DOI: 10.7860/JCDR/2014/7027.4614

Original Article

Microbiology Section

Identification and Differentiation of Carbapenemases in *Klebsiella Pneumoniae*: A Phenotypic Test Evaluation Study from Jaipur, India

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ABSTRACT

Background: Carbapenem resistance is one of the major threats faced in antimicrobial treatment of infections caused by gram negative organisms. In recent years, carbapenem resistance has emerged in *Klebsiella pneumoniae* isolates due to acquisition of carbapenemases which belong to Ambler class A KPC type enzymes or to Ambler class B metallo-β-lactamases (MBL). Routine lab detection of carbapenemase producing *K. pneumoniae* isolates is crucial, both for a therapeutic management and an efficient infection control.

Materials and Methods: A study was conducted on 60 carbapenem resistant *Klebsiella pneumoniae* strains which were isolated from various clinical samples over a period of one year (September 2010-August 2011), at a tertiary care hospital in Jaipur. Phenotypic confirmatory test was done by using discs of Meropenem alone and those with phenyl boronic acid (PBA) or Ethylenediaminetetraacetic acid (EDTA) or both, for detection of carbapenemase production and differentiation of KPC and MBL enzymes.

Results: Of the 60 carbapenem resistant *Klebsiella pneumoniae* isolates, 53 (88.33%) were found to be MBL producers, 4(6.66%) were found to be MBL and KPC co-producers and the rest of the 3(5%) isolates were negative for both MBL and KPC production, as was seen by combined disc testing.

Conclusion: The combined disc test is a simple test which can be used for differentiation of carbapenemases and it can be easily incorporated in routine microbiology lab testing.

Keywords: Carbapenem resistance, *Klebseilla pneumoniae*, Enterobacteriaceae, Modified hodge test, Carbapenemase inhibition tests

INTRODUCTION

Carbapenems are commonly used to treat infections which are caused by multidrug-resistant Enterobacteriaceae. Carbapenem -resistant Enterobacteriaceae (CRE) have been reported worldwide, mostly because of the acquisition of carbapenemase gene [1]. These organisms have become a great concern because of the frequency with which they cause infections, the associated high mortality and their potential to cause a widespread transmission of carbapenem resistance via mobile genetic elements [2]. The carbapenemases are classified according to their amino acid sequences: Ambler class A (serine carbapenemases); Class B (metallo-β–lactamases); and Class D (OXA carbapenemases). Moreover, rare chromosomal encoded cephalosporinases (Ambler class C) which are produced by Enterobacteriaceae may possess slight extended activities towards carbapenems, but their clinical roles have yet to be elucidated [3].

Antibiotic-resistant *K. pneumoniae* has been a noteworthy nosocomial pathogen for over 4 decades. Sequentially, aminoglycoside resistance seen in *K. pneumoniae* in the 1970s, third-generation cephalosporin resistance which occurred through ESBLs (extended-spectrum β-lactamases) which was seen in the 1980s and 1990s, and then, carbapenem resistance seen in recent times, have been major problems [4]. Carbapenem resistant *K. pneumoniae* cause considerable clinical problems because they are multidrug resistant and as they lack susceptibilities to β-lactam antibiotics, fluoroquinolones and to aminoglycosides. Thus, therapy for clinically significant isolates rests on the use of tigecycline or polymyxin, both of which have been associated with development of resistance during treatment.

In order to implement an effective infection control, and also to make appropriate choice of antimicrobial therapy, it is important that local microbiology laboratories should be able to detect carbapenem resistance in a timely manner and with high sensitivity at the point of care. Recommended phenotypic confirmation tests for carbapenemase production are the modified Hodge test as well as carbapenemase inhibition tests with use of boronic acid for Ambler class A carbapenemases and with use of ethylene diamine tetra-acetic acid (EDTA) or dipicolinic acid for metallo-β–lactamases [5]. A combined disc test done with use of discs of Meropenem alone and with those of phenyl boronic acid (PBA) or EDTA or both, was evaluated in this study, for the detection of carbapenemase production and the differentiation of KPC and MBL enzymes in *Klebsiella pneumoniae*.

