ORIGINAL ARTICLE

Characterisation of carbapenemase-producing Gram-negative bacilli among clinical isolates in a tertiary care centre in Kerala, South India

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ABSTRACT

Background: Infections caused by carbapenem-resistant Gram-negative bacteria are a cause for concern due to the limited choice of antibiotics available for their treatment. **Aims, Settings and Design:** This study screened multidrug-resistant (MDR) Gram-negative bacilli isolated from clinical samples over a period of one year, for carbapenem resistance and characterised them using phenotypic methods such as combined disc diffusion test (CDDT), modified Hodge test (MHT), E-test for metallo-beta-lactamase (MBL) and molecular method, PCR. **Materials and Methods:** Two hundred and ten MDR Gram-negative bacilli were screened for carbapenem resistance using Imipenem and Meropenem disc diffusion. These were further checked for carbapenemase production by CDDT, MHT and E-test for MBL. Those positive by E-test were subjected to PCR. Uniplex PCR for New Delhi metallo-beta-lactamase-1 was used for *Escherichia coli* and *Klebsiella pneumoniae*, and multiplex PCR for Imipenemase and Verona imipenemase was used for *Pseudomonas aeruginosa* and *Acinetobacter baumannii* isolates. **Results:** Twenty-three (11%) isolates were found to be carbapenem-resistant and included *E. coli* (six) *K. pneumoniae* (three), *P. aeruginosa* (five) and *A. baumannii* (nine). Seventeen (74%) isolates were positive by phenotypic methods and were subjected to PCR. Out of eight Enterobacteriaceae isolates subjected to PCR, all were positive for bla_{NDM} gene. All were negative for bla_{KPC} gene. All five *A. baumannii* isolates subjected to PCR were found to contain bla_{VIM} gene. Two out of four *P. aeruginosa* isolates were positive for bla_{IMP}, one was positive for bla_{VIM} gene. One *P. aeruginosa* isolate was positive for both bla_{IMP} and bla_{VIM} gene. **Conclusions:** In view of the increasing resistance of Gram-negative bacilli to carbapenems, rational use of antibiotics needs to be emphasised.

Key words: Carbapenemases, Imipenemase, *Klebsiella pneumoniae* carbapenemase, New Delhi metallo beta-lactamase-1, Verona imipenemase

INTRODUCTION

Carbapenems are β-lactam antibiotics with the broadest spectrum of activity and are often the most appropriate agents in the treatment of infections caused by multidrug-resistant (MDR) strains of Gram-negative bacteria. The emergence and spread of acquired carbapenem resistance are, therefore, a major concern and have been dubbed a 'global sentinel event'.[1] Although the first carbapenemases identified were chromosomally encoded, the newer ones are seen on mobile genes on plasmids and integrons, resulting in more rapid interspecies dispersion.

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The strong carbapenem-hydrolysing β-lactamases include (1) metallo-β-lactamases (MBL) that belong to molecular class B and encoded mainly by alleles of bla_{IMI}, bla_{VIM}, bla_{SPM} or bla_{GIM}, (2) carbapenem-hydrolysing class D β-lactamases encoded by various bla_{OXA} alleles and (3) class A, plasmid encoded *Klebsiella pneumoniae*

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carbapenemase (KPC)-hydrolysing β-lactamase enzymes encoded by bla_{KPC} alleles and chromosomally encoded NMC/IMI, SME.[2] Carbapenem resistance may also be due to AmpC enzymes or loss of outer membrane proteins.

