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MOLECULAR IDENTIFICATION OF VIRULENCE GENES OF PATHOGENIC ESCHERICHIA COLI ISOLATED FROM BROILERS CHICKEN

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ABSTRACT

This study was implemented to isolate, characterize the presence of *E coli* and study their antibiotic resistance and virulence genes in broiler chickens in Assiut city. A total of 120 samples (liver, heart, yolk sac and lung) were gained from 3 to 35 days old clinically and freshly dead broiler suffering from respiratory manifestation (CRD), omphalitis, septicemia and diarrhea in Assiut Governorates for the detection of pathogenic *E coli*. Isolation and phenotypic identification of the isolates were performed. Serology and detection of antibiotic sensitivity and resistance were done. Also, detection of genes accountable for virulence (ompA and iroN genes) and antimicrobial resistance were all performed on the samples. Also, resistance genes to antimicrobials (*blaTEM, blaVIM and qnrA* genes) were detected. *E coli* was detected and recognized in 31.7 % of the cases. According to the data, 11 of the 38 *E coli* isolates were identified using serology. The conventional disc diffusion method was used to assess the susceptibility and resistance of the isolated *E coli* to various antibacterial agents. A total of 81.5 % of isolates have a MAR index exceeding 0.2, whereas 18.5 % have a MAR index not more than 0.2. with an average MDR index of 0.485. Antimicrobial resistance genes were detected in 73.7 % of 19 serologically recognized virulence and antibiotic resistance genes in E. coli isolates such as ompA gene detected in 95%, blaTEM gene detected in 95 %, blaVIM gene detected in 73.7 %, and qnrA gene detected in 31.5 %, but the iroN gene was not detected.

***Keywords**: Escherichia coli*; antibiotic-resistance genes; virulence genes, PCR.

INTRODUCTION

Avian colibacillosis is deliberated as one of the greatest serious chickens' diseases causing high morbidity and mortality resulting in significant economic losses (Radwan *et al.,* 2021). *E coli* strains were identified by Russo and Johnson (2000) into 3 major strains including intestinal pathogenic strains, commensal strains and extra intestinal pathogenic strains *E coli* (ExPEC). This sickness has resulted in up to 30% of poultry mortality due to diminished output, high treatment costs, carcass rejection, and mortality (Radwan *et al.,* 2020).

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E coil resistance to antibiotics is prevalent and of concern to poultry veterinarians. Considerable consideration has been established for this resistance worldwide and especially in Egypt (Radwan *et al.,* 2021). The widespread use of antibiotics for disease control and avoidance has resulted in an extraordinary rise in antibiotic-resistant organisms (Ibrahim *et al.,* 2019). Horizontal gene transfer or gene mutations are responsible for this resistance (Hughes and Andersson, 2015).

Bacteria that are multidrug resistant (MDR) frequently have multiple drug resistance genes (Nikaido, 2009). The emergence of multidrug-resistant APEC poses a number of challenges, not only in terms of preventing and controlling APEC infection, but also in terms of allowing resistance to spread to additional infections (Li *et al.,* 2021). Antibiotic resistance is mainly linked to genetic alterations encoded by chromosomal and plasmid genes carried by bacterial organisms. Bennett (2008). Momtaz *et al.* (2012) show that all *E. coli* strains have one or more genes responsible for antibiotic resistance. PCR assays enable the detection of the regularity of the various virulence-associated genes that happen in the resident APEC population; consequently, these identified isolates were considered the most highly pathogenic *E.coli* using the PCR technique. This is used as the basis for the manufacture of the most powerful vaccine (Ewers *et al.,* 2004).

