British Microbiology Research Journal
5(4): 368-383, 2015, Article no.BMRJ.2015.038
ISSN: 2231-0886

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Phenotypic and Molecular Characterization of MBL Genes among Uropathogens Isolated in Mumbai City

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Authors' contributions

This work was carried out in collaboration between all authors. Both the authors equally contributed in the designing of the study and performing the statistical analysis. Author TM wrote the protocol, wrote the first draft of the manuscript and managed literature searches. Authors TM and KA managed the analysis of the study and literature searches. Both authors read and approved the final manuscript.

Article Information

DOI: 10.9734/BMRJ/2015/13762
Editor(s):
(1) Giuseppe Blaiotta, Department of Food Science, Via Università, Italy.
Reviewers:
(1) Jasmine Selvakumar, Biotechnology, Bishop Heber College, Trichy, India.
(2) Teresita Sainz-Espuñes, Departamento de Sistemas Biológicos, Universidad Autónoma Metropolitana-Xochimilco, México City, México.
Complete Peer review History: http://www.sciencedomain.org/review-history.php?iid=824&id=8&aid=6863

Original Research Article

Received 2nd September 2014
Accepted 30th September 2014
Published 6th November 2014

ABSTRACT

Aims: Growing incidences of the pathogens producing Metallo-β-lactamases (MBLs) has been observed in many countries including India. They are carbapenemases that are capable of hydrolysing carbapenems that are often considered as a last resort antibiotic, for infections caused by multiple drug resistant bacteria. The objective of the current study was to investigate the prevalence of New Delhi Metallo-β-lactamase (NDM-1) and other MBL producers among 747 uropathogens collected from Mumbai city.
Place and Duration of Study: The current study was carried out at Department of Microbiology, Wilson College, Mumbai 400007 between September 2011- January 2014.
Methodology: The screening of MBL producers was done using phenotypic detection tests such as antimicrobial susceptibility test, Modified Hodge Test and MBL E-Test. Molecular characterization of the test isolates was carried out using Molecular tests like Polymerase chain reaction and Restriction enzyme digestion.
Results: In the current study, 49 MBL producers were obtained, all of which harboured a plasmid of

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approximately 50 kb size. *Polymerase chain reaction* showed the presence of bla_{NDM-1} in 43/49, bla_{VIM-1} in 3/49, and bla_{IMP} gene in 3/49 MBL producers. Co-production of Extended Spectrum β-Lactamase namely, bla_{TEM} and bla_{CTX-M} genes were also observed among 25/49 MBL producers. Determination of Minimum Inhibition Concentration (MIC) showed only 35/49 MBL producers to be resistant to meropenem, indicating difficulty in routine MBL detection where it is generally carried out as a screening step. Plasmid borne resistance of MBL producers was confirmed by plasmid loss studies. Restriction enzyme analysis carried out with the help of Eco RI, Bam HI and HindIII revealed different band patterns among MBL producers, indicating clonal unrelatedness and more than one source of origin for spread of antibiotic resistance.

Conclusion: The current study may serve as a basis for better understanding of the resistance patterns of MBL producers, and thus restrict the spread of drug resistance.

Keywords: MBL, NDM-1, Modified Hodge test, PCR, E-test, restriction enzyme.

1. INTRODUCTION

Urinary tract infections (UTIs) represent the most frequent bacterial infections encountered in the community settings [1,2]. It is estimated that over 150 million people worldwide get infected with UTIs annually, making it one of the most common infectious disease [3]. UTI is characterized by the presence of organisms in the midstream urine sample, tested from patients. The most common uropathogens are *Escherichia coli, Pseudomonas aeruginosa, Enterococcus aerogenes, Klebsiella pneumoniae, Enterobacter clocae, Proteus vulgaris, Candida albicans* and *Staphylococcus aureus* [4]. Recently, antimicrobial resistance among uropathogens, causing uncomplicated cystitis has been on a rise; The main cause seems to be the prescription of the empirical therapy to the patients either without a urine culture examination or before the results become available [5,6]. Ecologically adverse effects of these continuous therapies have resulted in the emergence of extreme drug resistance which has only worsened with time. Recently there has been an increase in the number of Extended Spectrum β-Lactamase (ESBL) and carbapenemase-producing *Enterobacteriaceae* causing UTIs.

Carbapenemases belong to the molecular class A, B, and D β-lactamases. Class A and D enzymes have a serine-based hydrolytic mechanism for cleaving the β-lactam ring in antibiotics. Metallo β-lactamases (MBLs), on the other hand, are class B carbapenamases containing zinc at its active site [7]. They are the most common type of carbapenamase found to be associated with clinical infections worldwide [8].

The advent of β-lactamase producers has posed a great threat to the use of many classes of antibiotics, particularly cephalosporins. Amoxycillin (β-lactam antibiotic) was traditionally the first line therapeutic drug for the treatment of UTIs but with the spread of drug resistance, other options now used include amoxycillin-clavulanate and cephalosporins like cefixime, cefotaxime, and ceftazidime. Over the last 15 years, numerous outbreaks of infections with organisms producing β-lactamases have been observed worldwide [9].

The first MBLs detected and studied were chromosomal enzymes found in the environment and opportunistic pathogenic bacteria such as *Bacillus cereus* [10,11], *Aeromonas* spp. [12] and *Stenotrophomonas maltophilia* [13]. Although MBLs were originally found in bacterial species of limited clinical occurrence, they are now emerging as acquired resistance-determinants in major Gram negative pathogens such as members of *Enterobacteriaceae*, *Pseudomonas aeruginosa* and *Acinetobacter* spp., in which they can be responsible for ESBL resistance phenotypes. The most common transferable MBL families include the VIM, IMP, GIM, SPM and SIM-type enzymes which have been detected primarily in *Pseudomonas aeruginosa*, but were also found in other gram-negative bacteria including *Enterobacteriaceae*. Recently, 2 new subgroups of MBLs, designated NDM-1 and DIM-1, were identified in a clinical isolate from patients admitted in India and Netherlands respectively [14,15]. NDM-1 was first identified in 2008 in single isolates of *Klebsiella pneumoniae* and *E. coli*, both recovered from a patient repatriated to Sweden after treatment in a hospital in New Delhi, India [14]. DIM-1 gene was first identified in a clinical isolate of *Pseudomonas stutzeri* in Netherlands [15].

