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Original Research Article

Prevalence, antibiotic resistance pattern and identification of extended spectrum beta-lactamase producing Klebsiella pneumoniae from urine samples in a tertiary care hospital

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ABSTRACT

Aim: the present study aim is to know the prevalence, Antibiotic resistance pattern and frequency of extended spectrum beta lactamases producing Klebsiella pneumonia in urine sample.
Introduction: Klebsiella species are important opportunistic nosocomial pathogens particularly Klebsiella pneumoniae which causes infections like septicemia, urinary tract infections, wound infections, pneumonia. Out of total bacterial nosocomial infections it is estimated that Klebsiella species causes 5-7% of infections. To control the spread of extended spectrum beta lactamases it is necessary to know the prevalence of these extended spectrum beta lactamases (ESBL) and to characterize their epidemiology.
Materials and Methods: The present study is about ESBL producing Klebsiella pneumoniae which is diagnosed by phenotypic methods. Samples received in the Microbiology laboratory were processed following standard protocol. Total 227/3415 (6.6%) Klebsiella pneumoniae were isolated from urine sample among which number of ESBL producing isolates were 85/227. The isolates were tested by the Kirby-Bauer disc diffusion method on Muller Hinton agar (Hi-Media) using 0.5 McFarland's as the turbidity standard as per CLSI guidelines. Klebsiella pneumoniae isolated were tested for ESBL production by CLSI recommended screening and confirmatory methods like phenotypic confirmation method and MIC using E test.
Conclusion: It was noted that the prevalence of ESBL producing K. pneumoniae was fairly uniform during the study period. Maximum cases were seen in the age group of 31 – 40 years. The antibiotic resistance pattern of ESBL and non ESBL producing K. pneumoniae shows that all the ESBL producing Highly resistant drugs for Klebsiella pneumoniae ESBL isolates were Ampicillin (100%), Piperacillin (100%), Amoxycillin + clavulanic acid (66.0%). Non ESBL producing isolates were less resistant to the same antibiotics.

1. Introduction

Increase in extended-spectrum β-lactamases (ESBL) producing microbes in recent years has led to limitations of treatment options. Strains that produce ESBLs are widely distributed, especially in a hospital environment, where they may even cause an endemic. They are responsible for therapeutic failure, especially with the use of beta-lactam antibiotics, and increased morbidity and mortality of patients. The detection of strains producing ESBLs is a challenge for microbiologists, because of the difficulties mainly arising from the phenotypic differences of these strains. Emergence of beta lactamase producers due to abundant use of beta lactam antibiotics has become a major problem of concern. To control the spread of extended spectrum beta lactamases it is necessary to know the prevalence of these ESBL and to characterize their epidemiology. One of the most important resistant mechanisms in Gram-negative bacteria against beta-lactam antibiotics is induced by production of beta-lactamase enzymes (Shashwati N 2014[1] and Li et al, 2002[2]). The

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new broad-spectrum antibiotics such as Cephalosporins used in treatment of bacterial infections has led to the production of a new class of broad-spectrum enzymes called betalactamase (Pooja Shakya 2017[3] & Tenover et al, 2003[4]). Members of Enterobacteriaceae family have emerged as one of the major causes of both nosocomial and community acquired infections. Antibiotic like beta lactams (especially extended-spectrum cephalosporins and carbapenems) and flouroquinolones are the major therapeutic options to treat infections caused by the organisms of this family (Canton et al, 2008[5]). Due to continuous mutations of ESBLs changes in the amino acid configuration near the active site of these beta lactamases occur, which results in the development of new enzymes showing extended substrate profiles. The aim of the study is to detect extended spectrum beta lactamase producing *Klebsiella pneumoniae* isolated from urine samples by phenotypic methods.

2. Materials and Methods

The present study was conducted in Khaja Banda Nawaz Institute of Medical Sciences, Kalburagi, extending over a period of 3 year from July 2016 to June 2019. The study was approved by the Institutional Ethical Committee (IEC), of Khaja Banda Nawaz Institute of Medical Sciences with the ethical clearance number KES/KBNIMS/2016-17/203. In clean sterile container mid-stream clean catch urine, suprapubic aspirate and from Foley's catheter was collected. A total of 3415 properly collected, well labelled urine samples received in the Microbiology laboratory were processed following standard protocol. *Klebsiella pneumoniae* was isolated on the basis of grams staining, cultured on nutrient agar (NA), MacConkey's agar (MA), enriched media like blood agar (BA) by streak plate method under all aseptic conditions and incubated at 37°C for 24hours and were confirmed and identified to the species level by standard biochemical tests.

