Studies On Virulence Genes Of *E. coli* From Different Sources And Their Relation To Antibiotic Resistance Pattern

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ABSTRACT

In recent years, multidrug resistant virulent strains of *E. coli* are implicated in broiler cases of high mortalities causing significant economic losses in poultry industry. The current study aimed to determine the prevalence of *E. coli* isolates and to discuss the distribution of some virulence genes among multidrug resistance *E. coli* isolates. Bacteriological examination of 242 samples collected from different sources in Sharkia province, Egypt revealed a low recovery rate of *E. coli* isolates (20.2%). Serotyping of the representative *E. coli* isolates revealed 8 different serotypes with a dominance of O26:K60 and O114:K90 serotypes (20.4% for each one). Antimicrobial susceptiblility testing of *E. coli* isolates demonstrated that all the isolates were multidrug resistant and all *E. coli* isolates were resistant to more than 11 antimicrobial agents. Moreover, the highest resistance rates were recorded against trimethoprim sulfamethoxazole, amoxicillin clavulanic acid and erythromycin (100%), while the least resistance rate was detected against doxycycline (51.02%). With regard to the molecular detection of some virulence (*iss and pap*C) and resistance genes (*bla*TEM and *tet*B) on the extracted plasmid of 11 *E. coli* isolates were tested. Overall, these data indicated that the dissemination of resistance is associated with genetic mobile element such as plasmids that may also carry virulence determinants.

Key words: *E. coli*, broiler, virulence genes, MDR, plasmid.

INTRODUCTION

E. coli is one of the serious pathogen that can cause tremendous therapeutic problems. Pathogenic *E. coli* strains can be classified into intestinal (InPEC) and extra-intestinal (ExPEC) on the basis of their virulence factors and clinical symptoms *(1)*. Extra-intestinal *E. coli* infection is common in poultry farms causing colibacillosis which is the most frequently reported disease in surveys of poultry disease worldwide *(2)*. In the past few years, both the incidence and severity of colibacillosis have increased rapidly and current trends indicate that it is likely to continue and become a greater economic problem *(3)*.

Poultry, poultry by-products and water play an important role in the spread of *E. coli (4)*. Virulence traits among APEC have encouraged their zoonotic potential importance rendering ExPEC to cause disease in humans *(5)*.

ExPEC virulence potential is largely determined by the presence of specialized virulence factors such as fimbriae, adhesions, siderophores and invasions.These virulence factors help the microorganism to avoid or subvert host defenses, colonize key anatomical sites and/or incite a noxious host inflammatory response, therapy causing the disease *(6)*. It has been noted that the combination of these virulence factors will determine if a bacterium

can cause infection; the presence of a single factor rarely makes an organism virulent *(7)*.

Antibiotic therapy helps in reducing both incidence and mortality associated with avian colibacillosis. Unfortunately, unscrupulous use of antibiotics as food additives in modern poultry husbandry to prevent infections and promote growth results in the emergence of large numbers of drug resistance (**8**). Multidrug resistant *E. coli* can cause tremendous therapeutic problems in developing countries, where it is endemic is considered an alarming public health concern as a result of delays in appropriate therapy with subsequent increasing in morbidity and mortality *(7)*. Moreover, *E. coli* serves as reservoirs of resistance genes for potentially pathogenic bacteria and has been recognized as an increasing problem in the veterinary field *(9, 10)*.

Resistance to antimicrobial agents in *E. coli* may be due to a spontaneous mutation or could be acquired through transmission from other resistant *E. coli*. It is worthy of note that plasmids may contain resistance genes for single or multiple antimicrobial agents and they have been reported to transfer these resistance from one bacteria to another *(11, 12)*.

It is conceivable that virulence genetic determinants, if located on the same genetic platform as antimicrobial resistance genes (plasmid, transposons and integrons) may be co-mobilized under antimicrobial selection pressure. Furthermore, stable virulent clones or strains may be perpetuated if they acquire resistance determinants *(6)*.

In the last decades, the majority of virulence associated plasmids of *E. coli* possess transfer functions and often antimicrobial resistance determinants giving rise to the spread of antibiotic resistance among bacteria and plasmid facilitating the proliferation of resistance genes in bacteria *(13)*.

Hence, rapid identification of pathogenic *E. coli* strains and detection of their virulence and resistance genes allow the rapid diagnosis of pathogenic *E. coli* strains and understand their genetic and pathogenic relatedness *(14)*.

