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Serological identification of *Escherichia Coli* multidrug-resistance genes isolated originated from retailed Chicken meat

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Gene mutation on plasmids, integrons, and transposons causes *Escherichia coli* multi-resistance to a number of antimicrobials. The goals of this study were to 1) find antibiotic resistance genes in *E. coli* by genotyping and phenotype, and 2) use whole-genome sequencing to find *E. coli* gene multi-drug resistance in chicken flesh. Samples were collected, processed, and analyzed bacteriologically, as well as an antimicrobial sensitivity test and serological identification of *E. coli* isolates. From 100 chicken samples, a total of 9 *E. coli* strains were isolated from tested samples the antibiotics resistance phenotypes were recorded as following; AMP-CIP-NA-TET, FEB-AMC*,* GEN-CFZ-C-NA*,* AMP-NA-CTX*,* TET*.* The obtained results showed that the highest sensitivity of tested strains was to Amoxicillin-clavuanate 25 (50%). While, the highest resistance rate of tested strains was against Tetracycline 25 (50%). In our study we can referring the high resistance rate against tetracycline as this antibiotic is one of the most common used drug in poultry industry while the low resistance toward Amoxicillin-clavuanate may referring to the new trend combination of this recent generation family antibiotics. The measures should be taken to reduce the harm to public health, such as the reasonable use of antimicrobials specially (tetracycline) in animal husbandry.

Keywords: microbial resistance gene structure, STEC (O157: H7), enterotoxins, cephalosporins, Shiga toxin, ETEC (O142), sulfamethoxazole,

INTRODUCTION

Despite the fact that *E. coli* is a gram-negative typical non-pathogenic intestinal resident, it is considered a commensal for both animals and humans. Entero-invasive, entero-pathogenic, entero-haemorrhagic, and entero-toxigenic are the four classifications. However, pathogenic *E. coli* (Shiga toxin-producing *E. coli*) (STEC) can cause gastrointestinal disorders such as haemorrhagic colitis (HC), diarrhoea, and haemolytic-uraemic syndrome (HUS) by drinking contaminated water or eating infected food (Kim et al. 2022).

STEC are pathogenic microbes that are commonly transmitted to humans via poultry meat and are commonly transmitted after drinking contaminated water or eating contaminated food such as chicken, beef, or sheep meat, which primarily acquired contamination from the poultry's fecal intestinal contents during slaughter or from human handlers during storage and processing (Habib et al. 2021).

Antimicrobials are commonly used to treat any *E. coli* infection; however, the difficulty arises from the incorrect use of antimicrobials as *E. coli* therapy, resulting in the pathogen developing multi-resistance to a variety of antimicrobial medications. Antimicrobial resistance is mostly acquired via gene mutation, which results in the presence of resistance genes on integrons, transposons, and plasmids. Integron is a gene expression system that incorporates the gene cassettes that activate it. Integron class I consist of a variable region (VR) flanked by two conversed segments (CS), which is the common type among Enterobacteriaceae new generations (Yoon et al. 2020).

Antimicrobial resistance develops as a result of repeated antibiotic use, resulting in the emergence of new strains of microbes that are extremely resistant to a variety of antibiotics. This is regarded as one of the world's most serious public health crises. Scientists are exploring for new antibiotic generations to combat the microbe's resistance in animals, poultry, and humans (Cunrath et al. 2019).

As a result, the goals of this work are to 1) look at antibiotic resistance phenotype and genotype in Escherichia coli, and 2) use whole-genome sequencing to discover the structure of bacterial resistance genes in multi-drug resistant *Escherichia coli* in marketed poultry meat.

