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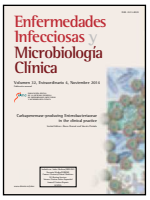

Carbapenemases in Enterobacteriaceae: Types and molecular epidemiology

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ABSTRACT

The most important mechanism of carbapenem resistance in Enterobacteriaceae is the production of carbapenemases, although resistance can also result from the synergistic activity between AmpC-type or (to a lesser extent) extended-spectrum beta-lactamases combined with decreased outer membrane permeability. Three major molecular classes of carbapenemases are recognized: A, B and D. Classes A and D are serine-beta-lactamases, whereas class B are metallo-beta-lactamases (their hydrolytic activity depends on the presence of zinc). In addition to carbapenems, carbapenemases also hydrolyze other beta-lactams, but the concrete substrate profile depends on the enzyme type. In general terms, class A enzymes are to some extent inhibited by clavulanic acid, and class B enzymes do not affect monobactams and are inhibited by zinc chelators. Given Enterobacteriaceae producing carbapenemases usually also contain gene coding for other mechanisms of resistance to beta-lactams, it is not unusual for the organisms to present complex beta-lactam resistance phenotypes. Additionally, these organisms frequently contain other genes that confer resistance to quinolones, aminoglycosides, tetracyclines, sulphonamides and other families of antimicrobial agents, which cause multiresistance or even panresistance. Currently, the most important type of class A carbapenemases are KPC enzymes, whereas VIM, IMP and (particularly) NDM in class B and OXA-48 (and related) in class D are the more relevant enzymes. Whereas some enzymes are encoded by chromosomal genes, most carbapenemases are plasmid-mediated (with genes frequently located in integrons), which favors the dissemination of the enzymes. Detailed information of the genetic platforms and the context of the genes coding for the most relevant enzymes will be presented in this review.

Carbapenemasas en enterobacterias: tipos y epidemiología molecular

Palabras clave:
Enterobacterias
Betalactámicos
Carbapenemasas
Multirresistencia

RESUMEN

El mecanismo más importante de resistencia a carbapenémicos en enterobacterias es la producción de carbapenemasas, aunque dicha resistencia también puede deberse a la combinación de betalactamasas tipo AmpC o, en menor medida, de espectro extendido, combinadas con disminución de la permeabilidad de la membrana externa. Se conocen 3 tipos moleculares de carbapenemasas: A, B y D. Las de las clases A y D son betalactamasas de serina, mientras que las de clase B son metalobetalactamasas (su actividad depende del cinc). Las carbapenemasas también hidrolizan otros betalactámicos, además de carbapenémicos, pero el perfil de sustrato concreto depende de la enzima considerada. En términos generales, las enzimas de clase A se inhiben en mayor o menor medida por ácido clavulánico y las de clase B no afectan a los monobactámicos y se inhiben por quelantes del cinc. Las enterobacterias que producen carbapenemasas, generalmente contienen otros genes de resistencia a betalactámicos y no es raro que presenten fenotipos de resistencia a betalactámicos complejos. Además, estos organismos frecuentemente contienen genes de resistencia a quinolonas, aminoglucósidos, tetraciclinas, sulfonamidas y otros antimicrobianos, causando multirresistencia o incluso panresistencia. Actualmente, las carbapenemasas más importantes de clase A son KPC, las de clase B son VIM, IMP, y en especial NDM, y las de clase D, OXA-48 y similares. Aunque algunas enzimas están codificadas por genes cromosómicos, la mayoría están mediadas por plásmidos (y los correspondientes genes con frecuencia se encuentran en integrones), lo cual favorece la diseminación de estas enzimas. En esta revisión se presenta información detallada sobre las plataformas y los contextos genéticos de los genes que codifican las enzimas más relevantes.

