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Phenotypic Detection of Metallo-β-lactamase in Carbapenem Resistant Clinical Isolates of *Klebsiella pneumoniae ssp. pneumoniae* at a Tertiary Care Hospital in Jaipur

Authors
Sonali Mittal[1], Ved Prakash Mamoria[2]*, Ashina Singla[3], Daisy Bacchani[4], Preeti Chaudhary[5]
[1]PG Resident III year, Department of Microbiology, Mahatma Gandhi Medical College& Hospital, Jaipur (Raj.)
[2]Professor and Head, Department of Microbiology, Mahatma Gandhi Medical College& Hospital, Jaipur (Raj.)
[3]Associate Professor, Department of Microbiology, Mahatma Gandhi Medical College& Hospital, Jaipur (Raj.)
[4,5] PG Resident II year, Department of Microbiology, Mahatma Gandhi Medical College& Hospital, Jaipur (Raj.)
*Corresponding Author
Ved Prakash Mamoria

Abstract
K. pneumoniae ssp pneumoniae is notorious for causing nosocomial as well as community-acquired infections. Over the decades, K. pneumoniae strains have increasingly acquired resistance to a wide range of antibiotics, one of the major mechanisms of resistance being expression of β-lactamases. Carbapenems have been the drug of choice for ESBL and Amp-C β-lactamase producing strains. The presence of carbapenemases like Metallo-β-lactamase renders the bacteria recalcitrant to treatment due to its broad-spectrum resistance profile. The MBL-positive strains are usually resistant to β-lactams, aminoglycosides and fluoroquinolones, leaving the treatment option with potentially toxic drugs like colistin. In the present study, 291 K. pneumoniae ssp pneumoniae clinical isolates were tested by Combined Disc Test for phenotypic detection of MBL production. A ≥ 7 mm increase in the inhibition zone diameter around imipenem+EDTA disk in comparison to imipenem-only disk was interpreted as a positive result for MBL production. Out of 191 MBL screen-positive isolates, 68 were confirmed to be MBL producers by CDT (23.37% prevalence of MBL producing isolates). MBL producing isolates were 98.5% resistant to amoxicillin-clavulanate combination,100% resistant to third-generation and fourth-generation cephalosporins, 100% resistant to carbapenems, 100% resistant to fluoroquinolone, 98.5% resistant to monobactams, 83-88% resistant to aminoglycosides and 75% resistant to cotrimoxazole. Maximum sensitivity was towards tigecycline (47.06%). This situation warrants a consistent surveillance of antimicrobial resistance of Klebsiella pneumoniae ssp pneumoniae and requires implementation of an efficient infection control programme.

Keywords: *Klebsiella pneumoniae, Metallo-β-lactamase, antimicrobial susceptibility testing, Combined disc test, multi-drug resistant.*

Introduction

Klebsiella pneumoniae is a facultative anaerobic, gram-negative, encapsulated, non-motile bacilli that belongs to the family Enterobacterales. It is ubiquitous in nature and readily colonizes human intestinal tract and nasopharynx[1] from where it

can gain entry to other tissues and cause infections. *K. pneumoniae* has gained notoriety for causing nosocomial as well as community-acquired infections. Over the decades, *K. pneumoniae* strains have increasingly acquired resistance to a wide range of antibiotics, as a consequence of which simple infections such as urinary tract infections (UTIs) have become recalcitrant to treatment, and more serious infections such as pneumonia and bacteremia have become increasingly life-threatening.[2]

One major mechanism of antibiotic resistance in *Klebsiella pneumoniae* involves the expression of β-lactamases. Carbapenems are the drug of choice to treat infections caused by extended spectrum β-lactamase (ESBL) and AmpC β-lactamase producing *K. pneumoniae*.[3] Expression of carbapenemase enzymes like plasmid mediated metallo-β-lactamase (MBL) in *K. pneumoniae* is even more troubling since it renders the bacteria resistant to almost all available β-lactams, including the carbapenems, aminoglycosides and fluoroquinolones.[4,5] This leaves the treatment option only with potentially toxic drugs, like colistin.[6] The spread of MBL producers and absence of novel agents in the future and may lead to therapeutic dead ends.

