International Research Journal of Medical Sciences _____ ISSN 2320 –7353
Vol. **3(8),** 1-6, August **(2015)** *Int. Res. J. Medical Sci.*

Prevalence of ESBL and AmpC β-Lactamase in Gram Negative Bacilli in various Clinical Samples at Tertiary Care Hospital

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Available online at: www.isca.in, www.isca.me
Received 27th June 2015, revised 18th July 2015, accepted 23rd August 2015

Abstract

The rapid dispersal of antibiotic resistance as extended spectrum beta lactamases (ESBLs) and AmpC β-lactamases in bacteria makes a major public health concern worldwide. It becomes essential to guide the clinicians about the Knowledge of their occurrence and the appropriate anti-microbial treatment. This study was done to evaluate the Prevalence of ESBL and AmpC β-lactamases and their antibiotic susceptibility in gram-negative clinical isolates were analyzed. Total 200 non repetitive clinical isolates of {Escherichia coli (n=101), Klebsiella spp (n=41), Citrobacter spp. (n=22), Pseudomonas spp. (n=20), Proteus spp.(n=5), Acinetobacter spp.(n=7) and Enterobacter spp.(n=4)} obtained over a period from (January to August, 2014), were screened by Kirby Bauer disc diffusion method for ESBLs and AmpC production and suspected isolates were confirmed by combined disc and AmpC disc tests. From 117(58.5%) and 62 (31%) screened out isolates, 87(43.5%) and 44(22%) were found to be ESBL and AmpC producers respectively. The distribution of ESBL and AmpC isolates organism wise showed E.coli (44.5% and 21.9%), Klebsiella spp (41.4% and 24.3%), Pseudomonas spp. (30% and 20%) and Acinetobacter spp (28.6 and 14.3%) respectively and they were found significantly multidrug resistance too. The co-existence phenotype of both ESBLs and AmpC were 23(11.5%) isolates. In our hospital moderate prevalence of ESBLs and AmpC was found. Combination disc test was effective for ESBL detection While the AmpC disc test, was found to be a convenient, specific and highly sensitive. Regular monitoring is necessary for the incidence of the ESBL and AmpC β-lactamase production by organisms.

Keywords: ESBLs, Amp-C β-lactamases, multidrug antibiotic resistance.

Introduction

In community and hospital settings resistant bacteria are emerging worldwide as a threat to the favourable outcome for common infections[1]. Resistance to antimicrobials is a natural biological phenomenon[2]. Bacterial infections are commonly treated by β-lactam antibiotics[3].

ESBLs are enzymes that are plasmid-mediated which hydrolyze the oxyimino β-lactams (3rd generation cephalosporins) and the monobactams (aztreonam) but they have no effect on the cephamycins (cefoxitin, cefotetan) and the carbapenems. They can be easily transferred from one organism to another as being plasmid mediated[4]. In the mid 1980s the first ESBL isolates were discovered in Western Europe[1]. They can be seen in a variety of Enterobacteriaceae species, however, ESBL producing strains mostly are found in *K. Pneumonia, K. oxytoca* and *E.coli*. The emergence of the ESBLs is due to widespread use of third generation cephalosporins and aztreonam leading to mutations in these enzymes[1]. Typically, ESBLs are plasmid-mediated mutant beta-lactamases derived from older broad-spectrum beta-lactamases (e.g.TEM-1, TEM-2, SHV-1), which have a extended substrate profile that allows hydrolysis of all cephalosporins, penicillins, and aztreonam[5]. More than 300 ESBLs have been identified based on their physical properties[4].

AmpC β-lactamase are group I cephalosporinases that are resistance to cephalosporins (e.g. ceftriaxone, cefotaxime, and ceftazidime), cephamycins (e.g. cefoxitin and cefotetan), aminopenicillins and monobactams. Cloxacillin and 3-aminophenylboronic acid also inhibit AmpC β-lactamases and these are not affected by inhibitors (clavulanic acid, tazobactam and sulbactam)[6,7]. AmpC β-lactamase production is chromosome or plasmid mediated in gram-negative bacteria[7]. There are limited therapeutic options for infections caused by Gram-negative organisms expressing plasmid mediated AmpC β-lactamases because these organisms are usually resistant to all β-lactams except of cefepime, cefepirome and carbapenems[8]. The increasing prevalence of bacterial pathogens producing both ESBLs and AmpC β-lactamases makes a requirement of methods for laboratory testing which can accurately detect the presence of these enzymes in clinical isolates[9]. All the methods utilize two properties of ESBLs that is reduction of susceptibility to extended spectrum cephalosporins and inhibition by clavulanate[10].

