New-Delhi Metallo-β-Lactamase

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A Global Review for a Global Threat

By

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ABSTRACT

New-Delhi metallo-β-lactamase (NDM) is one of the most worrying bacterial resistance markers, due to its worldwide dissemination and its ease to be transferred as gene or protein from resistant to susceptible bacteria. Currently, research focuses around potential inhibitors, some of which display high K_i on the enzyme alone, but display much weaker results against the bacteria or in *in vivo* tests. To avoid such disappointments, this review covers knowledge from all concerned areas, such as chemistry, microbiology, structural biology, genetics, and pharmacy. It also links the knowledge from these different domains to display a full picture of the current knowledge about NDM. The review highlights thus the evolution of NDM over time, its mechanism of action, its mutations and transfer pathways, as well as the currently known ways to inhibit NDM, just as much as genetic features which caused the anchoring of NDM to the outer membrane, endowing it with the capacities to overcome scarce zinc conditions, more stability, and several other characteristics. To find new tools against NDM, this review aims to gather all information about NDM from any research field, and to explain causes and consequences in a language understandable for all chemists.

INTRODUCTION

Since the work of Sir Alexander Fleming from 1928 showing the inhibiting effect of the *Penicillium notatum* mold on *Staphylococcus aureus*, and the large scale use of benzylpenicillin during the Second World War, the β-lactam antibiotics demonstrated their efficiency and their relative non-toxicity.[1,2] To date, they have become one of the most used drugs, representing about half of all prescribed antibiotics[3]. Their mechanism of action is based on the inhibition of the transpeptidase enzymes catalyzing the peptidoglycan cross-coupling by forming covalent complexes,[4] preventing them to counterbalance the action of cell wall hydrolases[1] during the bacterial cell wall biosynthesis,[4,5] thus leading to the destruction of the bacterial membrane. Hence, β-lactam antibiotics apply strong selective pressure on bacteria, which forced them to evolve and develop resistance pathways through β-lactamase gene expression in order to survive.[6,7]

In Gram-negative bacteria, the most recent of the β-lactamase enzymes was discovered in Sweden in 2008, in a *Klebsiella pneumonia* strain isolated from a patient that had been hospitalized in New-Delhi, India, not long before. Although nothing unusual was found when the patient came to the hospital in the first culture sample, two months later from the same sample scientists observed an unusual resistance to all tested antibiotics except colistin. After sequencing of the bacterial isolate by gene libraries and restriction profiling, three resistance domains were identified, containing a $bla_{\text{CMY-4}}$ gene responsible for the AmpC phenotype (a type of β-lactamase genes ; *bla* stands for β-lactamase), a complex class-1-integron carrying a unique set of resistant genes, and a genetical sequence that had never been seen before. Resistant to cloxacillin but susceptible to EDTA (ethylenediaminetetraacetic acid), this new gene was recognized as a β-lactamase enzyme containing metal ions. According to the rules for naming the genes, it was named New-Delhi Metallo-β-lactamase, NDM (or NDM-1 to speak about this first discovered variant).[8]

It turned out that an *Acinetobacter baumannii* isolate from India was documented in 2005, and further analysis showed that it carried this resistance gene. To date, it is the oldest reported identified NDM.[9] The retro analysis of 1443 Enterobacteriaceae isolated from Indian hospitals between 2006 and 2007 found 26 carbapenemase-producers, 15 of which (57.7 %, 2.7 % overall) expressed the NDM-1 gene.[10]

In the following years, this NDM-1 gene was observed several times, and its occurrence in India and Pakistan grew exponentially. In 2009, a Study for Monitoring Antimicrobial Resistance Trends (SMART) identified 235 ertapenem-resistant Enterobacteriaceae species isolated worldwide and concluded that 28 % had a carbapenemase gene, 14 % of which carried NDM-1.[11] Infected strains were mainly *Klebsiella pneumonia* and *Escherichia coli*, but some *Escherichia cloacae*, *Providencia rettgeri,* and *Morganella morganii* strains carried the gene too. All these NDM-1-producing strains had been isolated from Indian patients, suggesting that the gene is endemic in the Indian sub-continent.[12–16] Last but not least, a study revealed that out of 33 carbapenem-resistant *Escherichia coli* recovered in south India between November 2013 and February 2014, 15 isolates (45 %) were NDM-1-positive.[13] These are just a few examples clearly demonstrating the exponential spread of the NDM gene and its endemic status in the Indian subcontinent.[16,17]

Since its discovery fifteen years ago, more than 50,000 cases of NDM-producing bacteria strains were reported. Because the gene of NDM (noted bla_{NDM}) is often accompanied with other resistance markers,[17,18] bacteria often display a high resistance against several families of antibiotics at the same time. The rare therapeutic solutions to treat such infections can cause strong side effects, making it all the more urgent to find a solution to inhibit or get around this enzyme.

Many excellent reviews were written about NDM, highlighting either the genetic aspects, its different mutations, its catalytic mechanism which vary a little depending on the antibiotic substrate (penicillins, cephalosporins, or carbapenems), its structure, or the currently investigated inhibitors. However, rare are those reviews which link these pieces of information together. Indeed, the unique genetic characteristics of bla_{NDM} drive some

specificities in its structure, for instance making the enzyme more resistant to the absence of its zinc ions cofactor. Whereas chemists developed several zinc chelators because those are efficient on other metallo-β-lactamases, NDM is able to pick up new zinc ions, making such inhibitors much less efficient. Previous reviews on NDM generally focused on one or two aspects only because they target a specific audience, but ignore or only briefly mention other aspects without giving causes and consequences. This absence of bridges between the information from different scientific fields, and the use of specific terms for genetics, microbiology, or structural biology in the precedent reviews makes it difficult for chemists to fully understand the issues caused by NDM enzyme. Hence chemicals that act as good inhibitors *in vitro*, often display much weaker results *in vivo*.

