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New Delhi Metallo Beta Lactamase: Menace and its Challenges

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Abstract

Clinicians have been facing an enormous challenge of treating infections caused by multiple drug resistant (MDR) pathogens since long. The latest and most alarming of such challenge is the emergence of New Delhi Metallo-β-Lactamase-type 1 (*NDM-1)* producing clinical isolates. *NDM-1* is a metallo β-lactamase that confers resistance to all β-lactam antibiotics including carbapenems generally regarded as last resort to treat infections. *NDM-1* is part of a huge conjugative plasmid bla*NDM* capable of rapid dissemination via horizontal gene transfer, transposition and recombination. Therefore, it has become a matter of global concern now as these pathogens have surpassed all geographical barriers and are threatening the public health all over the world. In addition to this, *NDM-1* gene coexists with other resistance determinants such as other MBLs or porin mutations. Plasmid also carries genes conferring resistance to other antibiotic classes such as 16S RMTases, qcr, or mcr-1 gene imparting resistance to aminoglycosides, fluroquinolones and colistin respectively making current therapeutic recourse ineffective. If not addressed immediately, this resistance and its dissemination will bring us to a therapeutic dead end. In the present review, we have discussed the global spread of *NDM-1* and its variants, its structural challenges that currently limit inhibitor drug designing, along with focusing some light on immediate measures that can be adapted at healthcare facilities with review of recent pharmacologic agents under research effective against *NDM-1*.

Keywords: New Delhi-Metallo-Beta-Lactamases (NDM); Carbapenemase; Antibiotic resistance

Introduction

Rapidly emerging antimicrobial resistance associated with healthcare infections has always been a significant concern with evolving complications. Such constantly emerging 'Superbugs or Multi-Drug Resistant (MDR)' pathogens have always presented human community with intricately braining need of developing novel mechanisms to combat such adaptations. In this light, the learning of NDM challenges the effectiveness and usefulness of even most potent beta lactam group of antibiotics, such as carbapenem in infections caused by related pathogens [1]. These powerful enzymes are capable of hydrolyzing the beta-lactam ring comprising an essential part of these antibiotics rendering them ineffective. The possible reason for evolving resistance is mutations [2]. The overuse of antibiotics is one of the other reasons to cause resistance, as selective effectiveness against susceptible bacteria of specific population allows the resistant bacteria to grow. Several key players for antibiotic resistance have been described which help clear understanding of evolving status of β-lactamase enzymes [3].

Widespread use of Carbapenem class increased due to emergence of methicillin resistant *Staphylococcus aureus* (MRSA), vancomycin resistant Enterococci (VRE) or extended spectrum β-lactamases (ESBLs) producing Gram negative bacteria. However, this continuous clinical usage of this class such as meropenem and imipenem further lead to resistance development. The advancement to ESBL's was the *Klebsiella pneumoniae*. Carbapenemases (KPC) followed by *NDM-1*, altogether bestowing resistance to most effective carbapenems in gram negative bacteria [4]. The former class is a serine protease while NDMs are broad-substrate spectrum metallo-enzymes that are disseminated via large conjugative plasmids harboring genes conferring resistance to other antibacterial classes. Introduction of β-lactamase inhibitors effectively restored the activity of β-lactam class of antibiotics; clinical examples include the tazobactam –piperacillin combination. However, it has lost its usefulness against KPC and *NDM-1* class of Carbapenemases and ineffective against infections caused by carbapenem resistant enterobacteriaceae (CRE). According to a report released by the National Health-care Safety Network from the US Centers for Disease Control and Prevention (CDC) in the year 2009-2010, 12.8% of *K. pneumoniae* isolates associated with bloodstream infections showed carbapenem resistance [5]. Acquisition of *NDM-1* production also confers resistance to cephalosporins and beta-lactamase inhibitors such as clavulanic acid and tazobactam. Such pathogens are the toughest to handle being susceptible only to Colistin (MICs ≤ 4 mg/L), Tigecycline (MICs ≤1 mg/L) or Aztreonam (MICs 0.25-24 mg/L) and emerging to be the most powerful superbugs in existence [6,7]. However, new variant *NDM-9* was recently found to produce colistin resistant *E. coli* strain in a chicken meat sample in Guangzhou, China [8].

