Identification and Characterization of Carbapenem-Resistant Organisms in Ventilator Associated Pneumonia from clinical isolates and direct clinical samples by Molecular Methods

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Abstract:

Background: Globally, ventilator-associated pneumonia (VAP) is most prevalent hospital acquired infection for patients admitted to an intensive care unit (ICU).The estimated attributable mortality of VAP is around 10%, with higher mortality rates in surgical ICU patients and in patients with mid-range severity scores at admission. Carbapenems are considered to be the most effective antibiotics against many multidrug resistant bacteria.Carbapenemases are encoded by the blaNDM, blaOXA, blaVIM, blaIMP, and blaKPC genes, which are located on both the plasmid and the chromosome. This investigation sought to ascertain whether the (Carbapenem resistant organisms) CRO isolated from VAP patients at a significant tertiary care facility in Navi Mumbai, Maharashtra, were carbapenemase producers. **Objectives:** This study aimed for identification and characterization of Carbapenem resistance gene in direct specimen and from clinical isolated organisms by molecular methods. **Methods:** This study was carried in one tertiary health care unit in Navi Mumbai Maharashtra, between Jan 2020 to Dec 2022. Phenotypic detectionwere performedusingantimicrobial susceptibility test (Kirby Bauer method and Modified Hodge test). Carbapenem producers likeblaNDM1, OXA48, OXA181, VIM, IPM, and KPCwere detected using a polymerase chain reaction (PCR) assay. **Result:**121 ET samples were examined, and 100 (82.64%) of them revealed significant bacterial growth. *Acinetobacter* species made up 37 (37%) *Klebsellia* species 21 (21%), *Citrobacter*species 17 (17%), *Enterobacter*species 5 (5%), and *Proteus*species 5 (5%), with the remaining 2 (2%) being other bacteria. The association between the molecular testing results for carbapenem resistance on direct patient samples and clinically isolated samples was examined. For each gene, the Phi value was computed. For early detection, NDM (Phi= 0.766), OXA 48 (Phi=0.854), OXA 181 (Phi=0.858), VIM (Phi=0.581), KPC (Phi=0.492) and IPM (Phi=0) demonstrates the significance and association because all phi values were more than 0.05. We advise using molecular techniques in clinical laboratories.

Introduction:

Ventilator-associated pneumonia (VAP) is one of the most frequent (Intensive Care Unit)ICU-acquired infections. According on the environment and diagnostic criteria, reported incidences range greatly from 5 to 40%. Extended ICU stays and periods of mechanical ventilation are associated with VAP. It is thought that about 10% of VAP-related deaths are directly related, with higher mortality rates among surgical ICU patients and with mid-range severity scores at admission[(1)]. Carbapenems are considered to be the most effective antibiotics against many multidrug resistant bacteria[(2)]. Carbapenem resistant *Enterobacteriaceae* (CRE) bacteria are most common pathogens in VAP and have attracted attention because of their high rates of drug resistance, high rates of morbidity, and high rates of death[(4)]. Carbapenemases are encoded by the blaNDM, blaOXA, blaVIM, blaIMP, and blaKPC genes, which are located on both the plasmid and the chromosome[(3)]. VIM (Verona integron encoded metallo-lactamase), KPC (*K. pneumonia* carbapenemase), IMP (imipenemase), NDM (New Delhi metallo-lactamase), OXA-48 and OXA-181 (carbapenem hydrolysing Class D oxacillinases),

are the most common genes found in patients. These genes are primarily located on plasmids and can spread more easily since they are linked to a number of mobile genomic structures, such as the transposons and insertion sequences. While NDM is more common in India, KPC carbapenemases is more common in the United States[5-7]. NDM, OXA, IMP, VIM and KPC are frequently occurring carbapenemases[8-9].Each class of lactamases cannot be recognized concurrently by a single test. It is challenging, time-consuming, and labor-intensive to detect different classes. Although acknowledged valuable, phenotypic methods such as modified Hodge tests (MHTs) and inhibitor-based assays are not applicable to all carbapenemases. Additionally, manufacturers of carbapenemase use varyingminimum inhibitoryconcentration (MICs) of carbapenem; Moderate carbapenem resistance is commonly seen in *Enterobacteriaceae* [4].

