Turk J. Pharm. Sci. 4 (3) 151-158, 2007

DETERMINATION OF EXTENDED SPECTRUM BETA-LACTAMASE FREQUENCY OF *KLEBSIELLA PNEUMONIAE* STRAINS ISOLATED FROM URINARY TRACT INFECTIONS AND TYPING WITH ISOELECTRIC FOCUSING METHOD

Mine HOŞGÖR LİMONCU[1*], Şafak ERMERTCAN[1], Cengiz ÇAVUŞOĞLU[2], Bayrı ERAÇ[1]

[1] Ege University, Faculty of Pharmacy, Department of Pharmaceutical Microbiology, 35100 Bornova - İzmir, TURKEY
[2] Ege University, Faculty of Medicine, Department of Microbiology and Clinical Microbiology, 35100 Bornova - İzmir, TURKEY

Abstract

Extended spectrum beta-lactamase (ESBL) presence was investigated in Klebsiella pneumoniae strains which were the ethiological agents of urinary tract infections and typed with isoelectric focusing (IEF) method. The antibiotic sensitivities of 52 K. pneumoniae strains were determined by disc diffusion method and ESBL were found in 12 strains (23 %) by double disc synergy test. Minimum inhibitory concentration (MIC) values of ESBL (+) K. pneumoniae strains for ceftazidime (CAZ) and cefotaxime (CTX) were investigated by E-test and isoelectric points of ESBL enzymes were determined by polyacrilamide gel electrophoresis. When the strains' sensitivity patterns were investigated for 3rd generation cephalosporins and aztreonam, 6 strains had ESBL premise sign with at least one antibiotic. Band pattern which focused around pl 7.6 was determined in 11 of 12 strains by IEF method. MIC values were found 16 μg/mL and over for CAZ and 12 μg/mL and over for CTX in 5 of these strains. When pI's and antibiotic susceptibility patterns were analysed, 5 of 12 isolates had a band and susceptibility patterns suggesting SHV-2.

Keywords: *Beta-lactamase, Klebsiella pneumoniae, isoelectric focusing*

İdrar Yolu İnfeksiyonlarından İzole Edilen *Klebsiella pneumoniae* Kökenlerinde Genişlemiş Spektrumlu Beta-Laktamaz Varlığının Araştırılması ve İzoelektrik Odaklama Yöntemi ile Tiplendirilmesi

Bu çalışmada, idrar yolu infeksiyonlarından izole edilen Klebsiella pneumoniae kökenlerinde genişlemiş spektrumlu beta-laktamaz (GSBL) varlığı araştırıldı ve tiplendirmeleri izoelektrik odaklama yöntemi ile yapıldı. Elli iki K. pneumoniae kökeninin antibiyotik duyarlılıkları disk difüzyon yöntemi ile belirlendi. On iki (% 23) kökende çift disk sinerji yöntemi ile GSBL varlığı saptandı. Bu kökenlerin seftazidim (CAZ) ve sefotaksim (CTX) minimum inhibitör konsantrasyon (MİK) değerleri E-test yöntemi kullanılarak, GSBL enzimlerinin izoelektrik noktaları ise poliakrilamid jel elektroforezi yöntemi ile belirlendi. Kökenlerin üçüncü jenerasyon sefalosporinler ve aztreonam duyarlılık paternleri dikkate alındığında, altı kökende en az bir antibiyotik ile GSBL öncü belirtisi saptandı. İzoelektrik odaklama yöntemi ile 12 kökenin 11'inde pI 7.6 olan bantlar gözlendi. Bu kökenlerin beşinde CAZ MİK'i 16 μg/mL ve üzerinde, CTX MİK'i 12 μg/mL ve üzerinde bulundu. Sonuç olarak, antibiyotik duyarlılık paternleri ve izoelektrik noktaları gözönüne alındığında bu beş kökende bulunan enzimlerin SHV-2 olabileceği düşünüldü.

