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Carbapenem Resistance in Non-Fermenters: An Overview

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ABSTRACT

This study was conducted with interest in increasing carbapenem resistance in non-fermenters: an important causative agent of nosocomial infection and to standardize the methods for interpretation of their resistance. The aim of this study is to perform disk diffusion testing and minimal inhibitory concentration technique for the identification of carbapenem resistance for imipenem and meropenem. The isolates found resistant to carbapenems were confirmed with the modified Hodge test. The genes responsible for carbapenem resistance were identified by both phenotypic and genotypic methods. Out of 240 non-fermenters isolated 20% showed resistance to carbapenem by disk diffusion. Only 7% showed resistance by the micro broth dilution technique of minimum inhibitory concentration. 3% were panning drug-resistant. Out of 16 carbapenem-resistant isolates, 5 were found to have KPC (*Klebsiella pneumonia* carbapenem) genes, 9 had MBL (Metallo beta-lactamase) genes and 2 had KPC+MBL genes and none were found to have Amp C and OXA-48 genes phenotypically. Genotypically all the KPC strains had KPC genes and out of 9 MBL strains, 6 had VIM and the remaining 3 strains were negative for both IMP and VIM gene. In conclusion, the interpretation of susceptibility for carbapenems should not be made only with disk diffusion testing. Always check for Minimal inhibitory concentration methods and determination of genes responsible for carbapenem resistance, a double-disc synergy test goes in hand with genotypic detection.

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INTRODUCTION

Non-fermenters can cause a variety of infections in humans, which may be either community-acquired or nosocomial acquired. Infections with these pathogens were usually limited to nosocomial settings where carbapenem use is heavy and the patients had compromised immune systems (Thomson, 2010). However, in the last decade, there has been a dramatic increase in resistance to carbapenemases among these organisms (Nordmann and Poirel, 2014).

The problem is being compounded by the lack of readily available alternative drug development for Multi Drug-Resistant (MDR) non-fermenters (Walsh, 2010; Nordmann *et al.*, 2012; Livermore and Woodford, 2006). So, early detection and identification of these organisms is necessary for appropriate antimicrobial therapy for the timely introduction of infection control procedures and to limit the spread of these MDR organisms in hospital settings as well as in community and for epidemiological surveillance.

Hence, the present study was carried out to identify the prevalence of carbapenem resistance of non-fermenters in our setup as well as to determine the methodology to routinely do in the diagnostic laboratory for the detection of carbapenem resistance.

MATERIALS AND METHODS

A cross-sectional study during the period of April 2018 to October 2018 was conducted at Saveetha Medical College and Hospital, Thandalam, Tamil Nadu after getting approval from Human Ethical Committee and Institutional Review Board. Disk diffusion testing and micro broth dilution for minimal inhibitory concentration detection were done according to CLSI (Central laboratory standard institute) 2017 guidelines. A double-disc synergy test was done for phenotypic detection of resistant genes. Uniplex PCR was done for genotypic detection.

Double disk synergy test

Phenotypic methods for detecting carbapenemase activity and the differentiation of KPCs and MBLs was performed (Tsakris *et al.*, 2010; Prakash, 2006). Carbapenemase activity was assessed with the modified Hodge test (MHT) using meropenem disks according to the CLSI guidelines (CLSI, 2012). The phenotypic detection of KPC- and/or MBL-possessing nonfermenter isolates was carried out by a combined disk test with meropenem as a substrate without and with phenylboronic acid (PBA), Ethylene diamine tetraacetic acid (EDTA), or both (Tsakris *et al.*, 2009).

