ejpmr, 2016,3(9), 92-97.

SJIF Impact Factor 3.628

EUROPEAN JOURNAL OF PHARMACEUTICAL AND MEDICAL RESEARCH

www.ejpmr.com

Research Article

ISSN 2394-3211

EJPMR

EXTENDED SPECTRUM BETA LACTAMASES AND METALLOBETA-LACTAMASES: AN UPDATE FROM LAHORE, PAKISTAN

Noor Ul Ain[1], Samyyia Abrar[1], Kiran Nazeer[1] and Saba Riaz[1,2]*

[1,2]Department of Microbiology and Molecular Genetics, University of the Punjab, Lahore, Pakistan.
[2]Citi Lab and Research Center, Faisal Town Lahore, Pakistan.

***Corresponding Author: Saba Riaz**

Citi Lab and Research Center, Faisal Town Lahore, Pakistan.

Article Received on 08/07/2016 **Article Revised on 30/07/2016** **Article Accepted on 19/08/2016**

ABSTRACT

The objectives of the presented study were to update knowledge of the resistance burden in isolates from clinical settings in Pakistan, and assess the prevalence of ESBLs and MBLs in them. A total of 642 samples were processed in regional diagnostic center during October 2014 to December 2014. All the samples were subjected to Antimicrobial susceptibility testing (AST) by Kirby-Bauer disc diffusion method. Extended spectrum beta lactamases were screened by double disc synergism test (DDST) and Combination disc test (CDST). Strains resistant to carbapenems were further screened for metallo-β-lactamases (MBLs) using EDTA- imipenem disc synergism test and modified Hodge test. 498 Gram negative strains were obtained, most frequently from urine samples (61%), followed by pus (19%) and wound (6%) samples. There were 30% more infected samples from female patients than from male patients. The pathogens most frequently detected were *Escherichia coli* (50%) followed by *Klebsiella* spp. (18%), *Pseudomonas* spp. (11%) and *Citrobacter* spp. (11%). All of the *E. coli* and *Klebsiella* strains showed resistance to ampicillin and cefuroxime, and 97-98% of them showed resistance to cephradine, ceftrioxone, azeotrenum and amoxicillin. However, 85-92 and 82% of them were susceptible to carbapenems and amikacin, respectively. The DDSTs indicated that 58% of the strains carried ESBLs, and they were most prevalent in isolates from neonates (<1-year-old) followed by 21-40 year-olds. The commonest ESBL-carrying strains were *Escherichia coli* (54%) followed by *Klebsiella* spp. (23%) and *Citrobacter* spp. (11%). CDSTs detected ESBLs in more strains (73%) than the DDSTs. In addition, 57 and 85% of the carbapenem-resistant strains possessed MBL activity according to EDTA-imipenem CDST and EDTA-imipenem disc synergism tests. Use of ESBL- and MBL-phenotypic detection tests and antimicrobial susceptibility tests in routine screening may be a good strategy for managing infections in a country like Pakistan.

KEYWORDS: ESBLs, MBLs, AST and antibiotics resistance.

1. INTRODUCTION

Multi-drug resistant bacteria are causing increasingly severe clinical problems globally[1], including high rates of mortality and morbidity in Pakistan and other developing countries.[2] Thus, more information on their distributions and resistance patterns of bacteria expressing them is required. A major contributor to the problems is the emergence of resistance to β-lactam antibiotics: an important battery of agents that have high historic efficacy against many Gram-negative and Gram-positive bacteria.[3]

Resistance to these drugs can be attributed to beta lactamase enzymes, of which more than 850 have been identified[4], particularly so-called extended spectrum beta lactamases (ESBLs), which can confer co-resistance to major classes of antibiotics, including cephalosporins, aminoglycosides and fluoroquinolones.[5,6] Carbapenems were considered drugs of choice for many infections until the early 1960s, but resistance to them conferred by various metallo-beta lactamases (MBLs) is severely reducing therapeutic options. Furthermore, genes encoding them are readily transferred via plasmids among compatible hosts.

