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EVALUATION OF THE RELATIONSHIP BETWEEN VIRULENCE, ANTIBIOTIC RESISTANCE GENES AND DEVELOPMENT OF BIOFILM IN *ESCHERICHIA COLI* ISOLATED FROM BROILER CHICKEN

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ABSTRACT

A total of 100 diseased broiler chickens of average age 22-28 days old were collected from different farms located in *Dakahlia* Governorate. All samples were subjected to clinical and postmortem (P.M) examination as well as bacteriological examination for detection of infection. Thus, *E. coli* was recovered from examined organs of 37 chickens with an incidence rate of 37 % (37 out of 100). Accuratly, 10 *E. coli* serogroupes were identified in 28 positive samples and the most predominant serogroup were O_{125} 16%, O_{91} 14%, O_1 11%, O_{26} K_{60} 11% & O_{86} K_{64} 8 & O_{128} 5% & O_{55} K_{59} 3% & O_{166} 3% & O_{103} 3% and O_{144} 3% and untypable *E. coli* (24 %). Studying antibiogram resistance pattern of *E. coli* isolates revealed that all isolates demonstrated multidrug resistence pattern. The highest resistance rates were recorded against Lincomycin (100%) and lowest are Colistin (7.14%) and other tested antibiotics showed variations regarding their resistance patterns. The most predominant serotypes of this study have the ability to form biofilm on the inner wall of the glass. PCR was done for detection of resistant genes (bla_{TEM} and *tet*A(A)) and virulence genes (*csg*D, *adr*A, *eae*A and *fim*H) in the 10 tested isolated serotypes and the results revelead that, 100% and 90% &100%, 90%, 90% and 100% were positive for resistant genes and virulence genes, respectively and investigate the relationship between them was discovered. By studying the effect of Olive Leaf Extract on multidrug resistant isolates, results were revealed that all tested serotypes are sensitive to Olive Leaf Extract (10% conc.).

***Key words**: E coli*, resistant to antibiotic, resistant genes, virulent genes, biofilm, olive leaf extract.

INTRODUCTION

Avian colibacillosis caused by *E. coli* is serious infectious disease occurring in different types of chicken resulting in a significance losses in poultry industry. *Escherichia coli* (*E. coli*) is one of the normal bacterial flora in the gastrointestinal tract of poultry. About 10-15% of the intestinal coliforms in chickens are of pathogenic serotypes. Colisepticemia, respiratory tract infections, poultry cellulitis, swollen head syndrome, omphalitis/yolk-sac infection, pericarditis, peritonitis and salpangitis are important diseases caused by *E. coli* in birds *Barnes et al.* (1999). *Escherichia coli* recognized as major pathogen for public health problems in developing countries and represents leading etiological agent of diarrhea where several classes of enterovirulent *E. coli*, namely enterotoxigenic *E. coli* (ETEC), enteropathogenic *E. coli* (EPEC), enterohaemorrhagic *E. coli* (EHEC), enteroinvasive *E. coli* (EIEC), enteroaggregative *E. coli* (EAggEC), diarrhoea-associated haemolytic *E. coli* and cytolethal distending toxin (CLDT)-producing *E. coli* have been recognized by *Nataro and Kaper* (1998). The term biofilm is used to describe matrix-enclosed bacterial population adherent to each other and/or to surfaces *Costerton et al*. (1995). *E. coli* is one of many bacteria that can switch between planktonic form and biofilm form. Several reasons can explain the need of bacteria to create biofilm, in this way bacteria can avoid being washed away by water flow or, cells in biofilms are about 1000 times more resistant than their planktonic *Jefferson*, (2004). Several surface organelles, including various types of fimbriae, autotransporter proteins and extracellular polysaccharides, have been found to facilitate or enhance biofilm formation of *E. coli*, largely depending on the environmental conditions and the particular strains studied *Schembri et al*. (2002) which may be resistant to antibiotic or not. The aim of this study is to evaluate the relationship between virulence, antibiotic resistance genes and

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development of biofilm in *E. coli* isolated from broiler chicken farms in *Dakahlia* Governorate in Egypt.

MATERIALS AND METHODS

A total of 100 diseased broiler chickens of average age 22-28 days old were collected from different farms located in *Dakahlia* Governorate were subjected to clinical and postmortem (P.M) examination as well as for isolation and identification of *E. coli* from tissue samples including liver, caecum, spleen, lungs, kidneys and heart according to *Maram*, (2014).

1. *Detection of Escherichia coli by conventional method:* was done according to *Swayne et al*. (1998) and *Quinn et al*. (2002).

1.1. *Selective enrichment of E. coli in broth:*
Each sample was inoculated separately into buffer peptone water were incubated at 37 ºC for 24 hrs under aerobic condition.

1.2. *Colonization of E. coli on selective differential solid media:*
A loopful from the broth of each sample was streaked onto MacConkey's agar and Eosin Methylene Blue agar. The inoculated plates were incubated at 37 ºC for 24 hours. Suspected *E. coli* colonies were purified and kept for further identification.

1.3. *Identification of suspected E. coli colonies was done according to Quinn et al.* (2002)*:*
Culture characters, *Microscopic examination and motility*: were done according to *Cruickshank et al.* (1975)

2. *Biochemical Identification of E. coli:*
It was done according to *Quinn et al.* (2002) on indole reaction, methyl red test, voges proskauer test, citrate utilization test, catales test, sugar fermentation test, oxidase test, triple sugar iron and christener's urea agar test.

3. *Serological identification of E. coli isolates was carried out according Edwards and Ewing* (1972):
The obtained isolates were serogrouped in Animal Health Research Institute, Dokki, Giza using: Sifin antisera "Berlin, Germany" Polyvalent and monvalent diagnostic *E. coli* antisera.