The current review describes how and why bla_{NDM} emerges and disseminates around the world, and then highlights the structure of the enzyme. Structural investigations explain why the hydrolysis mechanisms display slight variations between the different β-lactam antibiotics. As medical doctors try to counteract NDM, some of the mutations which naturally and randomly appeared were favoured by selective pressure, resulting in certain structural changes. These changes enable the NDM-producing bacteria to better resist to e.g. scarce zinc conditions. This and the capacities of NDM to be transferred through all the possible ways from a resistant to a susceptible bacteria (vertical transfer, horizontal transfer, outer-membrane vesicles) resulted in an exponential increase of NDM-producing strains worldwide. Finally, we describe all potential inhibitors which were published to date, classifying and comparing structures, and pointing out the association of different chemical functions which are displaying certain antibacterial properties on their own. Readers will find in this review all relevant information about NDM from the past ten years, the discovery of NDM enzyme being relatively recent (15 years). Some papers from as early as 1972 are included to shed light on the timeline of antibiotic families' discoveries or to describe characteristics of metallo-proteins.

I. NDM RESISTANCE WORLDWIDE

1. β-lactamases emergence

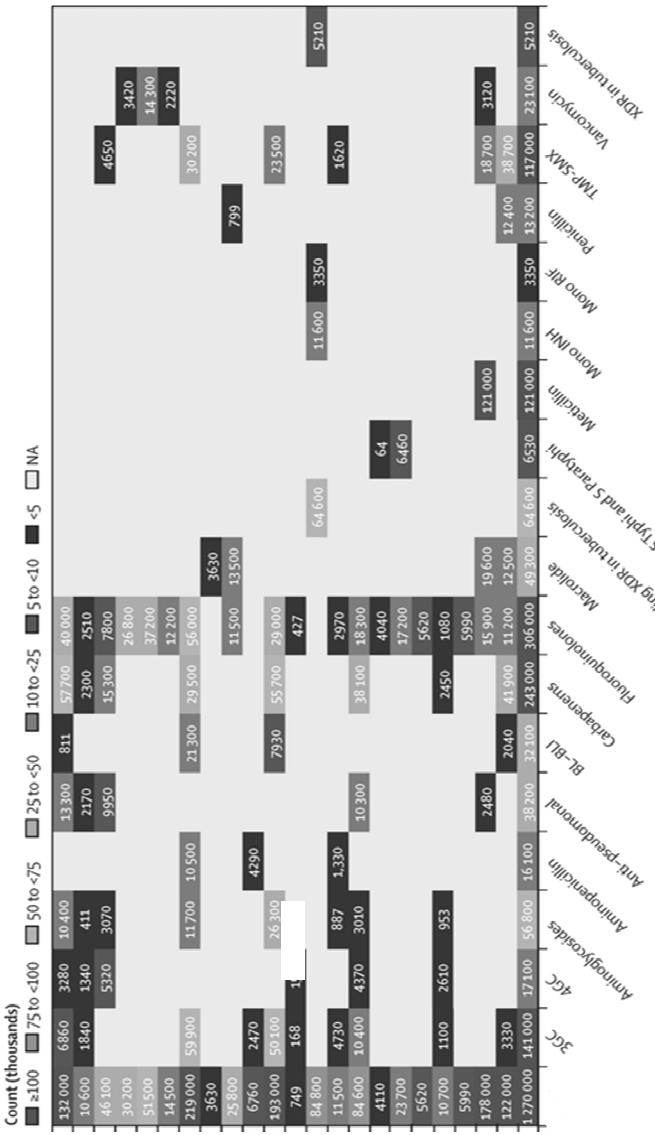
Confronted with an overwhelming presence of β-lactam antibiotics in the environment, bacteria have developed always more effective β-lactamase genes: for some years, their copy number and their diversity surged: in 2012 more than 1000 different β-lactamases were identified and classified,[19] and in 2017, there were more than 1300 different β-lactamase genes reported.[20] In parallel, the β-lactam antibiotics were steadily improved with penicillins, carbacephems, and then cephalosporins of 1st, 2nd, 3rd, and 4th generation. However, these antibiotics always encountered higher resistance from bacteria expressing "extended-spectrum β-lactamase" genes (ESβLs), able to hydrolyze all β-lactam antibiotics except monobactams.[21,22]

Interestingly, a study from 2013 linked patients infected with resistant bacteria to high socioeconomic status (skilled worker 86 %, access to electricity 78 %, no farmers 75 %), demonstrating the link between the access to health care services and the proliferation of these problematic pathogens. Additionally, 81 % of the contaminated patients reported an antibiotic treatment within the last 3 months,[6] leading to the selection of bacteria carrying resistance genes. This effect is aggravated by the wide use of these β-lactam antibiotics,[11,23] which can be uptaken without prescription even as a "precaution use".[7,16] Moreover, these antibiotics are often used for animal treatments. Thus, some studies have even found traces of β-lactam in rivers or seepage waters.[24,25]

