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Antimicrobial Susceptibilities of Commensal *Escherichia coli* Isolates Recovered from Cloacal Samples of Japanese Quails and Chickens in Turkey

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Authors' Contribution
MAB designed the study and directed the sampling procedure. RIA performed all laboratory experiments. EBB supervised the work and interpreted the microbiological data and prepared the manuscript.

Key words
***Escherichia coli*, Japanese quail, Chicken, Antimicrobial resistance, Poultry.**

ABSTRACT

Antibiotic resistance has been rapidly increasing all over the world. Presence of antibiotic resistant bacteria in food producing animals is considered an important transmission source for both direct and indirect transfer of those bacteria to human. For this reason, 45 cloacal samples comprising from 23 Japanase quails and 22 chickens were analyzed by conventional and molecular methods for *E. coli* isolation in this study. Subsequently, antimicrobial susceptibility profiles of *E. coli* isolates (n=71) were determined against thirteen agents; amoxicillin-clavulanic acid, piperacillin-tazobactam, ceftazidime, cefotaxime, ceftriaxone, cefpodoxime, aztreonam, ciprofloxacin, amikacin, ertapenem, imipenem, meropenem, trimethoprim-sulfamethoxazole by disk diffusion method. Double-disk synergy test (DDST) was also performed for detection of Extended Spectrum β-Lactamase (ESBL)-producing organisms. As a result, majority of poultry isolates was susceptible to most of the tested antibiotics and highest resistance rates were detected for ciprofloxacin (2.8%) followed by ertapenem, imipenem, meropenem with equal resistance rates of 1.4% with CLSI interpretative criteria. However, analyzing of the same data according to EUCAST interpretative criteria resulted in higher number of resistant isolates (n=16). In this case, isolates showed resistance to amoxicillin-clavulanic acid (7%), cefpodoxime (5.6%), ciprofloxacin (5.6%), ertapenem (2.8%) and aztreonam (1.4%). No ESBL-producing organism was detected among isolates. This is the first report on ertapenem resistant bacteria from various poultry sources in Turkey. Therefore, it is important to perform surveillance on the antibiotic resistant bacteria in healthy livestock animals for predicting their risk status to society.

INTRODUCTION

The widespread and uncontrolled usage of antibiotics has caused the development of antimicrobial resistant bacteria as a global threat. Antibiotic resistance frequencies seem to change in different countries mostly depending on antibiotic prescribing policies of countries. Currently, it has been established that antibiotic resistant bacteria or resistance genes are transmitted between humans and animals that is especially raised for human consumption (Aarestrup *et al*., 2000). For this reason, increasing antibiotic resistant bacteria from animal sources can also be a potential hazard to consumers via food chain. Besides from animal product contamination, direct contact or more indirectly environmental pathways may contribute to spread of antibiotic resistant bacteria (EFSA, 2009). In the transfer of resistance genes, mobile genetic elements such as plasmids have major roles, although mutations can also cause formation of resistance gene (Tenover, 2006).

Antibiotic resistance in bacteria can occur by different mechanisms. In the case of resistance to broad-spectrum of cephalosporins, production of Extended-Spectrum β-Lactamase (ESBL) can be the primary cause (Pitout, 2010). Cephalosporins fall within β-lactam group of antibiotics and has been regularly used against bacterial infections in both human and veterinary medicine. Moreover, Enterobacteriaceae members have been persistently identified from human infections. For these reasons, treatment and controlling of Enterobacterial infections have turned into difficult task.

Although more data is available on antimicrobial resistance profiles of commensal *E. coli* isolates from

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various poultry (turkey, layers and broiler) around the world (Martin *et al*., 2005; Jiang *et al*., 2011; Wasyl *et al*., 2013), few studies exist in Turkey (Mustak *et al*., 2012; Başaran-Kahraman *et al*., 2016). Besides that, to the best of our knowledge, this is the first work that focuses on commensal isolates from healthy Japanase quails. Eventually, aim of the present study was to gain more information on occurrence of antibiotic-resistant bacteria from various poultry including Japanase quails and chickens in Kahramanmaras province of Turkey. Moreover, the prevalence of ESBL-producing isolates was also assessed in order to determine the risk status of antibiotic resistance bacteria as a possible reservoir.