OXA-48 disk test

The test is based on the use of EDTA to permeabilize the bacterial cell and release _-lactamases into the external environment. EDTA was also used to inhibit the production of MBL carbapenemases (Franklin *et al.*, 2006), while a solution of PBA was used to inhibit the production of KPCs (Tsakris *et al.*, 2009). The stock solution of EDTA was prepared by dissolving anhydrous EDTA in distilled water at a concentration of 0.1M (CLSI, 2012). From this solution, 10 μl (containing 292 μg of EDTA) was dispensed onto two blank paper disks. The stock solution of PBA was prepared as previously recommended (Tsakris *et al.*, 2009) by dissolving PBA in dimethyl sulfoxide and water at a concentration of 60 mg/ml. From this solution, 10 μl (containing 600 μg of PBA) was dispensed onto the right of the two disks containing EDT. The disks were then dried and used within 60 min. The surface of a Mueller-Hinton agar plate was inoculated with a lawn of carbapenem-susceptible *E. coli* ATCC 25922 at turbidity of 0.5 McFarland standards. A 10-μg imipenem disk was placed on the inoculated surface of the Mueller-Hinton agar. By touching the tops of well-isolated colonies, 2 to 3 colonies of the tested microorganism were applied to coat the disks containing EDTA and EDTA plus PBA. The inoculated disks were placed with the bacterial inoculum (microorganism) side down on the lawn adjacent to the imipenem disk. The plate was then incubated overnight at 35°C in ambient air. After 18 h of incubation, the plates were examined for either an indentation or a flattening of the zone of inhibition, indicating enzymatic inactivation of imipenem (positive result) for either the disk adjacent to the imipenem or the absence of a distortion of the inhibition halo, indicating no significant inactivation of imipenem (negative result). Indentation of growth toward both EDTA and EDTA/PBA disks indicated the production of OXA-48 carbapenemase. Indentation of growth toward the EDTA disk and an absence of growth toward the EDTA/PBA disks was indicative of KPC or KPC plus MBL production. An absence of growth toward both disks containing EDTA and EDTA/PBA indicated production of MBL carbapenemase or nonsusceptibility to carbapenems due to ESBL/AmpC production plus porin loss.

Genotypic detection

Pure culture of carbapenem-resistant strains was further processed for molecular detection of drug-resistant genes targeting the following sequence (Murugan *et al.*, 2010).

Primer sequence targeting bla $_{KPC}$ gene :

KPC-F: CGGCAGCAGTTTGTTGATTG

KPC-R: CGCTGTGCTTGTCATCCTTG

Primer sequence targeting blaVim gene:

VIM -F: TGTCCGTGATGGTGATGAGT

VIM- R: GTGCTTCCGGGTAGTGTTGT

Primer sequence targeting bla $_{IMP}$ gene (Lari *et al.*, 2015)

IMP-F (5′- GAAGGCGTTTATGTTCATAC-3′)

IMP-R (5′-GTATGTTTCAAGAGTGATGC-3′)

RESULTS AND DISCUSSION

In this study, out of 240 non-fermenters isolated, 49 were found to be resistant to carbapenems by disc diffusion test. Out of which only 16 were confirmed to be resistant by minimum inhibitory concentration (MIC) technique. The disk diffusion susceptibility report is given in Table 1. MIC 50 and 90 are tabulated in Table 2. The antibiotic susceptibility profile

Table 1: Antibiotic susceptibility test of non-fermenters

Group of Antibiotics	Number of isolates Sensitive	Number of isolates Resistant
Aminoglycoside (AK)	201	39
Cephalosporin (CEPH)	117	123
Quinolone (Q)	156	84
Beta-lactam inhibitors (BLI)	183	57
Carbapenem (C)	191	49
	Chi-square=91.965, P<0.001	

Table 2: Minimum inhibitory concentration of non-fermenters for carbapenems

	Total carbapenem-resistant isolates by disc diffusion method = 49	
	MIC 50 (25^{th} no.)	MIC 90 (44^{th} no.)
Imipenem	2	8
Meropenem	1	8

Table 3: The antimicrobial susceptibility profile of 16 carbapenemase producers

Susceptibility profile		No. of isolates
(AK, G, CAZ, CPM, CIP, OF, PIT, IMP, MR)^{R}	(Nil)^{S}	7
(CAZ, CPM, CIP, OF, PIT, IMP, MR)^{R}	(AK, G)^{S}	5
(G, CAZ, CPM, CIP, OF, PIT, MR) ^{R}	(AK, IMP)^{S}	2
(G, CAZ, CPM, CIP, OF, PIT, IMP, MR)^{R}	(AK)^{S}	1
(AK, G, CAZ, CPM, CIP, OF, MR) ^{R}	(PIT, IMP)^{S}	1

