A study of phenotypic and genotypic virulence markers of *Escherichia coli* isolated from poultry

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Abstract

E. coli isolates (345 from chickens and 150 from ducks) were tested for phenotypic and genotypic virulence markers. The results revealed that 285 out of 345 examined *E. coli* strains from chickens (82.6%) were able to bind Congo red dye and appeared as red colonies, 112out of 150 examined *E. coli* strains from ducks were positive for Congo red test (74.7%). 302 *E. coli* strains from chickens (87.5 %) were HA^+, while 114 *E. coli* strains from ducks were HA^+. 252 *E. coli* strains from chickens (73%) were positive for haemolytic activity, in contrast to 85 *E. coli* strains from ducks (56.7%). *E. coli* serogroups O44 and O158 isolated from chickens cases were cytotoxic to vero cells. On the other hand, serogroups (O114, O91, O111, O125, O103, O142, O26, O78, O127, O164 and O119) obtained from chickens were non-cytotoxic for vero cells. The serogroups (O44, O158, O114, O91, O111, O125, O103, O142, O26, O78, O127, O164 and O119) obtained from ducks were non cytotoxic for vero cells. The testing of *E. coli* strains representing 13 O groups for virulence genes isolated from chickens revealed the detection of *Stx*1 gene in one isolate belonging to O158 and *Stx*2 gene was found also in one isolate of the O group 44. The *eae*A gene (intimin) was detected in *E. coli* isolates belonging to the O groups O44, O158, O125, O114 and O78. *Iut* A gene was detected in *E. coli* isolates belonging to the O groups O114, O44, O111, O26, O142, O78, O103 and O91. *ISS* gene was detected in *E. coli* isolates belonging to the O groups O44, O91, O111, O158, O125, O114, O142, O127, O103, O164 and O119. All of the tested 9 serogroups from ducks were negative for the *Stx*1 gene, and *Stx*2 gene, while *eae*A gene was detected in *E. coli* isolates belonging to the O groups O158, O78 and O26; *iut A* gene was detected in *E. coli* isolates belonging to the O groups O114, O44, O91, O111, O26, O158, O78, O103 and O125 and *iss* gene was detected in *E. coli* isolates belonging to the O groups O114, O44, O91, O111, O158, O125, O103 and O26. Concerning the plasmids of ***E. coli*** isolates recovered from chickens, three bands of plasmids were detected in four O groups (O44, O158, O111, O114), two bands in O groups (O78, O103 and O125), while only one band was seen in group (O142). In duck's isolates, three bands of plasmids were detected in two O groups (O158 and O114) and two bands in one O group O44. Intratracheal inoculations of one day old chicks of different isolates of ***E. coli*** revealed that serogroups (O44, O158, O114, O111, O91 and O78) caused a mortality rate that varied between (60%-80%), while in serogroups (O103, O125, O142, O26, O127, O164 and O119), the mortality rates were much lower (30%-40%).

Key words: *E. coli,* virulence markers, Congo red binding, haemagglutination, haemolytic activity, vero cell toxicity, *Stx*1, *Stx*2, *eae*A, *iut* A, *iss* genes, plasmids, pathogenicity.

Introduction

Escherichia coli infection causing colisepticaemia in poultry is an important disease of chicks. It is also associated with other disease conditions like acute enteritis, air–saculitis, peri-carditis, peritonitis, coligranuloma of liver, synovitis, swollen-head syndrome and osteomyelities **(Amara *et al.,* 1995 and Reddy *et al.*, 1994**). Although infections due to *E. coli* have been costly to the poultry industry, the exact virulence mechanisms used by these organisms to cause disease in birds remain interesting point of research.

The pathogencity of *E. coli* for chickens has been correlated with numerous extrinsic and intrinsic bird- related factors and condi-

tions .The extrinsic factors include environment, exposure to other infectious agents, virulence and duration of exposure. The intrinsic factors affecting susceptibility includes age, route of exposure and breed or strain of chicken. **(Piercy and West, 1976).**

The virulence factors of *E .coli* are usually complex and the contribution of individual determinants can not be considered in isolation, the relevant determinants may vary with the locus and nature of the infection. Virulence in microorganisms is associated with the capacity to attach and colonize at the site of infection, with subsequent damage to the host and is promoted by aggresins that interfere with the host defense **(Burrows, 1985).**

E. coli that causes infections usually possess one or more virulence properties that may help in establishment of the infection. Among these factors, Congo red binding activity, haemagglutination, haemolytic activity and toxin production are the most commonly studied ones. **Vinal (1988), Berkhoff and Vinal (1986) and Gjessing and Berkhoff (1989**) confirmed that, binding of Congo red dye has been associated with pathogenicity of the *E. coli*.

The ability of bacteria to cause haemoagglutination of different erythrocytes in the presence of D-mannose (Mannose resistance haemoagglutination, MRHA) has been used to screen for the presence of the colonization factor antigens (CFAS) (**Evans *et al.*, 1979**). Fimbriae have been well recognized as specific mediators and adhesion of different host epithelial surfaces **(Krogfelt, 1991**). The use of D-mannose with HA is considered to be more specific to clear up the virulent strains from avirulent strain **(Abd EL-Moti *et al*., 1993).**

Haemolysis of erythrocytes is considered as an important virulence factor for some strains of *E. coli* to overcome host defense mechanism through haemolysis production, which is related to the release of iron into the bacterial environment and cytotoxic effect on neutrophiles **(Cavalieri *et al*., 1984**).

Shiga toxin- producing *E. coli* (STEC), also called Vero toxin producing *E. coli* (VTEC), strains are the important recently emerged group of food- borne pathogens that can cause severe gastrointestinal disease in human and complication as haemolytic uremic syndrome (HUS). (**Paton and Paton, 1998**). **Levine 1987 and O'Brien *et al*. (1982)** found that VT plays a role in the pathogenesis of diarrhoea. **Peighambari *et al.* (1995)** found no isolates that showed cytotoxic effects.

The virulence mechanisms that characterize *E. coli* are genetically coded for by chromosomal, plasmid, and DNAs and include heat- labile (LTI, LTlla and LTllb) and heat- stable (STI and STI) toxins, verotoxin types 1, 2 and VT2e, respectively), cytotoxic necrotizing factors (CNF1 and CNF2), attaching and effecting mechanisms (*eae*A), enteroaggregative mechanisms (Eagg), and entroinvasive mechanisms (*Ein*v).

The aim of the present work was to determine both the phenotypic virulence factors, namely Congo red binding, haemagglutination, haemolysis and verotoxins, as well as virulence genes, such as *Stx*1, *Stx*2, *eae*A, *iut* A and *iss* genes in *E. coli* strains isolated from chicken and ducks with symptoms or post mortem signs of colibacillosis.