Due to lack of standard guidelines for detecting AmpC producing isolates efforts to detect β-lactamases such as AmpC enzymes in gram-negative rods are largely non-existent[11].

A convenient means of detection for plasmid-mediated AmpC β-lactamases is AmpC disc test, based on filter paper discs impregnated with EDTA and using cefoxitin insusceptibility as a screen was found to be a highly sensitive and specific[12].

Material and Methods

Clinical Specimens : Total 200 non-repetitive gram-negative clinical isolates over a period of Eight months (January to August 2014) were obtained from clinical specimens of urine, blood, stool, pus, wound swab, sputum, swabs (ear, nasal, throat, tracheal) and other respiratory tract specimen (endotracheal secretions, BAL) body fluids (ascitic, pleural, synovial), catheter tips, high vaginal swab and CSF etc. samples were collected from the patient of OPDs and admitted in wards and ICUs at hospital. Isolation and identification of the causative bacteria were performed using standard methods.

Antibiotic susceptibility testing[13]: The isolates were subjected to antimicrobial susceptibility testing using Kirby-Bauer disc diffusion method following CLSI guidelines, using commercially available 6mm discs (HIMEDIA, Mumbai, India) cefoxitin (30µg), ceftriaxone (30µg), ceftazidime (30µg), cefepime (30µg), imipenem (10µg), aztreonam (30µg), amikacin (30µg), piperacillin/tazobactam (75µg+10 µg), ciprofloxacin (5µg), gentamicin (10 µg), ceftazidime/clavulanate (30/10 µg) and cotrimoxazole (25 µg) on Mueller Hinton agar plate.

Screening for ESBLs and AmpC β-lactamases : According to CLSI guidelines, the isolates showing inhibition zone of size of ≤ 22 mm with ceftazidime (30µg), < 25 mm for ceftriaxone and ≤ 27 mm with cefotaxime (30µg) recorded were identified as potential ESBL producers and shortlisted for confirmation of ESBL production.

Isolates showing resistance or reduced sensitivity to cefoxitin were considered as a screen positive AmpC producer and subjected to AmpC disc test.

Combined disc test (Phenotypic confirmatory test)[14]: On the screened out isolates, disc of ceftazidime (30µg) alone and a disc of ceftazidime + clavulanic acid (30µg/10µg) were placed independently, 30mm apart center to center on a lawn culture of 0.5 Mc-Farland opacity of the screened test isolate on Mueller Hinton Agar (MHA) plate and incubated for 18-24 hours at 37°C. Isolates which showed an enhancement in zone of inhibition of ≥5mm diameter around the ceftazidime/clavulanic acid in comparison to ceftazidime alone confirmed ESBL production (figure-1).

Amp C disc test[12]: AmpC discs made from filter paper containing Tris-EDTA, were prepared in laboratory by applying 1:1 mixture of saline and 100× Tris-EDTA in 20µl volume to sterile filter paper discs, than allowing discs to dry, and stored at 2 to 8°C. A lawn from cefoxitin susceptible E. coli ATCC 25922 was made. On the inoculated surface of the Mueller-Hinton agar a 30µg cefoxitin disc was placed. AmpC discs were rehydrated with 20µl of saline and several colonies of each test organism were applied to a disc immediately prior to use. The inoculated AmpC disc with the test organism is inverted and is than placed on agar plate almost touching the cefoxitin antibiotic disc. After incubation, either an indentation or a flattening (distortion) of the zone of inhibition around cefoxitin antibiotic disc were examined on plates, indicating enzymatic inactivation of cefoxitin (a positive result), or the absence of a distortion, indicating no significant inactivation of cefoxitin (a negative result). (Figure-2).

Quality control: Sterility testing for 24 hours was done on every batch of media prepared. ESBL positive *K.pneumoniae* ATCC 700603 and ESBL negative and cefoxitin susceptible *E.coli* ATCC 25922 as reference strains of CLSI were included in the study.

Statistical Analysis[14]: In ESBL and non-ESBL, AmpC and non-AmpC isolates significance between the resistance level of various drugs were performed using the Proportion test (Z).

$$Z\ (obs) = \frac{p1 - p2}{\sqrt{p * q(\frac{1}{n1} + \frac{1}{n2})}}$$

Where $p1$ = Proportion of ESBL or AmpC isolates showing resistance to individual antimicrobial, $p2$ = Proportion of non-ESBL or non-AmpC isolates showing resistance to individual antimicrobial, $n1$ = No. of ESBL or AmpC isolates, $n2$ = No. of non-ESBL or non-AmpC isolates.

$p = \frac{n1p1+n2p2}{n1+n2}$
q = 1- p. obs = observed value of Z.

