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Original Article

Microbiology Section

Phenotypic and Molecular Characterisation of Carbapenemases in *Acinetobacter* Species in a Tertiary Care Centre in Tamil Nadu, India

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ABSTRACT

Introduction: *Acinetobacters* are significant nosocomial pathogens involved in outbreaks of infection in intensive care unit. Carbapenem resistance in *Acinetobacter* species is due to a variety of combined mechanisms and is a cause of great concern

Aims: The current study was conducted to determine the antimicrobial susceptibility pattern and prevalence of carbapenemases amongst the *Acinetobacter* species.

Materials and Methods: The study was conducted in a 1850 bedded university teaching hospital between November 2013 to April 2014. One hundred and fifty seven consecutive *Acinetobacter* isolates were subjected to Modified Hodge test & inhibitor potentiated disc diffusion test for screening of carbapenemases & metello-beta-lactamases. Antibiotic susceptibility was performed by Kirby-Bauer's disc diffusion method to detect resistance to various drugs. PCR was performed for detection of genes encoding OXA carbapenemases and metallo-beta-lactamases.

Study Design: Laboratory based cross sectional study.

Results: Out of 157 isolates (151 *A.baumannii* and 6 *A. lwoffii*), 110 (70%) exhibited carbapenem resistance. MIC_{50} to imipenem was 32 µg/mL. Modified Hodge test was positive in 90 (57.3%). Metallo-beta-lactamase screening test was positive in 126 (80.2%). All the 90 MHT positive isolates carried different OXA or the MBL genes. Of 126 MBL screen positive isolates VIM, IMP & NDM encoding genes were detected in 69 isolates. Of the total 157 *Acinetobacter* isolates, $bla_{OXA\ 23}$ was detected in 71, $bla_{OXA\ 24}$ and $bla_{OXA\ 58}$ in 6 each. $Bla_{VIM,}$ $bla_{IMP\ ,}$ bla_{NDM} were detected in 49, 4, 27 *Acinetobacter* isolates respectively.

Conclusion: $Bla_{OXA\ 23}$ was the most common OXA carbapenemase. Bla_{VIM} was the most common metallo-beta-lactamase. Coproduction of OXA and metallo-beta-lactamases (33.7%) is not an uncommon phenomenon. Production of carbapenemase is the most important reason for imipenem resistance in *Acinetobacter* species in our health care setting. Since the screening tests perform poorly, early detection of the drug resistance genes by molecular methods is necessitated.

Keywords: Inhibitor potentiated disc diffusion test, Metallo-beta-lactamases, Modified Hodge test, OXA carbapenemases

INTRODUCTION

Acinetobacters are significant nosocomial pathogens. Surgical-site infections, urinary tract infections, ventilator associated pneumonia & bacteremia are some notable infections caused by them especially in the Intensive Care Unit (ICU) patients [1]. Multi drug resistant and pan-drug resistant phenotypes in *Acinetobacter* species which have been reported over the past 20 years and is of great concern because they exhibit resistance to several classes of antibiotics thereby limiting the therapeutic options [2].

The most common mediator of carbapenem resistance is the production of carbapenem hydrolysing enzyme which include the class B metallo β-lactamase (bla_{VIM}, bla_{IMP} and bla_{NDM}) and class D OXA carbapenemases (bla_{OXA-23} like, bla_{OXA-24} like, bla_{OXA-51} like and bla_{OXA-58} like). Out of these bla_{OXA-51} is chromosomally mediated in *A. baumannii*. Bla_{OXA-24} like is both plasmid and chromosomally mediated. The rest are plasmid mediated [2]. There are non enzymatic mechanisms which operate through the presence of efflux pumps, porin defects & outer membrane protein alteration to bring about carbapenem resistance in *Acinetobacter* species, all of which result in an increase in minimum inhibitory concentration (MIC) to carbapenems [3].

This study was conducted to determine the presence of carbapenemases in *Acinetobacter* species by both phenotypic and genotypic methods.

MATERIALS AND METHODS

Study Isolates

The study was conducted in an 1850 bedded university teaching hospital (Sri Ramachandra Medical College and Research Institute, Chennai) for a period of six months from November 2013 to April 2014. It included 157 clinically significant consecutive, non-duplicate *Acinetobacter* isolates, recovered from various clinical specimens such as respiratory secretions (59), exudative specimens (53), urine (29) and blood (16). Isolates from out-patients, those deemed as colonisers and repetitive isolates from the same patient were excluded from the study. The organisms were identified up to species level by conventional and automated method (Microscan Walk Away 96).

