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Research Article

Phenotypic and genotypic detection of carbapenem-resistant Gram-negative bacteria from different clinical samples in a tertiary care hospital.

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Abstract

Carbapenems represent the sole class of beta-lactam antibiotics that remain effective against pathogens producing extended-spectrum beta-lactamases (ESBLs). This critical efficacy has led to an increasing reliance on carbapenems, not only for treating identified infections but also for providing empirical therapy to patients in intensive care units (ICUs) who acquire infections. Consequently, the frequent use of these medications has created a significant selective pressure within the ICUs of tertiary care hospitals, paving the way for the emergence of carbapenem-resistant strains. Infections caused by these resistant organisms are alarmingly linked to high rates of morbidity and mortality, underscoring the urgency of addressing this growing challenge in clinical settings.

Objectives: To identify carbapenemase-producing Gram-negative bacteria (GNB) using phenotypic methods and to confirm the presence of resistant genes via real-time polymerase chain reaction (PCR).

Materials & Methods: A total of 2,890 clinical samples were processed, yielding 1,293 instances of growth of Gram-negative bacilli. From these, 220 carbapenem-resistant strains were isolated and identified across diverse clinical specimens, including blood, urine, pus, endotracheal secretions, cerebrospinal fluid, body fluids, stool, and sputum. Of the 1293 GNB, 220 organisms exhibited resistance to carbapenem antibiotics, including imipenem and meropenem, as determined by using the Kirby-Bauer disc diffusion method. Combined Disc test(CDT) & Etest for carbapenemase production and subjected to multiplex PCR for detection of resistant gene, i.e., bla NDM-1, bla VIM, bla IMP and bla KPC. Follow-up of all the cases till the end of hospital stay was done, and the mortality rate was calculated.

Results: out of 1293 gram-negative isolates, 220 (17.0%) were resistant to carbapenems by both tested methods. Carbapenemase detection using the RAPIDEC®CARBA NP test indicated that 207 (94.0%) were carbapenemase producers, of which 189)91.2%(were MBL producers. The most common carbapenemase genes identified were New Delhi metallo-β-lactamase)NDM; 47.3%(, followed by the co-existence of genes in combination of NDM, with Verona integron-mediated metallo-β-lactamase)VIM; 39.6%(, VIM and oxacillin hydrolyzing enzymes-48)OXA-48; 4.3%(, and OXA 48)1.4%(.No gene encoding an active imipenem, Klebsiella pneumonia carbapenemase, VIM, or OXA-48 was detected.

Conclusions: This study emphasises the urgent need for routine carbapenem resistance testing in multidrug-resistant Gram-negative bacteria (GNBs), particularly within hospital environments where the incidence of these challenging infections is rising. The presence of highly antibiotic-resistant genes in these pathogens presents a significant threat, as they may not only persist in the hospital setting but also transfer to other bacterial species. This potential horizontal gene transfer could lead to a wider dissemination of resistance, making previously treatable infections more difficult to manage. Such a scenario underscores the critical importance of proactive measures in monitoring and controlling antibiotic resistance to safeguard public health and ensure effective treatment options remain available.

Introduction

Infections stemming from bacterial strains that exhibit resistance to antimicrobial agents are increasingly recognized as a formidable challenge within the healthcare landscape. The phenomenon of antimicrobial resistance poses significant risks, leading to adverse outcomes for both healthcare institutions and patients alike. These detrimental effects encompass heightened mortality rates, extended durations of hospitalization, and an escalation in medical costs. Additionally, delays in administering effective treatment protocols further exacerbate the issue of antimicrobial resistance, culminating in a cycle that jeopardizes patient recovery and strains healthcare resources. [1]

Carbapenem drugs represent a critical class of antibiotics in the fight against multidrug-resistant Gram-negative bacteria (MDR-GNB) infections, having been a cornerstone of treatment for over a decade. However, the emergence of carbapenem-resistant organisms poses a serious threat to public health, leading to increased morbidity and mortality. The carbapenems, which include key agents such as meropenem, ertapenem, and imipenem, are a subset of β-lactam antibiotics characterized by their distinctive β-lactam ring structure. This structural feature contributes to their broad antimicrobial spectrum and remarkable efficacy.

