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Mechanisms of Carbapenem Resistance in *K.pneumoniae* and *E. coli* from Bloodstream Infections in India

Archa Sharma[1], Yamuna Devi Bakthavatchalam[1], Radha Gopi[1], Shalini Anandan[1], Valsan Philip Verghese[2] and Balaji Veeraraghavan[1*]

[1]*Department of Clinical Microbiology, Christian Medical College, Vellore-632004, India*

[2]*Department of Child Health, Christian Medical College, Vellore-632004, India*

[*]**Corresponding author:** Balaji Veeraraghavan, Department of Clinical Microbiology, Christian Medical College, Vellore-632004, India, Tel: 9442210555; E-mail: vbalaji@cmcvellore.ac.in

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Abstract

Introduction: Emergence and global spread of carbapenemase producing Enterobacteriaceae (CPE) are of great concern in healthcare settings. Resistance to carbapenem is mostly conferred by metallo β-lactamase (IMP, VIM and NDM) and carbapenem hydrolyzing class D β-lactamase (OXA-48 like). The aim of this study was to characterise the molecular mechanism of resistance in the clinical isolates of Enterobacteriaceae causing bacteremia and showing resistance to β-lactams, including carbapenems.

Materials and Methods: Isolates of *E.coli* (n=42) and *K. pneumoniae* (n=134) from blood culture collected during 2013-2015 were screened for carbapenemase production by using carba NP test and the presence of carbapenem resistant genes (KPC, IMP, VIM, NDM and OXA- 48 like). Sequencing was performed for the randomly selected isolates positive for NDM and OXA-48 like.Results: Of the 176 isolates, 97% of the isolates were found to be positive with carba NP test. Carba NP test has the sensitivity, specificity, PPV and NPV of 98%, 50%, 99% and 20% respectively. Each of *bla*NDM and *bla*OXA-48 like was seen in 32% of the tested isolates. Co-production of *bla*NDM and *bla*OXA48 like and *bla*VIM and *bla*OXA48 were seen in 13% and 8% of isolates respectively. Noticeably, 3% of isolates were identified as co-producers of *bla*NDM, *bla*VIM and *bla*OXA48 like. All of the sequenced NDM and OXA-48 like were identified as NDM-1 and OXA-181 variants.

Conclusion: Increasing incidence of OXA-48 like is worrisome in developing countries. Because of its weak hydrolytic acivity against broad spectrum cephalosporin and carbapenems, these may go undetected in routine screening. In particular, *bla*OXA48 like gene is mostly identified on the plasmid and is implicated as the cause for silent spread and outbreaks in hospitalized patients.

Introduction

Carbapenemase producing *Enterobacteriaceae* (CPE) causing bacteremia is of great clinical concern. Carbapenemases are a versatile group of β-lactamases that are characterised by their resistance to virtually all β-lactam antibiotics including cephalosporins and carbapenems, complicating therapy and limiting treatment options. CPE infections are also associated with high mortality of 26%-44% [1]. The most common carbapenemase in *Enterobacteriaceae* belongs to class A carbapenemase (KPC), class B metallo β-lactamases (IMP, VIM, NDM) and class D oxacillinase (OXA-48 like) [2,3].

The SENTRY antimicrobial surveillance programme on antimicrobial resistance was conducted across India. The most common gene isolated in this surveillance study was NDM (38.4%) followed by OXA-48 like [4]. In particular, OXA-48 like carbapenemases has disseminated from Middle East region to European countries, Asia, and more recently from North America as well [5,6]. The prevalence of OXA-48 like carbapenemases is on the rise, and are the predominant carbapenemases in countries such as France and Belgium [7,8]. There are 11 known variants of OXA-48 like carbapenemases including OXA-48, OXA 54, OXA-162, OXA 163, OXA 181, OXA 199, OXA 204, OXA 232, OXA 242 and OXA 247 [9]. OXA-48 like carbapenemases has significant hydrolysing activity against penicillins, cloxacillin, and oxacillin and is not inhibited by β-lactamase inhibitors in clinical use [10]. They also demonstrate weak hydrolysing activity against 3rd and 4th generation cephalosporin and to carbapenems [11]. Most infections associated with OXA-48 like carbapenemases are described in nosocomial outbreaks in hospital settings [12-16]. The emergence of drug-resistant organisms both in the hospital environment and in the community is a major concern for health care providers. Continued monitoring of antimicrobial resistance patterns in hospitals is essential to guide effective empirical therapy.