From the above mentioned, the current study was undertaken to determine the prevalence of *E. coli* from different sources in Sharkia province, Egypt, in addition to the detection of their virulence and resistance genes located on the plasmid. Finally, an effort was made to correlate between the presence of virulence and resistance genes on the plasmid of MDR *E. coli* isolates.

MATERIAL AND METHODS

Clinical specimens

Total of 242 samples were collected from different sources in Sharkia province, Egypt during all seasons from March 2011 to July 2013 [Summer (84), autumn (54), winter (38) and spring (66)]. These samples included 150 specimen from liver, heart and lung of broilers with a history of respiratory manifestations and postmortem lesions (pericardiatis, perihepatitis and air saculitis). The broilers were from different breeds; cub (20) , sasso (35) and hubbard (95). Additionally, 61 samples were collected from burger (28) and lunchon (33) from different supermarkets and another 31 samples were collected from different water tanks of broiler farms. All the samples were transported immediately in an ice box for further bacteriological examination.

Bacteriological analysis

The collected samples were placed overnight in buffered peptone water, then cultivated on macConkey's agar and the lactose fermenting colonies were reinoculated onto EMB agar. Further identification of *E. coli* isolates were applied based on microscopical examination and biochemical activities (indole, citrate and urease tests) and on TSI agar medium *(15)*. Furthermore, *E. coli* isolates were biotyped using API 20E identification kit *(16)*. Identified *E. coli* isolates were serotyped by commercially available kits using polyvalent and monovalent antisera O and K (Test Sera

Enteroclon, Anti –Coli, SIFIN Berlin, Germany). All the isolates were stored in brain heart infusion broth with 30% glycerol at –70°C until required.

Antimicrobial susceptibility testing

Antibiotic susceptibility test was performed utilizing Mueller Hinton agar medium and antibiotic discs of 12 commonly antimicrobial agents including amoxicillin-clavulanic acid (20-10 µg), ceftriaxone (30 µg), chloramphenicol (30 µg), ciprofloxacin (5 µg), colistin (15 µg), doxycyclin (30 µg), gentamicin (10 µg), tetracycline (30 µg), trimethoprime- sulfamethoxazole (1.25-23.75 µg), streptomycin (10), erythromycin (15 µg) and rifamycin (15 µg) according to method mention previously *(17)*. It was applied by disc diffusion assay and the diameter of the inhibition zones were measured and interpreted according to CLSI criteria *(18)*.

Plasmid extraction

Plasmid DNA was isolated as described by QIAamp DNA Mini Kit (Catalogue No.51304).

PCR amplification of some virulence and resistance genes from different sources

Eleven MDR *E. coli* isolates were randomly selected from different sources were tested for the presence of some virulence and antibiotic resistance genes. Two primer sets were used for amplification of *iss* and *pap*C virulence genes and another two primers sets were used for ampilification of *tet*B and *bla*TEM antibiotic resistance genes in these *E. coli* isolates in a Biometra T3 thermal cycler. The primer squences of these genes are shown in Table (1). The PCR reaction was performed in a total reaction volume of 25 µl consisting of 12.5 µl of Emerald Amp GT PCR mastermix (Takara) Code No. RR310A kit, 0.25 µl of each primer (100 pmol), 2 µl of the DNA template and water nuclease-free up to 25 µl. The amplification cycling conditions are tabulated in Table (2).

Agarose gel electrophoresis

The amplified products were stained with 0.5 µg/ml ethidium bromide subsequently separated by electrophoresis on 1.5% molecular biology grade agarose gel, along with a 100-bp molecular weight DNA ladder (Applichem, Germany, GmbH).DNA bands were visualized and photographed under an ultraviolet transilluminator (Spectroline Model TC- 312 A, USA).

Table 1. Oligonucleotide primer sequences of some virulence and antibiotic resistance genes in *E. coli* isolates

Target Gene	Specificity	Primer	Oligonucleotide sequence 5´-3´	References
tet B	Tetracycline resistance	TETB Cs TETB as	F:CCTTATCATGCCAGTCTTGC R:ACTGCCGTTTTTTCGCC	(10)
*bla*TEM	Amoxicillin+clavulanic resistance	BLA Cs BLA as	F:ATCAGCAATAAACCAGC R:CCCCGAAGAACGTTTTC	(19)
papC	Adhesin	PAPCs PAPCas	F:TGATATCACGCAGTCAGTAGC R:CCGGCCATATTCACATAA	(20)
iss	Various	ISS Cs ISS as	F:ATGTTATTTTCTGCCGCTCTG R:CTATTGTGAGCAATATACCC	(21)

Table 2. Cycling conditions and predicted sizes of PCR products for virulence and antibiotic resistance genes.

