Phenotypic detection of Carbapenemase production and difference in antimicrobial susceptibility pattern in clinical isolates of *Klebsiella pneumoniae* at a Medical College Hospital in Vidarbha region

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Abstract

Introduction: Carbapenemases are one of the common β-lactamases seen in *Klebsiella pneumoniae* that are responsible for multi drug resistance. Detection of resistance in these bacteria is necessary for formulation of infection control policies. The hidden resistance of this bacteria is critical to diagnose. To address this problem, the present study aims to detect Carbapenemase production and antimicrobial susceptibility patterns in clinical isolates of *Klebsiella pneumoniae* at the present setting.

Materials and Method: A total of 438 strains of *Klebsiella pneumoniae* were isolated and subjected to Carbapenemase detection by Screening method using Imepenem (10 µg) disc. Modified Hodge test and Combined disc diffusion test along with E-test was done to confirm carbapenemase production followed by antimicrobial susceptibility testing. E-test was considered to be gold standard.

Results: Total 34/438 (7.76%) carbapenem resistant isolates were obtained. The carbapenemase positive isolates were predominantly isolated from Burns wards (14.61%). E-test considering it as gold standard test confirmed all 34 of these as carbapenemase producers. Out of 34, MHT detected 31and CDT detected 32 isolates as positive for carbapenemase production. They were highly resistant to cefotaxime, and ceftazidime (91.18%).

Conclusion: Screening for Carbapenemase production needs to be carried out routinely in every clinical diagnostic facility. There is a need for rational use and strict adherence to the concept of "reserve drugs" to minimize the misuse of available antimicrobials. The findings of this study emphasize the need for a continuous surveillance in the ICUs and different hospital wards to detect the resistant strains.

Keywords: Carbapenemase, *Klebsiella pneumoniae*, Modified Hodge test, Etest, Antimicrobial Susceptibility Pattern

Introduction

A global spread of resistance to antimicrobials has been seen in the strains of *Klebsiella pneumoniae* that is intensifying through time (Anderson et al., 2007). Diverse ranges of β-lactamases that are plasmid/ transposons mediated and horizontally transferred in these bacteria, lead to resistance. Carbapenemases are one of the common β-lactamases seen in these bacteria that are responsible for multi drug resistance (Bora et al., 2014). Antimicrobial resistance threatens the effective prevention and treatment of an ever increasing range of infections caused by it. It has led to prolonged hospital stay, complex and costlier treatment and bad prognosis (Brown, 1991). Detection of resistance in these bacteria is necessary for epidemiological purposes as well as formulation of infection control policies and antibiotic policies for the hospitals. Diagnostic microbiology is needed to meet all these requirements (Carvalhaes et al., 2010). The hidden resistance of this bacteria is critical to diagnose, as they seem to be under diagnosed with routine testing (Chakraborty et al., 2010). To address this problem, the present study aims to detect Carbapenemase production and antimicrobial susceptibility patterns in clinical isolates of *Klebsiella pneumoniae* at the present setting.

Materials and Method

The present observational descriptive study was carried out in the Department of Microbiology at Government Medical College, Akola during January 2015 to June 2016 by convenient sampling method. A total of 438 laboratory confirmed non-repetitive isolates *of Klebsiella pneumoniae* from clinical samples were included. The Data of patients regarding the age, sex, and brief history was recorded. Subsequently, the patients were counseled to obtain consent for being part of the study. The study was initiated after obtaining approval from the Institutional Ethical Committee. Depending on site of infection, two specimens of various samples like urine, pus, wound swabs, sputum, bronchoalveolar lavage, blood and other body fluids, were collected, transported immediately and processed as per the standard microbiological methods.(CLSI, 2014) Of the two specimens collected,one was used for microscopic examination and other was used for culture. All strains were then identified on the basis of microscopy, Gram staining and biochemical tests (Denisuik et al., 2013).

Antimicrobial Susceptibility Testing (Fattouh et al., 2015): Each isolate was subjected to antimicrobial susceptibility test as per CLSI guidelines by Kirby-Bauer disc diffusion technique. Antibiotic Discs were commercially available (HiMedia Lab, Mumbai) discs of 6 mm diameter with recommended potency. Discs

used were- Gentamicin (10 µg), Amikacin (30 µg), Ampicillin (10 µg), Amoxiclav (20/10 µg), Cefuroxime (30 µg), Ceftazidime (30 µg), Cefotaxime (30 µg), Cefepime (30 µg) and Aztreonam (30 µg). Susceptible, intermediate and resistant was interpreted with reference to CLSI guidelines. Isolates with intermediate susceptibility were included in resistant category. Each batch of Muller Hinton agar (MHA) and antibiotic discs were tested by using *Escherichia coli* ATCC 25922 strain.