Material and Methods

E. coli isolates

A total of 495 ***E. coli*** isolates (345 isolates recovered from chickens and 150 isolates recovered from ducks) were previously isolated from chickens and ducks suffering from colibacillosis by Roshdy *et al*. (2012). The chicken isolates were typed in 24 O groups with predominance of the O groups O44, O158, O114, O91, O111, O125, O103, O142, O26, O78, O127 and O164. Of the 15 O groups recovered from ducks, the O158, O103, O125, O44, O114, O91, O111 and O78 were the most predominant.

PCR Master Mix for conventional PCR: (**Thermo SCIENTIFIC**) with Cat. No. (AB0575/LD-A)

QIAamp®DNA Mini Kit (Cat. No. 51304-Qiagen)

Oligonucleotide primers : Two sets of primer pairs were used for amplification of DNA for the detection of *E. coli* virulence genes, as shown in the following Table (1).

Gene	Primer Sequence (5'- 3')	Expected fragment size
Iron uptake transport gene, *IUTA*	F: GGCTGGACATGGGAA CTG G R: CGTCGG GAACGG GTAGAA TCG	300
Increased serum survival gene*ISS*	F:ATGTTATTTTCTGCCGCTCTG R: CTATTGTGAGCAATATACCC	266
Intimin gene, *eae*A	F: GTGGCGAATACTGGCGAGACT R: CCCCATTCTTTTTCACCGTCG	890
Shiga toxin 1 gene, *Stx*1	F: ACACTGGATGATCTCAGTGG R: CTGAATCCCCCTCCATTATG	614
Shiga toxin 2 gene, *Stx*2	F:CCATGACAACGGACAGCAGTT R:CCTGTCAACTGAGCAGCACTTTG	779

Laboratory animals:

A number of 130 One -day -old chicks commercial were used in the pathogenicity experiment.

Detection of virulence factors of *E. coli* isolates:

Congo red binding test (Berkhoff and Vinal, 1986):

Strains of ***E. coli*** were grown in nutrient broth 37°C for 24hours. A loopful of each culture was grown on Cong red medium and incubated for 24 hours of at 37°C. Cong red positive (CR+) ***E. coli*** was indicated by the development of red colonies. Cong red negative (CR-) ***E. coli*** did not bind the dye and appeared white colonies.

Haemagglutination test (HA) (Evans *et al*., 1979):

1 ml of 3.8% citric acid in distilled water was to be added to 9.0ml of the blood obtained from healthy chickens. Blood was diluted 1:4 with phosphate buffered saline (PBS) PH 7.2 to test for HA and 1:4 with 1% D-mannose in PBS to test for mannose resistance haemagglutination (MRHA). Bacterial cells from ***E. coli*** isolates were obtained from cultures grown for 18 hours at 37°C on colonization factor agar (CFA) plates. ***E. coli*** colonies were picked up and mixed with a drop of the (chicken) blood on a glass slide at room temperature and observed for 1-2 minutes. Haemagglutination was observed by the naked eye supported by the aid of hand lens.

Haemolysine production (Beutin *et al*., 1993):

Production of haemolysine was assayed by different ***E. coli*** strains in nutrient broth at 37°C for 24h. A loopful from each broth culture was streaked onto blood agar medium containing 5% sheep blood. The culture was incubated at 37°C for 24h. Haemolysine production was detected by the presence of a clear haemolytic zone around the colony.

Vero cell toxicity

A confluent monolayer of vero cells was inoculated by the *E. coli* culture, incubated at 37°C in 5% CO_2 incubator and observed daily for cytopathogenic effect (CPE) up to 72hr. The cell culture supernatant was harvested for up to (72) hours and, then fixed with 10% formalin and stained with crystal violet and observed for cytopathic effect (**Giugliano *et al*., 1982).**

Detection of virulence genes using PCR

(Sambrook *et al.*, 1989 and **Sritharan and Barker, 1991):**

Bacteria were grown on tryptone soya agar for 18 hours at 37°C, then harvested, washed 3 times with PBS and pelleted by centrifugation at 3000 rpm for 10 minutes. The bacterial pellets were resuspended in 400 µl Tris-EDTA buffer (pH 8.0) and heated at 105°C in heat block for 25 minutes and the suspensions were left to cool at room temperature then centrifuged at 14,000 xg for 10 minutes. The supernatant was transferred to another tube with double volume of absolute ethanol and 0.1 volume 3 M sodium acetate (pH 5.2), and then the test tubes were kept at 20°C overnight. DNA was pelleted by centrifugation at 14,000 xg for 10 minutes, followed by washing with ice cold 70% ethanol and recentrifugation at 14,000 xg for 10 minutes. The DNA pellet was dried and resuspended in 20 µl sterile distilled water. The concentration and purity of DNA that had been extracted were determined by measuring the optical density (OD) at wave length of 260 and 280 nm using spectrophotometer. Amplification of genomic DNA was performed in 50 µl volumes in PCR tubes (Ojeniyi *et al*., 1994). The reaction mixture consisted of 1 µl (200 µg) of the extracted DNA templates from bacterial cultures, 5 µl 10x buffer, 1µl dNTPs, 0.5µl Taq polymerase enzyme, 4 mM MgCL2, 1 µl (20 PM) from each of forward and reverse primers and the reaction mixture volume were completed to 50 µl. Forty µl of paraffin oil were added to avoid evaporation of the reaction mixture. PCR was performed using AB Thermocycler, where the initial denaturation was done by heating to 94°C for 5 min and then subjected to 30 cycles of 1 min at 94°C, 1 min at 55°C and 1 min at 72°C. The final extension was set at 72°C for 5 min. Screening PCR products was done by agarose gel electrophoresis (Sambrook *et al*., 1989).Ten µl of each of PCR products samples were loaded to the gel after mixing with 1 µl loading buffer. The power supply was adjusted at 10 Volt/ cm of the tank length. The run was stopped and the gel was transferred to UV trans-illuminator. The gel was photographed using a digital camera.

Detection of Plasmids of *E. coli* strains:

The Plasmids were separated from different *E. coli* strains, by using QiAprep spin mini prep Kit. The specific DNA bands were observed linear plasmid, coiled plasmid or super coiled plasmid.

Pathogenicity of *E. coli* strains in one-day-old chicks:

The pathogeniciity of *E. coli* strains to one-day -old chicks was carried out using (130) one day -old-chicks. Pathogenicity was assessed by intratracheal inoculation with 0.1ml of a 24 hours nutrient broth culture containing 10 ^{7}CFU/chick of one of the *E. coli* isolate in susceptible one day-old-chicks. Based on mortality and lesions occurring during 21 days post inoculation period, isolates were characterized as being high, moderate or low in pathogenic.