According to a report in 2013 by Centre for Disease Control and Prevention, hospital acquired untreatable and hard to treat infections caused by CREs are considered to be an urgent threat [9]. Accordingly, >9000 such infections occur each year in clinical settings of which half of the patients died suffering untreatable CRE mediated blood stream infections [10]. Furthermore, CREs pose a major concern in infections associated with indwelling catheters, hospital acquired pneumonias/ ventilator-associated pneumonias (HAP/VAPs) and less frequent infections of CNS, skin and soft tissue. CREs result in morbid infections

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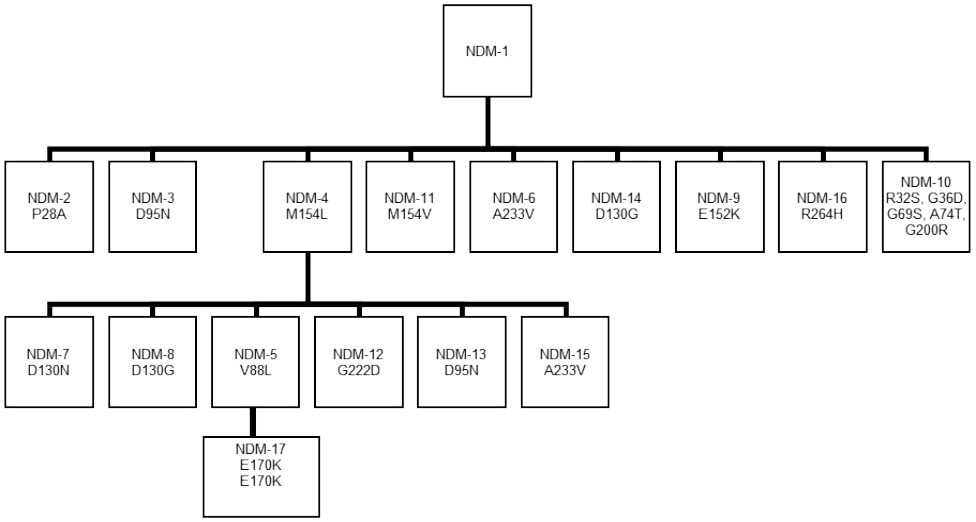

Figure 1: Variants of NDM-1 emerging due to single point mutations resulting in amino acid substitutions. Variants such as NDM-2 and NDM-3 share similar hydrolytic activity with NDM-1 as the mutations are not located in the active site of the enzyme. NDM-4, however, is hydrolytically activity than NDM-1 as the mutations lie with active pocket.

in old, immunosupressed or severely ill patients or the ones undergoing invasive procedures [11-13]. In later cases poor initial antimicrobial therapy result in the possible outcome of treatment failure [14].

Background and global spread

Increased carbapenem resistance can be credited to various factors such as increased use of carbapenems, simultaneous dosage of other antibacterial classes, acquisition of newer resistance markers and their spread among the bacteria through horizontal gene transfer, and global travel and migration. First detected in 2008 from *Klebsiella pneumoniae* strain isolated from Swedish patient hospitalized in India, *NDM-1* has widely spread to almost all parts of the world including United Kingdom, Pakistan, Japan, China, United States and Canada becoming a global concern [15]. Ever since its introduction in 2008, *NDM-1* infections are more often associated to medical-travel history to India or Pakistan. High sequence similarities between *NDM-1* genes from Enterobacteriaceae strains of United States and Pakistan have also been reported explaining crossing of Carbapenem resistance over geographic borders [16]. However, strain types that are not related to these sub-continents have also been detected [6]. Recent reports of infections in patients with no travel history to India or Pakistan describe emergence of newer unrelated clones [17]. Therefore, these subcontinents might appear to be the epicenters for its emergence, but newer clones are developing around the world with no travel history to these sub-continents.

