

Carbapenemases: A worldwide threat to antimicrobial therapy

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severe Gram-negative bacterial infections presenting resistance to most β -lactams but have become an empirical option in countries with high prevalence of Extended Spectrum β -lactamase-producing bacterial infections. Imipenem, the first commercially available carbapenem, was approved for clinical use in 1985. Since then, a wide variety of carbapenem-resistant bacteria has appeared, primarily *Enterobacteriaceae* such as *Escherichia coli* or *Klebsiella pneumoniae* (*K. pneumoniae*), *Pseudomonas aeruginosa* and *Acinetobacter baumannii*, presenting different resistance mechanisms. The most relevant mechanism is the production of carbapenem-hydrolyzing β -lactamases, also known as carbapenemases. These enzymes also inactivate all known β -lactams, and some of these enzymes can be acquired through horizontal gene transfer. Moreover, plasmids, transposons and integrons harboring these genes typically carry other resistance determinants, rendering the recipient bacteria resistant to almost all currently used antimicrobials, as is the case for *K. pneumoniae* carbapenemase - or New Delhi metallo- β -lactamases-type enzymes. The recent advent of these enzymes in the health landscape presents a serious challenge. First, the emergence of carbapenemases limits the currently available treatment options; second, these enzymes pose a risk to patients, as some studies have demonstrated high mortality associated with carbapenemase-producing bacterial infections; and third, these circumstances require an extra cost to sanitary systems, which are particularly cumbersome in developing countries. Therefore, emphasis should be placed on the early detection of these enzymes, the prevention of the spread of carbapenemase-producing bacteria and the development of new drugs resistant to carbapenemase hydrolysis.

Abstract

Carbapenems are potent β -lactams with activity against extended-spectrum cephalosporinases and β -lactamases. These antibiotics, derived from thienamycin, a carbapenem produced by the environmental bacterium *Streptomyces cattleya*, were initially used as last-resort treatments for

Key words: Carbapenemases; Origin; Epidemiology; Evolution; Multi-Drug-resistant bacteria

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Core tip: Carbapenemase-producing bacteria were a rare curiosity 15 years ago, as these bacteria were primarily detected in hospital settings. However, now carbapenemase-producing bacteria are observed in farms, companion or wild animals and even in distant glaciers, becoming an epidemic. The relevance this subject has acquired can be easily demonstrated through a search in any medical database; more than 1500 articles have been published depicting the exponential isolation of these bacteria since 1990, with an alarming acceleration in the last seven years.

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INTRODUCTION

Carbapenems are potent β -lactams with activity against extended-spectrum cephalosporinases and β -lactamases. These antibiotics, derived from thienamycin, a carbapenem produced by the environmental bacterium *Streptomyces cattleya*, were initially used as last-resort treatments for severe Gram-negative bacterial infections presenting resistance to most β -lactams but have become an empirical option in countries with high prevalence of extended spectrum β -lactamase (ESBL)-producing bacterial infections.

Imipenem, the first commercially available carbapenem, was approved for clinical use in 1985. Since then, a wide variety of carbapenem-resistant bacteria has appeared, primarily *Enterobacteriaceae* such as *Escherichia coli* (*E. coli*) or *Klebsiella pneumoniae*, *Pseudomonas aeruginosa* (*P. aeruginosa*) and *Acinetobacter baumannii* (*A. baumannii*), presenting different resistance mechanisms.

The most relevant mechanism is the production of carbapenem-hydrolyzing β -lactamases, also known as carbapenemases. These enzymes also inactivate all known β -lactams, and some of these enzymes can be acquired through horizontal gene transfer *via* conjugation, transduction or transformation through outer membrane vesicles. Moreover, plasmids, transposons and integrons harboring these genes typically carry other resistance determinants, rendering the recipient bacteria resistant to almost all currently used antimicrobials, as is the case for *Klebsiella pneumoniae* (*K. pneumoniae*) carbapenemase (KPC) - or New Delhi metallo- β -lactamase (NDM)-type enzymes.

The recent advent of these enzymes in the health landscape presents a serious challenge. First, the emergence of carbapenemases limits the currently available treatment options; second, these enzymes

pose a risk to patients, as some studies have demonstrated high mortality associated with carbapenemase-producing bacterial infections; and third, these circumstances require an extra cost to sanitary systems, which are particularly cumbersome in developing countries. Therefore, emphasis should be placed on the early detection of these enzymes, the prevention of the spread of carbapenemase-producing bacteria and the development of new drugs resistant to carbapenemase hydrolysis.

Carbapenemase-producing bacteria were a rare curiosity 15 years ago, as these bacteria were primarily detected in hospital settings. However, now carbapenemase-producing bacteria are observed in farms, companion or wild animals and even in distant glaciers, becoming an epidemic. The relevance this subject has acquired can be easily demonstrated through a search in any medical database; more than 1500 articles have been published depicting the exponential isolation of these bacteria since 1990, with an alarming acceleration in the last seven years.

The objective of this paper is to summarize the current understanding of the epidemiological, biochemical and diagnostic characteristics of carbapenemases.

CLASIFICACION

Carbapenem-hydrolyzing β -lactamases are classified according to Bush, Jacoby and Medeiros^[1], updated in 2010^[2], proposing a functional classification dividing β -lactamases into 4 groups (1-4) according to the target substrate and inhibition profile of these enzymes and the phenotypic expression of the clinical isolates (Table 1).

This functional classification correlates with the Ambler molecular classification^[3], which divides β -lactamases into 4 classes (A-D) according to the amino acid sequence and spatial structure of these molecules. Two large families have been determined based on the hydrolysis active site: serine- β -lactamases (classes A, C and D), possessing a serine residue at the active center, and metallo- β -lactamases (class B), requiring the presence of one or two divalent Zn²⁺ cations^[4].

Serine carbapenemases

Class A carbapenemases (functional group 2f):

Class A carbapenemases hydrolyze carbapenems, cephalosporins, penicillins and aztreonam and are typically inhibited by clavulanic acid and tazobactam. Characteristically, imipenem susceptibility widely varies from actual resistance to a slight susceptibility reduction that is typically undetected in routine microbiological tests.

Class A carbapenemases have been detected in *Enterobacter cloacae*, *Serratia marcescens* (*S. marcescens*), *Klebsiella* spp. and *E. coli* in the bacterial chromosome or in mobile elements^[5-8].

Among these class A enzymes, KPC, *S. marcescens*

Table 1 Carbapenemase classification

Bush-Jacoby (2010)	Bush-Jacoby-Medeiros (1995)	Molecular class	Distinctive substrate	Inhibited by		Characteristics	Representative enzyme
				CA or TZB	EDTA		
2f	2f	A	Carbapenems	Variable	No	Increased hydrolysis of carbapenems, oxyamino- β -lactams, cephamycins	KPC-2, IMI-1, SME-1
2df		D	Carbapenems	Variable	No	Hydrolyzes oxacillin and carbapenems	OXA-23, OXA-48
1e		C	Cephalosporins	No	No	Increased hydrolysis of ceftazidime and often other oxyimino- β -lactams	CMY-10
3a	3	B1	Carbapenems	No	Yes	Broad.spectrum hydrolysis including carbapenems but not monobactams	IMP-1, VIM-1, CcrA, IND-1
3a	3	B3	Cephalosporins	No	Yes	Broad.spectrum hydrolysis including carbapenems but not monobactams	L1, CAU-1, GOB-1, FEZ-1
3b	3	B2	Carbapenems	No	Yes	Preferential hydrolysis of carbapenems	CphA, SFh-1

CA: Clavulanic acid; TZB: Tazobactam; KPC: *Klebsiella pneumoniae* carbapenemase; IMI: Imipenem hydrolyzing B-lactamase; SME: *Serratia marcescens* enzyme; VIM: Verona integron-encoded metallo- β -lactamase; EDTA: Ethylenediaminetetraacetic acid.

enzyme (SME), imipenem hydrolyzing B-lactamase (IMI) and non metallo-carbapenemase of class A (NMC-A) are the most similar in structure, with the largest deviations observed in areas distant from the active site.

SME and the NMC-A/IMI group are the most important chromosomally encoded enzymes, sharing 97% amino acid sequence identity.

