The emergence and implications of metallo-β-lactamases in Gram-negative bacteria

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ABSTRACT

The increase in Gram-negative broad-spectrum antibiotic resistance is worrisome, particularly as there are few, if any, “pipeline” antimicrobial agents possessing suitable activity against Pseudomonas spp. or Acinetobacter spp. The increase in resistance will be further enhanced by the acquisition of metallo-β-lactamase (MBL) genes that can potentially confer broad-spectrum β-lactam resistance. These genes encode enzymes that can hydrolyse all classes of β-lactams and the activity of which cannot be neutralised by β-lactamase inhibitors. MBL genes are often associated with aminoglycoside resistant genes and thus bacteria that possess MBL genes are often co-resistant to aminoglycosides, further compromising therapeutic regimes. Both types of genes can be found as gene cassettes carried by integrons that in turn are embedded within transposons providing a highly ambulatory genetic element. The dissemination of MBL genes is typified by the spread of bla\textsubscript{VIM-2}, believed to originate from a Portuguese patient in 1995, and is now present in over 20 counties. The increase in international travel is likely to be a contributory factor for the ascendancy of mobile MBL genes as much as the mobility among individual bacteria. Fitness, acquisition and host dependency are key areas that need to be addressed to enhance our understanding of how antibiotic resistance spreads. There is also a pressing need for new, and hopefully novel, compounds active against pan-resistant Gram-negative bacteria – a growing problem that needs to be addressed by both government and industry.

Keywords Metallo-β-lactamase, Pseudomonas aeruginosa, gram-negative bacteria

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INTRODUCTION

The frequency of antibiotic resistance in Gram-negative bacteria is likely to continue to rise and, while new drugs such as linezolid and daptomycin can be employed against Gram-positive infections, new anti-Gram-negative drugs are conspicuous by their absence. Gram-negative resistance has probably now eclipsed Gram-positive resistance and has prompted similar calls for the implementation of infection control measures to negate the dissemination of these organisms [1]. A recent survey of Gram-negative resistance from North America showed that Pseudomonas aeruginosa has become increasingly resistant, with cefepime, imipenem and meropenem still maintaining good activity [2]. Global SENTRY data broadly support the previous study, in that P. aeruginosa showed moderate levels of resistance to cefepime (10%), imipenem (12%) and meropenem (10%) [3]. A longitudinal survey from the UK did not show an increase in P. aeruginosa resistance but did highlight a worrying and dramatic increase in resistance of Enterobacteriaceae and Acinetobacter spp. to cephalosporins, quinolones and aminoglycosides. The recent increase in Enterobacteriaceae acquiring the ‘newer’ extended-spectrum β-lactamase genes, e.g., bla\textsubscript{CTX-M}, bla\textsubscript{OXA} and bla\textsubscript{CMY}, while concurrently becoming resistant to quinolone antibiotics, is becoming a commonplace scenario [4]. While such studies have often been reported from ‘developed countries’, a similar trend in resistance is found elsewhere. A longitudinal study from India showed a dramatic increase in the resistance of P. aeruginosa to most anti-infectives, particularly ceftazidime (52–94%). The recent multinational neonatal infection survey by Zaidi et al. further highlights the increase in infections with and resistance in Gram-negative bacteria [5].
Treatment regimens for such bacteria are limited and may involve aminoglycosides and/or broader-spectrum β-lactams such as carbapenems. Resistance to carbapenems in Gram-negative bacteria may take on many guises, including permeability barriers and/or hydrolysis by β-lactamases. Recent studies have shown that outer-membrane mutations in Enterobacteriaceae and Acinetobacter spp. can singularly mediate low-level resistance to imipenem [6]. Enzymes mediating resistance to carbapenems can be divided into serine β-lactamases and those possessing a metal ion(s) at their active site. Serine carbapenemases include NmcA, Sme-type IMI-1, KPC type, GES type and OXA type [7–16]. Those that possess metal at their active site are termed metallo-β-lactamases (MBLs) and come from a disparate group of bacteria; these are classified as group 3 or class B [17–19]. There exists a plethora of reasons why MBLs are likely to displace serine carbapenemases as the key group of enzymes capable of hydrolysing carbapenems. This brief appraisal, while unavoidably parsimonious, will give an insight into the contribution of MBLs to Gram-negative resistance and why there is a growing concern about the lack of appropriate therapeutic regimens.

