

A Laboratory Study on the Prevalence of Extended Broad Spectrum Beta-Lactamases Producing *Klebsiella Pneumoniae* among Nosocomial Isolates

Dr.P.Subbulu¹, Dr.V.Nagamadhavi²

¹ (Microbiology, Katuri medical College, DR.N.T.R.University of Health sciences, India)

² (Microbiology, Katuri medical College, DR.N.T.R.University of Health sciences, India)

Corresponding Author: Dr.V.Nagamadhavi

Abstract: Introduction: The emergence and spread of resistance to antibiotics among common pathogenic bacteria is an important health care concern. Today the magnitude of the problem has become a threat to reverse the scientific progress made so far. Aims and objectives: To know the prevalence of extended broad spectrum beta lactamases (ESBLs) producing *Klebsiella pneumonia* strains among nosocomial infections and their antibiotic sensitivity pattern. Materials and methods: *Klebsiella pneumonia*, 300 strains isolated from various clinical samples namely urine, pus, sputum and blood collected from various admitted cases in Katuri Medical College and hospital during the period from June 2015-2016. The organisms cultured from specimens were identified as *Klebsiella pneumoniae* as per standard techniques. They were subjected to antimicrobial susceptibility testing. Result: By the sentinel disc diffusion method 144 (48%) *klebsiella pneumoniae* strains were resistant to ceftazidime though their sensitivity to other cephalosporins varied, indicating that they were extended spectrum betalactamase producer (ESBLs).

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I. Introduction

The introduction of third-generation cephalosporins into clinical practice in the early 1980s was heralded as a major breakthrough in the fight against β -lactamase mediated bacterial resistance to antibiotics. Soon after the introduction, the first report of plasmid-encoded β -lactamase capable of hydrolyzing the extended-spectrum cephalosporin was published in 1983 from Germany. Hence these new β -lactamases were coined as extended spectrum β -lactamases (ESBLs) ². Beta-lactam antimicrobial agents exhibit the most common treatment for bacterial infections and continue to be the prominent cause of resistance to β -lactam antibiotics among Gram-negative bacteria worldwide. The persistent exposure of bacterial strains to a multitude of β -lactams has induced dynamic and continuous production and mutation of β -lactamases in these bacteria, expanding their activity even against the newly developed β -lactam antibiotics. These enzymes are known as extended-spectrum β -lactamases (ESBLs) ². Beta-lactamases are commonly classified according to two general schemes: the Ambler molecular classification and the Bush-Jacoby-Medeiros functional classification³. The Ambler scheme classifies β -lactamases into four classes according to the protein homology of enzymes. Beta-lactamases of class A, C, and D are serine β -lactamase and class B enzymes are metallo- β -lactamases. The Bush-Jacoby-Medeiros functional scheme is based on functional properties of enzymes, i.e. the substrate and inhibitor profiles⁴. The SHV family of β -lactamases appears to be derived from *Klebsiella* spp. The progenitor of the SHV class of enzymes, SHV-1, is universally found in *K. pneumoniae*. In many strains of *K. pneumoniae*, the gene encoding SHV-1, or its apparent precursor, LEN-1, resides within the bacterial chromosome too; it may be that the gene for SHV-1 β -lactamase evolved as a chromosomal gene in *Klebsiella* and was later incorporated into a plasmid which has spread to other *enterobacteria* species. SHV-1 confers resistance to broad-spectrum penicillins such as ampicillin, tigecycline and piperacillin but not to the oxyimino substituted cephalosporins⁵. The SHV-1 β -lactamase is responsible for up to 20% of the plasmid-mediated ampicillin resistance in *K. pneumoniae* species⁶. *Klebsiella pneumoniae* is a successful opportunistic pathogen and has been associated with various ailments such as urinary tract infections, septicaemia, respiratory tract infections and diarrhoea⁷. The emergence of ESBL-producers along with multiple resistant isolates poses a serious problem in the hospital setting. The widespread use of antibiotics coupled with the transmissibility of resistance determinants mediated by plasmids, transposons, and gene cassettes in integrons are factors that contribute to the increase in antibiotic resistance in bacterial pathogens⁸. Increasing antimicrobial resistance, especially towards aminoglycosides, (fluoro)quinolones, third and fourth generation cephalosporins, cephamycins, and carbapenems have been reported in the last decade, and poses serious therapeutic problems when treating *Klebsiella* infections in humans^{9,10,11}.