Unsurprisingly, given their clinical significance, numerous methods have been developed for detecting ESBLs and MBLs, all of which have some limitations. The optimal choices depend on the required specificity and detection limits.[7] Strengths and limitations of tests applied in this study (described below) have been addressed in various reviews.[13,25] and are further considered here.

2. MATERIALS AND METHODS

2.1. Bacterial Isolation and Identification

A total of 642 non-duplicate strains were isolated from the Citi Lab and Research Centre, Lahore from samples collected during the period October 2014 to December 2014 were processed according to Clinical Laboratory

Standard Institute (CLSI) criteria.[8] Gram negative and Enterobacteriaceae isolates were selected by re-streaking on MacConkey's agar plates. All strains were identified using biochemical tests and some were confirmed using the API-20E Strip test (bioMerieux).

2.2. Antibiotic Susceptibility Testing

Initially, the susceptibility of all of the identified strains to 21 antibiotics was tested using the Kirby Bauer disc diffusion method, with Mueller Hinton agar, as recommended by the CLSI.[9] These antibiotics included: 8 cephalosporins (Ampicillin, 20 µg; Amoxicillin, 30 µg; Aztreonam, 30 µg; Ceftrioxone, 30µg; Cefuroxime, 5µg; cefepime, 30 µg; cefoperazone/sulbactam, 105 µg; carbenicillin, 25 µg), 2 Carbapenems (imepenum, 10 µg; meropenum,10 µg), 2 Aminoglycosides (gentamicin, 10 µg; Amikacin, 30ug), 2 Quinolones (levofloxacin, 5 µg; norfloxacin, 30 µg), 1 Sulphonamide (sulfamethoxazole, 1.25 µg) and tetracycline, 30 µg.

2.3. Phenotypic Detection of ESBLs and MBLs

To determine whether isolated strains expressed ESBLs they were subjected to DDSTs using test amoxicillin-clavulanate together with cefuroxime, aztreonam, ceftriaxone, cefotaxime and ceftazidime, as well as CDSTs with cefotaxime/cefotaxime+clavulanate and ceftazidime/ ceftazidime+clavulanate. As an initial screen for MBL expression, DDSTs were applied using imipenum (10 µg) and a blank filter paper disc dipped in 0.5 M EDTA solution.

To confirm MBL expression, CDSTs were applied with imipenem/imipenem and EDTA. The criteria for ESBL and MBL detection were differences in diameters of inhibition zones around combination and non-combination disks of >5 mm and >7mm, respectively. The Modified Hodge Test was used to confirm production of carbapenemases, manifested in a characteristic cloverleaf-like pattern of growth inhibition of the control organism.

2.4. *Ribotyping*

The taxonomic status of isolated strains was confirmed by 16S rRNA sequencing and comparison of the consensus sequences to sequences in the NCBI nucleotide database using NCBI nucleotide-BLAST (blastn). Stocks of all confirmed isolates were prepared in 70% glycerol and stored at -20°C.

3. RESULTS

3.1 Sampling

From the 642 analyzed samples, 498 gram negative strains were obtained, 82% of which were members of the Enterobacteriaceae .The clinical specimens included samples of urine (62%), pus (19%), body fluids (5%) and others (7%). The most frequent clinical isolates were *Escherichia coli* (50%) followed by *Klebsiella* spp. (18%), *Pseudomonas* spp. (11%) and *Citrobacter* spp. (11%). 58% of the strains were ESBL producers according to the DDSTs. *E. coli* (52%) was the most common ESBL producer, followed by *Klebsiella* spp. (22%), *Citrobacter* spp. (13%), *Proteus* spp. (7%) and *Morgenella* spp. (4%) (Table 1).

3.2 Demographic Data Analysis

Analysis of the demographic data (summarized in Table 2) showed that 57 and 43% of the samples infected with Gram negative bacteria were from female and male patients, respectively. The frequencies declined with age of the patients: 40% of infected samples were from 0-20 year-old patients (including 24% from neonates), followed by 20, 16, 13 and 2% from 21-40, 41-60, 61-80 and >80 year-old patients, respectively. In total, 46% of the isolates were found to express ESBLs. There were 30% more infected samples from female patients than from male patients. Infection with ESBL-producing bacteria was most frequent in samples from 41-60 year-old (51%), followed by samples from 61-80 year-old (48%), 1-40 year-old (45%) and neonate (40%) patients. 52% of the ESBL-producing isolates were from pus samples followed by samples from wounds (48%), urine (43%) and body fluids (37%). Urinary tract infections were the most common source of ESBL-associated infection in females (accounting for 68% of the total).