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Introduction

Resistance to beta-lactams in Enterobacteriaceae and other Gram-negative organisms is primarily mediated by beta-lactamases, although other mechanisms typically cooperate for an increased level of resistance. Multiple types of this family of enzymes are clinically relevant, but because of its production by multiresistant organisms, three are particularly important in Enterobacteriaceae: extended-spectrum beta-lactamases (ESBL), chromosomal or plasmid-mediated AmpC-type enzymes (AmpCs) and carbapenemases.

Both ESBL and AmpCs show (very) poor hydrolytic activity against carbapenems, but when associated with porin loss or modification or even other low level resistance mechanisms, they (particularly AmpCs) can determine carbapenem resistance of clinical importance.[1] On the other hand, some beta-lactamases efficiently hydrolyze carbapenems and are globally designated as carbapenemases. A few reports have also identified other mechanisms (such as altered penicillin-binding proteins), as a cause of carbapenem resistance in Enterobacteriaceae.[2] From a genetic point of view, some carbapenemases are related to other enzymes of the same molecular class lacking (relevant) carbapenemase activity. In 1980, Ambler proposed a beta-lactamase classification based on the sequence of these enzymes that included 4 groups named A to D.[3] The enzymes in groups A, C and D are serine-beta-lactamases, whereas those in group B (requiring Zn for hydrolyzing their substrates) are metallo-beta-lactamases (MBL). This classification is also of interest from a biochemical, clinical and epidemiological point of view.

Besides carbapenems, carbapenemases can also hydrolyze other beta-lactams, although the concrete spectrum of affected substrates depends on the nature of the specific enzyme (Table 1). In addition, the organisms producing these enzymes frequently contain other resistance genes also affecting beta-lactams (see below); thus, carbapenemase-producing Enterobacteriaceae do not necessarily present the phenotype corresponding to the presence of the carbapenemase alone. Multiple reports have documented Enterobacteriaceae producing more than one class of carbapenemase, which is not only important from a therapeutic point of view, but also for the epidemiological consequences of the (in) adequate recognition of these enzymes.

The exact causes of the emergence and abrupt spread of carbapenemase-producing Enterobacteriaceae are not completely understood. It is possible that inadequate and uncontrolled use of carbapenems due to other resistance problems (particularly that of ESBL) have translated into a selective pressure favoring the transfer of genes from chromosomes to plasmids, with the subsequent dissemination of plasmids between strains and of strains between patients. The intercontinental movement of patients has also undoubtedly contributed to the problem.

Class A carbapenemases

This class of enzymes was recognized sporadically during the 1980s, but became of major importance when KPC (*Klebsiella pneumoniae* carbapenemase) enzymes were identified in 1996 and spread worldwide after the 2000s.[4]

SME/IMI/NMC-A

These three groups of enzymes are encoded by chromosomal genes and hydrolyze a broad spectrum of substrates, including penicillins, some cephalosporins, aztreonam and carbapenems.

The *Serratia marcescens* enzyme (SME-1) was first detected in England in 1982 in two *S. marcescens*. The variants SME-2 and SME-3 have been reported in North America. The bla_{SME} genes are found in cryptic prophage genomic islands within the *S. marcescens* chromosome.[5,6]

Imipenem-hydrolyzing beta-lactamase enzymes (IMI) were first discovered in *Enterobacter cloacae* in North America in 1996.[7] The bla_{IMI-1} gene was located in the bacterial chromosome; however, a point-mutation derivative, bla_{IMI-2}, was later identified encoded on a plasmid in *Enterobacter asburiae* strains recovered from various USA rivers and in *E. cloacae* from China.[8] IMI carbapenemases are rare, and only 6 variants have been reported to date (IMI-1 to IMI-6) (http://www.lahey.org), most in *Enterobacter* spp. and exceptionally in *Escherichia coli*.[5,9]

The expression of the bla_{IMI} gene is regulated by a LysR transcriptional regulator, which is encoded by the bla_{IMI-R} gene, adjacent to bla_{IMI}. Upstream and downstream of the complex an IS*2*-like element and a transposase gene are encoded, respectively, which may have been involved in its mobilization.[5,9]