Class A carbapenemases enzymes occur rarely and spread depends on the clonal expansion of the bacteria harboring these enzymes. Class A carbapenemases are susceptible to broad-spectrum cephalosporins, but resistant to carbapenems, penicillins, aztreonam and first generation cephalosporins. The production of these enzymes is induced through imipenem and cefoxitin *via* an AmpC type LysR-dependent regulation system involving the proteins SmeR, ImiR and NmcR^[9,10].

SMEs have been detected in a small number of *S. marcescens* isolates, whereas IMI/NMC group enzymes have been detected in *Enterobacter* spp.^[10,11].

KPCs are clinically the most relevant among plasmid-encoded class A carbapenemases. Not only do these enzymes hydrolyze almost all known β -lactams^[12], but KPCs are also inserted in mobile genetic elements bearing resistance genes for antimicrobials other than β -lactams. Remarkably, *K. pneumoniae* accumulates and transfers virulence factors, such as plasmids, to other genera^[13-17].

To date, 20 different types of KPCs, differing by 1-3 amino acid substitutions and presenting different hydrolysis profiles (www.lahey.org/studies), have been described. Interestingly, these enzymes share 61% homology with SFC-1 from *Serratia fonticola*, another class A carbapenem^[18].

KPCs efficiently hydrolyze nitrocefin, cefalotin, cephaloridine, benzylpenicillin, amoxicillin and piperacillin and also imipenem, meropenem, cefotaxime and aztreonam, but with 10-fold lower activity. Remarkably, KPCs present weak hydrolyzing activity on cefoxitin and ceftazidime^[4].

Guiana extended spectrum (GES) enzymes were initially classified as ESBL, but subsequent studies have demonstrated that GESs hydrolyze imipenem,

although less efficiently. These enzymes have been detected in *P. aeruginosa* and *K. pneumoniae*, and to date, 24 GES types have been described (www.lahey.org/studies).

Class D (functional group 2df): Class D β -lactamases, also known as oxacillinases (OXAs) due to their ability to hydrolyze oxacillin, are classified according to their hydrolysis spectrum. Broad-spectrum OXA enzymes hydrolyze carbapenems and represent a heterogeneous group including more than 100 genetically different enzymes subdivided into 9 clusters based on amino acid sequence (Table 2)^[4]. Carbapenems are weakly hydrolyzed through OXA carbapenemases, rendering high minimum inhibitory concentrations (MICs) below the resistance cut-off value. Nevertheless, OXA-producing bacteria are typically resistant to carbapenems, reflecting concurrent resistance mechanisms, such as permeability defects or the production of other β -lactamases^[19,20]. OXA carbapenemases are variably inhibited by clavulanic acid, sulbactam and tazobactam.

The vast majority of OXA carbapenemases, except the OXA-48 cluster, have been detected in *Acinetobacter* spp. isolates, primarily *A. baumannii*, presenting high resistance to carbapenems^[21,22].

However, the OXA-48 cluster is the most important among class D carbapenemases. The activity of these enzymes is 10-fold higher than that of other OXA enzymes, and the OXA-48 cluster has been identified in *K. pneumoniae*, from which it has spread to other *Enterobacteriaceae*^[23-27].

OXA enzymes hydrolyze penicillins and first generation cephalosporins, but have a weak activity on oxyimino-cephalosporins. OXA-163, an OXA-48-like enzyme with a single amino acid substitution and a four-amino-acid deletion, exhibits lower affinity for carbapenems, but is active on extended-spectrum cephalosporins and partially inhibited by clavulanic acid, mimicking an ESBL phenotypic profile.

Class C (functional group 1e): CMY-10 is the only carbapenem-hydrolyzing enzyme within class C β -

Table 2 Oxacillinase-type clusters

Cluster	Subfamily	Other members	Microorganisms associated
1	OXA-23	OXA-27, OXA-29	<i>Acinetobacter</i> spp.
2	OXA-24/40	OXA-25, OXA-26, OXA-72	<i>Acinetobacter</i> spp. and <i>P. aeruginosa</i>
3	OXA-51	OXA-64-71, OXA-75-78, OXA-83, OXA-84, OXA-86-89, OXA-91, OXA-92, OXA-94, OXA-95	<i>Acinetobacter</i> spp.
4	OXA-58		<i>Acinetobacter</i> spp.
5	OXA-55	OXA-SHE	<i>Shewanella</i> spp.
6	OXA-48	OXA-54, OXA-181, OXA-163, OXA-204, OXA-232, OXA-247, OXA-SAR2	<i>Enterobacteriaceae</i>
7	OXA-50	OXA-50a-d, POXB	<i>P. aeruginosa</i> .
8	OXA-60	OXA-60a-d	<i>Ralstonia pickettii</i>
9	OXA-62		<i>Pandoraea pnomemusa</i>

P. aeruginosa: *Pseudomonas aeruginosa*.

lactamases. This enzyme is an extended-spectrum cephalosporinase, reflecting a three-amino-acid deletion in the R2-loop, and has been detected in *Enterobacter cloacae* (*E. cloacae*) in South Korea^[28].

Class B or metallo-β-lactamases (functional group 3):

Class B or metallo-β-lactamases (MBL) vary greatly in amino acid sequence and molecular structure, but share some features: a divalent cation, primarily Zn²⁺, for substrate hydrolysis; the hydrolysis of carbapenems, penicillins and cephalosporins, but not monobactams, such as aztreonam, reflecting low affinity interactions; and inhibition through metal chelators, such as ethylenediaminetetraacetic acid (EDTA), which inhibit the activity of these enzymes through Zn²⁺ sequestration.

In *Enterobacteriaceae*, the expected phenotype includes resistance to amino-, carboxy-, and ureido-penicillins, penicillin-clavulanate combinations, and cefoxitin; decreased susceptibility to piperacillin-tazobactam and oxyimino cephalosporins and elevated MICs compared with the epidemiological cut-off values^[19].

MBLs are subdivided into three subclasses, B1, B2 and B3, depending on the primary amino acid sequence and the structural characteristics of the active site, presenting low homology^[29].

B1 and B3 subclasses use two Zn²⁺ ions in the active site and have a broad hydrolysis spectrum, whereas subclass B2 employs a single Zn²⁺ ion in the active site and only shows activity on carbapenems^[30].

MBLs have been detected in the bacterial chromosome and mobile genetic elements. MBLs in the bacterial chromosome were the first type studied, as these enzymes have been detected in environmental organisms or opportunistic pathogens, such as *Bacillus cereus*, *Aeromonas hydrophila*, *Chryseobacterium* spp. or *Stenotrophomonas maltophilia*^[31,32].

Acquired MBLs are easily transferred among different species, as these enzymes can be inserted into mobile

genetic elements. These MBLs are primarily classified as B1 subclass enzymes, likely reflecting increased predisposition for insertion into gene cassettes, integrons, transposons or plasmids^[33,34].

NDMs have been recently described as MBLs with high dissemination tendencies and have become the most relevant enzymes within this family^[35]. NDM-1 has been identified in *Enterobacteriaceae*, and Gram-negative non-fermenters^[36,37]. Some variants, such as NDM-4, -5 and -7, present higher efficiencies of carbapenem hydrolysis^[38]. The systematical association of NMDs with other resistance determinants and spread rapidly to other bacterial strains poses a serious challenge to sanitary systems worldwide.

IMP (active on imipenem) was first detected in Japan during the 80's^[39]. Since then, 48 different subtypes have been described in *Enterobacteriaceae* and Gram-negative non-fermenting rods, particularly *Pseudomonas* and *Acinetobacter*, worldwide (www.lahey.org/studies). These enzymes exhibit a high specificity for hydrolyzing cephalosporins and carbapenems and characteristically, exhibit low activity on temocillin^[40]. Enzymatic kinetic differences have been identified among different subtypes, but with low clinical relevance.

Verona integron-encoded metallo-β-lactamase (VIMs), first identified during the 90's, are currently detected worldwide^[41], and 41 VIM subtypes have been described (www.lahey.org/studies). VIM-1 and VIM-2 are distributed worldwide and spread easily. Typically, these enzymes exhibit a high specificity for hydrolyzing carbapenems, and different from IMPs, these enzymes also hydrolyze temocillin. The hydrolysis profile varies among different VIM subtypes with no clinical relevance^[30].