3.3 Susceptibility Tests

All of the *E. coli* strains showed resistance to ampicillin and cefuroxime, and >95% showed resistance to azeotrenam, amoxicillin, ceftriaxone and cephredine. More than 80% also showed resistance to cotrimoxazole, tetracyclin and norflaxacin, but frequencies of resistant strains were successively lower for: ciproflaxacin (78%), gentamycin (47%), amikacin (18%), cefoperzone/ sulbactam (11%), nitrofurontoin (10%), tazocin (9%), meropenem (2.3%) and imepenem (1%). Of the *Klebsiella* spp. >95% showed resistance to ampicillin, amoxicillin, azeotranum, cefuroxime, ceftrioxone, cephredine and co-trimoxazole, but these strains showed less resistance to nitrofurontoin (78%), tetracycline (58%), ciprofloxacin (45%), gentamycin (37%), norflaxacin (35%), amikacin (18%), cefoperzone/ sulbactam (16%), tazocin (13%), meropenem (8%) and imipenem (3%). All of the *Citrobacter* spp. showed resistance to β-lactam drugs, but increasingly few were resistant to gentamycin (86%), nitrofurontoin (76%), ciprofloxacin and norflaxacin (>40%), tazocin (29%), amikacin (19%), cefoperazone/sulbactam (14%), meropenem (10%) and imipenem (1%). Most (>80%) of the *Morgenella* spp. and *Proteus* spp. also showed resistance to β-lactams. However, only 14% of the former and none of the latter showed resistance to carbapenems. Most *Pseudomonas* strains were susceptible to imipenem (84%), tazocin (78%) and meropenem (73%). More than 95% of strains of all genera were resistant to co-trimoxazole, except *Morgenella* spp. (71%). In contrast, <3% of strains of all genera were resistant to imipenem, except *Morgenella* and *Pseudomonas* spp. (>14%). Very few *E. coli* isolates were resistant to meropenem (<3%), but higher

frequencies of all other strains were resistant to it (Figure 1).

3.4 Phenotypic Detection Tests for ESBLs

The DDSTs and CDSTs respectively indicated that 58 and 73% of the strains expressed ESBLs. However, 76% and 50% of these strains were susceptible to ceftazidime + clavulanic acid and cefotaxime+ clavulanic acid, respectively. Similarly, 74 and 57% were susceptible to cefotaxime + amoxicillin and ceftazidime + amoxicillin, respectively. In more detail, 75, 71, 56, 50, 50 and 33% of ESBL-expressing *Pseudomonas*, *Klebsiella*, *E. coli*, *Proteus*, *Morgenella* and *Enterobacter* strains were susceptible to cefotaxime + amoxicillin. Ceftazidime had 100% efficacy with amoxicillin against *Morgenella* and *Proteus*, but successively less efficacy against ESBL-expressing strains of *Pseudomonas* (75%), *Enterobacter* (67%), *E. coli* and *Klebsiella* (both 29%).

3.5 Phenotypic Detection Tests for MBL

EDTA-imipenem disc synergism and EDTA-imipenem Combination Disc tests respectively indicated that 57 and 85% of the ESBL-expressing strains expressed MBLs (Figure 3).

3.6 Phylogenetic Analysis

The RNA sequences obtained have been submitted to GenBank under accession numbers K1 (KR905685), K2 (KR905686) and K3 (KR905687).

7. Tables

Table 1: Sources of processed samples and numbers of isolates of indicated taxa from them.

