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Original Research Article

Phenotypic and Molecular Characterization of Plasmid-Encoded Extended Spectrum Beta-Lactamases Produced by *Escherichia coli* and *Klebsiella* spp from Lahore, Pakistan

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Abstract

Purpose: *To investigate the distribution of plasmid-encoded extended spectrum beta-lacatamases (ESBLs) in Lahore, Pakistan using different phenotypic and molecular methods.*
Methods: *Escherichia coli and Klebsiella spp were obtained over a period of nineteen months (June 2007 to December 2008). Both were tested by the double disk synergy test, combined disk test and Epsiometer-test (E-test) to evaluate their ability to detect ESBLs. The genotypes of ESBLs were analyzed by monoplex polymerase chain reaction (PCR), multiplex PCR, DNA sequencing and isoelectric focusing.*
Results: *662 E. coli and 153 Klebsiella spp were analyzed. Among these isolates, 39.3 % E. coli and 26.1 % Klebsiella spp were positive for extended spectrum beta-lactamases (ESBLs).71.9 % E. coli and 79.6 % Klebsiella spp showed minimum inhibitory concentration (MIC) in the range > 32/0.064 = 500 µl/mL for cetatzidime/cetatzidime + clavulanic acid, while 66.5 % E. coli and 69.1 % Klebsiella spp revealed MIC in the range of > 16/0.016 = 1000 µl/mL for cefotaxime/cefotaxime + clavulanic acid. Antibiotic susceptibility testing revealed that imipemem, meropenem and tazocine were the most effective in the management of such infections. The most frequent genotype of ESBL was OXA (19.2 %) for E. coli and SHV (92.5 %) for Klebsiella spp. The highest genotypic combination found was the combination of TEM/OXA (44.2 %) for E. coli.*
Conclusion: *The resistance of E. coli and Klebsiella spp-producing ESBLs in Pakistan is a serious issue, and TEM, OXA and SHV type ESBL were the most common genotypes. Some isolates produced two or three genotypes at a time. Multiplex PCR of ESBL may help in early detection as well as phenotypic antibiotic therapy of these infections.*

Keywords: *Beta-lactamases, Escherichia coli, Klebsiella spp, Antibiotic susceptibility, Plasmid-encoded, Structural genes, Imipemem, Meropenem, Tazocine*

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INTRODUCTION

Antimicrobial resistance is a very serious issue all over the world. Pathogenic and opportunistic bacteria, which become highly resistant to drugs are causing untreatable infections [1]. Such bacteria are frequently reported as multidrug resistant bacteria (MDR) and some of the MDRs produce ESBL. The incidence and type of ESBL producer strains varies according to different geographical locations [2,3].

Plasmid-mediated extended-spectrum beta-lactamase (ESBL) confers resistance to cephalosporin group, such as ceftazidime, ceftriaxone and others. These enzymes occur in genera of the family *Enterobacteriaceae* for example *Citrobacter, Enterobacter* but predominantly present in *E. coli* and *Klebsiella* species [4]. The successful spread of ESBLs can be attributed to the fact that genes encoding for ESBLs are often located on self-transmissible or mobilizable broad range plasmids [5]. Change in the type of ESBL gene determines the activity of enzyme, for example if organisms producing *TEM* and *SHV* type ESBLs, apparent *in vitro* sensitivity to cefepime and to tazobactum is changed [6]. These drugs show an inoculum effect, with diminished susceptibility as the size of the inoculum was increased from 10^5 to 10^7 organisms. Strains having *CTX-M*–type and *OXA*-type ESBL are resistant to cefepime despite the use of standard inoculums [7].

Beta-lactams are important antibiotics in this part of world. Only few studies analyzing the extent of beta-lactam resistance based on their phenotypic features have been conducted. Very little is known about the molecular genotyping of extended spectrum beta-lactamases. The main objective of this work, therefore, was to determine beta-lactam resistant phenotypes and genotypes of *E. coli* and *K. pneumoniae* isolates from Lahore, Pakistan.

EXPERIMENTAL

Bacterial isolation and identification

A total of 1018 consecutive and non-duplicate lactose fermenting *Enterobacteriaceae* members were collected from Citi lab and Research Center during June 2007 to December 2008. All clinical specimens were processed according to standard operating procedures [8]. Lactose fermenting Gram-negative bacteria were characterized by colony morphology and biochemical tests. Confirmation of identification was performed by using API 20E (BioMerieux). Data were reported according to guidelines of Heath protection agency [9].