Antimicrobial Susceptibility Testing

Disc diffusion

Susceptibility to various antimicrobial agents – ceftazidime (30µg), amikacin (30 µg), ciprofloxacin (5 µg) piperacillin-tazobactam (100/10 µg), imipenem (10 µg)[Hi-media laboratories, Mumbai, India] was performed by disc diffusion method according to Clinical Laboratory Standard Institute (CLSI) guidelines [4]. Susceptibility to tigecycline (15 µg BBL™ BD, USA) was also performed. Interpretation for susceptibility to tigecycline was done using United States Food and Drug Administration (US-FDA) guidelines [5].

Minimum inhibitory concentration

MIC to imipenem was determined by agar dilution method - the range tested being 0.03 µg/mL to 256 µg/mL in accordance with CLSI guidelines [4].

Phenotypic Tests

All the isolates were subjected to screening test for carbapenemase production by Modified Hodge Test (MHT) using imipenem disc (10 µg) [4] and MBL screening was done using 5 µL Ethylene Diamine Tetra Acetic Acid (EDTA) impregnated ceftazidime (30 µg) and imipenem (10 µg) discs as described previously [6].

Molecular Detection of Genes

A single colony was inoculated into Luria bertini broth, incubated for 20 hours with shaking in between. 1.5 mL of this was centrifuged for 5 min. The pellets were suspended in 500 µL of distilled water and lysed by heating at 95°C for 5 min and centrifuged for 1 min. 5 µL of this extract was used as a template for amplification.

All the isolates were subjected to PCR using consensus primers targeting *bla* $_{VIM}$, *bla* $_{IMP}$ and *bla* $_{NDM}$ [7,8]. Multiplex PCR was performed for the detection of *bla*$_{OXA-23}$ like (*bla*$_{OXA-23}$, *bla*$_{OXA-27}$, *bla*$_{OXA-49}$), *bla*$_{OXA-24}$ like(*bla*$_{OXA-24}$, *bla*$_{OXA-25,}$ *bla*$_{OXA-26}$ *bla*$_{OXA-40}$), *bla*$_{OXA-51}$ like and *bla* $_{OXA-58}$ like gene as described previously [9]. The primers used are shown in the [Table/Fig-1,2]. For optimization of PCR, strains previously confirmed by PCR and gene sequencing were used as positive controls and *Escherichia coli* ATCC 25922 as negative control.

Primers	Primer Sequence 5' - 3'	Product Size
bla$_{OXA-23}$ like-F	GATCGGATTGGAGAACCAGA	501
bla$_{OXA-23}$ like-R	ATTTCTGACCGCATTTCCAT	
bla$_{OXA-51}$ like-F	TAATGCTTTGATCGGCCTTG	353
bla$_{OXA-51}$ like-R	TGGATTGCACTTCATCTTGG	
bla$_{OXA-24}$ like-F	GGTTAGTTGGCCCCCTTAAA	246
bla$_{OXA-24}$ like-R	AGTTGAGCGAAAAGGGGATT	
bla$_{OXA-58}$ like-F	AAGTATTGGGGCTTGTGCTG	599
bla$_{OXA-58}$ like-R	CCCCTCTGCGCTCTACATAC	

[Table/Fig-1]: Primers used for detection of OXA genes

Primers	Primer Sequence 5' - 3'	Product Size
bla$_{VIM}$-F	TTTGGTCGCATATCGCAACG	500
bla$_{VIM}$-R	CCATTCAGCCAGATCGGCAT	
bla$_{IMP}$-F	GTTTATGTTCATACWTCG	432
bla$_{IMP-}$R	GGTTTAAYAAAACAACCAC	
bla$_{NDM-}$F	GGGCAGTCGCTTCCAACGGT	475
bla$_{NDM}$-R	GTAGTGCTCAGTGTCGGCAT	

[Table/Fig-2]: Primers used for detection of metallo-beta-lactamase gene

RESULTS

Of 157 *Acinetobacters* tested, 151 were *A.baumannii &* 6 were *A.lwoffii*. They were distributed among various clinical specimens as follows: Respiratory secretions (59), exudative specimens (53), urine (29), blood (16).