Pathogenic *E. coli* have much genetic diversity and many virulence-related factors such as invasins, adhesins, iron acquisition factors, toxins and serum resistance factors (Kaper *et al.,* 2004). The virulence genes *iroN* and *ompT* are the most basic predictors of *E. coli* pathogenicity (Johnson *et al.,* 2008a). Iron acquisition (*iroN*) and adhesion are the key roles of these genes (*ompT*) (Mohamed *et al.,* 2018). It was established a close implication between the pathogenicity of E. coli and its virulence-associated components (Janßen *et al.,* 2001). Moreover, it is utilized to determine the clinical symptoms and the level of bacterial harm. Horizontal transfer of gene and antibiotic resistance mechanisms were also linked to virulence factors (Wang, 2002).

MATERIALS AND METHODS

Collection of samples:

A total of 120 samples were conveyed from diseased and freshly dead broilers distress from respiratory signs appearance (CRD), septicemia and diarrhea. Postmortem findings comprising general congestion. Characteristic fibrinous lesions (air sacs, pericardium and perihepatitis). Fatal septicemia samples were collected from lung, liver, heart, yolk sac and kidney.

Conventional method for isolation of E coli (Collee *et al.*, 1996):

Samples were injected into MacConkey broth then, incubated in aerobic conditions for 24 hours at 37°C. Then inoculated into MacConkey agar for 24 hours at 37°C. E. coli appears as pink colonies on into MacConkey media with a green metallic sheen on EMB medium. Microscopically it is Gram-ve rods.

Biochemical reactions (Quinn *et al.*, 2002).

E. coli strains showed fermentation of lactose, indole and methyl red positive. Isolates were negative for oxidase, urea hydrolysis, Voges-Proskauer, citrate utilization and did not produce hydrogen sulphide.

Antibiotic susceptibility testing (Mary and Usha 2013):

The single diffusion method was used to determine antimicrobial susceptibility using different concentrations of sensitivity discs (Oxoid Limited, Basingstoke, Hampshire, UK). 14 antibiotic discs were used including S: Streptomycin, AMX: Amoxicillin, EN: Enrofloxacin, DO: Doxycycline, T: tetracycline, AM: Ampicillin, SXT: Sulphamethoxazol, CP: Ciprofloxacin, G: Gentamicin, CN: Cephalothin, CO: Colistin, L: Levofloxacin, AK: Amikacin and M:

Meropenem. The interpretation of the results was done according to the National Committee for Clinical Laboratory Standards 'NCCLS' (2001).

O-Serotyping (Kok *et al.*, 1996):
Quick diagnostic *E coli* antisera sets (DENKA SEIKEN Co., Japan) were used to identify the strains serologically. Slide agglutination test was done and then confirmed with a tube agglutination test for O-serotype using 38 APEC isolates as recommended by the manufacturer's guidelines.

Detection of the virulence-associated genes by polymerase chain reaction test
PCR was used to detect antimicrobial resistance genes (5 genes) conferring resistance to b-lactamase (blaTEM) and *blaVIM* and quinolone antibiotics (qnr), 2 virulence genes *ompA* (outer membrane protein) and ***iron*** **(**iron acquisition**)**

Table 1: Oligonucleotide primers sequences.

Gene	Primer sequence (5'-3')	Length of amplified product	Reference
blaTEM	ATCAGCAATAAACCAGC CCCCGAAGAACGTTTTC	516 bp	Colom *et al*., 2003
ompA	AGCTATCGCGATTGCAGTG GGTGTTGCCAGTAACCGG	919 bp	Ewers *et al*., 2007
qnrA	ATTTCTCACGCCAGGATTTG GATCGGCAAAGGTTAGGTCA	516 bp	Robicsek *et al*., 2006
blaVIM	AGTGGTGAGTATCCGACA ATGAAAGTGCGTGGAGAC	280 bp	Xia *et al*., 2012
Iron	ATC CTC TGG TCG CTA ACT G CTG CAC TGG AAG AAC TGT TCT	847 bp	Ewers *et al*., 2007

RESULTS

Table 2: PCR amplification products for the different genes detected in *E. coli* serogroups.