**GENETIC LOCATION OF MBL GENES**

As with any molecular class of β-lactamase, MBLs can be broadly divided into those that are ‘mobile’ and those that are normally carried by the host organism on the chromosome. Those that are normally chromosomally encoded are usually derived from organisms whose habitat is predominantly environmental. Consequently, there is some debate as to why this is the case, but the most plausible explanation is that these enzymes perform a normal cellular function that is yet to be fully elucidated. Irrespective, these organisms are seldom pathogenic and, with the arguable exception of *Stenotrophomonas maltophilia* and *Bacillus anthracis*, rarely cause serious infections [20,21].

Most ‘mobile’ MBL genes are carried on various genetic elements that aid their movement from one genetic apparatus to another. Genes encoding IMP and VIM types as well as GIM-1 are found as gene cassettes in class 1 integrons [22–27], although IMP-type MBL genes are also found on class 3 integrons [28,29]. Integrons are capable of procuring gene cassettes via a site-specific recombination event between two DNA sites, one in the integron and one in the gene cassette. Integrons consist of three regions: the 5′-conserved region (5′CS), the 3′-conserved region (3′CS) and a variable region. Frequently, other gene cassettes are also sequenced and inserted, the most notable example being the *aacA4* gene, which encodes kanamycin, neomycin, amikacin and streptomycin resistance. Thus, one integron can carry resistance to one or more classes of antibiotic and, accordingly, either aminoglycosides or β-lactams could, potentially, select for the resistant element. However, the genetic plasticity is further enhanced when the mobility of integrons is facilitated by other genetic elements such as plasmids and transposons [30]. In 2003, the first account was published of an MBL gene (*blaIMP-13*) and its integron being embedded in a Tn5051-type transposon from an Italian *P. aeruginosa* isolate [31]. Additional studies showed that the elements containing *blaIMP-13* and *blaVIM-2* from a *P. aeruginosa* strain from Poland were inserted in an identical site. Moreover, the *tnpR* genes of the transposon from both sites are identical, suggesting that the transposon is responsible for dissemination of the class 1 integron, which then procured the different MBL genes [31].

While the majority of MBL genes are mobilised by integrons/transposons, a minority appear to be mobilised with mobile common regions (CRs) that have also been associated with other mobile elements called SXT regions. The gene encoding SPM-1, which appears to be restricted to Brazil, is associated with two different types of CR element [32]. Further analysis of the Brazilian *P. aeruginosa* isolates demonstrated that the upstream DNA contained common regions or CR elements, in this case CR4 [33]. Compared to integrons and transposons, very little is known about CR elements, particularly how they facilitate the mobilisation of resistance genes [34].

**ACQUIRED MBLS IN GRAM-NEGATIVE BACTERIA**

Those MBL genes that can be described as mobile include those encoding CfiA (or CcrA), IMP, VIM, SPM and GIM. The first of these to be characterised was the gene for CfiA, which is predominantly associated with *Bacteroides* spp. [35]. The enzyme has been well studied and has often been utilised as a paradigm for mechanistic studies [36,37]. The CfiA gene is often quiescent, and its
expression is dependent on the implantation of insertion elements such as IS942, IS1186 and IS4351, thereby providing a hybrid promoter and facilitating its expression [38–40].

The first ‘mobile’ MBL to be characterised outside Bacteroides spp. was IMP-1, with the discovery of P. aeruginosa strain GN17203 in Japan in 1988 [15]. The isolate possessed an imipenem MIC of 50 mg/L, as well as resistance to extended-spectrum cephalosporins, e.g., ceftazidime MIC of > 400 mg/L. The resistance allele was found on a transferable conjugative plasmid that could be readily mobilised to other Pseudomonas strains. Three years later, an identical gene was found in Serratia marcescens strain Tn9106 isolated from a urinary tract infection at Aichi Hospital, Okazaki, Japan [26], highlighting the potential dissemination of the genes encoding these enzymes. Hitherto, Japan has become a major reservoir for IMP-type MBLs, which now include IMP-1, IMP-2, IMP-3, IMP-6, IMP-10 and IMP-11, and these have spread to a number of strains of Pseudomonas spp., Acinetobacter spp. and Enterobacteriaceae.

