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Prevalence and characterization of carbapenemase producing isolates of *Enterobacteriaceae* obtained from clinical and environmental samples: Efflux pump inhibitor study

Radha Srinivasan, Maanasa Bhaskar, Ellappan Kalaiarasan and Harish Belgode Narasimha*

Department of Microbiology, Jawaharlal Institute of Post Graduate Medical Education and Research (JIPMER), Puducherry 605006, India.

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The present study was undertaken to screen for carbapenem resistance and the ability of efflux pump inhibitors to inhibit this resistance in enterobacteriaceae strains isolated from clinical specimens from patients attending Jawaharlal Institute of Post Graduate Medical Education and Research (JIPMER) hospital and water samples in and around Puducherry. A total of 425 carbapenem resistant isolates from clinical samples were studied by both phenotypic and genotypic methods. Two hundred and forty eight (248) strains were positive for metallo beta lactamase in the double disk synergy test (DDST) and 264 strains were positive for modified Hodge test (MHT). Multiplex PCR assays revealed that 262 of the 425 strains harboured *blaNDM-1* gene. Efflux pump inhibitory activity of Phenylalanine arginine beta-naphthylamide (PAβN) was detected for these strains. This study demonstrates that, 30 strains out of 163 blaNDM-1 negative strains were found to exhibit efflux pump activity. This study brings out the fact that such carbapenem resistant strains are limited only to clinical samples and not found in water samples in and around Puducherry.

Key words: *Enterobacteriaceae, bla*NDM-1, Efflux pump, carbapenem resistance.

INTRODUCTION

Antibacterial resistance continues to be a global public health concern, threatening the effectiveness of therapy, and challenging the efforts for developing novel antibacterial (Li and Nikaido, 2009). The introduction of carbapenem into clinical practice represented a great advancement in the treatment of serious bacterial infections caused by beta-lactam-resistant bacteria (Li and Nikaido, 2009; Walsh et al., 2002; Bush, 1998). Due to their broad spectrum of activity and stability to hydrolysis by most beta-lactamases, the carbapenem have been the drugs of choice for the treatment of infections caused by cephalosporin-resistant Gram

*Corresponding author. E-mail: drbnharish@yahoo.com. Tel: +91-944-3413502. Fax: +91-413-2272067.

negative bacilli, especially ESBL-producing Gram-negative infections (Li and Nikaido, 2009; Walsh et al., 2002; Bush, 1998; Kahan et al., 1983; Bradley et al., 1999).

Resistance to carbapenem is mediated by decreased outer membrane permeability (Walsh et al., 2005), efflux systems (Coyne et al., 2011), alteration of penicillin-binding proteins and carbapenem hydrolyzing enzymes-carbapenemases (Fernandez-Cuenca et al., 2003). Carbapenemases are class B metallo-β-lactamases or class D oxacillinases or class A clavulanic acid inhibitory enzymes. Metallo-β-lactamases which belong to class B, require divalent cations of zinc as cofactors for enzyme activity (Urban et al., 2003). They have potent hydrolyzing activity not only against carbapenem but also against other β- lactam antibiotics (Walsh et al., 2005; Bush, 1999).

The increase in resistance among bacteria, most notably *Klebsiella* spp. and *Escherichia coli*, by the production of extended-spectrum β-lactamases (ESBLs) has led to the increased use of carbapenem antibiotics (Paterson and Bonomo, 2005). Resistance to carbapenem among these bacteria remains remarkably rare in most countries. However, *Klebsiella* spp. that produce serine-based carbapenemase enzymes, referred to as *Klebsiella pneumoniae* carbapenemases (KPCs), have been identified in recent years (Bulik et al., 2010)

The emergence of carbapenem resistance constitutes an alarming development in the field of infectious diseases, with major public health implications. More intensive efforts are urgently required to elucidate the epidemiological and infection control issues related to these organisms and to improve the management of patients with infections. In this study, attempts have been made (i) to characterize the carbapenem resistant enterobacteriaceae species in clinical specimens and water samples and (ii) to detect the role of efflux pumps in mediating carbapenem resistance in *Enterobacteriaceae*.

