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The variants of New delhiMetallo –β lactamase-1: A Comparative Assessment

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ABSTRACT: *The discovery of antibiotic was a step forward in the medical science. Antibiotics is surely a boon for public health as it is a life saving drug, but surplus and unnecessary use of antibiotics is harmful as it is causing bacteria to develop resistance towards them. And therefore one of the biggest problem associated with antibiotic therapy is resistance. It is not a new issue and was seen shortly after the discovery of antibiotics. Recent studies have revealed that enterobacteriaceastrains like E.coli and Klebsiella pneumonia isolated from several Indian centers and other countries are resistant to many antibiotics including the most potent antibiotic like carbapenems. And this antibiotic resistance is due to thecarbapenemase enzyme called NDM-1 (New delhiMetallo –β lactamase-1) . The uncontrolled NDM-1 is now considered as a nightmare scenario and global threat due to its rapidly spreading power. They are referred as the "New Superbug". This review paper is an update of the current scenario of NDM enzyme in the world and the recent advancements made for its treatment. There is a need for active screening of microorganisms for NDM-1 and further researches should be done to develop new classes of safe antibiotics to fight these kinds of infections.*
Key words: *NDM enzyme, superbug, antibiotic resistance, NDM variants,cladogram,percentage identity matrix.*

I. INTRODUCTION

It was in 1942 that Selman Waksman for the first time used the term antibiotic to substances produced by microorganisms that inhibit the growth of other microorganisms.[1] However, today the term antibiotic is used for substances or antimicrobial agents from natural or synthetic sources (any class of organic molecule), that kill or inhibit the growth of microbes by specific interactions with bacterial targets, without harming the eukaryotic host harboring the infecting bacteria. An antimicrobial agent have the potency and access in order to exert its antimicrobial action. The major classes of antibiotics inhibit or kill the bacteria. The present paper endeavors to analyze the New delhimetallo*β lactamase-1*which is spreading all over the globe The medical practitioner believe that the medical tourism is one of the reasons of its multiplicity. New delhimetallo*β lactamase-1*provides evidence to be a serious threat to public healthin view of the fact that it is an enzyme that makes bacteria resistant to an extensive collection *ofβ* lactam antibiotic drug. Scholars have defined antibiotic resistance as "the ability of a microorganism to refuse to acceptthe antibiotic pressure and stay alive", in opposition to the vulnerable bacteriawhich will be eradicated. The consequence of an antibiotic can either be bacteriostatic orbactericidal based upon the antibiotic objective and attention. Bacterialvulnerability to a particular antibiotic can be defined from both a microbial and aclinical point of view. From a bacterial point of view, a susceptible bacteriumbelongs to a sub-population lacking mechanisms of resistence. Suchvulnerable bacteria may build up resistance by acquiring antibiotic resistancegenes, and for this reason being able to survive antibiotic exposure, andbecome resistant . From a clinical point of view, when the bacterium respondsto antibiotic therapy it is termed susceptible, and if the bacteria does not respondto antibiotic therapy it is categorized as resistant to that particular antibiotic.Therefore clinically, bacteria can be divided into susceptible, intermediatesusceptible, or resistant to antibiotics . Mechanisms of resistance are foundwithin bacteria either intrinsically or they may be acquired. The intrinsicresistance refers to existence of resistance genes as part of the genomeencoding mechanisms intrinsically found in the population of the bacteria . For instance, Gram-negative bacteria are intrinsically resistant toglycopeptides and macrolides due to their impermeable outer membrane.Further, due to the lack of a cell-wall, *Mycoplasma* intrinsically shows resistanceto β-lactams other cell-wall biosynthesis targeting antibiotics[2]. In contrast,the acquired resistance mechanisms are attained by bacteria through mutationsor mechanisms of horizontal gene transfer such as transformation, conjugation,and transduction . For instance, many β-lactamase genes are acquired bybacteria through mobile genetic elements such as plasmids, transposons, and insertion sequence common region (IS*CR*) elements.[3] Plasmids canreplicate independently within bacteria and also transfer between bacterial cellsand species, spreading resistance. Further, the rapid generation time ofbacteria support them to develop quickly and for this reason become resistant to antibioticswithin a short period of time.Generally bacteria show evidence of biochemical resistance by three different mechanisms[4]:

- by reducing their permeability into the cell and/or
- by active effluxmechanism
- by structurally altering the antibiotic targets byenzymatic modification or inactivation of the antibiotic before reaching the targets.