Anahtar Kelimeler: *Beta-laktamaz, Klebsiella pneumoniae, izoelektrik odaklama*

***Correspondence:** Tel: +90 232 388 0110 / 1358 fax: +90 232 388 5258
E-mail : minehosgorlimoncu@yahoo.com.tr

INTRODUCTION

Recently it was indicated that resistance had been developed against beta-lactam antibiotics through extended spectrum beta-lactamases (ESBL). These enzymes were first detected in the *Klebsiella pneumoniae* strains in Europe in 1983 and later in the other members of the *Enterobacteriaceae* family. Usually encoded on plasmids, the enzymes can mediate resistance against oxiimino beta-lactams such as cefotaxime (CTX), ceftazidime (CAZ), ceftriaxone and aztreonam (ATM). Due to the fact that ESBL encoding genes are on the easily transferable plasmids among bacteria, prevalence of high levels of resistance is enabled which result in major therapeutic problems (1-4). Although ESBL enzymes derive predominantly from TEM and SHV enzymes, recently new plasmids coded ESBL that did not derive from TEM and SHV were defined. These new enzymes were effective against all cephalosporins including cephamisins. There is a lot of epidemiological research on the prevalence of ESBL enzymes. Dominant types show geographical differences. The most prevalent ESBL enzymes are TEM-47 in Poland, SHV-3, SHV-4 and TEM-3 in France, TEM-52, SHV-12 and SHV-2a in Korea. ESBL enzymes were detected for the first time in the United States of America 5 years later than Europe and the most frequently isolated enzymes were TEM-10, TEM-12, and TEM-26. While worldwide the most prevalent ESBL enzymes are SHV-2 and SHV-5, including Turkey the most common ESBL type is SHV-2. It was seen that sporadic nosocomial cases affected by ESBL producing strains resulted in endemic problems in some hospitals. These strains are spreaded in the hospital by long term hospitalized patients. Epidemics are frequent among long term hospitalized patients, post-operation patients, arterial and urinary catheterized patients, and especially intensive care unit patients (3, 5 -10).

The aim of this study was detecting the frequency of ESBL in *K. pneumoniae* strains isolated from urinary tract infections (UTI) and typing them with polyacrylamide gel electrophoresis isoelectric focusing (PAGE-IEF) method.

EXPERIMENTAL

Determining the presence of ESBL by double disk synergy test

After the antibiotic susceptibility was determined by disk diffusion method in of 52 *K. pneumoniae* strains isolated from UTI, the presence of ESBL was determined by double disk synergy test (DDST). Bacterial suspension prepared according to McFarland 0.5 was inoculated on the plates. Amoxicillin-clavulanic acid (AMC) disk was placed at the center of the plate and around this disk at the distance of 25 mm from center the center ATM, CAZ and CTX disks were placed. Plates were incubated at 35°C for one night. The extension at the inhibition zones around CAZ, CTX, and ATM disks towards AMC and / or the zone diameters being 22 mm, 27 mm, and 27 mm or below for at least one of the for CAZ, CTX, and ATM disks respectively were interpreted as ESBL presence (11).

Determination of CAZ and CTX susceptibility by E-test

With this method the MIC values of *K. pneumoniae* strains against CAZ and CTX were investigated. Bacterial suspensions of 10^8 cfu/mL were inoculated on the surface of the Mueller-Hinton agar and then E-test strips were placed. MIC's of CAZ and CTX of the strains were determined after one night incubation.

Determination of isoelectric points of ESBL by PAGE-IEF

Obtaining raw beta-lactamase extracts from bacteria

Three to four colonies from the culture on the blood agar base were taken and inoculated into 10 mL Mueller-Hinton broth which were incubated at 35°C for 18 h. Bacterial suspensions were centrifuged at room temperature 5000 c/min for 10 min and the supernatant was extracted. The sediment was vortexed and centrifuged again by 5 mL 0.1 M phosphate buffer solution (PBS) of pH value 7 after which the supernatant was extracted and the same process was repeated twice. After 0.5 mL PBS was added, the sediment was vortexed and suspensions were poured into the eppendorf tubes. In order to extract beta-lactamase from cells, the bacteria was sonicated (ELMA, Transsonic Digital) for 15 min while the suspension was kept in cold to prevent the enzymes from the effects of heat. The obtained sonicates were centrifuged at 4°C at 10000 c/min for 2 min, the supernatant containing beta-lactamases was taken into the eppendorf tubes, and they were kept at -20°C.

Showing beta-lactamase presence

After the sonication, 5 µl of supernatant was mixed with 5 µl nitrocefin solution (Oxoid). Beta-lactamase presence was indicated by color change from yellow to pink-red in less than 2 min.