MATERIALS AND METHODS

Clinical isolates and susceptibility tests

This study was carried out in the Department of Microbiology, JIPMER, for a period of 2011 and 2012. A total of 425 carbapenem resistant bacterial isolates belonging to the family *Enterobacteriaceae* were collected from different clinical specimens such as wound swab, tracheal aspirate, blood, pus, peritoneal fluid, CSF and sputum during the study period. The isolates were 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.

Antimicrobial susceptibility test for all isolates were performed on Mueller Hinton agar (MHA) plates by the standard Kirby Bauer disk diffusion method. A panel of 9 antibiotics of different classes (in-house disks of amikacin (30 µg), gentamicin (10 µg), ciprofloxacin (5 µg), ceftazidime (30 µg), ceftriaxone (30 µg) and meropenem (30 µg) from Hi-Media, Mumbai, India) were encountered. The diameter of the zones of inhibition of growth was interpreted as per CLSI guidelines 2011. *E. coli* ATCC 25922 was used as a control organism. Strains found to be meropenem resistant by disc diffusion test were tested by both broth micro dilution and commercial E-strips (Biomerieux, France), in order to determine the MIC (µg/ml) level.

Phenotypic test for carbapenemase detection

Metallo β-lactamase (MBL) detection

In the present study, imipenem-EDTA (10 µg/750 µg) and Imipenem (10 µg) commercial disks (Hi-Media, Mumbai) were used. The test was performed on Muller-Hinton agar plate by disk diffusion method. A 0.5 McFarland adjusted suspension of the test organism was inoculated on MHA. A 10 µg imipenem disk was placed on the plate and the I-EDTA disk was placed at a distance of 20 mm, centre to centre, from the imipenem disk and the plate were incubated at 37°C overnight (Lee et al., 2001). An increase in the zone diameter of imipenem-EDTA disk of ≥5 mm as compared to imipenem disk alone indicates a positive test.

Modified Hodge's test (MHT)

This is a phenotypic test which can be used to determine if reduced susceptibility to carbapenems is mediated by a carbapenemase production. Mueller-Hinton agar plate was inoculated with a 0.5 McFarland 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 plates were incubated at 37°C 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 (Lee et al., 2001).

Water sample strains and susceptibility tests

A study was conducted to determine the prevalence of carbapenemase producing strains among enterobacteriaceae species from water samples in and around Puducherry. Five hundred milliliters of water samples were collected from lakes (4), ponds (2) and taps (9) in screw capped wide mouthed sterile bottle and were filtered through sterile membrane filters (0.2 µM, Millipore, India) and the membrane was placed upside down on the surface of sheep blood and MacConkey agar plates and allowed for few minutes before being taken off. Then, the surface of MacConkey agar and sheep blood agar were streaked for isolated colonies. Further, the membrane was introduced into McCartney bottles containing brain heart infusion broth using sterile forceps and the broth was sub-cultured on MacConkey agar and blood agar after overnight incubation at 37°C. The isolates were identified by standard methods and antimicrobial susceptibility testing was carried out by disk diffusion method as per CLSI guidelines 2011.

Multiplex PCR analysis

Genomic DNA was extracted from all the strains by boiling lysis method. Multiplex PCR was carried out in order to detect metallo-β-lactamase genes such as blaNDM-1, blaVIM, blaKPC and blaIMP. Primer sequences used are given in Table 1. Thermal cycler gradient, Eppendorf was used for multiplex PCR. An initial denaturation step of 15 min at 95°C was followed by 30 cycles of denaturation at 95°C for 1 min, annealing at 59°C for 1 min and extension at 72°C for 90 s. This was followed by a step of final extension at 72°C for 10 min. The amplified products were analyzed

Table 1. Primers used to identify *bla*NDM-1, *blaIMP, blaVIM* and *blaKPC* genes by multiplex PCR analysis (Michael et al., 2011).

