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Research Article

STUDY ON SOME GRAM NEGATIVE MULTI DRUG RESISTANT BACTERIA AND THEIR MOLECULAR CHARACTERIZATION

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ABSTRACT

Multidrug resistant (MDR) bacterial infections are rampant throughout the globe where MBLs (Metallo Beta Lactamases) and ESBLs (Extended Spectrum Beta Lactamases) are the major MDR related bacterial enzymes which are being studied widely. In Kolkata, the number of MDR bacteria are gradually increasing, so it has become a major concern to treat patients. Phenotypic and genotypic studies have been conducted with few MDR bacteria to get some idea about them.

Both phenotypic (three different methods) and genotypic methods (based on PCR) were utilized to characterize both MBLs and ESBLs of 100 MDR bacterial strains from clinical materials collected from patients admitted in ICCU and ITU of tertiary care hospitals of Kolkata. Other tests like Imipenem hydrolysis and isoelectric focusing tests were also done for confirmation of the findings.

Result: In this study it was found that majority of the MBL positive isolates were associated with urinary tract infections. The MBL VIM-2 variety was predominantly found in *Pseudomonas aeruginosa*. The main ESBL types was CTX-M1. A positive result in phenotypic methods should be confirmed by the genotypic methods as false positive phenotypic results were found in some of the cases. MBL status of isolated organisms from clinical materials collected from intensive care units should be studied at least by phenotypic methods in all hospitals along with their drug resistance pattern.

Keywords: Metallo Beta Lactamases, VIM-2, ESBL, Multi drug resistance.

INTRODUCTION

The prevalence of multi drug resistance (MDR) bacterial species has increased considerably since the introduction followed by arbitrary use of new generation extended spectrum antibiotics like third and fourth generation cephalosporins, carbapenems, monobactams, broad and extended spectrum penicillins etc[1].

Extended spectrum beta lactamase (ESBL, they are beta lactamase that hydrolyzes extended spectrum cephalosporins with oxymino side chain) and Metallo beta lactamase (MBL, that hydrolyzes beta lactam antibiotics including carbapenems like imipenem and meropenem) [2] classes of beta lactamases are produced generally by most of the MDR Gram negative bacteria.

So these bacteria presently create a very serious problem for antibiotic treatment especially in critically ill patients admitted in intensive care units. It has been seen that, ESBL producers showed reduced susceptibility to Imipenem due to the production of carbapenemase, so these bacteria can not be eliminated by carbapenems that are the usual drug of choice against them [3]. Different types of ESBLs (TEM, SHV, CTX-M and OXA beta lactamases) and MBLs (IMP, VIM, SPM, GIM and recently NDM-1) are the major enzymes that are increasing in pathogenic microorganisms worldwide[4]. MBLs, can be divided as chromosomal or plasmid mediated. VIM1 and VIM-2 were first described in Italy and France respectively from *Pseudomonas aeruginosa*[5].

VIM types are commonly seen among non fermenting bacteria in South and Eastern Europe [6,7,8,9]. New subtypes of VIM and IMPs are constantly appearing throughout the globe and to follow the local trend of this molecular characterization of MBL and ESBL producing MDR bacteria, this study has been conducted with some Gram negative bacteria (*E.coli, Pseudomonas aeruginosa and Klebsiella pneumoniae*) isolated from ITU and ICCU of two tertiary care hospitals.

The study of this type has not been done earlier in Kolkata, India. Our aim was to get acquaintance with the predominant classes of MBLs and ESBLs, which are prevailing in such bacteria in Kolkata hospitals. We also investigated the antibiotic sensitivity pattern of these MDR bacteria meticulously to understand the general drug resistance pattern of them.

MATERIALS AND METHODS

Strain collection

Gram negative bacterial strains (*E.coli, Pseudomonas aeruginosa* and *Klebsiella pneumoniae)* were collected from clinical isolates of patients admitted in ICCU and ITU of two tertiary care hospitals of Kolkata, India. Different specimens like tracheal aspirate, blood, pus, urine, stool, sputum, throat swab and pleural fluid were used as the source for isolating these bacteria. Analysis was done with 100 MBL producing strains to know in which source they were prevalent. Routine laboratory methods were employed to identify the bacterial strains as described elsewhere[10].