The spread of IMI-type plasmid-mediated carbapenemases is limited, and IMI-producing Enterobacteriaceae have been detected in North America, Argentina, France, Spain, Croatia, Finland and Ireland.[9,10]

The non-metallo-carbapenemase-A (NMC-A) enzyme was identified for the first time in *E. cloacae* in France in 1990.[11] This enzyme differs from IMI-1 and IMI-2 by 8 amino acid substitutions and possesses a Lys-R regulator like IMI-type enzymes.[5]

Guiana Extended-Spectrum beta-lactamase enzymes

Guiana Extended-Spectrum beta-lactamase enzymes (GES) were discovered in 2000 when the GES-1 beta-lactamase was reported. GES enzymes were originally considered ESBL, but some variants among the 24 currently recognized enzymes (http://www.lahey.org), including GES-2, -4, -5, -6, -11, -14 and -18,[12] are actually carbapenemases. GES-type enzymes have been detected worldwide in several Gram-negative bacteria. The Enterobacteriaceae GES-4, -5 and -6 are the GES enzymes that have been found to have carbapenemase activity.[8]

bla_{GES} genes are typically encoded as gene cassettes on class I integrons, which are located on transferable plasmids; however, chromosomally encoded bla_{GES} genes have also been found in Enterobacteriaceae.[5]

Table 1
Hydrolytic profiles of the carbapenemases described in Enterobacteriaceae[a]

Molecular class	Carbapenems	Penicillins	1st & 2nd cephalosporins	3rd & 4th cephalosporins	CLAV/EDTA	Monobactams
A	+	+	+[b]	+/(w)	±/–	+[c]
B	+	+	+	+	–/+	–
D	+	+	+	(–)[d]	–/(±)	±

CLAV/EDTA: inhibition by clavulanic acid and by EDTA; (w): weak hydrolytic activity.
[a]Class C enzymes can cause carbapenem resistance in Enterobacteriaceae when (over)expressed in strains with altered outer membrane permeability.
[b]Cephamycins are poor substrates for most class A enzymes.
[c]Some GES enzymes do not hydrolyze aztreonam.
[d]OXA-163 efficiently hydrolyzes expanded-spectrum cephalosporins.

KPC

KPC enzymes were first reported in 1996 from a *K. pneumoniae* isolated in North Carolina, North America.[4] KPC-producing bacteria have since spread worldwide. Twenty variants have been identified thus far (KPC-1 to KPC-20) (http://www.lahey.org). The two most frequent variants are KPC-2 and KPC-3 (differing in just one amino acid). Since its first report, KPC-enzyme producers have been detected primarily in *K. pneumoniae* but also in *E. coli, Citrobacter freundii, S. marcescens*, *Enterobacter* spp., and *Pseudomonas* spp.[5,8,13]

Enterobacteriaceae producing KPCs show high resistance to both penicillins and cephalosporins, but only low to moderate resistance to carbapenems (which could make their recognition difficult). The inhibitory effect of clavulanic acid and of related inhibitors against these enzymes is also lower than for other class A enzymes. It is not uncommon that KPC-producing enterobacteria also express other plasmid-mediated beta-lactamases.

bla_{KPC}-type genes are typically embedded in transposon Tn*4401*, a Tn*3*-based transposon that is able to mobilize this carbapenemase-encoding gene at a high frequency (Fig. 1A).[14] The transposon containing bla_{KPC} has been found in a large variety of transferable plasmids, including plasmids belonging to $IncFII_K$, IncA/C, IncN, IncI2, IncX, IncR and ColE incompatibility groups.[15,16] In addition to beta-lactam resistance, plasmids encoding bla_{KPC} usually harbor genes conferring resistance to other antimicrobial agents such as quinolones, aminoglycosides, tetracyclines, trimethoprim and sulphonamides. This situation has made most of the KPC-producing *K. pneumoniae* isolates multidrug- or even pandrug-resistant. Plasmid pKpQI is one of the most studied bla_{KPC}-carrying plasmids. This mobile genetic element and several derivatives primarily associated with *K. pneumoniae* ST258 have been identified in Israel, Italy and North America since 2006.[15,16]