In this study, *Escherichia coli* and *Klebsiella pneumoniae* causing bloodstream infection (BSI) were chosen to represent *Enterobacteriaceae* that are frequently associated with acquisition and spread of plasmid-mediated carbapenemase genes. The aim of this study was to characterize the mechanisms of carbapenem resistance in *E. coli* and *K. pneumoniae* and to study the susceptibility profile of these organisms to other classes of drugs.

Materials and Methods

Study design and ethics approval

This was an observational study conducted over a period of three years from 2013 to 2015 at Christian Medical College, Vellore, and a

2600 bedded tertiary level hospital in South India. This study was approved by Institutional Review Board of Christian Medical College, Vellore. (IRB Min no: 8201 dated 13.02.2013)

Study samples

Blood culture was performed with the BacTAlert automated system (bioMe´rieux, Durham, NC). Identification and characterization of *E. coli* and *K. pneumoniae* from positive blood cultures was performed according to the standard microbiological procedures including cultural characteristics and standard biochemical methods [17]. *Klebsiella spp.*, other than *K. pneumoniae* was excluded from this study.

Antimicrobial susceptibility testing

Antimicrobial susceptibility testing by disk diffusion was performed as a part of the routine testing and interpretation was done according to the clinical laboratory standard institute (CLSI) guidelines. Isolates of *E.coli* and *K. pneumoniae* were tested for susceptibility to cefotaxime (30 µg), ceftazidime (30 µg), cefoxitin (30 µg), cefepime (30 µg), piperacillin-tazobactam (100/10 µg), cefoperazone-sulbactam (75/30 µg), gentamicin (10 µg), amikacin(30 µg), netilmicin (30 µg), ciprofloxacin (5 µg), colistin (300 units) by Kirby Bauer disk diffusion method. Consecutive and non-repetitive isolates of *E. coli* (n=42) and *K. pneumoniae* (n=134) resistant to imipenem and/or meropenem by disk diffusion method were included in this study.

Carba NP test: Carba NP was performed for all carbapenem resistant *E. coli* and *K. pneumonaie* as previously described by Nordmann and Poirel [18]. Hydrolysis of the β-lactam ring of carbapenem is indicated by change in the colour of the indicator phenol red. Optical reading of the colour produced by each isolate was taken and interpreted.

Molecular detection resistant genes: Multiplex PCR was performed for detection of carbapenem resistant genes. Class A carbapenemase production was screened for by testing KPC (see ref. 4), Class B carbapenemase productionby testing for IMP [19], VIM [20] and NDM [21], as well as Class D carbapenemase (OXA-48 like) production [22]. Amplification of genes was performed using Veriti Thermal cycler (Applied Biosystems, USA) using the following cycling condition; initial denaturation at 95°C for 15 min, 30 cycles of 94°C for 30 seconds, 59°C for 1.5 minutes, 72°C for 1.5 minutes and final extension at 72°C for 10 minutes followed by 4°C. Amplicons were visualized on 2% agarose gel using electrophoresis. Ampilcons were subjected to direct DNA sequencing and was performed by with an automated sequencer ABI prism 3130 genetic analyzer from Applied Biosystems. Analysis of nucleotide sequences and enzyme variants were determined by using the software from the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov)

Results

Antimicrobial susceptibility testing

Antimicrobial susceptibility testing of the 176 carbapenem-resistant isolates, 24% (n=42) were *E. coli* and 76% (n=134) were *K. pneumoniae*. All were resistant to cefotaxime, ceftazidime, and β-lactam/β- lactamase inhibitor combinations including piperacillin-tazobactam and cefoperazone-sulbactam. Amikacin remained active against 24% (n=10) of *E. coli* but only 7% (n=9) of *K. pneumoniae* isolates. Susceptibility to gentamicin, netilmicin and ciprofloxacin in *E.coli* were 5% (n=2), 26% (n=11) and 7% (n=3) respectively. For *K. pneumoniae*, susceptibility to gentamicin, netilmicin and ciprofloxacin were 6% (n=8), 4% (n=6), 1% (n=2) respectively. A majority of the tested isolates, 100% (n=42) of *E. coli* and 99% (n=134) of *K. pneumoniae* were susceptible to colistin.

