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Original Research Article

DETECTION OF CARBAPENEM AND COLISTIN RESISTANT GRAM-NEGATIVE BACTERIA IN A TERTIARY CARE HOSPITAL, VISAKHAPATNAM, INDIA.

Running title: Antibiotic resistance

ABSTRACT:

Aims: Among the most clinically significant multidrug-resistant bacteria are the carbapenemase producing Enterobacteriaceae (CPE) which are detected all over the globe, with a marked endemicity according to enzyme type. These bacteria usually remain susceptible to polymyxins like colistin; however increasing use of colistin causes acquired colistin resistance which may now be added to the carbapenem resistance trait in Enterobacteriaceae. The present study was aimed to isolate and identify Gram-negative Enterobacteriaceae organisms from various clinical samples of patients suffering with various infections and to study the antibiogram of Gram-negative Enterobacteriaceae organisms with reference to carbapenem and colistin antibiotics. **Methodology:** Different clinical samples collected from both In-patients and Out-patients constituted the material for the study. Demographic variables of the patient were recorded include age, sex, type of patient (in-patient or out-patient) and type of samples. Standard microbiological techniques were used for the identification of pathogenic bacteria which include Gram staining, cultural and biochemical methods. Antibiotic susceptibility testing was performed according to Clinical Laboratory Standards Institute (CLSI) guidelines using Mueller-Hinton agar plates. Extended spectrum beta-lactamase (ESBL) production was tested with the CLSI confirmatory test using Ceftazidime (30 µg) disc alone and in combination with Clavulanic

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acid (10 µg). The bacterial isolates which were resistant to imipenem through disk-diffusion method were regarded to be screening positive and were further confirmed by EDTA combined disc test. Modified-Hodge test and E-test method were used to identify the carbapenem and colistin resistant strains. Frequency and percentages were calculated for categorical and ordinal variables. Chi-square test was carried out and p value ≤0.05 were considered statistically significant. **Results:** Male predominance (59%) is seen while compared to females (41%). The maximum cases were reported in the age group 51-60 years (31%). Majority of the isolates like Klebsiella species (30.6%) followed by *E.coli* (25.9%) and few isolates reported were Proteus species (5.8%). Extended spectrum beta-lactamase production was identified in test isolates at a range of 95.2% by using ceftazidime/clavulanic acid combination antibiotic susceptibility test. The prevalence rate of metallo-beta lactamase isolates was quite high i.e. 66.6% and carbapenemase producers was 77.3%. The major isolates were Klebsiella spp. and *E.coli* shows metallo-beta lactamase production. **Conclusion:** The study helps the clinicians in choosing the correct antimicrobial agent which contribute not only to better treatment but their judicious use will also help in preventing the emergence of drug resistant strains which are still sensitive.

Key words: Gram-negative bacteria, Carbapenems, Colistin, EDTA combined disc test Modified-Hodge test

INTRODUCTION: Gram-negative bacilli (GNB) are known causative agents of both community and hospital associated infections and responsible for high morbidity and mortality rates across all gender and ages. Clinical significance of GNB isolates is further heightened, because of their acquisition and dissemination of resistant genes in both community and hospital acquired infections and has been known to worsen the antibiotic treatment and management of patients. These organisms are highly responsible for causing urinary tract infections, pneumonia, peritonitis, meningitis, sepsis and medical device associated infections [1]. At the beginning of a new millennium, multidrug resistant Enterobacteriaceae producing Extended-spectrum Beta-lactamases (ESBLs) and acquired AmpC-type cephalosporinases have already spread worldwide, mainly as nosocomial pathogens, but also in the community [2]. Among the most clinically significant multidrug-resistant bacteria are

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carbapenemase producing Enterobacteriaceae. Carbapenemase producing Enterobacteriaceae (CPE) has already been detected all over the globe, with a marked endemicity according to enzyme type. The infections caused by CPE are associated with significant mortality. The mortality rates ranged from 22% to 72% [3]. The reasons for emergence of CPE is multifactorial, including underlying diseases, delay in initiation of effective therapy and lack of effective antimicrobials [4]. The choice of treatment for CPE producing Enterobacteriaceae is very limited. In vitro susceptibility to colistin, tigecycline and aminoglycosides is mostly preserved, but the impact of these antibiotics in vivo is still uncertain and the mortality rates remain high, despite treatment according to the results of susceptibility testing [5]. Some studies have suggested that combination therapy could be associated with a better outcome than monotherapy. However, there is still not an equal substitute for carbapenems in the treatment of severe infections caused by multidrug resistant Gram-negative bacteria [6]. Because these bacteria usually remain susceptible to polymyxins like colistin, an old class of antimicrobial drugs almost abandoned in the 1970s because of their potential toxicity, interest in polymyxins has been renewed worldwide. However, the increasing use of colistin explains why acquired colistin resistance may now be added to the carbapenem resistance trait in Enterobacteriaceae. So there is a need for tests that enable rapid detection of carbapenem and polymyxin resistance in Enterobacteriaceae and that may contribute to its containment. Hence the present study employs some rapid tests for the detection of carbapenem and colistin resistant strains of Enterobacteriaceae isolated from the various infectious patient samples for the disease management therapy and to prevent the dissemination of resistant genes among the Gram-negative bacilli. The present study is aimed to isolate and identify Gram-negative Enterobacteriaceae organisms from various clinical samples of patients and to study the antibiogram of Gram-negative Enterobacteriaceae organisms with reference to carbapenem and colistin antibiotics.

