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Determination of Antibiotic Susceptibility Profile and *Intl*1, *bla*SHV and *bla*TEM Genes of Raw Milk Origin Enterobacteriaceae Isolates

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Abstract

In this study, a total of 68 raw milk samples were used to investigate the prevalence of Enterobacteriaceae in milk samples obtained from different dairies and supermarkets in the province of Amasya (Turkey), and to determine the antibiotic resistance profile and presence of the *Intl1*, *bla*TEM and *bla*SHV genes. In this study, isolates were obtained using a classical culture technique. The detection of antibiotic resistance profile was carried out using disc diffusion methods. Twelve different antibiotics, including meropenem, cefotaxime, nalidixic acid, ceftriaxone, chloramphenicol, ceftazidime, streptomycin, ampicillin, gentamicin, tetracycline, levofloxacin and trimethoprim-sulfamethoxazole were employed. Single strain polymerase chain reaction (PCR) was created for the detection of extended spectrum beta-lactamase (ESBLs). For these aims, *bla*TEM and *bla*SHV genes were demonstrated by PCR assay. *Intl*1 was determined by using PCR assay. As a result, 50 isolates that belong to the Enterobacteriaceae family were obtained. A total of 41 (82%), 38 (76%), 7 (14%), 6 (12%), 2 (4%) and 1 (2%) isolates were determined to be resistant to ampicillin, trimethoprim-sulfamethoxol, ceftazidime, cefotaxime, meropenem and nalidixic acid as well as ceftriaxone and streptomycin respectively. In addition, 49 (98%), 50 (100%) 49 (98%) and 50 (100%) isolates were determined to be sensitive to chloramphenicol, gentamicin tetracycline and levofloxacin respectively. Among the Enterobacteriaceae isolates, 22 (44%), 6 (12%) and 2 (4%) rates of strains carried the *Intl*1, *bla*SHV and *bla*TEM genes, respectively. In conclusion, the resistance of Enterobacteriaceae isolates that were isolated from milk samples to many antibiotics poses a potential danger in terms of public health.

Keywords: Enterobacteriaceae, antibiotic resistance, *Intl*1, *bla*TEM, *bla*SHV, raw milk

1. Introduction

Milk is a foodstuff that contains almost all nutrients; it has with a unique taste, smell and consistency in a sufficient and balanced manner [1]. However, pathogenic microorganisms can easily contaminate milk regardless of the hygienic conditions and cause the quality of milk to deteriorate [2]. Species that belong to the Enterobacteriaceae family were frequently isolated from milk [3].

The Enterobacteriaceae family is a large and heterogeneous family of Gram-negative bacilli of medical importance. It is a group of facultative anaerobic, non-spore-forming catalase-positive and glucose-fermented microorganisms that are known to cause various infections such as urinary tract infections, cystitis, pneumonia and bacteremia [3, 4]. Many members of the Enterobacteriaceae family are detected in soil, water, and plants, and a significant proportion of Enterobacteriaceae are detected in the normal intestinal biota of humans and animals. However, there are also members of Enterobacteriaceae that cause hospital and community-borne bacterial infections, especially infections that affect the gastrointestinal system. In addition, Enterobacteriaceae that are detected in various foods cause foods to spoil faster. Although they are associated with fecal contamination, their presence can be seen as an indication that hygiene rules are not observed in post-milking processes as well as an indication environmental contamination [5, 6, 7].

Enterobacteriaceae detected in foods are considered an indicator of hygiene for quality and safety during the