Target gene	Initial denaturation °C/min	Actual cycles (35) °C/sec			Final extention °C/min	Amplified product Size (bp)
		Denaturation	Annealing	Extension		
*tet*B	94/5	94/60	50/60	72/60	72/10	773
*bla*TEM	94/5	94/45	54/45	72/45		516
*pap*C	94/5	94/45	59/45	72/45		501
Iss	94/5	94/30	54/30	72/30		255

RESULTS

Incidence of *E. coli* isolates from different localities of Sharkia province

The incidence of *E. coli* isolates among different samples in all seasons is presented in Table3, out of 242 specimens collected from different sources in Sharkia province, 49 *E. coli* isolates (20.2%) were recovered with a high recovery rate of *E. coli* from samples collected from broiler organs (22.7%). Furthermore, *E. coli* isolates were mostly recovered during summer season (33.3%). Regarding the prevalence of *E. coli* from different breeds, sasso breed revealed a higher incidence rate of *E. coli* isolates (25.7%).

Phenotypic characterization of *E. coli* isolates

E. coli isolates gave pink colonies (lactose fermenter) onto macConkey's agar medium. Subculturing onto EMB agar medium revealed characteristic greenish metallic sheen colonies. All *E. coli* isolates were Gram negative, medium sized bacilli. They were also indole test positive (red ring), citrate test negative (green colour), urease test negative and gave yellow slant and butt with gas formation and no H_2S production on TSI agar medium. Furthermore, API 20 E protocol carried out on 11 randomly selected *E. coli* isolates revealed the profile number 7144572 as the most prevalent one as it was referred to 6 isolates (55.5%) (Figure1).

Serotyping of *E. coli* isolates

Serogroup analysis of all *E. coli* isolates revealed eight different serotypes. The most predominant ones are O114:K90 and O26:K60 with a percentage of 20.4% for each one (Table4). Unfortunately, two *E. coli* isolates could not be typed by serotyping kits.

Results of antimicrobial susceptibility testing of *E. coli* isolates

All *E. coli* isolates showed the highest resistance rate (100%) against sulfamethoxazole trimethoprim, amoxicillin clavulanic acid and erythromycin, while the least resistance rate was detected against doxycycline (51.02%) (Table 6). Moreover, it was revealed that all *E. coli* isolates from different sources were multidrug resistant to 11 antimicrobials as shown in Table 4.

PCR detection of some virulence and antibiotic resistance genes among

E. coli isolates

Uniplex PCR revealed that all *E. coli* isolates were positive for *iss* gene (100%) which had a vital role in *E. coli* pathogenicity. It gave a characteristic band at 255bp (Figure 2A).

Furthermore, *pap*C gene which is involved in adhesion of pathogenic *E. coli* to the host cells was represented in most *E. coli* isolates (81.8%) with an amplican size of 501 bp (Figure 2B).

Additionally, it was revealed that *bla*TEM gene was the most prevalent resistance genes being recovered in all tested *E. coli* isolates (100%). Meanwhile *tet*B gene was the least detected antibiotic resistance gene among the examined isolates (45.5%). Amplifications of *bla*TEM and *tet*B genes produced PCR products at 516 and 773 bp, respectively (Figures 2C and 2D, respectively).

An obvious finding in this study is that, the results of phenotypic resistant pattern were in parallel with the results of PCR for detection of antibiotic resistance genes as isolates that showed resistance against certain antibiotic by disc diffusion were confirmed to have the relevant antibiotic resistance genes by PCR .