At 5% level, expected or tabulated value of Z for both sided test is 1.96. So, if the observed value of Z is more than the tabulated value then it is said to be significant at 5% level and the *P value* is < 0.05.

Results and Discussion

The present study was conducted in the Department of Microbiology, NIMS Medical College, Jaipur, Rajasthan from January 2014 to August 2014, to know the prevalence of ESBL producing gram negative bacilli in various clinical isolates at our tertiary health care centre. Out of the 200 non-repetitive gram-negative isolates included in the study, the isolated gram negative organisms were *E.coli* (n=101), *Klebsiella spp.*(n=41), *P.aeruginosa* (n=20), *Citrobacter spp.*(n=22), *Proteus spp.*(n=5), *Enterobacter spp.* (n= 4) *and Acinetobacter spp.* (n= 7).

ESBLs Detection : ESBL production was observed in 87(43.5%) isolates by combined disc test from 117 screened positive isolates, and amongst these 44.5%, 36.6%, 22.7%, 30% and 40 % isolates were *E.coli*, *Klebsiella spp.*, Citrobacter spp., *Pseudomonas spp.* and *Proteus spp* respectively.

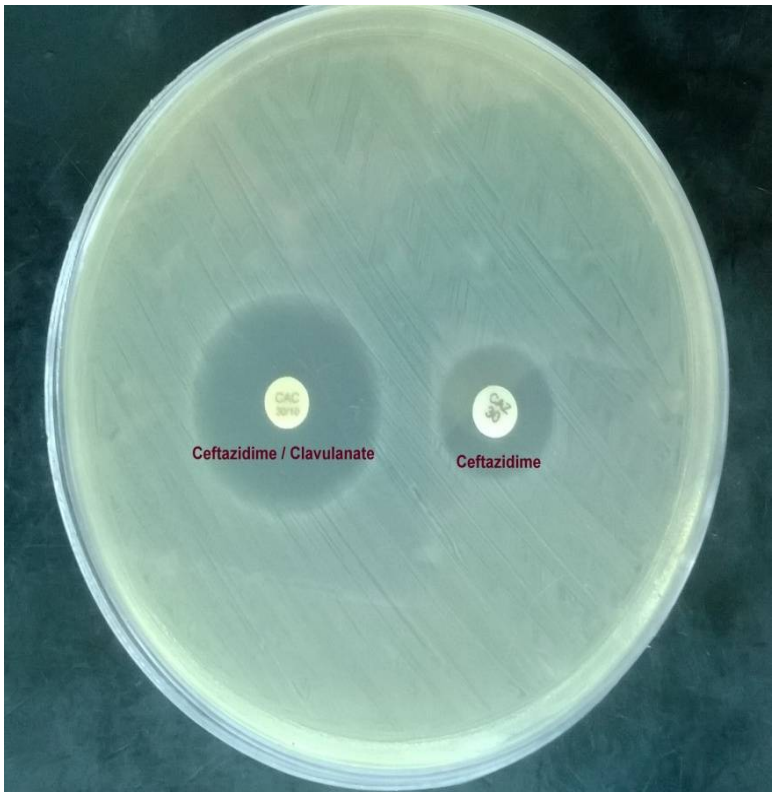

Figure-1
Phenotypic Confirmatory Test with combination disc using ceftazidime disc 30 µg and ceftazidime / clavulanate disc 30/10 µg.(ESBL Positive)

