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# ESBL PRODUCTION: RESISTANCE PATTERN IN ESCHERICHIA COLI AND KLEBSIELLA PNEUMONIAE, A STUDY BY DDST METHOD

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**ABSTRACT : Objectives:** Resistance to third generation cephalosporins in E. coli and K. pneumoniae are due to various factors. The present study was undertaken to detect resistance mediated by ESBL's. Multidrug resistance in isolates producing ESBL was also studied. **Methods:** The study included a total of 200 clinical specimens which include 95 urine, 45 pus, 32 sputum, 11 blood, 9 throat swabs, 6 suction tips and 2 vaginal swabs. The E. coli and K. pneumoniae isolates which were screen positive were studied for ESBL production by DDST method. **Results:** Culture of 200 samples yielded 200 isolates (117 E. coli and 83 K. pneumoniae). Out of these, 98 (49%) were screen positive for ESBL. Among them 79 (53 E. coli and 26 K. pneumoniae) were found to produce ESBL. Among them, 4 (7.6%) isolates of E. coli and 4 (15.3%) isolates of K. pneumoniae were positive by DDST method. The prevalence of 39.5% of ESBL production was noted in the present study. Among ESBL positive isolates, 98.1% were resistant to cefoxitin, however all of them were susceptible to imipenem. **Conclusion:** The prevalence of ESBL producing E. coli and K. pneumoniae was found to be high and routine screening of ESBL should be preformed on all isolates showing decreased susceptibility to one or more of third generation cephalosporins.

**Key words:** ESBL; DDST; cephalosporins; multidrug resistance

## INTRODUCTION

Escherichia coli (E. coli) and Klebsiella pneumoniae (K. pneumonia) are gram negative bacilli belonging to family enterobacteriaceae. E. coli mainly causes urinary tract infection, diarrhoea, pyogenic infection, sepsis and K. pneumonia causes UTI, pneumonia, other pyogenic infections and rarely diarrhea (Ananthanarayan et al., 2005). While both are common in community acquired infections, they have become notorious nosocomial pathogens (Kaye et al., 2004). Beta lactamase are enzymes that open the β-lactam ring of penicillin and cephalosporins and thus destroy their activity (Danel et al., 1997). The first plasmid mediated β-lactamase in gram negative isolates, TEM-1 was described in early 1960s (Danel et al., 1997). The TEM-1 β-lactamase was observed in different species of family enterobacteriaceae, pseudomonas aeruginosa, hemophilus influenzae and neisseria gonorrheae (Bradford, 2001). Another common plasmid mediated β-lactamase found in K. pneumoniae and E. coli is SHV-1, which is chromosomally encoded in majority of isolates (Bradford, 2001). It was reported that, with the introduction of each new β-lactam antibiotic, a new β-lactamase causing resistance to that antibiotic too emerged (Medeiros et al., 1997). Because of increased spectrum of activity, especially against expanded spectrum cephalosporins, these enzymes were called extended spectrum β-lactamases (ESBL) (Bradford, 2001).

ESBL are enzymes that confer resistance and hydrolyse the expanded spectrum cephalosporins like ceftriaxone, cefotaxime, and monobactams as well as to older penicillins and cephalosporins (Emery and Weymouth, 1997; Coudron et al., 1997). It was reported that, in Japan, the percentage of β-lactam resistance due to ESBL production in E. coli and K. pneumoniae remains low (Coudron et al., 1997). Elsewhere in Asia, the percentage of ESBL production in E. coli and K. pneumoniae varies from 4.8% in Korea to 1.5% in Taiwan and up to 12% in Hong Kong (Bradford, 2001). In India, it was reported that the ESBL prevalence ranges from 12.6% to 68% (Coudron et al., 1997).

It was opined that it is necessary to identify the prevalence of these ESBL strains in a hospital and to characterize their epidemiology to control their spread (Ananthakrishnan et al., 2000). These ESBLs were initially associated with nosocomial outbreaks caused by a single enzyme producing strain. Since the information regarding the occurrence of ESBL in India is scarce, this study was undertaken. The aim of the study was to identify various strains of E. coli and K. pneumoniae isolated from the clinical specimens and to study their ESBL production by double disk synergy test (DDST) method as well as to detect multiple antibiotic resistances. The antibiogram of E. coli and K. pneumonia was studied in detail.

