

## Prevalence of antibiotic resistance among clinical isolates of *Klebsiella pneumoniae* isolated from a Tertiary Care Hospital in Pakistan

Amin, A.<sup>1\*</sup>, Ghumro, P. B.<sup>3</sup>, Hussain, S.<sup>4</sup> and Hameed, A.<sup>2</sup>

<sup>1\*</sup>Adnan Amin Department of Microbiology, Microbiology Research lab (MRL) Quaid I Azam University Islamabad Pakistan.

<sup>2</sup>Abdul Hameed Department of Microbiology, Quaid I Azam University Islamabad.

<sup>3</sup>Pir Baksh Ghumro Department of Microbiology, Microbiology Research lab (MRL) Quaid I Azam University Islamabad.

<sup>4</sup>Shaguffa Hussain, Microbiology Laboratory, Pakistan Institute of Medical Sciences (P.I.M.S) Islamabad Pakistan.

E-mail: [dani\\_amin79@yahoo.com](mailto:dani_amin79@yahoo.com)

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### ABSTRACT

Antibiotic resistance pattern and extended spectrum  $\beta$ -lactamase production was investigated in cultures of forty *Klebsiella pneumoniae* isolates isolated in a Tertiary Care Hospital in Islamabad Pakistan towards novel cephalosporin (first, second and third generation), fluoroquinolones, carbapenems, amoxicillin/clavulanic acid and lincomycin by disc sensitivity, minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC). Cephalosporin was reported as highly resistant (cephradine 100%, cephalexin 75%, cefecloer 87.5%, ceftriaxone 85%, cefotaxime 82.5%), lincomycin (100%), followed by quinolones (ciprofloxacin 55%, ofloxacin 47.5%, nalidixic acid 42.5%, norfloxacin 35%, moxifloxacin 25%, gatifloxacin 15%), amoxicillin/clavulanic acid 12.5%, and carbapenams (imipenem, meropenem) with the least resistance at 7.5%. About 47.5% strains were found to be ESBLs positive among which 15.8% of the strains were producing carbapenamase, 26.5% with inducible cephalosporinase of bush functional gene 2e. No inhibitor resistant TEM- $\beta$  lactamases (IRT) positive strains were found. High antibiotic resistance rate against commonly used antibiotics is a disadvantage for health care system in countries like Pakistan as it can greatly effect patient management. Therefore physicians must change prescription priorities towards alternative antibiotics to reduce the burden of antibiotics resistance.

**Keywords:** *Klebsiella pneumoniae*, minimum inhibitory concentrations, ESBLs, IRT.

### INTRODUCTION

Bacteria from the genus *Klebsiella* causes numerous infections in human, which are often treated with  $\beta$ -lactam antibiotics. The fundamental mechanism of *Klebsiella* resistance to penicillin or cephalosporin involves the production of enzymes called extended spectrum  $\beta$ -lactamases (ESBLs), because of resistance of many *Klebsiella* sp. strains to  $\beta$ -lactamases; alternative antibiotic therapy can make use of aminoglycosides and quinolone (Sekosawa *et al.*, 2002).

A variety of nosocomial and community acquired (food borne) infections are caused by *K. pneumoniae*, one of the most deadly pathogens of *Enterobacteriaceae* (Podschun and Ullman, 1998). These pathogens possess  $\beta$ -lactamase, therefore they mediate high levels of resistance to  $\beta$ -lactam antibiotics and have become a global threat (Bouchillon *et al.*, 2004). Above 340  $\beta$ -lactamase have been detected, including an emerging class A SHV and TEM derived extended spectrum  $\beta$ -lactamases (ESBLs), Inhibitor Resistant Enzymes (IRTs), non-TEM and/or non-SHV class A ESBLs and carbapenemases, class B metallo- $\beta$ -lactamases and some of their novel inhibitors, plasmid and chromosomally

encoded class C enzymes and finally the OXA type oxacillinases, ESBLs and carbapenemases of class D (Helfand and Bonomo, 2003). The  $\beta$ -lactamase enhanced spectrum of activity towards penicillin, extended spectrum cephalosporin (ESCs) (e.g. ceftazidime [CAZ], cefotaxime [CTX], and ceftriaxone), and aztreonam is due to gene mutations encoding TEM and SHV  $\beta$ -lactamases (Bush *et al.*, 1995). It has been revealed by molecular and biochemical studies that many ESBLs are derivatives of older TEM-1, TEM-2, or SHV-1  $\beta$ -lactamases and some of which differ from the parent enzyme by only one or two amino acids (Dubois *et al.*, 1995). In addition, resistance to the ESCs has also arisen in *K. pneumoniae* and *Escherichia coli* via the acquisition of plasmids containing the chromosomally encoded AmpC  $\beta$ -lactamase found in *Enterobacter* sp., *Pseudomonas aeruginosa*, and *Citrobacter* sp. (Payne *et al.*, 1992).