PAGE-IEF method

Beta-lactamases were typed by using IEF method. For that reason polyacrylamide gel containing ampholyte (pH 3-10, Sigma Chem. Co.) was prepared. Twenty five microliter of bacterial sonicates including beta-lactamase were mixed with 5 µl 50% glycerol and 20 µl of this mixture was dropped into the pores of the gel. Five microliter from each of the reference enzymes, whose isoelectric points were known, such as TEM-1 (pI 5.4), OXA-1 (pI 7.4), SHV-1 (pI 7.6), and IEF standard marker was dropped into the gel. The IEF of polyacrylamide gel was performed for 1.5 h according to the instruction manuel (111 Mini IEF, Bio-Rad) **(12).** At the end of the IEF process nitrocefin solution was added on the gel and diffused in order to make beta-lactamase bands visible. The distance to the anode of beta-lactamase bands which appeared in red color was indicated on the paper and photographed.

RESULTS

The presence of ESBL was detected in 12 (23%) of 52 *K. pneumoniae* strains that are UTI agents. The antibiotic susceptibility investigated by disk diffusion method, CAZ and CTX MIC values determined by E-test and the isoelectric points of ESBL (+) *K. pneumoniae* strains were indicated in the Table 1.

A band pattern focusing around pI: 7.6 only was identified during the IEF in the 8 (67%) out of the 12 strains. In 2 (17%) strains there was a band pattern focusing around pI: 8 ↑ together with pI: 7.6. While 3 band patterns of pI: 5.4, 7.6 and 8 ↑ was observed in one strain (8%), in another strain (8%) only one band pattern focused at pI: 8 ↑ was observed (Figure 1).

DISCUSSION

In this study the presence of ESBL with DDST was determined in 12 (23%) of the 52 *K. pneumoniae* strains causing UTI. While the 3rd generation cephalosporins and aztreonam zone diameters of the strains were examined, it was seen that, with at least one antibiotic, ESBL preliminary indicator was present in 6 strains. A band pattern focusing around pI: 7.6 was

determined by IEF method in the 11 of 12 strains. CAZ and CTX MIC values in 5 of these strains were 16 µg/mL and over, and 12 µg/mL and over respectively. When their isoelectric points were considered, it was observed that these enzymes were suggesting SHV-2. SHV-2 with pI: 7.6, which is prevalent all over the world including Turkey, sustains high level resistance against broad spectrum cephalosporines (13, 14).

Table 1. The antibiotic susceptibility determined by disk diffusion method, CAZ and CTX MIC values identified by E-test and IEF patterns of *K. pneumoniae* strains producing ESBL.

Strains	AMC	ATM	CAZ	CTX	CXM	AMP	IPM	PRL	CF	CAZ µg/mL	CTX µg/mL	pI
1	I	S	S	S	R	R	S	R	R	0,25	0,03	7.6
2	S	S	S	S	S	R	S	R	R	1	0,03	7.6
3	I	S	S	S	S	R	S	R	R	0,75	0,03	7.6
4	S	R	R	I	R	R	S	R	R	128	12	7.6, 8 ↑
5	S	R	R	I	R	R	S	R	R	64	32	7.6
6	I	R	R	I	R	R	S	R	R	256	12	7.6
7	S	S	S	S	R	R	S	R	R	0,5	0,06	7.6, 8 ↑
8	S	S	S	S	I	R	S	I	R	0,5	0,01	7.6
9	S	S	S	S	S	R	S	R	R	0,023	0,01	7.6
10	S	R	R	R	R	R	S	R	R	32	64	7.6
11	R	S	S	S	R	R	S	R	R	1,5	0,09	8 ↑
12	S	I	I	I	R	R	S	R	R	16	16	5.4, 7.6, 8 ↑

AMC: Amoxicillin-Clavulanic acid, ATM: Aztreonam, CAZ: Ceftazidime, CTX: Cefotaxime, CXM: Cefuroxime, AMP: Ampicillin, IPM: Imipenem, PRL: Piperacillin, CF: Cephalothin
S: Susceptible, I: Intermediate, R: Resistant

