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Prevalence and antibiotic resistance of extended-spectrum beta-lactamase (ESBL) producing *Escherichia coli* and *Klebsiella* species isolated from foods of animal origin in Turkey

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The present study includes 50 *Klebsiella oxytoca*, 45 *Escherichia coli* and 13 *Klebsiella pneumoniae* isolated from raw meat (chicken drumsticks and minced meat), raw milk, white cheese and ice cream samples and was undertaken to determine the prevalence of extended-spectrum β-lactamase (ESBL)-producing isolates and antibiotic resistance. The double-disk synergy test was used to determine ESBL production. ESBL production was detected in 20 of 45 *E. coli* (44.4%), 5 of 13 *K. pneumoniae* (38.5%) and 13 of 50 *K. oxytoca* (26%) isolates. Resistance of all isolates to 14 antibiotics was determined by the disk diffusion method. All isolates showed resistance to ampicillin but none exhibited resistance to imipenem, ertapenem, cefepime and piperacillin/tazobactam. *E. coli* and *Klebsiella* spp. isolates have also been found resistant to cefotaxime, ceftazidime, ceftriaxone, aztroenam, tetracycline and ciprofloxacin. All isolates were resistant to two or more antimicrobial agents.

Key words: β-Lactamases, antibiotic resistance, *Escherichia coli*, *Klebsiella* spp., foods.

INTRODUCTION

Enterobacteriaceae are the significant causes of serious infection, and many of the most important members of this family are becoming increasingly resistant to currently available antimicrobials. Two organisms of concern are *Escherichia coli* and *Klebsiella pneumoniae*, an opportunistic pathogens of humans and animals responsible for a wide range of infections, such as urinary tract infections, pneumoniae, wound infections and septicemia (Slama et al., 2010). *Klebsiella oxytoca* was reported as an enterotoxigenic microorganism and causes hemorrhagic colitis (Gundogan et al., 2011). *E. coli* and *Klebsiella* species are commonly found in the environment and the gastrointestinal tracts of a wide range animals (Haryani et al., 2007), especially raised for human consumption. Worldwide studies have revealed that they can contaminate food of animal origin and contribute to disease and spoilage (Gundogan and Yakar, 2007; Haryani et al., 2007). Common sources of *E. coli* and *Klebsiella* are feces (of animal and human origin), personnel, water and containers (Slama et al., 2010). Extended-spectrum β-lactamases (ESBLs) are enzymes that are often plasmid mediated and confer broad resistance to penicillins, cephalosporins and monobactams. Because ESBL-producing strains often exhibit multidrug resistance, including resistance to aminoglycosides and fluoroquinolones, the therapeutic options associated with these strains are fairly limited. Resistance to beta lactam antibiotics is most commonly found in *E. coli* and *K. pneumoniae*, and today, this resistance mechanism is recognized globally (Jarlier et

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Table 1. Prevalence of *Klebsiella* spp., and *E. coli* isolates in food samples.

Sample	Number of samples	*K. oxytoca*	*K. pneumoniae*	*E. coli*
		n (%)	n (%)	n (%)
Calf meat, minced	15	8 (53.3)	3 (20)	9 (k20)
Chicken drumsticks	15	8 (53.3)	-	15 (33.3)
Raw milk	15	12 (80)	2 (13.3)	7 (15.5)
White cheese	15	9 (60)	7 (46.7)	11 (24.4)
Ice cream	15	13 (86.7	1 (6.7)	3 (6.7)
Total	75	50 (66.7)	13 (17.3)	45 (60)

al., 1988).

Food animals are increasingly recognized as a reservoir for ESBL-producing strains. These strains can be transmitted via the food chain. Fecal contamination might occur during animal slaughtering, milking, and/or processing, and the growth of the contaminating bacteria may occur during the product transport and storage phases. Consequently, without good hygienic practices, foods may act as a vehicle of transfer of β-lactam-resistant bacteria to the gastrointestinal tract of consumers (Overdevest et al., 2011). Some recent studies have documented frequent occurence of ESBL-producing *E. coli* isolates in poultry (Kolar et al., 2010; Overdevest et al., 2011) and ESBL-producing *Klebsiella* isolates in dairy and meat products (Gundogan and Yakar, 2007; Gundogan et al., 2011). The present study aimed to assess the occurence of *E. coli* and *Klebsiella* species in different types of meat and milk products consumed in Ankara, Turkey, as well as to investigate the antimicrobial resistance patterns and the prevalence of ESBL production by these bacteria.