Bacteria can combine these mechanisms to exhibit resistance towardsantibiotics.Increasing antibiotic resistance is a major problem especially multi drug resistance in bacterial strains which leads to a limited range of treatment options. When we take antibiotic against a bacterial strain, the bacteria sometimes fight back. When antibiotic resistance takes place another antibiotic is used. This is how bacteria builds up resistance to more than one antibiotic. The main reason for multi drug resistance is antibiotic overuseand it has become common rather than being a exception and thus it proves to be a challenge for the treatment of clinical infections.

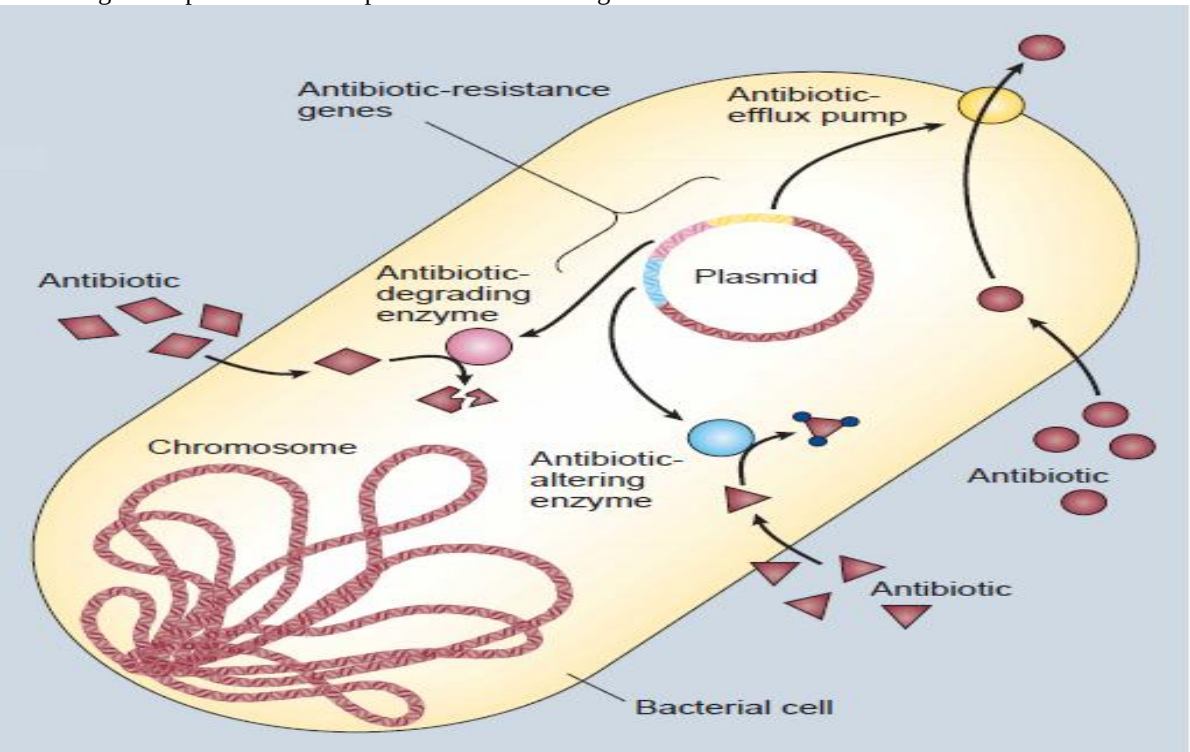


Figure 1: An overall representation of bacterial resistance mechanisms (modified from Levy, S.B et al., 2004)

NDM-1

NDM-1 was first detected in a Swedish patient from *Klebsiellapneumoniae* isolate in 2008.[5] Since then it has been in great attention of researchers and has made headlines for its ability of rapid spread worldwideand making the last antibiotics known as carbapenems incompetent. The NDM-1 is encoded by the bla_{NDM-1} novel gene.bla_{NDM-1} gene is also accompanied by other genes responsible for antibiotic resistance like, rifampicin, ciprofloxacillin, chloramphenicol and erythromycin . The NDM-1 was reported as the newest superbugin the scientific journal "*The Lancet Infectious Diseases"*2010,.[6] It is interesting to note that 37 strains of NDM-1 were reported in UK, and 99 in India. Gram negative bacteria such as E.coli and Klebsiella pneumonia but what makes NDM a superbug is the ability of NDM gene to spread from one strain of bacteria to another by horizontal gene transfer.[7] Walsh *et al* discovered the prevalence of *bla*NDM-1 in drinking water and seepage samples in New Delhi and hence named the enzyme "New Delhi Metallo-β lactamase".The genes of the patients of NDM 1 is by and large found worldwide and it advocates the wide increase and multiplicity of the enzyme. The medical practitioners believe that the medical tourism is one of the reasons of its multiplicity.

