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ESBL DETERMINATION AND ANTIBACTERIAL DRUG RESISTANCE PATTERN OF *KLEBSIELLA PNEUMONIAE* AMONGST PATIENTS AT PIMS ISLAMABAD

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Abstract

Background: A number of life threatening infections due to various bacterial pathogens are major cause of death in Pakistan. In a situation like the other developing countries, there is a general increase in the drug resistance due to the irrational use of the antibiotics. The present study was carried out to evaluate the prevalence and development of resistance in *Klebsiella pneumoniae* against commonly used antibiotics.
Methods and Materials: A total of 200 samples from different wards of the hospitals were received by the pathology laboratory of Pakistan Institute of Medical Sciences, Islamabad, which comprised of urine 65, blood 50 and other miscellaneous samples from various sources. *Klebsiella pneumoniae* was isolated and characterized using morphological, cultural, and biochemical methods and were further confirmed by using commercially available API 20E kit. The isolates were subjected to culture sensitivity test using Kirby Bauer disc diffusion method.
Results: The organism was found to be the most prevalent Gram-negative bacilli. *K. pneumoniae* showed highest resistance against co-amoxiclav and cephalosporins.
Conclusion: The study revealed that ESBL producing strains were resistant to most of the beta-lactam antibiotics. This trend therefore has become a significant problem and can be controlled with continued surveillance or monitoring for rationally improved antibiotics usage, in order to prevent the drug resistance and re-emergence of infection.

Key words: *Klebsiella pneumoniae*, ESBL (Extended-spectrum beta-lactamase), API (Analytical Profile Index)

Introduction

Antimicrobial resistance is the ability of bacteria to resist the effects of an antibiotic or a mechanism that blocks the inhibitory or killing effects of antibiotics (Popovic et al., 2007). Beta-lactam antibiotics have been used as therapeutic agents since the 1940 as they are excellent drugs because they are non-toxic and well tolerated by most of the patients. The broad-spectrum penicillins were soon followed by a large number of cephalosporin. (Conly, 2002). Antimicrobial resistance caused by ESBL has been found in many pathogenic Gram-negative bacteria but they are most common in nosocomial isolates of *K. pneumoniae* (Cheesbrough, 2005). Antimicrobial resistance can also occur as a result of random genetic mutations in bacteria, leading to variation in susceptibility within any bacterial population (Favre-Bonte and Licht, 1999, Feglo et al., 2010). *Klebsiella pneumoniae* is a non-motile Gram-negative rod-shaped bacterium, non-spore forming bacilli, which can grow both aerobically and anaerobically and are capsulated (Piranfar et al., 2014). *Klebsiella* species are known to cause a variety of human infections such as *pneumonia*, *septicaemia*, urinary tract infections, wound infection, meningitis, throat infection, intra-abdominal abscess, brain abscess, and device related infection (Andrade, 2014).

In recent studies most frequently identified bacteria were Escherichia coli (64.56%) and *Klebsiella pneumoniae* (13.78%). E-coli were 61.35% resistant to trimethoprim, sulfamethoxazole and 49.6% resistant for Klebsiella spp. Also intermediate resistance to nitrofurantion and tetracycline was also observed (Bergey's, 1994). There is association of important co-resistance and virulence factors in the *K. pneumoniae*, which makes this pathogen flourishing at infections and points to the quick evolution and expansion of this multi resistant as a virulent clone, leading to pan drug-resistant and persistent bacteria (Philipon et al 1989).

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Materials and Methods

Samples Collection

The study was conducted at Pakistan Institute of Medical Science Islamabad (PIMS) and a total of 200 clinical samples i.e., 65 urine, 50 blood, 22 pus, 20 tracheal secretions, 20 tissue and miscellaneous such as body fluids and infected wound swabs from out and in patients of different wards were collected . The collected specimens were labeled, including the sample type, date, and patient's gender, as per hospital record and transported to the laboratory within recommended time period and then processed for further studies.

