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Discussion

Metallo-β-lactamases as emerging resistance determinants in Gram-negative pathogens: open issues

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Abstract

The rapid spread of acquired metallo-β-lactamases (MBLs) among major Gram-negative pathogens is a matter of particular concern worldwide and primarily in Europe, one of first continents where the emergence of acquired MBLs has been reported and possibly the geographical area where the increasing diversity of these enzymes and the number of bacterial species affected are most impressive. This spread has not been paralleled by accuracy/standardisation of detection methods, completeness of epidemiological knowledge or a clear understanding of what MBL production entails in terms of clinical impact, hospital infection control and antimicrobial chemotherapy. A number of European experts in the field met to review the current knowledge on this phenomenon, to point out open issues and to reinforce and relate to one another the existing activities set forth by research institutes, scientific societies and European Union-driven networks.

Keywords: Metallo-β-lactamases; Antimicrobial resistance

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1. Introduction

Acquired carbapenemases represent a major threat to the clinical utility of all β-lactam antibiotics. They represent a heterogeneous group of β-lactamases belonging to different molecular classes (namely A, B and D). Carbapenemases belonging to either class A (such as NMC/IMI, SME and KPC) or class D (such as several OXA-types, mostly found in *Acinetobacter* spp.) are active site-serine enzymes, whilst those belonging to class B are metallo-enzymes whose activity is dependent on zinc ions [1].

The emergence of acquired metallo-β-lactamases (MBLs) among major Gram-negative pathogens (*Pseudomonas aeruginosa*, *Acinetobacter* spp., Enterobacteriaceae) has clinical and epidemiological implications and is a matter of particular concern worldwide. Their rapid spread, increasing diversity and the number of species involved has not been paralleled by accuracy/standardisation of detection methods, completeness of epidemiological knowledge or a clear understanding of what MBL production entails in terms of clinical impact, hospital infection control and antimicrobial chemotherapy.

As with previous experiences with other emerging resistance issues, this problem should elicit a prompt reaction, especially in the most affected countries. Europe has been one of the first continents where the emergence of acquired MBLs has been reported, from either individual isolates or nosocomial outbreaks [2–6]. A number of European experts in the field met to review the current knowledge on this phenomenon, to point out open issues and to develop a continental strategy for surveillance and control of these new resistance determinants. This position paper summarises the most relevant issues discussed during the meeting and the consensus opinion of the panel of experts on those issues.

2. Epidemiology and surveillance of acquired MBLs

Although there are now a multitude of reports on the detection of MBL-producing clinical isolates from various European countries, including a number of interesting studies on the molecular epidemiology of these resistance determinants (see for instance [4,7–27]), no satisfactory MBL surveillance system is currently active in Europe. This is likely related to the lack of standardisation in the methodologies used by clinical laboratories and to an overall limited awareness of this problem.

European-wide information regarding acquired MBLs is eagerly required and should be collected in order to analyse the current situation and to monitor trends. Surveillance systems such as the European Antimicrobial Resistance Surveillance System (EARSS) (http://www.rivm.nl/earss/) would appear to be the most suitable candidates for organising this network. As is always the case for antimicrobial resistance surveillance studies, absolute figures (i.e. how many cases are reported, broken down by species and type of enzyme), should be accompanied by data regarding denominators, with accurate data on sampling methods, collection areas and ratio to other (e.g. non-MBL-producing) drug-resistant isolates.

Although representing a first goal, comprehensive European data on MBL prevalence would not permit, by themselves, a sufficient appraisal of the problem. At the continental level, we also need to know how strains and genes are spreading. Molecular typing of strains (by polymerase chain reaction (PCR)-based methods and pulsed-field gel electrophoresis (PFGE)) is essential to recognise outbreaks caused by given strains in individual hospitals and to monitor their regional and international spread. More resources are required for the development and large-scale application of multilocus sequence typing (MLST) or other typing methods for Gram-negative organisms, thus facilitating comparison of results between different laboratories. Finally, further studies are required on the molecular epidemiology of MBL genes and on their association with mobile DNA elements (integrons, transposons, plasmids) in order to define the importance of intraspecies and interspecies horizontal spread of MBL genes.