MATERIALS AND METHODS

Sample collection

In this study, 15 samples of raw calf meat (minced), 15 samples of chicken drumsticks, 15 samples of raw milk, 15 samples of ice cream and 15 samples of Turkish white cheese were collected from various supermarkets, dairy plants and pastry shops in Ankara, Turkey, between July 2010 and March 2011. White cheese and meat samples were collected in sterile polyethylene packs and milk samples were collected in disposable plastic bottles, transported on ice to the laboratory, and analyzed within 2 h. Ice cream samples were collected in sterile jars and transported to the laboratory in a deep freezer and stored at -18°C. All samples were analyzed within 1 h after collection.

Isolation and identification

Meat and cheese samples were weighed into sterile stomacher bags, diluted with 225 ml of 1% sterile peptone water (Merck, Darmstadt, Germany), and homogenized for 10 min using a stomacher (Lab Lemco 400, Seward, Worthington, UK). Milk samples were diluted with 1% sterile peptone water. Ice cream samples were diluted with sterile saline. From each prepared sample, 0.1 ml was evenly spread on plates of eosin methylene blue (EMB) agar (Oxoid). For enrichment purposes, 0.1 ml of each sample of preparation was inoculated in tryptic soy broth. The inoculated media were incubated aerobically at 37°C for 24 to 48 h. Suspicious *Klebsiella* (large, viscid, dome-shaped, brownish) and *E. coli* (dark centred and flat, with or without metallic sheen) colonies were picked from the EMB plates and restreaked on fresh EMB agar to purify. After a 24 h incubation at 37°C, pure isolates were obtained. Such pure isolates were identified by colony and cell morphology, gram stain, oxidase and catalase activity, and indol reaction. Gram-and oxidase-negative rods were selected for further identification using the BBL® Crystal™ ENF system (Becton Dickinson and Company, Maryland, USA) (Dogru et al., 2010).

Antimicrobial susceptibility test

Antimicrobial susceptibility tests were performed as recommended by the Clinical and Laboratory Standards Guidelines (CLSI, 2006) on Mueller Hinton agar plates (Oxoid), using *E. coli* ATCC 25922 and *K. pneumoniae* ATCC 700603 as control strains. All disks for disk diffusion testing were obtained from Oxoid in the following concentrations: ampicillin (10 µg), cefotaxime (30 µg), ceftazidime (30 µg), ceftriaxone (30 µg), cefepime (30 µg), aztroenam (30 µg), imipenem (10 µg), ertapenem (10 µg), amoxicillin/clavulanic acid (30 µg), piperacillin-tazobactam (110 µg), gentamicin (30 µg), amikacin (30 µg), tetracyline (30 µg) and ciprofloxacin (5 µg).

Detection of ESBL by a double disk synergy technique

ESBL production was screened using a double disk synergy test as a standard disk-diffusion assay on Mueller-Hinton agar plates. The amoxicillin/clavulanic acid (30 µg) was placed in the center of the plate, and the following disks of β-lactam antibiotics were placed 30 mm apart from the center in order to observe the synergistic effect: cefotaxime (30 µg), ceftazidime (30 µg), cefepime (30 µg) and aztreonam (30 µg). ESBL presence was confirmed by demonstrating synergy between cephalosporin with reduced halo and clavulanic acid (Jarlier et al., 1988). *E. coli* NCTC 10418 was used as an ESBL-negative control, and *K. pneumoniae* ATCC 700603 was used as an ESBL-positive control.

RESULTS

The prevalence, antibiotic resistance and ESBL production of *Klebsiella* spp. and *E. coli* are given in Tables 1, 2 and 3. During the study period, 50 *K. oxytoca*, 45 *E. coli* and 13 *K. pneumoniae* isolates were recovered

Table 2. Number and percentage of *Klebsiella* spp. and *E. coli* isolates from raw meat (chicken and minced meat), raw milk, white cheese and ice cream samples that exhibited resistance to antimicrobial agents.