Antimicrobial susceptibility testing

Antimicrobial susceptibility of the selected bacteria was done by disc diffusion method according to CLSI guidelines[11]. The following antibiotics were used – Piperacillin, Amikacin, Meropenem, Imipenem, Cefotaxime, Ceftriaxone, Ceftazidime, Cefepime, Levofloxacin, Ofloxacin and Aztreonam which were obtained from Himedia Laboratories and Becton Dickinson diagnostic Pvt Ltd, India. Bacterial strains that demonstrated resistance to three or more antibiotics were considered as multi drug resistant bacteria (MDR).

Screening for MBL producers

MDR strains, isolated from clinical samples of patients admitted in ITU and ICCU, were tested for MBL production by three phenotypic methods- Imipenem-EDTA Double disc synergy test (DDST), Imipenem-EDTA Combined disc test (CDT) and MBL E-Test.

Imipenem-EDTA Double disc synergy test (DDST)

Test organism was inoculated on Mueller Hinton media according to the CLSI guideline. Two Imipenem discs were placed keeping farthest distance in a 90 cm culture plate and one blank sterilized filter paper disc was put 10-20 mm apart from one Imipenem disc. After this 5µL 0.5M EDTA was added onto the blank filter paper disc. Enhancement of the zone of inhibition in the area between Imipenem and EDTA discs in comparison with the Imipenem disc alone was considered as a positive result [12].

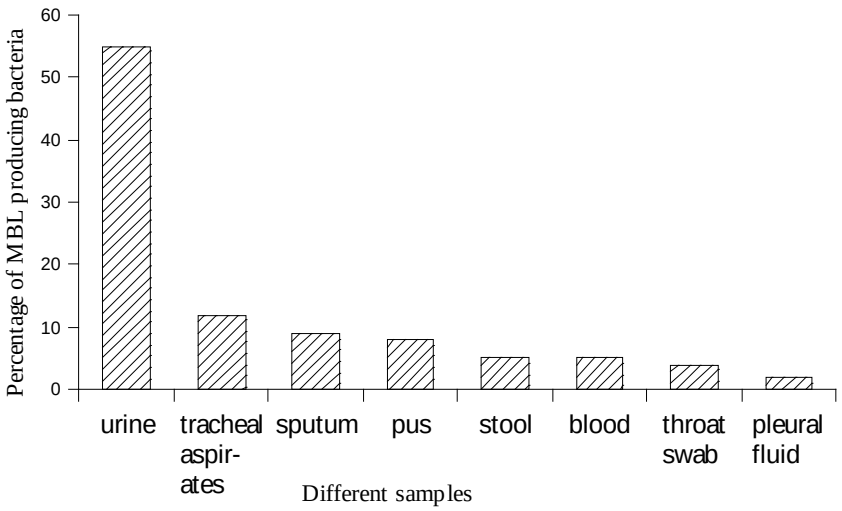

Fig. 1: Frequency of MBL Producers in Different Samples

Table 1: Antimicrobial resistance profiles

Strain No:	Different Antibiotics										
	Pip	Ami	Mero	Imi	Cefo	Ceftri	Cefta	Cefep	Levo	Ofl	Aztr
1	R(0)*	S(18)	R(09)	R(10)	R(0)	R(0)	R(0)	R(0)	S(26)	S(19)	R(11)
2	R(0)	R(09)	R(07)	R(06)	R(0)	R(08)	R(0)	R(0)	R(10)	R(10)	R(0)
3	S(20)	R(12)	S(27)	R(10)	R(0)	R(11)	R(12)	R(11)	R(0)	R(09)	R(09)
4	R(07)	I(15)	R(11)	R(09)	R(10)	S(27)	R(12)	R(12)	S(21)	R(O7)	S(26)
5	R(10)	S(20)	R(10)	R(08)	R(08)	S(25)	R(0)	S(25)	R(0)	R(11)	R(08)
6	R(0)	S(19)	S(28)	S(25)	S(23)	R(10)	I(15)	R(07)	S(26)	I(14)	S(27)
7	S(22)	R(12)	S(26)	R(11)	R(0)	R(0)	R(13)	R(12)	S(23)	S(21)	R(0)
8	R(12)	S(20)	R(10)	R(09)	R(09)	S(25)	R(0)	S(25)	R(0)	R(12)	R(08)
9	R(0)	R(0)	R(0)	R(11)	R(11)	R(0)	R(0)	R(0)	S(21)	S(26)	S(23)
10	S(19)	S(21)	R(0)	R(0)	R(0)	R(09)	R(0)	R(0)	S(22)	S(22)	R(0)
11	R(0)	S(23)	R(07)	R(10)	S(25)	R(11)	I(16)	R(0)	S(24)	R(11)	R(0)
12	S(23)	S(20)	R(10)	R(11)	S(24)	R(11)	R(09)	R(0)	S(25)	S(25)	R(0)
13	R(0))	R(0)	S(24.4)	S(23.5)	R(0)	R(0)	R(0)	S(20)	R(0)	R(0)	R(11)
14	R(0)	R(0)	R(08)	R(0)	R(0)	R(0)	R(0)	S(24)	S(26)	S(23)	R(09)
15	R(0)	R(0)	S(25)	S(25)	S(22)	R(0)	R(08)	R(0)	S(27)	R(09)	S(24)