II. Materials and methods

Klebsiella pneumonia, 300 strains isolated from various clinical samples namely urine, pus, sputum and blood collected from various admitted cases in Katuri Medical College and hospital during the period from June 2015-2016. The organisms cultured from specimens were identified as Klebsiella pneumoniae as per standard techniques. They were subjected to antimicrobial susceptibility testing.

Standard characteristics like motility, Gram's staining, capsule and biochemical reactions are studied by the following methods.

1. Motility by hanging drop preparations
2. Capsule by India ink preparation
3. Gram's staining
4. Sugar fermentation reactions by using sugar fermentation media
5. Indole formation using Tryptophan broth
6. Acetoin formation using MR/VP broth
7. Citrate utilisation using Simmons citrate medium
8. Urease production using Christensen's medium

Antibiotic susceptibility testing: By Kirby-Bauer Disc Diffusion Test using Mueller-Hinton agar. Broth was standardised to Mc Farland 0.5 standard. Antibiotic discs relevant to Klebsiella like tetracycline (30µg), Cotrimoxazole (25µg), Chloramphenicol (30µg), Gentamicin (10µg), Ampicillin (10 µg), Amikacin (30µg), Ciprofloxacin (5µg), Augmentin (30µg), Nalidixic acid (30µg), Norfloxacin (10µg), Cefotaxime (30µg), and Ceftazidime (30µg) etc.

Procedure

1. A sterile loop was taken, transferred the growth by touching the tops of colony of pure growth of the test organism in to 5ml of nutrient broth medium
2. The inoculum was inoculated at 37°C for 4 or 5 hours. At the end of incubation, the broth culture was adjusted to the turbidity of the MC Farland 0.5 standard.
3. After matching the turbidity a sterile non toxic cotton swab was taken and dipped into the suspension, rotated the swab several times on the inside wall of the tube to remove the excess fluid
4. Mueller-Hinton agar was previously brought to room temperature. The charged swab was inoculated on the dried surface of MHA, by streaking three times over the entire agar surface, by rotating the plate 60° approximately and ensured an even distribution of inoculum.
5. Agar surface was allowed to dry for 3-5 minutes. Antibiotic discs were applied with a sterile forceps at equal distance. Discs were gently pressed against the agar surface to ensure the uniform contact.
6. A single lot of Mueller-Hinton agar devoid of inhibitory effects as noticed by testing with standard E.coli (ATCC25922) was used.
7. Single batch of commercial antibiotic discs (Becton & Dickens) were used.
8. Inoculated plates were incubated at 37°C for 18 hours
9. After the incubation, the zones were measured to the nearest millimetres
10. Measured zones of inhibition were correlated with those of published guidelines (NCCLS) to interpret as susceptible, moderately susceptible and resistant.
11. Each strain was tested twice. Concurring and reproducible results were observed.

Extended spectrum beta lactamases (ESBLs) producing klebsiella pneumoniae strains can be detected in laboratory by two methods.

1. Sentinel disc diffusion method using ceftazidime
2. Double -disc synergy test (Thomas & Sanders).

Although the various conventional and commercial antimicrobial susceptibility testing methods provide accurate results in most instances certain clinically relevant resistance mechanism can be difficult to detect. In these instances, supplemental tests and alternative approaches are needed to ensure reliable detection of resistance. An approach that may be used to ensure accuracy in detection is the use of "Predictor" antimicrobial agents (i.e. the sentinel discs) that are most sensitive indicators of certain resistance mechanism. For example Staphylococcal resistance to oxacillin is used to determine and report resistance to all currently available beta lactams, including penicillins, cephalosporins and imipenems.

Likewise Ceftazidime is the best sentinel antibiotic for suspecting resistance against cephalosporins and this antibiotic may be the only one for which the resistance actually reaches the break point for interpretation of resistance in any commonly used testing system.