2.1. Antimicrobial susceptibility testing

The isolates were tested by the Kirby-Bauer disc diffusion method on Muller Hinton agar (Hi-Media) using 0.5 McFarland's as the turbidity standard as per CLSI guidelines. Antimicrobial disks used were Ampicillin (10μg), Amoxycillin-clavulanic acid (20/10μg), Piperacillin (100μg), Piperacillin-tazobactam (100/10μg), Nitrofurantoin (300μg), Norfloxacin (10μg), Ciprofloxacin (5μg), Ofloxacin, (5μg), tetracycline (30μg) Cefuroxime (30μg), Ceftriaxone (30μg), Ceftazidime (30μg), Gentamicin (10μg), Amikacin (30μg), Co-trimoxazole (1.25/23.75 μg), Aztreonam (30μg) and Imipenem (10μg).

2.2. Screening test for ESBLs

Screening of ESBLs was done as per CLSI guidelines, isolates showing inhibition zone size of $\leq$22 mm with Ceftazidime (30 μg), $\leq$25 mm with Ceftriaxone (30 μg), and $\leq$27 mm with Cefotaxime (30 μg) were identified as potential ESBL producers and shortlisted for confirmation of ESBL production. Resistance to at least one of the antibiotics was considered as positive in the screening test for possible ESBL production as per 2018 CLSI guidelines (CLSI, 2018).

2.3. Confirmatory Tests for ESBLs

2.4. Detection of ESBL by Double Disc Synergy Test (DDST)

The isolated organisms were inoculated on to peptone water and incubated at 37°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 were placed with the inter disc distance (edge to edge) of 15 mm from the amoxyclav disc. The plates were incubated at 37°C for overnight. Enhancement of zone of inhibition towards amoxyclav by any one of these drugs such as Ceftazidime, cefotaxime, ceftriaxone was considered as positive result.

2.5. Phenotypic Confirmatory Test (PCT) With Combination Disk

The isolated organisms were inoculated into peptone water and incubated at 37°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, Disk of Ceftazidime (30μg) and a disk of Ceftazidime +Clavulanic acid (30 μg/10 μg), cefotaxime (30mcg) and a disk of cefotaxime + clavulanic acid (30mcg/10mcg) were used. Both the disks were placed at least 25 mm apart, center to center, on a lawn culture of the test isolate on Muller Hinton Agar plate and incubated overnight at 37°C. Difference in zone diameters with and without clavulanic acid was measured. When there was an increase of $>$5 mm in inhibition zone diameter around combination disk with clavulanic acid versus the inhibition zone diameter in disk alone was confirmed positive for ESBL production. K. pneumoniae ATCC 700603 (an ESBL producer) and E. coli ATCC25922 were used as positive and negative controls respectively.

2.6. MIC reduction test: Epsilometer test (E test)

An absorbent strip E strip with a known gradient of drug concentrations along the length was placed on the Muller Hinton agar plate seeded with the test bacterium and incubated at 37°C in an incubator for another 24 hrs. The MIC was obtained by noting the lowest concentration of the gradient which inhibited bacterial growth. The

isolates positive with combination disk test were further confirmed for ESBL production by this test. Minimum inhibitory concentration of the isolates was determined by E strips. The values of range of concentration of antibiotics tested were as follows: CTX code indicates the cefotaxime (0.25-16 μg/mL) gradient and CTX+ code indicates the cefotaxime (0.016-1 μg/mL) plus 4 μg/mL clavulanic acid. CAZ code indicate the Ceftazidime (0.5-32 μg/mL) gradient and CAZ+ code indicates the Ceftazidime (0.064-4 μg/mL) plus 4 μg/mL clavulanic acid. A >3 two-fold decrease in MIC for Ceftazidime when tested in combination with clavulanic acid versus its MIC when tested alone indicates that the strain is an ESBL producer. K. pneumoniae ATCC 700603 (an ESBL producer) was used as control strain.

3. Results

3415 urine samples were tested for Klebsiella pneumoniae and 6.64% samples were found to be positive for Klebsiella pneumoniae. These positive samples were further tested for ESBL production.

