PHENOTYPIC DETERMINATION OF ESBL- and AmpC-PRODUCING ENTEROBACTERIACEAE IN CHEESE SAMPLES

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Abstract

The off-label over use of antibiotics results in development of antibiotic resistance in the bacteria. Beta-lactamase producing Enterobacteriaceae adversely affects the human health by leading to therapeutic failures against infections. Although microbiological criteria have been considered appropriate to the Food Codex, an inspection for antibiotic-resistant enterobacteria has not come into force yet. Therefore, the detection of foodborne beta-lactamases has gained significant importance for the human health. The objective of this study was to determine ESBL- and AmpC- producing *Enterobacteriaceae* in cheese phenotypically. In this study, a total of 83 cheese samples was examined by performing pre-enrichment, enrichment on selective media, and oxidase test according to the Criteria by ISO/DIS21528-2 microbiologically. Based on the microbiological results, a total of 18 isolates, including *Klebsiella pneumoniae* (27.8%), *Hafnia alvei* (27.8%), *Escherichia coli* (22.2%), *Klebsiella oxytoca* (11.2%), *Enterobacter cloacae* (5.5%), and *Citrobacter* spp. (5.5%) was identified by mass spectrometer. The phenotypic characterization of beta-lactamase type was conducted by disc diffusion, disc diffusion confirmation, and MIC determination according to the Guidelines of Clinical and Laboratory Standards Institute. The phenotypic results revealed that the most common beta-lactamase type was determined as ESBL in 9 isolates, followed by ESBL & AmpC in 4 isolates, and AmpC in 5 isolates, respectively. In conclusion, our study showed that ESBL- and Amp- type beta-lactamases were the most common phenotypes in Enterobacteriaceae from cheese. The cheese

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samples containing ESBL- and Amp- positive bacteria significantly presented a health risk for the consumers.

Keywords: *Antibiotic resistance, beta-lactamase, cheese, Enterobacteriaceae, food safety, public health.*

1. Introduction

The Global consumption of antibiotics in food-animals for growth promotion and disease prevention is twice that of humans [1]. The use of antibiotics cannot be controlled effectively due to economic concerns of the animal farming sector largely ignoring risks associated with human and animal health. Therefore, foods of animal origin are under suspicion for being transmission vectors for colonization and infection of the humans with antibiotic resistant bacteria [2].

Beta-lactamases are the most prevalent mechanism of antibiotic resistance that inactivate beta-lactam antibiotics, including penicillins, cephalosporins, and monobactams [3,4]. These enzymes are encoded by an extrachromosomal DNA fragment called plasmid. A plasmid can genetically be transferred between the same and/or different bacteria [5]. The beta-lactamases currently receiving the most attention are documented as extended spectrum beta-lactamases (ESBL) and aminopenicillin-deactivating cephalosporinase (AmpC), respectively [6].

The resistance to beta-lactams has been identified in the family of *Enterobacteriaceae*, including *Klebsiella* spp., *Escherichia* (*E.*) *coli*, *Proteus* spp., *Enterobacter* spp., *Citrobacter* spp., and *Salmonella* spp. [7,8]. But, the patterns of resistance vary among the species [9]. The recent studies have indicated that E. coli has gained increasingly beta-lactam resistance, and frequently observed in foods of animal origin [10]. However, their impact on the human health still remains incomplete across the World, including Turkey [11,12]. In this study, we determined ESBL- and AmpC-producing *Enterobacteriaceae* in cheese samples phenotypically.

2. Material and Methods

Reference cultures

An ESBL positive strain *K. pneumoniae* ATCC 700603 and an ESBL negative strain *E. coli* ATCC 25922 were used for control testing, respectively.

Food samples

During the year 2014, a total of 83 cheese samples was randomly collected from public bazaars and food chain markets located in İstanbul. All samples were put into sterile sampling bags, and taken to the laboratory in a sample carry case (JPB, UK) at 4ºC. The microbiological evaluation was started in the same day.