Note: R- Resistant, S- Sensitive, *- The number in parenthesis is an inhibitory zone diameter in mm.

Pip- Piperacillin, Ami- Amikacin, Mero- Meropenem, Imi- Imipenem, Cefo- Cefotaxime, Ceftri- Ceftriaxone, Cefta- Ceftazidime, Cefep- Cefepime, Levo- Levofloxacin, Ofl- Ofloxacin, Aztr- Aztreonam.

Table 2: Phenotypic and Genotypic Results of The Isolated Strains in Regarding MBL and ESBL Production

Strain no.	Bacteria	Phenotypic methods	MBL Production (IMP-1, IMP-2, VIM-1, VIM-2 Primers Were Used)	ESBL Production (TEM-1, OXA -2, OXA-4, CTX-M1, CTX-M9 Primers Were Used)
1	*E.coli*	DDST (-), CDT(+), E-test (+)	Unidentified	TEM-1, CTX-M1
2	*P.aeruginosa*	DDST (+), CDT(+), E-test (+)	VIM-2	NA
3	*P.aeruginosa*	DDST (+), CDT(+), E-test (+)	„	NA
4	*P.aeruginosa*	DDST (+), CDT(+), E-test (+)	„	NA
5	*P.aeruginosa*	DDST (+), CDT(+), E-test (+)	„	NA
6	*P.aeruginosa*	DDST (+), CDT(-), E-test (-)	Non-producer	Non-producer
7	*P.aeruginosa*	DDST (+), CDT(+), E-test (+)	„	NA
8	*P.aeruginosa*	DDST (+), CDT(+), E-test (+)	„	NA
9	*P.aeruginosa*	DDST (-), CDT(+), E-test (+)	„	NA
10	*P.aeruginosa*	DDST (-), CDT(+), E-test (+)	„	NA
11	*P.aeruginosa*	DDST (-), CDT(+), E-test (+)	„	NA
12	*K.pneumoniae*	DDST (-), CDT(+), E-test (-)	Non-producer	Non-producer
13	*K.pneumoniae*	DDST (+), CDT(-), E-test (-)	Non-producer	CTX-M1
14	*K.pneumoniae*	DDST (+), CDT(+), E-test (+)	Unidentified	CTX-M1
15	*K.pneumoniae*	DDST (+), CDT(-), E-test (-)	Non-producer	TEM-1, OXA-4, CTX-M1

DDST: double disk synergy test, CDT: combined disc test, NA: not applicable, not done in confirmed MBL producers.

Imipenem-EDTA Combined Disc Test (CDT)

Here test organism was inoculated as recommended by CLSI guidelines [Clinical and Laboratory Standards Institute] on Mueller Hinton (MH) agar and two 10µg Imipenem discs were placed keeping maximum distance from each other in a 90 cm culture plate. To one of the discs 10µL, 0.5 M EDTA solution was added. After 16 hours of incubation at 35°C, the zone of inhibition around Imipenem-EDTA discs if greater than 7mm, compared to the Imipenem discs alone, then the test organism was considered as MBL producing[13].

E-test

Bacterial suspension equivalent to of 0.5 McFarland standard was spread on MH agar medium by sterile swab stick. Then E- test strip which contained seven dilution range of Imipenem (4 to 256µg/ mL) on one side and seven dilution range of Imipenem (1- 64µg/ mL) with fixed concentration of EDTA on the other side, was placed on the surface of the medium and the plates were incubated at 35° C in air for 16-24hrs. After incubation the ratio of MIC values of Imipenem to Imipenem-EDTA was calculated. If it was greater than 8, then it was considered as a MBL producing strain. The extended zone between the junction of IMP and IMP-EDTA is called as phantom zone. If at phantom zone, inhibition of bacterial growth appeared, then it was also be considered as positive a result for MBL[14].

