ORIGINAL ARTICLE

Occurrence and phenotypic detection of class A carbapenemases among *Escherichia coli* and *Klebsiella pneumoniae* blood isolates at a tertiary care center

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Background: Resistance to carbapenems is a significant therapeutic threat. The increasing frequency of car bapenemase enzymes among Gram-negative bacilli makes their early detection and differentiation urgent. Carbapenemases belonging to Class A are most commonly produced by members of family *Enterobacteriaceae* and are inhibited to various degrees by clavulanic acid. The present study is aimed to determine the occurrence and phenotypic detection of Class A carbapenemases in *Escherichia coli* and *Klebsiella pneumoniae* blood isolates from septicemic patients.
Methods: A total of 75 isolates of *K. pneumoniae* and 25 *E. coli* were screened for resistance to carbapenems by using meropenem and imipenem discs and meropenem E-test. Positive strains were then subjected to a modified Hodge test combined with carbapenemase inhibition tests to phenotypically detect and differentiate Class A serine carbapenemases from other classes of carbapenem hydrolyzing enzymes.
Results: The screening test showing the number of isolates resistant to meropenem and imipenem were 41 and 35 for *K. pneumoniae* and nine and four for *E. coli*, respectively. A total of 25 (33.3%) *K. pneumoniae* isolates and two (8.0%) *E. coli* isolates were classified as Class A carbapenemase producers. Multidrug resistance with coexistence of extended spectrum-beta-lactamases occurred in 44.4% isolates. However, all of the isolates were susceptible to colistin, polymyxin B, and tigecycline by disc diffusion test.
Conclusion: We conclude from the present study that Class A carbapenemases appear to be the predominant cause of resistance to carbapenems in *Enterobacteriaceae* at our center and,

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thus, phenotypic detection based on simple methods should be employed routinely in clinical microbiology laboratories.

Introduction

Carbapenems are commonly used to treat infections caused by multidrug-resistant *Enterobacteriaceae*.[1] During the last decade, carbapenem resistance has emerged among clinical isolates of the *Enterobacteriaceae* family, and this is increasingly attributed to the production of carbapenemases.[2] The rapid emergence and dissemination of these enzymes poses a considerable threat to clinical patient care and public health. These enzymes confer resistance to virtually all β-lactam agents, including penicillins, cephalosporins, monobactams, and carbapenems.[3] The carbapenemases fall into three classes according to their amino acid sequence: (1) Ambler Class A serine carbapenemases (serine beta-lactamases, inhibited by clavulanic acid), (2) Class B metallo-carbapenemases (metallo-beta-lactamases [MBLs], inhibited by metal chelators), and (3) Class D oxacillinase-type carbapenemases (expanded-spectrum oxacillinases).[4] Among these enzymes, a new type of Ambler Class A β-lactamase, the *Klebsiella pneumoniae* carbapenemase (KPC), has been rapidly spreading among *K. pneumoniae* isolates and other *Enterobacteriaceae* in the northeastern regions of the United States and has now spread to several regions of North and South America, as well as in Israel, China, and Greece.[5–11] Class A carbapenemases (KPCs, Sme, not metalloenzyme carbapenemase (NMC)-A, imipenem-hydrolyzing β-lactamase (IMI), and some allelic variants of Guiana extended spectrum (GES)/integron-borne cephalosporinase (IBC)) have become more prevalent within the *Enterobacteriaceae* family.[12]

Of the Class A carbapenemases, the KPC family has the greatest potential for spread due to its location on plasmids, especially since it is most frequently found in *K. pneumoniae*, an organism notorious for its ability to accumulate and transfer resistance determinants. In addition, the clonal spread seen in several epidemics points to difficulties with infection control for this organism.[13]

In general, carbapenem resistance may be mediated by three major mechanisms: (1) the hyperproduction of a β-lactamase with weak carbapenem-hydrolyzing activity (such as AmpC-type cephalosporinase or an extended-spectrum β-lactamase [ESBL]) combined with decreased drug permeability through the outer membrane (i.e., outer membrane porin loss or hyperproduction of efflux pumps), (2) a decreased affinity of the penicillin binding proteins that constitute target proteins for carbapenems, and (3) carbapenem-hydrolyzing β-lactamase production.[4,14,15] Early recognition of producers of carbapenemases has now become mandatory, as recognition is crucial for controlling the spread of carbapenemase-producing bacteria. A uniform and standardized phenotypic tool for the detection of Class A carbapenemases is still lacking.[12] Despite these troubling

trends and the importance of this issue from both clinical and public health perspectives, epidemiologic studies are still lacking in this part of world. Keeping in view the high-level drug resistance in our setting, we conducted this study to determine the occurrence of Class A carbapenemases producing strains among *K. pneumoniae* and *Escherichia coli* strains isolated from septicemic patients at our center. In addition, we evaluated the susceptibility pattern of these isolates to newer antibiotics.