Phenotypic detections tests

Phenotypic test includes, double disk synergism augmentin (amoxicillin-clavulanate) was used to read the synergism with ceftriaxone (30 µg), ceftazidime (30 µg), aztreonam (30 µg), cefotaxime (30 µg). Combination disc (CD) test was performed using ceftazidime/ceftazidime+ clavulanic acid and cefotaxime/cefotaxime+ clavulanic acid. Combination disk test was considered positive when there was 5 mm increase in zone of inhibition as compared to non-combination disc. E-test was performed as final phenotypic confirmatory test. Detection of ESBL was determined based on ratio between MIC of ceftazidime and cefotaxime alone or in combination with clavulanic acid using E-test strips.

Confirmation by using ribotyping

Isolates were also confirmed using 16SrRNA sequences. The PCR products were subsequently sequenced by Macrogen Bioengineering Co., Ltd. The nucleotide sequences were analyzed using BLAST software, available from the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov). Phylogenetic analysis was performed using software seaview. Confirmed isolates were stored in trypticase soy broth containing 20 % glycerol at -80 °C until use.

Antibiotics susceptibility testing

The antibiotic susceptibility was determined by disk diffusion on Mueller-Hinton agar (Bio-Rad, Marnes-la-Coquette, France). Multiple antibiotic resistances (MAR) of ESBL producing isolates according to gender and age groups of patients were calculated. The panel used in this study consists of 21 antibiotics that belong to three major groups of antibiotics according to mechanism of action. Such as antibiotic disks containing amikacin 30 µg, ampicillin 10 µg , amoxicillin clavulanic acid (20 µg/10 µg) 30 µg, carbencilin 100 µg, cephredine 30 µg, cefaperazone 75 µg, ciprofloxacin 5 µg, gentamicin 10 µg, nitrofurontoin 300 µg, imepenem 10 µg, meropenem 10 µg, norfloxacin 10 µg, cotrimoxazole 25 µg, ceftizoxime 30 µg, cefotaxime 30 µg, cefoxitin 30 µg, cetazidime 30 µg, ceftriaxone 30 µg, cefixime 5 µg, aztreonam 10 µg, and pipracilin-tazobactam 100/10 µg were tested.

Genotyping using PCR and sequence data analysis

Colony PCR was performed following Jemima [10]. To differentiate bla_{SHV}, bla_{TEM}, bla_{OXA}, the published primers were used [11]. Initially, singleplex PCR was optimized and then multiplex PCR assay of three-primer set was optimized (*OXA, TEM* and *SHV* genes) and performed. ATCC 25922 *E. coli* (*SHV*-negative), ATCC 700603 *K. pneumoniae* (*SHV*-positive), ATCC

35218 *E. coli* (*TEM*-positive) and a strain of *E. coli* (*OXA*-positive) were used as control strains for PCR. Multiplex PCR was developed using primer set (*OXA*, *TEM* and *SHV*) in single reaction with maximum 25 µL volume of PCR mixture. The PCR products were purified using Qiagen kit Agarose Gel DNA Purification Kit Version 2.0. Subsequently, the amplicons were sequenced by Macrogen Bioengineering Co., Ltd. The nucleotide and deduced protein sequences were compared to those available in GenBank.

Isolectric focusing (IEF)

Beta-lactamase was isolated using Massidda protocol [12]. Isoelectric focusing was performed using 2D gel electrophoresis (BioRad) following manufacturer instructions. Spot editing, scanning, quantification and evaluation of isoelectric points (pI) was calculated by ImageMaster 2D Elite v3.1 software (Amersham Biosciences).