Infections by extended-spectrum beta-lactamases (ESBLs) producing Enterobacteriaceae have become a serious problem in India. Various authors have reported the prevalence of ESBLs to be in the range of 40%–90% in various hospitals,[3] especially among *K. pneumoniae* and *Escherichia coli*. The high incidence of ESBL has resulted in an increase in the usage of carbapenems, which has led to the emergence of carbapenem resistance among Gram-negative isolates. This is evidenced by the fact that the SENTRY surveillance study found no resistance to carbapenems in 2006, whereas in 2007, the study first reported five *K. pneumoniae* and four *E. coli* isolates from India that produced MBL and were resistant to carbapenems.[4]

KPCs, first identified in 2000 in the USA,[5] have been identified in many countries and need investigation. A new MBL, designated New Delhi metallo-beta-lactamase-1 (NDM-1), originating from New Delhi, has been reported in *K. pneumoniae*.[6] In 2010, Kumarasamy *et al*. reported NDM-1 among UK patients, many of whom had a history of travel to India or Pakistan within the past year.[7] In India and especially from Kerala, not much is known about the prevalence of carbapenem-resistant Gram-negative bacteria. In the present study, we screened MDR Gram-negative bacterial isolates from clinically significant cases for carbapenem resistance, to assess the prevalence of carbapenem resistance and to characterise carbapenemase enzymes.

MATERIALS AND METHODS

A prospective study was conducted in the Department of Microbiology, Government Medical College, Thrissur, from September 2011 to August 2012. A sample size between 150 and 200 was calculated anticipating a prevalence rate of 40% based on previous studies[8,9] to detect prevalence with 2% precision. The isolates included non-repetitive MDR Gram-negative bacteria recovered from different clinical specimens during the period of study. Biochemical identification of the isolates was done as per standard recommended procedures.[10] Those isolates which were found to be carbapenem-resistant phenotypically underwent molecular characterisation at Prof. Benjamin M Pulimood Laboratories for Infection, Immunity and Inflammation, Department of Medicine Unit I and Infectious Diseases, Christian Medical College, Vellore.

Antimicrobial susceptibility testing

Antimicrobial susceptibility of the isolates was performed using Kirby–Bauer disc diffusion using Mueller-Hinton agar and results interpreted as per Clinical and Laboratory Standards Institute (CLSI)[11] guidelines. For Enterobacteriaceae isolates, the antibiotics tested were as follows: Ampicillin (10 μg), Cephalexin (30 μg), Cefotaxime (30 μg), Co-trimoxazole (1.25/23.75 μg), Ciprofloxacin (5 μg), Gentamicin (10 μg) and Amikacin (30 μg). For non-fermenters, the antibiotic panel included Piperacillin (100 μg), Ceftazidime (30 μg), Ciprofloxacin (5 μg), Gentamicin (10 μg) and Amikacin (30 μg) (Hi-Media Laboratories Private Limited, Mumbai). *E. coli* ATCC 25922 and *Pseudomonas aeruginosa* ATCC 27853 were used for quality control. Those which were resistant to three or more classes of antibiotics were designated as MDR and were further evaluated for carbapenem resistance.

Screening for carbapenem resistance

This was done using Kirby–Bauer disc diffusion method using Meropenem (10 μg) and Imipenem (10 μg) discs as per 2012 CLSI guidelines.[11] Isolates which were resistant to Meropenem and Imipenem by disc diffusion (zone size ≤19 mm) were further checked for the production of MBL using combined disk diffusion test[12] and modified Hodge test (MHT).[11]

Phenotypic screening for metallo-β-lactamases

Combined disc diffusion test

Two Imipenem discs (10 μg), one containing 10 μl of 0.5 M (292 μg) anhydrous ethylenediaminetetraacetic acid (EDTA; Sigma Chemicals, St. Louis, MO, USA), were placed 25 mm apart (centre–centre). An increase in zone diameter of 7 mm or more around the Imipenem-EDTA disc compared to that of the Imipenem disc alone was considered positive for an MBL.[13] The test was done with Meropenem and Meropenem-EDTA also.

Modified Hodge test

In MHT, an Imipenem disc was placed on a Mueller–Hinton agar plate inoculated with 0.5 McFarland turbidity *E. coli* ATCC 25922. The test strain was then streaked radially from the edge of the disc to the periphery of the plate. After overnight incubation, the presence of a distorted inhibition zone indicated the carbapenem-hydrolysing activity of the test strain.[11]

Phenotypic confirmation of metallo-beta-lactamase

E-test

Here, an E-test strip containing Imipenem and Imipenem + EDTA was used. A reduction in MIC of Imipenem of three or more two-fold dilutions in the presence of EDTA was interpreted as a positive test

indicative of MBL production and further subjected to polymerase chain reaction (PCR) for the presence of genes, NDM-1, KPC, Verona imipenemase (VIM) and Imipenemase (IMP).