II. REVIEW OF LITERATURE

In this paper attempt has been made to make a systematic review of the literature on NDM 1 to summarize the descriptive features of the NDM-1. As discussed earlier NDM-1 is an enzyme reported worldwide from January 2008. In this part of the present paper some research studies have been selected to make a systematic review of New Delhi metallo-β-lactamase type 1 (NDM-1). For convenience these research studies have been further divided into two broad categories of

1. case series and case reports of NDM-1, or
2. active surveillance and environmental surveillance studies of NDM-1-producing bacteria.

A close analysis of these research studies suggests that sixty cases with NDM-1-producing bacteria were presented colonization without evidence of infection. In many of these studies urine was the most common specimen source for cases with infection and colonization. Seventeen cases had NDM-1-producing bacteria at more than one body site.In most of these reported casesKlebsiellapneumoniae and Escherichia coli were detected frequently, and the multilocus sequence type data from 34 E. coli and K. pneumoniae clinical isolates make available an unfinished, yet varied and diverse world-wideallocation of NDM-1-producing bacteria.[8]

It is interesting to note that the majority of thesecases had exposure to the Indian subcontinent of south Asia, and laboratory surveillance systems, as well as an environmental survey from India, suggest a presence of ecological and environmental reservoir for possible human infection and colonization with NDM-1-producing bacteria.

The knowledge of the 3-dimensional structure of *bla*NDM-1 gene will help revealing the mechanism and functional aspects of NDM-1 and its antibiotic resistance property. The $_{bla}$NDM-1 gene encoding *β*-lactamase isfound on a large 180-kb resistance. It is a genetic element extractedfrom *Klebsiella pneumonia*. It is plasmid borne which makes it easily transferable and rapidly spread to other bacteria.Furthermore, it controlled a multiplicity of other resistance determinants.It includes a genetic materialprogramming another broad-spectrum *β*-lactamases (CMY-4). It also holds genes which inactivate erythromycin, ciprofloxacin, rifampicin, and chloramphenicol. Adding up, the genetic element en- coded an efflux pump competent of providing added help toadditional antimicrobial resistance and growth promoters.[9]

The scientists have observed a possible rearrangement of *bla*NDM-1 *in vivo* either from *K. pneumoniae*to *E. coli* or *vice versa*, but what builds it interesting is that the plasmids carrying *bla*NDM-1 in these two species are of two different sizes. The fact that they are of two sizes suggeststhat there is rearrangement *in vivo* which could be either due to duplication or insertion. The *bla*NDM-1 open reading frame encodes a putative protein of 269 amino acids with a molecular mass of approximately 27.5 kDa and also contain N-terminal signal peptide with conserved LXXC like motif.

Multiple sequence alignment research have shown that NDM-1 have very modest similarity with other MBLs and is most intimately related to VIM-1/VIM-2, with which it has only 32% similarity. FascinatinglyNDM-1 also has a unique HXHXD pattern among the mobile MBLs, because it contains an alanine between the two histidines.

NDM-1have a Tyrosine at position 222 instead of tryptophan.NDM-1 have tight binding i.elow *Km* values to most cephalosporins. It also showed fairly tight binding to penicillins which is unusal forMBL .Phylogenetic studies is done to determine the origin of this antibiotic resistant NDM-1 gene by using BLAST.The results suggested that NDM-1 gene has nearestorthologues in marine bacteria The centerof NDM-1 is made of two *β*-sheets, one N-terminal which is further composed of 7 antiparallel strands named from *β*1 - *β*7 and the other is C-terminal which is composed of 5 antiparallel strands from*β*8 - *β*12. The seven linking helices are located below *α*1 - *α*4 and 310 helix 5 and above are *α*6 - *α*7 the plane of the *β*-sandwich. Hydrophobic interactions between these helices and *β*-sheets are important to understand the structural details. It also involves H bonds (Glutamine-96 with carbonyl of Tyrosine-64, Threonine-98 with carbonyl of Alanine-92, Tyrosine-229 with carbonyl of Leucine-209 and Serine-232 with carbonyl of Proline-187). These strands and helices are linked by flexible loops present above a 600 Å active site cavity. Similar unique features on primary sequence and unusually large cavity of the active site were also seen for the crystal structure of the 24-kDa, NDM-1[10]

The amino acid sequence of NDM-1 from Klebsiellapneumoniae is 241 amino acids long and is generated by taking the FASTA sequence of NDM-1 from ncbi and then cleaving it by using Peptide cutter for all possible enzyme.

