Relationship Between O-Serogroup, Virulence And Plasmids Profile In *E.coli* Isolated From Diseased Chickens

Mohammad, E. Enany[*]; Mervat, S. Hanafy[**] and Marwah, M. Rezk[***]

[*] Bacteriology, Immunity and Mycology Dept. , Fac. Vet. Med., Suez Canal Univ.

[**] Animal Health Research Institute, Dokki.

[***]Dept. of Bacteriology, Animal Health Research Institute, Ismailia.

ABSTRACT

A total of 197 *E.coli* strains were isolated from diseased chickens from different farms in Ismailia Governorate. The serotyped strains (n=40) were belonged to 10 serovars, O78(13), O153(6), O168(5), O26(4), O157 and O146(3/each), O20 and O114(2/each), O125 and O126(1/each). The results showed that 95% of *E.coli* serovars were Congo red positive, while 70% produced haemolysin. All investigated serovars produced cytotoxins for Vero cells (verocytotoxigenic). PCR assay was carried out for six serovars to detect the presence of eae A gene, only one serovar had intimin gene (O125:H7). The plasmids profile of the most important *E.coli* serovars were examined revealing that all contained plasmids. It was concluded that there was an association between plasmids containing strains and their high virulence.

INTRODUCTION

Shiga toxin-producing *E.coli* (STEC), also called verocytotoxin- producing *E. coli* (VTEC), have emerged as pathogens that can cause food-borne infections and severe potentially fatal illnesses in humans, such as haemorrhagic colitis (HC) and hemolytic uraemic syndrome (HUS). STEC strains that cause human infections belong to a large number of O:H serovars. Certain STEC strains belonging to serovars O26:H11, O103:H2, O111:H8, O145:H28, and O157:H7 have more frequently isolated from humans with severe illness. Furthermore, most outbreaks of HC and HUS have been attributed to strains of the enterohemorrhagic serovar O157:H7. However, as non-O157 STEC are more prevalent in animals and are food contaminants, humans are probably more exposed to these strains *(1)*.

Haemolysins form a family of lytic toxins can play a key role in the pathogenesis of disease. Strains of haemolytic *E.coli* produce either α or β haemolysins which are secreted and cell-associated *(2)*. Enterohaemolysin activity has been detected most frequently with washed sheep erythrocytes, but unlike α-haemolysin, enterohaemolysin does not lyse horse erythrocytes*(3)*. The role of haemolysins in the pathogenesis of VTEC infections is not known*(4)*.

The eae gene is chromosomally located which allows the bacteria to produce intimin, resulting in attaching and effacing lesions in the host intestinal mucosa cells. This has the effect of increasing the virulence of the bacteria to the host *(5)*.

Plasmids are bacterial extrachromosomal DNA elements that replicate autonomously *(6)*. Bacterial plasmids often carry genes for activities that allow their host to survive in adverse environments or to compete more successfully with other organisms. Selective advantage appears obvious in case of plasmids that confer resistance to antibiotics *(7)*.

The purpose of this study was to evaluate, some virulence factors in pathogenic *E.coli* isolated from diseased chickens in relation to plasmids profile.

MATERIAL AND METHODS

Collected samples, Samples of liver, heart, lungs and spleen were obtained from 275 sacrificed chickens which showed clinical signs suspected *E.*coli infections (loss of appetite, loss of body weight, pot belly and gasping the air. At postmortem examination, most of the examined cases showed clear colisepticaemia in the form of severe hemorrhage and congestion of intestinal tract, muscles of the thigh and breast, liver, kidney

and heart and fibrinous pericarditis, perihepatitis and airsacculitis) from different farms at Ismailia Governorate were subjected to bacteriological and serological examination.

Bacteriological examinations:-Bacterial isolation and identification

The obtained samples were cultured on MacConkey's and Eosin Methylene Blue agar (EMB) and incubated at 37°C for 24-48 hrs, and then inoculated onto Triple-Sugar Iron Agar, urea agar, Simmon's citrate agar and indole*(8)*. Analytical Profile Index (API 20E) (BioMerieux) system were used.