## MATERIALS AND METHODS

The present study included a total of 200 clinical specimens including 95 urine, 45 pus, 32 sputum, 11 blood, 9 throat swabs, 6 suction tips and 2 vaginal swabs. All the specimens were processed immediately within two hours of collection. A gram stained smear was made from all specimens except blood and urine. Smears were examined for pus cells and presence of gram negative bacilli. The specimens were inoculated on Mac Conkey's media and blood agar media. The blood samples were inoculated into brain heart infusion (BHI) broth, incubated at $37^0$C for 24 hours and then subcultured on to Mac Conkey's agar. Urine samples were inoculated on CLED media and incubated at $37^0$C for overnight.

### Identification of the strains:

On Mac Conkey's media lactose fermenting colonies (pink flat or mucoid colonies) were taken for identification. Gram staining was done to isolate the gram negative bacilli. Later hanging drop preparation was done, if the microorganism was motile it was confirmed as E. coli and nonmotile were grouped as K. pneumoniae. The catalase test was done after picking the small amount of isolated colonies with a clean sterile glass rod and keeping them inside a test tube containing 3% hydrogen peroxide ($H_2O_2$) solution. The test was considered positive if production of effervescence was seen. The strains were interpreted with IMViC test. The E. coli showed indole test and methyl red test positive, whereas K. pneumoniae showed both these tests negative. In contrast, K. pneumoniae showed VP test and citrate test positive and the E. coli showed these tests negative.

The strains were next tested with urease test by inoculating the isolated colonies onto entire slope of the Christensen's urease medium and incubating at $37^o$c overnight. The pink colour interpreted the test as positive and the strains were considered as K. pneumoniae. If the colour was yellow, the test was considered negative and strains were grouped as E. coli. In the triple sugar iron agar (TSI) test, both E. coli and K. pneumoniae produced acid slant / acid butt with gas. In the sugar fermentation test, pink colour indicates sugar fermentation and air bubbles in Durham's tube indicate gas production.

### Antibiotic susceptibility test:

E. coli and K. pneumoniae isolates were subjected for antimicrobial susceptibility test by Kirby-Bauer disk diffusion method. The isolated organism was inoculated onto peptone water and incubated at $37^o$c for 4-6 hours. The turbidity of growth was adjusted to 0.5 Macfarland's standard.

This suspension was inoculated onto Muller-Hinton agar plate by lawn culture. The antibiotic discs were placed using sterile forceps and pressed gently to ensure the contact of medium. The ampicillin, gentamicin, ciprofloxacin, norfloxacin, amikacin, cefoxitin, imipenem and cefepime antibiotic discs were used in the present study. The plates were incubated at 37°c overnight. The zone of inhibition was measured and interpreted, comparing zone with Kirby – Bauer chart.

### Detection of extended spectrum beta lactamase (ESBL)

### ESBL – screening test:

The E. coli and K. pneumoniae isolates were screened for possible ESBL production using ceftazidime, cefotaxime, aztreonam, ceftriaxone and cefpodoxime disks. The isolated organisms were inoculated on to peptone water and incubated at $37^0$C for 4-6 hours. The turbidity of growth was adjusted to 0.5 Macfarland's standard. This suspension was inoculated onto Muller-Hinton agar plate by lawn culture. The above five discs were placed at a gap of 20 mm each. These plates were incubated at $37^0$c for overnight. Then the reading was taken. The zone around the disks were measured and interpretated as sensitive and resistant. The isolates which showed resistance to any one of these drugs were considered as screen positive. These were further tested for ESBL production by double disc synergy test (DDST) method. The strains which were sensitive for these drugs were not included in the present study.

