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PHENOTYPIC DETECTION OF CARBAPENEMASE PRODUCTION AND MOLECULAR DETECTION OF *blaOXA-51* AND *blaNDM-1* GENES AMONG THE CARBAPENEM RESISTANT ENTEROBACTERIACEAE IN A TERTIARY CARE RURAL TEACHING HOSPITAL

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ABSTRACT

Introduction: CRE contribute to increased morbidity and mortality among hospitalized patients. A knowledge of CRE prevalence helps in treatment and prevention of further spread of resistance.

Materials and methods: All meropenem resistant Enterobacteriaceae isolates were subjected growth on KPC chrome agar, MHT, EDS test, CDT and genotyping for detection of baOXA-51 and blaNDM-1 genes.

Results and discussion: Prevalence of CRE was 3.5%(25/713). 24/25(96%) CRE grew on KPC chrome agar, 64%(16/25) were positive for MHT, 80%(20/25) each for EDS and CDT. 13/25 harboured blaNDM-1, one blaOXA-51 and 7 both. We found good consistency between phenotypic and genotypic results. Ours is the pioneer study in India in detecting blaOXA-51 among Enterobacteriaceae.

Conclusion: Even though the prevalence of CRE is low, appropriate infection control measures are needed to prevent further spread of resistance.

KEYWORDS : Carbapenemases, KPC chrome agar, Modified hodge test, Combined Disk Test, EDTA disk synergy test

Introduction:

Resistance to broad spectrum antibiotics is an emerging problem among Enterobacteriaceae. Carbapenems are being used as the drugs of last resort for treatment of infection with these resistant organisms.[1] But recently, resistance to carbapenems is becoming uncommon among the members of Enterobacteriaceae. The increased prevalence of carbapenem resistant Enterobacteriaceae (CRE) is mainly due to the production of betalactamases that hydrolyze carbapenems (carbapenemases).[1] Though CRE infections are more common in health care settings, they are also community acquired. This is because of the ability of CRE to transfer resistant genes to one another through plasmids and chromosomes. CRE infections are threats because they can lead on to increased morbidity, mortality, hospital stay and treatment expenditure.[1]

Among various carbapenemases, New Delhi metallobetalactamases(NDM) are widely distributed in India and in other countries.[2] NDM producing Enterobacteriaceae isolates are globally important as they can spread rapidly in hospital and community because *bla*NDM genes are located in mobile genetic elements, facilitating their rapid transfer.[2]

Oxacillinases are another class of betalactamases with OXA-48 being widely distributed across the world.[3] *bla*OXA-51 is distributed in *Acinetobacter baumanii* isolates.[4] Though there are studies on *bla*OXA-51 in *Acinetobacter*, there is hardly any study on the same gene in Enterobacteriaceae. That is the reason for us detecting *bla*OXA-51 in CRE.

The main disadvantages of molecular techniques are their high cost, requirement of trained personnel and inability to detect novel genes.[5] Phenotypic detection of carbapenemases is easier and cheaper. In this study, various phenotypic tests have been used for detection of carbapenemases in CRE and each test has been compared with genotyping results to determine the efficacy of the phenotypic tests.

Materials and methods:

It is a hospital based prospective cross sectional study conducted in the department of Microbiology, Chennai medical college hospital and research centre, Irungalur from May to December 2015 (8 months). Ethical committee clearance was not sought as it is a sample based study. Patient details like age, gender, comorbid conditions, duration of illness and hospital stay were collected from laboratory request forms. Clinical outcome of patients following treatment were gathered from the medical records department. All Enterobacteriaceae isolates from heterogenous clinical samples were isolated and identified by standard laboratory techniques and were subjected to antibiotic susceptibility testing for Enterobacteriaceae panel of drugs (according to CLSI) by Kirby bauer disk diffusion method.[6] All the isolates that were identified as CRE by intermediate susceptibility or resistance to meropenem according to CLSI guidelines were subjected to further phenotypic tests (growth on KPC chrome agar, modified hodge test, EDTA disk synergy test and combined disk test) and were genotyped for detection of *bla*NDM-1 and *bla*OXA-51 genes.

