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Molecular characterization of *Escherichia coli* resistance genes in chicken meat

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Abstract

Escherichia coli gene mutations (plasmids, integrons, and transposons) have instigated multidrug resistance (MDR) against various antimicrobials. This study detected antibiotic-resistance genes (genotypic and phenotypic) in *E. coli* and performed Whole-genome sequencing to discover MDR-associated *E. coli* genes in chicken meat. *E. coli* isolates were serologically identified, and their antimicrobial sensitivity was tested. *E. coli* presence was confirmed in 40% of the chicken samples. Cephalosporins, tetracyclines, and sulfonamides presented higher efficiency against *E. coli*. The serological investigation revealed the presence of STEC (O157:H7, 30%), ETEC (O142, 30%), EHEC (O26:H11, 10%), and EPEC (10%) in chicken meat samples. A symmetrical band represented the Subunit B of the Shiga-like toxin (SLT) gene whereas the Heat-labile toxin (LT) gene was found in plasmid and genomic DNA-detected strains. The results revealed the hazardous nature of STEC for chicken meat consumers. The study recommends improving the hygienic conditions during the chicken handling and processing steps, which will minimize antibiotic usage and resistance.

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Introduction

Escherichia coli is a gram-negative nonpathogenic commensal microorganism of human and animal intestines. They are mainly classified as Enterohaemorrhagic, Enteropathogenic, Enterotoxigenic, and Enteroinvasive. However, infectious Shiga toxin-producing *E. coli* (STEC) can cause various gastrointestinal disorders such as hemolytic-uraemic syndrome (HUS), hemorrhagic colitis (HC), and diarrhea through the consumption of contaminated food and water (Adeyanju and Ishola, 2014).

Poultry meat, contaminated food products, and water are the primary source of pathogenic STEC infection in humans. The chicken feces could contaminate the meats (mutton, chicken, and beef) with STEC during slaughtering, handling, processing, and storage (EL-Kholy *et al.*, 2020).

Antibiotic treatments are the main remedy to counter *E. coli* infections, which have led to the emergence ofmultidrug-resistant (MDR) *E. coli* strains. The antibacterial resistance of microorganisms is mainly attributed to gene mutation, and resistance genes found in integrons, transposons, and plasmids.

The activation cassette-containing integrons are associted with gene expressions. Two conversational segments (CS) are found in Integron Class I adjacent to the variable region (VR), which are common in the new generation of Enterobacteriaceae (CDC, 2019). The overuse of antimicrobials leads to the emergence of a new generation of mutated microbial strains with higher antibacterial resistance. This scenario is considered a serious global public health concern. Therefore, novel drugs are being investigated to treat MDR strains in poultry, animals, and humans (Cunrath *et al.*, 2014).

This study was aimed to explore the antimicrobial resistance (genotypic and phenotypic) and Whole-genome sequencing based identification of MDR-related gene structure of *E. coli* in commercial poultry meat.

Material and methods

Sampling, preparation, and bacterial testing

Chicken samples (100) were randomly collected from various markets. The samples were placed in polyethylene bags and immediately transferred to a refrigerator in the bacteriological laboratory for analysis. MacConkey broth was used to culture a homogenized chicken meat sample (2g) followed by incubation (37°C, 18 hours). Then, it was streaked into a MacConkey agar medium (Oxoid) plate and incubated at 37°C for 24 hours. The pink colonies were drawn on eosin methylene blue (Oxoid) and incubated at 37°C for 24 hours). The large *E. coli* colonies presented a blue-black-green metallic luster. *E. coli* colonies were identified through morphological and microscopic analyses and biochemical test kits (bioMerieux API, France) (CDC, 2020). Furthermore, serotyping was performed using a WHO-recommended antiserum set (Denka Seiken Co., Japan) (Ewing, 1986).

Antibacterial susceptibility

Disc diffusion method was adopted to assess antibacterial susceptibility using Mueller-Hinton agar and 12 antibiotic discs (Tetracycline, Gentamycin, Ciprofloxacin, Streptomycin, Trimethophrim, Cefepim, Doxycycline, Cefotaxim, Flumequine, Sulfamethoxazole, Penicillin, and Ampicillin). The concentrations of Penicillin and Ampicillin were 10 µg/disc whereas the concentrations of other discs were 30 µg/disc (Alderman and Smith, 2001).

