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Analysis of Extended Spectrum Beta Lactamase Frequency in Klebsiella spp Isolates

Klebsiella spp İzolatlarında Genişletilmiş Spektrumlu Beta Laktamaz Sıklığının Analizi

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ABSTRACT

The issue of increasing resistance to antibiotics in recent years has become an important problem all over the world. Our aim is to determine the antimicrobial resistance profile and Extended Spectrum Beta-Lactamase (ESBL) rates in *Klebsiella spp* isolates to prevent the gradual increase in multi-resistant isolates as a result of unconscious antibiotic use thereby contributing to the faster effective treatment of infections. A total of 100 Klebsiella spp were isolated and identified from various clinical specimens. Antibiotic susceptibility tests were performed using the Kirby-Bauer method. The presence of extended-spectrum beta-lactamases (ESBL) was detected using the Double Disc Synergy Test (DDST) and E-test methods. The rates of ESBL-producing strains were 46.1% in 6 *K. oxytoca* and 56.3% in 49 *K. pneumoniae*. These strains were found to be 38% in 38 adult patients and 17% in 17 pediatric patients, and this difference was statistically significant ($p < 0.05$). The ESBL rate was 31% in 31 male patients and 24% in 24 female patients, and this difference was not statistically significant ($p > 0.05$). This rate was found to be high in patients hospitalized in the pediatric service and intensive care unit. 67 out of 100 strains were found to be suspicious for ESBL by Disk Diffusion Test (DDT). DDST and E-tests were applied as confirmatory tests. The sensitivity of the DDST and E tests was 100%. Screening for ESBL in *Klebsiella spp* and other members of *Enterobacteriaceae* isolates is necessary to reduce further selection and spread of these increasingly broad-spectrum antimicrobial-resistant enteric pathogens.

Keywords: Klebsiella spp, Extended Spectrum Beta-lactamase (ESBL), Double Disc Synergy Test, E-Test

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ÖZET

Son yıllarda antibiyotik direncinin artması konusu tüm dünyada önemli bir sorun haline gelmiştir. Amacımız, *Klebsiella spp* izolatlarında antimikrobiyal direnç profilini ve Genişletilmiş Spektrumlu Beta- Laktamaz (ESBL) oranlarını belirleyerek bilinçsiz antibiyotik kullanımı sonucunda multi-dirençli izolatlarda kademeli artışı önlemek ve böylece enfeksiyonların daha hızlı etkin tedavisine katkıda bulunmaktır. Çeşitli klinik örneklerden toplam 100 Klebsiella türü izole edilmiş ve tanımlanmıştır. Antibiyotik duyarlılık testleri Kirby-Bauer yöntemi kullanılarak yapıldı. Genişletilmiş spektrumlu beta-laktamazların (GSBL) varlığı, Çift Disk Sinerji (DDST) ve E-test yöntemleri kullanılarak tespit edildi. GSBL üreten suşların oranları 6 *K. oxytoca* (%46,1) ve 49 *K. pneumoniae* (%56,3) idi. Bu suşlar 38 erişkin hastada %38, 17 pediatrik hastada %17 olarak bulundu. Sonuçlarımız istatistiksel olarak anlamlıydı ($p<0,05$). GSBL oranı 31 erkek hastada %31, 24 kadın hastada %24 idi ve cinsiyete göre bu fark anlamlı bulunmamıştır ($p>0,05$). Çocuk servisi ve yoğun bakım ünitesinde yatan hastalarda bu oran yüksek bulundu. Disk Difüzyon Testi (DDT) ile 100 suştan 67'si ESBL açısından şüpheli bulundu. Doğrulayıcı testler olarak DDST ve E-testleri uygulandı. DDST ve E testlerinin duyarlılığı %100 idi. *Klebsiella spp* ve *Enterobacteriaceae* izolatlarının diğer üyelerinde ESBL taraması, giderek artan geniş spektrumlu antimikrobiyal dirençli enterik patojenlerin daha fazla seçimini ve yayılmasını azaltmak için gereklidir.

