Characterisation of drug resistance of nosocomial ESBL-producing *E. coli* isolates obtained from a Turkish university hospital between 2009 and 2012 by pulsed field gel electrophoresis and antibiotic resistance tests

Alper Karagöz¹, Mahmut Sunnetcioglu², Mehmet Resat Ceylan², Yasemin Bayram³, Gozde Yalcin⁴,⁵, Nadir Kocak⁶, Burak Suvak⁷, Cenk A. Andac⁵

¹Molecular Microbiology Research and Application Laboratory, Refik Saydam National Public Health Agency, Ankara, Turkey;  
²Department of Clinical Bacteriology and Infections, School of Medicine Research Hospital, Yüzüncü Yıl University, Van, Turkey;  
³Department of Microbiology, School of Medicine Research Hospital, Yüzüncü Yıl University, Van, Turkey; 
⁴Biotechnology Institute, Ankara University, Ankara, Turkey;  
⁵Department of Medical Pharmacology, School of Medicine, Selçuk University, Konya, Turkey;  
⁶Department of Medical Genetics, School of Medicine, Selçuk University, Konya, Turkey;  
⁷Department of Gastroenterology, School of Medicine Research Hospital, Yüzüncü Yıl University, Van, Turkey

In this study, drug resistance of 28 ESBL-producing *Escherichia coli* isolates obtained from 144 patients hospitalized at the Yüzüncüyiil University Hospital at Van (YUH), Turkey, between 2009 and 2012 were characterized by pulsed field gel electrophoresis and antibiotic susceptibility tests. Antibiotic resistance profile was determined by a Phoenix automated system (BD, USA). The ratio of ESBL-producing *E. coli* strains was determined to be 19.4% (28 out of 144 *E. coli* isolates). It was determined that the anaesthesiology, pediatrics and thoracic medicine intensive care units in YUH were cross-contaminated between 2009 and 2012 by ESBL-producing *E. coli* strains, which is a sign of nosocomial infection in YUH. Analysis of PFGE results gave rise to two main PFGE profiles, profile-A with four subprofiles and profile-B with three subprofiles, where profile-A predominates over profile-B (14%). Comparison of the antibiotic resistance profile with the PFGE profile yielded similarities while some differences also exist due to either identical restriction enzyme cutting sites with slightly different genetic sequences in between the cutting sites or newly formed restriction enzyme cutting sites that do not affect antibiotic resistance genes. *Enterobacteriaceae*, particularly *E. coli*, have developed resistance in YUH by producing ESBLs against oxyimino and non-oxyimino cephalosporins, and penicillin-type antibiotics. Therefore, more effective antibiotics such as cefoxitin or cefoperazone-sulbactam should be used for the treatment of future nosocomial infections in YUH while hospital staff should take care with hygiene, such as hand washing.

Keywords: *E. coli*, ESBL, PFGE.

**INTRODUCTION**

*Escherichia coli* is a member of normal human flora that is a significant causative agent in community and hospitalized patients. A variety of virulence factors of *E. coli* such as mannose-sensitive type I fimbria, mannose-resistant type II fimbria (primarily P and S fimbria), hemolysin, K antigen, colicin V, and toxins are responsible for *E.coli* infections [1-6]. *E.coli* resistance to antibiotics has been a growing problem due to an increase in failures of antibiotic treatment [7]. In particular, the emergence of extended spectrum β-lactamase
(ESBL)-producing *E. coli* strains have reduced the number of antimicrobial treatment options. ESBLs are enzymes that hydrolyze the amide bond in the β-lactam ring of a broad class of β-lactam, such as penicillins, first, second and third generation cephalosporins as well as oxymino-cephalosporins (ceftazidime, cefotaxime and cefixime) and monobactams (such as aztreonam) [8]. ESBLs were first reported in Europe in the 1980s as products of several mutations in β-lactamase. Up to date, three classes of β-lactamase mutations in *Enterobacteriaceae* have been reported to evolve into ESBLs: TEM, SHV and CTX-M. The activity of these enzymes are blocked by β-lactamase inhibitors such as clavulanic acid, sulbactam and tazobactam. Moreover, increasing mutations on class C β-lactamase genes (AmpC) have led to carbapenem (such as imipenem) resistance [9, 10].

