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EMERGING CARBAPENEM RESISTANCE AMONG NOSOCOMIAL ISOLATES OF *KLEBSIELLA PNEUMONIAE* IN SOUTH INDIA

R. MOHAMUDHA PARVEEN*, B.N.HARISH AND S.C.PARIJA

Department of Microbiology, Jawaharlal Institute of Postgraduate Medical Education and Research, Pondicherry, India.

**** Corresponding Author*** mahamudhaparveen@gmail.com

ABSTRACT

Multidrug resistant *Klebsiella pneumoniae* are an increasingly difficult problem in Indian hospitals. Carbapenems have been the grounding of drug treatment for serious infections caused by these pathogens. Resistance to carbapenems has been unknown until now. The purpose of this study was to examine the prevalence of carbapenem resistance among *K. pneumoniae* in a tertiary care hospital. A total of 134 clinical isolates of *K. pneumoniae* were studied. Minimum inhibitory concentration for meropenem, colistin in resistant isolates, Metallo-beta-lactamases detection and modified Hodge's test for carbapenemase detection were done. About 43.6% percent isolates were resistant to meropenem, 32% to imipenem, 20.3% to ertapenem and 60% were resistant to colistin. None of them were Metallo-beta-lactamases producers. Six isolates showed Modified Hodge's test positive with dissimilar clonal types. This is the first report of *Klebsiella Pneumoniae* Carbapenemase (KPC) producing *K.pneumoniae* from India.

KEYWORDS

Klebsiella pneumoniae, carbapenems, multidrug resistance, KPC, RAPD typing.

INTRODUCTION

Klebsiella is an important human pathogen that has been associated in recent decades with nosocomial outbreaks. *Klebsiella* spp. primarily attack immunocompromised individuals who are hospitalized and suffer from severe underlying diseases such as diabetes mellitus, chronic pulmonary obstruction, urinary tract infections, pneumonia and intra-abdominal infections [1]. Nosocomial *Klebsiella* infections are caused mainly by *Klebsiella pneumoniae*, the medically most important species of the genus. After the introduction of extended- spectrum cephalosporins, extended spectrum β-lactamase (ESBL)-producing *Klebsiella pneumoniae* have become an increasingly serious problem worldwide [2].Their management has become complicated by the generation of a variety of ESBLs production and is considered as an important threat till now [3].ESBL-containing plasmids often carry resistance genes for other antibiotics thus; aminogycosides and fluoroquinolones may be ineffective [4]. Although β lactam/ β -lactamase inhibitors combinations have been suggested as an option for ESBL producers, these drugs must be given in high doses [5].

Presently, the members of the carbapenems are the most stable antibiotic class in the presence of

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ESBLs and are the most broadly active antibiotics available for systemic use in humans [6]. This is of great concern as presently to combat infections by multidrug resistant bacteria. Carbapenems were first introduced in 1980 and they are now frequently used as the last choice in treating serious infections caused by multidrug-resistant strains of Gram negative bacilli in intensive care units (ICUs) and in high risk wards. These are stable to β -lactamase including the ESBLs and AmpC produced by Gram negative bacilli [7, 8, 9]

The carbapenems are a class of beta-lactamase antibiotics that differ from the penicillins by the substitution of a carbon atom for a sulfur atom and by the addition of a double bond to the five membered ring of the penicillin nucleus. They bind the bacterial penicillin-binding proteins, which are responsible for elongation and cross link the peptidoglycan of the bacterial cell wall. This results in impairment of construction of the cell wall, inhibition of cell growth frequently, cell lysis and death [6]. Multi-drug resistance (including carbapenem) in *Klebsiella spp* is an increasingly difficult problem in Indian hospitals due to the lack of therapeutic options and the potential transfer of antibiotic resistance to other pathogens.

Carbapenems have been the grounding of drug treatment for serious infections caused by these pathogens. Meropenem and Imipenem are the two carbapenems available for use in India [16]. Recently, *Klebsiella spp* has developed resistance to carbapenems, an earlier unknown phenomenon and the literature concerning occurrence of resistance to carbapenem in *Klebsiella spp* is very limited from our country. Therefore we conducted this perspective study to evaluate the prevalence of carbapenem resistance in *Klebsiella spp.* isolated from clinical specimens.

