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RESISTANCE AND VIRULENCE GENES CONSTELLATION ASSOCIATED WITH BIOFILM FORMING AVIAN PATHOGENIC *E.COLI* RECOVERED FROM BROILER CHICKENS

By

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ABSTRACT

One of the most common infections in the world is colibacillosis that results in multimillion-dollar losses every year. In this study, out of 105 samples recovered from ailing chickens suffering from pericarditis, air saculitis, perihepatitis and cellulitis, 78 *E. coli* isolates were morphologically and biochemically identified. The antimicrobial profile of the isolates was determined and revealed that, the majority of the isolates were awfully resistant to amoxycillin (100%), lincomycin (100%), rifampicin (100%), clindamycin (100%), doxycycline (100%), spiramycin (100%), amoxicillin + clavulinic acid (100%), sulphamethaxzole + trimethoprim (73%), On the contrary,

E. coli strains were extremely sensitive to colistin sulphate (100%), gentamycin (84.46%). Selected 30 isolates were serologically identified. Phenotypic detection of some virulence traits revealed percentages of 100%, 26.92% and 73% for congored binding activity, mannose resistant haemagglutination (MRH) and biofilm production respectively. PCR was applied on 30 multi-drug resistant *E. coli* isolates for detection of 4 virulence genes (*Fim*H, *hly*, *pap*C and *adr*A) and 3 resistance genes (*Bl*a$_{TEM}$, *sul*1and *tet*A).The results of PCR denoted that *adr*A, *tet*A, *bla*$_{TEM}$ and *sul*1 genes were the most dominant genes as they were found in all isolates (100%) shadowed by *fim*H (80%), *papC* (60%) and *hly* (20%).

Keywords:

E. coli, Broilers, multidrug resistance, PCR, virulence, genes.

INTRODUCTION

Escherichia coli (*E. coli*) is a normal enteric inhabitant; however, it can be a pathogenic agent that colonizes the intestinal mucosa and causes diarrhea and extra intestinal diseases **(Croxen *et al.*, 2013).**

The most typical type of colibacillosis affects 3 - 6 week old broiler chickens and initially manifests as a respiratory illness. It is usually followed by a systemic infection with characteristic fibrinous lesions (Air Sacculitis, Perihepatitis, and Pericarditis) and fatal septicemia **(Ewers *et al.*, 2003; Roy *et al.*, 2006; Sharada *et al.*, 2010).** Detection of some *E. coli* serogroups as O1, O2, O8, O18 and O78, is a common method for determining pathogenic strains **(Ewers *et al.*, 2004)**. *E. coli* pathogenicity has been corelated with numerous extrinsic and intrinsic bird factors and conditions. The extrinsic factors include environment and exposure to other infectious agents. On the other hand, intrinsic factors affecting susceptibility to infection include age, route of exposure, active and passive immune status of the bird and its breed **(Zahid *et al.*, 2016)**. Potentially, pathogenic *E. coli* can be detected by different in vitro phenotypic assays of Congo red binding which is associated with presence of virulence genes such as *ompA, iss, crl* and *fimH*, and genes for multiple resistances to antibiotics **(Zahid *et al.* 2016 and Carniello *et al.* 2018)**.In addition, means of bacterial protection other than the expression of resistance genes include the production of a large quantity of extracellular polymeric substance (EPS) throughout the process of biofilm formation. This EPS is composed mainly of exopolysaccharides that form the main structure of biofilm and serves in bacterial resistance to antibiotics and host immunity **(Ostapska *et al.*, 2018).** Antimicrobial drugs remain important in reducing both incidence and mortality associated with *E.coli* infection **(Zakeri and Kashefi, 2012)** but there is increasing evidence that avian pathogenic *E. coli* (APEC) is becoming more resistant to antimicrobial agents **(Saidi *et al.*,2013).** The most virulent strains of *E. coli* can be found using the multiplex PCR approach. Those isolates can be used as candidates for the production of a powerful vaccines to be used against APEC infections **(JanBen *et al.*, 2001).**

In this investigation, the distribution of virulence and resistance genes in APEC isolates from broiler chickens was the main objective.

MATERIAL AND METHODS

Sampling and *E. coli* Isolation:

A total of 105 samples were recovered from ailing chickens suffering from colibacillosis and showing pericarditis, air saculitis, perihepatitis and cellulitis. Samples were processed for isolation of Gram-negative bacilli showing pink colonies on MacConkey agar (lactose Fermenter) and revealed that 85 cases were pure Gram-negative lactose fermenter bacilli with an incidence of 80.95 %.). Suspected *E. coli* isolates (n=78) with an incidence of 74.28% were confirmed by biochemical identification **(Quinn *et al.*, 2002).**

Serotyping of *E. coli* isolates:

Thirteen isolates that were preliminary identified biochemically as *E.coli*,taken randomly,and were subjected to serological identification **(Edward *et al.*, 1972)** using the slide agglutination test.

Phenotypic detection of virulence traits of *E. coli* isolates:

Congo red binding activity (Berkhoff and Vinal 1986):

Congo red positive *E. coli* was indicated by the development of red colonies after incubation for 24 hours at 35^0C while Congo red negative colonies did not bind the dye and appeardwhite to yellow.

Detection of haemagglutination and mannose resistance haemagglutination using chicken RBCs:

Hemagglutination test was performed in 96 -well plates. A volume of 50 µl of dense bacterial suspension (10^{10} colony-forming units/ml in PBS, pH 7.4), obtained from an Over- Night culture at 37 ^{0}C in colonization factor antigen (CFA) medium **(Evans *et al*., 1980),** was added to 50 µl of 3% erythrocyte suspension in either PBS alone or PBS containing 4% D-mannose. The plates were gently rocked before being incubated for 5 minutes at room temperature and 5 minutes at 4^0C. After repeated incubations at 4^0C and room temperature, the outcome was reported (30-45 min). If the hemagglutination occurred in the presence of mannose, the isolate was classified as mannose-resistant (MRHA), and if it was inhibited by mannose, it was classified as mannose-sensitive (MSHA).