Serological identification of E. coli using slide agglutination test

Monovalent and polyvalent standard anti-selenium revealed the tested microorganism as enteric pathogenic *E. coli* (EPEC) (Li *et al.*, 2013). The microbial colonies were emulsified by adding two saline drops to the glass slide. To test the monovalent serum, nutrient gradient agar was used to cultivate another colony (37°C and 24 hours) with the addition and aggregation of looped antiserum. Microorganisms' suspension was prepared in physiological saline and antigen was identified through a slide agglutination test.

DNA extraction

GeneJet Genomic DNA Purification Kit (ThermoFisher Scientific, USA) was used for DNA extraction. Briefly, microbial colonies were centrifuged (5000 µg for 10 minutes), and the cell pellet was resuspended in 180 µl solution for digestion. Then, K Proteinase was added (20 µl), mixed, and bathed in water followed by incubation for 30 minutes at 56°C with continuous shaking for complete dissolution. RNase solution (20 µl) was added to the mixture, incubated (10 minutes at 37°C), and vortexed followed by the addition of ethanol (50%, 400 µl) and vortexing. The lysed cells were transferred, purified, and centrifuged at 6000 µg for 1 minute. Washing buffer (I & II) (500 µL) was added to the column and centrifuged for 2 minutes at the maximum speed for the complete removal of ethanol. The purified DNA was stored at -20°C. Plasmid preparation was carried out using a DNA plasmid GeneJet miniprep kit (Thermofisher Scientific, USA). Microbial colonies were placed in a micro-centrifuge tube (1.5 ml) and centrifuged (12,000 xg /2 min). The ice-cold buffer solution (250 µl) was used for pellet resuspension. The tube was inverted 56 times for proper mixing and incubated for 5 minutes at 37°C. The supernatant was transferred and centrifuged for 30 seconds at 10,000 xg. Buffer (500 µl) was used for rinsing followed by centrifugation at 10,000 xg for 30 seconds. Preheated ddH_2O (50 µl) was used to elute the DNA, which was incubated at 37°C for 3 minutes. Then, centrifugation was carried out at maximum speed (14,000xg) for 30 seconds.

Gene amplification

The PCR reaction mixture (25 µl) consisted of purified genetic material (1 µl, genomic DNA / plasmid prep), $MgCl_2$ (2.5 µl), buffer (5 µl), primer (1 µl, Table 1), Taq polymerase (0.25 µl), dNTP (0.5 µl), and nuclease-free water. Agarose gel (1%) containing ethidium bromide (0.5 µg / ml) was used for the gel electrophoresis (80V for 50 minutes) of PCR products. DNA ladder of 100 bp was used to compare the target DNA size and imaged under a Gel documentation system (Biometra, Göttingen, Germany).

Table 1. Primer sequences for target gene amplification

Target Gene	Primer ID	Primer sequence
Shiga-like toxin (SLT)	SLT F:	5'-AAGAAGATGTTTATGGCGGTTT-3'
	SLT R:	3'-GTCATTATTAAACTGCACTTCAGCA-5'
Heat-labile toxin (LT)	LT F:	5'-ATTGACATCATGTTGCATATAGGTTAG-3'
	LT R:	3'-ACATTTTACTTTATTCATAATTCATCCCG-5'
Ciprofloxacin resistance gene	aac (6′)-F:	5'-TTTATTATTTTTAAGCGTGCATAATAAGCC-3'
	aac (6′)-R:	3'-TTAAGACCCTTAATTGTTGGGATTT-5'
Gentamycin resistance gene	aac C2-F:	5'-CATACGCGGAAGGCAATAAC-3'
	aac C2-R:	3'-ACCTGAAGGCTCGCAAGA-5'

Extraction of DNA fragments

DNA Extraction Kit (Thermofisher Scientific, USA) was used to extract the target DNA fragments from agarose gel under UV light and stored in a 1.5 ml tube. The tube containing extracted DNA fragment was centrifuged for 2 minutes at 13000 xg. Buffer (700 µl) was used to wash the column followed by centrifugation for 1 minute at 37°C. The buffer (50 µl) was again added to elute with a spin column filter, held for 1 minute at 37°C, and centrifuged for 2 minutes at 13,000 x g.

Statistical analysis

SPSS software was used to perform ANOVA to compare the means.

Results

E. coli prevalence

The results revealed *E. coli* prevalence in 40% of chicken meat samples (Fig. 1).