Material and Methods: Different clinical samples collected from both In-patients (IP) and Out-patients (OP) (GIMSR hospital, Visakhapatnam) constituted the material for this study. The clinical specimens include pus, urine, blood, endotracheal aspirates, sputum, ascitic fluid etc. Demographic variables of the patient recorded were age, sex, type of patient (in-patient or out-patient) and samples. Gram's staining was performed initially to study the morphological characteristics of the clinical isolates.

Inclusion and Exclusion criteria:

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Inclusion criteria:

- All bacterial isolates belonging to the family Enterobacteriaceae were tested.
- Any isolate which is resistant to at least two generations of cephalosporins.
- The patient Socio-demographic status was included in the study.

Exclusion criteria:

- Non-Enterobacteriaceae isolates were excluded.
- Patients with clinical evidence for any of the following clinical syndromes like endovascular infection including endocarditis, osteomyelitis, prosthetic joint infection, meningitis, renal abscess, fungal urinary tract infections, permanent obstruction of the urinary tract, and/or other central nervous system infections were excluded in the study.

Identification of bacterial isolates belonging to Enterobacteriaceae family:

The specimens were examined to detect, isolate and identify the pathogens by Microscopic examination, Culture methods and Biochemical characteristics. Microorganisms were tested for their motility through Hanging drop method. The morphological examination of the microorganisms was performed through Gram's staining reaction and the observations like shape and arrangement of organisms were noted. Pink coloured were gram-negative bacteria and violet coloured were gram-positive bacteria [7]. The culture of pathogens enables colonies of pure growth to be isolated for identification and antimicrobial susceptibility testing. The specimen is streaked on the culture plates (Blood agar, Mac Conkey agar) and incubated at 37^0C for 24 hours. Urine samples were inoculated onto CLED agar. The colony size, shape, margin, any pigmentation, whether the colony surface is dry or mucoid, whether growth from blood agar showed any associated haemolysis is noted. Following Culture methods, biochemical tests are often required to identify pathogens by using substrates and sugars to detect their enzymatic and fermentation reactions. The tests include carbohydrate fermentation tests with glucose, lactose, sucrose, xylose, mannitol and maltose etc. The organisms were also tested for Indole production, Methyl red test, Voges-proskauer test, Citrate utilization, Urease test, Oxidase test, Catalase test, Nitrate reduction test, Triple sugar iron agar test [7] etc.

Antibiotic susceptibility testing:

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Disk diffusion method: Disk diffusion is based on the determination of an inhibition zone proportional to the bacterial susceptibility to the antimicrobial present in the disk. The diffusion of the antimicrobial agent into the seeded culture media results in a gradient of the antimicrobial. Antibiotic susceptibility testing was performed according to Clinical Laboratory Standards Institute (CLSI) guidelines using Mueller-Hinton agar (MHA) plates using the concentration of antibiotics per discs, recommended by the WHO experts committee on biological standardization. The plates were incubated at 37°C for 16-18h hrs. The antibiotic discs used in this study were Piperacillin (100µg), Amikacin (30µg), Gentamicin (10µg), Ceftazidime (30µg), Ciprofloxacin (5µg), Imipenem (10µg), Ampicillin/Sulbactum (10/10 µg), Colistin E strips, Ampicillin (10 µg), Amoxyclav (20/10 µg), Co-trimoxazole (1.25/23.75 µg), Cefepime (30 µg), Cefuroxime (30 µg), Ceftazidime/Clavulanic acid (30/10 µg), Nitrofurantoin (300 µg), Tetracycline (30 µg). The inhibition zone was determined and compared with CLSI guidelines (CLSI Catalogue, 2016) [7].

Extended spectrum beta-lactamase production (ESBL): ESBL production was tested with the CLSI confirmatory test using Ceftazidime (30 µg) disc alone and in combination with Clavulanic acid (10 µg). At least 3cm distance was maintained between the disks. The test was considered positive when an increase in the growth-inhibitory zone around the CAZ disc with CA was 5 mm or greater of the diameter around the disk containing CAZ alone. The plates were incubated at 37°C for 18h [8].