Biofilm formation of identified *E. coli* isolates:

Congo red (CR) assay for bacteria, as described by **Zhou *et al.* (2013)** was used for the detection of biofilm formation on yeast extract-casamino acids(YESCA)CR agar plates after

pre-enrichment of the isolates on Luria-Bertani agar medium**.** For good induction of curli production, the isolates were grown on YESCA CR agar plates at 26°C for 48^{h}; after that, the color of the bacterial colonies was checked, where the red-stained colonies considered as positive for curli production, and on the other hand, pink or white colonies considered as negative.

Antimicrobial susceptibility testing of *E. coli* isolates:

The standard disk diffusion technique was used against 12 different antimicrobial disks, according to the protocol of the Clinical and Laboratory Standards Institute **(CLSI2018)**. Mueller Hinton agar plates were inoculated with suspensions of the isolates corresponding to 0.5 McFarland tube turbidity. Antimicrobial disks [Amoxicillin (10µg), Rifampicin (5µg), Enrofloxacin (5µg), Coli stinsulphate (25µg), Gentamicin (10 µg), Fosfomycin (300µg), Spectinomycin (100µg), lincomycin (10µg), Ceftriaxone (3oµg), Amoxycillin+Clavulinic acid (30µg), Doxycycline HCl (30µg), Sulphamethoxazole-Trimethoprime (25µg) (Oxoid, Basing Stoke, UK) were applied.

The tested isolates were categorized as Sensitive, Intermediate Sensitive, or resistant, according to **CLSI (2018).**

Detection of *E. coli* Virulence and resistance genes by PCR:

Thirteen virulent MDR *E. coli* isolates based on in vitro virulence and antimicrobial resistance were tested by PCR **(Bonnet *et al.* 2009)** for the prevalence of virulence and resistance genes, including bla_{TEM} **(Colom *et al*., 2003),** *Sul*1 **(Ibekwe *et al*., 2011),** *Te*tA **(Randall *et al.* 2004),** *fim*H **(Ghanbarpour and Salehi, 2010),** *hly* **(Piva *et al*., 2003),** *pap*C **(Wen-jie *et al*., 2008) and** *adr*A **(Bhowmick *et al*., 2011).**

RESULTS

The results of *E. coli* isolation showed that 78 out of 105 samples (74.28%) resulted in *E. coli* isolation and all 78 *E. coli* isolates (100%) showed congo red binding activity and were considered as congo red positive (CR^{+})**.** Of the isolates, 21 were mannose resistant haemaglutinating with an incidence of 26.92%. and 57 (73%) were moderate or strong biofilm on a YESCA CR agar (Table 1). Multidrug resistant and virulent *E. coli* isolates (n=30) were selected for serogrouping to the O somatic antigen. Seven seroroups were distinguished amongst the isolates viz, O_{119}, O_{78}, O_{25}, O_{55}, O111, O1 and O26 with an incidence of 30%, 20%, 20%, 10%, 10%, 3.33% and 3.33%, respectively (Table2).

Response of *E. coli* isolates recovered from broiler chickens to various chemotherapeutic agents in-vitro: Showed that, the majority of *E. coli* isolates were awfully resistant to amoxycillin (100%), lincomycin (100%), rifampicin (100%), clindamycin (100%), doxycycline (100%), spiramycin (100%), amoxicillin + clavulinic acid (100%), sulphamethaxzole + trimethoprim (73%), ceftifeur (53, 84%) and fosfofmycin (50%). On the contrary, *E. coli* isolates were extremely sensitive to colistin sulphate (100%), gentamycin (84.46%), and moderately sensitive for ciprofloxacin (42.23%) and apramycin (34.61%) (Table3).

The results of PCR of *E coli* isolates Fig. (1-14) revealed that *adr*A, *tet*A, bla_{TEM} and *sul*1 genes were the most dominant genes as they were found in all isolates (100%) shadowed by *fim*H (80%), *pap*C (60%) and *hly* (20%) (Table 4).

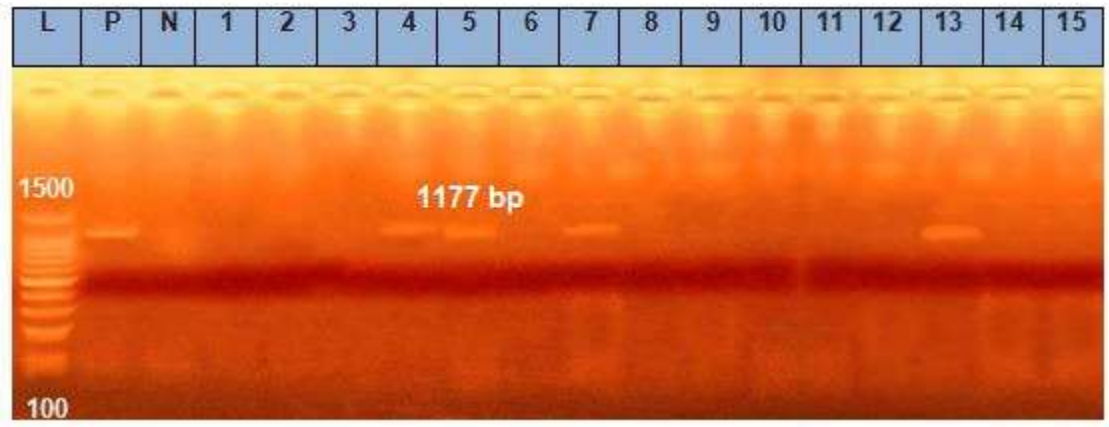


Fig. (1): PCR amplification of a 1500 bp fragment of the *hly* gene from *E. coli* isolates Lane P positive control Lane N: negative control Lanes (1-15): tested *E. coli* isolates.