2.1. Surveillance strategies

The introduction of systematic screening for MBL-producers in the routine diagnostic laboratory would seem a timely and important issue both for diagnostic and surveillance purposes, especially in areas where strains with acquired MBLs have already been reported.

To this purpose, it appears to be of the utmost importance to establish a network of reference laboratories using standard protocols and reagents (e.g. PCR primers) for detecting MBL-producers. Each laboratory should be provided with a set of control strains producing the various enzymes for quality control purposes and for testing new batches of reagents. Quality control procedures for the detection of MBL-producing clinical isolates should be regularly carried out in each reference laboratory. The reference laboratories should be of support to the routine diagnostic laboratories of the respective countries in the implementation of screening protocols and should carry out confirmatory testing of selected isolates and studies of molecular epidemiology.

The role of national central laboratories in pooling nationwide data should be considered. If national data are still not sufficiently informative, national central laboratories should promote two types of nationwide surveillance studies, namely: (i) prospective studies of all consecutive isolates (to assess the actual prevalence of MBLs in the surveyed centres); and (ii) comparative molecular analysis of either confirmed MBL-producers referred by reference laboratories or confirmatory analysis of MBL production of selected isolates when reference laboratories do not exist.

A broad discussion was carried out on the candidates to be considered for MBL screening in routine diagnostic labo-

ratories. Some resistance profiles may be suggestive of MBL production (e.g. resistance to all β-lactams except aztreonam in *P. aeruginosa*), however the high phenotypic diversity observed to date in MBL-producers would actually suggest the following, less stringent consensus proposal:

- *Pseudomonas aeruginosa*, other *Pseudomonas* spp. and *Acinetobacter* spp.
  - all isolates non-susceptible to carbapenems (imipenem and/or meropenem) and resistant to either ticarcillin, ticarcillin/clavulanic acid or ceftazidime;
- Enterobacteriaceae
  - for species not producing, or producing a small amount of, AmpC-type enzymes (e.g. *Escherichia coli*, *Klebsiella* spp., *Proteus mirabilis*, *Salmonella enterica*, *Shigella* spp.), all carbapenem-susceptible isolates that are resistant to cefoxitin and amoxicillin/clavulanic acid and are non-susceptible to ceftazidime;
  - in all other instances, all isolates non-susceptible to carbapenems.

For all of these isolates, screening for MBL production should be performed with the ancillary tests described below.

3. Detection of MBL-producing strains

Conventional susceptibility data are neither sensitive nor specific in detecting MBL-producing strains and specific tests are necessary for this purpose.

In particular, MBL-producing Enterobacteriaceae can be more difficult to detect than *P. aeruginosa* and *Acinetobacter*, since carbapenem minimum inhibitory concentrations (MICs) may fall within a broader range, and are often lower than the current susceptibility breakpoints [1,28–30]. Moreover, different automated systems have shown interpretation problems with MBL-producers with regard to their susceptibility to carbapenems [31].

Spectrophotometric measurement of ethylenediaminetetraacetic acid (EDTA)-inhibitable carbapenem hydrolysis, carried out with a crude cell extract [6], still stands as the reference method for confirming MBL production. However, this test is not suitable for routine use in the clinical microbiology laboratory.

In contrast, a number of simple phenotypic tests, based on diffusion or dilution formats, can be used as ancillary tests for specific detection of MBL-producers in the clinical microbiology laboratory, relying on the synergy between a MBL inhibitor (usually EDTA or a thiol compound) and an oxyimino cephalosporin or a carbapenem (see for instance [32–39]). Some of these assays require the testing of cell extracts instead of bacterial cultures and, although potentially useful, appear to be less practical for use in the clinical microbiology laboratory. However, it is worth noting that these tests have mostly been validated with *P. aeruginosa* and, to a lower extent, with *Acinetobacter* spp., whilst experience with other Gram-negative non-fermenters and with Enterobacteriaceae remains more limited. A more extensive validation and standardisation of these common ancillary tests remains an open and urgent issue.