Materials and methods

A total of 100 consecutive, nonduplicate clinical isolates of *K. pneumoniae* ($n = 75$) and *E. coli* ($n = 25$) from blood were collected in our tertiary care hospital over a period of 6 months (July 2009–January 2010). A retrospective record of demographic characteristics, including age and sex of the patient, and an association of the isolates with intensive care units (ICU), inpatient units, and outpatient clinics were maintained. All of the isolates were identified by standard biochemical tests and antibiotic susceptibility by Kirby-Bauer's disc diffusion method.[16,17]

Screening for the ESBL production was done by placing ceftazidime (30 μg), cefotaxime (30 μg), and cefepime (30 μg) to improve detection of ESBL in AmpC producers.[18,19] All of the isolates showing a zone diameter of ≤ 27 mm for cefotaxime or ≤ 22 mm for ceftazidime or ≤ 14 mm for cefepime were selected for ESBL production. ESBLs were confirmed by the method described in the 2009 *Clinical and Laboratory Standards Institute* (CLSI), also using positive and negative controls.[18] The results obtained were confirmed using E-test strips (BioMérieux India Ltd., bioMérieux, Marcy l'Etoile, France) containing ceftazidime at one end (Minimum inhibitory concentration (MIC) range, 0.5–32 μg/ml) and ceftazidime (MIC range, 0.064–4 μg/ml) plus 4 μg/ml clavulanic acid at the other end. A ≥ 3 two-fold concentration decrease in an MIC for ceftazidime plus clavulanic acid versus MIC for ceftazidime alone was inferred as positive for ESBL production.

Screening for carbapenemase production was done by placing meropenem (10 μg) and imipenem (10 μg) discs. Cut-off zone diameter breakpoints for carbapenemase production were taken as ≤ 23 mm and ≤ 21 mm for meropenem and imipenem, respectively.[20] All screening test positive isolates were tested for meropenem MIC by E-test (BioMérieux India Ltd.). The detection strategy/scheme of Class A carbapenemase, including screening steps and phenotypic confirmation, were followed (Fig. 1). We confirmed Class A carbapenemases phenotypically by combining a modified Hodge test (MHT) along with carbapenemase inhibition tests to overcome false positive results with MHT alone.[20,21] MHT does not distinguish between
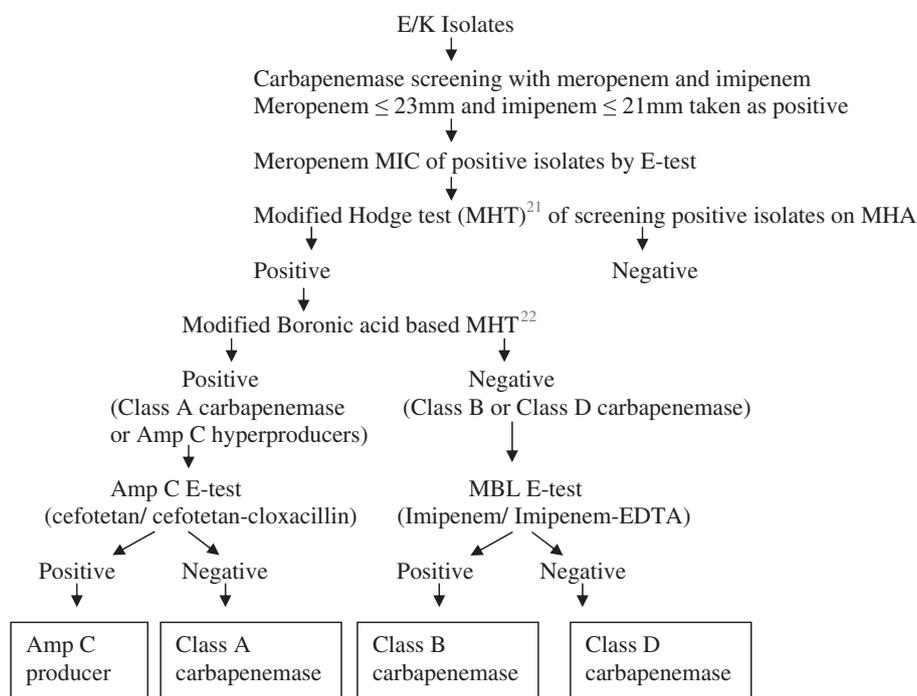
Figure 1. Algorithm followed for phenotypic detection of Class A carbapenemases in *Enterobacteriaceae*. E/K = *E.coli*/ *K.pneumoniae*; EDTA = ethylene diamine tetra acetic acid; MBL = metallo-beta-lactamase; MHA = Mueller Hinton Agar; MHT = modified Hodge test; MIC = minimum inhibitory concentration.