Table 1: Isolation ofKlebsiella pneumoniae from urine samples

Urine sample	Isolation of Klebsiella pneumonia
Positive	227(6.6%)
Negative	3188(93.4%)
Total	3415

Table 2: Distribution according to clinical sources ofK. pneumoniae isolates producing ESBL

	Number of ESBL producing isolates		
Sample	Klebsiella pneumonia		
	Tested	Positive (%)	Negative (%)
Urine	227	85 (37.4)	142 (62.6%)

Table 3 shows the age and sex distributions of ESBL positiveKlebsiella pneumoniae. Among the patients, from whom the clinical samples were collected, 60% were male and 40% were female. The age of the patients ranged from 0 to 70 years. Maximum cases were from males of age group 31-40 year (27.4%) followed by females of the same age group (26.4%). p – value was found to be insignificant.

Table 4Figure 2 displays the antibiotic resistance pattern of ESBL producing Klebsiella pneumoniae. Highly resistant drugs for Klebsiella pneumoniae ESBL isolates were Ampicillin (100%), Piperacillin (100%), Amoxycillin + clavulanic acid (66%). Non ESBL producing isolates were less resistant to the same antibiotics. Significant difference in resistant pattern between ESBL and Non ESBL isolates was found in case of beta lactam drugs Amoxycillin + clavulanic acid, Gentamicin, Amikacin, Ciprofloxacin, Tobramycin, Ofloxacin, Cefuroxime, Co – trimoxazole, Ceftazidime, Ceftriaxone, Piperacillin +

Table 3: Age and sex distribution of ESBL positiveK. pneumonia

Age group	Male	Female	Total
	No. (%)	No. (%)	No.
< 20	06 (11.7%)	04 (11.7%)	10
21-30	09 (17.6%)	06 (17.6%)	15
31-40	14 (27.4%)	09 (26.4%)	23
41-50	11 (21.5%)	07 (20.5%)	18
51-60	08 (15.6%)	04 (11.7%)	12
>60	03 (5.8%)	04 (11.7%)	07
Total	51 (60%)	34 (40%)	85
$c2 = 0.219$; $p = 0.999$			

Tazobactam, Nitrofurantoin. The ESBL isolates of K. pneumoniae were highly resistant to these set of antibiotics; however, non-ESBL isolates showed relatively higher sensitivity to them. ESBL producers showed high level of resistance to cephalosporins when compared to non-ESBL producers. About 41.1% ESBL producers were resistant to Cefuroxime were as only13.3% non-ESBL were resistant to the same drug. 35.2% and 10.5% ESBL and non-ESBL producers were resistant to Ceftazidime respectively. 31.7% ESBL and 11.9% non-ESBL were found to be resistant to Ceftriaxone. The most effective drugs found in antibiotic resistance testing against K. pneumoniae ESBL isolates were imipenem and Aztreonam showing 0% resistance each.

Screening test and phenotypic confirmatory test for ESBL: Out of 227 isolated 85 ESBL Producers (37.4%) and 142 (62.6%) were identified as Non-ESBL producers by double disc synergy test and phenotypic confirmatory test respectively Figure 2. Samples identified as ESBL producers were confirmed by E test.

4. Discussion

The spread of ESBL producing bacteria has become strikingly rapid worldwide, indicating that continuous monitoring systems and effective infection control measures are absolutely required. The therapeutic options for the infections which are caused by these organisms have also become increasingly limited (Kiratisin et al, 2008[6]). A number of nosocomial outbreaks which were caused by ESBL producing organisms, have been reported in the United States. (Meyer et al, 1993[7]) Although most of the outbreaks were limited to high risk patient care areas such as ICUs, oncology units etc., the first report of an outbreak in nursing homes appeared in the literature in the year (Wiener et al, 1999[8]). Therefore, now- a- days, the threat of ESBL producing isolates is not limited to ICUs or tertiary care hospitals only.