Like other acquired MBLs, NDM-1 hydrolyses all β-lactam antibiotics except for aztreonam which is usually inactivated by co-produced Extended-spectrum or AmpC β-lactamases. An association with other resistance mechanisms makes a majority of *Enterobacteriaceae* with bla_{NDM-1} gene extensively resistant to a vast group of antibiotics. Sometimes, simultaneous production of ESBL enzyme along with defects in the outer membrane porins, in members of Enterobacteriaceae, can be responsible for carbapenem resistance even in absence of carbapenamase production [16,17,18].

Carbapenemase-producing pathogens are considered as a serious nuisance as they have the ability to hydrolyze penicillins, cephalosporins, monobactams as well as carbapenems. In most cases, carbapenems are considered as agents of last resort antibiotics for the treatment of infections due to Metallo-β-lactamases producers [19].

Under such circumstances of extreme resistance towards antibiotics, the pathogens remain susceptible only to colistin and tigecycline combination therapy [20]. However, with its increased use, we may actually trigger the resistant mechanisms against these combinations in future leading to the end of the current era of pharmacopoeia [21].

Due to the continuous evolution of antibiotic resistance, regular monitoring of antibiotic resistance has become extremely necessary. In order to improve the antibiotic therapy, prior information about the most probable microorganisms and their susceptibilities according to the characteristics of the population must be known [22].

The objective of our study was to isolate and study the occurrence of NDM-1 and other MBL genes among uropathogens isolated from patients with UTIs obtaining treatment in Mumbai city.

2. MATERIALS AND METHODS

2.1 Bacterial Isolates

A total of 747 urine sample isolates were collected from 3 government tertiary-care hospitals, 4 private hospitals and 8 pathological laboratories in Mumbai city during September 2011- January 2014. The current study was carried out to estimate the frequency of occurrence of MBL producing uropathogens in Mumbai city. Urine-isolates were re-isolated on Mac-Conkey's (MAC) agar, a selective-differential medium, and Cystiene Lactose Electrolyte Deficient (CLED) Agar to ensure the purity of the culture. The isolates were further identified by means of standard morphological, cultural and biochemical tests carried out in the laboratory [23]. These isolates were grown on Nutrient agar slants at 37℃/24h and stored at 4℃ until further use.

2.2 Determination of Antimicrobial Susceptibility Test

All bacterial isolates were subjected to Antimicrobial Susceptibility Testing (AST) using the disc diffusion method as described by the Clinical and Laboratory Standard Institute [24]. *E. coli* ATCC 25922 was used as the quality control strain.

Dodeca discs (PBL-Bio-Disc- code # 612, PBL-Bio-Disc- code # 212, Pathoteq biological laboratories) were used for performing AST.PBL Biodisc 612 contained ticarcillin (85mcg), oxytetracycline (30mcg), ceftriaxone (30mcg), cefipime (30mcg), cefuroxime (30mcg), nalidixic acid (30mcg), norfloxacin (10mcg), amoxycillin-clavulanate (30mcg), cefadroxil (30mcg), cefoperazone (75mcg), ceftazidime (30mcg), polymyxine-B (300U) and PBL Biodisc 212 contained ampicillin (20mcg), co-trimoxizole (25mcg), cefotaxime (30mcg), piperacillin (100mcg), chloramphenicol (30mcg), ciprofloxacin (5mcg), ceftizoxime (30mcg), tetracycline (30mcg), ofloxacin (5mcg), gentamycin (10mcg), amikacin (30mcg), gatifloxacin (10mcg).

2.3 Carbapenem Susceptibility Screening and Confirmation

The susceptibility screening of the pathogens towards carbapenem antibiotic was performed by disc diffusion test using meropenem (10mcg) antibiotic [24]. CLSI guidelines were followed to interpret the breakpoints for screening of carbapenemase producers; all isolates with a zone diameter <23 mm were confirmed for carbapenamase production by the Modified Hodge test (MHT). To perform the MHT, a suspension of the carbapenem susceptible strain of *E. coli* ATCC 25922 was swabbed on Mueller Hinton (MH) agar plates. A 10 mcg meropenem disc was placed in the centre of the plate. The test isolates were streaked in a straight line from

the edge of the disc to the edge of the plate, and were incubated overnight. Positive test was indicated by presence of cloverleaf-like indentation at the intersection of the test organism and the standard strain, within the zone of inhibition of the carbapenem antibiotic [25,26]

2.4 Detection of MBL Producers by MBL E-test

Since MBLs contain zinc at their active site, the enzyme can be inactivated in presence of metal chellators like EDTA. Hence, confirmation of MBL production was done by the E-test method using Ezy-MIC™ Strips (Hi Media Laboratories Pvt. Ltd.) that contained a concentration gradient of imipenem (4-256mcg/ml) with and without EDTA (1-64mcg/ml) on either sides of the strip [27]. A standard MBL-producing *K. pneumoniae* strain provided by Hi Media laboratories and *E. coli* ATCC 25922 were used as positive and negative controls, respectively [27]. The interpretation of results was done as per CLSI guidelines mentioned in Table 1.

2.5 Determination of MIC

The MIC of meropenem against all MBL producers was determined using E-strip (Hi Media Laboratories Pvt. Ltd.) with a concentration gradient of 0.02-32 mcg/ml following CLSI guidelines [27]. Meropenem susceptibility determination criteria are indicated in Table 2.

2.6 Extraction of Plasmids

Plasmid extraction was carried out from overnight grown MBL producing uropathogens by SDS method. The extracted plasmids were treated with RNAase (10 mcg/ml) and then re-precipitated with 0.1 volume of 3M sodium acetate (pH 5.2) and two volumes of ethanol. The RNAase free plasmids were run on 1.2% agarose gel and visualized under UV, using Gel Documentation System to check for presence of plasmid DNA [28].

2.7 PCR Detection of MBL Genes

Gene sequences for variants of different MBL genes, found in the bacterial species were obtained from the GenBank Database (http://www.ncbi.nlm.nih.gov/pubmed). These variants were aligned using the Clustal W algorithm by means of the software MEGA 5.2 and a consensus sequence was generated [29]. The sequence was subjected to nBLAST (http://blast.ncbi.nlm.nih.gov) to check for any possible overlap of the consensus sequence with non-specific sequences. Both forward and reverse primers were synthesized by Sigma-Aldrich®. The primer sequences used in PCR are listed in Table 3.