### Detection of ESBL by Double disc synergy test (DDST):

The isolated organisms were inoculated on to peptone water and incubated at $37^0$c for 4-6 hours. The turbidity of growth was adjusted to 0.5 Macfarland's standard. This suspension was inoculated onto Muller-Hinton agar plate by lawn culture. A disc containing amoxyclav (amoxycillin + clavulanic acid) was placed at center of the plate. Ceftazidime, cefotaxime, ceftriaxone, aztreonam, cefpodoxime were placed with the interdisc distance (edge to edge) of 15 mm from the amoxyclav disc. The plates were incubated at $37^0$c for overnight. Enhancement of zone of inhibition towards amoxyclav by any one of these drugs such as ceftazidime, cefotaxime, ceftriaxone, aztreonam or cefpodoxime was considered as positive result.

## RESULTS

Among our specimens, 79 (39.5%) samples showed the ESBL positive. Maximum numbers of ESBL positive isolates were obtained from blood (63.6%) followed by vaginal swabs (50%), urine (42.1%), pus (37.7%), suction tip (33.3%), sputum (31.2%) and throat swab (22.2%). The 47% of samples of age group 16-30 years were ESBL positive. Though the number of isolates from infants was less, 50% of isolates from them were ESBL positive. The ESBL positive isolates were recovered in higher number (47.6%) from female patients than in male patients (32.2%). Maximum number of E. coli were isolated from vaginal swabs (100%) followed by equally from suction tips and throat swabs (66.7%) and by urine, pus, blood and sputum in the descending order. Maximum number of K. pneumoniae were isolated from sputum (56.3%) followed by blood, pus, urine in the descending order. From our specimens, 98 were screen positive isolates and 102 were screen negative. Only screen positive isolates were tested for ESBL production. Among the E. coli isolates 66 (56.4%) were screen positive and 32 (38.6%) isolates of K. pneumoniae were screen positive. We observed that, all the E. coli and K. pneumoniae isolates were uniformly resistant to cefpodoxime as well as aztreonam. However 100% of E.coli isolates were resistant to cefotaxime and only 93.7% of K. pneumoniae were resistant to it. A total of 53 E. coli and 26 K. pneumoniae isolates were identified as ESBL producer. Only 4 isolates each of E. coli (7.6%) and K. pneumoniae (15.3%) were identified as ESBL producer by DDST method. Remaining 92.4% of E. coli and 84.7% of K. pneumoniae were negative for ESBL by DDST. Table 1 shows the various antibiotic susceptibility pattern among ESBL positive E. coli and K. pneumoniae. All the E. coli and K. pneumoniae isolates were resistant to ampicillin (A), amoxyclav (Ac), aztreonam (Ao), cefpodoxime (Cep) and ceftazidime (Ca).

**Table 1 Showing the antibiotic pattern of ESBL positive in E. coli and K. pneumoniae**

| Antibiotics | E.Coli (n = 53) | | | K. Pneumoniae (n = 26) | | |
|---|---|---|---|---|---|---|
| | Susceptible | Moderately Susceptible | Resistant | Susceptible | Moderately Susceptible | Resistant |
| Ampicillin (A) | 0 | 0 | 53 | 0 | 0 | 26 |
| Amoxycillin + Clavulanic acid (Ac) | 0 | 0 | 53 | 0 | 0 | 26 |
| Norfloxacin (Nx) | 2 | 0 | 51 | 6 | 1 | 19 |
| Ciprofloxacin (Cf) | 3 | 1 | 49 | 9 | 2 | 15 |
| Gentamicin (G) | 18 | 2 | 33 | 4 | 0 | 22 |
| Amikacin (Ak) | 47 | 3 | 3 | 17 | 2 | 7 |
| Aztreonam (Ao) | 0 | 0 | 53 | 0 | 0 | 26 |
| Cefoxitin (Cn) | 1 | 0 | 52 | 1 | 0 | 25 |
| Cefotaxime (Ce) | 0 | 0 | 53 | 2 | 0 | 24 |
| Cefpodoxime (Cep) | 0 | 0 | 53 | 0 | 0 | 26 |
| Ceftazidime (Ca) | 0 | 0 | 53 | 0 | 0 | 26 |
| Ceftazidime + Clavulanic acid (Cac) | 17 | 0 | 36 | 7 | 0 | 19 |
| Ceftriaxone (Ci) | 1 | 0 | 52 | 1 | 0 | 25 |
| Ceftriaxone + Sulbactum (Cis) | 35 | 0 | 18 | 5 | 0 | 21 |
| Cefipime (Cpm) | 2 | 1 | 50 | 0 | 1 | 25 |
| Imipenem (I) | 53 | 0 | 0 | 26 | 0 | 0 |