The belief that IMP-type MBL genes were solely a distant Japanese problem was dispelled with the advent of blaIMP-2 in 1997 and blaimp-5 in 1998 in Italy and Portugal, respectively [41,42]. The absence of genetic data concerning these strains limits our ability to determine whether these IMP-like genes are actually evidence of dissemination from Japan or whether they have emerged separately in Europe. IMP-type MBLs have now been found in 12 countries; however, they still appear to be most prolific in Southeast Asia. The most recent IMP-like MBL (IMP-18) was found in a P. aeruginosa isolate from Las Cruces, North America [43].

Fig. 1. Phylogeny of chromosomally encoded MBLs. Represented sequences of various MBLs were obtained from GenBank. MBLs IMP-1, IMP-12 (most divergent from IMP-1), VIM-1 and VIM-7 (most divergent from VIM-1) were also added for comparison. Signal peptides were removed prior to alignment. Sequences were aligned and phylogeny trees constructed by Clustal W (PAM250 matrix—DNA Star) using the neighbour-joining method.

The ‘European MBL’ is likely to be that of VIM rather than IMP, and its global spread (probably from Europe) is rapid and worrisome. The VIM-type MBLs are the second dominant group, and there are currently 13 known derivatives. VIM-1 (Verona imipenemase) was first characterised from a P. aeruginosa strain isolated in 1997 in Italy [23]. Subsequently, a VIM-1 variant, VIM-2, was identified in the southern part of France from a P. aeruginosa isolate from a blood culture of a neutropenic patient in 1996 [25]. However, the ‘index’ case of VIM-2 would appear to be a P. aeruginosa strain isolated from a patient in Portugal in 1995 [44]. VIM-2 is closely related to VIM-1 (90% amino-acid identity) (Fig. 1), but shows appreciably different kinetic properties to most β-lactams. While there are now 13 variants of VIM-type MBLs, the dominant genotype is that of VIM-2, which has, hitherto, been reported from 20 countries in five continents. The most recent ‘outbreak’ to be reported was from the USA and involved four patients in an intensive care unit (ICU); typically, the P. aeruginosa harbouring VIM-2 was sensitive to aztreonam only [45].

The third type of acquired MBL in Gram-negative bacilli is SPM-1 (Sao Paulo MBL) [32], the index strain probably being a bloodstream isolate from a 4-year-old leukaemic girl who eventually succumbed to the infection. As is typical with other MBL-producing organisms, the isolate was shown to be highly resistant to all standard anti-Gram-negative anti-infectives except for colistin [46]. The sequence of SPM-1 possesses moderate identity with that of IMP-1 (35.5%) but is markedly different to that of VIM. Disturbingly, unsubstantiated reports from Brazil indicate that approximately 20–45% of P. aeruginosa isolates possess the SPM-1 MBL.

The latest and seemingly rarest MBL is GIM-1, which was recovered from five P. aeruginosa isolates in 2002 from Dusseldorf, Germany (Ger-
man ImiPenemase) [22]. Like most *P. aeruginosa* isolates possessing MBLs, the five isolates were only susceptible to polymyxin B. The amino-acid sequence of GIM-1 displayed most identity with IMP variants (43.1% with IMP-1), 29% similarity with VIM-1 and 28% similarity with SPM-1 (Fig. 1). Since 2002, there have been no further reports of GIM-1-possessing *P. aeruginosa* isolates.