There has been serious concerns regarding increased prevalence of ESBLs in different parts of the world, although exact prevalence is not known. ESBLs prevalence rate is 0 to 25% in USA, 0 to 40% in Netherlands, (Stobberingh *et al.*, 1999) 40% in France, (Branger *et al.*, 1998) 4.8% in Korea, (Jarlier *et al.*, 1998) and up to 12% in Hong Kong (Tsang *et al.*, 2000). The *K.*

*pneumoniae* and *E. coli* contribute 10 to 40% of ESBLs. Surveillance studies are valuable tool for assessing the changes in pattern of resistance of clinical isolates of antimicrobial agents (Monnet and Freney, 1994). Trend towards increased antimicrobial resistance shown by many of the Gram-negative bacteria is worrying, and has developed as a consequence of widespread and inappropriate use of various agents (Watterer and Wunderink, 2001). In conclusion, high ESBLs prevalence not only complicates antibiotic therapy but also interfere with empirical therapy resulting in increased morbidity and mortality (Podschn and Ullman, 1998).

## MATERIALS AND METHODS

### Bacterial strains

The study includes 40 clinical isolated *K. pneumoniae* obtained by screening about 200 samples of urine, blood, pus etc. Clinical isolates were isolated from Microbiology Lab, Pakistan Institute of Medical Sciences (P.I.M.S) Islamabad in 2006, which provides a tertiary level patient care and equipped with a capacity of 592 beds with 22 medical and surgical specialties. It serves as a referral hospital in Pakistan and provides health care facilities to people of different areas. Complete patient information was recorded by using hospital management information system (HMIS) in P.I.M.S. Initially, strains were identified morphologically by using MacConkey Inositol Penicillin Agar {modified with penicillin} (Seidler and Bagley, 1978), Simmon Citrate Inositol Agar (Kregten *et al.*, 1984), EMB and finally SIM agar. Based on the morphological behavior of *K. pneumoniae* on various differential media, a new identification scheme was designed (Figure 1). The species level identification was then carried out by standard biochemical test (Bergey's Manual of Determinative Bacteriology sixth edition) and confirmed by API 20E identification systems (Biomerix Inc).

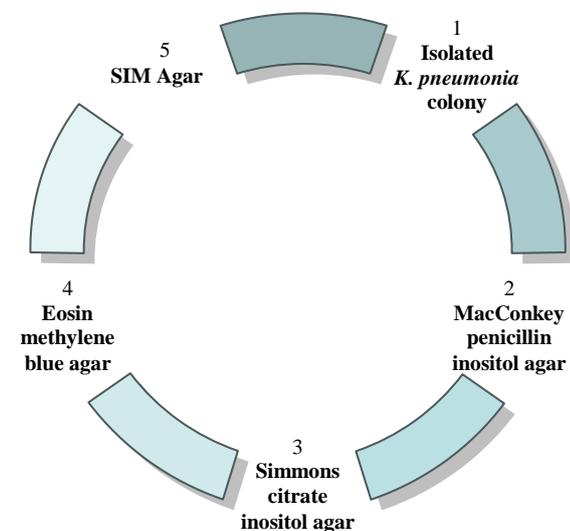


Figure 1: Morphological identification of *K. pneumoniae*

### Antimicrobial susceptibility testing

Susceptibility tests were performed by Bauer-Kirby (Bauer *et al.*, 1966) disc diffusion by using Muller Hinton Agar (CM337-Oxoid). The results were expressed as susceptible/resistant according to criteria developed by NCCLS 1996. The following antibiotic discs (Oxoid) were used with different specified concentrations: nalidixic acid (NA) 30 µg, ofloxacin (OFX) 5 µg, norfloxacin (NOR) 10 µg, moxifloxacin (MXF) 5 µg, ciprofloxacin (CIP) 10 µg, gatifloxacin (GAT) 5 µg, cephadrin (CE) 30 µg, cephalixin (CL) 30 µg, cefeclor (CEC) 30 µg, ceftriaxone (CRO) 30 µg, cefotaxime (CTX) 30 µg, imipenem (IMP) 10 µg, meropenem (MEM) 10 µg, amoxicillin/clavulanic acid (AMC) 10/20 µg, and kanamycin (KAN) 10 µg.