Alongside this global spread, *NDM-1* has been recoved in different genera of Gram negative bacteria *viz. Klebsiella, Escherichia, Enterobacter, Acinetobacter, Pseudomonas, Serratia, and Salmonella* species [18,19]. One of the reasons to such alarming situation can be attributed to horizontal gene transfer of plasmids harboring resistance gene. *NDM-1* is a huge conjugative plasmid being widely disseminated to related pathogens. Latest reports document interspecies horizontal transfer of *NDM-1* plasmid from *Escherichia coli* to *Citrobacter freundii* [20]. These genes are encoded or flanked by mobile genetic elements that not only facilitates in their transmission but also results in diverse genetic platforms rapidly dispersing worldwide. These genetic elements allow organism to acquire wider cassettes such as aminoglycoside modifying enzymes, fluoroquinolone-resistance determinants or other beta-lactamases. Therefore, transposition and recombination has led to novel drug resistance cassettes due to wide rearrangements imparting genotypic and phenotypic diversity to NDMs.

Another important concern is that the variants of *NDM-1* are increasing rapidly over time. Khan et al. have recently reported 17 different variants of the *NDM-1* gene carried on composite transposon Tn125 in *Acinetobacter baumanii* that have evolved by mutations in one or two amino acid residues and are not restricted to specific geographical locations [15]. The variants arise from point mutations within the gene encoding the β-lactamase resulting in variable activities. Many of the variants, such as *NDM-2* and *NDM-3,* show similar hydrolytic activity as *NDM-1* while *NDM-4* were catalytically more efficient as genetic alterations lied in the active site (Figure 1). In 2012, *NDM-6* gene harboring *E. coli* strain was reported from a patient infected with *Proteus mirabilis* harboring *NDM-1* positive plasmid [21]. This *NDM-6* differed from *NDM-1* by a point mutation at position 698 (C→T) causing alanine to valine amino acid substitution at position 233 producing a novel variant. In addition to these mechanisms, clonal expansion has also aided in the selection and spread of these genes globally [16].

Other alarming concern with *NDM-1* plasmid is that they also carry genes conferring resistance to other antimicrobial classes or other mechanisms of resistance such as redundant beta-lactamases *viz* Amp C or ESBL`s or porin mutations preventing intracellular drug accumulation. Production of carbapenamases however alone imparts resistance without need of any other accessory mechanism. The sequence of *NDM-1* plasmid has revealed that besides *NDM-1* gene, it also contains 14 other antibiotic resistance determinants [6] making other classes ineffective as well. Reports document presence of 16S RMTases (16S rRNA methyltransferase) in association with *NDM-1* in Enterobacteriaceae [22] and *P. aeruginosa* [23] making these pathogens resistant to aminoglycosides. Similarly, Gokmen et al. reported co-extience of rmtC, a type of 16S rRNA methyltransferase, and *NDM-1* genes imparting resistance to aminoglycosides. Likewise, *qepA* and *aac* genes have been reported on the bla_{NDM1} plasmid conferring resistance to fluoroquinolones [24,25]. Genetic elements conferring resistance to

macrolides (esterases), rifampicin (rifampicin-modifying enzymes), and sulfamethoxazole are also found to be associated with the *NDM-1* plasmid [26]. Most recently, colistin resistant *mcr-1* gene was detected co-existing with c gene in clinical isolates of *E. coli* raising the global concern [27]. Thus, *NDM-1* gene has been identified in a number of unrelated Gram-negatives and is no longer associated to a single species or to a specific clone. The plasmid varies in size, incompatibility group, and consists of different antibiotic resistance cassettes generating pandemic clones against which current antibiotics lose their effectivity.