MATERIALS AND METHODS

Sample collection

The experimental procedure of this study was approved by The Faculty of Agriculture, Animal Policy and Welfare Committee of the Kahramanmaras Sutcu Imam University (protocol number: 2014/01-3). A total of 45 cloacal swap samples comprising from healthy Japanase quails (n=23), domesticated chickens (n=13) and wild chickens (n=9) was randomly collected in April 2013 from Kahramanmaras Province, Turkey. Swab samples were then replaced into the sterile disposable culture tube containing 1 ml of sterile 1X Phosphate Buffered Saline (PBS) at pH 7.4. Buffer was contained 137 mmol of NaCl, 2.7mmol g of KCl, 10 mmol of $Na_2HPO_4.2H_2O$ and 2mmol of KH_2PO_4 per L. The collected samples were immediately transported and processed for bacteriological examination. Bacterial inoculation was initially made on eosin methylene blue (EMB) agar. Afterwards, inoculated plates were incubated for 24 h at 35°C. At the end of incubation, the plates were examined for selection of typical *E. coli* colonies (dark-centered and flat, with or without metallic sheen) and re-streaked on fresh EMB agar to purity. Grown pure isolates were transferred into Tryptic Soy Broth (TSB) and were grown at 35°C for 24 h. Grown cultures were then used for preparation of long-term bacterial stocks containing 15% of sterile glycerol at the final volume.

Phenotypic identification of E. coli *strains*

Identification of *E. coli* strains was done based on biochemical tests including oxidase, triple sugar iron (TSI), lysine decarboxylase, indole, methyl red, Voges-Proskauer test and citrate. Biochemical identification of the bacterial isolates was further verified using CHROMagar ECC (France). *E. coli* colonies appear in blue color on this selective medium.

PCR amplifications with E. coli *species-specific primers*

Preparation of the cellular DNA samples was done according to the procedure as described earlier (Buyukunal-Bal *et al*., 2010). The primers used for species-specific PCR amplifications were *E. coli* 1 5'-GCTTGACACTGAACATTGAG-3' and *E. coli* 2 5'-GCACTTATCTCTTCCGCATT-3' (Silva *et al*., 2009). Each PCR amplification was performed on 50 µl of total volume containing 5 µl of target DNA, 0.2 mM dNTP, 30 pmol of each primer, 5 µl of 10X PCR buffer solution (500 mM KCl, 100 mM Tris-HCl, pH 9.0), 1.5 mM $MgCl_2$ and 1 unit of Taq DNA Polymerase (Fermentas). PCR conditions were modified as following: denaturation at 95°C for 5 min, 40 cycles with denaturation at 95°C for 1 min, annealing at 55°C for 1 min and polymerization at 72°C for 2 min. After reactions were completed, final extension was performed at 72°C for 8 min. *E. coli* ATCC 25922 and an ESBL positive clinical *E. coli* isolate were used for quality control. PCR products were separated by electrophoresis on 1% agarose gels and visualized using a gel documentation system (Vilber Lourmat, France) after staining with Ethidium Bromide. GeneRuler 100 bp DNA Ladder (Fermentas) was used as molecular weight marker.

Antimicrobial susceptibility test

Antimicrobial susceptibility of the isolates was determined by Kirby-Bauer Disk Diffusion Susceptibility Test protocol as described by Clinical and Laboratory Standards Institute (CLSI) (2013). Thirteen different antimicrobial containing disks (Oxoid, UK) were used for the testing susceptibilities of *E. coli* isolates. Antimicrobial agent concentrations on each disk were as follows: amoxicillin-clavulanic acid (30 µg), piperacillin-tazobactam (110 µg), ceftazidime (30 µg), cefotaxime (30 µg), cefpodoxime (10 µg), ceftriaxone (30 µg), ertapenem (10 µg), imipenem (10 µg), meropenem (10 µg), aztreonam (30 µg), ciprofloxacin (5 µg), amikacin (30 µg) and trimethoprim-sulphamethoxazole (25 µg). *E. coli* ATCC 25922 was used as quality control strain. Results were interpreted according to CLSI and The European Committee on Antimicrobial Susceptibility Testing (EUCAST) criteria (CLSI, 2013; EUCAST, 2016).