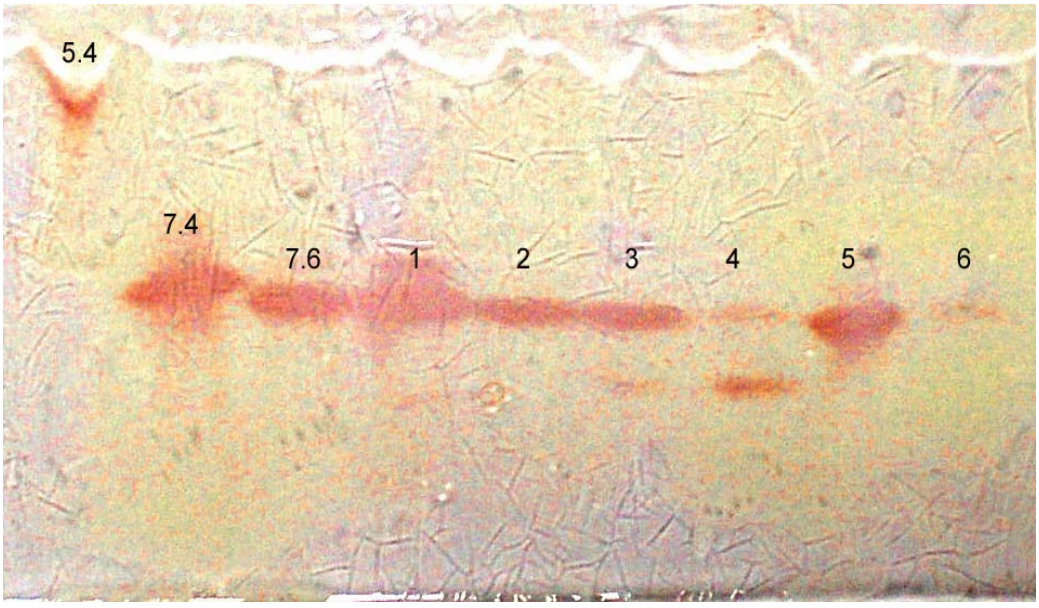

Figure 1. Beta-lactamases typing of TEM- 1, OXA-1, SHV-1 and 1 (positive control), 2 (strain 6), 3 (strain 10), 4 (strain 4), 5 (strain 5), 6 (negatif control) by using PAGE-IEF method.

In a study of Çavuşoğlu et al. (15), 11 of the 20 *K. pneumoniae* strains had band paterns pI: 7.6. As 7 of these strains had CAZ and CTX MIC values over 16 μg/mL, it was thought that they might have beta-lactamase bands in SHV-2 type. In another study from our country, Gülay et al. found the same band patterns in 1999 (16).

In this study, the MIC values of in 7 of CAZ or CTX the strains were below 16 μg/mL. SHV-2 gene in these strains might have been transferred by weak-promotor including genes. Although SHV-2 sustains high level resistance against broad spectrum cephalosporins, SHV-2 genes encoding weak promotor genes showed low level of ESBL activity and their MIC levels were lower. Another possibility was that those strains might have contained SHV-6 ESBL. SHV-6 with pI: 7 provides lower MIC values for CAZ and CTX. Nowadays, having 3-4 different beta-lactamases is frequent for the plasmid encoding ESBL containing microorganisms (13, 15).

In this study in 2 of the 11 strains, where a band pattern focusing around pI: 7.6 was identified, a second band pattern focusing over pI: 8 was observed. Three band patterns with pI: 5.4, 7.6, and over 8 were identified in one of the strains. In another strain only one band pattern focusing over pI: 8 was seen. MIC values for CAZ and CTX of the strain which had 3 beta-lactamase bands were 16 μg/mL. Beta-lactamase band focusing pI: 5.4 of that strain suggested TEM-7 which had low level resistance to 3rd generation cephalosporins. The MIC values of CAZ and CTX in strain no: 11 containing beta-lactamase with pI over 8 were below the susceptibility levels. Beta-lactamase type confirming these characteristics could not be identified.

In the study of Steward et al. (17), of the 139 *K. pneumoniae* strains, 117 strains were considered as ESBL producer by disk diffusion and 114 strains microdilution method. In 23 strains, although ESBL was not shown by microdilution method, TEM and SHV genes were detected by PCR. While in 138 of 139 strains, TEM and SHV genes were observed by PCR, 136 of 139 strains have TEM (pI: 5.2-6.5) and SHV (pI 7-8.2) type band patterns by IEF method. It was also detected that although three strains had SHV genes by PCR, their band patterns were not resemble to SHV enzymes. Researchers indicated that IEF method is diagnostic for investigation and typing of beta-lactamase enzymes.