10 20 30 40 50 60
ALMLSGCMPG EIRPTIGQQM ETGDQRFGDL VFRQLAPNVW QHTSYLDMPG FGAVASNGLI

70 80 90 100 110 120
VRDGGRVLVV DTAWTDDQTA QILNWIKQEI NLPVALAVVT HAHQDKMGGM DALHAAGIAT

130 140 150 160 170 180
YANALSNQLA PQEGMVAAQH SLTFAANGWV EPATAPNFGP LKVFYPGPGH TSDNITVGID

190 200 210 220 230 240
GTDIAFGGCL IKDSKAKSLG NLGDADTEHY AASARAFGAA FPKASMIVMS HSAPDSRAAI

T

The outline is thatNDM-1 adoptsheMBL fold that facilitates β-lactam antibiotic hydrolysis by a 2 zinc-catalyzed mechanism .Though, NDM-1 shows a larger active site and an electrostatic profile that can contain a wide variety of substrate molecules. Also NDM-1 reveals important rearrangements around the active site upon substrate binding and product formation.

Methods used for Detection of NDM-1

1. Screening of NDM-1 producers

ChromID ESBL is used as screening culture medium which contains cefpodoxime and is usually used toscreen for extended-spectrum β-lactamase producers bacteria. Theability of this medium to identify NDM-1 producers is due to the fact that producers arealso resistant to expanded spectrum cephalosporins in part due to the wide-spectrum hydrolyticproperties of the β-lactamase NDM-1. Hence , the detection is possible using ChromID ESBLeven though the strain may not express an ESBL.

2. Bacterial isolates and susceptibility testing

The bacterial isolates can be identified first by the disc diffusion technique on Mueller Hinton agarplates with beta-lactam and non beta-lactam antibiotic containing disc.

3. Modified Hodge test

Modified Hodge test is extensively used as phenotypic detection technique for detection of NDM-1 activity. A 1/10 dilution of 0.5 McFarland turbidity standard of fresh overnight growth of the NDM-1 producing organism was inoculated on Mueller Hinton Agar. Two points are marked a little apart in the centre of the plate. One was marked as 'Zn' and the other point was left unmarked and an imipenem disc was placed at this point. The test strains are heavily streaked from the edge of the disc to periphery. Three strains should be inoculated per plate. [11]

4. PCR amplification and sequencing

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. A real-time PCR assay is used to detect *bla*NDM-1. Justsmallamounts of total DNA isolated from one bacterial colony was sufficient to allow detection of *bla*NDM-1 by real-time PCR quickly. [12]

NDM and its variant

Since the discovery of first strain of NDM-1, new forms or variants of bla_{NDM-1}have been found which posses a slight variation from the standard NDM gene. Till date twelve variants of NDM-1 enzyme have been reported (NDM2-NDM13).[13]

- NDM-2

The first variant NDM-2 was reported by Kaase et al in 2011. It is a point mutation variant that had a Cytosine to Guanine substitution at position 82 which resulted in an amino acid substitution of a proline to an alanine residue at position 28 (Pro → Ala).The MIC values of lactams including carbapenems have no major difference between NDM-1 and NDM-2 producers as the point mutation is considered to be at the last amino acid. NDM-2 has been identified in several A. baumannii strains .

Pro ⟹ Ala

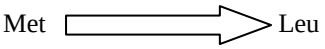
- NDM-3

The NDM-3 variant was isolated from an E.coli isolate and differs from NDM-1 by a single nucleotide change at position 95 which changes Aspartate to Asparagine(Asp → Asn).
NDM-3 does not change the hydrolytic activity of the enzyme.