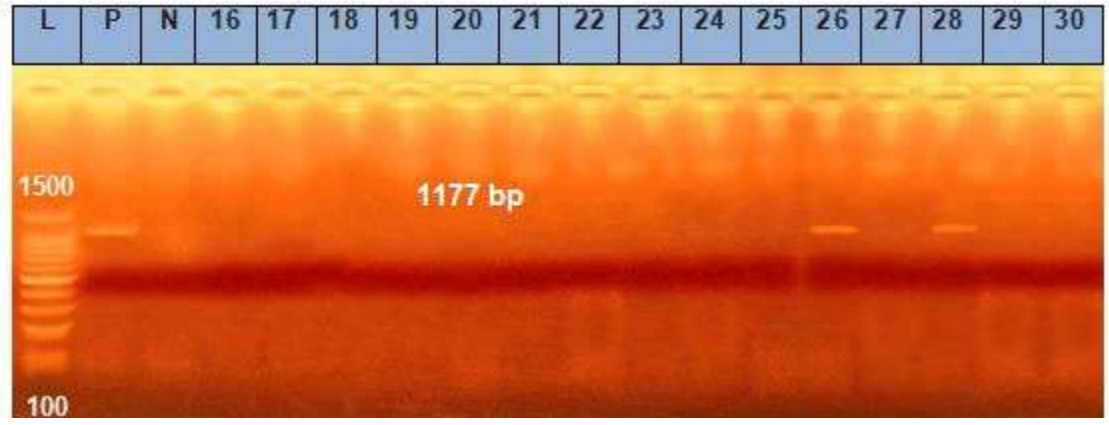


Fig. (2): PCR amplification of the 1177bp fragment of *hly* gene from *E. coli* isolates Lane P positive control Lane N: negative control Lanes (16-30): tested *E. coli* isolates.

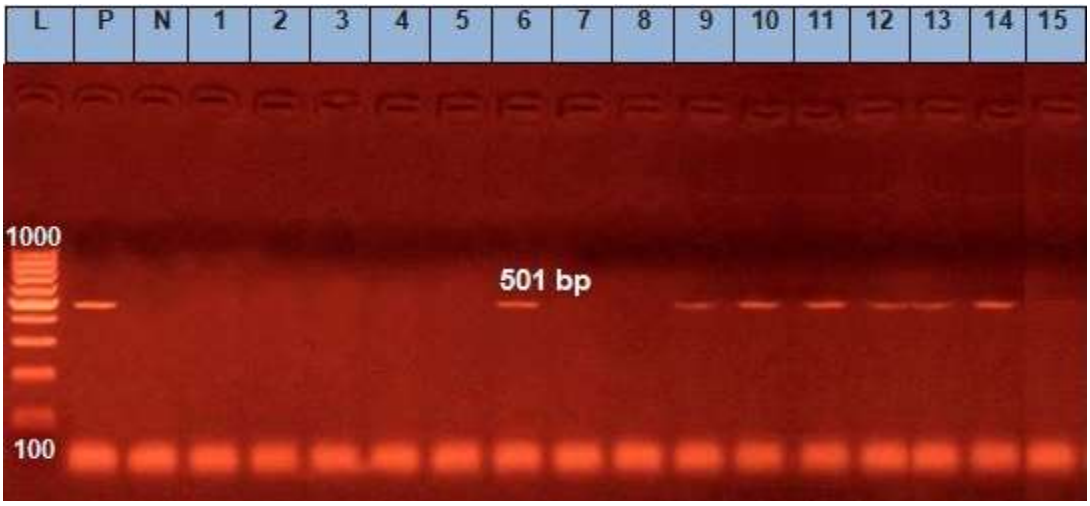


Fig. (3): PCR amplification of the 501bp fragment of *PapC*gene from *E. coli* isolates Lane P positive control Lane N: negative control Lanes (1-15): tested *E. coli* isolates.

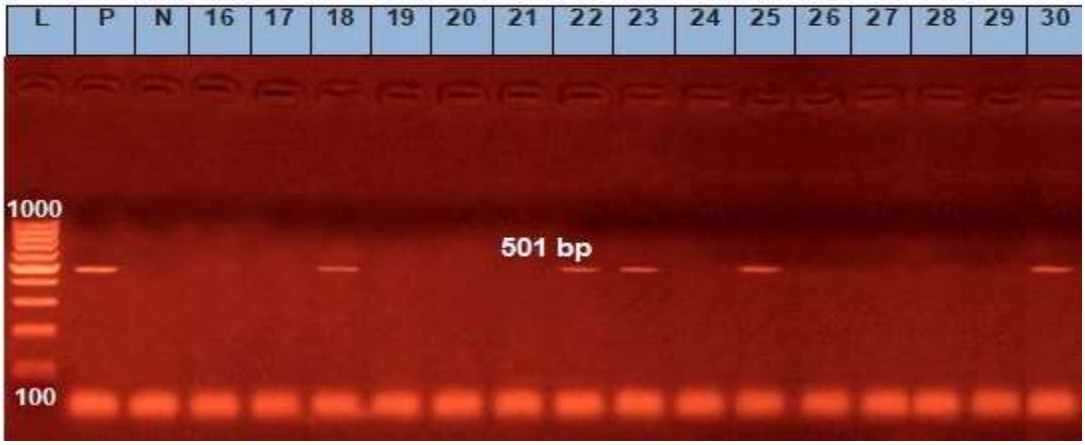


Fig. (4): PCR amplification of the 501bp fragment of *PapC* gene from *E. coli* isolates Lane P positive control Lane N: negative control Lanes (16-30): tested *E. coli* isolates

