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# Clinical and Molecular Epidemiology of Extended-Spectrum β-lactamase-Producing Klebsiellapneumoniae

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**ABSTRACT**

Infections caused by multidrug-resistant gram-negative bacilli that produce extended-spectrum *β*-lactamase (ESBL) enzymes have been reported with increasing frequency in intensive-care units and are associated with significant morbidity and mortality. Because of resistance to numerous antimicrobial agents, treatment can be challenging. Isolates of *Klebsiella pneumoniae* have been producing extended-spectrum β-lactamases, rendering them resistant to many classes of antibiotics such as β-lactam/β-lactamase inhibitor combinations, quinolones, and aminoglycosides. This limits the choice of treatment to carbapenems for the treatment of serious infections. However, there has been an increasing spread of carbapenem-resistant *Klebsiella pneumoniae* strains (KPC-Kp). This leaves limited therapeutic options against KPC-Kp (tigecycline and colistin). However, they are even starting to find KPC-Kp strains resistant to Collistin, and Tigecycine does not work as well against blood infections. Due to this reason, there is a need for the development of new antimicrobial agents that can fight off the multi-drug resistant strains of *Klebsiella pneumoniae*.

**Keywords**: Extended-spectrum β-lactamase, Klebsiella pneumonia

## INTRODUCTION

Infections caused by multidrug-resistant Gram negative bacilli that produce extended-spectrum β-lactamase (ESBL) enzymes have been reported with increasing frequency in intensive-care units and are associated with significant morbidity and mortality.Because of resistance to numerous antimicrobial agents, treatment can be challenging. The genus *Klebsiella* is a member of the Enterobacteriaceae family. *Klebsiella* spp are ubiquitous in nature and can be found in the natural environment (e.g., water and soil) and on mucosal surfaces of mammals. Common sites of colonization in healthy humans are the gastrointestinal tract, eyes, respiratory tract, and genitourinary tract.

*K. pneumoniae* has emerged as an important cause of hospital-acquired infections, especially among patients in the neonatal intensive-care unit and mortality rates can be as high as 70%. Over the last two decades, the incidence of infections caused by multidrug-resistant *Klebsiella* strains has increased.

Extended spectrum β-lactamase enzymes were first described in *K. pneumoniae* and *Serratia marcescens* isolates in 1983 in Europeand in *K. pneumoniae* and *Escherichia coli* isolates in 1989 in the United States.Since then, there has been a marked increase in the incidence of bacteria that produce ESBL enzymes. In the United States, the proportion of *K. pneumoniae* strains resistant to ceftazidime increased from 1.5% in 1987 to 3.6% in 1991, and by 1993 as many as 20% of strains were resistant to ceftazidime in some teaching hospitals[1,2]. Of 824 *K. pneumoniae* strains isolated from 15 hospitals in New York City during 1999, 34% expressed ESBL enzymes[3,4]. ESBL are plasmid-mediated enzymes that hydrolyze oxyimino- β lactam agents such as third-generation cephalosporins and aztreonam[5].These plasmids also carry resistance genes to other antibiotics including aminoglycosides,

chloramphenicol, sulfonamides, trimethoprim, and tetracycline. Thus, Gram negative bacilli containing these plasmids are multidrug-resistant[6-8,].Furthermore, these plasmids are mobile genetic elements and can be transmitted between Gram negative bacilli of different species *in vivo*[9]. During a 30-month outbreak of ESBL-producing *K. oxytoca* in an NICU, the plasmid from *K.oxytoca* spread to *K. pneumoniae*, *E. coli*, *Enterobacter cloacae*, and *Citrobacter freundii*[10].

Over 100 different ESBL enzymes have been identified, each with a preferential substrate. Thus, an ESBL-producing isolate may be resistant to ceftazidime, but susceptible to cefotaxime. As a result, ESBL-producing isolates may not be detected if susceptibility testing is limited to a single third-generation cephalosporin. The National Committee for Clinical Laboratory Standards (NCCLS) recommends routine screening for ESBL activity in *E. coli*, *K. pneumoniae*, and *K. oxytoca* isolates by determining susceptibility to several cephalosporins including cefpodoxime, cefotaxime, ceftriaxone, and ceftazidime[11,12]. If an isolate is resistant to any one of these agents, that is, MIC $\geq 2$ $\mu$g/ml, confirmatory tests for an ESBL enzyme are performed by demonstrating increased susceptibility to cefotaxime or ceftazidime in the presence of clavulanic acid, as clavulanic acid inhibits ESBL enzymes and lowers the MIC of the cephalosporins.