RESULTS

Bacterial isolates

Out of 1018 *Enterobacteriaceae* members, 662 *E. coli*, 153 *Klebsiella* spp, 122 *Enterobacter* and 81 *Citrobacter* were isolated. Clinical samples positive for ESBL were as: urine (53 %), blood (7.3 %), wound swabs (20.6 %), foley catheter tip (10.3 %) and others (8.6 %). Total 300 bacterial strains were positive for beta-lactamases, 39.2 % *E. coli* and 26.10 % *Klebsiella* species were found to express ESBLs. Regarding gender classification in the case of beta-lactamase producing *E. coli*, females (60 %) had a higher incidence as compared to males (40 %). Similarly, in case of *Klebsiella*, more females (55 %) than males (45 %) were affected. Comparison of hospitalized and community acquired infections revealed that in both cases > 55 % *E. coli* caused infection following *Klebsiella* > 45 %. Here data of *E. coli* and *Klebsiella* was arranged according to source of specimens. However, in the case of pus swabs, males were more infected by *Klebsiella* (71 %) than *E. coli* (69 %). In blood samples, *E. coli* showed higher infection rates (60 %) as compared to *Klebsiella* (40 %) for male patients. Frequency of infection of *E. coli* from Foley tip was less in males (29 %) than females (71 %). In *Klebsiella*, foley tip infections revealed more for females (62 %) than males (38 %). Other specimens had also high infection rate in females as compared to males.

Phenotypic characteristics

The isolates were further selected based on phenotypic detection test from hospitalized and community patients following Clinical and Laboratory Standard Institute (CLSI) guidelines [8]. Ceftazidime had high rate of synergism (93.4, 95 %) followed by cefotaxime (88.9, 88.7 %) aztreonam (86.2, 83.5 %) and ceftriaxone (84.5, 83.1 %). In *Klebsiella* spp highest synergism was found with ceftazidime (88.4, 89.1 %) followed by aztreonam (88.6, 86.2 %), cefotaxime (86.7, 89.6 %) and ceftriaxone (84.7, 88.5 %). Cephalosporin antibiotic ceftazidime, cefotaxime alone and combination disc were selected for further confirmation. *E. coli* was 99.2 % resistant to ceftazidime but was found 98 % sensitive, 1.34 % resistant and 0.74 % intermediate to ceftazidime+clavulanic acid (disc). *E. coli* was more resistant to cefotaxime (99.5 %) and less resistant to cefotaxime+ clavulanic acid (disc) (3.84 %).

Cefotaxime+clavulanic acid (disc) increased *E. coli* sensitivity to 96.12 %. *Klebsiella* spp was 99.5 % and 99.4 % resistance to ceftazidime and cefotaxime, respectively. Combination discs of ceftazidime+clavulanic acid and cefotaxime+clavulanic acid altered resistance against ESBL to 1.36 and 2.12 %, respectively. It was observed that 2.8 % *E. coli* and 1.4 % *Klebsiella* spp had non-determinable > 32/> 4 (ND), while a majority of ESBL *E. coli* (71.9 %) and *Klebsiella* (79.6 %) groups fell in the range of MIC > 32/0.064 = 500 µl/mL for ceftazidime/ ceftazidime+clavulanic. For cefotaxime/ cefotaxime+clavulanic (CT/CTL), maximum ESBL *E.coli* (66.5 %) and *Klebsiella* (69.1 %) isolates were in the MIC range of > 16/0.016 = 1000 µl/mL (Table 1).

Susceptibility of isolates

This panel is designed according to the CLSI guidelines [8]. This antibiotic panel is generally used in our hospitals and diagnostics labs. Out of 260 *E. coli* isolates > 97 % were resistant to ampicillin, carbencilin, cephredine, cefuroxime, ceftriaxone and ceftazidime. *E. coli* isolates were > 80 % and > 60 % resistant to cefoperazone and ceftizoxime respectively. *E. coli* isolates were 50 % resistant to amoxycillin/clavulanic acid.

Out of 40 *Klebsiella* isolates, 100 % were resistant to carbencilin, cephredine, cefuroxime and ceftriaxone. *Klebsiella* isolates > 90 %

Table 1: E-test with cetazidime/cetazidime+clavulanic acid (TZ/TZL) and cefotaxime/cefotaxime+clavulanic acid (CT/CTL) MIC ratio