Antimicrobial Susceptibility Profile

Resistance to ceftazidime, piperacillin-tazobactam, ciprofloxacin, amikacin imipenem, and meropenem was 100%, 97.1%, 81%, 80%, 70% and 54% respectively. Among the study isolates 3.2% were resistant to tigecycline.

Of the total 157 isolates 110 (70%) were resistant to imipenem by both disc diffusion & MIC determination. MIC_{50} of imipenem was 32 µg/mL.

Phenotypic Tests

MHT was positive in 90 isolates which included 88 *A.baumannii* and 2 *A.lwoffii*. MBL screen was positive in 126 isolates which included 124 *A.baumannii* and 2 *A.lwoffii.*

Results of PCR

Of the 151 *A. baumannii,* 85 carried one or more OXA carbapenemase encoding genes and in 66 isolates OXA and

MBL genes coexisted. Among *A.lwoffii* five isolates carried one or more of the genes looked for in the study. The distribution of carbapenemase encoding gene both OXA and MBLs among all study isolates is shown in [Table/Fig-3].

Genes	Number of Isolates	
	A.baumannii (151)	*A.lwoffii* (6)
OXA alone	85	2
MBL alone	0	2
MBL with OXA	66	1
None	0	1

[Table/Fig-3]: Distribution of OXA & MBL genes among study isolates

OXA Carbpenemase Encoding Genes

Of the 157 study isolates, OXA carbapenemase encoding genes were detected in 154. All the 4 OXA carbapenemase types were detected either alone (74) or in combination (80). The most common types were OXA 23 which existed in 71 isolates which included 2 *A. lwoffii*. [Table/Fig-4] shows the distribution of OXA genes in the study isolates.

Genes	Number of Isolates (154)	
	A.baumannii	*A.lwoffii*
bla_{OXA23} like alone ne	-	2
bla_{OXA51} like alone	71	-
bla_{OXA58} like alone	-	1
bla_{OXA23} like+ bla_{OXA51}	69	-
bla_{OXA24} like + bla_{OXA51}	6	-
bla_{OXA58} like + bla_{OXA51}	5	-

[Table/Fig-4]: Distribution of *bla* OXA genes among the isolates

MBL Genes

MBL genes were detected in total of 69 isolates. VIM was the most common type followed by NDM. MBL and OXA genes coexisted in 53 isolates. The distribution of MBL genes is shown in [Table/Fig-5].

MBL Genes	No. of Isolates (69)	
	A.baumannii	*A.lwoffii*
VIM alone	37	2
IMP alone	3	-
NDM alone	15	1
VIM+NDM	10	-
IMP+NDM	1	-

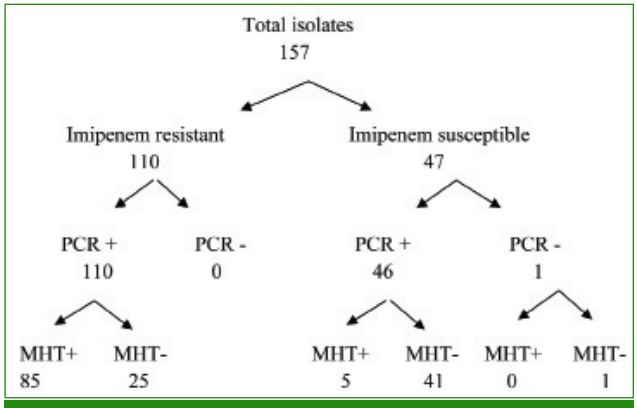
[Table/Fig-5]: Distribution of MBL genes (VIM,NDM,IMP)

Comparison of Imipenem Susceptibility, MHT & PCR

	Ceftazidime-EDTA MBL screen test	Imipenem-EDTA MBL screen test
Sensitivity	68.1%	39%
Specificity	23.8%	35.2%
Positive predictive value	41.2%	46.5%
Negative predictive value	48.8%	57.5%