Growth on KPC chrome agar:

KPC chrome agar preparation powder was obtained from Himedia and media was prepared according to manufacturer's instructions.[7] CRE isolates were plated onto the media and after overnight incubation, growth confirmed that the organism was resistant to carbapenems and specific colour indicated the specific genera of the organism (For example, *Escherichia* grew with magenta colour, *Klebsiella* grew with metallic blue colour.)[7]

Modified hodge test (MHT):

An overnight culture suspension of E.coli ATCC 25922 matched to 0.5 mcfarland turbidity standard was diluted ten times in peptone water and inoculated as a lawn over muller hinton agar(MHA) plate.[6] After drying for a few minutes, meropenem disk was placed over the centre of the plate and the test isolate was streak inoculated towards the four sides starting from the centre of the disk. Isolates producing a clover leaf shaped zone of inhibition around the meropenem disk, following overnight incubation of the plate were considered as carbapenemase producers according to CLSI guidelines.[6]

EDTA disk synergy test (EDS):

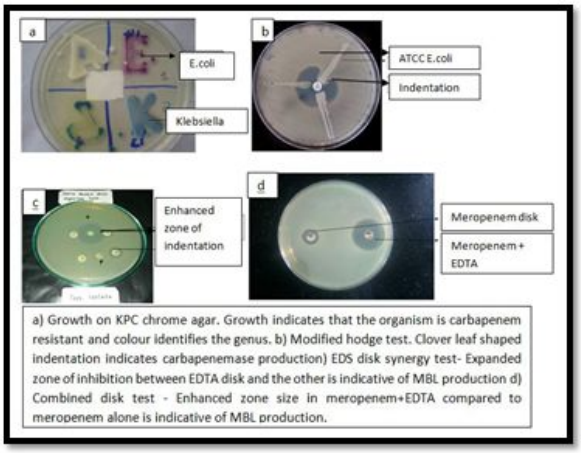
An overnight culture suspension of the test isolate matched to 0.5 mcfarland turbidity standard was inoculated as a lawn over MHA. Meropenem(10µg) and Ceftazidime(30µg) disks were placed over the lawn with EDTA disk in between them in such a way that the edge to edge distance was 10mm from EDTA.[8] Following overnight incubation, an expansion in the zone of inhibition between EDTA disk and either of the other disks was considered to be indicative of Metallobetalactamase production.[8]

Combined disk test (CDT):

An overnight culture suspension of the test isolate matched to 0.5 mcfarland turbidity standard was inoculated as a lawn over MHA.[8] A meropenem disk (10 µg) and another disk containing meropenem with EDTA were placed over the lawn. A 5mm increase in zone diameter of meropenem-EDTA disk compared to meropenem disk alone was considered to be indicative of metallobetalactamase

production.[8] The phentoypic tests are depicted in figure 1.

Fig1.Phenotypic tests performed in this study



All the CRE isolates were tested for tigecycline and colistin susceptibility.

Genotyping:

The CRE isolates were genotyped to detect blaNDM-1 and blaOXA-51 genes by multiplex PCR. Bacterial DNA was purified, extracted, mixed with specific primers (Helini ready to use NDM1 and OXA51 gene primer mix was used) and placed into PCR machine. An initial denaturation at 95°C for 5 mins, followed by 35 cycles of denaturation at 94°C for 30 sec, annealing at 58°C for 30 sec, extension at 72°C for 30 sec and a final extension at 72°C for 5 min were carried out. The products that were amplified were subjected to electrophoresis at 50 V in 2% agarose gel with ethidium bromide and the bands were visualized under ultraviolet light. The resistant genes were thus identified.

Results:

The number of samples that were processed was 3325, out of which 1019(46%) were gram negative bacilli. Out of those, 713 were Enterobacteriaceae isolates constituting 69.9%.

Out of 713 Enterobacteriaceae isolates, 25 were resistant to meropenem. So the prevalence of CRE was 3.5%. Out of 25 CRE, 12(48%) were *Klebsiella pneumoniae,* 10(40%) were *E.coli*, 2(8%) were *Proteus mirabilis* and 1(4%) was *Citrobacter freundii.*
Out of 25 CRE, 18(72%) were from pus samples, 5(20%) from sputum and 2(8%) were from urine.