Serotyping

Forty isolated strains of that proved to be *E.coli* were serologically typed with slide agglutination test for somatic "O" antigen using 8 polyvalent and 43 corresponding monovalent *E.*coli antisera "DENKA SEIKEN, Japan "*(9)* also some *E.coli* serovars were tested for flagellar "H" antigens(H4, H6, H7, H19, H21(DENKA SEIKEN, Japan) ***(10)***.

Virulence assessment

The following techniques were carried out for Virulence assessment

a) Congo red binding activity, *(11)*.

b) Haemolysin production, *(12)*

c) Production and detection of cytotoxins in Vero cells, *(13)*

d) PCR assays for detection of intimin gene

The predicted sizes of the PCR-amplified products were 384bp (eae A-F sequence 5\GACCCGGCACAAGCATAAG C3\) (eae A-R 5\CCACCTGCAGCAACAAGAGG3\) for eae A gene***(14)***.

Plasmids Profiling

six serovars were screened for plasmids content according to alkaline-lysis mini prep method and resolved on 0.7% agarose gels***(15)***.

RESULTS

Collected samples

A total of 197 strains of *E.coli* were isolated from diseased chickens.

Serotyping

The result of serotyping of 40 *E.*coli strains is depicted in Table,1 and Figure,1.

Table, 1. Incidence and frequency distribution of *E.coli* serovars isolated from examined chickens.

Bacterial Isolates	No. of serotyped isolates	*E.coli* serovars	No.	%
197	40	O78:H4	1	6.59
		O78:H21	2	
		O78:H?	10	
		O153	6	3.04
		O168	5	2.53
		O26:H19	1	2.03
		O26:H?	3	
		O157:H7	2	1.52
		O157:H?	1	
		O146	3	1.52
		O20	2	1.01
		O114	2	1.01
		O125:H7	1	0.50
		O126	1	0.36

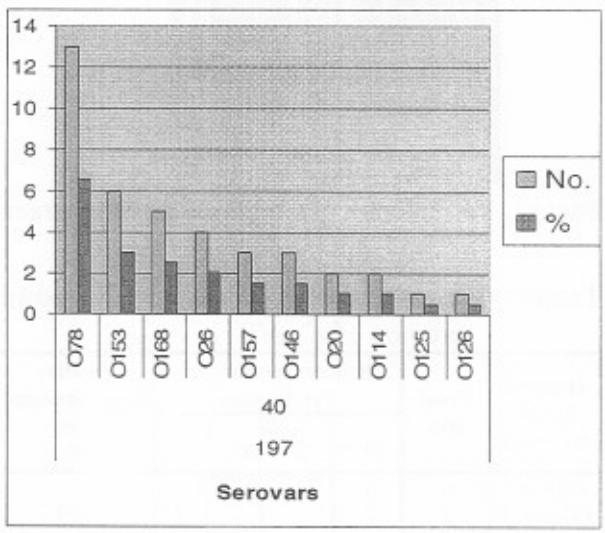
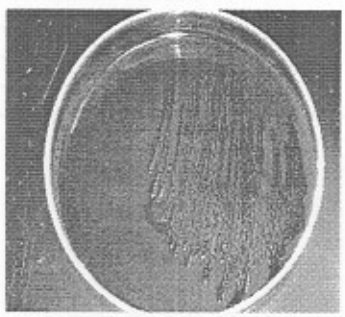


Figure 1. Incidence and frequency distribution of *E.coli* serovars isolated from examined chickens.

Virulence assessment:- The results of detection of virulence factors were Congo red binding activity (Table, 2, Photo, 1), haemolysin (Table, 3) verocytotoxins (Table, 4, Photos , 2 & 3) and intimin gene (eae A gene) (Table 5, Photo, 6).

Table 2. Congo red binding activity of some serovars of *E.coli.*

Isolated *E.coli* serovars	Total Number	+++	++	+	-	Positive Congo red %
O78:H4	1	1	-	-	-	92.3
O78:H21	2	1	1	-	-	
O78:H?	10	-	9	-	1	
O153	6	4	2	-	-	100
O157:H7	2	-	2	-	-	100
O157:H?	1	1	-	-	-	
O114	2	2	-	-	-	100
O20	2	-	-	1	1	50
O168	5	-	5	-	-	100
O146	3	-	2	1	-	100
O26:H19	1	1	-	-	-	100
O26:H?	3	1	2	-	-	
O126	1	1	-	-	-	100
O125:H7	1	-	-	1	-	100
Total	**40**	**12**	**23**	**3**	**2**	**95**



Photo,1. Red colonies of Congo red positive strains.