These powerful antibiotics are often reserved as the last line of defense for managing complex infections caused by MDR-GNBs, which are notorious for their ability to evade conventional treatments. Additionally, they are effective against bacteria that produce extended-spectrum β-lactamases (ESBLs), enzymes that facilitate antibiotic resistance. Laboratories routinely screen for carbapenem resistance, an important step in the management of infections caused by these formidable pathogens. The development of resistance can occur through genetic mechanisms, such as gene transfer between bacteria or spontaneous mutations, complicating treatment options and underscoring the urgent need for continuous monitoring and innovative therapeutic strategies. [2]

Carbapenemases are enzymes that hydrolyze carbapenems, undermining their effectiveness against bacterial infections. These enzymes contribute to resistance not only to beta-lactam antibiotics but also to other classes like fluoroquinolones and aminoglycosides. The genes encoding carbapenemases are typically located on mobile genetic elements called plasmids, which can easily transfer between microorganisms. This transfer facilitates the rapid spread of multidrug resistance, posing significant challenges for effective infection treatment. [3]

This study aimed to detect carbapenem-resistant and carbapenemase-producing Enterobacterales (CRE) in various clinical samples received in the department of microbiology from patients attending outpatient departments (OPDs) and admitted to wards and intensive care units (ICUs) at a tertiary care hospital.

Materials & Methods

This was an observational study carried out in the department of Microbiology, Index Medical College, Hospital & Research Centre, Indore, M.P., over a period of 2 years from 2023 after approval from the ethical committee.

During the study period, A total of 2,890 clinical samples were processed, yielding 1,293 instances of Gram-negative bacilli growth. From these, 220 carbapenem-resistant strains were

isolated and identified across diverse clinical specimens, including blood, urine, pus, endotracheal secretions, cerebrospinal fluid, body fluids, stool, and sputum. Of the 1293 GNB, 220 organisms exhibited resistance to carbapenem antibiotics, including imipenem and meropenem, as determined by using the Kirby-Bauer disc diffusion method.

The results were interpreted according to the Clinical Laboratory Standards Institute (CLSI) guidelines. [4] The organisms that were resistant to imipenem and meropenem were further processed to determine their MICs using the VITEK®2 compact system (bioMérieux, France). [2]

Screening of carbapenem production: Modified carbapenem inactivation methods (mCIM) were performed to detect carbapenemase production in all isolated Enterobacteriaceae. [5]

Phenotypic methods used for confirmation of MBL production

1. **Double Disk Synergy Test (DDST):** This test was performed with an overnight broth culture of the test strain inoculated on the MHA plate and allowed to dry. 10 µl of the 0.5 M EDTA solution was added to a 6-mm blank filter paper disk (Whatman filter paper, no. 1), which contained approximately 750 µg of EDTA. A 10 µg imipenem disk was placed in the centre of the plate, flanked by an EDTA disk at a distance of 20 mm centre-to-centre from a blank disc containing 10 µl of 0.5 M EDTA (750 µg). After overnight incubation, the presence of an enlarged zone of inhibition towards the EDTA disk was interpreted as positive for an MBL producer. [6]
2. **E-Test:** E-test Metallo-beta-lactamase strips consisted of a double-sided seven dilution range of imipenem IP (4 to 256µg/ml) and IP (1 to 64µg/ml) overlaid with a constant gradient of EDTA. Individual colonies were picked from overnight agar plates and suspended in 0.85% saline to a turbidity of 0.5 McFarland's standard. A sterile cotton swab was dipped into the suspension of the inoculum, and a lawn culture of the inoculum was performed on an MHA plate. The excess moisture was allowed to be absorbed for about 15 min before the E-test MBL (AB bioMerieux) strip was applied. Plates were incubated for 16 to 18 h at 37°C. The MIC endpoints were read where the

inhibition ellipses intersected the strip. A reduction of imipenem MIC=3 by two-fold in the presence of EDTA will be interpreted as suggesting MBL production. [7]

Phenotypic detection of CRE by CARBA NP test: The NG-Test CARBA 5 (Hi-media) is an in vitro, rapid, and visual multiplex immunochromatographic assay for the qualitative detection and differentiation of five common carbapenemases (KPC, OXA-48-like, VIM, IMP, and NDM) from carbapenem non-susceptible pure bacterial colonies. It is an in vitro diagnostic assay for professional use only, aiding in the rapid identification and control of infections by detecting carbapenemase-producing Enterobacteriaceae (including *Escherichia coli* and *Klebsiella pneumoniae*) and *Pseudomonas aeruginosa* in healthcare settings.[8]