Anahtar Kelimeler: *Klebsiella spp*, Genişletilmiş Spektrumlu Beta-laktamaz (ESBL), Çift Disk Sinerji Testi, E-Testi

1. Introduction

The issue of increasing resistance to antibiotics in recent years has become an important problem all over the world [1]. Beta-lactams have become the most prescribed antibiotics today due to their superior spectrum of action, high and selective toxicity to microorganisms, applicability in almost all age groups, relatively low incidence of side effects compared to other groups, and superior distribution to all body fluids. The numerical weight of these drugs among all licensed antibiotics is close to 70%. However, the resistance of bacteria to these antibiotics has increased rapidly over the years due to unnecessary-inappropriate-intensive use and insufficient application of infection control methods in hospitals [2]. The most important mechanism in the development of resistance to beta-lactam antibiotics in Gram-negative bacteria is beta-lactamase production [1]. Beta-lactamases; are enzymes that destroy the antibacterial effect of beta-lactam antibiotics by breaking the amide bonds in the beta- lactam ring and can be synthesized by many bacterial species, especially Enterobacteriaceae members [2]. More than 500 beta-lactamase enzymes have been identified to date. The most important beta-lactamase enzyme groups are plasmid-encoded cephalosporins, Metallo-beta- lactamases and ESBLs. About 200 beta-lactamases can be transferred between bacteria due to their plasmid properties [3,4].

Infections caused by ESBL-producing strains are frequently seen in patients who are hospitalized for a long time, undergo major surgery, have arterial and urinary catheters, and especially in intensive care units. However, in recent years, it has been observed that the incidence of community-acquired infections has increased [5]. ESBL enzymes have become an important resistance mechanism in today's hospitals because of their easy spread through plasmids, their ability to cause epidemics, and the emergence of serious clinical problems such as treatment failure and increased mortality in infections caused by these strains. Therefore, good identification of these enzymes in the laboratory is important in terms of directing the treatment. Our study aimed to prevent the gradual increase of multi- resistant strains, treat infections more rapidly and prevent unconscious antibiotic use.

2. Material and Method

This study was carried out with the approval of Harran University Clinical Research Ethics Committee, dated 14.12.2012, and numbered 05, in the Laboratory of Microbiology Department of Harran University Faculty of Medicine. A total of 100 *Klebsiella spp* strains were isolated from various clinical samples. These samples were sent to the Microbiology Laboratory of Harran University Research and Application Hospital between January 2014 and June 2015. *Klebsiella spp* strains were evaluated for extended spectrum beta-lactamase by determining their antibiotic susceptibility. Repeated samples from the same patient were excluded from the study. The tested antimicrobial discs were: amoxcillin clavulanate (AMC 10/20 µg), imipenem (IMP) 10µg, piperacillin-tazobactam (TZP) 10/100µg, cefepime (FEP) 30µg, Amikacin (AK) 30µg, ciprofloxacin (CIP) 5µg, gentamicin (CN) 120µg, cefotaxime (CTX) 30µg, ceftazidime (CAZ) 30µg, ceftriaxone (CRO) 30µg, cefoxitin (FOX) 30µg, and sulfamethoxazole-trimethoprim (SXT) 10µg.

Statistical Analysis

All statistical analyzes were performed using the "Windows Statistical Package for Social Sciences (SPSS)" (version 15.0; SPSS, Chicago, IL) program. For comparisons, the chi-square test was applied. Statistically, those with a p-value less than 0.05 were considered significant.

3. Results

In this study, the Kirby-Bauer method was performed. The zone of inhibition was measured and interpreted to the Clinical and Laboratory Standards Institute (CLSI) criteria. DDST (Figure 1) and E test methods (Figure 2) were used to detect the presence of ESBL. Some epidemiological information such as; age, gender, sample type, and risk factors for *Klebsiella* infection were recorded.