Table 3. Incidence of *E. coli* isolates among different samples in all year seasons

Samples source / Season	No. of *E. coli* isolates (%)						
	Broiler organs (150)			Broiler by-products (61)		Water (31)	Total (242)
	Sasso (35)	Cub (20)	Hubbard (95)	Lunchon (33)	Burger (28)		
Winter (38)	2	0	0	1	0	1	4 (10.5%)
Summer (84)	6	1	15	2	2	2	28 (33.3%)
Autumn (54)	1	0	3	1	1	2	8 (14.8%)
Spring (66)	0	3	3	0	2	1	9 (13.6%)
Total (242)	9 (25.7%)	4 (20%)	21 (22.1%)	4 (12.1%)	5 (17.9%)	6 (19.4%)	49 (20.2%)

Table 4. Percentages of different serotypes of 49 *E. coli* isolates among different collected samples

E. coli serotype	No. of *E. coli* isolates (%)	Isolate code No.
O26:K60	10 (20.4%)	56 Bo , 32Ln , 25W,4B,18W, 2Ln,149Bo,134Bo,140Bo,34Bo
O114:K90	10 (20.4%)	58Bo, 103Bo,77Bo,20W,21Bo, 66Bo,25Bo,26Bo,63Bo,28Bo
O111:K58	6 (12.2%)	129Bo, 87Bo,78Bo,18Bo,19Bo,26Ln
O78:K80	6 (12.2%)	137Bo, 88Bo, 122Bo, 5Bo, 15Bo,45Bo
O44:K74	4 (8.2%)	111Bo ,20B,27B,102Bo
O125:K70	4 (8.2%)	136Bo , 144Bo,10B,24Bo
O55:K59	4 (8.2%)	69Bo ,11W,9W,28Ln
O127:K63	3 (6.1%)	124Bo, 15B,10Bo
Non typeable	2 (4%)	123Bo,6W

Bo: Broiler organs B: Burger Ln:Lunchon W:Water

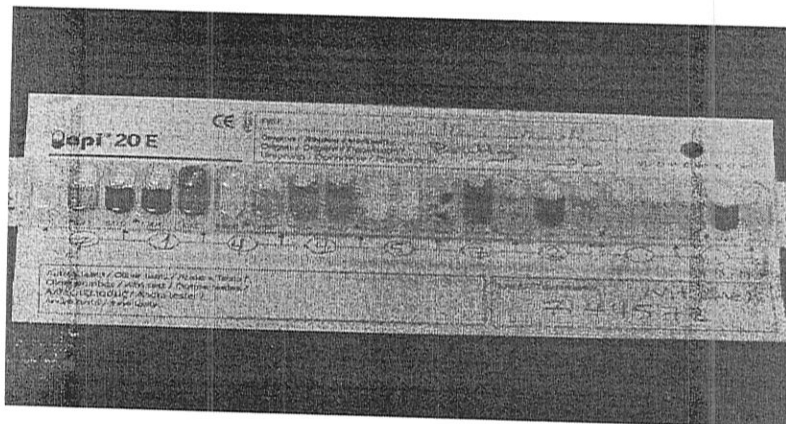
Table 5. Antibiotic Resistance profile of each antimicrobial agent aganist *E. coli* isolates from different sources

Resistant profile of AMA / Isolates source	AMC	CIP	SXT	CRO	S	TE	DO	C	E	CT	RF	CN
Broiler organs (34)	34	20	34	28	34	34	18	32	34	29	34	27
Lunchon (4)	4	3	4	3	4	3	2	3	4	3	4	3
Burger (5)	5	3	5	4	5	5	3	4	5	4	4	4
Water tank (6)	6	4	6	6	5	6	2	5	6	5	6	3
Total 49 (%)	49 (100%)	30 (61.2%)	49 (100%)	41 (83.67%)	48 (97.96%)	48 (97.96%)	25 (51.02%)	44 (89.49%)	49 (100%)	41 (83.67%)	48 (97.96%)	37 (75.51%)

AMA: Antimicrobial agent, AMC: Amoxicillin+clavulanic acid, CIP: Ciprofloxacin, SXT: Sulfamethoxazole + trimethoprim, CRO: Ceftriaxone, S: Streptomycin, TE: Tetracycline, DO: Doxycycline, C: Cholorumphenicole, E: Erythromycine, CT: Colistin, RF: Rifampicin, CN: Gentamicin.