Amplification reactions were performed in a 25µl reaction mixture volume, containing 2.5µl of 10X PCR reaction buffer, 1.5µl(1.5mM) $MgCl_2$,0.5µl (200µM) deoxynucleotide triphosphate-mix (dNTPs), 1µl(10pM) forward as well as reverse primers, and 1µl (3U/ml) of the Taq DNA polymerase (Bangalore Genie™, India). 1µl of the plasmid DNA extracted from the MBL producers were added to the reaction mixture as template and the reaction volume made was made up to 25µl using sterile double distilled water. The reaction mixture was subjected to PCR amplification using a MyCycler DNA thermal cycler (Eppendorf). The resultant PCR products were analyzed by Agarose gel-electrophoresis using a 2% Agarose gel prepared in TAE (Tris-Acetate-EDTA) buffer, and containing 5µlethidium bromide (5mg/ml). A molecular weight standard (100bp DNA ladder) was also included in the gel to confirm the amplicon size [28]. All results were documented using a Gel Documentation System post-electrophoresis.

2.8 Sequencing of the PCR Amplicon

The PCR amplicons were sequenced by using corresponding primers for specific genes listed in Table 2 and the entire sequence of each gene was compared with sequences in the GenBank nucleotide database (http://blast.ncbi.nlm.nih.gov/Blast.cgi).
Sequencing of the PCR amplicons was carried out at Sci Genomics Pvt. Ltd, Cochin, Kerela, India.

2.9 Plasmid Curing

The MBL producing uropathogens were incubated at 37℃ overnight under constant agitation with Nutrient broth containing acridine orange (50mcg/ml) to check for plasmid loss [30]. Curing was confirmed by carrying out plasmid extraction from the cured cells and checking for the absence of plasmids. Antibiotic sensitivity testing of the cured cells was also carried out to ensure plasmid mediated carbapenem resistance.

Table 1. Interpretative criteria for E-test

Report	Formula	Interpretative criteria
MBL positive strain	IPM: IPM+EDTA > 8	When the ratio of the value obtained for imipenem: (imipenem+EDTA) is greater than 8 or if a zone is observed on the side coated with imipenem+ EDTA and no zone is obtained on the side coated with imipenem.
MBL negative strain	IPM: IPM+EDTA ≤ 8	When the ratio of the value obtained for imipenem: (imipenem+EDTA) is less than or equal to 8.
MBL non-determinative	IPM: IPM+EDTA< 4:1	When no zone of inhibition is obtained on either side. In such cases resistance may be due to mechanisms other than MBL production. It should be further investigated before reporting or if the zones obtained are below the lowest concentration on both the sides, the strain should be tested with concentrations below the lowest concentration on the strips before concluding.

Table 2. Interpretative criteria for meropenem

Organisms	Susceptible	Intermediate	Resistant
Enterobacteriaceae	≤1mcg/ml	2mcg/ml	≥4mcg/ml
Pseudomonas aeruginosa	≤4mcg/ml	8mcg/ml	≥16mcg/ml

Table 3. List of primers used in the study

Target	Primer name	Primer sequence (5'-3')	Annealing temperature (°C)	Size of amplicon
bla_{NDM-1}	NDM-1-F	ATGGAATTGCCCAATATTATATGC	63	782 bp
	NDM-1-R	TCAGCGCAGCTTGTCGGCCATG		
bla_{IMP}	IMP-F	GTTTATGTTCATACTTCGTT	56	432 bp
	IMP-R	GGTTTAACAAAACAACCACC		
bla_{VIM}	VIM-F	TTTGGTCGCATATCGCAACG	62	500 bp
	VIM-R	CCATTCAGCCAGATCGGCAT		
bla_{TEM}	TEM-F	GGTGAAAGTAAAAGATGCTG	54	742 bp
	TEM-R	ACCAATGCTTAATCAGTGAG		
bla_{SHV}	SHV-F	ATGCGTTATATTCGCCTGTGTA	63	725 bp
	SHV-R	TTAGCGTTGCCAGTGCTCGATC		
bla_{CTX-M}	CTX-M-F	CTTAGCGCGGCCGCGCTACA	65	413 bp
	CTX-M-R	ACCAGAATCAGCGGCGCACGA		

2.10 Restriction Enzyme Analysis of Plasmids from MBL Producers

Restriction digestion of Plasmid DNA isolated from MBL producing uropathogens were carried out using Eco RI, HindIII, and Bam HI to compare the restriction pattern obtained and hence study the clonal relatedness between the pathogens. All the restriction enzymes were purchased from Bangalore Genie™, India. Restriction digestion was carried out in a 20µl reaction mixture containing 2µl of 10X restriction buffer, 2µl of restriction enzyme (10U/µl), 12µl Plasmid DNA, and 4µl deionized water. The reaction mixture was kept in a water bath maintained at 37°C for 3h, after which the digested plasmid fragments were analysed by agarose gel electrophoresis using 1.5 % agarose [28].

3. RESULTS

3.1 Bacterial Isolates Identified in the Study

In the present study, 747 gram negative urine isolates were collected from various healthcare centres. These isolates were identified as *Pseudomonas aeruginosa* (67), *Proteus mirabilis* (44), *Klebsiella pneumoniae* (121), *Escherichia*

coli (445), *Enterobacter aerogenes* (1), *Enterobacter cloacae* (1), *Enterococcus fecalis* (1), *Morganella morganii* (4), *Citrobacter diversus* (37), *Citrobacter amalonaticus* (7), *Acinetobacter baumanii* (4), and *Proteus vulgaris* (15).

Mumbai city has an area of about 233 sq. miles and a total population of over 20.5 million as per 2012 consensus. There are approximately 8.5 lakh people residing per sq. mile area of Mumbai city. In addition, the hot and humid climate observed in Mumbai city during most of the time period throughout the year allow diseases like UTI to become a major infectious disease.

In the current study the prevalence of *E. coli* and *K. pneumoniae* among total isolated uropathogens was found to be 59.57% and 16.19% respectively. *E. coli* is generally one of the most frequently encountered uropathogens in drug sensitive as well as drug resistant cases of UTI followed by *K. pneumonia* [31,32]. This fact is clearly reflected in the current study carried out in Mumbai city.

3.2 Determination of Antimicrobial Susceptibility test (AST)

Antibiotic sensitivity test of all the isolated uropathogens were carried out using Kirby Bauer method. Around 79.51 % (594/747) isolates were found to be resistant to more than 3 antibiotics. Among these cultures, 31.59 % (236/747) isolates showed resistance to over 70 % antibiotics used in the study including β-Lactam antibiotics (ampicillin, ticarcillin, amoxycillin, and piperacillin) and 3rd generation cephalosporins (ceftazidime, cefotaxime, and ceftriaxone).