[Table/Fig-6]: Sensitivity, specificity, PPV, NPV of the MBL screening test

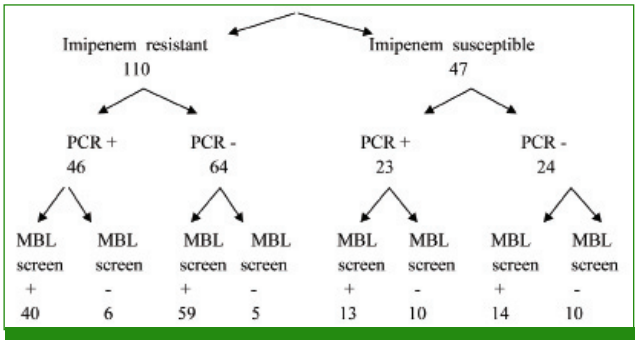
Comparison Of Imipenem Susceptibility, MBL Screen



[Table/Fig-7]: Comparison of imipenem susceptibility, MHT and PCR

Sensitivity and Specificity of Screening Tests

The phenotypic test for the detection of carbapenemase has a low sensitivity and specificity. As far as the MBL screening test is concerned, ceftazidime - EDTA method provided a better sensitivity whereas specificity, positive and negative predictive values were higher with imipenem - EDTA method. [Table/Fig-8] shows the sensitivity, specificity, positive predictive and negative predictive values of the screening tests.



[Table/Fig-8]: Comparison of imipenem susceptibility, PCR and MBL screening

DISCUSSION

The study constituted 157 *Acinetobacter* isolates, of which a major proportion 151 (96.1%) were *A. baumannii*. The organisms were isolated from the inpatients admitted in both non critical wards and intensive care units. The study isolates were recovered mostly from respiratory secretions which constituted 37.5% of the total clinical specimens. The respiratory specimens included endotracheal secretion (23) and non-directed broncho alveolar lavage fluid (23), bronchial wash (8) and sputum (5) indicating that most of the infections with *Acinetobacter* species are hospital acquired and tend to occur in debiliated patients on ventilatory support. It is well known that *Acinetobacter* colonise the respiratory tract in hospitalised patients and most infections follow colonisation. It is due to this fact that most of the authors have reported highest isolation rates of *Acinetobacters* from the respiratory secretions [10-14].

A substantially high proportion of resistance to carbapenem i.e. 110(70%) was observed in the study isolates. Similar high proportion of resistance has been reported in another Indian study [15]. Studies from Iran and Taiwan reported 62% and 91.7% resistance to carbapenem among *Acinetobacters.* [12,16]

Co resistance to various antimicrobials among the carbapenem resistant isolates varied widely: The highest resistance rate was observed to third generation cephalosporins followed by beta lactam - beta lactamase inhibitors. This indicates that carbapenemase production is frequently accompanied by high level resistance to various other antibiotics leading to the evolution of extremely drug resistant isolates. A minor proportion of isolates remained susceptible to meropenem by disc diffusion testing.

Susceptibility to tigecycline and colistin is retained by a majority of carbapenem resistant isolates [11]. Susceptibility to tigecycline in the study was 152 (95.2%). Previous study by Shanthi et al., from South India has reported 93.1% susceptibility to tigecycline. Emergence of resistance to tigecycline is a cause for concern.

Overall, OXA carbapenemases were encountered in 154 isolates. $Bla_{OXA\ 51}$ being a chromosomal gene was found in all isolates of *Acinetobacter baumannii.* $Bla_{OXA\ 23}$ was the most common gene encountered in the study isolates. Various studies have noted that $bla_{OXA\text{-}23}$ is the most common gene mediating carbapenem resistance. A study from South India revealed that 91.3% carried one or more OXA carbapenemase encoding gene namely $bla_{OXA\text{-}23}$(95), $bla_{OXA\text{-}24}$(2), $bla_{OXA\text{-}51}$(99) and $bla_{OXA\text{-}58}$(1).Another Indian study on 155 carbapenem resistant *Acinetobacter baumannii* also reported $bla_{OXA\text{-}23}$(55), $bla_{OXA\text{-}24}$(10), $bla_{OXA\text{-}51}$(47) and $bla_{OXA\text{-}58}$(15). Both the studies reported the coexistence of different OXA carbapenemase encoding genes [1,11]. In a study from Iran 81.3% of the isolates carried $bla_{OXA\text{-}23}$[10]. A korean study reported $bla_{OXA\text{-}23}$ as the most common gene found amongst the 36 isolates that caused an outbreak in the ICU [17]. Clonal nosocomial outbreaks of OXA-23 producing *A. baumannii* have been reported from Brazil, Colombia, Argentina, French Polynesia, China, and South Korea whereas non clonal outbreaks have been reported in the United Kingdom and Australia [18]. $Bla_{OXA\text{-}24}$ & $bla_{OXA\text{-}58}$ have been reported from other countries whereas not many Indian studies give information on prevalence and distribution of these genes [1].