Name	Forward	Reverse	Size (bp)
NDM-1	GGTGCATGCCCGGTGAAATC	ATGCTGGCCTTGGGGAACGS	660
VIM	GTTTGGTCGCATATCGCAAC	AATGCGCAGCACCAGGATAGAA	382
IMP	CCWAATITAAAAATYGAGAAGCTTG	TGGCCAHGCTTCWAHATTTGCRTC	522
KPC	ATGTCACTGTATCGCCGTC	AATCCCTCGAGCGCGAGT	863

Table 2. Clinical specimens from which meropenem resistant organisms were isolated.

Specimen	Number of organisms isolated	Percentage of organisms isolated
Wound swab	162	38
Blood	85	20
Tracheal aspirate	55	13
Pus	51	12
CSF	17	4
Sputum	17	4
Catheter tip	13	3
Pleural pus	9	2
Peretonial fluid	8	2
Synovial fluid	8	2

Table 3. Results of tests showing positivity with respect to different organisms isolated.

Organism	Number of organismsisolated	MBL positive (%)	MHT positive (%)	blaNDM-1 positive (%)	Efflux positive (number)
Klebsiella sp.	229	58	62	65	14
Enterobacter sp.	89	60	63	53	8
Escherichia coli	85	58	62	66	6
Citrobacter sp.	14	57	64	57	1
Providencia sp.	5	60	60	60	1
Proteus sp.	3	67	0	0	0
Total	425	58	62	62	30

in 1.5% agarose gel stained with ethidium bromide and visualized under UV.

Efflux pump inhibitors

The presence of efflux pump as the mechanism of carbapenem resistance was analyzed using MIC assay. The strains which were negative for carbapenemase genes by PCR were used for efflux pump studies. Meropenem trihydrate pure powder (Orchid, Chennai) was used for MIC assay. Broth micro dilution was performed using Muller Hinton broth in the presence and absence of 25 µg/ml of phenylalanine arginine beta-naphthylamide (PAβN) (Sigma Aldrich, US). A threefold or more decrease in the MICs of the strains in the presence of PAβN suggests efflux pump inhibition by PaβN, indicating the presence of an active efflux mechanism.

RESULTS

Among 425 meropenem resistant clinical isolates, 229 were *Klebsiella* sp., 89 were *Enterobacter* sp., 85 were *E. coli*, 14 were *Citrobacter* sp., 5 were *Providencia* sp. and 3 were *Proteus* sp. Table 2 shows the different clinical specimens from which meropenem resistant organisms were isolated. Two hundred and forty eight (58%) strains were positive for metallo beta lactamase double disk synergy test (MBL) and 264 (62%) strains were positive for modified Hodge test (MHT). Among 425 strains, 262 (62%) strains had blaNDM-1(660 bp) gene. Table 3 presents the positive percentage of these tests.

The efflux pump inhibitory studies showed that out of

Table 4. Organism isolated from water samples.

Organism	Number of isolates
Escherichia coli	5
Klebsiella sp.	3
Enterobacter sp.	2
Proteus mirabilis	1
Total	11

163 *blaNDM-1* gene negative strains, 30 (23%) isolates had shown three fold decrease in MIC values in the presence of PAβN, which suggests the presence of efflux pump-mediated carbapenem resistance.

The prevalence rate of carbapenem resistant enterobacteriaceae from water samples in 15 areas of Puducherry was studied. It was observed that the *Enterobacteriaceae* species isolated from lakes (4) and ponds (2) were all susceptible to meropenem, amikacin, ciprofloxacin, ceftazidime, ceftriaxone and gentamicin by disc diffusion method. The most common isolates were *E. coli* (5), *Klebsiella* sp. (3), *Enterobacter* sp. (2) and *Proteus mirabilis* (1) (Table 4). However, all the tap water samples (9) did not yield any Gram negative bacteria.