Table 3: Enterohaemolysin production of some serovars of *E.coli*

Isolated *E.coli* serovars	Total No.	Enterohaemolysin Production				Positive Enterohaemolysin %
		+++	++	+	-	
O78:H4	1	1	-	-	-	92.3
O78:H21	2	-	1	-	1	
O78:H?	10	10	-	-	-	
O153	6	-	-	4	2	66.6
O157:H7	2	2	-	-	-	100
O157:H?	1	-	-	1	-	
O114	2	-	-	-	2	0.0
O20	2	-	-	2	-	100
O168	5	3	2	-	-	100
O146	3	-	-	-	3	0.0
O26:H19	1	-	-	-	1	0.0
O26:H?	3	-	-	-	3	
O126	1	1	-	-	-	100
O125:H7	1	1	-	-	-	100
Total	**40**	**18**	**3**	**7**	**12**	**70**

+++ = Strong positive ++=Intermediate positive
+= Weak positive -= Negative

Table 4: Verocytotoxin production of isolated *E.coli* serovars with two fold dilution of toxins.

Isolated serovars	Verocytotoxin production					
	Two fold serial dilution of E.coli toxins					
	1/10	1/20	1/40	1/80	1/160	1/320
O78:H4	++	+	+	+	-	-
O153	++	+	+	+	-	-
O157:H7	++	+	+	+	-	-
O157:H7	++	+	-	-	-	-
O114	++	+	+	+	-	-
O168	++	+	+	+	-	-
O146	+++	++	+	+	+	-
O20	++	+	+	-	-	-
O126	+++	++	+	+	+	-
O26:H19	++	++	+	+	-	-
O125:H7	+++	++	+	+	+	-

+++= Destruction &degeneration of Vero cells &appearance of cell deformity with large gaps in between cells, ++ = Several cell shapes(round, spindle, small or detached), +=Aggregation of small round cells, - = No change in normal Vero cells.

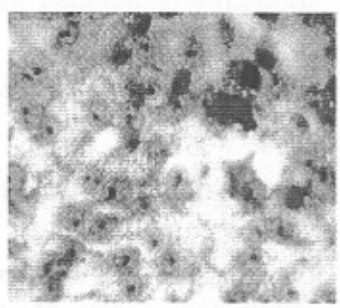


Photo 2. Verocytotoxic effect of *E.coli* (Enlargement, appearance of multinucleated cells) (Giemsa stain X 400)

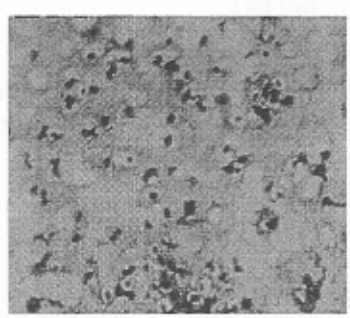


Photo 3· Verocytotoxic effect of *E.coli* (Destrucion and degeneration of cells and interstitial tissues) (Giemsa stain X200).

Table 5. Intimin gene (eae A) detection using PCR

Tested strains	Presence of eae A gene
O157:H7	–
O125:H7	+
O157:H7	–
O168	–
O78:H4	–
O26:H19	–

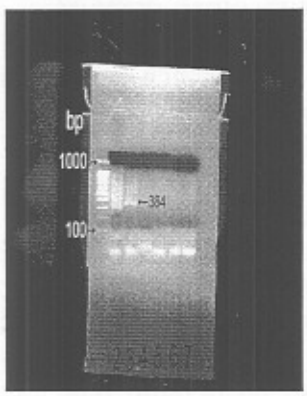


Photo 4. Agarose gel electrophoresis of PCR reaction of eae gene from some *E.coli* serovars.

Lane 1:Marker ladder(100-1000 bp),Lane 2:O157:H7,lane 3:O125:H7,lane4:O157:H7, lane 5:O168, lane 6:O78:H4 and lane7:O26:H19.

The data in Table 5 and Photo 4 revealed that only one serovar O125:H7gave positive result to eae A gene.

Plasmids Profiling:-Six serovars of isolated *E.coli* were examined for presence of plasmids. All the tested strains contained plasmids (Table,6, photo,5).

