RESEARCH ARTICLE

Effects of Misuse of Antibiotics on the Resistance of *Escherichia coli* Isolated from the Intestines of Broiler Chickens

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Received: 12th March, 2020; Revised: 27th April, 2020; Accepted: 16th May, 2020; Available Online: 25th June, 2020

ABSTRACT

The coliform bacteria is one of the most important bacterial diseases that threaten the poultry sector in Iraq, and the result of the excessive and indiscriminate use of antibiotics during the farming period to eliminate coliform bacteria has led to the development of antibiotics resistance. The current study aimed to investigate the *Escherichia coli* in the poultry intestines and to determine its ability to resist antibiotics to achieve this, 177 samples of poultry intestines were collected from local poultry farming houses in Babil province included 142 isolates (80.22%) of *E. coli* and 35 isolates (19.77%) for other intestinal bacteria. In this study, the bacterial susceptibility test for *E. coli* showed a significant difference at a statistical level of $p < 0.05$ by chi-square method for the antibiotics resistance ratios, respectively, rifampicin (100%), oxytetracycline (99.29%), cefixime (98.59%), sulfamethoxazole + trimethoprim (95.77%), norfloxacin (90.14%), cefepime (89.43%), nitrofurantoin (72.53%), gentamicin (71.12%), ceftazidime (70%). Also, Indian ink was used to detect the presence of the capsule around the *E. coli*, where the results of the microscopic examination showed 81 isolates (57.04%) were surrounded by the capsule and 61 isolates, 42.95% were not surrounded. Finally, the study concluded that the bacterial resistance of *E. coli* continues to rise and is alarming and is threatening public health.

Keyword: Broilers gut, *Escherichia coli,* Misuse of antibiotics, Resistance.

International Journal of Drug Delivery Technology (2020); DOI: 10.25258/ijddt.10.2.1

How to cite this article: Abed AR, Khudhair AM, Hussein IM. Effects of Misuse of antibiotics on the resistance of *Escherichia coli* isolated from the intestines of broiler chickens. International Journal of Drug Delivery Technology. 2020;10(2):190-194.

Source of support: Nil

Conflict of interest: None

INTRODUCTION

Misuse of antibiotics in poultry farming is a major problem that led to the resistance development of intestinal bacteria isolated from chickens broilers,[1] coliform bacteria are microorganisms that live in the intestines of poultry, and that contribute effectively too many vital events,[2] and as a result of exposure to antibiotics by mixing them with feed or with water in large quantities during the farming period, which led to the appearance of bacteria resistant to antibiotics.[3] *E. coli* are one of the bacteria that live in the intestines and form about 83% (freshly dead poultry) of intestinal bacteria.[4] Poultry is considered the most important sources for the *E. coli* development of resistance against antibiotics, and contributes significantly to its transmission to humans[5] and causing him urinary tract infections [*E. coli* (ExPEC) (Extraintestinal pathogenic)] through transmission through food.[6] The broilers chicken in Iraq is an important food source and is consumed by very large numbers of the community. During the farming in the fields, poultry breeders use antibiotics to keep them from the bacterial infection and continue this treatment until the day of marketing, so this study aimed at the laboratory detection of *E. coli* resistance to antibiotics in the broiler chickens.

MATERIALS AND METHODS

Sample Collection

One hundred seventy-seven samples of poultry intestines were collected for the period from January to May 2019 from local poultry farming houses in Babil province, Iraq and kept in a refrigerated container until they reached the microbiology laboratory, Technical Institute of Babylon.

Laboratory Examination of Samples

Once the intestinal specimens have arrived in the laboratory, a sterile incision is made in the colon area by sterile scissors, after which a sample is taken with a cotton swab from colon content, then disseminated to the coliform bacteria culture media (MacCkonkey and Eosin methylene blue agar) then diagnosed of *E. coli* and other coliform bacteria according to.[7]

E. coli counts

The bacterial count was calculated by using the spectrophotometer method (colometeric methods), which involves taking a

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sample of the bacterial colonies on the new medium (the age of the colony is 24 hours at 37°C), using a sterile bacterial loop, and then mixing well with 5 mL of the normal saline solution placed in a glass tube, then calculated the degree of turbidity solution (result from bacteria mixed with normal saline) by use adjusted according to the absorbance of 0.08-0.1 at 625 nm corresponding to 10^8 CFU/mL National Committee For Clinical Laboratory Standards (NCCLS).[8]