Microbiological evaluation

25 g of cheese in 225 mL of *Enterobacteriaceae* Enrichment Broth (LABM, UK) was homogenized in a sterile bag (Interscience, France) for 2 min by a stomacher (EasyMix, France). The suspension was then incubated at 37°C for 18-24 h under aerobic condition. After that, 10 µL of the suspension was directly streaked onto an ESBL selective media (Liofilchem, Turkey) by a sterile loop. The plate was again incubated at 37°C for 18-24 h under aerobic condition. The colonies were then sub-cultured onto Tryptic Soy Agar (Merck, Turkey), and allowed for incubation at 37ºC for 18-48 h. The green-colored colonies indicated ESBL-positive *K. pneumoniae*, whereas pink-violet-colored colonies indicated ESBL-positive *E. coli* according to the manufacturer's instructions. The suspected isolates were tested for oxidase activity by Bactident Oxidase Testing Kit (Merck, Turkey). Finally, oxidase negative colonies were identified by a mass spectrometer (Vitek® MS bioMérieux, France).

Disc screening and confirmation of ESBL suspicious isolates

After identification, the isolates were suspended in a sterile salt solution (0.85% NaCl) to 0.5 McFarland-standardized density by a densitometer (bioMérieux, France). After that, they were transferred onto Mueller–Hinton agar (Liofilchem, Turkey) using sterile swabs. Cefpodoxime (CPD; 10 µg), cefotaxime (CTX; 30 µg), and ceftazidime (CAZ; 30 µg) containing antibiotic discs (CPD10 Mast Group, UK) were placed on the plate. Disc diffusion confirmation test was performed by a

combination of CPD, CTX, and CAZ±Clavulanate (CLA, 10 µg) (D67C MAST Group). The disc inserted plates were then incubated at 37°C for 18-24 h. The breakpoints with zone diameters and zones of inhibition were evaluated according to the criteria described by the Guidelines of CLSI (2013) [13].

Antimicrobial susceptibility based on minimal inhibitory concentration (MIC)

MIC determination was performed for ESBL- and AmpC-type beta-lactamases according to the manufacturer's instructions on Micronaut-S Beta-Lactamase VII plate (Merlin Diagnostika, Germany). A 50 µL aliquot of 0.5 McFarland-standardized suspension of the isolate was vortexed in 10 mL of Mueller Hinton Broth (Merck, Germany).

After that, 100 µL of this suspension was pipetted into each well of the 96-well plate, followed by an incubation at 37ºC overnight. The plates were then measured by ThermoScientific™ Multiskan FC spectrometer. The readings were automatically analyzed by the MCN6 Software (Sifin, Germany).

3. Results

Microbiological results

A total of 83 cheese was microbiologically examined according to the Criteria by ISO/DIS21528-2. A total of 18 isolates, including *Klebsiella pneumoniae* (27.8%), *Hafnia alvei* (27.8%), *Escherichia coli* (22.2%), *Klebsiella oxytoca* (11.2%), *Enterobacter cloacae* (5.5%), and *Citrobacter* spp. (5.5%) was identified by mass spectrometer (Figure 1).

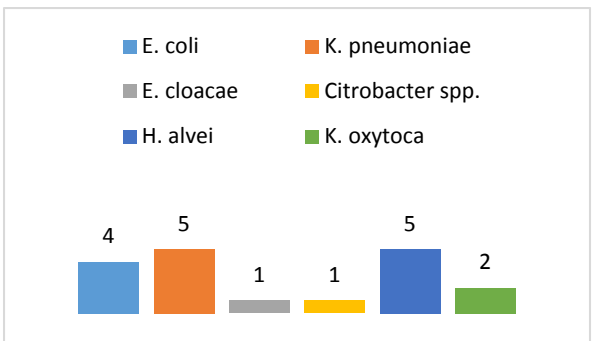

Figure 1. Types of ESBL- and AmpC-positive isolates based on cheese samples

4. *Phenotypic results*

The phenotypic characterization of ESBL- and AmpC- type beta-lactamases was conducted by disc diffusion, disc diffusion confirmation, and MIC determination, respectively according to [13]. The phenotypic results revealed that the most common beta-lactamase type was determined as ESBL in 9 isolates (5 *K. pneumoniae*, 2 *E. coli*, and 2 *K. oxytoca*), followed by ESBL &

AmpC in 4 isolates (2 *E. coli*, 1 *H. alvei*, and 1 *Citrobacter* spp.), and AmpC in 5 isolates (4 *H. alvei* and 1 *E. cloacae*), respectively. All the phenotypic results were presented in Table 1, Figure 1, Figure 2 and Figure 3.

