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The Relative Change in Expression Level of Avian Pathogenic *Escherichia coli* -Associated Virulence Genes after Antibiotic Treatment

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Abstract: This study aimed to determine which antibiotic is the most vulnerable and resistant to pathogen and to study the change of expression fold after antibiotic-treatment in compare to isolate without treatment by Reverse Transcription Quantitative Polymerase Chain Reaction RT-qPCR technique. A total of 55 samples with omphalitis symptoms in chicks in Baghdad province. These samples were cultivation, molecular analysis and antimicrobial susceptibility. Fifty isolates showed blue-black colonies on culture Eosin Methylene Blue and pink colonies on MacConkey agar and five isolates showed negative results. of Identification by PCR analysis for detection the *16S rRNA* gene. The results of nucleotide sequencing were submitted in GenBank/NCBI database through accession number (OR142657.1). Antimicrobial susceptibility tested showed that the isolates were resistant to ampicillin, amikacin. Four *E. coli* out 50 isolate chosen for the minimum inhibitory concentration (MIC) exhibited by the ampicilin against tested *E. coli* bacteria, neomycin and combination of ampicillin and neomycin In particular, the result of gene expression the change in expression fold was analyzed using the Livak formula. The results showed that treatment with neomycin had a higher suppression effectiveness on the studied genes compared to ampicillin. Additionally, the combination treatment (Amp/Neo) did not exhibit a clear suppression effect on the genes, which could be attributed to the antagonistic action of ampicillin when used with neomycin.

Keywords: *E. coli*; virulence genes; PCR; broiler hatcheres; Omphalitis.

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Introduction

Omphalitis may be defined technically as an inflammation of the navel. When used commonly the word indicates an incorrect navel closure that leads to bacterial infection. (Navel ill; mushy chick disease). It only occurs in the first few days of life, so it cannot be considered transmissible from bird to bird, It moves from dirty hatching to newly hatched birds that have their navels exposed(1). Avian pathogenic *Escherichia coli* (APEC) refers to strains of the bacterium *Escherichia coli* that are pathogenic to birds. These strains cause a range of diseases in poultry, such as chickens and turkeys, affecting various body systems and leading to significant economic losses in the poultry industry(2). These virulence factors may become useful for pathogenic strains of *E. coli* by enabling invasion, colonization, and adherence and protecting *E. coli* from host defenses (3). Among them, *tsh* (temperature-sensitive hemagglutinin) is associated with the production of adhesion-related factors to enable the adhesion of *E. coli* and is responsible for occurring infections (4), *irp2* (iron

acquisition systems) encoded by an iron-repressible protein gene (5) and *hlyF* (hemolysin) carried by plasmids were the most significantly associated with highly pathogenic APEC strains(6). A number of microorganisms, including *E. coli*. Proteus, *Salmonella*, and Enterobacter species. Different *Pseudomonas species. bacteria Klebsiella spp. Staphylococcus species. Streptococcus species. Clostridium species* have been isolated from the yolk sac infection of birds (7). In week-old chicks, yolk sac (YLS) results in reduced weight gain, delayed development, higher mortality, and low-quality carcasses in the survivors (8). Omphalitis can lead to a reduction in hatchability and an increase in the rate of culling due to growth retardation. The increased mortality rates associated with this condition cause significant financial losses in the poultry sector (2). The use of antibiotics reduces mortality when combined with proper care and cleanliness 5 to 10%(8-10). It's critical to identify bacteria early and accurately in order to take the best control measures. Antibiotic usage, together with good management and hygiene, helps lower mortality. The purpose of this work was to use the RT-qPCR technique to examine the change in expression fold following antibiotic treatment in comparison to an isolates that were not treated.

Materials and methods

Sampling: A total of 55 samples of yolk sac were collected from infected chicks, located in five areas (Abu-grab, Al – mahmoudia, Al-yousifiya, AL -Taj and Bub AL-sham) in a period (October 2022 to February 2023). suffering with clinical signs: (anorexia, depression and huddling near heat sources).

Isolation and identification of *E. coli*

The surface of MacConkey agar and Eosin Methylene Blue (11) agar were then streaked with a loop of each samples, and the media was then incubated for 24 hours at 37℃ (12, 13).