MATERIALS AND METHODS

Clinical isolates

This study was conducted in a 700 bedded tertiary care hospital in South India. A total of 103 *K. pneumoniae* were obtained in a period of 8 months (November 2008 to June 2009) from patients admitted in our hospital. The distributions of the sources of the isolates were: blood (n=23), urine (n=39), wound discharge (n=12), peritoneal fluid (n=6), ascitic fluid (n=11), tracheal aspirate (n=7), and sputum (n=5). All the isolates were identified as per the standard biochemical bacterial identification methods [10] and stocked in 0.2% semi-solid agar until analyzed.

Patients' demographic data, clinical diagnoses, and specimen types were recorded. Only one positive culture per patient was included. Infections caused by more than one organism and the isolates for which it was impossible to discriminate between contamination and infection were excluded. Hospital associated infection was defined as occurrence of infection 48 hours or more after hospital admission, without evidence that the infection was present or incubating on admission, in patients without prior history of stay in a healthcare facility [10].

Antimicrobial susceptibility testing

Antimicrobial susceptibility of all the isolates was performed on Mueller Hinton agar plates by the standard Kirby Bauer disk diffusion method [11]. The diameter of the zones of inhibition of growth were interpretated as per as CLSI guidelines [12]. *Escherichia coli* ATCC 25922 was used as control organism. Antibiotics included were ceftazidime (30µg), cephotaxime (30µg), ceftriaxone (30µg), cefepime (30µg), amikacin (30µg), ciprofloxacin (5µg), levofloxacin (5µg), doxycycline hydrochloride

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(30µg), gentamicin (10µg), cephoxitin (30µg), amoxycillin/clavulanate (20/10µg), ampicillin/sulbactam (10/10µg). Piperacillin/tazobactam (100/10µg), cefperazone/sulbactam (75/30µg), trimethoprim-Sulphamethoxazole (1.25/23.75µg), colistin (10µg), polymyxin-B (300 units), tetracycline (30µg), meropenem (10 µg), imipenem (10 µg), ertapenem (10 µg), and tigecycline (15µg). All the antibiotic discs were procured from Hi-media, Mumbai. .

Determination of Minimum Inhibitory Concentration (MIC)

MIC for cephalosporins, amikacin, ciprofloxacin, gentamicin (antibiotic powders from Hi-media, Mumbai) were tested by CLSI agar dilution method using Mueller Hinton agar. *E.coli* ATCC 25922 was used as the control strain. Similarly, strains found to be resistant to meropenem by disc diffusion test (zone of inhibition $\leq$ 13mm) were tested to determine the MIC level of meropenem. Meropenem powder (Astra Zeneca, UK) was used. Doubling dilutions for meropenem ranging from 0.25 µg/mL to 128 µg/mL were tested *P. aeruginosa* ATCC 27853 was used as the control strain. The NCCLS (CLSI) has defined meropenem MIC breakpoints for non-fastidious aerobes as < or = 4 (susceptible), 8 (intermediate) and > or = 16 mg/L (resistant), respectively [13].

MICs of colistin were obtained using colistin sulphate powder (Hi-media, Mumbai) by the agar dilution method performed according to CLSI methods[13] by a 2-fold concentrations ranging from 0.25 to 128 mg/L. *Escherichia coli* ATCC 25922 and *P. aeruginosa* ATCC 27853 were used as quality control, and test values obtained were in line with published standards [12].