4. *Sensitivity of E. coli isolates to antimicrobial agents:*
E. coli strains were tested for their antimicrobial sensitivity to various antibiograms (Amoxicillin, Enrofloxacin, Tetracycline, Doxycycline, Ampicillin, Flumequine, Gentamycin, Naldixic acid, Chloramphenicol, Erythromycin, Ciprofloxacin, Lincomycin and Colistin) by the agar disc diffusion method according to *finegold and martin* (1982) and interpretation of the results according to CLSI (2016)

5. *Biofilm formation of E. coli:*
According to *Maram*, (2011); it was done on 10 serotyped *E. coli* isolates represents to serotypes detected. A loopful of tested organisms was inoculated in 10 mL of trypticase soya broth with 1% glucose in test tubes. The tubes were incubated at 37ºC for 24 hours. One ml from the inoculated broth was transferred into another tube containing 4 ml trypticase soy broth with 1% glucose, one tube used as a control negative (not inoculated) and another tube was inoculated with *E. coli* (positive control). All test tubes were incubated at 37 ºC for 5 days. After incubation, tubes were decanted and washed with phosphate buffer saline (pH 7.3) and dried. Tubes were then stained with crystal violet (0.1%). Excess stain was washed with deionized water. Tubes were dried in inverted position. The results of tube method were compared with the control positive strain. Biofilm formation was considered positive when a visible film lined the wall and the bottom of the tube. The experiment was performed in triplicate and repeated three times.

6. *Molecular detection of E. coli resistant and virulence genes using Polymerase chain reaction (PCR):*

6.1. *DNA extraction:*

DNA extraction from 10 *E. coli* isolates represented 10 serotypes detected was performed using the QIAamp DNA Mini kit (Qiagen, Germany, GmbH) with modifications from the manufacturer's recommendations. Briefly, 200 µl of the sample suspension was incubated with 10 µl of proteinase K and 200 µl of lysis buffer at 56 ºC for 10 min. After incubation, 200 µl of 100% ethanol was added to the lysate. The sample was then washed and centrifuged following the manufacturer's recommendations. Nucleic acid was eluted with 100 µl of elution buffer provided in the kit.

6.2. *Oligonucleotide Primer:* Primers used were supplied from Metabion (Germany) are listed in Table (1).

6.3. *PCR amplification:* Primers were utilized in a 25 µl reaction containing 12.5 µl of DreamTaq Green PCR Master Mix (2X) (Thermo Scientific), 1 µl of each primer of 20 pmol concentration, 4.5 µl of water, and 6 µl of DNA template. The reaction was performed in an Applied biosystem 2720 thermal cycler.

6.4. *Analysis of the PCR Products:*
The products of PCR were separated by electrophoresis on 1% agarose gel (Applichem, Germany, GmbH) in 1x TBE buffer at room temperature using gradients of 5V/cm. For gel analysis, 20 µl of the PCR products were loaded in each gel slot. Gelpilot 100 bp, 100 bp plus plus DNA Ladders (Qiagen, Germany, GmbH), generuler 100 bp ladder (Fermentas, Thermo) and Genedirex 100 bp DNA ladder H3 RTU, Cat. No. DM003-R500 were used to determine the fragment sizes. The gel was photographed by a gel documentation system (Alpha Innotech, Biometra) and the data was analyzed through computer software.

Table 1: Primers sequence, target genes, amplicon sizes and cycling conditions.

Target gene	Primers sequences 5'-3'	Amplified segment (bp)	Primary denaturation	Amplification (35 cycles)			Final extension	Reference
				Secondary denaturation	Annealing	Extension		
***adr*A**	ATGTTCCCA AAAATAATG AA TCATGCCGC CACTTCGGT GC	1113	94˚C 5 min.	94˚C 30 sec.	50˚C 1 min.	72˚C 1 min.	72˚C 10 min.	Bhowmick *et al*. (2011)
***csg*D**	TTACCGCCT GAGATTATC GT ATGTTTAAT GAAGTCCAT AG	651	94˚C 5 min.	94˚C 30 sec.	50˚C 40 sec.	72˚C 45 sec.	72˚C 10 min.	
***fim*H**	TGCAGAACG GATAAGCCG TGG GCAGTCACC TGCCCTCCG GTA	508	94˚C 5 min.	94˚C 30 sec.	50˚C 40 sec.	72˚C 45 sec.	72˚C 10 min.	Ghanbarpour and Salehi, (2010)
***eae*A**	ATGCTTAGT GCTGGTTTA GG GCCTTCATC ATTTCGCTT TC	248	94˚C 5 min.	94˚C 30 sec.	51˚C 30 sec.	72˚C 30 sec.	72˚C 7 min.	Bisi-Johnson *et al*. (2011)
***tet*A(A)**	GGTTCACTC GAACGACGT CA CTGTCCGAC AAGTTGCAT GA	576	94˚C 5 min.	94˚C 30 sec.	50˚C 40 sec.	72˚C 45 sec.	72˚C 10 min.	Randall *et al.* (2004)
***bla*$_{TEM}$**	ATCAGCAAT AAACCAGC CCCCGAAGA ACGTTTTC	516	94˚C 5 min.	94˚C 30 sec.	54˚C 40 sec.	72˚C 45 sec.	72˚C 10 min.	Colom *et al*. (2003)

7. *In vitro sensitivity testing using Olive Leaf Extract on isolated E. coli:*
All *E. coli* isolates that showed resistance to antibiotics were subjected for testing the effect of Olive Leaf Extract.