Antibiotic sensitivity Test:

The Kirby-Bauer disk diffusion method was used to evaluate the isolates' antibiotic susceptibility profile in compliance with the standards set given by the National Committee for Clinical Laboratory Standards (14). A Muller-Hinton agar plate (HI Media) was used to test the antibiotic sensitivity of various commercial antibiotic disc types (Ampicillin, Gentamicin, Amikacin, Neomycin, Erythromycin, Chloramphenicol, Florfenicol, Levofloxacin, Tetracycline, and Furaltadone). The plates were then incubated for a full day at 37 °C. The manufacturer's instructions were followed when measuring the zone using a millimeter scale following an overnight incubation.

Preparation of antimicrobial Agents

A stock solution of antibiotic, was prepared in 1.5 ml microcentrifuge tubes (Eppendorff) by dissolving in DW to a final concentration of 400 µg/ml and filtered with 0.22 Millipore filter. Two-fold serial dilutions were done from the stock solution to gate concentrations range from 200 µg/ml to 0.7µg/ml using Muller-Hinton broth (MHB) in 96-well plate.100 µl of the stock solution will be transferred aseptically into A1 of microtiter plate, which will contain 100 µl aliquots of sterile MHB, thereby resulting in a 50 % dilution of the stock solution to 200 µg /ml. After adequate mixing of the contents of each well, 100 µl aliquots of A1 will be transferred to the corresponding wells in B1 (also containing 100 µl aliquots of MHB),

followed by mixing and resulting in yet another 50% dilution of the antibiotic (to 100 µg/ml). The above process was repeated for every row to obtain other dilutions: 50 µg/ml, 25 µg/ml, 12.5 µg/ml,6.3 µg/ml, 3.1 µg/ml, 1.5 µg/ml, and finally 0.7 µg/ml.

Determination of minimum inhibitory concentration (MIC) by Microtiter plate

Hundred microliter of the standardized bacterial suspension at a concentration of 1 x 108 cfu/ml suspension was added to each well containing the previously prepared 100 µl of diluted antibioticl agents, resulting in a final volume of 200 µl in each well. In order to determine the sensitivity of the microbial isolates, positive control was conducted in column no. 11 of microplate that consisted of broth, solvent of antimicrobial agents and the bacterial inoculation. On the other hand, a broth and antimicrobial agent solvent without inoculum, served as the negative control which was putting in column no. 12 of microplate . Then the microtiter plates were incubated overnight at 37 ºC for 18-24 hrs .After incubation MIC values was determined visually by pipetting 30ml of Alamar blue dye into microplate wells and incubated at 37 ºC for 1 hr. Alamar blue dye was used as indicator due to resazurin-based solution that functions as a cell health indicator by using the reducing power of living cells to quantitatively measure viability. Resazurin, the active ingredient of Alamar blue reagent, is a non-toxic, cell-permeable compound that is blue in color and virtually non-fluorescent. Upon entering living cells, resazurin is reduced to resorufin, a compound that is red in color and highly fluorescent. The lowest concentration of each fraction displaying no visible growth was recorded as the minimum inhibitory concentration, MIC values were determined at least in duplicate to confirm activity (15).

RT-qPCR protocol

This is the main step in our project have been divided into two phases, the first is done through synthesis of cDNA from RNA through specific primer for *irp2, hlyf, tsh* and *16S rRNA* transcripts as mentioned in (Table 1) and postscript II cDNA synthesis kit. This procedure has performed through steps:

1. Five microliters from each extracted total RNA sample added into new PCR tube.
2. Protoscript reaction mix that contain dNTPs, buffer and other essential components added as 10 ul for each sample.
3. MuLV Enzyme then added into reaction as 2ul per sample
4. Two microliter of random oligos was added, and the volume completed up to 20ul by adding nuclease free water.
5. This mixture was incubated for 1 hour at 42 ºC by using thermocycler and this followed by 80 ºC for inactivation of enzyme. The cDNA product quantification also done through Qubit 4.0 and stored until performing the second step (Relative quantitative PCR).

The second section of this protocol it's done by choosing the cDNA from each sample at the same run, for each sample there are 4 PCR tubes, three tubes for virulence factor genes *irp2, hlyF, tsh* and one tube for *16S rRNA* which is consider as a house keeping gene in this study. The detection of quantity based on fluorescent power of Syber Green. The reaction mix composed from

component with their quantity as mentioned in (table1) below:

Quickly spin for PCR tubes to remove the bubbles and collect the liquid (1 minute at 2000g, then the program for Real- Time PCR was setup with indicated thermocycling protocol as shown in (Table 2).

Table (1): Component of qPCR master mix used for RT-qPCR.