Table 1. Species-based distribution of ESBL- and AmpC- positive isolates

Type	ESBL	ESBL and Amp C	Amp C	Total
K. pneumoniae	5	-	-	**5 (27.8 %)**
H. alvei	-	1	4	**5 (27.8 %)**
E. coli	2	2	-	**4 (22.2 %)**
K. oxytoca	2	-	-	**2 (11.2 %)**
E. cloacae	-	-	1	**1 (5.5%)**
Citrobacter spp.	-	1	-	**1 (5.5%**
				)
Total	**9 (50.0 %)**	**4 (22.2 %)**	**5 (27.8 %)**	**18 (100 %)**

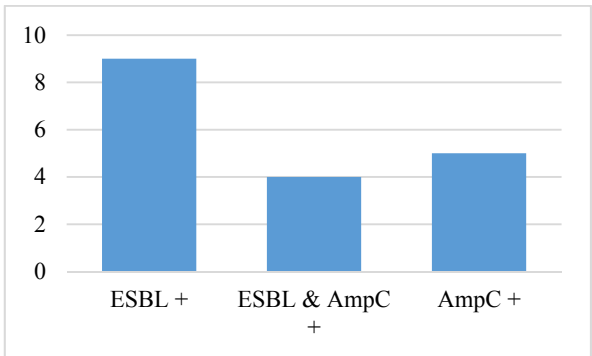

Figure 2. Distribution of ESBL- and AmpC type beta-lactamases in cheese samples

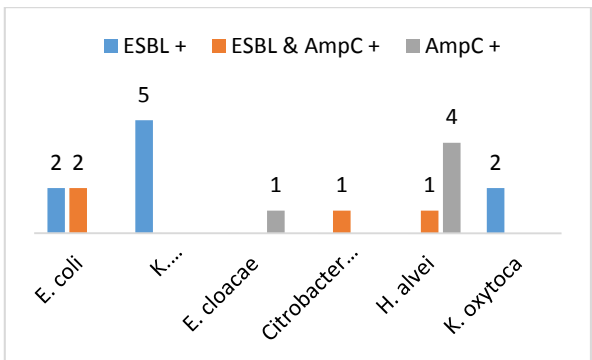

Figure 3. Dstributionj of beta-lactamases based on the type of Enterobacteriaceae

The average zone differences of CAZ±CVA, CTX±CVA and CPD±CVA were 26.3±3.2 mm, 28.6±7.4 mm and 24.4±11.7 mm in ESBL+ isolates, respectively, and 22.0±10.0 mm, 23.0±12.0 mm and 19.0±13.0 mm in

ESBL&AmpC+ isolates, respectively, while 15.5±1.9 mm for CPD±CVA in AmpC+ isolates.

Of 9 ESBL+ isolates, 2 were resistant to CTX (≥128 µg/mL), 2 to CAZ (MIC=16 µg/mL), 1 to COX (>32 µg/mL), 2 to CEP (=64 µg/mL), 1 to MER (MIC=8 µg/mL), and 1 to CMC (≤0.25/4 µg/mL).

Among ESBL & AmpC positive isolates, 4 were resistant to CTX (≥128 µg/mL), 4 to CAZ (MIC=16 µg/mL), 1 to MER (=64 µg/mL), 2 to COX (>32 µg/mL), 1 to ERT (>1 µg/mL), 3 to CEP (>128 µg/mL), and 2 to CMC (≤0.25/4 µg/mL), respectively.

The AmpC positive isolates showed resistance to CTX (=8 µg/mL) in 3, to CAZ (≥16 µg/mL) in 1, COX (≥32 µg/mL) in 4, and ERT (>1 µg/mL) in 1, respectively.

4. DISCUSSION

In this study, ESBL- and AmpC- type beta-lactamases were characterized in a total of 18 isolates phenotypically, including *K. pneumoniae* (27.8%), *H. alvei* (27.8%), *E. coli* (22.2%), *K. oxytoca* (11.2%), *E. cloacae* (5.5%), and *Citrobacter* spp. (5.5%) was microbiologically detected. The phenotypic results revealed that the most common beta-lactamase type was determined as ESBL in 9 isolates (5 *K. pneumoniae*, 2 *E. coli*, and 2 *K. oxytoca*), followed by ESBL & AmpC in 4 isolates (2 *E. coli*, 1 *H. alvei*, and 1 *Citrobacter* spp.), and AmpC in 5 isolates (4 *H. alvei* and 1 *E. cloacae*), respectively.