Molecular methods (PCR or DNA hybridisation approaches) are necessary to confirm the presence of MBL genes in clinical isolates and can also be adopted for screening purposes. However, these methods remain confined to reference or research laboratories and are currently not available to the majority of routine diagnostic laboratories for diagnostic or surveillance purposes. Moreover, the molecular methods will only detect MBL genes that are recognised by the repertoire of available probes and could miss detection of new MBL genes.

Owing to the transferable nature of MBL genes and the importance of plasmids in this event, typing methods for plasmids involved at least in incompatibility group level should apply to characterise dissemination and follow their evolution [5,40,41].

At present, it appears difficult to outline univocally one correct procedure for specific detection of MBL-producers. In principle, combination disk tests, MBL Etest and double-disk synergy tests all appear to be adequate for use in routine clinical microbiology laboratories, which are expected to be already familiar with these types of tests for detection of extended-spectrum β-lactamases (ESBLs) in Enterobacteriaceae [42,43]. Based on literature data and personal experience, any of the above tests would seem suitable for detection of MBL-producing *P. aeruginosa*, although double-disk tests may suffer from a certain lack of standardisation. Moreover, intrinsic EDTA susceptibility might complicate the interpretation of tests based on the simultaneous presence of EDTA and the antibiotic on the same disk/strip, leading to false MBL detection in *P. aeruginosa* [44]. With Enterobacteriaceae, combo-disk or double-disk can be used as first-line tests, although the usually low carbapenem MICs of these isolates make the interpretation of the MBL Etest results difficult. With *Acinetobacter* spp., experience is more limited, making it more difficult to compare the accuracy of different ancillary tests.

4. Clinical issues

4.1. Definition of the problem

The proliferation of MBLs and the spread of MBL-producing strains must be regarded as a potential public health problem and not as a mere laboratory finding of scarce clinical significance. Although both known and novel variants of these enzymes are being reported at an increasing rate, a prompt awareness of the problem might be helpful to limit their uncontrolled diffusion. Increased awareness and correct information could help microbiologists to detect outbreaks of MBL-producing strains early, both of clonal and polyclonal origin, as well as prompt clinicians to adopt proper measures in terms of antimicrobial use and infection control.

4.2. Breakpoints and laboratory reports

Although substantial clinical evidence is lacking (and little evidence is available from animal models, mostly limited to *P. aeruginosa* [45,46]), data from most clinical reports, enzyme kinetics and whole-cell physiology would support the view that MBL-positive strains must with caution be held as resistant to all carbapenems and should be reported as such. The strong inoculum effect observed in these strains [40,47] further supports this view. The same note of caution would presently apply to all non-carbapenem β-lactams, although the possibility of using high dosages of aztreonam, which is stable to MBLs, seems worth considering, following some data reported in animal studies [45], and requires further clinical evidence in humans.

Having accepted this, one must admit that current breakpoints—be they those issued by the Clinical and Laboratory Standards Institute (CLSI) [48] or those issued by the European Committee on Antimicrobial Susceptibility Testing (EUCAST) (http://www.escmid.org/sites/science/eucast/index.aspx)—are not useful for categorical assignment of β-lactam susceptibility of MBL-producers and should not be applied for this purpose. Results of susceptibility testing should be better reported according to an interpretative reading of the antibiogram, i.e. by pointing out and carefully analysing those phenotypes suggestive of a MBL.

All in all, reporting a MBL presents microbiologists with problems similar to those usually encountered with ESBLs and should be dealt with in a similar way.

A further insight into the clinical significance of a MBL, also taking into account the increasing diversities observed within this group, is eagerly needed and mandates more accurate investigations with animal models, pharmacokinetic/pharmacodynamic (PK/PD) studies and retrospective case–control studies of carbapenem use in infections caused by MBL-positive strains (including correlation of the observed MICs with the actual clinical outcomes).

4.3. Reporting MBL-positive isolates

Although non-MBL-producing multidrug-resistant (MDR) strains can also represent a serious therapeutic threat and are worthy of serious consideration, reporting the presence of a MBL holds a peculiar importance owing to the high-level resistance it may entail for all β-lactams and to the inherent implications for antibiotic and infection control policies.

Thus, MBL-producing strains should be reported and their isolation should be conveniently underlined—also when they occur as colonisers and are not isolated from pathological specimens, owing to their possible spread to other body sites and/or to other patients in the same ward, mandating careful monitoring of patients from whom they have been first isolated.

