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PREVALENCE AND VIRULENCE PROFILE OF AVIAN PATHOGENIC E.COLI ISOLATED FROM CLINICALLY DISEASED BROILER CHICKENS

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ABSTRACT

Escherichia coli plays a part in maintaining intestinal physiology. Although, there are pathogenic strains that cause different syndromes of diarrheal disease. E. coli strains are the cause of a number of diseases in broiler chickens, leading to death, decrease in production or condemning of carcasses. This study aimed to determine phenotypes and virulence associated genes of avian pathogenic Escherichia coli (APEC) recovered from diseased and freshly dead chickens including, shiga toxin 1, shiga toxin 2 and intimin using genotypic methods. A total of 200 chicken visceral organs (liver, lungs, spleen and heart) collected from diseased and freshly dead birds (four organs per each bird) were subjected for conventional culturing, serotyping and molecular characterization of virulence genes. 32 (16%) of isolates were biochemically identified as E.coli and confirmed by PCR using phoA gene. O78, .O8, O145 and O1 are the most prevalent serotypes from APEC isolates. stx1 and stx2 were determined by multiplex PCR , while eae gene was detected by uniplex PCR . stx2 gene was detected in 50% (16/32), while, 81.26% (26/32) of APEC isolates harbored eae. stx1 was not detected in all the tested isolates. The obtained results demonstrated the importance of studies in E. coli of avian origin in regions associated with intensive poultry industry, aiming to evaluating the predominant strains and also acquiring preventive measures to minimize losses due to APEC.

***Key words**: Phenotypes, Virulence genes, stx gene, eae gene, APEC.*

INTRODUCTION

Avian pathogenic *Escherichia coli* strains are known as APEC as described by (***Dho-Moulin & Fairbrother 1999).*** APEC may cause much diseases including extraintestinal diseases which causes an abundant losses in the poultry industry (***Gross 1994***).

Avian colibacillosis is a complex syndrome characterized by air sacculitis and associated pericarditis, peritonitis and perihepatitis being most typical with a great lesions in the organs (***Ewers et al., 2003***).The incidence and severity of colibacillosis caused by avian pathogenic *E. coli* (APEC) have increased rapidly and indicate that it seems to continue and become one of the most important reasons for economic losses in poultry production (***Altekruse et al ., 2002***).

Examination of the virulence genes by PCR has been used to differentiate avian pathogenic *Escherichia coli* (APEC) strains (***Ewers et al., 2005; Kimata et al., 2005 and Johnson et al., 2008***). Multiplex PCR technique is used for identifying the most

highly pathogenic *E. coIi* isolates in a flock. Thus, more studies have been applied to identify virulence factors of isolated pathogenic *E.coli* strain (***Kaipainen et al., 2002; Zaki et al., 2004 and Ewers et al., 2007***).

Mortality and morbidity rates associated with large infections of gastrointestinal diseases are produced by Shiga toxin *Escherichia coli* (STEC) indicating the hazard of these bacteria to public health (***Paton 1998***). STEC strains produce one or two types of Shiga toxins, designated *stx1* and *stx2*. Shiga toxigenic *Escherichia coli* (STEC), hemolytic uremic syndrome (HUS) and enteritis are associated with major mortality and morbidity rates, especially amongst patients with neurological and renal problems (***Borgattaa, 2012).*** The *stx2* is associated with increased risk of haemolytic uremic syndrome that leading to diarrhea, especially infants in the developing countries ***(Boerlin, 1999).***

The attaching and effacing (A/E) lesion that is caused by APEC strains is related to strains isolated from animals. These strains attach tightly to and colonize the epithelial cells of the villus in the small intestine and cause typical A/E lesions (***Kaper 1994***). Intimin, an outer membrane protein is a bacterial adhesion molecule, encoded by *eae* A. Intimin mediates the intimate bacterium host cell interaction characteristic of attaching and effacing lesions.

The overall objectives of this study was to investigate the presence of pathogenic *E. coli* in chicken viscera followed by determination of the virulence genes (*eae*, *stx1* and *stx2*) in the local isolates at Mansoura, Egypt.

MATERIALS AND METHOD

Samples collection:

A total of 200 chicken visceral organs (liver, spleen, lungs and heart) of clinically diseased broiler chicken (4 samples per each bird) were collected randomly from different poultry farms located in Mansoura City, Egypt, the common lesions detected in postmortem examination were pericarditis, air sacculitis, perihepatitis, ascites, splenitis and peritonitis. Samples were taken from diseased and freshly dead chickens after clinical and post mortem examination.