3.3 Carbapenem Susceptibility Screening and Confirmation of MBL Production

Screening of carbapenamase producers by disc diffusion test revealed 64 isolates to be potential carbapenamase producers. All of them showed < 23mm zone size of inhibition towards meropenem antibiotic. In the current study, 58 isolates tested positive for carbapenamase production by MHT (Fig. 1), However, only 49 isolates (6.55%) were confirmed to be MBL producers by MBL E-test (Fig. 2). This included *E. coli* (71.42 %), *K. pneumonia* (14.28 %), *P. mirabilis* (6.12 %), *P. aeruginosa* (6.12 %), and *C. amalonaticus* (2.04%).

Since MHT is a general test for screening of MBL as well as other carbapenamase enzyme producers, 49 isolates were reported as confirmed MBL producers in the study. However, MBL gene detection was carried out for all 64 isolates that showed susceptibility to meropenem.

In a study carried out in Pune, India, MBL production was reported among 300/1546 gram negative ICU cultures isolated from different medical samples; Maximum resistance was observed among *P. aeruginosa* (47.6%) and *Acinetobacter baumanii* (41.66%) followed by *E. coli* (6.66%) and *K. pneumoniae* (4%) [33]. Another study carried out in Bhubaneshwar, India, *reported* 17.85% uropathogens to be MBL producers where *E. coli* and *K. pneumoniae* were found to be the main causative agents [34]. Researchers from Kanchipuram and Tamil Nadu, India, identified MBL producing *A. baumanii* strains in 66.6% and 14.8% cases respectively by MHT [35,36]. The resistance in the other 9 isolates may be due to production of class A (IMI, SME and KPC enzymes) or class D (OXA enzyme) carbapenamases which were not detected in the current study [37].

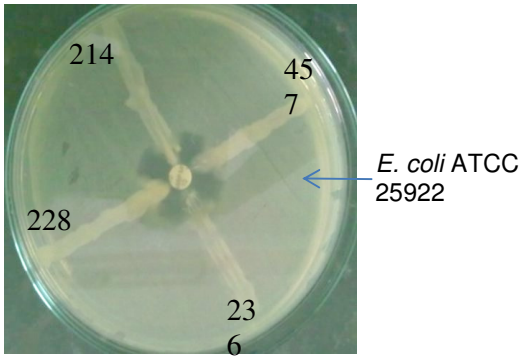

Fig. 1. Screening of MBL production by Modified Hodge Test. Isolate 214 (*P. aeruginosa*), 228 (*E. coli*), 236 (*E. coli*), and 457 (*K. pneumoniae)* showing a clover leaf indentation at the intersection of the test organism and the standard strain (*E. coli* ATCC 25922) indicating positive MHT

3.4 Determination of MIC

MIC of the identified MBL producers as well as the genes amplified from each organism is given in Table 4 below. MIC results showed 35 isolates to be resistant to meropenem. Eight isolates showed intermediate susceptibility, whereas 6 isolates were found to be sensitive to

meropenem. There was no co-relation observed between multiple gene production and increase in MIC.

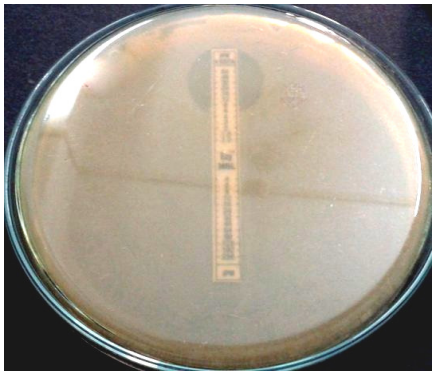

Fig. 2. Confirmation of MBL production by MBL E-test. Isolate 228 (*E. coli*) showing a zone of inhibition on the side coated with imipenem+ EDTA and no zone on the side coated with imipenem only indicating positive MBL E-test

The MIC of meropenem for six MBL producers i.e., *P. aeruginosa* (isolate 216), *E. coli* (isolates 238, 469, 611 and 623) and *K. pneumoniae* (isolate 614) was found to be in the sensitive range (< 1mcg/ml for Enterobacteriaceae and < 4mcg/ml for *P. aeruginosa*). Also, eight MBL producers including *E. coli* (isolates 234, 525, 537, 609, 621 and 625) and *P. mirabilis* (isolates 605 and 608) showed MIC of meropenem to be in the intermediate range (1-2 mcg/ml for Enterobacteriaceae and 4-8 mcg/ml for *P. aeruginosa*). This indicates that MBL production may not be associated with complete resistance to carbapenem antibiotic; Moderately resistant strains may also be potential MBL producers. The current data is also in accordance with many published studies. Franklin et al. (2006) reported 61% of MBL producing gram negative strains to have their MIC, of either imipenem or meropenem, in the susceptible range [38]. Another study reported 81% MBL producing *S. marcescens* strains to have their MIC of meropenem in susceptible range [39]. All the six meropenem susceptible isolates in this study showed an MIC of 0.64mcg/ml. There are reports of a possible co-relation between the hyper expression of MexCD-OprJ efflux system and imipenem hypersusceptibility in *P. aeruginosa* [40,41]. Hence the possible mechanism of susceptibility to meropenem in case of *P. aeruginosa* (isolate 216) can be explained. However, the mechanism of occurrence of such phenotype as observed in other organisms like *E. coli* and *K. pneumoniae* is not known. Eight isolates including *E. coli* (isolates 86, 220, 242, 522, 617, 624) and *K. pneumoniae* (isolates 202, 618) showed elevated MICs for meropenem (i.e >32mcg/ml) in the current study. An MIC of as high as 256mcg/ml of meropenem has been reported in *P. aeruginosa* [42] and *K. pneumoniae* [43] strains. Such high degree of resistance can be attributed to loss in porin, decreased outer membrane permeability and/or ESBL production, in addition to carbapenamase production [44].

3.5 Extraction of Plasmids

All 49 MBL producing uropathogens (Table 4) were found to possess approximately 50kb plasmid as shown in Fig. 3. The isolated plasmids were used as a template for PCR reactions.

