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Phenotypic and genotypic evaluation of ESBL- and AmpC-producing *Escherichia coli* isolated from chicken distributed in Birjand, East of Iran

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ABSTRACT

The aims of the present study were to detect *Escherichia coli* in chicken distributed in Birjand, to investigate the prevalence of ESBL and AmpC beta-lactamases producers among them, and to identify their antibiotic resistance patterns. The study was conducted on 150 chicken samples, and the antimicrobial susceptibility patterns were determined by the Kirby–Bauer disk diffusion method. Phenotypic identification of ESBL and AmpC was performed by the combined disk test (CDT). The specific genes of ESBL and AmpC beta-lactamases were detected using two multiplex PCR (m-PCR) assays. According to our results, 116 out of 150 chicken samples were contaminated with *E. coli*. Moreover, the highest resistance of *E. coli* isolates was observed to trimethoprim/sulfamethoxazole (46%), ampicillin (40%), and amoxicillin (29.33%). In the molecular confirmation step, among 17 (11.33%) beta-lactamase producers, five samples contained the $bla_{\text{CTX-M14}}$ gene (3.33%), two samples contained bla_{DHA} (1.33%) and $bla_{\text{CTX-M3}}$ gene (1.33%), and just one sample carried $bla_{\text{CMY-2}}$ gene (0.66%). The bla_{SHV} and bla_{TEM} genes were not detected in any strains isolated from the chicken samples. This study showed the contamination of chicken with antibiotic-resistant *E. coli*. Therefore, it is recommended that veterinarians be more precautious in prescribing antibiotics.

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KEYWORDS

Escherichia coli, chicken, drug resistance, anti-bacterial agents, beta-lactamases

1. INTRODUCTION

Escherichia coli as member of the normal intestinal flora of humans, poultry, and other animals has great medical importance due to pathogenic *E. coli* strains that cause intestinal diseases and food infection in humans. *E. coli* can cause various infections, including meningitis, endocarditis, urinary tract infections, sepsis, diarrhea, and cellulitis (Akond et al., 2009).

Nowadays, the use of antibiotics for treatment of bacterial infection as well as growth promotion has been remarkably increased in the broiler industry. This fact may lead to increased bacterial antibiotic resistance (Ojer-Usoz et al., 2017). Antibiotic treatment plays the most critical role in the global emergence and spread of antibiotic-resistant microorganisms. These strains may transfer their elements of antibiotic resistance to other human pathogens. *E. coli* strains are highly capable of acquiring and transferring resistance genes (Ryu et al., 2012a).

Beta-lactamase enzymes are the leading cause of antibiotic resistance in Gram-negative bacteria (Barilli et al., 2019). Nowadays, more novel beta-lactamases are emerging, including Extended-Spectrum Beta-Lactamase (ESBL) and AmpC beta-lactamases. ESBLs cause resistance to beta-lactam antibiotics by degrading the beta-lactam ring through hydrolysis (Ryu et al., 2012b). ESBLs are among the most critical determinants of resistance against oximino-cephalosporines in *Enterobacteriaceae* (Barilli et al., 2019), while AmpC beta-lactamases can lead to resistance against aminopenicillins, cephalosporins, oximino-cephalosporins, cephamycin, and monobactams. Cloxacillin and aminophenyl boric acid can inhibit AmpC beta-lactamases. Up to now, no standard method has been identified for detecting and identifying the bacterial strains producing AmpC beta-lactamases (Peter-Getzlaff et al., 2011).

ESBL genes have been reported and documented in food animals, especially poultry. Recent studies on broiler chicken in the UK have reported the $bla_{\text{CTX-M1}}$ as the most common ESBL gene, followed by bla_{TEM} and bla_{SHV} as the most extensively reported genes in Europe (Barilli et al., 2019). High similarities have been reported in the ESBL-producing *E. coli* strains and related genes between chicken and humans, highlighting the possibility of chicken as the potential source of ESBL-producing *E. coli* in humans (Biasino et al., 2018).

Isolation of antibiotic-resistant bacteria and evaluation of their local distribution is of great importance. The present study was the first attempt in Birjand, East of Iran, to detect both phenotypically and genotypically beta-lactamase producer *E. coli* in chicken. Considering the importance of this problem in human health and the high global prevalence of this specific antibiotic resistance type, the present study aimed to detect and isolate *E. coli* in chicken distributed in Birjand, to investigate the prevalence of *E. coli* strains producing ESBL and AmpC beta-lactamases, and to identify their antibiotic resistance profiles.