Antimicrobial Susceptibility Testing

The *E. coli* isolates from Broiler colon content samples were tested for antimicrobial susceptibility for nine antibiotics using the disc agar diffusion method, according to the Clinical And Laboratory Standards Institute (CLSI).[9] Standard discs were distributed on the surface of the Muller Hinton agar at the rate of five standard discs in a Petri dish, and the rest of the standard antibiotics discs were placed in the second Petri dish (Figure 1).

Standard Antibiotics Discs

Antibiotics were used in this study in the form of standard discs (diameter 5 mm), manufactured by the company Conda-Pronadisa, Spain, as shown in Table 1.

Capsule Stain

Duguid's method[10] was used to detect the bacterial capsule under microscope, with some modifications by researchers in the way they work.

A sample of colonies of *E. coli* (colonies ages 24 hours old cultured on the Muller Hinton agar) was taken by bacterial loop, and it placed on the surface of a glass slide, and added on it a drop of Indian ink, and well mixed by one corner of another glass slide. A thin smear worked on the surface of the glass slide and left until dry at room temperature, and then flood the smear with Locton phenol cotton blue dye (previously was used crystal violet stain) over the thin smear, and left for 5 minutes, then put a cover slide on smear and examined under the light microscope (100X). If a capsule is present, the appearance of a transparent halo (clear zones) around the bacterial cell will be observed, as shown in Figure 2.

Data Analysis

Chi-square (x^2) test was used for statistical data analysis in this study with SPSS (Statistical Package for the Social Sciences) program, version 16.0 software (SPSS Inc, Chicago, IL), to determine significant differences at a probability level of $p < 0.05$.

RESULTS AND DISCUSSION

One hundred and seventy-seven intestinal samples were collected from broiler chickens, of which 142 isolates for *E. coli* (80.22%), and 35 isolates from other bacteria (19.77%) (Table 2).

The total number of isolated *E. coli* in this study was about 142 isolates, divided into two types, depending on the presence

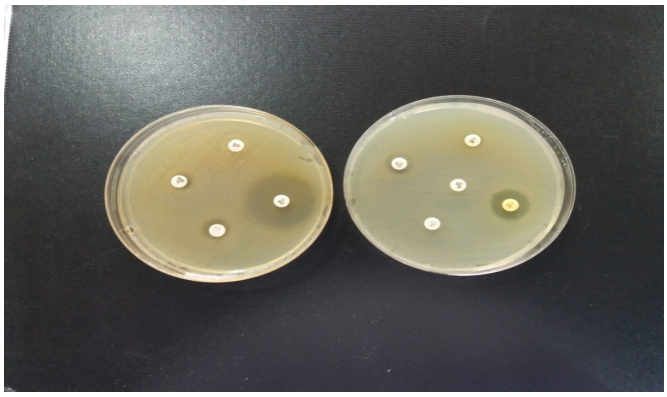

Figure 1: Anti-bacterial standard disc in Muller Hinton agar

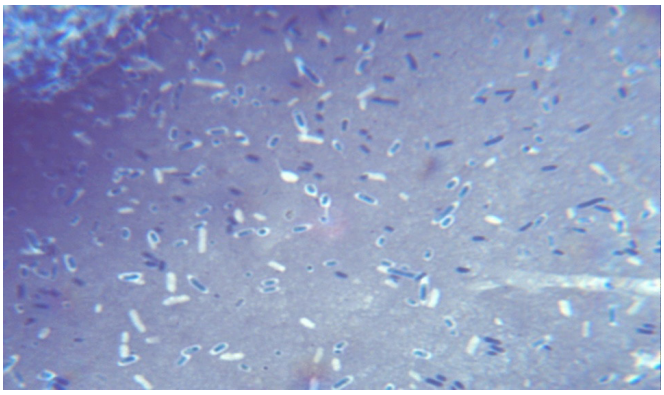

Figure 2: Capsule stain (Indian ink–lactophenol cotton blue dye).

Table 1: The concentration of antibiotics in the standard discs used and zone diameter interpretive (mm) standards for *E. coli*.