4.4. Correlation between MBLs and antibiotic use

Analysis of the antimicrobial chemotherapy received by patients before isolation of MBL-positive strains often revealed that many of them had not received therapy with carbapenems but had been given non-carbapenem β-lactams, mostly expanded-spectrum cephalosporins. In the Japanese multifocal epidemics of bla_{IMP}-positive Gram-negative bacilli, prior use of carbapenems could be confirmed for only 15% of patients, whilst 39% of patients were administered cephems prior to the isolation of bla_{IMP}-positive isolates and such strains were also isolated from antibiotic-free patients, suggesting that MBL-producing *P. aeruginosa* can spread as hospital infections without the use of antibiotics [49]. This finding has been confirmed on the occasion of the first appearance of VIM-1 in Italy, when only three patients had received therapy with imipenem, whilst the others had been given expanded-spectrum cephalosporins or, in one case, amoxicillin [2]. However, in an outbreak of IMP-4 producers recently reported in an Australian hospital, 75% of the colonised or infected patients had received carbapenems before isolation of the MBL-producing strains [50,51].

The frequent co-resistance to other classes of antibiotics observed in MBL-producers owing to the simultaneous presence of additional resistance determinants, often carried on integrons, such as genes for aminoglycoside-modifying enzymes and/or mutations that upregulate efflux systems, underlines the possibility that MBLs may be co-selected by clinical use of unrelated classes of antibiotics.

Many further reports emphasise the complex relationship between antibiotic resistance and antibiotic use and suggest that curbing antibiotic consumption cannot be the only strategy for controlling MBLs in hospitals, whereas it might be far more important to enhance the laboratory's ability to identify resistant strains as well as emphasising the need for early recognition of MBL-producing isolates and for the use of rigorous infection control precautions to prevent transmission [52,53].

4.5. Infection control procedures

The clinical data published to date do not offer a clear picture of the infection control measures to be set up whenever a MBL-producer is reported.

Controlling the use of those antibiotics that are likely to favour the spread of MBL-producers appears to hold an important role, with reference not only to carbapenems but also to other antibiotic classes—namely aminoglycosides and quinolones—that can be involved in the co-selection of MBLs. However, interesting evidence does exist on the importance of hospital hygiene rather than antibiotic selection [52,53]. Different actions should apply to different isolation sources (e.g. gut, catheter, respiratory tract), patient types (neutropenic, multiple antibiotic treatments, mechanically ventilated) and different locations (Intensive Care Unit (ICU), transplant unit, medical ward, surgical ward),

and should include withdrawal of indwelling devices, contact precautions and patient isolation. All other patients in the same ward should be investigated for the presence of colonising organisms. Specimens to be routinely investigated could be skin swabs, urine and sputum, and faecal carriage should be also checked (at least once a week). With this aim, the use of selective, carbapenem-containing plates may be worth investigating. Patients in the ICU or undergoing invasive procedures should be also investigated for their surgical drainages, catheters and others indwelling devices. One should consider cohorting patients, if possible.

MBL-producing *Pseudomonas* isolates have also been detected in the hospital environment, sometimes 6–12 months after the isolation of the MBL-positive strain from a clinical sample. Sources of the environmental MBL-positive isolates can be devices such as stethoscopes and wet surfaces such as sinks, water pipes, spillways, plugholes and wet plastic surfaces near the sinks [52,54].

It seems prudent that control procedures should apply to all proven MBL-producing isolates regardless of their actual level of susceptibility. Given that not every laboratory is presently capable of reporting a MBL with a sufficient likelihood, more stringent measures (such as patient isolation) should be only enforced on the basis of either molecular confirmation of the finding or the laboratory's proven experience in dealing with this problem.

Colonisation studies, performed both on colonisers and infecting strains, should record the length of colonisation and should include discharged patients not only as a follow-up to the study but to prevent any undue spread of MBL-producing strains to the community.

Mathematical modelling similar to that performed with vancomycin-resistant enterococci [55] should contribute to a better definition of this problem, including its transmission dynamics and the efficacy of different infection control actions.