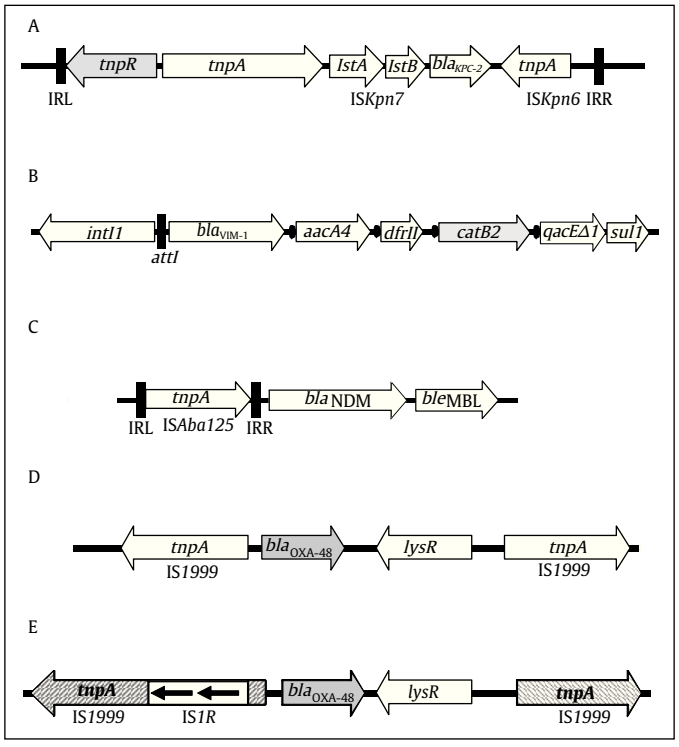

Figure 1. Schematic representation of several genetic structures associated with carbapenemase-encoding genes in enterobacterial isolates. A) Structure of a Tn*4401* encoding bla_{KPC-2} gene. B) Structure of a class I integron containing a bla_{VIM-1} gene. C) Structures of a bla_{NDM} gene associated with IS*Aba125*. D) Structure of a Tn*1999* encoding bla_{OXA-48} gene. E) Structure of a Tn*1999.2* encoding bla_{OXA-48} gene.

K. pneumoniae ST258 has significantly contributed to the worldwide dissemination of KPC-enzymes, specifically to the KPC-2 and KPC-3 variants. Despite ST258 having been the primary clone, many other *K. pneumoniae* strains and other enterobacterial species have been reported as KPC producers in different countries.[17] KPC-producing isolates are now considered endemic and predominate in several countries from the Americas, including some states in North America, Colombia, Brazil and Argentina. In Europe, Italy and Greece are the endemic countries, and in other countries, such as Spain and France, sporadic cases have been reported, some linked to imported isolates from endemic areas.[17] Among Asian countries, Israel and China are considered endemic. In India, KPC-producing isolates have only occasionally been reported, as well as in Australia, New Zealand and on the African continent.[17]

Class B carbapenemases (metallo-beta-lactamases)

MBLs hydrolyze nearly all beta-lactams except monobactams by a mechanism that depends on the presence of zinc ions; as a consequence, MBLs are inhibited by the zinc chelator EDTA. Unfortunately, they are not inhibited by clavulanic acid and similar inhibitors and are frequently found in strains also coding for other enzymes, particularly ESBL (which cause resistance to aztreonam). Three subclasses (B1, B2 and B3) have been proposed based on substrate preference and protein structure, of which B1 includes the enzymes of greatest clinical interest.[18] Among these enzymes, NDM-1 (see below) presents a lower hydrolytic rate than representative enzymes of other families such as IMP-1 or VIM-2,[19] but this is likely counterbalanced by having been encoded by a gene included in highly efficient genetic platforms.