Cetazidime/ cetazidime+clavulanic acid (TZ/TZL) MIC(µl/ml)						
MIC Ratio	**>32/>4 (ND)**	**>32/0.064=500**	**>32/0.125=256**	**24/0.19=126**	**16/0.38=42.1**	**4/0.25=16**
E. coli	2.8	71.9	14.6	7.4	1.3	2.0
Klebsiella spp	1.4	79.6	9.3	4.5	5.2	0.0
Cefotaxime/ Cefotaxime +clavulanic acid (CT/CTL) MIC(µl/ml)						
MIC Ratio	**>16/>1 (ND)**	**>16/0.016=1000**	**12/0.023=521**	**3/0.023=130**	**8/0.125=64**	**4/0.094=42.5**
E. coli	5.1	66.5	17.6	4.5	2.8	3.5
Klebsiella spp	1.5	69.1	14.8	8.1	5.3	1.2

resistant to ceftizoxime whereas > 80 % were resistant to cefoperazone. > 60 % resistance and 20 % intermediate sensitivity to amoxycillin/ clavulanic acid were seen in *Klebsiella* isolates. *E. coli* and *Klebsiella* isolates showed highest susceptibility to imepenem followed by meropenem and tazocine. There was very rare resistance found to meropenum and tazocine (< 2 %), but no resistance was found to imepenem in both types of isolates. Hence, the most effective antibiotics that can be used as a therapy of choice in both types of isolates were imepenem followed by meropenem and tazocine. Protein inhibiting drugs, gentamicin and amikacin were also used. *Klebsiella* isolates > 80 % and *E. coli* isolates > 60 % were resistant to gentamicin. On the other hand amikacin responded well and showed highest activity for *E. coli* and *Klebsiella* spp. In nucleic acid inhibiting drugs *E. coli* showed > 80 % resistance to ciprofloxacin and trimethoprim/sulfamethoxazole, while > 90 % resistance to nitrofurontoin and norfloxacin. *Klebsiella* isolates had > 70 % resistance to ciprofloxacin but > 90 % resistance to trimethoprim/sulfamethoxazole, nitrofurontoin, and norfloxacin.

Phylogenetic data

Phylogenetic relation was developed using neighbor joining method (NJ method). The tree length (sum of branch lengths) was 0.01111 in the case of *E. coli* (Figure 1) and 0.02171 in *Klebsiella* spp (Figure 2); branch lengths are proportional to the number of substitutions per site. Comparison of two bacterial groups was done using Parsimony analysis bootstrap 100 replicates along with control strains. K-TJW and K-ANT used as control *Klebsiella* spp while K12 that is used as control *E. coli*. Sequences were submitted to GenBank nucleotide database under accession numbers GU594294-GU594311.

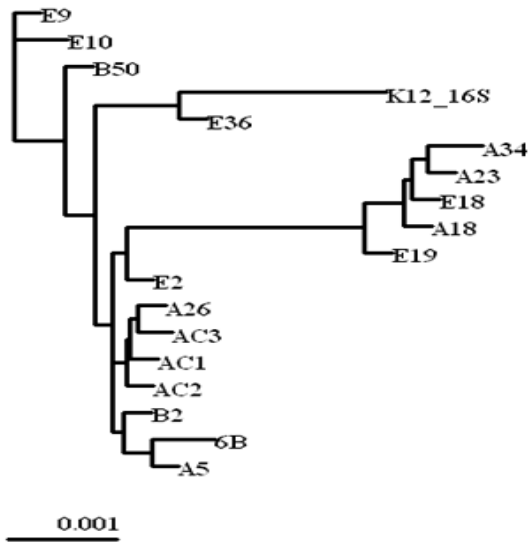

Figure 1: Phylogenetic tree from 14 aligned sequences of 1478 sites, based on the NJ method. The tree is rooted by outgroup B16

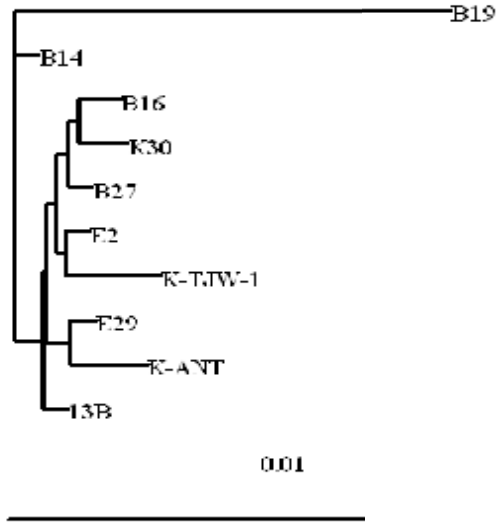

Figure 2: Phylogenetic tree from 10 aligned *Klebsiella* sequences of 1608 sites, based on the NJ. The tree is rooted by outgroup B19