Specimen type	N	*Citrobacter* spp.	*E. coli*	*Klebsiella* spp.	*Morgenella* spp.	*Proteus* spp.	*Pseudomonas* spp.	Others
		n=50	n=236	n=87	n=24	n=25	n= 53	
Urine	276	17	87	22	6	1	13	-
Pus	86	3	15	14	2	7	12	2
Wound	27	1	2	3	-	3	8	3
HVS	9	-	1	3	-	-	2	-
Body fluids	26	-	4	1	1	1	4	1
Sputum	8	-	-	2	-	-	3	-
Ear swabs	11	1	1	1	-	1	5	-
Tips and catheters	5	-	1	-	-	1	2	-

N= No. of samples

n= Total No. of isolates/Others*= *Enterobacter* spp., *Acinetobacter* spp., *Serratia* spp. and *Bacillus cepacia*

Table 2: Frequencies of infections by ESBL- and non-ESBL-producing strains (detected by DDSTs) by age and gender.

		<1 year	1-20 Years	21-40 Years	41-60 Years	61-80 Years	>80 Years	Total	Grand Total
Male	**ESBL**	19(21%)	11(12%)	18(20%)	22(24%)	17(19%)	3(3%)	90(47%)	193(43%)
	Non-ESBLs	30(29%)	16(16%)	22(21%)	20(19%)	14(14%)	1(1%)	103(53%)	
Female	**ESBL**	25(21%)	21(18%)	33(28%)	23(20%)	11(9%)	4(3%)	117(46%)	255(57%)
	Non-ESBLs	35(25%)	23(17%)	40(29%)	24(17%)	16(12%)	0(0%)	138(54%)	
General		109(24%)	71(16%)	113(25%)	89(20%)	58(13%)	8(2%)	**448**	

Legends to Figures

Figure 1. Resistance patterns of indicated strains to the applied antibiotics

AMP=Ampicillin, AMC=Amoxicillin, AZT=Aztreonam, CRO=Ceftrioxone, CE=Cephredine, MEM= Meropenem, IMP=Imipenem, CN=Gentamycin, TE=tetracyclin, AK=Amikacin, CIP=ciprofloxacin, F=Nitrofurantoin, NOR=Norfloxacin, SXT=Cotrimoxazole, CES= TZP Tazocin.

Figure 2. Phenotypic Detection Tests for (A) ESBLs-producers (B) MBLs-producers

Figure 3. (A) an EDTA-imipenem combination disc synergism test, showing a synergistic zone of inhibition around the IMP + EDTA disc and (B) a Modified Hodge Test showing the characteristic clover leaf like pattern arising from release of inhibition of the control strain by streaks of the test organisms (IP-1, 1P-2 and 1P-3) within the zone of diffusion from the impregnated disc.