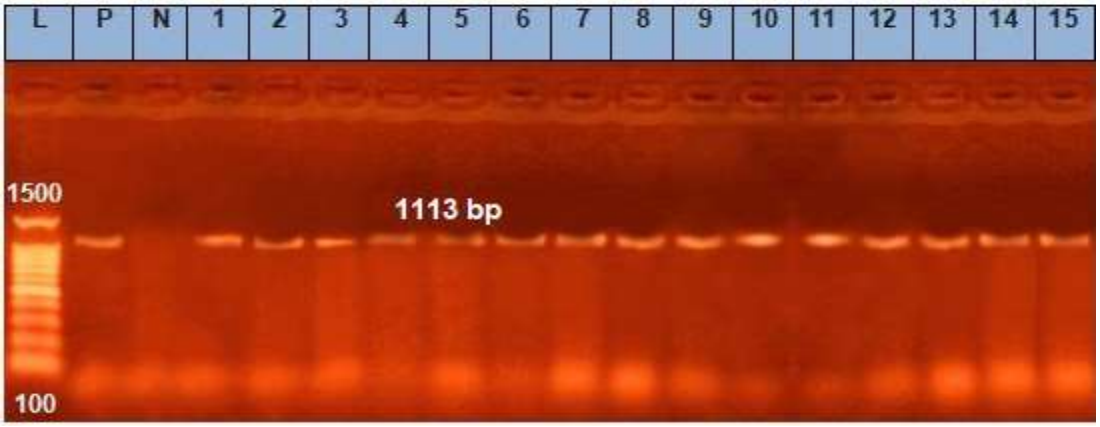


Fig. (5): PCR amplification of the 501bp fragment of *adrA* gene from *E. coli* isolates Lane P positive

control Lane N: negative control Lanes (1-15): tested *E. coli* isolates.

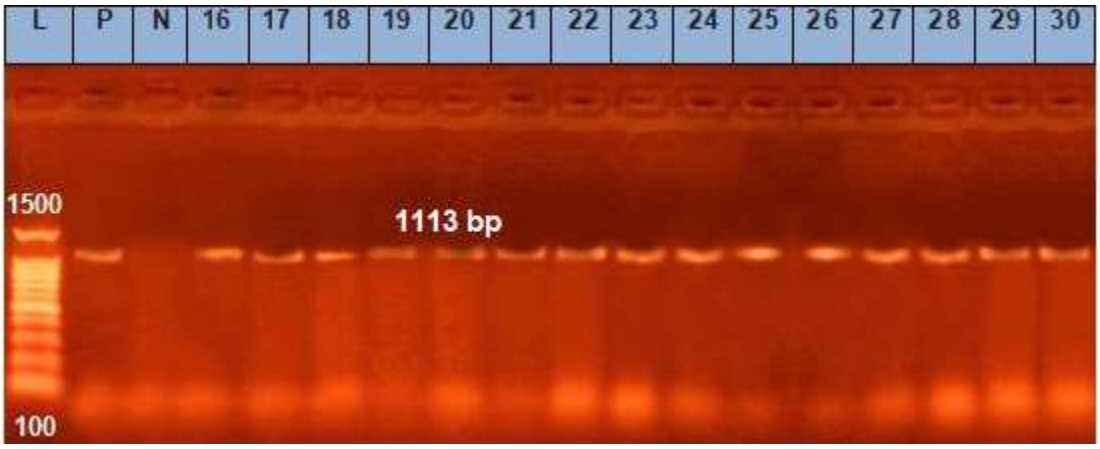


Fig. (6): PCR amplification of the 501bp fragment of *adrA* gene from *E. coli* isolates Lane P positive control Lane N: negative control Lanes (16-30): tested *E. coli* isolates.

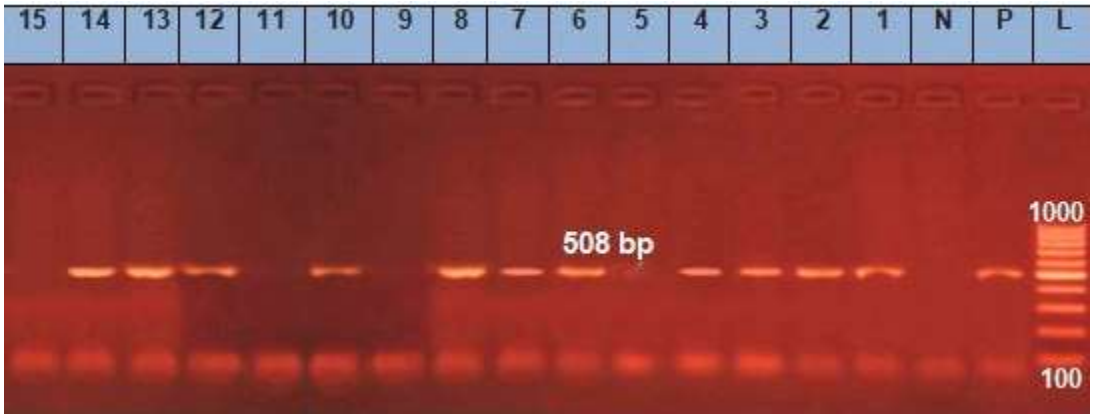


Fig. (7): PCR amplification of the 501bp fragment of *FimH* gene from *E. coli* isolates Lane P positive control Lane N: negative control Lanes (1-15): tested *E. coli* isolates.