89% CRE were from inpatients and 11% from out patients. Most of the CRE isolates(70%) were from patients above the age of sixty years. The isolates were more from surgical wards (71.43%) as against 28.57% from medical wards. Diabetes mellitus was the most common comorbid condition (43%) in patients infected with CRE, others being urinary tract catheterization, systemic hypertension, pulmonary tuberculosis and chronic kidney disease. 20 out of 25 (80%) CRE infected patients had been hospitalized for more than a week and all the hospitalized patients were on antibiotic treatment.

Four out of 25 *CRE* isolates (16%) were sensitive to gentamycin, which included 1 *E.coli* and 3 *Klebsiella* pneumoniae, 7/25 (28%) were sensitive to amikacin, which included 2 *E.coli*, 3 *Klebsiella pnuemoniae,* 1 *Proteus mirabilis.,* and 1 *Citrobacter freundii,* 13/25 (52%) were sensitive to cefoxitin, which included 7 *E.coli*, 2 *Proteus mirabilis,*. and 4 *Klebsiella pneumoniae*. All the isolates were resistant to ampicillin, ciprofloxacin, cotrimoxazole, ceftriaxone, cefotaxime, cefoperazone – sulbactam, aztreonam, imipenem and meropenem according to CLSI guidelines. All the isolates were found to be susceptible to colistin and tigecycline.

Growth on KPC chrome agar:

On testing all the meropenem resistant isolates for growth on KPC chrome agar, 24/25 isolates (96%) grew on KPC chrome agar showing that they were CRE, which included 12(50%) *Klebsiella pneumoniae*, 9(37.5%) *E.coli*, 2(8.3%) *Proteus mirabilis*, and 1(4.3%) *Citrobacter freundii.*

Modified Hodge Test (MHT):

Sixteen out of 25(64%) isolates were positive for MHT which included 7(43.75%) *Klebsiella pneumoniae,* 6 *E.coli (37.5%)*, 2(12.5%) *Proteus mirabilis and 1(6.25%) Citrobacter freundii.*

EDTA disk synergy (EDS) test:

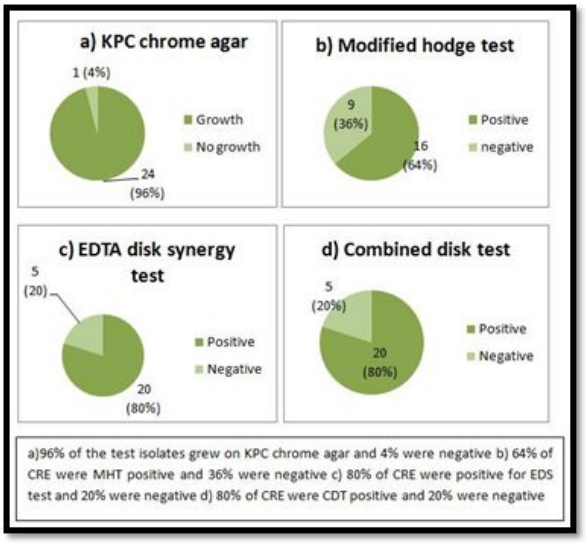
Twenty out of 25 CRE isolates (80%) were positive for EDS test which included 8 Klebsiellla pneumoniae, 9 E.coli, 2 Proteus mirabilis and 1 Citrobacter freundii.

Combined disk test (CDT):

Twenty out of 25 CRE isolates (80%) were positive for CDT which included 8(40%) Klebsiellla pneumoniae, 9(45%) E.coli, 2(10%) Proteus mirabilis and 1(5%) Citrobacter freundii.

The results of all the phenotypic tests performed in our study are shown in figure 2.