SPM-1s (São Paulo metallo-β-lactamases) were first identified in Brazil in 2001^[42]. These enzymes have a broad hydrolyzing profile, including penicillins, cephalosporins and carbapenems^[43]; but due to the associated mobile genetic elements, these enzymes exhibit a low tendency toward spreading.

GIM-1s (German imipenemases) have only been identified in Germany, originally associated with *P. aeruginosa*; however, these enzymes have recently been identified in *Enterobacteriaceae*^[4,38,44].

Other acquired MBLs, including SIM, AIM, DIM and the recently described KHM-1 in a Japanese isolate from 1997, exhibit a low dissemination range and are clinically less relevant.

CARBAPENEMASES: MECHANISMS OF ACTION

β-lactamases catalyze the hydrolysis of the β-lactam ring, using water in nucleophilic ring opening, generating an innocuous β-amino acid^[45].

Classes A, C and D possess a serine amino acid in the active site, while class B carbapenemases are metallo-enzymes possessing a zinc in the active site^[4].

The catalytic mechanism of serine β -lactamases involves acylation, which is common to serine- β -lactamases and penicillin-binding proteins (PBP), and deacylation, which is absent in PBPs and is the essence of antibiotic resistance through serine- β -lactamases^[46].

Class A carbapenemases

Class A β -lactamases possess four catalytic residues (Ser70, Lys73, Ser130 and Glu166) and use water as a nucleophile.

First, Lys73 is deprotonated through a concerted double proton transfer from Lys73N ζ to Ser130O γ and from Ser130O γ to C3-carboxylate in the substrate; Lys73N ζ -Ser130O γ -C3-carboxylate in the acyl-enzyme intermediate forms a hydrogen bond network. Second, the acyl-enzyme tetrahedral intermediate is formed, with Glu166 acting as a general base catalyst. This step is the rate-determining process. Third, Lys73 is protonated through the concerted double proton transfer from C3-carboxylate to Ser130O γ and from Ser130O γ to Lys73N ζ . Finally, the degraded substrate is detached from the enzyme in concert with the transfer of a single proton from Lys73N ζ to Ser70O γ to cleave the C7-Ser70O γ bond^[47].

Plasmidic class A carbapenemases: KPCs, GESs

The active site of KPC-2 contains the catalytic Ser70 residue and harbors the deacylation water molecule that is primed through an interaction with Glu166, Asn170 and Ser70. The oxyanion hole formed by the nitrogen backbones of S70 and Thr237 is partially occluded by the side chain of Ser70, which is somewhat unusual for class A β -lactamases. Cys69, adjacent to Ser70, is involved in a disulfide bond formation with Cys238. This disulfide bond formation is characteristic of class A carbapenemases.

A comparison of the KPC-2 structure with that of non-carbapenemases and previously determined NMC-A and SME-1 carbapenemase structures revealed several active-site alterations unique to carbapenemases. An outward shift of the catalytic Ser70 residue renders the active sites of carbapenemases more shallow, likely facilitating easier access for bulkier substrates. Further space for the α -substituents is likely provided through shifts in Asn132 and Asn170 and concerted movements in the postulated carboxyl-binding pocket to facilitate substrate binding at a slightly different angle to accommodate these α -substituents. Thus, the structure of KPC-2 provides key insights into the carbapenemase activity of emerging Class A β -lactamases^[48].

The importance of Asn170, a ligand for the hydrolytic water, is further supported by the lack of this residue in GES-1, an enzyme exhibiting characteristic active-site disulfide bond formation and possessing a structure similar to other known carbapenemases, but lacking carbapenem-hydrolyzing activity. Although Asn170 is replaced with a Gly in GES-1, this residue is present in GES-2 and other class A carbapenemases^[49-52].

Chromosomal class A carbapenemases: NMCs, SMEs, and IMIs

Chromosomal class A carbapenemases exhibit sequence differences compared with traditional class A β -lactamases, such as TEM-1 and SHV-1, likely contributing to the unique substrate specificity of these enzymes.

The activity of SME-1 reflects a highly distributed set of interactions that subtly alter the structure of the active-site pocket, although no single position uniquely contributes to carbapenem hydrolysis^[53]. Nonetheless, Ser-237 is significant to the carbapenemase activity of SME-1^[54]. SME-3 differs from SME-1 by a single amino acid substitution of Tyr for His at position 105 at the entrance of the active site, a position that plays a subtle role in the binding affinities of some substrates^[6,10,11,55].

Class D carbapenemases

OXA enzymes present markedly different amino acid sequences, however the most characteristic feature is the presence of an unusual N-carboxylated lysine post-translational modification, a general base for the activation of the serine nucleophile in the acylation reaction, and deacylating water^[56-58]. In the first step of the acylation reaction, nucleophilic Ser attacks the carbonyl carbon of the β -lactam ring, leading to ring cleavage and the formation of a stable carbapenem-enzyme acylate. In the second step of the acylation reaction, a water molecule attacks the same carbon, the Ser-carbapenem bond breaks and the inactivated ligand is released from the active site^[59].

In addition, some OXAs form dimers, and a dimer-monomer equilibrium has been implicated in the kinetic behavior of these enzymes^[60].

The strictly conserved residues that participate in the acylation and deacylation enzymatic reactions of class D β -lactamases include the Ser67 nucleophile^[59,61,62], the carboxylated Lys70, which activates Ser67 and is essential for the deacylation reaction^[63], and Lys212, which plays a role in the protonation of the antibiotic substrate in concert with the acylation reaction.

The active site of OXA-24 has two main elements. Catalytic Ser-81, Thr-82, Phe-83 and Lys-84 form the first element^[62]. As well as establishing contacts with water molecules in the cleft, the hydroxyl group of Ser-81 establishes a weaker hydrogen bond with the N ζ of Lys-84 (3.35 Å) than that observed in other oxacillinases. The relative weakness of this interaction is compensated by the slight movement of Lys-84 within the crevice, forming a hydrogen bond with the side chain nitrogen of the conserved Trp-167.

The conserved motif Ser-128/X/Val-Ile represents the second active site element of OXA-24. The lateral hydroxyl chain of Ser-128 is accommodated in the direction of active serine Ser-81, forming hydrogen bonds with the neighboring residues. Another characteristic feature within this second element is the presence of a non-polar residue (Val-130) unique to class D enzymes and contributing to the broad substrate specificity of

OXA-24.

A hydrophobic barrier established through the specific arrangement of Tyr-112 and Met-223 side chains, which define a tunnel-like entrance to the active site, determines carbapenem substrate specificity. This hydrophobic environment over the active site is stabilized through other hydrophobic contacts involving Thr-111, Trp-115, and Trp-221. The tunnel diameter regulates the shape and chemical nature of the antibiotics accessing the active site^[64].

Unexpectedly, the structure of OXA-48 was similar to that of OXA-10, an enzyme without carbapenemase activity, indicating that the hydrolysis of these compounds depends on subtle changes in the active site region. Moreover, the active site groove of OXA-48 is different in shape, dimensions, and charge distribution from that of OXA-24^[65].

In OXA-58, the deacylating water molecule approaches the acyl-enzyme species, anchored at Ser-83, from the alpha-face. Additional studies comparing OXA-24, OXA-48 and OXA-58 carbapenemases with OXA-10 support the theory that OXA carbapenemases retain the catalytic machinery associated with class D β -lactamases, and residues located close to the β 5- β 6 loop might play a role in the mechanism of carbapenem hydrolysis^[66,67].

Class B carbapenemases

MBLs require zinc ions for β -lactam ring hydrolysis. The active site has two potential zinc ion binding sites, often referred to as sites 1 and 2^[68-70]. His, Asn, Gln, Asp and Cys residues typically form the zinc ligands in the two binding sites, and these residues are not fully conserved between different MBLs.

It has been suggested that metal ions act as Lewis acids based on coordination to the peptide carbonyl oxygen, providing a more electron-deficient carbonyl carbon to facilitate nucleophilic attack. Thus, metal ions stabilize the negative charge developed on the carbonyl oxygen of the tetrahedral intermediate anion. In many other metallo-proteases, metal ions lower the pKa of the coordinated water, thereby increasing the concentration of the metal-bound hydroxide ion. Although different in nature from simple solvated ions, coordinated hydroxide ions are better nucleophiles than water^[71].

