Phenotypic methods for detection of metallobetalactamase in Meropenem resistant *Pseudomonas aeruginosa* and molecular detection of *bla*IMP & *bla*VIM genes carrying strains

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Abstract

Introduction and Objectives: *Pseudomonas aeruginosa* is one of the major organisms responsible for nosocomial infections such as pneumonia, UTIs, surgical site infections, and bloodstream infections.[1] High intrinsic and acquired resistance to many antimicrobial agents have allowed *P. aeruginosa* to persist in both community and hospital settings. This study was done to find the prevalence rate of Meropenem resistant *Pseudomonas aeruginosa* carrying metallobeta lactamase producing genes.

Materials and Methods: The study included a total of 100 non-duplicate clinically significant, random bacterial isolates obtained from a North Indian rural tertiary care hospital (Sri Guru Ram Das Institute of Medical Sciences and Research, Amritsar, India) from various clinical specimens, over a period of one year (January2012 to December 2012) . Identification of organisms was done using the standard microbiological techniques as described by Colle et al 1996.[7] The antimicrobial susceptibility testing was performed by Kirby Bauer disc diffusion method. To detect MBL producing isolates phenotypicaly, Disc potentiation test (DPT) and Double disc synergy test (DDST) were performed. PCR was performed on Meropenem resistant isolates to detect bla_{IMP} and bla_{VIM} genes.

Results: Out of 100 examined isolates, 36% isolates were resistant to Meropenem. Out of these Meropenem resistant strains, 44.4%(16) *P. aeruginosa* isolates were MBL producers as detected by DDST method and 69.44%(25) isolates were MBL producers phenotypically by DPT method . PCR revealed prescence of bla_{VIM} gene in 16.66%(6) of Meropenem resistant *Pseudomonas aeruginosa* isolates and 38.88% (14) isolates were found to be carrying bla_{IMP} gene . Three isolate were shown to carry both bla_{IMP} and bla_{VIM} genes.

Conclusion: Resistance of *P. aeruginosa* isolates to Meropenem due to MBL enzymes is increasing in rural area. Resistance due to MBL has clinical significance, rapid detection of MBL producing strains followed by appropriate treatment is necessary to prevent the spreading of these organisms.

Keywords: Pseudomonas aeruginosa, PCR, Metallobetalactamase.

Introduction

Pseudomonas aeruginosa is a ubiquitous Gram-negative bacteria present in many diverse environmental settings. The wide metabolic versatility and high intrinsic and acquired resistance to many antimicrobial agents have allowed *P. aeruginosa* to persist in both community and hospital settings. It is one of the major organisms responsible for nosocomial infections such as pneumonia, UTIs, surgical site infections, and bloodstream infections.[1]

According to data from the Centre for Disease Control and Prevention (CDC) National Nosocomial Infections Surveillace system, *Pseudomonas aeruginosa* can be rated as the most common cause of ICU related pneumonia. Second most common cause of nosocomial pneumonia. This organism is third most common cause of hospital acquired UTI and fourth in order to cause surgical wound infections .It is the most common gram negative organism to be isolated from corneal ulcer & endocarditis.[2]

Blood stream infections caused by *Pseudomonas aeruginosa* have 50% higher mortality rates than other gram negative bacteremia. Mortality because of pneumonia along with pseudomonal bacteremia occurs typically after 3-4 days of appearance of signs and symptoms of infection. Mortality rates due to VAP caused by this organism are approximately 68% higher than other organisms.[2]

Intrinsic resistance of *Pseudomonas aeruginosa* to many known antibiotics as well as acquired resistance to multiple drugs leading to multi drug resistant strains (MDR) are the important reasons of high mortality rates of this organism.[1]

The drugs of choice against MDR *Pseudomonas aeruginosa* and other non fermenters are Carbapenems.[3]

However, Carbapenem resistance in non-fermenting bacteria such as Acinetobacter spp. and P. aeruginosa is increasing worldwide and poses a major public health threat. The mechanisms of carbapenem resistance include the production of β-lactamases, efflux pumps, and mutations altering the expression and function of porins and PBPs.[4]

Instead of these intrinsic β-lactamases, several other acquired β-lactamases have been identified as inducing carbapenem resistance. These acquired enzymes belong either to the class B enzymes (also

known as metallo-β-lactamases, (MBLs) or to the class D enzymes (also known as oxacillinases).

Carbapenemases can be classified into two main molecular families: those with serine at their active site, known as serine carbapenemases, and those with at least one zinc atom at their active site known as metallo-carbapenemases, which are considered as subgroup of metallo-beta-lactamases (MBLs). The VIM, IMP and SPM types are the most clinically significant carbapenemases which encoded by *bla*IMP, *bla*VIM and *bla*SPM genes.[5] MBLs such as VIM and IMP confer a high level of carbapenem resistance in nonlactose fermenter gram negative bacilli, as well as resistance to all β-lactams except aztreonam.[6]

The high resistance for antibiotics and the rapid dissemination of metallobetalactamase producing isolates in hospital require an effective phenotypic method for identifying them as there are no prescribed CLSI guidelines available for detecting them. This study was conducted to find the prevalence of MBL and their resistance genes, namely bla_{VIM} and bla_{IMP} in MDR *Pseudomonas aeruginosa* strains isolated from specimens received in a rural tertiary care hospital.