Metallo β-lactamase (MBL) detection [14]

EDTA disks were prepared using 0.5M EDTA (Sigma Chemicals) solution dissolved in distilled water, pH was adjusted to 8 and sterilized by autoclaving.10µl of the EDTA solution was added to sterile blank 6mm disk (Whatman filter paper 4) and dried without overflowing. The disk contained approximately 1,900µg of EDTA. The test was performed on Muller-Hinton agar plate by disk diffusion method. A 0.5McFarland adjusted suspension of the test organism was inoculated on MHA plated. A 10 µg meropenem disk was placed at the center of the plate and the EDTA disk was place at a distance of 10mm, centre to centre, from the meropenem disk and the plate was incubated at 37ºC overnight. The zone around the meropenem disk would be extended on the side nearest the EDTA if the organism is a MBL producer.

Modified Hodge's Test [14]

This is a phenotypic test which could be used to determine if reduced susceptibility to carbapenems is mediated by a carbapenemase production. Mueller-Hinton agar plate was inoculated with a 0.5McFarland suspension of *E.coli* ATCC 25922 and streaked for confluent growth using a swab. A 10 µg imipenem disk was placed in the center, and each test isolate was streaked from the disk to the edge of the plate and the plate was incubated at 37ºC overnight. After incubation the plates were examined for a clover leaf-type indentation at the intersection of the test organism and the *E. coli* ATCC 25922, within the zone of inhibition of the carbapenem susceptibility disk. A positive test has a clover leaf-like indentation of the *E.coli* ATCC 25922 growing along the test organism growth streak within the disk diffusion zone. A negative test has no growth of the

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E.coli ATCC 25922 along the test organism growth streak within the disc diffusion.

AmpC detection [15]

AmpC enzyme production was tested by the AmpC disk . 0.5 McFarland suspension of ATCC *E. coli* 25922 was inoculated on the surface of Mueller-Hinton agar plate. A 30 µg cephoxitin disc was placed on the inoculated surface of the agar. A sterile plain disc inoculated with several colonies of the test organism was placed beside the cephoxitin disc almost touching it, with the inoculated disk face in contact with the agar surface. The plate was then inverted and incubated overnight at 37°C .After incubation, plates were examined for either an indentation or a flattening of the zone of inhibition, indicating enzymatic inactivation of cephoxitin (positive result), or the absence of a distortion, indicating no significant inactivation of cephoxitin (negative result).

Typing by Randomly Amplified Polymorphic DNA (RAPD) analysis

RAPD technique was used to investigate the relatedness of the strains. Two individual primers, AP4 (5'-TCA CGA TGC A-3'), HLWL74 (5'-ACG TAT CTG C- 3') [16] were used. The DNA was prepared by boiling method as above mentioned. The amplification condition was initial denaturation for 7 min at 94°C; followed by 30cycles of 1 min at 94°C, 1 min at 36°C, and 2 min at 72°C; and with a final extension for 10min at 72°C. The amplified products were separated by electrophoresis in a 2% agarose gel and stained with ethidium bromide. The band patterns were visually interpretated and a difference of more than 2 bands were considered a given major type, one to two bands difference was considered a minor variant.

RESULTS

Among the 103 isolates of *K. pneumoniae* from clinical specimens, 45 (43.6%) were resistant to meropenem by the disk diffusion test. The distribution of the clinical samples were; blood (n=9), urine (n=18), wound discharge (n=6), peritoneal fluid (n=3), ascitic fluid (n=5), tracheal aspirate (n=2), and sputum (n=2). The 45 meropenem resistant isolates exhibited high resistant (100 percent) to third and fourth generation cephalosporins, tetracycline, gentamicin, cephoxitin, amikacin and trimethoprim/sulfamethoxazole. They were 100 percent resistant to penicillin/inhibitor combinations such as amoxycillin/clavulanate, ampicillin/sulbactam and piperacillin/tazobactam. Only 6 (13.3%) were sensitive to cefperazone/ sulbactam combination and 14 (32.5 %) to polymyxin-B. Twenty seven also exhibited higher resistant (60 %) to colistin. 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 ICUs.

The MIC of meropenem showed a varied range. Eight isolates showed a maximum MICs of >128µg/mL, nine were MIC at 128µg/mL. Similarly 6 isolates showed MIC of 16µg/mL and five had MIC of 32µg/mL. The rest 17 isolates showed MIC level of 2µg/mL. Similarly, MIC of colistin showed a varied range from 16µg/mL, with a maximum of >128µg/mL for fourteen isolates.

