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Current Concept of New-Delhi Metallo Beta Lactamases (NDM)

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Abstract

The emergence of superbugs intermittently has always been a global threat for control and treatment of infectious disease caused by them over many decades which influence human population with their mutated genetic make-up and mechanics of resistance. However, bacteria have been developing resistance to antibiotics since they were introduced in 1930s. From the year 1961 till date, among some of the more common superbugs are methicillin-resistant Staphylococcus aureus (MRSA) and drug resistant tuberculosis (MDR-TB and XDR-TB). The latest superbug encodes the gene for New Delhi metallo-beta-lactamase (NDM-1) is an enzyme that can hydrolyze and inactivate carbapenems, which are used as a last resort for the treatment

of multi-resistant bacterial infection. Escherichia coli and Klebsiella pneumoniae commonly expresses the gene for NDM-1 moreover NDM-2 in Acinetobacter baumannii, NDM-4, NDM-5, NDM- 6, NDM-7 and NDM-8 from E.coli and other Enterobacteriaceae also have been reported. Research is on to develop new classes of antibiotic to handle these threats and it is worrying as there are very limited therapeutics available in the development pipeline that works effectively in the current situation.

Introduction

In current scenario Carbapenems are the drug of choice for treatment of infections by penicillin or cephalosporin resistant gram negative bacilli especially in extended spectrum beta lactamase (ESBL) producer. Over few years resistance to carbapenems due to production of carbapenemases have been reported. Carbapenemases may be defined as beta-lactamases that significantly hydrolyze at least imipenem or meropenem. Carbapenemases involved in acquired resistance are of Ambler molecular classes A, B and D. . Carbapenemases are specific beta-lactamases with the ability to hydrolyze carbapenems. Production of beta-lactamases appears to be the most widespread cause of carbapenem resistance, since the documentation of their distribution in different bacterial species is extensive. An increasing number of class A carbapenemases (e.g., KPC and GES enzymes), class B metallo-beta-lactamases (e.g., VIM, IMP, and NDM beta-lactamases), and class D carbapenemases (e.g., OXA-23) have recently emerged. In addition, overproduction of class C beta-lactamases, such as CMY-10 and PDC beta-lactamases, which are not robust carbapenemases, can lead to carbapenem resistance, especially when combined with other resistance mechanisms (e.g., porin loss)[10].

A bacterium carrying several antibiotic-resistant genes is called multi-resistant bacteria or informally, a "super bacteria" or "super bug" because, infections caused by them are difficult to treat.[1] These bacteria came to limelight when an article was published in The Lancet medical journal in August 2010, about multi-drug resistant "super bug" infection, which they named controversially as New Delhi metallo-beta-lactamase (NDM-1) based on their assumed origin.[2] Most probably, a very rare "genetic fusion" has occurred between two previously known

antibiotic-resistant genes that gave birth to mutant called NDM-1 gene. Subsequently NDM-2, NDM-3, NDM-4, NDM-5, NDM-6, NDM-7 and NDM-8 also have been found [3,4,5,6,7,8,9]. The emergence of bacteria carrying such genes threatened the mankind by putting a challenge for the medicine. NDM is an enzyme that makes the bacteria resistant to a broad range of antibiotics, including the carbapenems, which are reserved for the treatment of resistant bacterial infections. This carbapenem beta-lactamase enzyme, produced by the blaNDM-1 gene, can hydrolyze and inactivate the carbapenem antibiotics. The NDM-1 enzyme is one of the class B metallo-beta-lactamase; other classes of carbapenemase being class A (the most common carbapenemase) and class D beta-lactamases.