MATERIALS AND METHODS

2.1. Ethical approval: There is no ethical approval necessary.

2.2. Bacteriological examination:

A total of 100 samples of chicken meat were examined; they were chosen at random from a variety of markets, stored in a plastic bag, and then transported right away inside an ice box to the bacteriological laboratory for evaluation. Inoculated in MacConkey broth for approximately 18 hours at 37°C using two grams of a homogenized chicken sample. Then, for around 24 hours at 37°C, streaked onto MacConkey agar (Oxoid) plates. After around 24 hours at 37°C of incubation, the Rose pink colonies were streaked over Eosin Methylene Blue (Oxoid). typical Escherichia coli has a big colony with a blue-black figure and a green metallic sheen. Morphological, microscopic, and biochemical test kits (bioMerieux API, France) used to identify *E. coli* colonies (Wright, 2010). Serotyping and antisera sets for additional identification (Denka Seiken Co., Japan) as per Kitchel et al. (2009). The following 15 antibiotic discs were tested for antimicrobial sensitivity using the Muller-Hinton agar disc diffusion technique: Amoxicillin-clavulanate (30 mg/disk), Ampicillin (10 mg/disk), Cefazoline (30 mg/disk), Cefotaxime (30 mg/disk), Cefepime (30 mg/disk), Chloramphenicol (10 mg/disk), Ciprofloxacin (30 mg/disk), Gentamicin (30 mg/disk), Nalidixic acid (10 mg/disk), Tetracycline (30 mg/disk).

2.3. Serological identification of *Escherichia coli* isolates

(Gao et al. 2015) using slide agglutination test with conventional monovalent and polyvalent *E. coli* antisera sets for definition of the entero pathogenic kinds as follows; emulsified microbial colony by two drops of saline on a glass slide. Addition of a loop ful antiserum, Agglutination, and continued colony culture on nutrient agar slant followed by an incubation period of 24 hours at 37°C. 2.5. Nucleic acid extraction: DNA was isolated using the Gene Jet genomic DNA purification kit (ThermoFisher Scientific, USA). distilled into the following; Colonies of bacteria were selected by centrifuging them for 10 minutes at 5000 g, resuspending the cell pellet in 180 mL of digestion solution (provided in the kit), adding 20 mL of proteinase K, thoroughly mixing, and incubating the mixture at 56 oC in a water bath for about 30 minutes until complete lysis. After adding 20 L of RNase solution to the mixture, it was vortexed before being heated for 10 minutes at 37 C. 400 liters of 50 percent ethanol were added to the mixture after 200 liters of Lysis solution and vortexed. Lysated cells were transferred, purified, and centrifuged for 1 minute at 6000 g. Plasmid preparation using the GeneJet DNA miniprep kit (Thermofisher Scientific, USA). The handbook states that 1 to 3 ml of the growing culture should be placed in 1.5 ml microcentrifuge tubes before being centrifuged at 12,000 x g for two minutes. Re-suspend the pellet in 250 l of the kit's provided, ice-cold resuspension buffer, and mix it by inverting the tube roughly 5–6 times. Incubate tubes at 37 °C for 5 minutes. Transferred the supernatant, centrifuged at 10,000 x g for 30 seconds, washed with 500 l of the kit's provided buffer, and then repeated the process. DNA plasmids were eluted using pre-warmed 50 l of ddH2O, incubated for 3 min. /37˚C, and then recentrifuged at maximum speed for 30 sec. (14,000 x g). Gene Amplification: Purified genetic material (genomic DNA/plasmid preps) diluted to 1 l, 2.5 l MgCl2, 5 l buffer, 1 l primer (mentioned in table 1 below), 0.25 l Taq Polymerase enzyme mix, 0.5 l dNTPs, and 25 l free-nuclease water are used in PCR processes to amplify genes. 0.5 g/ml ethidium bromide and 1 % agarose gel are used to resolve PCR results, and a 100 bp DNA ladder is used to gauge their size. Run Gel at 80V/50 minutes after that, and document using the gel method (Biometra, Goettingen, Germany). DNA fragments can be extracted from agarose gel by utilizing a DNA extraction kit to elute them (Thermofisher Scientific, USA). Under UV lighting, fragmentation was done before being preserved in a 1.5 ml tube. then centrifuged for two minutes at 13,000 x g. the PCR primers used in molecular examination tabulated in table (1).