None of the *K. pneumoniae* was found to produce MBL by EDTA-meropenem disk approximation test. Six isolates (13.3%) were positive by Modified Hodge's test (figure 1) and they

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were resistant to imipenem and ertapenem also. Susceptibility patterns of the six KPC producing *Klebsiella pneumoniae* clinical isolates. Among the 45 meropenem resistant isolates 28(62.2%) were AmpC beta lactamase producers (figure 2).

The six KPC positive isolates were typed by RAPD analysis with primer AP4 (figure 3) and HLWL74 (figure not shown), showed no relationship between RAPD type and ward.

Table 1

Susceptibility patterns of the six KPC producing Klebsiella pneumoniae clinical isolates.

Category	Name of the antimicrobial agent	Disc diffusion test	Minimum Inhibitory Concentration
Penicillin/ enzyme inhibitors	Amoxycillin/clavulanate	Resistant(n=6)	ND
	Ampicillin/sulbactam	Resistant(n=6)	ND
	Piperacillin/tazobactam	Resistant(n=6)	ND
	Cefperazone/sulbactam	Resistant(n=6)	ND
Cephalosporins	Cefotaxime,	Resistant(n=6)	>1280 µg/mL(n=6)
	Ceftriaxone	Resistant(n=6)	>1280 µg/mL(n=6)
	Ceftazidime	Resistant(n=6)	>1280 µg/mL(n=6)
	Cefepime	Resistant(n=6)	ND
Cephamycins	Cefoxitin	Resistant(n=6)	ND
Carbapenems	Ertapenem	Resistant(n=6)	ND
	Imipenem	Resistant(n=6)	ND
	Meropenem	Resistant(n=6)	32 µg/mL (n=1)
			64 µg/mL (n=3)
			>128µg/mL (n=2)
Fluoroquinolone	Ciprofloxacin	Resistant(n=6)	32 µg/mL (n=3)
			64 µg/mL (n=2)
			128µg/mL(n=1)
	Levofloxacin	Intermediate (n=4)	ND
Polymyxin	Colistin	Resistant(n=3)	64 µg/mL (n=2)
			>128µg/mL (n=1)
	Polymyxin-B	Sensitive(n=2)	ND
Tetracyclines	Tetracycline	Resistant(n=6)	64µg/mL (n=3)
			128µg/mL (n=1)
			>128µg/mL (n=2)
	Doxycycline	Intermediate (n=4)	ND

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Others	Amikacin	Resistant(n=6)	32 µg/mL (n=2) 64 µg/mL (n=3) >128µg/mL(n=1)
	Trimethoprim/sulfamethoxazole	Resistant(n=6)	ND
	Tigecycline	Sensitive(n=6)	ND

n= total number of isolates, ND- test not done

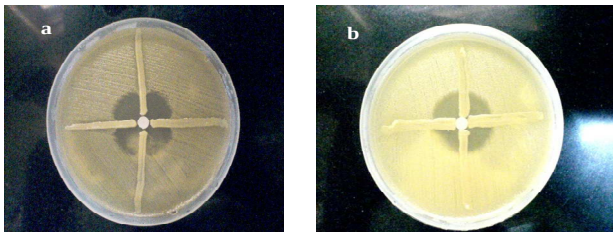

Figure 1. *Modified Hodge's Test for detection of carbapenemases production. a. Positive test with clover leaf-like indentation with ertapenem (10µg) disc, b. negative test with no indentation.*

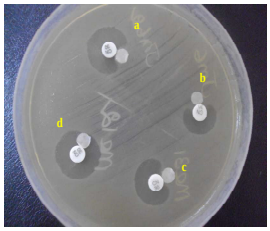

Figure 2. *AmpC disc test for detection of AmpC-β-lactamases production. Organism showing indentation of zone of inhibition near cefoxitin disc, indicating enzymatic inactivation of cefoxitin, positive test (isolate a, b and c). The absence of a distortion, indicating no significant inactivation of cefoxitin, negative result (isolate d).*