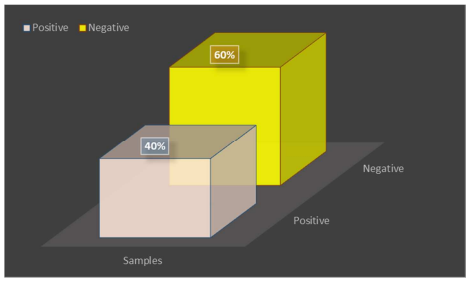

Fig. 1. Incidence of E. coli isolates in chicken meat samples

Table 2. Antibacterial susceptibility of *E. coli* isolated from chicken meat samples

Antimicrobial family	Antibacterial agents	Sensitive		Intermediate		Resistant	
		No.	Percentage	No.	Percentage	No.	Percentage
Sulfonamides	Trimethoprim	20	100	0	00	0	00
	Sulfamethoxazole	16	80	2	10	2	10
Cephalosporins	Cefepime	14	70	4	20	2	10
	Cefotaxime	13	65	4	20	3	15
Tetracycline	Tetracycline	10	50	0	00	10	50
	Doxycycline	8	40	2	10	10	50
Quinolones	Ciprofloxacin	4	20	4	20	12	60
	Flumequine	4	20	6	30	10	50
Aminoglycosides	Gentamicin	3	15	5	25	12	60
	Streptomycin	2	10	5	25	13	65
β-lactam	Penicillin	2	10	6	30	12	60
	Ampicillin	0	00	7	35	13	65

Antibacterial resistance pattern of E. coli

Antibacterial susceptibility of chicken meat-isolated *E. coli* strains (n = 20) against 12 antibiotics belonging to six different classes is presented in Table 2. Sulfonamides were the most effective antibiotics against isolated *E. coli* strains [trimethoprim (20/20) 100% and sulfamethoxazole (16/20) 80%]. The efficacy of cephalosporins such as Cefepime and Cefotaxime was noted as 70% (14/20) and 65% (13/20) against *E. coli* strains, respectively. Tetracyclines including Tetracycline and Doxycycline were effective against 50% (10/20) and 40% (8/20) *E. coli* strains, respectively. The other three antibiotic classes including Quinolones [ciprofloxacin (4/20) 20% and flumequine (4/20) 20%], aminoglycosides [gentamicin (3/20) 15% and streptomycin (2/20) 10%], and β-lactams [penicillin (2/20) 10%, and ampicillin (0/20) 0%] presented weak efficiency against isolated *E. coli* strains.

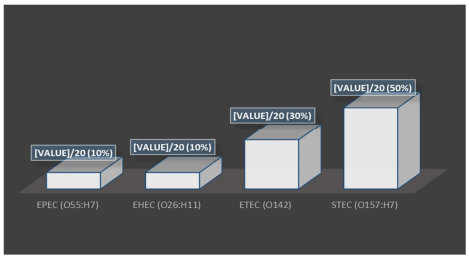

Fig. 2. Prevalence of various E. coli Serotypes in chicken meat samples.

Serological identification of E. coli isolates

Serological testing identified 20 *E. coli* isolates in chicken meat samples (Fig. 2). The presence of various *E. coli* isolates was noted as STEC [O157: H7,10/20 (50%)], ETEC [O142, 6/20 (30%)], EHEC [O26: H11, 2/20 (10%)], and EPEC [O55: H7, 2/20 (10%)].

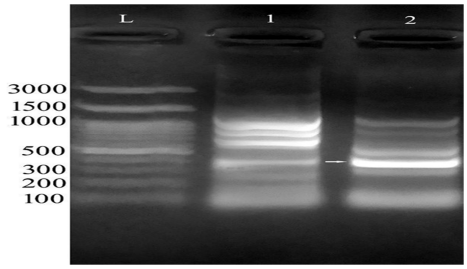

Fig. 3. Screening of SLT (B) subunit. Strain 1 has low amplified gene density. Lane (L) represents a DNA ladder of 100 - 3000 bp.