Hence, early detection of MBL is crucial, the benefits of which include implementation of proper antibiotic therapy and infection control policy. Metallo-β-lactamases (MBLs) belong to Ambler class B.[7] Molecular detection of these enzymes is the gold standard in their diagnosis but lack of availability of molecular methods like PCR in routine diagnostic laboratories due to factors like cost and expert technical skill is a major limitation in the developing countries. In the absence of molecular techniques, phenotypic methods if performed using stringent precautions, and by standard methods provide effective and reliable alternatives.[8] It is a challenge for clinical microbiologists to accurately detect MBL by employing chelating agents which inhibit the MBL activity. An imipenem disc with added ethylene-diamine-tetra-acetic acid (EDTA) (750 µg) was reported by Yong et al[9] to detect MBL-producing clinical isolates of *Pseudomonas spp*. and *Acinetobacter spp*. with high sensitivity.

Therefore, the present study was undertaken to estimate the burden of MBL β-lactamases producing *K. pneumoniae ssp pneumoniae* using Combined disk test (CDT) as the phenotypic method. Antimicrobial susceptibility pattern of these strains was analyzed to get a better understanding of the proper antibiotic therapy to be implemented in our hospital setting.

Material and Methods

This laboratory based observational study was carried out in the Department of Microbiology, Mahatma Gandhi Medical College, Jaipur (Rajasthan) over a period of one year. Institute ethics committee approval was obtained before start of the study.

Sample Size

A total of 291 non-repetitive clinical isolates of *Klebsiella pneumoniae ssp pneumoniae* recovered from various clinical specimens such as urine, sputum, pus, pus swab, endotracheal (ET) secretion, bronchoalveolar lavage (BAL), cerebrospinal fluid (CSF), pleural fluid, ascitic fluid and blood, which were received for bacterial culture & sensitivity from inpatients and outpatients at Microbiology laboratory, MGMCH, Jaipur, were included in this study. These isolates which were included in the study were identified by VITEK 2 Compact.

Antimicrobial Susceptibility Testing

Antimicrobial susceptibility testing was performed on Mueller-Hinton agar (MHA) by Kirby Bauer disk diffusion method.[10] The following antibiotic discs (drug concentrations in µg) were used: Amoxicillin/clavulanate (20µg/10µg), Ceftazidime (30µg), Ceftriaxone (30µg), Cefotaxime (30µg), Cefepime (30µg), Imipenem (10µg), Ertapenem (10µg), Aztreonam (30 µg), Gentamicin (10 µg), Amikacin (30 µg), Ciprofloxacin (5µg), Tigecycline (15µg) and

Trimethoprim-sulfamethoxazole (Co-trimoxazole) (1.25/23.75 µg). Zone diameters were recorded and results interpreted as per Clinical Laboratory Standards Institute (CLSI) 2019 criteria for zone diameter breakpoints.[11]

Escherichia coli American Type Culture Collection (ATCC®) 27922 was used as control strain.

Detection of Metallo-β-lactamases (MBL) by Combined Disk Test (CDT) [12]

Klebsiella pneumoniae ssp pneumoniae isolates which were resistant to imipenem (≤19mm) and/or ertapenem (≤18mm) (MBL screen-positive isolates) were tested phenotypically for confirmation of MBL production using the **imipenem-ethylenediamine-tetra-acetic acid (EDTA) combined disk test (CDT).** Lawn culture of MBL screen-positive isolate was made on Mueller-Hinton agar plate. Two disks, one of imipenem (10µg) and other disk of imipenem+EDTA (10/750µg) were placed approximately 30mm apart (centre to centre) on the MHA plate inoculated with the MBL screen positive isolate. The zones of inhibition of imipenem and imipenem+EDTA disks were compared after 16 to 18 hours of incubation at 37˚C. A ≥ 7 mm increase in the inhibition zone diameter around imipenem+EDTA disk in comparison to imipenem-only disk was interpreted as a positive result for MBL production. **(Figure 1)**

Klebsiella pneumoniae ATCC® 700603 was used as control strain.

All the media and antimicrobial discs were procured from Himedia Laboratories, Mumbai.