Origin and Spread

It was 2009 (December) when NDM-1 enzyme was first described in a Swedish national who fell ill with an antibiotic-resistant bacterial infection which he acquired in India. The infection was unsuccessfully treated in a New Delhi hospital, and after repatriation of the patient to Sweden, a carbapenem-resistant *Klebsiella pneumoniae* strain bearing the novel gene was identified. [11]

In March 2010, a study conducted in a hospital in Mumbai found that most of the carbapenem-resistant bacteria isolated from patients carried the blaNDM-1 gene. [12] Later on a patient having an infection with *Escherichia coli* expressing NDM-1 was reported in May 2010 in the United Kingdom, who had a history of visiting India 18 months ago for undergoing dialysis. [13] In June 2010, three isolates of Enterobacteriaceae family bearing this resistance mechanism were reported in the US, all of which were from patients who had received recent medical care in India. [14] In July 2010, isolation of three cases of *Acinetobacter baumanii* bearing blaNDM-1 from the intensive care unit of a hospital in Chennai, India, were reported by a team in New Delhi.[1] Though the first reported case had a history of Indian origin, its exact geographical origin, however, has not been conclusively verified. Moreover, bacteria carrying NDM-1 enzyme has been reported from patients from different countries also, suggesting its wide dissemination thus it has been proposed that the patients travelling to India for cost-effective cosmetic surgery,

cancer treatment and transplantation and returning to their native countries for further care, were responsible for spreading these bacteria. In addition to this, lack of antibiotic policy and registered data of hospital-acquired infections in most of the hospitals in India made it easy to name the bacteria as "New Delhi Bug." However, the nomenclature was strongly denied by the Indian Government saying that, it is unfortunate to attach the name of a nation to a new bug which is an environmental thing. On January 12, 2011, the editor of the Lancet apologized and acknowledged that naming a superbug after New Delhi was an error.

Number of isolates contains NDM 1 are as follows uk -50, usa -3, pakistan -25, hongkong -1, japan -1, australia -3, china -3, singapore -6, canada -8, denmark -1, southkorea -2, thirteen european countries -77, north india -26, south india -44, other parts of india -48 and these data is not stagnate. Most of the NDM-1 carrying bacteria reported were from patients, having a history of hospital admission. However, detection of NDM-1 gene carrying bacteria in drinking water and sewage samples by a point prevalence study conducted in New Delhi between, September 26 and October 10, 2010, concluded that these bacteria are also present in the environment. These genes were much more prone to swap between bacterial species at 30°C than at 25°C or 37°C suggesting that the bacteria have found environmental mixing more important than in the gut. In addition to Enterobacteriaceae, blaNDM-1 gene was detected in other 11 bacterial species in which NDM-1 were not previously reported, including *Shigella boydii* and *Vibrio cholerae*. Isolates of *Aeromonas caviae* and *V. cholerae* carried blaNDM-1 on chromosomes while others carried it on the plasmids [15]. But in current scenario NDM-1 prevalence is worldwide as they have been recovered from a number of cases all around the glove.

Other type of NDM which may poses threat against their treatment and control globally have also been found. The identification and dissemination of NDM-2 carbapenemase in Acinetobacter baumannii from Egypt and also from the Israeli rehabilitation center [3, 16]. Plasmid carrying blaNDM-3 in Australia and identification of a novel NDM-type β-lactamase, NDM-4, possessing a high ability to hydrolyze carbapenems and several bulky cephalosporins. A Novel Variant, NDM-5, of the New Delhi Metallo--Lactamase in a Multidrug-Resistant Escherichia coli ST648 Isolate Recovered from a Patient in the United Kingdom. In addition, the bla(NDM-6) gene,

which differed from bla(NDM-1) by a point mutation at position 698 (C→T), was also identified in an E. coli isolate from the same patient who harboured the bla(NDM-1)-positive P. mirabilis from New Zealand hospitals. Detection of NDM-7 in Germany, a new variant of the New Delhi metallo-β-lactamase with increased carbapenemase activity. Again a novel metallo-β-lactamase, NDM-8, was identified in a multidrug-resistant *Escherichia coli* isolate obtained from the respiratory tract of a patient in Nepal [4,5,6,7,8,9]. Identification of NDM variants may signal an ongoing and rapid evolution of the NDM genes resulting from their large spread, at least in the Indian subcontinent.

