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TO STUDY PHENOTYPIC AND MOLECULAR CHARACTERISTICS OF CARBAPENEM RESISTANT *KLEBSIELLA PNEUMONIAE* ISOLATED FROM TERTIARY CARE HOSPITAL

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ABSTRACT

Production of Metallo-Beta-lactamase (MBL) enzyme is most important resistant mechanism against carbapenems. Metallo-Beta lactamases comes under Ambler class B. This enzyme breaks the Beta-lactam ring of the drug thus making the drug inactive. New Delhi Metallo-Beta-lactamase is an enzyme which is active against compounds that possess Beta-lactam ring like Penicillins, Cephalosporins, Carbapenems except aztreonam and colistin. The gene that encode for NDM-1 is known as bla_{NDM-1} and has been identified on bacterial plasmids and chromosomes. Carbapenems are one of the very few therapeutic agents available for treating multidrug resistant infections caused by Enterobacteriaceae however, carbapenemases are increasingly being reported, with the latest threat being New Delhi metallo-β-lactamase (NDM-1). Transmission of plasmid carrying resistant gene has increased the incidence of multidrug resistance. Early detection of these genes will help in prevention and adequate infection control by limiting the spread of these organisms.

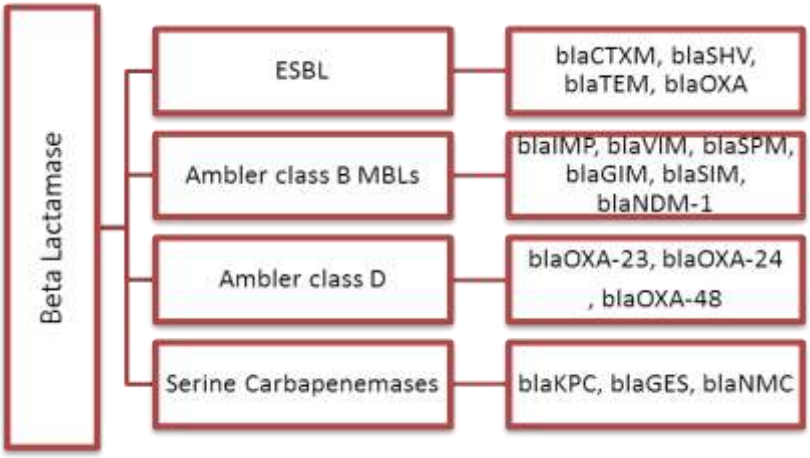
KEYWORDS: Metallo-Beta lactamases, $bla_{NDM-1,}$ Carbapenems.

INTRODUCTION

Klebsiella pneumoniae is Gram negative, non-motile, encapsulated, lactose fermenting, facultative anaerobe. It belongs to Enterobacteriaceae family. It can progress into severe

bacterial infections like Pneumonia, Bloodstream infections, Nosocomial infections, Urinary Tract infections, Wound infection, Meningitis. Patients who are administered with a course of broad spectrum antibiotics are at high risk of infection and even those who require equipments like ventilators or cathetors.

Beta-lactam group are named after the type of rings fused to beta-lactam rings which includes penems, carbapenems, oxapenems, cephems, carbacephems, oxacephems, monobactams, cephamycins. Carbapenemases are increasing concern because they often confer resistance to most beta-lactam antimicrobial agents. Carbapenemases are found in Ambler class A penicillinase or class B metallo-enzyme group.



The novel MBL, New Delhi Metallo-Beta-Lactamase-1 (NDM-1) confers resistance to all beta-lactam antibiotics except aztreonam, which was named after the city of origin. i.e. New Delhi. It was first reported in 2009 by Yong. et al in Swedish patient who travelled New Delhi and acquired urinary tract infection due to carbapenem resistant *Klebsiella pneumoniae* which was resistant to all antibiotics. There after the spread of NDM-1 have been reported from India, Pakistan and United Kingdom.

