Original Article

Carbapenem Resistant *Klebsiella Pneumoniae* – An Emerging Global Threat.

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Abstract

Introduction: The main concern in the 21st century is antibiotic resistance. Early detection of carbapenem-resistant Klebsiella pneumoniae infections can lower morbidity and death rates. Due to a shortage of alternative medicines, multidrug-resistant (MDR) and carbapenem-resistant *Klebsiella pneumonia* have become major therapeutic challenges in various countries. To ascertain the frequency of carbapenem-resistant *Klebsiella pneumoniae* isolates and their antibiogram profile is done in order to create therapeutic and infection-control advice.

Materials and Methods: A total of 366 *Klebsiella pneumoniae isolates* collected from various clinical samples are subjected to initial identification and they are screened and confirmed for carbapenemase production by phenotypic methods based on CLSI and for genotypic detection polymerase chain reaction is done.

Results: Among them, 90 isolates showed carbapenem resistance. 90 isolates of carbapenem-resistant Klebsiella pneumoniae were subjected to phenotypic detection of carbapenemase enzyme synthesis using MHT, mCIM, and eCIM. Out of which, 75 isolates showed positive for MHT, 80 isolates showed positive for mCIM, 70 isolates showed positive for eCIM. In genotypic detection 80 isolates were positive for mCIM. PCR is performed for mCIM positive isolates in which the NDM gene was positive for 70 isolates and KPC the gene was positive for 10 isolates these PCR findings correlate with the phenotypic detection method mCIM and eCIM respectively.

Discussion: In this study, confirmatory tests mCIM (Carbapenemase detection) were determined to be the most significant and reliable method. Also, it can be performed in routine microbiological testing. Since these correlate with results of PCR, this will aid in the detection of antibiotic resistance and judicious use of antibiotics.

Keywords: *Klebsiella pneumoniae*, Carbapenemase resistance, mCIM, eCIM, KPC, NDM.

INTRODUCTION:

Antibiotic resistance is the major threat to the world [1,2]. The European Commission and the world health organisation (WHO) has acknowledged the significance of drug resistance as well as the demand for effective control measures. Antibiotic resistance imposes additional pressure on developing nations [3]. Multidrug-resistant bacteria like Methicillin-resistant *Staphylococcus aureus*, multidrug-resistant *Mycobacterium tuberculosis*, carbapenem-resistant *Klebsiella pneumoniae*, and Carbapenem-resistant *Acinetobacter baumannii*, are the most common infectious agents causing a global threat [4, 5]. The rapid development of bacterial resistance in India is due to the result of several factors, including the country's, treatment uncertainty, self-medication, market forces, unqualified health professionals prescribing antibiotics, based on the availability of prescriptions given, and standard laboratory facilities that are difficult to access. As a result, poor compliance to infection control practices and universal hygiene, hence antibiotic stewardship programs are desperately needed in India [6,7]. NDM-1, a newly discovered carbapenem resistance, received a lot of media attention [8]. Although efforts are taken to focus on antibiotic development, actions to stop the spread and illnesses from these organisms are also equally important [9]. Local studies and integrated preventative actions can be effective even if additional research is required to determine the best approaches to stop the spread of Carbapenem-resistant Enterobacteriaceae [10]. This study seeks to describe the prevalence of carbapenem-resistant Klebsiella pneumoniae in this tertiary care hospital, as well as the antibiogram susceptibility pattern and gene responsible for resistance.

MATERIALS AND METHODS

A total of 366 *Klebsiella pneumoniae* isolates that were clinically significant, consecutive, non - duplicate are included and Institutional Ethics Committee IEC N0.1860(A)was approved at SRM medical college hospital and research Centre. These isolates have been isolated from variety of clinical samples, including blood, sputum, endotracheal aspirate, pus, wound swabs, vaginal swabs, and ear swabs. Preliminary tests were done which shows Gram-negative Short stout bacilli which were non-motile, Catalase (+ve), and Oxidase (-ve). In Nutrient Agar, colonies were mucoid grey colonies, and it

produces lactose fermenting colonies in MacConkey agar. Following this, biochemical identification was done for the confirmation of *Klebsiella pneumoniae* isolates.