processing of foodstuffs [8]. Members of the Enterobacteriaceae family have an important role in foodborne poisoning [9] Today, food poisoning cases that originate from members of the Enterobacteriaceae family can be fatal. The intense use of antibiotics in medical clinics, animal husbandry, aquaculture and soil cause the selection of resistance genes. Consequently, multidrug resistant (MDR) Enterobacteriaceae emerged [6]. Beta-lactams (mostly extended-spectrum cephalosporins and carbapenems) and fluoroquinolones are among the preferred antibiotic groups in preventing infections caused by Enterobacteriaceae [10]. However, the most important feature of the members of this family is that they produce beta-lactamase enzyme in their body and are resistant to antibiotics that contain beta lactam. Antibiotic resistance increases the cost of treatment of infections caused by bacteria that produce extended-spectrum beta-lactamase (ESBLs). The main genes responsible for ESBL production include TEM (*bla*TEM), SHV (*bla*SHV) and CTX-M (*bla*CTX-M) genes. ESBLs are a group of enzymes that are encoded by genes that are detected on the plasmid that is predominantly common among Enterobacteriaceae. In addition, genes that encode ESBL are associated with mobile genetic elements [11]. Integrons have an important role in the formation of resistance against antimicrobials. In particular, it is associated with the spread of antibiotic resistance to classes 1, 2 and 3 integrons, which are most commonly detected in pathogens. For the continued insertion and deletion of gene cassettes, it is important to obtain the integrase gene (*Intl*), which is an important part of the integrons [12].

Studies in the literature have focused on the investigation of ESBLs in human derived Enterobacteriaceae isolates; however, few studies investigate the presence of ESBLs in Enterobacteriaceae isolates that are isolated from food [4].
For this purpose, this study aimed to determine (i) the antibiotic resistance profiles of Enterobacteriaceae isolates that are isolated from raw milk samples using a classical culture technique, (ii) the molecular characterization of resistance genes (*bla*TEM and *bla*SHV) and (iii) the presence of integrase (*Intl*1) gene of the isolates.

2. Materials and Methods

2.1. Food Samples

A total of 68 raw milk samples were collected randomly from the different dairy farms between April and August in 2018. Milk samples were brought to the laboratory under the cold chain following purchase.

2.2. Bacteria Isolation and Phenotypic Identification

10 mL of samples were taken and 90 mL of buffered peptone water (Merck, Germany, 107228) was added (10^{-1} dilution) and homogenized. Subsequently, decimal dilutions were made and cultivated on Violet Red Bile Glucose Agar (Merck, Germany, 110275) using classic culture technique. Presumptive colonies (1-2 mm diameter dark pink or red colonies surrounded by precipitate zone by the formation of bile acids around the colony) were subcultured onto Tryptone Soy Agar (TSA, Merck, 22091). After incubation, colonies grown on TSA were transferred to Brain-heart Infusion (BHI, Merck, 53286) broth with 15% glycerol for the next analysis and stored at -20 °C [7].

2.3. Antimicrobial Susceptibility Tests

Antibiotic susceptibility properties of the isolates were determined using the disc diffusion method using Mueller Hinton Agar (MHA; Merck, Germany, RM347.20). Resistance profiles of the obtained isolates were tested against 12 different antibiotics. These antibiotic discs (Bioanalyse Ltd., Turkey): meropenem (MEM, ASD05400, 10 µg), cefotaxime (CTX, ASD01800, 30 µg), nalidixic acid (NA, ASD06000, 30 µg), ceftriaxone (CRO, ASD02300, 30 µg), chloramphenicol (C, ASD02800, 30 µg), ceftazidime (CAZ, ASD02100, 30 µg), streptomycin (S, ASD08400, 10 µg), ampicillin (AM-10 µg), gentamicin (CN, ASD04300, 10 µg), tetracycline (TE, ASD08900, 30 µg), levofloxacin (LEV, ASD04800, 5 µg) and trimethoprim / sulfamethoxazole (TMP / STX, ASD09320, 25 µg). For this purpose, the MHA medium was switched after each Enterobacteriaceae isolate subcultured at TSA was adjusted to a degree of turbidity of 0.5 McFarland. After the plates were incubated at 35° C for 24 hours, the zone diameters formed were measured Clinical and Laboratory Standards Institute (CLSI) criteria [13]. *Escherichia coli* ATCC 25922 was used as a positive control.