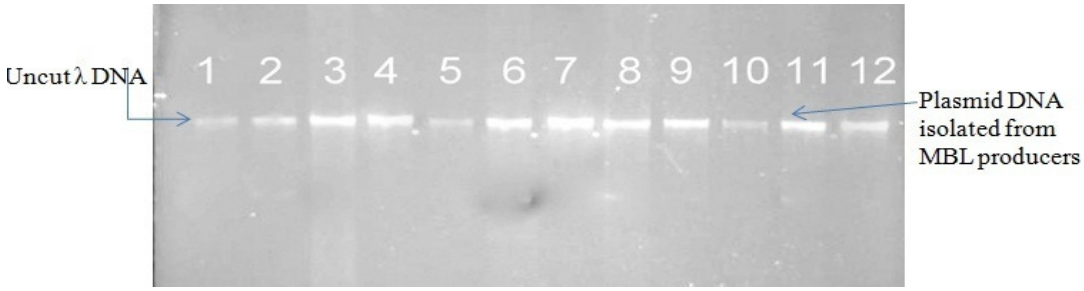

Fig. 3. Plasmid DNA extracted from MBL producing uropathogens. Lane 1: uncut λ DNA; lane 2: isolate 202 (*K. pneumoniae*); lane 3: isolate 213 (*E. coli*); lane 4: isolate 214 (*P. aeruginosa*); lane 5: isolate 216 (*P. aeruginosa*); lane 6: 135 (*C. amalonaticus*); lane 7: isolate 605 (*P. mirabilis*);lane 8: isolate 614 (*K. pneumoniae*);lane 9: isolate 608 (*P. mirabilis*);lane 10: isolate 237 (*E. coli*)

Table 4. MIC of meropenem against MBL producing uropathogens (n = 49)

Sr. no	Isolate no.	Identified as	MBL gene present	ESBL gene present	MIC (mcg/ml)	Susceptibility
1	75	*E. coli*	IMP	-	4	R
2	80	*E. coli*	NDM-1	CTX-M	8	R
3	85	*P. aeruginosa*	NDM-1	-	16	R
4	86	*E. coli*	IMP	-	>32	R
5	95	*K. pneumoniae*	VIM-1	TEM	8	R
6	101	*K. pneumoniae*	NDM-1	TEM, CTX-M	16	R
7	135	*C. amalonaticus*	NDM-1	TEM	8	R
8	202	*K. pneumoniae*	NDM-1	-	>32	R
9	213	*E. coli*	NDM-1	-	16	R
10	214	*P. aeruginosa*	NDM-1, VIM	CTX-M	16	R
11	216	*P. aeruginosa*	NDM-1	TEM	0.064	S
12	220	*E. coli*	NDM-1	CTX-M	>32	R
13	234	*E. coli*	NDM-1	-	2	I
14	236	*E. coli*	NDM-1	TEM, CTX-M	8	R
15	237	*E. coli*	NDM-1	-	8	R
15	238	*E. coli*	NDM-1	-	0.064	S
17	242	*E. coli*	NDM-1	TEM, CTX-M	>32	R
18	280	*E. coli*	NDM-1	CTX-M	16	R
19	457	*K. pneumoniae*	NDM-1	-	8	R
20	459	*E. coli*	NDM-1	TEM	4	R
21	461	*E. coli*	NDM-1	-	4	R
22	462	*K. pneumoniae*	IMP	-	16	R
23	463	*E. coli*	NDM-1	CTX-M	16	R
24	467	*E. coli*	VIM-1	TEM	16	R
25	469	*E. coli*	NDM-1	CTX-M	0.064	S
26	471	*E. coli*	NDM-1	-	4	R
27	473	*E. coli*	NDM-1	-	8	R
28	479	*E. coli*	NDM-1	-	8	R
29	493	*E. coli*	NDM-1, IMP	-	8	R
30	519	*E. coli*	NDM-1	-	4	R
31	520	*E. coli*	NDM-1	TEM	4	R
32	522	*E. coli*	NDM-1	CTX-M	>32	R
33	525	*E. coli*	NDM-1	TEM	2.5	I
34	526	*E. coli*	VIM	-	8	R
35	534	*E. coli*	NDM-1	TEM, CTX-M	8	R
36	537	*E. coli*	NDM-1	-	2	I
37	605	*P. mirabilis*	NDM-1	-	1.5	I
38	607	*P. mirabilis*	NDM-1	TEM	8	R
39	608	*P. mirabilis*	NDM-1	TEM	2	I
40	609	*E. coli*	NDM-1, VIM	CTX-M	2	I
41	611	*E. coli*	NDM-1, VIM	-	0.064	S
42	612	*E. coli*	NDM-1	-	4	R
43	614	*K. pneumoniae*	NDM-1	TEM, CTX-M	0.064	S
44	617	*E. coli*	NDM-1	-	>32	R
45	618	*K. pneumoniae*	NDM-1	-	>32	R
46	621	*E. coli*	NDM-1	TEM	2.5	I
47	623	*E. coli*	NDM-1	-	0.064	S
48	624	*E. coli*	NDM-1	CTX-M	>32	R
49	625	*E. coli*	NDM-1	TEM	2.5	I

3.6 PCR Detection of MBL Along with Co-produced ESBL Genes

Plasmid DNA obtained from MBL producing uropathogens was used as a template to amplify *bla*$_{TEM}$, *bla*$_{CTX-M}$, *bla*$_{SHV}$, *bla*$_{IMP}$, *bla*$_{VIM,}$ and *bla*$_{NDM-1}$ genes using its specific primers listed in Table 2. These were seen as discreet bands on 2% agarose gels when visualized under UV light (Fig. 4). PCR amplification showed presence of *bla*$_{NDM-1}$ (782 bp) gene in 43 out of 49 MBL producers, *bla*$_{VIM}$ (500 bp) gene in 3/49, and *bla*$_{IMP}$ (432 bp) gene in 3/49 MBL producers. 25/ 43 *bla*$_{NDM-1}$ gene was found to be co-produced along with either *bla*$_{TEM}$ or *bla*$_{CTX-M}$ gene. *bla*$_{SHV}$ gene was not detected in any of the isolates used in the study. Amplification of the MBL genes was not detected among the 15 MBL E-Test negative cultures. However, they showed presence of ESBL genes i.e *bla*$_{TEM}$ or *bla*$_{CTX-M}$.