### Minimum inhibitory concentration (MIC)

Minimum Inhibitory Concentration (MIC) (lowest concentration of an antimicrobial agent that inhibits growth of microorganism after overnight incubation) of antibiotics was determined by broth macro dilution method in the inocula  $10^6$  cfu/mL according to criteria developed by NCCLS, 1996. Active Pharmaceutical Ingredients (APIs) manufactured by different pharmaceutical companies were obtained with complete information regarding expiry date, potency, solubility, stability as a powder and in solution, storage conditions and relevant COSHH (Control of Substances Hazardous to Health) information. These were OFX (GlaxosmithKline Pakistan) CIP (Bayer Pakistan), CE (Bristol Mayer Squibb, USA), CEC (Elli Lilly Pakistan), CRO (Hoffmann-La Roche Basle, Switzerland), CTX (Aventis Parma Deutschland GmbH, AMC (GlaxosmithKline Pakistan), MEM (Astra Zeneca UK), and lincomycin (Pfizer Pakistan).

### Minimum bactericidal concentrations (MBC)

Minimum Bactericidal Concentrations (MBC) (lowest concentration of antimicrobial that will prevent the growth of an organism after sub-culture onto an antibiotic free media) was determined methodology developed by NCCLS, 1996. *K. pneumoniae* ATCC (700603) was used as a control strain.

### Determination of ESBLs

Double disc synergy method (Jarlier *et al.*, 1998) was used to detect the ESBLs. Disc of AMC (central disc); CTX, CRO, CAZ and azteronam were used for ESBL determination in inocula  $10^6$  cfu/mL. Briefly, disc of co-amoxicillin (20 µg amoxicillin/10 µg clavulanic acid) was placed in the centre of the agar surface containing the test strain. The discs of CTX, CRO, CAZ and azteronam were arranged in such a way that the distance between the central disk and the surrounding disks were approximately 20 to 30 mm. The isolates were incubated at 37 °C for 24 h. After an overnight incubation the zones around third generation cephalosporin disc and azteronam disc was observed. When inhibition zone around one or more

cephalosporin discs was extended on the side nearest to the co-amoxiclavate disc, organism showing this synergism was recorded as an ESBL producer

**Determination of inducible cephalosporinases of bush functional gene 2e**

The inducible cephalosporinases of bush functional gene were detected by Calibrated Dichotomous Susceptibility Tests (CDS 2004) (Bell *et al.*, 2004). Briefly IMP 10 µg disc was placed adjacent to (20 mm) the cephalosporin disc (CRO was used) on Muller Hinton agar having ESBLs positive strain and incubated overnight at 37 °C. Enhancement of growth between the IMP and the cephalosporin discs was noted and the flattened edge of the cephalosporin inhibitory zone was recorded as presence of inducible cephalosporinases of bush functional gene 2e.

**Inhibitor resistant TEM β-lactamases (IRTs)**

IRT producing strains are resistant to AMC but remain susceptible to CL. IRT positive strains were determined by using Calibrated Dichotomous Susceptibility Tests, (CDS 2004) (Bell *et al.*, 2004) criteria. Briefly disc of co-amoxicillin (AMC) (20 µg amoxicillin or 10 µg clavulanic acid and CL) were placed on agar plates having ESBLs positive strain and incubated overnight at 37 °C. Strains that are resistant to augmentin but remain sensitive to CL was recorded as TEM β-lactamases producing strain.

**Determination of carbapenamase**

The carbapenamase production was determined by using Calibrated Dichotomous Susceptibility Tests, (CDS 2004) (Bell *et al.*, 2004). Briefly disc of IMP was placed on agar plates having ESBLs positive strain and incubated overnight at 37 °C. Resistant strains were recorded as carbapenamase producing strain.

**RESULTS AND DISCUSSION**

*K. pneumoniae* is an enteric Gram-negative bacillus causing hospital-acquired infections and infections in debilitated or immuno-compromised patients (Podschun and Ullman, 1998) accounting for up to 10% of all nosocomial bacterial infections (Spencer, 1996). Mostly these infections are treated with β-lactam antibiotics, which are usually hydrolyzed by β-lactamases produced by such microorganisms resulting in failure of therapy (Bush *et al.*, 1995). Therefore this study was designed to determine the sensitivity of commonly used antibiotics against *K. pneumoniae* (β-lactam), prevalence of ESBLs (subclasses), to optimize empirical therapy and suggest a therapeutic regimen against it. Forty *K. pneumoniae* strains were isolated from various sources (i.e. pus, urine, sputum, blood, pleural fluid) obtained from both hospitalized (72.5%) and non-hospitalized patients (27.5%). Significant majority of patients were male (60%) then females (40%) (Table 1).