Only one isolate of E. coli and K. pneumoniae each were susceptible to cefoxitin. Only 2 isolates of E. coli and none of the K. pneumoniae isolates were susceptible to cefipime. Only 17 isolates of E. coli were rendered susceptible to ceftazidime (Ca) by the presence of clavulanic acid. Similarly only 35 E. coli isolates were rendered susceptible to ceftriaxone (Ci) by the presence of sulbactum. Likewise presence of clavulanic acid rendered 7 isolates of K. pneumoniae susceptible to ceftazidime (Ca) and 5 isolates susceptible to ceftriaxone (Ci) by the presence of sulbactum. All the isolates were uniformly susceptible to imipenem (I).

The table 2 shows that all ESBL positive isolates were resistant to penicillin and monobactum. These isolates were also resistant equally to third generation cephalosporins and cefamycin. From Table 3, we can observe that the role of β-lactamase inhibitor in protecting the 3rd generation cephalosporins used in combination.

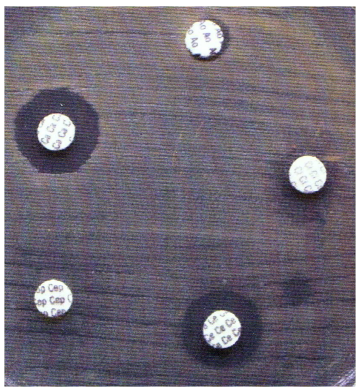

Fig. 1 Showing the screening for ESBL using ceftazidime (Ca), cefotaxime (Ce), ceftriaxone (Ci), Aztreonam (Ao) and Cefpodoxime (Cep).

While all the strains of E. coli and K. pneumoniae were resistant to ceftazidime (Ca), presence of clavulanic acid rendered 17 E. coli isolates (32%) and 6 isolates K. pneumoniae (23%) susceptible to ceftazidime (Ca). While all, but 1 isolate each of both E. coli and K. pneumoniae were resistant to ceftriaxone (Ci), presence of sulbactum rendered 35 E. coli isolates (67.3%) and 17 isolates of K. pneumoniae (68%) were rendered susceptible to ceftriaxone (Ci). Figure 1 shows the screening for ESBL using ceftazidime (Ca), cefotaxime (Ce), ceftriaxone (Ci), aztreonam (Ao) and cefpodoxime (Cep). Whereas in Figure 2, by double disk diffusion test (DDST), we can observe the enhancement of colonies towards amoxyclav (Ac) disk by cefotaxime (Ce) and ceftriaxone (Ci).

**Table 2 showing the pattern of multidrug resistance in ESBL positive E. coli and K. pneumoniae**

| Organism | | (A) Penicillins | (Nx, Cf) Flouroquinolones | (G, Ak) Aminoglycocides | (Cn) Cephamycin | (Ca, Ce, Ci, Cep) 3rd gen. Cephalosporines | (Cac, Cis) 3rd gen. cephalosporines + β-lactamase inhibitor | (Cpm) 4th gen. Cephalosporines | (Ao) Monobactam | (I) Carbapenem |
|---|---|---|---|---|---|---|---|---|---|---|
| E. Coli (n = 53) | No. | 53 | 51 | 36 | 52 | 52 | 27 | 50 | 53 | 0 |
| | % | 100 | 96.2 | 67.9 | 98.1 | 98.1 | 50.9 | 94.3 | 100 | 0 |
| K. pneumoniae (n = 26) | No. | 26 | 18 | 15 | 25 | 25 | 17 | 25 | 26 | 0 |
| | % | 100 | 69.2 | 55.7 | 96.1 | 96.1 | 65.3 | 96.1 | 100 | 0 |

(A = Ampicillin, Nx = Norfloxacin, Cf = Ciprofloxacin, G = Gentamicin, Ak = Amikacin, Cn = Cefoxitin, Ca = Ceftazidime, Ce = Cefotaxime, Ci = Ceftriaxone, Cep = Cefpodoxime, Cpm = Cefipime, Ao = Aztreonam, I = Imipenem).