7.1. *Extraction of Olive Leaf:*
The leaves were purchased from herbal market, cleaned from extraneous matter and properly washed then dried in hot air-oven for 24 h at 40 °C. The dried leaves were ground in a blender to form powder. Thereafter, 100 g of the powder were macerated in 1000 ml absolute ethanol (10% concentration) and allowed to extract for 48 h *Ahmed and Abolghait* (2014) with some modification. The resultant (dark green-brown mixture) was filtered and the filtrate was concentrated in a rotary evaporator under reduced pressure.

7.2. *Preparation of filter paper discs from olive leaf extract:*
Six millimeter filter paper was used to prepare discs. The discs were then sterilized in by autoclaving. The extract was diluted at different concentrations. A total volume of 250 µl was used to soak 50 discs without over or under wetting those *Hannan et al.* (2008).

Discs with concentration 10.0 mg per discs were obtained. Prepared discs were stored at 4 °C in the refrigerator till use. The discs were kept at room temperature for one hour to before use.

Antimicrobial susceptibility testing using filter paper discs from olive leaf extracts according to *Finegold and Martin* (1982).

RESULTS

1. *Prevalence rate of E. coli isolated from examined broilers:*

In 100 broiler chickens suffering from ruffled feathers, depression, off food were subjected for P.M. examination, revealed caseous masses on internal organs, cellulitis, entero-colitis and damage intestinal mucosa then examined by bacteriological methods during different seasons of the year, *E. coli* was recovered from 37 samples with a prevalence rate 37 % (37 out of 100) as shown in Table (2).

Table 2: Incidence of *E. coli* infection in broiler chickens.

Number of examined chicken	Number of positive	Percentage of positive%
100	37	37

2. *Prevalence of E coli in different chicken organs:*

The internal organs of each chicken were examined by bacteriological examination to determine the prevalence of *Escherichia coli* in each chicken organ as shown in Table (3) where *E. coli* was isolated as the following 30% (30 out of 100) from liver; 24% (24 out of 100) from lung; 20% (20 out of 100) from caecum; 14% (14 out of 100) from spleen; 11% (11 out of 100) from kidney and 9% (9 out of 100) from heart.

Table 3: Rate of *E coli* recovery from internal organs.

Examined organs in 100 chicken 6 organs/ Bird	Percentage of positive%*	Percentage of negative%*
Liver	30	70
Lung	24	76
Caecum	20	80
Spleen	14	86
Kidney	11	89
Heart	9	91
Total	18**	82**

***** calculated according to the number of tested birds (100).
****** calculated according to the number of examined samples (600).

3. *E. coli serotypes isolated from examined chickens:*

The isolated *E. coli* were serotyped using polyvalent and monovalent *E. coli* antisera to determine the *E. coli* serotype.

The serotyping of isolated biochemically identified *E. coli* revealed that the most predominant serotypes and the most predominant serogroup were O_{125} 6%& O_{91} 5%& O_1 4%& O_{26} K_{60} 4% & O_{86} K_{64} 3% & O_{128} 2% & O_{55} K_{59} 1% & O_{166} 1% & O_{103} 1% & O_{144} 1% and 9 strains untypable *E. coli* (9 %) as shown in Table (4).

Table 4: *E. coli* serotypes recovered from bacteriologically examined chickens.

The infected *E coli* serotype	Number of positive chicken	Percentage of positive %
O_{125} K-	6/37	16
O_{91} K –	5/37	14
O_1 K -	4/37	11
O_{26} K_{40}	4/37	11
O_{86} K $_{64}$	3/37	8
O_{128} K -	2/37	5
O_{55} K $_{59}$	1/37	3
O_{166} K -	1/37	3
O_{103} K -	1/37	3
O_{144} K -	1/37	3
Untypable	9/37	24
Total	37	100

4. *Sensitivity of E. coli serotypes to different antibiotic agents:*

As shown in Table (5), *E. coli* O groups (28) were found to be 100% resistant to Lincomycin antibiotic followed by Amoxicillin (82.14), Enrofloxacin (71.73%), Tetracycline (67.86%), Doxycycline (60.71%), Ampicillin (57.14%), Flumequine (42.86%), Gentamycin (32.14%), Erythromycin (32.14%), Naldixic acid (28.27%), Chloramphenicol (25%), Ciprofloxacin (10.7%) and Colistin (7.14%).

Table 5: Sensitivity of *E. coli* serotypes and to different antibiotic agents.

	Cl	L	C	CF	ENR	UB	NA	DO	T	A	E	G	Am
R	2	28	7	3	20	12	8	17	19	16	9	9	23
S	25	0	19	23	7	14	18	9	6	10	18	15	3
I	1	0	2	2	1	2	2	2	3	2	1	4	2

R: resistant. S: sensitive. I: intermediate.
CL: Colistin. L: Lincomycine. C: Chloramphenicol.
CF: Ciprofloxacine. ENR: Enrofloxacine. UB: Flomequine.
NA: Nalidixic acid. DO: Doxycycline. T: Tetracycline.
A: Amoxycilline E: Erythromycin. G: Gentamycine.
AM: Ampicilline.

5. *PCR Detection of resistant Genes of E. coli:*

PCR using primers fragments listed in materials and methods for amplification of *bla*$_{TEM}$ and *tet*A(A) genes from the isolated *E. coli* strains in this study.