DISCUSSION

Carbapenemase producing organisms display higher levels of resistance to almost all antibiotics (Li and Nikaido, 2009; Lee et al., 2001). Meropenem resistance is used in practice as an indicator of the presence of metallo beta lactamases. The present prospective study was carried out to reveal the prevalence rate of MBL-producing enterobacteriaceae from clinical and environmental isolates. It has been mostly reported that resistance to beta lactam antibiotics is on the rise among clinical isolates in different Indian hospitals, indicating the need for exhaustive research. Several studies have reported the risk role of MBL and ESBL-producing *Pseudomonas aeruginosa, Acinetobacter* species or enterobacteriaceae among hospitalized patients. The occurrence of an MBL-positive isolate in a hospital environment not only poses a therapeutic problem, but also is a serious concern for infection control management (Behera et al., 2008).

In the present study, MHT was positive in 62% of the isolates. This indicates that carbapenemase mediated mechanism of resistance is more frequent than the non-carbapenemase mediated mechanism of resistance. 62% of the isolates showed the presence of blaNDM-1 gene and the predominant is *K. pneumoniae.* NDM-1 which is a recent addition to the carbapenemase list, was the only carbapenemase identified in our isolates. In a recent study (Rahman et al., 2014) which evaluated the detection and molecular characterization of NDM variants in Enterobacteriaceae at a tertiary care hospital in India, all carbapenem-resistant isolates were bla_{NDM} positive by PCR.

Efflux pump inhibitor studies revealed that out of 163 clinical isolates negative for carbapenemase genes by PCR, 30 isolates showed efflux pump activity. Previous studies (Baroud et al., 2013) reported that efflux pump inhibition has significantly decreased MICs to carbapenems in *E. coli*. Carbapenem resistance in ESBL-producing *K. pneumoniae* and *E. coli* is due to the combined effect of β-lactamases with porin impermeability and/or efflux pump activity observed in these organisms, and in a number of isolates is due to the production of the carbapenemase-encoding genes and the newly emerging blaNDM-1 (Cunningham et al., 2013).

The prevalence of carbapenem resistant strains of *Enterobacteriaceae* in water samples from 15 places in Puducherry was studied. It was observed that the *Enterobacteriaceae* species isolated from lakes and ponds were susceptible to meropenem. Tap water samples were found to be negative for *Enterobacteriaceae* species.

Findings of this study emphasize the role of carbapenem resistance among hospital isolates and leads to consideration of empirical treatment for infection caused by these organisms especially in patients compromised by underlying disease or immunological status.

Conclusion

In this study, it was observed that efflux pumps play an important role in carbapenem resistant *Enterobacteriaceae* species apart from other resistance mechanisms such as metallo beta lactamases and outer membrane proteins. The results of environmental studies indicate the absence of carbapenem resistant enterobacteriaceae species in pond, lake and tap water from the area under study. This study provides reliable data for researchers working in the area of carbapenem resistant bacteria in clinical and environmental samples.

Conflict of interest

The authors did not declare any conflict of interest.

REFERENCES

Baroud M, Dandache I, Araj GF, Wakim R, Kanj S, Kanafani Z, et al (2013). Underlying mechanisms of carbapenem resistance in extended-spectrum β-lactamase-producing Klebsiella pneumoniae and Escherichia coli isolates at a tertiary care centre in Lebanon: role of OXA-48 and NDM-1 carbapenemases. Int. J. Antimicrobial Agents. Jan 41(1):75-79.

Behera B, Mathur P, Das A, Kapil A, Sharma V (2008). An evaluation of four different phenotypic techniques for detection of metallo-β-lactamase producing *Pseudomonas aeruginosa*. Indian J. Med. Microbiol. 26: 233-237.

