# Metallo-β-lactamases: structure, function, epidemiology, treatment options, and the development pipeline

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**Key words:** metallo-β-lactamase, treatment, pharmacology, drug development

**Running title:** Treatment options for infections caused by bacteria that produce metallo β-lactamases

## Abstract

Modern medicine is threatened by the global rise of antibiotic resistance, especially among -negative bacteria. Metallo-β-lactamase (MBL) enzymes are a particular concern and are increasingly disseminated worldwide, though particularly in Asia. Many producers have multiple further drug resistances, leaving few obvious treatment options. Nonetheless, and more encouragingly, MBLs may be less effective agents of carbapenem resistance *in vivo*, under zinc limitation, than *in vitro*. Owing to their unique structure and function, and their diversity, MBLs pose a particular challenge for drug development. They evade all recently licensed β-lactam- β-lactamase inhibitor combinations, although several stable agents and inhibitor combinations are at various stages in the pipeline. These potential therapies, along with the epidemiology of producers and current treatment options, are the focus of this review.

## Introduction

Antimicrobial therapy is threatened by the global rise of resistance, especially in gram-negative bacteria (1), where resistance to β-lactams is largely mediated by β-lactamases (2). Carbapenems evade most β-lactamases but are hydrolyzed by metallo-β-lactamases (MBLs) as well as by a few active-site serine β-lactamases (SBLs), notably members of the KPC and OXA-48-like groups. MBLs are chromosomal and ubiquitous in some non-fermenters, including *Stenotrophomonas maltophilia, Aeromonas* spp. and *Chryseobacterium* spp., which are of modest clinical concern. A minority of *Bacteroides fragilis* strains have a chromosomal MBL, CfiA or CcrA, but this is uncommon and only expressed strongly if an upstream insertion sequence provides an efficient promoter (3). More important are the acquired MBLs that are spreading among Enterobacterales and *Pseudomonas aeruginosa* (4); these are associated with extremely-drug-resistant (XDR) phenotypes, with the producers generally also resistant to multiple aminoglycosides, fluoroquinolones, and other agents as well as to β-lactams.

## Classification and diversity of metallo-β-lactamases

β-Lactamases are classified by two major systems. The first is based on substrate profiles and vulnerability to inhibitors (5), and places MBLs into its Group 3, whereas Groups 1 and 2 comprise SBLs. The second classifies β-lactamases according to their amino acid sequences, recognising four enzyme classes (6). MBLs form class B whilst SBLs divide among classes A, C and D (7). The MBLs are structurally and mechanistically dissimilar from SBLs, suggesting a separate evolutionary origin.

Class B is further divided into three subclasses, B1, B2 and B3, based on differences in amino acid sequence at the active site, zinc ligands, zinc stoichiometry, loop architecture, and substrate profiles (8). The important acquired MBLs, comprising the IMP, NDM and VIM types

62 fall into subclass B1. They hydrolyze all currently available β-lactam antibiotics except
63 monobactams (e.g. aztreonam) (9), as do most or all other sub-class B1 or B3 enzymes. In
64 contrast, the CphA (subclass B2) MBLs of *Aeromonas* spp. have narrow-spectrum activity
65 directed exclusively against carbapenems. Irrespective of subclass, MBLs are not inhibited by
66 clavulanic acid, sulbactam, tazobactam, avibactam or by developmental penicillanic acid
67 sulfones and diazabicyclooctanes.

68 The important acquired subgroup B1 MBLs (Table 1) are mostly named based on where
69 they were first described; thus, for example, Verona Integron-encoded Metallo β-lactamase
70 (VIM) and New Delhi Metallo β-lactamase (NDM). The first acquired MBL ('imipenemase', IMP-
71 1), was reported from clinical isolates of *P. aeruginosa* and *Serratia marcescens* in Japan in the
72 1990s (10) and its family now includes over 85 sequence variants (11). The first VIM enzyme
73 was found in *P. aeruginosa* in 1997 (12), with over 69 variants since described (11). NDM –
74 now the most prevalent MBL in Enterobacterales and *A. baumannii* – was first identified in
75 2008 in *Klebsiella pneumoniae* and *Escherichia coli* isolates from a patient who had travelled
76 to Sweden from New Delhi, India (13). Twenty-nine NDM variants have since been described,
77 (11).

78 It is easy to be dismissive of the chromosomal subclass B2 and B3 MBLs, but recent
79 reports highlight *Stenotrophomonas maltophilia* as a multidrug-resistant pathogen in
80 immunocompromised hosts (14). *S. maltophilia* carries a subclass B3 MBL (L1 enzyme), which
81 is unique among MBLs in having four identical subunits (15), in addition to a chromosomally-
82 mediated SBL (L2 enzyme). This combination confers resistance to almost all β-lactams,
83 although minimum inhibitory concentrations (MICs) vary with methodology, because media
84 affect the expression and/or function of these enzymes (16). *Elizabethkingia meningoseptica*

has two chromosomal MBLs, a B1 enzyme (BlaB) and a B3 type (GOB) with the former making the dominant contribution to resistance (17).

## Genetic support of acquired MBLs

Acquired IMP and VIM enzymes generally are encoded by gene cassettes within class 1 or class 3 integrons. These may be embedded within transposons, allowing insertion into the bacterial chromosome or plasmids (18). By contrast, the *bla*$_{NDM}$ gene is not integron-associated and has been observed on narrow-host-range plasmids belonging to incompatibility group IncF, in addition to wide-host-range plasmids belonging to IncA/C, IncL/M, IncH and IncN (19–22). *K. pneumoniae* and *E. coli* are the frequent hosts of these plasmids, and there are particular associations with *K. pneumoniae* sequence types (STs) ST11, ST14, ST15 or ST147 and *E. coli* ST167, ST410 or ST617 (23). These should not, however, be seen as global epidemic strains along the lines of *K. pneumoniae* ST258 variants with KPC carbapenemases, for many are common STs without carbapenemases. In *A. baumannii* the *bla*$_{NDM-1}$ gene is generally located within the composite transposon Tn*125* and embedded between two copies of a strong promoter gene IS*Aba125* (24, 25); it is much less prevalent in this genus than are OXA carbapenemases (Class D).

B2 and B3 MBLs are generally chromosomally encoded, ubiquitous in their host species and not transmissible. However, exceptions exist, with horizontal transfer observed. Thus, the AIM-1 MBL (B3) was initially reported, in 2012, to be encoded by a gene inserted in (and atypical of) the chromosome of a *P. aeruginosa* isolate; subsequently, in 2019, it was reported from *K. pneumoniae* (26). The *bla*$_{LMB-1}$ gene, encoding another subclass B3 enzyme, was reported to be located on a plasmid in *Rheinheimera pacifica* where it was flanked by ISCR

mobilization sequences, implying transfer from some other (unknown) source organism. (27). Mobilization of $bla_{SMB-1}$, encoding a third sub-class B3 enzyme, has occurred similarly (28).

## Structure and catalytic function of MBLs

Irrespective of subgroup, MBLs contain the αβ/βα fold typical of the metallo-hydrolase / oxidoreductase superfamily (29). The *S. maltophilia* enzyme has four identical subunits (15), whereas other MBLs are monomeric.

