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STUDIES ON ENTEROHAEMORRHAGIC *ESCHERICHIA COLI* (EHCE) STRAINS NON O157:H7 IN CHICKEN WITH REGARD TO ANTIBIOTIC RESISTANCE GENE ON PLASMID

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ABSTRACT

Shiga-toxin-producing *Escherichia coli* (STEC) are the most important recently emerged group of food-borne pathogens. Among fifty EHEC strains, thirty strains were found as non O157 EHEC (60%) by serotyping. Also the highest incidence of O26 and O111 were 16% and 14%, respectively, meanwhile the lowest was of O128 (2%). Antibiotic resistance profiles for these strains showed 100% resistance to: sulfamethoxazole, Trimethoprime, chlormphencal, colistin, gentamycin and tetracycline. However, they showed 85% resistance to streptomycin and doxycycline, 80% resistance to cefotraxone while their resistance for amoxicillin clavulanic acid was 75%. PCR examination for twenty strains of EHEC non O157 revealed *stx*1 gene in 45%, *stx*2 in 65% while *hly* in 80% of the examined strains. Antimicrobial resistance genes for these strains confirmed that *sul*I, *aad*A and *bla*TEM resistance genes were detected in percentages of 85%, 75% and 60%, respectively. Recommendation for minimizing an excessive use of antibiotics in the veterinary field and periodically use of different antibiotics could overcome the problem of increased bacterial resistance.

Key words: *EHEC, non O157, resistance, genes, virulence, antibiotic, serotyping.*

INTRODUCTION

Chicken meat is one of the most popular foods among population. However, the epidemiology and prevalence of EHEC strains in chicken meat is essentially unknown. Enterohemorrhagic *Escherichia coli* (EHEC) is considered recently as one of important emerged groups of food-borne pathogens. Although *E. coli* O157:H7 is currently the most common EHEC strains in many regions of the world (Armstrong *et al*., 1996) but serotypes O103, O26, O104,O111, O 113,O 128, O5 and O145 (which are now usually referred to as non-O157 EHEC) constituted also a serious threat to public health (Bettelheim,1996). They characterized by their specific virulence factors and its ability to produce potent cytotoxins (verotoxins). All strains produce hemolysin (which is encoded by *hly*A) (Schmidt *et al*., 1994) and at least one Shiga-like toxin (encoded by *stx*1 or *stx*2) (O'Brien *et al*., 1987). Shiga – like toxins have similar biological activities including cytotoxicity to Vero and HeLa cells, but they are different immunologically (Law, 2000).

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Antimicrobials are routinely used for disease prevention and growth promotion in poultry production however, they are not recommended for treating EHEC O157:H7 infection because antimicrobials may lyse bacterial cell walls, thereby liberating Shiga toxins (Wong *et al*., 2000 and Hedican *et al*., 2009), and/or cause increased expression of Shiga toxin genes in vivo (Zhang *et al.*, 2000).

Although EHEC infections are not aggressively treated with antimicrobial therapy and many isolates are susceptible to numerous antimicrobials, recent reports indicate that antimicrobial resistance of EHEC is on the rise (Farina *et al*., 1996; Galland *et al*. 2001 and Schroeder *et al*., 2002).Moreover, it is proven that repeated sublethal exposure to antibacterial agents not only promotes adaptative resistance but also confers decreased sensitivity to antibiotics (Braoudaki and Hilton, 2003). This practice leads to a selection of antimicrobial resistance among commensals in the intestinal tracts of chicken, which poses a public health threat (Witte, 1998).

Ideally, PCR-based detection methods are rapid and sensitive without need for extensive sample preparations. Large plasmids resembling carried the genetic information for most non-O157 EHEC strains which is responsible for the so-called enterohaemolytic phenotype. (Schmidt *et al*., 1995).

Due to the fact of definite zoonotic origin and pathogenic STEC/EHEC group. This paper will review the recentfinding on the most virulence factors of EHEC, trying to seek out what makes a STEC, an EHEC highly pathogenic to poultry production and evaluation of antibiotic resistance properties in EHEC serogroups isolated from chicken meat (Momtaz and Jamshidi, 2013).

MATERIALS AND METHODS

1. Bacterial strains

A total of 50 EHEC strains that were recovered from chicken and were collected from different sources in Sharkia province, Egypt. The majority of strains included in the present study had been obtained in previously published studies (Blanco *et al*., 2001; Blanco *et al.*, 2003 and Blanco *et al*., 2004) and the procedures for their isolation were described in detail in those studies.

