Virulence-associated genes in pathogenic *E.coli* isolated from ostriches

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Abstract
A total of 200 samples from ostriches at different ages (100 from diseased ostriches and 100 from freshly dead) were collected from different ostriches farms. All cases were subjected to post-mortem and bacteriological examinations. About 95 samples were positive for *E.coli* isolation with an incidence of 47.5%. Thirty (15%) out of 100 diseased ostrich were positive for *E.coli*, while from 100 freshly dead ostrich, 65 samples (32.5%) were positive for *E.coli.*
Escherichia coli isolates were serotyped as O2, O78, O91, 026, O103, O126 and untyped. The highest percentage of the isolates were belong to O2 (22.1%) then O78 (18.9%), followed by O91 (16.8%), O26 (13.6%), O103 (11.5%) and finally O126 (9.4%). While 7.3% of the isolated *E.coli* were untyped. Pathogenicity tests of the isolated *E.coli* were studied in one day old chicks. Symptoms, mortalities and postmortem lesions were recorded and discussed in details. The virulence genes (*IutA* and *Iss* genes) were detected by PCR in all isolated *E.coli* strains.
The more recent use of genetic approaches for the identification of new virulence factors will greatly enhance our knowledge of APEC pathogenic mechanisms.

Key words*: E.coli*- ostriches – virulence- isolation.

Introduction

In recent years ostrich farming is becoming popular in many countries namely UK, Kenya, Namibia, Zimbabwe, Australia, Europe and recently in Zambia **(Mellett, 1993; Pandey *et al*., 2001** and **Abdel-Razik, 2007)**. In Egypt, the development of ostrich farming is dramatically increased in the last five years, to meet local and international markets, particularly for meat and leather production. Its future success depends on its high quality and disease free products **(Youseif *et al*., 2001; El-Gohary, 2003** and **Ali and Ibrahim, 2004)**.
Colibacillosis is one of the serious problems for the ostrich industry, since it causes high morbidity and mortality with high economic losses. The etiology of colibacillosis is *Escherichia coli* which is present in the normal microflora of the intestinal tract and environment of ostrich, but certain strains designated as avian pathogenic *E.coli* (APEC) possess specific virulence factors and are able to cause avian colibacillosis. Avian pathogenic *Escherichia coli* (APEC) can induce a variety of diseases like air sacculitis, pericarditis, peritonitis, salpingitis, synovitis, osteomyelitis or yolk sac infections. The most common form is infection of the respiratory tract frequently followed by septicemia **(Knobi *et al*., 2001; Delicato *et al*., 2003** and **Abdel-Razik, 2007).** The lack of information regarding the pathogenesis of APEC infections and the recent finding that septicemic *E.coli* harber virulence genes, prompted us to investigate the presence and distribution of several virulence-associated genes in ostrich field strains to get more insight in their virulence genotypes and to improve the possibilities to characterize APEC **(JanBen *et al*., 2001)**.
The aims of the present study were to isolate the *E.coli* from the diseased and dead ostriches and then characterize the isolated *E.coli* biochemically and serologically. Study the pathogenicity of the isolated *E.coli* in one-day old chicks and then detect the virulence genes associated with the isolated *E.coli*.

Material and Methods

Samples

A total of 200 samples of ostriches (100 sam-

ples from diseased ostriches and 100 samples from recently dead ones) were collected from different ostrich's farms at different localities in Egypt. Samples were collected aseptically and were representative for different ages. Collected samples included cloacal swabs from diseased ostriches, and organs (heart, liver, lung, air sacs and bone marrow, showed lesions in postmortem examination) from recently dead ostriches. Postmortem examination was done for dead birds. Type and number of examined samples are shown in table (1).

Table (1). Type and number of examined ostriches samples.

Types of samples	No. of samples
Cloacal swabs	100
Heart	20
Livers	20
lungs	20
Air sacs	20
Bone marrow	20
Total	200

Bacteriological examination

All collected samples were inoculated in buffer peptone water and brain heart infusion broth (Oxoid), and incubated at 37 °C for 24 hours. Direct inoculation of different samples on sheep blood agar, nutrient agar and MacConkey agar plates then incubated all tubes and plates at 37 °C for 24 hours. A loopful from each broth culture was inoculated onto blood agar, MacConkey agar (Oxoid) and XLD agar (Oxoid) plates and incubated at 37°C for 24 hours. Isolated colonies were identified morphologically, microscopically and biochemically according to **Cruickshank *et al*., (1975).**

Serotyping of *Escherichia coli* isolates

E.coli isolates were serotyped according to **Lennette, (1980)** using the available O antisera.