carbapenemase types and lacks sensitivity for MBL detection.[13] We added boronic acid, a reversible inhibitor of Class A carbapenemases and AmpC cephalosporinases, to differentiate between types of carbapenemases. A negative result on MHT after the addition of boronic acid at a concentration of 3000 μg/disc on meropenem was interpreted as positive for the presence of Class A carbapenemases or AmpC enzyme in the isolates tested.[22] Further differentiation of these two types of enzymes was performed with the help of AmpC E-test (BioMérieux India Ltd.) with cefotetan at one end and cefotetan plus cloxacillin at the other end. Isolates giving a positive result (> three-fold reduction in MIC of cefotetan) were treated as AmpC hyperproducers and the rest were taken as true Class A carbapenemase—producing strains (Fig. 1).

For a MHT, *E. coli* ATCC 25922 was used as the indicator strain and *K. pneumoniae* ATCC BAA-1705 and *K. pneumoniae* ATCC BAA-1706 were used as positive and negative control strains, respectively.[21] All of the Class A carbapenemase positive isolates were also tested for susceptibility to ciprofloxacin (5 μg), amikacin (30 μg), piperacillin/ tazobactam (100/10 μg), cefoperazone/sulbactam (75/ 30 μg), colistin (10 μg), tigecycline (15 μg), and polymyxin B (300 units). *E. coli* ATCC 25922 was used as the susceptible control strain. Interpretation was done as per CLSI for amikacin, ciprofloxacin and piperacillin/tazobactam while for others manufacturer's interpretative criteria were used. The zone diameters interpreted as sensitive for these drugs are as follows: cefoperazone/sulbactam (≥27 mm), colistin (≥11 mm), tigecycline (≥19 mm), and polymyxin B (≥12 mm). All the antibiotic discs were procured from BD Diagnostics (BD, Gurgaon, Haryana, India) except for cefoperazone/sulbactam (HiMedia Laboratories, Mumbai,

India) and control strains were kindly provided to us by Ranbaxy India Ltd (Daiichi Sankyo Research Centre, New Delhi, India).

Results

A total of 100 isolates used in the study were from patients with a mean age of 35.6 years. Of these 100 patients, 61 were men and the rest were women. Out of a total of 75 *K. pneumoniae*, 51, 24, and none were from patients in ICUs, wards, and attending outpatient clinics (OPDs), respectively. A total of 25 *E. coli* isolates were distributed as nine (ICUs), 11 (inpatients), and five (OPD). Of the total 100 isolates, 83% were ESBL-screening-test positive with higher rates in *K. pneumoniae* (85.3%). There was a combined double disc diffusion test and E-test—confirmed ESBL production in 68% (51 *K. pneumoniae* and 17 *E. coli*) of the isolates (Table 1). A total of 15% of isolates (13 isolates of *K. pneumoniae* and two of *E. coli*) that demonstrated screening-test positive for ESBL did not show any zone of

Table 1 Extended spectrum-beta-lactamase detection in *E. coli*/*K. pneumoniae* strains

Test	*K. pneumoniae* (n = 75)		*E. coli* (n = 25)	
	Positive	Negative	Positive	Negative
Screening test	64	11	19	6
Combined disc diffusion test	51	13/64	17	2/19
E-test	51	13/64	17	2/19

inhibition by clavulanic acid either on the combined disc diffusion or E-test used for confirmation for ESBL production. These isolates were treated as ESBL nonproducers but were resistant to third generation cephalosporins alone as well as in combination with a β-lactamase inhibitor.

Of the total 100 isolates screened for carbapenemase production, 50% and 39% showed positive cut-off zone breakpoints for meropenem (≤23 mm) and imipenem (≤21 mm), respectively. All of the isolates positive with imipenem were also positive with meropenem but the opposite was not true. The results for carbapenemase production are shown in Table 2.

A total of 25 (33.3%) *K. pneumoniae* and two (8.0%) *E. coli* isolates were found to be positive for Class A carbapenemase enzyme by our detection strategy, of which 88.9% (24/27) were from the ICU, 11.1% (3/27) from ward/admitted patients, and none were from OPD patients. The Class A carbapenemase positive isolates were mainly from adults (77.8% [21/27]), and there was nearly equal sex distribution (men, 55.6% [15/27]; women, 44.4% [12/27]).

Susceptibility of Class A carbapenemase producers to other drugs was ciprofloxacin (11.1%; 3/27), amikacin (14.8%; 4/27), piperacillin/tazobactam (18.5%; 5/27), and cefoperazone/sulbactam (18.5%; 5/27). However, all of the isolates were sensitive to colistin, polymyxin B, and tigecycline for which only the disc diffusion method was employed.

