Burden of ESBL with antibiogram in clinical isolates of E.coli and *Klebsiella* species from tertiary care hospital in central India

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Abstract

Introduction and Objectives: The ESBLs producing organisms are reported worldwide in increasing numbers for which Clinical Laboratory Standard Institute (CLSI) recommends screening for ESBLs producing Escherichia coli and Klebsiella species by phenotypic methods. The study was undertaken to assess the burden of ESBL and comparison of Double Disc Synergy Test (DDST) and Phenotypic Confirmatory Test (PCT) with gold standard E-Test for ESBL detection in clinical isolates of tertiary care hospital, Bhopal.

Materials and Methods: 223 clinical isolates of *E.coli and Klebsiellae species* were included in the study and those showing zone size of < 22 mm for Ceftazidime were selected as potential ESBL producers. The ESBL were confirmed with gold standard E-test and later DDST & PCT were performed over the suspected strains were compared with E-test. The data was maintained in MS Excel and appropriate tests of proportion and significance were applied.

Result: Out of 223 isolates 124 were potential ESBL producers with preponderance of *E.coli* 58.3%. E-Test showed confirmed cases of ESBL 72/124(58%) which was much higher than by DDST 44/124 (35.5%) but close to PCT 70/124 (56.4%) among suspected clinical isolates. Overall ESBL burden out of 223 isolates was 32.28% which is quite alarming. ESBL producing strain showed maximum sensitivity to Colistin, polymyxin-B & Imipenem nearing 100% whereas sensitivity ranging from 60 to 85% for beta lactamase inhibitor combinations. Non-ESBL producers were more sensitive to Amikacin, Fluoroquinolones, Nitrofurantoin, Cotrimoxazole with sensitivity ranging between 40 to 70%.

Conclusion: With rise in ESBL producing strains with multi drug resistance, screening with Ceftazidime should be done to detect probable ESBL producer. PCT can be used with confidence with comparable results with that of MIC tests in resource poor microbiology laboratories which will save human, technical and monitory resources in terms of its confirmation by molecular genotypic methods.

Keywords: DDST-Double Disc Synergy Test, PCT-Phenotypic confirmatory test, E-Test-Epsilometer Test.

Key message: Screening with Ceftazidime and PCT for confirmation of ESBL should be routinely employed for resource poor microbiology laboratories for *E.coli* and *Klebsiella* species

Introduction

The most common organisms responsible for infections are multidrug resistant gram negative bacilli, particularly among members of the family *Enterobacteriaceae* & non fermenting gram negative rods. Among the wide array of antibiotics, β-lactams are the most widely used agents. The most common cause of resistance to β-lactam antibiotics is the production of β-lactamases. The first plasmid-mediated β-lactamase in gram-negatives, TEM-1, was described in the early 1960s, originally found in a single strain of *E. coli* isolated from a blood culture from a patient named Temoniera in Greece, hence the designation TEM. Latter SHV-2, was found in a single strain of *Klebsiella ozaenae* isolated in Germany. Because of their increased spectrum of activity, especially against the oxyimino-cephalosporins, these enzymes were called extended-spectrum β-lactamases (ESBLs).[1]

Extended-spectrum β-lactamases (ESBLs), which hydrolyse extended-spectrum cephalosporins and are inhibited by β-lactamase inhibitors such as clavulanic acid, are spreading among *Enterobacteriaceae*.[2] Plasmids coding for ESBLs may also carry additional β-lactamase genes as well as genes conferring resistance to other antimicrobial classes. They are usually associated with resistance to multiple unrelated antibiotics such as aminoglycosides, chloramphenicol, trimethoprim-sulfamethoxazole, tetracycline, and fluoroquinolones, leaving few therapeutic choices.[3] Phenotypic detection of ESBLs among *Enterobacteriaceae* species is important for epidemiological purposes as well as for limiting the spread of resistance mechanisms. ESBL producing *Enterobacteriaceae* are now found in ambulatory patients without recognized risk factors for multidrug-resistant organisms. ESBLs have emerged within the community, particularly among *E.coli and K. pneumoniae.* Various authors have reported the prevalence of ESBLs to be in the range of 6–88% in various hospitals, especially among *Klebsiella pneumoniae* and *Escherichia coli.*[4] So the study was carried in *E.coli and Klebsiella* to look for the burden

of ESBL in clinical isolates in central India with their antibiogram to aid the treating physicians in empirical therapy.

