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ORIGINAL ARTICLE

Metallo-β-lactamase production among Pseudomonas species and Acinetobacter species in costal Karnataka

SHOBHA KL* , LENKA PR ** , SHARMA M K*** , RAMACHANDRA L **** , BAIRY I *****

ABSTRACT

Background and objectives: The emergence of multidrug resistance among Pseudomonas species and Acinetobacter species is a notable threat. Acquisition of the metallo-ß-lactamase (MBL) gene is an important mechanism of broad-spectrum-ß-lactam resistance. The aim of the study is to detect the prevalence of MBL among Pseudomonas spp. and Acinetobacter spp. isolated from various clinical samples collected from different age groups.

Materials and Methods: A total of 54 meropenem resistant Pseudomonas spp. and Acinetobacter spp., tested by the disc diffusion method, were included in the study .The strains were isolates obtained from burn wounds, ulcers, sputum and urine collected from the patients aged from one year to 90 years. The strains were identified up to the species level and the EDTA disk synergy test was used, with simultaneous testing of different ß-lactams (meropenem and aztreonam).

Results: Of the 54 meropenem resistant strains, 16(30%) were MBLs producing isolates, of which 13(81%) were isolates of samples from male patients and 3(19%) were those from female patients. More strains of MBLs [13(81%)] were seen in the age group of 40-75 years and only few strains of MBLs [3(19%)] were isolated from samples in the age group below 40 years and above 75 years. MBLs were more prevalent in respiratory specimens 4(45%) and less prevalent in urine specimens 3(21%) when compared to other specimens. Pseudomonas putida 7(64%) had more number of MBLs producing organisms as compared to other species of Pseudomonas. The isolates producing MBLs were more resistant to Tobramycin [16(100%)] and Gentamicin [15 (94%)] and were less resistant to Piperacillin [10(63%)] than meropenem -non susceptible isolates which did not produce MBLs.

Conclusion: The rapid discrimination of MBL producers is worrisome and necessitates the implementation of not just surveillance studies, but also the proper and judicious selection of antibiotics, especially carbapenems.

Key Words: Metallo-ß-lactamase, EDTA disk synergy test, carbapenems.

*Prof., Dept. of Microbiology, Melaka Manipal Medical College, **, *** P.G. student, Dept. of Microbiology , ****Prof., Dept. of Surgery, (Kasturba Medical College), *****Prof., Dept. of Microbiology, Melaka Manipal Medical College, Manipal

Corresponding Author:
Shobha K L
E.mail: shobhamicro@yahoo.com

Introduction

The emergence of multidrug resistance among gram-negative bacteria is a notable threat. Clinically relevant species of gram-negative bacilli are often resistant to β-lactam antibiotics, including extended spectrum cephalosporins, but rarely to carbapenems [2]. Carbapenems are often used as last resort antibiotics for treating infections caused by multidrug-resistant gram-negative bacilli, as they are stable and respond only to extended spectrum and AmpC β-lactamses.

However, emergence of acquired carbapenemases, particularly Ambler class B metallo-β-lactamases (MBLs), IMP and VIM, have been increasingly reported in Asia , Europe [2], Canada [5] and in many geographical locations [10]. Another type (SPM-1) has been reported in South America [9]. Five enzymes have been identified (IMP, VIM, SPM, GIM and SIM types) in various host organisms, the most common ones being found in Pseudomonas spp. and Acinetobacter spp [3]. Pseudomonas aeruginosa producing metallo-β-lactamases (MBLs), was first reported from Japan in 1991 [5] and then the resistance spread to other species. Recently, IMP-2-producing Acinetobacter baumannii [14] and VIM-1 and VIM-2 producing strains of P.aeruginosa have been reported in Europe [8]. A particular concern is that acquired MBL genes are located on integron structures that reside on mobile genetic elements such as plasmids or transposons, thus enabling wide spread dissemination. Clinical infection with such organisms poses serious therapeutic challenges, with increasing reports of poor patient outcomes and death [19].

With the worldwide increase in the occurrence, types and rate of dissemination of MBLs, early detection is critical. The benefits of such include timely implementation of strict infection control practices, as well as clinical guidance regarding the potential risk for therapeutic failure. As seen with extended-spectrum β-lactamases (ESBLs) and AmpC type β-lactamases with cephalosporins (12), MBL carrying Pseudomonas spp. and Acinetobacter spp. can appear susceptible to carbapenems using current Clinical and Laboratory Standards Institute or British Society For Antimicrobial Chemotherapy break points [1]. Pitout and colleagues reported the presence of IMP-non susceptible P.aeruginosa in clinical isolates and showed that 46% (110/241) of the strains were MBL positive [13]. Similar findings have been reported by others [15].