2.4. Detection of Resistance Genes:

E. coli DNA was extracted using Bacterial Genomic DNA Extraction Kit (Tiangen, Beijing, China) and then the resistance genes associated with beta-lactams (bla_{CTX-M}, bla_{TEM}), the quinolone-resistance genes (*qnr*S), the sulfonamides-resistance genes (*sul1*, *sul2*), were amplifed by PCR, using the previously reported (Valat, et al. 2016; Moawad, et al. 2017 and Navajas-Benito, et al. 2017).

2.5. Detection of Integrons:

universal primers for the amplifcation of Class I integron gene cassette genes were detected by PCR. Te primers of cassette FP and cassette RP were designed according to reference: 5-TCATGGCTTGTTATGACTGT-3 and 5-GTAGGGCTTATTATGCACGC-3. The amplifcation consisted of an initial denaturation at 94∘C /5min, 30 cycles of denaturation at 94∘C /60 s, annealing at 56∘C /55 s, and extension at 68∘C /6min. A final extension for 10 min/72∘C was also applied. PCR products were purified and sequenced for the further analysis (White, et al. 2000).

Table 1: List of PCR Primers Used in Molecular examination

Antibiotics	Sequence	Reference
Amoxicillin-clavulanate	F: TGTCATTTACGGCATACTCG R: ATCAGGCATCCCATTCCCAT	Navajas-Benito, et al. (2017)
Ampicillin	F: TTGCGATGCTCTATGAGTGGCTA R: CTCGAATGCCTGGCGTGTTT	
Cefazoline	F: GCAGGTCCAGCAGCGGGTAG R: CTTCCTGCCCGAGTATCGTG	Moawad, et al. (2017)
Cefotaxime	F: CGCTTTGCGATGTGCAG R: ACCGCGATATCGTTGGT	Valat, et al. (2016)
Cefepime	F: TTGCGATGCTCTATGAGTGGCTA R: CTCGAATGCCTGGCGTGTTT	Moawad, et al. (2017)
Chloramphenicol	F: CACTCAAGGATGTATTGTG R: TTAGCGTTGCCAGTGCTCG	Navajas-Benito, et al. (2017)
Ciprofloxacin	F: ACACAATACATATCAACTTCGC R: AGTGTGTTTAGAATGGTGATC	
Gentamycin	F: GCGCCTTTCCTTTGGGTTCT R: CCACCCGTTCCACGTTGTTA	
Nalidixic acid	F: ATTCTTGAAGACGAAAGGGC R: ACGCTCAGTGGAACGAAAAC	
Tetracycline	F: CATTAATAGGCGCATCGCTG R: TGAAGGTCATCGATAGCAGG	

RESULTS

3.1. incidence of isolated *E. coli*:

figure (1) A total of 100 tested *E. coli* about 45% were positive and 55% were negative, from this positive detection about 20 isolates were tested serologically and reported the following serotypes in figure (2); 3/20 (15%) Enteropathogenic *E. coli* (O55:H7) (EPEC), 4/20 (20%) Enterohemorrhagic *E. coli* (O26:H11) EHEC, Enterotoxogenic *E. coli* (O142) 5/25 (12%) ETEC, 8/20 (40%) Shiga toxin- producing *E. coli* (O157:H7) STEC.

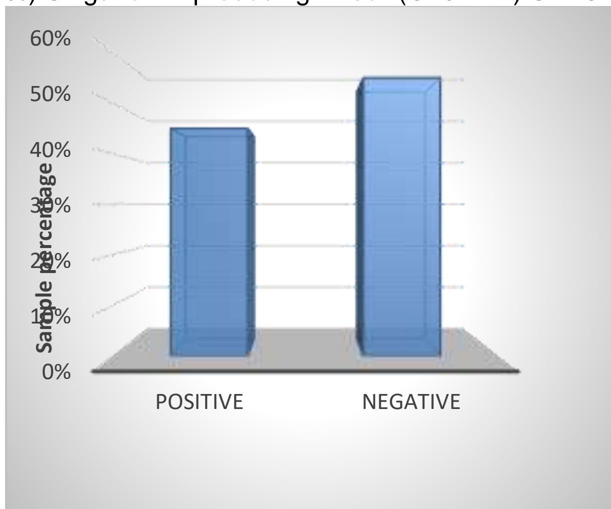

Figure 1: Incidence of *E. coli*

Table 2. List of phenotypic resistance and integrons resistance in *E. coli* from chicken meat samples