2. MATERIALS AND METHOD

2.1. Sampling

In the present study, 150 chicken samples were purchased from markets of Birjand from October to November 2020. The meat collected included 50 samples of whole chicken, 50 samples of

sliced non-spicy chicken, and 50 samples of sliced, spicy chicken. The samples were put in a container filled with ice and were immediately transferred to the laboratory.

2.2. Isolation and identification of *E. coli*

Three grams from each chicken sample was homogenised with 27 ml of peptone water (Merck KGaA, Darmstadt, Germany) and enriched at 37 °C for 24 h (Feng et al., 2020; Saei et al., 2022). Afterward, 100 µL of the enriched sample was cultured on EMB agar (Merck KGaA, Darmstadt, Germany) at 37 °C for 24 h. Dark colonies with a green metallic luster were considered as suspected *E. coli* isolates, which were further identified based on Gram staining and the results of biochemical tests like SIM (sulphide, indole, motility), triple sugar iron, citrate, and Methyl-Red Voges-Proskauer (MR-VP) (Feng et al., 2020; Saei et al., 2022).

2.3. Antibiotic susceptibility testing

The antibiotic susceptibility of the isolates was performed using the Kirby-Bauer disk diffusion method according to Clinical and Laboratory Standards Institute guidelines (CLSI, 2018). The antimicrobial agents (Rosco, Taastrup, Denmark) used in this study included ampicillin (10 µg), cefepime (30 µg), trimethoprim/sulfamethoxazole (1.25 µg + 23.75 µg), amoxicillin/clavulanic acid (20 µg + 10 µg), gentamycin (10 µg), azithromycin (15 µg), imipenem (10 µg), meropenem (10 µg), and ceftazidime (30 µg). For quality control of the antibiotic susceptibility test, *E. coli* ATCC 25922 was used (Su et al., 2016). Isolates that had resistance to more than three antimicrobial agents were considered multidrug resistant (MDR) (Motallebi et al., 2011).

2.4. Screening and identification of ESBL and AmpC producing *E. coli* isolates

ESBL production was detected utilising the combined disk test (CDT) method based on CLSI recommendations. Briefly, susceptibility to ceftazidime (30 µg), ceftazidime/clavulanic acid (30 µg + 10 µg), cefotaxime (30 µg), and cefotaxime/clavulanic acid (30 µg + 10 µg) was determined on Muller-Hinton agar (Merck KGaA, Darmstadt, Germany). ESBL-producing strains were recognised by an at least 5-mm increase in zone diameter around cefotaxime/clavulanate and ceftazidime/clavulanate disks compared with disks without clavulanic acid. *E. coli* ATCC25922 and *Klebsiella pneumonia* ATCC700603 were used as control strains. Moreover, the isolates with a zone of inhibition lower than 19 mm around the cefoxitin (30 µg) disk were considered as suspected carriers of the AmpC gene (Peter-Getzlaff et al., 2011; Ogbolu et al., 2013).

2.5. DNA extraction from *E. coli* isolates

The *E. coli* isolates were inoculated into the microtubes containing 150–200 µL of sterile deionised distilled water and heated at 100 °C for 10 min. Afterward, the microtubes were centrifuged at 10,000 r.p.m. for 10 min, and the DNA-containing supernatant was transferred to a new microtube (Hosseini et al., 2013; Jamshidi et al., 2015).

2.6. Detection of ESBL or AmpC genes

The primers used for the amplification of the beta-lactamase genes are listed in Table 1. The specific genes of ESBL and AmpC were amplified using two multiplex polymerase chain reaction (m-PCR) tests. The first m-PCR test was used to detect the $bla_{\text{CTX-M3}}$, $bla_{\text{CTX-M14}}$, and bla_{SHV} genes, and the second m-PCR for the $bla_{\text{CMY-2}}$, bla_{DHA}, and bla_{TEM} genes.