C-N bond fission is the most energetically difficult process in peptide hydrolysis. This fission reaction could be facilitated through the direct coordination of the departing amine nitrogen to the metal ion^[72]. Alternatively, a metal-bound water could act as a general acid catalyst, protonating the amine-nitrogen-leaving group to facilitate C-N bond fission.

The catalytic mechanism suggested for BCII from *Bacteroides fragilis* was based on the crystal structure of this enzyme at a resolution of 2.5 Å, showing only one zinc-ion bound to the enzyme at the His site. Following substrate binding, the zinc-bound water molecule, deprotonated by the Asp120 residue, attacks

the carbonyl, forming a negatively charged tetrahedral intermediate, which is stabilized through interactions with the metal ion. The Asp120 residue donates a proton to the nitrogen, and C-N bond cleavage occurs, followed by product dissociation from the enzyme active site^[73,74].

In this mechanism, the bridging hydroxide ion is responsible for the nucleophilic attack, generating a negatively charged intermediate, stabilized through the oxyanion hole of the enzyme. The apical water molecule bound to zinc is optimally positioned to donate a proton to the leaving nitrogen, and the newly formed hydroxide ion moves to occupy the vacated Wat1 site, followed by product dissociation from the enzyme active site. This mechanism was initially proposed to involve zinc coordination to the β -lactam nitrogen, which is unlikely because of the relatively low electron density of zinc due to amide-resonance. However, the increased basicity of this ion, suggests that nitrogen binding to zinc likely occurs once the tetrahedral intermediate is formed^[72].

An unusual mechanism has been proposed for subclass B2 CphA from *Aeromonas hydrophila* based on the crystal structures of the free enzyme and a reaction intermediate between the enzyme and the substrate biapenem^[75]. Nucleophilic attack is performed through a water molecule, which is not coordinated to zinc, but activated through His118 catalysis, followed by C-N bond fission, which occurs prior to nitrogen protonation. This proposed mechanism uses zinc as a Lewis acid to facilitate C-N bond fission and stabilize the leaving group^[72,73], whereas most mechanisms use the zinc ion as a Lewis acid to stabilize oxyanion formation from the β -lactam carbonyl oxygen. Rearrangement of the proposed intermediate amine anion, whose negatively charged nitrogen is stabilized through an interaction with the zinc ion, generates a bicyclic intermediate, which is observed in the crystal structure, although this rearrangement might not occur in the active site of the enzyme. A solvent molecule replaces the water molecule used in the nucleophilic attack, protonates the nitrogen and releases the final product^[71].

ORIGIN OF CARBAPENEMASES

Bacteria appeared on Earth approximately 3.5 billion years ago. Subsequently, these evolved, differentiated and adapted to new environments, initiating competition among different species for the many resources needed for survival.

One of the survival strategies of early microorganisms involved the production and secretion of antimicrobial molecules that would eliminate any competitor but not affect the producer. To this end, bacteria synthesize both antimicrobials and antimicrobial neutralizers from the molecules already present in the metabolic networks of these organisms. The next step in this biochemical warfare involved the development of resistance mechanisms in non-antimicrobial-producing bacteria.

It has been suggested that antimicrobials developed from signaling molecules, such as those used in *quorum* sensing, while β -lactamases are primarily derived from PBPs, enzymes for the production and maintenance of the bacterial wall^[76,77].

Currently, many different biochemical products with antimicrobial or resistance properties are found ubiquitously, as this biochemical warfare has been ongoing for billions of years. Thus, it is only now that we are beginning to understand antimicrobial resistance. β -lactams and β -lactamases regularly occur in nature, therefore to understand the origin and evolution of the carbapenemase enzymes, it is necessary to introduce and explain the term "resistome", *i.e.*, the global set of molecules that confer antimicrobial resistance to microorganisms^[78]. According to this theory, antimicrobials act as selectors and mutation accelerators of the resistance mechanisms present in nature; then, these resistance mechanisms are selected and transferred from one bacteria to another, as horizontal gene transfer is advantageous in dynamic environments^[79-81]. Moreover, the level of antimicrobial pressure will determine the evolutionary rate^[78].

Origin of serine β -lactamases

PBPs are a group of transpeptidases, transglycosylases and carboxypeptidases belonging to the active-site serine family of enzymes, except for one Zn²⁺-dependent PBP. These enzymes have been divided into low- and high-molecular-weight molecules responsible for building and maintaining the peptidoglycan structure of the bacterial cell wall. To this end, PBPs are fixed to the inner bacterial membrane with the active site facing the periplasmic space^[77]. Interestingly, these enzymes cluster into six different groups closely related to different β -lactamases, suggesting that these enzymes developed independently from PBPs under different selection pressures in a two-step evolution to acquire acylation/deacylation properties^[77,82-84]. Hall *et al.*^[85,86] showed that serine β -lactamases are ancient enzymes, over two billion old, which form three different clusters: classes A and B are sister taxa, while class C separated earlier from a common ancestor of classes A and B^[85,86]. Recently, a new family of PBPs, PBP-A, has been described in *Cyanobacteria*, and these enzymes represent an intermediate step between class A β -lactamases and PBPs^[84].

Phylogenetic analysis revealed that class A carbapenemases generate six related branches sharing 32%-70% amino acid sequence identity and group together with other β -lactamases and low-molecular-weight class C PBPs^[77]. This observation suggests that these clusters evolved from different ancestors^[18].

Class A carbapenemases existed before carbapenems were clinically used, as chromosomally encoded SME-1 and IMI-1 were detected in *S. marcescens* and *E. cloacae*, respectively, a few years before imipenem was launched^[7,10]. Thus, carbapenem use acted as a

selector. Indeed, Walther-Rasmussen *et al.*^[18] conducted a phylogenetic analysis, and the resulting phylogram showed that NMC-A, SME, KPC and SFC-1 cluster together and could share a common ancestor with carbapenemase activity.

In contrast, GES and SHV-38 have different origins, and only SHV-38 has a known ancestor^[87]. Both enzymes cluster with other ESBLs, indicating that the carbapenemase activity of these enzymes evolved through various mutations in response to carbapenem use^[18].

Class D β -carbapenemases might have also been derived from low-molecular-weight class C PBPs, and similar to class A, these enzymes predated carbapenem clinical use, as the first member of this family, ARI-1 (subsequently referred to as OXA-23), was identified in 1985 from an *A. baumannii* isolate^[88]. Interestingly, BlaR and MecA transducers are included in branches belonging to these β -lactamases, potentially representing reverse evolution from β -lactamases^[77,83]. Most OXA carbapenemases are primarily chromosomally encoded in *Acinetobacter* spp. and might represent a natural component of some subgroups^[89]. Other OXA carbapenemases have been identified in bacteria, such as *P. aeruginosa* or *Shewanella* spp. Notably, the most clinically relevant enzyme in this family is the OXA-48 cluster, which exhibits the highest carbapenem hydrolysis rate, is plasmid encoded and has been detected in *Enterobacteriaceae*. Interestingly, OXA-48 might have originated from *Shewanella xiamensis*, an environmental bacterium isolated from seepage water that harbors an OXA-48-like chromosomal gene, namely OXA-181. However, the genetic structure of bla_{OXA-181} is different from that of bla_{OXA-48}, indicating an unrelated origin. Thus, *Shewanella* spp. might represent a reservoir for OXA-type carbapenemases^[90-93].

Origin of MBL

MBLs belong to a superfamily whose members exhibit a four-layered $\alpha\beta/\alpha\beta$ structure and share five conserved motifs. Many enzymes of this superfamily are involved in hydrolysis and redox processes, mRNA maturation and DNA repair, suggesting that MBLs could represent divergent evolution from an early ancestor^[94].

Bayesian phylogenetic studies indicate that subclasses B1 and B2 form one cluster that split approximately 2.2 billion years ago and developed β -lactamase activity approximately 1 billion years ago, whereas B3 is older, splitting earlier from a common ancestor and developing β -lactamase activity approximately 2.2 billion years ago^[94,95]. Therefore, although the 3-dimensional structure of these enzymes is similar, with structural features consistent with the enzymatic activity profiles, the amino acid sequences of MBLs significantly diverge^[94,95].