Resistance phenotype	Integrons/ resistance
AMP-NA-TET	bla_{CTX-M}, bla_{TEM}, *sul2*
AMP-TET	bla_{CTX-M}, bla_{TEM}, *sul2*
AMP-CIP-NA-TET	bla_{CTX-M}, bla_{TEM}, *sul2*
FEB-AMC	bla_{CTX-M}, bla_{TEM}, *sul1*
AMP-GEN- CFZ -TET	bla_{CTX-M}, bla_{TEM}, *sul2*
AMP-C-NA-TET	bla_{CTX-M}, bla_{TEM}, *flor*, *sul2*
AMP-NA-CTX	bla_{CTX-M}, bla_{TEM}, *sul2*
TET	bla_{CTX-M}, bla_{TEM}, *sul2*
AMP-NA	bla_{TEM}, *qnrS, sul1*

AMP: Ampicillin AMC: Amoxicillin-clavulanate CIP: ciprofloxacin CFZ: Cefazoline CTX: Cefotaxime C: Chloramphenicol GEN: Gentamycin FEP: Cefepime NA: Nalidixic acid TET: tetracyclin

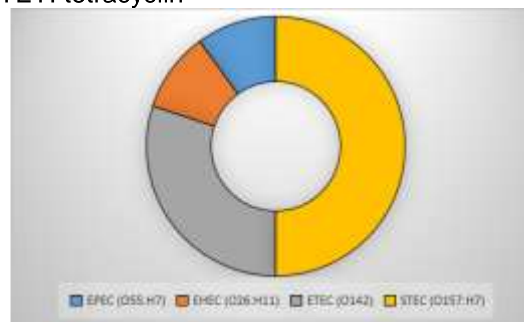

Figure 2: Different serotypes isolates of *E. coli*

The antibiotics resistance phenotypes were recorded in table (2) as following; AMP-NA-TET (*bla*$_{CTX-M}$, *bla*$_{TEM}$, *sul2)*, AMP-TET (*bla*$_{CTX-M}$, *bla*$_{TEM}$, *sul2),* AMP-CIP-NA-TET (*bla*$_{CTX-M}$, *bla*$_{TEM}$, *sul2),* FEB-AMC (*bla*$_{CTX-M}$, *bla*$_{TEM}$, *sul1),* AMP-GEN-CFZ-TET (*bla*$_{CTX-M}$, *bla*$_{TEM}$, *sul2),* AMP-C-NA-TET (*bla*$_{CTX-M}$, *bla*$_{TEM}$, *flor*, *sul2),* AMP-NA-CTX (*bla*$_{CTX-M}$, *bla*$_{TEM}$, *sul2),* TET (*bla*$_{CTX-M}$, *bla*$_{TEM}$, *sul2),* AMP-NA (*bla*$_{TEM}$, *qnrS, sul1).*

3.2. phenotypic resistance and integrons resistance in *E. coli*:

A total of 9 *E. coli* strains were isolated from tested samples the antibiotics resistance phenotypes were recorded in table (2) as following; AMP-NA-TET (*bla*$_{CTX-M}$, *bla*$_{TEM}$, *sul2)*, AMP-TET (*bla*$_{CTX-M}$, *bla*$_{TEM}$, *sul2),* AMP-CIP-NA-TET (*bla*$_{CTX-M}$, *bla*$_{TEM}$, *sul2),* FEB-AMC (*bla*$_{CTX-M}$, *bla*$_{TEM}$, *sul1),* AMP-GEN-CFZ-TET (*bla*$_{CTX-M}$, *bla*$_{TEM}$, *sul2),* AMP-C-NA-TET (*bla*$_{CTX-M}$, *bla*$_{TEM}$, *flor*, *sul2),* AMP-NA-CTX (*bla*$_{CTX-M}$, *bla*$_{TEM}$, *sul2),* TET (*bla*$_{CTX-M}$, *bla*$_{TEM}$, *sul2),* AMP-NA (*bla*$_{TEM}$, *qnrS, sul1).*