Epidemiological studies suggest that the increasingly widespread use of third-generation cephalosporins is a major risk factor that has contributed to the emergence of ESBL-producing *K. pneumoniae*[13-15]. Several additional risk factors for colonization and infection with ESBL-producing organisms have been reported and include: arterial and central venous catheterization, gastrointestinal tract colonization with ESBL-producing organisms, prolonged length of stay in an intensive-care unit, low birth weight in preterm infants, prior antibiotic use, and mechanical ventilation[16-18]. Carriage of this organism increases dramatically among hospitalized patients, as colonization rates increase in direct proportion to the length of stay[19].

Outbreaks of ESBL-producing organisms have been described. Asymptomatic patients colonized with ESBL-producing *K. pneumoniae* can serve as reservoirs for this pathogen with subsequent patient-to-patient spread via the hands of health-care workers. In addition, contaminated patient-care items and artificial fingernails worn by health-care workers have been implicated in transmission[20-23]. Most studies have demonstrated a poor adherence to infection control policies as an important factor. Outbreaks of ESBL-producing *K. pneumoniae* in NICUs have been notable for high attack rates and large numbers of colonized infants. The neonates at greatest risk for colonization are those with a longer length of stay, a lower estimated gestational age and/or a lower birth weight. To control the spread of ESBL-producing pathogens, appropriate infection control interventions should be implemented for all patients who are infected or colonized with ESBL-producing bacteria. These interventions include effective hand hygiene and instituting contact precautions for all colonized and infected patients. Health-care workers in NICUs should not be permitted to wear artificial fingernails. During a prolonged outbreak, a broader investigation to search for additional reservoirs in colonized patients, health-care workers, and the environment may be needed. Also important is the judicious use and control of antimicrobials, particularly the restricted use of broad-spectrum cephalosporins[24-26]. This is particularly important in NICUs that use cefotaxime to treat late-onset sepsis.

## MATERIALS AND METHODS

The study was undertaken from November 2004 to April 2005 in the Department of Microbiology, SriRamachandra Medical College and Research Institute, a 1500-bedded tertiary care centre. During this period all clinically significant, consecutive, non-repetitive isolates of the genus *Klebsiella* from hospitalized patients were included in the study. The isolates were collected from various specimens like blood, urine, pus, wound swab, sputum, bronchial wash, endotracheal secretions and body fluids from patients admitted in medical, surgical wards and intensive care units (medical, surgical, cardiothoracic, cardiology, neurosurgery and burns units). A detailed clinical history was taken and recorded from the patients whose culture grew *Klebsiella* from any of the above clinical specimens. The proforma included the patient's age, sex, date of admission, admitted ward, brief clinical history, diagnosis, presence of any risk factors (DM, intake of steroid or immunosuppressant, HIV, HBV), presence of associated illness and antibiotic therapy [Proforma enclosed in Annexure I]. The samples were collected aseptically by standard techniques [2]. Information on the isolated strains, including etiology and susceptibility to antibiotics, was also obtained.

**Methodology**

**Specimen processing**

A direct smear for assessment of the cellularity and presence of bacteria was carried out in all cases. The media for the study were procured from Himedia, Mumbai.The media and the biochemicals were prepared by following standard procedures [20] **[Annexure II].**

The isolates, which showed resistance to any one of the third generation cephalosporin by the disc diffusion method, were subjected to minimum inhibitory concentration (MIC) determination for third generation cephalosporin's, Double Disc Synergy Test (DDST), Inhibitor potentiation to confirm ESBL production.

The MIC for the third generation cephalosporin was performed by the agar dilution technique in accordance with the CLSI guidelines with reference strains ATCC. *E.coli* 25922 and ATCC *Klebsiella pneumoniae* 700603. A serial two fold drug dilution spacing the expected and normal range of MIC values was incorporated. The range of coverage included the expected values for ATCC strains. Commercially available therapeutic antibiotic vials were used for the study. The drugs were provided by the manufacturers.

- Ceftazidime: Orchid chemicals and pharmaceuticals Ltd, Chennai.
- Ceftriaxone: Lupin Ltd, Mandideep, Madhya Pradesh.
- Cefotaxime: Alkem laboratories, Mumbai.