MBLs are chromosomally encoded in bacteria from different phyla, including δ -*Proteobacteria*, which are primarily soil inhabitants and bacterial predators, harboring

as many as 30 gene copies, *Firmicutes*, *Bacteroidetes*, and α -, β - and γ -*Proteobacteria*^[96,97].

Environmental bacteria exposed to antimicrobial pressure might be a plausible source of MBLs. For example, the sequence analysis of the bla_{NDM-1}-positive IncA/C plasmid revealed that the plant pathogens *Xanthomonas* spp. or *Pseudoxanthomonas* spp. might be the origin of the NDM-1 β -lactamase^[98], the pLD209 plasmid containing the *Pseudomonas putida* bla_{VIM-2} is a mosaic of both clinical and environmental bacteria^[99], or the bla_{IMP} gene, found in bacteria isolated from glaciers^[100].

GENETIC CONTEXT

The expansion of chromosomally encoded carbapenemases, such as SME, IMI or NMC, depends on clonal replication; therefore, although common pathogens can easily spread among humans, the clinical impact of these microorganisms is typically limited to sporadic and local outbreaks that can be controlled.

However, mobile genetic elements carrying carbapenemases are a greater threat and challenge. These elements can be interchanged between different species and rapidly spread beyond clinical control. Indeed, horizontal gene transfer can occur in natural environments and remain unknown.

Among class A, KPCs are the most successful enzymes^[101]. bla_{KPC} has been identified in *Enterobacteriaceae*, *Pseudomonas* and *Acinetobacter* species and is typically inserted in TN4401, a TN-3 based transposon that evolves and mobilizes genes at a high frequency^[102-105]. Interestingly, bla_{KPC} success has been associated with a single *K. pneumoniae* sequence type, ST-258, which harbors the TN4401 transposon. Naas *et al* suggested that the transposition of these elements into various-sized plasmids is a recent event^[95].

GES has also been identified in different species worldwide, but this enzyme is associated with single outbreaks^[4], although a new type of mobile genetic element, namely the integron mobilization unit, has been described for bla_{GES-5}^[106]. IMI-2 has been identified in plasmids in *Enterobacter asburiae* and *E. cloacae*, but the clinical import of this enzyme is minimal^[107,108].

Class D carbapenemases are transferred from plasmids to bacterial chromosomes and vice versa^[109]. bla_{OXA-23} has been identified in certain *A. baumannii* clones and is generally located in a Tn2006 transposon, bracketed by the ISAbA1 insertion sequence and associated with other antimicrobial resistance islands^[110]. bla_{OXA-48} is associated with TN1999 in *K. pneumoniae* and Tn1999.2 in *E. coli*, where an ISR1 insertion sequence transfers this enzyme from an IncL/M plasmid to the bacterial chromosome. The high conjugative frequency of this plasmid among *Enterobacteriaceae* accounts for its overall success^[109,111,112].

Until the emergence of bla_{NDM} type MBLs, bla_{VIM}

and bla_{IPM} were the most successful enzymes in this family. These enzymes are associated with IncA/C, IncF, IncHI1, IncL/M, IncN, and IncK incompatibility group plasmids and intercountry, interhospital, intrahospital, interspecies, and intracolonial spread^[113]. These genes have primarily been identified on class I integrons as gene cassette arrays with other antimicrobial and antiseptic resistance genes^[114,115]. In contrast, in a surprisingly short period of time, the bla_{NDM} gene has become the most disseminated MBL among countries and species^[35,116]. However, bla_{NDM} is harbored in highly transferable plasmids containing resistance genes to almost all known antimicrobials^[116,117]. Indeed, bla_{NDM-1} is considered a chimera constructed in *A. baumannii* through the initial capture of a precursor gene by ISCR27 and subsequent fusion to the aphA6/ISAbA125 promoter, which was incorporated into the existing large multi-resistance regions of different *Enterobacteriaceae* plasmids. The gene bla_{NDM-1} has been associated with the remnants of the Tn125 transposon, which was identified in the chromosomes or plasmids of *Acinetobacter* spp. and is positioned adjacent to a complete or truncated ble_{MBL} gene^[118-121]. The high genetic mobilization of bla_{NDM-1} has been demonstrated through reports of a new chromosomally located class I Tn402-like structure in *P. aeruginosa*^[122].

CMY-10, the only class C carbapenemase, has recently been associated with a new complex class 1 integron within a conjugative plasmid^[123].

EPIDEMIOLOGY

bla_{SME} has been identified in some subpopulations of *S. marcescens* located primarily in the United Kingdom and sporadically in North America. This gene has been associated with single cases or small outbreaks, but the spread is most likely hindered through fitness costs associated with the expression of SME protein. However, recently, bla_{SME} has been detected on a genomic island, SmarGI1-1, which can be excised and circularized, likely contributing to the dissemination of this gene among *S. marcescens*^[4,18,124,125].

bla_{IMI/NMC-A} has been identified in *Enterobacter* spp. in sporadic cases in the United States, France, Finland, Croatia, Argentina and China^[4,38,126].

bla_{KPC} was first described in *K. pneumoniae* in cases in the east coast of the United States, but has now been identified worldwide in other *Enterobacteriaceae* and Gram-negative non-fermenters^[12,18,127]. The spread of bla_{KPC} has become an endemic problem in the east coast of the United States, Puerto Rico, Colombia, Greece, Italy, Israel and the east coast of China, and regional or local outbreaks have been reported worldwide^[101,128,129]. The high adaptability and inter-genera spread of KPC has been demonstrated by the first KPC-3 outbreak in Canada, involving five different *Enterobacteriaceae* in eight patients^[130].

bla_{GES} genes have been detected worldwide in both

Enterobacteriaceae and Gram-negative non-fermenters, but these genes pose a minor challenge compared with *bla_{KPC}*. Nevertheless, *bla_{GES}* has been identified in plasmids recovered from environmental samples easily replicated in *E. coli* and *P. aeruginosa*^[4,131].

OXA-48-like carbapenemases, a source of frequent nosocomial outbreaks, were first detected in Turkey, but has recently spread primarily across North Africa^[132,133]. In France, The Netherlands and Morocco, OXA-48-like carbapenemases have been associated with *K. pneumoniae* ST395, suggesting geographical dispersion through immigration. OXA-181 is a growing concern in the Indian subcontinent, and sporadic cases have been reported worldwide, although most cases have been associated with this geographical area^[38]. OXA-163 has also been identified in Argentina and Egypt^[38].

Chromosomally encoded MBLs are ubiquitous in environmental bacteria, but acquired MBLs have recently become a global concern. IMP type carbapenemases were first reported in Japan in the late 1980s in *P. aeruginosa*, subsequently these enzymes were identified in *S. marcescens* and are currently primarily detected in *Enterobacteriaceae* and *Pseudomonas* across Eastern Asia^[38,39,134-136].

Similarly, VIM-type enzymes have primarily been detected in *Enterobacteriaceae* and *Pseudomonas*. These enzymes were first detected in the 1990s in Italy and France and are now endemic in Greece, Italy, Spain, South Korea and Taiwan^[38,41,137,138].

However, the most challenging MBLs are undoubtedly, the NDM-type carbapenemases. These enzymes were first detected in *K. pneumoniae* and *E. coli* in a Swedish patient returning from India, showing the propensity for interspecies spread^[117,139]. The Indian subcontinent has been identified as both the origin and reservoir, where the prevalence of these enzymes is approximately 5%-18%^[140,141], but the Balkans has also been recently identified a reservoir for NDM-type carbapenemases^[142]. NDM producers have been reported worldwide, associated with intercontinental travel^[38]. Disturbingly, NDM producers are involved in nosocomial infections and community-acquired infections.

CARBAPENEMASE COSTS

The emergence of carbapenemase-resistant bacteria is a global health threat and a medical challenge, implicating an extremely high cost for hospitals. This cost is associated with prolonged hospital stays, higher health care expenses, and increased mortality, particularly when a second antibiotic is needed for better coverage of the causative pathogen^[143]. Significantly, the length of stay in hospital, the hours of mechanical ventilation and the time spent on an intensive care unit also contribute to increased costs^[144].