**MECHANISM OF MBL-MEDIATED HYDROLYSIS**

The mechanism of hydrolysis of the amide bond within the \( \beta \)-lactam ring is different for MBLs than for serine \( \beta \)-lactamases. MBLs invariably possess the signature sequence HXHXD, which facilitates the coordination of zinc ions (usually two) at the ‘active site’ (Fig. 2). Despite the fact that MBLs may share less than 25% amino-acid identity with one another, they all share the unique \( \alpha/\beta \)β\( \alpha \) fold and their active site architectures are virtually superimposable. While not completely elucidated, the proposed mechanism of hydrolysis suggests that this active site orients and polarises the \( \beta \)-lactam bond to facilitate nucleophilic attack by zinc-bound water/hydroxide [47]. Most MBLs possess a wide plastic groove and, accordingly, can accommodate most \( \beta \)-lactam substrates, giving rise to their broad spectrum of activity (Table 1). Moreover, because MBLs, unlike serine \( \beta \)-lactamases, do not form highly populated stable covalent intermediates with their substrate, it would be difficult to arrest the hydrolytic mechanism with use of a \( \beta \)-lactam-type compound. Accordingly, MBLs are impervious to the impeding effects of compounds such as clavulanic acid and sulbactam [10,48]. Notwithstanding the above, none of the mobile MBLs are capable of hydrolysing aztreonam particularly well, indicating that it might be used as a possible therapeutic inhibitor. However, animal pneumonia models infected with VIM-2 producing *P. aeruginosa* could not be eradicated with aztreonam even when challenged with high doses [49].

Since the introduction of amoxycillin–clavulanate in the 1980s, the potentiation between a \( \beta \)-lactam and a \( \beta \)-lactamase inhibitor could be theoretically used to treat any infection with \( \beta \)-lactamase-producing bacteria. However, inhibiting all clinically relevant MBLs will be a colossal undertaking and there are many toxicity issues that may arise, due to human binuclear enzymes possessing an almost identical active site, e.g., glyoxalase II [50]. Moreover, many studies exploring the potentiation by experimental inhibitors have not used whole-cell assays. This is likely to be a major obstacle with organisms such as *P. aeruginosa* and *Acinetobacter* spp.

![Fig. 2. Direct comparison of Bacillus cereus BCII MBL (left) with IMP-1 (right) [75,76]. While these enzymes possess little homology (< 25% similarity), their structures possess the unique \( \alpha/\beta \)β\( \alpha \) fold and are virtually superimposable. The grey spheres represent zinc ions, which would normally coordinate water/hydroxide molecules necessary for the hydrolysis of the \( \beta \)-lactam amide bond.](image)

<table>
<thead>
<tr>
<th>MBL</th>
<th>IMP-1</th>
<th>VIM-1</th>
<th>VIM-2</th>
<th>SPM-1</th>
<th>GIM-1</th>
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<tr>
<td>Moxalactam</td>
<td>2.3</td>
<td>400</td>
<td>104</td>
<td>30</td>
<td>19</td>
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</table>

Hydrolytic rates were determined by the Michaelis-Menten steady-state equation where \( V = \frac{K_{cat} [E][S]}{K_m + [S]} \). [S] was kept at a 150 \( \mu \)M for all \( \beta \)-lactam substrates and imipenem was standardised at a relative rate of 100.

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THE DISSEMINATION OF MBLS
Within the realm of resistant genes that bacteria procure and evince, some genotypes appear to dominate while others make only a fleeting appearance. The reasons for this may be multiple but are likely to involve the genetic elements on which the genes are carried, as well as the promiscuity and subsequent ‘fitness’ of the recipient bacteria. Moreover, there is little doubt that the increase in human travel has further enhanced the spread of resistance, as exemplified by the first Scandinavian *P. aeruginosa* (*bla*VIM-4) isolate, which was introduced into Sweden via a Greek patient [51]. Notwithstanding stringent infection control policies, the MBL gene pool in some countries may already be firmly established. For example, the first *P. aeruginosa* isolate possessing *bla*SPM-1 was isolated in 1997 and now appears to contribute approximately 25–40% of the pseudomonal population, severely limiting antimicrobial regimens in some hospitals. This may also be the case with the *P. aeruginosa* carrying VIM-2 that possibly arose from a patient residing in Portugal in 1995 [44]. Since 1995, *bla*VIM-2-positive *P. aeruginosa* has appeared in France, Italy, Greece, Spain, Poland, Croatia, Germany, Belgium and Russia, and has asserted itself as the dominant European MBL [31,52–56]. It has also been found in Venezuela, Chile, Argentina, Korea, Japan, Taiwan, Saudi Arabia and, most recently, from an outbreak in the USA [57–61]. It is quite probable that the gene pool is more extensive than that which we can detect and is either in a quiescent state or is not being detected, or both, as demonstrated by the Taiwanese study, in which 140 multiresistant *K. pneumoniae* isolates were examined for IMP- and VIM-type genes with reference to hybridisation studies, in which 40 were positive for IMP, yet only 5/40 were carbapenem-resistant [62]. Such studies provide an excellent insight into the silent gene pool which is undetected by standard microbiological testing regimens.