Pathogenicity tests of different isolated bacteria :

A total of seventy, one-day old chicks were used for testing the pathogenicity of different *E.coli* strains. The chicks were kept in cages and observed for a period of a week and proven to be healthy. Chicks were divided into 7 groups (10 chicks per group). Chicks in group 1 were kept as negative non-infected control group, while chicks in groups 2, 3,4,5,6 and 7 were inoculated orally with 1 ml of 3X 10^7 C.F.U/ml of 24 hours broth culture of each *E.coli* isolated **strain (Abdel-Razik, 2007).** The chicks in all groups were fed on unmedicated ration and clean water and kept under observation for one week. Symptoms, mortalities and postmortem lesions were recorded. Re-isolation of the experimentally infected *E.coli* strain in each group was carried out.

Detection of virulence genes in *E.coli* isolates by PCR technique

1. Extraction of DNA by boiling method **(Croci *et al*., 2003).** One ml of broth was centrifugated for 10 min at 16000 xg and the pellet was washed twice with sterile PBS, resuspended in 25 µL sterile PBS, incubated at 120 °C for 15 min and then cooled on ice. After a final centrifugation for 2 min at 16000xg, supernatants were cooled at 4 °C and directly used for PCR.

2. DNA amplification. The amplification reaction was performed by using DNA thermal cycler (T3 thermalcycler Biometra, Germany). All reactions were carried out in a final volume of 25 µl in PCR tubes. Base sequences and predicated sizes of amplified products for the specific primers used in this study are shown in table (2). Oligonucleotide primers (metabion, 20 pmol concentration) according to (Delicato *et al*., 2003) for the DNA amplification of the 300bp of *iutA* gene of *E.coli*. 1.5 µl of the purified DNA was added to 12.5 µl of the prepared master mix (Thermoscientific ready mix). Each reaction mixture was overlaid with 1 or 2 drops of mineral oil to avoid evaporation of the reaction mixture. The samples were subjected to preincubation at 94°C for 5 min., followed by 30 cycles of denaturation (94 °C for 1 min), annealing (63 °C for 1 min) and extension (72°C for 2min). For the DNA amplification of the *Iss* gene of *E.coli* (Yaguchi *et al*., 2007), add 1µl of forward primer (20 p Mol) (Lena-

christ-strass Metabion international AG) and 1µl of reverse primer (20 p Mol) (Lena-christ-strass Metabion international AG) each primer pair was used separately. Each reaction mixture was overlaid with 1 or 2 drops of mineral oil to avoid evaporation of the reaction mixture. The samples were subjected to preincubation at 94 °C for 5 min., followed by 35 cycles of denaturation (94 °C for 1min), annealing (54 °C for 1min) and extension (72 °C for 2min). The PCR products were stored in the thermal cycler at 4 °C until they were used.

3. Screening of PCR products by agarose gel electrophoresis **(Sambrook *et al*., 1989).** 10 µL of PCR product was analyzed by standard submarine gel electrophoresis (2% lonza agarose, 5v/cm) and 0.5µg/ml ethedium bromide. Visualization of the resulted product was done by location on gel documentation system (UV star 312nm, Biometra). PCR was performed, with a quality control for the test using *Salmonella enteritidis* DNA which served as negative control and *E. coli* NCIMB 50034 as a positive control.

Table (2). Primers sequences used for amplification of DNA for the detection of *E.coli* recovered strains.

Target gene	Bp fragment	Primer sequence (5´-3´)	Source
Iron uptake transport gene *IUTA*	300	F(GGC TGG ACA TGG GAA CTG G) R(CGT CGG GAA CGG GTA GAA TCG)	Lena-christ-strass Metabion international AG
Increased serum survival gene *Iss*	266	F(ATG TTA TTT TCT GCC GCT CTG) R(CTA TTG TGA GCA ATA TAC CC)	Lena-christ-strass Metabion international AG

