemiological studies have shown the emergence of other KPC-producing high-risk clones, such as ST307, which competes with ST258 in some geographical areas (Bonura et al., 2015; Villa et al., 2017; Wyres et al., 2019). Nowadays, *K. pneumoniae* ST307 is widely reported from human clinical samples, with high proportions of both KPC-3- and KPC-2-producing strains and lower proportions of VIM-1- and OXA-48-like-producing isolates (Loconsole et al., 2020; Magi et al., 2021).

4.4 Multidrug resistance in animal and human isolates

Another public health concern arising from this study is the association between genes encoding for carbapenemases and resistance factors to classes of antibiotics other than β-lactam antibiotics. The emergence of resistance to multiple AMs in pathogenic bacteria has become a significant threat to public health as there are fewer, or even sometimes no, effective antimicrobial agents available for infection treatment. Many studies have previously highlighted the correlation between the presence of genes encoding carbapenemases and other AM resistance genes (Katchanov et al., 2018; Kanj et al., 2022).

All isolates carrying carbapenemase-producing genes were subjected to a MIC test using the Sensitrite^{TM} EUVSEC3 plates, and all of them were found to be MDR strains resistant to five different antimicrobial classes other than β-lactam antibiotics. In particular, all the isolates were resistant to ampicillin, sulfamethoxazole, trimethoprim and tetracycline. Resistance to ampicillin is most likely due to the presence of β-lactamases (both carbapenemases and ESBLs), as these enzymes are able to hydrolyze many penicillins. In *Enterobacterales,* resistance to sulfamethoxazole, tetracycline, trimethoprim and ampicillin is often observed (Pavelquesi et al., 2021; Stoltidis-Claus et al., 2023; Valentine-King et al., 2024).

Apart ampicillin, sulfamethoxazole, trimethoprim and tetracycline, the most common resistance was observed against ciprofloxacin, with five isolates (71.43%) having a MIC above the EUCAST

2023 breakpoint values. Resistance to chloramphenicol, nalidixic acid, colistin, tigecycline and gentamycin was observed less frequently, while none of the isolates showed resistance to azithromycin and amikacin.

Interestingly, the porcine isolates were phenotypically resistant to meropenem but not to third-generation cephalosporins, despite resistance to carbapenems and cephalosporins are often linked together due to the capability of carbapenemases of hydrolyzing both AM classes (Hayer et al., 2022). To investigate their AMR genotype, the strain SCRE 213 was sequenced and *bla*$_{OXA-181}$ was the only β-lactamase-encoding gene found. Considering that OXA-48-like β-lactamases usually have a strong activity against penicillins but hydrolyze carbapenems at low levels and are ineffective against broad-spectrum β-lactam antibiotics (Poirel et al., 2019), the absence of β-lactamase-encoding genes other than *bla*$_{OXA-181}$ could explain its AMR phenotype. Moreover, a study on recombinant *E. coli* strains expressing various OXA-48-like carbapenemases showed that a *bla*$_{OXA-181-}$ carrying isolate could have MIC values for cefotaxime, ceftazidime and meropenem comparable to those observed in SCRE 213 (Ouselati et al., 2015).

The phenotypically AMR data were confirmed by the WGS results in the four sequenced isolates, where resistance genes to seven AM classes other than β-lactam antibiotics were found (*i.e.,* aminoglycosides, fluoroquinolones, trimethoprim, phosphonic acids, sulfonamides, tetracyclines and phenicols). The detection of resistance genes to the above-mentioned AMs, in combination with carbapenemase-producing genes, was also observed in other studies (Gentile et al., 2020; He et al., 2022; Yang et al., 2023).

Noteworthy, while 2NEF 120, 2NEF 154 and FL 172 harbored quite similar AMR genes, with nearly overlapping resistance profiles, SCRE 213 shared only two genes (*sul2* and *tetA*) with the other three isolates. Nevertheless, its phenotypic resistance profile was quite similar to the other three. The main difference was that SCRE 213 carried a chloramphenicol resistance gene (*cmlA1*) and no resistance genes to phosphonic acids.

Interestingly, in the retrospective study by Gentile et al. (2020), 27 carbapenem-resistant MDR *K. pneumoniae* strains were isolated from hospitalized patients in Modena province, northern Italy. These isolates carried genes encoding for carbapenemases (*bla*$_{KPC-2}$, *bla*$_{KPC-3}$ and *bla*$_{OXA-9}$)

and for aminoglycosides (*aadA2, aph(3')-Ia, aac(6')-Ib*), quinolones (*oqxA, oqxB, aac(6')-Ib-cr, gyrA-B*, *parC-E*), macrolides (*mphA*), sulfonamides (*sul1*), fosfomycin (*fosA*), colistin (*mcr-1*) and trimethoprim *(dfrA-12*) as also observed in our study. These findings confirm the widespread distribution of CP and MDR K. pneumoniae among hospitalized patients in northern Italy.

In general, the wide variety of AMR genes found in the carbapenem-resistant isolates of our study, definitively confirm the results obtained in other studies on animal and human *Enterobacterales* strains.

5. Conclusions

The spread of carbapenemase-producing *Enterobacterales* has become a major threat to healthcare facilities worldwide. In particular, some high-risk clones of *K. pneumoniae*, such as ST 307 KPC-3-producing, are among the most important opportunistic pathogens responsible for nosocomial infections. In addition, the detection of carbapenemase-producing bacteria in samples collected from food-producing animals represents a wake-up call for possible contamination of products thereof. The ability of these genes to be transferred horizontally even between bacteria of different species is also a major concern.

In the present study, many AMR genes have been identified in both human and animal samples. Of particular interest is the finding, in animal isolates, of genes encoding for different carbapenemases. These includes genes encoding for the production of class D carbapenemases (bla_{OXA-48}-like), for metallo-β-lactamases (bla_{VIM}) and for a carbapenemase typically found in healthcare settings (bla_{KPC-3}). To the best of our knowledge, our study reports the first detection of *K. pneumoniae* carrying bla_{KPC-3} from bovine milk in Italy. Furthermore, carbapenemase-producing genes in our isolates were always harboured together with resistance genes to several antimicrobials, including tetracyclines, sulfonamides, ampicillin, trimethoprim and fluoroquinolones in a MDR scenario.

As the use of carbapenems is banned in veterinary medicine, our findings suggest that animals may carry opportunistic pathogens harboring resistance genes to carbapenems even if not treated with such antimicrobials. A plausible hypothesis is that animal contamination has an environmental origin, with carbapenemase-producing pathogens deriving for human sources.

The increase and spread of bacterial resistance to antimicrobial agents is a natural expression of bacterial evolution, resulting from the selective pressure exerted by the use of certain molecules, as well as from the natural transmission that occurs between micro-organisms. Of course, prudent use of AMs in veterinary medicine is necessary and has already been implemented at EU level. Nevertheless, strategies to reduce the contamination of food of animal origin by resistant bacteria include the application of good hygiene practices. Adherence to hygiene rules during

milking and slaughter, as laid down in the European regulations, is of the greatest importance to prevent the spread of AMR bacteria from shedding animals to food.

Other mechanisms to control the spread of AMR microorganisms range from practical measures, such as adopting of high standards of hygiene in hospitals and communities, to tough challenges for researchers, such as developing new AM molecules. It is also necessary to limit the use of antibiotics in both veterinary and human medicine, with particular attention to the use of CIAs. Nowadays, the main challenge for humans is to implement new guidelines for the prudent use of antibiotics, including the use of targeted rather than broad-spectrum molecules performing antimicrobial sensitivity testing before administration of AMs to human and animal patients.

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