Phenotypic detection in extended spectrum β-lactamase Producing Multidrug Resistance *Klebsiella Pneumoniae* Isolated in various Clinical Samples

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Abstract

Introduction: *Klebsiella pneumoniae* is a gram-negative, encapsulated, non-motile bacterium found in the environment and has been associated with pneumonia in patient populations with alcohol use disorder or diabetes mellitus. The bacterium typically colonizes human mucosal surfaces of the oropharynx and gastrointestinal (GI) tract. Once the bacterium enters the body, it can display high degrees of virulence and antibiotic resistance. Today, *K. pneumoniae* pneumonia is considered the most common cause of hospital-acquired pneumonia in the United States, and the organism accounts for 3% to 8% of all nosocomial bacterial infections.

Material and Methods: This cross-sectional study was carried out in the Department of Microbiology of Santosh Medical College & Hospital, Ghaziabad in association with Govt. Medical College & super facility Hospital, Azamgarh. All clinical samples including pus, urine, sputum, blood culture and fluid etc. If isolated *K. pneumoniae* from various clinical samples was shown the ESBL production along with showing MDR was included in the proposed study. Various clinical samples were cultured on appropriate culture media like Blood Agar MacConkey Agar, Chocolate Agar and CLED Agar and incubated at 37°C for 18–24 hours. Isolates will be confirmed by using standard IMVIC biochemical test (Indole, Methyl Red, Voges Proskauer, Citrate) Triple Sugar Iron, Urease, Motility Test, Catalase, Oxidase, Coagulase test etc.

Results: It is found that 256 isolates collected from samples, it is found in the study that male to female ratio is 1.7:1 (163 males as compared to 93 males. In this study, the maximum

number of patients were in the age group of 51-60 years which were 50.7% (n =130) of total followed by age group 41–50 years having 28.5% (n = 73) followed by age group 30-40 years with 20.7% (n=53). Out of 256 *K. pneumoniae* isolates, distribution of ESBL screening positive *K. pneumoniae* isolates in various clinical samples are shown in the Table 4. Among the isolates screened for ESBL production, 54.2% of urine sample and least were blood sample 13.6%. the antimicrobial susceptibility test against for *K. pneumoniae* which revealed 98% senstivity to Amikacin followed by Imipenem, Meropenem respectively and 75% to Gentamicin and Nitrofurantoin, 55% to piperacillin, 65% to Ciprofloxacin . *K. pneumoniae* isolates showed Cefepime was highest resistant 75% followed by Cotrimoxazole (70%), Ceftazidime (65%), Amoxicillin+Clavulanic acid (60%).

Conclusion: The present study highlights the emergence of MDR and ESBL producing K. pneumoniae isolates with high antibiotic resistant rates to commonly used antibiotics Since the spread of MDR and ESBL producing K. pneumoniae has been increasing rapidly worldwide including developing country like India.

Keywords: Extended spectrum β-lactamase, Multidrug Resistance *Klebsiella Pneumoniae*, Clinical Samples**.**

Introduction

Klebsiella pneumoniae belongs to the *Enterobacteriaceae* family and is described as a gram-negative, encapsulated, non-motile bacterium found in the environment and has been associated with pneumonia in patient populations with alcohol use disorder or diabetes mellitus. [1] The bacterium typically colonizes human mucosal surfaces of the oropharynx and gastrointestinal (GI) tract. Once the bacterium enters the body, it can display high degrees of virulence and antibiotic resistance.[2] Today, *K. pneumoniae* pneumonia is considered the most common cause of hospital-acquired pneumonia in the United States, and the organism accounts for 3% to 8% of all nosocomial bacterial infections. [3]

Virulence of the bacterium is provided by a wide array of factors that can lead to infection and antibiotic resistance. The polysaccharide capsule of the organism is the most important virulence factor and allows the bacteria to evade opsono-phagocytosis and serum killing by the host organism. [4]

To date, 77 different capsular types have been studied, and those *Klebsiella* species without a capsule tend to be less virulent. A second virulence factor is lipopolysaccharides that coat the outer surface of a gram-negative bacteria.[5] The sensing of lipopolysaccharides releases an inflammatory cascade in the host organism and has been a major culprit of the sequela in sepsis and septic shock. Another virulence factor, fimbriae, allows the organism to attach itself to host cells. Siderophores are another virulence factor that is needed by the organism to cause infection in hosts. Siderophores acquire iron from the host to allow propagation of the infecting organism.[6]