The detection of microbes resistant to carbapenem requires 3 days by conventional procedures viz., Microscopy, culture, biochemical and antibiotic sensitivity testing. Rapid identification of patients harbouring carbapenemases is beneficial since it helps for the early implementation of suitable infection control measures and the beginning of the proper antibiotic medication to stop spread of infection. It is usual practise to use molecular amplification techniques for fast detection of respiratory tract infections. This study sought to ascertain the frequency of the carbapenem resistance bacteria and to assess phenotypic and genotypic approaches for the identification and characterization of the carbapenem resistant isolates. The comparative analysis of carbapenem resistant genes identification in clinical isolate and in direct patient specimens by (polymerase chain reaction)PCR method will be highlighted in this study.

Method:

Study setting: The study was carriedout during a period of two years from Jan 2020 to Dec 2022, in a tertiary care facility in Navi Mumbai, India. This study was approved by the institution's ethics committee. The study was approved by the MGMIHS Institutional Ethics Committee (IEC) (Ref: MGMIHS/RES./02/2020-21/71), and sample collection were done after each participant or their family members provided written informed consent.

Identification and susceptibility testing: The isolates were obtained from endotracheal secretions samples from patients with VAP hospitalised to the hospital's ICU. Organism identification was based on morphological studies, microscopy (Gram Staining), and biochemical tests such as oxidase, catalase, motility, and metabolic processes [10].

Phenotypic testing: Antibiotic discs of HIMEDIA DE738-1PK, DE737-1PK and other antibiotics like Polymyxin, Tigecycline, Aztreonam and Etrapenem were used for the preliminary identification of CRO (Carbapenem Resistant Organisms) in compliance with (Clinical and Laboratory Standard Institute) CLSI M100recommendations. Antibiotic susceptibility was interpreted in line with the guidelines set by the (European Centre for Diseases Control) ECDC and (Centre for Diseases Control)CDC. For additional analysis, isolates were preserved at -80 °C. According to the CLSI's recommendations, the modified Hodge test (MHT) was carried out. Muller Hinton agar was inoculated with standard suspension of E. coli ATCC 25922. At the center of the plate, a disk of 10 g of ertapenem was placed.As control strains, E. coli ATCC 25922 and test isolates were streaked in straight line from the edge of the disk towards end. The appearance of an inhibitory zone that was "cloverleaf shaped" after an overnight incubation period was finally deemed positive [11-12].

Molecular characterization of carbapenemase- encoding genes:

In order to produce high quality DNA, HiPurA® Genomic - DNA Purification kits (Hi-Media®, India) were used to extract the DNA. These kits are primarily based on the enhanced silica gel membrane's reversible nucleic acid binding capabilities and the Miniprep spin columns' speed and adaptability. With one polymerase chain reaction (PCR) for each carbapenemase-encoding genomes in the specimens were extracted and amplified using a panel of primers (EUROFINS GENOMICS INDIA PVT. LTD) with their predicted amplicon sizes as shown in Table 1 Agarose Gel Electrophoresis was used to analyse the PCR results from direct patient specimens and

clinically isolated specimens[13-16]. A 1.5% agarose gel (Promega, USA) of 0.5 g/l Ethidium - Bromide was used to separate the resultant amplicons. The gel was electrophoresed in an electrophoresis machine with 1x TBE buffer at 100 V for 55 minutes. The molecular size marker was of 50 bp ladder. Using a gel documentation system, DNA bands were seen.

Each gene's run gel and associated band sizes were visible using Gel Doc.

Table 1: TA: Annealing temperature, R: Reverse, F: Forward, VIM: Verona integron-encoded metallo-β-lactamase, KPC: Klebsiella pneumoniae carbapenemase, NDM: New Delhi metallo-beta-lactamase, OXA: Oxacillinase, IMP: Imipenemase and their amplicon size.