B1 and B3 MBLs have a shallow active-site groove containing 1 or 2 catalytically functional divalent zinc ions, flanked by flexible loops (29). In contrast, the B2 enzymes have an active site that is less accessible and flanked by a helix (30). Except for these consistencies, MBLs are highly divergent even within subclasses, and have as little as 20% sequence identity between subclasses (7).

Mechanistically, the zinc ion(s) activate a water molecule, which acts to open the β-lactam ring (31). There is no covalent intermediate, as with SBL-mediated catalysis. Anionic intermediates have been characterized when MBLs hydrolyze carbapenems (32), but not when NDM-1 enzymes hydrolyze penicillins or cephalosporins (33). In general, imipenem and meropenem are similarly good substrate for MBLs: for example, NDM-1 enzyme displays similar catalytic activity, reflected in values of $k_{cat}/K_m$ ratio, for imipenem ($0.09 \mu M^{-1} s^{-1}$) and meropenem ($0.06 \mu M^{-1} s^{-1}$) (34); biapenem is a weaker substrate, owing to high $K_m$ values, but seems unsuitable for high-dose development (35).

Figure 1 illustrates the amino acid residues that bind zinc at the active sites of B1, B2, and B3 MBLs (8). Crystal structures of B1 enzymes, including IMP-, VIM-, NDM-, and *B. fragilis* CcrA, (panel A) reveal two zinc-binding sites (Zn1 and Zn2). The Zn1 site contains three histidine residues (His116, His118, and His196), whereas the ligands for the Zn2 site are aspartic acid

(Asp120), cysteine (Cys221), and histidine (His263) (8). There is only one zinc ion in the active site of the *A. hydrophila* enzyme (subclass B2, panel B), and two in the active site of the *S. maltophilia* enzyme (subclass B3, panel C).

Differences in assay methodology between workers make it difficult to compare hydrolytic efficiencies for different MBLs. Variation within e.g. the VIM, IMP, SPM and GIM family appears largely inconsequential (36). Nevertheless, subtle but important evolution may be ongoing, as illustrated in the NDM family. Here, experimental data do not define major differences in the catalytic efficiencies among NDM -1, -3, -4, -5, -6, -7 and -8 enzymes (37) under standard conditions, but differences are seen under zinc deprivation. Thus, studies comparing NDM-1, NDM-4 (Met154Leu) and NDM-12 (Met154Leu, Gly222Asp) demonstrate that the Met154Leu substitution, present in 50% of clinical NDM variants in some locales, enhances the ability to confer resistance at low $Zn^{++}$ concentrations (38, 39). This is potentially important because, as discussed later, zinc is restricted in infection (40) and its scarcity may impede the ability of classical NDM-1 enzyme to confer clinical resistance. NDM variants that have increased affinity for zinc (up to ~10-fold decreased $K_{d,\,Zn2}$) display selective advantages in experiments that mimic zinc scarcity imposed by the host immune system (41). Perhaps driven by similar pressures, the NDM-15 variant has evolved to function efficiently as a mono- rather than a bi-zinc enzyme (41). In addition, there are suggestions that NDM enzymes are evolving to develop greater thermodynamic stability (37).

## Epidemiology and distribution of acquired MBLs

Bacteria with IMP, VIM and NDM enzymes have been identified in a range of community, hospital, and environmental settings (42). Their prevalence, and importance relative to serine carbapenemases varies greatly by country.

156

157 Indian Subcontinent, Asia and Russia. The greatest burden of acquired, plasmid-mediated,
158 MBLs lies in south and south-east Asia (43), where NDM types are prevalent. As already noted,
159 *bla*$_{NDM-1}$ was first identified in bacteria isolated in 2008 from a patient who had travelled to
160 Sweden from India (44). NDM variants have subsequently been spread worldwide via patient
161 transfers and travel (45). Epidemiological surveillance has confirmed that NDM-1 and its
162 variants are widely disseminated throughout India, Pakistan and Bangladesh (46, 47);
163 moreover, a review of 39 carbapenem-resistant Enterobacterales (CRE) collected in India in
164 2006-2007 by the SENTRY Antimicrobial Surveillance Program found that 15 harboured *bla*$_{NDM-1}$
165 (48), indicating that it was circulating prior to its 'discovery' in 2008. Enterobacterales with
166 *bla*$_{NDM}$ were isolated from public tap water in India (49) and in river systems around pilgrimage
167 sites (42) demonstrating the gene has become established beyond healthcare environments.

168 In India there is frequent co-carriage with other carbapenemases in Enterobacterales
169 (50); thus, in 2012, among 113 non-clonal CRE isolates at a Mumbai hospital, 106 produced
170 NDM enzymes and 21 of these also have a second carbapenemase, most often an OXA-48-like
171 (n=17) or VIM-type (n=4). Surprisingly, given that most international reports of NDM enzymes
172 relate to Enterobacterales, *P. aeruginosa* was the most common MBL host (24%) among 3414
173 carbapenem-resistant gram-negative bacteria collected from community and hospital settings
174 in North India (51), with *bla*$_{NDM-1}$ (36%) the most prevalent carbapenemase gene followed by
175 *bla*$_{VIM}$ (18.4%).

176 Although KPC is the principal carbapenemase among Enterobacterales (CPE) in China,
177 a survey across 25 provinces showed that 32% of phenotypic carbapenem resistance in
178 Enterobacterales was linked to *bla*$_{NDM-1}$ (52) whilst a study (2012-16) of clinical *Enterobacter*
179 *cloacae* across three tertiary hospitals found *bla*$_{NDM-1}$ to be the most common carbapenemase

gene (80%), followed by *bla*$_{\text{IMP-26}}$ (8%) and *bla*$_{\text{IMP-4}}$ (6%) (53). The importance of IMP MBLs, particularly IMP-4, in China has been underscored by others; thus, multiple Enterobacterales species carrying a plasmid encoding IMP-4 enzyme were identified from patients with epidemiological links to China (54), and surveillance at a Beijing hospital highlighted both IMP-4 and NDM-1 in *K. pneumoniae* (55). Co-localisation of *bla*$_{\text{NDM-9}}$ and the plasmid-mediated colistin resistance gene *mcr-1* was seen in an *E. coli* strain recovered from retail chicken meat in Guangzhou, China (56). Having been recognized 30 years ago in Japan, IMP-type enzymes are now endemic there, though not highly prevalent (57).

NDM MBLs are the second-most-prevalent carbapenemases after OXA-48 in the Middle East, excepting Israel (58, 59). This probably reflects extensive interactions with the Indian subcontinent. As in India, there is significant penetration of *bla*$_{\text{NDM}}$ into *P. aeruginosa*, where a much greater proportion of carbapenem resistance appears to be carbapenemase-mediated than in Europe or the USA. Thus, in the Gulf Cooperation Council countries, *bla*$_{\text{VIM}}$ was found in 39% of carbapenem-resistant *P. aeruginosa* isolates (60), with most hosts belonging to internationally-disseminated high risk clones, including ST235, ST111, ST233, ST654 and ST357 (60). These lineages seem unusually adept at acquiring extrinsic resistance genes. In Dubai, 32% of resistant *P. aeruginosa* isolates produced VIM-type MBLs (61), though a larger proportion had outer membrane impermeability.