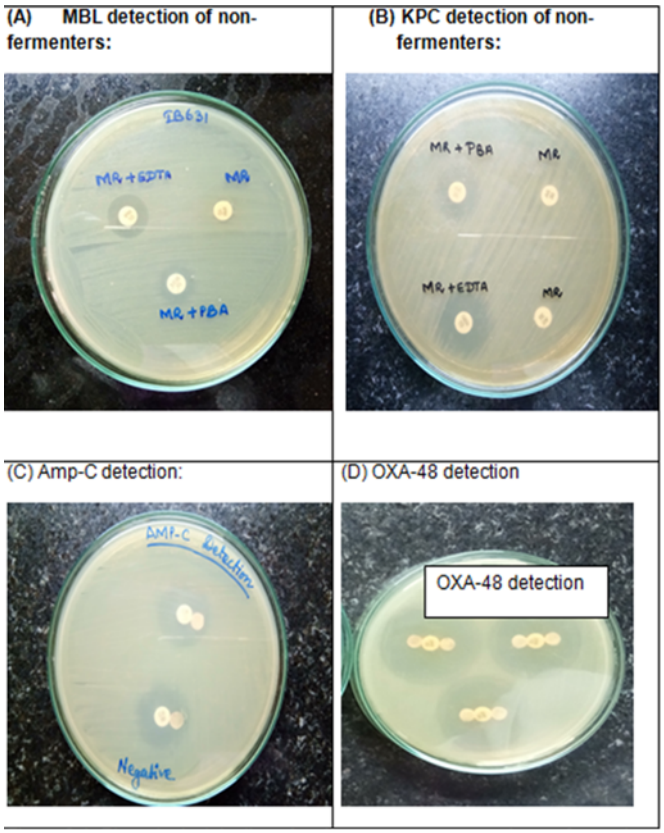

Figure 1: Phenotypicdetection of Carbapenem-resistant gene

Table 4: Comparison of phenotypic and genotypic identification of carbapenem-resistant genes

Organism	Imipenem MIC (μg/mL)	Meropenem MIC (μg/mL)	phenotypic gene detection	Genotypic detection
Pseudomonas aeruginosa	8	4	KPC	KPC
Pseudomonas aeruginosa	4	8	MBL	VIM
Pseudomonas aeruginosa	8	8	MBL	VIM
Pseudomonas aeruginosa	4	8	KPC+MBL	KPC+VIM
Acinetobacter baumannii	4	2	KPC	KPC
Acinetobacter baumannii	8	8	MBL	VIM
Acinetobacter baumannii	8	8	KPC	KPC
Acinetobacter lwoffii	8	8	MBL	VIM
Acinetobacter lwoffii	8	8	MBL	Negative for IMP,VIM and KPC
Acinetobacter lwoffii	8	8	MBL	Negative for IMP, VIM and KPC
Acinetobacter lwoffii	8	16	KPC	KPC
Stenotrophomonas maltophila	4	2	MBL+KPC	VIM+KPC
Achromobacter xylosoxidans	8	4	MBL	VIM
Pseudomonas stutzeri	4	2	KPC	KPC
Pseudomonas putida	8	4	MBL	Negative for IMP, VIM and KPC
Moraxella atlantae	8	8	MBL	VIM

*KPC-Klebsiellspneumonia carbapenemase, MBL- metallo beta lactamase, VIM- Verona integrinencoded metallo beta lactamase, IMP- active on imipenem

Table 5: Comparison of phenotypic andgenotypic identification of carbapenem-resistant genes

Genotypic Identification		Phenotypic Identification		
		KPC Positive (5 isolates)	MBL Positive (9 isolates)	KPC+MBL Positive (2 isolates)
KPC	POSITIVE	5	NA	NA
	NEGATIVE	0	NA	NA
MBL(VIM,IMP)	POSITIVE	NA	6	NA
	NEGATIVE	NA	3	NA
KPC+MBL	POSITIVE	NA	NA	2
	NEGATIVE	NA	NA	0

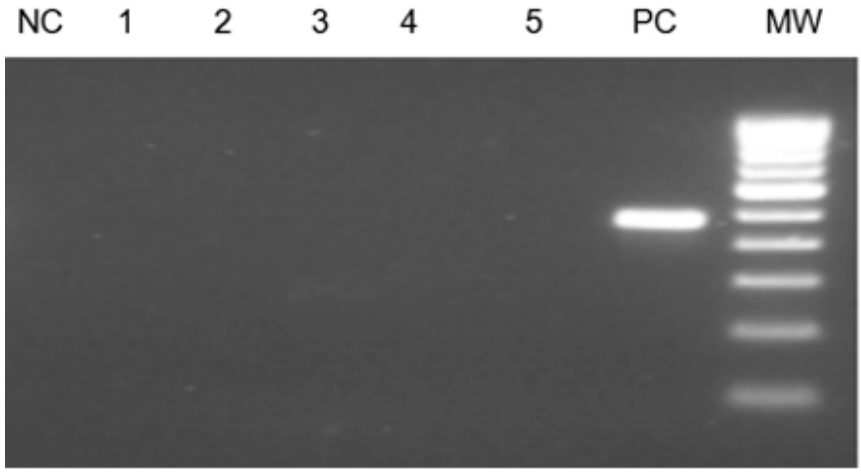

Figure 2: Genotypic detection of IMP gene, Lane 1,2,3,4 and 5 showing strains negative for IMP gene, NC- Negative control, PC-Positive control, MW-DNA ladder