Isolation and identification of *E. coli* isolates:

From each chicken visceral organ, about 2 g was directly enriched in Macconkey's broth and incubated for 18 hours at 37°C. Then, a loopful was streaked onto MacConkey's agar plates from the overnight enriched culture. After overnight incubation at 37°C, rose pink colonies were picked up and streaked onto eosin methyelene blue (EMB; Becton Dickinson, Sparks, USA) and incubated overnight at 37°C. The identification of *E. coli* isolates depends upon colony characters morphological criteria and biochemical testing ***(Ewing, 1986).***

Serotyping:

E.coli isolates were serologically identified according to ***Kok et al. (1996)*** at the Department of Food Hygiene Control, University of Benha, Egypt, by using rapid diagnostic *E.coli* antisera sets (Denka Seiken Co., Japan).

DNA extraction:

DNA template was prepared from *E.coli* isolates according to ***Bridge (1996).*** 1ml distilled water was added to *E.coli* growth on slope agar then shacked well. The suspension was centrifuged and the pellet was resuspended in distilled water. The genomic DNA was extracted by boiling of the suspension for 10 minutes in water bath then the supernatants were used as DNA template for polymerase chain reaction for the confirmation of *E.coli* isolates and virulence genes identification.

Molecular identification of *E.coli*:

The biochemically identified *E.coli* was molecularly confirmed by uniplex PCR using *pho* A primer, The sequence and PCR product were summarized in Table(1). uniplex PCR was performed in a volume of 25 µL consisting of 12.5 µL of 2X PCR master mix (PROMEGA, MADISON, USA) 1 µl of each primer (100 µm each), and 6 µl DNA template. The PCR condition for the *pho* A was summarized in table (2) as described by ***Hu et al., (2011).***

Molecular identification of *stx1* and *stx2* genes using multiplex PCR:

A multiplex PCR was carried out using 2 sets of oligonucleotide primers for *stx1* and *stx2* genes. The primer pairs used (target gene, sequence and PCR product) are summarized in Table (1). PCR was performed in a volume of 50 µL consisting of 25µL of 2X PCR Master Mix (Promega, Madison, USA), 1 µL of each primer (100 µM each), and 12 µL DNA template. PCR program for both *stx1* and *stx2* genes was similar to that done by **Dipineto *et al.*, (2006)** as demonstrated in table (2).

Molecular identification of *eae* gene:

For *eae* gene (sequence and PCR product) was mentioned in table (1). The PCR condition for the *eae* gene was similar to that described by ***Bisi-Johnson et al., (2011*)** as described in table(2). Using uniplex PCR**,** PCR was performed in a volume of 25 µL consisting of 12.5 µL of 2X PCR master mix (PROMEGA, MADISON, USA) 1 µl of each primer (100 µm each), and 6 µl DNA template.

Table (1): Oligonucleatide primers sequences

Target gene	Sequences of primers	Amplified segment (bp)	References
stx1	ACACTGGATGATCTCAGTGG	614	*Dipineto et al., (2006)*
	CTGAATCCCCCTCCATTATG		
stx2	CCATGACAACGGACAGCAGTT	779	
	CCTGTCAACTGAGCAGCACTTTG		
phoA	CGATTCTGGAAATGGCAAAAG	720	*Hu et al., (2011)*
	CGTGATCAGCGGTGACTATGAC		
eaeA	ATG CTT AGT GCT GGT TTA GG	248	*Bisi-Johnson et al., (2011)*
	GCC TTC ATC ATT TCG CTT TC		