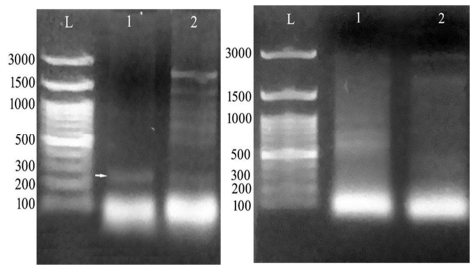

Fig. 4. (A) Heat-labile toxin (LT) gene (B) plasmid. The molecular size of the target gene was about ~ 200 bp in both strains (1 & 2)

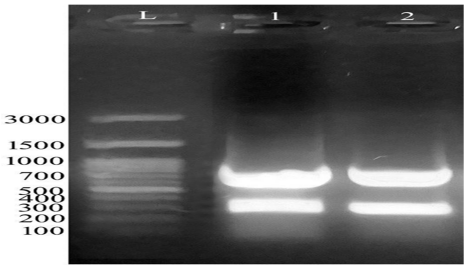

Fig. 5. Gentamycin-resistance gene (aac C2) of ~ 856 bp. A minor band appeared at 300 bp in both strains (1 & 2).

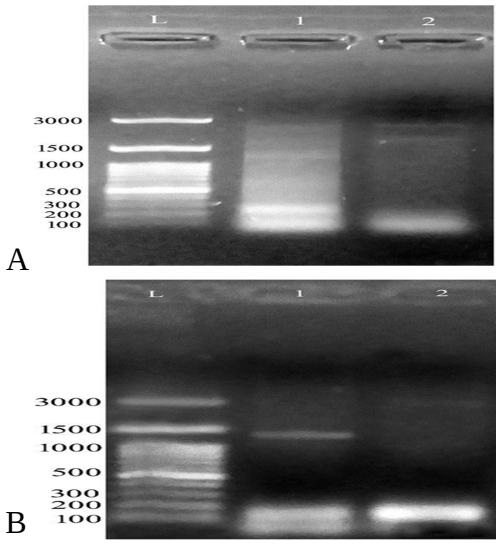

Fig. 6. Ciprofloxacin-resistance gene of 1 kb. (A) genomic amplification of strain (1), and (B) plasmid amplification

Fig. 3 reveals the screening of Shiga-like toxin (SLT). Subunit B of the SLT gene presented a uniform genomic DNA band of 300 bp. Results demonstrated minimal amplification of strain 1 as compared to strain 2. Both strains (1 and 2) contained fragments of approximately 200 bp. Heat-labile toxin (LT) gene was found in plasmid (Fig. 4). A gentamicin resistance gene (aac C2) segment of 856 bp was found in both strains whereas a minor band was detected at 300 bp (Fig. 5). Fig. 6 depicts a low prevalence of the Ciprofloxacin resistance gene in plasmid and genomic preparations. A sharp band of 1 kb was detected in strain 1 that was absent in strain 2. Contrarily, the target resistance gene was absent in the plasmid preparations of both strains.

Discussion

The findings of this study are in accordance with Partridge *et al.* (2018) and Wu *et al.*, (2018) who reported *E. coli* prevalence in 35.5% and 35.0% of tested chicken samples, respectively. The Indian chicken analysis revealed a 31% *E. coli* occurrence whereas *E. coli* presence in 20% of chicken samples has been reported in the US (Ngullie *et al.*, 2011; Sato *et al.*, 2010). Shaltout *et al.* (2020) reported *E. coli* isolation from 13.33% of Egyptian chicken meat samples whereas *E. coli* was noted in 11.1 % of Nigerian chickens (Tomova, *et al.*, 2018). Liu *et al.* (2016) have demonstrated the presence of *E. coli* in 10.60% of Croatian chicken samples whereas Jakabi, *et al.* (2002) observed a 9% occurrence of *E. coli* in chicken meat. Multiple studies have investigated Saudi Arabian chicken meat and revealed an *E. coli* prevalence of 5.92% (Deng *et al.*, 2016; Schulz *et al.*, 2015). The lowest *E. coli* occurrence (1.56%) has been reported in Moroccan chicken (Collins, 2000).

These reports indicate inadequate hygienic practices during slaughtering, handling, and transportation. The unhygienic meat processing could lead to *E. coli* presence in processed chicken meat indicating post-processing contamination (Collin, 2000). *E. coli* inhabits the gastrointestinal tracts of humans and animals. *E. coli* presence in prepared foods is associated with fecal contamination, which could also accompany other harmful microorganisms (*Salmonella, Campylobacter,* and *Shigella*) (Younis *et al.*, 2017).

