Original Article

Phenotypic Detection of Carbapenem Resistance in Clinical Isolate of *Klebsiella Pneumoniae* in Tertiary Care Hospital

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Abstract

The emergence and rapid spread of Multidrug resistant isolates of *Klebsiella pneumoniae* causing nosocomial infections, which require the great afford for the treating the patients by the clinician. The emergence of Metallo β-lactamase (MBL) producing isolates poses a challenge to routine microbiology laboratories. The purpose of this study was to assess the accuracy of various phenotypic methods for detecting MBL production among *K. pneumoniae isolate.* The current study may provide the necessary data to formulate a hospital antibiotic policy and to prevent the spread of multidrug resistance in the community. Total 240 *K. pneumoniae* isolates were included in our study which was isolated from various clinical samples from patients visiting a tertiary care hospital in central India. A total of 148 (61.7%) of *K. pneumoniae* were isolated from male patients whereas 92 (38.3%) isolates were from female patients. Out of 240 *K. pneumonia*e, 102 (42.5%) was detected Imipenem resistance by conventional (Kirby-Bauer) disc diffusion method which was confirmed by minimum inhibitory concentration (MIC) test (Epsilometer test). By the MIC Carbapenem resistance *K. pneumonia*e were confirmed for MBL producer by using phenotypic test. Combined disc synergy test (CDST) (Imipenem 10 mcg and Imipenem 10mcg + 0.5 m EDTA) and Modified Hodge test (MHT).The MHT test found most sensitive approach for detecting MBL in *K. pneumonia*e. This procedure is simple and clear and it may use in routine clinical labs for detection of MBL because carbapenems are last resort for treating bacterial infections. In recent years, carbapenem resistance has rampantly increased across the globe.

Keywords: *Klebsiella Pneumoniae,* carbapenem resistance, MBL

INTRODUCTION

Klebsiella is an important nosocomial pathogen among hospitalized patients throughout the world. Among *Enterobacteriaceae* members *Escherichia coli* is the primary pathogen followed by *Klebsiella* which causes predominantly UTI, pulmonary infections and primary bacteremia with high mortality rate.[1.2, 3,4,5,6]

Extensive use of broad-spectrum antibiotics leads to multidrug-resistant nosocomial infections especially in *Klebsiella*. *Klebsiella pneumoniae* that produce extended spectrum beta-lactamase (ESBL), Ampicillinase C (AmpC) and *Klebsiella pneumoniae* carbapenemase (KPC), Metallo - beta lactamase (MBL) causes drug resistant in broad spectrum beta-lactams, including carbapenems and monobactams, which is difficult to treat the infections.[7,8,9,10,11] The major health concern is with transmissible carbapenemase, which can be acquired unpredictably by important nosocomial pathogens such as *Klebsiella pneumoniae*, *Acinetobacter baumanni* and *Pseudomonas aeruginosa.* [12]

In India 65.4% isolates were ESBL producers, 28.5 were AmpC producers, 9.4% were combined ESBL and AmpC producers and 48.6% were Carbapenamase producers in which 25.6% were KPC and 23% MBL producers and 8.2% were KPC and MBL coproducers [8, 10, 11]. So the detection of Carbapenem resistance is important in proper and early treatment of patients and also preventing the spread of resistant strains.[13]

The emergence and rapid spread of Multidrug resistant isolates of *Klebsiella Pneumoniae* causing nosocomial infections, which require the great afford for the treating the patients by the clinician .[14,15] The routine susceptibility tests may not detect the resistance pattern, therefore special tests are required to detect the resistance mechanisms[16]. Considering the rapid spread of infection spread and the high rate of morbidity and mortality associated with infections caused by *K. pneumoniae*, it is essential that susceptibility methods identify carbapenem resistance rapidly and reliably.[17,18]

Hence the current study was taken up to assess the most prevalent species among *Klebsiella pneumoniae* and their antibiotic susceptibility pattern for Carbapenem antibiotic by using Modified Hodge test, which is recommended as

phenotypic techniques by CLSI guideline.[12,19] The automated method such as VITEK-2 is not reliable because Carbapenemase-producers have MIC below the clinical carbapenems break point.[20,21]

Rapid detection and typing of these bacteria are critical especially for surveillance purposes, to prevent outbreaks and optimize antibiotic therapy. The current study therefore aimed to determine the effectiveness of Modified Hodge test as one of the most sensitive phenotypic tests for detection of KPC producers among *Klebsiella pneumoniae*. The purpose of this study was to assess the accuracy of various phenotypic methods for detecting MBL production among *K. pneumoniae* isolate *to* formulate a hospital antibiotic policy and prevent the spread of multidrug resistance strains in the community.