MATERIALS AND METHODS

A total of 340 *Klebsiella pneumoniae* isolates obtained from 8178 clinical samples were collected over a period of one year (September 2010-August 2011) at the Microbiology lab of Fortis Escorts Hospital, Jaipur, India. Of these 340 isolates, 60 *Klebsiella pneumoniae* strains which tested resistant to Meropenem (30mcg) on routine disc diffusion testing were included in the study. The antibiotic sensitivity profiles of these 60 CRKP strains, as seen on routine disc diffusion testing, were noted for the following antibiotics-amikacin(30mcg), gentamycin (30mcg), colistin (50mcg), polymyxin B (300 units), ceftriaxone(30mcg), cefepime(30mcg), amoxyclav (30/10mcg), piperacillin/tazobactam (100/10mcg), ciprofloxacin (5mcg), levofloxacin (5mcg), tetracycline (30mcg) and tigecycline (15 mcg). The 60 CRKP isolates were screened for carbapenemase production by using Modified Hodge test. A combined disc test was performed as a confirmatory phenotypic method, to differentiate between MBL and KPC productions.

Procedure for Modified Hodge test [6]: A Mueller Hinton Agar plate was inoculated with a 0.5Mc Farland's suspension of *E. coli*,

ATCC 25922 and it was streaked to obtain confluent growth, by using a swab. A 10µg Imipenem disk was placed at its centre, and each isolate was streaked from the disk to the edge of the plate and plate was incubated at 37°C overnight. After incubation, the plates were examined for a clover leaf type of indentation at the intersection of growth of the test organism and the *E. coli* ATCC 25922, within the zone of inhibition of the carbapenem susceptibility disc.

Procedure for Confirmatory Phenotypic test [7]: A phenotypic detection test was done by using combined discs of Meropenem alone and with those of phenyl boronic acid (PBA) or EDTA or both PBA and EDTA, for the detection of carbapenemase production and differentiation of KPC and MBL enzymes. The stock solution of PBA was prepared by dissolving phenyl boronic acid in DMSO at a concentration of 20µg/mL. 20µL of the stock solution (containing 400µg of PBA) was dispensed onto commercially available Meropenem discs. The stock solution of EDTA was prepared by dissolving anhydrous EDTA in distilled water at a concentration of 0.1M. From this solution, 10 µL (containing 292µg of EDTA) was dispensed onto Meropenem discs. The discs were dried and used within 60 minutes.

The test was performed by inoculating the test organism on Mueller Hinton Agar and placing one disc of Meropenem without any inhibitor and three discs of Meropenem, each containing containing 400µg of PBA ,292µg of EDTA or both i.e. 400µg of PBA and 292µg of EDTA on it. The agar plates were incubated at 37°C overnight. The diameter of the growth inhibitory zone seen around the Meropenem disc with PBA, EDTA, PBA+EDTA was compared with that seen around the plain Meropenem disc.

Interpretation: Modified Hodge test: A positive test shows a clover leaf like indentation of *E. coli* ATCC 25922 which grows along the growth of test organism within the disc diffusion zone. A negative test shows no growth of *E. coli* ATCC 25922 along the growth of test organism within the disc diffusion zone.

Combined disc test: Production of KPC was considered when the growth inhibitory zone diameters seen around the Meropenem disc with PBA and the Meropenem disc with PBA+EDTA had increased to >=5mm as compared to the growth inhibitory zone diameter seen around the disc containing Meropenem alone. Production of MBL was considered when the growth inhibitory zone diameters seen around the Meropenem disc with EDTA and the Meropenem disc with PBA+EDTA had increased to >=5mm as compared to the growth inhibitory zone diameter seen around the disc containing Meropenem alone. Productions of both KPC and MBL were considered when the growth inhibitory zone diameter seen around the Meropenem disc with both PBA+EDTA had increased to >=5mm, as compared to the growth inhibitory zone diameter seen around the disc containing Meropenem alone. When none of the three combined disc tests was positive, the isolate was considered to be negative for MBL and KPC carbapenemase productions.