Materials and Methods

All the *P. aeruginosa* isolates were obtained from a North Indian rural tertiary care hospital (Sri Guru Ram Das Institute of Medical Sciences and Research, Amritsar, India) from various clinical specimens, such as endotracheal aspirates, cerebrospinal fluid, wound swabs, urine and blood culture specimens, from patients admitted to various wards from over a period of one year (January2012 to December 2012)

Identification of organisms was done using the standard microbiological techniques as described by Colle et al 1996.[7] The study included a total of 100 non-duplicate clinically significant, random bacterial isolates.

The antimicrobial susceptibility testing was performed by Kirby Bauer method. Antimicrobial agents and their disc concentrations used are as follows**;** (HIMEDIA) Amikacin 30µg, Ciprofloxacin5µg, Ceftazidime 30µg, Piperacillin-tazobactam100/10µg, Imipenam10µg, Meropenem10µg, Polymyxin 300 units, Chloramphenicol 5µg, Gentamicin 10µg, Norfloxacin10µg.

The results were interpreted as per CLSI (Clinical Laboratory Standards Institute) guidelines.[8]

The carbapenem resistant MDR *Pseudomonas aeruginosa* were further screened for MBL production.[9]

Minimum inhibitory concentration (MIC) of the isolates was determined by Epsilometer test (E-test) for Meropenem. E strips (AB bioMerieux, Sweden) containing predefined gradient of antibiotic were used.[10]

To detect MBL producing isolates Disc potentiation test (DPT) as described by Yong D et al[11] and Double disc synergy test (DDST) as described by Lee K et al.[12] Combination used for DDST was Me-EDTA. For DPT combination used in this test is Me (10µg) and Me-EDTA for zone enhancement. All the MDR Meropenem resistant isolates were checked genotypically by PCR for the presence of predominant genes bla_{IMP} and bla_{VIM}. Primers for the PCR were procured from BIOSERVE (Hyderabad)

MBL GENE IDENTIFICATION

MULTIPLEX PCR FOR *IMP* and *VIM*[13]

Duplex PCR was performed for *IMP* AND *VIM* genes.

The primer sequence for *IMP*-F(5'-3');TTGACACTCCATTTACDG

The primer sequence for *IMP*-R(5'-3');GATYGAGAATTAAGCCACYCT

This amplified a 139bp amplicon.

The primer sequence for *VIM*-F(5'-3');GATGGTGTTTGGTCGCATA

The primer sequence for *VIM*-R (5'-3');CGAATGCGCAGCACCAG

This amplified a 390 bp amplicon.

The data thus obtained was tabulated, compiled and analysed.

Results

Among the hundred study isolates, maximum number of isolates were obtained from pus samples i.e 58 isolates.(Table-1).Table-2 shows the distribution of isolates from various wards . Antimicrobial sensitivity pattern shows that maximum number of isolates were resistant to Chloramphenicol i.e 69 isolates and 36 isolates were found to be resistant to Meropenem. (Table 3).

All the isolates were sensitive to polymyxin B. Meropenem resistant isolates were also found to be MDR. MIC of Meropenem by Epsilometer test revealed 29% isolates with intermediate to complete resistance. (Table 4). Comparison of resistance of isolates by disc diffusion method and E Test shows 36% and 29% resistance respectively.7% isolates detected resistant in disc diffusion method were having their MIC in the sensitive range.

Out of 36 Meropenem resistant strains, 44.4%(16) *P. aeruginosa* isolates were MBL producers as detected by DDST method and 69.44%(25) isolates were MBL producers by DPT method. (Table 5)

PCR revealed amplification of a 390bp fragment corresponding to bla_{VIM} gene in 16.66%(6) isolates and 38.88% (14) isolates were found to be carrying bla_{IMP} gene corresponding to 139bp. (Table 6)

In our study bla_{IMP} was the predominant gene.

Three isolate were shown to carry both bla_{IMP} and bla_{VIM} genes.

Table 1: Distribution of *P. Aeruginosa* in indoor patients

Sample	*P. Aeruginosa*
Blood	2
Body Fluids	1
ETT sec	13
Pus	58
Sputum	8
Urine	18
Total	100

Table 2: Source of *P. aeruginosa* isolates in various wards of institute

Ward	*P. aeruginosa*
Emergency/W	5
Eye/W	2
Gynae/W	1
ICU	25
Med/W	10
Ortho/W	9
Paed/W	3
Surgery/W	45
Total	100

Table 3: Antibiotic susceptibility pattern of *P. aeruginosa.*

Antimicrobials	P. aerugenosa		
	Sensitive	Intermediate	Resistant
AK	91	3	6
G	52	0	48
CIP	41	0	59
CAZ	38		62
PIT	82	1	17
IMP	75	0	25
MP	64	0	36
CHL	13	0	69
NX	7	0	11
P-B	100	0	0