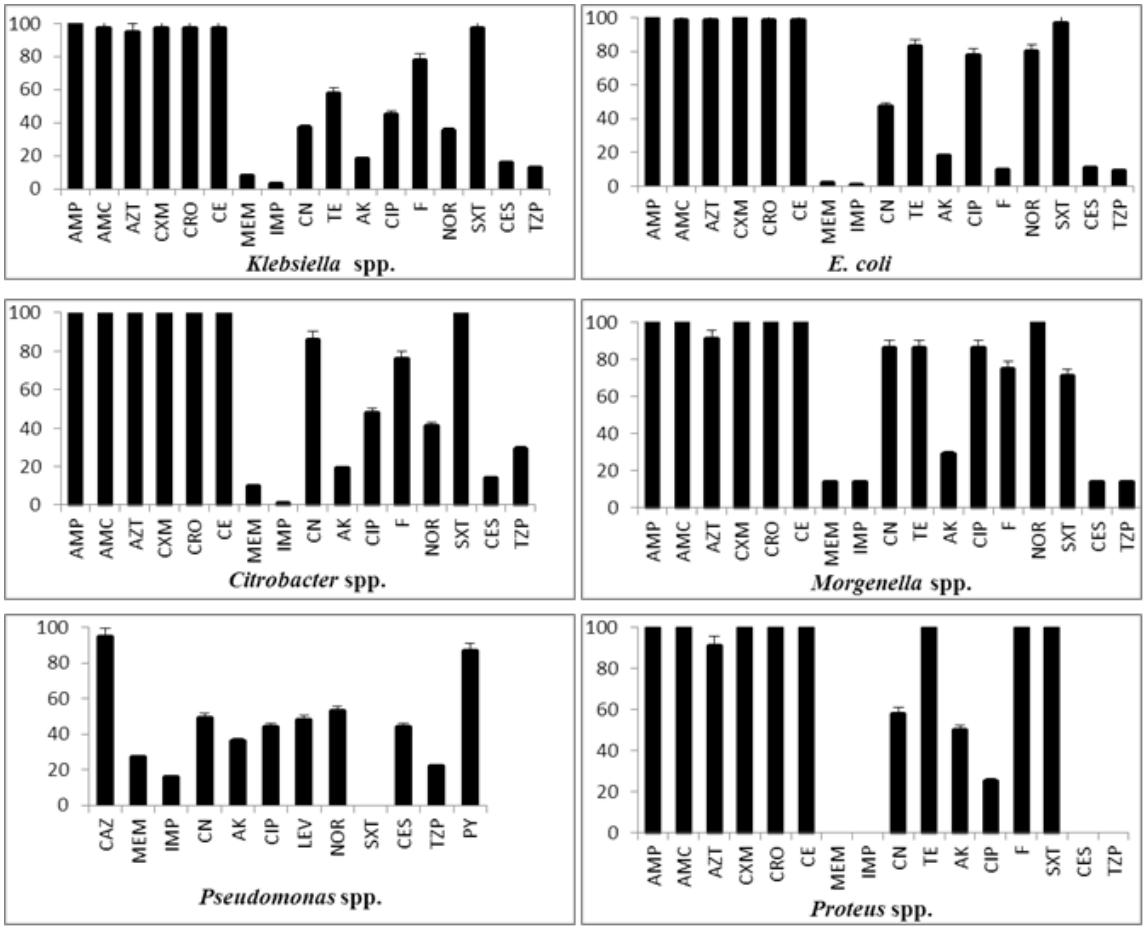

Figure 1

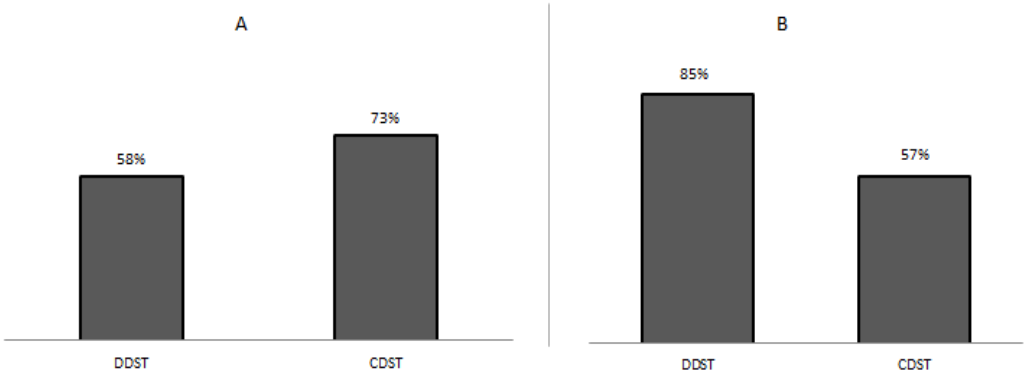

Figure 2.