As mentioned previously, molecular epidemiology data are clearly of the utmost importance for tracing the clonal spread and for correctly setting up infection control measures.

4.6. MBL-producers as a therapeutic challenge

Although MBL-producing strains represent a serious therapeutic challenge, to date clinical data are surprisingly scarce with regard to both prevalence and outcome of infections caused by these strains. This prompts the need for ad hoc clinical studies but also for carefully reporting clinical management and output data, even when related to individual experiences.

Consequently, therapeutic options to be recommended in these cases have never been reviewed or compared with one another. Table 1 lists these options alongside some comments stemming from individual case reports and from what is known or may be inferred from pre-clinical studies. The aforementioned list shows once more the paucity of clinical data regarding possible therapeutic options but also the insufficient contribution of pre-clinical data.

Nevertheless, the low susceptibilities of MBL-producing strains to many different classes of antibiotics appear to

Table 1
Potential therapeutic options for metallo-β-lactamase (MBL)-producing strains

Antimicrobial	Comments
Carbapenems	No clinical evidence for this recommendation except for a single report [56]. One recent report from an animal model [57]. Evidence against the use of carbapenems: inoculum effect [40], isolation of carbapenem-resistant mutants following carbapenem therapy [2,50,51]. This should be applied to all carbapenems despite some in vitro data regarding different killing effects (killing curves) of the various carbapenems on MBL-producers (Giamarellou, personal communication).
Aztreonam	In vitro data would support its use but there are limited clinical data [56]. Conflicting results have been reported from animal models [51]. It is worth mentioning that MBL-producers may often be endowed with other resistance mechanisms affecting aztreonam [58–61]. Might be useful in combination therapy. Ad hoc studies are needed.
Piperacillin/tazobactam	In vitro data would support its use with some *Pseudomonas aeruginosa* strains, but clinical data are lacking.
Fluoroquinolones and aminoglycosides	Clinical data would support their use in susceptible strains [51]. Might be useful in combination therapy. Ad hoc studies are needed.
Colistin	Sole therapeutic choice in many instances [62]. Resistance is now reported [63,64] and should be investigated in the laboratory.
Colistin + rifampicin	There are in vitro data supportive of this combination (synergic effect) [65,66].
Tetracyclines and glycylcyclines	Clinical data supporting their use in susceptible strains are missing. In vitro data suggest that tigecycline is also active against MBL-producing Enterobacteriaceae and *Acinetobacter* spp. [67].
Fosfomycin	Clinical data supporting its use in susceptible strains are missing. Possible use in association with other compounds [68].

limit greatly the possibilities of any single-drug regimen in favour of combination therapies. Possible combinations and their dosages look promising but they represent an almost completely unexplored field of investigation, with special reference to their actual effectiveness on different bacterial species and on isolates endowed with different MBLs (and/or additional resistance mechanisms).

PK/PD studies should also warrant correct information about administration routes and dosages. Of paramount importance is the possibility for microbiologists to test these combinations by means of rapid and standardised methods and to produce standardised, quantitative and reproducible reports, also using newly developed technologies.

An open question is whether to treat only infected patients or also subjects colonised by MBL-producers and selected at-risk individuals (to be properly detected), with the aim of eliminating the isolate before it becomes virulent in the same patient or can spread to other patients. Needless to say, the present absence of clinical guidelines confers this debate a merely academic interest.

In conclusion, therapeutic options look extremely scarce and are often not sufficiently documented, which constitutes a most disquieting development in the field of antibiotic therapy and makes mandatory careful management of the drugs used to treat severe Gram-negative infections and the adoption of rigorous infection control precautions to prevent their transmission.

Although carbapenem resistance can also result from the interplay between porin loss and production of some serine β-lactamases with weak carbapenemase activity (see for instance [69–72]), MBL-mediated resistance is a matter of serious concern for several reasons. First, porin loss often results in low-level carbapenem resistance only, whereas MBL production normally results in high-level resistance to most β-lactams, with the exception of aztreonam. Second, MBL production is typically associated with resistance to aminoglycosides and quinolones. The first case often relates to the co-presence in the same integrons of MBL genes and genes coding for aminoglycoside-modifying enzymes [1]. Still to be fully clarified are the roles that are played by different efflux pumps in determining this co-resistance as well as in contributing to other β-lactam resistance mechanisms. All in all, multidrug resistance often leaves colistin as the only antibiotic agent available, which might be a poor alternative in view of the alleged toxicity of this agent and its unfavourable pharmacokinetics [62].