Table 4: MIC (E test Meropenem) in *P.aeruginosa* isolates

E Test	*P.aeruginosa*
Sensitive	71
Intermediate	1
Resistant	28
Total	100

Table 5: Results of DDST and DPT

Test	Performed on isolates	MBL producers	NON MBL producers
DDST	36	16(44.4%)	20
DPT	36	25(69.44%)	11

Table 6

Sample	Total Isolates Obtained	Meropenem Resistant	MBL Producers by DPT Method	IMP	VIM
PUS	58	18	12	6	1
Urine	18	7	5	3	2
ET Tube Secretions	13	10	8	5	3
Sputum	8	1	0	0	0
Blood	2	0	0	0	0
Body fluids	1	0	0	0	0
Total	100	36	25	14	6

Discussion

P. aeruginosa isolated from pus were 58%, urine 18%, ETT secretions 13%, sputum 8%, blood 2%, body fluids 1%. While Attal RO et al isolated 28.6% & 22.9% *P. aeruginosa* in pus and urine respectively, and

25% in sputum, 12.1% in blood, 5% in ett secretions and 6.4% in body fluids.[14]

Total isolates from the surgery ward were 45%, followed by ICU 25% while in a study of Attal RO et al 2010 only 9.28% isolates of *P. aeruginosa* were from ICU.[14]

Resistance to Meropenem in *P. aeruginosa* is 36% .This is in accordance with the study by Sarkar et al which reports 36.36% resistance to carbapenems,[15] study by Ramakrishna K[16] shows 84% resistance to Meropenem. Our study revealed resistance of *P .aeruginosa* to Imipenem 25%,while in another study 40% resistance to Imipenem was detected,[16] Ceftazidime showed (62%) resistance in this study while in a previous study by Aggarwal et al resistance to Ceftazidime was 10.35%, while in the study of Shahid et al and Pitt et al resistance to Ceftazidime was 20% & 39.6% respectively.[17] Among aminoglycosides, Amikacin showed least resistance in our study (6%). This is in accordance with the study by Aggarwal et al.[17] while in the study by Yilmaz NO et al,[18] it is 82%. Gentamicin resistance was (48%) in our study which is in accordance with Sarkar et al (45%) while Yilmaz NO et al, reports much higher i.e(91%).[18] We observed (59%) resistance to Ciprofloxacin while in the study of Sarkar et al it was slightly lower i.e (50%).[15] and as high as (82%) by Yilmaz NO et al.[18]

All the isolates were sensitive to Polymyxin B, same results were also recorded in other studies.[15-18] In present study MIC of Meropenem by epsilometer test revealed that 29% of *P .aeruginosa* were moderate to complete resistant, while these results are different in a south Asian study, which recorded 86% moderate to complete resistance.[19]

This may be due to the fact that Meropenem might be a commonly used antibiotic for treatment of various infections in their hospital setup.

So the sensitivity pattern of epsilometer test have been found to be more discriminatory as compared to disc diffusion method as 5% resistant isolates were also found to have their MIC in the sensitivity range .

Meropenem resistant isolates were subjected for the detection of MBL production by DDST and DPT and revealed that among *P. aeruginosa* isolates 44.4% were MBL producers by DDST method and 69.44% isolates of *P.aeruginosa* were MBL producers by DPT method. While In study by Attal RO et al both DDST & DPT were performed and prevelance of MBL producing strains in *P .aeruginosa* was 11.4% and it was 8.5% in a study by Aggarwal et al,[17] while acc. to study by Yilmaz NO et al 71.05% are MBL producing by DDST.[18] This upward trend seems to be due to the evolving scenario of indiscriminate antibiotic use in India and free access to purchase antibiotics over the counter. Results by DPT are more in accordance with the disc susceptibility test. Also study by Manoharan et al supported the present study regarding the results of DPT.[20]

All 36 Meropenem resistant isolates were further subjected to investigate predominant MBL coding gene like bla_{IMP} &bla_{VIM} by multiplex PCR.

16.66% *P. aeruginosa* in our study harboured bla_{VIM}, in contrast to 88.8% in study by Manoharan et al.[20] In study by Amudhan et al, 55.5% *P. aeruginosa* were carrying bla_{VIM} gene. Presence of bla_{IMP} gene was detected in 38.88% isolates in contrast to 3.2% by Amudhan et al.[21] Of the above PCR positive isolates 3 *P. aeruginosa* isolates were showing the presence of both bla_{IMP} and bla_{VIM}. Suggesting that they are coproducers of *IMP* and *VIM* genes.

The isolates that were MBL positive by DPT but negative for either bla_{IMP} or bla_{VIM} amplification, may have genes other than these two.

Conclusion

It can be concluded from the study that *P. aeruginosa* are leading hospital pathogens. Resistance to commonly used antibiotics is posing therapeutic challenge to the clinicians. Such strains have been implicated in many recent out breaks mostly in ICUs where extensive use of antibiotics has contributed to the selection of highly resistant strains .These strains are proficient in acquiring resistance determinants and are well accustomed to survive for prolonged periods in hospital environment.

The organisms are resistant due to production of various MBLs and by detecting genes for these antibiotic resistance will help in the study of epidemiology of these organisms and hospital infection control programme.

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