2.4. Genomic DNA Extraction and Detection of *Intl* 1, *SHV* and *TEM* Genes

DNA was obtained using the boiling method. For this purpose, first Enterobacteriaceae isolates were removed from -20 °C and passaged in a 5% sheep blood agar and incubated at 24 hours 37 °C. A loopful of isolate was suspended in 500 µl of sterile distilled water for DNA isolation. The solution was kept in boiling distilled water for 10 minutes and then centrifuged at 10,000 x g for 5 minutes. The supernatant containing genomic DNA was taken into a fresh tube [14]. The primer sequences used are given in Table 1.

Table 1. Primer sequences used in this study

Target gene	Primer sequence 5′-3′	Reference
***intl* 1 F**	CCT CCC GCA CGA TGA TC	[15]
***intl* 1 R**	TCC ACG CAC TGT CAG GC	
***bla*SHV F**	AAGATCCACTATCGCCAGCAG	[16]
***bla*SHV R**	ATTCAGTTCCGTTTCCCAGCGG	
***bla*TEM F**	GAGTATTCAACATTTCCGTGTC	
***bla*TEM R**	TAATCAGTGAGGCACCTATCTC	

PCR mixture for *Intl* 1 gene; 2.5 mM $MgCl_2$, 1X PCR buffer, 200 µM dNTP, 0.2 µM primer (Forward and Reverse) were completed with 1.25 U Taq Polymerase, 5 µL template DNA and the remaining volume bidistilled water and the final volume was 25 µL. Amplification, 5 minutes pre-denaturation at 94 °C, 30 cycles followed by 1 minute at 94 °C followed by 1.5 minutes of primer at 55 °C, and 1 minute extension at 72 °C and 7 minutes at 72 °C. elongation step was performed (TurboCycler Lite 9020, Blue-Ray, Biotech). PCR products were carried out in an electrophoresis device with 1.5% agarose gel at 90 V for 60 minutes and bands corresponding to 280 bp were evaluated as positive for the *Intl* 1 gene [14, 15, 16].

Mixture of PCR components for bla_{SHV} and bla_{TEM} genes: 2.5 mM $MgCl_2$, 1X PCR buffer, 200 µM dNTP, 0.2 µM primer (Forward and Reverse) were completed with 1.25 U Taq Polymerase, 5 µL template DNA and the remaining volume bidistilled water and the final volume was 25 µL. The thermal reactions were performed as follows: the primary denaturation for 5 min at 95 ºC; followed by 40 cycles as 30 sec for 94 ºC, 30 sec at annealing temperature (58 and 56 ºC respectively), and 30 sec at 72 ºC; and the final extension phase for 5 min at 72 ºC. Amplified samples were done electrophoresis on 2% agarose gel in TBE (Trisma base, Boric acid, EDTA) buffer at 90 V for 60 minutes.

3. Results and Discussion

The presence and number of enteric pathogens that may pose a health hazard to consumers is always taken as an absolute index of fecal contamination of milk and its products. This index is an indication of the lack of health measures during milk production and processing [8]. In this study, 68 raw milk samples that are presented for consumption in Amasya within a period of 4 months were analyzed by a phenotypic method (classical culture technique), and 50 isolates that belong to the Enterobacteriaceae family were obtained. The presence of a high rate of Enterobacteriaceae in the milk samples is consistent with other studies in the literature. The isolates in the milk samples can be attributed to their access to milk during milking in the animal's environment, skin and udders [8, 10, 17]. This situation shows the necessity of taking measures to improve milking and product processing hygiene in farms where milk samples are obtained.

There are reports of high-level antibiotic resistance in community and hospital acquired Enterobacteriaceae species in Turkey [14, 16, 18]. In the last few years, antibiotic-resistant ESBL-producing Enterobacteriaceae have been reported in patients hospitalized in various hospitals in Turkey [19]. Nevertheless, few studies address the antibiotic resistance profile of Enterobacteriaceae species and ESBL production in food animals. Intense and indiscriminate use of antibiotics in animal production has led to the emergence of many antibiotic-resistant strains, including ESBL production in animals. Therefore, animals not only act as a reservoir but also directly or indirectly transmit these pathogens to humans. For this reason, antibiotic resistance is important in food samples. Antibiotic resistance profiles of Enterobacteriaceae isolates in this study are shown in Table 2.