Epidemiological Information

Sent to the microbiology laboratory; Of a total of 100 strains, 87 identified as *K. pneumoinae* and 13 as *K. oxytoca*, were included with biochemical tests and API 20 E test. ESBL production was detected in 49 (56.3%) of *K. pneumoinae* strains and 6 (46.1%) of *K. oxytoca* strains. The distribution of the presence of ESBL according to the isolated *Klebsiella species* is shown in Table 1.

Table 1: Distribution of ESBL presence by isolated Klebsiella species

Klebsiella spp.	**ESBL Positive**		**ESBL Negative**	
	n	**%**	**n**	**%**
***K. pneumoniae* (87)**	49	56.3	38	43.7
***K. oxytoca* (13)**	6	46.1	7	53.9

Urine (38.1%), 18 blood (32.7%), 5 wounds (9%), 4 drain tip (7.2%), 4 Tracheal aspirate (7.2%), 2 sputum (%3.6), and 1 throat (1.8%) were detected. The distribution of isolates according to clinical samples is shown in Table 2.

Table 2: Distribution of ESBL presence according to clinical samples from which strains were isolated

Clinical specimens (n)	ESBL Positive		ESBL Negative		Total
	n	%	n	%	(n)
Urine	21	38.1	16	35.5	37
Blood	18	32.7	10	22.2	28
Wound	5	9	5	11.1	10
Tracheal aspirate	4	7.2	4	8.8	8
Surgical drain	4	7.2	3	6.6	7
Sputum	2	3.6	3	6.6	5
CSF	0	0	2	4.4	2
Throat	1	1.8	1	2.2	2
Ear	0	0	1	2.2	1
Total	55		45		100

Of the 100 patients included in the study, 54 male and 46 female patients were isolated from their culture. ESBL positivity was detected in 31 (57.4%) of the strains isolated from males and 24 (52.1%)of the strains isolated from females. Although the frequency of ESBL was higher in males than females, the difference was not statistically significant (p> 0.05) Fig1.

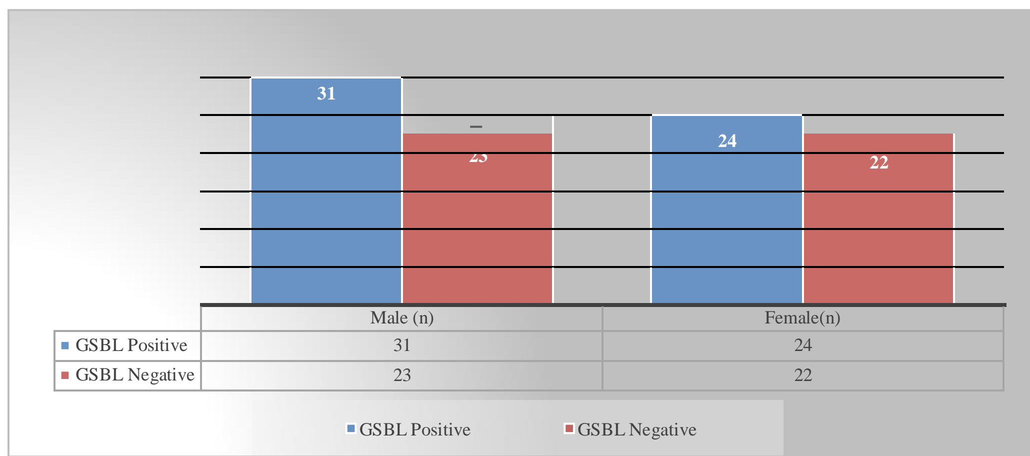

Figure 1: Distribution of ESBL presence by gender

Of the patients, 67 were adults and 33 were children. The rate of ESBL-producing strains in adult patients was 38%, and the rate of ESBL-producing strains in pediatric patients was 17%. The frequency of ESBL was higher in adults than in children, and it was statistically significant (p <0.05) Fig2.