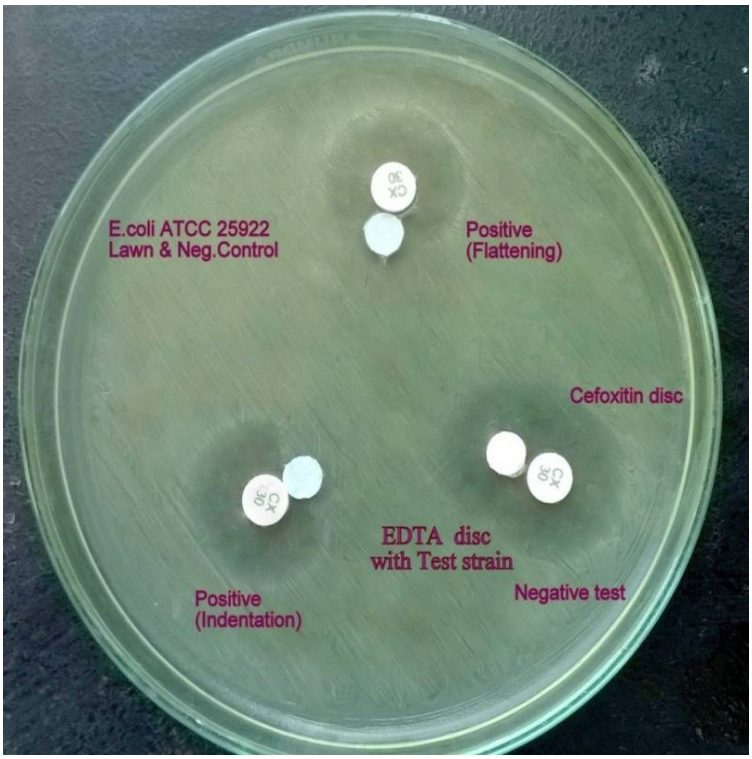

Figure-2
Representative results obtained with the AmpC disc test

AmpC β-lactamases Detection : AmpC disc test detected AmpC enzymes in 44(22%) isolates, among these were *E.coli* (21.9%), *Klebsiella spp* (24.3%), *P.aeruginosa* (20%). Citrobacter spp. (18.2%) and *Acinetobacter spp* (14.3%). Indentation indicating strong AmpC producer was observed in 17 isolates whereas flattening (weak AmpC) in 27 isolates.

Detection of ESBLs in presence of AmpC β-lactamases: This study demonstrated the co-existence phenotype of both ESBLs and AmpC in 23(11.5%) isolates of which 12(11.8%) and 4(9.8%) isolates were *E.coli* and *Klebsiella spp* respectively.
The comparison of anti-microbials resistance for ESBL and non-ESBL, AmpC and non-AmpC isolates producing strains is shown in (table-2 and 3).

Table-1
Detection of ESBLs, AmpC β-lactamase and ESBL+Amp C

Microorganisms (n= no.of isolates)	Screening +ve ESBL (%)	AmpC Screening +ve (%)	ESBL +ve by CDT	AmpC Disc Test			ESBL + AmpC
				Indentation (%)	Flattening (%)	No Distortion (%)	
E.coli (n= 101)	51(50.5)	30(29.7)	45(44.5)	6(6)	16(15.9)	8(8)	12(11.8)
Klebsiella spp. (n=41)	27(65.9)	15(36.6)	17(41.4)	5(12.1)	5(12.2)	5(12.2)	4(9.8)
Citrobacter spp. (n=22)	18(81.8)	5(22.7)	11(50)	3(13.6)	1(4.6)	1(4.5)	2(9)
Pseudomonas spp. .(n=20)	8(40)	6(30)	6(30)	1(5)	3(15)	2(10)	2(10)
*Aceinetobacter spp. .(*n=7)	4(57.1)	3(42.8)	2(28.6)	1(14.3)	0(0)	2(28.6)	1(14.3)
Proteus spp. .(n=5)	5(100)	2(40)	4(80)	0(0)	2(40)	0(0)	1(20)
Enterobacter spp. .(n=4)	4(100)	1(25)	3(75)	1(25)	0(0)	0(0)	1(25)
Total (n=200)	117(58.5)	62(31)	87(43.5)	17(8.5)	27(13.5)	18(9)	23(11.5)

Table-2
Resistant Pattern of ESBL (n=87) and non-ESBL isolates (n=113)

Antibiotic	**ESBLs**	**NON ESBLs**	***p* value**
Amikacin	37	44	>0.05
Amoxiclav	44	46	>0.05
Aztreonam	59	58	< 0.05
Ceftriaxone	74	34	< 0.05
Ciprofloxacin	49	40	< 0.05
Cefoperazone/sulbactum	34	11	< 0.05
Ceftazidime	66	51	< 0.05
Cefoxitin	30	31	>0.05
Cefepime	71	13	< 0.05
Cotrimoxazole	76	51	< 0.05
Imipenem	9	7	>0.05
Piperacillin/tazobactum	32	25	< 0.05
Gentamicin	40	46	>0.05

Table-3
Resistant Pattern AmpC(n=44) and non-AmpC (n=156) isolates

Antibiotic	**AmpC**	**Non-AmPC**	***p* value**
Amikacin	24	57	<0.05
Amoxiclav	35	55	<0.05
Aztreonam	38	79	<0.05
Ceftriaxone	38	70	<0.05
Ciprofloxacin	28	61	<0.05
Cefoperazone/sulbactum	16	29	<0.05
Ceftazidime	39	78	<0.05
Cefoxitin	40	21	<0.05
Cefepime	21	57	>0.05
Cotrimoxazole	36	90	<0.05
Imipenem	4	12	>0.05
Piperacillin/tazobactum	18	39	<0.05
Gentamicin	24	62	>0.05

Discussion: In the present study, the prevalence of ESBLs (43.5%) was lower in comparison to reports from different parts of the country (17% to 70%)[15-18.] It has been analyzed that prevalence of the ESBLs among the clinical isolates varies from country to country and institution to institution within the same country. This might be due to judicious usage of cephalosporins and adopting appropriate infection-control measures in our hospital.

