Introduction

Third generation cephalosporins (TGC) were introduced to the clinical use in the early 1980s. Soon after their introduction, bacterial resistance to extended spectrum cephalosporins have been reported [13]. TGC resistance in Enterobacteriaceae is generally related to extended-spectrum β−lactamases (ESBLs). ESBLs are plasmid-encoded β−lactamases capable of hydrolyzing penicillins, cephalosporins and monobactams (but not cephamycins and carbapenems) and they are usually inhibited by β−lactamase inhibitors [4]. ESBLs have been studied in detail in terms of their prevalence and types in different clinical settings in human medicine. However there are only a few reports of ESBLs in bacterial isolates of animal origin [14, 20, 24] and there is no data about ESBLs prevalence and types in animals in Turkey. Such data are important to improve our understanding about the complex epidemiology of ESBLs. In this study we would like to evaluate the presence and types of ESBL producing Enterobacteriaceae from healthy food animals in Turkey.

Materials and Methods

SAMPLING AND BACTERIAL IDENTIFICATION

Between February-July 2007, we visited and obtained fecal samples from three slaughterhouses and two farms in Istanbul and Tekirdag, Turkey. Istanbul and Tekirdag are in the Northwest part of Turkey and they are neighbour cities. Individual fecal samples were taken from adult cows and rectal swabs were taken from calves and sheep. All samples were obtained from healthy animals. Swabs were sent to the microbiology laboratory in the transport medium.

For detection of the presence of ESBL producing Enterobacteriaceae, fecal samples (approximately 0.1 g feces)
and rectal swabs were directly streaked onto the MacConkey agar plates containing 2µg/ml ceftazidime or cefotaxime using a cotton swab incubated at 37°C for 48h under ambient air. Negative samples were enriched in buffered broth for 18h at 37°C before being plated onto the same selective medium. One representative colony for each morphotype per plate was selected and identified by both conventional methods and Vitek2 GNI card (bio Merieux, St. Louis, MO, USA).

**SUSCEPTIBILITY TESTING AND ISOELECTRIC FOCUSING**

Minimum inhibitory concentrations (MICs) of cefoxitin, ceftiofur, cefotaxime, ceftriaxone, ceftazidime, cefepime, aztreonam, amikacin, imipenem and ciprofloxacin were determined by the agar dilution method and interpreted as described by the Clinical and Laboratory Standarts Institute (CLSI)[8]. Additional antimicrobial susceptibilities for gentamicin, streptomycin, tetracycline, nalidixic acid, trimethoprim-sulphamethaxazole, chloramphenicol were performed by the disk diffusion method as recommended by the CLSI. MICs for ceftazidime and cefotaxime were determined alone and in combination with 4µg/ml clavulanic acid for phenotype detection of ESBLs according to the CLSI.

Crude extracts of β−lactamases were subjected to isoelectric focusing (IEF) using a method described by MATHEW et al.[19] with modifications [2] using a model 111 Mini IEF Cell (Bio Rad, Hercules, CA, USA). β−lactamase bands were visualized by staining with nitrocefin. Gels were run over a pH range of 3-10. The pl values used as controls were 5.4 for TEM-1, 5.8 for TEM-8, 7 for SHV-2, 7 for CMY-1 and 9 for CMY-2.

**RESISTANCE TRANSFER**

Conjugation experiments were carried out with all ESBL producing isolates [11]. *E.coli J53Az2* was used as the recipient strain. Equal volumes (1ml) of cultures of the donor and the recipient strain (10^9 cfu/ml) grown with agitation in triptic soy broth were mixed and incubated statically for 18 h at 35°C. Transconjugants were selected on Mac Conkey agar supplemented with sodium azide (150 µg/ml). Frequency of transfer was calculated by dividing the number of the transconjugants by the number of donors.

**DETECTION AND SEQUENCING OF β−LACTAMASE GENES**

Bacterial DNA was extracted using a commercial kit according to the manufacturer’s instructions (High Pure PCR template preparation kit-Roche Diagnostics, Mannheim, Germany). Previously described (16) primer pairs were used for the partial amplification of and bla TEM, bla SHV, bla OXA and bla CTX-M genes. Same primers were used for the bidirectional sequencing of PCR amplicons using ABI 310 sequencer (AppliedBiosystems, USA). The sequences were compared using Blast.2.0 software with those sequences deposited at the GenBank.