Materials and Methods

This cross sectional prospective analytical study was carried out in Department of Microbiology of Peoples College of Medical Sciences between November 2012 to April, 2014 over a period of one and half years. Clearance from research advisory committee and waiver of consent from Institutional Ethics committee for using the clinical samples received in department of Microbiology was obtained before processing of the samples. All the clinical samples were cultured and isolates identified according to standard microbiological techniques.[5] A total of 223 Non-repetitive isolates of *E.coli and Klebsiella* species from various clinical samples of urine, blood, pus, wound swab, sputum, intravenous catheter etc. from outpatient and inpatient units of all age groups and both sexes were included in the study.

The Antibiotic Sensitivity pattern was assessed using Kirby Bauer Disk Diffusion method for the drugs as per CLSI guidelines 2013. All the isolates having zone size of ≤22 mm for Ceftazidime were selected as potential ESBL producers being resistant to 3rd generation cephalosporin for further processing confirming the ESBL producers by various phenotypic methods as per CLSI 2013 guidelines.[6]

All potential ESBL producers were subjected to three methods for detection of ESBL -
1. Double Disc Synergy Test (DDST)
2. National Committee for Clinical Laboratory Standards (NCCLS) Phenotypic confirmatory test (PCT)
3. MIC by Epsilometer Test (E-Test)

The test strain to be tested was inoculated in peptone water and incubated for 2 to 3 hrs. to be in log phase, The inoculum was adjusted to 0.5 McFarland Turbidity standard using the Densimat® from BIOMERIEUX. The test strain was inoculated on 3 Muller Hinton Agar (MHA) Plates and disk placement done as follows and the plates were incubated overnight at 37° C & read. The strain of *Klebsiella pneumoniae* ATCC 700603 was used as positive control whereas *Escherichia coli* ATCC 25922 was used as negative control while performing the tests.
1. For Double Disc Synergy Test (DDST) Amoxyclav disc (30µg-20 µg of amoxicillin & 10 µg clavulanic acid), Ceftazidime (30µg) and Cefotaxime (30µg) were used. With the help of a sterile forceps, Amoxyclav disc was placed in center of Muller Hinton agar plate. Ceftazidime and Cefotaxime discs were placed at 16 mm from Amoxyclav disc on either side. Accentuation of zone of cephalosporin disks towards clavulanic acid indicates ESBL production.[7]

2. For Phenotypic Confirmatory Test Ceftazidime (30 µg) and Combination of Ceftazidime and Clavulanic acid (30/10 µg) were placed opposite to each other in inoculated MHA. The measured zone of inhibition around Ceftazidime-clavulanic acid by more than 5mm than that of Ceftazidime alone, confirms the isolate to be ESBL producer.[7]
3. For MIC by Epsilometric Test (E-test), E-test strips (TZ/TZL) obtained from BIOMERIEUX was placed on the third plate. It carries two gradients Ceftazidime (0.5-32 µg/ml) on one end & Ceftazidime-clavulanic acid (0.064-41 µg /ml) in a different concentration gradient on other end. It had fixed concentration gradient of clavulanic acid (4 µg /ml). Manufacturer's instructions were followed for performing and interpreting the result. Presence of ESBL was confirmed by-[8]
   i. Appearance of phantom zone.
   ii. Or by deformation of Ceftazidime (TZ) eclipse.
   iii. Or when MIC is reduced by $>3\log_2$ dilutions.
   iv. Or ratio of TZ/TZL >8 in presence of clavulanic acid as per manufacturer guide lines.

The E-test results were taken as confirmatory for detection of ESBL strain. The DDST and PCT were then compared with the gold standard test for their overall utility as phenotypic tests in routine bacteriology practices. All the data was maintained in Microsoft office Excel and was analyzed using test of proportion and test of significance.