5.1. *Detection of bla$_{TEM}$ gene of E coli:*

bla$_{TEM}$ gene responsible for resistant of the isolated *E. coli* strains to Beta-lactames antibiotics. Our results showed amplification of 516 bp of (*bla*$_{TEM}$) gene from the extracted DNA of all tested *E. coli* strains (10) as shown in Figure (1)

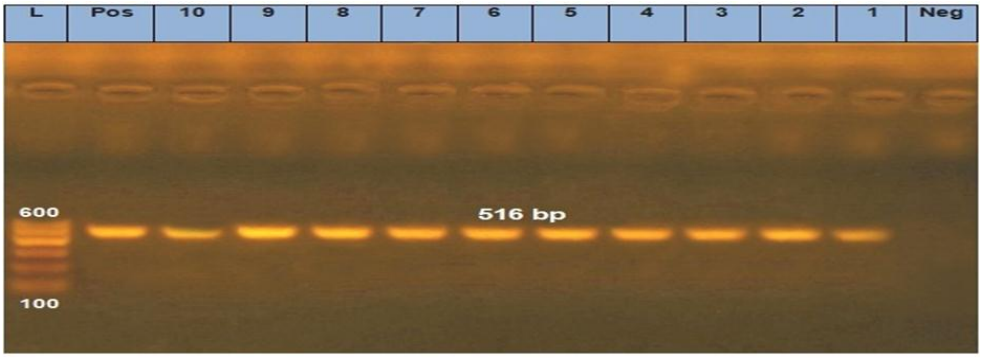

Figure (1) amplification of *bla*$_{TEM}$ gene of *Escherichia coli* strains: Amplification of 516 bp was observed in the extracted DNA of O_1, O_{26}, O_{55}, O_{86}, O_{91}, O_{103}, O_{125}, O_{128}, O_{144} and O_{166}, (in lane number 1, 2,3, 4, 5, 6, 7, 8, 9, 10, respectively).

5.2. *Detection of tet (*A) *gene of E. coli:*

*tet*A (A)gene responsible for resistant of the isolated *E. coli* strains to tetracycline antibiotics. Our results showed amplification of 576 bp of *tet*A(A) gene from the extracted DNA of all isolated *E. coli* strains except O_{55} (No. 3) not have this gene as shown in Figure (2)

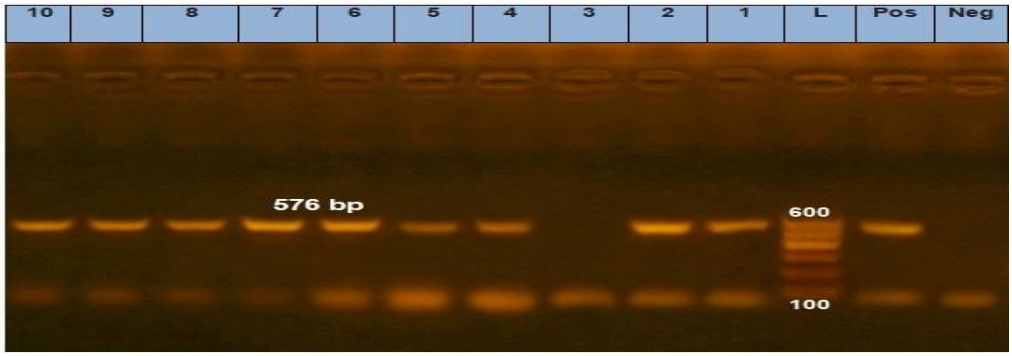

Figure (2) amplification of *tet*A(A) gene of *Escherichia coli* strains: Amplification of 576 bp was observed in the extracted DNA of O_1, O_{26}, O_{86}, O_{91}, O_{103}, O_{125}, O_{128}, O_{144} and O_{166} (in lane number 1, 2, 4, 5, 6, 7, 8, 9 and 10, respectively). No amplification in O_{55} (in lane number 3).

6. *PCR for Detection of virulence Genes of E. coli:*
PCR using primers fragments listed in materials and methods for amplification of *eae*A, *fim*H, *csg*D and *adr*A from the isolated *E coli* strains in this study.

6.1. *Detection of eae*A *gene of E. coli:*
Ettaching and effacing mechanisms gene (*eae* A) is responsible for attachement and effacing of *E. coli* to the enterocytes of the intestine of chicken. Our results showed amplification of 248 bp of (*eae* A) gene of all isolated serotypes except O_{125} (in lane No. 7) from the extracted DNA of *E coli* strains as shown in Figure (3)

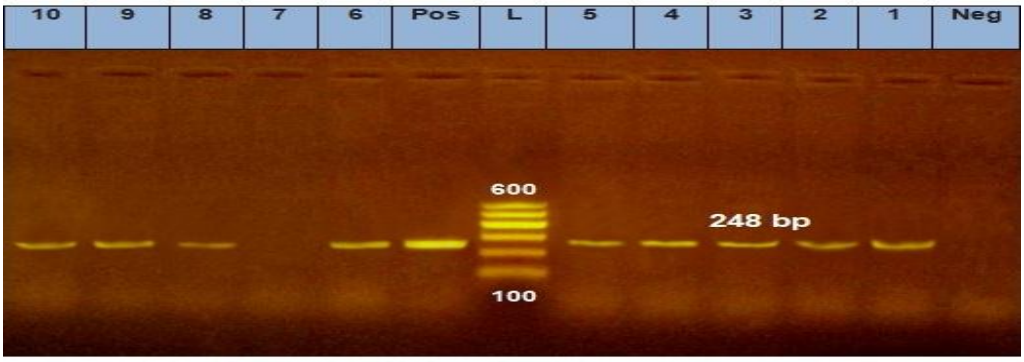

Figure (3) amplification *eae*A gene of *Escherichia coli* strains: Amplification of 248 bp was observed in the extracted DNA of O_1, O_{26}, O_{55}, O_{86}, O_{91}, O_{103}, O_{128}, O_{144} and O_{166} (in lane number 1, 2,3, 4, 5, 6, 8, 9 and 10, respectively). No amplification in O_{125} (in lane number 7).