2. Serotyping

EHEC strains were serotyped by slide agglutination test with commercially available polyvalent and monovalent anti E coli O and K sera (Test Sera Enteroclon, Anti –Coli, SIFIN Berlin, Germany).

3. Antimicrobial susceptibility testing

The susceptibility of identified EHEC strains to a panel of ten commonly used antimicrobial agents was performed by the standard Kirby–Bauer disc diffusion method (Bauer *et al*., 1966) and the results were interpreted according to the criteria recommended by the Clinical and Laboratory Standards Institute for antimicrobial susceptibility testing (CLSI, 2011) Isolates resistant to three or more antibiotics were classified as MDR strains.

4. Molecular detection of antibiotic resistance and virulence genes

Plasmid DNA from 10 MDR EHEC strains was isolated using QIAprep Spin Miniprep Kit (QIAGEN GmbH, Hilden, Germany). Screening for the presence of some antibiotic resistance and virulence plasmid-associated genes was carried out by PCR amplifications using specific primers and different cycling conditions as previously described by (Clark *et al.*, 1999; Colom *et al*., 2003; Ewers *et al.*, 2007 and Santos *et al*., 2014). The PCR products were tested for positive amplification by agarose gel electrophoresis (Sambrook *et al*., 1989). For each PCR experiment, appropriate positive and negative controls were included. The primer sequence from Metabion (Germany) of virulent and resistance genes and the amplification cycling conditions are listed in tables (1 & 2).

Table1: Oligonucleotide primer sequences of virulence and antibiotic resistance genes of *E.coli*.

Target gene		Primer sequence (5'-3')	References
***Stx*1**	F	CAGTTAATGTGGTGGCGAAG	**Sahilah *et al*., 2010**
	R	CTGTCACAGTAACAACCGT	
***Stx*2**	F	CCATGACAACGGACAGCAGTT	**Dipineto *et al*., 2006**
	R	CCTGTCAACTGAGCAGCACTTTG	
hly	F	AACAAGGATAAGCACTGTTCTGGCT	**Piva *et al*., 2003**
	R	ACCATATAAGCGGTCATTCCCGTCA	
***sul*I**	F	CGG CGT GGG CTA CCT GAA CG	**Kerrn *et al*., 2002**
	R	GCC GAT CGC GTG AAG TTC CG	
***aad*A1**	F	TGATTTGCTGGTTACGGTGAC	**Clark *et al*., 1999**
	R	CGCTATGTTCTCTTGCTTTTG	
***bla*TEM**	F	ATCAGCAATAAACCAGC	**Colom *et al*., 2003**
	R	CCCCGAAGAACGTTTTC	

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

Target gene	Initial denaturation °C/min	Cycle	Actual cycles °C/sec			Final extention °C/min	Amplified product Size (bp)
			Denaturation	Annealing	Extension		
***Stx*1**	94/7	94	94/30	51/30	72/30	72/7	180
***Stx*2**	94/10	94	94/60	58/60	72/60	72/10	779
hly	94/15	94	94/60	60/60	72/90	72/12	1177
***sul*I**	94/5	30	94/15	69/30	72/60	72/7	443
***aad*A1**	95/10	30	94/30	60/30	72/30	72/10	284
***bla*TEM**	94/3	32	94/30	54/30	72/60	72/10	516

RESULTS

This study was done on fifty chicken *E.coli* strains isolated from avian origin as shown in Table 3, thirty five (70%) strains were confirmed to be EHEC positive, also there were significant differences between the presence of attaching and effacing *E.coli* EPEC and EHEC subgroups in strains. Among the 35 EHEC included in this study, 5 (10%) strains were serotyped as O157:H7 and 30 (60%) non-O157. Non-O157 EHEC strains were belonged to 8 different O:H serotypes (O26:H-, O111:H-,O 103:H3,O104, O145,O5,O113,O128). Based on the results, O26 (16%) and O111(14%) were of high incidence ratio among serogroups whereas O128 had the lowest incidence in EHEC strains 2%.

Table 4 shows the distribution of virulence factors of non-O157EHEC strains. All of the EHEC-positive samples had *stx*1, *stx*2, and *hly* virulence genes with significant differences as shown in Table 5.