Figure2. Results of phenotypic tests



Results of genotyping:

On testing for *bla*NDM-1 and *bla*OXA-51 genes, 21/25 CRE isolates (84%) were positive for atleast one of the target genes. 13/25(61.9%) were positive for *bla*NDM-1 alone, 1(4.8%) for *bla*OXA-51 alone and 7(33.3%) for both *bla*NDM-1 and *bla*OXA-51 genes.=

So the total prevalence of *bla*NDM-1 among CRE is 80% (20 out of 25) which included 8(40%) *Klebsiella pneumoniae*, 9(45%) *E.coli*, 2(10%) *Proteus mirabilis* and 1(5%) *Citrobacter freundii*. The total prevalence of *bla*OXA-51 among CRE in this study is 32% (8 out of 25) which includes 4(50%) *Klebsiella pneumoniae*, 2(25%) *Ecoli* and 2(25%) *Proteus mirabilis.*

Combined results of phenotyping and genotyping are summarized in table 1.

Table1. Results of phenotyping and genotyping

S.NO	CRE ISOLATE	KPC	MHT	EDS TEST	CDT	blaNDM-1	blaOXA-51
1	*Klebsiella pneumoniae*	+	+	-	-	N	P
2	*Klebsiella pneumoniae*	+	+	+	+	P	P
3	*Klebsiella pneumoniae*	+	+	+	+	P	P
4	*Klebsiella pneumoniae*	+	+	+	+	P	N
5	*Klebsiella pneumoniae*	+	+	+	+	P	P

6	*Klebsiella pneumoniae*	+	-	-	-	N	N
7	*Klebsiella pneumoniae*	+	-	-	-	N	N
8	*Klebsiella pneumoniae*	+	-	+	+	P	N
9	*Klebsiella pneumoniae*	+	-	+	+	P	N
10	*Klebsiella pneumoniae*	+	-	+	+	P	N
11	*E.coli*	+	-	+	+	P	N
12	*E.coli*	+	-	+	+	P	N
13	*E.coli*	+	-	+	+	P	P
14	*E.coli*	+	+	+	+	P	N
15	*E.coli*	+	+	+	+	P	N
16	*E.coli*	+	+	+	+	P	P
17	*E.coli*	+	+	+	+	P	N
18	*E.coli*	+	+	+	+	P	N
19	*E.coli*	+	+	+	+	P	N
20	*E.coli*	-	-	-	-	N	N
21	*Proteus mirabilis*	+	+	+	+	P	P
22	*Proteus mirabilis*	+	+	+	+	P	P
23	*Citrobacter freundii*	+	+	+	+	P	N
24	*Klebsiella pneumoniae*	+	+	-	-	N	N
25	*Klebsiella pneumoniae*	+	+	+	+	P	N

Drug resistant Enterobacteriaceae cause many community and hospital acquired infections, carbapenemase producing Enterobacteriaceae being the most resistant, leading to increased morbidity and mortality among the hospitalized patients.

69.9% of gram negative bacilli were Enterobacteriaceae in our study, which is concordant with the study results of Lockhart et al and Sankarankutty et al.[9] The prevalence of carbapenem resistant Enterobactericaeae according to our study is 3.5%. The prevalence of CRE according to studies by Xu et al[10](2012), Alice et al[11](2015), Dutta et al [12]and PK Nair et al[13] were 1.6%, 2.93%, 7.8% and 12.26% respectively, which shows that prevalence of CRE is gradually increasing over years warranting immediate corrective action.

Patients above the age of sixty years are more commonly affected (70%) according to our study. The high susceptibility of this age group may be due to higher prevalence of co-morbid conditions like diabetes, chronic kidney disease (CKD), cancer and other immunocompromised states.

Out of 25 CRE, 12(48%) were *Klebsiella pneumoniae,* 10(40%) were *E.coli*, 2(8%) were *Proteus mirabilis* and 1(4%) was *Citrobacter freundii* which is concordant with the study results of P Nair et al.[13]Out of 25 CRE, 18(72%) were from pus samples, 5(20%) from sputum and 2(8%) were from urine. The higher prevalence of CRE from pus samples shows that the surgical and post-operative wards of our healthcare setting are lagging behind in infection control practices, as most of the pus isolates in our study were received from surgical and post-operative wards.

89% CRE were from inpatients and 11% from out patients. This indicates that most of the drug resistant infections are hospital acquired rather than community acquired. Prolonged hospitalization is a strong risk factor for acquiring these resistant bugs, as most of the patients infected with CRE in our study had been hospitalized for more than a week. All the CRE infected patients in our study were on antibiotic treatment. So, irrational antibiotic usage must be avoided in hospitalized patients to prevent emergence of resistant mutants.