Verona integron-encoded metallo-beta-lactamases

Verona integron-encoded metallo-beta-lactamase (VIM)-type enzymes were first identified in a carbapenem-resistant *Pseudomonas aeruginosa* clinical isolate in 1997 in Verona, Italy.[20] To date, 41 variants have been reported and this type of enzymes has become one of the most worldwide prevalent plasmid-mediated MBLs, being identified in several bacterial species. VIM enzymes are primarily produced by *P. aeruginosa* followed by *E. coli, K. pneumoniae* and to a lesser extent *E. cloacae* within the Enterobacteriaceae family.

bla_{VIM} have been found embedded primarily in class I integrons as gene cassettes, typically harbored in transposons, with more than 100 different arrangements in the integron structure, including additional gene cassettes encoding resistance to aminoglycosides, chloramphenicol and sulphonamides, resulting in multidrug resistance (Fig. 1B). Initially, bla_{VIM}-integrons were found to be located in the IncN plasmid type; however, they have recently also been found on large-size transferable plasmids belonging to other incompatibility groups, such as Inc A/C, IncR, IncHI2, IncI1 and IncW, as well as being integrated in the bacterial chromosome.[15]

The dissemination of *E. coli* and *K. pneumoniae* isolates harboring bla_{VIM} is primarily polyclonal, and isolates carrying these enzymes have been reported in a large number of countries from Europe, Asia and America in sporadic occurrence or causing significant outbreaks.[8,21] It has been found in several Mediterranean countries (e.g., Greece, Italy) and in several countries from Southeast Asia (e.g., Taiwan, Japan), where a high prevalence of VIM-producing bacteria is found.[22,23]

New Delhi metallo-beta-lactamase

New Delhi metallo-beta-lactamase (NDM) was detected for the first time in 2008 in a *K. pneumoniae* isolate causing a urinary tract infection in a Swedish patient of Indian origin who had been previously hospitalized in New Delhi.[19] It has since rapidly

disseminated and has been detected increasingly in several countries, primarily in Enterobacteriaceae (particularly in *E. coli* and *K. pneumoniae*), and to a lesser extent in *Acinetobacter* spp. Currently, 12 different variants of the NDM enzymes (NDM-1 to NDM-12) have been reported (http://www.lahey.org).

Reports on NDM enzymes have a social impact beyond their clinical aspects: the name given to the enzyme suggests a link to the health system in India, and this has been claimed to cause major economic losses in this country, where health care offered to foreign patients is provided at a lower cost than in many Western countries. From this point of view, NDM (multiresistance in general) can be perceived as a major menace with important economic consequences.

In Enterobacteriaceae, bla_{NDM} are primarily carried on heterogeneous conjugative plasmids of varying sizes and belonging to different incompatibility groups, including IncL/M, IncA/C, IncF and IncHI1.[15] These plasmids have spread between different Enterobacteriaceae strains, species and genera.[24] Despite being found in plasmids, *Acinetobacter* spp. bla_{NDM} genes are most often located on the chromosome, particularly in *Acinetobacter baumannii*. The genetic structures identified surrounding the bla_{NDM} genes in Enterobacteriaceae are primarily associated with the insertion sequence IS*Aba125* on the upstream side, found as a full or truncated element, and with the ble_{MBL} gene (bleomycin resistance encoding gene) on the downstream side, which can also be present as a complete or truncated structure (Fig. 1C). Both bla_{NDM} and ble_{MBL} genes are coexpressed under the control of the same promoter, which is located at the 3'-end of IS*Aba125*. IS*Aba125* had previously been identified in *A. baumannii,* and in this species the bla_{NDM} gene is flanked downstream by a second copy of IS*Aba125,* forming the composite transposon Tn*125*.