Results and Discussion

Bacteriological examination in the present study revealed that *Escherichia coli* were isolated from the examined samples. Ninety five samples were positive for *E.coli* isolation with an incidence of 47.5%. Thirty (15%) out of 100 diseased ostrich were positive for *E.coli*, while, from 100 freshly dead ostrich (internal organs showed lesions in postmortem examination), 65 samples (32.5%) were positive for *E.coli* as in table (3). This result was nearly agreed with **Nasef *et al*., (2003) and Ali and Ibrahium (2004)** who isolated *E.coli* from ostriches by 40.4% and 40.3%, respectively. On the other hand, this is lower than those obtained by **Ali and Moursi (2003)** and **Moursi *et al*., (2003)** who isolated *E.coli* with an incidence of 55.7% and 86.6%, respectively. At the same time it was higher than those of **Kamel *et al*., (2001)** and **Afifi (2003)** who detected 34.2% and 25.6%, respectively. The sick birds showed respiratory distress included nasal discharge, coughing and hard respiration, in addition to abdominal enlargement and general symptoms (depression, recumbency, diarrhea, loss of weight and holding their heads downward). Similar clinical signs were recorded in *E.coli* infection by **Afifi (2003)** and **Ali and Moursi (2003).** The postmortem examination of dead birds revealed congestion of subcutaneous blood vessels, and of the internal organs, enteritis, pericarditis, airsacculitis, pneumonia, omphalitis and impaction of proventriculus. These lesions were similar to those recorded by **Kamel *et al*., (2000)** and **Abdel-Razik (2007)**.

Serotyping of the investigated *E.coli* isolates revealed that they belonged to O2, O78, O91, 026, O103, O126 and untyped (Table 4). The highest percentage of the isolates were belong to O2 (22.1%) then O78 (18.9%), followed by O91 (16.8%), O26 (13.6%), O103 (11.5%) and finally O126 (9.4%), while 7 isolates (7.3%) were untyped. Similar results were recorded by different investigators **(Kamel *et al*., 2001; Nasef *et al*., 2003 and Abdel-Razik, 2007)**.

The results of pathogenicity tests (Table 5) revealed that *E.coli* strains isolated from ostriches in general, were highly pathogenic to one-day chicks, where it caused mortalities range between 60% and 100% with symptoms of se-

vere diarrhea. The lesions revealed severe watery enteritis. The highest percentage of the mortality were belong to O2 (100%) then O78 (90%), followed by O91, O26 (80%), O103 (70%) and finally O126 (60%). The *E.coli* strains were reisolated from all the internal organs of the dead chicks. These results were in agreement with that obtained by **Youseif (1995)** who found that *E.coli* strains isolated from local and imported one day old chicks were highly pathogenic to three days old chicks (100% mortalities) after subcutaneous route of inoculation. They also agreed in a great extent with that obtained by **Nasef *et al*., (2003)** who found that pathogenicity test of *E.coli* in one-day old chicks caused 90% mortalities.

Table (3). Incidence of *E.coli* isolated from diseased and dead ostriches.

Samples	No. of examined ostriches	No. of isolation	%*	%**
Diseased bird	100	30	31.5 %	15%**
Dead bird (internal organs)	100	65	67.4%	32.5%**
Total	200	95	100%	47.5%**

*Percentage according to total number of *E.coli* isolates
** Percentage according to total number of the samples.

Table (4). Serotyping of *E.coli* strains isolated from ostriches

O serotype	No. of isolates	Percentage of isolation
O2	21	22.1%*
O78	18	18.9%*
O91	16	16.8%*
O26	13	13.6%*
O103	11	11.5%*
O126	9	9.4%*
untyped	7	7.3%*
Total	95	100%

*Percentage according to total number of *E.coli* isolates.

Table (5) Result of pathogenicity tests of *E.coli* strains isolated from ostriches in orally infected one-day old chicks.

Group No.	Bacterial agent	No. of infected chicks	Mortality rate	
			No.	%
1	Control negative	10	0	0
2	O2	10	10	100
3	O78	10	9	90
4	O91	10	8	80
5	O26	10	8	80
6	O103	10	7	70
7	O126	10	6	60

Results of PCR for detection of virulence genes associated with *E.coli* strains isolated from ostriches revealed that ***IutA*** gene and ***Iss*** gene were detected in all of the field isolates (6 serovars) examined with an incidence of 100% (figure 1, 2 and table 6, 7). These results nearly agreed with that obtained by **Hassan, (2007)** who recorded that the ***IutA*** gene and ***Iss*** gene were presented in all of the *E.coli* strains isolated from chicken suffered from colibacillosis (11/11 serovars) with an incidence of 100%. In addition to, **Delicato *et al*., (2003)** mentioned that these virulent genes were detected significantly more often amongst colibacillosis-derived *E.coli* isolates than amongst fecal isolates from healthy chickens. Moreover, **Johnson *et al*., (2008)** reported that the increased serum survival gene *Iss* has long been recognized for its role in extraintestinal pathogenic *Escherichia coli* (EXPEC) virulence and has been identified as a distinguishing trait of avian EXPEC but not of human EXPEC. On the other hand, **Mellata *et al*., (2003)** found that the ***Iss*** gene did not play a major role in resistance to serum in strain x7122. Also, **Rocha *et al*., (2008)** mentioned that the property of invading and multiplying presented by pathogens was influenced by iron availability, which is essential for growth in living cells and the aerobactin system enables microorganisms to grow in iron free media at low concentration,

Finally, it may be concluded from the present investigation that there were association between pathogenicity and the presence of virulent genes in *E.coli* strains isolated from ostriches. The more recent use of genetic approaches for the identification of new virulence factors will greatly enhance our knowledge of APEC pathogenic mechanisms.