In order to adapt specific infection control measures against nosocomial infections, hospitals must intermittently determine and keep a record of antibiotic susceptibility profiles as well as molecular epidemiologic characteristics of infectious strains. Pulsed field gel electrophoresis (PFGE) has been adopted as a key method for molecular typing, which involves separation of very large bacterial DNA fragments (up to around 2 Mbp) by agarose gel electrophoresis running on an alternating voltage periodically switched among three directions; each at an angle of 60 degrees. Due to high resolution, PFGE has been reliably used for molecular typing of *Enterobacteriaceae* such as *E. coli* and *Klebsiella* spp. [11-16]. This study aims to determine phenotypically an antibiotic susceptibility pattern as well as genotypic PFGE typing of *E. coli* isolates from clinical specimens collected from hospitalized patients at the Yüzüncüyıl University Hospital (YUH) at Van, Turkey, over more than three years between April 2009 and August 2012, and sent to the microbiology laboratory of the same hospital (n=420; 1 specimen per patient) for microbial isolation and identification.

### Isolation and determination of ESBL-producing *E. coli* isolates

Specimens submitted to the microbiology laboratory at YUH were inoculated on Eosine-Methylene Blue (EMB) (bioMérieux, France) and Sheep Blood Agar (bioMérieux, France), followed by incubation at 37 °C for 18-24 hours. Microbial identification was performed by means of the Phoenix automated system (Becton Dickinson, USA). ESBL-producing *E. coli* isolates were then identified by minimum inhibitory concentration (MIC) method using the same automated system. All *E. coli* (both producing-ESBL and not producing-ESBL) isolates were then stored at -80 °C.

### Antibiotic resistance studies

Antimicrobial susceptibility (ampicillin, ampicillin-sulbactam, piperacillin, piperacillin-tazobactam, cefazolin, cefoxitin, cefoperazone-sulbactam, cefotaxime, ceftazidime, cefepime, imipenem, meropenem, trimethoprim-sulfamethoxazole, ofloxacin, amikacin, gentamicin, and netilmicin) was determined by the Phoenix automated system (BD, USA) and the isolates were classified as resistant (R) or intermediate-resistant (I) or susceptible (S).

### Sample preparation for Pulsed Field Gel Electrophoresis

Bacterial isolates were suspended in 3 mL cell suspension buffer (CSB) solution, which contained 10 mM Tris-Cl, 50 mM EDTA and 20 mM NaCl (pH 7.2). The suspended cells were then centrifugated at 2500xg at 4°C for 15 minutes, followed by pipeting out the CSB solution. The cell pellets were added 1 mL of CSB solution and mixed by pipeting in and out several times. The cell concentrations were adjusted at absorbance 1 at 590 nm by UV/Vis Spectrometer (Beckman Coulter, USA).

100 μL aliquots of pre-heated 2% low-melting temperature agarose (LMA), dissolved in CSB solution by microwave heating, were pipetted into Eppendorf tubes mounted on a waterbath (45-50°C). A 100 μL aliquot of each bacterial suspen-
sion, prepared as described above, was pipetted into each Eppendorf tube, containing CSB solution, placed on water bath. Finally the Eppendorf tubes were added 1 mL of sodium dodecyl sulfate solution (10%) and mixed slowly by pipeting in and out several times. 100 μL of the bacterial isolates in liquid agar in each Eppendorf tube were carefully transferred into small molds (10 mm x 5 mm x 1.5 mm, Bio-Rad, Hercules, CA, USA), which were then kept at 4 °C for 10 minutes until they homogenously solidified without cell lysis and endonuclease activity. The agarose molds were taken out of their molds and transferred into 5 mL tubes containing 0.5 mL Cell Lysis Solution-I [CLS-I = 10 mM Tris-HCl (pH 7.2) + 50 mM NaCl +50 mM EDTA +0.2% sodium deoxycolate + 0.5% sarcosine], which were then shaken at 37°C for one hour in a water bath. After removal of CLS-I from the tubes, 0.5 mL of Cell Lysis Solution-II [CLS-II =250 mM EDTA (pH 9.0) +1% sarcosine + Proteinase K (1.5 mg/ml)] was then added into each tube, followed by shaking at 50°C for half an hour in a water bath. After CLS-II was completely removed, the agarose molds were washed three times with TE buffer [10 mM Tris-HCl (pH 7.6) +0.1 mM EDTA] at 50°C over three hours (3 x 1 hour). After removal of TE buffer, the tubes with agarose molds were added 100 μl of FastDigest XbaI restriction enzyme in TE buffer and incubated at 37°C for 2 hours. After removal of the enzyme buffer, the agarose molds were stored in refrigerator for 15 minutes before use in Pulsed Field Gel Electrophoresis.