METHODOLOGY

Characterization of Bacterial Isolates

Sputum, Pus, Endotracheal tube, Cerebrospinal Fluid and Blood samples were aseptically inoculated on to Blood and MacConkey's agar plates. Urine samples were aseptically inoculated on Cystine Lactose Electrolyte Deficient (CLED agar) and MacConkey's agar plates and incubated at 37^0C for 24 hours. *Klebsiella pneumoniae* isolates were identified by their morphology and biochemical characteristics. Morphology of *Klebsiella* identified were

large dome shaped colonies on Blood agar and lactose fermenting mucoid colonies on MacConkey's agar. In Gram staining, Gram negative, short, stout, blunt rods were seen.

The suspected colonies were identified based on their morphological, physiological and biochemical characters using microscopic observation, standard biochemical methods and cultural characteristics.

Antibiotic Susceptibility Test

A total of 30 positive isolates were screened for antimicrobial susceptibility testing by Kirby-Bauer disc diffusion method on Mueller-Hinton agar (Hi-Media) and interpreted as per CLSI guidelines. A log phase broth culture inoculums of the isolate with a turbidity equivalent to McFarland 0.5 standard (1.5x108 CFU/ml) was prepared and lawn cultured on the Mueller-Hinton agar and allowed to dry. Antibiotic discs were applied to the Mueller Hinton agar surface with the help of sterile forceps.

Antibiotics chosen for the study were

Antimicrobial Agent	Symbol	Disc Content
Pipercillin	PI	100mcg
Amox/Clav	AMC	30mcg
Pipercillin/Tazobactum	PIT	100/10mcg
Cephalothin	CEP	30mcg
Cefuroxime	CXM	30mcg
Cefotaxime	CX	30mcg
Ceftazidime	CAZ	10mcg
Cefepime	CPM	30mcg
Aztreonam	AT	30mcg
Imipenem/Meropenem	IPM/MRP	10/10mcg
Amikacin	AK	30mcg
Gentamicin	GEN	10mcg
Netilmicin	NET	10mcg
Tobramycin	TOB	10mcg
Ciprofloxacin	CIP	5mcg
Chloramphenicol	C	30mcg
Co-Trimoxazole	COT	25mcg
Polymyxin-B	PB	300mcg
Colistin	CL	10mcg
Ampi+Sulbactam	A/S	10/10mcg
Ceftriaxone	CTR	30mcg
Norfloxacin	NX	10mcg
Nitrofurantoin	NIT	100mcg

Minimum Inhibitory Concentration

MIC was checked with the help of Vitek 2 radiometric techniques.

Phenotypic tests

a. Imipenem-EDTA Double disc synergy test (DDST)

- **Test Procedure**

Test organism was inoculated on Mueller Hinton media according to the CLSI guideline. A Imipenem disc was placed and one 5µL 0.5M EDTA blank filter paper disc was put 10-20 mm apart from the Imipenem disc. Enhancement of a synergistic inhibitory zone was regarded as MBL positive.

b. Imipenem-EDTA Combined Disc test (CDT)

- **Test Procedure**

Here test organism was inoculated as recommended by CLSI guidelines [Clinical and Laboratory Standards Institute] on Mueller Hinton (MH) agar and two 10µg Imipenem discs were placed keeping maximum distance from each other in a 90 cm culture plate. To one of the discs 10µL, 0.5 M EDTA solution was added. After 16 hours of incubation at 35°C, the zone of inhibition around Imipenem-EDTA discs if greater than 7mm, compared to the Imipenem discs alone, then the test organism was considered as MBL producing.

c. Modified Hodge's Test

Step 1	Prepare a 0.5 McFarland dilution of the *E.coli* ATCC 25922 in 5 ml of broth or saline**.**
Step 2	Dilute 1:10 by adding 0.5 ml of the 0.5 McFarland to 4.5 ml of MHB or saline.
Step 3	Streak a lawn of the 1:10 dilution of *E.coli* ATCC 25922 to a Mueller Hinton agar plate and allow to dry 3–5 minutes.
Step 4	Place a 10 µg imipenem susceptibility disk in the center of the test area.
Step 5	In a straight line, streak test organism from the edge of the disk to the edge of the plate. Up to four organisms can be tested on the same plate with one drug.
Step 6	Incubate overnight at 35^0C ± 2^0C in ambient air for 16–24 hours

MHT Positive test has a clover leaf-like indentation of the *E.coli* 25922 growing along the test organism growth streak within the disk diffusion zone.