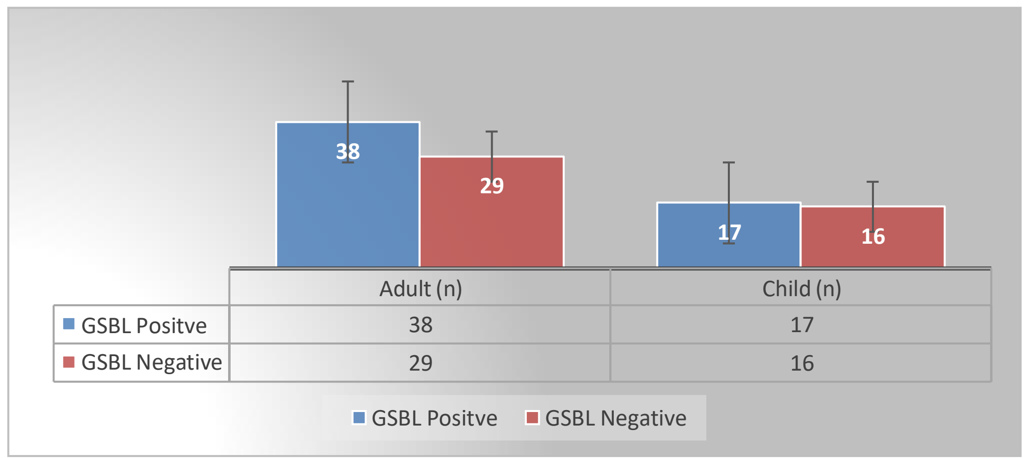

Figure 2: Distribution of ESBL presence by child and adult age groups

Of the 100 strains, 48 were isolated from the intensive care unit (ICU) and 52 from various clinical services. 32 (66.6%) of the strains isolated from the intensive care unit and 23 (44.2%) of the strains isolated from the clinical services were found to be ESBL-positive. Of the strains included in the study, 15 Pediatric ICUs, 13 Internal Medicine ICUs, 12 Surgical ICUs, 11 Surgical Services, 8 Pediatric Services, 8 Urology Services, 8 Infection Services, 8 Cardiology ICUs, 7 Internal Medicine Services, 7 Chest Services, and 3 Cardiology Services. The ratios of ESBL positive strains were calculated according to clinics services. They were as follows: Pediatric ICU 16.3%, Internal Medicine ICU 16.3%, Surgical ICU 14.5%, Cardiology ICU 10.9%, Urology Serv 9.0%, Surgical Serv 7.2%, Infection Serv 7.2%, Internal Medicine Serv 7.2%, Pediatric Serv 5.4%, Chest Serv 3.6%, Cardiology Serv was found to be 1.8%. The highest frequency of ESBL was found in the Pediatric and Internal Medicine intensive care units. Although the frequency of ESBL was higher in the ICU than in the wards, the difference was statistically significant ($p<0.05$) Fig 3.