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Sample Processing

Freshly drawn 3-5 ml blood was immediately transferred to 50 ml of Brain Heart Infusion (BHI) broth, and incubated at 37°C for 24 hours. Growth was sub-cultured on blood agar and MacConkey agar plates, and incubated for 24 hours at 37°C. For broth with no growth even after 48 hours were further incubated up to ten days, and observed the result. All urine samples were transferred to sterile tubes for centrifugation and streaked on Cysteine-Lactose-Electrolyte Deficient (CLED) medium. The plates were incubated for 24 to 48 hours at 37°C. The bacterial growth was sub-cultured on blood agar and MacConkey agar for further processing. Body fluids, pus, swabs samples were cultured on blood agar, MacConkey agar and XLD and incubated for 24 to 48 hours at 37°C. The bacterial isolates from all type of samples were identified up to species level and the susceptibility testing was done using disc diffusion method. For short term storage isolates were preserved on the nutrient agar slants. These slants were incubated for overnight at 35ºC and placed at 4ºC. For long term preservations, the bacterial isolates were preserved at -70ºC on nutrient broth containing 30% (v/v) glycerol in screw capped tubes.

Identification of Bacterial Pathogens

Isolated colonies, after purification, were initially Gram stained by using Bergey's Manual of Bacteriology (Jarlier et al., 1988). The isolates were characterized using biochemical tests i.e., triple sugar iron test, indole test, voges-Proskauer test (VP), motility test**,** citrate utilization test and oxidase test. API 20E was used for the micro standard system of specific identification of *K. pneumoniae* from the rest of the Gram negative rods using 20 microtubules with dehydrated substrates with the addition of bacterial suspension as inoculums (Al-Charrakh et al 2011). An anaerobic environment was created in tubules containing ADH, LCD, ODH, H2S, and UREA with a drop of mineral oil overlay as per manufacturer's instructions. The incubation box was then incubated at 37C for 24 hours.

Antibiotic Sensitivity Test

Antimicrobial susceptibility testing was carried out by the standard Kirby-Bauer disk diffusion method on Muller-Hinton agar following guidelines provided by Clinical and Laboratory Standards Institute (CLSI) 2010.

Detection of ESBL

For detection of ESBL-production, double disc method of (Jarlier et al 1988) was used to observe the ESBL production. The identified Gram negative organisms were swabbed onto a Mueller-Hinton agar plate. A susceptibility disc of co-amoxiclav (20µg amoxicillin/ 10µg clavulanic acid) was placed in the center of the agar surface. The discs of ceftriaxon and ceftazidime (30µg each) were arranged in such a way that the distance between the central disc and surrounding discs was approximately 30 mm. After 30 minutes of pre-incubation time, the plates were incubated aerobically at 35 C for 24hrs. If the inhibition zone around one or more cephalosporin discs was extended on the side nearest to the co-amoxiclav disc, the organism showed an ESBL production. If no extension of zones of third generation of cephalosporin towards co-amoxiclav disc was observed, the organisms were considered as non-producer of ESBLs.

Results and Discussion

ESBL Production in *K. Pneumoniae*

Out of 200 ß-lactamase (ESBL) producing isolates, 175 (87.5%) ESBL positive and 25 (12.55%) ESBL negative organisms were detected by double disk diffusion method (Figure 1) and this pattern was similar as in another finding by Al-Charrakh et al., 2011.