Klebsiella pneumoniae is one of a handful of bacteria that are now experiencing a high rate of antibiotic resistance secondary to alterations in the core genome of the organism. Alexander Fleming first discovered resistance to beta-lactam antibiotics in 1929 in gram-negative organisms. [7] Since that time, *K. pneumoniae* has been well studied and has been shown to produce a beta-lactamase that causes hydrolysis of the beta-lactam ring in antibiotics. Extended-spectrum beta-lactamase (ESBL) *K. pneumoniae* was seen in Europe in 1983 and the United States in 1989. [8]

ESBLs can hydrolyze oxyimino cephalosporins rending third-generation cephalosporins ineffective against treatment. Due to this resistance, carbapenems became a treatment option for ESBL. [9] However, of the 9000 infections reported to the Centers for Disease Control and Prevention (CDC) due to carbapenem-resistant *Enterobacteriaceae* in 2013, approximately 80% were due to *K. pneumoniae.* [10] Carbapenem resistance has been linked to an up-regulation in efflux pumps, alteration of the outer membrane, and increased production of ESBL enzymes in the organism. [11]

Material and methods

This cross-sectional study was carried out in the Department of Microbiology of Santosh Medical College & Hospital, Ghaziabad in association with Govt. Medical College & super facility Hospital, Azamgarh.
All clinical samples including pus, urine, sputum, blood culture and fluid etc.

Sample size: 256 clinical isolates of K. *pneumonia*. (Calculated at end)

Inclusion Criteria: If isolated *K. pneumoniae* from various clinical samples was shown the ESBL production along with showing MDR were included in the proposed study.

Exclusion criteria: If isolated *K. pneumoniae* from various clinical samples was not show the ESBL production along with MDR that was excluded from the study.

SAMPLE COLLECTION AND IDENTIFICATION:

- This study was done after getting approval from ethical committee and written informed consent from patients.
- All the samples were processed and identification was as per the standard microbiological protocols and procedures.

Specimen processing

Various clinical samples were cultured on appropriate culture media like Blood Agar MacConkey Agar, Chocolate Agar and CLED Agar and incubated at 37°C for 18–24 hours. Isolates was confirmed by using standard IMVIC biochemical test (Indole, Methyl Red, Voges Proskauer, Citrate) Triple Sugar Iron, Urease, Motility Test, Catalase, Oxidase, Coagulase test etc.[13]

Antibiotic sensitivity test: CLSI (2021)

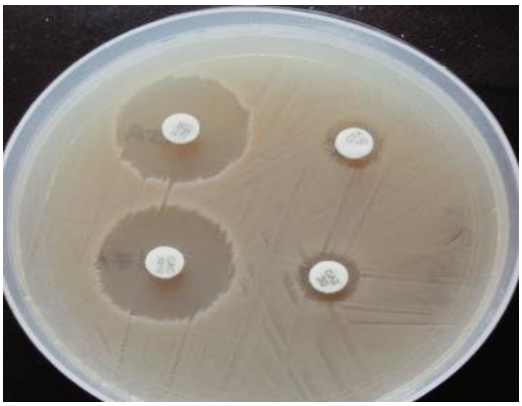
(Amikacin, Amoxicillin-Clavulanic acid, Cefixime, Ceftazidime, Cefepime, Cefotaxime, Ciprofloxacin, Imipenem, Meropenem, Piperacillin, Gentamicin, Cotrimoxazole, Nitrofurantoin, Norfloxacin),

Phenotypic method

All clinical samples were routinely cultured on appropriate culture media like Blood Agar, MacConkey Agar, Chocolate Agar and CLED Agar and incubated at 37°C for 18–24 hours. Isolates was confirmed by using standard IMVIC biochemical test (Indole, Methyl Red, Voges Proskauer, Citrate) Triple Sugar Iron, Urease, Motility Test, Catalase, Oxidase, Coagulase test etc
[13]

Identification of ESBL by Double-Disk Synergy Test (DDST) (DDST)

The phenotypic characterization of ESBL-producing strains of *K. pneumoniae* was performed on Mueller-Hinton by a double-disk synergy test (DDST) and combined-disc test (CDT). Both of these tests use cephalosporin antibiotics individually and in combination with clavulanate.[14] The agar plates were incubated overnight at 35°C-37°C. The β-lactamase resistant clavulanate mediates a keyhole or a zone of enhancement to visualize the results phenotypically. The bacterial strains of *K. pneumoniae* were preserved in brain heart infusion broth supplemented with 15% glycerol and stored at -85°C until used.