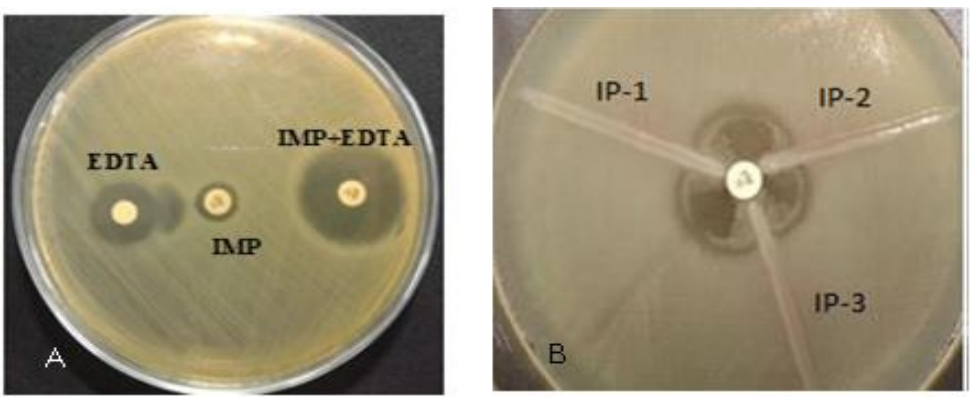

Figure 3

4. DISCUSSION

Antimicrobial resistance is causing a severe public health crisis across the entire globe. In this study, Gram negative strains were isolated from 78% of the examined clinical samples, indicating that they are the most common causes of resistant infections. There was also a high prevalence of *Enterobacteriaceae*, corroborating high reported levels of their dissemination in the study region.[9,10] The prevalence of ESBL-producing strains varies substantially among different countries, and even different hospital settings in the same country.[11] It is generally high in Asian countries, particularly India, where up to 75% of Gram negative clinical isolates are reportedly ESBL-producers[12,13], compared to just 15% in Korea and Taiwan.[14] According to a previous study the frequency is high in Pakistan (35-40%)[15], and we found somewhat higher frequencies (46 and 47% of Gram negative strains isolated from samples from males and females, respectively, expressed ESBLs).

The most frequently detected pathogens in this study were *E. coli* and *Klebsiella* spp., in accordance with previous findings that these strains are prominent clinical isolates.[16] Infectivity rates vary depending on patients' gender, age and nature of infection. There were 30% more infected samples from female patients than from male patients. Similarly, the infectivity rate was previously found to be 38% higher among female patients than among male patients in a hospital in Karachi, Pakistan.[17] Excluding neonates, the groups most vulnerable to infections by ESBL-expressing bacteria are 21-40 year-old females and 41-60 year-old males, according to both the cited study and the present study. However, neonates appear to be highly susceptible to infection by these organisms, which has alarming implications for neonatal nurseries. According to results presented here, ESBL-positive strains were most frequent among pus samples followed by wound swab, urine and body fluid samples, but several previous studies have found them to be most frequent in urine samples.[18,19] According to a previous study 76 and 32.6% of ESBL-producing strains isolated from a hospital in Rawalpindi, Pakistan, were resistant to amikacin and gentamycin, respectively.[20] The overall proportions recorded here differ by 18% and 63%, respectively, and the results indicate that the pathogens were most susceptible to the floroquinolone levofloxacin and carbapanem imipenem, which could thus be regarded as drugs of choice for treating deadly infections caused by ESBL-producing strains (Figure 1). Carbapenem resistance has been gradually increasing due to its use as it is the only remaining choice left for treating lethal infection of ESBL-producing strains.[21] For example, the first lethal cases of infection by carbapenem-resistant *Klebsiella pneumoniae* in Saudi Arab were recently reported.[22] However, high susceptibility rates to combinations of drugs like sulzone (cefoperazone/sulbactam) were observed here and in previous studies in both India (>80%)[23] and Nepal.[24]

Regarding methodology, CDSTs were found to be more sensitive than DDSTs (detecting ESBLs in 73 and 58% of the Gram negative isolates, respectively), in accordance with findings of previous studies in Lahore[25] and India.[13] Moreover, CDSTs with impipenem and EDTA detected metallo β-lactamases in more strains (85-86% of the ESBL-producers in this study and a previous study in China[26]) than DDSTs. Finally, strains K-1 and K-2 exhibited 100% similarity with NCBI reference strain (NR_074913.1), designated *K. pneumonia* MGH78578 ATCC700721[27]: a human pathogen harboring a gene encoding the ESBL CTX-M-15. K3 is an *E. coli* strain with 100% similarity to NBRC 102203 in its 16S ribosomal RNA gene sequence.