Primer	5'-3' sequence	Amplicon size (base pair)	TA (°C)
NDM-1 F:	CGA CGA TTG GCC AGC AAA TG	551	62
NDM-1 R:	ACT TGG CCT TGC TGT CCT TG		
KPC F:	ATG TCA CTG TAT CGC CGT CT	893	58
KPC R:	TTT TCA GAG CCT TAC TGC CC		
IMP F:	TTG AAA AGC TTG ATG AAG GCG	615	60
IMP R:	ACC GCC TGC TCT AAT GTA AG		
VIM F:	AGT GGT GAG TAT CCG ACA G	261	52
VIM R:	ATG AAA GTG CGT GGA GAC		
OXA-48 F:	GCG TGT ATT AGC TTA TC	760	55
OXA-48 R:	CGC GGT TCG GTA GTG TGT TT		
OXA-181 F:	ATG CGT GTA TTA GCC TTA TCG	798	55
OXA-181 R:	AAC TAC AAG CGC ATC GAG CA		

Statistical analysis:

All information was kept in Microsoft Excel 2016, version. Excel was also used for statistical analyses and data management. The frequencies, percentages, and correlations represent the descriptive statistics of the data and variables. Version 23 of SPSS. Thesignificance and association was interpreted using phi- values more than 0.05.

Result:

Demographic Profile of VAP Patients: From 100 patient's specimens incorporated in study, 78% were men and 22% were women. A mean age was found to be 53.81 withpatients age ranging from 18 years to >80 years.

Bacterial isolates of VAP Patients: 121 ET samples were examined, and 100 (82.64%) of them revealed noticeable bacterial growth. *Acinetobacter* and *Klebsiella* were the two most prevalent species found in the population, accounting for 37% and 21% respectively followed by *Citrobacter*species (17%) and *Pseudomonas*species (13%). The least

common were *Enterobacter*species, *E. coli* and *Proteus*with 5%, 4% and 3% respectively.

Antimicrobial Susceptibility: Out of 121 isolates 100 (82.6%) tested positive for carbapenem based on phenotypic detection. The majority of the (Carbapenem resistant) CR isolates showed predicted resistance to the majority of beta lactams. Carbapenem (90%) and cephalosporin (85%) resistance werepresent in the majority of the samples. On the other hand, 100% of them were sensitive to Tigecycline and Polymyxin. Modified Hodge test showed (98%) positive results.

Molecular Analysis:

Molecular testing of direct patient samples and clinical isolates were carried out for carbapenem resistance genes and their correlation was studied. The highest percentage of prevalence is seen in NDM1 (75% & 73%) in both direct ET samples and clinical isolates followed by OXA-48 (30% & 37%) and OXA 181 (24% & 31%), (Fig 1 & 2) VIM (4% & 11%) and KPC (1% & 4%) whereas the least percentage of prevalence is seen in IPM (0% & 0%). In this study maximum *Klebseilla* species harboured (5 gene type NDM, OXA 48, OXA 181, VIM, KPC) followed by *Acinetobacter* species (4 gene type NDM, OXA 48, OXA 181, KPC), *Pseudomonas* species&*Citrobacter* species(4 gene type NDM, OXA 48, OXA 181, VIM).

The Phi value for respective genes were calculated. NDM (Phi= 0.766), OXA 48 (Phi=0.854), OXA 181 (Phi=0.858), VIM (Phi=0.581), KPC (Phi=0.492), IPM (Phi=0) shows significance and strong relation since all phi values were more than 0.05. This interprets that there is strong positive relation between both variables.