6.2. *Detection of fim*H *gene of E. coli:*
*fim*H gene which responsible for adhesion of *E. coli*. The results showed amplification of 508 bp of *fim*H gene of all isolated serotypes from the extracted DNA of *E. coli* strains as shown in Figure (4).

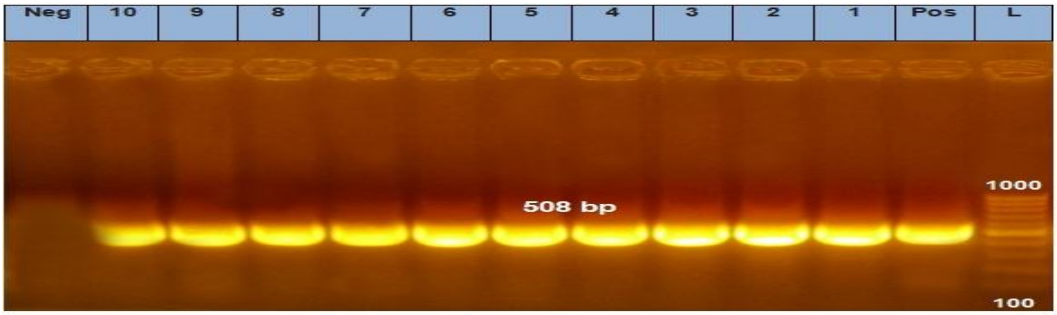

Figure (4) amplification of *fim*H gene of *Escherichia coli* strains: Amplification of 508 bp was observed in the extracted DNA of O_1, O_{26}, O_{55}, O_{86}, O_{91}, O_{103}, O_{125}, O_{128}, O_{144} and O_{166}, (in lane number 1, 2,3, 4, 5, 6, 7, 8, 9, 10, respectively).

6.2. *Detection of csg*D *gene of E. coli:*
*csg*D gene which is the master regulator for adhesive curli fimbriae expression, plays a positive role in biofilm formation of *E. coli*. The results showed amplification of 651 bp of *csg*D gene of all isolated serotypes from the extracted DNA of *E. coli* strains as shown in Figure (5).

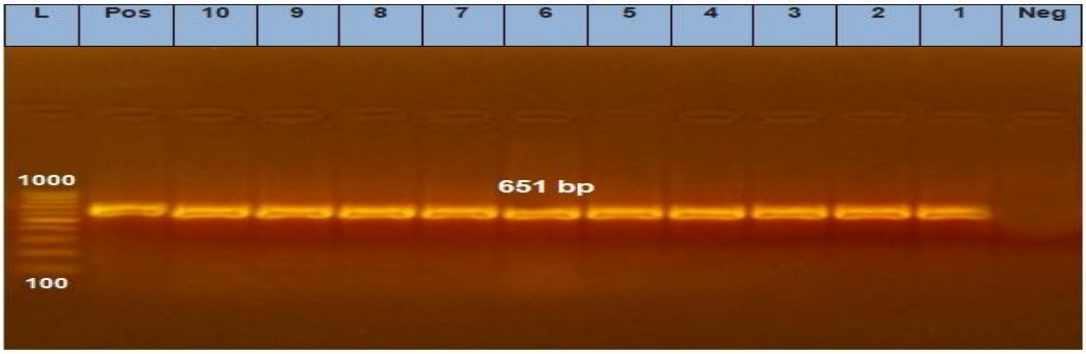

Figure (5) amplification of *csg*D gene of *Escherichia coli* strains: Amplification of 651 bp was observed in the extracted DNA of O_1, O_{26}, O_{55}, O_{86}, O_{91}, O_{103}, O_{125}, O_{128}, O_{144} and O_{166}, (in lane number 1, 2,3, 4, 5, 6, 7, 8, 9, 10, respectively).

6.3. Detection of *adr*A gene of *E. coli*:
*adr*A gene which responsible for cellulose synthesis. The results showed amplification of 1113 bp of *adr*A gene of all isolated serotypes from the extracted DNA of *E. coli* strains except O_{103} as shown in Figure (6).

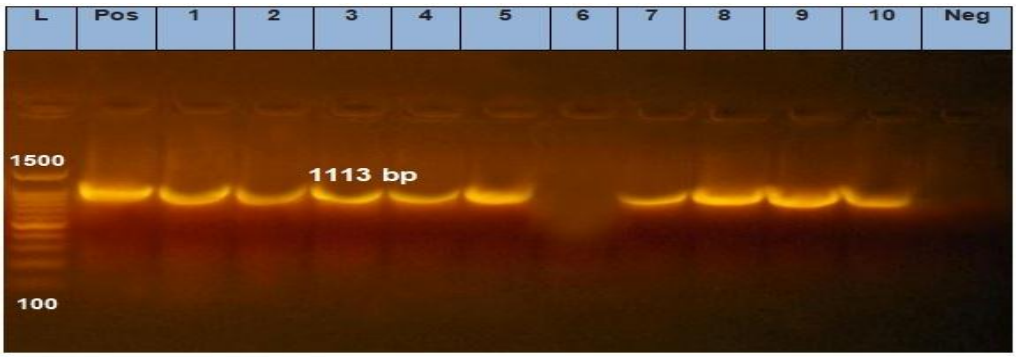

Figure (6) amplification *adr*A gene of *Escherichia coli* strains: Amplification of 1113 bp was observed in the extracted DNA of O_1, O_{26}, O_{55}, O_{86}, O_{91}, O_{125}, O_{128}, O_{144} and O_{166} (in lane number 1, 2,3, 4, 5, 7, 8, 9 and 10, respectively). No amplification in O_{103} (in lane number 6).