Detection of Metallo-beta-lactamase production (MBL) by EDTA combined disc test: In the present work, the bacterial isolates which were resistant to imipenem through disc-diffusion method were regarded to be screening positive and were further confirmed by EDTA combined disc test. Initially Muller-Hinton agar (MHA) plates were prepared and inoculated with the test isolates through spread plate technique and later two imipenem (10µg) discs were placed in a MHA plate at a distance of 20mm from center to center on it. Then 10µl of freshly prepared EDTA solution at a concentration of 0.5M was added to one of the imipenem disc and incubated at 37°C for 16-18h. A difference in the zone diameter between Imp and Imp with EDTA of ≥7mm was considered to be positive for carbapenemase production [8].

Detection of Carbapenemase producing organisms by Modified Hodge test: In this test, the production of carbapenemase was identified if the tested microbial isolate was able to produce enzyme and permits the growth of standard strain *E.coli* ATCC 25922 towards a carbapenem disc.

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The results were noted based on the observation of clover leaf-like indentation. Initially 0.5 McFarland dilution of the standard strain *E.coli* ATCC 25922 was prepared in 5 ml of Mueller Hinton broth (MHB). Now 1:10 dilution of the culture was prepared by adding 0.5 ml of the 0.5 McFarland to 4.5 ml of MHB broth. A lawn culture was made by streaking an aliquot amount of diluted standard culture on MHA plate and allowed to dry 5 minutes. Imipenem disc at a concentration of 10 µg was placed in the center of the MHA plate. Now a swab from each test culture sample was streaked from the edge of the disc to the edge of the plate [9]. Nearly four organisms were streaked on the same plate with one drug. The plates were incubated at 35°C for 24 hours.

Detection of Colistin resistant organisms by E-test method: Initially a suspension of each test bacterial isolate in Mueller-Hinton broth was prepared and adjusted to 0.5 McFarland standards. The suspensions were now swabbed onto MHA plates. When once the surface of the agar was completely dry, a colistin E-strip (concentration ranging from 0.06 to 1,024 µg/ml was applied to each plate and incubated at 35°C for 16-20h. The results were noted as MIC where inhibition of growth intersected the E-strip. A ≥4 µg/ml colistin concentration was used as the breakpoint to select as resistant isolates [10].

Statistical analysis: Data were statistically analyzed using SPPS software version 20.0. Frequency and percentages were calculated for categorical and ordinal variables. Chi-square test was carried out and p value ≤0.05 were considered statistically significant.

RESULTS:

Socio-demographics and isolate characteristics: The study used 100 clinical samples that were obtained from 59 males and 41 females (Figure-1). Table-1 showed that maximum number of cases were recorded in the age group 51-60 (31%) followed by 31-40 (16%). Among the various clinical samples collected, maximum numbers of samples were urine (34%) followed by sputum (26%) and pus (17%) (Table-2). Out of 100 samples collected, 85 samples showed the mono-microbial growth and remaining 15 were culture sterile. The organisms were identified based on the morphological, cultural and biochemical characteristics as *E.coli*, Klebsiella spp., Citrobacter spp., *Pseudomonas aeruginosa*, Proteus spp., Enterobacter spp., Providencia (Table-3). All are gram-negative organisms belonging to *Enterobacteriaceae* family. In an overall percentage, majority of the isolates were

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Klebsiella spp. (30.6%) followed by *E.coli* (25.9%) and few isolates reported were Proteus spp. (5.8%) (Figure-2).

Figure 1: Gender-wise distribution of clinical cases

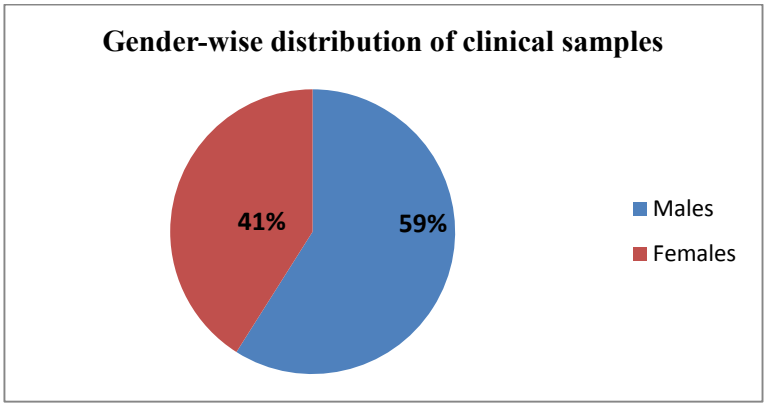

Table 1: Distribution of clinical cases according to their age groups

Age groups	Frequency and percentage
<10	1
11-20	2
21-30	14
31-40	16
41-50	13
51-60	31
61-70	12
71-80	11
Total	**100**