Sample	serotype	*ompA*	*iroN*	*blaTEM*	*blaVIM*	*qnrA*
1	O17	+	-	+	-	+
2	O78	+	-	+	-	-
3	O78	+	-	+	-	-
4	O146	-	-	+	-	-
5	O78	+	-	+	+	+
6	O127	+	-	+	+	+
7	O26	+	-	+	+	-
8	O78	-	-	+	+	-
9	O159	+	-	+	+	-
10	O78	+	-	+	+	-
11	O78	-	-	+	+	-
12	O91	+	-	+	+	+
13	O55	+	-	+	+	-
14	O78	+	-	+	+	-
15	O2	+	-	+	+	+
16	O128	-	-	-	+	-
17	O78	-	-	+	+	+
18	O1	+	-	+	+	-
19	O128	+	-	+	-	-
		79%	0%	95%	73.6%	31.5 %

Table 3: Antimicrobial resistance outline of *E. coli* strains (n=38).

NO	*E. coli* Strains	Antimicrobial resistance profile	MAR index
1	O17 : H18	S, AMX	0.143
2	O78	S, AMX, EN, T, DO, AM	0.428
3	O78	S, AMX	0.143
4	O146: H21	S, AMX, EN, T, DO, AM	0.428
5	O78	S, AMX, EN, T, DO, AM, SXT, CP, G, CN, CO, L, AK, M	1
6	O127: H6	S, AMX, EN, T, DO, AM, SXT, CP	0.571
7	O26: H11	S, AMX, EN, T, DO	0.357
8	O78	S, AMX, EN, T, DO	0.357
9	O159	S, AMX, EN, T, DO, AM, SXT, CP	0.571
10	O78	S, AMX, EN	0.214
11	O78	S, AMX, EN, T, DO, AM, SXT, CP	0.571
12	O91: H21	S, AMX, EN, T, DO, AM, SXT, CP, G, CN, CO, L, AK	0.928
13	O55: H7	S, AMX, EN, T, DO, AM, SXT	0.5
14	O78	S, AMX, EN, T, DO, AM, SXT, CP, G, CN	0.714
15	O2: H6	S, AMX, EN, T, DO, AM, SXT, CP, G, CN	0.714
16	O128: H2	S, AMX, EN, T, DO, AM, SXT, CP	0.571
17	O78	S, AMX, EN, T, DO, AM, SXT, CP, G, CN	0.714
18	O1: H7	S, AMX, EN, T, DO, AM, SXT, CP	0.517
19	O128: H2	S, AMX, EN, T, DO, AM	0.428
Average	0.485		

S: Streptomycin AMX: Amoxicillin EN: Enrofloxacin DO: Doxycycline
T: tetracycline AM: Ampicillin SXT: Sulphamethoxazol CP: Ciprofloxacin
G: Gentamicin CN: Cephalothin CO: Colistin L: Levofloxacin
AK: Amikacin M: Meropenem