RESULTS

From Out Patient 56.66% of the carbapenem resistant *Klebsiella pneumoniae* strains were isolated from ICU patients, 40% were isolated from ward patients and 3.33% were isolated Department (OPD).

A majority (25%) of the carbapenem resistant *Klebsiella pneumoniae* isolates were isolated from blood specimens, followed by endotracheal secretions (18.33%) [Table/Fig-1].

70% of the CRKP isolates were obtained from males, with a majority being isolated from patients who were in the age group of 21-30 years [Table/Fig-2]. 100% sensitivities to colistin, polymyxin B and tigecycline were noted among the CRKP isolates [Table/Fig-3]. These 60 CRKP isolates were subjected to screening for carbapenemase by Modified Hodge test. All these 60 strains (100%) showed positive results on doing Modified Hodge test.

On the basis of combined discs test, 53 strains (88.33%) were found to be MBL producers i.e. the growth inhibitory zone diameter around the Meropenem disc with EDTA and the Meropenem disc with PBA+EDTA had increased to >=5mm as compared to the growth inhibitory zone diameter seen around the disc containing Meropenem alone. Four (6.66%) isolates were found to coproduce both MBL and KPC, as the growth inhibitory zone diameters seen around the Meropenem disc with both PBA+EDTA had increased to >=5mm as compared to the growth inhibitory zone diameter seen around the disc containing Meropenem alone .The rest of the 3(5%) isolates were negative for both MBL and KPC productions, as none of the three combined disc tests was positive.

S. No.	Clinical Specimen	No. (%)
1	ET SECRETION	11(18.33)
2	BLOOD	15(25.00)
3	SPUTUM	7(11.66)
4	URINE	3(5.00)
5	PUS	5(8.33)
6	STOOL	5(8.33)
7	CSF	2(3.33)
8	BODY FLUIDS	711.66)
9	VAGINAL SWAB	2(3.33)
10	I.V. CATHETER TIP	2(3.33)
11	THROAT SWAB	1(1.66)
	TOTAL	**60(100)**

[Table/Fig-1]: Distribution of clinical samples yielding CRKP on culture

Age	Males	Females	Total No. of Patients (%)
0-10	6	2	8(13.33)
11-20	5	2	7(11.66%)
21-30	5	5	10(16.67%)
31-40	5	2	7(11.66%)
41-50	8	1	9(15.00)
51-60	3	1	4(6.67)
61-70	4	3	7(11.66)
71-80	5	2	7(11.66)
81-90	1	0	1(1.66)
>91	0	0	0(0)
TOTAL	42(70%)	18(30%)	60(100)

[Table/Fig-2]: Age and sex distribution of patients harbouring CRKP isolates

Antibiotic	Sensitive (No.)	% Sensitive
Amikacin	11	18.33
Gentamicin	0	0
Cefuroxime	0	0
Ceftriaxone	0	0
Cefepime	0	0
Aztreonam	0	0
Ampicillin	0	0
Amoxyclav	0	0
Piperacillin/Tazobactam	0	0
Ciprofloxacin	0	0
Levofloxacin	0	0
Tetracycline	19	31.66
Colistin	60	100
Polymyxin B	60	100
Tigecycline	60	100

[Table/Fig-3]: Antibiotic sensitivity profile of CRKP

DISCUSSION

Carbapenems are used as an empiric therapy for the treatment of life threatening infections in hospitals. The clinical use of carbapenem drugs have increased after the emergence and dissemination of Extended Spectrum β-Lactamase (ESBL) producers, which were capable to hydrolyze all β-lactams except the carbapenems [8]. The emergence of carbapenem-hydrolyzing beta-lactamases has threatened the clinical utility of this antibiotic class and it has brought us closer to the challenge of extreme drug resistance in gram-negative bacilli.