The proportion of carbapenem-resistant *P. aeruginosa* harboring MBLs in Russia rose from 4.5% between 2002-04 to 28.7% between 2008-10 (62), largely reflecting the spread of an XDR *bla*$_{\text{VIM-2-}}$positive ST235 high-risk clone, also present in Belarus and Kazakhstan (62). NDM is reported as the predominant carbapenemase among Enterobacterales in St Petersburg (63, 64), whereas OXA-48 is predominant in Moscow (65).

**Europe**. Although Italy had earlier reported both IMP and VIM enzymes (66), Greece was the first European country to report extensive dissemination of Enterobacterales with MBLs. Specifically , *K. pneumoniae* with VIM carbapenemases were reported from multiple hospitals in 2003–7, and multi-locus sequence typing identified three major clonal complexes (CCs); CC147, CC18 and CC14 among the producers (67). By 2006, 20% of *K. pneumoniae* isolates collected from hospital wards and 50% of those from ICUs monitored by the Greek System for the Surveillance of Antimicrobial Resistance were carbapenem-resistant, largely owing to the spread of the $bla_{\text{VIM-1}}$ cassette (68). By 2010, KPC had displaced VIM to become the dominant carbapenemase in Greece, largely through the spread of a *K. pneumoniae* ST258 variant (69). Nonetheless, VIM-types remained scattered, and may now be re-emerging due to suppression of the KPC carbapenemases via the use of ceftazidime-avibactam (70).

Elsewhere in Europe concern about carbapenemases grew following a flurry of press interest in NDM enzymes from 2008-10, and with the spread of *K. pneumoniae* ST258/512 lineages with KPC carbapenemases in Italy from 2010. The UK, taken as an exemplar, recorded a few *P. aeruginosa* and Enterobacterales with IMP and VIM MBLs before 2008. Thereafter, Enterobacterales with NDM enzymes increased (46). Most early cases were imports via patients who had travelled to (and often been hospitalized in) the Indian sub-continent. Multiple NDM variants have subsequently been reported in the UK, with NDM-1 the most frequent among Enterobacterales, followed by NDM-5 and NDM-7 (71). In contrast, VIM variants account for 91% of the (uncommon) MBLs in *P. aeruginosa*, again associated with international high-risk clones ST235, ST111, ST233 and ST357 (72).

While referral of CPE isolates to the national reference laboratory has increased 100-fold since 2008, many producers are from screening rather than clinical samples. OXA-48 is now the fastest-spreading carbapenemase but isolates with NDM enzymes account for 20-25%

228 of CPE submitted. A growing minority of these, particularly *E. coli*, have both NDM- and OXA-
229 48-like enzymes (71, 73).

230 In 2012, the European Centre for Disease Prevention and Control launched its
231 'European Survey of Carbapenemase-Producing Enterobacteriaceae (EuSCAPE)' project. The
232 geographic distribution of enzyme types were estimated by national experts across 38
233 European countries in 2015 (74). A random sample of carbapenem-susceptible and -non-
234 susceptible *K. pneumoniae* and *E. coli* subsequently were collected prospectively to determine
235 the occurrence of carbapenemases (75). The results, published in 2017, revealed SBLs (KPC or
236 OXA-48 enzymes) were more prevalent than MBLs in most countries but that MBLs were
237 widely scattered and were the most prevalent carbapenemases among Enterobacterales in a
238 few countries. Thus, VIM enzymes were the dominant carbapenemases in Hungary and NDM
239 in Serbia and Montenegro. The prevalence of NDM enzymes in the latter countries tallies with
240 early descriptions of producers linked to these Balkan states. It is unclear whether these
241 originated as imports from India or as independent local gene escapes from the unknown
242 source organism (76).

243

244 North America. Infections due to Enterobacterales carrying $bla_{\text{VIM-2}}$, $bla_{\text{VIM-7}}$, $bla_{\text{IMP-4}}$ and $bla_{\text{IMP-18}}$ genes were recorded in the USA prior to 2005 but, in general, MBLs remained extremely rare
246 (1, 77). In 2010, Enterobacterales harboring NDM-1 were isolated from three patients in
247 different states (78) and, as with many contemporaneous cases in the UK and elsewhere, the
248 source patients had all recently been in India or Pakistan (21). Subsequent expansion of NDM
249 enzymes in the US has been less marked than the UK, with KPC carbapenemases becoming
250 considerably more prevalent. Nevertheless, up to December 2017, 379 CPE with NDM

carbapenemases were reported to the CDC from 34 States, with just under a third (109) from Illinois (79), where an outbreak was associated with contaminated endoscopes.

Enterobacterales with NDM enzymes have been increasing in Canada since 2008 and these MBLs are now the second-most-common carbapenemases in the country, with a higher prevalence in the Western Provinces (80). Surveillance conducted between 2007-2015 in Toronto revealed that, among 291 clinical CPE, 51% had NDM enzymes, and 24% of these patients had never received healthcare abroad nor travelled to high-risk areas (81), suggesting the enzymes are established locally. In 2019 a novel MBL, *bla*$_{\text{CAM-1}}$, was identified from isolates that were collected in 2007 (82). No subsequent isolates harboring this gene have been reported.

Africa. Paucity of data means the prevalence of CPE carrying MBLs in Africa is difficult to estimate. Apparent infrequency may reflect true rarity, limited sampling, or a lack of infrastructure for accurate detection. CPE with VIM MBLs nonetheless have been identified in Nigeria, Morocco, Algeria, Tunisia, Tanzania, and South Africa; and those with NDM enzymes in Kenya, Nigeria, Morocco, Algeria, Tunisia, Tanzania, and South Africa (83, 84). Infections caused by Enterobacterales producing MBLs are reported from both imported and local cases, raising concerns regarding emerging endemicity (85). Those with IMP-type enzymes have been identified in small numbers in Morocco, Tunisia, and Tanzania, and appear genuinely uncommon (84). An outbreak caused by *Klebsiella* spp. carrying *bla*$_{\text{NDM-5}}$ was reported from a neonatal unit in Nigeria (86). A concern is that African patients are strongly represented in medical tourism to India, which is a risk factor for colonisation with Enterobacterales producing MBLs (87).

**Rest of the world**. KPC enzymes dominate among carbapenemases from Enterobacterales in Latin America, with (unusually) some penetration also into *P. aeruginosa*. Nonetheless, Enterobacterales with NDM enzymes are endemic in Brazil, with several outbreaks reported (88). Early case reports of MBL-producing Enterobacterales in Latin America often concerned Proteeae, including *Providencia* spp. and *Morganella* (89, 90), which are infrequent hosts of $bla_{NDM}$ elsewhere. This creates a treatment issue since these genera are inherently resistant to polymyxins and newer-generation tetracyclines, which remain options against other MBL-producing Enterobacterales (below).

Unique to South America is the wide distribution in Brazil of *P. aeruginosa* with SPM-1 MBL (91), principally associated with an ST277 clone. Outcomes of severe infections with this clone are often poor, reflecting a lack of good treatment options (92).

Carbapenemases are rare in Australasia, but there is spread of $bla_{IMP-4}$ among Enterobacterales (93), as in parts of China. *E. cloacae* is a major host, with dissemination mediated by an IncHI2 plasmid (94). Production of IMP-4 enzyme has also been recorded in *Salmonella* spp. from domestic pets (95) and seagulls (96), but the significance of this is uncertain.