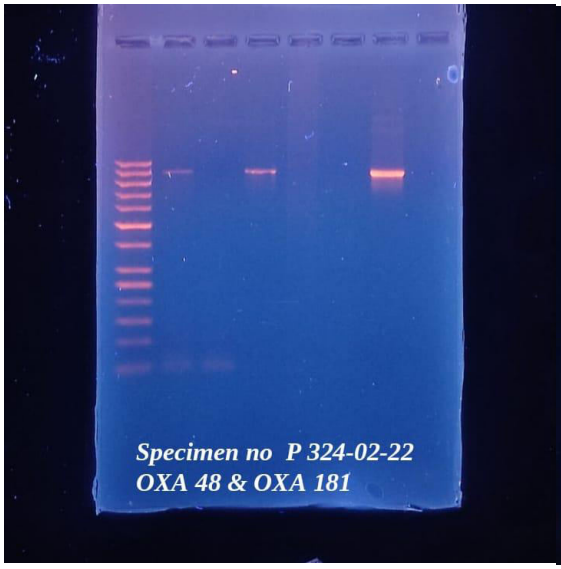

Fig 1. Presence of OXA 48 & 181gene

Fig 2. Presence of NDM gene

Table 2.Descriptive Statistics

Genes	ET Direct sample Percentage (%)	ET Clinical isolate Percentage (%)	Phi value	Significance
NDM1	73	75	0.766	0.000
OXA48	30	37	0.854	0.010
OXA181	24	31	0.858	0.000
VIM	4	11	0.581	0.000
KPC	1	4	0.492	0.000
IPM	0	0	0	0.000

Discussion:

Carbepenem Resistant Organisms has emerged as a key health care pathogen that causes infection during hospitalisation in the current setting. Carbapenems are beta-lactam antibiotics that are used to treat infections caused by bacteria that have developed resistance to various medications, including penicillin and cephalosporins. The majority of CRO isolates tested positive for resistance to the majority of Beta lactams.In our study, we also concentrated mostly on ICU patients. Furthermore, for molecular analysis, carbapenem resistance in direct and cultured samples was compared. The carbapenemase-encoding genes NDM1, OXA 48, OXA 181, VIM, KPC, and IMP were discovered and their prevalence was investigated using PCR. Similarly study done by McCann E and Srinivasan A on ICU patients studies also showed that there is considerably higher carriage of carbapenem-resistant organisms, putting them at a higher risk of infection and transmission [17].

Statistical analysis revealed that NDM1 was the most prevalent in both direct ET samples and cultured ET samples. Same results was reported by Nordmann's study found that NDM and OXA were the most widespread in nations such as India, Vietnam, and China [18].

In a global surveillance survey conducted between 2008 and 2012 by Biedenbach D et al., NDM carbapenemases dominance was also verified in India, Vietnam, and Serbia, among the nine participating nations. Further molecular analysis of the NDM positive isolates from India (n = 71) revealed that 94.3%, 2.8%, and 1.4%, respectively, carried NDM 1, 4, and 6 [19].

In our investigation, direct samples revealed 73% (blaNDM), 30% (blaOXA-48), 24% (blaOXA-181), 4% (blaVIM), 1% (KPC), and 0% (blaIMP), whereas clinical isolates showed 75% (blaNDM), 37% (blaOXA-48), 31% (blaOXA-181), 11% (blaVIM), 4% (KPC), and 0% (blaIMP). Delarampour A et al. examined 110 non-repetitive isolates of *K. pneumoniae*, 75% blaNDM-1 positive. Other resistance genes blaVIM, blaIMP, blaOXA-48, and KPC were not found[20].

Alraddadi BM et al. did a study with 189 patients from which OXA-48 (69.3%) being the most common gene in which*A. baumannii*(98.90%) had the blaOXA-51-like gene, which is unique to *A. baumannii*[21].

The prevalence of carbapenemases generating organisms is a dilemma for both doctors and microbiologists. It is necessary to identify a colonised or infected patient in areas where NDM and OXA 48/181 carbapenemases have become endemic. The increased turnaround time of phenotypic testing and molecular assays addressing focused mechanisms do not resolve the problem. In the current scenario, we cannot choose between phenotypic and molecular assays; nonetheless, a combination of both would aid in the identification of carbapenem resistance.

Much research has already indicated that there is an urgent need for the implementation of stringent prevention strategies, such as improving methods for early detection, identifying patient populations at high risk, changing antibiotic treatment regimens, and implementing hygiene measures to combat the looming threat of increasing antibiotic resistance. It is necessary that CRO screening should be performed on a regular basis in VAP patients since failure will eventually lead to a condition in which all empirical treatment approaches are futile.

Conclusion:

Comparative study of carbapenem resistant genes in direct patient specimen and clinically isolated specimen showed the PCR results to be more significance than traditional microbiological assays.Despite the fact that the genetic method is not always indicative of phenotypic resistances, in some circumstances it may be useful for optimizing therapeutic care, implementing infection control measures, and preventing subsequent transmission. Hence, the clinical isolated organisms by microbiology assays can be considered over direct samples for PCR method for detection of carbapenem resistance.

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