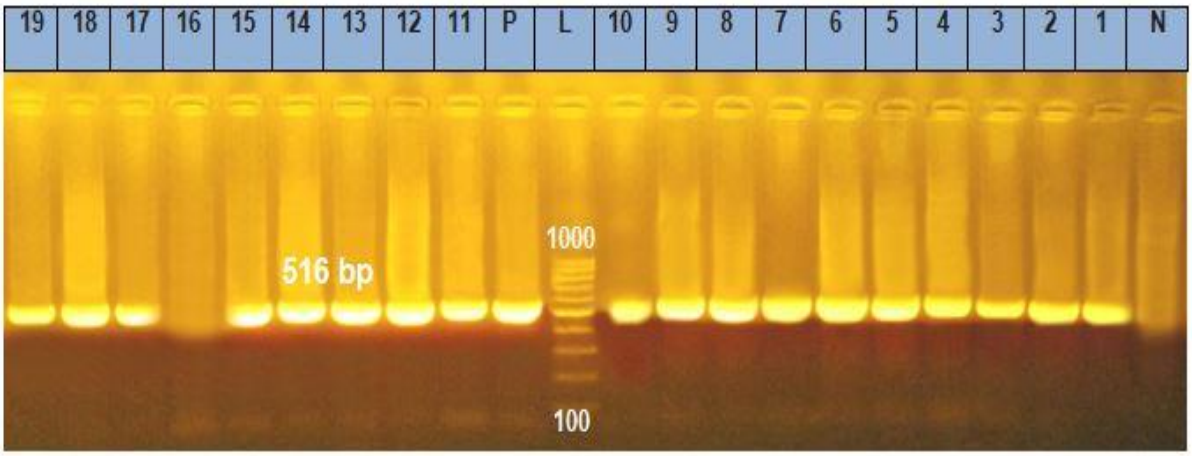

Fig.1: Agarose gel electrophoresis of PCR produced after amplification of *blatTEM* gene (516 bp)
Lane L: Ladder marker 100:1000 bp
Lane P: Control positive
Lane N: Control negative
Lanes (1-19): positive E. coli strains
Lane 16: Negative E. coli strain

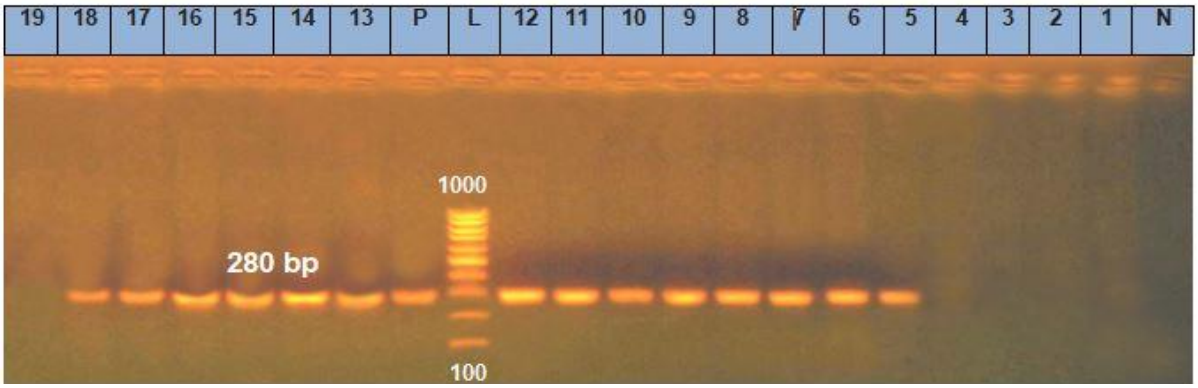

Fig. 2: Agarose gel electrophoresis of PCR produced after amplification of *blatVIM* gene (280 bp)
Lane L: Ladder marker 100:1000bp
Lane P: Control positive
Lane N: Control negative
Lanes (1,2,3,4 and19): positive E. coli strains
Lane 16: Negative E. coli strain