## MBL function in resistance, *in vitro* and *in vivo*

For many years MBLs were perceived as clinically unimportant chromosomally-encoded enzymes from non-pathogenic organisms, notably *Bacillus cereus* (97, 98). This perception changed with recognition that MBLs confer much of the resistance seen in *Chryseobacterium* spp. and *E. meningoseptica* (99) and with heightened awareness of the morbidity and mortality associated with *S. maltophilia* bacteraemia (100, 101). Interest then escalated with the

298 discovery and proliferation of acquired MBLs, especially NDM-1, which drew extensive press
299 coverage in 2010.

300 Many MBL producers are broadly resistant *in vitro* and, on this basis, real concern exists
301 about lack of treatments. On the other hand, there is evidence that *in vivo* resistance to
302 carbapenems may be less than it appears *in vitro*, because susceptibility tests are
303 conventionally done in media (e.g. cation-adjusted Mueller-Hinton broth) with high zinc
304 concentrations (102), whereas the host immune system imposes a state of zinc deprivation in
305 infection (40, 103). This lack of zinc may not only impede the catalytic function of MBLs but
306 may also interfere with their protein folding (102) and may promote degradation of the
307 enzyme in the periplasm (104).

308 Several preclinical studies suggest a disconnect between high-level *in-vitro* resistance
309 to carbapenems associated with NDM-1 enzymes, but a weak ability to protect against
310 carbapenems in standard murine infection models (105). Moreover, NDM enzymes appear less
311 effective than other carbapenemases in causing resistance to carbapenems in patients (106,
312 107). Thus, mortality in severe infections due to Enterobacterales with *bla*$_{NDM}$ appears
313 relatively low, ranging from 13% (108) - 55% (109), when compared to that seen with bacteria
314 expressing other MBLs (18% to 67%) (13), or KPC carbapenemases (41% to 65%) (110, 111).
315 Good clinical outcomes have been reported despite treatment with agents to which NDM
316 enzymes confer resistance *in vitro* (106, 107, 112). As yet, there are no studies that confirm or
317 refute whether the higher numbered NDM alleles, encoding variants with their greater affinity
318 for zinc (above), are better able to cause clinical resistance than NDM-1 (39, 41).

319 Finally, it should be underscored that whilst these indications that NDM MBLs are less
320 potent *in vivo* are intriguing, they should be approached with caution. Double-blinded
321 randomized-controlled trials have not been conducted, and existing outcome data are subject

to various biases (113, 114). For VIM MBLs, clinical outcomes correlate with carbapenem MICs, implying little or no such *in vitro/in vivo* discordance (115).

## Current treatment options

Limited data exist to inform clinicians on the optimal treatment for infections caused by MBL-producing gram-negative bacteria (106). Co-trimoxazole remains the standard of care for infections due to *S. maltophilia,* but most Enterobacterales with acquired MBLs also have *sul* and *dfr* genes, conferring resistance. Resistances to fluoroquinolones and aminoglycosides are often present alongside genes encoding acquired MBLs. In particular, $bla_{NDM}$ genes are often linked to the genes encoding ArmA or RmtB methyltransferases, which modify ribosomes to block binding of aminoglycosides, including plazomicin; $bla_{IMP}$ and $bla_{VIM}$ generally occur within integrons that often also carry *aac(6')*, encoding an acetyltransferase that compromises amikacin and tobramycin, though not gentamicin or plazomicin (116). A thorough review of treatment options for MDR and XDR Enterobacterales is available (117). This highlights observational studies comparing monotherapy to combination therapy for bloodstream infection (BSI) involving CRE, although few of these were specifically identified as having MBLs (118, 119).

**Colistin**. Colistin is the current mainstay of treatment for infections due to MBL-producers. A multinational survey of MBL-producing Enterobacterales and *P. aeruginosa* conducted from 2012-2014 found >97% susceptibility among MBL-producing *P. aeruginosa* (variously with IMP-, VIM- and NDM- enzymes), and >85% for MBL-producing Enterobacterales (>86.1% NDM-type, >88.9% IMP-type >88.9% IMP-type) (83). Exceptions are *Proteeae* and *Serratia* spp., which have intrinsic polymyxin resistance.

346 For bacteria harboring KPC and OXA-48 carbapenemases, colistin has recently been
347 shown less effective than microbiologically-active β-lactamases inhibitor combinations (120),
348 making it plausible that an active β-lactam likewise would be more efficacious than colistin
349 against MBL producers. Of note, the emergence of colistin resistance during treatment, with
350 secondary transmission of resistant variants is a concern (121, 122).
351
352 Tigecycline, omadacycline and eravacycline. These tetracyclines have strong *in vitro* activity
353 against many MBL-producing Enterobacterales, except *Proteeae*, although not against *P.*
354 *aeruginosa*. During November 2018, 275 unique Enterobacterales isolates carrying $bla_{NDM}$
355 collected by the US Centers for Disease Control were tested with tigecycline (86.5%
356 susceptible, based on a ≤2 μg/ml FDA breakpoint), eravacycline (66.2% susceptible, based on
357 a ≤0.5 μg/ml FDA breakpoint) and omadacycline (59.6% susceptible, based on a ≤4 μg/ml
358 breakpoint) (123). The higher susceptibility rate for tigecycline than eravacycline reflects the
359 higher FDA breakpoint for Enterobacterales; in Europe both agents have an identical 0.5 μg/ml
360 breakpoint and eravacycline is the more active on a simple gravimetric basis, though it is
361 unclear whether this confers clinical advantage (124). Merits of omadacycline are its minimal
362 known drug interactions and that it can be administered orally (125), however, it has the least
363 relevant license (for community-acquired bacterial pneumonia and acute bacterial skin and
364 skin structure infections) in relation to the clinical burden of MBL producers.
365 Whilst the *in vitro* activity of these tetracyclines is encouraging, there are multiple
366 caveats. First, tigecycline carries an FDA 'black box' warning of increased mortality when the
367 drug was used as monotherapy (126); second, both tigecycline and eravacycline have failed to
368 achieve non-inferiority to comparators in one or more clinical trials (VAP and diabetic foot
369 infection for tigecycline, cUTI for eravacycline); third, there is little provenance for tetracyclines

as monotherapy in the severely-ill patients who commonly develop infections due to MBL-producing opportunists; fourth, particularly for tigecycline, the disparity between EUCAST (S ≤0.5 μg/ml) and FDA (S ≤2 μg/ml) breakpoints creates categorization uncertainty; last, the lack of anti-*Proteeae* activity is important in Latin America, where *Providencia* spp. are frequent hosts of $bla_{NDM}$ (127). Given these uncertainties, the best advice is to consider these tetracyclines in combination against MBL producers, not as monotherapy.

Aztreonam. Aztreonam is stable to MBLs, though activity is lost against organisms that co-produce ESBLs or AmpC enzymes (128), which are common in MBL-producing Enterobacterales. Clinical experience as monotherapy is lacking for MBL producers, although some success has been recorded when aztreonam was used in combination with ceftazidime-avibactam (129, 130), with avibactam serving to inhibit ESBLs. Six out of ten patients survived following treatment with this combination during an outbreak of *K. pneumoniae* with NDM-1, OXA-48, and CTX-15 β-lactamases in Barcelona (129). Although no adverse events were reported, the safety is unclear, and it is difficult to match the 1.5g +0.5g q6h regimen of aztreonam-avibactam that is presently being developed (below).