Results

Congo red binding, haemagglutination and haemolytic activity of *E. coli* strains isolated from poultry samples.

As shown in Table (2), 285 out of 345 examined *E. coli* strains from chickens (82.6%) were able to bind Congo red dye and appeared as red colonies, while 112 out of 150 examined *E. coli* strains from ducks bound Congo red (74.7%). The haemagglutination was positive (HA^{+}) in (87.5 %) of chicken isolates, in contrast to (76%) of *E. coli* isolates from ducks. On the other hand, the haemolytic activity of *E. coli* strains isolated from chickens was (73%), while that of ducks was (56.7%).

Table (2). Phynotypic virulence markers: Congo red binding, haemagglutination and haemolytic activity of *E. coli* strains isolated from poultry samples.

Birds	No. of *E. coli* strains tested	Congo red binding		Haemagglutination				Haemolytic activity	
				MR		HA^+			
		No.	%	No.	%	No.	%	No.	%
Chickens	345	285	82.6	254	73.6	302	87.5	252	73
Ducks	150	112	74.7	88	58.7	114	76	85	56.7

Correlation between *E. coli* O groups and virulence factors

As shown in (Table3), 220 typed strains of *E. coli* isolated from chickens samples that belonged to 24 serogroups were able to bind Congo red dye (83.6%). The highest Congo red binding rate was in strains of *E. coli* O142 (94.4%), followed by O158 (92.6%), while strains of the O groups O57, O6, O148, O18 and O8, did not bind to Congo red. On the other hand, 62 typed strains of *E. coli* isolated from ducks that belonged to 15 serogroups, were able to bind Congo red dye. The highest Congo red binding rate was in strains of *E. coli* O114 (85.7%), followed by O103 and O158 (83.3%), while strains O86, O118, O145, O157, O126, O55, O57, O6, O148, O18 and O8 did not bind to Congo red.

In *E. coli* isolates recovered from chickens, the rate of HA^+ among the serotyped *E. coli* was (91.2%), in contrast to (75.6%)of untyped isolates. Most of the isolates showed MRHA (78.7% and 57.3%), while the rates of MS were (12.5% and 18.5%) in the serotyped and untyped isolates, respectively. The rate of HA^+ isolates among the various O groups varied between 50% and 100%. The rate of HA^+ among the serotyped ***E. coli*** isolated from ducks was slightly lower (80.7% and 70.1%) for typed and untyped strains, respectively. Also, the MRHA in both groups was lower than the of chickens (89.8% and 45.4%, respectively). The percentage of HA^+ strains among the different O groups did not exceed (91.7%).

The haemolytic activity was detected in only 217 *E. coli* isolates recovered from chickens samples belonging to 24 serogroups, in addition to 35 untyped and in 55 *E. coli* isolates recovered from ducks samples belonging to 15 serogroups, in addition to 30 untyped strains .The highest haemolytic activity in chickens was observed in strains of the O103 (94.7%) and O78 (94.4%). In case of *E. coli* isolated from ducks, the highest haemolytic activity was recorded in the strains of the O103 and O158 (83.3%). Some strains did not have haemolytic activity, particularly in the O157, O57, O6, O18 and O8 from chickens and ducks.

Determination of vero cell activity of *E. coli* strains from different poultry (chickens and ducks):

Only *E. coli* serogroups O44and O158 isolated from chickens were cytotoxic to vero cells. In contrast, other serogroups (O114, O91, O111, O125, O103, O142, O26, O78, O127, O164 and O119) obtained from chickens were non-cytotoxic for vero cells. Also, serogroups (O44, O158, O114, O91, O111, O125, O103, O142, O26, O78, O127, O164 and O119) obtained from ducks were non- cytotoxic for vero cells.

Detection of virulence genes in *E. coli* isolates belonging to different O serogroups recovered from chickens and ducks:

As shown in Table 4), *E. coli* isolates belonging to 13 O serogroups recovered from chickens and 13 O serogroups recovered from ducks were tested by PCR for the presence of 5 virulence genes.

In chicken's isolates, the 13 O groups contained one or more of the genes looked for in the study. 4 genes were detected in one O group (O44), 3 genes in two O groups (O158 and O114), 2 genes in six O groups (O111, O125, O103, O142, O78 and O91), while only one gene was detected in four O groups (O26, O119, O127 and O164). The most commonly

detected gene was ISS gene, as it was found in 11 different O groups, followed by the gene IUTA gene, found in 8 different O groups. The gene eae A was found in 5 different O groups, while the genes STX1 and STX2 were found each in 1 group.

In case of duck isolates, only 9 of the 13 O groups contained one or more of the five genes looked for in the study. The reaming 4 O groups were all negative for these genes. Three genes were detected in two O group (O158 and O26), while two genes were detected in seven O groups (O44, O114, O91, O111, O125, O103 and O78). The most commonly detected gene was IUTA gene, as it was found in 9 different O groups, followed by the gene ISS gene, found in 8 different O groups. The gene eae A was found in 3 different O groups, while the genes STX1 and STX2 were not found in any of the 13 O groups

Table (3). Relation beween serogroups and Congo red binding, haemagglutination and haemolytic activity of *E. coli* strains isolated from poultry samples.

Sero-groups tested	Chickens							Ducks						
	No tested	CR		HA^+		HLy^+		No tested	CR		HA^+		HLy+	
		No.	%	No.	%	No.	%		No.	%	No.	%	No.	%
O44	32	28	87.5	30	93.7	29	90.6	9	7	%	7	77.8	6	66.7
O158	27	25	92.6	26	96.3	24	88.9	12	10	83.3	11	91.7	10	83.3
O114	23	20	86.9	22	95.6	21	91.3	7	6	85.7	6	85.7	5	71.4
O91	20	18	90.0	19	95.0	17	85.0	6	4	66.7%	5	83.3	4	66.7
O111	20	17	85.0	20	100	18	90.0	6	4	66.7%	5	83.3	3	50.0
O125	19	16	80.0	17	89.5	17	89.5	11	9	81.8%	10	90.9	8	72.7
O103	19	17	89.5	18	94.0	18	94.7	12	10	83.3%	11	91.7	10	83.3
O142	18	17	94.4	16	88.9	16	88.9	3	2	66.7%	2	66.7	1	33.3
O26	15	13	86.7	14	93.3	11	73.3	3	2	66.7%	2	66.7	2	66.7
O78	18	16	88.9	18	100	17	94.4	5	4	80%	4	80.0	3	60.0
O127	11	9	81.8	10	90.9	8	72.7	2	1	50%	1	50.0	1	50.0
*Other O groups	41	24	58.5	30	73.2	21	51.2	8	3	37.5	3	12.5	2	25.0
Typable	263	220	83.6	240	91.2	217	82.5	84	62	73.8	67	80.7	55	66.3
Untypable	82	65	79.3	62	75.6	35	42.7	66	50	75.7	47	70.1	30	44.8
Total No.	345	285	82.6	302	87.5	252	73	150	112	74.7	114	76	85	56.7