There is difficulty in detecting such organisms, which poses significant risks, particularly due to their role in unnoticed spread within institutions and their ability to participate in horizontal MBL gene transfer with other pathogenic hospital-related organisms, as the MBL genes reside in mobile gene cassettes inserted integrons [16]. The rapid detection of MBL-positive gram-negative bacilli is necessary to aid infection control and to prevent their dissemination [6]. A PCR method was simple to use in detecting MBL producing isolates initially (16), but it became more difficult with the increased number of types of MBL [21].

Currently, no standardized method for MBL detection has been proposed. Several non-molecular techniques have been studied, all taking advantage of the zinc dependence of the enzymes, by using chelating agents such as EDTA or 2-mercaptopropionic acid to inhibit their activities. The commercially available MBL-E test is simple to perform, but is highly insensitive at detecting carbapenem-susceptible MBL carrying organisms [3] and is costly. Also, poor specificity has been described with carbapenem-resistant Acinetobacter baumannii carrying bla_{oxA-23} [17]. A double disk synergy test (DDST) using imipenem (IPM) and 0.5M EDTA [9] and a combined-disk test either using two IPM disks or two meropenem (MEM)disks, one containing 930µg [10] or 750µg [15] of EDTA, have

both been reported as reliable methods for the detection of MBLs in carbapenem-resistant Pseudomonas and Acinetobacter strains. When the latter method was studied using carbapenem-susceptible isolates, the sensitivity was found to be poor, ranging from 10% to 86% [22]. Thus far, no method has been reported to show adequate sensitivity and specificity for the detection of carbapenem resistant MBL-positive isolates. The aim of this study was to determine the feasibility of the method which involved the use of an MEM disk with added EDTA to confirm the presence of MBL-producing clinical isolates of Pseudomonas spp. and Acinetobacter spp.

Materials and Methods

Bacterial Strains and Isolate Collection

Consecutive non-duplicate isolates of Pseudomonas spp. and Acinetobacter spp. which are resistant to MEM (MIC > 8 µg/ml), were taken from various clinical specimens, which included samples of wounds (purulent), urine, respiratory tract specimens, blood and others like ascetic fluid, synovial fluid, throat swab and ear swab, which were collected from in- patients and out-patients of various departments. The departments included the medicine ward, the surgery ward, the paediatric ward, the orthopaedic ward and the ENT ward of Kasturba Medical College Hospital (KMCH), Manipal, India and the samples studied were from patients who attended these hospitals from February 2007 to January 2008. The total number of patients included in our study was 54, which included both males (41/54) and females (13/54). The age group of the patients was between 1 year and 90 years. 44 Pseudomonas species and 10 Acinetobacter species were isolated .The strains were identified up to the species level according to W.winn et al (2006) [20] [Table/Fig 1].

(Table/Fig 1) Results of the identification of strains to the species level and of the phenotypic method in the detection of MBL-producing species.

Species	No. (%) of isolates	No. (%) of isolates positive on phenotypic plate
Pseudomonas aeruginosa	11(20)	1(9)
Pseudomonas putida	11(20)	7(64)
Pseudomonas alkaligens	10(19)	3(30)
Pseudomonas fluorescens	9(17)	3(33)
Pseudomonas sp. Grp-1	2(4)	0(0)
Pseudomonas maltophila	1(2)	0(0)
Acinetobacter baumani	4(8)	1(25)
Acinetobacter junii	3(6)	1(20)
Acinetobacter lowffii	3(6)	0(0)

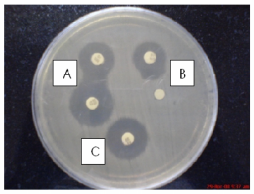
Antimicrobial Susceptibility Testing

Antimicrobial susceptibility testing was performed using Mueller-Hinton (MHA) agar. The antimicrobial susceptibility tests of the following drugs were determined by the Kirby-Bauer disc diffusion method: Piperacillin (PIP) (100µg), Ceftazidime (CAZ) (30µg), Ciprofloxacin (CIP) (5µg), Gentamicin (GEN) (10µg), and Tobramycin (TOB) (10µg). The quality control strains used for this part of the study were *Escherichia coli* ATCC 25922 and *Pseudomonas aeruginosa* ATCC 27853. Throughout the study, the results were interpreted using the Clinical and Laboratory Standards Institute (CLSI) guidelines criteria for the disc diffusion method [14]. Before inoculation into Mueller Hinton agar, the strains were compared to those from McFarland standard tube no. 0.5. Antibiotic disks used in this study were obtained from Span Diagnostics Ltd .Surat, India.