Comparative structural assessment analysis of *NDM-1*

Based on sequence homology β-Lactamases have been grouped into 4 major classes (A–D) [28]. Serine nucleophile acts as an active site in Classes A, C, and D enzymes. However, bound zinc atoms act as an active site in the class B metallo-β-lactamase (MBL) enzymes to help ionize and coordinate a nucleophilic hydroxide ion to mediate hydrolysis [29]. The mature form of *NDM-1* (M27-R270) is synthesized by cleaving first 26 residues of a signal polypeptide chain [30]. It shares an average sequence identity of 30-40% with other MBL`s but in 3D space *NDM-1* shares higher structural similarities with VIM-2, VIM-4, IMP-1 and other MBL enzymes. The *NDM-1* exhibits αβ/βα like structural fold conserved across the family of MBL enzymes. The interior sandwich like β-sheets are flanked by exposed exterior solvent surface flanked by five α-helices [30]. The binding cavity of

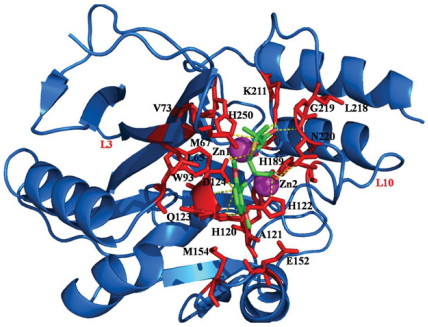

Figure 2: The key structural fragment representation of NDM-1 (blue color cartoon format). The Zinc metal ions are shown as magenta colored spheres; functional residues interacting with hydrolyzed methicillin (green color stick) are represented as red sticks. The key functional residues and structural fragments are labeled. Yellow color dotted lines show hydrogen bond interactions with active site residues.

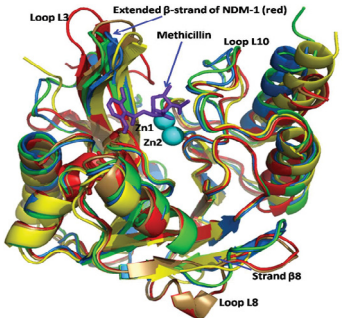

Figure 3: The Cα atoms stereo superimposition of NDM-1 (4EY2, red color) and NDM-4 (4TYF, sand color) with other MBL enzymes VIM-2 (5N4T, green color), VIM-4 (2WHG, blue color) and IMP-1 (1JJE, yellow color). The key fragment loop regions viz extended N-terminus β-strand, L3, L8, L10 and β-strand regions are highlighted. The bound methicillin (with NDM-1) is shown in purple stick and zinc metal ions (Zn1 and Zn2) are shown in cyan color spheres.

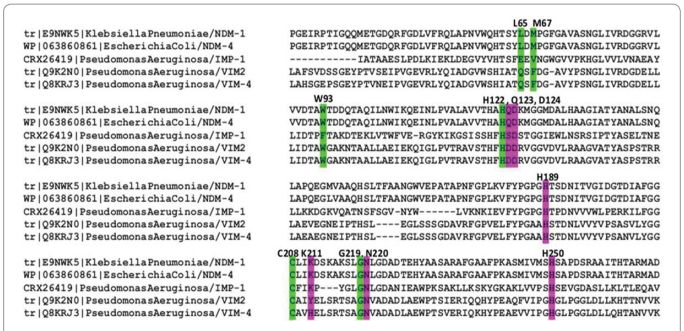

Figure 4: The multiple sequence alignment of NDM-1, NDM-4, VIM-2, VIM-4 and IMP done using Clustal Omega. The known substrate binding residues in binding cavity of NDM-1 are highlighted in magenta and green colour. The residues directly involved in tight binding interactions with antibiotics or other substrates are highlighted in magenta and the other functional sites are highlighted in green colour. All residues are levelled according to NDM-1.

NDM-1 exists in binuclear bound state with zinc metal ions Zn1 and Zn2 flanked respectively by loops L3 and L10. The catalytic activation mechanism of *NDM-1* is metal dependent which is activated through these zinc metal ions (Figure 2).