The origin of the bla_{NDM} gene is still unknown, but it has been postulated that it was integrated into the chromosome of *A. baumannii* from an environmental species and later transposed onto Enterobacteriaceae plasmids of a broad host range of replication.[8]

Strains carrying plasmid-encoded bla_{NDM} frequently coproduce other beta-lactamases such as oxacillinases (OXA-1, OXA-10), plasmid-mediated AmpCs (CMY-type, DHA-type), ESBLs (CTX-M-type, SHV-type) and additional carbapenemases (VIM-type, OXA-48), as well as other non-beta-lactamase enzymes conferring resistance to other antimicrobials (e.g., aminoglycosides, macrolides, quinolones). These elements can be found encoded in the same or in a different plasmid.[25] This situation threatens public health because only a few treatment options, such as colistin and tigecycline, remain available.

NDM-type carbapenemase-producing Enterobacteriaceae have been identified progressively on all continents. However, India and Pakistan are the countries with the higher prevalence for what has been proposed as the primary reservoir of NDM-producing bacteria. In countries near the Indian subcontinent, such as China, a recent report has revealed a high incidence of NDM-producing Enterobacteriaceae isolated from patients in specific provinces.[26] It has also recently been reported that NDM has become the most common class B carbapenemase in Enterobacteriaceae in the countries of the Gulf Cooperation Council.[27] Outside Asia, the Balkans have been identified as another reservoir of NDM-producing bacteria.[25]

International dissemination of bla_{NDM}-producing bacteria has been strongly associated with travel and receipt of medical care in South or Southeast Asia.[24,25] The UK is currently one of the countries with a high incidence of patients with NDM-producing bacteria, and in most cases they are epidemiologically linked to the endemic areas with which the country has had historically close bonds.[24] However, intra- and inter-country dissemination of NDM-positive strains within individuals who have not traveled to high risk areas have been also documented.[24]

IMP

IMP-1 carbapenemase was the first plasmid-encoded MBL detected, identified in a Japanese *P. aeruginosa* isolate in 1988.[28] In 1993, it was identified in a *S. marcescens* isolate in the same country.[29] bla_{IMP} has since spread worldwide, and more than 40 variants have been identified. The IMP-type beta-lactamase has been reported primarily in *P. aeruginosa, Acinetobacter* spp. and in several enterobacterial species, including *E. coli, K. pneumoniae, Klebsiella oxytoca, E. cloacae* and *Citrobacter* spp.

As with bla_{VIM}, bla_{IMP} is found as a gene cassette integrated in class 1 integrons and different integron structures harboring gene cassettes which confer resistance to diverse antibiotic families (e.g., aminoglycosides, sulfonamides, chloramphenicol). In addition to class 1 integrons, bla_{IMP} gene cassettes have been found sporadically on class 3 integrons. These integrons are usually located in transposons and in conjugative plasmids, which enable its horizontal dissemination.[21,30] Plasmids carrying bla_{IMP} are not associated with a specific backbone and belong to diverse incompatibility groups that include IncL/M, IncN and IncHI2.[15] Chromosomal location (primarily in Gram-negative non-fermenting bacilli) of bla_{IMP} genes has also been reported, but appears to be uncommon.[21]

IMP-producing Enterobacteriaceae have been identified in several countries around the world causing outbreaks or sporadic cases, the highest prevalence has been found in Southeast Asia, Japan and Taiwan).[21,30]

Class D carbapenemases

Class D carbapenemases include the oxacillinase (OXA)-type enzymes, some of which are narrow-spectrum beta-lactamases, others of which are ESBLs and a few that are carbapenemases. It has recently been suggested that OXA enzymes traditionally not considered to be carbapenemases (e.g., OXA-2, OXA-10) have some catalytic activity against carbapenems, and their ability to determine resistance to these agents actually depends on the host in which they are expressed.[31]

OXAs show a lower hydrolytic efficiency of carbapenems than other carbapenemases, and carbapenem-resistant isolates with these enzymes often contain other resistance mechanisms (e.g., porin loss). In contrast to class A carbapenemases (and to class C beta-lactamases), class D enzymes cause high-level resistance to temocillin;[32] although this compound can also be affected by class B enzymes, the latter are easily differentiated by their inhibition by zinc chelators.