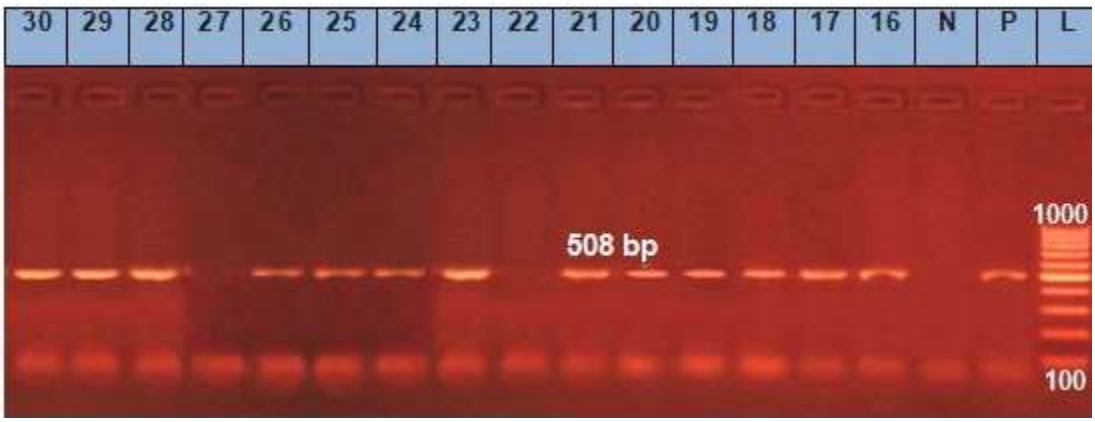


Fig. (8): PCR amplification of the 501bp fragment of *FimH* gene from *E. coli* isolates Lane P positive control Lane N: negative control Lanes (16-30): tested *E. coli* isolates.

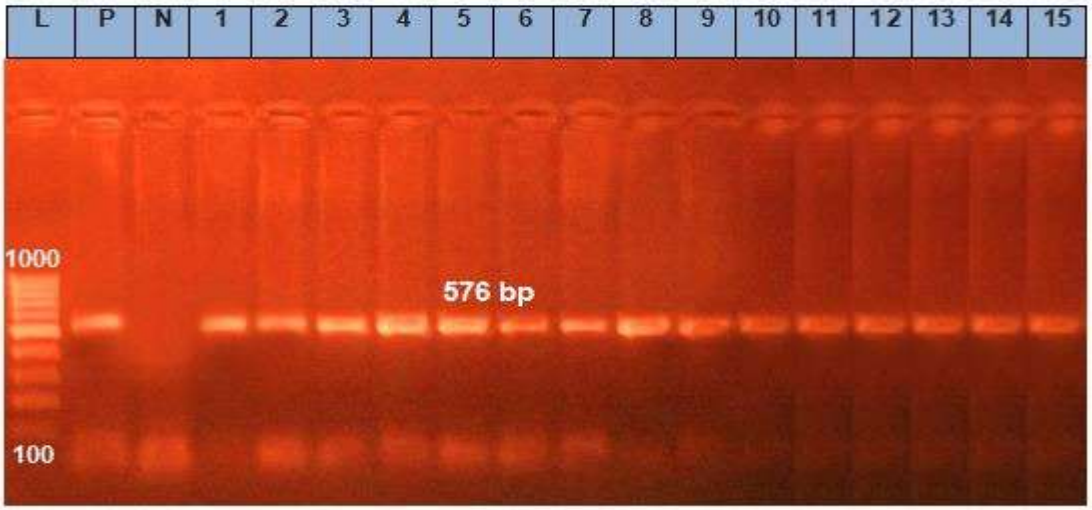


Fig. (9): PCR amplification of the 576bp fragment of *Tet (A)* gene from *E. coli* isolates Lane P positive control Lane N: negative control Lanes (1-15): tested *E. coli* isolates.

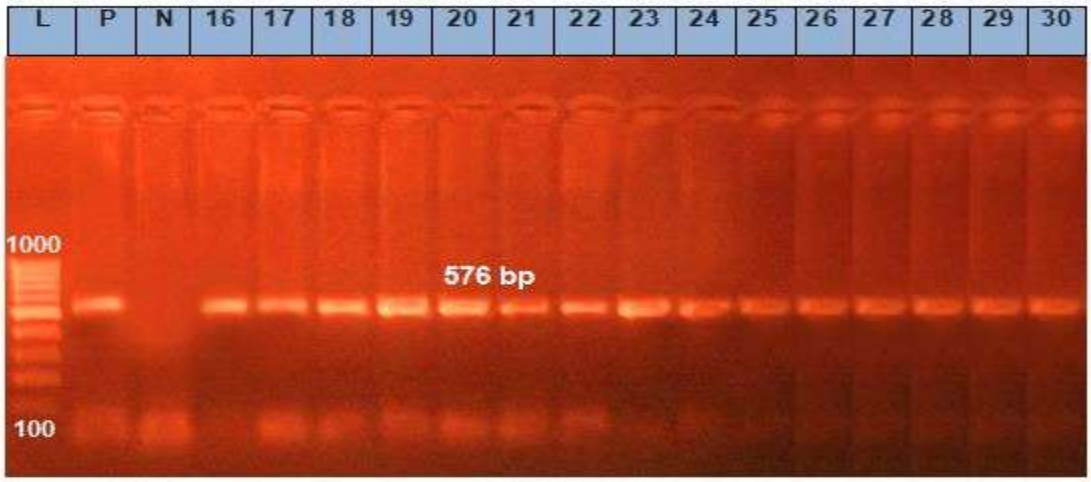


Fig. (10): PCR amplification of the 576 bp fragment of *Tet* (*A*) gene from *E. coli* isolates Lane P positive control Lane N: negative control Lanes (16-30): tested *E. coli* isolates.