Antimicrobial agent	*K. oxytoca* (n = 50)	*K. pneumoniae* (n = 13)	Total *Klebsiella* (n = 63)	*E. coli* (n = 45)
Ampicillin	50 (100)	13(100)	63 (100)	45 (100)
Cefotaxime	9 (18)	2 (15.5)	11 (17.5)	15 (33.3)
Ceftazidime	6 (12)	1 (7.7)	7 (11.1)	4 (8.9)
Ceftriaxone	8 (16)	3 (7.7)	11 (17.5)	4 (8.9)
Cefepime	0	0	0	0
Aztroenam	24 (48)	3 (23.1)	27 (42.9)	13 (28.9)
Imipenem	0	0	0	0
Ertapenem	0	0	0	0
Amoxicillin/clavulanic acid	2 (4)	0	2 (3.1)	3 (6.7)
Piperacillin/tazobactam	0	0	0	0
Gentamicin	4 (8)	0	4 (6.3)	3 (6.7)
Amikacin	3 (6)	0	3 (4.8)	2 (4.4)
Tetracycline	35 (70)	9 (69.2)	44 (69.8)	35 (77.8)
Ciprofloxacin	12 (24)	3 (23.1)	15 (23.8)	14 (31.1)

Table 3. The number and percentage of ESBL-producing *Klebsiella* spp., and *E. coli* strains in raw meat (chicken and minced meat), raw milk, white cheese and ice cream samples.

Bacterial species	Chiken meat no. (%)	Minced meat no. (%)	Cheese no. (%)	Milk no. (%)	Ice cream no. (%)
E. coli (n = 20)	10 (50)	5 (25)	2 (10)	2 (10)	1 (5)
K. pneumoniae (n = 5)	-	1 (20)	2 (40)	1 (20)	1 (20)
K. oxytoca (n = 13)	5 (38.4)	4 (30.8)	2 (15.3)	2 (15.3)	-
Total (38)	15 (39.5)	10 (26.3)	6 (15.8)	5 (33.3)	2 (5.2)

from 75 samples of minced meat, chicken meat, raw milk, white cheese and ice cream. *Klebsiella* spp., and *E. coli* isolates isolated from minced meat, chicken meat, raw milk, white cheese and ice cream samples were analyzed in terms of their resistance to various types of antibiotics. *Klebsiella* spp. isolates have been found resistant to ampicillin (100%), tetracycline (69.8%), aztroenam (42.9%), ciprofloxacin (23.8%), cefotaxime (17.5%), ceftriaxone (17.5%), ceftazidime (11.1%), gentamicin (6.3%), amikacin (4.8%) and amoxcillin/clavulanic acid (3.1%). All isolates were susceptible to cefepime, imipenem, ertapenem and piperacillin/tazobactam. *E. coli* isolates were resistant to ampicillin (100%), tetracycline (77.8%), cefotaxime (33.3%), ciprofloxacin (31.1%), aztroenam (28.9%), ceftazidime (8.9%), ceftriaxone (8.9%), amoxcillin/clavulanic acid (6.7%), gentamicin (6.7%) and amikacin (4.4%). All *E. coli* isolates which were studied have been found sensitive to cefepime, imipenem, ertapenem and piperacillin/tazobactam. Multiple resistance to antimicrobial agents was very common in the present study. Thirty five (55.6%) of the 63 *Klebsiella* isolates were resistant to one or more antibiotics, and 20 (31.7%) of the 63 isolates were resistant to three or more antibiotics. Thirty (66.7%) of the *E. coli* isolates were resistant to one or more antibiotics, and multiple resistance to three or more antibiotics was 37.8% (data not shown).

ESBL production was detected using a double disk diffusion test to determine the presence of ESBL in *Klebsiella* spp. and *E. coli* isolates that were resistant to third-and fourth-generation cephalosporins or aztroenam. Prevalence of ESBL per species was 20 of 45 *E. coli* (44.4%), 5 of 13 *K. pneumoniae* (38.5%) and 13 of 50 *K. oxytoca* (26%). Of the 20 ESBL-producing *E. coli*, 10 (50%) were from chicken meat, 5 (25%) were from minced meat, 2 (10%) were from cheese, 2 (10%) were from milk and 1 (5%) were from ice cream samples. Of the 13 ESBL-producing *K. oxytoca* isolates, 5 (38.4%) were from chicken meat, 4 (30.8%) were from minced meat, 2 (15.3%) were from cheese and 2 (15.3%) were from milk samples. Of the 5 ESBL-producing *K. pneumonia*, 2 (40%) were from cheese, 1 (20%) were from minced meat, 1 (20%) were from milk and 1 (20%) were from ice cream samples.