The actual cost of controlling infection has been associated with several factors, and some of these factors, such as new antimicrobial development

(estimated at U\$ 1 billion per drug), the need for increased surveillance within each hospital to determine problematic pathogens, enforced isolation procedures to control spread, and education (primarily interns, residents, and medical students who have not yet developed specific treatment habits), are unsuspected.

Other costs involve the aging population and the delivery of quality health care at reasonable prices^[143]. For optimal economic understanding, considering that high-cost drugs can also be cost-effective, the clinicians, pharmacists and economists at each hospital should work together to achieve cost-savings with effective antibiotic therapy, rather than making choices based on drug-acquisition costs^[144].

With regard to the host, particularly in multi-pathological patients, there is a synergy between the severity of the underlying disease and infection with a resistant microorganism. The consequences are higher mortality rates, extended hospitalization and increased costs^[145]. A common problem in endemic settings or during outbreaks is that therapeutic options become limited and are not always optimal^[146]. Consequently, the appropriate selection of the initial antibiotic therapy before susceptibility tests are available is difficult. Thus, patients under these conditions have poorer clinical outcomes. Indeed, the death rates associated with KPC producers are higher than 50%, while rates associated with MBL producers range from 18%-67%^[101].

Other challenges include clinical laboratory testing and the obtained results. Ideally, rapid and optimal susceptibility tests are in demand, but each pathogen varies, even within the same family, reflecting delayed resistant pathogen identification, treatment errors and higher costs^[143].

The ability to efficiently treat carbapenemase-producing bacterial infections requires a multifactorial approach, including continuous investigation, the development of new antibiotics and the adequate use currently available treatments, considering that the first approach starts with an appropriate means of infection control^[147].

CARBAPENEMASE DETECTION

A series of non-molecular-based tests have been proposed for the detection and identification of carbapenemases, but none of these analyses have 100% specificity or sensitivity. Therefore, the use of routine susceptibility tests to detect carbapenem resistance should be followed by genotypic and phenotypic confirmation.

Accordingly, Clinical Laboratory Standards Institute (CLSI) and European Committee on Antimicrobial Susceptibility Testing (EUCAST) breakpoints for carbapenems have been significantly lowered to improve the detection of carbapenem-resistant isolates^[148,149].

Both organizations recommend reporting carbapenem MICs, regardless of carbapenemase production, and suggest that special tests for carbapenemase detection

Table 3 Breakpoint and screening cut-off for carbapenemase-producing *Enterobacteriaceae*

	MIC (mg/L)				Disk-diffusion zone diameter (mm) using 10 µg disks			
	S breakpoint		Screening cut-off		S breakpoint		Screening cut-off	
	EUCAST	CLSI	EUCAST	CLSI	EUCAST	CLSI	EUCAST	CLSI
Meropenem	≤ 2	≤ 1	> 0.12	2-4	≥ 22	≥ 23	< 25	16-21
Imipenem	≤ 2	≤ 1	> 1	2-4	≥ 22	≥ 23	< 23	-
Ertapenem	≤ 0.5	≤ 0.5	> 0.12	2-4	≥ 25	≥ 22	< 25	19-21

S: Susceptibility; MIC: Minimum inhibitory concentration; CLSI: Clinical Laboratory Standards Institute; EUCAST: European Committee on Antimicrobial Susceptibility Testing.

be performed only for epidemiology purposes and infection control issues. However, partial or total susceptibility to carbapenems has been observed in carbapenemase producers, and carbapenem elevated MICs have been associated with decreased outer membrane permeability or the overexpression of other β-lactamases or chromosomally encoded AmpC cephalosporinases^[101,150].

The detection of carbapenemase producers in clinical specimens is based on a careful analysis of the susceptibility results obtained from automated systems, liquid media or disc diffusion tests according to standardized guidelines. The CLSI and EUCAST have defined cut-off values for carbapenemase detection in *Enterobacteriaceae* because carbapenem MICs for carbapenemase-producing bacteria might be below the clinical breakpoints. However, while the CLSI values lie within the intermediate category, the EUCAST values lie within the susceptible range. According to EUCAST epidemiological cut-off values, meropenem offers the best sensitivity and specificity balance (Table 3). Imipenem shows a narrow range between the wild-type bacteria and carbapenemase producers, and ertapenem, although it presents excellent sensitivity, has a variable positive predictive value, depending on the carbapenemase prevalence and presence of different resistance mechanisms^[151,152].

Day *et al.*^[153] recently evaluated the effectiveness of disc diffusion using faropenem to predict carbapenemase activity in *Enterobacteriaceae*. These authors concluded that a zone diameter of 6 mm for a 10 µg faropenem disc predicted carbapenemase activity with a sensitivity (99%) and superior specificity (94%) equivalent to any recommended screening indicator of carbapenemase activity. Moreover, most carbapenemase-producing isolates grow up to the disc, and consequently these bacteria are easily detected. Interestingly, a double inhibition zone with colonies growing up to the disc was observed for isolates carrying OXA-48 carbapenemase.

Automated systems

Automated systems present poor specificity and cannot distinguish carbapenemase producers from isolates with porin loss and ESBL or AmpC overproduction^[152]. These systems might not reliably detect all carbapenemase producers^[154], particularly when a single carbapenem

is tested^[153]. Additionally, discrepancies arise with automated systems, such as Vitek2, which reported meropenem MICs for KPCs that were significantly lower than those obtained through broth microdilution^[155]. OXA-48 producers are also poorly detected.

The modification of antibiotic testing panels for the inclusion of synergy tests between carbapenems and β-lactamase inhibitors, such as boronic acid for KPCs, cloxacillin for AmpCs and dipicolinic acid or EDTA for MBLs, would facilitate more stringent interpretive criteria and improve specificity^[156].

Modified hodge test

The cloverleaf or modified hodge test (MHT) is based on the inactivation of a carbapenem using carbapenemase-producing organisms. Briefly, an inoculum of the indicator organism *E. coli* ATCC 25922, adjusted to 0.5 McFarland turbidity standards, is used to inoculate the surface of a Mueller-Hinton agar plate. After incubation for 10 min at room temperature, a disc containing 10 µg of meropenem is placed onto the agar plate. Subsequently, 3-5 colonies of the test organisms are inoculated in a straight line from the edge of the disc to the periphery of the plate. The growth of the indicator strain toward the meropenem disc is interpreted as a positive result for carbapenem hydrolysis.

CLSI recommends this test for screening purposes, except for *P. aeruginosa*. An optimization of MHT (PAE-MHT) using *K. pneumoniae* ATCC 700603 as an indicator has demonstrated 100% sensitivity and 98% specificity for detecting carbapenemase activity without indeterminate results, including KPC and carbapenem-susceptible MBL-producing *P. aeruginosa*^[157].

The MHT works well for the detection of KPC and OXA-48 producers, but is time consuming, cannot distinguish the type of carbapenemase involved and, most importantly, false positive results have been observed with isolates producing CTX-M-type ESBL or AmpC β-lactamases at high level. Moreover, false negative results may occur, primarily among NDM producers. Adding zinc to the culture medium significantly improves the sensitivity in detecting NDM producers by increasing the stability of the enzyme or modifying porin expression^[158]. The replacement of Mueller-Hinton agar with MacConkey agar also increases the sensitivity for MBL or OXA producers. The

enhanced detection reflects the presence of bile salts in the medium, which improve the release of periplasmic enzymes^[159].

A modification of the MHT using discs containing carbapenem supplemented with boronic acid, which inhibits both class A carbapenemases and AmpC β -lactamases, can be used to reduce the number of false positives compared with the MHT results obtained using discs containing carbapenem alone or carbapenem supplemented with cloxacillin, which only inhibits AmpC enzymes. The proposed procedure results in high sensitivity and specificity for class A carbapenemase detection, regardless of the carbapenem tested. A low performance level is observed only among mucoid phenotype strains^[160].

Overall, MHT, although remaining a convenient screening assay, cannot be used as the sole method for the detection of carbapenemase-positive isolates in the clinical laboratory.