7. *Biofilm formation:*
PCR amplifications of *eae*A, *fim*H, *csg*D and *adr*A genes from the isolated *E. coli* strains in this study were assayed for biofilm formation in vitro using tube biofilm assay. Result revealed that, all the examined serogroups (O_1, O_{26}, O_{55}, O_{86}, O_{91},O_{103}, O_{125}, O_{128}, O_{144} and O_{166} have the ability to make biofilms on the inner walls of the glass tubes after crystal violet staining. On the other hand, no biofilm was observed with negative uninoculated tube. These results might point to the role that these genes play during expression of proteins involved in biofilm formation.

8. *Effect of olive leaf extract on the multiresistant strains:*
Results of antimicrobial susceptibility testing for 10 *E. coli* serotypes revealed that olive leaf extracts had inhibitory effect at a concentration of (10 mg) on tested serotypes as shown in Figure (7).

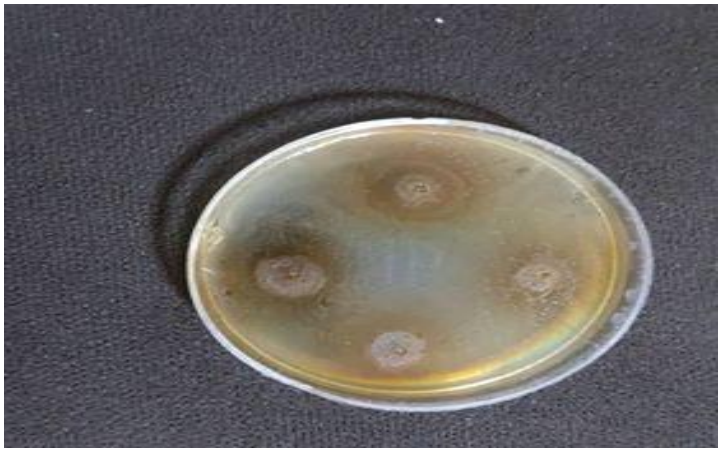

Figure (7): effect of olive leaf extraction *E. coli* multiresistant strains.

DISCUSSION

In this study, the incidence of *E. coli* in broiler chickens from *Dakahlia* governorate was 37 %. These results were agreed with that of *Robert et al.* (2002) and *Ružauskas et al.* (2010), who isolated *E. coli* with percentage of 36.8% and 41.7%, respectively. Higher rates were recorded by *El-Sukhon et al.* (2002) and *Alimehr et al.* (1999) who recovered *E. coli* in 88.2% and 100% of the examined samples, respectively.

The obtained results of this study revealed that all the most *E. coli* isolates obtained from liver of the examined chickens followed by lung, caecum, spleen, kidney and heart 30%, 24%, 20%, 14%, 11% and 9%, respectively which agreed with *Otaki*, 1995 and the explanation of these results is due to infection with APEC generally begins as a localized infection of the air sacs commonly referred as airsacculitis or the air sac disease which in turn may spread to other internal organs resulting in systemic infection *Barnes et al.* (1999). These results were agreed with *Ogunleye et al.* (2008) who reported that, the most *Escherichia coli* isolates obtained from liver 67% then lung and intestine10%. Also, *Sharada et al.* (2010) recovered highest percent of isolates from cases of hepatitis 44.6%, enteritis

33.8%, pericarditis 16.9% followed by air saculitis 7.7%.

More than 1000 *E. coli* serotypes have been reported but only small percentages have been implicated in poultry diseases *Cloud et al.* (1985). In this study, 10 *E. coli* serogroupes were identified in 28 positive samples and the most predominant serogroup were O_{125} 16%, O_{91} 14%, O_1 11%, O_{26} K_{60} 11% & O_{86} K_{64} 8 & O_{128} 5% & O_{55} K_{59} 3% & O_{166} 3% & O_{103} 3% & O_{144} 3% and untypable *E.coli* (24 %). These results nearly go hand to hand with the previous studies of *Abd El Tawab et al.* (2015). The occurrence of a specific serotype and its role in disease production depends upon the health status of the birds, climatic conditions, geographical situations and managemental strategies *Srinivasan et al.* (2013).

The Results for Antibiotic sensitivity showed that most of the isolates were multidrug resistant as they resist at least 3 antibiotics as these results agreed with that reported by Momtaz *et al.* (2012). *E. coli* isolates were found to be 100% resistant to Lincomycin almost similar resistance were detected by *Ngeleka et al.* (1996) who reported 100% resistant to lincomycin. Lower rate 39.50% were detected by *Sharada et al*. (2010).

The Amoxicillin resistance of the *E. coli* isolates in the present study were 82.14%, these results go hand to hand with the previous studies of Anthonia (2012) who showed 80% resistant to amoxicillin in free range chickens. On the other hand Salehi and Bonab (2006) reported medium resistance AM 53% in *E. coli* isolates from chickens of Colisepticemia.

The present study showed resistance percentages to Enrofloxacin (71.73%). Almost similar resistance were detected by Salehi and Bonab (2006), 76% however less percentages 23%, 34.8 were detected by Amara *et al.* (1995) and Alimehr *et al.* (1999), respectively.

In this study, there was high resistance rate of Tetracycline (67.86%) in the isolated *E. coli* which agreed with earlier reports of Alhaj *et al.* (2007) and Morad (2013) to these antibiotics 81.4%, 85.1%, respectively in chicken isolates and disagreed with those of Kolar *et al.* (2005) who showed less resistant to tetracycline about 48%.