Table 1. Target genes and their primers used in this study (Su et al., 2016)

Genes	Primer	Sequences of primers (5–3)	Size (Bp)
$bla_{\text{CTX-M3}}$	F	AATCA CTGCG CCAGT TCACG CT	479
	R	GAACG TTTCG TCTCC CAGCT GT	
$bla_{\text{CTX-M14}}$	F	TACCG CAGAT AATAC GCAGG TG	355
	R	CAGCG TAGGT TCAGT GCGAT CC	
bla_{SHV}	F	ATGCGTTATATTCGCCTGTGTAT	868
	R	TTAGCGTTGCCAGTGCTCGATCAG	
bla_{TEM}	F	ATGAGTATTCAACATTTCCG	868
	R	CTGACAGTTACCAATGCTTA	
bla_{DHA}	F	AACTT TCACA GGTGT GCTGG GT	405
	R	CGTAC GCATA CTGGC TTTGC	
$bla_{\text{CMY-2}}$	F	CTGAC AGCCT CTTTC TCCAC A	1,100
	R	CTACG TAGCT GCCAA ATCCA C	

The first m-PCR reaction was in the total volume of 20 µL (10 µL of 2X Hot Star Taq PCR Master Mix (Amplicon, Odense, Denmark), 2 µL of the DNA template, primers (10 pmol μL^{-1}; 0.5 µL of $bla_{\text{CTX-M3}}$, 1 µL of $bla_{\text{CTX-M14}}$, and 0.6 µL of bla_{SHV} primers), and 3.8 µL of ddH_2O. The second m-PCR mixture was also included 20 µL (10 µL of 2X Hot Star Taq Master Mix, 2 µL of the DNA template, primers (10 pmol μL^{-1}; 0.5 µL of each bla_{DHA}, $bla_{\text{CMY-2}}$, and bla_{TEM} primers), and 5 µL of ddH_2O). The positive control included *K. pneumonia* ATCC700603 and *E. coli* ATCC25922.

PCR schedule was as follows: 1) Initial denaturation at 95 °C for 45 s, 2) 30 cycles including denaturation at 95 °C for 45 s, annealing at 63 °C for 45 s, and extension at 72 °C for 45 s, and 3) a final extension at 72 °C for 10 min. Finally, the amplified products were electrophoresed on 1.5% agarose gel containing 1X green viewer DNA stain (Pasrzama, Tehran, Iran) (Su et al., 2016).

2.7. Statistical analysis

Data analysis was performed using the SPSS software version 16 and Fisher's exact test. The significance level was considered at $P < 0.05$.

3. RESULTS AND DISCUSSION

According to our results, 116 (77.33%) out of 150 chicken were contaminated with *E. coli*, including 34 (22.66%) samples of whole chicken, 36 (24%) samples of sliced non-spicy chicken, and 46 (30.66%) samples of sliced-spicy chicken. The results of the antibiotic susceptibility test are presented in Table 2. About 28.81% of the *E. coli* isolates had MDR. The highest antibiotic resistance of *E. coli* isolates was observed to trimethoprim/sulfamethoxazole (46%), ampicillin (40%), and amoxicillin/clavulanate (29.33%). The most effective agent was meropenem (0%). Moreover, no significant relationship was seen between chicken preparation (sliced or whole and spicy or non-spicy) and antibiotic susceptibility ($P > 0.05$).

In the CDT, 17 *E. coli* isolates (11.33%) were considered ESBL producers. Moreover, only one isolate (0.66%) showed complete resistance to cefoxitin and was considered as potentially positive for AmpC.

Table 2. Antibiotic susceptibility patterns in *E. coli* isolates from chicken based on preparation types

Antibiotic	Whole chicken			Sliced, non-spicy chicken			Sliced, spicy chicken			*P* value
	R × n (%)	I × n (%)	*S* × n (%)	*R* × n (%)	I × n (%)	*S* × n (%)	*R* × n (%)	I × n (%)	*S* × n (%)	
Cefotaxime	4 (3.39%)	2 (1.69%)	28 (23.72%)	2 (1.69%)	3 (2.54%)	31 (26.27%)	1 (0.84%)	0 (0%)	47 (39.83%)	0.07
Cefepime	0 (0%)	1 (0.84%)	33 (27.96%)	1 (0.84%)	1 (0.84%)	34 (28.81%)	0 (0%)	5 (4.24%)	43 (36.44%)	0.3
Amoxicillin/ Clavulanate	15 (12.71%)	8 (6.78%)	11 (9.32%)	14 (11.86%)	7 (5.93%)	15 (12.71%)	15 (12.71%)	11 (9.32%)	22 (18.64%)	0.7
Trimethoprim/ Sulfamethoxazole	25 (21.18%)	0 (0%)	9 (7.62%)	20 (16.94%)	0 (0%)	16 (13.56%)	26 (22.03%)	2 (1.69%)	20 (16.94%)	0.2
Meropenem	0 (0%)	1 (0.84%)	33 (27.96%)	0 (0%)	0 (0%)	36 (30.5%)	0 (0%)	0 (0%)	48 (40.67%)	0.3
Imipenem	3 (2.54%)	12 (10.17%)	19 (16.1%)	2 (1.69%)	6 (5.08%)	28 (23.72%)	2 (1.69%)	13 (11.01%)	33 (27.69%)	0.3
Ampicillin	18 (15.25%)	2 (1.69%)	14 (11.86%)	17 (14.4%)	10 (8.47%)	9 (7.63%)	26 (22.03%)	12 (10.17%)	10 (8.47%)	0.07
Gentamicin	1 (0.84%)	1 (0.84%)	32 (27.12%)	1 (0.84%)	1 (0.84%)	34 (28.81%)	0 (0%)	0 (0%)	48 (40.67%)	0.4

