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Research Article

Occurrence of ESBL-Producing Enterobacteriaceae in Red Meat Samples

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Abstract

Extended spectrum beta-lactamase (ESBL)-producing Enterobacteriaceae is becoming a worldwide concern for the public health. They can adversely affect the treatment based on modern beta-lactam antibiotics against bacterial infections as well as possibly becoming a source for the spread of ESBL-encoding genes among different and/or the same bacterial species. The objective of this study was to determine the occurrence of ESBL-producing Enterobacteriaceae from a total of 110 red meat samples sold in İstanbul. The samples were initially homogenized in Enterobacteriaceae broth. Subsequently, the homogenized suspensions were exposed to pre-enrichment at 37°C for 18-24 h. After that, Chromogenic ESBL agar was used for selective enrichment at 37°C for 18-24 h again. The presumptive ESBL-producers were sub-cultured on Trypton Soy agar, followed by an overnight incubation at 37°C. The isolates were identified by Vitek®MS (bioMérieux). Finally, the identified isolates were subjected to agar disc diffusion testing, disc diffusion confirmation testing and MIC determination testing using a combination of cefotaxime (CTX), ceftazidime (CAZ), and cefpodoxime (CPD) discs ± clavulanic acid (CV) according to the guidelines by Clinical and Laboratory Standards Institute. The results revealed that a total of 23 isolates were confirmed to be positive for ESBL-production. Of 23 isolates, the most common species was determined as *E. coli* (30%), followed by *C. brakii* (22%), *E. cloacae* (17%), *K. pneumoniae* (9%), *C. freundii* (9%), *S. fonticola* (4%), *K. intermedia* (4%), and *M. wisconsensis* (4%). In conclusion, the results of this study provided that the red meat samples harbored ESBL-producing Enterobacteriaceae, and they may, therefore, hold a potential risk for the colonization of the consumers with ESBL-producers.

Keywords: *Antibiotic resistance, Enterobacteriaceae, ESBL, Red Meat, Public Health*

Kırmızı Etlerde GSBL-Üreten Enterobacteriaceae Suşlarının Varlıkları

Özet

Genişlemiş spektrumlu beta-laktamaz (GSBL)-üreten Enterobacteriaceae tüm Dünya'da halk sağlığı açısından ciddi endişelere yol açmaktadır. GSBL-üreten bakteriler, bakteriyel infeksiyonlara karşı kullanılan modern beta-laktam antibiyotikleri işlevselliklerini olumsuz şekilde etkilemekte ve aynı zamanda farklı ve/veya türdeş bakteri türleri arasında direnç kodlayan genlerin yayılmalarında rol oynamaktadırlar. Bu çalışmada, İstanbul ilinde satışa sunulan toplam 110 adet kırmızı et örneklerinde GSBL-üreten enterobakterlerin tespiti amaçlanmıştır. Örnekler, Enterobacteriaceae buyyonda homojenize edilmişlerdir. Homojenize edilen süspansiyonlar ilk olarak 37°C ve 18-24 saat ön zenginleştirme işlemine, devamında ise kromojen GSBL agarda tekrar 37°C ve 18-24 saat selektif zenginleştirme işlemine alınmışlardır. Şüpheli izolatlar saflaştırma için trypton soy agara ekimi yapılmış ve gece aşırı 37°C'de inkübe edilmişlerdir. İnkübasyon sonunda izolatlar Vitek®MS (bioMérieux) cihazı kullanılarak tiplendirilmiştir. Tiplendirilen şüpheli GSBL-üreten enterobakteri izolatlar Clinical and Laboratory Standards Institute talimatları takip edilerek ve sefotaksim (CTX), seftazidim (CAZ) ve sefpodoksim (CPD) ± klavulanik asit (CV) diskleri kullanılarak sırasıyla disk difüzyonu, disk difüzyon doğrulama ve MİK tespiti testlerine alınmışlardır. Sonuçlara göre toplam 23 adet enterobakteri izolatları kesin GSBL pozitif tespit edilmiştir. Aralarında en yaygın tip *E. coli* (%30) olarak belirlenirken, bunu *C. brakii* (%22), *E. cloacae* (%17), *K. pneumoniae* (%9), *C. freundii* (%9), *S. fonticola* (%4), *K. intermedia* (%4) ve *M. wisconsensis* (%4) izlemiştir. Sonuç olarak, kırmızı et örneklerinin GSBL-üreten enterobakteriler içerdikleri ve bu nedenle tüketicilerin GSBL-pozitif türler ile kolonize olmaları bakımından potansiyel risk taşıdıkları görülmüştür.