Table 2. Results of antimicrobial resistance rate of Enterobacteriaceae isolates

Antibiotics	R (n) %	I (n) %	S (n) %
MEM (10 µg)	2/50 (%4)	-	48/50 (%96)
CTX (30 µg)	6/50 (%12)	2/50 (%4)	42/50 (%44)
NA (30 µg)	2/50 (%4)	4/50 (%8)	44/50 (%88)
CRO (30 µg)	1/50 (%2)	3/50 (%6)	46/50 (%92)
C (30 µg)	-	1/50 (%2)	49/50 (%98)
CAZ (30 µg)	7/50 (%16)	3/50 (%6)	40/50 (%80)
S (10 µg)	1/50 (%2)	3/50 (%6)	46/50 (%92)
AM (10 µg)	41/50 (%82)	-	9/50 (%18)
CN (10 µg)	-	-	50/50 (%100)
TE (30 µg)		1/50 (%2)	49/50 (%98)
LEV (5 µg)	-	-	50/50 (%100)
TMP/STX (1,25/23,75 µg)	38/50 (%76)	10/50 (%20)	2/50 (%4)

*MEM: Meropenem, CTX: Cefotaxime, NA: Nalidixic acid, CRO: Ceftriaxone, C: Chloramphenicol, CAZ: Ceftazidime, S: Streptomycin, AM: Ampicillin, CN: Gentamycin, TE: Tetracycline, LEV: Levofloxacin, TMP/STX: Trimethoprim-sulfamethoxazole. R: Resistant, I: Intermediate, S: Sensitive

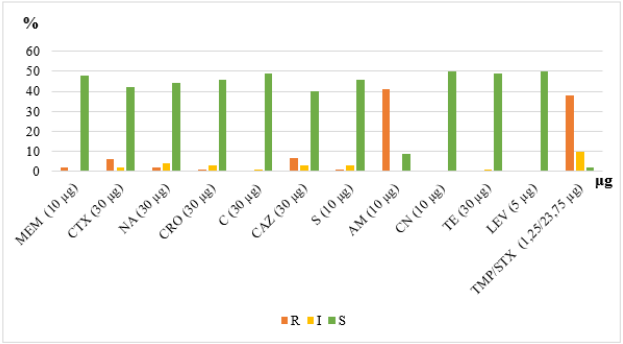

Figure 1. Antibiotic resistance profile of Enterobacteriaceae isolates (R= resistant, I =intermediate, S= Sensitive)

It was determined that 50 (100%) of the isolates were susceptible to levofloxacin and gentamicin; 49 (98%) of the isolates were susceptible to chloramphenicol and also tetracycline; and 48 (96%) of the isolates were susceptible to meropenem (Fig 1).

In contrast to our results, which showed that 76% of Enterobacteriaceae isolates were resistant to trimethoprim-sulfamethoxazole, Vitas et al. found that only 26.5% of Enterobacteriaceae isolates from chicken were resistant to trimethoprim-sulfamethoxazole, and Seedy et al found that 97.6% of Enterobacteriaceae isolates from milk were susceptible to trimethoprim-sulfamethoxazole [12, 18]. Some Enterobacteriaceae strains isolated from raw milk samples exhibited multiple resistance profiles against different antibiotic groups similar to finding reported by other authors [22].

When the isolates in this study were evaluated in terms of multiple antibiotic resistance, the following resistance outcomes were observed 2 (4%) isolates were resistant to 5 different antibiotics (Meropenem, Ceftriaxone, Streptomycin, Ampicillin, and Trimethoprim-sulfamethoxazole); 1 (2%) isolate was resistant to 5 different antibiotics (Ceftazidime, Ampicillin, Trimethoprim-sulfamethoxazole, and Cefotaxicin); 1 (2%) isolate was resistant to 4 different antibiotics (Streptomycin, Nalidixic acid, Ampicillin, and Trimethoprim-sulfamethoxazole), 2 (4%) isolates were resistant to 3 different antibiotics (Ceftriaxone, Ampicillin, and Trimethoprim-sulfamethoxazole) and 1 (%2) isolate was resistant antibiotics to 3 different antibiotics (Tetracycline, Ampicillin, and Trimethoprim-sulfamethoxazole) (Table 3). Increasing resistance to antibiotics in environmentally derived bacteria due to intensive use of antibiotics or contamination of their waste into the environment is an important problem that threatens public health [23, 24].