Fosfomycin. Fosfomycin commonly retains full *in vitro* activity against MBL-producing Enterobacterales, and has been successful trialed, very recently, as an IV agent in cUTI (131). It may be an option against MBL producers - particularly *E. coli*, which is more susceptible than other Enterobacterales - but it is mainly advocated for use in combination due to concerns about emergence of resistance, particularly in *Klebsiella* spp. (132). Fosfomycin has little direct antipseudomonal activity, with typical MICs above breakpoints. However, *in vitro* synergy is

393 seen when fosfomycin is combined with meropenem against MBL-producing *P. aeruginosa*
394 strains (133), suggesting a need for *in-vivo* exploration.
395
396 ## Development Pipeline
397 The development pipeline represents four main strategies against MBL producers: (i)
398 protection of MBL-stable-monobactams from other co-produced β-lactamases, as e.g. with
399 aztreonam-avibactam; (ii) development of β-lactams stable to MBLs as well as SBLs, as with
400 e.g. cefiderocol and BOS-228, (iii) combinations of cephalosporins and carbapenems with
401 triple-action diazabicyclooctanes (DBOs), and (iv) direct inhibition of MBLs with cyclic
402 boronates, thiols, chelators, dicarboxylic acids, and other agents.
403
404 ## Aztreonam-avibactam
405 Aztreonam-avibactam is the first antibiotic to be developed under a public-private partnership
406 agreement (134, 135), with partial finance from the European Union's Innovative Medicine's
407 Initiative and, latterly, also the US Biomedical Advanced Research and Developmental
408 Authority (BARDA). A prospective randomized phase 3 study (NCT03580044) begins in 2020
409 to determine efficacy, safety, and tolerability versus best available therapy (BAT) for
410 hospitalized adults with complicated intra-abdominal infections (cIAI), nosocomial pneumonia
411 (NP), complicated UTI, or BSI due to MBL-producing gram-negative bacteria (135).
412 Aztreonam evades hydrolysis by MBLs (128) but is compromised by the ESBL and AmpC
413 enzymes that are co-produced by many MBL-positive CPE. These SBLs are inhibited by
414 avibactam, a diazabicyclooctane (DBO) (136, 137) and, consequently, MBL-producing
415 Enterobacterales that also carry ESBLs or AmpC are susceptible to aztreonam-avibactam *in*

416 *vitro* (138) and *in vivo* (139). The combination is less reliably active against MBL-producing *P.*
417 *aeruginosa* (140), because aztreonam has weak anti-pseudomonal activity.

418 Considerable interest exists, because the safety and efficacy of aztreonam are well
419 established, and because avibactam was established to be effective at inactivating ESBLs and
420 AmpC enzymes during trials with ceftazidime. Moreover, case reports suggest success against
421 infections caused by MBL producers when aztreonam was co-administered with ceftazidime-
422 avibactam (see aztreonam section above) (129, 130).

423

424 MBL-stable β-lactams

425 **Cefiderocol (S-649266)**. Cefiderocol (S-649266) is a novel parenteral siderophore
426 cephalosporin designed by Shionogi & Co. Ltd., with a catechol linked to its 3-position side
427 chain. It is licensed in the USA for cUTI and in the EU and UK for 'treatment of infections due
428 to aerobic gram-negative organisms in adults with limited treatment options' (141). It is
429 retained among developmental agents here, rather than being included in the established
430 treatments, because there is little published experience with MBL producers to date (142,
431 143).

432 Critically, the catechol moiety forms a chelation complex with ferric iron and this
433 complex is actively accumulated by gram-negative bacteria, which are forced to scavenge this
434 essential element (144). Cefiderocol has good activity *in vitro* under iron starvation, against
435 gram-negative bacteria, including CPE, *P. aeruginosa* and *A. baumannii* (145). It is relatively
436 stable to both SBLs and MBLs (144), however, the MICs for Enterobacterales and non-
437 fermenters with NDM carbapenemases tend to be slightly higher than those for isolates of the
438 same species with other carbapenemase types (146). Cefiderocol proved effective against
439 carbapenem-resistant *P. aeruginosa* (expressing IMP-1 enzymes), *A. baumannii* (expressing

OXA-51-like enzymes) and *K. pneumoniae* (expressing NDM-1 enzymes) in immunocompetent rat respiratory tract infection models, achieving a ≥3-log reduction in the number of viable bacteria in the lungs when dosed over 4 days so as to recreate the human exposures of a 2g q8h 3h-IV infusion regimen (147). Efficacy reduced when the infusion time was reduced to 1h, owing to a lower percentage of the dosing interval during which free-drug concentrations were above the MIC (% $T_f$ >MIC) (147). Interestingly, the mean % $T_f$ >MIC required for a 1-$\log_{10}$ reduction was 18-24% greater for *A. baumannii* isolates (expressing OXA-23 or OXA-24) in the murine lung infection model than for Enterobacterales expressing NDM-1, NDM-4 or KPC-2 enzymes and for *P. aeruginosa* isolates expressing IMP-1 or VIM-10 MBLs (148).

In humans, the 2g IV q8h 3h-infusion regimen provided >90% probability of target attainment (PTA) with 75% $T_f$>MIC for MICs of ≤4μg/ml for patients with normal renal function (149). A phase 3 trial (NCT03032380) has shown non-inferiority to meropenem in nosocomial pneumonia (150). Less encouragingly, another trial (NCT02714595), found excess deaths in the cefiderocol arm, compared with 'best available therapy,' for patients with severe infections caused by carbapenem-resistant gram-negative pathogens (151). Full analysis is awaited but, notably, deaths were mostly associated with *Acinetobacter* infections (152), not Enterobacterales.

BOS-228 (formerly LYS228). BOS-228 is a monobactam and, like aztreonam, is stable to MBLs (153). Unlike aztreonam, it is also stable to many potent SBLs, including carbapenemases, ESBLs, and AmpC types (154); moreover it binds strongly to PBPs1a and 1b of Enterobacterales as well as to PBP3, which is the sole target of aztreonam (155). BOS-228 had an $MIC_{90}$ of 2 μg/ml for a clinical panel of 88 Enterobacterales isolates expressing ESBLs, KPCs and MBLs (153) and no single-step mutants were selected from 12 β-lactamase-expressing

Enterobacterales exposed to the drug at 8 x MIC, though mutants were selected from 2/12 strains, neither of which expressed MBLs, at 4 x MIC (155).

A randomized evaluator-blinded multi-center phase 2 trial (NCT03354754) to evaluate pharmacokinetics, clinical responses, safety, and tolerability of BOS-228 in cIAI commenced in 2018. The drug is being administered as IV monotherapy (without metronidazole) q6h for at least 5 days and compared to standard of care, with outcomes evaluated at day 28. A randomized controlled evaluator-blinded multi-center trial (NCT03377426) in cUTI has also been initiated.