Bradley JS, Garau J, Lode H, Rolston KV, Wilson SE, Quinn JP (1999). Carbapenems in clinical practice: a guide to their use in serious infection. Int. J. Antimicrob. Agents 11:93-100.

Bulik CC, Christensen H, Li P, Sutherland CA, Nicolau DP, Kuti JL (2010). Comparison of the Activity of a Human Simulated, High-Dose, Prolonged Infusion of Meropenem against Klebsiella pneumoniae producing the KPC Carbapenemase versus That against Pseudomonas aeruginosa in an In Vitro Pharmacodynamic Model. Antimicrobial Agents Chemother. 1:54(2):804-810.

Bush K (1998). Metallo- β -lactamases: a class apart. Clin. Infect. Dis. 2(Suppl.1):S48-S53.

Bush K (1999). β-Lactamases of increasing clinical importance. Curr. Pharm. Des. 5:839-845.

Coyne S, Courvalin P, Pe´richon B (2011). Efflux-Mediated Antibiotic Resistance in *Acinetobacter* spp. Antimicrobial Agents Chemother. pp. 947-953.

Cunningham SA, Noorie T, Meunier D, Woodford N, Patel R (2013). Rapid and Simultaneous Detection of Genes Encoding Klebsiella, pneumoniae Carbapenemase (blaKPC) and New Delhi Metallo-β-Lactamase (blaNDM) in Gram-Negative Bacilli. 51(4):1269-1271.

Fernandez-Cuenca F, Martinez-Martinez L, Conejo MC, Ayala JA, Perea EJ, Pascual A (2003). Relationship between beta-lactamase production, outer membrane protein and penicillin-binding protein profiles on the activity of carbapenems against clinical isolates *Acinetobacter baumannii*. J. Antimicrob. Chemother. 51:565-574.

Kahan FM, Kropp H, Sundelof JG, Birnbaum J (1983). Thienamycin: development of imipenem-cilastatin. Antimicrob. Agents Chemother. 12(Suppl. D):S1-S35.

Lee K, Chong Y, Shin HB, Kim YA, Yong D, Yum JH (2001). Modified Hodge and EDTA-disk synergy tests to screen metallo-b-lactamase producing strains of Pseudomonas and Acinetobacter species. Clin Microbiol Infect. 7:88-91.

Li X Z, Nikaido H (2009). Efflux-Mediated Drug Resistance in Bacteria: an Update, Drugs 20;69(12):1555-1623.

Michael RM, Jennifer MG, Katherine P, Diane R, David AB (2011). New Delhi Metallo-β-Lactamase in Klebsiella pneumoniae and Escherichia coli, Canada, Emerging Infectious Dis. 17(1):103-106.

Paterson DL, Bonomo RA (2005). Extended-spectrum beta-lactamases: a clinical update. Clin. Microbiol. Rev. 18:657-686.

CLSI guidelines (2011). Performance Standards for Antimicrobial Susceptibility Testing; Twenty-First Informational Supplement, M100-S21, 31:1.

Rahman M, Shukla SK, Prasad KN, Ovejero CM, Pati BK, Tripathi A, et al. (2014). Prevalence and molecular characterisation of New Delhi metallo-β-lactamases NDM-1, NDM-5, NDM-6 and NDM-7 in multidrug-resistant Enterobacteriaceae from India. International J. Antimicrobial Agents 44(1):30-37.

Urban C, Segal-Maurer S, Rahal JJ (2003). Considerations in control and treatment of nosocomial infections due to multidrug-resistant *Acinetobacter baumannii*. Clin. Infect. Dis. 36:1268–1274.

Walsh TR, Bolmstrom A, Qwrnstrom A, Gales A (2002). Evaluation of a New Etest for Detecting Metallo-β-Lactamases in Routine Clinical Testing, J. Clin. Microbial. 40:(8).

Walsh TR, Toleman MA, Poirel L, Patrice N (2005). Metallo--Lactamases: the Quiet before the Storm? Clin. Microbiol. Rev. 18(2):306-325.