Pulsed Field Gel Electrophoresis (PFGE), monitoring and data analysis
Pulsed Field Gel Electrophoresis (PFGE) experiment was implemented on a BioRad CHEF-DR III PFGE (Bio-Rad, Hercules, CA USA) instrument at the Biotechnology Institute at Ankara University, Ankara, Turkey.
1% pulsed-field certified agarose (Bio-Rad, Hercules, CA USA) in 0.5 x TBE buffer (pH 8.4) was casted in a PFGE chamber. Agarose molds treated with XbaI restriction enzyme, as described in the previous subsection, were then implanted into the wells of the pulsed field agarose gel using a PFGE comb.

The agarose gel implanted with bacterial DNA was then placed into the chamber of the PFGE instrument filled with 1900-2000 mL. 0.5 x TBE buffer. PFGE experimental parameters used were the following: restriction enzyme FastDigest XbaI; pulse angle 120°; current 6 V/cm²; temperature 14°C; time 20 hrs.

Following the PFGE experiment, the agarose gel was transferred into a solution of ethidium bromide (5 μg/mL) in 400 mL of distilled water, where it was kept for 20 minutes for staining. The gel was then destained for 20 minutes in distilled water. DNA bands which became visible on the destained agarose gel were pictured by GeneGenius Gel Bioimaging Unit (Sygene), which was then saved in TIFF picture format.

DNA band profiles were analyzed by Gel Compar II Software v.3.0 (Applied Maths, Sint-Martens-Latem, Belgium). The pictures were normalized based on reference DNA bands from E. coli strain ATCC 25922 (standard). Dendogram analysis of the PFGE profiles was carried out by an unweighted pair group method with mathematical averaging (UPGMA) [17]. E. coli strains were correlated on PFGE dendogram by a suitable Dice correlation coefficient, which utilized a band and profile tolerance of 1-1.5%, for the corresponding DNA bands.

The relatedness of PFGE bands were evaluated based on four categories proposed by Tenover et al.: indistinguishable, closely related, possibly related and unrelated [17]. Here, genetically indistinguishable isolates are epidemiologically identical isolates, referring to the same number of bands with the same size. Closely related isolates differ by a single genetic event, i.e., a point mutation or insertion or deletion of DNA, leading to two or three fragment differences. Possibly related isolates are less likely to be epidemiologically related, involving two independent genetic events that give rise to four to six band differences brought about by insertions/deletions on DNA or the gain/loss of restriction sites. Isolates with more than two genetic events, leading to more than 7 fragments, are considered to be genetically unrelated.

RESULTS
Out of a total of 420 specimens, 144 clinical isolates were identified as E. coli. Amongst the whole E. coli isolates, 28 (19.4%) were found to be ESBL-producers whose sources were tracheal aspirators (n=10), urine (n=8), wound (n=2), blood (n=7),...
Characterisation of drug resistance of nosocomial ESBL-producing E. coli isolates obtained and abscess (n=1) of patients hospitalized at YUH during 2009-2012 (Figure 1).

Antimicrobial susceptibility results are reported in Figure 1. In general, all the ESBL-producing E. coli isolates were completely resistant to penicillins and cephalosporins, strongly suggesting that these isolates are definitely ESBL-producing E. coli isolates, that is, they are likely to carry chromosomal class C (AmpC) for non-oxymino cephalosporins as well as plasmid encoded TEM- or SHV- or CTX-M-type ESBLs for oxymino cephalosporins.