The isolation rate of carbapenem resistant *Klebsiella pneumoniae* in this study was found to be 60/340 (17.64%). A study done in Puducherry, southern India reported 43.6% carbapenem resistance in nosocomial isolates of *Klebsiella pneumoniae* [9]. Datta et al., (2012) reported an increase in carbapenem resistance from 2.5% in 2002 to 52% in 2009 in *Klebsiella pneumoniae* strains which were isolated from blood stream infections in Delhi [10]. A recent study done in Dhaka (Bangladesh) reported the incidence of CRKP to be 4.8% [11].

This study found that a majority (25%) of the carbapenem resistant *Klebsiella pneumoniae* strains were isolated from blood, followed by from endotracheal secretions (18.33%). Consistent to our findings, Braykov N et al., in their study done on CRKP which was isolated over a decade in the USA, also reported that isolates which were recovered from respiratory or blood sites of adult male patients who were admitted to the ICU were most likely to be CRKP [12]. However, a study done in Bangalore, India reported that most of the carbapenem-resistant isolates of *Klebsiella pneumoniae* were obtained from urine samples [13].

100% sensitivities to colistin, polymyxin B and tigecycline were noted for these CRKP isolates. Our findings are in sync with those which have been reported in literature, which have stated that tigecycline and polymyxins remained the most active in-vitro [14,15]. However, emerging tigecycline resistance in *Klebsiella pneumoniae* has been reported [16].

We found all the carbapenem resistant *Klebsiella pneumoniae* strains to be positive on Modified Hodge test (MHT), despite the fact that 3 isolates were negative for both KPC and MBL carbapenemase productions. This can be explained by recent reports which have questioned the specificity of MHT and have reported that 25% of results seen among carbapenemase non producers, mainly among strains harbouring CTX-M and Amp C hyperproducers, were observed to be false positive on MHT [17].

Using the phenotypic confirmatory combined disc method, 53 (88.33%) CRKP were found to be MBL producers, 4(6.66%) isolates were found to coproduce both MBL and KPC and the rest of the 3(5%) isolates were negative for both MBL and KPC productions. The prevalence of MBL type of carbapenemase in *Klebsiella pneumoniae* in our set up was 53/340(15.58%). Using this simple phenotypic combined disc test, a recent study done in north India found 75% CRKP strains to have MBL type carbapenemases, 25% CRKP isolates to be negative for both MBL and KPC and no KPC type of carbapenemases were detected [18]. A recent study done by Bansal et al., which evaluated this combined disc test, found 61.97% of the *Klebsiella pneumoniae* isolates to harbour KPC, 23.94% to harbour MBL and 14.04% to harbour both MBL and KPC [19]. Tsakris et al., reported this combined disc diffusion method to be 100% sensitive for detecting MBL and KPC carbapenemases and to be 98.6% sensitive for reporting MBL and KPC coproductions [7].

Molecular methods like PCR, DNA hybridization and sequencing are the gold standard for detection of carbapenemase production. But these are used in research settings and are of no use in the routine diagnostic laboratories.

CONCLUSION

The need of the hour is a simple, rapid and cost effective test that can provide accurate identification of carbapenemase producers in the clinical laboratory. The proposed methodology will provide fast and useful information which is needed for targeting antimicrobial therapy and appropriate infection control, especially in regions where carbapenem resistance which is mediated by carbapenemases is high or is increasing.