Those bacteria that showed positive result for at least one of the three phenotypic tests, were selected for PCR to detect different types of MBL genes present in them. In this study those bacteria in which MBL producing genes were detected by PCR, were not tested for the presence of ESBL genes thereafter. However, if MBL gene was not determined then further study was done to see for the presence of ESBL genes as all of these bacteria belonged to MDR variety. Primer pairs specific for each MBL gene (IMP-1, IMP-2, VIM-1, VIM-2) and for each ESBL gene (TEM-1, OXA-2, OXA-4, CTX-M1 and CTX-M9) were selected for PCR.

RESULTS

Out of 100 MBL producing bacteria, which were randomly selected, 55%,12%, 9%, 8%, 4%, 5%, 5% and 2% were isolated from urine, tracheal aspirates, sputum, pus, stool, blood, throat swab and pleural fluid respectively and it was seen that urine was the most important source of MBL producers (Fig- 1).

Drug resistance characteristics of isolated MDR strains

Antimicrobial susceptibility was done with aforementioned antibiotics for all collected strains and from resistance patterns we selected 15 MDR bacteria (Table-1) for PCR studies. They were mostly resistant to Cephalosporins (73.3-87%), Imipenem (80%) and Meropenem(66.6%), Piperacillin and Aztreonam (73.3% each), while least resistance was seen with Levofloxacin (33.3%). Amikacin and ofloxacin showed moderate resistance around 50%.

Phenotypic methods and PCR study (Table-2)

Strain 1 was identified as *E.coli.* Among three phenotypic methods we have studied, it showed positive result for combined disk test and E-test. No MBL producing gene was found in PCR amplification method with the four primer pairs but as the two phenotypic methods detected MBL production and the Imipenem hydrolysis activity was 0.288unit/mg, so further studies with other MBL primers could be done. As the bacterium was MDR, the presence of ESBL genes was determined with all primer pairs for ESBL genes and it has been seen that TEM1 and CTX-M1 genes got amplified.

Strains 2-11 were identified as *P. aeruginosa*. Here, only one strain (strain no: 6) was a non MBL and non ESBL producer, it showed positive DDST test and negative CDT and E-test. The Imipenem hydrolysis activity for the rest of the strains was ranged between 0.25 to 1.48 units /mg and they gave positive result for all three or at least two phenotypic detection methods. The PCR amplification method was employed for the presence of MBL genes carried by these strains and all strains were found to amplify VIM-2 primer, so they were VIM-2 producing. Two strains (strain no.5 and strain no.8) out of these nine *P.aeruginosa* strains, were identified as identical strains by antimicrobial susceptibility and isoelectric focusing test, these two strains were collected from two different patients admitted at the same time in ITU ward.

Four strains (strains: 12-15) were detected as *K. pneumoniae.* Strain 12 was an MBL non producer. It only showed a positive result to CDT among the three phenotypic tests. As it was a MDR bacteria PCR was done for ESBL genes but no amplified product was seen. Strains 13 and 15 were found to be ESBL producers. These two strains showed positive result against only one of the phenotypic tests. After a negative result of PCR analysis for MBL genes, ESBL production was verified for these two strains and result showed a confirmed ESBL production. Strain 15 was a TEM-1, OXA-4 and CTX-M1 producer, whereas strain 13 was responsible for the production of CTX- M1 enzyme.

Strain 14 was able to produce both MBL and ESBL enzymes. It clearly showed positive result for all three phenotypic methods and Impenem hydrolysis activity of this strain was 0.245unit/mg, although against the four MBL primers used in PCR, no genes got amplified, may be genes other othan IMP-1, IMP-2, VIM-1 and VIM-2, were present. PCR analysis for the presence of ESBL gene was done and it was found that the strain was a CTX-M1 producer.

Analysis of the MBL phenotypes and genotypes:

We used both phenotypic and genotypic methods for detecting MBL genes that were being carried by those MDR bacteria. Among the 15 strains, all strains showed positive result to minimum one and maximum three out of the three phenotypic methods but all of them were not identified as MBL producers after PCR amplification. Four of them were MBL non producing strains when verified by PCR.