R: resistant; I: intermediate; S: sensitive.

In the molecular step, among 17 (11.33%) strains, five (3.33%) *E. coli* strains had $bla_{\text{CTX-M14}}$ gene, two samples were contaminated with the strains having bla_{DHA} gene (1.33%), two samples had *E. coli* strains with $bla_{\text{CTX-M3}}$ gene (1.33%), and just one sample contained $bla_{\text{CMY-2}}$ gene (0.66%). The bla_{SHV} and bla_{TEM} genes were not detected in any isolates. Moreover, there was no significant difference between the chicken preparation type and the frequency of the mentioned genes ($P > 0.05$). Figure 1 shows the amplified genes in *E. coli* strains.

In the present study, trimethoprim/sulfamethoxazole showed the highest resistance rate (46%), while the lowest resistance was observed for meropenem (0%). Moreover, ampicillin (40%) and amoxicillin/clavulanic acid (29.33%) showed high resistance rates. Al-Ghamdi et al. (1999) worked on 119 samples of healthy broiler and 100 samples of sick broiler and reported high resistance to ampicillin, gentamicin, and trimethoprim/sulfamethoxazole. In addition, the resistance to ceftazidime was 0% and 8.70% in healthy and sick broiler samples, respectively. There was also a low resistance to amoxicillin/clavulanic acid (Al-Ghamdi et al., 1999). In the present study, the resistance to gentamicin was very low, which is incompatible with the study by Al-Ghamdi et al. However, the resistance to amoxicillin in our study was similar to the mentioned study. Another study by Sáenz et al. (2001) on human and animal models and food samples reported high resistance to gentamicin, ampicillin, and trimethoprim/sulfamethoxazole in the bacteria isolated from the broiler faecal samples (Sáenz et al., 2001). In addition, resistance to imipenem, cefoxitin, cefotaxime, and ceftazidime was almost zero (Ryu et al., 2012a). A study by Gregova et al. (2012) reported high resistance to ampicillin (89%). Moreover, 43% of the samples were resistant to gentamicin (Gregova et al., 2012). These two studies were incompatible with the present study in the resistance to gentamicin, which can be due to different geographical areas, used antibiotics in the farms, and samples type (Kargar et al., 2013).

A study by Klimienė et al. (2017) on chicken showed high resistance to ceftazidime and ampicillin, while the resistance to cefoxitin, imipenem, meropenem, and trimethoprim/sulfamethoxazole was 0% (Klimienė et al., 2017). However, the resistance to trimethoprim/sulfamethoxazole was relatively high in the present study. Another study by Miles et al. (2006) reported that 20% of the samples were resistant to ampicillin, while the resistance to gentamicin was 0% (Miles et al., 2006). Another study by Ryu et al. (2012a) on food samples reported that