Antibacterial drugs prevent bacterial infections and serve as chicken growth promoters. The selection of a proper antibacterial agent is crucial for better output. The testing of antibacterial susceptibility against *E. coli* strains (n = 20) isolated from chicken meat samples revealed varying efficacy of different antibiotics such as sulfonamides [trimethoprim (20/20) 100%, sulfamethoxazole (16/20) 80%], cephalosporins [Cefepime (14/20) 70%, Cefotaxime (13/20) 65%], tetracyclines [Tetracycline (10/20) 50%, Doxycycline (8/20) 40%], Quinolones [ciprofloxacin (4/20) 20%, flumequine (4/20) 20%], aminoglycosides [gentamicin (3/20) 15%, streptomycin (2/20) 10%], and β-lactam [Penicillin (2/20) 10%, ampicillin (0/20)0%]. These results are in line with the recommendations of the CDC (2019). Younis *et al.* (2017) have also reported almost complete *E. coli* resistance against penicillin, cefepime (95.8%), and amoxicillin (94.5%). Ammar *et al.* (2015) have associated *E. coli* antibiotic resistance with plasmid genes. Other studies have also demonstrated *E. coli* resistance (90%) against trimethoprim, ampicillin, cephalexin, gentamicin, tetracycline, sulfamethoxazole, and streptomycin (Bie, *et al.*, 2018; Adeyanju *et al.*, 2014).

Multidrug resistance of *E. coli* against sulfonamides, aminoglycosides, β-lactams, and tetracyclines is a well-known phenomenon (Ramadan *et al.*, 2016). Different studies have reported the prevalence of β-lactam resistant *E. coli* (Mohamed *et al.*, 2015; Eid and Erfan 2013). Li *et al.* (2020) have reported high resistance of *E. coli* towards tetracycline, sulfadiazine, chloramphenicol, gentamicin, ceftriaxone, amoxicillin, sulfadiazine, and ampicillin. Zhang *et al.* (2012) have revealed that 60% of chicken meat-isolated *E. coli* were resistant to fluoroquinolones. Tang *et al.* (2011) have also reported considerable *E. coli* resistance to ciprofloxacin (35.0%), norfloxacin (36.8%), and enrofloxacin (34.1%).

Serological test of isolated *E. coli* strains (20) revealed the presence of STEC [O157: H7 (10/20) 50%], ETEC [O142 (6/20) 30%], EHEC [O26: H11 (2/20) 10%], and EPEC [O55: H7 (2/20) 10%]. The subunit B of the SLT gene presented a uniform DNA segment with a molecular size of 300 bp.

The results depicted a lower amplification of strain (1) than Heat Labile toxin (LT). Fragments of approximately 200 bp were noted in both strains (1 and 2). The gentamicin resistance gene (aac C2) fragment was documented with a molecular size of 856 bp whereas a small band appeared at 300 bp. Ciprofloxacin-associated resistance genes were also screened in plasmid and genomic preparations of both strains (1 and 2). A band of 1 kb was only detected in strain (1). Contrarily, the target gene was not detected in the plasmid of tested strains. These results are in line with the findings of Momtaz and Jamshidi (2013), who have reported various O serotypes (O2, O35, O1, O8, O109, O18, O88, O15, O115, and O78). Ying *et al.*, (2020) isolated an eaeA gene from enteropathogenic *E. coli*, which resembled the eae genes of EHEC, O157: H7, and O55: H7. Kakoullis *et al.* (2019) detected a SLT gene producing false negative results whereas HECO157 contained 60MDa plasmid. Lagerqvist *et al.* (2020) have reported the eaeA and SLT (I, II) genes, which indicate the occurrence of EHEC O157 strain whereas Yang *et al.* (2020) have documented the Stx gene in EHEC strains. The virulence genes include extra-intestinal infectious genes such as traT, afaD8, Eisen, Cdt2, bmaE, cdt3, iutA, and iucD. The presence of the etpD gene in the ETEC strain has been reported (Villegas *et al.*, 2013). RIMD 0509952 and EDL933 are known to cause intestinal hemorrhage whereas the fmH gene is considered a non-virulent gene in various *E. coli* strains (Momtaz and Jamshidi, 2013).

Conclusion

The study demonstrates *E. coli* pathogenicity-related genes in chicken meat samples, which included antigenic genes and different somatic capsules. STEC management is crucial as it could be hazardous to chicken meat consumers.

Recommendation(s)

The common presence of *E. coli* in daily meals could be harmful to food biosafety and public health. Therefore, enhanced hygiene measures should be adopted during the slaughtering, handling, and processing of chicken carcasses. Furthermore, unnecessary antibiotic administrations should also be avoided in humans and chickens to counter the emergence of new antibacterial resistance.

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