ANTIMICROBIAL SUSCEPTIBILITY TESTING (AST)

According to Clinical and Laboratory Standard Institute (CLSI) standards, AST was performed on each isolate using cation-adjusted Muller-Hinton agar (MHA) by Kirby-Bauer disc diffusion technique [11]. Antibiotics discs as follows - ampicillin (10µg), amoxicillin-clavulanate (20µg /10 µg), ceftazidime (30µg), ceftriaxone (30µg), cefipime (30µg), cefotaxime (30µg), piperacillin-tazobactam(100µg/10µg), chloramphenicol (30µg), ciprofloxacin (5µg), cefoxitin (30µg), cefazolin (30µg), gentamycin (10µg), imipenem (10mg), amikacin (30µg), tetracycline (30µg), trimethoprim/sulfamethazole (1.25/23.75µg). In addition, nitrofurantoin-300g discs were used for urine isolates. All of these antibiotic discs were purchased from Mumbai's Hi Media. According to CLSI recommendations, the zones of inhibition's diameters were interpreted.

CONFIRMATION OF CARBAPENEMASE PRODUCTION BY PHENOTYPIC METHODS:

(A)Modified Hodge test:

Test procedure:

According to CLSI recommendations, this test was conducted for confirmation of carbapenemase enzyme production. The indicator organism, Escherichia coli ATCC 25922, was streaked over MHA as a lawn culture, and a 10 g Ertapenem disc was positioned in the middle. The test isolate is streaked and cultured for 24 hours together with positive and negative controls.

(B) Modified Carbapenem Inactivation Methods:

Test procedure:

A loopful of isolate was emulsified in two mL TSB. The ten-g meropenem disc was well immersed in TSB before being vortexed for 10 to 15 seconds in each test tube. The test tube is incubated for four hours and 15 minutes at 35°C 2°C in ambient atmosphere. A lawn culture of Escherichia coli ATCC 25922 is inoculated on cation-adjusted Muller Hinton agar, and the meropenem disc is gently withdrawn from the TSB. Excess liquid from the disc is then drained from the disc by dragging and compressing it along the inside edge of the tube. Using the loop, the disc was removed from the tube and set on the MHA plate that had previously received an inoculum of the meropenem-susceptible strain of E. coli ATCC 25922. The meropenem disc and MHA plates were incubated for overnight at 35 °C 2 °C in ambient air. [12].

(B.1) Test interpretation:

Positive:

If zone diameter is 6 to 15 mm or the presence of pinpoint colonies inside a zone diameter of 16 to 18 mm is considered to be positive.

Negative:

If zone diameter is 19 mm or less

(C) EDTA Carbapenemase inactivation test:

Test procedure:

A 2-mL TSB (Trypticase soy broth) tube was filled with 20 mL of 0.5 M EDTA to produce a final concentration of 5mM EDTA. The meropenem-susceptible *Escherichia coli* ATCC 25922 strain was used to inoculate the MHA plate, and the mCIM method was used. Test tubes containing mCIM and eCIM were incubated concurrently.

(C.1) Test interpretation: The isolates which showed positive for mCIM are subjected to eCIM

(D) Metallo-ß-lactamase positive:

Compared to mCIM, the zone diameter of eCIM is 5 mm greater. Ignore pinpoint colonies inside any zone of inhibition for the eCIM test. The action of carbapenemase is inhibited by EDTA, which stops the hydrolysis of the meropenem disc. As a result, the meropenem-sensitive *Escherichia coli* is suppressed, and as opposed to the mCIM zone diameter, the eCIM zone diameter grows.

(D.1) Negative:

Comparing the zone diameter of the eCIM to that of the mCIM, there is an increase of 4 mm. If the test isolate generates serine carbapenemase, EDTA will have no impact on the enzyme's activity, and the zone diameter can't increase more than 4 mm over the mCIM zone diameter.

Genotypic:

(E) Molecular Method:

DNA was extracted by inoculating 5-10 colonies of pure culture of *Klebsiella pneumoniae* in the 1ml of distilled water in a test tube and boiled for 10 minutes in the water bath and centrifuged at 1000rpm.5 microliters of the supernatant fluid were used for PCR

Resistant gene identification by polymerase chain reaction:

(E.1) Materials used:

2U of Taq DNA polymerase, 5X Taq reaction buffer, 2mM $MgCl^{2+}$, 1µl of 10mM dNTPs mix and SYBR green dye.