No.	Component	20 ul Reaction
1	Luna Universal qPCR Master Mix	10 ul
2	Forward primer (10 µM)	0.5 ul
3	Reverse primer (10 µM)	0.5 ul
4	Template DNA	5 ul
5	Nuclease-free Water	4ul

Table 2: RT-qPCR condition target genes *tsh-irp2-hlyF*

Cycle Step	Temperature	Time	Cycles
Initial Denaturation	95°C	60 seconds	1
Denaturation Extension	95°C 60°C	15seconds 30 seconds (+plate read)	45
Melt Curve	60-95°C	40 minutes	1

Results and discussion

All isolate of *E. coli* gave pink color on MacConkey agar Lactose fermentation resulted in the production of acidic byproducts, which lower pH and cause the pH indicator to turn pink. Second stage was done for *E. coli* isolates on Eosin Methylene Blue (11) agar which served as a means of identifying *E. coli* from other Gram-negative bacteria. It used to identify *E. coli*, which produced a metallic green colony on the media, Figure (1 and 2) The results agree with (16).

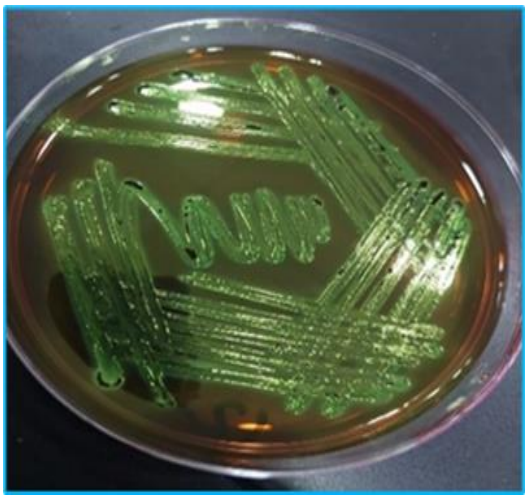

Figure (1): Colonies of *E. coli* on EMB agar appeared as green metallic –sheen

Figure (2): Colonies *E. coli* on MacConkey Agar shows rounded, pink color.

Molecular diagnosis of pathogenic *E. coli*

The *16S rRNA* gene's results showed that *E. coli* primers had successfully targeted the corresponding gene. Sequencing of *16SrRNA* gene of *E. coli* was performed to the isolate, the nucleotide sequence of chicken *E. coli 16SrRNA* gene was submitted in GenBank database and have accession number: ID: OR142657, the phylogenetic analysis post sequencing of the Iraqi *E. coli* isolate that was placed in the NCBI and based on the nucleotide phylogenetic tree of*16S RNA* gene. three virulence genes were found in the PCR analysis of 50 *E. coli* isolates, and these genes were then assigned to specific pathotypes based on the results *(tsh, hlyf and irp2*). In the 50 strains of ill birds studied, *tsh* was found to be 6 % positive, *hlyf* was found to be 9% positive, *irp2\hlyf* was found to be 8 % positive, *tsh\hlyf* was found to be 13 % positive, and *tsh\hlyf\irp2* was found to be 14 % positive. The results of antimicrobial activity in MIC exhibited by the ampicilin against tested *E. coli* bacteria were explained in (figure 3), MICs ranged at (100- 6.2 µg/ml). The concentration 100.µg/ml was inhibiting the growth of *E. coli* isolates in percent of 50%(2/4), while concentrations 6.3µg/ml were inhibitory for a of isolates 1 (25%) (1/4), while concentrations 12.6µg/ml were inhibitory for a of isolates 1 (25%) (1/4) while concentrations 6.3µg/ml were inhibitory for a of isolates 2 (50%) (2/4). and the The results of antimicrobial activity in MIC exhibited by the combination against tested *E.coli* bacteria were explained in (fig 4) ,MICs ranged at (12.5- 6.3 µg/ml) The concentration 3.12.µg/ml was inhibiting the growth of *E.coli* isolates in percent of 3(37.5%)(3/8), while concentrations 1.5 µg/ml were inhibitory for a of isolates 5 (62%) (5/8). As bacterial cells are viable at these concentrations, RNA can be extracted from them to be used in the expression part of the experiment. Sub-MIC values are given for the amp concentration (100-6.2 g/ml), Neo concentration (12.5-6.3 g/ml) as well as the Amp-Neo concentration (12.5-6.3 g/ml).