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Table 2: Distribution of clinical samples collected from various infectious patients

Sample	Frequency and percentage
Blood	14
Urine	34
Pus	17
Sputum	26
Ascitic fluid	2
Others	7
Total	100

Table 3: Distribution of pathogenic microorganisms isolated from various infections

Organism	Frequency
E.coli	22
Klebsiella spp.	26
Citrobacter spp.	9
P.aeruginosa	7
Providencia spp.	10
Proteus spp.	5
Enterobacter spp.	6
Total	85

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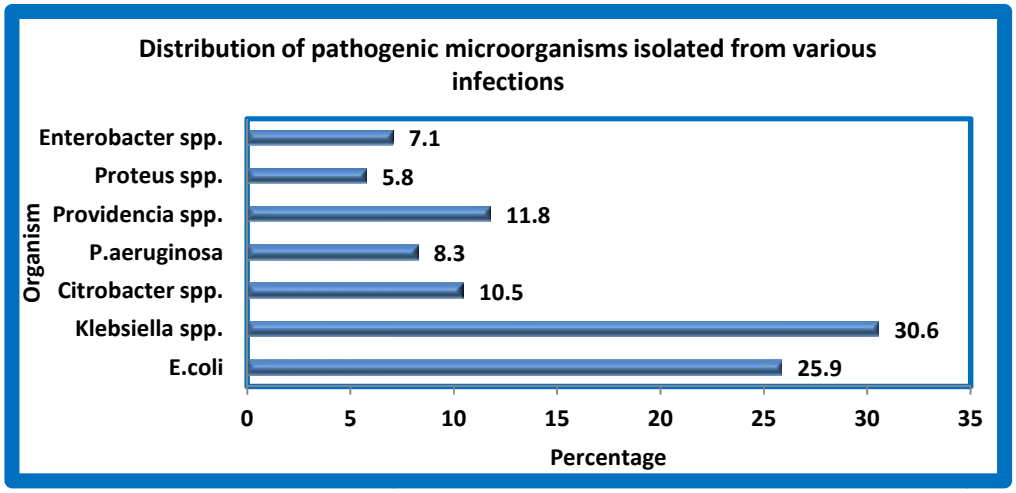
193 **Figure 2: Overall percentage of pathogenic microorganisms isolated from various infections**



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195 **Antibiotic susceptibility pattern and Detection of carbapenem and colistin resistant**
196 **microorganisms:** Table-4 shows that, among the 85 isolated microbial strains, all the strains showed
197 maximum resistance to cefuroxime i.e. 100%, 97.6% resistant to ceftazidime, 95.2% to
198 ceftazidime/clavulanic acid, 91.7% to cefipime, 88.2% to imipenem (Figure-3). However all the strains
199 were 100% sensitive to colistin followed by amikacin (54.2%) (Figure-4). ESBL detection was
200 identified in 81 test isolates out of 85 isolated strains at a range of 95.2% by using CAZ/CA
201 combination antibiotic susceptibility test (Figure-5). Majority of the cultures were resistant to second
202 and third generation cephalosporins and few isolates showed sensitivity to fourth generation
203 cephalosporin cefepime (8.3%) (Table-5). All the 75 test isolates which showed resistance to
204 imipenem by Kirby-Bauer disc diffusion method were now tested for the metallo-beta-lactamase
205 production by EDTA combined disc method (Figure 6). The results from the Table-6 revealed that, out
206 of 75 bacterial isolates 50 isolates showed MBL production (66.6%) and 25 were non-MBL producers
207 (33.4%). Maximum MBL production was seen in Klebsiella spp., (20%) and *E.coli* (20%) followed by
208 Providencia (16%), Citrobacter (14%) (Figure-7). This difference was found to be statistically
209 significant (p=0.03). All these 75 strains were again screened for carbapenemase production by
210 Modified Hodge test. The results from the Table-7 showed that 58 strains were positive for
211 carbapenemase producers (77.3%) and 17 strains were negative for carbapenemase production
212 (22.7%) (Figure-8). This difference was found to be statistically insignificant (p=0.9). Majority of the

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strains were *Klebsiella* spp. followed by *E.coli* However all these 58 strains were 100% sensitive to colistin which was detected by 'E' strip method (Figure-9).