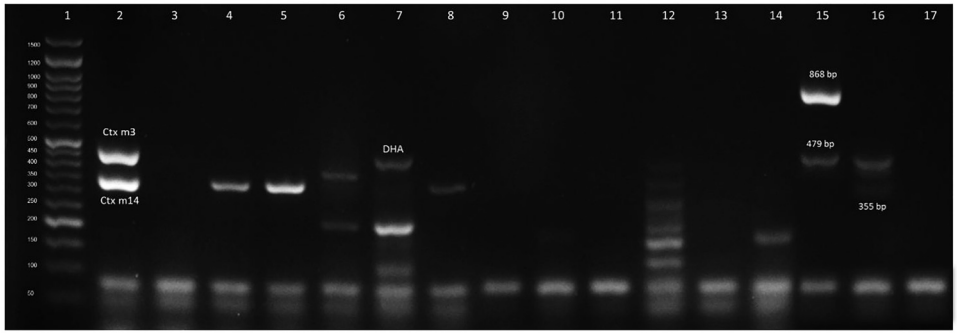

Fig. 1. Detection of B-lactamase genes in *E. coli* isolates; Lane 1: 50 base-pair DNA ladder; Lane 2 to 14: chicken samples; Lane 15: Positive control of *K. pneumoniae* ATCC700603; Lane 16: Positive control of *E. coli* ATCC25922; Lane 17: Negative control

from a total of 96 samples contaminated with *E. coli*, only 10% were resistant to ampicillin, while the resistance to other antibiotics was negligible (Ryu et al., 2012a). These findings are not the same as in the present study in the rate of ampicillin resistance, which was reported to be 52%. The present study agreed with most of the mentioned studies in the high resistance to ampicillin and trimethoprim/sulfamethoxazole and low resistance to imipenem, meropenem, and cefotaxime. However, our findings were disagreed with most previous studies on the resistance to gentamicin, which can be due to different samples, diversely used antibiotics in each area, the prevalence of contamination with *E. coli*, and others (Kargar et al., 2013).

In the current study, 3.33% of *E. coli* strains contained $bla_{\text{CTX-M14}}$ gene, 1.33% carried bla_{DHA} gene, 1.33% had $bla_{\text{CTX-M3}}$ gene, and just 0.66% of *E. coli* had $bla_{\text{CMY-2}}$ gene. In total, 4.66% were positive for ESBL, and 1.33% was positive for AmpC. Moreover, bla_{SHV} and bla_{TEM} genes were not observed in any strains isolated from the chicken samples. Batabyal et al. (2018) reported that 100% of the samples had the $bla_{\text{CTX-M}}$ gene (Batabyal et al., 2018), while Müller et al. (2018) showed that 22% of the samples carried the $bla_{\text{CMY-2}}$ gene (Müller et al., 2018), which was inconsistent with the present study. Moreover, Klimiené et al. (2017) reported that almost half of the samples had the bla_{SHV} gene, while the prevalence of bla_{TEM} and $bla_{\text{CTX-M}}$ was 70% and 80%, respectively (Kargar et al., 2013). According to Overdevest et al. (2011), 14% of the chicken samples had bla_{TEM} gene, 15% carried the bla_{SHV} gene, and 66% had the $bla_{\text{CTX-M}}$ group genes (Overdevest et al., 2011), which was incongruous with the present study. According to the study of Su et al. (2016) that investigated milk samples, prevalence of bla_{SHV}, bla_{TEM}, $bla_{\text{CTX-M3}}$, $bla_{\text{CTX-M14}}$, bla_{CMY}, and bla_{DHA} were reported as 9%, 40%, 9%, 4%, 40%, and 2%, respectively (Su et al., 2016). In the study of Ojer-Usoz et al. (2017) on food samples, $bla_{\text{CTX-M14}}$, bla_{TEM} and bla_{SHV} was reported as 25%, 12.5%, and 18.5%, respectively (Ojer-Usoz et al., 2017). Another study by Ryu et al. (2012b) reported no samples carrying the bla_{SHV} gene, while the prevalence of bla_{TEM} genes was slight (Ryu et al., 2012a, 2012b), which is compatible with the present study in the low prevalence of bla_{SHV} and bla_{TEM} genes. In the mentioned studies, the prevalence of bla_{SHV} and bla_{TEM} genes was low or even 0%, while the prevalence of $bla_{\text{CTX-M}}$, $bla_{\text{CMY-2}}$, and bla_{DHA} genes was higher, which disagreed with the present study. This can be due to the presence of different bacterial colonies in those environments and specific types of samples (Kargar et al., 2013).

The present study showed a high prevalence of contamination with resistant *E. coli* in the chicken present in the markets of Birjand. This problem can be due to various reasons, including not following the hygiene by the related staff, unsanitary processing, packaging, storage, and use of water contaminated with *E. coli* for washing the chicken.

4. CONCLUSIONS

The present study showed the high contamination of chicken with antibiotic-resistant *E. coli*, including ESBL- and AmpC-producing strains. So, use of antibiotics in poultry production must be more precautious.

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