Table (6) Antibiotic Resistance pattern of *E. coli* isolates from different sources

No. of antibiotics to which the isolates were resistant \ Isolates source	Broiler organs (34)	Lunchon (4)	Burger (5)	Water tanks (6)	Total 49 (%)
12	7	0	1	0	8 (16.3%)
11	12	3	0	2	17 (34.7%)
10	10	0	3	2	15 (30.6%)
9	3	0	1	2	6 (12.2%)
8	1	0	0	0	1 (2%)
7	1	1	0	0	2 (4%)
Less than 7	0	0	0	0	0 (0%)



1	2	4	1	2	4	1	2	4	1	2	4	1	2	4	1	2	4	1	2
ONPG	ADH	LDC	ODC	CIT	H2S	URE	TDA	IND	VP	GEL	GLU	MAN	INO	SOR	RHA	SAC	MEL	AMY	ARA
+	+	+	+	-	-	-	-	+	-	-	+	+	-	+	+	+	+	-	+
	7			1			4			4			5			7			2

Fig.1. Biotyping result of an isolate of *E. coli* using API 20E kits showing very good *E. coli* identification (Seven-digit profile number: 7144572, id %: 99.8, Tindex=0.63)

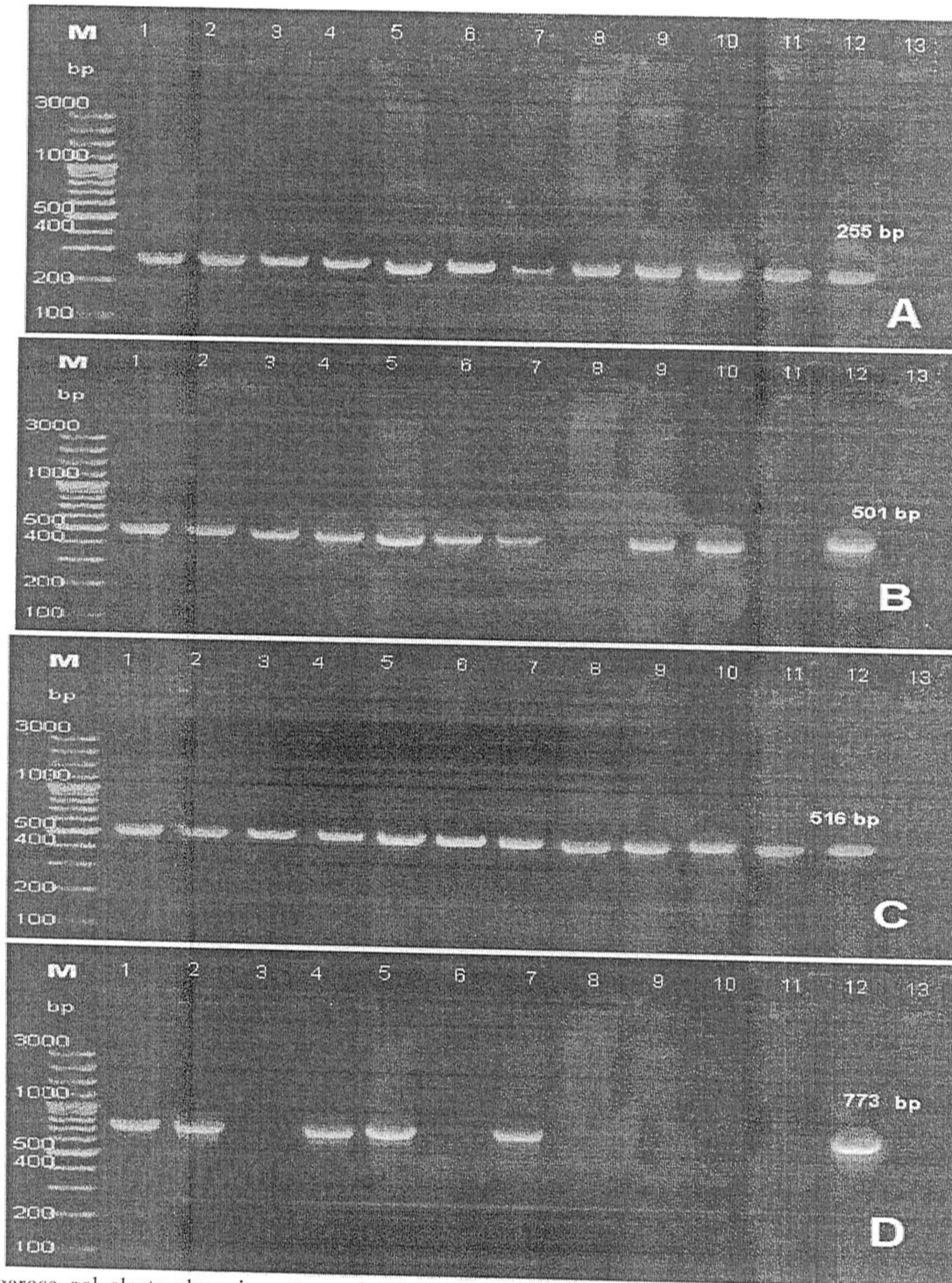


Fig.2. Agarose gel electrophoresis patterns showing typical amplification products sources for *iss* **(A)**, *pap*C **(B)**, *bla*TEM **(C)** and *tet*B **(D)** genes on extracted plasmid from 11 *E. coli* isolates from different samples (lanes 1- 8, broiler organs with code no. 111Bo,123Bo,122Bo,124Bo,5Bo,69Bo,87Bo and 58Bo; lanes 9, burger samples with code no. 10B; lanes 10, Lunchon samples with code no. 26Ln; lane 11, water sample with code no. 6W) ; Lane M, DNA molecular size marker (100-bp); Lane 12,positive control; Lane 13: negative control.

Fig 2A lanes 1-11, *E. coli* isolates showing *iss* positive PCR bands.

Fig 2B lanes 1-7, 9 and 10 showing *pap*C positive PCR bands.

Fig 2C lanes 1-11, *E. coli* isolates showing *bla*TEM positive PCR bands.

Fig 2D lanes 1, 2,4,5,7 showing *tet*B positive PCR bands.

Amplification products and DNA fragment sizes are marked in base pairs (bp) on the right of the bands.

DISCUSSION

E. coli has been recognized as a major pathogen in poultry industry worldwide. The majority of virulence associated plasmids in *E. coli* which have transfer functions and often antimicrobial resistance determinants giving rise to the spread of antibiotic resistance among *E. coli* ***(13)***.

Therefore, the present study aimed to detect the prevalence of *E. coli* isolates from different sources in Sharkia province, Egypt to determine the correlations between the presence of virulence genes and resistance genes on the plasmid of some *E. coli* isolates. In the present study, 49 *E. coli* isolates were isolated from 242 collected samples from different sources (20.2%).The prevalence of *E. coli* isolates differed among countries; (23%) in Canada ***(22)***, and (89%) in the West of Romania ***(23)***. Regarding the prevalence of *E. coli* isolates from broiler organs, broiler by-products and water, it was isolated with a lower