Diabetes mellitus was the most common comorbid condition (43%) in patients infected with CRE, others being urinary tract catheterization, systemic hypertension, pulmonary tuberculosis and chronic kidney disease. This is concordant with the study results of Alice et al.[11] Immune compromised states make patients easily susceptible to infections with drug resistant organisms.

Some of the isolates resistant to imipenem showed sensitivity to aminoglycosides like gentamycin and amikacin. All the CRE isolates in our study were sensitive to tigecycline and colistin. This finding suggests that these two drugs can be used to treat CRE infections. More than 90% of the patients responded well to treatment with these two drugs. However, we must be cautious enough to reserve these antibiotics for carbapenem resistant infections alone. Inappropriate use can make bacteria resistant even to these drugs.

Phenotypic tests:

In this study, growth on KPC chrome agar was used for detecting CRE. Modified hodge test, combined disk test and EDTA disk synergy test were used for detection of carbapenemase producers among the CRE.

Growth on KPC chrome agar:

96% of isolates grew on KPC chrome agar showing that they were CRE. KPC CHROM agar has a high sensitivity of 100% and specificity of 98.4% for detecting CRE. KPC, IMP, VIM, NDM, and OXA-48-producing CRE can be detected by KPC chrome agar.[7]

Modified hodge test:

Sixteen out of 25(64%) isolates were positive for MHT in our study. MHT positive CRE included 7(43.75%) *Klebsiella pneumoniae,* 6 *E.coli (37.5%)*, 2(12.5%) *Proteus mirabilis and 1(6.25%) Citrobacter freundii,* which is similar to the findings of a study by Amjad et al.[14] MHT is the phenotypic confirmatory test for carbapenemases according to CLSI. It is most sensitive for detection of KPC carbapenemases.[6] It has varying sensitivity for other types of carbapenemases. It is not done as a routine, but only for epidemiological purposes. It can also give false positives with ESBL and Amp C producers.[6] Only 64% MHT positivity in our study may be due to the other CRE isolates producing non class A carbapenemases.

EDTA disk synergy test:

Twenty out of 25 CRE isolates (80%) in our study were positive for EDS test. Thus the prevalence of metallobetalactamase(MBL) producing CRE according to our study was 80%. MBL producers included 8 *Klebsiella pneumoniae*, 9 *E.coli*, 2 *Proteus mirabilis* and 1 *Citrobacter freundii*. The sensitivity and specificity of EDS test to detect MBL producers were 95% and 92% respectively according to a study by Yong et al.[15] Lee et al. have proved 100% sensitivity and specificity for MBL detection.[16] Many bacterial isolates that produce MBL are not detected by the MHT, but can be detected by the sensitive EDS test.

Combined disk test:

Twenty out of 25 CRE isolates (80%) were positive for CDT. The ability of the test to detect MBL producers is similar to that of EDStest according to our study. Behera et al.[17]reported equal efficacy of both combined disk test and EDS test which is concordant with our test result.

On comparing the four phenotypic tests with each other, growth on KPC chrome agar has picked up 96% of CRE showing it to be highly efficient in detection of CRE. Modified hodge test has the lowest sensitivity for detection of carbapenemases among all the four tests. EDTA disk synergy test and combined disk test detects carbapenemases better than MHT does. This finding is concordant with the study results of Shivaprasad et al.[18]

Out of 25 CRE isolates, 13(61.9%) were positive for *bla*NDM-1(Newdelhi metallobetalactamse) alone, 1(4.8%) was positive for *bla*OXA-51(oxacillinase producers) alone and 7(33.3%) were positive for both the genes, ie. co-producers of Newdelhi metalobetalactamase and oxacillinase. This prevalence is concordant with the study results of Shenoy et al.[19] So the total prevalence of blaNDM-1 among CRE is 80% (20 out of 25). Majority of the NDM producing isolates were *Klebsiella spp.,* and *E.coli,* which is also in concordance with the study by Bora et al.[20]The total prevalence of *bla*OXA-51 among CRE in this study is 32% (8 out of 25).