MHT Negative test has no growth of the *E.coli* 25922 along the test organism growth streak within the disc diffusion.

Molecular Tests

- **Isolation of DNA and PCR Amplification of extracted DNA**

DNA extraction was done using Invitrogen Kit following manufacturer's protocol. The bla_{NDM-1} gene sequences were amplified from the extracted DNA of culturable diversity by

using the respective primers. The reaction was performed in a 50 µl of volume, comprising 3 µl of template DNA (~50 ng/ µl), 1.5 µl of each primer (20 pM), 25 µl of dream taq PCR master mix, 19 µl of nuclease-free water (provided with the kit.

- **PCR assay for *bla*$_{NDM-1}$ gene**

DNA from bacterial isolates was extracted; presence of *bla*$_{NDM-1}$ was established by PCR with specific primers targeting the gene. The primers from Eurofin Genomics, Banglore were used in the study which amplified an internal fragment of 264 bp of *bla*$_{NDM-1}$ gene.

Primer	Sequence
***bla*$_{NDM-1}$ forward**	5'- GGTTTGGCGATCTGGTTTTC-3'
***bla*$_{NDM-1}$reverse**	5'-CGGAATGGCTCATCACGATC-3'

- **PCR Protocol followed were**

Initial Denaturation 94^0c for 5 minutes.

Final Denaturation 95^0c for 30 seconds.

Annealing 58^0c for 30 seconds.

Initial Extension 72^0c for 30 seconds.

} 35 cycles

Final Extension 72^0c for 10 minutes.

Freezing 4^0c for 10 minutes

- **Purification**

Purification was done using QIAquick PCR purification kit (QIAGEN catalogue number 28106) following manufacturer's protocol.

- **Sequencing**

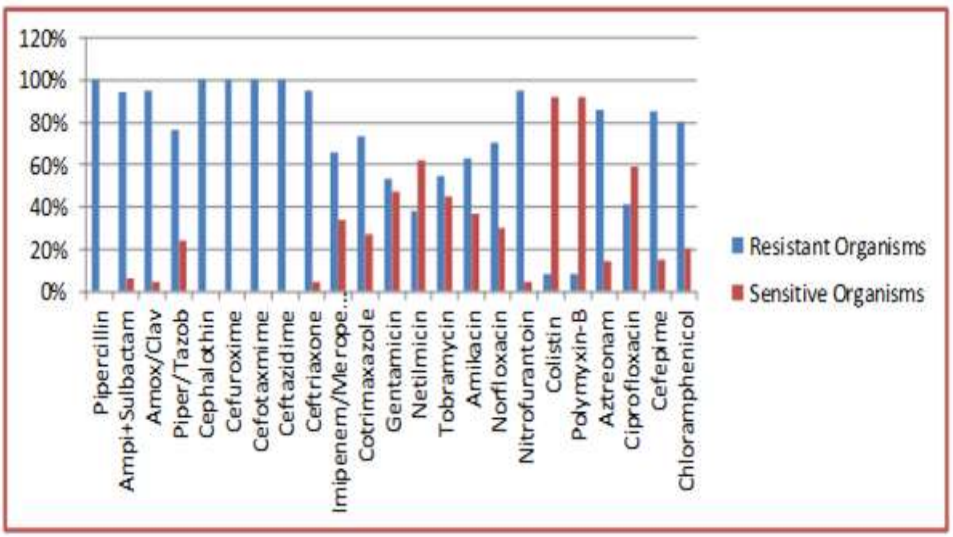
After purification, PCR products were sequenced by ABI 3130 XL DNA sequencer, using the same set of primers. The sequencing was done using Genetic Analyzer 3130 XL (ABI) based on the Big Dye terminator v 3.1 (Chain terminator) chemistry. Aligned contiguous consensus sequences of nucleotides were used for homology search by the Basic Local Alignment Search Tool (BLAST) software algorithm at National Centre for Biotechnology Information (NCBI).