DISCUSSION

Klebsiellae are ubiquitous in nature and have two common habitats, one being the environment, where they

are found in surface water, sewage, and soil and on plants, and the other being the mucosal surfaces of mammals such as humans, horses, or swine, on which they colonize. *E. coli* is accepted as a fecal contamination indicator in foods because of its presence in the intestinal tract. The gastrointestinal tract and the hands of personnel were reported as principal reservoirs of *Klebsiella* spp. and *E. coli* (Gundogan and Yakar, 2007). During the study period, 50 *K. oxytoca*, 45 *E. coli* and 13 *K. pneumoniae* isolates were recovered from 75 samples of minced meat, chiken meat, raw milk, white cheese and ice cream. Similar isolation rates were also reported by Cook et al. (2009), Uddin et al. (2011), Haryani et al. (2007) and Gundogan and Yakar (2007) in turkey meat, raw milk, street foods, and milk and milk products, respectively. During the past decade, drug resistance in *Enterobacteriaceae* has increased dramatically worldwide. This increase is mainly the result of an increased prevalence of ESBL-producing *Enterobacteriaceae* and has increased the use of last-resort antimicrobial drugs (that is, carbapenems) (Kuzucu et al., 2011). According to our results, all of the *E. coli* and *Klebsiella* spp. isolates were susceptible to imipenem and ertapenem. This result is consistent with those obtained in previous studies (Bindayna et al., 2009; Kolar et al., 2006). However, there remains a need for continued surveillance and judicious use of these antibiotics as a recent report from our country has documented the occurence of carbapenem resistant *E. coli*, *K. pneumoniae* and *K. oxytoca* isolates (Kuzucu et al., 2011).

Previous studies reported that ampicillin was the least effective among the antimicrobials tested against *E. coli* (Nijssen et al., 2004). In this study, all of the *E. coli* and *Klebsiella* spp. isolates were resistant to ampicillin. Similar findings have been reported in a recent study of swine *K. pneumoniae* isolates in China, where 100% of all the isolates were resistant to ampicillin (Li-Kou et al., 2011). In the present study, among the combinations with β-lactamase inhibitors, the most effective combination was piperacillin/tazobactam, followed by amoxicillin/clavulanic acid. Susceptibility to β-lactamase inhibitors was similar to the results of Bindayna et al. (2009). Aminoglycosides are active against clinically important gram-negative bacilli (Ramirez et al., 2010). A low prevalence of gentamicin and amikacin resistance was detected in *K. oxytoca* and *E. coli* isolates. None of the *K. pneumoniae* isolates had resistance to amikacin and gentamicin. Low level of resistance to gentamicin and amikacin in *Klebsiella* and *E. coli* isolates was also reported by Gundogan and Yakar (2007), Apun et al. (2008) and Gundogan et al. (2011). A exception was reported in China, where 44.83% of *K. pneumoniae* isolated from swine were resistant to gentamicin (Li-Kou et al., 2011).

In our study, 77.8 and 69.8% of *E. coli* and *Klebsiella* spp. isolates, respectively, were resistant to tetracycline. Other than β-lactams, the high resistance to tetracycline may be related to the extensive use of tetrcycline in veterinary practise. Recently, tetracycline resistant *E. coli* and *Klebsiella* strains in raw milk samples (Uddin et al., 2011) and in broiler farms (Smet et al., 2008) have also been described. Ciprofloxacin is a broad spectrum fluoroquinolone antimicrobial agent that is highly effective for the treatment of a variety of infections in humans and animals (Periti et al., 1998). The observed resistance of *E. coli* and *Klebsiella* spp. isolates to ciprofloxacin was 31.1 and 23.8%, respectively. High prevalence of ciprofloxacin resistance (84%) has been reported in *E. coli* in Chinese (Yang et al., 2004). van den Bogaard et al. (2001) speculated that high percentages of quinolone resistance of the animal isolates were probably due to the therapeutic use of such antibiotic in animals and/or widespread addition of it to the animal feed. As resistance to ciprofloxacin emerged, resistance to β-lactam antibiotics became prominent. This resistance was largely a result of ESBLs, which mediate resistance to newer β-lactam agents, such as ceftazidime, ceftriaxone, cefotaxime, and aztreonam, that have an oxyamino group (Gundogan et al., 2011).