In one of the study coproduction of the ESBL/MBL/ AmpC β- lactamases was observed. The ESBL and MBL coproduction was detected was found to be maximum in Escherichia coli, Pseudomonas aeruginosa and Klebsiella pneumoniae, while the ESBL and the AmpC co-producers were commonly isolated from Escherichia coli, Klebsiella pneumoniae and Acinetobacter baumannii. The co-production of AmpC and MBL was detected mostly in Escherichia coli.

Classification and pattern of resistance

NDM is a type of ambler class B carbapenemases – metallo B-lactamases and referred to as MBLs and require a metal ion, usually zinc, for β-lactam hydrolysis (Walsh et al., 2005). Due to the dependence on Zn2+, catalysis is inhibited in the presence of metal-chelating agents like ethylenediaminetetraacetic acid (EDTA). MBL expression in Gram-negative bacteria confers resistance to penicillins, cephalosporins, and carbapenems. MBLs are not inhibited by the presence of commercially available β-lactamase inhibitors and susceptibility to monobactams (e.g., aztreonam) appears to be preserved in the absence of concomitant expression of other resistance mechanisms (e.g., ESBL production). The more geographically widespread MBLs include IMP (imipenem-resistant), VIM, and New Delhi metallo-β-lactamase (NDM). Chromosomal MBLs were the first to be identified and are the cause of carbapenem resistance observed in Bacillus cereus, Aeromonas spp., and Stenotrophomonas maltophilia (Walsh et al., 2005). However, of growing concern are the “mobile” MBLs that have been reported since the

mid-1990s. Although most frequently found in carbapenem-resistant isolates of P. aeruginosa and occasionally Acinetobacter spp., there is growing isolation of these enzymes in Enterobacteriaceae. Prior to the description of NDM-1, frequently detected MBLs include IMP-type and VIM-type with VIM-2 being the most prevalent. These MBLs are embedded within a variety of genetic structures, most commonly integrons. When these integrons are associated with transposons or plasmids they can readily be transferred between species.

Although blaNDM-1 was initially and repeatedly mapped to plasmids isolated from carbapenem-resistant E. coli and K. pneumoniae, reports of both plasmid and chromosomal expression of blaNDM-1 has been noted in other species of Enterobacteriaceae as well as Acinetobacter spp. and P. aeruginosa (Moubareck et al., 2009; Bogaerts et al., 2010; Bonnin et al., 2011; Nordmann et al., 2011; Patel and Bonomo, 2011). Recently, bacteremia with a NDM-1 expressing V. cholerae has been described in a patient previously hospitalized in India colonized with a variety of Enterobacteriaceae previously known to be capable of carrying plasmids with blaNDM-1 (Darley et al., 2012).

NDM-1 shares the most homology with VIM-1 and VIM-2. It is a 28-kDa monomeric protein that demonstrates tight binding to both penicillins and cephalosporins (Zhang and Hao, 2011). Binding to carbapenems does not appear to be as strong as other MBLs, but hydrolysis rates appear to be similar. Using ampicillin as a substrate, allowed for detailed characterization of the interactions between NDM's active site and β-lactams as well as improved evaluation of MBLs unique mechanism of β-lactam hydrolysis. More recent crystal structures of NDM-1 reveal the molecular details of how carbapenem antibiotics are recognized by dizinc-containing MBLs (King et al., 2012). To date, NDM-1 remains the most common NDM variant isolated. Eight variants (NDM-1 to NDM-8) exist. It is currently held that blaNDM-1 is a chimeric gene that may have evolved from A. baumannii (Toleman et al., 2012). Contributing to this theory is the presence of complete or variations of the insertion sequence, ISAba125, upstream to the blaNDM-1 gene in both Enterobacteriaceae and A. baumannii (Pfeifer et al., 2011a; Poirel et al., 2011a; Dortet et al., 2012; Toleman et al., 2012). This insertion sequence has primarily been found in A. baumannii.