Double disk synergy test

Detection of ESBL producing isolates was performed with double disk synergy test using following antibiotic disks: amoxicillin/clavulanic acid (30 µg), aztreonam (30 µg), cefotaxime (30 µg), ceftazidime (30 µg), ceftriaxone (30 µg) and cefpodoxime (10 µg) (Oxoid, UK) (CLSI, 2013). Briefly, inoculums were prepared in sterile saline solutions from grown cultures. When 0.5 McFarland turbidity value was obtained for each bacterial inoculum, they were inoculated onto Mueller Hinton Agar (Merck) by sterile cotton swab. Then, amoxicillin/clavulanic acid was placed at the center and the others around it that

were far from each other 24 mm from center to center. Subsequently, they were incubated at 37ºC for 18-20 h. A clear extension or protrusion (synergistic effect) of the edge of the inhibition zone of any of the antibiotics toward the disk containing amoxicillin/clavulanic acid was interpreted as positive for ESBL production. Three clinical isolates of ESBL-producing *E. coli*, possessing bla_{TEM}, bla_{SHV} and bla_{CTX-M} β-lactamase genes, were used as control strains (Durmaz *et al*., 2015).

RESULTS AND DISCUSSION

In this study, 45 cloacal samples from Japanase quails and chickens (domesticated and wild) collected from Kahramanmaras province were used for the isolation of *E. coli*. Identification was performed by application of both phenotypic and molecular methods. Based on the results of biochemical tests and growth on ChromAgar ECC, seventy-one *E. coli* isolates composed from Japanase quail (n=44), domesticated chicken (n=24) and wild chicken (n=3) were identified. Only few isolates (n=3) were appeared colorless on ChromAgar ECC plates among isolates that shared typical biochemical test results of *E. coli*. This result is compatible with the fact that approximately 97% of *E. coli* strains can produce β-D-glucuronidase enzyme which can cleave chromogenic substrates (Moberg, 1985). Four isolates from quails had positive citrate test result and also produced H_2S on TSI. Except the results of those two tests, all of their test results resemble typical biochemical test results of *E. coli*. This result is consisted with the observation of citrate positive variants of *E. coli* that have been isolated from domestic pigeons, pigs, cattles and horses (Ishiguro *et al*., 1978). The H2S-positive *E. coli* strains were also reported in earlier studies (Darland *et al*., 1974; Park *et al*., 2015). Those strains were identified as *E. coli* since they appeared in blue on ChromAgar ECC and gave amplification products with *E. coli* specific primers.

DNA samples from seventy-one isolates were also screened with *E. coli* specific primers for confirmation of phenotypic identification. Table I summarize the results of two repeats of PCR analyses. In the first PCR analysis, amplification products were obtained for DNA samples from 34 isolates (48%), whereas in the second PCR, amplification products were observed for 24 DNA samples (34%). During molecular conformation of phenotypic identification results, DNA samples from less number of isolates (n=24) yielded positive amplification results in the second PCR. This result was explained with possible degradation of DNA samples by time. Therefore, formation of amplification product from each DNA sample with either single or both PCR was scored as positive result. As a result, phenotypical identification was confirmed for 41 isolates (58%) based on PCR amplification with *E. coli* specific-primers. At this point, lower level of molecular confirmation of phenotypic identification results might also cause from presence of mutation in primer binding site of species-specific primers in some strains and definitely needs to be investigated.

Table I.- Results of confirmatory PCR analyses with *E. coli* specific-primers.

Number of samples after PCR amplification (%)				Total number of positive samples (%)
First		Second		
Positive	Negative	Positive	Negative	
34 (48%)	37 (52%)	24 (34%)	47 (66%)	41 (58%)

Table II.- Antimicrobial susceptibilities of *E .coli* isolates from poultry interpreted according to CLSI criteria.

Antimicrobial agent	CLSI zone diameter breakpoints (mm)*			Total no. of isolates	Number of isolates (%)		
	S	I	R		S	I	R
AMC	≥18	14-17	≤13	71	69 (97.2)	2 (2.8)	0 (0)
TZP	≥21	18-20	≤17	71	66 (93)	5 (7)	0 (0)
CAZ	≥21	18-20	≤17	71	70 (98.6)	1 (1.4)	0 (0)
CTX	≥26	23-25	≤22	71	48 (67.6)	23 (32.4)	0 (0)
CPD	≥21	18-20	≤17	71	66 (93)	5 (7)	0 (0)
CRO	≥23	20-22	≤19	71	71 (100)	0 (0)	0 (0)
ETP	≥23	20-22	≤19	71	69 (97.2)	1 (1.4)	1 (1.4)
IMP	≥23	20-22	≤19	71	68 (95.8)	2 (2.8)	1 (1.4)
MEM	≥23	20-22	≤19	71	68 (95.8)	2 (2.8)	1 (1.4)
ATM	≥21	18-20	≤17	71	70 (98.6)	1 (1.4)	0 (0)
CIP	≥21	16-20	≤15	71	67 (94.4)	2 (2.8)	2 (2.8)
AK	≥17	15-16	≤14	71	64 (90.1)	7 (9.9)	0 (0)
SXT	≥16	11-15	≤10	71	71 (100)	0 (0)	0 (0)