The antibiotic resistance profiles of the thirty non-O157 strains were significantly different with respect to the levels of resistance of *E. coli* to the tested antibiotics. Non-O157 EHEC serogroups demonstrated high rates (100%) for sulfamethoxazole, trimethoprim, chlorumpheneol, colistin, gentamycin and tetracycline while 85% for streptomycin, doxycycline, 80% for cefotraxone and 75% for amoxicillin clavulanic acid.

Phenotypic resistance of non-O157 to amoxicillin-clavulanic acid, sulfamethoxazole- trimethoprim and streptomycin antibiotics could be explained by the presence of *bla*TEM, *sul*I and *aad*A resistance genes among the tested strains (60%,85% and 75%), respectively.

The frequencies and combinations of virulence genes carried on plasmid (*stx*1, *stx*2 and *hly*) of twenty non-O157 EHEC strains were assessed. Among the detected virulence genes, *stx*1 and *stx*2 were the most prevalent gene (45% and 65%), followed by *hly*gene (80%) fig. (1).

Finally, characterization of the selected non-O157 EHEC according to their serotypes, virulence potential and corresponding antibiotic resistance pattern were shown in table (5). It was demonstrated that MDR *E. Coli* serovars possessed at least two virulence genes accompanied by attendance of antibiotic resistance genes. The co-occurrence of these concerning trends confirmed that the acquisition of antimicrobial resistance by E. coli has been accompanied by increased virulence.

Table 3: Prevalence of detected serotypes based on total number of *E.coli* isolates.

Types of *E.coli* strains	Serotypes	% No. of strains	
EPEC	O2:H6	12%(6)	24%(12)
	O1:H7	8%(4)	
	O127:H6	4%(2)	
EHEC	O26:H11	16%(8)	60%(30)
EHEC	O111:H4	14%(7)	
	O103:H2	6%(3)	
	O104	8%(4)	
	O 145	6%(3)	
	O5	4%(2)	
	O 113	4%(2)	
	O128	2%(1)	
	O157:H7	10%(5)	
Untypable		6%(3)	
Total		100%(50)	

Table 4: The prevalence of virulence genes and antibiotic resistance genes among examained EHEC**.**

Genes		% No. of isolate strains
Virulence genes	*Stx*1	45%(9/20)
	*Stx*2	65%(13/20)
	Hly	80%(16/20)
Resistance genes	*Sul* I	85%(17/20)
	*aad*A1	75%(15/20)
	*bla*TEM	60%(20/20)