Result

A total of 223 isolates of *Escherichia coli* and *Klebsiella species* from different clinical samples were enrolled for the study. Maximum isolates were received from young adults between 21-30 year (24.7%) and patients aged >61 year (22.9%), with insignificant gender distribution. Maximum isolates were from admitted patient's i.e. 80.7%.

A total of 124 of 223 (55.60%) isolates were suspected to be possessing the ESBL based on the reduced susceptibility shown to Ceftazidime with ≤ 22 mm zone of inhibition. Speciation of the same along with the total number of confirmed cases are as depicted in Table 1. A total of 72/223 (32.28%) strains were confirmed to be ESBL producer by Gold standard test i.e. MIC by E-test.

Table 1: Speciation of isolated Gram negative bacilli and suspected and confirmed ESBLs by gold standard

Clinical Isolates	Speciation of Isolated GNB Percent (%)	Suspected ESBL producers	Confirmed ESBL by Gold Standard
Escherichia coli	144 (64.6%)	84 (58.3%)	50
Klebsiella pneumoniae	74 (33.2%)	38 (51.3%)	22
Klebsiella oxytoca	05 (02.2%)	02 (40%)	00
Total	(223) 100.0%	124 (55.6%)	72

The clinical sample wise distribution of the enrolled, suspected and confirmed cases of the E.coli and Klebsiella species is depicted in Table 2. Urine was the most predominant sample received which yielded 95/223 (42.60%) followed by 51/223 (22.86%) and 30/223 (13.45%) for pus and sputum respectively.

Table 2: Clinical sample wise distribution of the enrolled, suspected and confirmed cases of the E.coli and *Klebsiella* species

Sample	Urine	Pus	Sputum	Tips	HVS	Blood	Body fluids	Total
E.coli detected	75	19	18	15	12	03	02	144
Suspected ESBL	43	10	09	07	10	03	02	84
ESBL by Gold standard test	29	07	06	01	05	00	02	50
Klebsiella species detected	20	32	12	09	05	00	01	79
Suspected ESBL	08	17	07	04	03	00	01	40
ESBL by Gold standard test	03	09	06	02	02	00	00	22

The maximum samples were received from intensive care unit 51(22.86%) followed closely by Surgical and Outpatients department with 42(18.83%) and 43(19.28%). The department wise distribution of the samples received and enrolled, their species wise isolation and response to various phenotypic tests along with the gold standard is shown in table 3.

The maximum isolation of confirmed cases of ESBL were from the Surgery department with 18/72(25%) with 15(20.83%) & 12(16.66%) cases from OPD and pulmonary medicine department. Whereas from Medicine department there were 10/72 (13.88%) confirmed cases.

Table 3: Department wise distribution of samples yielding E.coli and *Klebsiella* species along with suspected ESBL producing strains tested and confirmed by various phenotypic methods.

Department	Sample No (%)	E.coli		Klebsiella species		Total	E Test	PCT	DDST
		Isolated	Suspected	Isolated	Suspected				
I.C.Us.	51(22.86%)	39	15	12	03	51	07	07	04
Burn	05(02.2%)	01	01	04	03	05	02	02	03
Medicine	19(08.5%)	13	09	06	04	19	10	10	06
Surgery	42(18.83%)	21	18	21	11	42	18	17	10
Gynecology	26(11.7%)	17	09	09	03	26	04	04	05
Pulmonary medicine	27(12.1%)	14	10	13	08	27	12	12	07
Pediatric	10(04.4%)	06	04	04	04	10	04	04	02
OPD	43(19.28%)	33	18	10	04	43	15	14	07
Total	223	144	84	79	40	223	72	70	44

The DDST and PCT when compared with the gold standard test i.e. E-test and though both the tests showed p value of less than 0.001 and were highly significant for detection of the ESBL, the PCT with sensitivity of more than 97% and specificity of 100 percent is found to be comparable to gold standard with Positive predictive value of 100% and negative predictive value of 96.2% as depicted in Table 4.