Many antibiotics that were formerly effective against bacterial infections are no longer effective because of resistant strains [14]. Off-label over use of antibiotics has fueled exchange of resistance-coding genetic elements making a bacteria resistant to antibiotics [15,16]. This situation contributes to circulation of antibiotic-resistant strains and resistance-coding genes among humans, animals, food, water and the environment [17]. The average consumption rate of antibiotics per kilogram for food animal produced annually will globally increase nearly double by 2030 [1,18]. By 2050, the infections associated with antibiotic resistant bacteria could kill 10 million people a year all over the World with a burden of $100 trillion: more than the size of the current World economy [19]. Despite of these facts, there is not actual data about the use of antibiotics in food animals in Turkey [17]. The related studies from Turkey in this area

are quite limited [18,20,21]. Our study, therefore, contributed to an underestimation of the antibiotic resistance patterns in foodborne Enterobacteriaceae.

The foods of animal origin easily gets contaminated by enterobacteria [22]. Their unhygienic consumption could be an important health issue in terms of food safety and antibiotic resistance. But, it should be essentially free from Enterobacteriaceae, including the resistant ones [23,24]. The beta-lactamase producing Enterobacteriaceae are considered as major agents of many foodborne infections, and confer to penicillins, 1st, 2nd and 3rd-generation cephalosporins, and aztreonam [25,26]. These strains may contaminate foods, and so colonize in the intestinal tract, or exchange their resistance-coding genes with commensal bacteria of the humans [27]. The recent studies indicated that beta-lactamases observed in human and foods of animal origin were the same to each other [28].

In this study, we phenotypically detected ESBL- and/or AmpC-type beta-lactamases in *K. pneumoniae, H. alvei*, *E. coli*, *K. oxytoca*, *E. cloacae*, and *Citrobacter* spp. The frequency rates of the beta-lactamase positive phenotypes in cheese were similar to Belgium [29], Germany [30], China [31], Holland [23], Poland [32], and Denmark [33], respectively. According to the Ministry of Health in Turkey (www.uhes.saglik.gov.tr), the antibiotic resistance patterns from clinical isolates have spread particularly in *E. coli* (33.2% in 2008 and 48.83% in 2013) and *K. pneumoniae* (40% in 2008 and 49.69% in 2013). But, the rapidly increasing frequency rate of beta-lactamase positive enterobacteria could be a result of the foods of animal origin, and this suspected risk factor has not been seriously addressed so far in Turkey [34]. Therefore, our study is extremely important for the purpose of detecting the presence of resistant bacteria in cheese.

AmpC-type beta-lactamase is associated with multiple antibiotic resistances, leaving limited therapeutic options [35]. In our study, we detected AmpC- production in *E. cloacae* and *H. alvei*. If raw milk flavor is required, the best culture to add is H. alvei. However, we determined that a flavoring food culture could even be resistant to anbiotics. Because co-existence of ESBL- and AmpC- is a growing concern all over the world [15]. Failure to detect these multi-resistance

pattern has contributed to their uncontrolled spread [2].

The MIC results revealed that ESBL positive isolates were resistant to CTX, CAZ, COX, CEP and CMC while ESBL & AmpC positive ones were resistant to CTX, CAZ, MER, COX, ERT, CEP and CMC. As seen the documented antibiotic agents, a co-existing pattern of ESBL with AmpC suggested two different agents, including MER and ERT. For alone AmpC producers, the antibiotic agents were CTX, CAZ, COX and ERT. All these beta-lactam agents are of importance in veterinary medicine [36]. Our results showed that the resistance patterns of these isolates were remarkable [29].

The detection of ESBL co-presence with AmpC in an isolate with ERT susceptibility could be considered as one of the indicators of KPC activity. But, we could not detect it by MIC determination. This means that carbapenem non-susceptible ESBL isolate is a potent problem in the future if it is not precisely detected.

5. CONCLUSIONS

Even though important food safety-indicator microorganisms are routinely checked by legal authorities based on directives, the inspection for antibiotic-resistant enterobacteria has not come into force yet [37,38]. There have been multiple studies reporting the spread of resistant bacteria from animals to humans through food [39]. Each transmission may not cause an illness, but it is still extremely important in mediating the spread of resistance-coding genes to humans [21].

In conclusion, our study revealed that ESBL- and Amp- were the most common phenotypes in Enterobacteriaceae from cheese samples, presenting a foodborne health risk for the consumers. Accordingly, excessive and/or unconscious use of antibiotics in farming animals should be considered, but there also is a need for advanced molecular studies to understand whether resistant bacteria are transferred from animals to humans or the other way around.

CONFLICTS OF INTEREST

The authors declare that there is no conflicts of interest.

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