*O16, O86, O119, O118, O145, O157, O126, O55, O57, O6, O148, O18,O8

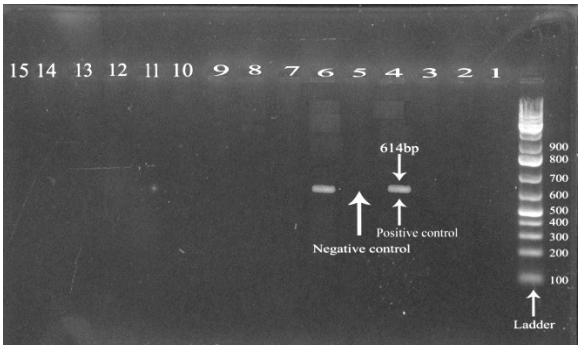
CR: Congo red binding, $HA^{+:}$ positive haemagglutination, $HLy^{+:}$ positive haemolysis

Table (4). Results of the PCR in the detection of the five virulence genes in *E. coli* O serogroups isolated from chickens and ducks:

Serogroups	Chickens						Ducks					
	Stx1	Stx2	eaeA	Iuta	Iss	Total genes	Stx1	Stx2	eaeA	Iuta	Iss	Total genes
O44	-ve	+ve	+ve	+ve	+ve	4	-ve	-ve	-ve	+ve	+ve	2
O158	+ve	-ve	+ve	-ve	+ve	3	-ve	-ve	+ve	+ve	+ve	3
O114	-ve	-ve	+ve	+ve	+ve	3	-ve	-ve	-ve	+ve	+ve	2
O91	-ve	-ve	-ve	+ve	+ve	2	-ve	-ve	-ve	+ve	+ve	2
O111	-ve	-ve	-ve	+ve	+ve	2	-ve	-ve	-ve	+ve	+ve	2
O125	-ve	-ve	+ve	-ve	+ve	2	-ve	-ve	-ve	+ve	+ve	2
O103	-ve	-ve	-ve	+ve	+ve	2	-ve	-ve	-ve	+ve	+ve	2
O142	-ve	-ve	-ve	+ve	+ve	2	-ve	-ve	-ve	-ve	-ve	0
O26	-ve	-ve	-ve	+ve	-ve	1	-ve	-ve	+ve	+ve	+ve	3
O78	-ve	-ve	+ve	+ve	-ve	2	-ve	-ve	+ve	+ve	-ve	2
O127	-ve	-ve	-ve	-ve	+ve	1	-ve	-ve	-ve	-ve	-ve	0
O164	-ve	-ve	-ve	-ve	+ve	1	-ve	-ve	-ve	-ve	-ve	0
O119	-ve	-ve	-ve	-ve	+ve	1	-ve	-ve	-ve	-ve	-ve	0

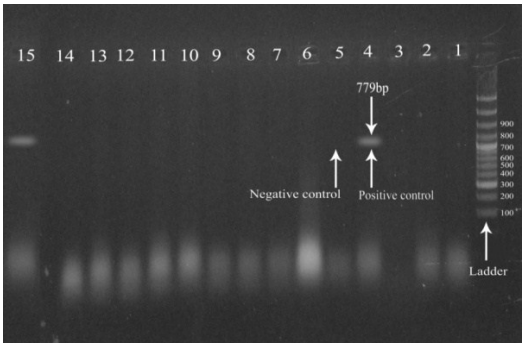
Detection of Stx1 and Stx2 genes (Shiga toxins 1& 2).

All tested isolates representing 13 *E. coli* serogroups isolated from chickens were negative to amplification of the Stx1and Stx2 genes, except one isolate belonging to serogroup O158 was positive for Stx1 gene (Photo 1, Lane 6) and the isolate representing the O group 44 Stx2 gene (Photo 2, Lane 15). All of the tested 9 serogroups of ducks origin were negative for amplification of the Stx1 and Stx2 genes.



1

Photo (1). Agarose gel electrophoresis showing amplification of 614 bp fragment using PCR, performed with primer specific for Stx1gene (Shiga toxin 1) gene of *E. coli* in chickens. Lane (5). Negative control (Salmonella Enteritidis ATCC 13076). Lane (4). Positive control (*E. coli* ATCC 43894). Lane (6). was positive for Stx1 gene.

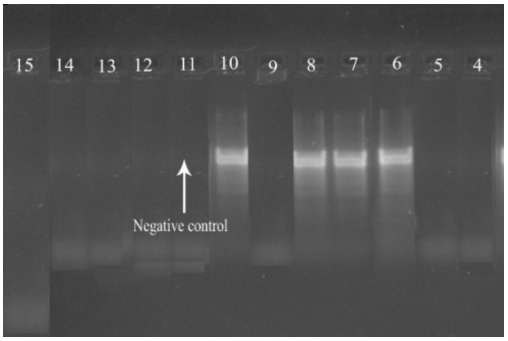


2

Photo (2). Agarose gel electrophoresis showing amplification of 779 bp fragment using PCR, performed with primer specific for Stx2 gene (Shiga toxin2) gene of *E. coli* in chickens. Lane (5). Negative control (Salmonella Enteritidis ATCC 13076).
Lane (4). Positive control (*E. coli* ATJCC 43894). Lane (15). was positive for Stx2 gene.

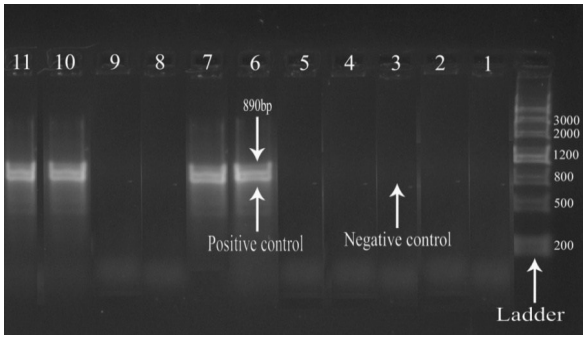
Detection of eae A gene (intimin).