A recent evaluation of the genetic construct associated with blaNDM- has led to the discovery of a new bleomycin resistance protein, BRPMBL. Evaluation of 23 isolates of blaNDM-1/2 harboring Enterobacteriaceae and A. baumannii noted that the overwhelming majority of them possessed a novel bleomycin resistance gene, bleMBL (Dortet et al., 2012). Co-expression of blaNDM-1 and bleMBL appear to be mediated by a common promoter (PNDM-1) which includes portions of ISAba125. It is postulated that BRPMBL expression may contribute some sort of selective advantage allowing NDM-1 to persist in the environment. A contemporary evaluation of recently recovered NDM-1 producing A. baumannii isolates from Europe demonstrates that blaNDM-1 and blaNDM-2 genes are situated on the same chromosomally located transposon, Tn125 (Bonnin et al., 2012). Dissemination of blaNDM in A. baumannii seems be due to different strains carrying Tn125 or derivatives of Tn125 rather than plasmid-mediated or clonal (Bonnin et al., 2013; Poirel et al., 2012a).

Mechanisms of resistance to carbapenems include production of b-lactamases, efflux pumps, and mutations that alter the expression and/or function of porins and PBPs. Combinations of these mechanisms can cause high levels of resistance to carbapenems in certain bacterial species, such as Klebsiella pneumoniae, P. aeruginosa, and A. baumannii. A distinction exists between resistance to carbapenems in Gram-positive cocci and Gram-negative rods. In Gram-positive cocci, carbapenem resistance is typically the result of substitutions in amino acid sequences of PBPs or acquisition/ production of a new carbapenem-resistant PBP. Expression of b-lactamases and efflux pumps, as well as porin loss and alterations in PBP, are all associated with carbapenem resistance in Gram-negative rods. [10]

Detection method

(1) Screening for carriers of ndm-1 producers

The screening culture medium ChromID ESBL contains cefpodoxime, which is routinely used to screen for extended-spectrum β-lactamase producers, may also be used to detect carbapenemase-producing bacteria. Using the CHROM agar medium, carbapenemase-producing isolates with MICs of <4 μg/ml are detected with much higher detection limits. An inoculum of 2×10^6

(range, 1.5×10^5 to 3.5×10^7) CFU/ml are used, serial 10-fold dilutions are made in normal saline buffer, and then 100 μl are plated onto sheep blood-containing tryptic soy agar, ChromID ESBL medium. Viable bacteria are counted after 24 and 48 h of culture at 37°C, and growth on selective medium are compared to growth on standard culture medium. The lower limit of detection of NDM-1 producers ranged from 8×10^0 to 5×10^2 CFU/ml for ChromID ESBL. The ability of that medium to detect NDM-1 producers is based on the fact that those producers are also resistant to expanded-spectrum cephalosporins, in part due to the broad-spectrum hydrolytic properties of the β-lactamase NDM. Therefore, this detection was possible using ChromID ESBL even though the strain may not express an ESBL. The lower limit of detection of NDM-1 producers ranged from 1×10^1 to 4×10^5 CFU/ml. Those detection limits were higher than those of the ChromID ESBL medium, especially for several strains with low MICs of carbapenems (*E. coli* B, *E. coli* H, *and E. coli* J, *E. cloacae* C, *Klebsiella oxytoca* A, *Providencia rettgeri* A). After the screening of putative NDM-1 producers, definitive identification of the NDM-1 producers is needed using the techniques developed above (susceptibility testing, and PCR).

(2) Bacterial isolates and susceptibility testing

The bacterial isolates can be identified. The antibiotic susceptibilities of the isolates and their Trans-conjugants can be determined first by the disc diffusion technique on Mueller–Hinton agar plates with beta lactam and non-beta lactam antibiotic-containing discs and interpreted according to CLSI guidelines. Then, precise MIC values are determined by using Etest strips.