Table 4: Comparative evaluation of DDST & PCT against E-Test with Sensitivity, Specificity, NPV& PPV

	GSP GSN	Total	Chi Square Value & P-Value	Sensitivity, Specificity, Positive & Negative Predictive value
DDSTP[@]	38 06	44	22.43 P< 0.0001	SN-52.7%
DDSTN[#]	34 46	80		SP-88.4% PPV-86.3%
Total	72 52	124		NPV- 57.5%
PCTP[$]	70 00	70	116.09 p= < 0.0001	SN-97.2%
PCTN[*]	02 52	54		SP-100% PPV-100%
Total	72 52	124		NPV-96.2%

@DDSTP- Double disk synergy test positive # DDSTN- Double disk synergy test negative
$ PCTP- Phenotypic confirmatory test positive *PCTN- Phenotypic confirmatory test negative,
SN-Sensitivity, SP- Specificity, PPV- Positive predictive value & NPV- Negative predictive value

The antibiogram of the confirmed ESBL producing strain was compared with the Non- ESBL producing strain (Graph 1) ESBL producing strain showed maximum sensitivity to Colistin, polymixin-B & Imipenem nearing 100% whereas sensitivity ranging from 60 to 85% for beta lactamase inhibitor combinations. These strains were not much sensitive to Amikacin, Fluoroquinolones, Nitrofurantoin, Cotrimoxazole with a sensitivity below 30%.

The Non-ESBL producer strains when compared to ESBL producing strain showed comparable sensitivity to Colistin, polymixin-B &Imipenem as well as beta lactamase inhibitor combinations. They were more sensitive to Amikacin, Fluoroquinolones, Nitrofurantoin, Cotrimoxazole with sensitivity ranging between 40 to 70%. (Graph 1)
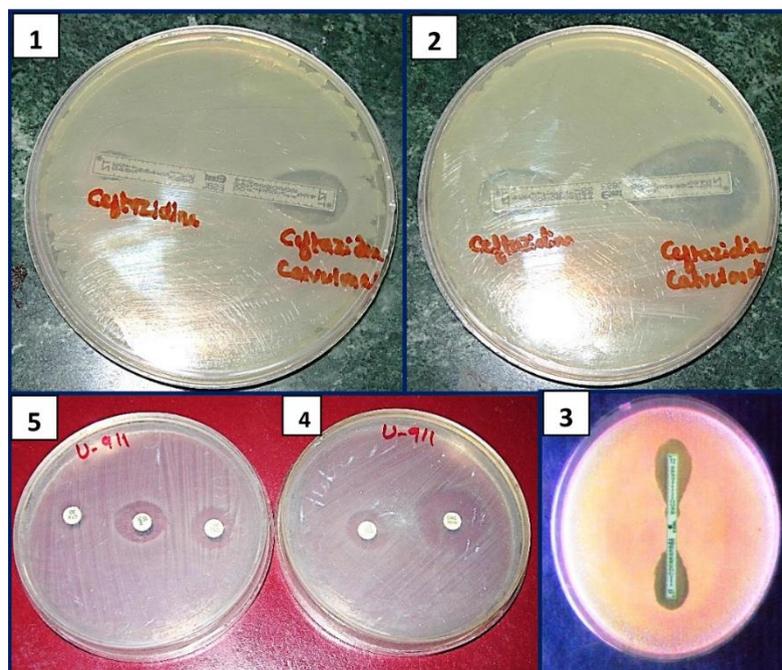
Graph 1
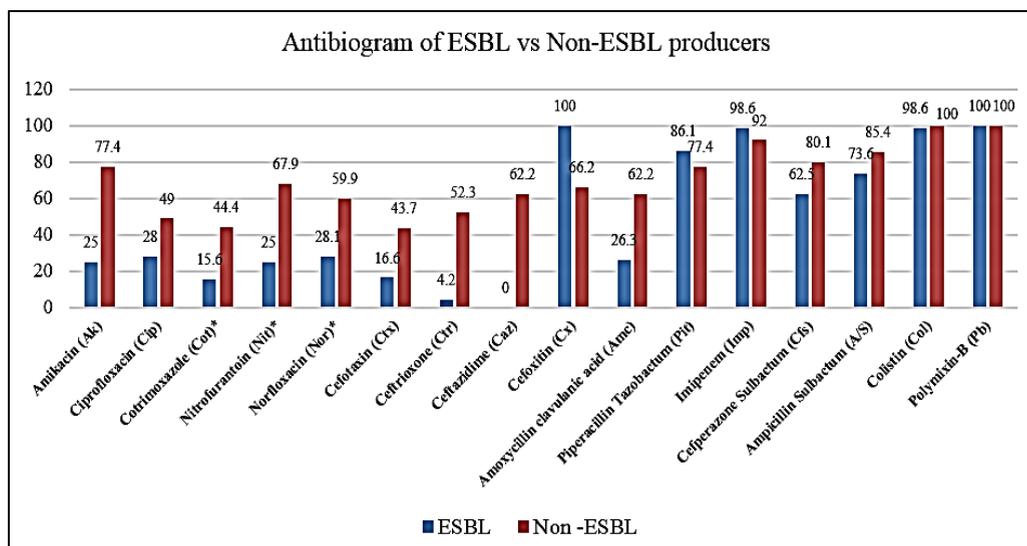
Fig. 1-3: E test and 4-DDST test and 5-PCT test positive for ESBL