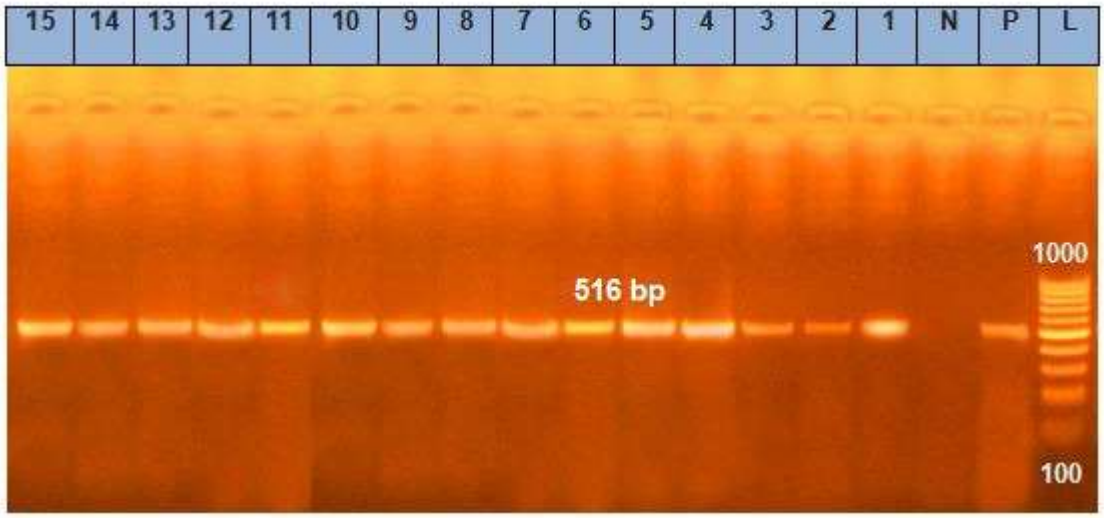


Fig. (11): PCR amplification of the 516bp fragment of *bla*$_{TEM}$ gene from *E. coli* isolates Lane P positive control Lane N: negative control Lanes (1-15): tested *E. coli* isolates.

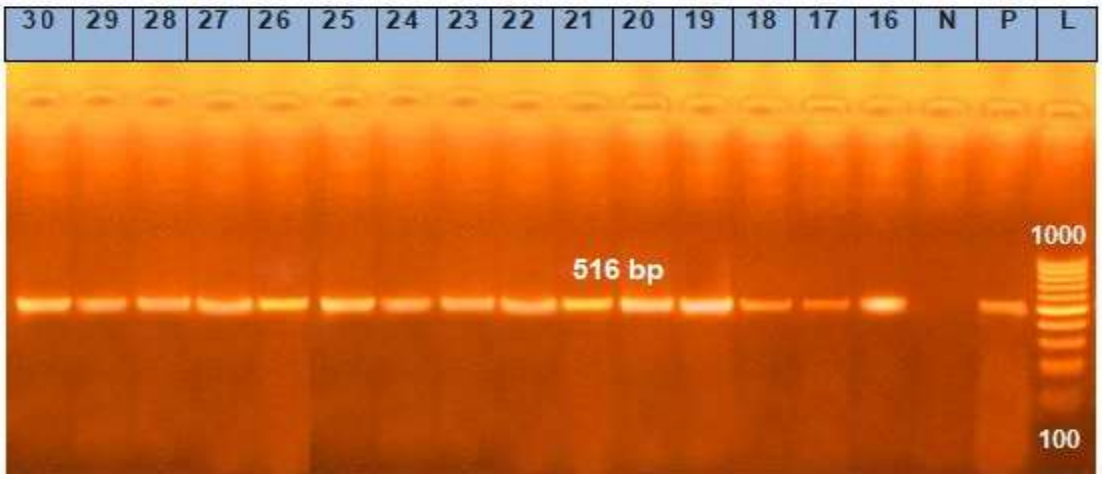
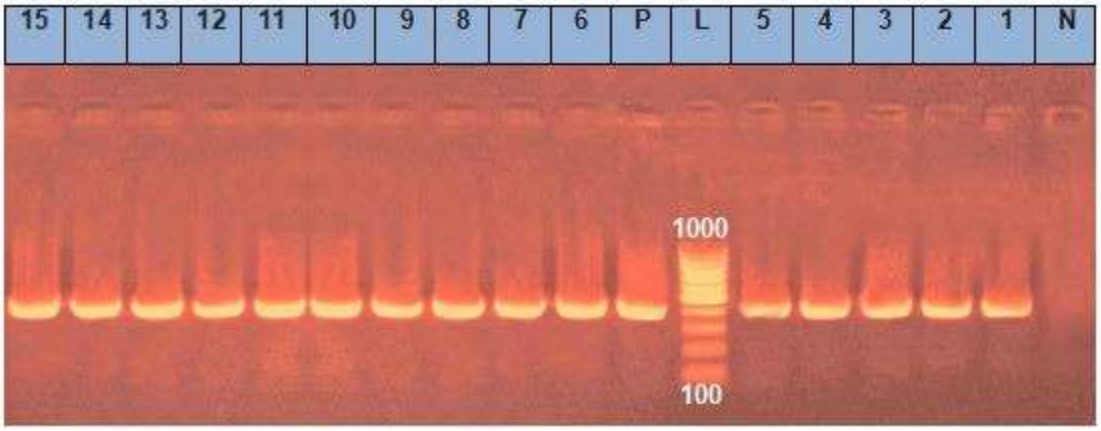


Fig. (12): PCR amplification of the 516 bp fragment of *bla*$_{TEM}$ gene from *E. coli* isolates Lane P positive control Lane N: negative control Lanes (16-30): tested *E. coli*



isolates.

Fig. (13): PCR amplification of the 433bp fragment of *sul1* gene from *E. coli* isolates Lane P positive control Lane N: negative control Lanes (1-15): tested *E. coli* isolates.