Biochemical tests

Biochemical tests based on the detection of β -lactam ring hydrolysis have been developed for the early identification of carbapenemases in *Enterobacteriaceae* and *P. aeruginosa*.

The Carba NP test is a colorimetric test based on the detection of acidification resulting from imipenem hydrolysis coupled with inhibitors that reliably identify carbapenemase producers and discriminate between the three main types of carbapenemases (Ambler classes A, B and D). Ambler classes A and B are inhibited through tazobactam and EDTA, respectively, while class D production is deduced from the lack of inhibition.

This test is highly sensitive and specific, easy to perform and interpret, cost-effective, adaptable to any laboratory, and the results are obtained within 2 h. Additionally, the Carba NP test is cheaper and faster than molecular techniques and can be used to select isolates for subsequent analyses using polymerase chain reaction (PCR) methods^[161,162]. False negative results have been observed using this test, particularly for OXA-48 and GES^[163].

The Carba NP test has been evaluated to directly detect carbapenemase-producing *Enterobacteriaceae* from positive blood cultures, yielding high sensitivity and specificity, which might be useful for the rapid detection of carbapenemase producers in endemic countries and outbreak situations and for the early implementation of appropriate antimicrobial therapies^[164,165].

The Rapid CARB Screen Kit (Rosco Diagnostica A/S, Taastrup, Denmark) is another method based on the identification of β -lactam ring hydrolysis in the presence of an indicator. This kit is technically easier to use, but the results are more difficult to interpret, and this assay does not discriminate carbapenemase types. Furthermore, the number of false positives and uninterpretable results present another major

disadvantage of this assay. Thus, this kit can be used for the direct detection of carbapenemases in positive urine and blood samples.

Both tests should be used with caution in areas with high OXA-48 prevalence and should be evaluated in epidemiological settings where carbapenemases with lower hydrolytic activity might be detected, *i.e.*, IMP, GES or OXA-198^[166].

The Blue-Carba test is a variation of the Carba NP test, validated for the direct detection of carbapenemase-producing strains from bacterial cultures. This test uses tienam instead of the more expensive imipenem monohydrate. The sensitivity and specificity of the Blue-Carba test are similar to those of the Carba NP test, but the former has an additional advantage of detecting *Acinetobacter* OXA-type carbapenemases^[167].

Detection of MBLs based on chelating agents

The phenotypic detection of MBL producers in the clinical laboratory is based on the specific inhibition of MBLs using zinc-depriving compounds, such as EDTA, dipicolinic acid, and 1,10-phenanthroline or thiol compounds, such as 2-mercaptopropionic and mercaptoacetic acid.

The double-disc synergy test (DDST) employs a disc containing hydrolyzable β -lactam (typically a carbapenem, although ceftazidime has also been widely used) placed near a disc containing an MBL inhibitor. The formation of a synergistic inhibitory zone indicates MBL production^[168].

Alternatively, in the combined disc test (CDT), the inhibition zone of a disc containing β -lactam combined with an inhibitor is compared with that of a disc containing β -lactam alone. An increase in the inhibition zone diameter above a predefined cut-off value indicates MBL activity^[169].

Based on similar principles, gradient diffusion methods (*e.g.*, IP/IPI Etest, bioMérieux, France) use strips containing imipenem and imipenem plus EDTA. A MIC reduction greater than or equal to eight-fold in the presence of EDTA indicates MBL activity^[170,171]. New E-test strips containing other inhibitors or carbapenem molecules would facilitate MBL detection, but these strips are not yet commercially available.

Another strategy to facilitate easy interpretation, regardless of the carbapenem MIC, is the use of an inhibitor-impregnated agar. EDTA-impregnated agar is prepared by spreading 2 mL of a 5 mmol/L EDTA solution on the surface of a mueller Hinton agar plate (MH). Differences in the inhibition zone of the carbapenem discs are compared between inhibitor-impregnated and a regular MH agar, searching for differences ≥ 10 mm. This technique facilitates the easy detection of simultaneously associated mechanisms of β -lactam resistance and carbapenemase production in clinical isolates^[172].

When using methods based on β -lactam-chelator combinations, it is important to consider the potentially detrimental effects of chelating agents on bacterial

growth, which might yield false positive results.

Detection of class A carbapenemases based on boronates

The phenotypic detection of class A carbapenemase production is based on the susceptibility of these enzymes to boronic acid and boronic acid derivatives, *i.e.*, phenylboronic and 3-aminophenylboronic acid (PBA). For MBL detection, DDST, CDT and carbapenemase PBA-impregnated agar techniques are used.

PBA and PBA-combined discs are prepared as previously described^[173]. To prepare PBA-impregnated agar, 750 µL of PBA at 10 mg/mL is spread onto a Mueller Hinton agar plate^[172].

Because boronic acid derivatives also inhibit AmpC-type β-lactamases, the simultaneous use of cloxacillin, which only inhibits AmpC but not class A enzymes, enhances the specificity of these tests. MH agar containing cloxacillin and discs containing boronic acid alone or meropenem in combination with boronic acid or cloxacillin are also commercially available (MAST, United Kingdom; Rosco, Denmark).

Notably, isolates with both KPC and metallo-beta-lactamase enzymes might not show synergy in inhibitor-based assays and appear highly resistant to carbapenems.

Detection of OXA-48 based on temocillin-resistance

CLSI and EUCAST interpretative criteria exhibit poor sensitivity in the detection of OXA-48 producers. The zone diameters for meropenem in OXA-48-producers can be up to 26 mm, thus < 27 mm must be used as a screening cut-off in countries where OXA-48 is endemic, at the expense of lower specificity^[149].

Because OXA-48 carbapenemases confer high-level temocillin resistance, a disc diffusion assay using discs containing 30 µg of temocillin provides a reliable phenotypic test for the detection of OXA-48 in *Enterobacteriaceae*^[174].

The combination of both temocillin and piperacillin/tazobactam diameters, < 12 mm and < 16 mm, respectively, is used to identify OXA-48 *Enterobacteriaceae* producers with high sensitivity and specificity^[175].

Detection using chromogenic media

Several selective chromogenic media, in which species are distinguished according to colony color, are commercially available.

CHROMagar-KPC medium lacks sensitivity, as this medium only detects carbapenemase producers resistant to high levels of carbapenems. Thus, the detection of low-level resistance, as observed with KPC, IMP, VIM, NDM, and OXA-48, might not be achieved using this medium.

Supercarba medium is a Drigalski agar-based culture medium containing a low concentration of ertapenem, cloxacillin to prevent the growth of non-carbapenemase-producing carbapenem-resistant isolates, and zinc

sulfate to improve the expression of MBLs. This medium might be used for the detection of not only KPC and MBL producers but also OXA-48 producers that do not co-express ESBLs. Supercarba medium exhibits higher sensitivity and specificity than other media and is useful for the specific selection of carbapenemase-producers in stools, as this medium inhibits the growth of ESBL producers.

CHROMagar-KPC and Supercarba medium are only suitable for *Enterobacteriaceae* testing because non-enterobacterial non-carbapenemase-producing Gram-negative rods grow on these media^[176].

ChromID CARBA is also designed for the specific detection of carbapenemase-producing *Enterobacteriaceae*, and the sensitivity and specificity of this medium is high. ChromID CARBA inhibits all ESBL producers and has demonstrated the excellent detection of CPE, with MICs from 2 to > 32 mg/L, however OXA-48 was only detected at a high inoculum (10⁷ CFU/mL)^[177].

Oxid Brilliance CRE Agar exhibits high sensitivity for the detection of KPC and MBL-producing *Enterobacteriaceae*, but lower sensitivity if observed for the detection of OXA-48 producers. The specificity of this agar is relatively low, reflecting the growth of AmpC and ESBL producers that require further confirmation^[178].

ChromID CARBA SMART is a new bi-plate combining two chromogenic media to rapidly and reliably identify all carbapenemase-producing *Enterobacteriaceae*, particularly KPC, NDM-1 and OXA-48; however, this medium has not yet been evaluated^[179].