Table 6. Plasmids DNA profile of the some *E.coli* isolates.

Bands	Lane 1 Ladder	Lane 2 O157:H7	Lane 3 O125:H7	Lane 4 O157:H7	Lane 5 O168	Lane 6 O78:H4	Lane 7 O26:H19
	(mol.w.) bp	(mol.w.) bp	(mol.w.) bp	(mol.w.) bp	(mol.w.) bp	(mol.w.) bp	(mol.w.) Bp
1	23130	19912	10937	10937	10937	10937	10937
2	9416	5432	2194	1870			1870
3	6557	2219	1468				
4	4361	1870					
5	2322	1396					
6	2027	1001					
7	564						

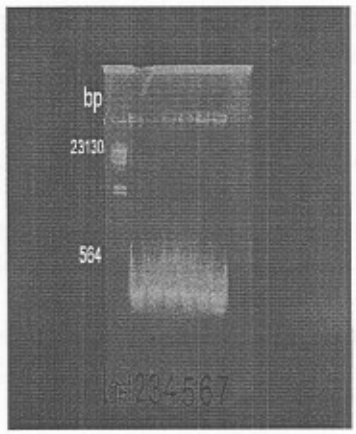


Photo 5. Plasmids DNA profile of isolated *E.coli* serovars.

Lane 1: Marker ladder(564-23130 bp),Lane 2:O157:H7,lane 3:O125:H7, lane4:O157:H7, lane 5: O168, lane 6: O78:H4 and lane7: O26:H19.\

DISCUSSION

In the current study *E.coli* isolates recovered from diseased chickens were serologically examined with available agglutinating sera***(9)***. Ten only antisera were found to agglutinate with the 40 isolated *E.coli* serovars. Similar serovars were obtained by several investigators ***(16,17)*** who isolated O26, O114, O125 and O126 and O20, O78 and O153 from *E.coli* diseased chickens. Also O20, O78 and O26 were isolated from cases of colisepticaemia***(18)***. The predominant serovar was O78 which coincide with the results and showed in Table,1 and Figure,1.

Table, 2 and Photo, 1 showed that 95% of the strains were Congo red positive. Similarly previous investigation showed that all tested *E.coli* were Congo red positive***(19)***. The high percentage of ability of *E.coli* to bind with Congo red dye is due to the isolated *E.coli* were from diseased chickens. Positive Congo red isolates were recovered from diarrhoeic chickens while negative isolates were from apparently healthy cases***(19)***.

As shown in Table (3), 70% of the *E.coli* serovars produced haemolysin, the same percentage (70%) was recorded previously ***(20)***. The elaboration of haemolysin by *E.coli* was frequently associated with diarrhoea and play critical role in extra-intestinal

infection*(21)*, without an evidence to suggest the elaboration of haemolysin increases the potential for causing diarrhea. There was an association between Shiga toxin production and a novel haemolysin *(22)*,this may explain the high percentage of positive verocytotoxin strains in this study. All serovars of O157 (3 strains), O20 (2 strains), O168 (5 strains), O126 (one strain) and O125 (one strain) produced enterohaemolysin while 12 out of 13 O78 and 4 of 6 O153 produced enterohaemolysin.

Concerning the verocytotoxins production by *E.coli,* as shown in Table,4 and Photos,2 & 3, all tested serovars (O20, O26:H19, O78:H4, O114, O125:H7, O126, O146, O153, O157:H7and O168) were cytotoxic for Vero cells (100%). A high percentage (72%)of VTEC isolated *E.coli* from swollen head syndrome produced cytotoxin that was active on Vero and Hela cells *(23)*. The high percentage of verocytotoxin *E.coli* strains in this investigation may be due to that, subinhibitory concentration of some antimicrobial agents may stimulate verotoxin production*(24)*. Production of intimin is not essential for pathogenesis, because a number of sporadic cases of hemolytic uremic syndrome have been caused by eae-negative non-O157 STEC strains. Thus STEC O104:H21 and O113:H21 strains lacking the eae gene were responsible for an outbreak and a cluster of three HUS cases in United Sates and Australia *(25)*. Further more recently it has been shown that a novel mega plasmid-encoded adhesion might be an important virulence factor of eae-negative STEC strains capable of causing severe disease in humans*(26)*.