### Cephalosporins or carbapenems combined with triple-action DBOs

**Zidebactam and nacubactam.** Unlike with cyclic boronates (see below), it has not been possible to discover DBOs that directly inhibit MBLs. However, nacubactam and zidebactam are DBO analogs that combine inhibition of SBLs with direct antibacterial activity by inhibiting PBP2 (156). When combined with PBP3-targetted β-lactams, this attack on PBP2 leads to an 'enhancer' effect, with further β-lactamase-inhibition-independent synergy observed (156, 157). Consequently, cefepime-zidebactam and cefepime- or meropenem- nacubactam combinations are active *in vitro* against >75% of MBL-producing Enterobacterales and, in cefepime-zidebactam's case, also against many MBL-producing *P. aeruginosa* (158).

Although the direct antibacterial activity of nacubactam and zidebactam is readily lost via mutations compensating for inhibition of PBP2 (159), the enhancer effect is retained, with many of the mutants consequently remaining susceptible to e.g. cefepime-zidebactam or meropenem-nacubactam at low concentrations (156, 157). Cefepime-zidebactam is currently the most advanced of these combinations, with a phase 3 trial due to commence (160).

Direct inhibitors of MBLs.

Cyclic boronates - VNRX-5133 (taniborbactam) and QPX7228. Inhibitors that target both SBLs and MBLs are of great interest but have proved difficult to obtain owing to structural and functional differences between and among these enzymes. This combination of inhibitory activities nonetheless has recently been achieved with several cyclic boronates, notably taniborbactam and QPX7228. These mimic the tetrahedral anionic intermediate common to SBL and MBL catalysis (161) and additionally inhibit some penicillin-binding proteins (e.g. PBP-5, which is non-essential) by the same mechanism (162). They represent a considerable expansion in spectrum over vaborbactam, their progenitor, which inhibits only few class A β-lactamases, notably KPC types (163).

Taniborbactam (VenatoRx) is the more advanced of these two 'second-generation' boronates, and is in Phase III trials combined with cefepime (164). It irreversibly inhibits class A, C, and D SBLs, and is a reversible competitive inhibitor of VIM and NDM MBLs, though not of IMP types (165). Safety has been established in healthy volunteers (NCT02955459), and the FDA has allowed cefepime-taniborbactam to proceed via fast track pathway for the clinical indications of cUTI and cIAI. QPX7728 (QPEX) likewise inhibits both SBLs and MBLs: 50% inhibitory concentrations [$IC_{50}$], for KPC enzymes are around 2.9 ± 0.4 nM, compared with 22 ± 8 nM for the class C cephalosporinase of *E. cloacae* P99, 55 ± 25 nM for the NDM-1 MBL and 14 ± 4 nM for VIM-1 enzyme. As with taniborbactam, the $IC_{50}$ for IMP-1 enzyme is considerably higher, at 610 ± 70 nM) (166). An IV combination of QPX7728 with meropenem is being explored. This significantly lowered bacterial counts in murine thigh and lung infection models with carbapenem-resistant *K. pneumoniae*, *P. aeruginosa* and *A. baumannii* when compared to meropenem alone, although strain genotypes were not reported. Unlike taniborbactam,

511 QPX7228 is orally bioavailable and combinations with ceftibuten and tebipenem were
512 evaluated *in vitro* against CPE, including those with MBLs (167).
513
514 Thiol-containing MBL inhibitors and chelating agents. Small molecules that bind and/or
515 chelate zinc ions include thiols, dicarboxylates, hydroxamates, and tetrazoles; these are widely
516 reported to inhibit MBLs, but human metallo-proteases are vulnerable too, so toxicity may
517 preclude clinical development.
518 Thiol-containing compounds inhibit all MBL subtypes (B1, B2 and B3) (168), with strong
519 competitive inhibition of IMP-1 by thioester derivatives first reported in 1999 (169). The
520 dipeptide L-captopril deserves mention in context. It is used as an ACE inhibitor in the
521 treatment of hypertension and is reported also to inhibit MBLs by chelating the active site zinc
522 ions via its thiol group (170); the corresponding D- stereoisomer is a more potent inhibitor and
523 can potentiate meropenem against strains with VIM-2 MBLs (170). Both captopril isomers act
524 via zinc chelation and repurposing is attractive given the known safety of the L-isomer at its
525 licensed dose; however the economic model for development is yet to be established and
526 safety issues for the D-isomer need exploration. Other thio-carbonyl compounds, such as
527 thiomandelic acid, exhibit synergy with meropenem against Enterobacterales with VIM, NDM,
528 and IMP enzymes (171).
529 Bisthiazolidines are carboxylate-containing bicyclic compounds, considered to be
530 penicillin analogs that inhibit MBLs through a zinc-bridging thiol group and a carboxylate that
531 interacts with K224 (172). The orientation of the carboxylate and thiol moieties create diverse
532 binding that is observed on X-ray crystal structures and has been shown to inhibit all MBL types
533 (173). The bisthiazolidine scaffold inhibits NDM-1 enzymes *in vitro*, with $K_i$ values in the low

534 micromolar range (from 7 ± 1 to 19 ±3 μM); they restore imipenem activity against *E. coli*
535 producing NDM-1 (172).

536 The divalent cation chelator EDTA has raised interest, too, both as an inhibitor of MBLs,
537 and also because it disrupts the gram-negative outer membrane and neutralizes various
538 bacterial enzymes and toxins (174, 175). It is widely used in identification tests for MBLs.
539 Sodium calcium EDTA, which is licensed for use for treatment of lead poisoning, reportedly
540 restored imipenem's activity *in vivo* against *P. aeruginosa* producing IMP- and VIM- enzymes
541 and against *E. coli* producing NDM-1 enzyme (176, 177), raising the issue of whether it might
542 be used to potentiate carbapenems in human infections. Elores®, which is marketed in India,
543 combines ceftriaxone, sulbactam and EDTA (178, 179) and reportedly achieved cures of
544 infections due to MBL producers in multiple patients, with no serious adverse events (178).
545 However, prospective and controlled studies are lacking, the dose of EDTA is low, and there
546 remains uncertainty (above) about the function of NDM-1 enzyme *in vivo*. More negatively,
547 the FDA has placed strict limits on the amount of EDTA permissible even in food (180) and
548 sodium calcium EDTA is capable of producing toxic effects that can be fatal (181). High
549 concentrations of EDTA are likely to strip divalent cations from human metalloenzymes,
550 including matrix metalloproteinases, carbonic anhydrase and carboxypeptidases, thus limiting
551 clinical applicability.

552 Aspergillomarasmine A (AMA) is a fungal natural product discovered in the 1960s (182),
553 and re-evaluated in the 1980s as an inhibitor of the human metalloproteinase angiotensin-
554 converting enzyme (ACE). AMA inhibits MBLs via a metal ion sequestration mechanism and
555 displays rapid and potent inhibition of NDM-1 and VIM-2 enzymes *in vitro* (183). It restored
556 the activity of meropenem against a *K. pneumoniae* strain expressing NDM-1 enzyme in an

intraperitoneal murine infection model (184). Again, the hazard of inhibiting human metallo-enzymes requires careful investigation.