OXA-48-like carbapenemases

The OXA-48 class D carbapenemase was first reported in a *K. pneumoniae* isolate recovered in Istanbul, Turkey, in 2003.[33] OXA-48-like carbapenemases have since been increasingly reported among Enterobacteriaceae in Turkey, the Middle East and North Africa. These have been considered the most important reservoirs.[34]

bla_{OXA-48} is located in a composite transposon named Tn*1999,* which is constituted of two copies of IS*1999* that flank the carbapenemase gene (Fig. 1D). These insertion sequences primarily confer two important functions to bla_{OXA-48}; (I) the IS*1999* located upstream of bla_{OXA-48} drives its expression by an outward-directed promoter and (II) they allow gene mobilization by transposition.[35] In addition to Tn*1999*, other genetic backgrounds have been reported for bla_{OXA-48}. One is Tn*1999.2,* which differs from the previous by the insertion of IS*1R* within the IS*1999* located upstream of bla_{OXA-48} (Fig. 1E).[36]

The gene bla_{OXA-48} is harbored in a broad-host-range conjugative IncL/M plasmid of 62-kb.[34,37] A particular feature of this plasmid is that Tn*1999* disrupts its *tir* gene, which encodes a plasmid cell-to-

cell transfer inhibitor protein. This leads to an increase in the transfer frequency of the plasmid, which is the proposed reason for the successful spread of *bla*$_{OXA-48}$.[38]

Since the discovery of OXA-48, several variants differing by a small number of amino acid substitutions have been reported, such as OXA-162, OXA-163, OXA-181, OXA-204, OXA-232, OXA-244 and OXA-245. All but OXA-163 possess similar hydrolytic properties as OXA-48.[34,39] OXA-163 differs from OXA-48 by a nucleotide substitution and by 4 amino acid deletions. This structure makes OXA-163 able to hydrolyze expanded-spectrum cephalosporins but not carbapenems, the opposite hydrolytic properties as those of OXA-48.[40]

The origin of OXA-48 has been proposed to be from *Shewanella* spp., a waterborne bacterium. *In silico* analysis of the entire genome of *Shewanella oneidensis* initially suggested that this was the species from which *bla*$_{oxa-48}$ emerged given it encoded in its chromosome a class D carbapenemase (OXA-54), which shared 84% nucleotide identity and 92% amino acid identity with OXA-48.[41] It has recently been suggested, however, that *bla*$_{OXA-48}$ is likely to have originated from *Shewanella xiamenensis* where a *bla* gene with only 4 silent nucleotide differences from *bla*$_{oxa-48}$ and a *bla* gene encoding an OXA enzyme (OXA-199) with only 3 amino acid substitutions from OXA-48, were found in one strain each in China.[42] Genetic elements such as IS*1999* might have been involved in the mobilization of *Shewanella* chromosomal carbapenemases to plasmids, which have then spread to other bacterial species.

The emergence and rapid spread of OXA-48 producers in European countries has been observed in recent years, probably related to a high level of human population exchanges with the endemic areas (e.g., Turkey, the Middle East and North African countries).[34] Spain, France, Germany, Switzerland, the Netherlands and the UK are the primary European countries in which a substantial increase in the number of Enterobacteriaceae producing OXA-48 carbapenemases have been reported, and in some countries it has become the predominant carbapenemase.[10,23,43] In India (a country that recently has been proposed as an endemic area of this type of enzymes), OXA-181 is the primary identified OXA-48-like carbapenemase. In the Americas, Russia, China and Australia, only sporadic reports of OXA-48-like carbapenemases have been reported.[10]

Other OXA-type carbapenemases have also been reported in Enterobacteriaceae, including those of groups OXA-23, OXA-40, OXA-51 and OXA-58; additional details can be found in the recent review of these enzymes.[44]

Conflicts of interest

The authors have no conflicts of interest to declare.

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