Molecular detection of carbapenemase genes

Molecular techniques, primarily based on PCR, are considered the reference standard for the identification and differentiation of carbapenem resistance genes based on the excellent specificity, sensitivity, accuracy and rapidity of these methods. If identification of a carbapenemase is required for epidemiological purposes, then the PCR products are subjected to additional sequencing. These techniques generate results within 4–6 h, or even less when real-time PCR techniques are used. The main disadvantages to the use of these techniques are high costs, requirement of trained technicians, and inability to detect novel carbapenemase genes.

Simplex PCR assays, using various primers and several in-house and commercial multiplex PCR tests, facilitate the simultaneous identification of multiple carbapenemase types^[4,180,181], but the development of methods that further shorten the detection time, such as real-time systems, is needed^[182,183].

The Xpert MDRO assay (Cepheid, Sunnyvale, CA) uses GeneXpert cartridges for the detection of carbapenemase-producing Gram-negative bacterial colonization directly from rectal swab samples in 47 min^[184].

For the detection of the main carbapenemase gene types, PCR and hybridization-based kits, such as Hyplex MBL ID and Hyplex CarbOxa ID kits (BAG Health Care,

Lich, Germany), are commercially available.

Microarray technology facilitates the rapid and reliable identification of multiple resistance determinants. Check-KPC ESBL and Check-MDR CT102 microarray analyses (Check-Points Health BV, Wageningen, Netherlands) have been successfully used for the detection of most clinically relevant carbapenemases within a single reaction^[185].

Spectrometry

Spectrometry is based on a reduction in imipenem absorbance in the presence of carbapenemase-containing extracts of bacterial enzymes at a wavelength of 297 nm. This extract is obtained from an overnight culture of the isolate through mechanical lysis using sonication. Spectrometry accurately differentiates between carbapenemase producers and carbapenem-resistant bacteria with non-carbapenemase-mediated resistance mechanisms. Moreover, this method is cheap compared with molecular techniques and has excellent sensitivity and specificity (100% and 98.5%, respectively). However, spectrometry does not discriminate between different types of carbapenemases, is time-consuming and requires trained microbiologists^[186]. Therefore, this technique is considered a reference method for the confirmation of carbapenemase activity: however, spectrometry is laborious and technically demanding, which limits the use of this method in reference laboratories.

Matrix-assisted laser desorption ionization-time of flight mass spectrometry

Matrix-assisted laser desorption ionization-time of flight mass spectrometry (MALDI-TOF) is based on the detection of enzyme activity through the direct detection of hydrolysis degradation products, which show a different molecular mass from that of the native molecules^[187].

Antibiotics and their degradation products are typically small molecules (< 1000 Da), making the analysis of these compounds difficult because the matrix is also visible in mass spectra and produces interference with high background levels. Thus, for the detection of these molecules, different matrices and modified approaches for sample preparation have been described^[188,189].

The MALDI-TOF methodology involves the suspension of a fresh bacterial culture in a buffer, followed by centrifugation. The pellet is subsequently re-suspended in a reaction buffer containing carbapenem. After 1-3 h of incubation at 35 °C, the reaction mixture is centrifuged, and the supernatant containing carbapenem and carbapenem metabolites is assessed through MALDI-TOF. Carbapenem activity is determined based on the disappearance of the peak corresponding to native carbapenem and the appearance of peaks corresponding to metabolites as a result of carbapenem hydrolysis^[190]. All reported studies have presented similar results, demonstrating the excellent sensitivity

and specificity for all carbapenemases, including OXA-type carbapenemases in *Enterobacteriaceae* and *A. baumannii*^[191,192].

Manual measurements and analyses of raw spectra, however, can be difficult and require trained microbiologists. Therefore, the availability of software for the automatic acquisition and interpretation of results is desirable in diagnostic laboratories.

The direct detection of carbapenemase activity from positive blood culture is currently being evaluated, having achieved adequate sensitivity^[193].

FIGHTING CARBAPENEMASES

The fight against carbapenemase-producing bacteria should be based on three pillars: preventing transmission, avoiding antimicrobial selective pressure and developing new treatments.

Preventing transmission is presently the starting point to win this war. Therapeutic options are limited, particularly for NDM and KPC producers, making prevention the best strategy to avoid life-threatening infections, prolonged hospital stays and increased health care costs^[146].

The first step in the prevention of transmission is to emphasize adherence to hand hygiene and standard precautions in daily clinical practice. In addition, the early identification of carriers is needed to apply control measures. Early detection can be achieved passively, selecting potential carriers through risk factor evaluation, or actively, through microbiological screening techniques.

The risk factors for carbapenem-producers are similar to those other multi-drug-resistant bacteria (MDR), including hospitalization, critical illness, surgery, ICU stays, the use of invasive devices and prior exposure to antimicrobial treatments. Recent travel to endemic countries is also important, particularly for medical tourism and military conflicts^[194-196].

The active screening of patients is the gold standard to identify carriers. The number and type of patients investigated, the use of cultures, biochemical or molecular tests, and the duration of the surveillance program will depend on the economical characteristics of the country and whether the infection represents an outbreak event or an endemic situation^[146].

Once the carriers have been identified, contact precautions and pre-emptive contact isolation must follow. Additionally, environmental decontamination and patient decolonization are recommended^[129,197].

Antimicrobial stewardship to avoid antimicrobial selective pressure should be mandatory in sanitary institutions. Antimicrobials act as selectors and accelerators of diversity in resistance mechanisms, promoting MDR and selecting new resistance enzymes^[78,198]. Thus, the high prevalence of ESBL-carriers in India encouraged the use of carbapenems as empirical treatment and selected carbapenemase-producers^[199]. Nevertheless, this issue is controversial and more research is needed^[146].

Finally, the third and most important pillar for

clinical use against carbapenemase producers involves the development of new molecules that are either active against these pathogens or act as inhibitors of carbapenemases, which might extend the life of existing antimicrobials.

New antimicrobials are being developed to fight MDR. Sulfactams are monocyclic β -lactams structurally related to monobactams, such as BAL30072, which exhibit potent activity against MDRs, particularly when combined with colistin or meropenem^[200]. Plazomicin is a new-generation aminoglycoside with bactericidal effects on carbapenem-resistant bacteria, although more studies are needed to assess the activity of this antimicrobial^[201,202]. TP-434 and GSK2251052 are antimicrobials that target protein synthesis and exhibit a spectrum encompassing multidrug-resistant Gram-negative pathogens^[203].

Moreover, carbapenemase inhibitors have also been developed, including Penem derivatives, such as BRL 42715, which inhibit serine β -lactamases, and BLI-489, which inhibits KPC; 1- β -methylcarbapenem compounds, such as J-110,411 and J-11,225, which inhibit classes A and C and IMP-type enzymes; Thiols, such as thiomandelic acids, which inhibit MBLs; and avibactam, which inhibits serine β -lactamases. These compounds will likely extend the life and increase the activity of existing antimicrobials and bring hope in the carbapenemase era^[19,204].

CONCLUSION

Carbapenem-resistant bacteria, particularly *Enterobacteriaceae*, *Pseudomonas* and *Acinetobacter*, have become a challenging health issue worldwide. Fourteen years have passed since Livermore *et al.*^[205] published the article entitled "Carbapenemases: a problem in waiting?". These authors discussed the emergence of new carbapenem-hydrolyzing enzymes and the possibility that these molecules could pose a threat to infection treatment in the near future. Five years later, Walsh *et al.*^[206] warned about the perils of MBLs, remarking on the spread of these enzymes through horizontal gene transfer and resistance mechanisms to other antimicrobial families. These authors suggested the development of both surveillance programs and new inhibitors.

Moreover, in 2012, Nordmann *et al.*^[207] published an article entitled "Carbapenem resistance in *Enterobacteriaceae*: here is the storm!", stating that carbapenemase-producing *Enterobacteriaceae* constituted a growing threat to public health.

To paraphrase John Donne, "no man is an island", in this case "no country is an island". Thus, now that the storm has arrived, international organizations, such as the World Health Organization, Centers for Disease Control and Prevention and European Centre for Disease Prevention and Control, have called for a worldwide collaboration to contain this epidemic^[146]. Migration fluxes, vacations and medical travels or

distant military conflicts likely contribute to the spread of resistance genes across the globe, and only through a collaborative global effort, will we successfully address this problem.

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