## Challenges for the development of inhibitors of MBLs

One of the biggest challenges in designing MBL inhibitors is the diversity among these enzymes, which share less than one third sequence identity at their active sites. Thus, for example, taniborbactam and QPX7728 target NDM and VIM enzymes, but not IMP types (185). Development of inhibitors that bind remotely from the active site might overcome this limitation, but possible target areas also vary within class B1 and seem even better able to tolerate mutations than the active site (29). Another challenge is the shallow binding site in B1 enzymes, meaning that inhibitors can only make limited interactions (29). Specificity for bacterial MBLs is a further recurring challenge; interactions with human metallo-enzymes and contingent toxicity are major concerns. Molecules that solely inhibit MBLs are limited by the fact that many MBL producers also co-produce SBLs, including carbapenemases, meaning that the partner β-lactam must evade these enzymes, that the inhibitor must inactivate both MBLs and SBLs, or that a second inhibitor is required.

Preclinical development is challenging, too, because it is difficult to establish reliable animal models in which MBL-mediated resistance is expressed, perhaps owing to the already-mentioned lack of essential zinc at infection sites. Moreover bacteria are prone to lose MBL-encoding plasmids, or fail to reliably express them, in murine models, resulting in pharmacodynamic data that suggest meropenem susceptibility (186, 187). Consequently it is difficult to establish the efficacy of candidate MBL-stable drugs or inhibitor combinations. It is unclear if the same phenomena occur in patients (188), and this requires further research. Irrespective of this aspect, it is also challenging to find and recruit the required number of

patients with MBL-producing pathogens to clinical trials. Rapid diagnostics should help, but their use is complicated by cost and the need to deploy them to all trial sites, including in countries where they are not licensed or are licensed only to inform infection control, not treatment.

## Conclusion

MBLs are disseminating internationally, particularly in Asia, and often are produced by gram-negative bacteria with extremely broad spectra of *in vitro* resistance. Unlike for KPC and OXA-48-like carbapenemases, producers are typically not susceptible to recently licensed β-lactamase inhibitor combinations such as ceftazidime-avibactam, meropenem-vaborbactam, imipenem-relebactam, although cefiderocol may be a potential answer. The ability of MBLs to confer resistance to carbapenems may not be so great *in vivo* as *in vitro*, though this is uncertain and may vary by enzyme type even within MBL subclasses.

Inhibitors are known, and the developmental boronates, taniborbactam and QPX7728 are of particular interest. Nonetheless, the quest for effective inhibitors is complicated by differences in active site structure and zinc ligand interactions among MBLs, and by difficulties in the design of appropriate preclinical and clinical trials. Non-boronate inhibitors face toxicity issues, particularly if they interact with other metallo-enzymes or are general chelators. Other approaches to overcoming MBLs include, avibactam-protected aztreonam; stable β-lactam, notably BOS-228 as well as cefiderocol, and combinations of β-lactams with-triple action DBOs, notably cefepime-zidebactam and meropenem-nacubactam.

And *that* is the positive aspect on which to close: there is now a diverse and exciting pipeline of potential agents for the treatment of infections caused by bacteria that produce MBLs. It remains to be seen what will be the most effective of these agents.

## Transparency declaration.

DML: Advisory Boards or ad-hoc consultancy Accelerate, Allecra, Antabio, Centauri, Entasis, GlaxoSmithKline, J&J, Meiji, Melinta, Menarini, Mutabilis, Nordic, ParaPharm, Pfizer, QPEX, Roche, Sandoz, Shionogi, T.A.Z., Tetraphase, Venatorx, Wockhardt, Zambon, Paid lectures – Astellas, bioMérieux, Beckman Coulter, Cardiome, Cepheid, Merck/MSD, Menarini, Nordic, Pfizer and Shionogi. Relevant shareholdings or options – Dechra, GSK, Merck, Perkin Elmer, Pfizer, T.A.Z, amounting to <10% of portfolio value. William Hope holds or has recently held research grants with F2G, Astellas Pharma, Spero Therapeutics, Antabio, Allecra, Bugworks, and NAEJA-RGM. He holds awards from the Medical Research Council, National Institute of Health Research, FDA and the European Commission. David Hooper: consultancy for Selux Diagnostics, Day Zero Diagnostics, Wockhardt Pharmaceuticals and Shionogi Pharmaceuticals. William Hope has received personal fees in his capacity as a consultant for F2G, Amplyx, Ausperix, Spero Therapeutics, VenatoRx, Pfizer and BLC/TAZ.

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1284 Table 1 Examples of chromosomal and plasmid-associated MBLs (11)

1285

| Chromosomal MBLs | | | Plasmid-associated MBLs | |
|---|---|---|---|---|
| Species | Enzyme | Subclass | Enzyme | Subclass |
| *Bacillus cereus* | BcII | B1 | Verona integron-encoded (VIM) | B1 |
| *Chryseobacterium indologenes* | IND | B1 | New-Delhi metallo-β-lactamase (NDM) | B1 |
| *Elizabethkingia meningoseptica* | BlaB | B1 | Imipenemase (IMP) | B1 |
| *Myroides odoratimimus* | MUS/ MYO | B1 | Sao Paulo metallo-β-lactamase (SPM) | B1 |
| *Bacteriodes fragilis** | CfiA / CcrA | B1 | German imipenemase (GIM) | B1 |
| *Aeromonas* spp. | CphA | B2 | KHM | B1 |
| *Stenotrophomonas maltophilia* | L1 | B3 | Dutch imipenemase (DIM) | B1 |
| *Elizabethkingia meningoseptica* | GOB | B3 | Adelaide Imipenemase (AIM) | B1 |

1286

1287 *Unlike most other chromosomal MBLs, the *Bacteroides fragilis* enzyme is rare in the species

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1289

**Figure 1. Structure of amino acid residues in metallo-β-lactamase enzyme subclasses** (8) (Reproduced with permission from John Wiley and Sons Publishers, sourced from Palzkill T et al. 2013. Metallo-β-lactamase structure and function. Ann N Y Acad Sci 1277:91–104)