Molecular phylogeny of the bla_{SHV}, bla_{TEM} and bla_{OXA} beta-lactamase genes

Distributions of *TEM, OXA* and *SHV* in bacterial isolates were observed (Table 2). Of the 260 clinical isolates of *E. coli* suspected to be ESBL producers 13.2 %, 19.2 % and 7.6 % demonstrated *TEM, OXA* or *SHV*-specific PCR products respectively. Combination of ESBL showed 44.2 %, 3.8 %, 1.5 %, 1.9 % of *TEM/OXA, TEM/SHV, OXA/SHV, TEM/SHV/ OXA*-specific products respectively. Sequence determination of PCR products obtained from *E. coli* and *Klebsiella* spp confirmed the identity of the genes (Figure 3). Of the 40 clinical isolates of *Klebsiella* spp 92.5 % demonstrated *SHV*-specific products, 5 % *TEM/OXA* and 2.5 % *TEM/SHV* (Table 2). The nucleotide sequences reported in this paper have been submitted to the GenBank nucleotide database under accession numbers HM063036-HM063040 = *SHV*, HM063041-HM063050 = *TEM*, HM063051-HM063064 = *OXA*.

IEF data

Amino acids calculation showed that in *SHV* enzyme most frequent amino acid was Arginine following glutamine with average pI 8.11, where as in *TEM* beta-lactamase most frequent amino acid was Glutamine following Arginine with 4.47 pI. In *OXA* beta-lactamase most frequent amino acid was lysine, glutamine, aspartic acid following other amino acids. Average pI of *OXA* beta-lactamase calculated was 9.03.

Table 2: Distribution of *TEM*, *SHV* and *OXA* in bacterial isolates (n = 300: ***Note:*** Data in parenthesis indicate percent

Bacterial isolate	No	*TEM*	*OXA*	*SHV*	*TEM* / *OXA*	*TEM* /*SHV*	*SHV*/ *OXA*	*TEM*/*SHV*/*OXA*	Others
E. coli	260	36(13.8)	50(19.2)	20(7.6)	115(44.2)	10(3.8)	4(1.5)	5(1.9)	0(0)
Klebsiella spp	40	0(0)	0(0)	37(92.5)	2(5)	1(2.5)	0(0)	0(0)	0(0)

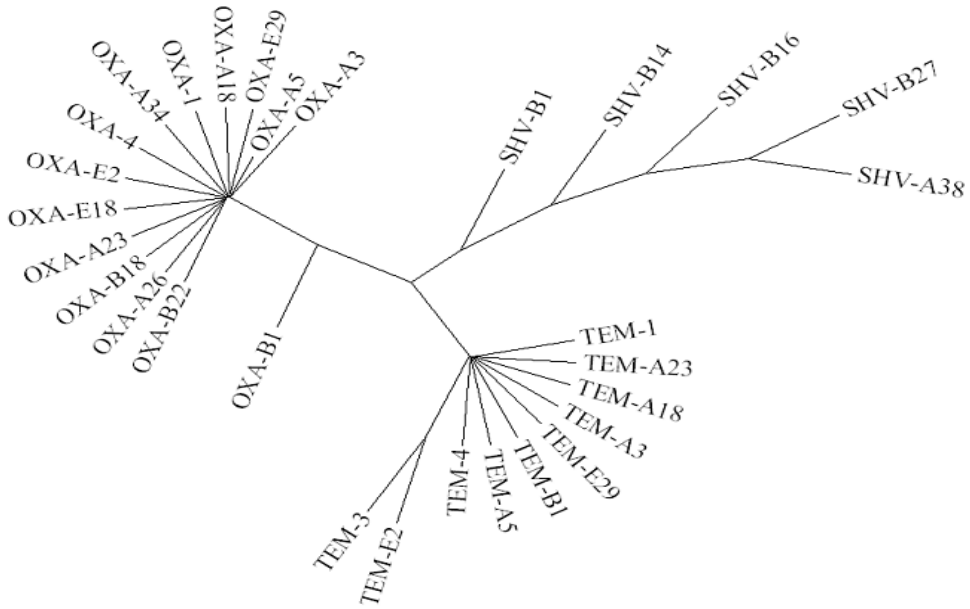

Figure 3: Parsimony analysis of *OXA*, *TEM* and *SHV* sequences. Analysis of *OXA*, *TEM* and *SHV* aligned sequences with bootstrap100 replicates, randomize sequence order 5