prevalence rates (22.7%, 14.8% and 19.4%, respectively) than those recorded in Cairo, Spain and El-sharkia (80%, 80% and 63.75%) ***(1, 24, 25)***. Moreover, the prevalence of *E. coli* isolates was detected in sasso breed (25.7%) while the prevalence rate during summer season (33.3%) which is lower than that in El-sharkia and El-dakahlia provinces isolated from sasso bread (66.67%) while high prevalence during summer season (76.1%) ***(26)***. Results of conventional methods for isolation and identification of *E. coli* was consistent with several authors ***(27)***.Moreover, API 20E protocol confirmed the identification of all selected *E. coli* isolates as was commonly reported ***(28, 29)***. Serotyping of 49 *E. coli* isolates revealed 8 different serogroups with O26:K60 and O114:K90 as the most prevalent serotypes (20.4% for each one). This finding is exactly in conformity with that recorded in Egypt ***(1)***, where detected a higher prevalence rate of O26 and O114 serotype were also recorded. Other serotypes were also detected in the present study (O125:K70, O44:K74, O55:K59, O127:K63, O111:K58 and O78:K80). Several researchers detected at least one of the obtained serotypes in this work ***(8, 30, 31)***.

In this research, disc diffusion test of *E. coli* isolates from different sources showed higher resistance rates against amoxicillin/ clavulanic acid, erythromycin and trimethoprim sulfamethoxazole (100% for each), followed by tetracycline, streptomycin and rifampicin (97.96%) for each. The resistance of *E. coli* isolates to these drugs is due to their regular usage in poultry industry for control of pathogenic avain colibacillosis ***(8)***.

Several investigators documented higher resistance rates of *E. coli* isolates against tetracycline (96.43%) in China ***(32)***; erythromycin (94.19%) in Bangalore ***(8)*** and streptomycin (84.2%) in Korea ***(13)***. On the other hand, several researches recorded lower resistant rates of *E. coli* isolates against different antibiotics in Bangladesh than those in the current research (45.5, 26.7 and 20.8%) against tetracycline, trimethoprim-sulphamethoxazole and streptomycin, respectively ***(33)***. In the current study, all 49 tested *E. coli* isolates from different sources showed multidrug resistance and 34.7% of them showed multi drug resistance against 11 drugs only. MDR *E. coli* isolates were highly detected by several researches in Swiss (87.5%) ***(34)***.Our findings showed that there is an emerging drug resistance problem in APEC associated with colibacillosis in Egypt. The observed high level of multidrug resistance could hamper the treatment of colibacillosis ***(35)***.