3.3. Antibiotics susceptibility

profile viewed in table (3) as following; about 25 (50%) of tested strains were sensitive to Amoxicillin-clavuanate, followed by 20 (40%) sensitive to Ampicillin, 18 (36%) to Cefazoline, 17 (34%) to (Cefotaxime, Cefepime, Chloramphenicol), 15 (30%) to (Ciprofloxacin, Gentamycin), 14 (28%) to (Nalidixic acid, Tetracycline). While, the resistant percentage was as following; 25 (50%) against Tetracycline, 22 (44%) against (Cefotaxime, Gentamycin, Nalidixic acid), 20 (40%) against (Chloramphenicol, Ciprofloxacin), 19 (38%) Cefazoline, 18 (36%) against Cefepime, 17 (34%) against Ampicillin, 11 (22%) against Amoxicillin-clavulanate.

Table 3: Antibiotics resistance profile of *E. coli* isolates from chicken meat

Antibiotics	Number of samples (50)		
	Sensitive (S)	Intermediate (I)	Resistant (R)
Amoxicillin-clavulanate	25 (50%)	14 (28%)	11 (22%)
Ampicillin	20 (40%)	13 (26%)	17 (34%)
Cefazoline	18 (36%)	13 (26%)	19 (38%)
Cefotaxime	17 (34%)	11 (22%)	22 (44%)
Cefepime	17 (34%)	15 (30%)	18 (36%)
Chloramphenicol	17 (34%)	13 (26%)	20 (40%)
Ciprofloxacin	15 (30%)	15 (30%)	20 (40%)
Gentamycin	15 (30%)	13 (26%)	22 (44%)
Nalidixic acid	14 (28%)	14 (28%)	22 (44%)
Tetracycline	14 (28%)	11 (22%)	25 (50%)

The obtained results showed that the highest sensitivity of tested strains was to Amoxicillin-clavuanate 25 (50%). While, the highest resistance rate of tested strains was against Tetracycline 25 (50%).

DISCUSSION

Chicken meat is becoming a more important source of animal protein food and it is one of the most popular foods, nonetheless, that are frequently linked to outbreaks of foodborne illness. By coming into contact with chicken excrement or eating infected poultry products, pathogenic microorganisms can be transmitted to humans. *Escherichia coli* has been hypothesized to reside in the avian intestine and be capable of being transmitted from birds to people (Vuthy et al. 2017). In the digestive systems of people, animals, and birds, the Gram-negative bacterium *Escherichia coli* typically functions as a natural commensal, although some strains are important intestinal and extraintestinal pathogens (Shobrak & Abo-Amer, 2014 and Abo-Amer et al. 2018).

the obtained results from a total of 100 tested *E. coli* about 45% were positive and 55% were negative, from this positive detection about 20 isolates were tested serologically and reported the following serotypes; 3/20 (15%) Enteropathogenic *E. coli* (O55:H7) (EPEC), 4/20 (20%) Enterohemorrhagic *E. coli* (O26:H11) EHEC, Enterotoxogenic *E. coli* (O142) 5/25 (12%) ETEC, 8/20 (40%) Shiga toxin- producing *E. coli* (O157:H7) STEC. Lower results reported by Moawad et al. (2017) whom studied about 180 chicken examined samples in Egypt and observed about 21 *E. coli* (11.7%), another lower incidence rate reported in Canary Islands (16.5% in chicken meat) (Hernandez et al. 2005), Northwestern Spain (17.9% in chicken) (Capita et al. 2007), On the other hand, it was higher than reported previously in prevalence of *E. coli* in chicken meat which was lower than in Nigeria (43.4%) in frozen poultry meat (Adeyanju and Ishola, 2014) but higher than in Korea (4.9% in poultry meat) (Lee et al. 2009). Prevalence of *E. coli* in poultry meat was 19.0% while in China and Iran were found 60.0% 4.1 and 29.0%, respectively (Momtaz et al. 2013 and Wang et al. 2014).