In the current study, *bla*$_{NDM-1}$ gene was found among 87.75% of MBL producing uropathogens. Many studies have been carried out to report the prevalence of various MBL genes by PCR according to which *bla*$_{IMP}$ and *bla*$_{VIM}$are considered to be the most clinically important genes till now, due to their ability to spread among other major pathogens [45]. However, with the emergence of *bla*$_{NDM-1}$ gene, its prevalence has outnumbered the presence of other MBL genes. Since its outbreak in 2008, many studies have been published on its emergence and spread [14,15,25,44]. In a study done by Islam et al. (2012), it was found that around 3.5% of gram-negative organisms in Bangladesh were *bla*$_{NDM-1}$producers, based on a prospective surveillance of consecutive clinical samples tested in 2010 [46]. A study carried out in Mumbai city reported 22 of the 24 *K. pneumoniae* isolated from ICU patients to be *bla*$_{NDM-1}$producers [25]. Kumarasamy et al. [47] identified 44 isolates with *bla*$_{NDM-1}$in Chennai, 26 in Haryana, 37 in the UK, and 73 in other sites in India and Pakistan. In these studies *bla*$_{NDM-1}$ was mostly found among *E. coli* (36) and *K. pneumoniae* (111). A recent study in Saudi Arabia reported presence of *bla*$_{NDM-1}$ gene in 20% *K. pneumoniae* whereas *bla*$_{OXA-48}$ was present in 78.33% isolates [48]. NDM-1 is strongly linked to India and Pakistan and has been accumulating swiftly probably due to efficient plasmid transfer [25]. *P. aeruginosa* (isolate 214) and *E. coli* (isolates 493, 609, 611) showed presence of multiple MBL genes. However, they showed MIC of meropenem in the range of 0.064-16 mcg/ml. Presence of multiple MBL genes has also been reported by Dwivedi et al. (2009) in 3 of the 13 Enterobacteriaceae isolated in North India. The co-production of ESBL genes along with MBL genes has been also been observed in this study. Vidya Pai et al. [49] has reported co existence of ESBL and MBL genes in 1.2% cases in a medical hospital in Mangalore, India [49]. Another researcher has reported co-production ESBL and MBL genes in 8.79% of the cases observed in a tertiary care hospital in Amritsar which included 33.34% *E. coli*, 25% *P. aeruginosa* and 16.67% *K. pneumoniae* [50].

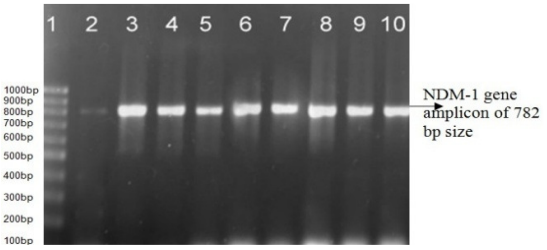

Fig. 4. Amplification of NDM-1 gene (782bp) by PCR. Lane 1: 100bp Molecular weight ladder; lane 2: isolate 234 (*E. coli*); lane 3: isolate 457 (*K. pneumoniae*); lane 4: isolate 605 (*P. mirabilis*); lane 5: isolate 608 (*P. mirabilis*); lane 6: isolate 242 (*E. coli*); lane 7: isolate 280 (*E. coli*); lane 8: isolate 457 (*K. pneumoniae*); lane 9: isolate 135 (*C. amalonaticus*); lane 10: isolate 214 (*P. aeruginosa*)

3.7 Sequencing of MBL Genes

PCR products of amplified NDM-1 genes were sequenced by Sci Genomics Pvt. Ltd, Cochin, Kerela, India. The resulting sequence was compared to gene sequences submitted in NCBI site using BLAST program which showed 100% identity to either one of the 2 sequences registered as Genbank accession no. KJ150693.1 or KJ131191.1.

3.8 Antibiogram Pattern of MBL Producing Uropathogens

The antibiogram pattern of the confirmed MBL producers indicated in Table 5 were analyzed, which showed them to possess almost complete resistance towards antibiotics that are routinely prescribed against UTIs, as compared to other uropathogens (Fig. 5). MBL producers showed more than 95% resistance towards all β-lactam antibiotics (ticarcillin, amoxycillin, ampicillin, and

piperacillin) and above 90% resistance towards 2nd and 3rd generation cephalosporins (ceftriaxone, cefepime, cefuroxime, cefadroxil, cefoperazone, ceftazidime, and cefotaxime). Twelve isolates showed complete resistance to all 24 tested antibiotics. Resistance to ciprofloxacin was observed in 85.71 % (42/49) of the MBL producers. However, resistance to amikacin (36.73%) and gatifloxacin (44.89%) was found to be comparatively lower than other antibiotics among MBL producers (Fig. 5).

Antibiogram of MBL producers showed high degree of resistance to β-lactam as well as non β-lactam antibiotics. Occurrence of MBL producers are associated with the use of carbapenems that is often used as a last resort antibiotic i.e., when the pathogens do not respond to treatments using other group of antibiotics [36]. However, resistance to amikacin (aminoglycoside) and gatifloxacin (flouroquinolone) was observed among 36.73% and 44.89% of MBL producers in this study. This suggests the use of carbapenem antibiotics even in non-complicated cases of UTI. A similar study from Puducherry, India, reported 36 strains of *A. baumanii* from non ICU patients to be completely resistant to imipenem but resistance to amikacin and ciprofloxacin (flouroquinolone) was reported in 80% and 72% strains respectively [51].

As carbapenems are used more frequently now days, a larger number of these enzymes can be expected in resistant populations [52]. There have also been reports of presence of NDM-1 producers in sewage water as well as drinking water in a study carried out in New Delhi [53]. Many studies have revealed that increased use of carbapenems to treat ESBL-producing organisms especially in ICU patients has been associated with the emergence of carbapenem-resistant organisms [54,55]. The exact status of prevalence of carbapenemase-producing bacterial strains remains unknown because many countries worldwide do not report rates of antibiotic susceptibility.

3.9 Plasmid Curing

The acridine orange treated uropathogens were tested for presence of plasmid and antimicrobial resistance. The extracted plasmids from cured cells were run on 1.2 % agarose gel along with a control plasmid isolated from test culture. All the cured cells showed absence of plasmids and susceptibility to meropenem antibiotic indicating plasmid borne carbapenem resistance. Plasmid curing results also showed the possibility of presence of β-lactam genes on the Plasmid DNA of MBL producing uropathogens as the cured cells showed sensitivity to most of the β-lactam and cephalosporin antibiotics. In addition, all the cured cells showed resistance to gentamicin and oxacillin, further indicating that the genes responsible for its resistance may be chromosomal. More than 50 % of the cured cells also showed resistance to polymixin-B and amoxycillin. Resistance to amikacin, tetracycline, ceftriaxone, ticarcillin, gatifloxacin, and ceftizoxime was lost in 90 % of the plasmid cured cells (Fig. 6). This indicates that the resistance to these antibiotics are due to acquiring of plasmid in majority of the cases. All MBL producers in this study harboured a 50 kb plasmid. Plasmids of varied molecular size have been observed among MBL producers.