In a study from China (18), ESBL were investigated by DDST and E-test and typed by IEF method in 14 *K. pneumoniae*. These strains produced beta-lactamases with pI: 5.4-8.4. It was seen that although ceftazidime MIC's of ten isolates were below 2 μg/mL, the enzymes produced by these strains had pI values of 7.6 and or 8.4. It was reported that beta-lactamases with pI: 7.6 might be identical to SHV-1, 2, 6, 7, 8, and 11.

In a hospital in Taiwan, ESBL were detected in 31 of 104 *K. pneumoniae* by DDST and E-test. It was found that 22 of these strains had beta-lactamases identical to SHV-5 and 2 strains had beta-lactamases identical to SHV-2 (19).

In another study (20), ESBL were identified in 13 of 21 *K. pneumoniae*. It was seen that six strains produced beta-lactamases with pI 7.6 and seven produced beta-lactameses with pI 8.2. It was though that these enzymes were consistent with SHV-2a and SHV-12, respectively.

In a study performed in Mexico, TEM (pI: 5.4), SHV-1 (pI: 7.6), and SHV-5 (pI: 8.2) type ESBL were detected in 31 *K. pneumoniae* (21).

CONCLUSION

In this study out of 12 *K. pneumoniae* strains, ESBL presence was determined in 5 strains using DDST and in 7 strains by evaluating their ATM, CAZ and CTX zone diameters. The MIC values of CAZ and CTX of the strains producing ESBL by DDST were comparatively higher than the MIC values of the other strains. The evaluation of the IEF patterns of the strains indicated that except one strain, 11 of the strains had bands of pI: 7.6. The results of the study

indicated that the use of DDST alone was not sufficient in the determination of ESBL in *K. pneumoniae* strains and that beta-lactamase genes should be identified initially in matching the beta-lactamase bands with corresponding enzymes in IEF method.

REFERENCES

1. **Gürdoğan, K., Arslan, H.,** "Hastane kökenli ve hastane dışı *Escherichia coli*'lerde çift disk sinerji yöntemiyle genişlemiş spektrumlu beta-laktamaz araştırılması ve izolatların çeşitli antibiyotiklere duyarlılık durumu" *Flora,* 4(3), 177-180, **1999**.

2. **Hoşgör, M., Özkan, F., Yapar, N., Tünger, A., Özinel, M.A.,** "Genişlemiş spektrumlu beta laktamazların belirlenmesinde çift disk sinerji testi ile üç boyutlu yöntemin karşılaştırılması" *Klinik Derg.,* 11, 59-60, **1998.**

3. **Özsoy, M.F., Öncül, O., Yıldırım, A., Pahsa. A.,** "Genişletilmiş spektrumlu beta-laktamazlar: klinik önemi ve getirdiği sorunlar" *Flora,* 6 (Ek:1), 1-23, **2001**.

4. **Philippon, A., Labia, R., Jacoby, G.,** "Extended spectrum beta-lactamases" *Antimicrob. Agents Chemother*., 33, 1131-1136, **1989**.

5. **Heritage, J., M'Zali, F.H., Gascoyne-Binzi, D., Hawkey, P.M.,** "Evolution and spread of SHV extended-spectrum β-lactamases in gram-negative bacteria" *J. Antimicrob. Chemother.,* 44, 309-318, **1999**.

6. **Gür D.,** "β-laktamazlar" *Hacettepe Tıp Derg.,* 33, 102-109, **2002**.

7. **Gniadkowski, M., Palucha, A., Grzesioski, P., Hryniewicz, W.,** "Outbreak of ceftazidime-resistant *Klebsiella pneumoniae* in a pediatric hospital in Warsaw, Poland: clonal spread of the TEM-47 extended-spectrum β-lactamase (ESBL)-producing strain and transfer of a plasmid carrying the SHV-5-like ESBL-encoding gene" *Antimicrob. Agents.Chemother.,* 42, 3079-3085, **1998**.