MATERIALS AND METHODS

Study Design

This study was descriptive, cross-sectional conducted in the Department of Microbiology of Index Medical College, Indore, Madhya Pradesh. The protocol of the study was approved by Institutional Ethics Committee. The study was conducted during the period of July 2019 to December 2020. A total of 240 clinical samples were received. *K. pneumoniae* isolates from clinical samples from patients visiting a tertiary care hospital in central India.

Inclusion criteria- *K. pneumoniae* isolated from various clinical specimens collected from various outpatient departments (OPDs), wards and critical care units.

Exclusion criteria- Repeat isolate from same clinical specimen of same patient. Except *K. species* other isolated organism from clinical specimen.

Identification of *K. Pneumoniae*

K. pneumoniae isolated from various clinical specimens was identified using standard microbiological procedure. It included characteristic colony morphology on culture media, Gram staining and pattern of biochemical reactions[22, 23]
After identification *K. pneumoniae* was sub-cultured onto nutrient agar media. These colonies were used for antibiotic susceptibility testing and detection of carbapenem resistance by phenotypic method.

Antibiotic susceptibility testing of *K. pneumoniae*.

Antimicrobial susceptibility testing was carried out on Muller-Hinton agar plates (Hi-Media Laboratories Pvt Ltd, Mumbai) by using Kirby-Bauer disk-diffusion method. Antibiotic discs (Hi-Media Laboratories Pvt Ltd, Mumbai) used in the study were Amikacin (30µg), Gentamicin (10µg), Ceftazidime (30µg), Ciprofloxacin (5µg), Imipenem (10µg), Meropenem (10µg), Aztreonam (30µg), Piperacillin / Tazobactum (100/10µg). Amoxy-Clavulinic acid (30µg), Ceftriaxone (30µg), Cefoperazone (75µg), Cefepime (30µg), Tigecycline (15µg), CO-Trimoxazole (25µg), Cefotaxime(30µg), Tetracycline(30µg), Clostin (10µg), Ampicillin (10µg). The interpretation of the results as per Clinical and Laboratory Standards Institute guidelines (CLSI 2018).[24] *E. coli* ATCC 25922 was used as a control strain. For urinary isolates, additional antibiotic Nitrofurantoin (300µg) were tested.
The *K.pneumoniae* isolate which were resistance and intermediate susceptibility to Imipenem were further subjected to phenotypic methods for detection of carbapenemase production.[24]

Phenotypic tests for detection of carbapenemase production

Combined Disc test (with Imipenem and Imipenem + EDTA) and Modified Hodge test were used for screening tests in the present study.

A. Combined Disc Synergy test (CDST)

In this method, a 10-µg Imipenem disk and Imipenem (10-µg) and EDTA (0.5M) combined disc was placed on the plate of agar plate with lawn culture of tested bacterium and incubated for 24 hrs. at 35°C. The increase in inhibition zone with the Imipenem and EDTA disc 7 mm than the Imipenem disk alone was considered as a Metallo -Lactamase (MBL) positive strain.

B.Modified Hodge Test (MHT)

All carbapenem resistant *K. pneumoniae* isolates were tested by MHT. In this method 0.5 McFarland standard matched suspension of *E. coli* ATCC 25922 was prepared in broth. A Mueller Hinton agar plate was inoculated as for the routine disk-diffusion procedure.[25]
The plate was allowed to dry for 10 minutes. Meropenem disc was placed in the center of the plate. Using a 10 µL loop, three to five colonies of test organism grown overnight on a blood agar plate were picked and inoculated in a straight line out from the edge of the disk. The streak was at least 20-25 mm in length. Following incubation, Mueller Hinton agar was examined for enhanced growth around the test streak at the intersection of the streak and the zone of inhibition.[25]
Positive MHT showed a clover leaf-like indentation of the *E. coli* 25922 strain growing along the test strain growth streak within the disc diffusion zone indicating production of carbapenemase while a negative test was indicated when there was no growth of the *E. coli*25922 along the test strain growth streak within the disc diffusion.

Phenotypic confirmatory test

E-test was the phenotypic confirmation test. In this method an E-test strip containing Imipenem and Imipenem + EDTA was used. A reduction in MIC of Imipenem of 3 or more 2-fold dilutions in the presence of EDTA was interpreted as a positive test indicative of carbapenemase production.