5. CONCLUSION

In conclusion, high resistance to available antibiotics is drastically reducing options for treating potentially lethal infections. However, pathogenic Gram negative bacteria still have low degrees of resistance to some combinations of drugs like sulzone and tazocin, which can be recommended for treating infections. Infection rates by ESBL- and MBL-expressing bacteria are particularly (and alarmingly) high among neonates, so thorough training of hospital personnel and neonatal nursery staff is essential. In addition, infection rates appear to be higher among females than among males, and the causes of the gender bias require further attention. As frequencies of the pathogens are increasing daily in countries like Pakistan, where health facilities are limited, there is also an urgent need to include additional tests in routine microbiological screening to detect ESBL- and MBL-expressers at early stages. Better antibiogram profiling and monitoring of resistant strains is also highly important to enhance management of the increased risks they pose in Pakistan. It should be noted that this is a pilot-scale, and further investigation of the focal problems with more advanced techniques is required.

ACKNOWLEDGEMENT

The authors are thankful to Department of Microbiology and Molecular Genetics University of the Punjab, Lahore Pakistan. Sampling and basic identification was done in Punjab Institute of Cardiology (PIC), Lahore, Pakistan.

6. REFERENCES

1. Pfaller MA, Jones RN, Marshall SA, Coffman SL, Hollis RJ, Edmond MB, *et al.* Inducible Amp C β-lactamase producing Gram-negative bacilli from blood stream infections: Frequency, antimicrobial susceptibility, and molecular epidemiology in a national surveillance program (SCOPE). Diagn Microbiol Infect Dis., 1997; 28(4): 211-9.
2. Chaudhary M, Payasi A. Molecular characterization and in vitro susceptibilities of β-lactamase producing *Escherichia coli*, *Klebsiella* species, *Acinetobacter baumannii*, *Pseudomonas aeruginosa* and *Staphylococcus aureus* to CSE1034 and other β-lactams. Asian Pac J Trop Med., 2014; 7: S217-S23.