*CLSI, 2013. S, Sensitive; R, Resistant; I, Intermediate; AMC, amoxicillin-clavulanic acid; TZP, piperacillin-tazobactam; CAZ, ceftazidime; CTX, cefotaxime; CPD, cefpodoxime; CRO, ceftriaxone; ETP, ertapenem; IPM, imipenem; MEM, meropenem; ATM, aztreonam; CIP, ciprofloxacin; AK, amikacin; SXT, trimethoprim-sulphamethoxazole.

Antibiotic resistance can be recognized for various groups of antibiotics. Among those groups, β-lactams are significant, since they are widely used in veterinary and human clinical practices. Resistance to β-lactams is generally achieved by β-lactamases which are capable of inactivating the effects of β-lactams (Bradford, 2001). Production of these enzymes in clinical Enterobacteriaceae isolates represents an increasing problem resulting in higher patient morbidity and mortality. In this regard, possible spreading ways of those resistant bacteria have to be investigated. Poultry animals possessing resistant bacteria might be important reservoirs for transmission to humans via food chain and environment. Therefore, investigation of antibiotic resistance levels among *E. coli* originated from poultry animals is valuable for assessing the risk status of those animals for human. This will also aid for taking precautions against those bacteria in the future. For this reason, antibiotic-resistant bacteria occurrence from various poultry of Kahramanmaras province in Turkey was addressed in the present study.

As a result, among studied antimicrobials, highest level of resistance among *E. coli* isolates were observed for ciprofloxacin (2.8%), followed by ertapenem (1.4%), imipenem (1.4%), meropenem (1.4%) when antibiotic data was interpreted according to CLSI criteria (Table II). Moreover, rate of intermediate level of resistance to cefotaxime (32.4%) was quite high. However, when data was analyzed with the EUCAST interpretive criteria, higher number of resistant isolates (n=16) was observed against amoxicillin-clavulanic acid (7%), cefpodoxime (5.6%), ciprofloxacin (5.6%), ertapenem (2.8%) and aztreonam (1.4%) (Table III).

Analyses of antimicrobial resistance rate in commensal *E. coli* from different countries or even from different parts of the same country may result in substantial variation. This situation has been attributed to antimicrobial usage strategy and methodology differences in the detection of antimicrobial resistance of each country, therefore it makes comparison difficult. However, our results somewhat agreed with the results of Wasyl *et al*. (2013) which high resistance to ciprofloxacin (40.1%-82.4%) and low resistance to cephalosporins (0.6%-10%) were detected in commensal *E. coli* isolated from slaughtered broilers, laying hens, turkeys in Poland between 2009 and 2012.

When one or more than one antibiotic resistance was investigated among sixteen resistant isolates, most of them (n=14) were found to be resistant to one antimicrobial and only 2 isolates (one from Japanase quail and one from domesticated chicken) were resistant to two antibiotics. The detection rate of *E. coli* isolates showing resistance to two antibiotics was 2.8% in the present study, which was much lower than detected for turkeys (16%) and broilers (16%), but higher than for laying hens (%0) reported from the south of The Netherlands (van den Bogaard *et al*., 2001). Similarly, *E. coli* isolates from chickens displayed higher rates of resistance to one or two antibiotics (12.2% and 13.25%) in Chile compared to the results of the present study (Martin *et al*., 2005).

Table III.- Antimicrobial susceptibilities of *E. coli* isolates from poultry interpreted according to EUCAST criteria.

Antimicrobial agent	EUCAST zone diameter breakpoints (mm)†		Total number of isolates	Number of isolates (%)	
	S ≥	R <		S	R
AMC	19	19	71	66 (93)	5 (7)
TZP	20	17	71	71 (100)	0 (0)
CAZ	22	19	71	71 (100)	0 (0)
CTX	20	17	71	71 (100)	0 (0)
CPD	21	21	71	67 (94.4)	4 (5.6)
CRO	23	20	71	71 (100)	0 (0)
ETP	25	22	71	69 (97.2)	2 (2.8)
IMP	22	16	71	71 (100)	0 (0)
MEM	22	16	71	71 (100)	0 (0)
ATM	24	21	71	70 (98.6)	1 (1.4)
CIP	22	19	71	67 (94.4)	4 (5.6)
AK	18	15	71	71 (100)	0 (0)
SXT	16	13	71	71 (100)	0 (0)

†EUCAST (2016). For abbreviations, see Table II.