Detection of carbapenemase genes by using the multiplex real-time RT- PCR assay

The overnight bacterial culture was used for DNA extraction using the Hi Per Bacterial Genomic DNA Extraction Kit & High-Media Specific sections of the genes encoding the carbapenemase enzyme were detected using a Hi-PCR carbapenemase Gene (multiplex) probe-based kit. It is an uncomplicated, easy, fast, and powerful technique for detecting genes such as blaVIM, blaNDM, blaKPC, *bla*OXA-48, and *bla*IMP within a large and diverse bacterial pathogen. This kit identifies single and multiple carbapenemase genes in a single tube reaction without compromising the sensitivity and specificity.

The cycling method consisted of initial denaturation at 95°C for 10 minutes, followed by denaturation at 95°C for 5 seconds, and 45 cycles of annealing and extension at 60°C for one minute, and the final holding stage was carried out in Quant Studio real-time PCR (ThermoScientific). A cycle threshold value of ≤40 and a band was considered positive.

Results

Among 1293 Gram-negative bacilli identified, 220 (17%) by using the disc diffusion methods and VITEK2 methods, they were shown to be resistant to carbapenems.

Out of 220 isolates, 207(94%) showed carbapenemase production by the Carba NP test (colour change after 2 hrs of incubation from red to yellow/orange/thick orange), and 13(5.9%) were negative without colour change (red colour). Among these, 189 (91.2%) were MBL producers

and 18 (8.6%) were non-MBL producers, as determined by the double disk synergy test (DDST) method and E-test methods.

The MBL production in Enterobacter was 100% (9 out of 9), followed by Klebsiella spp. with 94.8% (110 out of 116), Escherichia coli with 91.1% (31 out of 34), and Acinetobacter spp. with 88% (22 out of 25). Among the tested bacteria, the highest minor MBL production was observed in *Pseudomonas spp*., with 60.7% (17 out of 23). However, the incidence of non-MBL is more in *Pseudomonas* at 26%.

Prevalence of carbapenemase organisms in different wards

The highest incidence of CRGNB was isolated from patients from the neonatal and paediatric care unit78(38%), followed by the ICU 57(27%), and the Wards 46 (22%), and the RICU with26(13%).

Geno typic detection of carbapenemase genes

Using real-time PCR, all 207 isolates were screened for carbapenemase genes; 192 (92.7%) of these isolates tested positive for carbapenemase genes, while 15 (7.24%) tested negative. The most common gene found in 98 isolates (47.3%) was NDM, and VIM in combination with NDM in 82 isolates (39.6%), followed by NDM, VIM, OXA-48 in 9 isolates (4.3%) and NDM, OXA-48 in three isolates (1.4%). However, other noted carbapenemase genes such as; IMP and KPC were not detected in this study, whereas, in the case of VIM and OXA-48, other than their co-existence in combination with NDM, they were not detected singly.

Table 1: Prevalence of genes in different isolates

	Genes				
Organisms	NDM	NDM,0XA48	NDM, VIM,0XA 48	NDM, VIM	Grand Total
Acinetobacter	8		3	10	21
E.coli	23			12	35
Enterobacter	2			4	6
Klebsiella	57	1	4	47	109

Pseudomonas	8	2	2	9	21
GrandTotal	98	3	9	82	192

In the 207 carbapenemase-producing isolates, carbapenemase genes were detected in 192 (92.7%() using real-time PCR. The most common genes identified were NDM (47.3%), followed by the co-existence of genes in combination of NDM, with VIM (39.6%), VIM and OXA-48)4.3%(, and OXA-48 (1.5%). No gene of IMP, KPC, VIM, or OXA-48 alone was detected.

Out of 192 samples, the most predominant organism is *Klebsiella spp., with 109(56.8%), and this Klebsiella spp. was isolated more from endotracheal secretion, 27 (24.8%), followed by wound &pus, 26 (23.9%), and blood,* 23(21.1%). The second most common organism is *E. coli* with 35 (18.2%), isolated more from stool samples 11 (31.4%). Among the non-fementers, both *Acinetobacter spp.,* and *Pseudomonas spp.,* with with21(11%)and among which *Acinetobacter spp.,* was isolated more from blood 10 (47.6%) and *pseudomonas aeruginosa* from endotracheal secretions (6 (28.6%) The less common organism is *Enterobacter spp.*, with 6 (3.1%), and it was isolated more from wound pus (50%) (Table 2)