Cephalosporins are an important class of antibacterial agents in use for both humans and animals. The use of cephalosporins in food-producing animals could be selective factor for the appearance of ESBL-producing and multiple-antimicrobial-resistant bacteria in such animals (Cavaco et al., 2008). In this study, all isolates were susceptible to cefepime. Higher susceptibility to cefepime of the ESBL-producing *E. coli* and *K. pneumoniae* isolates was also reported in North America (Sader et al., 2007). On the other hand, increasing resistance to third-generation cephalosporins (for example, cefotaxime, ceftazidime, ceftiriaxone) has become a cause for concern about *Enterobacteriaceae* (Okesola and Makanjuola, 2009). According to our results, *E. coli* isolates were resistant to cefotaxime (33.3%), ceftazidime (8.9%) and ceftriaxone (8.9%). *Klebsiella* spp. isolates were resistant to cefotaxime (17.5%), ceftiriaxone (17.5%) and ceftazidime (11.1%). Compared with earlier reports which have examined the susceptibility to third-generation cephalosporins of *Klebsiella* spp. and *E. coli* in Turkey (Gundogan and Yakar, 2007; Gundogan et al., 2011), and in Serbia (Knezevic and Petrovic, 2008), there is a clear tendency towards decreased susceptibility for aforementioned antibiotics.

Aztroenam is a syntetic monocyclic β-lactam in the family of monobactams and is exclusively active against the aerobic gram-negative bacilli (Nijsen et al., 2004). In the present study we observed that 42.9 and 28.9% of *Klebsiella* spp. and *E. coli* isolates, respectively, were resistant to aztroenam. According to Gundogan et al. (2011), aztroenam had moderate activity against *K. pneumoniae* and *K. oxytoca* (resistance rates of 29 and 21%, respectively). Multiple resistance to antimicrobial

agents was very common in the present study. Thirty five (55.6%) of the 63 *Klebsiella* spp. isolates were resistant to one or more antibiotics, and 20 (31.7%) of the 63 isolates were resistant to three or more antibiotics. Thirty (66.7%) of the *E. coli* isolates were resistant to one or more antibiotics, and multiple resistance to three or more antibiotics was 37.8% (data not shown). Similar results were obtained with milk and milk products. This implies that among *Klebsiella* isolates, the prevalence of resistance to two or more antimicrobial agents was in 35% (Gundogan et al., 2011).

Cook et al. (2009) reported that 71% of the *E. coli* isolates isolated from turkey meat samples were resistant to one or more antimicrobials, and 18% of isolates were resistant to five or more antimicrobials. In the last few years, different reports have alerted about the dissemination of ESBL-positive *E. coli* and *Klebsiella* spp. to the food-producing animals and also food products. These resistant bacteria could enter into the food chain, creating a problem for food safety because they can transfer resistant genes to the pathogenic bacteria (Slama et al., 2010).

In the present study, the majority of the ESBL producers were *E. coli* (44.4%), followed by *K. pneumoniae* (38.5%) and *K. oxytoca* (26%). Chicken meat had the highest prevalence of ESBL-producing *E. coli* and *Klebsiella* spp. isolates among the products. These results are appeared to be higher than those in several studies; for example Duan et al. (2006) reported a 3.1% prevalence of ESBL producers among *E. coli* isolates from cattle. In a Turkish study reported a 2.1% prevalence of ESBL producing *Enterobacteriaceae* isolated from cattle (Kucukbasmaci et al., 2008). On the other hand, our results are much lower than those in a recent study conducted by Stuart et al. (2012) who found that all conventional retail chicken meat and 84% of organic chicken meat samples were contaminated with ESBL-producing *E. coli*.

In conclusion, due to the intensive use of antimicrobial agents for food animal production, meat and milk products are frequently contaminated with antimicrobial-resistant bacteria. The present study reveals that multidrug resistant and ESBL-producing *E. coli* and *Klebsiella* species can be transmitted by different foods, including meat, milk and their products. The increasing prevalence of resistance in the isolates from animal origin may have important therapeutic implications. Thus, monitoring of ESBL-producing enterobacteria should be continued at various level (animals, human, and environment), while investigating the factors that contribute to their selection and dissemination.

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