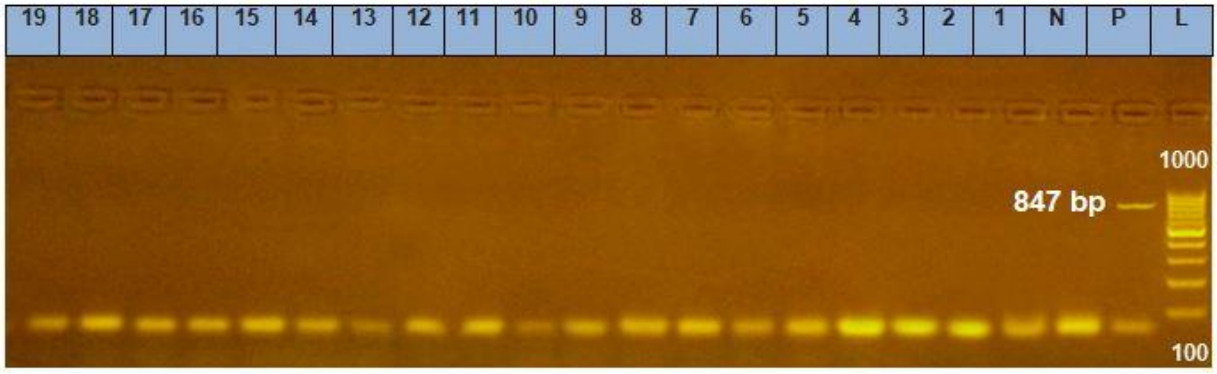

Fig. 3: Agarose gelelectrophoresis of PCR produced after amplification of *iroN* gene (847 bp)
Lane L: Ladder marker 100:1000bp
Lane P: Control positive
Lane N: Control negative
Lanes (1-19): negative E. coli strains

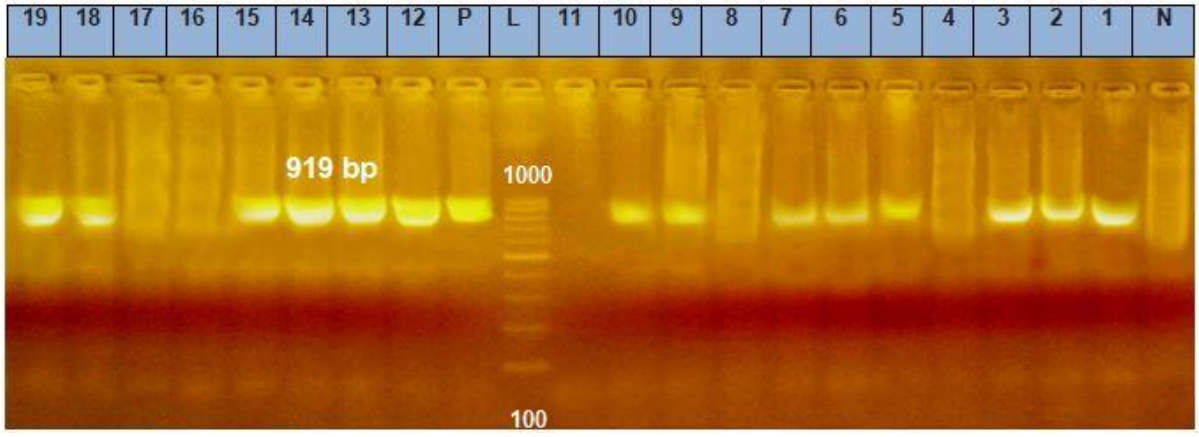

Fig. 4: Agarose gelelectrophoresis of PCR produced after amplification of *ompA* gene (919 bp)
Lane L: Ladder marker 100:1000 bp
Lane P: Control positive
Lane N: Control negative
Lanes (4,8,11,16 and17): Negative E. coli strains