Also *E. coli* isolates were found to be resistant to Doxycycline with a percentage of (60.71%) which similar to that of *Ngeleka et al.* (1996) (more than 50%) but disagree with the results of *Salehi and Bonab* (2006), who showed high resistant to Doxycycline (88%).

The *E. coli* isolates in this study expressed resistance to ampicillin at (57.14%) percentage, these results go hand to hand with the previous studies of *Akond et al.* (2009) who reported that 58% of *E. coli* strains isolated from poultry and poultry environment in Bangladesh were resistant to ampicillin on the other hand resistance rate to ampiclllin in this study was higher than those reported by *Idrees et al.* (2011) from poultry in Pakistan.

In this study, about (42.86%) of the isolated *E.coli* were resistant to Flumequine which represent a lower percentage than those reported by Morad, 2013 and Alimehr *et al.* (1999) who reported percentage of 81.8% and 67.5%, respectively.

It was also reported that 32.14% resistant rate was found to Gentamycin. Lower rate (0%) was detected by Momtaz *et al*. (2012). Higher resistant rates 74.3% were detected by *Alhaj et al.* (2007).

This result revealed Erythromycin resistant rate (32.14%) in the isolated *E. coli* however higher level detected in previous studies by Salehi and Bonab (2006) who reported 97% resistant to Erythromycine.

The present study revealed that 28.27% of the *E. coli* isolates were resistant to Nalidixic acid. This finding was agreed with those of Johnson *et al.* (2003) who recorded resistance rates of (37%) and disagreed with the results reported by Kmet and Kmetova (2010), (87/85/67 %) from *Escherichia coli* isolated from healthy chicken broilers during (2006/2007/2008).

The present study showed intermediate percentage of Chloramphenicol resistant (25%) between those of Alhaj *et al.* (2007) who reported high percentage of resistant 75.7% and Miles *et al.* (2006) with low percentage rate 2.9% from broiler chichens.

However, the level of resistance to ciprofloxacin (10.7%) of isolated serotypes observed in this study was similar to that reported by Anthonia, (2012), 12 %in free range chicken. This low resistance rate may also be associated with the low usage of this drug by poultry farmers.

The highest sensitivity rate detected in this study was to colistin 89.28%. This result agreed with that of Filali *et al.* (1988) and Amara *et al.* (1995) who reported that colistin exhibited excellent activity against *Escherichia coli* isolates.

Among food animals that act as reservoirs of ESBL-producing *E. coli*, broilers were considered to be the most potent reservoir Pacholewicz *et al.* (2015).In this study the percentage of bla_{TEM} gene from the isolated *E .coli* strains was 100 % (10 out of 10 strains) which nearly go hand with the results of

Maram (2014) who detected *bla*$_{TEM}$ gene in 18 out of 19 *E.coli* isolates with 94.73%. But these results disagreed with Ghosh *et al.* (2017) who obtained lower percentage about 10%from isolated *E. coli*.

In recent years, *tetracycline* resistance has emerged among many pathogenic and nonpathogenic species of bacteria. This resistance is mainly due to different efflux pump and ribosomal protection genes, mostly associated with mobile components such as plasmids or transposons Roberts (2012). Screening of the tetracycline resistance gene showed that *tet*(A) gene was detected in all isolated *E. coli* strains except O_{55} not have the gene with a percentage 90% which nearly agreed with the results reported by Zibandeh *et al.* (2016), 72.5% of *E. coli* isolated from the chickens on the day before slaughter.

virulence-associated genes and pathogenicity islands of bacteria play an important role in the pathogenicity of bacteria and that they are important parameters to clarify the mechanism of bacterial pathogenicity Vandekerchove *et al.* (2005) and Cheng *et al.* (2006).

This study showed many different genes which are responsible for virulence and biofilm formation of *E. coli*. Intimin, an outer membrane protein, encoded by *eae*A, is a bacterial adhesion molecule that mediates the intimate bacterium host cell interaction characteristic of A/E (attaching and effacing) lesions of avian pathogenic *E. coli* Kilic *et al.* (2007). In this study, high incidence rate (90%) of *eae*A gene detection was recorded, as it was detected by PCR in 9 out of the 10 tested isolates and these high results agreed with Ramadan *et al.* (2016) and disagreed with Kilic *et al.* (2007) who reported the incidence rate 48% of the *E. coli* isolates.

Surface virulence factors of the pathogens including different adhesion factors may promote bacterial adhesion and biofilm development Schembri *et al.* (2003). *fim*H consists of a fimbria- associated pilin domin and a mannose binding lactin domin, receptor-ligand specific adhesion is among the most fundamental of biological phenomena in nature. This phenomenon underlies eukaryotic cell-cell or cell-surface attachment, initiates recognition and signaling events, binds bacteria to target cells and mediates biofilm formation on medical implants Aprikian *et al.* (2007). In this study the prevalence of *fim* H gene in the isolated *E. coli* strains was 100 % (10 out of 10 strains) and these results were agreed with the results reported by Trkov *et al.* (2014) as seventy-four (88.1 %) isolates carried the type 1 fimbriae gene *fimH* and Ghanbarpour *et al.* (2011) also reported 96.4% of fecal isolates positive for *fim*H compared to 95% of isolated *E. coli* from cases of colibacillosis. The results were disagreed with the results of Eftekharian *et al.* (2016) who detected less percentage 41.7% of the intestinal isolates only positive for *fim* H gene.