Table 5. Total number of MBL producers isolated in the study

Pathogens	Total no. of isolates	MBL producers	NDM-1 gene
E. coli	445	35	31
K. pneumoniae	121	7	5
P. mirabilis	44	3	3
P. vulgaris	15	-	-
P. aeruginosa	67	3	3
C. amalonaticus	7	1	1
C. diversus	37	-	-
E. aerogenes	1	-	-
E. cloacae	1	-	-
E. fecalis	1	-	-
M. morganii	4	-	-
A. baumanii	4	-	-
Total	747	49	43

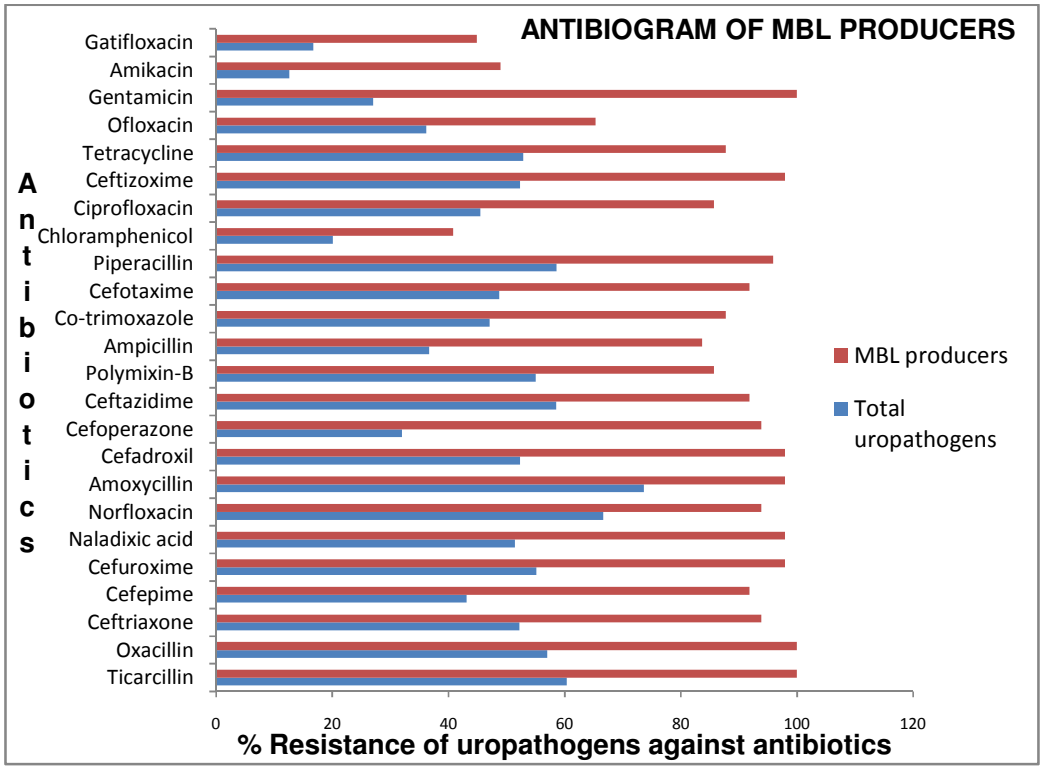

Fig. 5. Antibiogram pattern of MBL producers as compared to other uropathogens isolated in the study

A study carried out by Poirel et al. [56] reported presence of 4 plasmids (160kb, 130kb, 50kb and 80kb) in a MBL producing *E. coli* isolated from a patient shifted to Australia from Bangladesh. Another study by Kumarasamy et al. [47] reported 50-350kb plasmids among bla_{NDM-1} producers isolated in Chennai. Plasmid borne resistance may be one of the reasons in increased spread of MBLs as transmission of carbapenemase genes may occur readily when the gene is located within mobile elements such as plasmids and integrons [57]. Many published data has defined MBL producers as epidemic strains due to the fact that they show presence of plasmids and transposons bearing carbapenemases [58,36].

Plasmid cured cells of MBL producers showed sensitivity towards most of the antibiotics indicating plasmid borne resistance of the same. On the contrary, they showed sensitivity to gentamicin and oxacillin suggesting the genes for the resistance of these antibiotics may be chromosomal. Resistance to polymixin-B, ceftazidime, cefadroxil, and amoxicillin was observed among 40-60 % of the cured isolates. This indicates that resistance to these antibiotics may be mediated both chromosomally and/or by acquiring of plasmids. Plasmid mediated β-lactam and cephalosporin resistance has been reported by Philipon et al. [59] in gram negative bacteria. Aminoglycoside resistance is mostly encoded on plasmids but it also found to be present on other mobile genetic elements that may or may not be chromosomal [60,61]. In case of other antibiotics used in the study, which mostly consisted of cephalosporins and quinolones, resistance was observed only among 2-17% of the cured cells. Hence, the resistance to these antibiotics can be attributed to plasmids in most of the cases.

3.10 Restriction Enzyme Analysis of Plasmids from MBL Producers

Restriction digestion analysis of plasmids using EcoR1, Hind111 and BamH1 showed different band patterns for each of the 7 MBL isolates, indicating clonal unrelatedness and different source of acquiring MBL genes. Restriction digestion pattern of plasmids from MBL producers using EcoR1 enzyme is shown in Fig. 7.