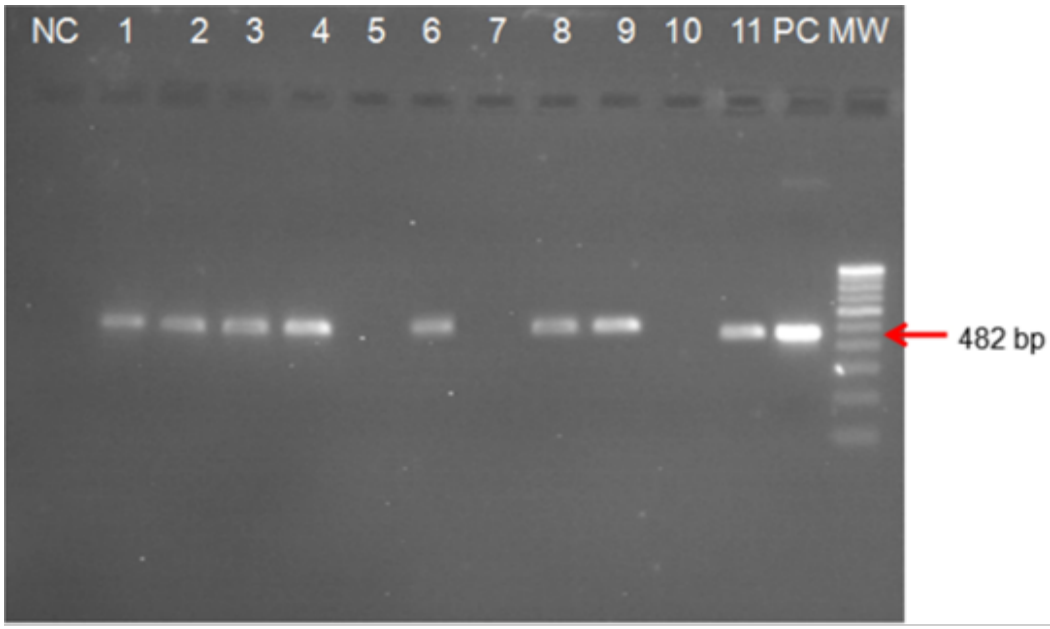

Figure 3: Genotypic detection of VIM gene MW – DNA ladder,
NC=Negative Control (no template),
Lane 1=Pseudomonasaeruginosa strain 1, Lane 2=Pseudomonasaeruginosa strain 2,
Lane 3=Pseudomonasaeruginosa strain 3, Lane 4=Pseudomonasaeruginosa strain 4,
Lane 5=Acinetobacter lwoffii strain 1, Lane 6 =Acinetobacter lwoffii strain 2,
Lane 7=Acinetobacter lwoffii strain 3, Lane 8=Acinetobacter lwoffii strain 4,
Lane 9=Pseudomonas stutzeri, Lane 10=Pseudomonas putida,
Lane 11 =Moraxellaatlantae, PC= PC (VRFPA04) -Positive Control

of 16 carbapenem-resistant strains was tabulated in Table 3.

The correlation between phenotypic and genotypic detection is explained in Figures 1, 2, 3 and 4 and also in Table 4 and Table 5. This explains that 81% correlation was there between both methods.

The present study deals with the analysis of 240 non-repeatable non-fermentative gram-negative bacilli isolated from various clinical samples that came to the clinical microbiology laboratory of Saveetha Medical College and Hospital for the prevalence of carbapenem-resistant of those nosocomial infections causing organisms in our set up. In this study minimum, inhibitory concentration was proved to be the highly sensitive methodology for screening the resistance to imipenem and meropenem. Where carbapenem resistance prevalence rate is only 7%, which can be controlled by proper surveillance, disinfection and