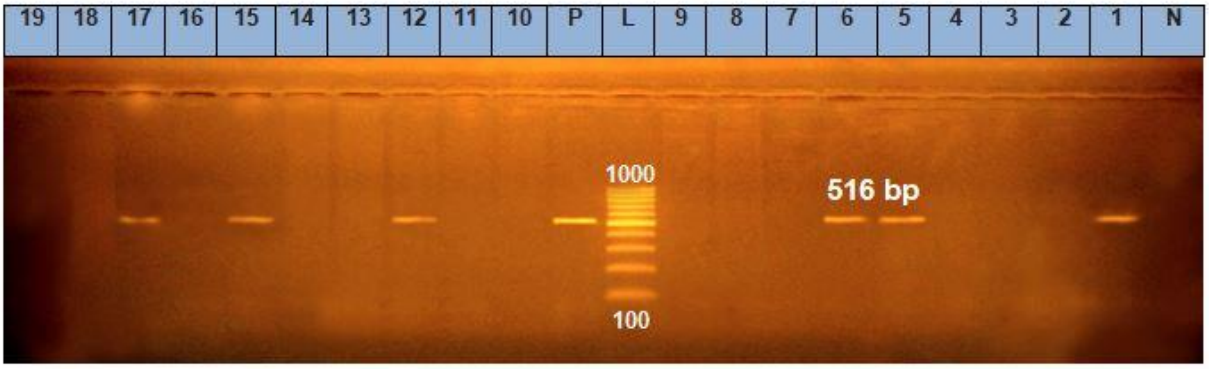

Fig.5: Agarose gelelectrophoresis of PCR produced after amplification of *qnrA* gene (516 bp)
Lane L: Ladder marker 100:1000 bp
Lane P: Control positive
Lane N: Control negative
Lanes (1,5,6,12,15 and 17): Positive E. coli strains

DISCUSSION

Clinical signs such as depression, loss of appetite, retardation of growth, huddling together, ruffled feathers, respiratory discomfort and diarrhoea which is white and pasty were all seen in broiler hens examined in many areas of the Assiut Governorates. Enteritis, Pericarditis, peritonitis and congestion in internal organs were seen. Petechial haemorrhages on parenchymatous organs were noticed and air sacs with varying degrees of turbidity and thickness, air sacculitis, and omphalitis were the most common post-mortem findings.

E. coli was isolated and the percentage of isolation was (31.7%), 38/120 while, 11 from 38 *E. coli* isolates were verified by serology. The O-serotyping results for 38 (31.7%) APEC strains are reviewed in Table (2) 11 serotypes from a total 38 *E.coli* isolates that have been serotyped the most predominant serotype was O78 (8 isolates), followed by O91: H21(6 isolates), O1:H7 & O128: H2 (5 isolates), O2:H6 (4 isolates), O146:H21 (3 isolates), O55:H7 & O26 & H11. (2 isolates), O127:H6, O159 & O17:H18 (1 isolate each). Our results were consistent with (Nolan *et al.*, 2013) who reported that the O serotypes found in the majority of the *E. coli* isolates achieved from the affected birds. Also, Dho-Mulin and Fairbrother, 1999) discovered that the main serotypes recovered from colibacillosis infections in chickens were O1, O2, and O78. A study by Chinese researchers revealed that the two most common *E. coli* groups detected in birds in distress from colibacillosis were te O78 and O2 groups (Dou *et al.*, 2016). On the other hand, In Egypt, the most predominant serotypes were O125, O114 and O44 as each represents and the very low percent Serotypes O2 and O78 may probably be due to variation in serotypes over a period of time in a particular area (Amer *et al.*, 2011).

Antimicrobial Susceptibility:

The disc diffusion test was used to assess susceptibility of antibiotics in 19 avian E. coli isolates. Table 3 shows the antimicrobial resistance profiles of the examined *E. coli* strains, 89.5 percent of isolates have a MAR index greater than 0.2, while 10.5 percent have a MAR index less than or equal to 0.2 with an MDR index of 0.485 on average. A MAR index score of higher than 0.2 denotes high-risk sources contamination, as many antibiotics are frequently used to prevent disease (Chitanand *et al.*, 2010). From the antimicrobial susceptibility results, we found that *E.coli* strains were greatly resistant to **S,** T, DO AMX, EN, AM, G SXT, CP and **CN**. These results are in agreement with Xu *et al*. (2019) who found that the isolated a pathogenic E. coli from diseased chickens with mainly typical lesions had great

resistance to T, AMP, cefotaxime, GEN, STR and SXT. However, Xia *et al.* (2009) observed in China the avian *E.coli* strains were resistant to enrofloxacin 99%, ciprofloxacin 100%, norfloxacin 100%, amoxicillin/ clavulanic acid 87.4%, ampicillin 99.5%, gentamicin 97% and amikacin 27.8%.