Table-4: Antibiotic susceptibility pattern of isolated microorganisms by Kirby-Bauer method

Antibiotic susceptibility pattern (N=85)		
Antibiotic discs	**Number of resistant strains**	**Number of sensitive strains**
AMP	85	0
CXM	85	0
CAZ	83	2
CAC	81	4
CPM	78	7
IMP (Carbapenem antibiotic)	75	10
COL	0	85
CIP	65	20
GEN	52	33
AK	39	46
TE	75	10
COT	55	30
NIT	55	30
A/S	77	8
PIP	65	20

N=Number of isolates Piperacillin (PIP), Amikacin (AK), Gentamicin (GEN), Ceftazidime (CAZ), Ciprofloxacin (CIP), Imipenem (IMP), Ampicillin/Sulbactum (AS), Ampicillin (AMP), Co-Trimoxazole (COT),Cefepime (CPM),Cefuroxime (CXM), Ceftazidime/Clavulanic acid (CAC), Nitrofurantoin (NIT), Tetracycline (TE), Colistin (COL)

Table 5: Identification of antibiotic sensitive microorganisms by Kirby-Bauer method

	Microorganisms (Total isolates = 85)						
Antibiotics Sensitivity	Citrobacter Spp. (n=9)	Enterobacter Spp. (n=6)	Klebsiella spp. (n=26)	E.coli (n=22)	Proteus (n=5)	P.aeruginosa (n=7)	Providencia (n=10)
Ampicillin	0 (0.0%)	0 (0.0%)	0 (0.0%)	0 (0.0%)	0 (0.0%)	0 (0.0%)	0 (0.0%)
Ampicillin/sulbactam	2 (22.2%)	1 (16.6%)	2 (7.6%)	1 (4.5%)	1 (20%)	1 (14.2%)	0 (0.0%)
Cefuroxime	0 (0.0%)	0 (0.0%)	0 (0.0%)	0 (0.0%)	0 (0.0%)	0 (0.0%)	0 (0.0%)
Ceftazidime	0 (0.0%)	0 (0.0%)	0 (0.0%)	1 (4.5%)	0 (0.0%)	1 (14.2%)	0 (0.0%)
Cefipime	1 (11.1%)	1 (16.6%)	0 (0.0%)	2 (9.0%)	0 (0.0%)	2 (28.5%)	1 (10%)
Ceftazidime/ clavulanic acid	0 (0.0%)	1 (16.6%)	1 (3.8%)	1 (4.5%)	0 (0.0%)	1 (14.2%)	0 (0.0%)
Ciprofloxacin	1 (11.1%)	1 (16.6%)	3 (11.5%)	10 (45.4%)	2 (40%)	2 (28.5%)	1 (10%)
Colistin	9 (100%)	6 (100%)	26 (100%)	22 (100%)	5 (100%)	7 (100%)	10 (100%)

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Co-Trimoxazole	4 (44.4%)	3 (50%)	10 (38.4%)	7 (31.8%)	2 (40%)	2 (28.5%)	2 (20%)
Gentamycin	3 (33.3%)	4 (66.6%)	6 (23%)	9 (40.9%)	3 (60%)	5 (71.4%)	3 (30%)
Imipenem	1 (11.1%)	1 (16.6%)	2 (7.6%)	3 (13.6%)	1 (20%)	1 (14.2%)	1 (10%)
Nitrofurantoin	3 (33.3%)	4 (66.6%)	9 (34.6%)	8 (36.3%)	1 (20%)	2 (28.5%)	3 (30%)
Amikacin	4 (44.4%)	5 (83.3%)	14 (53.8%)	11 (50%)	4 (80%)	4 (57.1%)	4 (40%)
Tetracycline	1 (11.1%)	1 (16.6%)	2 (7.6%)	3 (13.6%)	1 (20%)	1 (14.2%)	1 (10%)
Piperacillin	3 (33.3%)	1 (16.6%)	5 (19.2%)	4 (18.1%)	2 (40%)	2 (28.5%)	3 (30%)

Figure 3: Percentage of antibiotic susceptible microorganisms by Kirby-Bauer method