Results

The highest percentage of *K. pneumoniae ssp pneumoniae* isolates among the processed specimens was found in urine (32.65%) followed by ET secretion (20.96%), pus & pus swab (17.18%), sputum (14.43%), blood (7.90%), sterile body fluids (5.15%) and BAL (1.72%). 191 out of 291 isolates (65.63%) were resistant to imipenem and/or ertapenem. These MBL screen-positive isolates were further subjected to MBL phenotypic confirmatory test by CDT. Among 191 screen-positive isolates, 35.60% (68 out of 191) were confirmed as MBL producers in combined disk test (CDT).

Hence, the prevalence of MBL in the present study of 291 *K. pneumoniae ssp pneumoniae* isolates was found to be 23.27%. Majority of MBL producing isolates were recovered from in-patients as compared to out-patients. **(Table1)**

Urine and ET secretions were the most common specimens from which MBL producing isolates were recovered**. (Table 2)**

In the present study, MBL producing isolates were 98.5% resistant to amoxicillin-clavulanate combination,100% resistant to third-generation and fourth-generation cephalosporins, 100% resistant to carbapenems, 100% resistant to fluoroquinolone, 98.5% resistant to monobactams, 83-88% resistant to aminoglycosides and 75% resistant to cotrimoxazole. Maximum sensitivity was towards tigecycline (47.06%). **(Table 3, Chart1)**

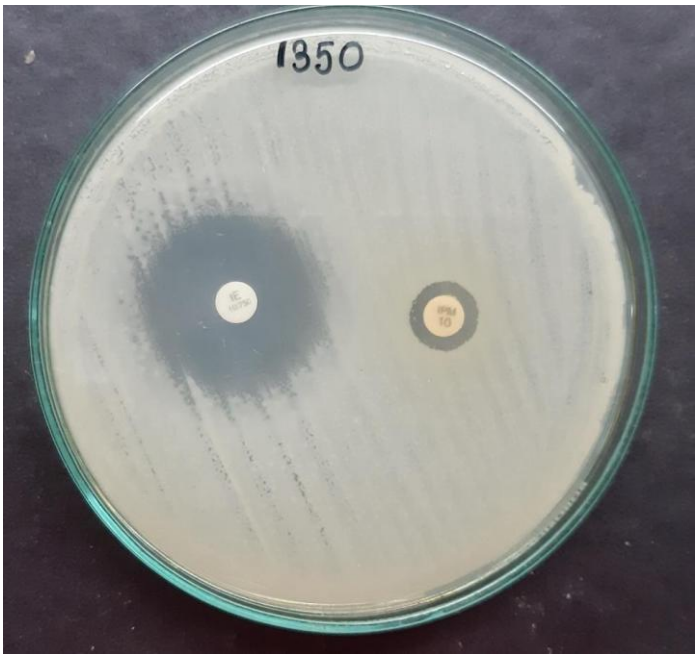

Figure 1: Shows a positive Combined disk test (CDT) for MBL production with >7mm increase in imipenem/EDTA disk in comparison to imipenem alone.

Table 1: Distribution of MBL producers among IPD & OPD patients

Location	MBL producing isolates n(%)
IPD	63 (92.65)
OPD	5 (7.35)
Total isolates	68 (100)

Table 2: Distribution of MBL producing isolates among various clinical specimens

Specimen	MBL producing isolates n(%)
Urine	22 (32.35)
Et secretion	21 (30.88)
Pus & swab	8 (11.76)
Sputum	6 (8.82)
Blood	5 (7.35)
Sterile body fluids (Ascitic fluid, CSF & Pleural fluid)	5 (7.35)
BAL	1 (1.47)
Total isolates	68 (100)

Table 3: Antimicrobial susceptibility pattern of MBL producing *K. pneumoniae ssp pneumoniae* isolates (R= Resistant, S= Sensitive)