**Table 1:** Comparison of patient clinical profile and antibiotic resistance data

Clinical data (before trial patient data)			Resistance data (after trials patient data) Rf*		
Variable	No of subjects	% age	Variable	No of subjects	% age
Male	24	60	Male	21	53
Female	16	40	female	19	47
A (0-20) yrs	5	12.5			
B (21-40) yrs	19	47.5			
C (41-60) yrs	13	32.5			
D (61-80) yrs	3	7.5			
Hospitalized	29	72.5	Hospitalized	29	72
Non hospitalized	11	27.5	Non hospitalized	11	28
Pus	21	52.5	Pus	23	58
Urine	11	7.5	Urine	10	25
Tracheal secretions	1	2.5	Tracheal secretions	4	10
Others <sup>1*</sup>	7	17.5	Others <sup>1*</sup>	3	7
		10			
U.T.I	11	27.5	U.T.I	4	10
R.T.I	4	10	R.T.I	9	24
Post operative	15	37.5	Post operative	23	57
Others <sup>2*</sup>	10	25	Others <sup>2*</sup>	4	9

Note. Patient clinical data is expressed as percentage while patient resistance data is expressed as relative frequency.

Rf\* - relative frequency.

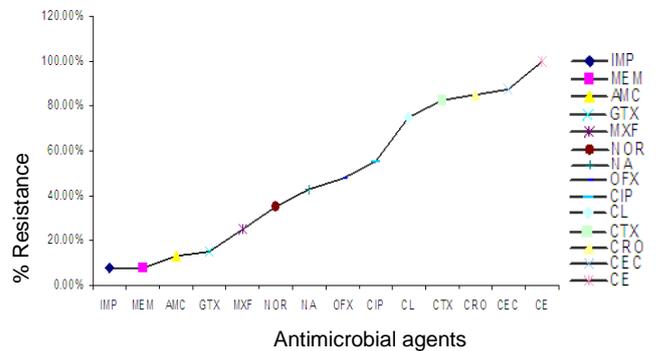
Others<sup>1\*</sup> - pleural fluid, blood, catheter tips, sputum.

Others<sup>2\*</sup> - septicemia, post burn, diabetics.

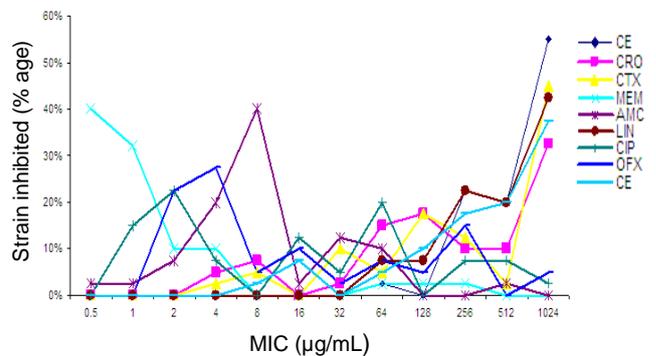
**Table 2:** Susceptibility of 15 antibiotics against clinical isolates of *K. pneumoniae*

Antimicrobial agent	Susceptibility test results	
	Susceptible (% age)	Resistant (% age)
	(S)	(R)
1. Imipenem	92.5	7.5
2. Meropenem	92.5	7.5
3. Amoxicillin/clavulanic acid	87.5	12.5
4. Gatifloxacin	85.0	15.0
5. Moxifloxacin	75.0	25.0
6. Norfloxacin	65.0	35.0
7. Nalidixic acid	57.5	42.5
8. Ofloxacin	53.0	47.0
9. Ciprofloxacin	45.0	55.0
10. Cephradin	0.0	100.0
11. Cephalexin	25.0	75.0
12. Cefeclor	12.5	87.5
13. Ceftriaxone	15.0	85.0
14. Cefotaxime	17.5	82.5