Table 2: Prevalence of carbapenemase genes producing organisms in different clinical samples

	Organisms					
ClinicalSample	*Acinetobacter*	*E coli*	*Enterobacter*	*Klebsiella*	*Pseudomonas*	Grand Total
Blood	10	6	2	23	2	43
CSF	1			1	1	3
Endotrachealsecreation	6	6	1	27	6	46
sputum&Bal		4		19		23
Stool	1	11		3		15
Urine		6		10	2	18

wound&pus	3	2	3	26	10	44
GrandTotal	21	35	6	109	21	192

The antibiotic sensitivity pattern amongst isolates. 76% were resistant to ceftriaxone, followed by 73.5% to ceftazidime, 73% to Amikacin, 68.5% to cefepime, 63.5% to Pipracillin/tazobactam, 66.5% to ciprofloxacin, and 17% to meropenem & imipenem. Colistin and Polymyxin B were found to be 100% sensitive, followed by meropenem & Imipenem 83%, Pipracillin/tazobactam 73(36.5%), cefepime 63(31.5%), ciprofloxacin 33.5%, amikacin 27%, ceftazidime 26.5%, and aztreonam 23.5%. (Table 3)

Table 3 : Antibiotic susceptibility and resistance patterns of all 220 isolates of CRE.

Antibiotics	Resistant	Sensitive
Amikacin	73%	27%
Ceftazidime	73.5%	26.5%
Ceftriaxone	76%	24%
Cefepime	68.5%	31.5%
ciprofloxacin	66.5%	33.5%
Aztreonam	67.5%	23.5%
Meropenem	17%	83%
Imipenem	17%	83%
Pipracillin/Tazobactam	63.5%	36.5%
Colistin	0	100
Polymyxin B	0	100

Discussion:

Carbapenems are considered the primary choice for treating infections caused by extended-spectrum β-lactamases (ESBLs) and multidrug-resistant (MDR) organisms due to their broad-spectrum efficacy and effectiveness against resistant bacteria. However, increasing resistance

to carbapenems has been observed, particularly in healthcare-associated infections (HAIs), which complicates treatment strategies. These resistant infections pose significant challenges for healthcare systems, leading to prolonged hospital stays, increased healthcare costs, and ultimately higher rates of morbidity and mortality among affected patients. As such, the emergence of carbapenem resistance highlights the urgent need for ongoing surveillance, effective infection control measures, and the development of novel antimicrobial agents to combat these formidable pathogens.

In our study the prevalence rate of carbapenem resistance was 17%, which is similar to Vasmi et al and Haji et al. [2,9]

The incidence of prevalence in various parts of India varies from 14-69%. This was mostly owing to nfection control practices, hospital infrastructure, and the number of antibiotics used.[10]

Among the total of 220 isolates studied, an impressive 207, accounting for 94.0%, exhibited phenotypic positivity. This rate closely aligns with the findings reported by Diwakar et al. Furthermore, our results revealed a genotypic positivity rate of 87.2%, which is in close correspondence to the 90.3% genotypically positive rate documented by Garg et al. and Vasmi et al. This consistency reinforces the reliability of our findings in relation to previous research. [11,2].

In our comprehensive study, we observed a genotypically positive rate of 87.2%, which is closely aligned with the 90.3% positivity rate reported by Garg et al. [11] This similarity suggests that our findings are consistent with previous research. However, the notable differences in these rates can likely be attributed to several factors, including geographic variations, the specific testing methodologies employed, and the strains of the organism being examined. These elements highlight the complexity of our results and emphasize the importance of context in interpreting genotypic data.[12]

In this study, the New Delhi metallo-β-lactamase gene emerged as the predominant resistance gene, identified in 47.3% of the isolates. This finding aligns closely with the results reported by Naim et al. [13]. Furthermore, a higher prevalence of resistance genes was observed in Klebsiella spp., which accounted for 56.7% of the cases, while E. coli showed a comparatively lower prevalence at 17.7%. These results underscore the concerning trend of antibiotic resistance among these pathogens.