Table (6). Results of detection of ***IutA*** gene of *E.coli* strains isolated from ostriches

Lane	Serotypes	*IutA* gene
1	*E. coli* NCIMB 50034	Positive
2	O2	Positive
3	O78	Positive
4	Ladder (100-600bp)	
5	O91	Positive
6	O103	Positive
7	O26	Positive
8	O126	Positive
9	*Salmonella enteritidis* ATCC-13076	Negative

Table (7). Results of detection of ***Iss*** gene of *E.coli* strains isolated from ostriches

lane	Serotypes	***Iss*** gene
1	*Salmonella enteritidis* ATCC-13076	Negative
2	O2	Positive
3	O78	Positive
4	Ladder (100-600bp)	
5	*E. coli* NCIMB 50034	Positive
6	O103	Positive
7	O26	Positive
8	O91	Positive
9	O126	Positive

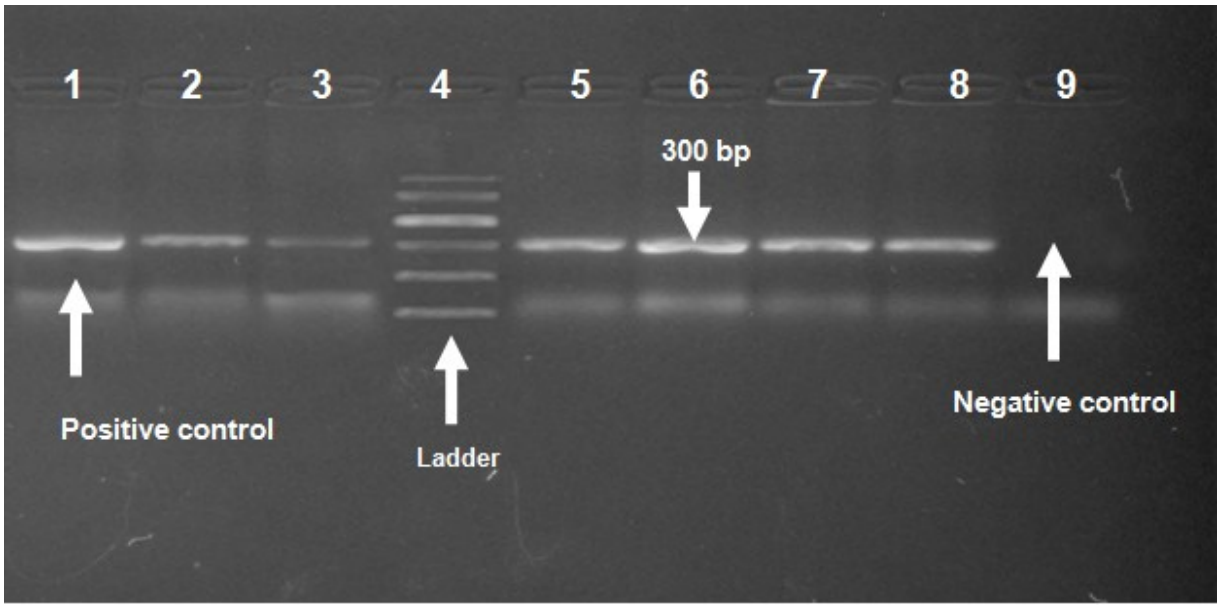

Fig. (1). Agarose gel electrophoresis showing positive amplification of 300 bp fragments using PCR was performed with primer specific for *IUTA* gene of *E. coli.* Lane 9: Negative control (*S.* Enteritidis ATCC 13076); Lane 1: Positive control (*E. coli* NCIMB 50034); Lane 4: 100 bp molecular weight marker; Lanes 2, 3, 4, 5,6,7 and 8 show positive amplification of 300 bp fragment of *E. coli* field isolates.