Asp ⟹ Asn

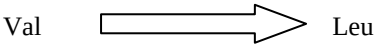
- NDM-4

NDM-4 variant is also isolatedfrom E.coli strains and it differs from NDM-1 by a single amino acid substitution at position 154 changing Methionine into Leucine (Met → Leu). This amino acid substitution results in increased hydrolytic activity of NDM-4 than NDM-1.[14]



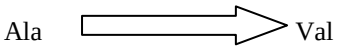
- NDM-5

The NDM-5 variant changesValine to Leucineat position 88 and has been identified in E.coli.[15]



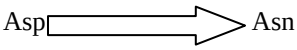
- NDM-6

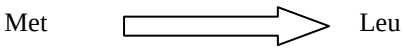
The NDM-6 variant differs from NDM-1 by a single amino acid substitution at position 233 replacing Valine by Alanine (Ala → Val) and is found in E.coli.



- NDM-7

The NDM-7 variant was isolated from E.coli strain recovered from a French patient who travelled to Burma and an E. coli isolate recovered from a Yemeni patient. The gene differs from NDM-1 gene by two amino-acid substitutions at positions 388 (G→A) and 460 (A→C) leading to amino acid substitutions at position 130 (Asp → Asn) changing Aspartate into Aspargine and at 154 methionine into leucine. (Met → Leu).[16]

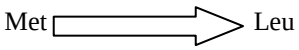




- NDM-8

In NDM-8, substitutions occur at positions 130 changing Aspartate into Glycine (Asp → Gly) and 154 (Met → Leu) compared with NDM-1. [17]

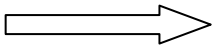
AspGly



- NDM-9

NDM-9 variant was isolated from ST107 Klebsiellapneumoniae strain found in China.

- NDM-10



NDM-10 is also found in Klebsiellapneumoniae.

- NDM-11

NDM-11 is a variant of NDM-1 which is found from E.coli.

- NDM-12

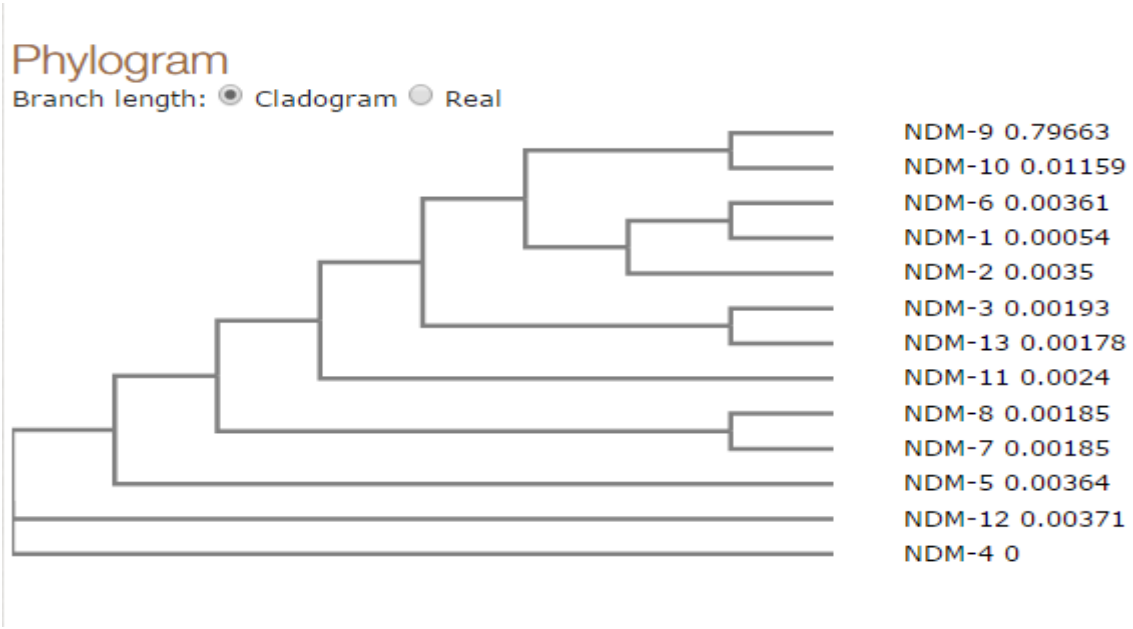
NDM-12 was identified in E.coli clinical isolate from the urine sample from a patient of Nepal. It differs from NDM-1 by two amino acid substitution at position 222. The 2 substitutions G222D and M154L in NDM-12 affect the activity of the enzyme.[18]

- NDM-13

NDM-13 is the most recent discovered variant of NDM-1 which is isolated from a Multidrug resistance E.coli clinical isolate in Nepal.It is obtained from the urine sample of the patient. It has been observed that there is no change in the enzymatic activity of NDM-13from NDM-1. However, it showed comparatively high K_{cat}/K_m ratio.[19]

In all 12 variants known the substitution take place at 13 amino acid positions including28, 32, 36, 69, 74, 88, 95, 130, 152, 154, 200, and 222.