Anahtar sözcükler: *Antibiyotik direnci, Enterobacteriaceae, GSBL, Kırmızı Et , Halk Sağlığı*

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INTRODUCTION

Antibiotics are the chemical agents that were first developed in the early 1940 against bacteria-causing infections in humans. The antibiotics have been widely used in both human and veterinary medicine [1]. However, over- and improper-use of antibiotics have increasingly caused the dissemination of foodborne resistant-bacteria, not only in healthcare and community settings, thereby leading to higher resistant-bacteria-associated morbidity and mortality rates in the recent years. Therefore, the international health authorities are warning about the emergence of antibiotic resistance, including the non-medicinal use of antibiotics in the food animals [2].

Antimicrobial resistance is mainly caused by mechanisms of resistance. Within them, beta-lactam antibiotics are enzymatically inactivated by the specific enzymes, which are encoded by specific plasmid-mediated genetic materials. Especially, extended spectrum beta-lactamases (ESBL) are widely produced by the bacteria belonging to the family of Enterobacteriaceae. To date, more than 400 beta-lactamases were identified. ESBL-encoding genes can be transferred between the different and/or the same species through their plasmid-mediated characteristics [3]. Therefore, unpredictable and uncontrollable dissemination of ESBL-producing Enterobacteriaceae, including *E. coli*, *K. pneumoniae*, *Citrobacter* spp., *Salmonella* spp., and *Enterobacter* spp. are adversely affecting the human health [4-6].

Antibiotics are also widely used in the veterinary medicine, especially in the food animals for therapeutic purposes, not only in clinical and community settings [7,8]. The recent studies have showed that the foods of animal origin contain ESBL-producing enterobacteria and their ESBL-encoding *bla*-genes transferable to the human's intestinal microflora [9-11]. Except for the controlled therapeutic purposes, the antibiotics are, therefore, banned as growth promoters used in the farm animals by European Union, including Turkey. Also, the researchers are challenging on alternative antibiotic-replacers [12]. Probiotics and prebiotics, which are naturally occurring species and substances have been used for prevention of emerging resistant bacteria [7,8]. The leading international health and food organizations such as WHO and FAO are enhancing the surveillance programs for monitoring food, clinical, and community-related antibiotic resistance all over the [10,11].

The red meat is an important source in the human nutrition. It contains essential nutrients such as proteins and fatty acids, including minerals and vitamins [13]. For the human nutrition, slaughtering of the farm animals under unhygienic conditions threats the human health if contaminated with Enterobacteriaceae strains, especially with ESBL-producing enterobacteria. The recent studies have indicated that the red meat and the red-meat-derived foods may possibly be reservoirs for ESBL-producing Enterobacteriaceae, and their ESBL-encoding genetic elements [9-11].

The objective of this study was to determine the occurrence of ESBL-producing Enterobacteriaceae from a total of 110 red meat samples sold in İstanbul.

MATERIAL and METHODS

Sampling

A total of 110 red meat samples were randomly collected from butchers, supermarkets and slaughter-houses located in İstanbul from October 2014 to December 2014.

Reference Strains

ESBL-positive *K. pneumoniae* ATCC®700603 and ESBL-negative *E. coli* ATCC®25922 were used for control purpose in ESBL-screening testing.

Microbiological Evaluation of Samples

25 g of sample was added in 225 ml of Entero-bacteriaceae Enrichment Broth (LABM, England). The mixture was homogenized in a sterile blender-bag (Interscience, France) for 2 min by stomacher (EasyMix, France), and exposed to aerobic incubation at 37°C for 18-24 h [11]. After that, 10 µl of the pre-enriched suspension was inoculated to Chromatic ESBL agar (Liofilchem, Italy). The inoculated plates were incubated again at 37°C for 18-24 h under aerobic conditions [14]. After that, the colonies were selected according to the manufacturer's instructions by a sterile loop, i.e. the blue-green one as *Klebsiella* spp., red-pink-purple one as *E. coli*, and white-yellowish colony as *Proteus* spp. The selected presumptive ESBL-producing colonies were subcultured on Tryptone Soy Agar (LABM), followed by an overnight incubation at 37°C [14]. The subcultures were identified by a mass spectrometer (Vitek®MS, bioMérieux, France).

Screening and Confirmation of ESBLs

Disc Diffusion Testing: The suspected ESBL-producers were subjected to disc diffusion testing according to the guidelines by Clinical and Laboratory Standards Institute (CLSI) [15]. The isolate was spiked in a sterile saline physiological solution to get a density expressed as 0.5 McFarland standard. Using a cotton swap, it was spreaded over Mueller Hinton Agar (MHA) (Merck, Germany) [15,16]. The ESBL screening was performed by cefotaxime (CTX; 30 µg), ceftazidime (CAZ; 30 µg), and cefpodoxime (CPD; 10 µg) discs (TurkLab, Turkey). The disc inserted plates were then incubated at 37°C for 18-24 h. After that, the zone measurements were taken by a milimetric ruler. The breakpoints with zone diameters were evaluated

according to the criteria by CLSI (2013), so that CTX ≤22 mm, CAZ ≤17 mm, and CPD ≤17 mm were considered to be positive for ESBL-production [15].