3. Jonathan N. Screening for extended-spectrum beta-lactamase-producing pathogenic enterobacteria in district general hospitals. J Clin Microbiol., 2005; 43(3): 1488-90.
4. Drawz SM, Bonomo RA. Three decades of β-lactamase inhibitors. Clin Microbiol Rev., 2010; 23(1): 160-201.
5. Shahid M, Singh A, Sobia F, Rashid M, Malik A, Shukla I, *et al.* bla (CTXM), bla (TEM), and bla (SHV) in Enterobacteriaceae from north-indian tertiary hospital: high occurrence of combination genes. Asian Pac J Trop Med., 2011; 4(2): 101-5.
6. Adeyankinnu FA, Motayo BO, Akinduti A, Akinbo J, Ogiogwa JI, Aboderin BW, *et al.* A multicenter study of beta-lactamase resistant *Escherichia coli* and *Klebsiella pneumoniae* reveals high level chromosome mediated extended spectrum β lactamase resistance in Ogun State, Nigeria. Interdiscip Perspect Infect Dis., 2014; 2014.
7. Paterson DL, Bonomo RA. Extended-spectrum β-lactamases: a clinical update. Clin Microbiol., Rev., 2005; 18(4): 657-86.
8. Wayne, P.A. Clinical Laboratory Standard Institute. Performance strandards for antimicrobial disk susceptibility tests In: Institute CLS, editor. Approved standard-9th ed., ed: 2013.
9. Livermore DM. Current epidemiology and growing resistance of gram-negative pathogens. Korean J Intern Med., 2012; 27(2): 128-42.
10. Abhilash K, Veeraraghavan B, Abraham O. Epidemiology and outcome of bacteremia caused by extended spectrum beta-lactamase (ESBL)-producing *Escherichia coli* and *Klebsiella spp*. in a tertiary care teaching hospital in south India. J Assoc Physicians India., 2010; 58(Suppl): 13-7.
11. Rupp ME, Fey PD. Extended spectrum β-lactamase (ESBL)-producing Enterobacteriaceae. Drugs., 2003; 63(4): 353-65.
12. Aruna K, Mobashshera T. Prevalence of extended spectrum beta-lactamase production among uropathogens in south Mumbai and its antibiogram pattern., 2012.
13. Dalela G. Prevalence of extended spectrum beta lactamase (ESBL) producers among gram negative bacilli from various clinical isolates in a tertiary care hospital at Jhalawar, Rajasthan, India. J Clin Diagn Res., 2012; 6(2): 182-7.
14. Pai H, Lyu S, Lee JH, Kim J, Kwon Y, Kim J-W, *et al.* Survey of extended-spectrum β-lactamases in clinical isolates of *Escherichia coli* and *Klebsiella pneumoniae*: prevalence of TEM-52 in Korea. J Clin Microbiol., 1999; 37(6): 1758-63.
15. Jabeen K, Znfar A, Hasan R. Frequency and sensitivity pattern of extended spectrum beta lactamase producing isolates in a tertiary care hospital laboratory of Pakistan. J Pak Med Assoc., 2005; 55(10): 436.
16. Quershi M, Asif N, Baig S. Evaluation of extended spectrum beta–lactamase mediated resistance in *Escherichia coli* and *Klebsiella* in urinary tract infection at a tertiary care hospital. Biomed., 2013; 29: 78.
17. Afridi FI, Farooqi BJ, Hussain A. Frequency of extended spectrum beta lactamase producing enterobacteriaceae among urinary pathogen isolates. J Coll Physicians Surg Pak., 2011; 21: 741-4.
18. Riaz S, Faisal M, Hasnain S. Prevalence and comparison of Beta-lactamase producing *Escherichia coli* and *Klebsiella spp.* from clinical and environmental sources in Lahore, Pakistan. Afric J Microb Res., 2012; 6(2): 465-70.
19. Metri BC, Jyothi P, Peerapur B. The prevalence of ESBL among Enterobacteriaceae in a tertiary care hospital of North Karnataka, India. J Clin Diagn Res., 2011; 5(3): 470-5.
20. Mumtaz S, Ahmad M, Aftab I, Akhtar N, ul Hassan M, Hamid A. Extended spectrum beta-lactamases in enteric gram-negative bacilli: related to age and gender. J Ayub Med Coll Abbottabad., 2007; 19(4): 107-11.
21. Kaul S, Brahmadathan KN, Jagannati M, Sudarsanam TD, Pitchamuthu K, Abraham OC, *et al.* One year trends in the gram-negative bacterial antibiotic susceptibility patterns in a medical intensive care unit in South India. Indian J Med Microbiol., 2007; 25(3): 230-5.
22. Balkhy H H E-SA, Al Johani, SM Francis C, Al-Qahtani, Al-Ahdal MN, Altayeb HT, Arabi Y, Alothman A, Sallah M. . The epidemiology of the first described carbapenem-resistant *Klebsiella pneumoniae* outbreak in a teritary care hospital in Saudi Arabia : How far do we go? Euro J Clin Micro Infec Disease., 2012; 31: 1901-9.
23. Poudyal N, Gyawali N, Gurung R, Bhattarai N, Baral R, Khanal B, *et al.* In vitro activity of cefoperazone-sulbactam combination against gram negative bacilli. Nepal Med Coll J., 2012;14(1):5-8.
24. Kumar D, Singh AK, Ali MR, Chander Y. Antimicrobial Susceptibility Profile of Extended Spectrum β-Lactamase (ESBL) Producing *Escherichia coli* from Various Clinical Samples. Infect Dis (Auckl)., 2014; 7: 1.
25. Ejaz H, Zafa A, Mahmood S, Javed MM. Urinary tract infections caused by extended spectrum β-lactamase (ESBL) producing *Escherichia coli* and *Klebsiella pneumoniae*. J Biotechnol., 2013; 10(73): 16661-6.
26. Yong D, Lee K, Yum JH, Shin HB, Rossolini GM, Chong Y. Imipenem-EDTA disk method for differentiation of metallo-β-lactamase-producing clinical isolates of *Pseudomonas* spp. and *Acinetobacter* spp. J Clin Microbiol., 2002; 40(10): 3798-801.
27. Brink A, von Wintersdorff C, van der Donk C, Peeters A, Beisser PS, Stobberingh EE, *et al.* Development and validation of a single-tube multiple-locus variable number tandem repeat analysis for *Klebsiella pneumoniae*. PloS one., 2014; 9: e91209.