Molecular detection of antimicrobial resistance genes in *E. coli* isolated from slaughtered commercial chickens in Iran

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ABSTRACT: This study was carried out to detect the distribution of antibiotic-resistant genes in *Escherichia coli* isolates from slaughtered commercial chickens in Iran by PCR. The investigated genes included *aadA1, tet*(A), *tet*(B), *dfrA1, qnrA, aac(3)-IV, sul1, bla*$_{SHV}$, *bla*$_{CMY}$, *ere*(A), *catA1* and *cmlA*. According to biochemical experiments, 57 isolates from 360 chicken meat samples were recognized as *E. coli*. The distribution of antibiotic-resistance genes in the *E. coli* isolates included *tet*(A) and *tet*(B) (52.63%), *dfrA1, qnrA, catA1* and *cmlA* (36.84%) and *sul1* and *ere*(A) (47.36%), respectively. Nine strains (15.78%) were resistant to a single antimicrobial agent and 11 strains (19.29%) showed resistance to two antimicrobial agents. Multi-resistance which was defined as resistance to three or more tested agents was found in 64.91% of *E. coli* strains. The results indicate that all isolates harbour one or more of antibiotic resistance genes and that the PCR technique is a fast, practical and appropriate method for determining the presence of antibiotic-resistance genes.

Keywords: *Escherichia coli*; chicken meat; antibiotic-resistance genes; PCR; Iran

There is worldwide concern about the appearance and rise of bacterial resistance to commonly used antibiotics. In this regard, programs for monitoring resistance have been implemented in many countries for the purpose of protecting the health of humans as well as animals (Cizman 2003; Aarestrup 2004; Li et al. 2010). These programs usually monitor indicator bacteria such as *Escherichia coli*.

E. coli is commonly found in human and animal intestinal tracts and, as a result of faecal contamination or contamination during food animal slaughter, is often found in soil, water, and foods. A number of *E. coli* strains are recognised as important pathogens of Colibacillosis in poultry and some of them can cause severe human diseases such as haemorrhagic colitis and haemolytic uremic syndrome (Riley et al. 1983; Chansiripornchai 2009; Ferens and Hovde 2011).

The treatment of illnesses caused by this bacterium often requires antimicrobial therapy. The decision to use antimicrobial therapy depends on the susceptibility of the microorganism and the pharmacokinetics of the drug for achieving the desired therapeutic concentration at the site of infection and thus clinical efficacy (McKellar et al. 2004). However, veterinary practitioners have a limited choice of antimicrobials for use in the poultry industry, due to antimicrobial resistance issues and human health concerns. Moreover, the repeated and unsuitable use of antibiotics has led to an increasing rate of antimicrobial resistance (Mooljuntee et al. 2010). Antibiotic usage selects for resistance not only in pathogenic bacteria but also in the endogenous flora of exposed individuals or populations. Therefore, the antibiotic selection pressure for resistance in bacteria in poultry is high and consequently, their faecal flora contains a relatively high proportion of resistant bacteria (Piddock, 1996; van den Bogaard and Stobberingh, 1999).

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Integrons are important players in the dissemination of antimicrobial resistance among Gram-negative bacteria. Integrons are genetic structures able to capture, excise and express genes, frequently included in mobile elements such as plasmids, that allow their dissemination among bacteria (Fluit and Schmitz 2004). Recently, molecular techniques, especially polymerase chain reaction (PCR), have been widely used to study antimicrobial resistance genes. Due to the excessive use of antibiotics in the poultry industry in Iran, the purpose of this study was to determine antimicrobial resistance patterns in *E. coli* isolated from slaughtered Iranian commercial chickens.

MATERIAL AND METHODS

Sample collection and identification of *E. coli*

In this study, a total of 360 chicken meat samples were collected randomly from July to September 2010 at the Shahrekord abattoir (located in Chaharmahal va Bakhtiari province, Iran). Before collecting chicken muscle meat samples, the external surfaces were disinfected with 70% alcohol to minimize surface contamination. Using sterile scissors and tissue forceps, pieces of the muscles were collected separately into sterile bags and transported in a cooled box for further processing.

The surfaces of chicken muscles were seared with a hot spatula, incised and cultured on 5% sheep blood and MacConkey agar (Merck, Germany) and incubated for 18 to 24 h at 37 °C. Colonies with the typical colour and appearance of *E. coli* were picked and streaked again on blood agar plates and re-streaked on EMB agar (Merck, Germany). Green metallic sheen isolates were considered to be *E. coli* and the presumptive colonies were biochemically tested for growth on triple sugar iron agar (TSI) and lysine iron agar (LIA), and for oxidative/fermentative degradation of glucose, citrate utilization, urease production, indol fermentation, tryptophan degradation, glucose degradation (methyl red test) and motility. The *E. coli* isolates were stored in tryptic soy broth (Merck, Germany) with 15% glycerol at –20 °C (Mooljuntee et al. 2010). Colony confirming was performed using molecular methods (PCR). Molecular confirmation of clones was determined according to the 16S rRNA gene region from *E. coli* described by Sabat et al. (2000) and identification of *E. coli* O157:H7 isolates was performed as described by Fode-Vaughan et al. (2003). Primer design and PCR conditions were optimised for DPCR using recommendations reported previously. The PCR conditions for amplification of *stx1* and *stx2* were those used for *pmoA*.