To treat these highly β-lactam resistant bacteria, another type of β-lactam antibiotics was developed: named carbapenems, they are considered as drugs of last resort.[1,15] Although imipenem and meropenem are considered as the most potent carbapenems thanks to their high affinity for penicillin-binding proteins and the non-affinity of ESβL enzymes (extended-spectrum-β-lactamases) for them, their weak ability to pass through the bacterial outer membrane, and the increasing emergency of carbapenemase

enzymes such as NDM are creating a major issue, letting only a few therapeutic options for treating this type of resistant bacteria. The infections caused by, e.g. pathogenic carbapenemase-producing-Enterobacteriaceae, may lead to death in more than 40 % of the cases.[11] Thus, one of the widest studies focusing on the cause of hospitalizations and deaths worldwide showed an extreme prevalence of deaths attributable to β-lactams resistance (3GC, 4GC, aminopenicillin, anti-pseudomonal, βL-βLI, carbapenems, and penicillin columns on **Figure I-1**), which lead to half a million of deaths in 2019 (39 % of the studied cases), and the most worrying resistances concern the carbapenems and the cephalosporins of 3rd generation.[26]

Figure I-1 *(next page): Global deaths (counts) attributable to bacterial antimicrobial resistance by pathogen–drug combination in 2019*
Reprinted from Murray et al., The Lancet, 2022, 399 (10325), pp. 629-655, © CC-BY License[26] For this figure, only deaths attributable to resistance, not deaths associated with resistance, are shown due to the very high levels of correlation for resistance patterns between some drugs. 3GC: 3rd-generation cephalosporins. 4GC: 4th-generation cephalosporins. Anti-pseudomonal: anti-pseudomonal penicillin or beta-lactamase inhibitors. BL-BLI: β-lactam or β-lactamase inhibitors. MDR: multidrug resistance. Mono INH: isoniazid mono-resistance. Mono RIF: rifampicin mono-resistance. NA: not applicable. Resistance to 1+: resistance to one or more drug. TMP-SMX: trimethoprim-sulfamethoxazole. XDR: extensive drug resistance. ABau: *Acinetobacter baumannii* ; Citro: *Citrobacter* species; Entero: *Enterobacter* species; EFaeca: *Enterococcus feacalis*; EFaeci: *Enterococcus faecium*; Enteroc: Other *Enterococci*; Strept A : Group A *Streptococcus*; Strept B: Group B *Streptococcus*; HInf : *Haemophilus influenzae*; KPneu : *Klebsiella pneumoniae*; Morg : *Morganella* species; MycoTub : *Mycobacterium tuberculosis*; Proteus : *Proteus* species; PAeru : *Pseudomonas aeruginosa*; SPara : *Salmonella enterica serotype paratyphimurium*; STyphi : *Salmonella enterica serotype typhimurium* ; Salmo : Non-typhoidal *Salmonella* ; Serra : *Serratia* species; Shig: *Shigella* species; SAureus: *Staphylococcus aureus*; SPneu: *Streptococcus pneumoniae*
Please see the centrefold for this image in colour.


Count (thousands)
≥100
75 to <100
50 to <75
25 to <50
10 to <25
5 to <10
<5
NA
3GC
4GC
Aminoglycosides
Aminopenicillin
Anti-pseudomonal
BL–BLI
Carbapenems
Fluoroquinolones
Macrolide
MDR excluding XDR in tuberculosis
MDR in S Typhi and S Paratyphi
Meticillin
Mono INH
Mono Rif
Penicillin
TMP-SMX
Vancomycin
XDR in tuberculosis

2. Worldwide presence

a. Tourism and medical tourism

In 2010, almost all cases of infections caused by NDM-1 in the UK can be linked to India or Pakistan.[16] However, frontiers do not stop the NDM-1 gene dissemination and clonally-linked NDM-producing bacteria isolates can be observed in neighboring countries.[27] The exponential spreading of NDM-1 (and other β-lactamase genes) is worsened in these times by global trends of tourism and frequent long-distance travels.[28] Indeed, traveling in exotic countries exacerbates the risk to be contaminated by an unusual bacterium and to bring it back, or to contaminate the population with a locally unknown pathogen. Bacteria are disseminating worldwide at a much higher rate than ever before.[11,17,28,29] For instance, in 2014 several pilgrims returning from Saudi Arabia were tested positive for *bla*$_{\text{NDM-5}}$ gene, whereas they were negative before their travel.[30]

Isolates harboring this gene have been identified outside the Indian sub-continent, and although some years ago, *Klebsiella Pneumoniae* Carbapenemase (KPC) was the most frequent carbapenemase in some countries (USA, Israel, Turkey, China, India, UK, or in Scandinavia), NDM-1 has now replaced it.[7,8,31] Already in 2014, the gene was reported in more than 40 countries.[32] To date, NDM is currently present on all the continents (**Figure I-2**),[33] and its presence was reported further in Asia (Viet-Nam, Japan[15], China[7,15,34], South Korea[35], Malaysia[35], Hong Kong[7], Taiwan[15,36], Bangladesh, Singapore[15]), in Oceania (Australia[15], New-Zealand), in the Middle-East (Sultanate of Oman (11 NDM-1-positive isolates between October 2010 and March 2011)[37]), Lebanon[7], Israel[15], Turkey[15], EAU), in Africa (South Africa, Kenya[7,15], Maghreb[31], Madagascar), in Europe[7] (France[15,35], Serbia, Germany[35], Netherlands[7], Italy[7,31], UK[7], Belgium[7], Balkans[7], Spain[29], Denmark), in the USA[7,15,35] and Canada. It is noted that the less infected regions are for now Central and South America, and Antarctica.[7,33,38]