Strains resistance to ceftazidime indicates resistance to all newer cephalosporins. All the common ESBLs remain sensitive to inhibition by Clavulanic acid. Therefore any strain that is resistant to ceftazidime or cefotaxime but becomes sensitive in the presence of clavulanic acid is likely to contain genetic material for

coding extended spectrum betalactamases. This can be readily demonstrated by a double-disc synergy test, but no discs of clavulanic acid are commercially available. However, it is possible to use the clavulanic acid content of co-amoxyclav discs.

A double –disc synergy test with discs of cefotaxime (30µg) and Amoxycillin/clavulanic acid (Augmentin-20/10µg) placed 30mm apart from centre to centre was effective at detecting resistance mediated by ESBLs type of enzymes.

I. Sentinel disc diffusion method:

Sentinel disc diffusion method was done on Mueller-Hinton agar using third generation cephalosporin ceftazidime (30µg) and incubated at 37°C over night.

If the strain is ESBLs producer, then the organisms grow up to the edge of ceftazidime disc.

II. Double disc synergy test:

1. Mueller –Hinton agar plates were prepared. The test culture inoculated by standardized method (corresponding to 0.5McFarland’s tube) to form a lawn culture with a sterile forceps. Cefotaxime (30µg) disc was placed on the agar near the centre. Giving a centre to centre distance of 15mm, amoxycillin/clavulanic acid (Augmentin 20/10µg) disc was placed in line with cefotaxime. The plates were incubated for 16-18 hours.

2. Escherichia coli ATCC25292 was used as the negative control and in house ESBLs producer as the positive control

3. If the strain was an ESBLs producer, then the zone around cefotaxime was extended on the side nearest amoxycillin-clavulanic acid disc.

III. Results

Klebsiella pneumoniae, 300 strains were studied to know the prevalence of ESBLs producing among them. Screening for ESBLs production was done by Sentinel disc diffusion (SDD) and Double-disc synergy (DDS) methods.

By the sentinel disc diffusion method 144 (48%) klebsiella pneumoniae strains were resistant to ceftazidime though their sensitivity to other cephalosporins varied, indicating that they were extended spectrum betalactamase producer (ESBLs)

In double disc synergy test all those Klebsiella strains (144) that were identified as ESBLs producers by sentinel disk diffusion method have shown distortion of zone sizes in synergistic fashion. Another 24 strains out of 156 Klebsiella pneumoniae which were previously sensitive to ceftazidime by sentinel disc diffusion method have shown distortion of the zone sizes in a synergistic fashion indicating that they were also ESBLs producers. Thus increasing the number of ESBLs producing Klebsiella pneumoniae strains detected to 168 (56%) i.e., 144 (48%) by sentinel disc diffusion method and 24 (8%) more after double - disc synergy test.

Table I: Prevalence of ESBLs producing Klebsiella pneumoniae strains among Hospital infections

S.no	Specimen	No.of tested strains	No.of positive by SDD method	Percent positive	No.of positive by DDS method	Percent positive
1.	Urine	148	74	50	86	58.10
2.	Sputum	54	16	29.6	19	35.8
3.	Pus	82	45	54.84	53	64.63
4.	Blood	16	9	56.25	10	62.50
Total		300	144	48.0	168	56.0

Out of 300 Klebsiellae pneumoniae strains 148 strains were isolated from UTI, 54 from RTI, 82 from post operative wound infections and others 16 from septicaemia cases.

Among strains isolated from hospital infections, maximum number of ESBLs producing Klebsiella pneumoniae strains were isolated from post operative wound infections (64.63%), followed by those among septicaemia cases (62.5%), UTI cases (58.8%). Least were isolated from sputum (35.8%)

The antibiogram of ESBLs producing Klebsiella pneumoniae strain has been shown in the Table 2 and 3.