Regarding the intimin (eae) gene using PCR, 6 serovars were chosen for detection of eae A gene. As observed(Table,5 and Photo,4), positive amplification of 384 bp frequent of intimin gene from extracted DNA of O125:H7 while serovars O26:H19, O78:H4, O168 and O157:H7 didn't contain the gene. O157:H7 and O78:H4 were negative for intimin*(20)* which confirm our finding. The low incidence of intimin gene in poultry in this work corresponded with that which indicating that *E.coli* strains originated from birds were carrying a low percentage of eae A gene*(27)*. STEC associated virulence genes including eae were primarily detected in isolates from humans and cattle*(28)*.

Bacterial plasmids often carry genes for activities that allow their host to survive in adverse environments or to compete more successfully with other organisms. Selective advantage appears obvious in case of plasmids that confer resistance to antibiotics *(7)*.Tables,6 and Photo,5 showed that all tested serovars(O26:H19, O78:H4, O125:H7, O157:H7 and O168) contained one or more plasmids bands ranged from 19.912 to 1.001 kbp. This result similarly 22 out of 30 *E.coli* isolates were originated from poultry contained plasmids DNA bands of 2.3 to 102 kbp, From the plasmids DNA profile of *E.coli* serovars, it was observable that there was a relationship between large plasmids containing strains and their virulence *(29)*. The high molecular weight plasmids was associated with pathogenic strains of *E.coli(30)*. The differences in plasmids profiles of the same serovar are also recorded*(31)*and dissimilarity in plasmids profile among the same serovar was recorded. Concerning the serovar O157:H7 it has been observed that the variations in the plasmids sizes in between serovar O157:H7 were recorded*(32)*. The plasmids molecular weights of examined *E.coli* strains were more than 2 kbp in general and only one isolate had low molecular weight plasmids more than 0.8 kbp*(33)*. The author found that some serovars contained 4 plasmids, and others had 3 plasmids. The plasmids vary in size but in general it depends on the size of the bacterial genome and the plasmids sizes are usually less than one tenth of the size of a bacterial genome*(8)*.

CONCLUSSION

Great attention must be reflect on non-O157:H7 *E.coli* strains as they show the presence of virulence factors as O125:H7, O26:H19 and O78:H4.

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الملخص العربى

العلاقه بين التصنيف السيرولوچى والضراوه وصورة البلازميد فى ميكروب الإشيريشياكولاى المعزوله من الدجاج المريض

محمدالسيد عنانى ,مرفت سيد حنفى** ,مروه محمود رزق****

* أستاذ ورئيس قسم البكتيريولوچى والمناعه والفطريات- كلية الطب البيطرى- جامعة قناة السويس

** رئيس بحوث ورئيس وحدة السيرولوجي-معهد بحوث صحة الحيوان بالدقى

*** قسم البكتيريولوجى –معهد بحوث صحة الحيوان بالإسماعيليه

لإيجاد العلاقه بين التصنيف السيرولوجى و بعض عوامل الضراوه وصورة البلازميد لميكروب الإشيريشياكولاى تم عزل ١٩٧عتره من الميكروب من الدجاج المريض بمحافظة الإسماعيليه وبتصنيف ٤٠(3)O146,(3)O157,(4)O26,(5)O168,(6)O153,(13)O78عتره منهم كانتالنتائج كالاتى:-

وأوضحت النتائج أن ٩٥٪ من عترات الميكروب القولونىO20,(2)O114,(2)O125,(1)O126,(1) كانت قادره على النمو على صبغة الكونجو الحمراء فى حين أن ٧٠٪ فقط كانت قادره على خاصية ذوبان الدم. وأن جميع العترات المختبره كانت لديها القدره على إنتاج سموم الفيرو. وبإستخدام تفاعل البلمره المتسلسل للكشف عن وجود چين الإنتيمين فى ٦ عترات (فقط O168,7H:O125,19H:O26,4H:O78 هى التى إحتوت على هذا الچين. كما تم إختبار صورة 7H:O125فوجد أن 7H:O157) و٢من البلازميد لهذه العترات ال ٦أيضاً وقدإحتوت كل هذه العترات على بلازميد. كما وجد إرتباط طردي بين الحجم الكبير للبلازميد والضراوه العاليه لهذه المعزولات.