RESULTS:

Total 240 isolates were positive for *Klebsiella pneumoniae*

Table No.1: Sex wise distribution of patients with *K. Pneumoniae* infection

Gender	No of Patients	Percentage
Male	148	61.7
Female	92	38.3
Total	240	100

Table No. 2:-Ward wise distribution of patients showing isolation of *K. pneumoniae*

Ward/Critical care area	Number of isolates	Percentage
ICUs (NICU, PICU, MICU, SICU, RICU)	95	39.5
Surgery	54	22.5
Obstetrics &Gynaecology	20	8.3
Orthopaedics	18	7.5
Medicine	45	18.7
Paediatrics	6	2.5
ENT	2	0.8

Table No.3:-Specimen wise isolation of *K. pneumoniae*

Clinical specimen	Number of isolates	Percentage
Urine	70	29.2
Pus	52	21.7
Blood	28	11.7
Sputum	29	12.9
Wound swab	19	7.9
Tracheal aspirate	27	11.3
CSF	07	2.9
Vaginal swab	08	3.3
Total	240	100

Table No 4. Antimicrobial Susceptibility Pattern of 240 isolated *K. Pneumoniae* by Kirby –Bauer disc diffusion method.

Antimicrobial agent (240 isolates)	Susceptible		Resistant	
	No. of isolates	Percentage%	No. of isolates	Percentage
Ampicillin	05	2.1	235	97.9
Gentamicin	87	36.2	153	63.8
Amikacin	88	36.7	152	63.3
Cefepime	16	6.7	224	93.3
Cefotaxime	07	2.9	233	97.1
Ceftazidime	34	14.2	206	85.8
Piperacillin+Tazobactum	20	8.3	220	91.7
Imipenem	138	57.5	102	42.5
Meropenem	142	59.2	98	40.8
Tigecycline	195	81.3	45	18.7
Ciprofloxacin	46	19.2	194	80.8
Amoxyclav	05	2.1	235	97.9
Aztreonam	04	1.7	236	98.3
Cefoperazone	08	3.3	232	96.7
Nitrofurantoin (n=70)	25	35.7	45	64.3
Ceftriaxone	10	4.1	230	95.8
Cotrimoxazole	78	32.5	162	67.5
Tetracycline	42	17.5	198	82.5
Colistin	240	100	-	-

A total of 102 *K. pneumoniae* showed resistance to Imipenem by Kirby-Bauer disc diffusion method (zone size≤19mm). These isolates were subjected to phenotypic screening and confirmatory tests for detection of Metallo β- lactamase (MBL) mediated carbapenem resistance.

Combined Disc test (with Imipenem and Imipenem + EDTA) and Modified Hodge test were used for screening. E-test was used as a phenotypic confirmatory test.

Table No.5:-Comparison of phenotypic is screening methods for detection of carbapenemase production in 102 *K. pneumoniae.*

Phenotypic test	Positive (%)	Negative (%)	Total
Combined Disc Test	86 (84.3)	16 (15.7)	102
Modified Hodge test.	88 (86.3)	14 (13.7)	102

When results of two phenotypic screening methods were compared, there was no significant difference was observed between these two tests for detection of carbapenemase resistant in *K. pneumoniae* (Fisher exact test, *P* value <0.05).

Table No. 6:-Sex wise distribution with carbapenem (Imipenem) resistant and susceptible *K. pneumoniae* infection confirmed by E test.

Susceptibility to carbapenem	Male (%)	Female (%)	Total
Susceptible	04 (66.7)	02 (33.3)	06
Resistant	63 (65.6)	33 (34.4)	96
Total	67 (65.7)	35 (34.3)	102

Out of 102 carbapenem resistance *klebsiella pneumoniae* isolates 96 where resistant for MBL by E test. In which 65.6% resistant shows by male and 34.4% resistant shows by female for MBL (Table no 6). Although carbapenem resistant *K. pneumoniae* was more commonly isolated in males (65.6%) compared to female (34.4%), this difference was not statistically significant (Fisher exact test, P value >0.05)

DISCUSSION

Health and diseases have always been the most important matter of concern to human beings. The ever escalating want of better health resulted in discovery of various novel techniques for diagnosing and treating diseases. However, even in the golden era of medicine, where diagnostic and therapeutic modalities have progressed by leaps and bounds, infectious diseases still continue to be one of the major causes of morbidity and mortality in clinical setup worldwide.[26]