Table II: Antibiogram of 168 ESBLs producing strains of Klebsiella pneumoniae

S.no	Antibiotic	No. of Resistant strains	Percent
1.	Co-trimoxazole	144	85.7
2.	Chloramphenicol	57	33.9
3.	Gentamicin	105	62.5
4.	Ampicillin	168	100
5.	Amikacin	54	32
6.	Ciprofloxacin	84	50
7.	Cefotaxime and other cephalosporins	54	32
8.	Nalidixic acid	78	46.4

9.	Norfloxacin	42	25.0
10.	Amoxyclav	69	41.0
11.	Netilmicin	12	7.0
12.	Ceftazidime	144	87.7
13.	All antibiotics tested	54	32.1

From the table 2 it can be seen that all the ESBLs producing *Klebsiella pneumoniae* strains are exhibiting resistance to commonly used antibiotics like co-trimoxazole (85.7%) and nalidixic acid (46.4%) respectively. This indicates that standard empirical treatment with such drugs can often result in therapeutic failures, HAI cases.

Only 7% of ESBLs producing *Klebsiella pneumoniae* strains showed resistance to Netilmicin.

Table 3: Antibiogram of 168 ESBLs producing strains of *K.pneumoniae* to non-beta lactam antibiotic

S.no	Antibiotics	Sensitive strains	percent	Antibiotics	Sensitive strains	Percent
1.	Chloramphenicol	111	66.9	Gentamycin+ Chloramphenicol	93	55.3
2.	Gentamycin	63	37.5	Gentamycin +Ciprofloxacin	75	44.6
3.	Ciprofloxacin	84	50	Chloramphenicol+ Ciprofloxacin	96	57.17

It can be noticed from the above ESBLs producing *K.pneumoniae* strains a low percentage of resistance to non-betalactam antibiotics like Chloramphenicol (33.1%) which is less frequently used, but 62.5%, 50% resistance to frequently used gentamicin and ciprofloxacin respectively.

IV. Discussion

In the present study, out of 300 *Klebsiella pneumoniae* strains, which were identified as per standard methods, only 144 (48%) could be detected as ESBL producers by sentinel disk diffusion method and the number has increased to 168 (56%) when the same strains were further tested by double disk synergy test. A total number of 24 (8%) ESBL producing *Klebsiella pneumoniae* strains were missed by sentinel disc diffusion method. So, double disk synergy test is more sensitive than the sentinel disk diffusion method in detecting ESBLs producing *Klebsiella pneumoniae* strains.

The prevalence of ESBLs producing *Klebsiella pneumoniae* strains in the present study by double disk synergy test is 56% (168 strains), correlating with the study conducted by Ali Abdel Rahim KA et al¹² in 2014, showing 46.15% of ESBLs production by double disk synergy test.

Among 168 ESBLs producing strains 62.5% (105 strains) were shown resistance against Gentamicin, which is almost equal to that of the study done by Shukla I et al showing 70% resistance against gentamicin¹³. The resistance pattern of non-betalactam antibiotics among *Klebsiella* strains showed 33.1% resistance against chloramphenicol which is almost similar to that of studies done by Padmaja et al⁴.

V. Conclusion

ESBLs are Gram-negative bacteria that produce an enzyme; beta-lactamase that has the ability to break down commonly used antibiotics, such as penicillins and cephalosporins and render them ineffective for treatment. If ESBL-producing bacteria cause an infection, a different antibiotic may need to be used to treat the infection. People who carry ESBL-producing bacteria cause an infection, without any signs or symptoms of infection are said to be colonized. The most common ESBL-producing bacteria are some strains of *Escherichia coli* and *Klebsiella pneumoniae*.

ESBLs are spread via direct and indirect contact with colonized/infected patients and contaminated environmental surfaces. ESBLs are most commonly spread via unwashed hands of health care providers. Risk factors for ESBL-producing bacterial acquisition include: Direct transfer from another hospital, nursing home, retirement home or other health care facility, including between facilities in the same health care corporation Any hospital, nursing retirement home or other health care facility admission in the, past year Patient receiving home health care services or hemodialysis, Patient living in a communal living setting (e.g. shelter, halfway house), Patient who previously had an antibiotic-resistant organism (e.g., MRSA, VRE).

Good Hand Hygiene Practices should follow to limit the spread of ESBLs strains.

Hand hygiene should occur:

- Before client/patient/resident or environment contact
- Before performing aseptic procedures
- After care involving body fluids
- After client/patient/resident or environment contact

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