All the isolates that were found to be positive and negative for *bla*NDM-1 by genotyping were found positive and negative respectively for EDS test and CDT. This shows that EDS and CDT are 100% sensitive and specific for the detection of metallobetalactamses, concordant with Lee et al.[15] This proves EDS test and CDT to be the two most reliable phenotypic tests for detection of metallobetalactamase producers.

All the *CRE* isolates that were found to harbour *bla*NDM-1 or *bla*OXA-51 grew on KPC chrome agar which shows that KPC chrome agar is highly efficient in detecting NDM-1 and OXA-51 producers.

All the *bla*OXA-51 positive CRE isolates in our study were found MHT positive, which shows that MHT has 100% sensitivity for picking up oxacillinase producers. 14 out of 20(70%) *bla*NDM-1 positive isolates were MHT positive. Thus the sensitivity of MHT for detection of NDM according to this study is 70%. However, 7 out of 14 isolates were found to harbour both *bla*NDM-1 and *bla*OXA-51 genes, making the specificity of MHT for metallobetalactamase detection doubtful, as the MHT positivity in those 7 isolates might have been due to oxacillinase production and may not be due to metallobetalactamase alone. In a study by Jyothi et al conducted in Bangalore, published in 2014, 76.47% of metallobetalactamase producers were identified by MHT, which is concordant with our results.

*bla*NDM-1 gene located on plasmid carries many other resistance determining genes (upto 14) conferring resistance to other group antibiotics in addition to carbapenem resistance.[19] This is the reason why the bacterial isolates resistant to carbapenems in our study has been non susceptible to many other groups of drugs too.

In our study, 40% (8 out of 25) of the isolates were positive for *bla*OXA-51 gene. Niu et al (2015) identified *bla*OXA-51 in all the 93(100%) *Acinetobacter* isolates that were tested.[21] Budak et al suggested *bla*OXA-51 to be intrinsic to *Acinetobacter baumanii.* A patient with Ventilator associated pneumonia co-infected with *Klebsiella pneumoniae* and *Acinetobacter*, with the *Klebsiella* harbouring *bla*OXA-51 gene was reported in their study.[22] The reason for *bla*OXA-51 gene in that *Klebsiella* isolate was suspected to be due to chromosome or plasmid mediated transfer from *Acinetobacter*. However, PCR and other conjugation experiments proved their suspicion false.[22]The reason behind the *Klebsiella pneumoniae* harbouring *bla*OXA-51 remains obscure. In a study by Leski et al, in Sierra Leone *bla*OXA51 like gene was detected in *Klebsiella pneumoniae*, *Enterobacter cloacae* isolates and *E.coli* suggesting that the gene is no longer confined to *Acinetobacter* alone[23]. Though there are a considerable number of studies on *bla*OXA-51 in *Acinetobacter* in India, there are hardly any studies on *bla*OXA-51 in *Enterobacteriaceae* in India, and hence molecular detection of *bla*OXA-51 gene has been carried out in this study making our study the pioneer one in India to detect *bla*OXA-51.

With all these results, it is obvious that carbapenemase producing *Enterobacteriaceae* are prevalent in our setting.

Conclusion:

The prevalence of CRE in our setting is considerably low, probably due the rural population that is relatively naive to antibiotics. Even then, the presence of these superbugs itself strongly suggests the need to prevent their further spread, which can be done by appropriate use of antibiotics, hand hygiene measures, health education of the patients regarding strict compliance to prescribed antibiotic regimen and to avoid over the counter antibiotics, up to date vaccinations and isolation of patients carrying drug resistant organisms. All these measures can prevent us from reaching the post antibiotic era where common diseases can kill us once again. Carrying out researches/studies on drug resistant organisms, formulation of antibiotic policies for health institutions and antibiotic stewardship programs can aid to a great extent in proper antibiotic usage and prevention of further spread of resistance, thereby paving a way for a healthy community.

Relationship between the phenotypic and genotypic tests when analysed showed that the consistency between the two were considerably good. KPC chrome agar is highly efficient in picking up CRE. EDTA disk synergy test and combined disk test are 100% sensitive and specific for metallobetalactamse detection. So simple phenotypic tests can be used in laboratory for detection of carbapenemase producers.

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