**Table 3 Showing comparison of Ceftazidime (Ca), Ceftazidime - Clavulinic acid (Cac) and Ceftriaxone (Ci), Ceftriaxone – Sulbactum (Cis) in ESBL positive E. coli and K. pneumoniae**

| Antibiotics | E. coli (n = 53) | | K. pneumoniae (n = 26) | |
|---|---|---|---|---|
| | Sensitive | Resistant | Sensitive | Resistant |
| Ceftazidime (Ca) | 0 | 53 | 0 | 26 |
| Ceftazidime with Clavulanic acid (Cac) | 17 | 36 | 6 | 20 |
| Ceftriaxone (Ci) | 1 | 52 | 1 | 25 |
| Ceftriaxone with Sulbactum (Cis) | 35 | 18 | 17 | 9 |

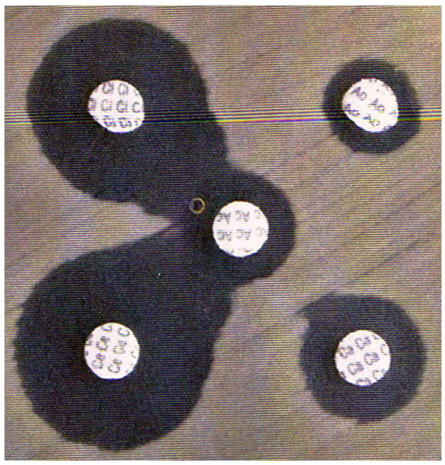

Fig. 2 Double disk diffusion test (DDST) showing enhancement towards amoxyclav (Ac) disk by cefotaxime (Ce) and ceftriaxone (Ci).

## DISCUSSION

Beta lactamase production by several gram negative and gram positive organism is perhaps the most important mechanism of resistance to penicillins and cephalosporins (Chaudhary et al., 2004). Over the last 20 years, many new β-lactam antibiotics have been developed which were specifically developed to be resistant to hydrolytic action of β-lactamases. However, with each new class that has been used to treat patients, new β-lactamases emerged that caused resistance to that class of drug (Bradford, 2001). Extensive use of newer generation cephalosporins has been the strong factor for evolution of newer β-lactamases such as ESBLs. The ESBL was first detected in E. coli, in 1982 at Germany (Coudron et al., 1997) and isolates producing it have been steadily being reported world over (Babypadmini et al., 2004). Initially they were detected in clinical isolates of K. pneumoniae and E. coli but are now increasingly seen in other members of enterobacteriaceae such as enterobacter, citreobacter, salmonella, serratia etc. (Bradford, 2001). The fact that ESBLs are coded on transmissible plasmids makes them to easily transfer to other species. E. coli and klebsiella species that have adopted themselves to the hospital environment are more at risk of acquiring these enzymes. Such isolates could be responsible for outbreaks of infections in hospitalised patients resulting in higher morbidity and mortality. Since ESBL confer resistance to all third generation cephalosporins and since these antibiotics are predominantly used once in several hospitals, such a resistance can lead to treatment failures. Hence detection of isolates expressing ESBLs is crucial in patients health care as well as limiting the spread of ESBL producing isolates. Though ESBLs might be produced by several members of enterobacteriaceae, the present study was restricted only to detect their presence in clinically significant E. coli and K. pneumoniae isolates. There are few studies available in the literatures which are similar to the present investigation. Shukla et al. (2004) screened the isolates by using cefotaxime (Ce), ceftazidime (Ca), ceftriaxone (Ci) discs and found 88.3% of isolates which were resistant to one of the above mentioned third generation cephalosporins and 72% were resistant to all the three drugs. Rodrigues et al. (2004), screened the isolates by using aztreonam (Ao), cefotaxime (Ce), ceftazidime (Ca), ceftriaxone (Ci) and cefpodoxime (Cep) and found that cefpodoxime (Cep) is the most sensitive screening agent. In present study we have used five cephalosporins to screen possible ESBL production. Resistance to one or more of these screening agents might indicate ESBL production. Since there are variations among the ESBLs in their ability to hydrolyse various cephalosporins, it is difficult to pickup any one as the best screening agent. However we observed that cefpodoxime (Cep) is the best screening agent. Aztreonam (Ao) too has faired well in demonstrating resistance against these isolates. Use of single or 2 screening agents might sometimes miss the detection of resistant isolates. Hence we state that use of three or more screening discs improves the rate of detection. The present study correlates with Rodrigues et al. (2004) study.