DISCUSSION

This research work was conducted because there was little available information regarding antimicrobial susceptibility and molecular characterization of ESBL genotypes of *E. coli* and *Klebsiell*a spp in Pakistan. These members of *Enterobacteriaceae* are well known ESBLs producers and are responsible for nosocomial and community acquired infections [13]. Depending upon the rate of infecting isolates, *E. coli* and *Klebsiella* were considered as important research work. There are many reasons for the spread of infections, and these include misuse of

antibiotics, animals and hospitals cross infection [4]. The prevalence of ESBLs can be different based on geographical areas. In Europe, ESBLs positivity was 1.3 % of *Escherichia coli* and 18.4 % for *Klebsiella pneumonia* [14]. Several other reports are available for other areas like Latin America, Asia and also North America [15]. In 2003 about 30 % ESBL producing *E. coli* were found in Aga Khan University research [12]. ESBLs producers were more commonly isolated from urinary tract infections (UTI) both in hospital and community-acquired infections [16]. In Iran, UTI causing ESBL producers were in high frequency [17]. Similarly in Japan the increased frequency of UTI caused by ESBL producers have been reported [18].

When data were analyzed according to gender and age groups it depicts that females were more infected. This complexity is because of lack of medical facilities in rural areas. High frequency of infected patients were observed either from young or old age group, this might be because of low immunity [13]. Data revealed that in Pakistan multidrug resistance was more common in the age group of 50+ in both males and females [19]. Resistance appeared to be correlated with the old age as observed in another report [20]. In susceptibility profile imepemem, meropenem and tazocine were effective against ESBL isolates. Meropenem was considered as best option for therapy against gram-negative bacilli especially for cephalosporin resistant gram-negative bacilli [21]. The good activity of imepenem, meropenem and tazocin was also previously known [22]. Another work that supports this was conducted at King Fahd Hospital, Saudi Arabia. However, variability with other drugs such as amoxiclar augmentin, cefoperazone, ceftizoxime, gentamycin and ciprofloxacin was also observed [23].

Our data also suggest that majority of cases are positive for ESBLs as earlier reported [24]. Overall, phenotypic detection of clinical ESBL was 39 % in *E. coli* and 26 % in *Klebsiella* spp. In previous report, 53 % *K. pneumoniae* and 44 % *E. coli* were identified as suspected ESBL producer [25]. Comparative study of different phenotypic techniques revealed that double disc synergism (DDS) test was the most economical method of detection.

In current study multiplex PCR was developed for *TEM, OXA* and *SHV* genes. This method of diagnosing ESBL is a one-step procedure, which is very significant in diagnostics. There are a number of studies that showed the significance of using multiplex PCR in ESBL. Multiplex PCR for *SHV* and *CTXM* was developed in another study [26]. Multiplex PCR for detection of plasmid mediated quinolone resistance ESBL has also been developed [27]. The change in the antibiotic phenotype pattern in different ESBL, clearly differentiated the resistance mechanism of beta-alactamase producing isolates. This was solved by PCR, which accurately distinguished between different types of ESBL genes [28]. On sequence similarity analysis, it was found that tested isolates had 96 to 100 % similarity to sequences of ESBL isolates from China. *Klebsiella pneumoniae* strain B27 (HM063040) *SHV* beta-lactamase producer had 98 % similar sequence to clinical *Klebsiella pneumoniae* strain HS94 beta-lactamase [28]. *Escherichia coli* strain B1 (HM063048) *TEM*-beta-lactamase was 100 % similar to *Escherichia coli* strain SA-Y1-39 [29].

CONCLUSION

ESBLs are an increasing problem in healthcare facilities, especially in rural areas. Imepemem, meropenem and tazocine remain good choices for treating infections. Addition of a combination disc in routine susceptibility testing gives satisfactory results for ESBLs in less time. *TEM, OXA* and *SHV* type ESBLs were the most common genotype in this region.

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