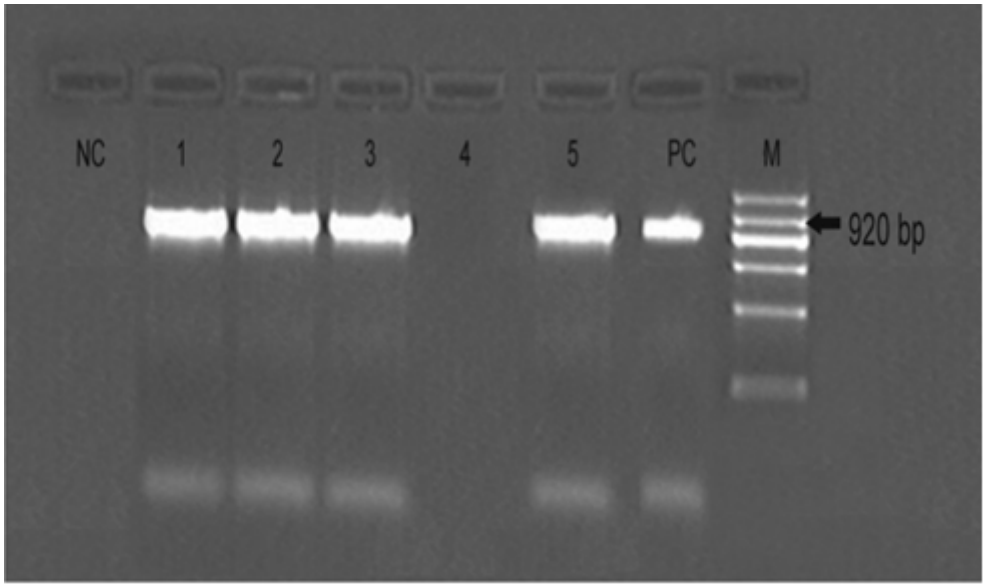

Figure 4: Genotypicdetection of KPC gene Lane 1, 2, 3 and 5 showing positiveKPC genes. Lane 4 was negative for the KPC gene

following proper regimen for patients; moreover, phenotypic methods for gene detection can be done when there is no availability of polymerase chain reaction. Because 81% was well correlated with genotypic detection, the remaining 19% also may be correlated if the study would have identified for NDM (new Delhi Metallo carbapenemase) or GIM (German Imipenamase) or SPM (Sao Paulo Metallo beta-lactamase). As the genotyping is expensive to perform couldn't do for other genes which were not prevalent in this locality. This is a drawback of this study.

Carbapenem resistance

The present study shows the prevalence of Carbapenem resistance among non-fermenting gram-negative bacilli is 7%, which is less when compared to the study done by (Noyal *et al.*, 2009). Which was found to be 14.3% and 15% by Shivesh P *et al* and 10.9% by (Shashikala *et al.*, 2006) respectively. In our study, of the total carbapenem-resistant non-fermenters isolated, 31% were KPC producers, 56% were MBL producers and 13% were both KPC and MBL producers. None were found to be positive for OXA-48. While the study done by (Datta *et al.*, 2012), reported a 5.75% MBL-type Carbapenemase among *Enterobacteriaceae* strains and KPC production among Enterobacteriaceae was found to be 2.51% (13 out of 516). Most of the Indian studies reported carbapenemase production in non-fermenters like *Pseudomonas aeruginosa* and *Acinetobacter baumannii,* where the incidence ranged from 7% to 65%. (Singh *et al.*, 2014). However, very few studies that showed carbapenemase production, including MBL and KPC in *Enterobacteriaceae,* have been conducted in India so far and according to those reports, the occurrence of these enzymes ranged from 1% to 18%. (Deshmukh *et al.*, 2011). The bacteria having MBL has the potential to spread rapidly (horizontal MBL gene transfer) within the hospital environment and also across continents posing both therapeutic and control management problems.

BlaKPC, BlaIMP and Bla IMP detection

Molecular detection was done for the KPC and MBL producing isolates where for three isolates bla$_{IMP}$ and bla$_{VIM}$ gene were not detected. This could be explained by the fact that this isolate show MBL resistance by the presence of genes other than bla$_{IMP}$ and bla$_{VIM,}$ which needs further evaluation. This is contrary to the study done by Fatemeh Fallah et al. (Fallah and Borhan, 2013), where out of 48 MBL producers, all were found to be positive for bla$_{IMP}$ and negative for bla$_{VIM}$.

CONCLUSION

Minimal Inhibitory Concentration technique needs to be done for carbapenem instead of Kirby Bauer disk diffusion assay before reporting resistance to carbapenems. This will help the patients by preventing the treatment with third-line drugs like Colistin, Polymixin B and fosfomycin. Genotyping and phenotyping can go in hand for the detection of resistant genes for research and diagnostic purposes.

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