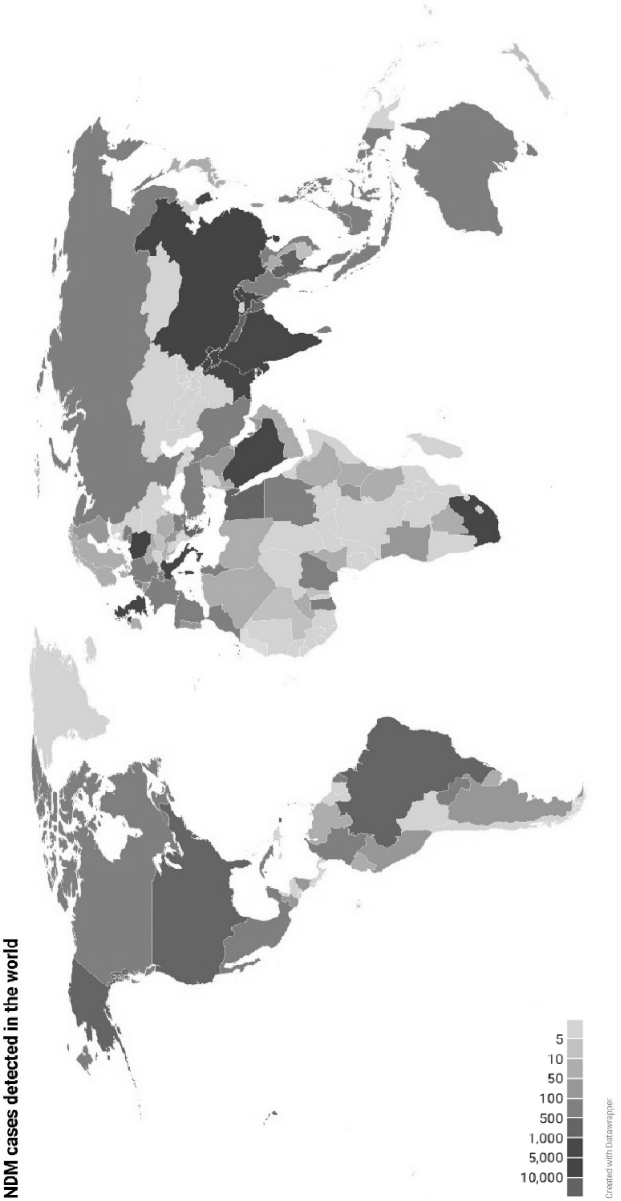

NDM cases detected in the world
5
10
50
100
500
1,000
5,000
10,000
Created with Datawrapper

Figure I-2 *(previous page): Number of New-Delhi-Metallo-β-lactamase positive bacteria detected in the world, per country.*
Method: research on Google Scholar, with key words NDM bacteria AND "Country" between June and October 2022. The resulting papers were considered according to the relevance of their title with the research, and research was stopped when no results were identified on two consecutive full pages. Papers were then taken in account only if information such as the number of cases and the size of the initial samples were provided, regardless in the abstract or in the text if available. Please see the centrefold for this image in colour.

Another cause of bacterial propagation is medical tourism. As carbapenemases may cause severe infections with long hospitalization times, hospitals are a place where cross-infections can easily occur. Special procedures should be applied to isolate the patients in case of resistant-bacterial infections to avoid any cross-infections.[15] Sometimes these procedures may not be strictly applied, either due to a lack of knowledge, of equipment, or because the resistant bacteria are not observed immediately.[8,35] This results in nosocomial infections, proved by the identification of clonally related cultures in different patients.[7]

Moreover, the use of antibiotics and biocides in hospitals leads to the development of strong resistance factors.[39] Thus in a population of *K. pneumoniae* bacteria, the majority of which expresses the KPC carbapenemase and a minority NDM-1, the treatment with gentamycin (KPC-bacteria are susceptible, NDM-1-bacteria are resistant) led, after 24h, to a complete overthrow of KPC-bacteria by NDM-1-bacteria.[40] Hospitals are therefore a place of choice to select strong multi-resistant isolates: the study of some *A. baumannii* isolates from clinical origin showed high expression of resistance markers, resulting in multiresistant isolates: among those expressing metallo-β-lacatamases (NDM-1 or VIM, 40% of the isolates), 54 and 77 % displayed higher MIC to respectively chlorhexidine and cetrimide biocides (and not antibiotics!) than the recommended concentrations for disinfecting.[41]

In a general way, India is one of the major consumers (and producers) of drugs, especially β-lactams, even if there was in the last decade awareness of the need to reduce antibiotic consumption. It is henceforth not surprising that it is one of the countries where the rate of resistant bacteria is the highest.[42] Among all these cases, the first ones observed in a country are

often linked with recent traveling and/or hospitalization in the Indian subcontinent.[35] Following a study on 29 European countries between 2008 and 2010, 77 infections (7 lethal) caused by NDM-1-positive isolates were reported in 13 countries. Authors related that over the 77 source patients, more than 70 % had recently traveled, 43 % had traveled or been hospitalized in India or Pakistan, 6.5 % were hospitalized in the Balkans, and 13 cases were due to nosocomial infections.[7]