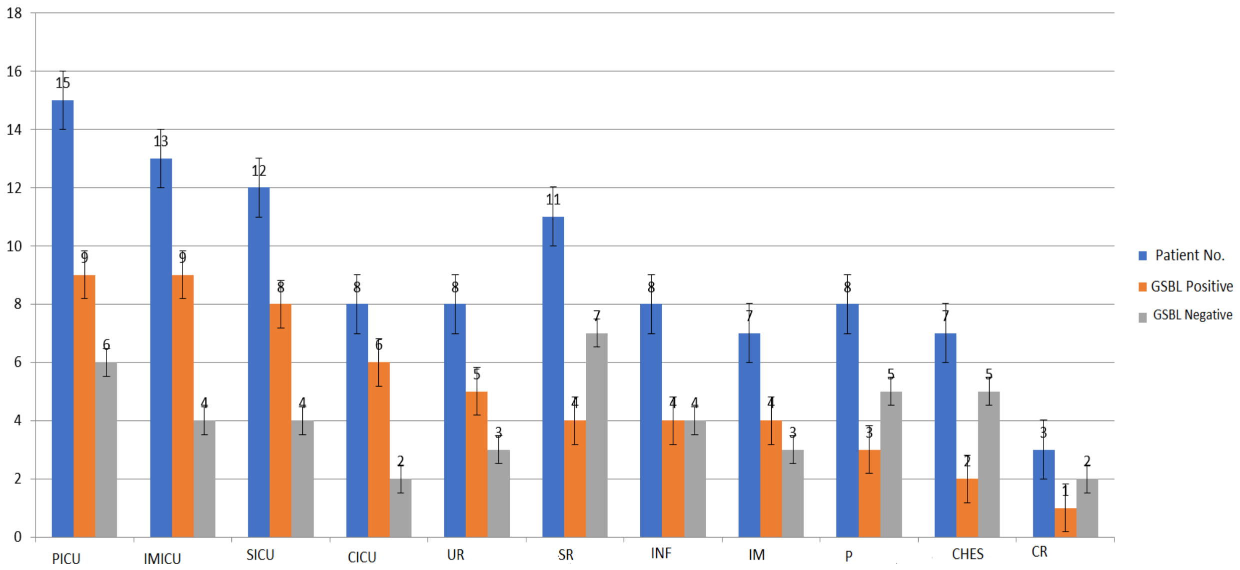

Figure 3: Distribution of ESBL presence by ICU and clinics

Antibiotic Susceptibility Test Results

The resistance of strains to 11 different antibiotics with DDT was investigated. The results obtained according to the CLSI criteria were interpreted. According to the results of antibiotic susceptibility tests, Klebsiella spp. 16% of the strains were sensitive to all antibiotics; highest resistance; It is seen against SXT with a rate of 80%, followed by 68% CRO, 65% CTX, 62% ATM, 52% CAZ, 50% CIP, 35% AMC, 30% CN, 14% PRP, 13% AK and 1%. IPM resistance followed. *Klebsiella spp.* the most sensitive antibiotic of the strains was IPM with 99%. The susceptibility and resistance rates of the strains to antibiotics are shown in Table 3.

Table 3: *Klebsiella spp*. antibiotic susceptibility results of strains

Antibiotic	SXT	CRO	CTX	ATM	CAZ	CIP	AMC	CN	TZP	AK	İMP
Resistant	80	68	65	62	52	50	35	30	14	13	1
Sensitive	20	32	35	38	48	50	65	70	86	87	99

Results of ESBL Screening and Validation Tests

According to CLSI recommendations, 100 Klebsiella spp. ESBL production was detected in 67 (67%) with DDT, 55 (55%) with DDST, and 55 (55%) with E-test (Table 4).

Table 4: Comparison of DDT and Confirmation tests

	ESBL Positive	ESBL Negative	Total
DDT	67	33	100
DDST	55	45	100
E - TEST	55	45	100

In our study; Samples found as "ESBL suspicious" with CRO, CTX, CAZ, and ATM by disk diffusion test were accepted as DDST reference test and compared with E-test. When DDT and four antibiotics were used together, ESBL was positive in 67 strains, while ESBL was positive in 55 strains with confirmatory tests DDST and in 55 strains with E-test, suggesting that DDT might cause false positivity. When the resistance status of ESBL positive and ESBL negative strains to antibiotics according to DDST was investigated; While ESBL positive strains were most resistant to CRO (87%), CTX (85%), and ATM (83%), no resistance to IPM was observed. In addition, ESBL-negative strains were most resistant to SXT (80%), CRO (46%), and CAZ (41%), while they were least resistant to IPM (3%) Fig 4.