8. **Kim, J., Kwon, Y., Pai, H., Kim, J.V., Cho, D.T.,** "Survey of *Klebsiella pneumoniae* strains producing extended-spectrum β-lactamases: prevalence of SHV-12 and SHV-2a in Korea" *J. Clin. Microbiol.,* 36, 1446-1449, **1998**.

9. **Pai, H., Lyu, S., Lee, J.H., Kim, J., Kwon, Y., Kim, J.W., Choe, K.W.,** "Survey of extended-spectrum β-lactamases in clinical isolates of *Esch-erichia coli* and *Klebsiella pneumonia*e: prevalence of TEM-52 in Korea" *J. Clin. Microbiol.,* 37, 1758-1763, **1999**.

10. **Nordmann P.,** "Trends in β-lactam resistance among *Enterobacteriaceae" Clin. Infect. Dis.,* 27, 100-106, **1998**.

11. **National Committee for Clinical Laboratory Standards**. "Methods for dilution antimicrobial disk susceptibility tests". Approved standard M2-A6. National Committee for Clinical Laboratory Standards, Wayne, Pa, **1997**.

12. **Shanahan, P.M.A., Thomson, C.J., Amyes, S.G.B.** "Beta-lactam resistance in aerobic commensal flora. *Int. J. Antimicrob. Agents.* 3, 259-266, **1994**.

13. **Gür D.,** "β-Laktamazlar" *Flora,* 2 (Ek:3), 1-18, **1997**.

14. **Livermore DM.,** "β-lactamases in laboratory and clinical resistance" *Clin. Microbiol. Rev.* 8 (4), 557-584, **1995**.

15. **Çavuşoğlu, C., Dibek, M.A., Çiçek Saydam, C., Göksel, S., Özkalay. N., Tümbay, E**. "Genişlemiş spektrumlu beta-laktamaz üreten *Klebsiella pneumoniae* izolatlarında plazmit analizi ve beta-laktamaz tiplendirmesi" *Flora,* 7(1), 38-43, **2002**.

16. **Gülay, Z., Amyes, S.G.B., Yuluğ, N.,** "Genişlemiş spektrumlu beta-laktamaz tanımlanmasıda çift disk sinerji testi ve izoelektrik odaklama yöntemi ile elde edilen sonuçların karşılaştırılması" *İnfeks. Derg.,*; 13(3), 381-384,**1999**.

17. **Steward, C.D., Rasheed, J.K., Hubert, S.K., Biddle, J.W., Raney, P.M., Anderson, G. J., Williams, P.P., Brittain, K.L., Oliver, A., McGowan, J.E., Tenover, F.C.,** "Characterization of clinical isolates of *Klebsiella pneumoniae* from 19 laboratories using the National Committee for Clinical Laboratory Standards extended spectrum beta-lactamase detection methods" *J. Clin. Microbiol.,* 39(8), 2864-2872, **2001**.

18. **Shen, D., Winokur, P., Jones, R.N.,** "Characterization of extended spectrum beta-lactamase-producing *Klebsiella pneumoniae* Beijing-China" *Int. J. Antimicrob. Agents*, 18, 185-188, **2001**.

19. **Liu, P.Y.F., Tung, J.C., Kee, S.C., Chen, S.L.,** "Molecular epidemiology of extended-spectrum beta-lactamase producing *Klebsiella pneumoniae* isolates in a district hospital in Taiwan" *J. Clin. Microbiol.,* 36(9), 2759-2762, **1998**.

20. **Howard, C., van Daal, A., Kelly, G., Schooneveldt, J., Nimmo, G., Giffard, P.M. ,** "Identification and minisquencing-based discrimination of SHV beta-lactamases in nosocomial infection-associated *Klebsiella pneumoniae* in Brisbane, Australia" *Antimicrob. Agents Chemother*., 46(3), 659-664, **2002**.

21. **Silva, J., Gatica, R., Aguilar, C., Becerra, Z., Garza-Ramos, U., Velázquez, M., Miranda, G., Leaños, B., Solórzano, F., Echániz, G.,** "Outbreak of infection with extended spectrum beta-lactamase-producing *Klebsiella pneumoniae* in a Mexican hospital" *J. Clin. Microbiol.,* 39(9), 3193-3196, **2001**.

Received: 06.02.2007
Accepted: 17.04.2007