DNA extraction

E. coli were subcultured overnight in Luria-Bertani broth (Merck, Germany) and genomic DNA was extracted using a Genomic DNA purification kit (Fermentas, Germany) according to the manufacturer's instructions.

Primers and PCR assay

The presence of genes associated with resistance to streptomycin (*aadA1*), tetracycline [*tet*(A), *tet*(B)], trimethoprim (*dfrA1*), quinolones (*qnr*), gentamicin [*aac(3)-IV*], sulfonamides (*sul1*), beta-lactams (bla_{SHV}, bla_{CMY}), erythromycin [*ere*(A)] and chloramphenicol (*catA1, cmlA*) were determined by PCR and the set of primers used for each gene is shown in Table 1.

PCR reactions were performed in a total volume of 25 µl, including 1.5mM $MgCl_2$, 50mM KCl, 10mM Tris-HCl (pH 9.0), 0.1% Triton X-100, 200 µm of each dNTP (Fermentas), 1 µm primers, 1 IU of Taq DNA polymerase (Fermentas), and 5 µl (40–260 ng/µl) of DNA. Amplification reactions were carried out using a DNA thermo-cycler (Eppendrof Mastercycler, Eppendorf-Nethel-Hinz GmbH, Hamburg, Germany) as follows: Three min at 95 °C, 35 cycles each consisting of 1 min at 94 °C, 90 s at ~55 °C (show in Table 1) and 1 min at 72 °C, followed by a final extension step of 10 min at 72 °C. Amplified samples were analyzed by electrophoresis in 1.5% agarose gel and stained by ethidium bromide. A molecular weight marker with 100 bp increments (100 bp DNA ladder, Fermentas) was used as a size standard. Strains of *E. coli* O157:K88ac:H19, CAPM 5933 and *E. coli* O159:H20, CAPM 6006 were used as positive controls.

Antimicrobial susceptibility testing

Antimicrobial susceptibility testing was performed by the Kirby-Bauer disc diffusion method using Mueller-Hinton agar (HiMedia Laboratories, Mumbai, India, MV1084), according to the Clinical

Table 1. *Escherichia coli* antimicrobial resistant genes and primer sequences used for PCR identification

Antimicrobial agent	Resistance gene	Sequence	Size (bp)	Annealing temperature (°C)	References
Streptomycin	*aadA1*	(F) TATCCAGCTAAGCGCGAACT (R) ATTTGCCGACTACCTTGGTC	447	58	Van et al. 2008
Gentamicin	*aac(3)-IV*	(F) CTTCAGGATGGCAAGTTGGT (R) TCATCTCGTTCTCCGCTCAT	286	55	Van et al. 2008
Sulfonamide	*sul1*	(F) TTCGGCATTCTGAATCTCAC (R) ATGATCTAACCCTCGGTCTC	822	47	Van et al. 2008
Beta-lactams	bla_{SHV}	(F) TCGCCTGTGTATTATCTCCC (R) CGCAGATAAATCACCACAATG	768	52	Van et al. 2008
	bla_{CMY}	(F) TGGCCAGAACTGACAGGCAAA (R) TTTCTCCTGAACGTGGCTGGC	462	47	Van et al. 2008
Erythromycin	*ere*(A)	(F) GCCGGTGCTCATGAACTTGAG (R) CGACTCTATTCGATCAGAGGC	419	52	Van et al. 2008
Chloramphenicol	*catA1*	(F) AGTTGCTCAATGTACCTATAACC (R) TTGTAATTCATTAAGCATTCTGCC	547	55	Van et al. 2008
	cmlA	(F) CCGCCACGGTGTTGTTGTTATC (R) CACCTTGCCTGCCCATCATTAG	698	55	Van et al. 2008
Tetracycline	*tet*(A)	(F) GGTTCACTCGAACGACGTCA (R) CTGTCCGACAAGTTGCATGA	577	57	Randall et al. 2004
	tet(B)	(F) CCTCAGCTTCTCAACGCGTG (R) GCACCTTGCTGATGACTCTT	634	56	Randall et al. 2004
Trimethoprim	*dfrA1*	(F) GGAGTGCCAAAGGTGAACAGC (R) GAGGCGAAGTCTTGGGTAAAAAC	367	45	Toro et al. 2005
Quinolones	*qnrA*	(F) GGGTATGGATATTATTGATAAAG (R) CTAATCCGGCAGCACTATTTA	670	50	Mammeri et al. 2005

and Laboratory Standards Institute guidelines (Anonymous 2006). The antimicrobial agents tested and their corresponding concentrations were as follows: sulfamethoxazol (25 µg/disk), trimethoprim (5 µg/disk), chloramphenicol (30 µg/disk), enrofloxacin (5 µg/disk), tetracycline (30 µg/disk), gentamycin (10 µg/disk), cephalothin (30 µg/disk), ampicillin (10 µg /disk), and streptomycin (10 µg/disk). After incubating the inoculated plates aerobically at 37 °C for 18 to 24 h, the susceptibility of the E. coli isolates to each antimicrobial agent was measured and the results were interpreted in accordance with criteria provided by CLSI (Anonymous 2006). *E. coli* ATCC 25922 was used as quality control organisms in antimicrobial susceptibility determination.