The Clinical and Laboratory Standards Institute (CLSI) has issued recommendations for ESBL screening, for the confirmation of the isolates of Klebsiella spp., and for reporting the confirmed organisms (Thompson et al,

Table 4: Antibiotic resistance pattern ofKlebsiella pneumonia

Antibiotic	Code	ESBL Producer (n= 85)	Non-ESBL Producer (n=142)	P- value based on Chi – square test
		n (%)	n (%)	
Ampicillin	A	85 (100)	39 (27.0)	<0.001
Pipecillin	B	85 (100)	47 (33.0)	<0.001
Amoxycillin + clavulanic acid	C	56 (66.0)	23 (16.1)	<0.001
Gentamicin	D	41 (48.2)	35 (24.6)	<0.001
Amikacin	E	40 (47.0)	31 (21.8)	<0.001
Ciprofloxacin	F	40 (47.0)	53 (39.4)	<0.001
Tobramycin	G	39 (45.8)	27 (19.0)	<0.001
Ofloxacin	H	37 (43.5)	43 (30.2)	<0.001
Cefuroxime	I	35 (41.1)	19 (13.3)	<0.001
Co-trimoxazole	J	33 (38.8)	23 (16.1)	<0.001
Ceftazidime	K	30 (35.2)	15 (10.5)	<0.001
Ceftriaxone	L	27 (31.7)	17 (11.9)	<0.001
Piperacillin + tazobactam	M	23 (27.0)	6 (4.2)	<0.001
Nitrofurantoin	N	15 (17.6)	29 (20.4)	<0.001
Aztreonam	O	0	0	-
Imipenem	P	0	0	-

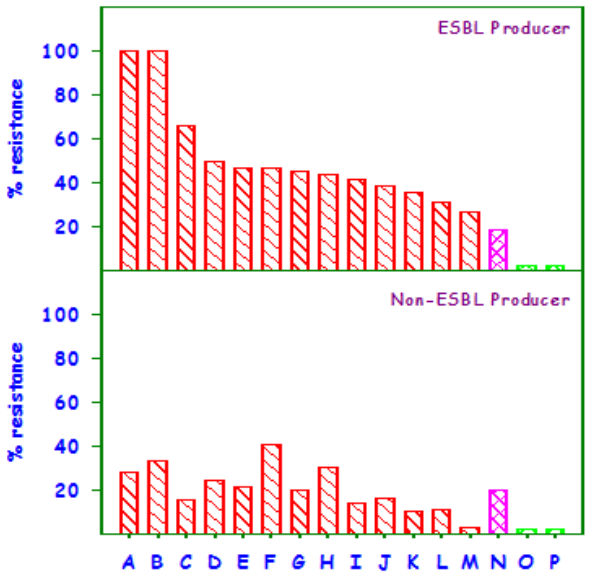

Fig. 1: Percent resistance of ESBL and Non-ESBL producers of K.pneumonia isolates. The X-axis label is given in Table 4

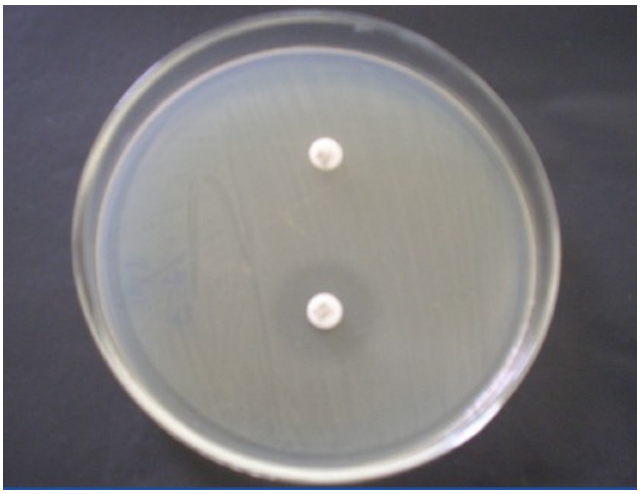

Fig. 2: Phenotypic confirmatory testshowing >5 mm increase in zone of inhibiton for Ceftazidime/clavulanic acid (CAC) verses its zone diameter when tested alone by Ceftazidime confirmed an ESBL producing organism.

2001[9]). Previous studies from India have reported the prevalence of the ESBL producers to be 6.6 to 68%. In south India, 6.6% ESBL producers among *Klebsiella pneumoniae* from children were reported (Subha et al, 2002[10]), whereas (Babypadmini et al, 2004[11]) reported 40.3% ESBL production. The ESBL production which was reported among gram negative bacteria by (Mathur et al, 2002[12]) was 68%. (Singhal et al, 2005[13]) detected ESBL positivity in 64% isolates and (Rodrigues et al, 2004[14]) reported 53% ESBL production and 61.7% ESBL positivity among *K. pneumoniae*. These findings correlated well with those of our study. The occurrence of ESBL producers among *Klebsiella pneumoniae* in the current study was 85/227 (37.4%), (Table 2).