Name of antimicrobial agent	MBL Positive isolates, n (% within 68 MBL) R= Resistant; S= Sensitive
Amoxicillin/Clavulanate	**R** 67 (98.53) **S** 1 (1.47)
Ceftazidime	**R** 68 (100) **S** 0
Cefotaxime	**R** 68 (100) **S** 0
Ceftriaxone	**R** 68 (100) **S** 0
Cefepime	**R** 65 (95.60) **S** 3 (4.41)
Aztreonam	**R** 67 (98.53) **S** 1 (1.47)
Imipenem	**R** 68 (100) **S** 0
Ertapenem	**R** 68 (100) **S** 0
Amikacin	**R** 60 (88.23) **S** 8 (11.76)
Gentamicin	**R** 57 (83.82) **S** 11 (9.40)
Ciprofloxacin	**R** 68 (100) **S** 0
Tigecycline	**R** 36 (52.94) **S** 32 (47.06)
Trimethoprim/ Sulfamethoxazole (Co-trimoxazole)	**R** 51 (75) **S** 17 (25)

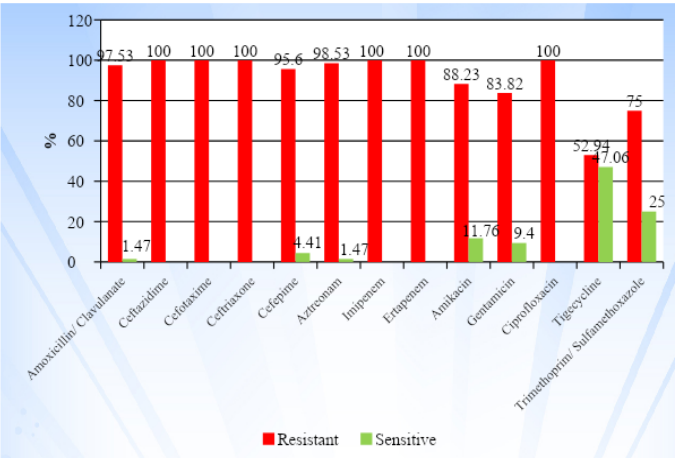

Chart 1: Antimicrobial susceptibility pattern MBL producing *K. pneumoniae ssp pneumoniae* isolates

Discussion

In the present study out of 291 isolates, 68 (23.37%) were found to be MBL producers. Supportive results were seen in a study conducted by Oberoi L et al[13] where the prevalence of MBL producing *K. pneumoniae* was 22.72%. Another study which showed similar observation was conducted in Egypt by Shahandeh Z et al[14], which reported the MBL prevalence to be 23.1%. Various studies from different parts of India like Karnataka[15] (30.69%), Pune[16] (19.91%), Dehradun[17] (4.34%) have reported a varied percentage of MBL producing *K. pneumoniae ssp pneumoniae*.

In the present study, maximum number of MBL producing isolates (32.35%) was recovered from urine followed by ET secretions (30.88%). Study conducted by Singh M et al.[17], reported that maximum percentage of MBL producing isolates were recovered from ET secretions (62.5%) where as another study conducted by Kamble D et al[16] reported highest percentage of MBL producers from pus (42.55%), which was closely followed by urine (40.42%).

In the present study, MBL producing isolates are 100% resistant to all third-generation cephalosporins used in the study, 100% resistant to both imipenem and ertapenem, 100% resistant to ciprofloxacin, 98.53% resistant to aztreonam and amoxicillin/clavulanate. Tigecycline is the

only drug which showed a moderate resistance of 53%. Similar resistance to third-generation and fourth-generation cephalosporins was observed in studies conducted by Bora A et al[4], Deshmukh DG et al[18], Franco MRG et al[19] and Souli M et al.[20]. Fluoroquinolone resistance was very high in the present study which correlated with findings on Franco MRG et al[19] and Bora A et al.[4] Aminoglycoside resistance findings of the present study were comparable with other studies.[4,19,20] However, Deshmukh DG et al[18] reported much lower aminoglycoside resistance. Toleman MA et al[21] demonstrated that a large proportion of MBL genes are associated with one or more aminoglycoside- or β-lactam resistant genes, partially explaining multi-drug-resistant cases. Tigecycline resistance is even higher in a few other studies.[4,19]

Conclusion

The results of this study revealed a worrying situation concerning *Klebsiella pneumoniae ssp pneumoniae* that is resistant to antibiotics commonly used to treat infections. The findings of this study also revealed that the prevalence of MBL was high in *Klebsiella pneumoniae ssp pneumoniae.* This situation warrants a consistent surveillance of antimicrobial resistance of *Klebsiella pneumoniae* and requires implementation of an efficient infection control programme.

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