In the Balkans, where there is a large medical tourism to the Indian subcontinent for kidney transplantation, a secondary cluster of NDM-1 seemed to evolve with the first case reported in 2007.[17,43] To our knowledge, no bacteriological study specifically targeting NDM-1 isolates was conducted for now on these patients after their return, so the hypothesis cannot be directly confirmed. Yet, N. Ivanovski and colleagues reported for 36 patients frequent cases of *K. pneumoniae* and *E. coli* infections linked to these unregulated transplantations, causing for example 13 wound infections, 6 abscesses, 9 hepatitis C, leading to more than 42 medical issues and 7 deaths (compared to only 4 urinary infections reported for patients transplanted in Skopje, Macedonia).[43,7]

b. Environment and water wastes

Resistant bacteria do not only disseminate in hospitals, as not all NDM-1-positive tourists in India have a hospitalization history. Acquisition of resistant bacteria can occur from the environment.[17]

Indeed, sewage and wastewaters raise a specific challenge worldwide. The resistant bacteria causing urinary infections or present in the intestine tracks are found in the water sewage,[6,7,17] and if the wastewaters are not well decontaminated, bacteria can easily spread. These bacteria are therefore present in the influent wastewater of treatment plants, and although the wastewaters should be microbiologically neutralized, this is not always the case. Several NDM-producing isolates were found in wastewater samples, treatment plants, and their receiving rivers.[6,44–57] Thus, *K. pneumoniae* carrying bla_{NDM} were documented in the seepage of a hospital in Switzerland.[57] Two studies led in China between 2013 and 2016 highlighted the risk of environmental contamination, showing the presence of NDM-1-

positive *Achromobacter* species in a wastewater treatment plant, including the final effluent (between $1.3*10^3$ and $2.7*10^3$ cfu/mL, used for farm irrigation and aquaculture) and the waste dewatered sludge ($5*10^7$ cfu/mL, used as fertilizer),[6] and demonstrating at the same time that some NDM-1 species found downstream in the river were not observed upstream of the wastewater treatment plant.[58] And after the first observation around New-Delhi in 2010, one-third of the surface waters and seepage in New Delhi and more than 60 % of the environmental waters in Bangladesh seem to be now contaminated by NDM-1-harboring bacteria.[44,59]

The presence of NDM-1 in the water is an advantage for its dissemination into the environment in tropical countries, e.g during the monsoon period with floods or drains overflowing.[17] Moreover, the dissemination of NDM-harboring pathogens in the environment is a vicious circle, as in reaction to the spread of NDM-1 in the river, some studies have isolated NDM-1-positive bacteria in tap water.[7] For instance, 4 % of the tap water samples taken in New Delhi contained NDM-1-carrying bacteria, concerning wide varied bacteria species, opportunistic ones as well as pathogen ones, like *E. coli*, *K. pneumoniae*, *Shigella boydii*, *Aeromonus cavia*, *Vibrio cholera*, *Stenotrophomonas maltophilia*, *Citrobacter freudii,* or *P. aeruginosa*.[17] This situation is highly worrying, as the most infected countries are China, India, and surrounding countries, which are also the most populated ones, containing half of humanity,[44] showing the potential for a medical disaster.

c. Communities and hygienic factors

This is why NDM-1-expressing bacteria may be also acquired through the community. A patient hospitalized in China was hence infected by an NDM-1-harboring isolate, although she did not report any travel, and no other NDM-1-positive bacteria was found in the hospital over a period of two months, suggesting contamination during daily life.[60]

In low-income countries where some inhabitants have no access to clean water or sewage services, the resistant bacteria cause high mortality,[16] particularly among newborns and young mothers.[61,62] Hence, research on ESβLs transmission in Madagascar highlighted the case of the first NDM-1-contaminated Malagasy patient, not reporting any travel neither recent

hospitalization or β-lactam antibiotics uptake. The authors concluded that contamination occurs probably via a feco-oral transmission.[61] *E. coli* is indeed the main pathogen acquired inside communities, and the first cause of diarrhea in children on the Indian subcontinent. The risk to be contaminated with this kind of transmission is strongly dependent on access to sanitation.[17] Associated with the lack of toilets and sanitation in some regions (according to the United Nations Organization, about 650 million of the Indian inhabitants "do not have access to a flush toilet, and even more probably do not have access to clean water, […] and only 60 % of the New Delhi population is served by the sewerage system"),[17] it can participate in the dissemination of bacteria and NDM in the environment and into the community. Moreover, in tropical countries, a higher prevalence of resistant genes is reported during the monsoons season, associated with an increase in bacterial diarrhea and feco-oral transmission.[16,61] This fact confirms the community acquisition,[31] increasing with the rate of overpopulation.[7,16]

Moreover, β-lactams as penicillins count among the three most used families of antibiotics in animal-intensive farming.[63] The close proximity of animals in such kind of farms promotes the circulation of diseases: when transferring NDM-negative broilers into a contaminated farm, almost 19 % were contaminated in less than 24 h.[64–66] Antibiotics are then widely used even in a prophylactic way or as a growth factor, as an animal in good health produces more meat, milk, or eggs. Antibiotic residues can later be found in animal products, but also in the environment,[6,36,72] however, some resistant bacteria isolates could carry so many resistance factors that a standard cleaning and disinfection process does not succeed to eliminate all of them.[67] NDM-positive isolates were also discovered in fruits, vegetables, and (wild) animals,[48,68–77] and thus in food, such as chicken,[6,36] swine,[75,78] or seafood.[78]

Some transmission to humans could occur, as some NDM-positive isolates enable maintainence even when animal-origin food is conserved in cold conditions or sterilized by irradiation.[79] Moreover, farms are not closed systems, and the surrounding area can be contaminated: workers through contact,[80–84] waters and soils through faeces,[77,85,86] and wild animals (mice, birds) through contact.[85] Thus, the high use of antibiotics in these intensive farms leads to the large contamination of their environment.[6,36,75,87–91] It was

also established that the prevalence of resistant bacteria around them is higher than their overall presence,[64–66] confirming the cause-and-effect relationship between antibiotic misuse and the apparition of resistant bacteria that embodies once more the importance of the hygienic rules for food conservation, food consumption, and the need to promote proper food hygiene by taking effective measures.[78]