Discussion

In recent years there has been an increase incidence and prevalence of ESBLs; enzymes that hydrolyze and cause resistance to oxyimino-cephalosporin's and aztreonam.[2] These enzymes are most commonly produced by Klebsiella pneumoniae, Klebsiella oxytoca and E.coli.[9] So ESBL detection was done in isolates in Klebsiella species and E.coli in this study. CLSI standard disc diffusion test was used as a screening test to detect probable ESBL producer, followed by confirmatory Gold standard test i.e. E-test and comparison of Double disk synergy test (DDST) & Phenotypic confirmatory test (PCT) with E-test.

Detection of Potential ESBLs: In our study, we found 55.6% potential ESBLs out of total 223 Gram negative isolates *(E.coli and Klebsiella species)* (Table 1) which was in accordance with Veena Krishnamurthy et al[10] who found 59.7% potential ESBLs, with predominance of *E.coli.* As per CLSI guidelines we used Ceftazidime for screening ESBLs, Katsanis GP et al[11] also says Ceftazidime is the best single test antibiotic for detecting ESBL production.

Confirmation test for ESBL –by E-test: In this study E-test was used as a gold standard method for confirmation of ESBL producers by MIC method. E-test was able to detect 72/223 (32.28%) enrolled strains whereas 72/124(58.06%) of the suspected ESBL strain. 50/72(69.44%) were E.coli whereas 22/72(30.55%) were *Klebsiella pneumoniae.* No *Klebsiella oxytoca*isolate was detected as ESBL producer. (Table 2) Maurine et al[12] and Anandkumar et al[13] have also used E-Test as the reference for detection of ESBL with up to 94% accuracy.

The overall prevalence of ESBL producers was found to vary greatly in different geographical areas and in different institute in India, based on various risk factors, local reasons and rationale use of β-Lactam antibiotics. It is high in referral centers where antibiotic use is profuse. In a study by wattle C. et al.[14] in Delhi, the prevalence was as high as 91.7% which was probably due to high use of cephalosporin's, high rate of patient transfer from peripheral centers and associated patient risk factors like chronic ill health etc.

In the present study, out of 223 isolates 32.28% were ESBL producers which was in accordance with Ranjan S[15] (2012), 34.8% but less than that of Babypadmini et al (2004),[16] C Rodrigues et al (2004)[17] and G Dalella(2011)[18] with 40.3%, 53% and 61.6% respectively.

Male to female ratio observed was 1:1.4 which was higher as compared to Babypadmini et al[16] (2004) 1:1.3 and Nema Shashwati[19] (2014)1:1.2 but common in all studies were female predominance.

In the present study, maximum number of ESBL producers were inpatients (79.1%) and minimum were outpatients 20.8%, which was in accordance to Avinash Laghawe et al[20] (2012) where they were 85.94% and 14.06% respectively.