(E.2) PRIMERS USED: (OPRL laboratory)

Table 1:

PRIMERS	SEQUENCE	GENE DETECTED
KPC-F	CGGCAGCAGTITGTTGATTG	**KPC**
KPC-R	CGCTGTGCTTGTCATCCTTG	
NDM-F	GGTTTGGCGATCTGGTTTTC	**NDM**
NDM-R	CGGAATGGCTCATCACGATC	

(E.3) PCR Reaction Mix:

DNA Template: 1µl, Master Mix: 10µl, Primer (0.1µM) (F&R): 1+1µl , Nuclease Free Water: 7µl, Total Reaction Volume: 20µl,Thermocycler Temperature: Initial denaturation (94˚C for 10 mins),Denaturation (94˚C for 30 Sec),Annealing (52˚C for 40 Sec)Extension (72˚ C for 50 Sec)Final Extension (72˚C for 1 mins)Agarose Gel Electrophoresis was done .1% agarose gel was prepared products are added to the wells and subjected to electrophoresis at 100V. The bands were observed under a UV transilluminator and Gel documentation was done.

RESULTS:

From various clinical samples, 366 nonduplicate Klebsiella pneumoniae were isolated. Among the total isolates 90 isolates (24%) were resistant to imipenem, 66.6% are isolated from males and 33.34% were isolated from females. Most of the carbapenem-resistant *Klebsiella pneumoniae* are isolated from the age group 60-70 years (19%) followed by 50-60 years (16%.) In the present study maximum *Klebsiella pneumoniae* are isolated from pus (23%) followed by urine (21%).

Table 2 Antibiotic susceptibility pattern in *Klebsiella pneumoniae*

Drug	NO.OF ISOLATES	PERCENTAGE
Amoxicillin- clavulanate (20µg/ 10µg)	183	50%
Cefazolin (30µg)	175	48%
ceftriaxone (30µg)	172	47.5%
Cefotaxime (30µg),	169	46.52%
cefepime (30µg)	168	46%
nitrofurontion (300 µg),	161	44.5%
cefoxitin(30µg)	157	43.47%
Ceftazidime(30 µg)	140	38.25%
Piperacillin Tazobactam (100µg/10µg)	125	34.15%
ciprofloxacin (5µg)	113	31.30%
Ertapenem (10µg)	109	30%
Trimethoprim/Sulfamethox azole (1.25/23.75µg	107	30%
Amikacin (30 µg)	107	30%
Gentamicin(30 µg),	106	29.13%
Imipenem (10µg)	90	25%
Chloramphenicol(30 µg)	54	15.21%
Tetracycline (10 µg)	43	12.06%

Among 90 isolates that were imipenem resistant, 75 isolates showed positive for the modified Hodge test and 80 isolates showed positive for mCIM and 70 isolates showed positive for eCIM.

In phenotypic detection of Metallo beta-lactamase enzyme production by using mCIM and eCIM. Among 90 isolates, 80 isolates showed positive for mCIM and the mCIM positive isolates are subjected to eCIM in which 70 isolates showed a positive test. Therefore 70 isolates showed positive for Metallo beta-lactamase production and 10 isolates that are eCIM negative indicate the production of serine carbapenemases.

Genotypic detection is done using polymerase chain reaction 80 isolates positive for Mcim are subjected to it in which 70 isolates showed positive for NDM gene and 10 isolates showed positive for KPC gene. The PCR findings correlate with the phenotypic detection method mCIM and eCIM.

Table no 3:

	MHT	mCIM	eCIM
Klebsiella pneumoniae	75	80	70

MHT-Modified Hodge Test, mCIM- Modified carbapenem Inactivation test eCIM-EDTA carbapenem inactivation test.

DISCUSSION:

One of the foremost threats to patients hospitalized to an ICU and extended hospitalization is the formation of multidrug-resistant (MDR) gram-negative bacteria [13]. Since carbapenems have historically been the last resort antibiotic class for the treatment of multidrug-resistant bacterial infections, the advent of carbapenem resistance among pathogenic bacteria has been viewed as a global sentinel event [14]. In the present study, 24% of isolates resistant to imipenem were as 76% of isolates were sensitive to imipenem The findings of the present study were in accordance to a study done by Patel JB et al [15]. According to research conducted in New York City and Israel by Debby et al [16], the prevalence of carbapenem-resistant Klebsiella pneumonia was 26% and 27%, respectively.