Acceptable MIC ranges of various drugs for ATCC *Escherichiacoli*

- Ceftazidime 0.06 µg/ml – 0.5 µg/ml
- Ceftriaxone 0.03 µg/ml – 0.12 µg/ml
- Cefotaxime 0.03 µg/ml – 0.12 µg/ml

MIC of Ceftazidime was done as ESBL screening test & MIC value of ≥ 2µg/ml was taken as probable ESBL producer. MIC of other third generation cephalosporin was estimated to reveal the high MIC values exhibited by ESBL producing organism.

**Procedure for drug dilution**

Commercially available antibiotic vials containing the antibiotic powder were used. They were diluted with sterile distilled water as all the drugs used in the study were soluble in the above solution. The drug was diluted in such a way that 10ml of distilled water contained 1gm of the drug. The required amount of the drug solution was taken for the corresponding antibiotic concentration and mixed with sterile distilled water to make the final 2ml volume, which then added to 18 ml of molten agar while setting at $50^0$C. A serial two fold drug dilutions were prepared and the range of dilutions varied for each antibiotic as shown below,

Ceftazidime: 0.125 µg – 1024 µg
Ceftriaxone: 0.03 µg – 1024 µg
Cefotaxime: 0.03 µg – 1024 µg

**[Drug dilution enclosed in Annexure V]**

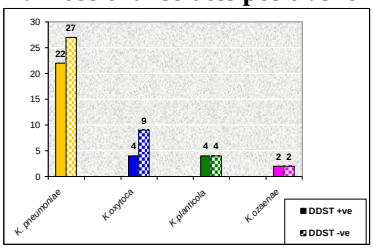
**Preparation of agar plates**

18ml of MHA was prepared, sterilized and cooled to 50°C. To this 2ml of the drug in required dilution was added to make up the volume to 20ml and poured in the glass petridish. To test the viability of the control organism and the test organism, a control plate without the antibiotic was included. The plates were allowed to set and the same were incubated overnight for quality checking. All the plates were inoculated the next day. After the plates with the media were set, they were allowed to dry for few minutes at 37°C before use.

## RESULTS AND DISCUSSION

**Figure 1 : *Klebsiella* isolates positive for DDST**

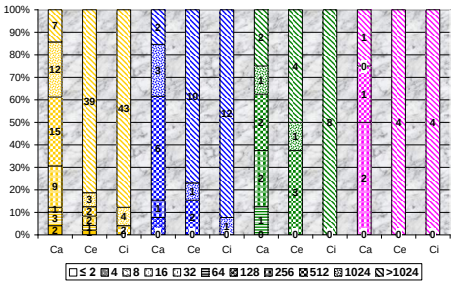



MIC determination done for third generation cephalosporin by agar dilution technique showed a very high MIC values suggestive of ESBL production **(Table 1).** The MIC values of individual *Klebsiella* isolates is shown in **Figure 1.**

**Table 1: MIC determination of *Klebsiella* isolates**

| Drug | Minimum inhibitory concentration in µg/ml | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | ≤ | 4 | 8 | 16 | 32 | 64 | 128 | 256 | 512 | 1024 | >1024 |
| Ceftazidime | 2 | - | - | - | - | 4 | 2 | 14 | 24 | 16 | 12 |
| Cefotaxime | 2 | - | - | - | - | 1 | - | 2 | 7 | 5 | 57 |
| ceftriaxone | - | - | - | - | - | - | - | - | 2 | 5 | 67 |

**Figure 2: MIC determination of *Klebsiella* isolates**




Inhibitor potentiated disc diffusion test was done using three drug/inhibitor combination. An increase in zone size by ≥5mm with Ceftazidime/Clavulanic acid, Piperacillin/Piperacillin-Tazobactam and Cefaperazone/Cefaperazone-Sulbactam was observed in 92% (n=68), 99% (n=73) and 96% (n=71) ($p<0.05$) of the isolates respectively **(Figure 2).**

### Inoculation of the test organism

The test organism was inoculated into peptone water and incubated at 37°C until the turbidity matched 0.5 Macfarland standards. Then a platinum loop calibrated to deliver 0.001ml of the inocula was used to spot inoculates the culture. The control strains ATCC *E.coli* 25922 and ATCC *Klebsiellapneumoniae* 700603 were included in all the plates. Inoculated plates were left undisturbed until the spot of inoculums had dried. The plates were then inverted & incubated at 37°C for 16 to 18 hours.

### Interpretation

The concentration of the drug at which there was no visible growth of the organism at the spot of inoculation was taken as the MIC for that particular drug.