In our study, ESBL producers were more often isolated from urine (44.4%) followed by pus (22.2%) and sputum (16.7%). Yazdi et al[21] by phenotypic method confirmed 44.3% ESBL among uropathogens.

There is a rising incidence of UTI with ESBL producing bacteria. Hence, routine ESBL testing for uropathogens along with conventional antibiogram would be useful for all cases of UTI. (Table 2)

Comparative Evaluation

Other Phenotypic methods like DDST and PCT were then compared to the Gold standard method for detection of the ESBL. (Table 4) DDST could detect 44/124 (35.48%) suspected strain to be ESBL as against 72 (58.06%) by E-test. Our study showed that 28 stains could not be detected by DDST. Similar results were found by Renuka Rampure et al[22] & Martin et al[23] whereas Jasmine et al[24] found equivocal result between E-test and DDST. Cormican et al[25] reported DDST 79% sensitive as compared to E-test, having itself 100% sensitivity. Although E-test is expensive, yet many investigators reported it to have high sensitivity and to be convenient. Though comparable with p value of < 0.001, the sensitivity of 52.7% doesn't make it a good test to rely upon.[18] DDST lacks sensitivity because of optimal disc spacing and incorrect storage of clavulanate containing disc.[26]

On comparing E-test with PCDT, only 2 more isolates were detected by E-test and performance of PCT was found to be in agreement with the Gold standard test. (Table 4) Present study showed at par result similar to Gaurav Dalela[18] and Derek et al[27] and Anbumani Narayanaswamy[28] found 100% agreement of the two methods- phenotypic confirmatory test and E-test strips in detection of ESBL producers. Enas Sh. Khater et al[29] (2014) also says E-test to be more sensitive.

The discovery and development of antibiotics was undoubtedly one of the greatest advances of modern medicine. Unfortunately, the emergence of antibiotic resistance bacteria is threatening the effectiveness of many antimicrobial agents which has not only increased hospital stay but also economic burden of patients. The production of beta-lactamase may be of chromosomal or plasmid origin. Plasmid mediated production is often acquired by transfer of genetic information from one organism to another. Such transferable plasmid also codes for resistant determinants to other antimicrobial agents. Hence multidrug resistance is expected to be more common in ESBL producing organisms.[30]

In our study, resistance among ESBL producer for cephalsporins, Cefotaxime 83.3%, Ceftrioxone 95.8% and Ceftazidime 100% where as Ciprofloxacillin and Amikacin showed 81.9% and 44.4% respectively. Resistance among uropathogen ESBL producers were for Cotrimoxazole 84.3%, Nitrofurantoin 75% and Norfloxacin 71.8%. The non ESBL producers had different mechanisms for their resistance pattern. (Graph 1) For other antibiotics, the resistance pattern was found to be more in the ESBL producers as compared to their non-ESBL producing counterparts. Hence forth, ESBL producing isolates were resistant to more antimicrobial agents than non-ESBL producing isolates.

In our study Susceptibility for Imipenemwas 98.6% and for piperacillin/tazobactum 76.3%. Henceforth, Imipenem is the most active drug for the treatment of infections caused by ESBL producers, followed by piperacillin/tazobactum and amikacin. We need to keep in mind that carbapenem must be kept in reserve for life-threatening infections where other susceptible antibiotics can be used. Carbapenems are the mainstay of therapy but, they are expensive and require prolonged intravenous administration and there is growing concern of carbapenem resistance in clinical isolates.

Limitations of Study

The important limitations of all the phenotypic tests based on synergy is their inability to detect OXA and Amp C enzymes which are of growing concern. Hyper production of TEM and/or SHV β-lactamases if accompanied with ESBL can cause false negative result by phenotypic test.

Conclusion

Keeping in view the growing incidences of the Multi Drug Resistant Organisms, the Gram negative clinical isolates should be looked for the presence of ESBL production and associated other resistance mechanisms. Resistance to Ceftazidime should be taken as a probable production of ESBL which can be confirmed by PCT effectively in resource poor microbiology laboratories or by MIC by E-test which is a bit costlier but definitely will save human, technical and monitory resources in terms of its confirmation by molecular genotypic methods.

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