Present study highlights the most alarming situation of highly diverse antibiotics resistance rates against cephalosporin (first, second and third generation) ranging from 82.5 to 100% (MIC90 1024 µg/mL). No doubt this represents very critical situation as compared to investigations from other regions of the world reporting resistance towards first, second and third generation antibiotics (Murray *et al.*, 1990; Fish and Piscitell, 1995), however there are reports covering high levels of resistance of *K. pneumoniae* towards these antibiotics in many countries (Subha and Ananthan, 2002). This may be due to the production of ESBLs which cause the hydrolysis of β-lactam ring resulting in inactivation of cephalosporin and penicillin antibiotics (Yano *et al.*, 2001) and overuse of cephalosporin antibiotics in developing nations (i.e. Pakistan) results in high levels of resistance. Antibiotic activity spectrum (MIC<sub>90</sub>, MIC<sub>50</sub> and MIC 50/90) of the 9 antibiotics (Figure 2, 3 and Table 2), is evidence for this fact. On the other hand, quinolone represented much less worrying condition with 15 to 55% resistance (MIC90 256 µg/mL) (CIP and OFX). Although the resistance rates are lower than cephalosporin but they are slightly higher than its class as shown from SENTRY surveillance report (Diekema *et al.*, 1997) with 10 to 40% resistance among quinolone resistance towards *K. pneumoniae*. As discussed earlier, excessive and overuse of cephalosporin antibiotics lead to emergence of resistance and therefore there are trends towards alternate therapy of aminoglycosides and quinolones in other countries (Sekowska *et al.*, 2002). Less use of quinolone and structural dissimilarities is one of the reasons covering issue of comparatively low resistances that cephalosporin. AMC combination represented a better picture with 12.5% resistance, which is very low as compared to certain investigations (Ndugulile *et al.*, 2005) which highlight the low level of resistance and effectiveness of combination therapy against *K. pneumoniae* infections. Carbapenems (IMP and MEM) however, was found to be the most effective drug



**Figure 2:** IMP Imipenem, MEM meropenem, AMC co amoxiclave, GTX gatifloxacin, MXF moxifloxacin, KAN kanamycin, NOR norfloxacin, NA nalidixic acid, OFX ofloxacin, CIP ciprofloxacin, CL cephlexine, CTX cefotaxime, CRO ceftriaxone, CEC cefeclor, CE cefradine.



**Figure 3:** MEM meropenem, AMC co amoxiclave, OFX ofloxacin, CIP ciprofloxacin, CTX cefotaxime, CRO ceftriaxone, CEC cefeclor, CE cefradine, LIN lincomycin.

against *K. pneumoniae* showing only 7.5% resistance with MIC<sub>90</sub> 4 µg/mL (Figures 2, 3 and Table 2). The resistant strains were proven to be carbapenamase producers as confirmed by CDS (Bell *et al.*, 2004) testing methodology. High efficacies of IMP and MEM have been determined by many investigations in different parts of the world (Bradford *et al.*, 1997; Jones and Pfaller, 2003; Hernandez *et al.*, 2005) with high (100%) susceptibility to carbapenems in *E. coli* and *K. pneumoniae*. Although these have a β-lactam ring they are provided with a Zwitterionic structure that protects these antibiotics from hydrolysis by β-lactamases. Moreover, limited use of these antibiotics is one of the reasons for the low levels of resistance towards *K. pneumoniae*. The hospitalized patients were found highly resistant (72%) than non-hospitalized patients (28%), which confirms the involvement of *K. pneumoniae* in nosocomial infections (Spencer, 1996). Similarly, prevalence of antibiotic resistance was high in strains isolated from pus (52.5%).

Overall ESBLs prevalence reported was 47.5% which is very high as compared to developed countries such as 0 to 25% in USA, 0 to 40% in Netherlands (Stobberingh *et al.*, 1999) 40% in France (Branger *et al.*, 1998). However, results are significantly better compared to China (51%) (Xiong *et al.*, 2002) and India (86.6%) (Jain *et al.*, 2003), whereby antibiotic overuse, prescription of drugs with proper sensitivity test and over dosing may have created this problem in developing nations. Moreover among ESBLs positive strains, there were carbapenamase producers (15.8%) and inducible cephalosporinase of bush functional gene 2e (26.3%) which are one of the most important reasons for prevalence of resistance against novel cephalosporin and carbapenems. Male gender was considered a risk factor in ESBLs prevalence as shown by previous investigations (Colodner *et al.*, 2004). As emphasized by various authors, prevalence of ESBLs positive strains in a particular region or even hospital is variable and is associated with frequency of treatment of bacterial infections with β-lactam antibiotics as well as with colonization of patients hospitalized for over 10 days by ESBLs positive strains (Agata *et al.*, 1998). In summary, high antibiotic resistance and ESBLs rates among *K. pneumoniae* towards commonly used antibiotics are the major reasons for prolonged infections, increased hospitalization, increased cost of therapy and enhanced morbidity mortality rates. Special care must be taken regarding treatment of infections especially in developing countries like Pakistan. Moreover physician must change their prescription priorities towards alternative treatments in management of enteric infections specially *Klebsiella* sp.

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