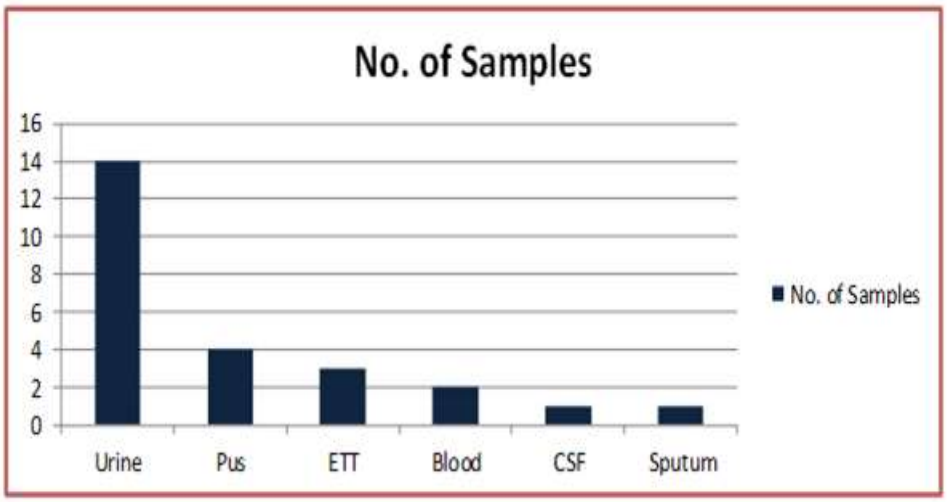
RESULTS

- **Antibiotic Susceptibility Test**

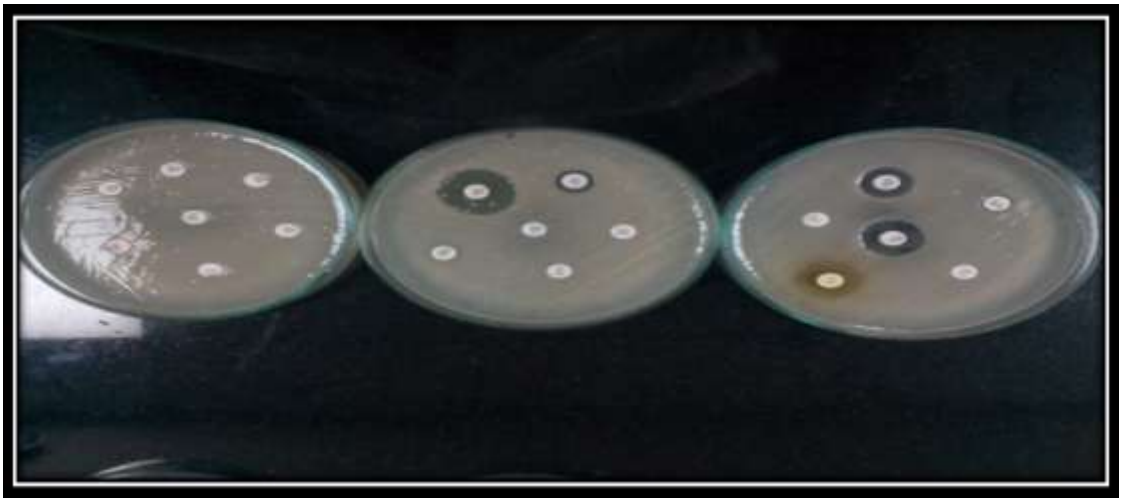
Sr no	Antibiotics	Resistant Organism	Sensitive Organism
1	Pipercillin	30/30 (100%)	0/30 (0%)
2	Ampi+Sulbactam	17/18 (94%)	1/18 (6%)
3	Amox/Clav	19/20 (95%)	1/20 (5%)
4	Piper/Tazob	20/26 (76%)	6/26 (24%)
5	Cephalothin	6/6 (100%)	0/6 (0%)
6	Cefuroxime	30/30 (100%)	0/30 (0%)
7	Cefotaxmime	30/30 (100%)	0/30 (0%)
8	Ceftazidime	24/24(100%)	0/24 (0%)
9	Ceftriaxone	23/24 (95%)	1/24 (5%)
10	Imipenem/Meropenem	20/30(66%)	10/30 (34%)
11	Cotrimaxazole	14/19 (73%)	5/19 (27%)
12	Gentamicin	16/30 (53%)	14/30 (47%)
13	Netilmicin	7/18 (38%)	11/18 (62%)
14	Tobramycin	10/18 (55%)	8/18 (45%)
15	Amikacin	19/30 (63%)	11/30 (37%)
16	Norfloxacin	14/20(70%)	6/20(30%)
17	Nitrofurantoin	20/21 (95%)	1/21 (5%)
18	Colistin	2/28(8%)	26/28 (92%)
19	Polymyxin-B	2/28(8%)	26/28 (92%)
20	Aztreonam	13/15(86%)	2/15 (14%)
21	Ciprofloxacin	7/17(41%)	10/17 (59%)
22	Cefepime	24/28(85%)	4/28 (15%)
23	Chloramphenicol	4/5 (80%)	1/5 (20%)