				Zone diameter interpretive standards (mm)		
Antibacterial groups	*Antibacterial*	*Disc content*	*Abbreviation*	*R*	*I*	*S*
Cephalosporins group	Cefepime	30 µg	FEP	≤26	27–31	≥32
	Cefixime	5 µg	CFM	≤15	16–18	≥19
	Ceftazidime	30 µg	CAZ	≤14	15–17	≥18
Aminoglycosides group	Gentamicin	10 µg	CN	≤12	13–14	≥15
Tetracycline group	Oxytetracycline	30 µg	OXT	≤15	16–25	≥30
Quinolones group	Norfloxacin	10 µg	NOR	≤12	13–16	≥17
Nitrofuran	Nitrofurantoin	300 µg	F	≤14	15–16	≥17
Rifamycins	Rifampicin	5 µg	RP	≤16	17–19	≥20
Folic acid synthesis inhibitors	Sulfamethoxazole/trimethoprim	25 µg	SXT	≤10	11–15	≥16

R = Resistance; I = Intermediate resistance; S = Susceptible

Table 2: The number and ratio of *E. coli* appearance in broiler chicken colon content specimens compared with other coliform bacteria.

Coliform bacteria	*Number*	*Appearance ratio*
E. coli	142	80.22 %
*Other bacteria	35	19.77 %
Total	177	99.99 %

*Other bacteria (*Klebsiella* spp , *Enterobacter aerogenes*, *Pseudomonas aeruginosa*).

Table 3: The number and ratio of capsulated *E. coli* appearance in Broiler chicken colon content specimens compared with other coliform bacteria

Coliform bacteria	*Number*	*Appearance ratio*
Capsulated *Escherichia coli*	81	57.04 %
Non-capsulated *Escherichia coli*	61	42.95%
Total	142	99.99%

Table 4: The number and ratio of resistance, intermediate resistance, susceptible of isolated *E. coli* from broilers colon content specimens to the anti-bacterial by disc diffusion method

	Inhibition zone diameter (mm)						
		R		*I*		*S*	
Anti-bacterial	*Disc content*	*No.*	*%*	*No.*	*%*	*No.*	*%*
Cefepime	30 µg	127	89.43	9	6.33	6	4.22
Cefixime	5 µg	140	98.59	0	0	2	1.4
Ceftazidime	30 µg	70	49.29	10	7.04	62	43.66
Gentamicin	10 µg	101	71.12	3	2.11	38	26.76
Oxytetracycline	30 µg	141	99.29	1	0.7	0	0
Norfloxacin	10 µg	128	90.14	5	3.52	9	6.33
Nitrofurantoin	300 µg	103	72.53	21	14.78	18	12.67
Rifampicin	5 µg	142	100	0	0	0	0
Sulfamethoxazole/trimethoprim	25 µg	136	95.77	0	0	6	4.22

Number of *Escherichia coli* isolated = 142; R = Resistance; I = Intermediate resistance; S = Susceptible; There was a significant difference at $p < 0.05$ between the resistance of *E. coli* and the antibiotic used in this study.

of the capsule, the first type was surrounded by a capsule (their number 81, representing 57.04 (%), and the second type was not surrounded by capsule (their number 61, representing 42.95%) (Table 3).

Resistance of *E. coli* to antibiotics used in this study showed different resistance ratios as follows: rifampicin has the highest resistance ratio (100%) followed by oxytetracycline (99.29%), cefixime (98.59%), sulfamethoxazole + trimethoprim (95.77%), norfloxacin (90.14%), cefepime (89.43%), nitrofurantoin (72.53%), gentamicin (71.12%), ceftazidime (70%), while nitrofurantoin (14.78%) present medium level of resistance, at the same time *E. coli* strains showed the highest sensitivity to ceftazidime, compared with other antibiotics used in this study, Table 4 and Figures 3-5.