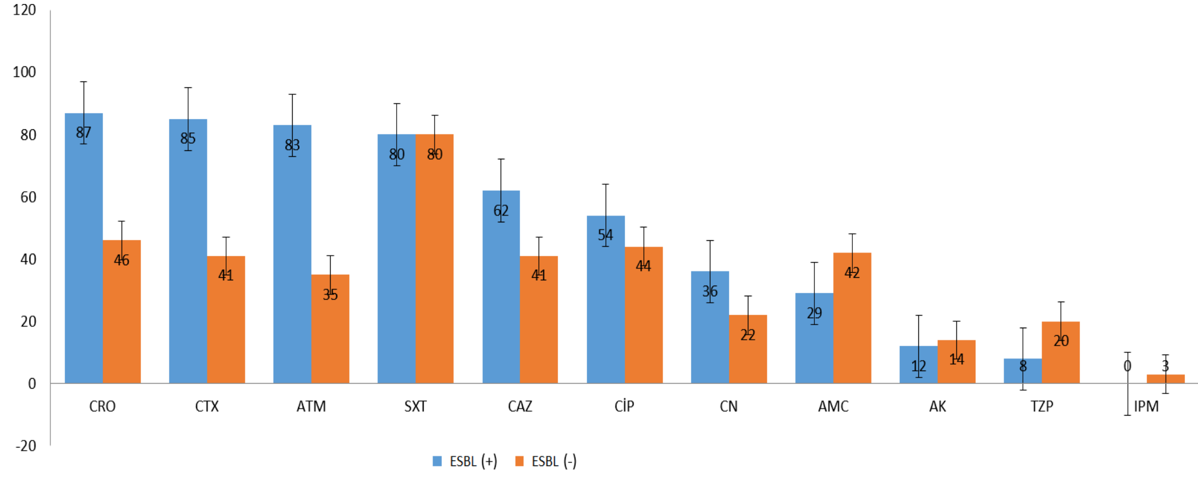

Figure 4: Antibiotic resistance rates of ESBL positive and negative samples by double disc synergy test

The mean hospital stay of the patients included in the study was 41.97±62.41 days. While the mean hospitalization period of ESBL-positive patients was 43.89±59.46 days, the mean hospitalization period of ESBL-negative patients was 20.79±34.78 days. When ESBL positive and negative isolates were compared, the difference between the length of hospital stay, antibiotic use, hospitalization in the ICU, presence of a central venous catheter and/or urinary catheter, presence of severe underlying disease (malignancy, sepsis, and others), which are considered risk factors for ESBL. The difference was significant ($p<0.05$).

4. Discussion and Conclusion

Nosocomial infections cause an increase in morbidity, mortality rates as well as in hospital costs. They constitute a serious public health problem [6]. In one study, in a 24-hour point, prevalence study conducted in 1150 centers, infection was proven in 54% of the patients in the intensive care unit; 70% of all patients were receiving at least 1 antibiotic (prophylactic or therapeutic). Hospital mortality has been reported to be 30% in patients with proven infection [7]. In our study, the most isolated samples of *Klebsiella spp* were urine.

According to studies conducted in various centers in Turkey, the rate of nosocomial infections was found to be 9% to 11.1%, while gram-negative bacteria causing nosocomial infections were reported to be 36.8% [8,9]. The prevalence of ESBL-producing Enterobacteriaceae varies widely between hospitals. Less than 1% to more than 70% of ESBL producers have been reported worldwide [10]. There are significant geographical differences in the occurrence of ESBLs. In a large study, ESBL producing Klebsiella spp. The rate of isolates was found to be in a high range from a low value suchas 4.2%, Canada 4.9%, Spain 20.8%, Taiwan 28.4%, Turkey 78.6%, Algeria 20%, China 51%, and Germany 1.5% [11]. The reason for these differences is the high socioeconomic and cultural variability in different regions, the use of different diagnostic methods in different patient groups, and the use of different and common antibiotics. A similar study of our study was conducted in England, using CDST and E-test, and the sensitivity of the E-test and MDST was determined to be 93% [12]. Antibiotic- resistant Klebsiella spp. Isolates of ESBL and/or carbapenemase producers resistant to third/fourth generation cephalosporins and carbapenems are of great concern [12] In one study, ESBL production in K. pneumoniae was 85.4% and the highest resistance levels are SXT (77.0%), AMC (71.6%), CRO (62.2%), FEP (60.3%), and CAZ (60.8%), it was seen as [13] In our study, it eas found that Klebsiella spp were the most resistant to SXT with 80%, whereas they were most susceptible to IPM with 99%. Resistance rates to third-generation cephalosporins are 68% for CRO, 65% for CTX, 52% for CAZ, and 62% for ATM. Both ESBL positive and negative isolates were susceptible to IPM