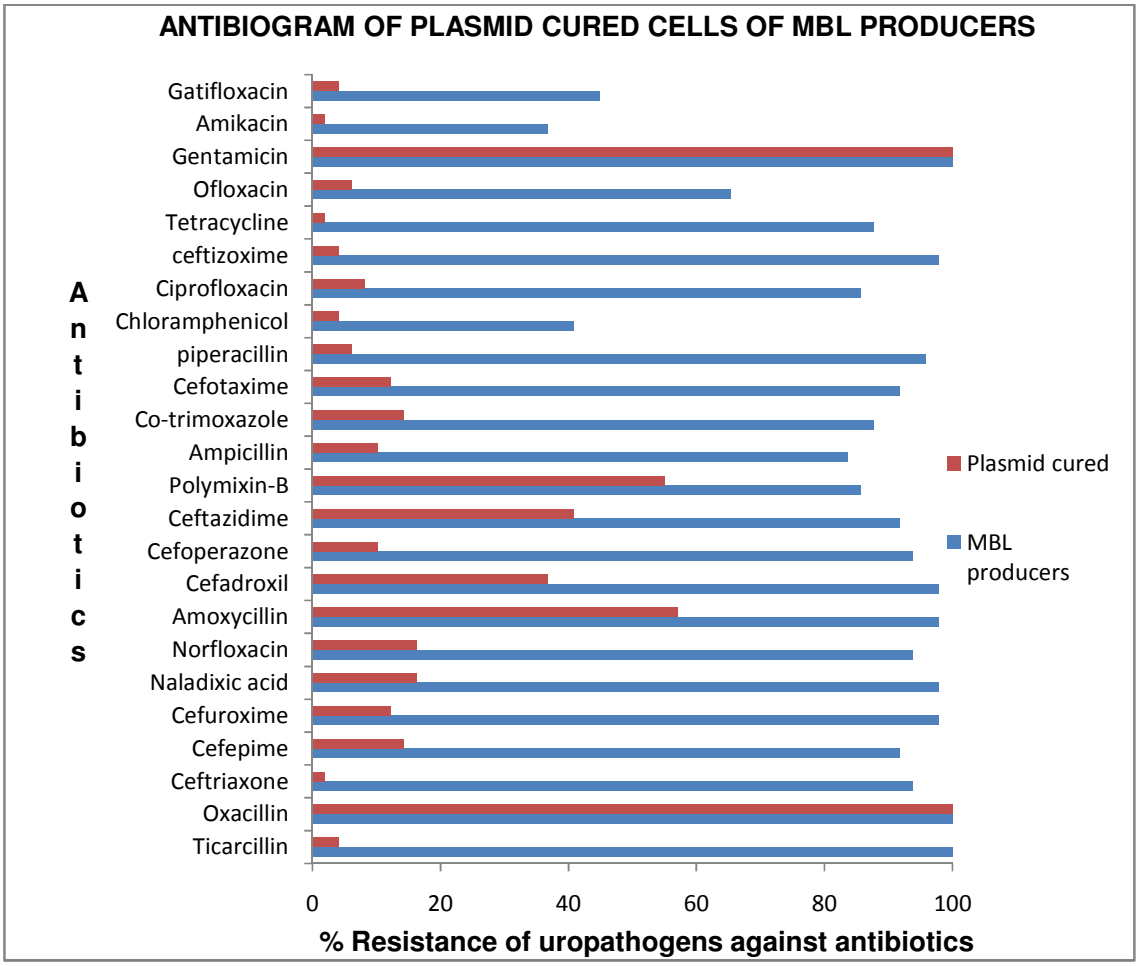

Fig. 6. Antibiogram pattern of Plasmid cured and Plasmid harbouring MBL producers

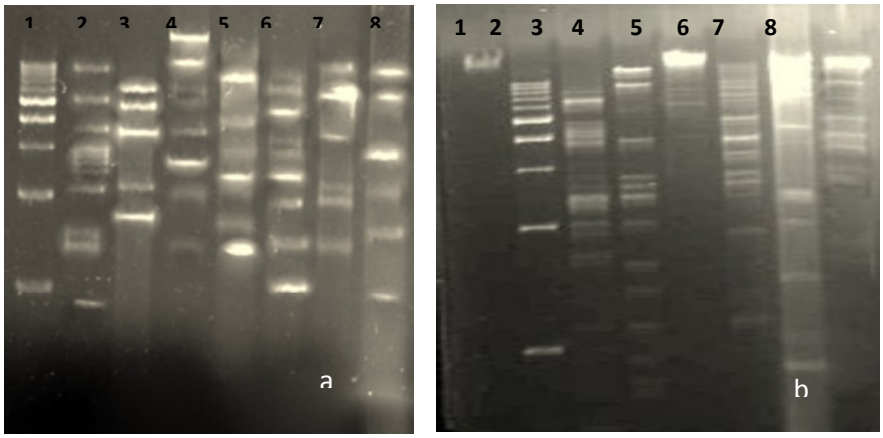

Fig. 7. Restriction digestion of plasmids from MBL producing isolates using EcoR1 enzyme. In Fig. 7a, lane1: 1Kb Molecular weight ladder; lane2: isolate 75 (*E. coli*); lane 3: isolate 80 (*E. coli*); lane 4: isolate 85 (*P. aeruginosa*); lane 5: isolate 86 (*E. coli*); lane 6: isolate 95 (*K. pneumoniae*); lane 7: isolate 101 (*K. pneumoniae*); lane 8: isolate 135 (*C. amalonaticus*). In Fig. 7b, lane 1: uncut λ DNA; lane 2:1Kb Molecular weight ladder;lane 3: isolate 214 (*P. aeruginosa*); lane 4: Isolate 216 (*P. aeruginosa*); lane 5: 135 (*C. amalonaticus*); lane 6: isolate 605 (*P. mirabilis*) lane 7: isolate 202 (*K. pneumoniae*); lane 8: isolate 213 (*E. coli*)

In this study, restriction enzyme analyses of plasmid DNA isolated from MBL producing uropathogens suggested that the strains have heterogeneous genetic backgrounds, although the gene responsible for carbapenem resistance in 87.75% (NDM-1 producing) isolates were identical. These findings suggest that MBL genes are clonally unrelated and there is more than one source of origin of carbapenem resistance. A previous study carried out in India showed that the bla_{NDM-1} producing *K. pneumoniae* isolates from North India are clonal whereas those isolated from Southern India are clonally diverse [47].

4. CONCLUSION

A worldwide spread of Enterobacteriaceae expressing carbapenemases represents a significant threat for the public health. It therefore requires proper and early detection and infection control strategies [62]. Fundamental studies based on the routine sampling and detection of MBL producers will help in better management of the disease by understanding the antibiogram pattern of the pathogens. It will also help in understanding the origin of resistance through molecular studies.

ACKNOWLEDGEMENT

This study is a Major Research project assigned and funded by the University Grants Commission (F. No. 40-111/2011(SR) dated 4th July 2011.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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