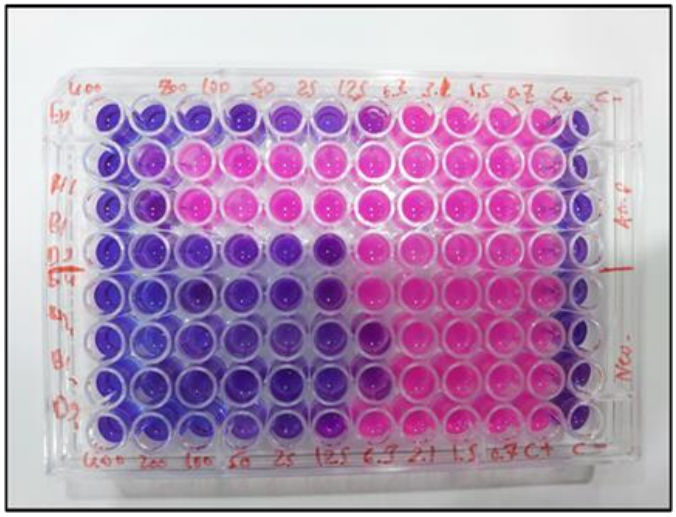

Figure (3): The results of Broth microdilution method to determination minimum inhibitory concentrations (MIC) values of *ampicillin and neomycin* against *E. coli* (C-)" Negative control (only broth and solvent), (C+)" Positive control (only bacteria, broth and solvent.

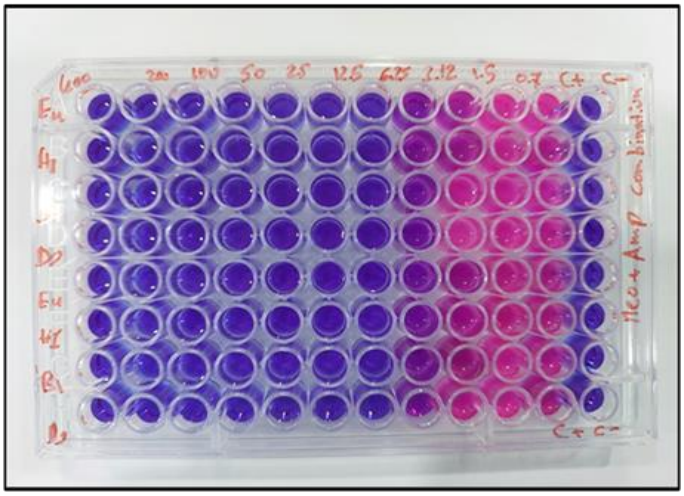

Figure (4): The results of Broth microdilution method to determination minimum inhibitory concentrations (MIC) values of ampicillin and neomicin combination aginst E.coli . (C-)" Negative control (only broth and solvent), (C+)" Positive control (only bacteria, broth solvent).

Transcriptional analysis by Real-Time PCR Relative quantitative technique was performed by Real Time PCR to determine the change of expression level for some genes after antibiotic treatment in compare to untreated isolates. Four *APEC isolates* were selected according to the results of AST and virulence factors, samples were treated with sub-MIC of ampicillin, neomycin and Amp/Neo combination. The amplification was recorded as a Ct value (cycle threshold) indicating that high Ct values indicate low gene expression and low Ct value indicates a high gene expression. The housekeeping gene is used in molecular studies because says that its expression remains constant in the cells or tissues investigation and under different conditions (17). The change of expression fold were analyzed in depend on Livak formula (18). The results showing that treatment with

neomycin have higher suppression effectiveness than ampicillin and Amp/Neo treatment as showing in (Table 3), The amplification curve showing the perfect S-shape pattern (Fig 5) and the results was confirmed by adding melting stage after PCR stage as showing in (Figure 6) there is only single peak for each gene and that mean the primers in PCR stage are bind specifically to target. Some sample showing the induced *hlyF* gene after treatment and this could be due to synergistic effect with ampicillin, this result totally agreed with Moores and his colleagues (19).