Carbapenemase Production: Carbapenemase production in *Klebsiella pneumoniae* isolates was tested by performing screening test and phenotypic confirmatory test. For screening test, lawn culture of 0.5 McFarland inoculum of the test strain was exposed to a disc of imipenem (10 µg). *Escherichia coli* ATCC 25922 was used as quality control. After overnight incubation, the zone diameter between 16-21 mm indicated Carbapenemase production (Fattouh et al., 2015).

Phenotypic confirmatory test: Modified Hodge test (MHT): A 0.5 McFarland standard suspension of *Escherichia coli* ATCC 25922 was prepared in a saline and it was further diluted in 1:10 in saline. The MHA plate was inoculated as per the routine disc diffusion procedure. The plate was allowed to dry for 3 to 10 minutes. A single disc of Meropenem was placed on the plate at the centre. Using a 10-µl loop, 3–5 colonies of test or quality control (QC) organism grown overnight on a blood agar plate were picked up. They were inoculated perpendicular to the disc in a straight line (at least 20–25 mm in length). *Klebsiella pneumoniae* ATCC BAA-1705 and ATCC BAA-1706 were used as positive and negative control. The plate was incubated at 37^0C for 16-20 hours. Following incubation, the MHA plate was examined for enhanced growth at the intersection of the streak and the zone of inhibition. The enhanced growth suggests positive test for the carbapenemase production.

Combined disc test (Disc potentiation test) (Forbes et al., 2002): It was used to detect Metallo-β-lactamase (MBL) production. In this test, the lawn culture of 0.5 McFarland inoculum of the test strain was exposed to a disc of imipenem (10 µg) and imipenem-EDTA (10/750 µg). The difference in diameter of ≥7mm in zones of inhibition of two discs after incubation period of 16-18 hours at 35ºC indicated MBL production.

ETEST: All imipenem resistant isolates were subjected to ETEST® strip impregnated with Meropenem (4-256 µg/ml) (MRP) at one side of strip with Meropenem (1-64 µg/ml)-EDTA (MRPE) on the other side. The MIC end points were read where the inhibition ellipses intersected the strip. A reduction of MRP by ≥3 twofold dilutions in the presence of EDTA was interpreted as being suggestive of Carbapenemase production. Equally, the presence of a "phantom" zone between the two gradient sections or deformation of MRP ellipse was indicative of Carbapenemase.

Results

Total 34 (7.76%) carbapenemase producing isolates of *Klebsiella pneumoniae* were recovered from 438 specimens. Female predominance (58.82%) with sex ratio of 1:1.4 was seen in this study. Most infected patients belonged to 11 to 20 years and 51 to 60 years (20.59%) of age group. (Table 1)

Table 1: Showing sex wise distribution of Carbapenemase producing isolates

Years	Male	Female	CP isolates (%)
<1	1	0	1 (2.94)
1 to 10	1	0	1 (2.94)
11 to 20	0	7	7 (20.59)
21 to 30	1	4	5 (14.71)
31 to 40	2	3	5 (14.71)
41 to 50	2	2	4 (11.76)
51 to 60	4	3	7 (20.59)
>60	3	1	4 (11.76)
Total	14 (41.18%)	20 (58.82%)	34

The carbapenemase positive isolates were predominantly isolated from Burns wards (14.61%) and ICUs (13.33%) followed by Medicine wards (12.26%). However, isolates from other wards including ENT, ophthalmology, and skin wards did not show carbapenemase production. (Fig. 1). Among various samples, highest number of isolates were obtained from blood (23.53%) and pus (11.11%).

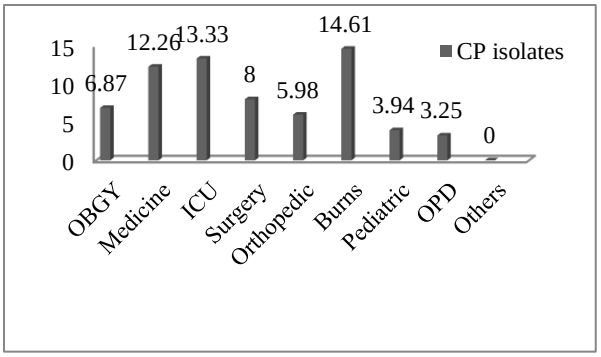


Fig. 1: Showing ward wise distribution of Carbapenemase producing (CP) isolates

Table 2: Sample wise distribution of carbapenemase producing isolates

Sample	Carbapenemase producing isolates (n=34)	Percentage
Pus	15	44.12
Urine	6	17.65
Sputum	4	11.76
Blood	4	11.76
Stool	5	14.71