Phenotypic confirmatory disc diffusion test — showing an increase in zone size of >5 mm for ceftazidime and ceftazidime-clavulanic acid

Determination of antibacterial resistance

The well-isolated colonies of *K. pneumoniae* were picked up with a sterile loop and emulsified in normal saline (0.9% sodium chloride) to produce a suspension equivalent to the 0.5 McFarland standard to report the antibacterial drug resistance in vitro. Antibiotic disks belonging to the cephalosporin (30µg each of the ceftazidime, cefixime, cefotaxime), aminoglycoside (30µg amikacin and 10µg gentamicin), fluoroquinolone (5µg ciprofloxacin), and carbapenems (10µg each of imipenem and meropenem) were used. Other antibiotics used in the study included co-trimoxazole (1.25/23.75µg), co-amoxiclav (20/10µg), and piperacillin (100 µg).[13]

The suspension was streaked with a sterile swab onto a petri disc plate containing Mueller Hinton agar and incubated at 37°C for 18 hours after the acclimation of various antibiotic disks. The organisms were reported as sensitive, intermediate sensitive, or resistant according to the zones of inhibition provided by the Clinical and Laboratory Standards Institute (CLSI). [15]

Quality control bacterial strains

Quality control (QC) strains of American Type Culture Collection (ATCC), *Klebsiella pneumoniae* (700603, ESBL-positive), and *E. coli* (25922, ESBL-negative) were used to ensure the quality of bacterial cultures and in vitro antibacterial sensitivity testing following CLSI guidelines. [15] An enhancement zone of ≥5 mm for ceftazidime-clavulanate and ≥3 mm for cefotaxime-clavulanate in comparison with ceftazidime and cefotaxime alone, respectively, were taken as ESBL-positive QC *K. pneumoniae* (700603) in CDT. A zone size of ≤2 mm enhancement for the same disks alone and in combination with clavulanate was taken as ESBL-negative QC *E. coli* (25922) in CDT. [16]

Statistical analysis

- The collected data was compiled in MS Excel sheet for analysis.
- Data analyzed in Statistical Package for the Social Sciences (SPSS) version 25th was applied.
- The qualitative data was represented in the form of frequencies and percentages also represented in visual impressions like bar diagrams.
- p value <0.05 indicates Statistically significant.

Results

Table 1 Distribution of Gender

S.No	Gender	Isolates n (%)
1.	Male	163 (63.6%)
2.	Female	93 (36.3%)
	Total	256 (100%)

Table 2: Distribution of the number of subjects according to age group

S.No	Age group	Isolates n (%)
1.	30-40 years	53 (20.7%)
2.	41-50 years	73 (28.5%)
3.	51-60 years	130 (50.7%)
	Total	256 (100%)

In this study, the maximum number of patients were in the age group of 51-60 years which was 50.7% (n =130) of total followed by age group 41–50 years having 28.5% (n = 73) followed by age group 30-40 years with 20.7% (n=53)

Table 3: Samples-wise distribution.

S.No	Sample	*K. pneumoniae* n (%)
1.	Urine	139 (54.2)
2.	Sputum	43 (16.7)
3.	Pus	39 (15.2)
4.	Blood	35 (13.6)

Out of 256 *K. pneumoniae* isolates, distribution of ESBL screening positive *K. pneumoniae* isolates in various clinical samples is shown in the Table 3. Among the isolates screened for ESBL production,54.2% of urine samples and 13.6% of *K. pneumoniae* were from blood samples.

Table 4. Antibiotic resistance pattern of *K. pneumoniae* isolates.

S.No	Name of Antibiotic	Symbol	*K. pneumoniae*	
			Susceptible (%)	Resistance (%)
1.	Amikacin,	AK	98	02
2.	Amoxicillin-Clavulanic acid	AMC	40	60
3.	Cefixime	CFM	40	60
4.	Cefotaxime	CTX	45	55
5.	Ceftazidime	CAZ	35	65
6.	Ciprofloxacin	CIP	65	35
7.	Cotrimoxazole	COT	30	70
8.	Cefepime	FEP	25	75
9.	Gentamicin	CN	75	25
10.	Imipenem	IPM	98	02
11.	Meropenem	MEM	98	02
12.	Nitrofurantoin	F	75	25
13.	Norfloxacin	NOR	85	15
14.	Piperacillin	PI	55	45

Discussion

In our study various clinical samples were processed in the Microbiology department of GMC Azamgarh. 256 ESBL producing isolates were isolated including 139(54.2%) isolates

from urine culture 43(16.7%) isolates from sputum, 39(15.2%) isolates from pus and 35(13.6) isolates from blood culture from patients. Study done by Sushma Koirala et al documented that the highest number of *K. pneumonia* isolates were recovered from urine (67.76%). (11.57%) from pus, (12.39%) from sputum, (5.78%) from blood, and (2.47%) from body fluids.[17]

Table 1: It is found that 256 isolates collected from samples male to female ratio is 1.7:1 (163 (63.6%) males as compared to 93(36.3%) males. This was almost similar to study conducted by Rakesh Prasad Shah et al where 56% of males were affected as compared to 44% of females. [18] and in a study done by Sahoo S et al.[19] 59.42% of males were affected compared to 40.57% of females.