Given below is the cladogram of NDM-1 and its variants which depicts the relationship between them which is generated by Clustal omega.



Given below is the Percent Identity Matrix- created by Clustal omega which depict what percentage of the two sequences have functional similarity or identity.

NDM-9 NDM-10 NDM-6 NDM-1 NDM-2 NDM-3 NDM-13 NDM-12 NDM-11 NDM-8 NDM-7 NDM-4 NDM-5

1: NDM-9	100.00	19.18	18.98	19.14	19.18	19.18	19.18	18.72	19.18	19.18	19.18	19.18	19.18
2: NDM-10	19.18	100.00	97.73	97.93	97.78	97.78	97.41	97.41	97.78	97.41	97.41	97.78	97.41
3: NDM-6	18.98	97.73	100.00	99.59	99.24	99.24	98.86	98.86	99.24	98.86	98.86	99.24	98.86
4: NDM-1	19.14	97.93	99.59	100.00	99.59	99.59	99.17	99.17	99.59	99.17	99.17	99.59	99.17
5: NDM-2	19.18	97.78	99.24	99.59	100.00	99.26	98.89	98.89	99.26	98.89	98.89	99.26	98.89
6: NDM-3	19.18	97.78	99.24	99.59	99.26	100.00	99.63	98.89	99.26	98.89	98.89	99.26	98.89
7: NDM-13	19.18	97.41	98.86	99.17	98.89	99.63	100.00	99.26	99.26	99.26	99.26	99.63	99.26
8: NDM-12	18.72	97.41	98.86	99.17	98.89	98.89	99.26	100.00	99.26	99.26	99.26	99.63	99.26
9: NDM-11	19.18	97.78	99.24	99.59	99.26	99.26	99.26	99.26	100.00	99.26	99.26	99.63	99.26
10: NDM-8	19.18	97.41	98.86	99.17	98.89	98.89	99.26	99.26	99.26	100.00	99.63	99.63	99.26
11: NDM-7	19.18	97.41	98.86	99.17	98.89	98.89	99.26	99.26	99.26	99.63	100.00	99.63	99.26
12: NDM-4	19.18	97.78	99.24	99.59	99.26	99.26	99.63	99.63	99.63	99.63	99.63	100.00	99.63
13: NDM-5	19.18	97.41	98.86	99.17	98.89	98.89	99.26	99.26	99.26	99.26	99.26	99.63	100.00

Variant	Source organism	Place	Position of a.a substitution	Change in amino acid	Feature
NDM-2	Acinetobactorbaumanni	Egypt	28	Pro →Ala	No difference from NDM-1
NDM-3	E.coli	Australia	95	Asp → Asn	No change in hydrolytic activity
NDM-4	E.coli	India	154	Met → Leu	Increased hydrolytic activity than NDM-1
NDM-5	E.coli	UK	88	Val→ Leu	
NDM-6	E.coli	NewZealand	233	Ala → Val	
NDM-7	E.coli	French patient, Germany	130 154	Asp → Asn Met → Leu	
NDM-8	E.coli IOMTU11	Nepal	130 154	Asp → Gly Met → Leu	
NDM-9	ST107 K.pneumoniae	China			
NDM-12	E.coli (urine sample)	Nepal	222 154	G222Dand Met → Leu	Have low k_{cat}/k_m Ratio
NDM-13	Multidrug resistant E.coli	Nepal			High k_{cat}/k_m Ratio

The depicted results do not show major differences in the hydrolytic and catalytic efficiencies of the studied NDM variants.From the results it is revealed that the clinically observed substitutions can make substantial differences in thermodynamic stability of the enzyme.Earlier reports point out that the substitutions affect NDM activity even though being located outside the active site.

III. CONCLUSION

A closed look at the table given above suggests that all the variants emerging out of NDM-1 is the result of some slight change in the sequence of the gene of this enzyme. Furthermore, the frequency of change from "Methionine→Leucine" is much more higher in comparison of other variations in the gene sequence. However, it should be mentioned here that what make these variants differ is the position at which these mutations occur .

As shown in the table the frequency of occurrence of "Methionine→Leucine" is much more higher than other patterns. With a high frequency of 33.3% it enjoys the top position followed by Aspartate→ Asparagine with 22,2 percent of occurrence. All others have only 11,1 % of the frequency of occurrence..

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