Bacterial antimicrobial resistance is a global emerging problem of public health concern. In the current study showed that the highest sensitivity of tested strains was to Amoxicillin-clavuanate 25 (50%). While, the highest resistance rate of tested strains was against Tetracycline 25 (50%). Higher results reported by Kim et al. (2022) who studied the antimicrobial resistance and molecular characterization of *Escherichia coli* isolates from layer breeder farms in Korea and observed that ampicillin and tetracycline had the highest resistance rates among the 55 *E. coli* strains tested (65.5% and 78.2% respectively). Another study performed by Johnson et al. (2008) in Portugal whom recorded about 11.4% resistance rates from poultry genes against ampicillin and tetracycline. Kagambègaab et al. (2012) detected the most resistance against ampicillin from *E. coli* isolates from Spain foods. Kim et al. (2019) isolated about 98.2% resistance to tetracycline and 61.8% resistance to ampicillin from south Korean chicken meat. The results which recorded by Moawad et al. (2017) which showed that the antimicrobial resistance of *E. coli* isolated from poultry was higher than from beef to the most of tested antibiotics. The high

resistance to tetracycline, ampicillin, amoxycillin–clavulanic acid, trimethoprim/sulphamethoxazole and streptomycin in this study was in agreement with previous reports conducted in Egypt (Ahmed & Shimamoto, 2015 and Ahmed et al. 2013) and Algeria (Laarem et al. 2017). While in Spain, most of *E. coli* isolated from diarrhoeic and healthy lambs were highly resistant to tetracycline and streptomycin but show lower resistance to ampicillin, Most of *E. coli* isolates in this study were sensitive to enrofloxacin, chloramphenicol and ceftriaxone which is in agreement with previous results in Spain (Medina et al. 2011). In this study *E. coli* isolates were multidrug resistant. Similar results reported previously in Ghana and US (Rasmussen et al. 2015 and Zhao et al. 2012). Moawad et al. (2017) showed also resistance to ampicillin (87.0%) and cefotaxime (80.0%) and all were susceptible to chloramphenicol, colistin and ciprofoxacin. *E. coli* isolates showed resistance to tetracycline, ampicillin, streptomycin, trimethoprim/sulphamethoxazole and amoxicillin–clavulanic acid with 80.9, 71.4, 61.9, 61.9 and 61.9%, respectively. Ten (47.6%), 9 (42.8%) and 9 (42.8%) isolates were susceptible to ciprofoxacin, enrofoxacin and ceftriaxone, respectively. All tested isolates were susceptible to colistin. In *E. coli* isolates, 5 of 8 screened resistance-associated genes were detected by PCR as following; Eleven (52.4%) out of 21 isolates harboured bla_{TEM} (1 and 104), while bla_{CTXM} (1 and 14) was detected in 9 isolates (42.9%). On the other hand, other studies detected bla_{CMY} in 89.0% *E. coli* isolated from poultry meat in Denmark (Hansen et al. 2016).

CONCLUSION

Our obtained results exhibit the molecular characterization of antimicrobial resistance in Escherichia coli from chicken meat and declared the highest sensitivity of tested strains was to Amoxicillin-clavuanate 25 (50%). While, the highest resistance rate of tested strains was against Tetracycline 25 (50%). In our study we can referring the high resistance rate against tetracycline as this antibiotic is one of the most common used drug in poultry industry while the low resistance toward Amoxicillin-clavuanate may referring to the new trend combination of this recent generation family antibiotics. The measures should be taken to reduce the harm to public health, such as the reasonable use of antimicrobials specially (tetracycline) in animal husbandry.

CONFLICT OF INTEREST

The authors declared that present study was performed in absence of any conflict of interest.

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AUTHOR CONTRIBUTIONS

The authors take responsibility for the content of the publication. Author can only be a person who participated in the original scientific research as following; NTE: Corresponding author of the manuscript, study design, drafted, shared in PCR test, write and revised the manuscript, and data analysis. HAA: revised the manuscript. All authors read and approved the final manuscript.

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