Discussion

With the spread of AmpC-, ESBL-, and carbapenemase-producing strains across the world, it is necessary understand the prevalence of these strains in hospitals. Detection of resistant isolates would allow physicians to formulate a policy of empirical therapy in high-risk units. Optimal use of microbiology laboratories is essential to combat the spread of multiple antibiotic-resistant pathogens. In the lab, an intermediate- or resistant-carbapenem result should always raise the suspicion of possible carbapenemase production.[13]

Recently, the CLSI issued recommendations[21] for the phenotypic screening of carbapenemase producers among species of *Enterobacteriaceae*: MICs of ertapenem, meropenem, and imipenem of 2, 2 to 4, and 2 to 4 μg/ml,

respectively (or a zone of inhibition by ertapenem or meropenem of ≤21 mm in diameter in the disk diffusion assay), may indicate isolates with carbapenemase production, and this phenotype should be confirmed by the Hodge method. However, ertapenem is not advised as an indicator carbapenem since it has lower specificity than imipenem and meropenem and thus its use can cause the prevalence of carbapenemases to be potentially high. Ertapenem is less specific because isolates with AmpC/ESBL and decreased permeability have higher MICs for ertapenem than for imipenem or meropenem.[12,20] To overcome this problem, we have used meropenem and imipenem in our study. Ideally, for an isolate with a positive carbapenemase screen test, a polymerase chain reaction—based molecular test should be performed to confirm the presence of carbapenemase genes. However, if genotypic confirmation is not readily available in routine clinical microbiology laboratories, delayed reporting of potential carbapenemase producers to the clinicians should be avoided by performing phenotypic confirmation tests. In our study, we have confirmed Class A carbapenemases phenotypically by combining MHT along with carbapenemase inhibition tests to overcome false positive results with MHT alone.[20] This kind of a strategy can be followed to detect Class A carbapenemases phenotypically in *Enterobacteriaceae*.

Coexistence of ESBL and Class A carbapenemase enzymes in *E. coli* and *K. pneumoniae* in septicemic isolates is more threatening (12/27 in our study) because the presence of carbapenemases increases both mortality and morbidity. Mouloudi and others[23] from Greece found KPC in 32.2% of *K. pneumoniae* isolates responsible for blood stream infections among ICU patients, which is in accordance with our study. By contrast, none of the isolates possessing Class A carbapenemase were from patients attending outpatient clinics. Thus, at present in India, carbapenemase harboring isolates are largely restricted to only hospitalized patients.

Most worrisome, treatment of infections caused by these organisms is extremely difficult because of their multidrug resistance, which results in high mortality rates. In our study, a high degree of co-resistance to ciprofloxacin, amikacin, ceftazidime, cefotaxime, cefepime, piperacillin/tazobactam, and cefoperazone/sulbactam was observed in Class A carbapenemase positive strains. All such strains, however, showed complete susceptibility to colistin,

Table 2 Detection of class A carbapenemase in *E. coli/K. pneumoniae* strains

Test	*K. pneumoniae* (n = 75)		*E. coli* (n = 25)	
	Positive	Negative	Positive	Negative
Screening test				
Meropenem	41	34	9	16
Imipenem	35	40	4	21
Meropenem E-test (positive ≥ 4 μg/ml)	41	34	9	16
MHT	28/41	13/41	2/9	7/9
Modified BA-MHT	26/28	2/28	2/2	0/2
AmpC E-test (cefotetan/cefotetan-cloxacillin)	1/26 (AmpC)	25/26	0/2 (AmpC)	2/2
MBL E-test (imipenem/imipenem-EDTA)	0/2 (Class B)	2/2 (Class D)	0 (Class B)	0 (Class D)
Class A carbapenemase positive	25	50	2	23

EDTA = ethylene diamine tetra acetic acid; MBL = metallo-beta-lactamase; MHT = modified Hodge test.

tigecycline, and polymyxin B by disc diffusion method, which needs further confirmation by employing MIC methodology. These drugs may act as substitutes to treat these infections either alone or in combination as supported by various studies from New York, Brazil, and India.[24—27]

To conclude, microbiologic excellence is a timely detection of resistant pathogens and can help formulate effective prevention and infection control strategies and help make better patient outcomes possible. This phenotypic method is very helpful to detect carbapenemase production and provides a simple algorithm for the differentiation of Class A carbapenemases and MBL enzymes in *Enterobacteriaceae* in routine clinical microbiological laboratories. In the present study, Class A carbapenemase appears to be a predominant cause of carbapenem resistance in *Enterobacteriaceae* in this part of India. To the best of our knowledge, this constitutes the first report on the prevalence and detection of Class A carbapenemases from India.

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