Phenotypic Detection of Mbls

An MBL phenotypic detection method was designed using a single agar plate and it comprised of three components [Table/Fig 2] .(I) In the combined-disk test, two MEM disks (10µg) (one containing 10 µl of 0.1 M (292 µg) anhydrous EDTA) were placed 25mm apart (center to center). An increase in the zone diameter of >4 mm around the MEM-EDTA disk as compared to that of the MEM disk alone, was considered positive for an MBL. (ii) In the DDST, an MEM(10 µg) disk was placed 20 mm (center to center) from a blank disk containing 10 µl of 0.1 M (292 µg) EDTA. Enhancement of the zone of inhibition in the area between the

two disks was considered positive for an MBL [Table/Fig 2]. (iii) The final component was an aztreonam (30 µg) disk. Given the unique sensitivity of MBLs to this antibiotic, we studied the inhibition zone sizes of all isolates to determine the utility of this component in phenotypic MBL detection. The disks were placed on the surface of the inoculated lawn culture agar plate as shown in [Table/Fig 2] and plates were incubated overnight at 37°C.



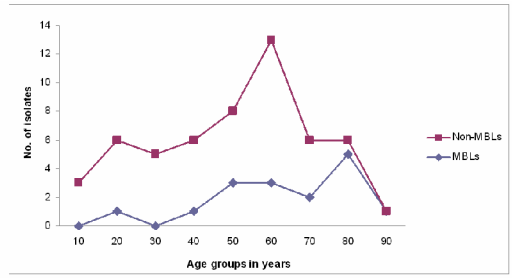
(Table/Fig 2) (A) Combined-disc test, using two MEM (10 µg) disks, one with 292 µg EDTA, showing an increase in zone inhibition of >4 mm around the disk with EDTA.

(B) Double disk synergy test, using a MEM (10 µg) disk placed 20 mm (center to center) from a blank filter disk containing 292 µg EDTA.(C) Aztreonam (30 µg) disk with a >22 mm zone of inhibition.

Results

Clinical Bacterial Strains

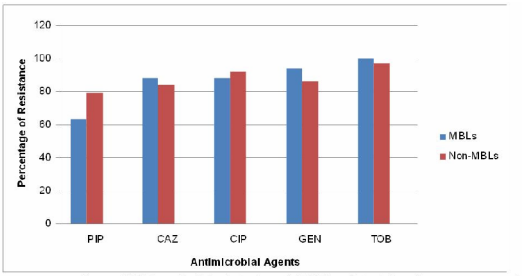
A total of 54 non duplicate isolates of Pseudomonas spp. and Acinetobacter spp. were included in the study. Of the 54 MEM-non susceptible strains, 14(26%) were isolated from urine, 4(8%) from blood, 19(35%) from wounds (purulent), 9(17%) from respiratory tract specimens, and the remaining 8(15%) from various other specimens (like ascetic fluid, synovial fluid, throat swab and ear swab). Of the 54 meropenem resistant strains, 16(30%) were MBLs producing isolates, of which 13(81%) were isolates of samples from male patients and 3(19%) were from female patients. Production of MBLs was seen more [13(81%)] in the age group of 40-75 years and only few strains of MBLs were isolated from samples [3(19%)] in the age group below 40 years and above 75 years . [Table/Fig 3] The isolates producing MBLs were more prevalent in respiratory specimens 4(45%) and less prevalent in urine specimens 3(21%). Pseudomonas putida 7(64%) was found more in MBLs producing organisms [Table/Fig 1].



(Table/Fig 3) Age wise distribution of clinical strains of MEM-resistant Pseudomonas spp. and Acinetobacter spp. MBLs (n =16), isolates producing metallo-β-lactamases; non-MBLs (n=38), MEM-non susceptible isolates not producing MBLs.

Antimicrobial Susceptibilities of Clinical Strains

Of the 54 clinical isolates included in this study, 40(74%) were resistant to PIP, 46(85%) to CAZ, 53(98%) to TOB, 48(89%) to GEN, and 49(91%) to CIP. The isolates producing MBLs were more resistant to TOB [16(100%)], GEN [15(94%)], CIP [14(88%)] and CAZ [14(88%)] and were less resistant to PIP [10(63%)] than MEM-non susceptible isolates which did not produce MBLs [Table/Fig 4]. A particularly important feature was that all the MBL producers were resistant to TOB as compared to 97% of the MEM-non susceptible isolates which did not produce MBLs [Table/Fig 4].



(Table/Fig 4) Susceptibilities of clinical strains of MEM-resistant Pseudomonas spp. and Acinetobacter spp. PIP (piperacillin), CAZ (Ceftazidime), CIP (Ciprofloxacin), GEN (Gentamicin), TOB (Tobramycin). MBLs (n =16), isolates producing metallo-β-lactamases; non-MBLs (n=38), MEM-resistant isolates not producing MBLs.