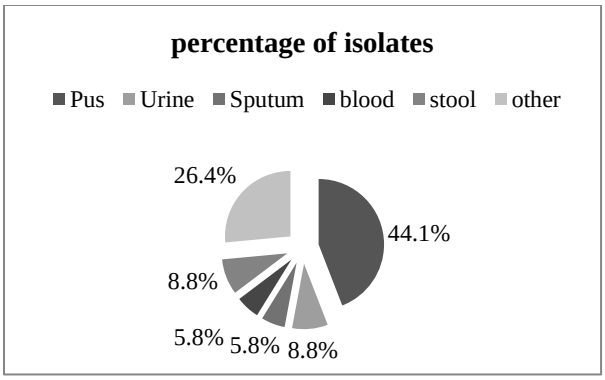


Fig. 2: Sample wise distribution of carbapenemase producing isolates

All carbapenemase producing isolates were completely resistant to ampicillin, amoxiclav, cefuroxime. They were highly resistant to 3rd and 4th generation cephalosporins i.e., cefotaxime, and ceftazidime (91.18%); and cefipime (88.24%). Aminoglycosides, predominantly amikacin was sensitive to 23 (67.65%) isolates (Table 3). Most Non-carbapenemase producers were resistant to ampicillin (81.68%), amoxiclav (73.76%). Minimal resistance was shown to imipenem (3.71%), amikacin (16.58%) (Table 4).

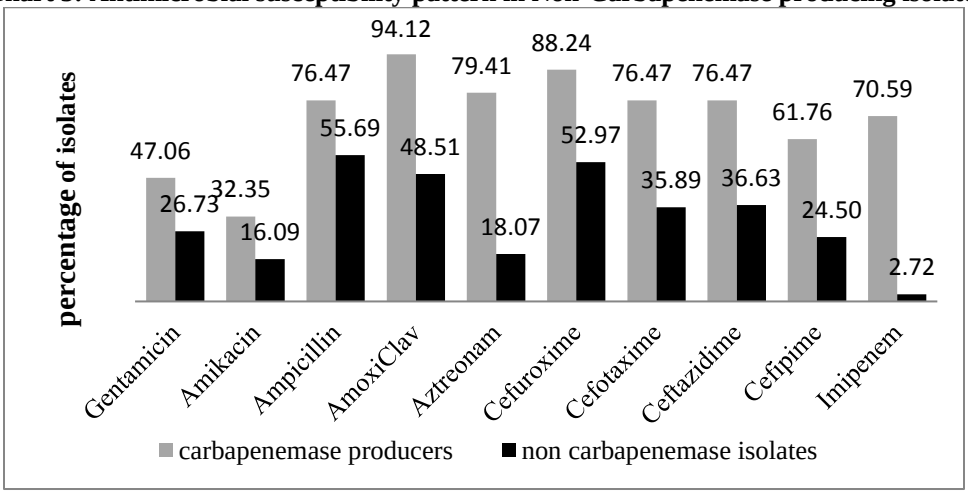
Table 3: Antimicrobial susceptibility pattern in Carbapenemase producing isolates

Antimicrobials	Sensitive (n=34)	Resistant (n=34)
Gentamicin	18 (52.94)	16 (47.06)
Amikacin	23 (67.65)	11 (32.35)
Ampicillin	0 (0)	34 (100)
Amoxiclav	0 (0)	34 (100)
Aztreonam	4 (11.76)	30 (88.24)
Cefuroxime	0 (0)	34 (100)
Cefotaxime	3 (8.82)	31 (91.18)
Ceftazidime	3 (8.82)	31 (91.18)
Cefipime	4 (11.76)	30 (88.24)
Imipenem	0 (0)	34 (100)

Table 4: Antimicrobial resistance pattern in Non-Carbapenemase producing isolates

Antimicrobials	Sensitive n (%)	Resistant n (%)
Gentamicin	285 (70.54)	119 (29.46)
Amikacin	337 (83.42)	67 (16.58)
Ampicillin	74 (18.32)	330 (81.68)
Amoxiclav	106 (26.24)	298 (73.76)
Aztreonam	268 (66.34)	136 (33.66)
Cefuroxime	185 (45.79)	219 (54.21)
Cefotaxime	299 (74.01)	105 (25.99)
Ceftazidime	254 (62.87)	150 (37.13)
Cefipime	251 (62.13)	153 (37.87)
Imipenem	389 (96.29)	15 (3.71)

Chart 3: Antimicrobial susceptibility pattern in Non-Carbapenemase producing isolates



Total 34/438 (7.76%) carbapenem resistant isolates were obtained. Etest considering it as gold standard test confirmed all 34 of these as carbapenemase producers. Out of 34, MHT detected 31 (91.18%) and CDT detected 32 (94.18%) isolates as positive for carbapenemase production. Both the tests were compared with the gold standard test (Etest).