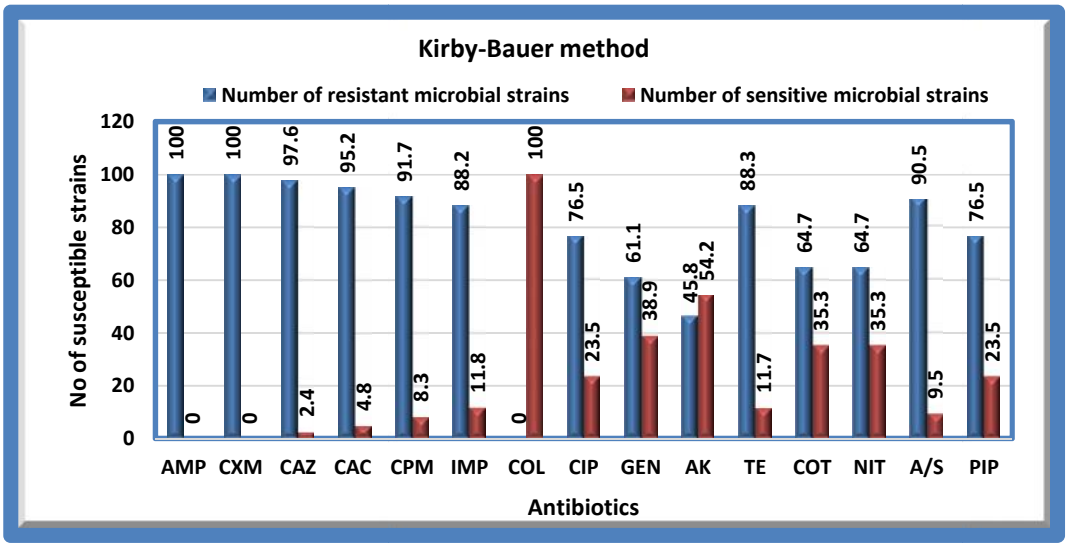
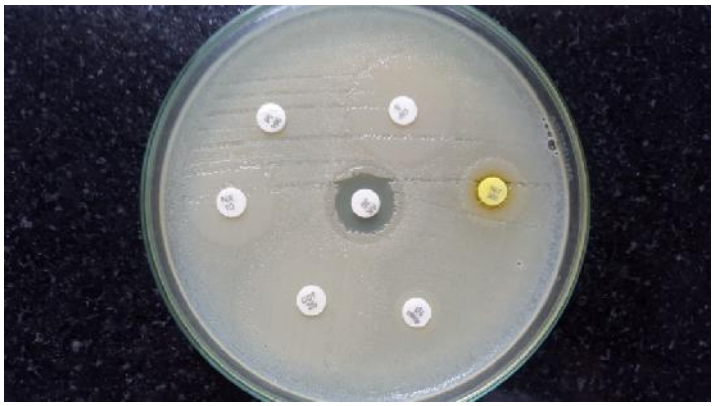


Figure 4: Antibiotic susceptibility pattern of isolates by Kirby-Bauer method



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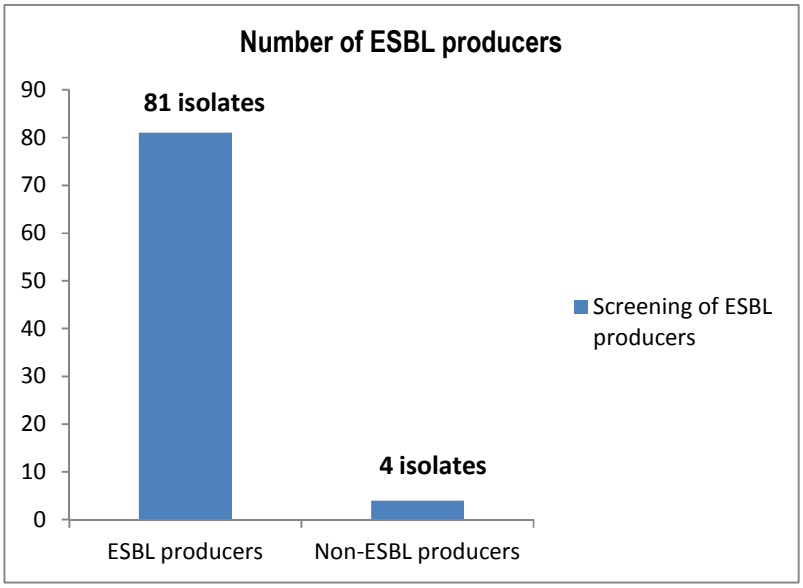
228 **Figure 5: Screening of ESBL (Extended spectrum beta-lactamase) producers among the**
229 **isolated strains**



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231 **Table-6: Distribution of MBL producing clinical isolates by using EDTA combined disc**
232 **diffusion method**

Organism	MBLproducer			
	Positive		Negative	
	Frequency	%	Frequency	%
E.coli	10	20.0%	8	32.0%
Klebsiella spp.	10	20.0%	10	40.0%
Citrobacter spp.	7	14.0%	2	8.0%
P.aeruginosa	6	12.0%	1	4.0%
Providencia spp.	8	16.0%	2	8.0%
Proteus spp.	4	8.0%	1	4.0%
Enterobacter spp.	5	10.0%	1	4.0%
Total	50 (66.6%)	100.0%	25 (33.4%)	100.0%
N=75 Chi-square=13.76 df=6 P-value=0.03 Significant				