Amongst the MBLs, VIM was the most common gene which was found in 49(31.2%) of all isolates followed by NDM in 27(17.1%). Several authors have identified VIM as the most common MBL gene [19]. In two other studies from Greece and Korea VIM has been identified as the most common MBL gene [20,21]. In Asia, bla_{VIM} and bla_{IMP} are the most prevalent. Bla_{IMP} is a common mediator of carbapenem resistance mainly in Japan, Korea, China, Taiwan. In a study from Iran VIM and IMP were not the common genes mediating resistance which is in contrast to our study [22].

Coexistence of all these genes has also been observed. A study from China has reported the coexistence of OXA & MBL [23]. Another study has evidenced the coexistence of different types of OXA genes [24].

On comparing the MHT results with PCR (the gold standard) (refer [Table/Fig-7]), among 110 imipenem resistant PCR positive isolates, MHT provided both true positive (85) and false negative (25).The false negative MHT has been observed in the isolates producing metallo beta lactamases especially NDM [25].

Out of 46 imipenem susceptible PCR positive isolates five were true positive. The rest 41 harbour the genes that are not phenotypically expressed. Out of these 41 silent carriers, two were *Acinetobacter lwoffii* and the rest were *A. baumannii.* Among the two *A. lwoffii,* one harboured VIM alone and the other harboured VIM along with OXA 58. Among the 39 *A. baumannii* 20 isolates harboured a combination OXA and MBL genes whereas in the rest (19) only OXA 51 was detected. OXA and MBL genes may be present in imipenem susceptible isolates. T.W. Boo in his study has reported the presence of OXA genes in imipenem susceptible isolates [26]. Similarly, previous studies have also reported the presence of NDM and VIM in phenotypically carbapenem susceptible isolates [27].

The silent carriers of the genes are of great concern. The presence of carbapenemase genes in carbapenem-susceptible *Acinetobacter* isolates highlights the threat of undetected reservoirs of carbapenemase-encoding genes, since laboratory detection of such genes and subsequent infection control measures in hospitals generally phenotypically targets multidrug-resistant organisms. This also reiterates the need for early detection of the genes by molecular methods.

Similarly, on comparing MBL screen test results with PCR (refer [Table/Fig-8]), among the 46 imipenem resistant PCR positive isolates, majority of them (40) were true positive and the rest were false negative. Among the imipenem resistant

PCR negative isolates screen test positivity (59) indicates the presence of MBLs that were not looked for in the study like SIM, SPM, GIM [28]. The rest five indicate the possibility of other mechanisms (efflux pumps, porin defects) which operate to bring about carbapenem resistance.

Out of 23 imipenem susceptible MBL PCR positive isolates, 13 were true positive and 10 harbour genes that are not phenotypically expressed. Of the 24 PCR negative imipenem susceptible isolates 10 were true negative and the rest were false positive. This high false positivity of the screen test may also be attributed to two other reasons, the presence of CHDLs in *A. baumannii* [29] and that EDTA itself may increase bacterial cell wall permeability and zinc (chelated by EDTA) accelerates imipenem decomposition and decreases outer membrane porin expression [29,30]. The low sensitivity and specificity of the MBL screen tests may be attributed to the high false positivity due to various reasons already described.

CONCLUSION

Among the *Acinetobacters*, *A.baumannii* is the most common species associated with infections in hospitalised patients. The phenotypic tests for carbapenemase detection have a low sensitivity and specificity. For the MBL screen test use of ceftazidime as a substrate provides a better sensitivity and imipenem provides a better specificity. OXA 23 is the most common gene mediating resistance. Among the MBL, VIM is the most common gene followed by NDM. Co-production occurs in a substantial proportion of isolates (33.7%). Emergence of resistance to tigecycline is a cause for concern. It should be addressed with alternative and newer therapeutic strategies, strict infection control measures and continuous surveillance.

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