Table 5: Results of various Carbapenemase detection methods

Method	Screening	MHT	CDT	Etest (Gold standard)
CP positive isolates	34	31	32	34

Discussion

Prevalence of Carbapenemase producing isolates: Carbapenems are the antibiotics of choice for treatment of infections caused by ESBL producing bacteria. However, several studies have reported worldwide increased production of β-lactamases, which hydrolyze all β-lactam antibiotics including carbapenems (Gelbrand et al., 2015) Carbapenemase mediated resistance is a serious cause for concern in the therapy of critically ill patients (Hirsch and Tam, 2010). This study has revealed a prevalence of the carbapenemase phenotype of 7.76% in *Klebsiella pneumoniae*. Our findings are comparable to study done by (Kumarasamy et al., 2010) in Haryana, where they reported a prevalence of 13% in a hospital setting. Similar findings were seen in study done in 2014 by (Sood, 2014) with 15.58% prevalence (Lee et al., 2003). The similarity in the results is probably due to similar antimicrobial utility patterns in India. These observations present a worrying trend of antimicrobial resistance. (Trepanier et al., 2016) observed a very low phenotypic prevalence (0.02%) as compared to our findings.(Nagaraj et al., 2012) A lower prevalence was also seen in study done by (Denisuik et al., 2013) (0.04%) from Canadian hospitals in 2013(Nordmann et al., 2012).

Carbapenemase producing isolates from various wards: The carbapenemase positive isolates were predominantly isolated from burn ward (26.47%) and medicine ward (20.59%) followed by OBGY (14.71%) (Table1). (Nagaraj et al., 2012) isolated most carbapenemase producing isolates from general surgery, general medicine, and ICU.(Parveen et al., 2010). Robert et al., 2014) found ICU (34.6%) had the largest burden of carbapenemase producing isolates followed by surgery ward (30.8%).(Robert et al., 2014) All these studies show colonization and dissemination of these strains carrying plasmids/transposons within wards; in patients with prolonged hospital stay and clustering of critical patients in ICU resulting in such scenario.

Carbapenemase producing isolates from various samples: Among various clinical samples, highest number of isolates was obtained from blood (23.53%) and pus (11.11%). Sood et al also found Carbapenemase producing organisms from blood (25%) which is similar to our study.(Lee et al., 2003) Urine was the most common sample in studies by Singh et al (39.4%) and Robert et al (42.8%).(Robert et al., 2014; Singh et al., 2016) (Souli et al., 2010) found stool (46%) to be most common sample.(Sood, 2014) Maximum Carbapenemase producers were isolated from the patients with indwelling devices with long hospital stay and prolonged antibiotic treatment in a study by (Denisuik et al., 2013) This could be due to the resistant hospital strains. However, their association with hospital-acquired infections could not be ascertained due to time constraints and unavailability of records (Nordmann et al., 2012).

Antimicrobial susceptibility pattern in Carbapenemase positive isolates: All carbapenemase producing isolates were 100 percent resistant to ampicillin, amoxiclav, cefuroxime and imipenem. They were highly resistant to 3rd and 4th generation cephalosporins i.e. Cefotaxime, and ceftazidime (91.18%) and cefipime (88.24%). Aminoglycosides, predominantly amikacin was sensitive to 23 (67.65%) isolates (Table 3).

In a study done in South India by (Parveen et al., 2010) it was found that 45 carbapenem resistant isolates exhibiting high resistance (100%) to third and fourth generation cephalosporins, tetracycline, gentamicin, cefoxitin, amikacin. They were 100% resistant to penicillin/inhibitor combinations such as amoxicillin/ clavulanate, ampicillin/ sulbactam, and piperacillin/ tazobactam. Among the meropenem resistant isolates, 33 and 21 were resistant to imipenem and ertapenem respectively. Nearly, ninety percent of the multidrug resistant isolates were from the patients admitted in ICU.(Souli et al., 2010)

Imipenem resistance was seen in all 34 isolates in our study which correlates with (Fattouh et al., 2015) (Chakraborty et al., 2013) reported 78.25% and 59.5% resistance which has lower results than the current findings.(Srinivasan et al., 2015; Thomson, 2010) Revised CLSI carbapenem breakpoints were used in this study to determine sensitivity or intermediate/resistance for Imipenem.