It is perhaps not coincidental that IMP, VIM, SPM and GIM first appeared in *Pseudomonas* (usually *P. aeruginosa*) before appearing in *Acinetobacter* spp. and Enterobacteriaceae. The reason for this is not clear but is likely to involve the universality, or lack thereof, of the genetic apparatus carrying the MBL genes, i.e., broad host range plasmids. Over a period of time it is highly likely that these genes will find mobile elements that can be accommodated in most Gram-negative bacteria. Unfortunately, little information concerning the plasmids that carry MBL genes is available and no fitness studies have been undertaken using native plasmids carrying MBL genes, which may provide a benchmark as to how successful they will be in spreading to Enterobacteriaceae. Moreover, no β-lactam efficacy animal studies have been undertaken to examine eradication of MBL-positive Enterobacteriaceae, the data from which would provide a prediction of their clinical importance and an indication of whether altered dosing regimens may prove efficacious.

THERAPEUTIC REGIMES FOR PAN-RESISTANT GRAM-NEGATIVE BACTERIA
The dissemination of MBL genes is thought to be driven by the regional consumption of extended-spectrum cephalosporins or carbapenems [63,64], and therefore care must be taken that these drugs are not used unnecessarily. Furthermore, given that MBL-possessing bacteria often appear sensitive or intermediate sensitive to carbapenems, MIC testing is advisable, as disk diffusion is unlikely to detect borderline cases.

The MBL producers that are most clinically significant are primarily those in which the gene encoding the enzyme is transferable. These MBL-positive strains are usually resistant to β-lactams, aminoglycosides and quinolones, and, as has recently been shown, inappropriate therapy can be associated with adverse outcome, particularly in high-risk patient groups [65]. Hitherto, there has been no suitable therapy for treating these infections. However, in a recent clinical study by Lee et al., the therapy and clinical outcome of six patients infected with MBL-positive Gram-negative bacilli were tracked, and in each case the infection was eradicated. In most cases, a β-lactam was used in combination with one or more other antibiotics; however, no detailed information was provided concerning the organism’s susceptibility to the other compounds or what resistance genes were associated with the MBL genes [66].

At the present time, no MBL inhibitors are available for treating patients, and there are not
likely to be any in the foreseeable future. Co-resistance with other compounds, such as aminoglycosides, is likely to further diminish other therapeutic options [48]. The only therapeutic alternative may be the administration of polymyxins, which have been shown recently to be efficient in treating multidrug-resistant Gram-negative bacilli [67]. It has been claimed recently that polymyxins are not as toxic as previously thought; however, it would be prudent to consider polymyxins only as part of combination therapy [68,69].

Clearly, there is a need to produce new and efficacious compounds to combat MBL-positive Gram-negative bacteria. However, there appear to be very few new compounds demonstrating potent activity against *P. aeruginosa* and *Acinetobacter* spp., even without an MBL [70]. The reasons for this are multifactorial, but largely centre on the lack of net return profits that companies will receive once an anti-pseudomonal drug has been developed [38,71]. Such a compound may require the better part of $800 million to develop and will only be used in highly specialised circumstances, even in life-threatening situations. Sadly, net returns to large pharmaceutical companies are far higher for lifestyle drugs than for those mitigating serious infections, and it is these that are seen as commercially attractive. This crisis heralds the desperate need for governmental support in drug research and development programmes if longevity is to be guaranteed [71]. Non-fermenting Gram-negative bacteria are particularly difficult to eradicate, as their outer-membrane provides a formidable barrier that is difficult to breach. Accordingly, attention appears to be increasingly turning to small proteins as anti-infectives, some of which are highly cidal and may not pose the toxic risks first feared [72]. Given the lack of new compounds that are likely to be efficacious against pan-resistant Gram-negative bacteria, news reports heralding the ‘end of the antibiotic era by 2015’ appear to be increasingly less dubious.

**REFERENCES**