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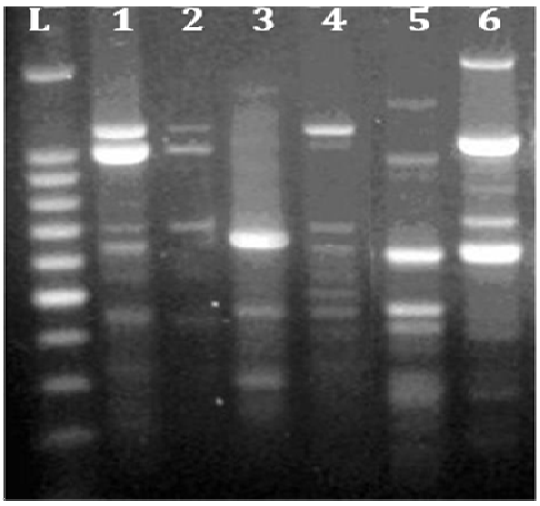

Figure 3. ***RAPD patterns of six KPC producing Klebsiella pneumoniae isolates with primer AP4. Lane L- 100 bp molecular weight marker, Lane 1-6 RAPD patterns of KPC positive isolates.***

DISCUSSION

Carbapenem resistance in *Klebsiella spp*. is an emerging problem and is a cause of concern as many nosocomial *Klebsiella spp*. are detected to be resistant to most other antibiotics. There is a limited literature available regarding the prevalence of resistance to carbapenems in *Klebsiella spp* from clinical isolates in our country. Gupta et al., from Delhi [17] reported 6.9% of meropenem resistance and 4.3% of imipenem resistance in *Klebsiella* and a study from Kanpur reported no carbapenem resistance among *K.pneumoniae* tested [18]. In a previous study from our hospital in 2007, among 126 ESBL producing *Klebsiella pneumoniae* tested, nearly half of them around 49% were susceptible to amikacin and 85% were susceptible meropenem[19]. In our hospital, meropenem came to use in 2006. Amikacin and meropenem were the frequently used antibiotics to treat infections by multidrug resistant bacteria in ICUs and high risk wards and soon within a short period 43.6% of resistance to meropenem, 32% to imipenem, 20.3% to ertapenem and 67.5% resistance to cefoperazone/sulbactam were developed, thus rendering the treatment options handicapped.

Similarly, studies have shown that a shift in empirical therapy to the carbapenems, due to the presence of ESBL producers, is associated with emerging resistance in and the ESBL-producing organisms themselves [20,21]. Recently, colistin, an older polymyxin antibiotic with a reputation for nephrotoxicity and neurotoxicity, has emerged as a salvage therapy for nosocomial infections caused by multidrug-resistant pathogens in the ICU [22]. However, colistin-resistant strains have recently been reported. Eighteen specimens containing colistin-resistant *K. pneumoniae* were cultured from 13 ICU patients in 2004 and 2005 [23]. Similarly, results were noticed in our study where a maximum of 60% of colistin-resistance was recorded. All those patients had long hospitalization and long duration of ICU stay. Tigecycline was the only drug to which 100 percent

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susceptibility was seen, but this was not introduced to clinical use so far in our hospital.

In this study, majority of the carbapenem resistant multi drug resistant isolates (Pan resistant) were from Neonatal Intensive Care Unit (NICU) and Medicine Intensive Care Unit (MICU). ICUs are the epicenter for spawning multidrug resistance within hospitals .Many patients are transferred to the ICU from other healthcare facilities, where they have acquired resistant pathogens. Patients within the ICU undergo invasive procedures, treatment with antibiotic combinations, and exposure to other patients with resistant pathogens [24]. Multiple mechanisms exist for ICU pathogens to acquire antibiotic resistance. These mechanisms include enzymatic inhibition of drugs, alteration of proteins targeted by antibiotics, changes in metabolic pathways, antibiotic efflux, alterations in porin channels, and changes of membrane permeability [25].