Among the ESBL-producing E. coli isolates (n=28), 100% was found to be resistant to penicillin-type antibiotics (AMP, AMS, PIP, TZP), and CFZ whereas 14% (4 out of 28 isolates) and 57% (16 out of 28 isolates) were reported as resistant (R) and resistant-intermediate-resistant (R+I) to CFX, respectively; 71% (20 out of 28 isolates) and 75% (21 out of 28 isolates) of the ESBL-producing E. coli isolates were R and R+I to SCP, respectively; 96% (27 out of 28) was R to CTX, 96% (27 out of 28 isolates) and 100% (28 out of 28 isolates) were R and R+I to CFT, respectively; 96% (27 out of 28 isolates) was R to CTX, 96% (27 out of 28 isolates) and 100% (28 out of 28 isolates) were R and R+I to CFPM, respectively; 0% (0 out of 28 isolates) was R to IPM and MEM, and 100% (28 out of 28) was R to SXT, OFX, AMK, NET and GEN. Overall, the ESBL-producing E. coli isolates were exceptionally found to possess the highest percentage of susceptibility against carbapenems (100%) as well as to CFX (43%) between 2009 and 2012.

A PFGE dendogram for the 28 ESBL-producing E. coli isolates is shown in Figure 1. Based on PFGE analysis criteria proposed by Tenover et al., two profiles were found to be epidemiologically different from each other: profile A that is the most commonly identified profile (lines 5-28 in Figure 1, 86% of all isolates), profile B that is the least common profile (lines 1-4 in Figure 1, 14% of all isolates) [17]. Profile A was found to possess four subprofiles; profiles A1, A2, A3 and A4. PFGE dendograms shown in lines 5-18, 20-21 and 23-27 in Figure 1 reveal an indistinguishable DNA band profile, profile A1, which are said to be epidemiologically identical isolates. Indeed, profile A1 is the most commonly identified subprofile (21 out of 28 isolates, 75%). DNA bands profile in line 21 in Figure 1 belongs to profile A2 (4% of all isolates studied), which was found to be epidemiologically closely related to profile A1 with >85% clonal similarity by a mutation that leads to appearance of a larger DNA fragment.
and disappearance of two smaller DNA fragments as compared to profile A1. In addition, isolate in line 28 in Figure 1 possesses a slightly different DNA profile, profile A3 (4% of all isolates studied), which is also epidemiologically closely related to profile A1 with >85% clonal similarity by a mutation that gives rise to disappearance of a larger DNA fragment and appearance of two smaller DNA fragments as compared to profile A1. The DNA bands shown in line 21 account for profile A4, which is closely related to profile A1 with >85% clonal similarity by a mutation that leads to disappearance of a larger DNA fragment and appearance of two smaller DNA fragments as compared to profile A1. There appears to be three subprofiles that are epidemiologically related within profile B: profiles B1, B2 and B3. PFGE dendogram in lines 1-2 in Figure 1 possess identical DNA bands and account for profile B1 (7% of all isolates studied). Line 3 in Figure 1 accounts for profile B2 (4% of all isolates studied), which is closely related to profile B1 with >85% clonal similarity by a mutation that gives rise to disappearance of a larger DNA fragment and appearance of two smaller DNA fragments as compared to profile B1. Line 4 in Figure 1 addresses profile B3 (4% of all isolates studied), which is possibly related to profile B1 with <80% clonal similarity by two genetic events that lead to at least four DNA band differences by gain and loss of XbaI restriction sites.