On the basis of formula used for sample size calculation, a total of 240 non-duplicate *K. pneumoniae* recovered from a range of clinical specimens were included in the study. A total of 148 (61.7%) of *K. pneumoniae* were isolated from male patients whereas 92 (38.3%) isolates were from female patients. (**Table No. 1**) A similar Indian study of Bashir *et al* (2014) reported isolation rates of *K. pneumoniae* from male and patients as 60.3% and 39.7% respectively.[27]

Although in present study, when statistical analysis was conducted there was significant difference observed between isolation of *K. pneumoniae* and gender of the patient. Similar observation was noted in the study of Moini*et al.* (2015).[28] Therefore, it can be commented that *K. pneumoniae* can cause infection in either of the sex in the presence of risk factors.

A total of 39.5% of *K. pneumoniae* isolates were from patients admitted to various ICUs of the hospital. Bshabshe *et al* (2020), reported that *K. pneumoniae* was a major pathogen from ICU patients.[29] Many studies worldwide including those from India by Gafur *et al*., Goel *et al*., and Italy by Mammina and Monaco have reported *Klebsiella* as the most common organism causing infections in ICU.[30,31] It is one of the important causes of HCAI especially for immunocompromised individuals admitted in the ICU[32] (**Table No. 2).**

In recent years, *K. pneumoniae* infections have emerged as an important public health problem. The clinical spectrum of *K. pneumoniae* is broad. It is implicated in various infections like pneumonia, blood stream infection, urinary tract infection and hepatic abscess.[33]

In the current study majority of *K. pneumoniae* were isolated from urine cultures (29.7%) (**Table No. 3**.)This observation is in accordance to that of Leavitt *et al*. (2010)[34], Parveen *et al.* (2010)[35] Bashir *et al*. (2014)[27] and Vivan *et al*. (2017)[36] who reported maximum isolation of *K. Pneumoniae* from urine samples. *K. pneumoniae* is the second most common cause of UTI after *E. coli*. It is one of the important causes of catheter associated urinary tract infections (CAUTI). Similar to *Proteus mirabilis, K. pneumoniae* produce urease and form crystalline biofilms on urinary catheters.[34] The enzyme urease breaks down the urea, release ammonia and increase urine pH. This leads to formation of calcium magnesium phosphate crystal within the biofilm matrix. Crystal formation creates blockage of the catheter leading to bladder distension, urine leakage and bladder reflux into the kidney.[34]

In recent years, various strains of *K. pneumoniae* is showing a high resistance to a broad spectrum of drugs including beta-lactam antibiotics, fluoroquinolones, and aminoglycosides. Similar observation was noted in the present study,

where *K. pneumoniae* isolates demonstrated resistance to various classes of antibacterial agents like cephalosporins, aminoglycosides, fluroquinolones and monobactam (**Table No. 4**). As compared to other classes of antibacterial agents, *K. pneumoniae* demonstrated maximum susceptibility to tigecycline, polymyxin B and colistin. This observation was in accordance to that of Bashir *et al* (2014) and Pandurangan *et al* (2015).[27, 37]

Combined disc test (with Imipenem and Imipenem + EDTA) and Modified Hodge test were screening methods used for detection of carbapenem resistance in *K. pneumoniae.* In combined disc test carbapenem disc alone and with those of phenyl boronic acid (PBS) or EDTA or both is used. The concept of utilizing combined disc test for detection of KPC producing isolates was first put forward by Pasteran *et al.* in 2008. In this study as shown in (**Table No.5)** a total 86 (84.3%) out of 102 *K. pneumoniae* tested, isolates were positive by modified Hodge test. Whereas 16 (15.7%) were found to be negative.[38] Solanki *et al*. (2014) reported combined disc test to be positive in 65 out 100 isolates tested.[39] In the same year, Sood *et al*. reported combined test to be positive in 88.3% of *K. pneumoniae* isolates.[40]A total of 88 (86.3%) out of 102 *K. pneumoniae* isolates were positive by modified Hodge test. The rate of carbapenem resistance by modified Hodge test was higher than that reported by Dahab *et al*. [41] In their study, modified Hodge test was performed for 75 isolates which were resistant to carbapenem by disc diffusion method, a total 42 (56%) were positive while 33 (44%) were negative for carbapenemase production. Similar to our observation Bashir *et al*. (2014) reported modified Hodge test to be positive in 85.9% of *K. pneumoniae* that were carbapenem resistant by disc diffusion method.[27]