In the present study, AmpC production was found to be 22%. On the contrary in various other studies AmpC production rate varies from 8% to 50%[8,19-23]. In the present study higher prevalence of AmpC producing organisms are seen probably because of multidrug resistant strains are used in the study. Different geographic areas and sample variation can also be a cause. The present study correlates with the study of Bandekar N *et al* (22.9%) *and* B. Sasirekha *et al* (20.4%).

In the AmpC producing β lactamase organism the shape of Zone of inhibition around cefoxitin disc was flattening (weak AmpC) in 26 (59.09%) of total 44 AmpC Producing organism and 18 (40.09%) was showing indentation (strong AmpC). This study relates with the study of Parul sinha *et al*[24].

In the present study 43.5% isolates were ESBL producers, 22% were AmpC producers, 11.5% were ESBL + AmpC Producers (co-existence of phenotype). The present study correlates well with the study of Charu Kothari *et al* (11.2%)[29] and Ritu nayar *et al* [25] (13.8%) in prevalence of ESBL + AmpC Phenotype. In our study there is not high prevalence of coexistence of phenotype (ESBL +AmpC β-lactamase) compared to other studies. Multidrug resistance was significantly ($P < 0.05$) higher in ESBL and AmpC β-lactamase producers than non-ESBL and non- AmpC producers in this study. In ESBLs Producer group maximum resistance is seen in Cotrimoxazole (87.36%), followed by Ceftriaxone (85.06%), Cefepime (81.61%) while in Non-ESBLs producers group it is seen in Aztreonam (51.33%) followed by Cotrimoxazole and ceftazidime (45.13% both) Amoxiclav (40.71%).6% to 10% resistance was seen for Imipenem in both ESBL and Non ESBL producers.

In AmpC Producer group maximum resistance is seen in Cefoxitin (90.91%) followed by Ceftazidime (88.64%), Aztreonam and Ceftriaxone (86.36% both), while in Non-AmpC producers group it is seen in Cotrimaxazole (57.69%) followed by Aztreonam (50.64%) and Ceftazidime (50.00%). However 7 to 10% resistance was observed in Imipenem in both AmpC and Non AmpC producers.

Interstingly, ESBL and AmpC producers also shown concurrent result to ceftriaxone (85.06% and 86.36%), Cefoperazone/ sulbactum (39.08% and 36.36%), Ceftazidime (75.86% and 88.64%), and Imipenem (10.34% and 9.09%) and Piperacillin/tazobactum (36.78% and 40.91%) and Gentamicin (45.98% and 54.55%) respectively. Similar findings was reported by Singh RKM *et al*[14], Dalela G *et al* [26.], GuptaV *et al*[27] and Jain A *et al*[28]. However, all the ESBL and AmpC producing isolates were 90 to 95% sensitive to Imipenem, thereby repeatedly making the continued efficacy of carbapenems as the first line agents for treatment of infections caused by *Enterobacteriaceae* producing ESBL and AmpC beta lactamases. They were also sensitive to piperacillin tazobactum (70% to 80%) and to cephoparazone sulbactum (60% to 90%). For prevention of ESBLs and AmpC resistance use of third generation cephalosporins should be limited.

Conclusion

In conclusion, 43.5%, 22% and 11.5% of ESBL, AmpC producers and co-production were detected respectively in our hospital. Combination disc test was effective for ESBL detection. While AmpC disc test was simple, easy to perform and require less expertise for the rapid detection of AmpC isolates. By adopting this test it would become possible to learn more about the clinical implications of AmpC ß-lactamases and to controling the spread of organisms having these type of resistance mechanism. There were limitation to the presnt study due to lack of infrastructure so, advance molecular methods were not been accessed.

In routine susceptibility testing it should be made essential to report ESBL and AmpC β-lactamase production, it will help the clinicians in prescribing proper antibiotics. In the reporting of resistant organisms, addition of ceftazidime and ceftazidime/clavulanic acid for the detection of ESBL and the addition of cefoxitin for the detection of AmpC β-lactamase must be done, because the restricted use of antibiotics on the resistant bacteria will no longer have a survival advantage against these antibiotics and can lead to the withdrawal of selective pressure. The detection of ESBLs and AmpC β-lactamases by this method is simple and any microbiology laboratory can do it along with the routine antibiotic susceptibility testing.

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