Photo 3 records the amplification of 890 bp fragment of eae A gene (intimin) from chicken's *E. coli* isolates belonging to the O groups O44, O158, O125, O114 and O78. Isolates representing the O groups O26, O91, O111, O142, O127, O103, O164 and O119 were negative to this eae A gene. Photo 4 demonstrates the amplification of 890 bp fragment of eae A gene (intimin) from duck's ***E. coli*** isolates belonging to the O groups O158, O78 and O26. Isolates representing the O groups 114, O44, O91, O111, O103 and O125 were all negative to this eae A gene.



3

Photo (3) : Agarose gel electrophoresis showing amplification of 890 bp fragment using PCR, performed with primer specific for eae A gene of *E. coli* in chickens.
Lane: (11) Negative control (Salmonella Enteritidis ATCC 13076).
Lane: (2) Positive control (*E. coli* ATCC 43894).
Lanes (3, 6, 7, 8, 10) were positive for eae A gene.

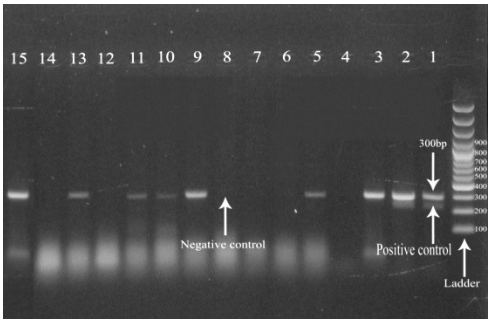


4

Photo (4). Agarose gel electrophoresis showing amplification of 890 bp fragment using PCR, performed with primer specific for eae A gene of *E. coli* in ducks.
Lane: (3) Negative control (Salmonella Enteritidis ATCC 13076). Lane: (6) Positive control (*E. coli* ATCC 43894). Lanes (7, 10, 11) were positive for eae A gene

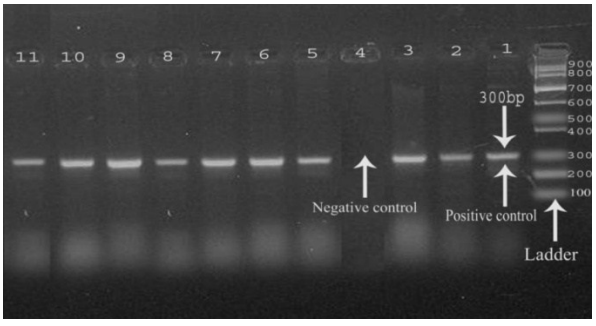
Detection of iron uptake transport IUTA gene of *E. coli* in chickens:

Photo 5 illustrates amplification of 300 bp fragment of IutA gene from the extracted DNA of *E. coli* in 8 serogroups, O114, O44, O111, O26, O142, O78, O103 and O91, while Photo 6 indicates positive amplification of 300 bp fragment of IutA gene from all *E . coli* isolates of duck origin belonging to the tested serogroups, O114, O44, O91, O111, O26, O158, O78, O103 and O125.



5

Photo (5). Agarose gel electrophoresis showing positive amplification of 300 bp fragment using PCR, performed with primer specific for IUTA gene of *E. coli* in chickens. Lane: (8) Negative control 6). Lane: (1) Positive control). Lanes (2, 3, 5, 9, 10, 11, 13, 15) were positive for IUTA gene.

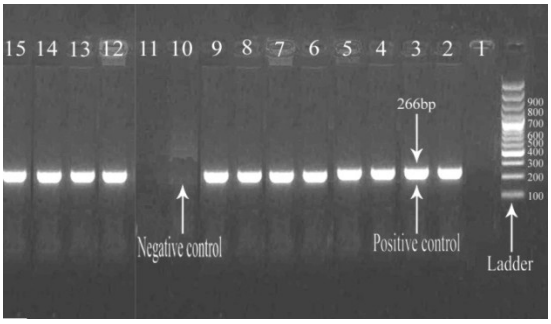


6

Photo (6). Agarose gel electrophoresis showing positive amplification of 300 bp fragment using PCR, performed with primer specific for IUTA gene of *E. coli* in ducks.Lane: (4) Negative control.
Lane: (1) Positive control.

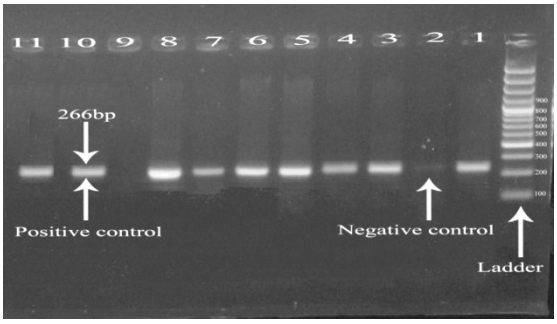
Detection of increased serum survival ISS gene of *E. coli:*

Photo 7 shows positive amplification of 266 bp fragment of ISS gene from ***E. coli*** isolates of chickens representing 11 serogroups (O44, O91, O111, O158, O125, O114, O142, O127, O103, O164 and O119). Only isolates of the O groups 26 and 78 were negative. Photo 8 illustrates positive amplification of 266 bp fragment of ISS gene from the extracted DNA of ducks's *E. coli* in 8 serogroups O114, O44, O91, O111, O158, O125, O103 and O26 in addition to that of the standard strain only O78 with negative.



7

Photo (7);Agarose gel electrophoresis showing positive amplification of 266 bp fragment using PCR, performed with primer specific for ISS gene of *E. coli* in chickens. Lane: (10) Negative control. Lane: (3) Positive control. Lanes (2, 4, 5, 6, 7, 8, 9, 12, 13, 14, 15) were positive for Iss gen

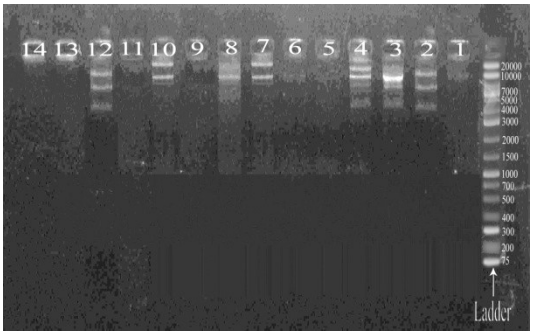


8

Photo (8). Agarose gel electrophoresis showing positive amplification of 266 bp fragment using PCR, performed with primer specific for iss gene of *E. coli* in ducks. Lane: (2) Negative control. Lane (10) positive control. Lanes (1, 3, 4, 5, 6, 7, 8, 11) were positive for ISS gene.