Disc Diffusion Confirmation Test: A combined combination of CTX, CAZ, and CPD ± clavulanic acid (CV; 10 µg) (Turklab, Turkey; Alkim, Turkey) was used for the confirmation of disc diffusion testing. The disc-inserted plates were incubated at 37ºC for 18-24 h. The zones of inhibition was evaluated according to the criteria by CLSI (2013) [16,17]. A difference of ≥5 mm between the zone measurements of the identical discs ± CV were considered to be ESBL-producing species [15,17,18].

Minimal Inhibitory Concentration (MIC) Determination

The MIC values of the previously conducted disc diffusion testing were finally obtained using Micronaut-S beta-lactamase VII kit (Merlin Diagnostika, Germany). A 50 µl of 0.5 McFarland suspension of the isolate was spiked in 10 ml of Mueller Hinton Broth (Merck, Germany), vortexed for a couple of seconds, and 100 µl of this suspension was inoculated into the plate. Following that, the inoculated plate was allowed for an incubation overnight at 37ºC. A Thermofischer Multiskan FC Spectrometer was used for readings. MIC analysis was automatically conducted by MCN6 Software (Sifin, Germany).

RESULTS

This study revealed that a total of 23 ESBL-producing Enterobacteriaceae was detected in a total of 110 red meat samples. The most common ESBL-producing species was determined to be *E. coli* (30%), followed by *C. brakii* (22%), *E. cloacae* (17%), *K. pneumoniae* (9%), *C. freundii* (9%), *S. fonticola* (4%), *K. intermedia* (4%), and *M. wisconsensis* (4%). All the ESBL-combined-disc screening and MIC confirmatory results were presented in *Table 1* and *Table 2*.

Table 1. *ESBL-screening results*
Tablo 1. *GSBL tarama sonuçları*

Type of Isolate	No of ESBL (+) Isolates
E. coli	7
C. braakii	5
E. cloacae	4
K. pneumoniae	2
C. freundii	2
S. fonticola	1
K. intermedia	1
M. wisconsensis	1
Total	23

DISCUSSION

Extended spectrum beta-lactamase (ESBL)-producing Enterobacteriaceae is becoming a worldwide concern for the public health. The different profiles of antibiotic resistance are arised from use of diverse antibiotics [19]. They may adversely affect the treatment based on modern beta-lactam antibiotics against bacterial infections and possibly becoming possibly a source for spread of ESBL-encoding genes among the different and/or the same species. However, epidemiological data related to this biohazard situation are very limited in different geographical regions, including in Turkey. The Standart Commitee of European Doctors and the Federation of Veterinarians of Europe issued a common press release for keeping all the authorities on fighting against ESBL-producing Enterobacteriaceae in 2014. This study was, therefore, the first reporting from Turkey based on the methods used for determination of ESBL-producing enterobacteria occurring from red meats.

The foods sold in Turkey had widely poor microbial quality and represented a potential health risk to customers [20]. Especially, foodborne ESBL bacteria has not been well-examined in various foods from Turkey. Most of the studies related to this field have been limited to the clinical isolates and veterinary medicine. The food-related works were restricted with screening of ESBL-producing bacteria based on only standard disc-approximation testing, including a diverse combination of antibiotics. On the other hand, disc diffusion confirmation testing and MIC testing were not simultaneously conducted, and not supported by auto-analyzers such as Vitek® MS and BD Phoenix instruments.

In Turkey, a study showed that 22.3% of the red meats were positive for ESBL-producing *E. coli*, *C. youngae, E. cloacae* [21], another study determined 26.3% of the red meat samples harbored *E. coli*, *K. pneumoniae*, *K. oxytoca* [22], and the analyzed red meat samples mostly contained ESBL-producing *E. coli* in concordance with our study and other regional findings [23]. The transfer of ESBL-encoding genetic elements between resistant Enterobacteriaceae and non-resistant strains was proved in both animal and human intestinal tractus [24]. All these studies showed that disc-approximation testing was a golden method in detection of resistant bacteria [25]. However, they cannot be used for all species of Enterobacter [26,27].