RESULTS AND DISCUSSION

Out of 360 specimens collected, 57 (15.8%) *E. coli* isolates were identified. None of the *E. coli* strain isolates was identified as *E. coli* O157:H7.

The resistance to tetracycline was found in 52.6% and to both sulfonamides and erythromycin in 47.4% of isolates. Our results showed that the genes *qnrA*, *dfrA1* and *catA1* genes were identified in 36.8% of isolates. No genes known to be associated with resistance to streptomycin, cephalothin and ampicillin were detected (Table 2).

Table 3 summarizes the resistance pattern of *E. coli* isolates to nine antimicrobial agents tested in this study. Of the 57 *E. coli* isolates tested, all were resistant to one or more antimicrobial agent. Resistance to tetracycline was the most common finding (91.2%), followed by resistance to sulfamethoxazol (45.6%), chloramphenicol and trimethoprim (29.8%). All *E. coli* isolates were susceptible to streptomycin, cephalothin, gentamicin and ampicillin.

The mechanism of spread of antibiotic resistance from food animals to humans remains controversial. However, colonisation of the intestinal tract with resistant *E. coli* from chickens has been shown in human volunteers (Linton et al. 1977) and resistance to the same drugs has been described previously

Table 2. Distribution of antibiotic resistance genes in strains of *E. coli* isolated from chickens

Gene	*aadA1*	*tet*(A)	*tet*(B)	*dfrA1*	*qnrA*	*aac(3)-IV*	*sul1*	bla_{SHV}	bla_{CMY}	*ere*(A)	*catA1*	*cmlA*
Presence	0	30 (53.63%)	30 (53.63%)	21 (36.84%)	21 (36.84%)	0	27 (47.36%)	0	0	27 (47.36%)	21 (36.84%)	21 (36.84%)

in programs undertaken in different countries that monitor bacterial resistance in veterinary medicine (Heuer and Hammerun 2005; Asai et al. 2006).

E. coli isolates are frequent contaminants of food of animal origin, and in this study, this microorganism was recovered from 57 tested poultry meat samples; in addition, most of the isolates showed a multi-resistant phenotype. The presence of genes that confer resistance to some antimicrobial agents (erythromycin, sulphonamides, chloramphenicol and tetracycline) were especially high (36 to 52%), indicating that *E. coli* isolates originating from meat could be a reservoir of antimicrobial resistance. In a similar study carried out in Thailand, all isolated *E. coli* from Thai broilers were found to be resistant to tetracycline, ampicillin and erythromycin in agar disk diffusion assays and these resistance properties were associated with a 90%, 93.3% and 73.3% prevalence of the *tet*(A), bla_{CMY} and *ere*(A) genes, respectively. Lower resistance in Thai broilers was observed to cephalothin (73.3%) and sulphonamide + trimethoprim (26.7%) and these resistances were in 86.4% of cases associated with bla_{SHV}, and in 100% of cases with the *sul1* and *dfrA5* genes (Mooljuntee et al. 2010). The percentage of faecal samples containing resistant *E. coli* and the proportion of resistant faecal *E. coli* were determined in three different poultry populations: broilers and turkeys commonly given antibiotics, and laying hens treated with antibiotics relatively infrequently. The results of this study documented resistance to nearly all tested antibiotics in faecal *E. coli* of turkey and broilers farmers, and a lower prevalence of antibiotic-resistant *E. coli* in laying hen farmers (van den Bogaard et al. 2001). Lietzau et al. (2006) reported 15.7% and 19.4% prevalence of ampicillin resistance in women and men, respectively, and 10% and 15% of all isolates were resistant to cotrimoxazole and doxycycline, respectively. In agreement with the above mentioned studies, our results confirmed a large percentage of antibiotic resistance in indicator strains of *E. coli* isolated from commercial chickens in Iran. Thus, we conclude that these commercial chickens represent an important reservoir of resistance genes.

Table 3. Antimicrobial resistance profiles of *Escherichia coli* isolated from chickens

Antimicrobial agent	*Escherichia coli* (*n* = 57)
Streptomycin	–
Tetracycline	52 (91.22%)
Trimethoprim	17 (29.82%)
Enrofloxacin	18 (31.57%)
Gentamicin	–
Sulfamethoxazol	26 (45.61%)
Cephalothin	–
Ampicillin	–
Chloramphenicol	17 (29.82%)

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