Out of 102 carbapenem resistance *klebsiella pneumoniae* isolates 96 where resistant for MBL by E test. In which 65.6% resistant shows by male and 34.4% resistant shows by female for MBL. Datta *et al* (2012) using this method reported 75% carbapenem resistance *K. pneumoniae* strains to have MBL type carbapenemases.[42] Bansal *et al.* (2013) by using this test, noted that 61.97% of *K. pneumoniae* isolates harboured KPC whereas MBL was detected in 23.94% of isolates and 14.04% possessed both MBL and KPC.[43] Mate *et al* (2014) reported E test as a sensitive method for detection of carbapenem resistance.[44] E test can detect metallo β- lacatamases both chromosomally and plasmid mediated. Diwakar *et al*. (2017) reported E test to be positive in 15 (50%) of *K. pneumoniae* isolates.[45] In the study of Shahi et al. (2019), by using E-test method for determination of MIC, out of 38 carbapenem resistant *K. pneumoniae,* 27 (71.1%) isolates had Imipenem MIC higher than CLSI resistance criteria whereas 3 isolates (7.9%) were found intermediate resistant.[46] In the present study, no isolates were found to be intermediate resistant to carbapenem.**(Table No.6)**

In the study of Tsakris *et al*. (2010) combined disc test was found to be 100% sensitive for detection of MBL and KPC carbapenemases whereas it was 98.6% sensitive for detection of MBL and KPC coproductions.[47] Giakkoupi *et al* (2009) reported boronate-based assays to be ineffective in detecting KPC producing *K. pneumoniae* isolates in the case of co-production of VIM β–lactamase.[48]
Among various phenotypic methods for detection of carbapenem resistance, modified Hodge test (the Clover leaf test) is relatively simple and easy. It can be easily incorporated as a routine technique in laboratories with heavy workload. Since this test is recommended by CLSI, it is extensively used as a phenotypic method for detection of carbapenem resistance.[27, 41]

In the study of Amjad *et al*. (2011), 17% of *K. pneumoniae* showed positive modified Hodge test.[49] Anderson and colleagues at CDC evaluated 45 isolated by modified Hodge test and further validated by PCR for detection of KPC activity.[50] Modified Hodge test was found to be 100% sensitive and specific for detection of carbapenem resistance in *K. pneumoniae*. Bashir H *et al.* reported modified Hodge test as a reliable and sensitive laboratory method for detection of carbapenem. Bashir H *et al*. also concluded that use of modified Hodge test avoid treatment failure and development of resistance due to unnecessary use carbapenem.[27]
A total of 16 isolates (15.7%) were negative by combined disc test whereas 14 (13.7%) were negative by modified Hodge test. Similar observation was reported by researchers like Doyle *et al*. (2012),[51] Solanki *et al.* (2014),[39] Swaminathan *et al*. (2016)[52] and Dahab *et al*. (2017).[41]

In the present study, when results of two phenotypic screening methods were compared, there was no significant difference was observed between these two tests for detection of carbapenemase production in *K. pneumoniae*. Researchers have expressed difference of opinion on utilization of combined disc test and modified Hodge test for detection of carbapenem resistance. Kumar and Mehra *et al*. (2015) reported combined disc test as a useful technique compared to modified Hodge test. Combined disc test was able to differentiate between KPC and MBL enzymes.[53] These authors recommended combined disc test for detection of carbapenem resistance in isolates from the geographical area where KPC and MBL enzymes are highly prevalent among the isolates from *Enterobacteriacae* family. In a recent study conducted by Manisha and Nagamani (2021) reported combined disc test to be more sensitive than modified Hodge test among phenotypic detection tests for carbapenemase production.[54]Aishwarya and Illamani (2021)reported modified Hodge test as a method of choice for screening of carbapenem resistance in *Enterobacteriaceae* as compared to combined disc test. The results of phenotypic screening methods vary geographically and from study to study.[55]

CONCLUSIONS

So, updating ourselves, exploring new diagnostic options and implementing new testing strategies can help to detect these carbapenem for early and proper patient management. Emergence of antimicrobial resistance in bacteria has

serious effects like high morbidity and mortality rates, increased length of hospital stays and additional financial burden on patients. *Klebsiella pneumoniae* is one among the few bacteria that are currently experiencing a significantly high rate of acquired or secondary antimicrobial resistance. Carbapenems are considered as reliable and often the last resort for treating bacterial infections. In recent years, carbapenem resistance has rampantly increased across the globe.

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