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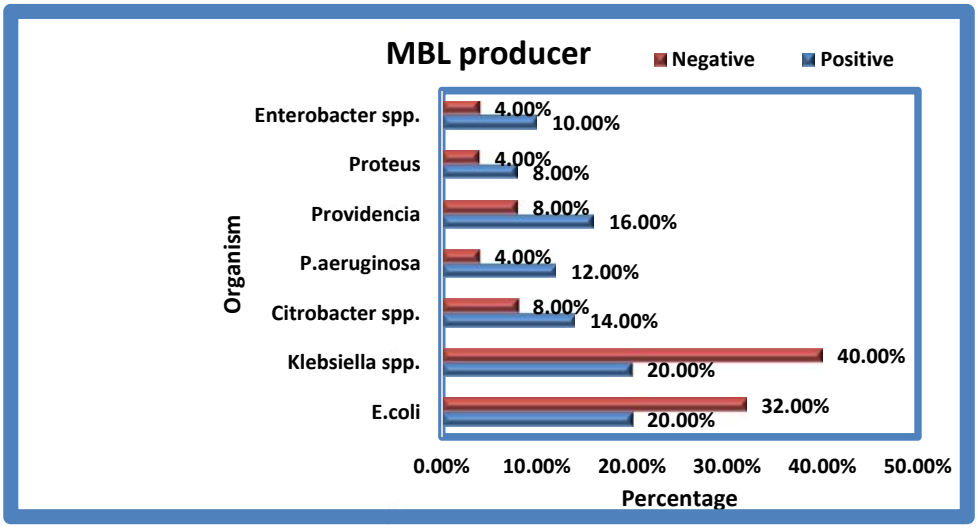
236 **Figure-6: Percentage of MBL producing strains**



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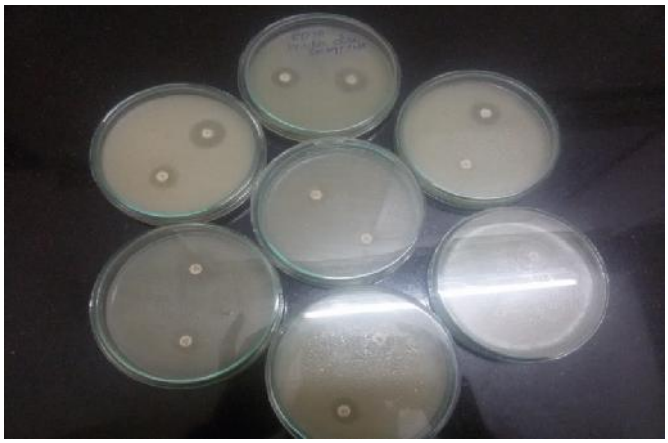
238 **Figure 7: Detection of MBL producers by EDTA combined disc method**



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240 **Table-7: Distribution of carbapenemase producing microbial strains by Modified hodge test**

Organism	Carbapenemase producer			
	Positive		Negative	
	Frequency	%	Frequency	%
E.coli	12	20.7%	6	35.3%
Klebsiella spp.	15	25.9%	5	29.5%
Citrobacter spp.	8	13.8%	1	5.9%

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P.aeruginosa	6	10.4%	1	5.9%
Providencia spp.	8	13.8%	2	11.7%
Proteus spp.	4	6.8%	1	5.9%
Enterobacter spp.	5	8.6%	1	5.9%
Total	58 (77.3%)	100.0%	17 (22.7%)	100.0%
N=75 Chi-square=1.29 df=6 P-value=0.97 NS				

Figure-8: Percentage of carbapenemase producing strains by MHT method

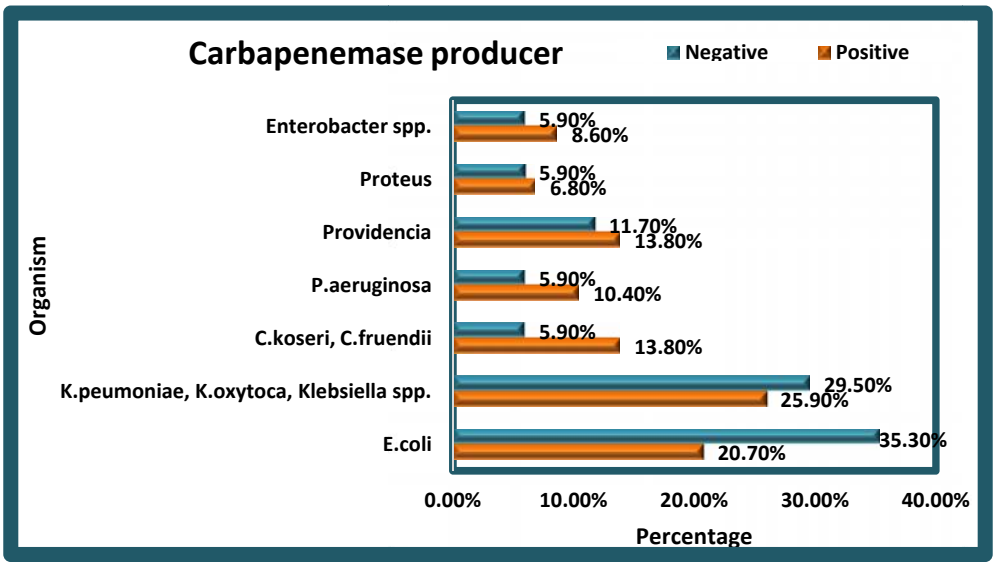
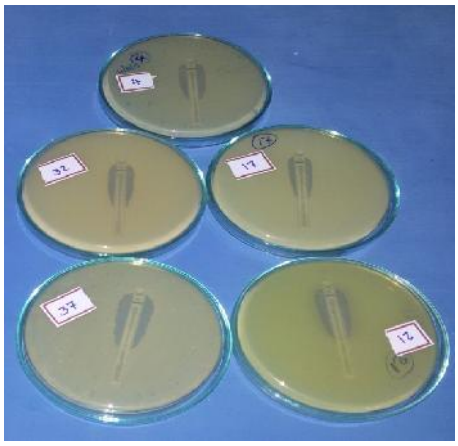