These last years, there have been therefore serious calls to ban several recently discovered antibiotics from veterinary use.[92] The first among those countries which adopted measures to decrease antibiotic usage was Sweden which has prohibited the usage of antibiotics as growth factors since 1986.[93] In the 90s, Denmark forbid the use of antibiotics for preventive treatments and restricted the availability of antibiotics for disease treatments. Then, the European Union started to ban the use of certain antibiotics, and, in 2006, forbade their use as a growth factor, establishing a strict control over their use.[94,95] Some countries also decided to take the last step by forbidding the use of certain drugs for animal purposes, like France which bans the use of "critical antibiotics" such as the last β-lactamase-resistant cephalosporins, for animal treatment.[95,96] In other countries, laws are more flexible, like in Brazil, Canada, India, and Russia, which adopted only certain specific prohibitions or constraints,[42,95] and the USA "suggested" not to use human antibiotics as growth factors. On this subject, the Canadian Journal of Law and Society published a comparative study of the legislation of different countries, and part of their results.[95]

To conclude this chapter, the NDM-1 bacterial enzyme has emerged within the context of a sprint between bacterial survival, misuse of antibiotics, and the development of new β-lactam inhibitors. Product of gene recombination in A. baumannii, the gene was first observed in India, and to date, it has become endemic in certain parts of the world. Its existence in the environment is now acted, and its dissemination occurs rapidly, helped by wide-use of β-lactams, long-distance travel, medical tourism, difficult access to sanitation, tropical climate, and high population densities. As bla_{NDM-1} *has one of the fastest spreads among the MBLs genes, it could therefore be interesting to focus more on its possible dissemination ways.*

II. STRUCTURE AND MECHANISM

1. Classification

The β-lactamases can be classified into the three Bush-Jacob categories based on their functions, but are more often referenced according to the four Amber classes, based on their sequence.[15,97] While classes A, C, and D are nicknamed "serine-β-lactamases" due to the nucleophilic serine residue in their active site, class B is named "metallo-β-lactamases" because of their need for metal ions as co-factors. Class B is then divided into three subclasses, depending on the number of zinc ions in their active site. Whereas B3 enzymes need two zinc(II) ions to hydrolyze β-lactams and B2 subclass gathers proteins working only with one ion, the proteins which belong to the B1 class, such as NDM, VIM, IMP, or SPM, contain two zinc ions but are able to work with only one, exhibiting, in this case, less efficiency in their catalytic activities.[97,98]

Weighting 28 kDa, NDM-1 is a monomer composed of 269[8] or 270[1,44,97–99] amino acid residues. As all B1 proteins, NDM possesses an αβ/βα sandwich fold structure, consisting of four α-helices surrounding two antiparallel β-sheets in the middle (**Figure II-1**).[97] Nevertheless, the secondary structure and zinc(II) ion co-factors are the only two characteristics shared between NDM and the other subclass B1-proteins, as they have very different primary sequences, with only 32.4 % identity between NDM-1 and VIM-1/-2, the closest one.[8,97]

On certain crystal structures of NDM-1, a small α-helix is found between β11 and α6, gathering Gly207, Cys208, Leu209, and Ile210 (PDB structure 5WIG, for instance), where it should be noticed that Cys208 plays a role in the active site (see next subchapter, II.3 The active site). Its absence on other crystal structures can be explained by its short size (4 amino acids against theoretically 3.6 residues per turn). However, the replacement of the Leu209 by a phenylalanine residue results in a drop in the catalytic activity of NDM-

1. This suggests more significant consequences than just the influence of the neighbor, for instance the breaking of this potential α-helix.[100]

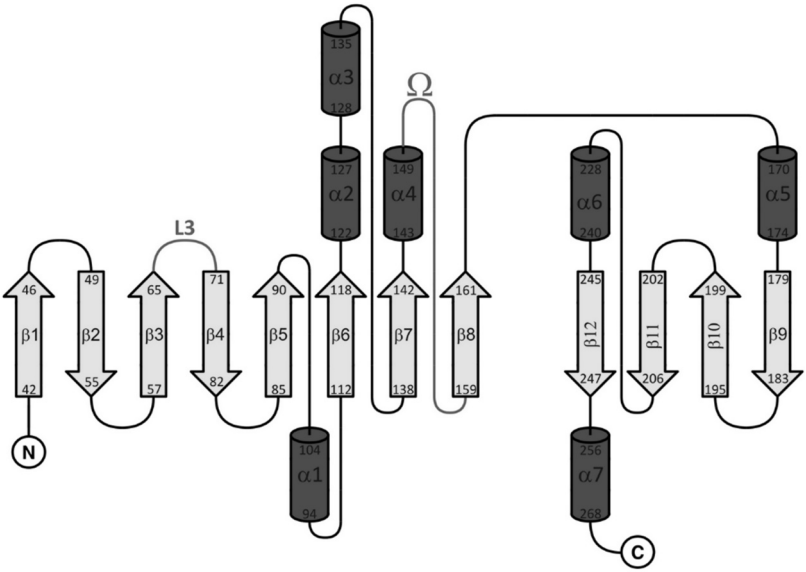


Figure II-1: *Topology diagram of NDM-1*
from Raczynska et al., Int. J. Biol. Macromol., 2020, 158, pp104-115, © 2020 Elsevier B. V.[101] The β-strands (yellow arrows) and helices (red cylinders) are numbered consecutively. The inclusive residue numbers correspond to secondary structure assignments in PDBsum database. Two accessory loop regions, namely L3 and Ω, that are present in numerous variants of NDM are highlighted in blue and green, respectively. This topology diagram was prepared in TopDraw.