Phenotypically determination of the enzyme activity using Modified Hodge Test and Re-Modified Hodge Test.

The cloverleaf technique, or modified Hodge test (MHT), has been extensively used as a phenotypic technique for detecting carbapenemase activity is available in clinical microbiology routine settings and recommended by the CLSI. It is based on the inactivation of a carbapenem by carbapenemase-producing strains that enables a carbapenem-susceptible indicator strain to extend growth toward a carbapenem-containing disk, along the streak of inoculum of the tested strain. MHT technique is highly sensitive for detecting class A, B, and D carbapenemases after addition of zinc in the culture medium. However, the limitations of the MHT in terms of clinical

performance remain its lack of specificity and the delay in obtaining the results (24 to 48 h) after isolation of a bacterial colony [19].

Modified Hodge test

The test can be performed briefly, a 1/10 dilution of 0.5 McFarland turbidity standard of fresh overnight growth of the indicator organism *Escherichia coli* ATCC 25922 was inoculated on Mueller Hinton Agar. Two points were marked approximately 3 cm apart in the Centre of the plate. One of the points was marked 'Zn' and the other point left unmarked and an imipenem disc (10 µg) was placed at the unmarked point. The test strains are heavily streaked from the edge of the disc to periphery. Three strains are inoculated per plate. The re-modified Hodge test was also done on the same plate.

Re-Modified Hodge test

Addition of zinc has been known to increase the activity of metallo beta lactamases. On the same plate as mentioned above, on the second point marked 'Zn', another Imipenem disc are placed and the same test strains were heavily streaked in an approximately equal amount as the mirror images of the strains previously inoculated from the unmarked point. A total of 10 µl of 50 mM (140 µg per disc) of zinc sulphate solution are added to this second disc. This was done so as to have a better comparison of the effect of zinc on the same plate and to avoid plate to plate variation. The plates are incubated overnight. In the case of MBL production a positive test was interpreted as an inward indentation of the growth of the indicator strain along the streak line of the test strain. These are read as negative, equivocal, positive and strongly positive [20].

(3) PCR amplification and sequencing

Multiplex PCR approaches can be used to detect the blaNDM-1 carbapenemase gene in addition to genes encoding narrow-spectrum b-lactamases (TEM-1, SHV-11, OXA-1, OXA-9 and OXA-10), extended-spectrum b-lactamases (CTX-M-15, CMY-16 and CMY-30). The blaNDM-1 gene are located on conjugative IncA/C or IncF type plasmids. Experiments can be performed to identify the upstream and downstream located regions of the blaNDM-1 gene. All amplified DNA fragments are purified using the PCR purification kit and sequenced using a sequencer. The nucleotide and deduced protein sequences can be analysed with software available over the

Internet at the National Center for Biotechnology Information web site (www.ncbi.nlm.nih.gov).

Plasmid analysis

Conjugation assays are performed between clinical isolates as donors and an azide-resistant E. coli J53 as the recipient strain, using a selection based on ceftazidime (30 mg/L) and azide (100 mg/L). Plasmid DNAs are extracted using the method of Kieser. Then, the plasmid incompatibility groups are determined by a PCR-based replicon typing method [17].

Strain genotyping

Multilocus sequence typing (MLST) methods can be used for E. coli, K. pneumoniae and other isolates.

By conventional PCR

blaNDM-1 gene can be detected by conventional PCR using forward (5′-GAA GCT GAG CAC CGC ATT AG-3′) and reverse (5′-GGG CCG TAT GAG TGA TTG C-3′) primers, and all positive PCR results are confirmed by sequencing the obtained amplicon. With the same isolates we can also screens for extended-spectrum b-lactamase (ESBL) genes (blaSHV, blaTEM and blaCTX-M), metallo-b-lactamase genes (blaIMP, blaVIM, blaSPM-1, blaGIM-1 and blaSIM-1) and plasmid borne ampC genes.