**Results**

**ESBL PREVALENCE AND ANTIMICROBIAL SUSCEPTIBILITY**

A total of 349 samples from 239 cattle, 38 calves and 72 sheep from three different slaughterhouses and two farms were tested. Of these five (2.1%) from cattle had Enterobacteriaceae members including three *Escherichia coli*, one *Citrobacter freundii* and one *Citrobacter brakii* with ESBL phenotypes. We have not detected any ESBL producing Enterobacteriaceae in two farms and one slaughterhouse. No ESBL producing bacteria was isolated from sheep and calves (Table 1).

All five isolates showed at least 3 twofold decrease in MICs for ceftazidime and/or cefotaxime in combination with clavulanic acid suggesting that these isolates are ESBL producers.

All five isolates showed higher MIC values of ceftazidime than those of cefotaxime and ceftiofur. Ceftazidime MICs were found between 64µg/ml and >128µg/ml whereas cefotaxime MICs varied from 4 µg/ml to 16 µg/ml. Ceftiofur MICs were slightly higher than that of cefotaxime changing between 8µg/ml and 32µg/ml. The most active cephalosporin was cefepime. Tetracycline resistance was observed in all isolates. All of the ESBL producing isolates were susceptible to imipenem and ciprofloxacin but two isolates were resistant to nalidixic acid. Four of the five isolates were resistant to gentamicin, streptomycin and trimethoprim-sulphamethaxazole. Four isolates were resistant to three or more non-β−lactam antibiotics. MIC results of antibiotics are given in table 2.

<table>
<thead>
<tr>
<th>No of samples</th>
<th>No. ESBL positive Isolates</th>
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<tbody>
<tr>
<td>Slaughterhouse1 (Istanbul) Cattle</td>
<td>54</td>
</tr>
<tr>
<td>Slaughterhouse2 (Tekirdag) Cattle</td>
<td>48</td>
</tr>
<tr>
<td>Slaughterhouse3 (Tekirdag) Cattle</td>
<td>36</td>
</tr>
<tr>
<td>Farm1 (Istanbul) Cattle</td>
<td>53</td>
</tr>
<tr>
<td>Farm2 (Tekirdag) Cattle</td>
<td>48</td>
</tr>
<tr>
<td>Calves</td>
<td>18</td>
</tr>
<tr>
<td>Sheep</td>
<td>72</td>
</tr>
<tr>
<td>Calves</td>
<td>20</td>
</tr>
</tbody>
</table>

**Table I**: Origin and occurrence of ESBL positive isolates.
RESISTANCE TRANSFER EXPERIMENTS AND CHARACTERIZATION OF THE ESBL PRODUCING ISOLATES

Conjugation experiments were performed with all five ESBL-producing Enterobacteriaceae isolates. Transconjugants were obtained for three isolates (E. coli-S1, C. freundii-S1, E. coli-S2b). Conjugation efficiency of about $10^{-4}$-$10^{-7}$ recombinant per donor cell was observed and all transconjugants expressed the ESBL-related resistance phenotypes.

All five ESBL producing isolates harboured TEM-1 gene combined with blaSHV (in four isolates SHV-5 and in one isolate SHV-12). Three of the ESBL positive isolates also harboured OXA-10 type genes. No blaCTX-M genes were detected in ESBL positive strains.

MICs, isoelectric points, the β-lactamase genes detected by PCR and sequencing, and coresistances against non β-lactam antibiotics in five ESBL positive isolates are summarized in table 2.

**Discussion**

This paper describes the first detection of an ESBL in Enterobacteriaceae members isolated from food animals in Turkey. Expanded spectrum cephalosporins are β-lactams with a broad spectrum of activity against most Gram negative bacteria. However they are sensitive to hydrolysis by ESBLs. Most ESBLs belong to class A according to the Ambler classification which possess an active site serine and are mostly susceptible to inhibition by clavulanic acid [21]. The dissemination of these enzymes is currently a global problem [21,23]. It is also well known that plasmids carrying genes encoding ESBLs may also carry genes encoding resistance to non β-lactam antibiotics such as aminoglycosides, chloramphenicol and trimethoprim-sulphamethoxazole [22].

Most ESBL producing bacteria are also resistant to fluoroquinolones which even further restricting the treatment options [29]. Relatively very little is known about the epidemiology of ESBLs in veterinary medicine. There have been a few studies reporting ESBLs from farm animals and pets [5,6,9,10,18,20,26]. In fact plasmid encoded ESBLs which are once rarely detected of animal origin bacteria are more frequently being observed in the recent years. The factors leading to the emergence of ESBLs among bacteria of animal origin are not fully elucidated. The use of ceftiofur which is a third generation cephalosporin in veterinary medicine may have contributed to selection and maintenance of ESBL producing bacteria. Nevertheless, certain mechanisms such as dissemination of these highly mobile genetic elements of ESBL determinants by horizontal gene transfer or clonal spread of resistant microorganisms may be other important factors [7,17]. In Turkey ceftiofur can easily be purchased even without a veterinary prescription and unfortunately reliable data are lacking on the antibiotic consumption in veterinary medicine in Turkey. We have not detected any ESBL producing Enterobacteriaceae isolate in two farms and one slaughterhouse. There was no history of ceftiofur use in these two farms and both farms were located in regions far from any populated areas.