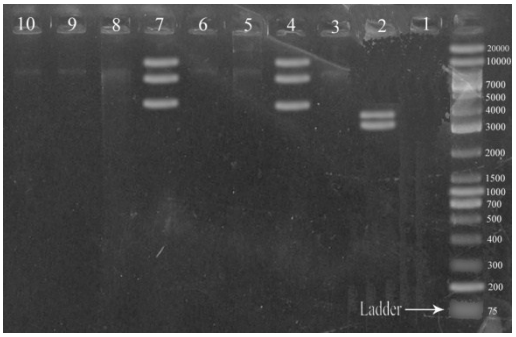
Detection of Plasmids of isolated *E. coli* strains:

The Plasmids were separated from different *E. coli* strains, by using Qiprep spin mini prep Kit. The specific DNA bands were linear plasmid, coiled plasmid or super coiled plasmid. Photo (9) demonstrates the plasmid profiles of *E. coli* isolates belonging to 14 O serogroups recovered from chickens. Three bands were detected in four O groups (O44, O158, O111, O114). Two bands were found in O groups O78, O103 and O125, while only one band was seen in group O142. No bands were detected in six O groups (O91, O127, O26, O164, O119 and O86). Photo (10) demonstrates the plasmid profiles of *E. coli* isolates belonging to 10 O serogroups recovered from ducks, Three bands were detected in two O groups (O158 and O114), two bands in one O group O44. No bands were detected in O groups (O103, O91, O111, O125, O78, O26 and O119).



9

Photo (9). 1.5% Agarose gel electrophoresis showing positive molecular weight of plasmids detected in various *E. coli* O groups: (Lane.1:10Kb). (Lane.2:4Kb, 7Kb, 10Kb). (Lane.3:4Kb, 7Kb, 9Kb). (Lane.4: 4Kb, 9Kb, 10Kb). (Lane.7:9Kb, 10Kb). (Lane.8:9Kb, 10Kb). (Lane.10: 9Kb, 10Kb). (Lane. 12: 4Kb, 7Kb, 10Kb).



10

Photo (10).1.5% Agarose gel electrophoresis showing positive molecular weight of plasmids detected in various *E. coli* O groups: (Lane.2:3Kb, 4Kb). (Lane. 4:4.5Kb, 8Kb, 10Kb). (Lane. 7:4.5Kb, 8Kb, 10Kb).

Results of pathognicity test of *E. coli* strains in one day old chicks

Intra tracheal inoculations of one day old chicks with 0.1 ml of 24 hour nutrient broth culture containing (10^7) CFU/chicks of different isolates of *E. coli* were carried out to detect their pathogencity. Dead birds showed pericarditis, perihepatitis, air sacculitis and liver congestion. The results indicated that the serogroups (O44, O158, O114, O111, O91 and O78) caused mortality rates varied between (60%-80%), while in serogroups (O103, O125, O142, O26, O127, O164 and O119), the mortality rates were much lower (30%-40%).

Discussion

E. coli strains that cause infections usually possess one or more virulence properties that may help in establishment of the infection; among these factors is Congo red binding activity. Congo red is a simple dye that can be readily incorporated into an agar gel media. Congo red positive (CR+) *E. coli* colonies are dark red due to binding of the dye and colonies that do not bind the dye (CR-) demonstrate a smooth white colonial morphology. **Vinal (1984), Berkhoff and Vinal (1986) and Gjessing and Berkhoff (1989)** confirmed that, binding of Congo red dye has been associated with pathogenicity of the *E. coli*.

In the present study, the highest Congo red binding rate was in strains of *E. coli* O142 (94.4%) isolated from chickens, followed by O158 (92.6%), while strains of the O groups O57, O6, O148, O18 and O8 , did not bind to Congo red . In case of *E. coli* isolated from ducks ,the highest Congo red binding rate was in strains of *E. coli* O114 (85.7%), followed by O103 and O158 (83.3%), while strains O86, O118, O145, O157, O126, O55, O57, O6, O148, O18 and O8 did not bind to Congo red. **Hussein (1998**) stated that the in vitro pathogenicity test using Congo red medium revealed that all serogroups strains O78, O25, O128, O158, O119, O126, O115 and O125 bound Congo red, giving red colonies, which was considered as CR positive after 18-24 hrs of incubation.

On the other hand, it was reported that not all the pathogenic *E. coli* serogroups absorb the dye and produce Congo red positive colonies **(Quadri *et al*, 1988, Panigrahy and Ling, 1990** and **Marwah *et al*., 2010).**

Corbett *et al*. (1987) and Sluggard *et al*. (1989) used Congo red dye to differentiate systemically invasive from non invasive *E. coli*. Also Harry and Yoder (1989) used Congo red binding to determine the pathogenicity of *E. coli* isolates from poultry. In fact, the nature of Congo red binding phenomenon has not yet been understood but it is suggested by **Berkhoff and vinal (1986**) to be associated with the presence of B-D-glycan in the bacterial cell wall.

The correlation between Congo red binding of *E. coli* and pathogencity in poultry was confirmed **by Stebbins *et al*. (1992), Zou El-Fakar (1994), Sanjiv *et al*. (1996) and Hussein (1998).** In this respect**, EL-ashker (2006)** reported the 70% of *E. coli* isolated from poultry were (CR+), while **Hassan (2009)** reported (CR+) a rate of 100%.

The ability of bacteria to cause haemoagglutination of different erythrocytes in the presence of D-mannose (Mannose resistance haemoagglutination, MRHA) has been used to screen for the presence of the colonization factor antigens (CFAS) **(Evans *et al*., 1979**). Fimbriae have been well recognized as specific mediators and adhesion of different host epithelial surfaces **(Krogfelt, 1991**).

It was proposed that adherence of fimbriated bacteria is blocked by the presence of D-mannose, suggesting that the host cell receptor contains mannose or mannose like residues (**Pearce and Bachmann, 1980**). Moreover, it was declared that the majority of *E. coli* strains (pathogenic or non-pathogenic) express mannose sensitive haemoagglutination (MSHA) for various animal species **(Ørskov and Ørskov, 1983).** The use of D-mannose with HA is considered to be more specific to clear up the virulent strains from most a virulent strain (**Abd EL-Moti *et al*., 1993**). Examination of isolated *E. coli* strains from (Chickens and Ducks) in the present study for Mannose resistance hae-

magglutination (MRHA) revealed that (87.5%) chicken isolates and (77.3%) of duck's isolates were (MRHA).

Haemolysis of erythrocytes is considered as an important virulence factor for some strains of *E. coli* to overcome host defense mechanism through haemolysis production, which is related to the release of iron into the bacterial environment and cytotoxic effect on neutrophiles **(Cavalieri *et al*, 1984**). In the present study, (73%) of isolates obtainted from chickens and (56.7%) of isolates obtained from ducks were haemolytic on sheep blood agar. The highest haemolytic activity in chickens was observed in strains of the O103 (94.7%) and O78 (94.4%). In case of *E. coli* isolated from ducks, the highest haemolytic activity was recorded in the strains of the O103 and O158 (83.3%). Some strains did not have haemolytic activity, e.g. strains of the O157, O57, O6, O18 and O8 from chickens and ducks.