REFERENCES

[1] Queenan AM, Bush K. Carbapenemase: the versatile β–lactamases. *Clin Microbiol Rev.* 2007; 20: 440-58.
[2] Gupta N, Limbago BM, Patel JB, Kallen AJ. Carbapenem-resistant Enterobacteriaceae: epidemiology and prevention. *Clin Infect Dis.* 2011; 53(1), 60–7.
[3] Bush K, Jacoby GA. Updated functional classification of β-lactamases. *Antimicrob Agents Chemother.* 2010; 54(3):969-76.
[4] Li B, Yi Y, Wang Q, Woo PCY, Tan L, Jing H, et al. Analysis of Drug Resistance Determinants in *Klebsiella pneumoniae* Isolates from a Tertiary-Care Hospital in Beijing, China. *PLoS One.* 2012; 7(7): e42280.
[5] Stuart CJ, Leverstein-Van Hall MA. Dutch Working Party on the Detection of Highly Resistant Microorganisms. Guideline for phenotypic screening and confirmation of carbapenemases in Enterobacteriaceae. *Int J Antimicrob Agents.* 2010; 36(3):205-10.
[6] Clinical and Laboratory Standards Institute. Performance Standards for antimicrobial susceptibility testing. Twenty first Informational Supplement. M100-S21, CLSI Wayne PA: USA, 2011.
[7] Tskaris A, Poulou A, Pournaras S, Voulgari E, Vrioni C, Themili-Digalaki K, et al. A simple phenotypic method for the differentiation of metallo-β-lactamases and class A KPC carbapenemases in Enterobacteriaceae clinical isolates. *J Antimicrobial Chemother.* 2010; 65:1664-71.
[8] Sekar R, Mythreyee M. Carbapenem Resistant Enterobacteriaceae – A Growing Threat to Public Health. *Open Access Scientific Reports.* 2012; 1(6):324.
[9] Parveen RM, Harish BN, Parija SC. Emerging carbapenem resistance among nosocomial isolates of *Klebsiella pneumoniae* in South India. *International Journal of Pharma and Biosciences.* 2010; 1(2).
[10] Datta S, Wattal C, Goel N, Oberoi JK, Raveendran R, Prasad KJ. A ten year analysis of multi-drug resistant blood stream infections caused by Escherichia coli and *Klebsiella pneumoniae* in a tertiary care hospital. *Indian J Med Res.* 2012, 135: 907-12.
[11] Hayder N, Hasan Z, Afrin S, Noor R. Determination of the frequency of carbapenemase producing *Klebsiella pneumoniae* isolates in Dhaka city, Bangladesh. *Stamford Journal of Microbiology.* 2012; 2(1):28-30.
[12] Braykov N, Eber M, Klein E, Laxminarayan R. Carbapenem Resistance among Clinical Isolates of *Klebsiella pneumoniae* in the United States, 1999-2010. https://idsa.confex.com/idsa/2012/webprogram/Paper37551.html.
[13] Nagraj S, Chandran SP, Shamanna P, Macaden R. Carbapenem resistance among Escherichia coli and *Klebsiella pneumoniae* in a tertiary care hospital in South India. *Ind J Med Microbiol.* 2012; 30:93-95.
[14] Kumarasamy KK, Toleman MA, Walsh TR, Bagaria J, Butt F, et al., Emergence of a new antibiotic resistance mechanism in India, Pakistan, and the UK: a molecular, biological, and epidemiological study. *Lancet Infect Dis.* 2010; 10: 597-602.
[15] Nordmann P, Naas T, Poirel L. Global spread of carbapenemase-producing Enterobacteriaceae. *Emerging Infect Dis.* 2011; 17(10):191-97.
[16] Neonakis IK, Stylianou K, Daphnnis E, Maraki S. First case of resistance to Tigecycline by *Klebsiella pneumoniae* in a European University Hospital. *Ind J Med Microbiol.* 2011; 29:78-9.
[17] Carvalhaes CG, Picão RC, Nicoletti AG, Xavier DE, Gales AC. Cloverleaf test (modified Hodge test) for detecting carbapenemase production in *Klebsiella pneumoniae*: be aware of false positive results. *J Antimicrob Chemother.* 2009; 65:249–51.
[18] Datta P, Gupta V, Garg S, Chander J. Phenotypic method for differentiation of Carbapenemases in Enterobacteriaceae: Study from North India. *Ind J Pathol Microbiol.* 2012;55:357-60.
[19] Bansal M, Vyas N, Sharma B, Maheshwari RK. Differentiation of Carbapenemase producing Enterobacteriaceae by Triple disc Test. *Indian Journal of Basic and Applied Medical Research.* 2013; 3(1):314-20.

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FINANCIAL OR OTHER COMPETING INTERESTS: None.

Date of Submission: **Aug 21, 2013**
Date of Peer Review: **Jan 06, 2014**
Date of Acceptance: **Apr 13, 2014**
Date of Publishing: **Jul 20, 2014**