**Double Disc Synergy Test (DDST)**

In DDST, either enhancement of the zone size (for the III generation cephalosporins) of the antibiotic in the presence of clavulanate or clear extension of the edge of the inhibition zone of any of the antibiotic towards the disc containing clavulanic acid was interpreted as an indication of ESBL production.

**Procedure**

The test organism was grown overnight at 37°C on nutrient agar plate. Isolated colonies of organism was inoculated into peptone water and incubated at 37°C and the turbidity was adjusted to 0.5 Macfarland standards. A lawn culture of the test organism was made on MHA plate with a sterile cotton swab soaked in the broth, after removing the excess broth by pressing against the sides of the test tube. A disc of Amoxyclav (20µg Amoxycillin/10µg Clavulanic acid) was placed in the center of the lawn culture, on the three sides of this disc at a distance of 30mm from the edge of the above disc; discs containing Ceftazidime, Cefotaxime and Ceftriaxone were placed. Plates were then incubated at 37°C for 18 to 24 hours.

**Interpretation**

The isolates interpreted as ESBL if the inhibition zone around one or more cephalosporin disc was extended on the side nearest to the Amoxyclav disc or clear extension of the edge of the inhibition zone of any of the antibiotic disc towards the Amoxyclav disc. If there is no extension of the zone, the test was repeated by reducing the distance between the discs to 20mm. The test was considered negative if there was no distortion or synergy.

**Inhibitor Potentiated Disc Diffusion Technique**

The test organism was grown at 37°C on a nutrient agar plate incubated overnight. Isolated colonies of the organism were inoculated into peptone water and incubated at 37°C and the turbidity adjusted to 0.5 Macfarland standards.

**Procedure**

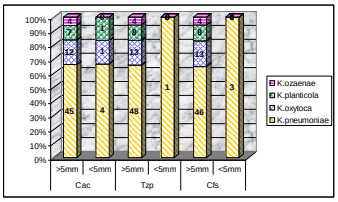
A lawn culture of the test organism was made on the MHA plates with a sterile cotton swab soaked in the broth, after removing the excess broth by pressing against the sides of the test tube and the following discs were placed,

Ceftazidime (30µg) / Ceftazidime-clavulanate (30µg + 10µg) (Himedia)
Cefaperazone (30µg) / Cefaperazone-sulbactam (30µg + 75µg) (Himedia)
Piperacillin (10µg) / Piperacillin-tazobactam (10µg + 100µg) (BBL, USA)
After placing these discs, the plates were incubated at 37°C for 18 to 24 hours.

**Interpretation**

Zone diameter of the antibiotic (alone) and antibiotic with the inhibitor combination were compared. If the difference in zone size was ≥ 5mm it was indicative of ESBL production.

**Table 3: Inhibitor Potentiated Disc Diffusion Test**




Out of the 74 isolates screened for Amp C production using Cefoxitin disc 38% (n=28) strains showed resistance **(Figure 3)**

**Figure 4: Screened for AmpC production using Cefoxitin**

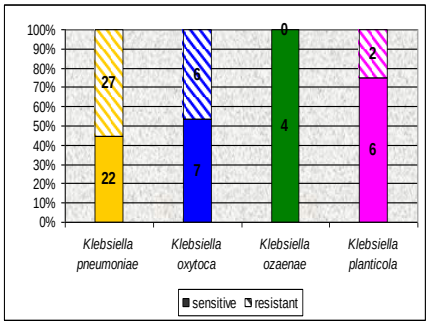



**Table 4**: Correlation of clinical samples & species of Klebsiella

| Sample | Total | Klebsiella pneumoniae | | | Klebsiella oxytoca | | | Klebsiella planticola | | | Klebsiella ozaenae | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | ICU | Non ICU | Total | ICU | Non ICU | Total | ICU | Non ICU | Total | ICU | Non ICU | Total | Total |
| Blood | 18 | 14 | 1 | 15 (15%) | 1 | 0 | 1 (1%) | 1 | 0 | 1 (5%) | 1 | 0 | 3 | 1 (5%) |
| Exudate | 31 | 1 | 18 | 19 (61%) | 2 | 5 | 7 (22%) | 1 | 2 | 3 (10%) | 0 | 2 | 3 | 2 (6%) |
| Respiratory | 20 | 14 | 0 | 14 (70%) | 3 | 0 | 3 (15%) | 2 | 0 | 2 (10%) | 1 | 0 | 3 | 1 (5%) |
| Total | 127 | 35 (41%) | 51 (59%) | 86 (68%) | 8 (38%) | 13 (62%) | 21 (16%) | 5 (42%) | 7 (58%) | 12 (9%) | 2 (25%) | 6 (75%) | 2 | 8 (6%) |