Shiga toxin- producing *E. coli* (STEC), also called Vero toxin producing *E. coli* (VTEC), strains are the important recently emerged group of food- borne pathogens that can cause severe gastrointestinal disease in human and complication as haemolytic uremic syndrome (HUS**). (Paton and Paton, 1998). Levine 1987 and O'Brien *et al*. (1982**) found that VT plays a role in the pathogenesis of diarrhoea. **Peighambari *et al*. (1995**) found no isolates that showed cytotoxic effects.

The commonly used method for the detection of Shiga toxin production of *E. coli* is the testing of their activity on vero cell and HeLa cell and hence the name vero cytotoxic (VT) and vero toxin producing *E. coli* (VTEC). The test was used by many authors **(Valente *et al*., 1984, Levine ,1987, Irwin *et al*., 1989, Emery *et al*. ,1992, Heuvelink** *et al*., 1999**, Chart *et al*., 2001, and Singh *et al*., 2009).**

Irwin *et al*. (1989) determined the prevalence of verocytotoxin producing *E. coli* in chickens, while **Emery *et al*. (1992**) reported that (22.5%) of chickens isolates produced a distinct labile toxin that was cytotoxic for vero cells. On the other hand, Singh *et al*. (2009) found only 2 out of 22 *E. coli* isolates to produce VT.

The present study showed that *E. coli* serogroups O44and O158 isolated from chickens were cytotoxic to vero cells. In contrast, other serogroups (O114, O91,O111, O125,O103, O142, O26, O78, O127, O164 and O119) obtained from chickens were non- cytotoxic for vero cells. On the other hand, serogroups (O44, O158, O114, O91, O111, O125, O103, O142, O26, O78, O127, O164 and O119) obtained from ducks were non- cytotoxic for vero cells. O'Brien *et al*. (1982) found that about 90% of *E. coli* produces VT and Blanco *et al*. (1997) found VTEC to range from (0-100%).

The most commonly detected virulence genes in *E. coli* strains are heat- labile (LTI, LTlla and LTllb) and heat- stable (STI and STI) toxins, verotoxin types 1, 2 and VT2e, respectively), cytotoxic necrotizing factors (CNF1 and CNF2), attaching and effecting mechanisms (eaeA), enteroaggregative mechanisms (Eagg), and entroinvasive mechanisms (Einv). The virulence genes, such as iutA, iss were detected significantly more often amongst colibacillosis -derived *E. coli* isolates than amongst faecal isolates from healthy chickens **(Delicato *et al*., 2003).**

In the present study, *E. coli* isolates belonging to 13 O groups were tested for 5 virulence genes. The 300 bp fragment specific of iut A gene could be detected in *E. coli* of 8 serogroups in chickens (O114, O44, O111, O26, O142, O78, O103 and O91) and 9 serogroups in ducks (O114, O44, O91, O111, O26, O158, O78, O103 and O125). Moreover, the amplification of 266 bp fragment of iss gene was successful in 11 *E. coli* serogroups in chickens (O44, O91, O111, O158, O125, O114, O142, O127, O103, O164 and O119) and 8 serogroups in ducks (O114, O44, O91, O111, O26, O158, O103 and O125).

The obtained results were similar to those reported by **Eliana *et al*. (2003**), who examined by PCR the presence of 16 of those genes in 200 colibacillosis isolates from their region, and **Zhao *et al*. (2005),** who reported that eighty-four percent of isolates of

E. coli from birds were PCR positive for the increased serum survival (iss) gene; **Hassan (2009**) reported that the iss gene was detected in all of the 11 serogroups isolated (100%).

Intimin is encoded by the chromosomal gene eae A, which is part of pathogencitiy termed the locus for enterocyte effacement, and closely associated with STEC types carrying the eae A gene for intimin (Schmidt *et al*., 1995, Paton and Paton, 1998). The amplification of the 890 bp fragment of eae A gene (intimin), in the present work, was successful from the extracted DNA of *E. coli* isolates belonging to the O groups(O44, O158, O125, O114 and O78), whereas isolates representing the O groups (O26, O91, O111, O142, O127, O103, O164 and O119) were all negative to this eae A gene from chickens. The gene was also detected in duck's isolates belonging to the O groups (O158, O78 and O26), but not isolates representing the O groups (O114, O44, O91, O111, O103and O125). These results were similar to those reported by **Janben *et al*. (2001)**, who examined 150 *E. coli* strains isolated from visceral organs of poultry having died from colibacillosis for the presence of virulence-associated genes by PCR. **EL-ashker (2006**) examined 10 strains for the presence of intimin gene and found 1 out of 10 (10%) *E. coli* isolates contained the gene.

The increased serum survival gene iss has long been recognized for its role in extra intestinal pathogenic *E. coli* virulence; iss has been identified as a distinguishing trait of avian but not of human **(Johnson *et al*., 2008). Parreira and Gyles. (2002**) determined the presence of Stx genes in avian pathogenic *E. coli* (APEC) in 52 out of 97 *E. coli*. In the present study, positive amplification of 266 bp fragment of iss gene from *E. coli* isolates of chickens was obtained in 11 serogroups (O44, O91, O111, O158, O125, O114, O142, O127, O103, O164 and O119). Only isolates of the O groups 26 and 78 were negative. Positive results were also obtained in ducks's *E. coli* isolates belonging to 8 serogroups: O114, O44, O91, O111, O158, O125, O103, while only O78 was negative.

In the present work, all 13 serogroups of *E. coli* of chicken origin were negative for amplification of the Stx1 gene (Shiga toxin1) except one isolate belonging to serogroup O158, which was positive for this gene. Similarly all isolates representing the tested O groups were negative to the Stx2 gene (Shiga toxin 2), except one isolate belonging to serogroup O44 which was positive for this gene. All of the tested 9 serogroups of duck origin were negative for amplification of the Stx1 gene (Shiga toxin1) and the Stx2 gene (Shiga toxin 2). These results are of great importance and need further studies of large number of avian pathogenic *E. coli*, because of the increasing role of non -O157 (STEC) infection in humane, e.g. O103 which was reported by **Tzschoppe *et al*. (2012**) to cause problems in Germany and O26 was also incriminated to cause disease in man in Italy. In the present study 5 isolates of O 103 isolated from chickens were found to be positive to CR, HA and haemolysis but negative to verotoxins. They possessed only iuta and iss genes. It is to be noted, that both serogroups were isolated in the present study from chickens and ducks, but they were negative for both toxins and showed no toxic effect on vero cells.