Percentage of MBL producers

Three phenotypic methods (DDST, CDT, E-test) were used to screen MBL producing strains in laboratory for preliminary detection and afterwards PCR was done for confirming the presence of MBL genes. For this genotypic determination , 15 phenotypically detected MBL producers were selected and it was found that 4 (26%) of them actually had no MBL genes present in them, indicated false positive test. Out of 15 MDR Gram negative bacteria, 11(73.3%) were identified as MBL producers, among them the MBL type of two strains were unidentified and rest of the strains (81.8%) were VIM-2 of type MBL.

DISCUSSION

Generally extended spectrum beta lactam antibiotics are the drugs of choice for ITU and ICCU patients. However, their widespread implication raising the drug resistance dramatically among bacteria that causes most hospital acquired infections[15].

Drug resistance due to the frequent use of Carbapenems is getting worse gradually, therefore to treat MDR bacterial infection clinicians must restrict the random prescription of these antibiotics. Our study revealed that the presence of such deadly MBL and ESBL producing MDR bacteria in hospitals of Kolkata, India is very significant. In the present study we found that the MDR bacteria are highly resistant to 3rd and 4th generation Cephalosporin groups. Among beta lactam antibiotics Piperacillin has also showed high resistance to these bacteria. Beta lactam resistance of bacteria has been correlated to frequent use of 3rd generation Cephalosporins[16]. No effective MBL inhibitor has been discovered till date, therefore, the drug resistance in bacteria should be monitored throughout the globe.

Two strains (strains 5 and 8) that found identical, were collected from two adjuscent beds in the same ward. The bacteria was MBL producer and probably spread from one patient to the other, indicating a probable hospital cross infection. To reduce this type of hospital cross infection, infection control program should be strengthened for all hospitals.

We found different ESBL genes (CTX-M1, TEM-1, OXA-4) in our tested bacteria. CTX-M enzyme producing bacteria are mostly seen in South America and Europe among them CTX-M3 and CTX-M2 are mostly found. In our study we detected CTX-M1 producing *Klebsiella pneumoniae* strains. CTX-M type beta lactamase are increasing nowadays that gives resistance to Cefotaxime and Ceftriaxone. Nearly 40 different types of CTX-M enzymes have been identified [17, 18] and spread of this beta lactamase producing Gram negative bacteria is becoming a great concern as they cause outbreak of nosocomial infection [19, 20, 21, 22]. OXA type enzyme production are generally seen in *Pseudomonas aeruginosa,* however, in this study we found OXA-4 producing *Klebsiella pneumoniae* also.

Out of the four tested *Klebsiella pneumoniae* strains three were carrying ESBL genes, one *E.coli* was also an ESBL producer. *Klebsiella pneumoniae* also is an important pathogen involving nosocomial infection. From this genetic study it can be stated that these genes are spreading worldwide from one species to other probably through travelers. Travelers having infection with drug resistant bacteria visiting other countries if admitted to the hospitals there, may spread these bacteria and thus the intra and inter species spread of these drug resistant genes occur.

So, to restrict the overspread of drug resistant genes, special care must be executed in case of patients who has traveled out of a country recently, hence detailed patients' history should be taken regarding this. Spread of these bacteria may happen due to frequent transfer of patients between wards or it may be from contaminated hands of nursing stuffs.

The prevalence of MBL genotypes varies from one country to other [23, 24]. Two genotypes (VIM and IMP) of MBL producing bacteria are most common in Asian countries. VIM predominates in MDR bacteria present in Korea, Taiwan and China [25, 26]. In this study VIM-2 producers were noted and all were found in *Pseudomonas aeruginosa*. However, the genotypes of two strains were other than VIM or IMP types and remained unidentified.

After phenotypic and genotypic methods were compared it has been observed that 26% bacteria showed false positive test in phenotypic method. From the data of our study it can be suggested that for screening MBL producing bacteria, three phenotypic methods should give positive results, and we should not rely solely only on one method (especially DDST). However, for confirmation three methods should be followed by PCR.

Strain no.12 which gave negative result to DDST and E-test, was resistant to Imipenem, although it was a MBL non-producer. It seems that this resistance to Imipenem may be due to the alteration of outer membrane protein, Porin OprD.

In summery we can say that in Kolkata, India, MDR strains are increasing in an alarming rate, most of them are either MBL or ESBL producers. In all hospital laboratories three phenotypic methods should be followed for detecting MBL production by laboratory technicians and a drug resistance profile should also be made to lower the spread of these diseases and to decrease the morbidity and mortality rate in intensive care units.

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