Carba NP test

Carbapenemase production by Carba NP test was noted in 95% (n=167) of the isolates. The test was repeated on negative isolates (n=9) after an increased incubation time of the bacterial isolate in the lysis buffer. With this modification, four of the isolates that previously tested negative retested as positive by Carba NP test for a detection rate of 97% (n=171). Of the isolates tested, 93% (n=42) of *E. coli* and 99% of *K. pneumoniae* isolates were positive on Carba NP test. Five isolates (three *E. coli* and two *K. pneumoniae* isolates) were negative with modified Carba NP test. Noticeably, Carba NP negative *K. pneumoniae* (n=2) were positive for *bla*OXA48 like gene. Of the Carba NP negative *E.coli* (n=3) isolates, two were positive for *blaNDM* and one isolate was also negative by PCR. The sensitivity, specificity, positive predictive value (PPV) and negative predictive value (NPV) of carba NP were 98%, 50%, 98% and 100% respectively.

Molecular characterisation: PCR and sequencing

On PCR testing, 165 isolates (94%) were found to possess at least one of the tested carbapenemase genes. Each of the bla_{NDM} and bla_{OXA48} like gene was seen in 32% (n=56) of isolates. Interestingly, 3% (n=6) of isolates had a triple combination of bla_{NDM}, bla_{VIM} and bla_{OXA48} like carbapenemase genes and all of them were *K. pneumoniae* (Table 1). All the tested isolates were negative for the KPC gene.

Carbapenem resistant genes	*E. coli* (n=42) n (%)	*K. pneumoniae* (n=134) n (%)	Total n (%)
NDM	20 (48)	36 (27)	56 (32)
OXA-48 like	8 (19)	48 (36)	56 (32)
VIM	0 (0)	2 (1)	2 (1)
NDM + OXA-48 like	2 (5)	20 (15)	13 (13)
VIM + OXA-48 like	0 (0)	14 (10)	8 (14)
NDM + VIM	7 (17)	2 (1)	5 (9)
NDM+VIM+ OXA-48 like	0 (0)	6 (4)	6 (4)
*All negatives	5 (12)	6 (4)	11 (6)
*All negative includes the isolates negative for the tested carbapenemase genes (IMP, VIM, NDM, OXA-48 like and KPC)			

Table1: Distribution of carbapenem resistant genes in *E. coli* and *K. pneumonia*.

Randomly, isolates which were positive for the $bla_{OXA48\ like\ s}$ gene (n=20) and positive for bla_{NDM} gene (n=7) were selected, sequenced, BLAST matched with the reference sequences in GenBank. Noteably, all the bla_{OXA48} like (n=20) producers were identified as bla_{OXA181} variant. Similarly, all the isolates (n=7) with bla_{NDM} gene were found

to be NDM- 1 variant. The sequences showed 100% identity and query coverage with the reference sequences deposited in NCBI website (www.ncbi.nlm.nih.gov).

Discussion

Carbapenem resistant *Enterobacteriaceae* (CRE) cause outbreaks of hospital acquired infections and are associated with high mortality and morbidity. There was a pronounced variation in the distribution of carbapenemase in different geographical region includes KPC in United States and Greece, metallo β-lactamase such as IMP and VIM were predominantly reported from southern Europe and Asia. In addition, oxacillinase-48 type carbapenemase was most commonly reported from Mediterranean and European countries and in India [19].

In this study, most of the tested isolates were resistant to first line drugs. Most of the CRE are multi-drug resistant, but may remain susceptible to one or more aminoglycosides. Aminoglycosides may be an appropriate component of combination therapy for CRE induced infection. *In-vitro* study on activity of aminoglycosides against CRE, has reported that higher susceptibility (80%) of CRE to amikacin [23]. In particular, amikacin is the preserved antibiotic and has the profound activity against *E. coli* but not on *K. pneumoniae*. Susceptibility of CRE to aminoglycosides has been reported from USA and European countries [24-27], although susceptibility of CRE to aminoglycosides is less likely to be reported from India as the NDM and its variants that are endemic in the Indian subcontinent are usually already resistant to aminoglycosides [24]. In addition, KPC and other MBLs (IMP, VIM) are more prevalent in the USA and European countries and are occasionally reported from India [28,29]. Most of the tested isolates were susceptible to colistin. However, colistin resistance in these organisms has begun to emerge [30,31].