**Table 5: Antibiotic Susceptibility Pattern of *Klebsiella* species**

| Antibiotic | *Klebsiella pneumoniae* | | *Klebsiellaplanticola* | | *Klebsiella oxytoca* | | *Klebsiella ozaenae* | |
|---|---|---|---|---|---|---|---|---|
| | S | R | S | R | S | R | S | R |
| Ampicillin | 0(0%) | 86(100%) | 0(0%) | 12(100%) | 0(0%) | 21(100%) | 0(0%) | 8(100%) |
| Piperacillin | 0(0%) | 86(100%) | 0(0%) | 12(100%) | 0(0%) | 21(100%) | 0(0%) | 8(100%) |
| Amikacin | 45(52%) | 41(48%) | 7(58%) | 5(42%) | 10(48%) | 11(52%) | 5(63%) | 3(38%) |
| Ciprofloxacin | 40(47%) | 46(53%) | 4(33%) | 8(67%) | 11(52%) | 10(48%) | 6(75%) | 2(25%) |
| Cefazolin | 15(17%) | 71(83%) | 2(17%) | 10(83%) | 4(19%) | 17(81%) | 0(0%) | 8(100%) |
| Cefuroxime | 25(29%) | 61(71%) | 2(17%) | 10(83%) | 5(24%) | 16(76%) | 2(25%) | 6(75%) |
| Ceftazidime | 48(56%) | 38(44%) | 4(33%) | 8(67%) | 9(43%) | 12(57%) | 4(50%) | 4(50%) |
| Cefotaxime | 41(48%) | 45(52%) | 4(33%) | 8(67%) | 8(38%) | 13(62%) | 4(50%) | 4(50%) |
| Ceftriaxone | 37(43%) | 49(57%) | 4(33%) | 8(67%) | 9(43%) | 12(57%) | 4(50%) | 4(50%) |
| Cefaperazone | 37(43%) | 49(57%) | 4(33%) | 8(67%) | 9(43%) | 12(57%) | 4(50%) | 4(50%) |
| Cefaperazone-Sulbactam | 52(60%) | 34(40%) | 7(58%) | 5(42%) | 14(67%) | 7(33%) | 6(75%) | 2(25%) |
| Piperacillin-Tazobactam | 55(64%) | 31(36%) | 8(67%) | 4(33%) | 15(71%) | 6(29%) | 6(75%) | 2(25%) |
| Imipenem | 86(100%) | 0(0%) | 12(100%) | 0(0%) | 21(100%) | 0(0%) | 8(100%) | 0(0%) |