Curli fibers (also known as thin aggregative fimbriae) are a major factor in adhesion to surfaces, cell aggregation, and biofilm formation in many enterobacteria Cookson *et al.* (2002) and Prigent-Combaret *et al.* (2001). Expression of both curli and cellulose depends on the *csg*D protein Arnqvist *et al.* (1994) in this study, 100% of the tested strains have the *csg*D gene which go hand with the results of Wang *et al.* (2016) in 36 non-O157 Shiga toxin–producing *Escherichia coli* (STEC) strains.

Cellulose synthesis regulation is a very complex phenomenon. In enterobacteria, this phenomenon involves a AgfD-regulated protein (*adr*A) that contains four N-terminal units of the GGDEF domain. These domains may be involved in the regulation of a second messenger molecule, called cyclic di-guanosine mono phosphate (c-di-GMP), the interaction between the GGDEF domain of *adr*A and the c-di-GMP molecule could initiate cellulose production Romling (2002). In the present study PCR detection of *adr* A gene in the isolates revealed that, all strains had the gene except O_{103} and this result agreed with that reported by Yin *et al.* (2018) as present exceeded 75% among all biofilm producer strains.

The result of in vitro tube biofilm assay revealed that, all the examined serogroups (O_1, O_{26}, O_{55}, O_{86}, O_{91}, O_{103}, O_{125}, O_{128}, O_{144} and O_{166} have the ability to make biofilms on the inner walls of the glass tubes after crystal violet staining and these results were similar to that of Kot *et al.* (2016) who reported the ability of *E. coli* strains to make biofilm in 81.1% of the isolates and differ from the results of Marhova *et al.* (2010) as biofilms were detected *in vitro* from 24% of investigated *E.coli* strains only.

From the results of PCR for detection of virulence and resistant genes we found that, the more resistant and more virulent strains and biofilm forming strains which disagreed with the results of Pavlickova *et al.* (2017) who reported that, the highest prevalence of antibiotic resistance was observed in weak biofilm producers. Biofilm formation was not statistically associated with any virulence determinant. In this study we found that, all multi resistant strain were sensitive to olive leaf extract and these results were agreed with the results reported by Liu *et al.* (2017) who demonstrated that at a concentration of 62.5mg/ml, OLE almost completely inhibited the growth of *E. coli*, so it will be good for controlling the resistant strains of *E. coli*.

CONCLUSION

In this study we found that, all multi resistant strain were more virulent strains also more biofilm forming strains. Moreover, all multi resistant strain were sensitive to olive leaf extract.

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تقييم العلاقه بين جينات الضراوة والمقاومة للمضادات الحيوبه وتكوين البيوفيلم فى الايشيريشيا كولاى المعزوله من مزارع دجاج التسمين بمحافظة الدقهليه فى مصر

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تم تجميع ١٠٠ دجاجة تسمين من مزارع مختلفة تقع في محافظة الدقهلية. خضعت جميع العينات لفحص الاعراض الاكلينيكية والصفه التشريحيه والفحص البكتريولوجى لاكتشاف العدوى وتم اكتشاف العدوى بالميكروب القولونى من الاعضاء التى تم فحصها. وقد تم عزل ٣٧ عينة بمعدل ٣٧٪ (٣٧ من ١٠٠). بشكل دقيق ، تم تحديد ١٠ انواع من الايشيريشيا كولاى في ٢٨ عينة إيجابية وكانت أكثر الانواع هي O_{125} ١٦٪ وO_{91} ١٤٪ و O_1 ١١٪ و $O_{26}K_{60}$ ١١ ٪ و $O_{86}K_{64}$ ٨ ٪ و O128 ٥٪ و $O_{55}K_{59}$ ٣٪ و O_{166} ٣ ٪ وO103 ٣ ٪ و O_{144} ٣ ٪ و ٩ انواع غيرمصنفه القولونية (٢٤ ٪). كشفت دراسة مقاومة المضادات الحيوية لعترات الايشيريشيا كولاى أن جميع العترات أظهرت مقاومة متعددة للمضادات الحيويه. تم تسجيل أعلى معدلات المقاومة ضد اللينكومايسين١٠٠٪و اموكسيسيلين ٨٢,١٤% وانروفلوكساسين ٧١,٧٣% وتيتراسيكلين ٦٧,٨٦% ودوكسسيكلين ٦٠,٧١% وامبسيلين ٥٧,١٤% وفلوموكوين ٤٢,٨٦% وجينتاميسين ٣٢,١٤% واريثرومايسين ٣٢,١٤% وحمض النااليدكسك ٢٨,٢٧% وكلورامفنيكول ٢٥% وسيبروفلوكساسين ١٠,٧% واخيرا كوليستين ٧,١٤% وفى دراسه قدرة العترات المعزوله على عمل فيلم كيميائى على الجدار الداخلي للزجاج وجد قدرة كل المعزولات على حدوثه. تم إجراء PCR للكشف عن الجينات المقاومة (*bla*TEM و (*tet*A (A)) وجينات الضراوة *csg*D، *adr*A، *eae*A و *fim*H في العترات المعزولة وكانت النتائج كالاتى ١٠٠٪ و ٩٠٪ و ١٠٠٪ ، ٩٠٪ ، ٩٠ كانت نسبة٪ ١٠٠ إيجابية بالنسبة للجينات المقاومة وجينات الضراوة، على التوالي ، والتحقق من العلاقة بينهما ثم دراسة تأثير مستخلص أوراق الزيتون على المعزولات المقاومة للمضادات الحيويه ، أوضحت النتائج أن ١٠٠٪ من المعزلات حساسة لمستخلص أوراق الزيتون) تركيز١٠ ٪.