The *NDM-1* is comparatively an extended β-strand at its N-terminal and has an elongated loop region L8 instead of β8 as in other MBL enzymes (Figure 3). The loop L8 is known to be involved in dimer formation by interface interactions with α3 helix. The residues A143, L144, N146 and Q147 of α3 helix interact with the residues T162, F163, A165 and G65 of loop region L8 by hydrophobic and Vander Waals interactions. Biologically, this dimer formation is a key feature leading to resistant phenotypes [30]. In particular with another loop L3, the structural and catalytic mechanism studies suggest that it has the plasticity feature necessary to form substrate-specific hydrophobic interactions. The residues L65, M67 and W93 of this loop L3 show hydrophobic interactions with aromatic phenyl group of antibiotic drugs, further enhancing their binding affinity to such substrates. Accordingly, either mutations at L3 loop residues or altering functional groups at the aromatic ring of β-lactam antibiotics might alter the binding of *NDM-1* and other MBLs. In the catalytic mechanism of *NDM-1,* another loop L10 plays a key role through the involvement of residue N220 (Figure 2). The N220 is an oxy-anion residue, conserved throughout the MBL family. It interacts with carbonyl group of lactam ring during the formation of product complex [31]. It is known to facilitate nucleophilic attack and in polarizing carbonyl group for binding [32].

The flexibility of loops L3 and L10 allow *NDM-1* to accommodate variable range of antibiotic substrates in its catalytic site. Therefore, the structural similarity of the active site does not determine the substrate specificity but is determined by the structures flanking, the common MBL fold. This suggests that along with interactions with zinc atoms in the active site, an inhibitor with additional interactions with the loops L3 and L10 can block their flexibility and binding. Accordingly, even beta-lactam antibiotic drug molecules can be modified to bypass the wide substrate specificity generated due to L3 and L10.

Comparative binding cavity specificity analysis of *NDM-1*

A comparative study of the binding sites of *NDM-1* with other MBLs can suggest insights into substrate binding specificity with perspective utility in drug designing. Therefore, a comparison of key sites was performed by multiple sequence alignment between *NDM-1, NDM-4,* VIM-2, VIM-4 and IMP using online tool Clustal Omega [33]. The sequence alignment among the MBL enzymes including

NDM-1 revealed that few of functionally known residues are conserved (H122, D124, H189, C208, G219, N220 and H250) whereas others are variable (L65, M67, W93, Q123 and K211) throughout the family of MBL (Figure 4). The MBL family conserved residues include oxy-anion whole essential in stabilizing the intermediate stage of product release. The other functionally conserved residues should play similar functional features during the catalysis mechanism. In contrast to that, variability at the functional sites of *NDM-1* suggests substrate binding specificity differences.

Further, we performed vacuum charge electro-potential surface analysis of *NDM-1, NDM-4*, VIM-2, VIM-4 and IMP-1 to visualize and compare the actual shape of the binding cavity. The 3D structures were retrieved for *NDM-1* (PDB id: 4EY2), *NDM-4* (PDB id: 4TYF), VIM-2 (PDB id: 5N4T), VIM-4 (PDB id: 2WGH) and IMP-1 (PDB id: 1JJE) from PDB database (13-16, 18). The vacuum charge electro-potential surfaces were generated for each by protein molecular visualization tool Pymol (Figure 4). The detailed electro-potential surface analysis of MBL enzymes suggested that the shape and charge potential nature of *NDM-1* is distinct as compared to other members of MBL family (Figure 4). The cavity shape analysis of *NDM-1* suggests that *NDM-1* has an open-broad cavity, whereas VIM-2, VIM-4 and IMP-1 have comparatively small and slightly closed-shape cavity (Figure 5). Interestingly, we observed random changes in cavity shape of *NDM-4* (generated by single point mutation M154L in *NDM-1*) resulting in a partial-open cavity and variations in charge potential distribution compared to *NDM-1* (Figure 6). The binding cavity of *NDM-1* is hydrophobic being rich in negatively charged characters while the binding cavity of *NDM-4* looked slightly more positively charged as compared to binding cavity of *NDM-1* (Figure 6). Further, the binding cavities of VIM-2, VIM-4 and IMP-1 are also comparatively less hydrophobic and have distinct charge distribution in cavities of these enzymes. The clinical isolates that produce *NDM* also possess genes for other MBLs such as IMPs and VIMs. In this light, these findings suggest that variable hydrophobicity and negative charge potential further difficult the designing of pan-inhibitor drug molecule; one that can target a whole-bacterial cell by inhibiting the pool of MBL concoction that the resistant phenotype is capable of producing.