The problem of bacterial resistance to antibiotics has become a global problem, and it threatens the poultry industry in many countries of the world, as well as, affect human health through the possibility of the transmission of bacteria resistance of poultry with their genes to the intestines of humans, causing the failure to respond to treatment when infected with bacterial pathogens.[11]

In this study, it was observed that all isolates *E. coli* have multiple resistances to the anti-bacterial used, the causes of resistance are attributed to: the first reason is the wide spread of *E. coli* in the digestive system of humans and animals, where the current study showed that the ratio of the frequency of *E. coli* compared with other bacteria isolated from chicken colon content, constitutes about 80.22% Table 2, while other bacteria formed about 19.77% of the total number of isolates in this study. This result agreement with D. Scholl, *et al*.,[17] who recorded in their study that the prevalence rate of *E. coli*

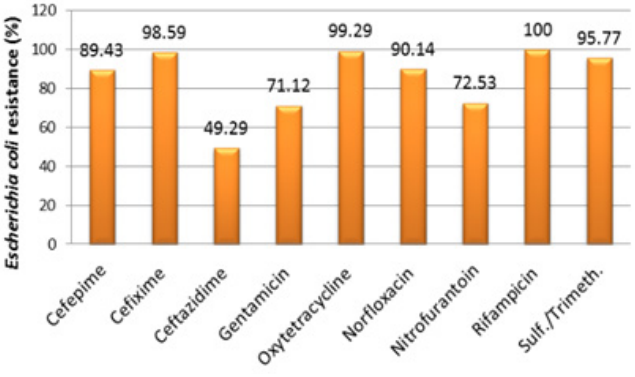

Figure 3: *E. coli* resistance ratio to antibacterial used; Sulf. = Sulfamethoxazole; Trimeth.= Trimethoprim.

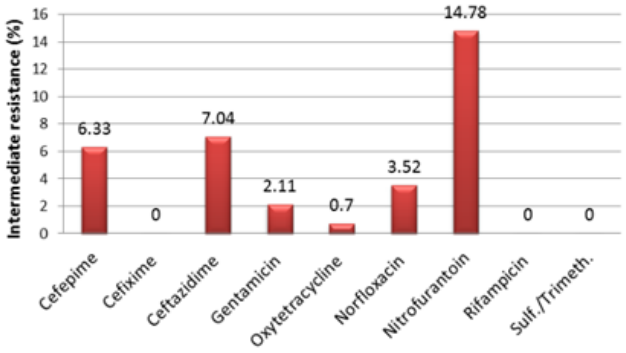

Figure 4: *Escherichia coli* intermediate resistance ratio to antiBacterial used.
Sulf. = Sulfamethoxazole, Trimeth.= Trimethoprim.

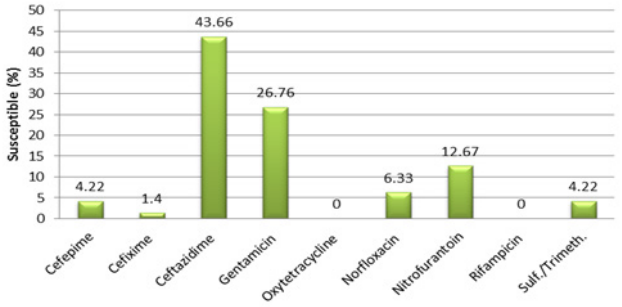

Figure 5: *Escherichia coli* susceptible ratio to antiBacterial used.
Sulf. = Sulfamethoxazole, Trimeth. = Trimethoprim.

was 83% percent in poultry feces. Another study found that the prevalence rate of *E. coli* in chicken feces was 98.5%. All previous studies, which indicated the high rates of the presence of *E. coli* in the intestines due to the competitiveness of these bacteria on nutrients in the intestines, which takes three ways: the first possible use of food cannot be used by other bacteria live in the same intestines,[12] secondly, it is possible for *E. coli* to overcome other bacteria through its rapid growth, third: it is possible to benefit from anaerobic fermentation of intestinal sugars, which are energy sources that can be exploited.[13] Other causes that increase the number of *E. coli* in the digestive system compared to other microflora is the presence of mucus in the intestines, where the latter provides the necessary nutrients (glycoprotein, protein, glycolipids, and lipids). In addition the mucous layer that works in the intestines to provide a layer preventing the arrival of defensive cells to *E. coli*.[14]

The results of the current study showed that the ratio of capsule appearance around the bacterial cells was higher than the cells that are not surrounded by a capsule (Table 3). the presence of capsule increases the chance of the survival of *E. coli* in the host, despite the presence of the immune system, and especially the phagocytosis, allowing it to propagate and spread easily.[15]