In this study, an 2.1% ESBL producing Enterobacteriaceae prevalence from cattle was found. This prevalence appeared to be concordance with several studies; for example DUAN et al. [10] reported a 3.1% prevalence of ESBL producers among E. coli isolates from cattle. SHIRAKI et al. [26] found 1.5% resistance to cefotaxime in Enterobacteriaceae members isolated from cattle and these E. coli strains were CTX-M-2 type ESBL producers. In 2003 a Spanish study reported a 3% prevalence of ESBL producers seen among E. coli isolates with reduced susceptibility to cefotaxime. Among the 619 E. coli isolates of this collection, most of the ESBL producing
isolates carried the CTX-M-14 variant and the rest had CTX-M-9 variant [5]. Currently ESBL producers are not frequent seen in animals, however true prevalence can be underestimated because of the fact that the studies on beta-lactamases including ESBLs in food and companion animals are still limited. These studies can also help us to elucidate what extent the use of antimicrobials in veterinary medicine contribute the antimicrobial resistance in humans [7,17].

The SHV type ESBLs were considered until recently to be the most frequent ESBLs where TEM types are also common, but very recently the CTX-M type ESBLs have become by far the most successful in terms of dissemination and they are the most common ESBL types in several settings [21, 25]. Unlike some studies on animal isolates [3,5,10,26] and most recent studies on human isolates reporting the CTX-M family group of β−lactamases is the predominant ESBL types, we have not detected any bla CTX-M gene in our isolates. GONULLU et al. [12], recently reported that CTX-M type ESBLs are highly disseminated among E.coli isolates in a large university hospital in Istanbul, Turkey and 86.5% of the E.coli strains harboured bla CTX-M genes. In our study all of five isolates harboured SHV and TEM-1 type ESBLs. SHV and TEM types of ESBLs are very common among both human and animal isolates. SHV-5 produced by K.pneumoniae isolates from Turkey was first reported by PATERSON et al. [22]. SHV-5, SHV-12, SHV-2 in clinical E.coli, Klebsiella pneumoniae and Enterobacter spp. strains had been also reported by TASLI et al. [28] in Izmir, Turkey. Absence of bla CTX-M genes in the isolates we studied appears to be an important finding since these results remind the early days of ESBL epidemic which SHV and TEM classes of ESBLs were more common.

Three isolates harboured bla OXA-10 gene which is not very common ESBL in Enterobacteriaceae. In fact, there are very few epidemiologic data on dissemination of OXA-type ESBLs among Enterobacteriaceae [21]. On the other hand it is well documented these type ESBLs are highly prevalent among Pseudomonas aeruginosa isolates in Turkey [1]. OXA-10 is occasionally found in human E.coli isolates in Turkey [15]. Recently OXA-10 type ESBLs are also found in an Enterobacter cloacae strain isolated from dogs in Australia [27].

An other important mechanism for TGC resistance in Enterobacteriaceae is AmpC type beta-lactamases. Several members of the Enterobacteriaceae including Citrobacter spp. and Enterobacter spp. have chromosomal encoded AmpC type beta-lactamases. AmpC type beta-lactamases confer resistance to TGC and cephamycins but unlike ESBLs they are not inhibited by beta-lactamase inhibitors [17]. ESBL production is particularly high in some enterobacterial species (e.g. K. pneumoniae, E. coli), but it has also been spread to other species like Enterobacter spp., Citrobacter spp., Salmonella spp.. In our study we have demonstrated that two Citrobacter isolates harbour ESBL genes so these relatively uncommon enterobacterial species might harbour these genes and play a role in their rapidly dissemination. To the best of our knowledge these Citrobacter isolates are the first description of ESBL producing Citrobacter isolates of animal origin.

In this study we aimed to provide an informative basis on the presence and types of ESBLs in food animals in Turkey. To our knowledge this is the first time ESBL encoding Enterobacteriaceae have been detected from food animals in Turkey. Larger scale surveillance studies both in veterinary and human medicine are needed to track the evolution and epidemiology of this type β−lactamases.

References