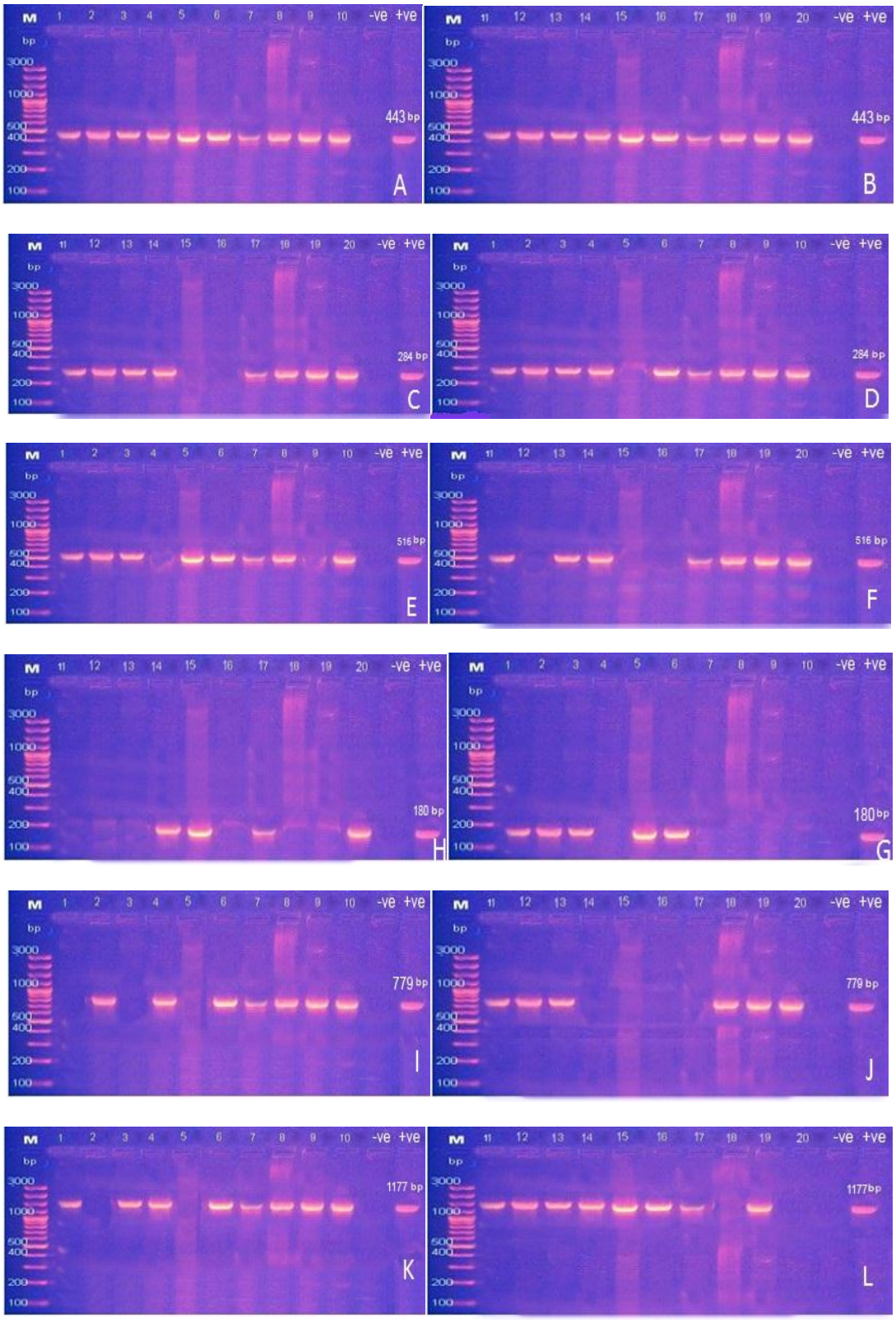

Fig. (1): Shows agarose gel electrophoresis of PCR amplified products of (A,B): *sul*I, (C,D):*aad*A1, (E,F) *bla*TEM resistant genes, (G,H):*stx*1,(I,J):*stx*2 and (K,L): *hly* virulence genes. Lane M: DNA molecular size marker (100 bp), lanes 1-20: *E.coli* isolates, lane (+ve): positive control and lane (-ve): negative control. The size in base pairs (bp) of each PCR product is indicated on the right of the bands

Table 5: Resistance profiles and genotypic characterization of some virulence and antibiotic resistance genes of EHEC strains on their plasmid.

ID	*E. coli* serotype	Resistance profile	Genotypic characterization					
			Virulence genes			Antibiotic resistance genes		
			Stx1	Stx2	hly	*sulI*	*aadA*1	*bla*TEM
1	O 26	SXT,CIP,AMC,CRO,S,TE,DO,C, CT, CN	+	-	+	+	+	+
2	O111	SXT,CIP,AMC,CRO,S,TE,DO,C, CT, CN	+	+	-	+	+	+
3	O103	SXT,CIP,AMC,S,TE,DO,C, CT, CN	+	-	+	+	+	+
4	O145	SXT,CIP, CRO,S,TE,DO,C, CT, CN	-	+	+	+	+	-
5	O111	SXT,CIP,AMC, TE,DO,C, CT, CN	+	+	-	+	-	+
6	O103	SXT,CIP,AMC,CRO,S,TE,DO,C, CT, CN	+	-	+	+	+	+
7	O26	SXT,CIP,AMC,CRO,S,TE,C, CT, CN	-	+	+	+	+	+
8	O145	SXT,CIP,AMC,CRO,S,TE,C, CT, CN	-	+	+	+	+	+
9	O26	SXT,CIP,CRO,S,TE,DO,C, CT, CN	-	+	+	+	+	-
10	O104	SXT,CIP,AMC,CRO,S,TE,DO,C, CT, CN	-	+	+	+	+	+
11	O5	SXT,CIP,AMC,S,TE,DO,C, CT, CN	-	+	+	+	+	+
12	O26	SXT,CIP,CRO,S,TE,DO,C, CT, CN	-	+	+	+	+	-
13	O113	SXT,CIP,AMC,CRO,S,TE,DO,C, CT, CN	-	+	+	+	+	+
14	O111	SXT,CIP,AMC,CRO,S,TE,C, CT, CN	+	-	+	+	+	+
15	O5	SXT,CIP, CRO, TE,DO,C, CT, CN	+	-	+	+	-	-
16	O26	SXT,CIP, CRO, TE,DO,C, CT, CN	-	-	+	+	-	-
17	O104	SXT,CIP,AMC,S,TE,DO,C, CT, CN	+	-	+	+	+	+
18	O111	SXT,CIP,AMC,CRO,S,TE,DO,C, CT, CN	-	+	-	+	+	+
19	O26	SXT,AMC,CRO,S,TE,DO,C, CT, CN	-	+	+	+	+	+
20	O128	SXT,AMC,CRO,S,TE,DO,C, CT, CN	+	+	-	+	+	+