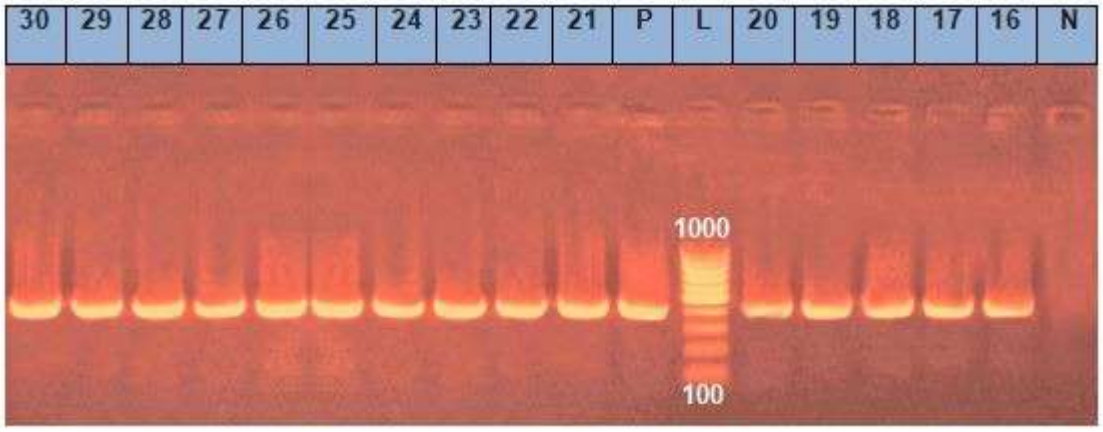


Fig. (14): PCR amplification of the 433 bp fragment of sul1gene from *E.coli* isolates Lane P positive control Lane N: negative control Lanes (16-30):tested *E. coli* isolates.

Table (1): Phenotypic characterization of some virulence factors in *E. coli* isolates recovered from broilers.

Congo red binding		Haemagglutination activities						Biofilm formation			
		HA		MRHA		MSHA		positive		Negative	
No.	%	No.	%	No.	%	No.	%	No.	%	No.	%
78	100	66	84.61	21	26.92	57	73	57	73	21	26.92

Table (2): Serogrouping of *E. coli* isolates recovered from diseased chickens.

Seroroup	N=isolates	Percentage (%)
E. coli O_{119}	9	30%
E. coli O_{78}	6	20%
E. coli O_{25}	6	20%
E. coli O_{55}	3	10%
E. coli O_{111}	3	10%
E. coli O_{1}	1	3.33%
E. coli O_{26}	1	3.33%
Un typed	1	3.33%
Total	30	100%

Table (3): Response of *E. coli* isolates to various chemotherapeutic agents.

Table (4): Virulence and antibiotic resistance genes prevalence in *E. coli* isolates**.**

Sample ID number	serogroup	bla_{TEM}	*tetA*	*Sul1*	*adrA*	*PapC*	*hly*	*fimH*
1	O_{119}	+	+	+	+	-	-	+
2	O_{119}	+	+	+	+	-	-	+
3	O_{55}	+	+	+	+	-	-	+

Chemotherapeutic discs	Investigated isolates n=78					
	Resistant		Intermediate		Sensitive	
	No.	%	No.	%	No.	%
Colistin sulphate	-	-	-	-	78	100
Gentamycin	9	11.53	3	3.846	66	84.46
Ceftiofeur	42	53.84	6	7.69	30	38.46
Ciprofloxacin	18	23.07	33	42.23	27	34.61
Sulfamethoxzol+trimethoprim	57	73	-	-	21	26.92
Doxycycline hydrochloride	78	100	-	-	-	-
Amoxicillin+Clavulinic acid	78	100	-	-	-	-
Fosfomycin	39	50	6	7.69	33	42.23
Amoxycillin	78	100	-	-	-	-
Apramycin	24	30.7	27	34.61	27	34.61
Clindamycin	78	100	-	-	-	-
Lincomycin	78	100	-	-	-	-
Spiramycin	78	100	-	-	-	-

4	O_{78}	+	+	+	+	-	+	+
5	O_{78}	+	+	+	+	-	+	-
6	O_{78}	+	+	+	+	+	-	+

7	**O_{119}**	+	+	+	+	-	+	+
8	**O_{119}**	+	+	+	+	-	-	+
9	**O_{25}**	+	+	+	+	+	-	-
10	**O_{111}**	+	+	+	+	+	-	+
11	**O_{119}**	+	+	+	+	+	-	-
12	**O_{25}**	+	+	+	+	+	-	+
13	**O_{78}**	+	+	+	+	+	+	+
14	**O_{111}**	+	+	+	+	+	-	+
15	**O_{1}**	+	+	+	+	-	-	-
16	**O_{119}**	+	+	+	+	-	-	+
17	**O_{111}**	+	+	+	+	-	-	+
18	**O_{25}**	+	+	+	+	+	-	+
19	**O_{78}**	+	+	+	+	-	-	+
20	**O_{25}**	+	+	+	+	-	-	+
21	**O_{119}**	+	+	+	+	-	-	+
22	**O_{25}**	+	+	+	+	+	-	-
23	**O_{55}**	+	+	+	+	+	-	+
24	**O_{119}**	+	+	+	+	-	-	+
25	**O_{25}**	+	+	+	+	+	-	+
26	**O_{78}**	+	+	+	+	-	+	+
27	**O_{55}**	+	+	+	+	-	-	-
28	**O_{119}**	+	+	+	+	-	+	+
29	**$_{26}$**	+	+	+	+	-	-	+
30	**untyped**	+	+	+	+	+	_	+
Total	**30**	**100%**	**100%**	**100%**	**100%**	**60%**	**20%**	**80%**

DISCUSSION

E. coli is one of the most significant bacteriological risk factors in poultry as it causes colibacillosis. In this study, *E. coli* was isolated from 74.28% of samples collected from diseased broilersIn a similar study, **Abd El Tawab *et al.* (2014)** isolated *E. coli* from 75.7% of diseased chickens.

In this study, 100 % of *E. coli* isolates from diseased broilers were Congo red (CR)-positive as a phenotypic pathogenicity marker **(Zahid *et al,* 2016; Reichhardt and Cegelski, 2018).**

This result is matched with that of **Berkhoff and Vinal (1986)**, who reported a correlation between CR expression and *E. coli* virulence.