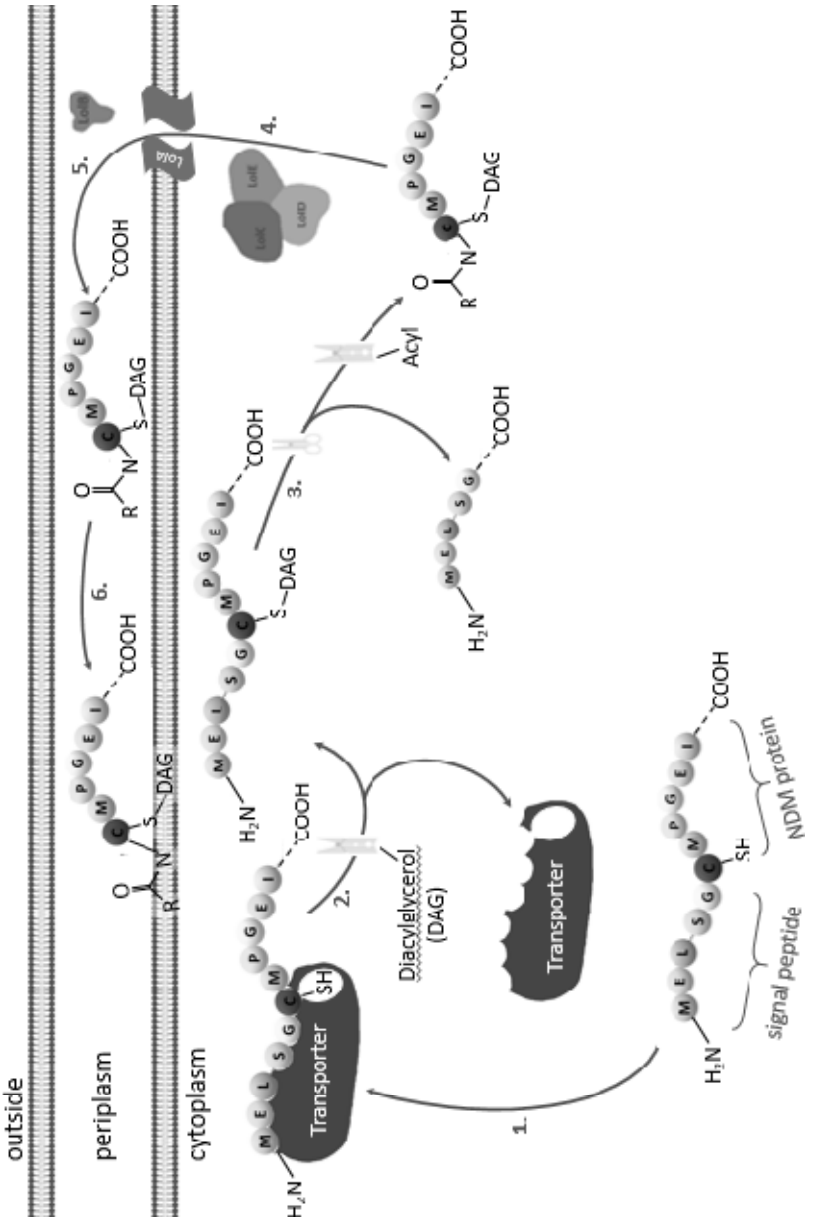
2. Anchored to the outer membrane

Production of NDM starts with a precursor, synthesized in the cytoplasm.[102] As with all newly synthesized proteins, it possesses a signal peptide. This short sequence should be recognized by some transporters and/or enzymes into the bacteria, inducing migration of the enzyme from its synthesis place to its final destination (**Figure II-2**). Its signal peptide contains mainly hydrophobic residues, and it is recognized and exported either by the secretion pathway or sometimes by the twin-arginine translocation system to the inner membrane (**Figure II-2**, 1st step).[44,102,103] A second enzyme adds a diacylglycerol to the thiol of the conserved cysteine in the signal

peptide part (**Figure II-2**, 2nd step). Then, a third enzyme cleaves the protein just before this cysteine, and the free amine is bound to an acyl group (**Figure II-2**, 3rd step).[103] The protein is then taken over by the *LolCDE* enzyme, which brings it to the *LolA*, an enzyme shuttling across the inner membrane layer (**Figure II-2**, 4th step). Finally, *LolB* recovers the protein and takes it out of the inner membrane (**Figure II-2**, 5th step).[103] NDM is in the periplasm, where it folds and binds zinc ions. Its N-terminal part, starting by the acylated-cysteine, anchors it to the outer membrane (**Figure II-2**, 6th step).[103] This anchoring, through hydrophobic interactions (the protein is not detached by the addition of KCl or Na_2CO_3) is resilient and resists centrifugation.[44]

Anchoring to the periplasmic inner membrane is a particularity of NDM, shared with only two other β-lactamases. Indeed, most B1 MβLs, such as VIM, IMP, or SPM are soluble periplasmic proteins.[44,102,104] Lipoproteins are common in Gram-positive bacteria to avoid the loss of enzymes in the medium as they possess only a single-layered membrane, but is considered as a curiosity in Gram-negative bacteria that possess a bi-layered membrane. However, anchoring favors a homogeneous dispersion of the enzyme along the bacterial membrane, although soluble MβLs tend to accumulate at the cell poles, forming inclusion bodies.[44] In this regard, NDM has an advantage, and even displays a strong efficiency[44]: it got 100 % anchoring to the periplasmic face of the outer membrane,[104] shared with PenA (100 % anchoring in *Escherichia coli*), but the third one BRO-1 is much less efficient (10 % of anchoring in its natural host *Moraxella catarrhalis*, 45 % in *Escherichia coli)*.