The growth and spread of bacteria that were multidrug-resistant has lowered the antimicrobials' efficacy, posing major health risks (Mellata, 2013). This is a clear evidence of indiscriminate and abusive antibiotic usage for disease or prevention. In an antibiotic-saturated environment, antibiotic-resistant bacteria eventually supersede drug-sensitive germs (Van den *et al.*, 2001). MDR microorganisms carriage a direct hazard to consumers as suppliers of antibiotic resistance genes to other bacteria (Nhung *et al.*, 2017).

Distributions of virulence-associated genes.

Results reviewed in Table 2 and figures 1, 2, 3, 4 and 5 showed that *ompA* gene was detected in 73.7%, *blaTEM* gene was detected in 95%, *blaVIM* gene was identified in 73.7%, *qnrA* gene was discovered in 31.5%, *iroN* gene not noticed in any *E. coli* serotype 0%. PCR results showed that antibacterial resistance genes were found in 19 serologically identified E. coli isolates. The two genes accountable for resistance of *B- lactamase* (*blaTEM* and *blaVIM* genes) were found at a rate of 95% and 73.6%, respectively in E.coli isolates. This genotypic pattern is similar to the present pattern of phenotypic This genotypic pattern is similar to the observed pattern of phenotypic resistance of *E. coli* isolates detected with the disk diffusion method and these results were steady with (Hiki *et al.*, 2013) who discovered the blaCTX-M-2, blaCTX-M-14, blaCTX-M-65, or blaCMY-2 genes in all strains of E. coli that were isolated from broiler chickens.

The *b-lactamase*–encoding gene *blaTEM* was noticed in 16 (11.1%) APEC-like strains, which have resistance to *b-lactam* antibiotics such as ampicillin, amoxicillin, cephalothin and clavulanic acid while the plasmid-borne quinolone resistance gene *qnr* was distinguished in 47 isolates (32.6%) (Li *et al.*, 2021).

Abd El Tawab *et al.* (2015) detected *bla*TEM gene in 22 (73%) of *E. coli* isolates. In addition, (Jiang *et al.*, 2011) detected 88.9% of *bla*TEM gene among *E. coli* strains detected from broilers in China. Also, *bla*TEM gene was detected by Domínguez *et al.* (2002) 20 (48.7%) of *E. coli* distinguished from broilers. In addition, Ivan *et al.* (2010) recorded that *E. coli* strains which were resistant to broad-spectrum cephalosporins and carried multiple *bla*TEM genes were responsible for the ESBL phenotype in gulls. Furthermore, 9 *E. coli* isolates harboring *bla*TEM genes that produce ESBL. *blaTEM* gene was detected in 11.1% (Li *et al.*, 2021).

Regarding quinolone (*qnrA)* encoding gene, it was presented by rate of 31.5% in *E. coli* isolates. The genotypic pattern is nearly parallel to the observed phenotypic resistance of *E. coli* isolates that were detected with disk diffusion method 11/19 (58%) of *E. coli* strains proved to be resistant to Quinolone and these results were consistent with Li *et al.* (2021). qnrA (36.84 %) was the greatly common antibiotic resistance gene in *E. coli* isolates, while blaSHV and blaCMY were not found in any *E. coli* isolates (Momtaz *et al.*, 2012). The incidence of *qnr* gene that confers resistance to Quinolone in *E. coli* isolated from broilers was (32.6%). The other gene, the plasmid-mediated quinolone resistance gene, *oqxAB* was found in 2/117 *E.coli* isolates from broilers by Ozaki *et al.* (2017).

Our results of detection of virulence gene *(ompT)* encoding gene verified that virulence genes were detected in 79% of the *E.coli* isolates. On the other hand, (iroN) virulence genes were not identified in any *E.coli* isolates, on the same context, *ompT* were the most prevalent virulence genes in the APEC-

like *E.col* isolated from broilers with a 100% frequency, while virulence genes iron were detected in 15.8% of the isolates (Li *et al.,* 2021). Outer-membrane protein A (OmpA), which is encoded by a plasmid, plays a key role in pathogenicity and has a substantial impact on bacterial illness therapy, with functions such as antiphagocytes, antialexin, and antiserum (Zhang *et al.,* 2003).