In the present study, plasmids extracted from 11 multidrug resistant *E. coli* strains were examined by uniplex PCR for the presence of two virulence genes (*iss* and *pap*C) that play an important role in the pathogenicity of *E. coli* and two resistance genes (*tet*B and *bla*TEM) that are responsible for resistance against tetracycline and amoxicillin- clavulinic acid antibiotics, respectively. During this study, with regard to the prevalence of both virulence genes among *E. coli* isolates, it was recorded that all *E. coli* isolates were positive for *iss* gene (100%).This result is consistent with a previous report in Germany, where *iss* virulence gene was detected in APEC with a higher percentage (95.5%) ***(36)*** suggesting that this gene has a

vital role in *E. coli* pathogenicity and could be a potential target for developing novel therapeutics and prevention strategies. Moreover, *pap*C gene, the main functional gene of P pilus, is involved in adhesion of pathogenic *E. coli* to the host cells. The prevalence of *pap*C positive *E. coli* isolates detected in this study (81.8%) was much higher than that observed in Germany (30%) *(37)*. PRC amplifications of *iss* and *pap*C genes yielded characteristic bands at 255 and 501bp as was previously reported *(20, 38)*.

Furthermore, the results of antibiotic disc diffusion test actually agreed with the results of uniplex PCR for detection of the relevant antibiotic resistance genes. *tet*B gene was detected in 45.5% of *E. coli* isolates. This finding was reported by other investigators who stated that 42% of *E. coli* isolated form poultry possessed *tet*B resistance gene *(39)*. Amplification of this gene yielded a characteristic band at 773 bp as was reported in Grenada *(10)*. Additionally, PCR amplification *bla*TEM gene in this study yielded an amplied product at 516 bp in the represented *E. coli* isolates (100%). This result is consistent with the literature that also documented a higher prevalence carriage of this gene in *E. coli* isolates from poultry (88.2%) *(19)*.

In conclusion, plasmid carry virulence and resistance genes and transmission of resistant clones and resistance plasmids of *E. coli* from food animals (especially poultry) to human can occur, thus introduction of surveillance programs to monitor antimicrobial resistance in pathogenic *E. coli* is strongly needed in developing countries *(1)*.

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الملخص العربي

دراسات عن جينات الضراوة و علاقتها بنمط المقاومة للمضادات الحيوية فى ميكروب الاشيرشيا كولاى المعزول من مصادر مختلفة

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تعتبر الاشيريشيا كولاى الضاريه و المقاومة للمضادت الحيوية هى المسببه لنفوق الدواجن مع الخسائر اقتصاديه كبيرة، لذا تهدف هذه الدراسه لتحديد مدى انتشار الاشيريشيا كولاى و مناقشه توزيع جينات الضراوة عبر الاشيريشيا كولاى المقاوم للمضادات الحيويه، فى هذه الدراسه تم عزل ٢٠,٢% من ٢٤٢ عينه من دجاج محافظة الشرقيه-مصر، اظهر التصنيف السيرولوجى ٨ انواع سيرولوجيه مختلفة من الميكروب و كان السائد فيها O26:K60 وO114:K90 بنسبه ٢٠,٤% لكل واحد ، اختبارات الحساسه للمضادات الحيويه و ان كل العترات المعزوله كانت مقاومة لاكثر من ١١ نوع من المضادات الحيويه مع العلم بأن كل العزلات كانت مقاومة تماما سلفاميثوكسازول تراى ميثوبريم – اموكسسلين/كلافولينك اسيد – الاريثروميسين و اتضح ان ٥١,٠٢% من العزلات كان مقاوما للدوكسي سيكلين، فيما يخص التحديد الجزيئى لجينات الضراوة *iss* و *papC* وجينات المقاومة *bla*TEM و *tet*B على البلازميدات تم اختبارهم لـ ١١ عزله ، ومن السابق اتضح انتشار المقاومة للمضادات كان مرتبطا بجينات مركبه مع جينات الضراوة على البلازميدات.