All carbapenemase producing isolates demonstrated in vitro resistance to amoxicillin/clavulanate, cefazolin, ceftriaxone, ceftazidime in (Denisuik et al., 2013) (Nordmann et al., 2012)

Most Non-carbapenemase producers were resistant to ampicillin (81.68%), amoxiclav (73.76%) in the present study. Minimal resistance was shown to imipenem (3.71%) and amikacin (16.58%) (Table 6).

Detection of Carbapenemases: Total 34/438 (7.76%) carbapenem resistant isolates were obtained. E-test (Forbes et al., 2002) considering it as gold standard test confirmed all 34 of these as carbapenemase producers. Out of 34, MHT detected 31 (91.18%) and CDT detected 32 (94.18%) isolates as positive for carbapenemase production. Both the tests were compared with the gold standard test (E-test).

The E-test MBL results as proved in study by Walsh et al in 2002 were in 100% agreement with the

results from the genotypic and biochemical methods for detection of Carbapenemase.(Forbes et al., 2002) E-test Meropenem plus Meropenem-EDTA with Mueller-Hinton agar had a sensitivity of 94% (79 of 84) and specificity of 95% (124 of 130). The E-test MBL strip appears to be an acceptable diagnostic reagent to detect MBL phenotypes in the clinical microbiology laboratory. The implementation of a simple MBL detection method that is quick, specific, sensitive, and reproducible is attractive, particularly where carbapenem and other β-lactam therapeutic regimens are indicated or preferred. The E-test MBL strip has the ability to detect MBL, both chromosomally and plasmid mediated, in aerobic and anaerobic bacteria. This method can be used by clinical laboratories to monitor the emergence of MBL in a range of clinically significant bacteria.(Trepanier et al., 2016) The 'E-test' is a method for measuring MICs of antimicrobial agents against bacteria and is based on diffusion of a pre-formed antibiotic gradient from a plastic strip (Vasoo et al., 2013).

(Nagaraj et al., 2012) recovered 51 carbapenem resistant isolates that also correlated with reduced MIC of Meropenem. The presence of the MBL was best detected by CDT as compared to MHT.[17] MHT gave positive results in 52/59 (88.41%) carbapenem resistant isolates and 20 were CDT positive in a study carried out by (Fattouh et al., 2015). (Srinivasan et al., 2015) She also quoted that MHT is cost effective reliable assay and could be applied in routine microbiology laboratories in detecting carbapenemase producers. In oppose to this the MHT, while a useful screen for carbapenemases, suffers from lack of specificity, poor sensitivity for MBL detection, and a long turnaround time, findings corroborated by (Vasoo et al., 2013) (Walsh et al., 2002).

When 200 isolates were tested for carbapenemase production by Singh et al in spite of carbapenem susceptibility 46, were MHT positive out of which 17 were found to be carbapenem sensitive. This indicates that even though the strains have not exhibited Carbapenem resistance by Disk Diffusion method they had the capacity to produce Carbapenemase that may give altered results in vivo. However, this could be a matter of concern as these isolates may resist antibiotic treatment in vivo leading to treatment failure. (Singh et al., 2016)

Klebsiella pneumoniae contribute a part of the gut flora. Just like other bacteria, carbapenemase producing *Klebsiella pneumoniae* are capable of colonizing the gut of patients. They in turn serve as reservoirs for spreading infection or contaminating the environment and fomites, especially in healthcare settings. In order to control the spread, disinfection measures need to be followed as contact isolation of these infected/ colonized patients which is not a routine and may not be feasible in all healthcare institutions, especially in the developing countries. Microbiological surveillance of the rectal flora at the time of admission (especially in patients who have already been exposed to antibiotics and healthcare interventions) and contact isolation of potentially colonized/ infected patients will go a long way in preventing contamination of the environment and spread to other patients. In addition, appropriate use of carbapenems will also prevent selecting resistant bacteria in a given geographical area.(Parveen et al., 2010)

Conclusion

β-lactamase producing strains of bacteria escalating world-wide is a concern. Screening for Carbapenemase production needs to be carried out routinely in every clinical diagnostic facility with standard methods to guide clinicians in proper selection of antimicrobials. There is a need for rational use and strict adherence to the concept of "reserve drugs" to minimize the misuse of available antimicrobials. For the detection of Carbapenemase, the phenotypic confirmatory disc diffusion test is simple, sensitive, and cost effective. The findings of this study emphasize the need for a continuous surveillance in the ICUs and different hospital wards to detect the resistant strains.

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