While the frequency of *the omp T* gene in APEC strains from poultry samples averaged from 78.6 to 94.1% the frequency of the *ompT* gene in APEC strains from poultry samples averaged from 78.6 to 94.1% (Jeong *et al.,* 2012; Ahmed *et al.,* 2013). In another study De Carli *et al.* (2015) reported a high frequency of virulence genes *iroN*, and *ompT, which* were 98% and 100% respectively among the *E. coli* strains that were isolated from broilers.

IroN and other iron acquisition genes were detected in 60 percent of pathogenic *E. coli* isolates from broilers (Xi *et al.,* 2016). The gene ompA is detected in 80 percent of LPEC isolates, and iron is vital in the innate immune system (Paauw *et al.,* 2009).

A total of 71.4 % of avian pathogenic E. coli strains identified from broilers suffering from septicemia in Egypt have five distinct virulence genes (Ahmed *et al.,* 2013). According to these studies, the occurrence of virulence genes varies depending on the isolation source and geographic origin of the samples. (Li *et al.,* 2021).

The present study concluded that *E.* coli identified from broiler chickens in Assiut were resistant at high rates to antibiotics with an average MDR index of 0.485 by the disk diffusion method. In E. coli strains from broiler chickens, genotypic resistance for *blaTEM, blaVIM* and *qnrA* resistance genes was discovered, and the genotypic pattern is nearly identical to the practical pattern of phenotypic resistance of *E. coli* isolates that were detected by disc diffusion method.

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التعرف الجزيئى لجينات الضراوه للميكروب القولونى الممرض (إشيرشا كولاى) المعزول من بدارى التسمين

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أجريت هذه الدراسة لعزل وتوصيف وجود الميكروب القولونى ودراسة جينات المقاومة للمضادات الحيوية والضراوه في دجاج التسمين في أسيوط. تم الحصول على 120 عينة (كبد، قلب، كيس صفار، رئة) من عمر 3 إلى 35 يوم سريرياً ولاحم نافق حديثاً يعاني من مظاهر تنفسية (CRD) وإلتهاب السرة وتسمم الدم والإسهال في محافظه أسيوط للكشف عن إي كولاي المسببة للأمراض. وتم تحديد العزلات على النمط الظاهري. تم عمل الأمصال والكشف عن حساسية المضادات الحيوية ومقاومتها. كما تم الكشف عن الجينات المسؤولة عن الضراوه (جينات ompA و iroN) ومقاومة مضادات الميكروبات على العينات. كما تم الكشف عن جينات المقاومة لمضادات الميكروبات (جينات blaTEM و blaVIM و qnrA). تم الكشف عن الميكروب القولونى والتعرف عليها داخل 31.7٪. وفقًا للبيانات ، تم تحديد 11 عزلة من أصل 38 عزلة من الميكروب القولونى باستخدام علم الاختبارات السيرولوجيه. تم استخدام طريقة انتشار القرص التقليدية لتقييم قابلية ومقاومة الميكروب القولونى المعزولة للعديد من العوامل المضادة للبكتيريا. 81.5 في المائة من العزلات لديها مؤشر MAR أكثر من 0.2 ، في حين أن 18.5 في المائة لها مؤشر MAR لا يزيد عن 0.2. بمتوسط مؤشر MDR يبلغ 0.485. تم اكتشاف الجينات المقاومة لمضادات الميكروبات في 73.7٪ من 19 جين الضراوه ومقاومة المضادات الحيوية المعترف بها مصليًا في عزلات الميكروب القولونى مثل جين ompA المكتشف بنسبة 95٪ ، اكتشاف جين blaTEM بنسبة 95٪ ، اكتشاف جين blaVIM في 73.7٪ ، واكتشاف جين qnrA في 31.5 في المئة ، ولكن لم يتم الكشف عن الجينiroN