High-Level carbapenem resistance in a *K.pneumoniae* is due to the combination of β - Lactamase production, porin OmpK35/36 Insertional inactivation, and down-regulation of the phosphate transport porin and changes in penicillin-binding proteins [26]. *Klebsiella pneumoniae* carbapenemases (KPC) are among the most common β-lactamases mediating carbapenem resistance among isolates of Enterobacteriaceae. They are most commonly associated with *Klebsiella pneumoniae*. They are class A β-lactamases that can inactivate all penicillins, cephalosporins, aztreonam and most importantly carbapenems. KPC resistance can co-exist with other gram-negative resistance mechanisms including ESBL, fluoroquinolone, and aminoglycoside resistances cephalosporins in addition to carbapenems; the genes for the enzymes are usually on plasmids and therefore can be spread readily among members of the same species or even among different genera [27]. KPC was first described in 2001 in an isolate of *Klebsiella* from a hospital in North Carolina. KPC-producing organisms have continued to spread over time and have now been reported in 27 states in the United States, and in many countries around the world, including China, Colombia, Brazil, France, and Israel [28].

According to the CLSI recommendations, Enterobacteriaceae that are resistant to the expanded-spectrum cephalosporins and have a carbapenem MIC ≥ 2 µg/ml or a carbapenem intermediate or resistant zone of inhibition by disk diffusion may produce a KPC β-lactamase. For KPC detection, ertapenem is the most sensitive carbapenem while meropenem and imipenem are more specific than ertapenem. It is important to recognize small resistant colonies growing inside the ertapenem disk zone. The definitive detection of a KPC encoding organism can be performed using PCR and/or by modified Hodge's test [29].

In this study, none of the isolates tested were positive for MBL which indicates the absence of Metallo-beta lacatamases mediated resistance in them but 62.2% of AmpC production was there. A high MIC range of >128ug/mL was noted for meropenem which could be because of selective pressure due to extensive or inadequate meropenem use. The high MIC and resistance prevalence rate for colistin, is worrying as it limits the antibiotic options available in the treatment of bacterial infections.

The modified Hodge's test differentiates carbapenemases (MHT positive) from *Klebsiella* strains showing resistance to carbapenems by a combination of porin loss and ESBL or AmpC production (MHT negative). Six strains were modified Hodge's test positive which optimistically indicate that they could posses the KPC gene which mediated the carbapenem resistance. It is needed to be studied by

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further molecular analysis. Four out of the six patients expired in ICU. It is worrisome that, the KPC producers were of dissimilar clonal patterns and their spread to other nosocomial isolates may cause potential outbreaks in future, if not controlled.

Microbial drug resistance is a growing problem of global magnitude. Emerging antibiotic resistance has created a major public health problem, compounded by a shortage of new antibiotic options. Physicians must select antibiotics with the specific needs of an individual patient in mind but also in a manner that does not breed further drug resistance. Selection of an appropriate initial antibiotic regimen for empiric therapy, rotation of different antibiotic classes and judicious use of antibiotics are needed.

CONCLUSION

The carbapenemases render the marketed agents (ertapenem, meropenem and imipenem) inactive. Because the carbapenems are regcognized as the treatment of choice for ESBL-positive isolates, misidentification of KPC-positive strains as ESBL-producers can be expected to have a negative impact on the patient care. Hence, given the limited choices available in antimicrobial therapy for these patients, laboratories should be prepared to test accurately for agents such as tigecycline, colistin and polymyxin B in addition to their usual antibiogram pattern.

In this study a maximum of 20.3% ertapenem resistance and 60% colistin-resistance among *Klebsiella pneumoniae* was recorded and KPC producing *Klebsiella pneumoniae* was reported from nosocomial isolates. To the best of our knowledge this is the first report in India.

Thus, this report on carbapenem resistance among the *Klebsiella spp* is an emerging phenomenon of great clinical and public health importance. Similar studies should be recommended in each part of the country to avail the prevalence. Also, further research to better define the mechanism of the resistance and spread of these strains are required, and enhanced measures to control the spread of this resistant strain are warranted.

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