Polymerase chain reaction

The DNA template was prepared from fresh subculture of organism suspended in 500 µl saline, lysed by heating at 100°C for 10 min and centrifugation at 8000 rpm for 5 min to remove cellular debris.[13] Uniplex PCR amplification for detection of genes was carried out on Thermal cycler 9700 instrument (Applied Biosystems, Norwalk, USA). For Enterobacteriaceae isolates, NDM-1 and KPC PCRs were done.[14] For non-fermenters, multiplex PCR for VIM and IMP was done.[14] Quality control strains for PCR were NDM-1 positive 2ADB65, *K. pneumoniae* NCTC13438 (KPC positive), *P. aeruginosa* C7 (IMP7) and C-10 (VIM2).

RESULTS

Two hundred and ten MDR Gram-negative bacterial isolates were included in the study. The isolates were obtained from different specimens such as blood (n = 7), urine (n = 108), pus aspirates (n = 28), swabs (n = 35), sputum (n = 23) and body fluids (n = 9). Antibiotic susceptibility pattern of the 210 MDR isolates is given in Table 1.

Out of 210 MDR Gram-negative bacilli screened for carbapenem resistance, 23 (11%) were found resistant to Meropenem and Imipenem by disc diffusion. These isolates included *E. coli* (six), *K. pneumoniae* (three), *P. aeruginosa* (five) and *Acinetobacter baumannii* (nine). These isolates were obtained from patients admitted to various Departments in the Hospital including general medicine (five), pulmonary medicine (three), general surgery (five), burns ward (two), urology (two) and nephrology (two) wards. One isolate each was obtained from newborn Intensive Care Unit, neurosurgery, orthopaedics and radiotherapy units. The proportion of carbapenem resistant isolates among the various clinical specimens is depicted in Figure 1.

Out of the 23 carbapenem-resistant isolates, 17 (74%) showed an increase in zone size by the combined disc diffusion test, suggestive of MBL mediated carbapenem resistance. These were further confirmed by the E-test. MHT was found to be negative in our isolates. All 17 isolates were subjected to PCR and found to contain at least one carbapenemase gene. Eight Enterobacteriaceae isolates were subjected to NDM and KPC PCRs. All Enterobacteriaceae isolates were bla_{NDM} positive [Figure 2] and were negative for the bla_{KPC} gene. *P. aeruginosa* and *A. baumannii* isolates found to be positive for MBL were subjected to PCR for bla_{IMP} and bla_{VIM}. Out of four *P. aeurginosa* isolates, two were positive for bla_{VIM} and one was positive for bla_{IMP}. One *P. aeurginosa* isolate was positive for both bla_{IMP} and bla_{VIM} [Figure 3]. All five *A. baumannii* isolates were positive for bla_{VIM} [Figure 4]. The phenotypic and genotypic characterisation results are summarised in Table 2. The six isolates which were not positive for MBL could not be further characterised.

DISCUSSION

The present study was aimed at identifying carbapenem resistance in Gram-negative bacterial isolates from clinical samples received at the hospital laboratory. We were able to isolate carbapenem-resistant isolates from patients admitted in different units of the hospital, showing the widespread distribution of carbapenem resistance among our hospitalised patients. Our study was done only on MDR Gram-negative isolates and the prevalence was 11%. The

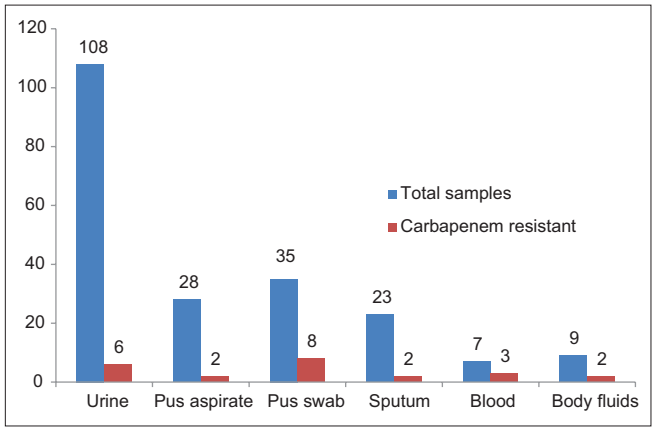

Figure 1: Distribution of carbapenem-resistant isolates among various clinical samples