Table 3. Distribution of multiple drug resistant Enterobacteriaceae isolates

Number of isolates	Ratio (%)	Number of antibiotics	Antibiotics names
2	4	5	Meropenem, Ceftriaxone, Streptomycin, Ampicillin, Trimethoprim/sulfamethoxazole
1	2	5	Ceftazidime, Ampicillin, Trimethoprim/sulfamethoxazole, Cefotaxime, Nalidixic acid
1	2	4	Streptomycin, Nalidixic acid, Ampicillin, Trimethoprim/sulfamethoxazole
2	4	3	Ceftriaxone, Ampicillin, Trimethoprim-sulfamethoxazole
1	2	3	Tetracycline, Ampicillin, Trimethoprim-sulfamethoxazole

Antibiotic resistance genes are transferred by the horizontal transfer of plasmids, transposons, and integrons [25]. In this study, among the studied strains, 22 isolates (44%) contained *Int*1 while 28 isolates (56%) were negative for the *Int*1 gene. In addition, all isolates that carry the *Int*1 gene were determined to be resistant to ampicillin and trimethoprim-sulfamethoxazole. Thongkao and Sudjaroen, investigated whether a correlation exists between the presence of the *Int*1 gene and antibiotic resistance acquisition by the Enterobacteriaceae isolate [26]. Similar to our study, they found that all isolates with the positive *Int*1 gene were also resistant to ampicillin. Among the 22 isolates with the *Int*1 gene, 7 isolates were MDR. In the light of this information, antibiotic resistance properties of Enterobacteriaceae isolates can be associated with a class 1 integron.

One of the most important consequences of the introduction of beta-lactams is the plasmid mediated spread of beta-lactamases. It is reported that TEM-1 is the most common plasmid-mediated enzyme in the Enterobacteriaceae species. It has been reported that this enzyme is frequently encountered in ampicillin-resistant isolates [25]. In this study, all 50 isolates were PCR performed to detect beta-lactamase genes. blaSHV and *bla*TEM genes were detected in 2 (4%) isolates and 6 (12%) isolates, respectively. In addition, all *bla*TEM positive isolates were determined to be resistant to ampicillin. In China a study by Ye et al. revealed that the frequency of *bla*TEM and *bla*SHV were 81.9%, 38.9%, respectively [4]. In another study of Turkey by Pehlivanoğlu et al. the frequency of *bla*TEM and *bla*SHV were 77.4% and 9.7%, respectively [26]. In a survey performed by Shahid et al. the frequencies of *bla*SHV and *bla*TEM among ESBL-producing strains were 13.7%, and 10.9%, respectively [17]. In research in Turkey, Tekiner and Özpınar showed that 20% of the isolates were positive for both *bla*SHV and *bla*TEM [19].

4. Conclusion

These results show that the prevalence of *bla*TEM and *bla*SHV genotypes is high in ESBL-producing Enterobacteriaceae isolates. Considering the data obtained in this study, it is seen that the isolates carrying the *bla*SHV, *bla*TEM and *Int*1 encoding genes are interrelated. Accurate detection of food pathogens that originate from MDR Enterobacteriaceae and the increasing prevalence of beta-lactamase and determination of antibiotic susceptibility patterns are important. To prevent the emergence of drug-resistant strains, attention should be paid to the use of antibiotics in the growth and feeding conditions of food animals.

Author's Contributions

Ceren Başkan: drafted the manuscript, compiled information from the literature, and designed the figures and tables.

Ethics

There are no ethical issues in the publication of this manuscript.

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