Majority of the clinical samples yielding the isolates used in this study were 60% male and 40% were female. Maximum cases were from males of age group 31-40 year (27.4%) followed by females of the same age group (26.4%) Table 3 . In 2009, CLSI introduced two-step procedure for the detection of ESBL producers. In the first step, isolates of *K. pneumoniae* should be screened for resistance to

one or more of third-generation indicator cephalosporin (ceftriaxone, cefotaxime, ceftazidime). Since ESBLs vary in their hydrolysis of these cephalosporins as substrates, resistance to at least one of them was considered as positive in the screening test.

In the present study prevalence of ESBL producing *Klebsiella pneumoniae* was found to be 37.4%. In the present study, all ESBL producers were uniformly resistant to ceftriaxone, Ceftazidime, cefotaxime, rendering them inappropriate for treatment. None of the ESBL producers tested in this study exhibited susceptibility MIC breakpoint of $\leq$1 μg/ml to cefotaxime and ceftriaxone and $\leq$4 μg/ml to Ceftazidime. While ESBL-KP was similarly resistant to cefotaxime and ceftriaxone at MIC level of $\geq$128 μg/ml in this study, significantly more number of ESBL-KP were resistant to Ceftazidime. In studies from India, susceptibility of ESBL-KP to ceftazidime ranged from 2.5% to 70%. Susceptibility of ESBL-KP to cefotaxime ranged ranged from 10.1% to 30% In few other studies, none of the ESBL producers were found to be susceptible to cefotaxime or ceftazidime (Vijayakanthi et al, 2013 [15]). These observations suggests that ESBL producers exhibit significantly high level of resistance towards cefotaxime and ceftriaxone than to ceftazidime, which may be because of presence of CTX-M-15 enzymes that hydrolyze cefotaxime and ceftriaxone better than ceftazidime.

Since ESBLs do not confer resistance to carbapenems, carbapenems have become the antibiotics of choice in the treatment of serious infections by beta-lactamase producing gram negative bacteria. In this study, 100% of ESBL-KP were susceptible to Imipenem. Similarly, high susceptibility to Imipenem has been shown across several studies. Clinical failures with carbapenem have been reported to occur during the course of treatment, which have been attributed to selection of porin mutants and co-production of AmpC beta-lactamase or KPC enzymes (Martínez-Martínez et al, 1999 [16] and Kitchel et al, 2010 [17]).

Oxyimino-cephalosporins such as ceftazidime, cefotaxime and ceftriaxone had been the mainstay of treatment of infections caused by Enterobacteriaceae members since their introduction. Their utility in treatment of severe infections has drastically fallen in the recent times due to increase in ESBL mediated resistance (Coenen et al, 2006 [18]). Cephalosporins may be used in the treatment as along as their MIC clearly fall in the susceptible category. Hence, it is vital that diagnostic laboratories determine the MICs of cephalosporin antibiotics among ESBL producers. It is believed that inappropriate and rampant usage of these antibiotics has led to emergence and selection of ESBL producers.

5. Conclusion

The present study shows the prevalence of antibiotic susceptibility pattern and identification of ESBL producing K. pneumoniae by phenotypic method. Clinical sample (urine) Klebsiella pneumoniae isolates obtained were studied for ESBL production. ESBLs in these isolates were detected by two-step method as recommended by the CLSI guidelines. In the first step, isolates of K. pneumoniae were screened for resistance to one or more of third-generation indicator cephalosporin (Ceftriaxone, Cefotaxime, Ceftazidime). Resistance to at least one of them was considered as positive in the screening test. It was noted that the prevalence of ESBL producing K. pneumoniae was fairly uniform during the study period. Maximum cases were seen in the age group of 31 – 40 years. The antibiotic resistance pattern of ESBL and non ESBL producing K. pneumoniae shows that all the ESBL producing Klebsiella pneumoniae isolates were highly resistant to Ampicillin and Piperacillin and least resistance was observed with Imipenem and Aztreonam. By estimating MIC values, high-level resistance was observed among the ESBL producers to cefotaxime than to Ceftazidime. At the same time, resistance to Ceftazidime was prominent among K. pneumoniae isolates. All the ESBL producing K. pneumoniae were susceptible to Imipenem and Aztreonam followed by Piperacillin plus Tazobactam suggesting that these drugs continue to be effective against ESBL producers.

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None.

8. Conflict of interest

None.

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