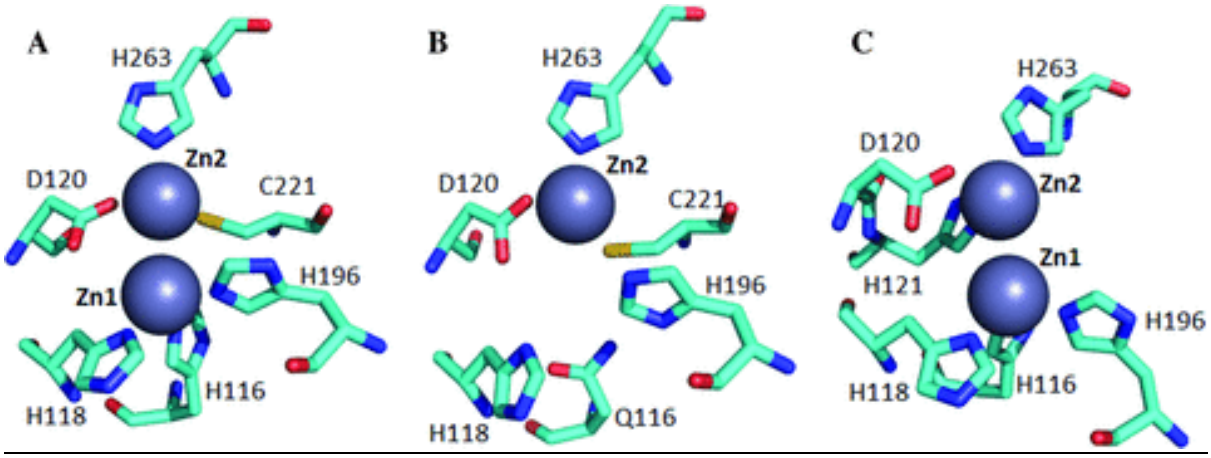
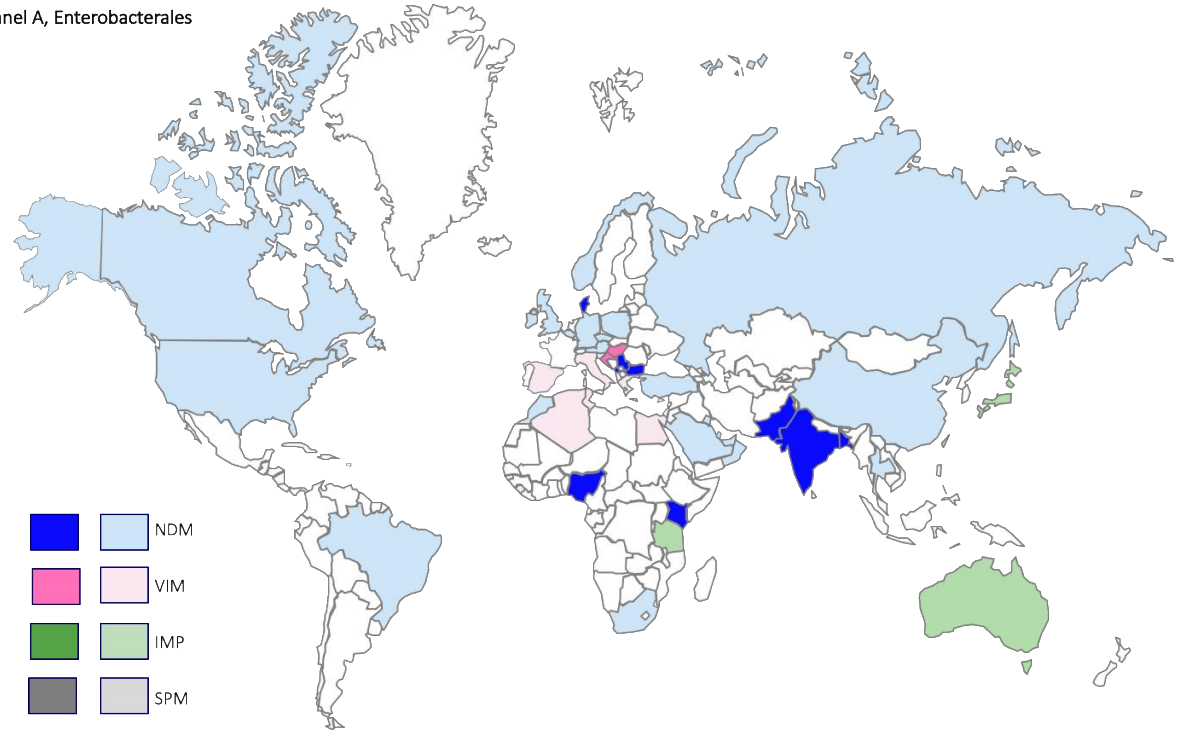



Figure 1 illustrates the amino acid residues that bind zinc at the active sites of B1, B2, and B3 MBLs. Crystal structures of B1 enzymes, including IMP-, VIM-, NDM-, and *B. fragilis* CcrA, (panel A) reveal two zinc-binding sites (Zn1 and Zn2). The Zn1 site contains three histidine residues (His116, His118, and His196), whereas the ligands for the Zn2 site are aspartic acid (Asp120), cysteine (Cys221), and histidine (His263). There is only one zinc ion in the active site of the *A. hydrophila* enzyme (subclass B2, panel B), and two in the active site of the *S. maltophilia* enzyme (subclass B3, panel C).

Figure 2. Global distribution of acquired metallo-β-lactamases

Panel A, Enterobacterales




Full-tone color is used when the indicated MBL is the most prevalent carbapenemase in the country. The lighter tone is used to indicate the most prevalent MBL group in countries where serine carbapenemases (KPC or OXA-48-like) are more prevalent. Panel A, Enterobacterales; Panel B, *P. aeruginosa*

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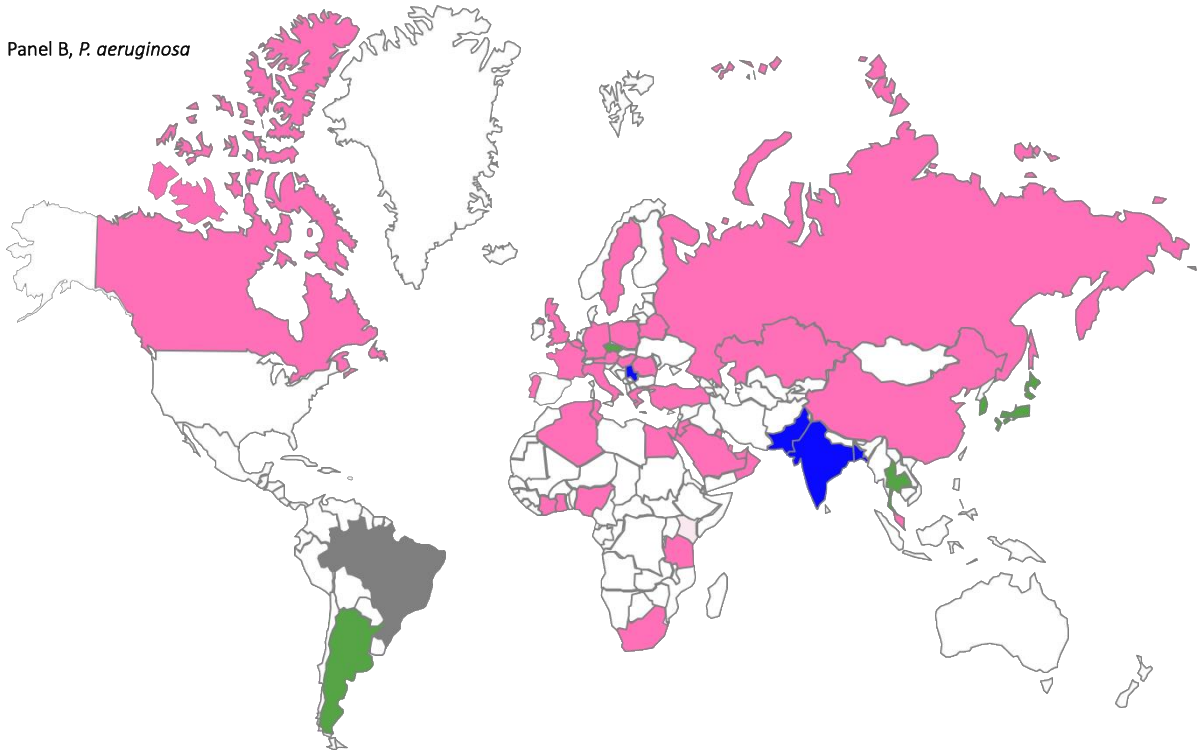
Panel B, *P. aeruginosa*



*In the USA there are just a few reports of *P. aeruginosa* with either IMP or VIM MBLs

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