On these grounds, all MBL-producing strains would well deserve the name of 'Gram-negative MRSA' recently coined for *Acinetobacter*, if it were not that they appear to carry a much greater therapeutic risk and deserve greater attention than their Gram-positive counterpart.

The limited therapeutic options for infections caused by MBL-producers and the virtual absence in the pipeline of short- and mid-term therapeutic alternatives for these infections—and for those caused by MDR Gram-negatives as a whole—should mandate that all efforts be made to limit the spread and clinical threat posed by these strains, besides warranting a careful re-consideration of the actual priorities in the field of antimicrobial research and a prompt redistribution of the intellectual and financial resources available to this aim.

5. Nomenclature of acquired MBLs

Five major lineages of acquired MBLs (IMP, VIM, SPM, GIM and SIM) have been discovered during the past decade, with a growing number of variants for the IMP- and VIM-type enzymes [28,73]. Different names have been assigned to acquired MBLs showing notable sequence divergence, whilst the same name with different suffix numbers has been assigned to allelic variants of each lineage that differ from each other by a limited number of amino acid substitutions and are clearly related to that lineage. However, with the discovery of an increasing number of IMP- and VIM-type variants, the amino acid sequence identity between alleles of these types has reached values as low as 78% and 73%, respectively.

It is our opinion that, at this stage, it would be useful to define a precise criterion for assignment of new names to acquired MBLs based on a fixed cut-off value for sequence diversity. We are aware that since these enzymes are orthologues, with detailed functional characterisation lacking for most of them, this cut-off value is necessarily somewhat arbitrary. However, we feel that this approach would be of help to prevent the development of a confounding nomenclature, as has happened with other classes of β-lactamases.

The following criterion is herewith proposed. Any new enzyme with documented MBL activity will be considered as an allelic variant of a known type if the degree of amino acid identity to the reference enzyme of that type is >70%, whilst it will be assigned to a new type if identity is ≤70%. For each known type (or lineage), an enzyme is selected as the reference enzyme for that type. The proposed reference enzymes are IMP-8 for IMP-type enzymes, VIM-2 for VIM-type enzymes, and SPM-1, GIM-1 and SIM-1 for the other types (for which a single allele is currently known). The choice of IMP-8 and VIM-2 as reference enzymes for the IMP- and VIM-types, respectively, is based on the fact that they are the variants most similar to the consensus generated following alignment of all known variants of each type.

To avoid confusing duplications, new enzyme names should only be assigned on application to Karen Bush and/or George A. Jacoby, who manage the database of β-lactamase names (http://www.lahey.org/Studies/).

6. Minimum requirements recommended for characterisation of new MBLs

Identification of a new MBL type or of a new allelic variant of a known type by sequencing should be followed by char-

acterisation of the enzyme. Characterisation should include at least:

(i) determination of the resistance profile shown by the clinical isolate and by a laboratory *E. coli* strain carrying the cloned gene in a vector that allows its expression (following the CLSI recommendations [74]); and
(ii) confirmation of the presence of MBL activity in crude extracts of the clinical isolate and of the *E. coli* strain carrying the cloned gene [6].

The minimum set of β-lactam compounds included for susceptibility testing with the recombinant clone should be: ampicillin, piperacillin, cefalothin (or cefazolin), cefotaxime, ceftazidime, cefepime, cefoxitin (or cefotetan), aztreonam, imipenem, meropenem and ertapenem.

Further characterisation of a new enzyme should ideally include analytical isoelectric focusing, sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS-PAGE) analysis, mass spectrometry and determination of kinetic parameters with the purified enzyme. The minimum set of substrates included for kinetic characterisation is the same as those listed for susceptibility testing of the recombinant clone (see above).

The availability of data for a core set of representative substrates collected under homogeneous experimental conditions will facilitate comparisons between different enzymes, which have often been difficult to perform owing to the differences in the substrates tested and in the conditions adopted for experimental measurements.

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