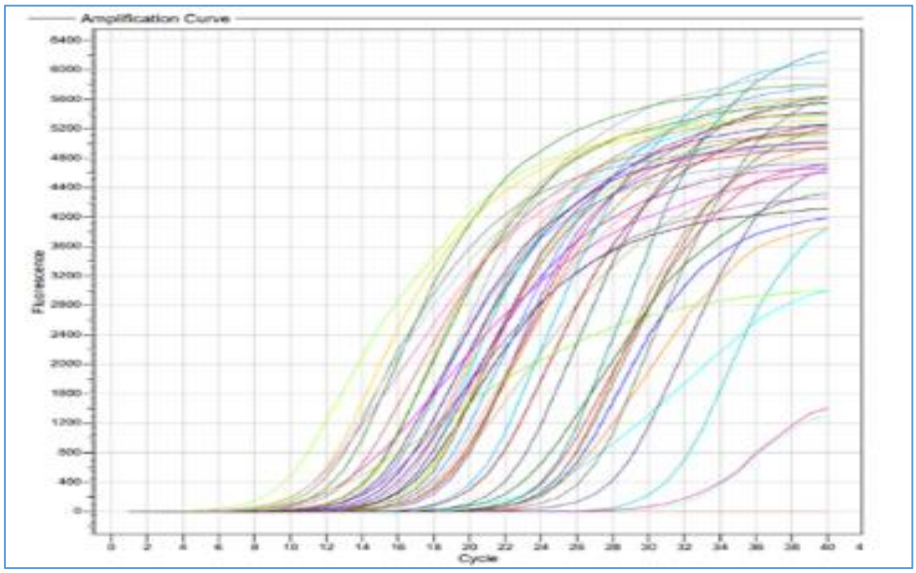

Figure (5): The results of amplification curve of *hlyf, irp2, tsh* and*16S rRNA* genes

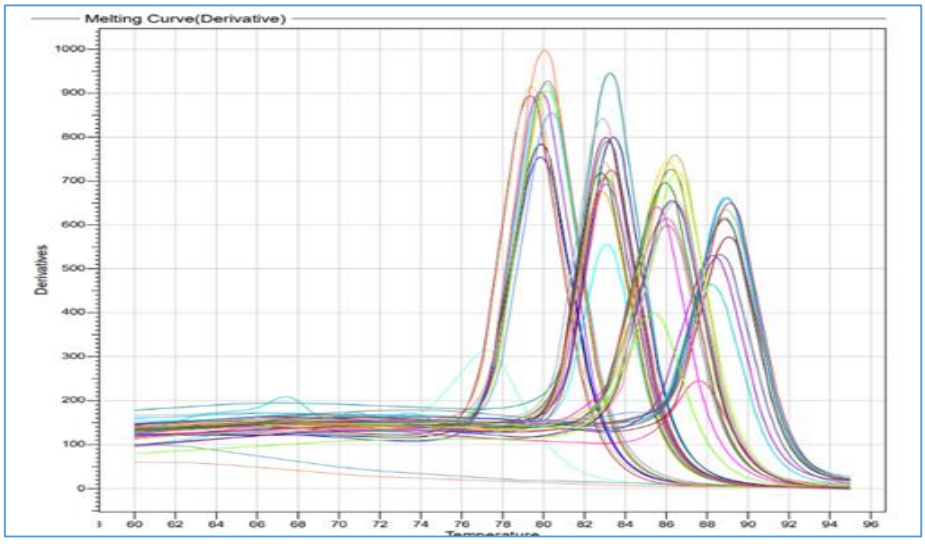

Figure (6): The melting curve showing unique and single peak for each gene *irp2-hlyf- tsh*

Tables (3): The level of fold change among selected *E. coli* isolates.

Group	AST	Virulence factors	Samples	Fold of expression
H	1/16	*hlyf* gene	TA	1.3 ↓[a]
			TN	9 ↓
			TC	0.7 ↑[b]
D	5/16	*hlyf* gene	TA	2.7 ↑
			TN	0.74 ↑
			TC	4.6 ↑
		tsh gene	TA	4 ↓
			TN	5.8 ↓
			TC	44.2 ↑
E	6/16	*hlyf* gene	TA	No result
			TN	
			TC	
		irp2 gene	TA	4.2 ↑
			TN	0.8 ↑
			TC	16.1 ↑
B	8/16	*hlyf* gene	TA	3.3 ↓
			TN	1.25 ↓
			TC	12.19 ↓
		irp2 gene	TA	4.2 ↓
			TN	0.8 ↓
			TC	16.1 ↓
		tsh gene	TA	2 ↓
			TN	66 ↓
			TC	20 ↓

(a: This arrow present the down-regulated , b: This arrow present for up-regulated)

Conclusion

Chicks with omphalitis harbored E. coli pathogens which could be considered a source of infection during the successive days of life in broiler chickens. Microdilution method to determination minimum inhibitory concentrations (MIC) values of ampicillin and neomycin against E. coli and values of ampicillin and neomycin combination against E. coli. the level expression of virulence genes hlyf, hylf\tsh, hlyf\irp2 and hlyf\tsh\irp2 was declined after neomycin treatment in most of isolates

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