**Table 6: Antibiogram of Resistant *Klebsiella* Isolates**

| Antibiotic | *Klebsiella pneumoniae* | | *Klebsiellaplanticola* | | *Klebsiella oxytoca* | | *Klebsiella ozaenae* | |
|---|---|---|---|---|---|---|---|---|
| | **S** | **R** | **S** | **R** | **S** | **R** | **S** | **R** |
| Ampicillin | 0(0%) | 49(100%) | 0(0%) | 8(100%) | 0(0%) | 13(100%) | 0(0%) | 4(100%) |
| Piperacillin | 0(0% | 49(100%) | 0(0%) | 8(100%) | 0(0%) | 13(100%) | 0(0%) | 4(100%) |
| Amikacin | 13(27%) | 36(73%) | 3(38%) | 5(63%) | 2(15%) | 11(85%) | 1(25%) | 3(75%) |
| Ciprofloxacin | 11(22%) | 38(78%) | 3(38%) | 5(63%) | 3(23%) | 10(77%) | 2(50%) | 2(50%) |
| Cefazolin | 0(0%) | 49(100%) | 0(0%) | 8(100%) | 0(0%) | 13(100%) | 0(0%) | 4(100%) |
| Cefuroxime | 0(0%) | 49(100%) | 0(0%) | 8(100%) | 0(0%) | 13(100%) | 0(0%) | 4(100%) |
| Ceftazidime | 1(2%) | 48(98%) | 0(0%) | 8(100%) | 1(8%) | 12(92%) | 0(0%) | 4(100%) |
| Cefotaxime | 4(8%) | 45(92%) | 0(0%) | 8(100%) | 0(0%) | 13(100%) | 0(0%) | 4(100%) |
| Ceftriaxone | 0(0%) | 49(100%) | 0(0%) | 8(100%) | 1(8%) | 12(92%) | 0(0%) | 4(100%) |
| Cefaperazone | 0(0%) | 49(100%) | 0(0%) | 8(100%) | 1(8%) | 12(92%) | 0(0%) | 4(100%) |
| Cefoxitin | 29(59%) | 20(41%) | 6(75%) | 2(25%) | 7(54%) | 6(46%) | 4(100%) | 0(0%) |
| Cefipime | 16(33%) | 33(67%) | 4(50%) | 4(50%) | 2(15%) | 11(85%) | 2(50%) | 2(50%) |
| Cefaperazone-Sulbactam | 15(31%) | 34(69%) | 3(38%) | 5(63%) | 6(46%) | 7(54%) | 2(50%) | 2(50%) |
| Piperacillin-Tazobactam | 18(37%) | 31(63%) | 4(50%) | 4(50%) | 8(62%) | 5(38%) | 2(50%) | 2(50%) |
| Aztreonam | 0(0%) | 49(100%) | 0(0%) | 8(100%) | 0(0%) | 13(100%) | 0(0%) | 4(100%) |
| Imipenem | 49(100%) | 0(0%) | 8(100%) | 0(0%) | 13(100%) | 0(0%) | 4(100%) | 0(0%) |

**CONCLUSION**

127 isolates of *Klebsiella* were taken for the study which was carried out at SriRamachandra Medical College & Research Institute, a tertiary care centre from hospitalized patients. The speciation was carried out as per procedure in Bergey's Manual of Determinative Bacteriology.*K. pneumoniae* (86%) was found to be the most common isolate recovered, followed by *K. oxytoca* (17%), *K.planticola*(9%) and *K.ozaenae*(6%).*K. pneumoniae* is the species most frequently isolated in clinical laboratories. In the present study, *K.pneumoniae* was the most common species isolated from clinical samples. Among the total 127 isolates, the isolation rate of *K. pneumoniae* was 68% followed by *K.oxytoca* 17%, *K.planticola* 9% and finally *K.ozaenae* 6%. In our study, the rate of isolation of various *Klebsiella* species from the intensive care units includes 70% (35) *K.pneumoniae*, 16% (8) *K.oxytoca*, 10% (5) of *K.planticola* and only 4% (2) isolates of *K.ozaenae*. In our study *K.pneumoniae* was isolated at the rate of 68% from various clinical samples that included urine 44%, exudates 22%, and blood 17%, and respiratory tract specimen's 16%.Among the *Kpneumoniae* isolates 41% (35) were from ICU and 59% (51) were from Non-ICU wards.

Out of the 44% *K.pneumoniae* isolated from urine in our study, 16% (*n*=6) were from ICU and 84% (*n*=32) were from Non-ICU wards. The presence of indwelling catheter was observed in 48% (*n*=41) of the patients in both ICU and Non-ICU setting. Age, presence of urinary catheters, BPH, CRF, surgical procedures, urogenital abnormality, prolonged hospital stay and exposure to antibiotics were found to be the associated risk factors for isolation from urinary tract. The organism was isolated in significant numbers from postnatal women and in women who underwent catheterization during surgical procedures. In male patients the risk factors were age, underlying illness like diabetes, CRF, urogenital tract abnormality and BPH with indwelling catheter. Of the 16% (n=14) *K.pneumoniae* respiratory isolates, 93% (n=13) of them were obtained from patients on ventilator support. Among the total 127 *Klebsiella* isolates, 57% were found to harbour ESBL. DDST and IPDD test could identify 43% and 95% of the *Klebiella* isolates producing ESBLs respectively.The outcome in some of these patients was fatal which is attributed to the underlying illness. The risk factors observed in these patients were prolonged stay in ICU, intubation, exposure to multiple antibiotics and immune-compromised states like diabetes, steroid therapy. Total *K.pneumoniae*, 17% (*n*=15) was isolated from blood samples, 60% (*n*=9) were from newborn infants and 67% (*n*=6)

of them were preterm. Of the 6 preterm infants, inborn error of metabolism was found in one patient, respiratory distress in 3 patients. The predisposing risk factors in these patients were the presence of vascular access, intubation, ICU stay, and antibiotic therapy.

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