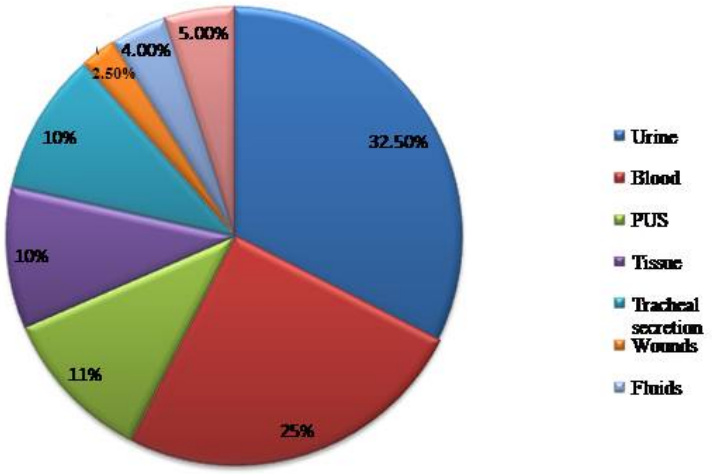

Figure 1: The total samples distribution pattern for *K. Pneumoniae* isolates

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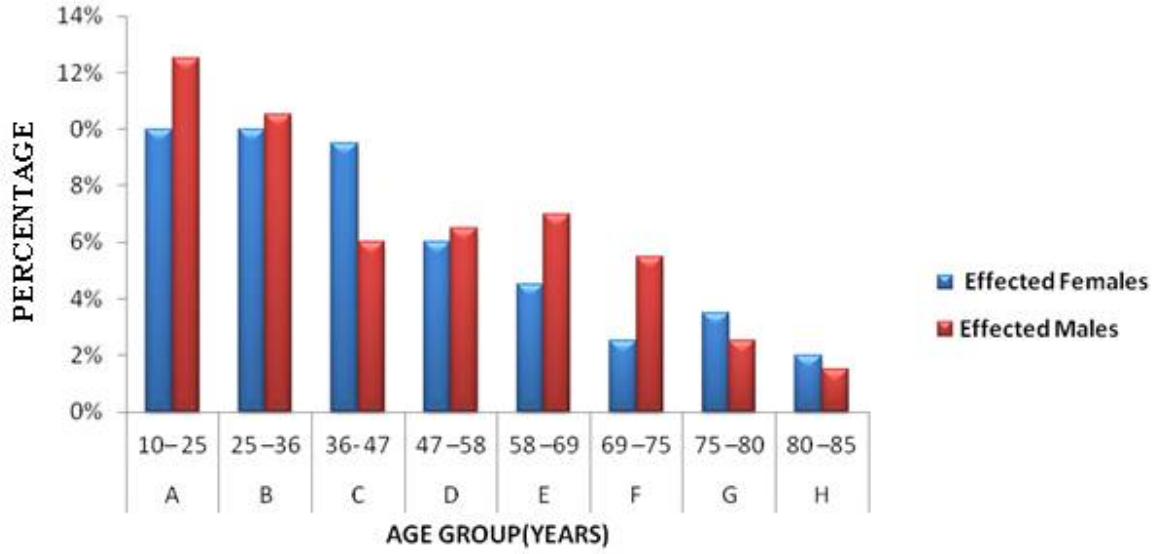

Figure 2: Gender wise distribution of *K. Pneumoniae* infection

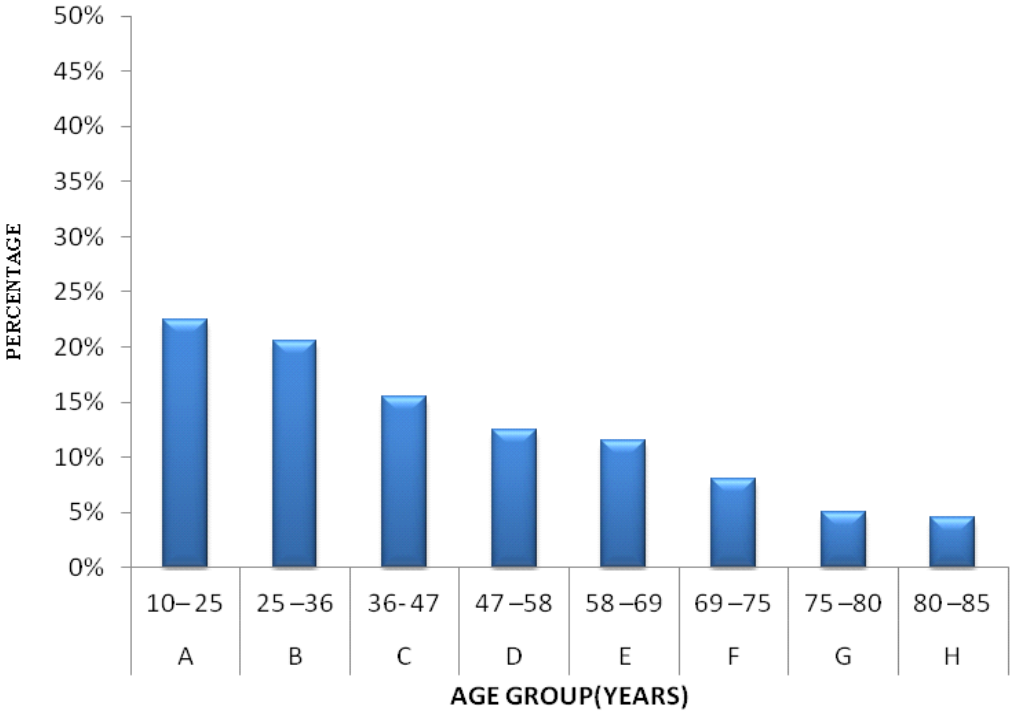

Figure 3: Age wise distribution of *K. Pneumoniae* infection

Microbial Analysis

Isolates were identified by their growth on different media, MacConkey agar, blood agar, xylose lysine deoxicholate (XLD) and cysteine lactose electrolyte deficient (CLED). Colonies were observed after 24 hours of incubation as pink , bright yellow and whitish grey colonies on MacConky agar, XLD, CLED and blood agar; respectively. These observations are generally noted as similarly observed in a study by (Shah *et al*., 2000, Mumtaz *et al*., 2008).