Table 1: Antibiotic susceptibility pattern of multidrug resistant isolates

Antibiotic tested	*Escherichia coli* (n=80)	*Klebsiella pneumoniae* (n=68)	*Pseudomonas aeruginosa* (n=39)	*Acinetobacter baumannii* (n=23)	Total (n=210), sensitive (%)
Ampicillin	0	0	0	0	0
Cephalexin	0	0	0	0	0
Third generation cephalosporin	2	1	1	0	4 (2)
Ciprofloxacin	7	3	1	0	11 (5)
Gentamicin	10	6	8	4	28 (13)
Amikacin	64	45	18	7	134 (64)
Carbapenem	6	3	5	9	23 (89)

Table 2: Results of phenotypic and genotypic characterisation of carbapenem-resistant isolates

Total MDR isolates	Imipenem resistant by disc diffusion (*n*=23)	MBL by EDTA, *n*=17 (74%)	MBL by E-test (*n*=17)	PCR positive
210	*Escherichia coli* (6)	6	6	6 (NDM)
	Klebsiella pneumonia (3)	2	2	2 (NDM)
	Pseudomonas sp. (5)	4	4	4 (2 IMP, 2 VIM)
	Acinetobacter sp. (9)	5	5	5 (VIM)
	Total 23 (11%)	17	17	17

MDR: Multidrug resistant; MBL: Metallo-beta-lactamase; EDTA: Ethylenediaminetetraacetic acid; PCR: Polymerase chain reaction; NDM: New Delhi metallo-beta-lactamase; IMP: Imipenemase; VIM: Verona imipenemase

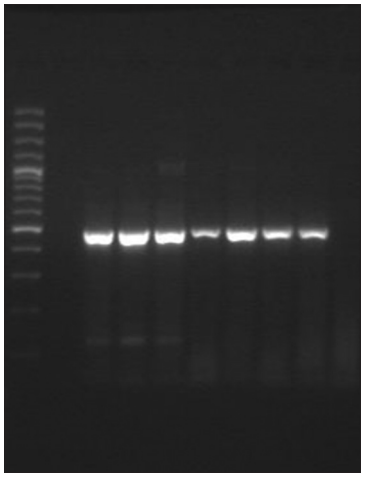

Figure 2: Uniplex polymerase chain reaction for New Delhi metallo beta-lactamase-1 detection Lane 1: 100 bp ladder, Lanes 2 and 10: Negative controls, Lane 3: Positive control, Lanes 4–9: Positive Enterobacteriaceae isolates. New Delhi metallo beta-lactamase-1 base pair size 475 bp

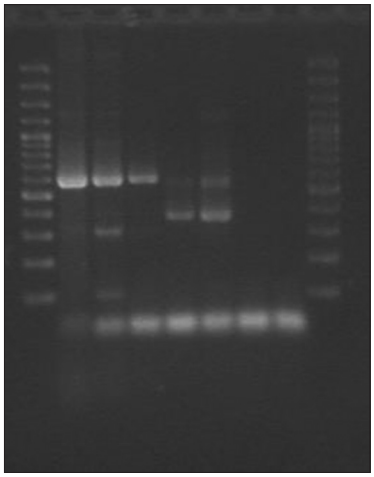

Figure 3: Multiplex polymerase chain reaction for Imipenemase, Verona imipenemase in *Pseudomonas* Lanes 1 and 9: 100 bp ladder, Lane 2: Positive control, Lanes 3–5: Isolates positive for imipenemase, Lane 5: Verona imipenemase positive isolate, Lane 6: Imipenemase and Verona imipenemase positive isolate, Lane 7: Negative, Lane 8: Milli-Q (negative). Imipenemase base pair size - 587 bp; Verona imipenemase base pair size - 382 bp

prevalence may be different if all Gram-negative bacteria including the sensitive ones were assessed. MDR isolates were selected since identification of carbapenem resistance among them would be more significant clinically both for treatment purposes and for infection control.