Overcoming the 'acquisition' barrier

Though at this point NDM appears to be exceptionally difficult

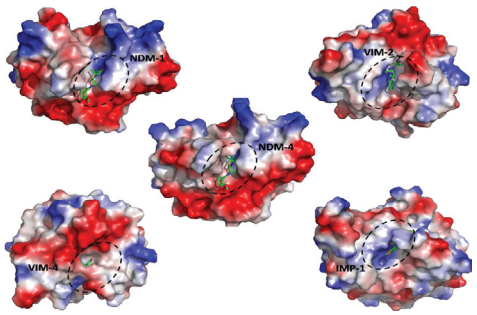

Figure 5: Three-dimension structural electro charge potential surface representation of NDM-1, NDM-4, VIM-2, VIM-4 and IMP-1. The bound anitibiotic/inhibitors in binding cavity are highlighted in green stick and probable view of cavity volume is highlighted in black dotted circle. The surface of NDM-1, NDM-4, VIM-2, VIM-4 and IMP-1 is coloured according to charge distribution with blue representing positive and red representing negative potential. The charge density distribution showed that the open and extended cavity of NDM-1 richer in negative charge potential comparative to NDM-4 and other three MBL enzymes which revealed the comparatively higher hydrophobic cavity of NDM-1.

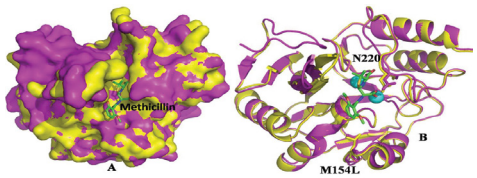

Figure 6: The Cα atoms stereo superimposition of NDM-1 and NDM-4 (M154L) in 3D space. The superimposition of NDM-1 and NDM-4 revealed that single point mutation results in simultaneous changes in the variability of their surface feature (A) and also slight displacement in their overall protein folding (B). NDM-1 and NDM-4 are shown in purple and yellow colour respectively. The point mutation site M154L is represented as stick.

to target, various surveillance measures can be adapted to reduce its spread. *NDM-1* is detected in isolates that are virtually pan antimicrobial resistant and most of such cases, not exclusively, are through nosocomial acquisition of infections that becomes a major concern with long term hospitalization or invasive procedures. Therefore, effective initial measures adapted during hospitalization could reduce incidence and prevalence of the bug known to affect the vulnerable. However, poor sanitation is the mainstay of transmission via oro-fecal route. Accordingly, most of the current measures aim to prevent the spread of the resistance by discouraging over-the-counter sale of antibiotics; improving knowledge on hygiene and the wide-scale detection of *NDM-1*. Identification and surveillance of *NDM-1* producers in clinical infections is a mandate that could be anticipated owing to increased Carbapenem MIC but can be detected using the Modified Hodge Test (MHT), Imipenem MIC in presence of EDTA (IMP/IMP+EDTA), BluCarba test or recent Carbapenem Inactivation method (CIM) and PCR confirmed using *NDM-1* specific primers [34]. Williamson et al. reported that Imipenem/EDTA MIC showed Carbapenemase negative even though molecular analysis detected presence of *NDM* gene [21]. Therefore, relying alone on such phenotypic interpretations can be misleading. Furthermore, surveillance of environmental contamination or that of equipments used during healthcare procedures along with educating health care personnel could be beneficial. Alongside, screening of infected individuals and possible asymptomatic carriers returning from Asia must become a quarantine mandate as India and Pakistan appear to be the harboring epicenter.