On Carba NP testing, 95% of the isolates demonstrated carbapenemase production. The sensitivity of this test has been described to vary from 72% to 100%, but with 100% specificity [32–34]. According to the CLSI guidelines (M100-S26), sensitivity and specificity of Carba NP was >90% in detecting class A (KPC) and class B carbapenemases (IMP, VIM, and NDM), but demonstrated a lower sensitivity in detecting OXA-48 like carbapenemases [35,36]. False negative reactions on Carba NP testing have been documented with mucoid strains and/or enzymes with weak carbapenemase activity such as OXA-48[32,33]. Similarly in this study, false negative result with OXA-48 producing *K. pneumonaie* (n=2) and NDM producing *E. coli* (n=2) isolates was seen in carba NP test.

For detection of carbapenemases, Modified Hodge test (MHT) works well for KPC and OXA-48 like carbapenemases but not for NDM. The fact that one isolate was negative in CarbaNP test as well by PCR could be attributed to the presence of other mechanisms of resistance such as efflux pumps or loss of porin channels. The mechanisms usually observed are plasmid encoded AmpC enzymes in combination with loss of porin channels OmpK35/36, OmpF or OmpC for *E. coli* [37].

The global antimicrobial resistance surveillance programme, Study for Monitoring Antimicrobial Resistance Trends (SMART) study in 2009 has documented NDM-1 as the predominant gene responsible for carbapenem resistance in isolates from India [38]. The SENTRY antimicrobial surveillance program from India has reported that although NDM-1 was the most common carbapenemase encoding gene, the OXA-181 variant was the next most common among carbapenemase-resistant isolates in 2006-2007 [4]. However our isolates from 2013-2015 showed an equal distribution of NDM (32%) and OXA-48 like (32%) genes. As there is a lack of national surveillance data, the prevalence of carbapenem resistant genes can only be compared with other single-centre studies from India. Khajuria et al. reported NDM-1 (100%) as the foremost gene encoding carbapenem resistance from urinary isolates of *E. coli* and in 55% of these isolates were found with OXA-48 like gene [39]. In contrast, Shanthi et al. reported very low prevalence of 1.8% $bla_{\text{OXA-48}}$ like gene among carbapenem resistant isolates [40]. All the sequenced NDM (n=7) and OXA-48 like (n=20) genes in this study were identified as NDM-1 and OXA-181 variants respectively. Similarly, Anandan et al. have reported that OXA-181 is the most common variant of OXA-48 like gene next to NDM which are endemic in India [41]. Remarkably, most of the reported OXA-48 like carbapenemases is co-produced with the extended spectrum β-lactamase (ESBL), CTX-M-15. Regardless of molecular characterisation, all the isolates included in this study were identified as ESBL producers with double disc diffusion method. In addition, co-production of OXA-48 with metallo β-lactamase such as NDM or VIM has also been reported [42].

KPC production was not observed in any of our study isolates. Nordmann et al. in their review stated that though KPC enzymes have been reported from India they are mainly responsible for sporadic outbreaks [43]. Although Shanmugam et al. found aprevalence of 67.4% of KPC carbapenemases [44], this could be due to the diverse nature of their study specimens and a small number of blood stream isolates.

Increasing prevalence of OXA-48 like carbapenemases in *Enterobacteriaceae* is worrisome as they are increasingly reported with outbreaks of nosocomial infection across the world [45]. OXA-48 like carbapenemases weakly hydrolyse carbapenem but spare extended spectrum cephalosporins [46]. This heterogeneous hydrolytic property, with the isolate being either susceptible or resistant to extended spectrum cephalosporins and to carbapenems, may lead to non-detection on routine diagnostic testing that is critical for therapy and infection control. The optimum treatment option for this multi-drug resistant pathogen remains uncertain. For ESBL negative OXA-48 producers, broad spectrum cephalosporins are the preferred treatment choice [47,48]. A triple combination regimen of colistin, a cephalosporin (ceftazidime or cefepime) and an aminoglycoside is the likely treatment option for ESBL positive OXA-48 producers [49]. However, further studies on the efficacy of such treatment options are necessary before such recommendations can be made.

Conclusion

The rapid emergence and widespread dissemination of the NDM-1 producing *Enterobacteriaceae* is now well known. The high prevalence of OXA-181 enzyme in this study could be an indication of changing epidemiology of carbapenemases. The carbapenemase OXA-48 like are implicated as a significant cause for silent spread and outbreak in hospital. Alarmingly high prevalence of these enzymes poses a definitive threat for antimicrobial chemotherapy. There is an urgent need for a rigorous antimicrobial policy to prevent emergence of carbapenem resistant strains. Formulating a robust hospital infection control policy and implementing it to prevent the spread of CRE is the most effective way to control the storm caused by these multidrug resistant organisms.

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