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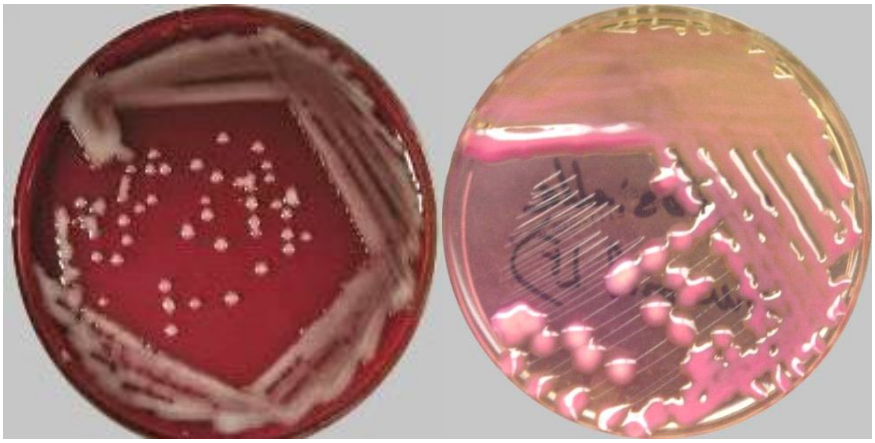

Figure 4: Growth of *Klebsiella pneumoniae* on MacConky and Blood agar

Figure 5: Growth of *Klebsiella pneumoniae* on CLED and XLD culture media

Gram Staining Result

All 200 samples were Gram stained to observe the morphological characteristics of *K. Pneumoniae* under microscope. It was observed that *K. Pneumoniae* was Gram negative as it appeared thick pink rod as shown in (Figure 6), these results are supported by other researchers and are in according to the Bergey's Manual of Determinative Bacteriology.

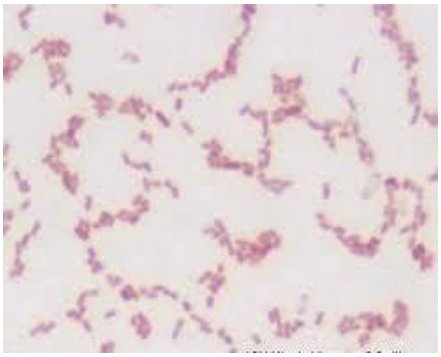

Figure 6: Microscopic appearance of *K. Pneumoniae*

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Biochemical Characteristics of *K. Pneumoniae*

For biochemical characterization, a series of biochemical tests for *K. pneumoniae* were manually performed such as citrate, VP, TSI, motility, indole and oxidase and were compared with API 20 E analysis. Our results were compatible with (Winn *et al*., 2006, Murray *et al*., 2007).

Table: 1. Biochemical Characteristics of *K. pneumoniae*

Biochemical Characteristics		
Test	**Results**	**Change In Growth Media**
Citrate	+ive	Green to Blue
Motility	-ive	No Turbidity no Growth
Indole	-ive	Oily Band on Top Not Red In Colour
Voges-Proskauser	+ive	Pink-Red in colour
Oxidase	-ive	No color change
Triple Sugar Iron	+ive	Light Pink(alkaline),