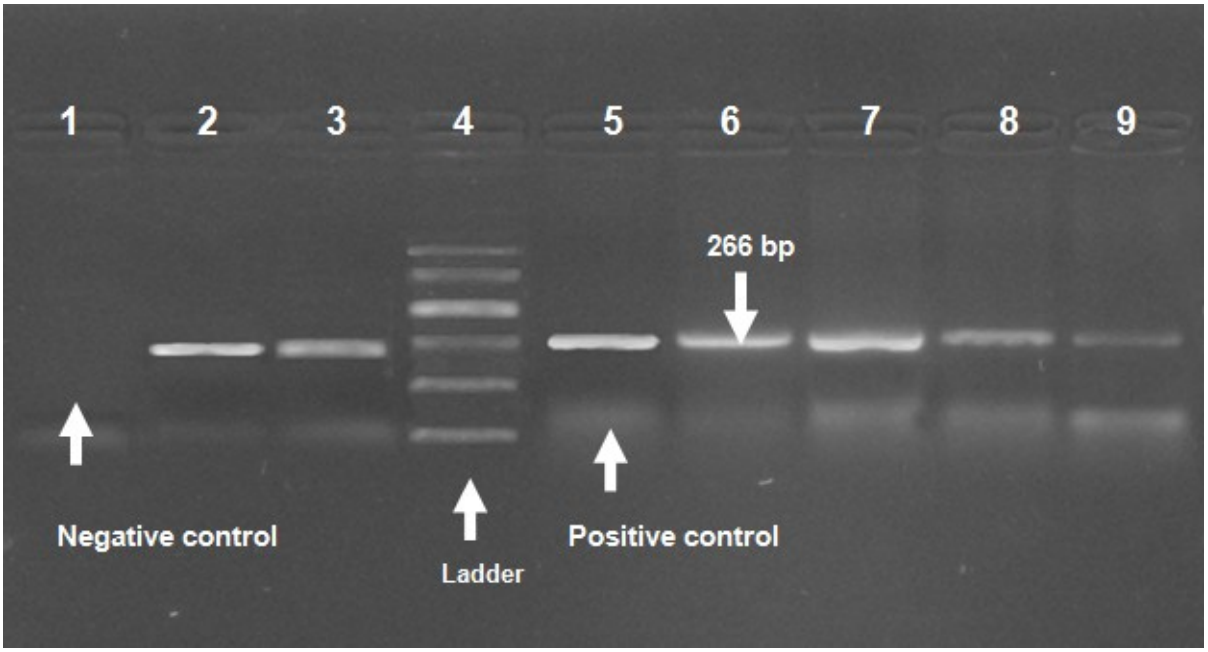

Fig. (2). Agarose gel electrophoresis showing positive amplification of 266 bp fragments using PCR was performed with primer specific for *iss* gene of *E. coli.* Lane 1: Negative control (*S.* Enteritidis ATCC 13076); Lane 5: Positive control (*E. coli* NCIMB 50034); Lane 4: 100 bp molecular weight marker; Lanes 2, 3, 6-9 show positive amplification of 266 bp fragment of *E. coli* field isolates.

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الجينات المصاحبة للضراوة في عترات الميكروب القولوني الممرضة المعزولة من النعام

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الملخص العربى

نظرا لتعدد الحالات المرضية الناتجة عن الإصابة بالميكروب القولوني في النعام مما يمثل مشكلة كبرى في مزارع النعام تودي إلى نقص في الأوزان وتكلفة علاجية ضخمة ووفيات بنسبة عالية لذلك لزم معرفة العترات المسببة لتلك الحالات المرضية مع توصيفها كيميائيا و سيرولوجيا و معرفة بعض جينات الضراوة المصاحبة لها. تم فحص 200 عينة من النعام المريض و النافق حديثا من مزارع النعام المختلفة في مصر و تم إجراء الصفة التشريحية و تجميع العينات اللازمة للعزل البكتيري.

أوضحت الفحوص البكتيريولوجية انه تم عزل الميكروب القولوني من 95 عينة بنسبة عزل 47.5% . وقد تم تصنيف عترات الميكروب القولوني المعزولة سيرولوجيا إلى O2, O78, O91, 026, O103, O126 و غير مصنف. تم إجراء اختبارات الضراوة للعترات المعزولة و تمت مناقشة الأعراض و نسب النفوق والصفة التشريحية بالتفصيل. تم الكشف عن وجود بعض جينات الضراوة في عترات الميكروب القولوني المعزولة (*IutA* and *Iss* genes)باختبار تفاعل إنزيم البلمرة المتسلسل.