Resistance to carbapenems occurs by different mechanisms. These mechanisms are changes in the active sites of penicillin-binding proteins (PBPs), decreased expression of outer membrane proteins (OMPs), efflux pumps, and production of β-lactamase enzymes. Production of β-lactamase enzymes from all four mechanisms is the most clinically important resistance mechanism. This may result from horizontal gene transfer of β-lactamase genes responsible for the production of β-lactamase enzymes [13,14]. β-lactamase specifically targets the β-lactam ring and breaks the bond in the ring, rendering the antibiotic inactive. Based on their activity profile, β-lactamases are grouped into four types: Penicillinases inactivate penicillins, but cephalosporins, aztreonam, or carbapenems are not. Cephalosporinases inactivate cephalosporins and aminopenicillins, but not other penicillins, aztreonam, and carbapenems. ESBL inactivates all β-lactams except carbapenems. Carbapenemasesinactivate carbapenems as well as other β-lactam antibiotics [15,16].

It is difficult to detect the presence of ESBL with standard antimicrobial disk susceptibility tests routinely performed in most microbiological laboratories. The presence of resistance or decreased susceptibility to these antibiotics in susceptibility tests may be a stimulus for ESBL production. However, routine susceptibility testing may not yield drug resistance or moderate susceptibility results, as some bacteria producing these enzymes may have low resistance (MIC 4-16 µg/ml). Failure to detect a resistance mechanism by susceptibility testing results in latent resistance that can be transferred by plasmids to other bacteria and causes serious problems in treatment [17,18]. Enterobacteriaceae species should be identified correctly by special methods because of the increase in the prevalence of ESBL production, their prevalence in clinical isolates, their easy spread through plasmids, the fact that they cause serious clinical problems such as epidemics, treatment failure, increased mortality, and that they are difficult to identify with routine susceptibility tests. Although

ESBL-producing bacteria are resistant to broad-spectrum cephalosporins and aztreonam, they can be found sensitive in routine antibiotic susceptibility tests and may cause problems during treatment [19].

Many methods have been proposed to detect ESBL-producing bacteria. They were: Ceftazidime resistance control, DDST, Combined Disk Test (CDT), three-dimensional test, E-test, using higher bacterial density, disk diffusion in media with clavulanic acid, MIC with a combination of clavulanic acid, automatic Vitek and Micro screening methods [20,21].

Beta-lactamases act by cleaving the cyclic amide bond in the beta-lactam ring. Beta-lactamase genes are encoded in chromosome control in bacteria or in genes found in plasmids or transposons. Plasmid-derived beta-lactamases such as TEM-1, TEM-2, and SHV-1 are common enzymes among members of the Enterobacteriaceae and are transferred to other bacteria via plasmids. Although ESBLenzymes are mainly derived from TEM and SHV enzymes, new plasmid-derived ESBLs such as CTX- M, OXA-1, PER-1, and PER-2, which are not from TEM and SHV, have also been identified [22-24].

In our study, the specificity of DDT (CAZ, CRO, CTX, and ATM together) was 65.3%, the sensitivity 93.1%, and CAZ (82%) was the indicator that revealed the most 'ESBL suspect' isolate. The high sensitivity of CDST and E-test in our study suggests that mostly TEM or SHV-type enzymes were produced in our hospital strains. Therefore, inhibitor combination tests for diagnosing ESBL are also insufficient and an additional test is definitely needed. In conclusion, factors such as changes in outer membrane protein profiles (such as OmpF and OmpC deficiency), the presence of beta-lactamases not inhibited by clavulanic acid, and secretion of beta-lactamases due to low-level AmpC chromosome contribute to resistance [25].