DISCUSSION

E.coli strains that cause entric infections are generally called diarrheagenic *E.coli* strains, and their pathogenesis is associated with a number of virulence attributes which vary according to pathotypes (Vidal *et al*., 2005). Currently, Diarrheagenic *E.coli* strains are classified into 6 pathotypes based on their distinct virulence determinants and pathogenic features, including Enterohemorrhagic *E.coli* (EHEC) that produceshiga-like toxins (Xia *et al*., 2010).

In this recent investigation, high recovered rate of EHEC strains (70%) was recorded. Similar results were reported by (Lyhs *et al*., 2012; Eid and Erfan, 2013 and Peer *et al*., 2013) where *E.coli* was isolated in percentage of 94.5%, 80% and 84%, respectively. Serotyping of EHEC strains confirmed the identification of O157 (10%) and non O157 serotypes (60%). The non-O157 STEC serotypes were: O26 (16%), O103 (6%), O111 (14%), O145 (6%), O5(4%), O113(4%), O128(2%) and O104 (8%). This result was in agreement with that recorded by (Hedican *et al*., 2009). However, another study in Korea showed that (41 / 900) from poultry samples were *E. coli* positive and non O157 serogroups were included in a percentage of 4.6% (Lee *et al*., 2009).

Because of the high indiscriminate use of antibiotics, especially in veterinary medicine, antibiotic resistance against most effective antibiotics was recorded. Several recent studies have documented antibiotic resistance especially among non-O157 EHEC (Farina *et al*., 1996; Horii *et al.*, 1998; Galland *et al*., 2001 and Khan *et al*., 2002). This study is matching with our study which recorded high levels of resistance for some antimicrobial agents (sulfamethoxazole- trimethoprim, cholorumphenicol, colistin, gentamycin, tetracycline). Likewise, higher resistance rates have been reported for avian *E. coli* isolates to tetracycline (100%) in Brazil (Lima-filho *et al*., 2013) and 84% in Korea (Kim *et al*., 2007). Meanwhile, streptomycin, doxycycline and amoxicillin clavulanic acid antibiotics rates were (70%, 70% and 75%), respectively, while doxycycline was recorded in another study in Egypt (51%) (Eloksh, 2014).

The increased prevalence of resistance of *E.coli* isolates to these antibiotics is due to their regular usage in poultry industry for control of pathogenic avain colibacillosis in many districts in Egypt because of their low cost and availability. Also, it would seem that the discrepancies in the rate of *E.coli* resistance among different countries are due to differences in the level of dependence on antimicrobial usage and management practices in poultry production as well as variations in legislation guiding the use of antimicrobials from region to region. Therefore, non-

O157 EHEC strains could be a potential reservoir of antimicrobial resistance genes.

As shown in figure (1), the highest incidence of antimicrobial resistance genes was for *sul*1 (100%), followed by *aad*A1 (85%), *bla*TEM (75%), however some researchers in Iran detected these genes in percentage of 64.63%and 62.19%, respectively. (Momtaz and Jamshidi, 2013).

While the serogroups are important for determining potential pathogens, the presence of virulence attributes, such as *stx*1, *stx*2 and *hly*, are important parameters for pathogenicity of the strains. In addition, the virulence factors associated with these strains are linked to plasmids which detected by PCR. Consequently, they are likely to be subjected to horizontal gene transfer between the species as exhibited by dissemination of plasmids (Magwedere *et al*., 2013).

In prevalence of virulence genes (*stx*1 and *stx*2) in the present study were 45% and 65%, respectively whereas nine only out of 987from poultry samples were positive for the same genes (Lukásová *et al*., 2004).