In our study, the maximum number of infections was found in patients aged 51-60 years. In the literature, the maximum number of infections was reported in patients aged 51-60 years by Ejaz H et al. [20] and in patients aged 60-65 years by Heinz E et al.[21] This may be attributed to the high prevalence of comorbid conditions in this age group. When compared with the recent study of Gaynes R et al, age difference was almost similar to the present study conducted on hundred patients. [22]

In our study according to table 4, the results of the antimicrobial susceptibility test against for *K. pneumoniae* which revealed 98% senstivity to Amikacin followed by Imipenem, Meropenem respectively and 75% to Gentamicin and Nitrofurantoin, 55% to piperacillin, 65% to Ciprofloxacin . *K. pneumoniae* isolates showed Cefepime was highest resistant 75% followed by Cotrimoxazole (70%), Ceftazidime (65%), Amoxicillin+Clavulanic acid (60%), Cefixime (60%), Cefotaxime (55%). In contrast to our result, Perez et.al reported *K. pneumoniae* isolates were 65% resistant to Ceftazidime.[23] This may be due to the irrational use of third generation cephalosporins.[24] However; a significant degree of susceptibility was found to nitrofurantoin (75.0%) followed by amikacin (80.7%) and gentamycin (73.9%) which have been reported in a study done by Malloy AM et al. [25] This may be due to the rational use of these drugs in UTIs cases since it is the drug of choice for UTIs. With regard to urinary tract infection, *E.coli* showed great extent of resistance to co-trimoxazole and third generation cephalosporins. *K. pneumoniae* were highly resistant to gentamicin and cotrimoxazole which was documented in a study done by S. Sageer abanoo et al.[25] Nevertheless, *K. Pneumoniae* showed a different sensitive rate to Amikacin, Imipenem, Meropenem, Nitrofurantoin with 98%, 98%, 98%, and 75% respectively in contrast to a study by Sinha P et al.[26]

ESBL-producing *K. pneumoniae* was found to be a little higher (53.1%) among the patients in our study. Similarly, a study done by Rakesh et al found 47.5% isolates of *klebsiella* species were ESBL producers.[18] A significant contribution to the dissemination of ESBL-producing strains is the large-scale use of antimicrobials, especially in unskilled workers, and holding healthy and infected persons in close contact. ESBL carriage rate among patients could be due to misuse of antimicrobials, close contact with other patients, or the use of contaminated instruments. In the current study, high prevalence of MDR isolates of *K. pneumoniae* was noticed in the clinical samples. The overall prevalence of MDR phenotypes

in *K. pneumoniae* isolates was respectively 75% and 87.5%. Among the MDR isolates of K. pneumoniae, a majority of them were producers of ESBL. Similar to the results of the present study, also in research by Akpaka PE et al reported the same ratios. [27]

In contrast, broader drug resistance was observed against cephalosporins and other antibiotics in a Korean study. ESBL-producing isolates exhibited extremely low or no resistance to carbapenems, and amikacin. [28] This is not similar to our study due to the variation in geography, study design and selection of type of antimicrobial agents. The indiscriminate use of β-lactam antibiotics leads to the generation of selective pressures which have led to the selection of a variety of mutated forms of β-lactamases. [29]

Conclusion

The present study highlights the emergence of MDR and ESBL-producing *K. pneumoniae* isolates with high antibiotic-resistant rates to commonly used antibiotics. Since the spread of MDR and ESBL-producing *K. pneumoniae* has been increasing rapidly worldwide including developing countries like India, treatment options for resistant bacteria have been increasingly sorted. In the present study, no resistance was documented to Amikacin, Imipenem, and Meropenem suggesting the suitable drug of choice for treating ESBL producing *K. pneumoniae* causing life-threatening infections. Our findings emphasize the need for implementation of strict antibiotic policy, clinical care management and antibiotic stewardship programs absolutely required in each and every health sector by all concerned authorities.

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