DDST method has not been standardised and the results were subjected to variation in the interdisk distance. Several studies have been conducted with interdisk distances being 30 mm (Subha et al., 2002; Nath et al., 2006), 25 mm (Ho et al., 2000) and 15 mm (Ananthakrishnan et al., 2000; Babypadmini et al., 2004; Menon et al., 2006). In the present study, an interdisk distance of 15 mm was choosen as per recommendation of Coudron et al. (1997). The enhancement of zone of inhibition between amoxyclav and ceftazidime (Ca), cefotaxime (Ce), ceftriaxone (Ci) and aztreonam (Ao) was performed in the present study. The present study observed better enhancement of zone of inhibition seen by ceftazidime (Ca) and cefotaxime(Ce). Babypadmini et al. (2004) and Ananthakrishnan et al. (2000) studied ESBL production by performing DDST method using amoxyclav (Ac) and cefotaxime (Ce) disk placed at a distance of 15mm. Subha et al. (2002) and Nath et al. (2006) performed DDST method by using amoxyclav (Ac) disk and each third generation cephalosporins test antibiotic placed at a distance of 30mm. In contrast, Menon et al. (2006) performed DDST method using aztreonam (Ao), cefotaxime (Ce), ceftazidime (Ca) and ceftriaxone (Ci) placed 15 mm from an amoxyclav (Ac) disk. The present study correlates with the findings of Menon et al. (2006) study. In the present study only 4 isolates of each E.coli and K. pneumoniae were positive by DDST method. The ability of DDST method to detect ESBL producer was surprisingly low (10.12%) in the study. Menon et al. (2006) reported 14.2% of isolates which were ESBL positive were detected by DDST method. Shukla et al. (2004) reported detection of ESBL production by DDST method was 27.3%. Khurana et al. (2002) reported a prevalence rate of 26.6% for ESBL production among the urinary isolates. ESBL producing strains are common in K. pneumonia, 38.5% and 24.7% of E. coli. Babypadmini et al. (2004) reported that ESBL production was 41% of E. coli and 40% of K. pneumoniae isolates. Tankhiwale et al. (2004) reported a prevalence rate of 48.3% of ESBL production from urinary isolates and found 25.6% of Klebsiella isolate and 18.5% of E. coli isolates were ESBL producers. The present study correlates with Babypadmini et al. (2004) and Tankhiwale et al. (2004) studies.

In the present study, all the ESBL producing isolates of E. coli and K. pneumoniae were uniformly resistant to cefpodoxime (Cep) and aztreonam (Ao) making those good indicates of possible ESBL production. Jain and Mondal (2007) reported 93.10% of ESBL positive isolates were resistant to cefpodoxime and 84.4% to aztreonam (Ao). All the isolates were resistant to ampicillin (A) and amoxyclav (Ac) in the present study. Only the aminoglycosides like gentamicin (G) and amikacin (Ak) were to some extent effective against these isolates. In the present study, 88.6% of E. coli and 65.3% of K. pneumoniae were susceptible to amikacin (Ak). Flouroquinolones like norfloxacin (Nx) and ciprofloxacin (Cf) were to some extent resistant to ESBL positive E. coli and K. pneumoniae in the present study. About 51 E. coli isolates were resistant to norfloxacin (Nx) and 2 were sensitive. Whereas in case of K. pneumonia, 19 were resistant and 6 were sensitive to norfloxacin (Nx).

## CONCLUSION

DDST method detected ESBL production in only 7.6% of E. coli and 15.3% of K. pneumonia. ESBL positive isolates also exhibited high levels of multidrug resistance. The prevalence of ESBL producing E. coli and K. pneumoniae was found to be high and we suggest that routine screening of ESBL should be preformed on all isolates showing decreased susceptibility to one or more of third generation cephalosporins.

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