Figure 9: Detection of colistin sensitivity by 'E' strip method



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Discussion: Now-a-days most of the *Enterobacteriaceae* members develop resistance to most of the commonly used antibiotics, because of acquisition of genes encoding ESBLs [11]. These ESBL producing organisms also might acquire resistance to other classes of antibiotics and thus become multi-drug resistant and limits the treatment options. In general, carbapenems like imipenem, meropenem etc. are used as the drugs of choice for ESBL producers [12]. They are the last line of defence against many organisms that are resistant to other antimicrobial agents. However there is an increase in the world-wide production of beta-lactamase enzymes by the resistant microbial strains, thus hydrolyze all the β-lactam antibiotics along with carbapenems. *Enterobacteriaceae* are particularly adapted to produce beta-lactamases like TEM-1, AmpC, ESBLs, carbapenamases like metallo-beta-lactamases and KPCs to defend themselves against beta-lactam antibiotics [13]. However colistin is one of the older drugs but now has become a popular choice of clinicians faced with few options in the treatment of MDR gram-negative bacteria [14]. In the present study, almost more than half of the tested isolates were phenotypically positive for carbapenemase activity therefore it is recommended that, isolates be phenotypically tested if resistant to atleast two and third generation cephalosporins so as to inform patient care. The present research had isolated 66.6% carbapenem (imipenem) resistant strains among the 85 test isolates, recently Okoche *et al.* (2015) [15] had reported the prevalence of carbapenamase producing organisms is 22.4% only. The maximum numbers of MBL producers were identified from the genus *E.coli* and Klebsiella spp. (20%) and least number were from Proteus spp. (8%). By concluding the above findings, the current study had isolated more number of carbapenem (imipenem) resistant isolates with EDTA combined disk test while comparing to the previous studies by Varaiya and Kulkarni *et al*. (2008) [16] who showed 26% resistance to imipenem, Basik and Attal *et al*. (2009) [17] reported 12% resistance. The present results 66.6% coincides with the results of Mendiratta *et al.* (2005) [18] who reported 67% resistance to imipenem through EDTA combined disc diffusion method. The modified hodge test utilized for the carbapenemase production gave good results while comparing to combined disc diffusion method. The MHT detects 58 (77.3%) cases of carbapenemase producers. The other 17 (22.7%) strains gave negative results. These results were similar to the findings of Amudhan *et al.* 2011 and Bartolini *et al.* 2014 [19], [20] and indicates that MHT is very sensitive and reliable method than other screening tests. According to Galani *et al.* 2008 [21] MHT is 98% sensitive method keeping PCR as the gold standard assay method. The test isolates of the current study showed high sensitivity to antibiotics like colistin

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(100%), amikacin (54.2%) followed by gentamycin (38.9%) similar to the reports of Hirsh and Tam (2010) [22]. Anupurba Sarkar *et al*. (2007) [23] reported least maximum sensitivity to amikacin and gentamycin 45.45%. Majority of the studies had report dismal outcomes among patients with carbapenem resistant entero bacteria ranging from 40-70% [24] which is in close agreement to the present study. These poor outcomes are due to patient co-morbidities, severity of illness, treatment with ineffective empiric antibiotics before carbapenem resistance is identified by the laboratory.

CONCLUSION:

Emerging multidrug resistant isolates is one of the main concerns as they pose challenge to the clinicians in the treatment of various infectious diseases. The present study had isolated somewhat quite high isolates of drug resistant organisms from various clinical samples. The early detection of such drug resistant microorganisms may help in the prompt antimicrobial therapy while beginning and thus evade the development and dissemination of these multidrug resistance strains in the hospital as well as community. The study helps the clinicians in choosing the correct antimicrobial agent which contribute not only to better treatment but there judicious use will also help in preventing the emergence of drug resistant strains which are still sensitive.

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