**DISCUSSION**

Although ESBLs have been identified in almost all *Enterobacteriaceae*, they are most frequently found in *E. coli* and *Klebsiella pneumoniae*. A noteworthy fact is that the prevalence of ESBL positive nosocomial infections have significantly increased over the last decade in Turkey. It was reported by Public Health Institution of Turkey (PHI-Turkey, http://www.thsk.gov.tr/en/) in 2011 that the rate of ESBL-producing *E. coli* strains is 21% in Turkey. Although it should not be generalized to other hospitals throughout Turkey, the rate of ESBL-producing *E. coli* isolates at YUH between the years of 2009 and 2011 was 19.4%, which seems to be a slightly lower value than that of Public Health Institution of Turkey. Based on an earlier worldwide survey carried out between 2004 and 2009 using data for over 23,000 clinical isolates collected from intensive care units (ICU) worldwide, Bertrand et al. reported that ESBL production amongst the *E. coli* isolates was 4.7% in North America, 12.9% in Africa, 13.9% in Europe, 25.9% in Latin America, 26.3% in the Asia-Pacific rim, and 32.4% in the Middle East [18]. Over the study period, an increase in the proportion of ESBL producing *E. coli* was shown for North America, Europe and the Asia-Pacific rim. According to a more recent study implemented in 2011 by the Annual Report of the European Antimicrobial Resistance Surveillance Network (EARS-Net), which involved analysis of a total of 23915 *E. coli* isolates from blood or cerebrospinal fluid throughout 29 European countries, it was reported that 9.8% of the *E. coli* isolates were found to be resistant to the third generation cephalosporins, 71-100% of which was ESBL producers (based on data for only 17 countries). That value is further supplemented by another finding that 18 out of 28 European countries reported an increase in resistance to those antibiotics while other countries did not report a decrease in bacterial resistance [19]. Differences reported for the ratio of bacterial ESBL production in Turkey and in other countries rely on varying circumstances, such as prescription of different extended-spectrum beta-lactam antibiotics in different countries that affect the frequency of the ESBL production. It is noteworthy that infections caused by ESBL-producing *Enterobactericeae* have increased in Turkey and worldwide. Although there seems to be many factors leading to the increase in ESBL-producing bacteria throughout the world, the increase in ESBL-producing bacteria in Turkey is thought to be mainly due to non-prescriptional sales of antibiotics in Turkish Pharmacy Stores, which lead to self-mistreatment or redundant self-treatment, insufficient education of the Turkish community regarding careless and uncontrolled use of antibiotics as well as insufficient hygiene such as ignorance of hand-washing. Antimicrobial susceptibility tests revealed that *E. coli* isolates obtained from the anesthesiology intensive care unit (AICU) at YUH between the years of 2009 and 2011, lines 1-14, 21 in Figure 1, were ESBL-producing isolates that were resistant to almost all antibiotics tested, except for carbapenems (100% susceptibility) and CFX (43%
susceptibility). Majority of the E. coli isolates obtained from AICU possesses the same genotypic profile, profile A1 in Figure 1, suggesting a nosocomial E. coli infection of the same origin. Interestingly, E. coli isolated in 2010 in AICU, lines 1-4 in Figure 1, possess different genotypic profiles (profiles B1-B3) that exhibit up to 20-25% genetic variations from profile A1, suggesting the possibilities that either a new type of E. coli infection has emerged into AICU or profile A1 managed to mutate into profiles B1-B3, in which cases the same antibiotic resistance profile is preserved as that of profile A1. Despite the genetic mutations observed with profiles A2-A4 (as compared to profile A1) in the PFGE dendogram in Figure 1, profiles A2-A4 were all found to be resistant to all antibiotics listed in Figure 1, similar to majority of profile A1. This finding suggests that newly formed (or lost) XbaI cutting sites in profile A2-A4 and profile B2-B3 do not affect antibiotic resistance genes.

ESBL-producing E. coli isolates obtained from the thoracic medicine intensive care unit (TMICU) at YUH between December 2010 and May 2011, shown in lines 15-18, 20 in Figure 1, possess the most common PFGE profile, profile A1, similarly to those collected in AICU (lines 22-25, 28 in Figure 1), suggesting that there is cross-contamination between different units in the hospital. ESBL-producing E. coli isolates obtained from the paediatric intensive care unit (PICU) at YUH between May 2010 and August 2012 seem to possess slightly different antibiotic profile and yet closely related PFGE profiles including profile A1 for the isolates in lines 23-25 in Figure 1, profile A2 for the isolate in line 22 in Figure 1, and profile A3 for the isolate in line 28 in Figure 1, suggesting that ESBL-producing E. coli may be responding to different antibiotic treatments in PICU by genetic mutations over time.