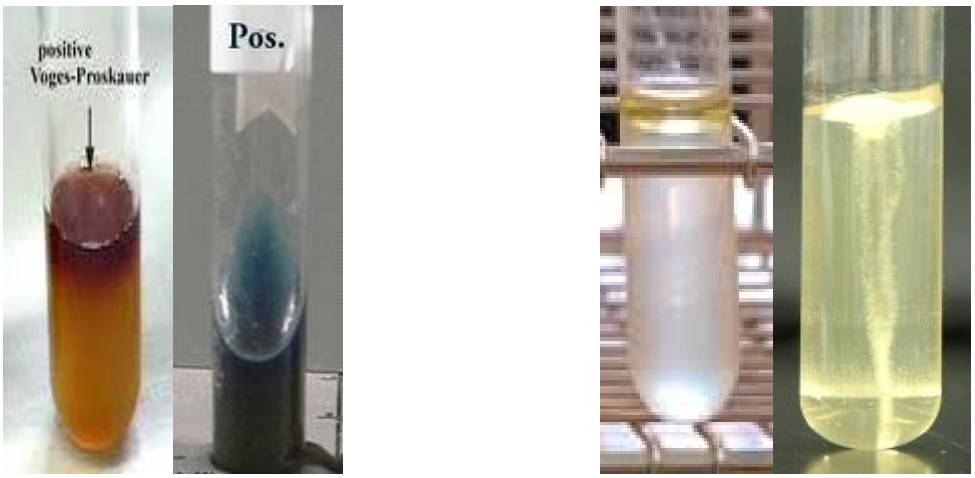

Figure 7: Voges-Proskauer , Citrate , Indole and Motility tests

Results on API 20E System

The biochemically identified samples of *K. pneumoniae* when analyzed on API 20E system on an API strip, a conversion numerical value was obtained. The generated number from API 20E system for *K .pneumoniae* was then checked in the catalogue book and was identified 95 percent up to species level. (Figure 8) Results showed similarity with findings by (Carson et al., 2001).

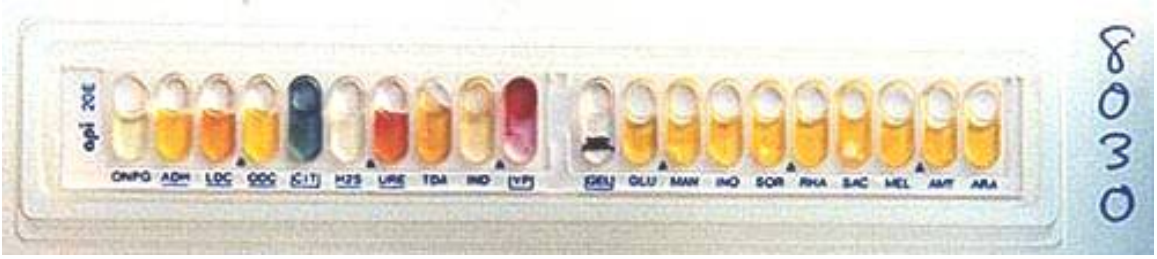

Figure 8: Results of API20E for confirmation of *K. Pneumoniae*

Antibiotic Susceptibility Pattern of Isolates

In the present study, most commonly used antibiotics discs were subjected to determine the *Klebsiella pneumonia* sensitivity. Antibiotic sensitivity of *K. pneumoniae* was observed by disc diffusion method in which fourteen different antibiotics were applied. The sensitivity of *K. pneumoniae* was checked with help of list of break points of zone of inhibition and to consider resistant one when zone of inhibition did not fall into the values of break points. The results showed that among fourteen conventional drugs *K. pneumoniae* showed highest

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resistance against co-amoxiclav, i.e., 100%. The next highest resistance was for cephalosporin, such as ceftazidime, ceftriaxone followed by Piperacillin-tazobactum, norfloxacin, cefoperazon+sulbactum, gentamycin, tobramycine, amikacin, piperacillin, imipenem and meropenem. Isolates were found to be resistant to all classes of antibiotics except polymixin B. The (Al-Zahrani *et al*., 2005), also determined antimicrobial sensitivity test by using different antibiotics and found that *K. pneumoniae* was sensitive to meropenem, imipenem. The resistance against different similar drugs was also reported by (Chaikittisuk *et al*., 2007).

Table 2: Antibiotic sensitivity pattern of specimens referred from surgical ward

Antibiotics	Resistance Count (%age)	Intermediat Count (%age)	Sensitive Count (%age)	Total Count
Amoxicillin	52(100)	0(0.00)	0(0.00)	52
Ceftazidime	44(89.7)	2(4.0)	3(6.10)	49
Ceftriaxone	54(100)	0(0.00)	0(0.00)	54
Tazobactum	48(84.2)	3(5.2)	6(10.5)	57
Norithindrone	35(83)	2(4.7)	5(11.5)	42
Gentamicin	30(60)	0(0.00)	20(40)	50
Tobramycin	24(40)	1(1.6)	35(58)	58
Amikacin	19(38)	0(0.00)	31(62)	50
Piperacillin	17(34.6)	10(20.0)	22(44.8)	49
Levofloxacine	5(9.0)	0(0.00)	50(90)	55
Meropenem	2(3.8)	0(0.00)	50(96)	52
Polymixine B	0(0.00)	0(0.00)	50(100)	50