Among the carbapenemase, nonproducers extended-spectrum beta-lactamase was the most predominant resistance followed by AmpC beta-lactamase resistance. Among the carbapenem, producers 66.66%are isolated from males and 33.34% isolated from females The above findings are consistent with a study conducted by Amin A et al [17] in Pakistan, in which males (60 %) than females (40 %) respectively. The prevalence of carbapenem-resistant klebsiella pneumonia isolates is around 24%. The result is consistent with research by Patel JB et al [18] in New York City and Debby et al in Israel, which found 26% and 27%, respectively, prevalence of carbapenem-resistant Klebsiella pneumonia.

A maximum number of *Klebsiella pneumoniae* isolates are obtained from pus samples (23%) followed by urine(21%) The finding of the above study are in contrast with a study done by Gaibani P et al [19] and Leavitt A et al [20] in which the maximum number of isolates are from a urine sample.AST pattern of *Klebsiella pneumoniae* highest resistance was shown by Amoxicillin clavulanate with a percentage (50%), followed by cefazolin (48%) and Ceftriaxone (47.5%) and lowest drug resistance was showed Tetracycline (12.06%) *Klebsiella* In this study *Klebsiella pneumonia* showed highest resistance against amoxicillin clavulanic acid (50%)followed by cefazolin(48%), ceftriaxone(47.5%) and lowest resistance against tetracycline(12.06%) followed by chloramphenicol (15.21%)and imipenem(21.43%) similar study conducted by mwangi joseph kibuchi et al.in which *Klebsiella pneumoniae* isolates showed the least resistance against meropenem followed by amikacin.

In this study nearly 90 isolates showed positive for carbapenemase enzyme production by screening method and 75 isolates showed positive by Modified Hodge test and 80 isolates showed positive by mCIM and 70 isolates came as positive by eCIMtest hence these 70 isolates were a Metallo-beta-lactamase producer. Additionally, 15 isolates with false-negative MHT results also had positive mCIM results, which was consistent with studies that found MBL producers to have a high proportion of false-negative MHT results [21,22].

Additionally, MHT results may be challenging to interpret, and for isolates producing AmpC and ESBL false-positive results have been reported, mCIM is more accurate than MHT and has a 100% sensitivity to detecting MBLs [23]. The several carbapenemases can be concurrently detected and distinguished using the eCIM/mCIM test. Previous results revealed that in the presence of 5 mM EDTA, eCIM's sensitivity and specificity were both 100% [24] .In this study since out of 90 isolates, 80 isolates showed positive for mCIM out of which 70 isolates showed positive for eCIM so phenotypically it confirms 80 isolates are Metallo-carbapenemase producers and 10 isolates are serine carbapenemase producers.

In our study, nearly 70 isolates showed bla NDM gene positive followed by 10 isolates that showed positive for bla KPC gene [25,26]. NDM-1 was first discovered in 2008 and quickly spread to other countries, earning it the designation of India's capital. Microbes evolve methods to evade the action of antimicrobials, which leads to AMR. Irrational and overuse of antibiotics are two factors that contribute to AMR [27,28].

CONCLUSION:

Our hospital has a significant problem with the frequency of 24% carbapenem-resistant Klebsiella pneumoniae. Due to their resistance to almost all antimicrobial drugs now in use, the spread of these strains could result in treatment failures and higher rates of morbidity and mortality. Understanding the risk factors connected to infections brought on by these bacteria may help clinicians deal with patients more effectively. In this study, confirmatory tests mCIM (Carbapenemase detection) were determined to be the most significant and reliable and can be performed simply in routine microbiological testing. This will aid in the detection of antibiotic resistance, which will aid in the judicious use of antibiotics.

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CONFLICTS OF INTEREST:

Authors declare there is no conflict of interest.

AUTHORS CONTRIBUTION:

All authors have substantial, intellectual and direct contribution to the work and approved it for publication.

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DATA AVAILABILITY:

All databases generated or analyzed during this study are included in the manuscript.

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