The rising prevalence of New Delhi metallo-beta-lactamase (NDM) in India is posing significant public health challenges, largely driven by its considerable medical complications and economic implications. In our study, we did not observe the presence of the blaKPC, blaSIM, and blaIMP resistance genes, aligning with the findings reported by Garg et al. This absence underscores the need for ongoing surveillance and intervention strategies to combat the spread of antibiotic resistance in the region.[11]

In this study, the co-existence of the antibiotic resistance genes VIM and NDM in Pseudomonas aeruginosa was found to be 39.6%. In contrast, previous research conducted by Mohanam et al. reported a significantly lower co-existence rate of 14.6%, while Ellappan et al. documented a rate of 17.3% in similar isolates from Southern India. These variations highlight the differing prevalence of these resistant strains in various geographical regions and underscore the importance of ongoing surveillance of antibiotic resistance patterns.

In this study, the co-occurrence of NDM and OXA-48 was observed to be 1.4%. In contrast, a previous study conducted by Garg et al. reported a significantly higher prevalence of 20.0% for the same combination. This indicates a notable discrepancy in the rates of these resistant strains, highlighting potential variations in bacterial resistance patterns across different research settings. Grag et al [11] identified the NDM and OXA-48 co-existance pre-dominantly in E.coli, followed by Klebsiella, and Enterobacter. None of the nonfermenters in his study exhibited OXA-48 gene. Whereas, according to Vatansever et al [16] the co-harboring of OXA-48 and NDM in colistin-resistant Pseudomonas aeruginosa was 88.8%. Males were more likely to be resistant to carbapenem (66.8%), similar to that reported by Esther et al. [17]

The prevalence of New Delhi metallo-β-lactamase (NDM-1) in stool samples was found to be approximately 7.8%. This figure is notably higher than the 3.6% prevalence documented by Pan et al. [18], yet it remains lower than the 18.5% reported by Esther et al. [16]. These varying rates underscore the importance of ongoing surveillance and research into the spread of antibiotic resistance in different populations.

A significant percentage of carbapenem-resistant Gram-negative bacteria (CRGNB) demonstrate resistance to many commonly used antimicrobial agents. Despite this alarming trend, current research indicates that most CRGNB remain susceptible to tigecycline and colistin, which are regarded as the last lines of defence in the treatment of severe infections caused by these resistant organisms. In our evaluation of various clinical samples, we identified that 18 out of the total samples, representing 8.6%, tested positive for phenotypic resistance to

carbapenems. However, it's noteworthy that no resistance genes were detected when employing real-time polymerase chain reaction (PCR) methods for genetic analysis. This discrepancy raises important questions about the mechanisms of resistance and points to the need for further investigations to understand the underlying factors contributing to the phenotypic resistance observed.

The study conducted was a single-center investigation carried out at a tertiary care facility, focusing exclusively on the resistance mechanisms to carbapenems associated with the production of carbapenemases. While this approach provided valuable insights into one specific aspect of antibiotic resistance, it is important to note that the study did not evaluate the role of other contributing mechanisms that may also play a significant part in causing carbapenem resistance. Specifically, factors such as the loss of porins, which can affect the permeability of the bacterial cell membrane, and the activity of efflux pumps, which can actively expel antibiotics from the cell, were not addressed. This omission suggests that a comprehensive understanding of carbapenem resistance may require further exploration into these additional mechanisms to provide a more holistic view of the challenges posed by resistant pathogens.

Conclusion

In conclusion, the rapid global spread of carbapenemase genes is a significant public health concern, driven largely by the increased prevalence of horizontal gene transfer among bacteria. This phenomenon allows resistant genes to move between different bacterial species, complicating efforts to control infections. Currently, there are no new antibiotics available that effectively combat infections caused by carbapenemase-producing organisms, making the situation even more dire.

Given the variability in the incidence of these resistant strains across different regions, the implementation of real-time PCR probe-based detection methods for identifying carbapenemase genes is crucial. Such advanced diagnostic techniques not only facilitate the early detection of these genes but also play a pivotal role in the development of effective infection control protocols. Furthermore, they enable healthcare professionals to promote the judicious use of antibiotics, thereby curbing the potential for further resistance.

The presence of co-existing carbapenemase genes in bacterial populations poses an additional challenge, as it may enhance the overall resistance profile of pathogens, complicating treatment options. Moreover, the high transmissibility of these antibiotic-resistant genes raises the

alarming possibility that they could spread to other bacteria. This not only increases the potential for further dissemination of resistance but also threatens to undermine the effectiveness of existing antibiotic treatments, highlighting the urgent need for comprehensive surveillance and strategic public health interventions to address this escalating crisis.

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