In this study, ESBL-screening was performed by disc diffusion testing, combined-disc diffusion testing, and disc diffusion confirmation based on MIC determination, including auto-identification of the isolates by mass spectrometer. Our results showed that 60 pre-assumptive isolates were initially determined to be positive for ESBL-production. However, MIC analysis revealed that only 23 (38.3%) of the 60 presumptive isolates were actually real

Table 2. *Disc diffusion and MIC results*
Tablo 2. *Disk tarama ve MİK sonuçları*

Isolate no	Type of Isolate	Zone Diameter (mm)						MIC (µg/ml)						
		CAZ	CAZ CV	CTX	CTX CV	CPD	CPD CV	CAZ	CAZ Reference	CAZ Actual	CTX	CTX Reference	CTX Actual	Result ESBL +/-
1	*E. cloacae*	22	26	23	26	16	17	?	-	=8/4	?	-	=8/4	+
2	*S. fonticola*	26	27	27	28	20	23	S	<=1	<=0.25/4	R	=128	<=0.25/4	+
3	*E. coli*	18	25	13	28	0	21	S	32	≤0.25/4	R	>128	≤0.25/4	+
4	*K. pneumoniae*	21	30	15	31	13	21	R	16	=4/4	R	128	=4/4	+
5	*M. wisconsensis*	19	28	22	32	14	21	R	128	-	R	32	=0.5/4	+
6	*C. freundii*	24	25	24	25	16	20	S	2	1	R	16	=8/4	+
7	*C. braakii*	22	22	15	25	16	20	R	64	<=0.25/4	R	128	=16/4	+
8	*K. intermedia*	21	22	20	22	18	20	S	<=1	<=0.25/4	S	<=1	<=0.25/4	+
9	*E. coli*	15	27	10	28	10	16	R	128	<=0.25/4	R	>128	<=0.25/4	+
10	*E. coli*	11	29	25	27	10	13	R	128	<=0.25/4	S	<=1	<=0.25/4	+
11	*E. coli*	12	18	10	19	7	9	R	=64	=2/2	R	>128	=4/4	+
12	*C. freundii*	18	24	19	22	14	16	R	=64	=8/4	R	=32	=4/4	+
13	*E. cloacae*	20	12	10	9	-	-	S	=4	>32/4	R	=32	>8/4	+
14	*C. braakii*	9	18	17	24	14	16	R	>128	=8/4	R	=128	=8/4	+
15	*C. braakii*	22	14	25	26	16	18	R	=64	=8/4	R	=4	=1/4	+
16	*K. pneumoniae*	16	25	10	24	7	9	R	=32	-	R	>128	=4/4	+
17	*C. braakii*	23	31	10	28	13	16	R	=16	=2/4	R	>128	=8/4	+
18	*E. cloacae*	21	26	21	27	18	19	R	=128	=4/4	R	=128	>32/4	+
19	*E. coli*	15	25	9	25	7	10	R	>128	=0.5/4	R	>128	=0.5/4	+
20	*E. cloacae*	16	22	16	22	6	9	R	=64	-	R	>128	-	+
21	*E. coli*	19	14	27	23	17	19	R	=64	=16/4	R	32	=8/4	+
22	*E. coli*	22	31	21	24	17	18	R	=128	=32/4	R	=64	=4/4	+
23	*C. braakii*	20	27	25	31	18	20	R	=128	<=0.25/4	R	=64	-	+

R=Resistant, ***I***=Intermediate, ***S***=Suspected

ESBL-producers. Therefore, we concluded that there was not still a common agreement on which confirmatory test was the most sensitive. Fast and accurate detection of ESBL-producers are, therefore, important for epidemiological surveillance and infection control of ESBL-producing enterobacteria.

A study in Spain reported that the frequency rate of ESBL-producing bacteria was 25% in the red meat samples, confirmed ESBL-producing *E. coli* as the most common species within the examined red meat samples [28]. Also, some further studies mainly focused on understanding the dissemination ways of ESBL-producing bacteria from numerous sources by epidemiological research [24,25,29]. The epidemiological studies have been arised due to spreadable and transferable properties of ESBL-encoding genetic elements among the same and/or different bacterial species through a diverse of mechanisms of antibiotic resistance. That's why, the studies should be extended for further genotypic testing.

The red meat and red meat derived-products should be supplied to the customers under hygienic conditions because of their importance for the human nutrition as well as including food safety concerns. To date, the number of the studies related to the occurrence of ESBL-producing Enterobacteriaceae are still limited worldwide, including in Turkey.

In conclusion, the results of this study indicated that the red meat samples harbored ESBL-producing Enterobacteriaceae, and they may hold a potential risk for the colonization of the consumers with ESBL-producers. Therefore, antibiotic use in the veterinary medicine should be intensively monitored.

Conflicts of Interest

The authors declare that there is no conflict of interests regarding the publication of this paper.

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