The high ratio of multidrug resistance to antibiotics in this study, indicates the misuse of these drugs as a result of their frequent and randomly use by poultry breeders for either disease prevention or prophylaxes, which in turn increases the risk of multiple resistance to *E. coli* in poultry. The antibiotic resistance mechanism used by bacteria to survive in the host body includes: alteration of the structure antimicrobial, enzymatic degradation, genetic mutation, reduction of bacterial cell permeability, increased active efflux of antibiotic across bacterial cell membrane.[16]

Isolates of *E. coli* showed high resistance to the Cephalosporin group antibiotics in compare with another study D Scholl, *et al*.,[17] who observed that *E. coli* resistance was high for Cephalosporins group this is due to the ability of *E. coli* to produce beta-lactase enzyme, which analyzes the beta-lactam ring in the chemical structure of cephalosporins drugs.[18] Also, *E. coli* showed resistance to gentamicin at a ratio 71.12%. Many previous studies have a deal with the resistance of *E. coli* to gentamicin as in study,[19] who noted the resistance ratio 79.6%. The difference in resistance of bacteria to the antibiotic may be caused by it: the country in which the study was conducted, type of *E. coli* strain, a system of poultry farming, and methods of antibiotic treatment**.** Explain the cause of *E. coli* resistance to gentamicin due to the presence of the aminoglycoside-3-N-acetyltransferase [AAC(3)]-III enzyme. This enzyme is linked to a gene that moves from one bacteria to another.[20] Oxytetracycline is widely used in Veterinary medicine to treat many bacterial diseases affecting small and large animals. In this study, *E. coli* showed very high levels of resistance to antibiotics, and this is supported by many researchers, who have conducted research on this subject[21] (*E. coli* resistance to oxytetracycline at ratio 82 and 95%, respectively). There are three mechanisms of *E. coli* to resist oxytetracycline: first mechanism is associated with the bacterial gene, which responsible for increasing the flow of this drug outside the cell; the second mechanism is to prevent protein binding with the bacterial ribosome; the third mechanism involves the manufacture of a bacterial enzyme that destroys the *E. coli* bacteria.[22] As a result of the random use of antibiotics by poultry breeders in Iraq, this result led to the development of bacterial resistance, especially *E. coli*. In this study, bacterial resistance of norfloxacin was recorded at a ratio 90.14%, while previous studies recorded a lower ratio.[23] The ratio of *E. coli* resistance to norfloxacin 78% explained the cause of *E. coli* resistance to norfloxacin, by its ability to alter the bacterial cell protein (OmpF protein) found in the outer cell membrane of the *E. coli*, thus, reducing the permeability of this membrane to the passage of this antibiotics.[24]

Antibiotic resistance varies from one country to another. The reason for this is the treatment programs. For example, in this study, *E. coli* was resistant to nitrofurantoin at ratio 72.53%, but in other studies, the ratios were low 2%, and in another study 30.8%.[25]

Because of the possibility of a genetic mutation in the DNA chain of *E. coli*, this led to the development of these bacteria against nitrofurantoin, through a gene called *oqxAB*, it is able to increase the efflux pump of nitrofurantoin to extracellular of bacteria.[26]

E. coli isolated from broiler chicken feces had the highest resistance to rifampicin (100%) compared to other antibiotics used in this study, this is agreement with AA Moawad, *et al*.,[27] who found that the highest percentage of resistance recorded were same as to this study. The reason for the high resistance to this drug is due to the containment of *E. coli* on a gene called *arr,* capable of ADP ribosylating resistance to rifampicin.[28]

Also, the results of the present study show that *E. coli* showed a 95% resistance to sulfamethoxazole/trimethoprim. This is an agreement with H Hizlisoy, *et al*.,[29] who carried out a study on antibiotic residues in poultry, where it was found that the resistance ratio of *E. coli* was (85.7%), sulfonamide resistance in *E. coli* generally rises from the presence of the *sul1*, *sul2*, and or *sul3* genes.[30]

ACKNOWLEDGEMENTS

I would like to thank Dr. Zahraa Norri Abbas who helped me in the completing my research work.

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