Maximum isolates were showing sensitivity to Polymyxin B and Colistin





Distribution of *Klebsiella pneumoniae* in various clinical isolates



Antibiotic Susceptibility Test

- **Phenotypic test**

DDST

CDT

MHT

Out of 30 MDR *Klebsiella pneumoniae*, 3 isolates where giving positive Phenotypic Confirmation Test

Electrophoretic banding pattern on 1.5% agarose gel showing PCR amplified products of bla_{NDM-1}
Lane 1 = Ladder
Lane 2 = Sample number 40 (Positive for NDM-1)
Lane 3 = Sample number 197(Negative for NDM-1)
Lane 4 = Sample number 216 (Positive for NDM-1)

- **Sequencing of amplified gene was carried out with accession numbers**

Sr no.	Isolate no.	Closest identity	Similarity	Accession no.
1	216	*Klebsiella pneumoniae* subsp. Pneumoniae strain ST147 metallo-beta-lactamase (blaNDM-5) gene	99%	MF033365.1
2	40	*Klebsiella pneumoniae* strain Kb43 metallo-beta-lactamase NDM-1 (blaNDM-1) gene	93%	JN697592.1

DISCUSSION

Klebsiella pneumoniae is a common cause of community-acquired and health-care–acquired infections. Carbapenems are being increasingly used to treat infections due to multi drug resistant Enterobacteriaceae. This has got a major impact in the emergence of multi drug resistance which can be easily transmitted from one species to another by transferable elements such as plasmid [Khajuria *et al*] Colistin is the main stay of therapy. Almost all the isolates were sensitive to colistin.

Phenotypic methods like combined disk test, Modified Hodge's test and MIC of imipenem and imipenem- EDTA were also used for enhanced detection of NDM-1 producers as has been recommended by other studies [Girlich *et al*]. Here, 3 isolates were showing Positive Phenotypic Confirmation Test.

In the present study, out of 30 Multidrug resistant Gram-negative bacteria, 15 were imipenem resistance out of which 2 (13.33%) *bla*NDM-1 producers were detected by PCR. In Indian studies, the prevalence of *bla*NDM-1 producers among carbapenem-resistant Enterobacteriaceae ranged between 31.2% and 91.6%. [Kumarasamy *et al*].

CONCLUSION AND INTERPRETATION

The study showed the presence of clinical isolates expressing NDM-1 in Bharati Hospital, Pune. These isolates harbour plasmid mediated multiple drug resistant determinants and can disseminate easily across several unrelated genera. To halt their spread, early identification of these isolates is mandatory. Phenotypic and molecular screening should be employed along with routine Antimicrobial Susceptibility Testing to reflect the true number of metallo-beta-lactamase producers.

An international effort is essential to control the spread. Scientists need to develop new antibiotics but again it will be a temporary option. To get rid of this problem scientists have to design an ingenious tool so that it will correct and reliable option to combat. At present there is urgent need of global monitoring and infection control.

Future Prospects

- Purification and Sequencing of all the samples.
- siRNA (synthetic RNA duplex designed to specifically target a particular mRNA for degradation.)
- Curing of the cryptic plasmid and block the NDM-1 gene and to check the behavior of the organism.
- Followed by working on nanoparticles and their derivatives because antibiotics will be one time use and we are on a stage that we all need to think one step ahead at a time.

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