Many reports indicate that ESBL-producing enterobacteria have increased significantly worldwide (Pitout, 2010). However, less information is available on the prevalence of ESBL-producing *E.coli* isolates from Japanase quails. Only few studies addressed antibiotic resistance in *E. coli* isolates from Japanase quails that were mainly recovered from sick animals (Salehi and Ghanbarpour, 2010). Therefore, as far as our knowledge, the present study represents the first one. The ESBL producing *E. coli* from various poultry was not detected in this study. However, ESBL-producing *E. coli* was detected at higher rates (2.3%) in Czech Republic and (3.6%) in Great Britain as compared to the results of our study (Kolar *et al*., 2010; Randall *et al*., 2011). In addition, ESBL-producing *E. coli* was detected more frequently in broilers compared to laying hens from Japan (Hiroi *et al*., 2012) and from Thailand (Boonyasiri *et al*., 2014).

Carbapenem antibiotics have never been licensed for veterinary use in any country worldwide (OIE, 2015). Probably due to this restriction, carbapenems were

excluded from antibiotic susceptibility testing of isolates originated from animals in most of the times; however, this group of antibiotics provides an alternative treatment option when cephalosporin resistance is present in clinical settings. As in the case of emergence of antibiotic resistance bacteria against any type of antibiotics, excessive usage of carbepenems in clinical cases have provoked increase of carbapenem resistant *E. coli* (CRE) in hospitals from many countries including Turkey (Baran and Aksu, 2016), but the research on the prevalence of CRE in foods, food animals and the environment is limited from Turkey. In two studies, no imipenem resistance was observed among *E. coli* isolates recovered from raw chicken meat (Arslan and Eyi, 2011; Gundogan and Avci, 2013). In addition, no ertapenem resistant isolates were observed among *Klebsiella* spp. and *E. coli* isolates from raw chicken meat (Gundogan and Avci, 2013). In another study, imipenem resistant isolates were detected at the rate of 7.14% among cattle isolates from Turkey. In addition, intermediate level of resistance was observed among cattle isolates for ertapenem, imipenem and meropenem (Buyukunal *et al*., 2016). In the present study, ertapenem resistant *E. coli* isolates from poultry were detected for the first time in Turkey with the application of both CLSI and EUCAST criteria. Moreover, higher number of resistant and intermediate level of resistant isolates was selected for carbapenems with the application CLSI criteria.

The presence of β-lactam resistance among the *E. coli* isolates from poultry origin in Turkey in the present study might indicate low risk status of poultry products as reservoir of β-lactamase resistant bacteria, however, the recent report on prevalence of ESBL producing *E. coli* from faecal samples of broilers in different areas of the Marmara Region in Turkey indicated higher prevalence rate of ESBL-producing *E. coli* isolates (Başaran-Kahraman *et al*., 2016). Detection of no ESBL producing *E. coli* in the present might be also related to number of *E. coli* isolates that have been analyzed as compared to other studies (Dierikx *et al*., 2010, 2012; Başaran-Kahraman *et al*., 2016). Therefore, proper decision about prevalence of ESBL-producing *E. coli* should be done after examination of higher number of *E. coli* isolates. Another important point for making more decisive conclusion can be related to sampling procedure. Definitely, sampling should represent more dispersed regions from Turkey for the future monitoring studies. At this point, detection of high rate of intermediate level of cefotaxime resistance in this study according to CLSI criteria should be noteworthy, since initial screening of ESBL-producing isolates have been done in cefotaxime containing MacConkey agar during examination of poultry faecal samples in the recent study from Turkey (Başaran-Kahraman *et al*., 2016).

CONCLUSION

Assessment of the prevalence of ESBL-producing and antibiotic resistant *E. coli* from animal sources in Turkey has great importance. For this reason, further investigation on antibiotic resistance profiles of *E. coli* isolates from poultry origin should be performed periodically. The results of more intensive studies will also demonstrate the possible transmission ways of antibiotic determinants from animals to human. In relation to those results, selection of best treatment options for both animal and human infections caused by *E. coli* can be achieved for controlling bacterial infections in the future.

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Statement of conflict of interest

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

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