Boerlin *et al*. (1999) found non-O157 EHEC serotypes O26, O103, O111, and O145 expressing *stx*2 gene concluding that this makes the organism significantly more likely to cause serious disease, while Friedrich *et al*. (2002) found that of 87 isolates of non-O157 (including O26, O103, O121, and O145) harbored *stx*2 gene.

Hemolysin (*hly*) gene is also one of important virulence factors (Gyles, 2006) which is more common in EHEC strains and in this study recorded in 80% of total non-O157 EHEC strains. It is widely distributed among non-O157 strains and could cause lysis of red blood cells in vitro. Approximately 90% of all EHEC strains possess genes encoding hemolysin (Kilic *et al*., 2007)

Plasmid profiles is one of the several useful methods for determining the relatedness or unrelatedness of bacterial strains that contain plasmid DNA. A large number of plasmids had been detected in *E.coli* as well as a large number of other bacterial species and their role in antibiotic resistance and other variable characters has been well established. The occurrence of multiple antibiotic resistances has also been shown to be due to a greater genetic mobilization of the antibiotic resistance genes carried by the plasmids (Gaddad and Shivannavar, 2011). In fact, plasmid profiles of the isolates are generally a useful tool for obtaining knowledge about resistance of the isolates to the antimicrobial substances and transfer of a plasmid among closely related isolates from different sources. Our results showed the high presence of virulence factors and multiple antibiotic-resistant properties among *E.coli* serotypes that were isolated from chicken meat samples (Momtaz and amshidi, 2013).

In conclusion there is an increase in detection of EHEC non O157. As well as there is an increase in antimicrobial resistance among the examined EHEC non O 157 so paying attention to this group of *E.coli* is required to avoid the risk to human. To the authors´ knowledge, excessive prescribing, crowding and poor sanitation are the primary factors responsible for the high antibiotic resistance in *E.coli* isolated from chicken meat. The sanitation conditions, especially in poultry slaughter houses and supermarkets could help to reduce the contamination rate of poultry meat. Finally, to prevent antibiotic resistance in bacteria, we have to prescribe antibiotics more cautiously in animals and periodically use different antibiotics.

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دراسات على عترات الايشيرشيا كولاى المعويه النزفيه غيرO 157:H7 من الدجاج مع الاشارة لجينات المقاومه للمضادات الحيويه المحمله على البلازميد

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تعتبر الايشيرشياكولاى المنتجه للسموم هي الأكثر أهمية والتى برزت في الآونة الأخيرة والمسببه للامراض المعديه التى تنقلها الاغذيه الملوثه بهذا الميكروب. من بين خمسين عتره ايشيرشياكولاى المعويه النزفيه، ومن خلال الفحص السيرولوجى تبين وجود ثلاثين عترة ايشيرشياكولاى المعويه النزفيه غيرO157:H7 بنسبه (٦٠%) وكانت أعلى نسبة من (O26) بنسبه ١٦% و(O111) بنسبه ١٤% وفي الوقت نفسه كان اقل نسبه (O128) ٢%. وقد أظهر اختبار الحساسيه للمضادات الحيوية المختلفه لهذه العترات مقاومه بنسبه ١٠٠٪ لكلا من سلفاميثوكسازول ، تريميثوبريم، كلورم فينيكول، كوليستين، الجنتاميسين والتتراسيكلين. وفى الوقت نفسه فقد تم تسجيل نسبه ٨٥٪ للستربتومايسين والدوكسيسيكلين، و٨٠٪ لسيفوتراكسون بينما ٧٥٪ للحمض كلافيونك أموكسيسيلين. وباستخدام تقنيه تفاعل اختبار انزيم البلمرة المتسلسل لعشرين عترة من غير O157:H7 ايشيرشياكولاى المعويه النزفيه كشف الجينات (*stx*1) بنسبه ٤٥٪، (*stx*2) بنسبه ٦٥٪ بينما (*hly*) بنسبه ٨٠٪ من اجمالى عدد العترات. تم الكشف عن جينات المقاومة للمضادات الحيويه (*sul*I)، (*aad*A) و(*bla*TEM) بنسبه (٨٥%، ٧٥% و ٦٠%) على التوالى. ولهذا فإنه يوصى بالحد من الاستخدام المفرط للمضادات الحيوية في مجال المعالجات البيطريه والمتابعه الدوريه لاستخدام المضادات الحيوية المختلفة حتى يمكن التغلب على مشكلة زيادة المقاومة الميكروبيه.