Biofilm formation is a mechanism for bacterial resistance and also for bacterial virulence **(Cepas *et al.*,2019)**, where it increases the antimicrobial resistance up to 1,000 folds on antimicrobials to inactivate organisms developing inside a biofilm, and high antimicrobial concentrations are required **(Hall and Mah,2017).** In addition to the existence of active antibiotic degradation mechanisms that contribute to the cessation of drug accumulation to a sufficient concentration, this resistance may be brought on by insufficient concentrations of antimicrobials that reach specific areas of the biofilms and metabolic inactivity.

According to research by **Reichhardt *et al.* (2015)**, CR dye can bind to curled whole cells without impairing growth and can be used to compare the degree of whole cell curliation, where *E. coli* accumulate extracellular adhesive amyloid fibres known as curli that facilitate bacterial adhesion and promote biofilm formation.

According to the results of the current study, biofilm formation was detected in 73% of the recovered *E. coli* isolates. This outcome is consistent with the finding by **Skyberg *et al.* (2007)** that 75.7% of intestinal *E. coli* isolates from healthy broilers could generate biofilm. Meanwhile, 53.3% and 16.6% of *E. coli* isolates were reported by **(Moori Bakhtiari *et al.* 2018)** to be moderately and highly biofilm producers, respectively.

The ability to colonise the intestine conferred by mannose resistant haemaggluting (MRHA) adhesins may allow commensal flora members like *E. coli* to serve as a protective barrier against pathogenic bacterial colonization **(Wooley *et al.*., 1994 and Snoeyenobos *et al.*, 1982).**

In our study, MRHA was observed in *E. coli* isolates, where 21 isolates had a 26.92% rate of being mannose resistant to haemaglutination.

Multidrug resistant and virulent *E. coli* isolates (N=30) were subjected for serogrouping to the O somatic antigen. Seven seroroups were distinguished amongst the isolates, namely, O_{119}, O78, O_{25}, O_{55},O111, O1 and O_{26} with an incidences of 30%, 20%, 20%, 10%, 10%, 3.33% and 3.33%, respectively. Similar *E. coli* serotypes had been previously isolated from cases of chickens in Egypt as previously reported **(Sharada *et al.*, 2010; Ammar *et al.*, 2011; Shimaa, 2013).**

In the current study,response of *E coli* isolates recovered from broiler chickens to various

chemotherapeutic agents in-vitro: showed that, the majority of *E.coli* isolates were awfully resistant to amoxycillin (100%) which is supported by the findings of **Li-ming *et al*. (2016)** who found that *E.coli* was highly resistant to amoxicillin and doxycycline (100%). Also, **Zhao *et al*. (2014)** recorded a high resistance, to a certain degree, against amoxycillin.

The high resistance recorded in this study may be related to the widespread use of the broad-spectrum antibiotics in colibacillosis Also, in this study resistance rates were recorded against lincomycin (100%), rifampicin (100%),clindamycin (100%), spiramycin (100%), amoxicillin + clavulinic acid (100%), sulphamethaxzole +trimethoprim (73%),ceftifeur (53,84%) and fosfofmycin (50%). On the contrary, *E. coli* strains were extremely sensitive to colistin sulphate (100%) and gentamycin (84.46%). **Raheel *et al*., (2020)** found *E. coli* to be 91.4% sensitive to gentamicin and moderately sensitive for ciprofloxacin (42.23%) and apramycin (34.61%). All of the biofilm-producing *E. coli* isolates in the current investigation were identified as MDR, indicating a connection between antibiotic resistance and biofilm production. Similar findings were recorded by **Neupane *et al.* (2016) and Karigoudar *et al.* (2019).** Cephalosporin resistance is linked to the genes that encode for β-lactamases such as *bla*TEM, *bla*CTX-M, and *bla*CMY **(Li *et al.*2007).** In this study, *bla*TEM was identified in all tested *E. coli* isolates, whereas **Zhao *et al.* (2013**) detected the gene in 30.4%. of their *E. coli* isolates. The well-known and ubiquitous sulfonamide resistance in Gram-negative bacteria is linked to the presence of the sulfonamide resistance gene (*Sul*1) on plasmids **(Zhang *et al*., 2019).** All of the tested *E. coli* isolates in this study had this gene. Additionally, the *tet*A gene, which is responsible for the creation of the protein involved in the efflux pump process, which is the most prevalent method of resistance to tetracycline and its analogues, was found in all of the isolates under study **(Ozgumus *et al*., 2007).**

Biofilm development is regarded as a key factor in the bacterial pathogenicity. Compared to planktonic cells, biofilm cells were found more resistant to adverse environmental conditions, cleaning agents, antibiotics, and the host's immune system **(Jensen *et al*., 2010).** Studies have shown that bacteria can cling to and create biofilms on a variety of food contact surfaces, including metal, plastic, and rubber **(Joseph *et al*., 2001; Stepanovic *et al*., 2004).**

As the source of the microbial contamination that can cause food spoiling and disease transmission, biofilm that forms in food processing environments is of particular significance. According to **Costerton *et al.* (1999)**, a biofilm is a group of bacterial cells that attach to a surface and are enmeshed in a polymeric matrix that they have formed themselves.

In the current study, PCR on *E. coli* isolates proved that *adr*A, was found in all isolates (100%). Similar result were obtained by **Bhowmick *et al.*, (2011) and Abd El-basit *et al.* (2019).**

CONCLUSION

The present study highlights the high incidence of MDR *E. coli* associated with avian colbacillosis. The detection of *adr*A gene is circumstantial evidence that this gene is crucial for the development of pathogenic *E. coli* biofilms. In addition to its link with biofilm develop ment, *E. coli* isolates also had high levels of antibiotic resistance.

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