Molecular characterisation of isolates revealed different genotypes including NDM-1, VIM and IMP. The prevalence rate of NDM-1 carbapenemase in this study is similar to that in other studies such as that of Deshpande *et al.* from Mumbai[15] and Nagaraj *et al.* from Bangalore.[16] In their studies, NDM was detected mainly from Enterobacteriaceae and those were primarily from urine samples. In the present study, all nine Enterobacteriaceae isolates subjected to PCR were found to be NDM-positive. The study by Nagaraj *et al.* found 27 out of 36 *K. pneumoniae* isolates (75%) and 10 out of 15 *E. coli* isolates (66%) to be NDM-positive by PCR. The study could not detect any KPCs among carbapenem-resistant isolates. KPCs could not be identified in the present study also. Dwivedi *et al.*, in 2009, reported 12 carbapenem-resistant Enterobacteriaceae isolates harbouring metallo-β-lactamases (5 IMP, 4 VIM, 2 IMP and SIM and 1 VIM and SIM).[17]

A study by Solanki *et al.*, in 2014, from Hyderabad, showed bla_{NDM} to be the most common gene (59/100), followed by bla_{KPC} (15/100) and least frequently bla_{VIM} (6/100). Their study could not detect bla_{IMP} in any isolates.[18] This is slightly different from our study which could not detect KPCs but had two *P. aeruginosa* isolates positive for bla_{IMP} gene. MHT is found to perform better for detection of KPCs and not for other carbapenemases and could explain the negative result for MHT in our isolates. Further, the six isolates that were carbapenem-resistant but negative by molecular tests may probably have other carbapenemases such as OXA, SPM or may be carbapenem-resistant as a result of efflux changes due to AmpC production or porin loss.

When the study was carried out, carbapenems had not yet been included in the routine antibiotic testing panel in our laboratory. The identification of carbapenem resistance has resulted in their inclusion in the testing panel. This study was meant as a pilot study to identify the presence of carbapenemases in our centre. Since the sample size

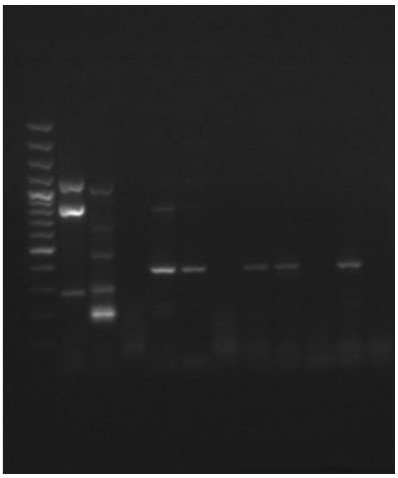

Figure 4: Multiplex polymerase chain reaction for Imipenemase, Verona imipenemase in *Acinetobacter* sp. Lane 1: Blank, Lane 2: Ladder of 100 bp, Lane 3: Imipenemase 1, Lane 4: Positive control for Imipenemase, Lanes 6–12: Verona imipenemase positive isolates of *Acinetobacter*, Lanes 5, 8, 11 and 13: MilliQ (negative). Imipenemase - 188 bp, Verona imipenemase - 390 bp size

is small and the isolates are from hospitalised patients, a clonal pattern cannot be ruled out.

CONCLUSIONS

The present study reveals the emergence of carbapenem resistance among hospitalised patients in a tertiary care teaching centre in Kerala. The diverse mechanisms of carbapenem resistance, including genes for NDM, VIM and IMP, identified among these isolates, are a major cause for concern. This highlights the need for a proactive approach by both clinicians and microbiologists to control these resistant organisms in the long run.

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Conflicts of interest

There are no conflicts of interest.

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