Discussion

MBLs have been identified from clinical isolates worldwide, with an increasing frequency over the past few years and strains producing these enzymes have been responsible for prolonged nosocomial outbreaks that were accompanied by serious infections, as reported by Senda K, et al

(1996) [15]. A case-controlled study from Japan showed that patients infected with MBL-producing P. aeruginosa were more likely to receive multiple antibiotics and more importantly, that infection-related deaths due to IMP-producing P. aeruginosa were more frequent than deaths caused by bla_{IMP} negative P. aeruginosa, as reported by Hirakata et al (2006) [7]. The occurrence of an MBL-positive isolate in a hospital setting poses a therapeutic problem, as well as a serious concern for infection control management. The accurate identification and reporting of MBL-producing P. aeruginosa will aid infection control practitioners in preventing the spread of these multidrug-resistant isolates, as reported by Senda et al (1996) [15]. Acinetobacter spp. is also notorious, both for its ability to acquire antibiotic resistance and for the ability of some strains, mostly strains of *A. baumannii*, to cause nosocomial outbreaks. Therefore, early laboratory detection is of great clinical importance.

Our study included the use of the CLSI disk methodology that developed an EDTA disk screen test with MEM disks alone and in combination with 292 µg of EDTA and Aztreonam (30 µg) per disk. All the three methodologies showed positive results for the same 16 strains .The above methods were simple to perform and the materials used were cheap, nontoxic, and easily accessible, making it highly applicable to routine clinical laboratories. In a similar study by Pitout et al.(2005) [13], they showed that the results with MEM alone and in combination with EDTA showed 100% sensitivity and 97% specificity in detecting well-characterized MBL-producing clinical strains of P. aeruginosa and that this test worked better than IPM and the MBL- E test. PCR was the most simple method which was used in detecting MBL producing isolates. Initially Senda et al (1996) [16] used this method, but it became more difficult for Yong D.K .Lee et al (2002) [21] to use it, due to the increased number of types of MBL. We do recommend however, that MEM can be used as a substrate for the EDTA disk screen test. The MEM-EDTA disks can be stored at 4°C or -20°C for 12 to 16 weeks without significant loss of activity, as suggested by Yong D.K .Lee et al (2002) [21]. The EDTA disk screen test is simple to perform and interpret and since it uses the CLSI methodology, it can be easily introduced into the workflow of a clinical laboratory.

This study illustrates that the MBL-producing isolates of Pseudomonas spp. and Acinetobacter spp. are important causes of MEM resistance among this species which were isolated in our hospital [Table/Fig 1] **.** Of the 54 meropenem resistant strains, 16(30%) were MBLs producing isolates, of which 13(81%) were isolates of samples from male patients and 3(19%) were from female patients. The production of MBLs was seen more [13(81%)] in the age group of 40-75 years and only a few strains of MBLs were isolated from samples [3(19%)] in the age group below 40 years and above 75 years. The MBL-producing isolates were more resistant to various antimicrobial agents [Table/Fig 4] and were more prevalent in respiratory specimens 4(45%) than MEM-resistant isolates which did not produce MBLs. Clare Franklin et al (2006) [3], in their study, reported the presence of more number of MBL producing isolates in respiratory tract specimens than in other specimens. Our results support the notion that clinical microbiology laboratories must be able to distinguish MBL-producing Pseudomonas spp. and Acinetobacter spp. from strains with other mechanisms which are responsible for carbapenem resistance. Resistance to other antimicrobial agents like Tobramycin was 100% and resistance to Gentamicin was 94%.This was in concordance with the study conducted by B.V .Navaneeth et al (2004) [11], where it was seen that resistance to piperacillin-tazobactum, cefoperazone-sulbactam and ticarcillin-clavulanic acid was 12%, 20% and 36%, respectively. In the absence of novel agents for the treatment of infections caused by multidrug-resistant gram negative bacteria in the near future, the uncontrolled

spread of MBL producers may lead to treatment failures, with increased morbidity and mortality. The early detection of MBL-producing Pseudomonas spp. and Acinetobacter spp. may avoid the future spread of these multidrug- resistant isolates.

Conclusion

The emergence of carbapenamase producing strains represents a serious therapeutic and epidemiological problem which can be circumvented only by the early detection and control of such multidrug resistant pathogens. The rapid detection of carbapenemases and metallo-β-lactamase producing isolates must be followed up in the laboratory on a routine basis, as the number of metallo-β-lactamase producing isolates are increasing and are posing a problem for the clinicians during the treatment of patients. The routine detection of MBLs will ensure optimal patient care and the timely introduction of appropriate infection control procedures.

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