Figure II-2 *(next page)**:** Pathway of NDM from synthesis in cytoplasm to the anchoring inside the outer membrane*
Please see the centrefold for this image in colour.


outside
periplasm
cytoplasm
Transporter
Diacylglycerol (DAG)
Transporter
Acyl
LolA
LolB
LolC
LolD
signal peptide
NDM protein
1.
2.
3.
4.
5.
6.

3. The active site

The two metal sites, commonly noted Zn1 and Zn2, are surrounded by amino acid residues able to form interactions with the zinc(II) ions (**Figure II-3**). Zn1 is bound with three histidine residues (H120, H122, H189), and Zn2 is bound by one histidine residue (H250), one aspartate (D124), and one cysteine (C208). One water molecule and one *in situ*-formed hydroxide ion are also involved in coordination, one bridging both zinc ions, and the other coordinating only Zn2, but there are some discussions about knowing which of both is bridging the metal ions (see subchapter II.4.c, Mechanistic adjustements). This results in a tetrahedral coordination for Zn1[97,105] and a coordination number of five for Zn2 with an approximative trigonal bipyramidal geometry, even though the active site being on the surface of the protein, the number of coordinated water molecules varies in the known crystal structures that have been determined for NDM so far. Thus, the Zn2 site is discussed as having a trigonal bipyramidal[98,101,106,107] or a tetrahedral[5,108] environment.

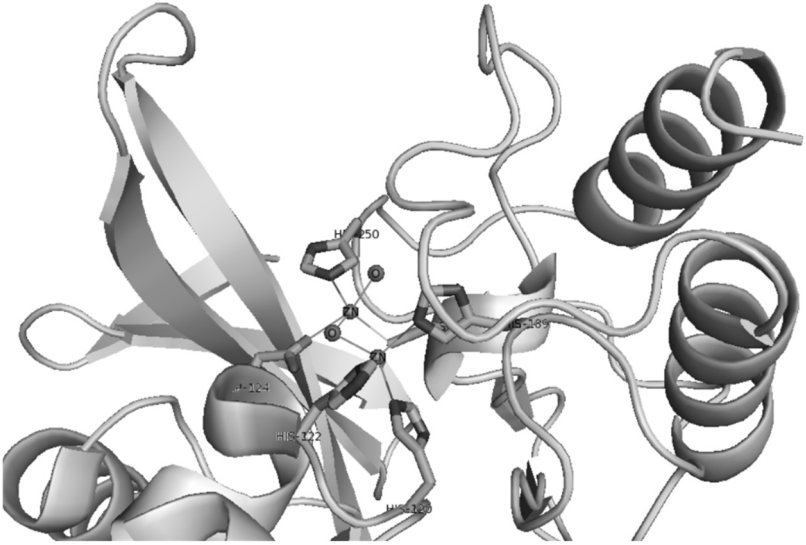


Figure II-3: *Active site of NDM protein*
RCS PDB structure 5ZGZ, Zhang et al. Antimicrob. Agents Chemother., **2018**, 62

The main roles of Zn1 are the stabilization of the negative charge which is formed on the oxygen atom of the β-lactam amide during the hydrolysis,[105] and the coordination of a water molecule which is nevertheless not always found in the X-ray structures. Zn2 is there to stabilize the carboxylate of the β-lactam antibiotic.[1]

a. Zinc affinity

Between the two zinc ions, Zn1 is bound tighter by three histidines, and the dissociation constant of the one within Zn2 is estimated to be 2 μM.[98,109] No precise values were found in the literature about the affinity of zinc ions for the two Zn1 and Zn2 sites of NDM proteins. However, Wommer *et al.* determined the binding constants of zinc ions of the B1 metallo-β-lactamase *Bacillus cereus II* (BcII): in absence of any substrate, the dissociation constants are 1.8 nM and 1.8 μM for Zn1 and Zn2, respectively.[110] As the active site of BcII and NDM are similar (HAHAD – H – C – H and HAHQD – H – C – H respectively, the A and Q did not participate in the zinc-binding itself), and due to the similar values found in the reference 109 compared to references 98 and 108 (1.8 μM versus about 2 μM), it seems reasonable to extend the dissociation constant values for BcII to NDM enzymes, while noting the enhancements brought by some mutations to tight zinc ion binding (see III.2.a Overcome zinc scarcity).

However, these values can vary depending on the presence or the absence of a substrate. When binding to imipenem, variations for Zn2 site are slight (1.8 to 0.8 μM), but Zn1 site encounters a tighter binding with a 100-fold improvement factor, from 1.8 nM to 13.9 pM.[110] This is due to some conformational changes around the zinc ions caused by the presence of a β-lactam antibiotic in the binding pocket. Among others, the binding of the carboxylic acid of the β-lactam release the water molecule which is sometimes presented as binding to Zn2, and the coordination geometry changes: as will be discussed later, penicillins accommodation results in pentahedral coordination around Zn2, whereas carbapenem binding leads to an octahedral conformation.[5]