Rapid detection of the blaNDM-1 gene by real-time PCR

For the NDM-1 real-time assay, primers and a probe were designed to amplify a 127 bp region based on currently available published sequences of blaNDM-1; GenBank accession numbers AB571289, FN396876, HM853678, HQ171206, HQ259057.1, HQ451074.1, AB614355.1, HQ738352.1, HQ284043.1, HQ284042.1 and HQ256747.1. DNA isolation can be performed using the Genomic DNA Kit from bacterial colonies according to the manufacturer's instructions. In conclusion, this real-time assay provides rapid and accurate detection of the blaNDM-1 gene in test population, and is a viable method for rapid screening of the NDM-1 gene in carbapenem-resistant Enterobacteriaceae [18].

Comparative analysis of NDM-1 and NDM-2

In order to compare the relative contributions of NDM-1 and NDM-2 to carbapenem resistance, the corresponding genes were cloned and expressed in an isogenic E. coli background under the

control of the same promoter (E. coli TOP10; Invitrogen). Cloning experiments can be performed using the PCR-BluntII-TOPO vector (Invitrogen) following the manufacturer's instructions with 984 bp PCR amplicons as targets that had been generated using external primers Pre-NDM-A (5′-CACCTCATGTTTGAATTCGCC-3′) & Pre-NDM-B (5′-CTCTGTCACATCGAAATCGC-3′) encompassing the entire blaNDM genes with the same promoter sequences [3]. And NDM-1 and NDM-3 can be compared only by one nucleotide mutation for example *E. coli,* typed as ST 443, had a variant of $bla_{\text{NDM-1}}$, $bla_{\text{NDM-3}}$, which differed by one nucleotide mutation [4].

Treatment

Few antimicrobials are currently available to treat infections with carbapenemase-producing Gram-negative bacteria are the polymyxins (including colistin), tigecycline, and fosfomycin but susceptibilities to these agents are unpredictable. Growing resistance to both the polymyxins and tigecycline has resulted the revisiting of older drugs including chloramphenicol, nitrofurantoin, and temocillin. Fosfomycin may be a potential therapeutic option for patients infected with carbapenemase-producing Enterobacteriaceae if the infection is localized to the genitourinary tract. Unfortunately, fosfomycin does not demonstrate reliable activity against non-urinary pathogens. A contemporary pre-clinical evaluation of ME1071 in combination with various type 2 carbapenems (i.e., biapenem, doripenem, meropenem, imipenem) confirms remarkable decreases in the carbapenem MICs for Enterobacteriaceae and A. baumannii harboring IMP, VIM, and NDM-type MBLs (Livermore et al., 2013). Irrespective of the candidate carbapenem, ME1071 activity against NDM MBLs was less than that of VIM-type and IMP-type MBLs. Of note, biapenem was the carbapenem with the lowest baseline MICs to the MBLs, but it is commercially unavailable in many countries including the USA. Other MBL-specific inhibitors are in pre-clinical development (Chen et al., 2012). These bacteria are sensitive to older generation antibiotics like colistin and tigecycline, which can produce toxic side effects in kidney disease patients. But death of a Belgian patient despite administration of colistin in August 2010, which was the first reported death due to bacteria expressing NDM-1 enzyme. Discovery of the

chemical compound GlaxoSmithKline Pharmaceuticals Ltd. (GSK) 299423 seems to bring a ray of hope, which does not allow the antibiotic-resistant to reproduce, citing a likely treatment for the NDM-1 strain. To find out new drugs that can attack NDM-1, Infectious Disease Society of America has launched a "bad bugs need drugs" campaign to promote development of new antibiotics by 2020.

Control and Prevention

The emergence of antibiotic-resistant bacteria comes from inappropriate prescriptions or overuse, rather abuse of antibiotics in the absence of proper surveillance in the community. According to center for disease control infection control guidelines, all the carbapenem-resistant bacteria should be identified among the isolates. Surveillance should also be done to identify undetected asymptomatic carriers of carbapenem-resistant bacteria.

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