Plasmids play an important role in virulence of *E. coli* (**Kovudzhiiski *et al*. 1982).** The R-plasmids have been extensively studied in view of the prevalence of multiple antimicrobial resistances. **(O'Brien *et al*. 1982).** Several virulence-associated genes were reported on plasmids of *E. coli* isolated from diseased poultry (Kelly *et al*., 2009). Tivendale *et al*. (2004) demonstrated that highly virulent strains of *E. coli* carried at least 4 virulence-associated genes on their largest plasmids. These large plasmids play an important role in invasion of chickens' tissue through the air sac. The strains which had lost their plasmid showed a low virulence against chickens **(Sekizaki *et al*., 1989).**

In the present study, *E. coli* isolates belonging to 14 O serogroups isolated from chickens were examined for their plasmid profiles .*E. coli* isolates of the O groups O44, O158, O111

and O114 in chickens and O158 and O114 in ducks harboured 3 plasmids of molecular weights nearing between (4.5kb and 10kb). Two plasmids were found in O groups O78, O103 and O125 in chickens with molecular weights (9Kb, 10Kb) and O44 in ducks with molecular weights (3Kb, 4Kb) and one band of (10kb) was present in isolates of O142. No plasmids were detected in 6 serogroups in chickens (O91, O127, O26, O164, O119 and O86) and in 7 serogroups in ducks (O103, O91, O111, O125, O78, O26 and O119).

It was concluded that there was an association between large plasmids containing strains and their high virulence (**Marwah *et al*., 2010). (Spears *et al*., 1990)** reported high molecular weight plasmids to be associated with pathogenic strains of *E. coli*. **Kelly *et al*. (2009)** examined a series of strains, the pathogenicity of which had previously been determined by aerosol exposure, for possession of large plasmids and found that all isolates carried at least one large plasmid, regardless of the level of virulence. Virulence-associated genes carried on these plasmids were also examined, and it was shown that highly virulent strains carried at least four virulence-associated genes on their largest plasmid. Two of the virulence-associated genes were shown to be chromosomally located in a strain of intermediate virulence, while no virulence-associated genes were carried by the low-virulence strain.

The pathogenicity of *E. coli* isolates recovered from chickens in the present work was confirmed in day old chicks and all of them were highly pathogenic to chicks and mortality rate reached from 50-100%. These findings were confirmed by **Bassiouni *et al*. (1979), Khalid (1990) and Ayman (1999**), who stated that the majority of *E. coli* strains that were isolated from colisepticaemic chickens were pathogenic for baby chicks. These findings support the statement of **Sinha *et al*. (1985**), who recorded a similar observation in artificially inoculated one day old chicks. It was reported that 60% of *E. coli* (45 strains isolated from chickens with air sacultitis) were pathogenic to 1- day-old. **Pourbakhsh *et al*. (1997) and Tudela (1999)** reported that after inoculation of pathogenic chickens *E. coli* strains, the bacteria could be re -isolated from kidney, liver and spleen at high levels. Macroscopic findings showed severe hypertrophy and congestion of the spleen. Clinical signs observed among chicks were depression, dropping of wing, dullness, closed eyes and diarrhea. Chicks which died at 48 hrs post infection showed no clinical symptoms. Most of the examined serovars produced the symptoms. The post mortem lesions of the dead chicks were congestion of internal organs; petechial hemorrhages on the heart, liver and spleen (Septicaemic picture), diarrhoea with pasty vent began to appear. These results come in agreement with **Abdel Ghafar (1979), Youssef *et al*. (1983) and EL-Shamy (1994).**

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دراسة على علامات الضراوة الظاهرية والجينية على الميكروب القولونى المعزول من الدواجن

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*** المعمل القومى للرقابة البيطرية على الانتاج الداجنى**

**** قسم الميكروبيولوجى كلية الطب البيطرى - جامعة القاهرة**

الملخص العربى

تم دراسة علامات الضراوة على عدد 495 معزولة الميكروب القولونى (345 من الدجاج و 150 من البط) وكانت النتائج كالاتى :-

285 عينه من 345 عينه المعزولة من الدجاج بنسبة 82.6% قادرة على الاتحاد بصبغة الكنجورد فى حين كانت النسبة فى العينات المعزولة من البط 74.7% بالنسبة لاختبار تلازن الدم كانت المعزولة من الدجاج الايجابية 302 ومن البط كانت العينات الايجابية 114 عينه

بالنسبة لنشاط خاصية ذوبان الدم كانت نسبة العينات الايجابية المعزولة من الدجاج 73% وبينما العينات الايجابية المعزولة من البط كانت 56.7% كانت المعزولة O44 ، O 158 أنها تأثير ضار على خلايا الفيرو وباقى المعزولات من الدجاج كانت غير ضارة على خلايا الفيرو وكانت جميع المعزولات من البط ليس لها تأثير ضار على خلايا الفيرو

بالنسبة بالكشف عن الجينات المسئولة عن الضراوة فى المعزولات من الدجاج وجدان جين STX1 موجود فى واحدة O158 وجين Stx2 موجود فى معزولة O44

بالكشف عن جين eae A وجد أنه موجود فى O44 ، O 158 ، O125 ، O114 ، O78

بالكشف عن جين iut A وجد فى العترات O114 ، O44 ، O111 ، O26 ، O78 ، O103 ، O91

بينما جين ISS كان موجود فى O44 ، 091 ، 0111 ، 0158 ، 0125 ، 0114 ، 0142 ، 0142 ، 0127 ، 0103 ، 0164 لا يوجد جينStx1،Stx2 فى جميع المعزولات ومن معزولات البط تم الكشف عن الجينات الاتية:-

جين eaa A موجود فى O 158 ، O26 ، O78

جين iut A موجود فى معزولات O114 ، O44 ،091 ، 0111 ،O26 ،0158 ،O78 ، O103 ، 0125

بينما جين ISS كان موجود فى O44 ،O114 ، 091 ،0111 ، 0158 ، 0125 ،0103 ،O26

بالكشف عن البلازميد وجد ثلاث بلازميد فى O114 ، 0111 ، 0158 واثنين فى عترات O78 ، 0103 ، O125 وواحد فقط فى 0142 فى المعزولات من الدجاج بينما فى البط 3 بلازميد فى 0158 ،O114 واثنين فى O44 باجراء العدوى الاصطناعية فى الدجاج وجد أن معزولات الميكـروب القولونـى O44 ، 0158 ، O114 ، 0111 ،091 ، O78 سببت نسبة وفيـات (60% - 80%) بينما عترات 0103 ،0125 ،0142 ، O26،0127 ،0164 ،0119 سببت نسبة وفيات أقل(30- 40%)