When we investigated the risk factors for ESBL colonization and infection, the incidence of ESBL was found to be significant in mechanically ventilated patients. In patients with a nasogastric tubes, new surgery was found to be associated only with the E-test method, while ESBL positivity was found to be significant only with DDST. There was no significant difference between other factors and ESBL. In one study, risk factors for ESBL (age, gender, length of hospital stay, severity of disease, presence of urinary catheter or mechanical ventilator, and antibiotic use up to two weeks before bacteremia) were investigated. Previous treatment with third-generation cephalosporins was the only independent risk factor (p=0.008). A similar study found the use of antibiotics containing oxyimino ring for *K. pneumoniae* strains as a risk factor for ESBL production [25,26]. According to one view, the reason for the differences in terms of risk factors in other studies, as in our study, is; The retrospective characterof the study is seen as insufficient number of patients, lack of consensus in distinguishing colonization from real infection, insufficient data on antibiotic use of patients before admission to hospital or infection, and isolates were collected only from certain services [27].

Three-dimensional testing and dilution methods for detecting the presence of ESBL are difficult and time-consuming tests to be applied in practice. DDST and CDT are tests that are frequently used in laboratories, difficult and costly to read as a result of diffusion of beta-lactamase inhibitor to the beta-lactam antibiotic side, and have close sensitivity to each other. Therefore, as phenotypic confirmatory tests, both the DDST and the CLSI -recommended DST are methods that are practical and easy to apply in every laboratory. One advantage of CDT is that it provides the convenience of using only two discs. The high level of ESBL production in our laboratory suggests that most of the isolates in our hospital are susceptible to inhibition by clavulanic acid.

The high level of ESBL production in our laboratory suggests that most of the isolates in our hospital are susceptible to inhibition by clavulanic acid. Two tests other than the Disk Diffusion Test seem to be good options for determining ESBL production in routine laboratories. However, the application of the E-test requires meticulousness, it is an expensive method compared to others, and sometimes the diffusion of beta-lactamase inhibitor to the beta-lactam antibiotic side creates difficulties in evaluating the result. The Double Disk Synergy Test, on the other hand, has disadvantages such as the distance between the disks affecting the result. Our recommendation; According to literature data, CDT seems to be an excellent test for reaching results when cefamycin and beta-lactamase inhibitor combinations of ESBL-producing strains are tested, fourth generation cephalosporins for AmpC enzyme, and ceftazidime for K1 enzyme overproduced in *K. oxytoca* are tested.

Abbreviations

ESBL: extended-spectrum beta-lactamases; DDST: Double Disk Synergy Test; DDT: Disk Diffusion Test; SPSS: Windows Statistical Package for Social Sciences; CLSI: Clinical and Laboratory Standards Institute; CDT: Combined Disk Test; PICU: Pediatric Intensive Care Unit; IMICU: Internal Medicine Intensive Care Unit; SICU: Surgical Intensive Care Unit; CICU: Cardiology Intensive Care Unit; UR: Urology Service; SRS: Surgical Service; İnf: Infection Service; IMS: Internal Medicine Service; PS: Pediatrics Service; CHES: Chest Diseases Service; CRS : Cardiology Service; ICU: intensive care unit; CRO: Ceftriaxone; CTX: Cefotaxime; ATM: Aztreonam; SXT: sulfamethoxazole- trimethoprim; CAZ: Ceftazidime; CIP: Ciprofloxacin; GN: Gentamicin; AMC: Amoxicillin/Clavulanic Acid; AK: Amikacin; TZP: Piperacillin-tazobactam; IPM: Imipenem; FEP: Cefepime; FOX: Cefoxitin; PBP: penicillin-binding proteins; OMP: outer membrane proteins.

Author Contributions

EC: Data Collection; Methodology
MB: Planning of the study; Formal Analysis; Statistical Analysis
BI: Writing and editing the article; Analysis of data

Declaration of Ethical Code

In this study, we undertake that all the rules required to be followed within the scope of the "Higher Education Institutions Scientific Research and Publication Ethics Directive" are complied with, and that none of the actions stated under the heading "Actions Against Scientific Research and Publication Ethics" are not carried out.

This study was carried out with the approval of Harran University Clinical Research Ethics Committee, dated 14.12.2012, and numbered 05, in the Laboratory of Microbiology Department of Harran University Faculty of Medicine.

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