Table: 3: Antibiotic sensitivity pattern of specimens referred from medical ward

Antibiotics	Resistance Count (%age)	Intermediat Count (%age)	Sensitive Count (%age)	Total Count
Amoxicillin	50(100)	0(0.00)	0(0.00)	50
Piperacillin	47(95.5)	1(2.0)	1(2.0)	49
Ceftriaxone	45(93.7)	2(4.1)	1(2.0)	48
Tazobactum	35(89.7)	1(2.5)	3(7.6)	39
Norithindrone	40(83)	1(2.0)	7(14)	48
Gentamicin	20(39)	1(1.9)	28(54)	49
Tobramycin	22(48)	2(4.4)	23(51)	47
Amikacin	20(41)	0(0.00)	28(58)	48
Piperacillin	20(34)	9(15)	29(50)	58
Levofloxacine	5(9.0)	0(0.00)	50(90)	55
Meropenem	3(6.5)	0(0.00)	43(93)	46
Polymixine B	0(0.00)	0(0.00)	49(100)	49

Table: 4: Antibiotic sensitivity pattern of specimen's referred from outpatient department

Antibiotics	Resistance Count (%age)	Intermediat Count (%age)	Sensitive Count (%age)	Total Count
Amoxicillin	55(100)	0(0.00)	0(0.00)	55
Ceftazidime	50(89)	2(3.5)	4(7.1)	56
Ceftriaxone	49(96)	1(1.9)	1(1.9)	51
Tazobactum	48(87.5)	2(4.0)	4(8.0)	54
Norithindrone	57(93)	1(1.6)	3(4.9)	61
Gentamicin	26(53)	0(0.00)	23(46.9)	49
Tobramycin	24(52)	1(2.0)	27(58)	52
Amikacin	22(36)	0(0.00)	39(63.9)	61
Piperacillin	19(38)	22(11)	20(40)	61
Levofloxacine	4(8.6)	1(2.1)	41(89)	45
Meropenem	4(7.8)	0(0.00)	53(92)	57
Polymixine B	0(0.00)	0(0.00)	50(100)	50

Table: 5: Antibiotic sensitivity pattern of specimens referred from intensive care unit

Antibiotics	Resistance Count (%age)	Intermediat Count (%age)	Sensitive Count (%age)	Total Count
Amoxicillin	43(95)	0(0.00)	2(4.4)	45
Ceftazidime	43(97)	0(0.00)	1(2.0)	44
Ceftriaxone	47(100)	0(0.00)	0(0.00)	47
Tazobactum	51(91)	2(3.5)	3(5.3)	56
Norithindrone	43(87)	1(2.0)	5(10)	49
Gentamicin	24(45)	2(3.7)	26(46)	52
Tobramycin	27(57)	0(0.00)	20(42)	47
Amikacin	14(34)	0(0.00)	27(65.8)	41
Piperacillin	14(32.5)	10(23)	19(44)	43
Levofloxacine	6(10.9)	0(0.00)	49(89)	55
Meropenem	3(6.6)	0(0.00)	52(93.3)	55
Polymixine B	0(0.00)	0(0.00)	50(100)	50

Acquired bacterial resistance is common among clinical isolates from healthy persons as well as patients having community-acquired infections in developing countries. *K. pneumoniae* was observed to be resistant against ciprofloxacin, amoxacilline-clavulanic acid and gentamicin. Polymixin B and meropenem were the drugs considerably effective against *K. pneumoniae* along with other high spectrum third generation antibiotics. In conclusion, antimicrobial resistance is widespread among hospital and community-acquired pathogens in Pakistan. Monitoring ESBL production and antimicrobial susceptibility testing are necessary to reduce the burden of increasingly resistant pathogens (Asti 2013). Therefore, the antibiotic stewardship guidelines need to be recommended and strictly adopted in a comprehensive control program to reduce the high levels of bacterial antibiotic resistance (Mohanalakshmi et al. 2014). In this study, the prevalence of the ESBLs was highest in *K. pneumonia* which is an indication of increased resistance. However, in the industrialized world, education of the medical professionals regarding the use of antimicrobial agents seems to be the single most important tool in avoiding further development of resistance and to minimize the problems.,

Conclusions

The study revealed that ESBL producing strains were resistant to most of the beta-lactam antibiotics. This trend, therefore, has become a significant problem and can be controlled with continued surveillance or monitoring for rationally improved antibiotics usage, in order to prevent the drug resistance and re-emergence of infection.

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