ESBL-producing E. coli obtained from general surgery, urology, and internal diseases (lines 19, 26, and 27 in Figure 1, respectively) departments in YUH possess slightly different antibiotic resistance profiles with close clonal similarities. As additional data regarding antibiotic resistance and PFGE profiles for more ESBL-producing E. coli in the aforementioned hospital departments are not available, it is not plausibe at this point to make a conclusion whether the E. coli above are a product of a nosocomial infection in YUH or were just presented to the hospital by patients. In terms of profile A1, the first specimen was collected in AICU in YUH, dating back to April 2009, which followed collection of some other specimens in the same unit during 2009 (Figure 1). In 2010, ESBL-producing E. coli isolates showing profile A1 were obtained mainly from TMICU, AICU, and PICU in YUH, suggesting that ESBL-producing E. coli strains with profile A1 had already spread over other departments in the hospital in 2010. Specimen for profile A2, a single-mutation variant of profile A1, was collected in PICU in YUH at the end of the year of 2010, indicating the first case of profile A1→A2 mutation. Specimen for profile A3, another single-mutation variant of profile A1, was collected in PICU in YUH in May 2012, revealing an incidence of profile A1→A3 mutation. An ESBL-producing E. coli specimen for profile A4 was collected in the general surgery department in YUH on January 2011, showing a genetic mutation for profile A1→A4. Specimens for profile B1 were collected in AICU department in YUH between March 2010 and June 2012. Additional specimens were also collected in AICU on August 2010, two months after profile B1 specimens were collected, that gave rise to profile B2 which is epidemiologically closely related to profile B1 as well as profile B3. It is highly likely that intra-profile mutations may have occured due possibly to gaining bacterial resistance as a follow up to antibiotic treatments in the hospital.

Analysis of the hospital epidemiologic data indicate that all the hospitalized patients, from which specimens were collected, resided in local areas in the vicinity of YUH at Van, Turkey and do not possess a record of the E. coli isolates collected before 2009. Therefore, it is unfortunately not known whether or not profiles A and B are of hospital origin.

The experimental data presented here strongly support the notion that Enterobacteriaceae, and particularly E. coli, developed resistance by producing extended-spectrum β-lactamase enzymes against cephalosporin type broad-spectrum antibiotics at YUH. Therefore, it should be taken into a serious consideration that prescription of such cephalosporin antibiotics as a first-line treatment may fail in curing E. coli as well as Klebsiella and A. Baumannii infections at YUH. Moreover, it is anticipated that such bacterial resistance would
spread over other bacterial species in the near future, which may pose problems in dealing with non-E. coli infections at YUH.

 Currently, the most plausible treatment of E. coli infections at YUH will rely on imipenem antibiotics with the highest priority as well as CFX with lower priority. A noteworthy fact is that the prevalence of ESBL positive nosocomial infections have significantly increased over the last decade in Turkey [20]. Our laboratory data also indicate that ESBL-producing E. coli isolated at YUH have gained almost complete resistance to the antibiotics listed in Figure 1, which poses an imminent treat in the antibiotic treatment of Gram-negative enterobacterial (such as E. coli and Klebsiella spp) infections at YUH. The decreasing efficiency of the nosocomial infection treatments by penicillin derivatives in combination with beta-lactamase inhibitors as well as third-generation cephalosporins against ESBL-producing Enterobacteriaceae, should urge the hospital health care officials at YUH to take further steps in terms of prescription of more suitable antibiotics against the E. coli isolated at YUH. In addition, education of the hospital staff at YUH on hygiene such as more frequent hand washing and hospital cleaning is needed.

 It should also be kept in mind that nosocomial enterobacterial antibiotic resistance at YUH may cross contaminate other bacterial species at YUH in the following years, which require taking further precautions in the hospital. Therefore, it is suggested that more specimens be taken from hospitalised patients at YUH to monitor imminent treats regarding ESBL-producing bacterial infections.

ACKNOWLEDGEMENTS

Ethics approval was received from the Ethics Committee at Mevlana University-Konya, Turkey. We would like to thank the Institute of Biotechnology at Ankara University-Ankara, Turkey for allowing us to use the Pulsed Field Gel Electrophoresis facility therein.

REFERENCES


Characterisation of drug resistance of nosocomial ESBL-producing E. coli isolates obtained