Recourse to clinical treatment

Detection of NDM guides a clinician towards treatment which unfortunately yet has not many options. Presently, treatment is attained using aztreonam, tigecycline, colistin or a combination therapy such as colistin or tigecycline. However, combination therapy proves its effectiveness on a case to case basis depending on the immune state of patient, site of infection or the antibiotic resistance profile of the pathogen. Recent broad spectrum diazabicyclooctaones (DBO) β-lactamase inhibitor of KPC enzyme *viz* avibactam was entered clinical development in combination with ceftazidime and aztreonam [35,36]. Resistance to ceftazidime-avibactam was observed in a panel of clinical isolates of *P aeruginosa [37]*. Aztreonam being a monobatam does not fit in the active site of NDM rendering the organism susceptible to it. This does not however hold true due to co-presence of other resistance mechanisms such as ESBLs or alteration in penicillin binding proteins (PBPs) that easily leave aztreonam ineffective. Co -administration of avibactam restores the activity of aztreonam against NDM [35,36]. However, recently resistance to aztreonam-avibactam was detected in a panel of clinical isolates of *E coli* producing *NDM-1* [38].

Other antibacterial, colistin is used in treating *NDM-1* infections

but often is the last resort due to its devastating toxic side effects. This infrequent use at least appeared to be the last resort until *mcr-1* plasmid conferring colistin resistance was detected along with *NDM* plasmid [8,27,39]. Infections caused by such extreme superbugs leave us with no last resort jeopardizing the treatment. It is important to determine the resistance/susceptibility profile of individual pathogenic isolate in each infection to further the antibiotherapy accordingly. Determining this however takes at least two days and is very difficult in case of patients with guarded prognosis such as the ones on ventilators or in intensive care units.

Results and Discussion

Recently, there have been development and research of some new pharmacologic agents against *NDM-1*. Thiols, including antihypertensive agent l-captopril, have shown effective inhibition of *NDM-1* and subclass B1, B2 and B3 enzymes [40,41]. Aspergillomarasmine A from *Aspergillus* has been reported to inhibit *NDM-1* [42]. Though, its non-toxicity and effectiveness in humans still needs to be validated. New class dipicolinic acid (DPA) derivatives when tested in combination, restored imipenem susceptibility against clinical isolates of *Eschericia coli* and *Klebsiella pneumoniae* harboring *NDM-1* [43]. *NDM-1* however needs a lot of focus due to limited clinical advancements and widely spreading threat.

Conclusion

NDM-1 is an explosive metallo-carbapenemase enzyme which since its origin has created a haphazard. It remains guilty of causing almost untreatable morbid infections owing to its complexity of metal binding. The gene resides on a notorious plasmid capable of swift dissemination via transposition and recombination. Therefore, it is no longer restricted to a particular geographical location or to a particular bacterial species. Besides, the organism harboring *NDM-1* gene also contains other antibiotic resistance markers rendering fluoroquinolone and aminoglycoside classes ineffective. In the current scenario emergence of colistin resistance elevates the alarming need of novel mechanisms to treat such dreadful infections. Furthermore, single point mutations leading to amino acid substitutions have generated newer variants. These variants also harbor genes producing other MBLs such as IMP and VIM. All of this has lead us to a difficult situation where we need an inhibitor simulating structure that can effectively fit into the active sites of varying metallo lactamases concoction. This however is too much to ask for with no such inhibitor in developmental pipeline. Currently the research in the field of *NDM-1* is very active but novel carbapenems that can bypass these powerful enzymes appear to be in no closer view yet. It is essential to match the pace between development of novel targets and spread of carbapenem resistance and the fact that no treatment alternative is currently available may jeopardize the modern medical techniques. For this reason, "bad bugs need drugs" campaign has been launched by Infectious Disease Society of America, to promote development of new antibiotics by 2020 [44].

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