Table (2): Cycling conditions of the different primers during PCR

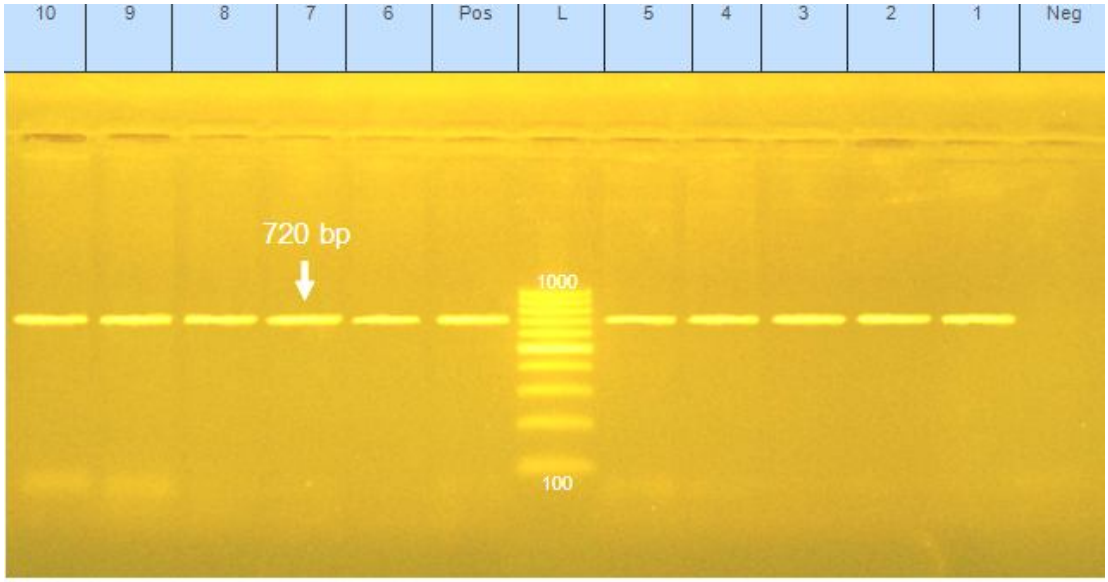
Target gene	Primary den.	Sec. den.	Ann.	Ext.	Final ext.
Stx1,2	94˚C 5 min.	94˚C 30 sec.	58˚C 45 sec.	72˚C 45 sec.	72˚C 10 min.
***pho*A**	94˚C 5 min.	94˚C 30 sec.	58˚C 45 sec.	72˚C 45 sec.	72˚C 10 min.
*eae*A	94˚C 5 min.	94˚C 30 sec.	51˚C 30 sec.	72˚C 30 sec.	72˚C 7 min.

Table (3): Distribution of APEC serotypes among examined organs

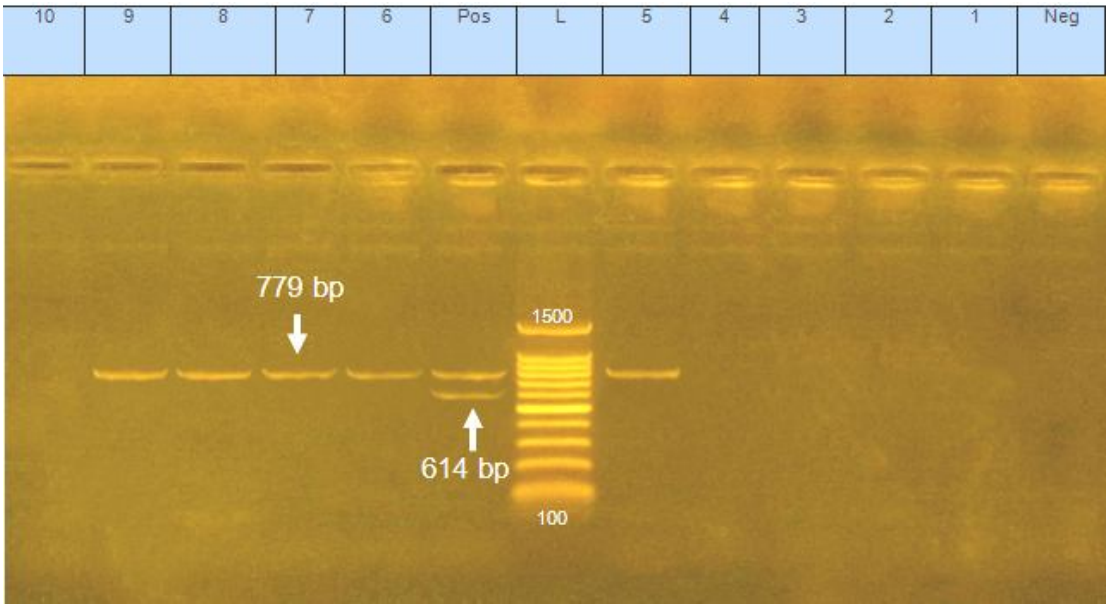
Serial No.	Serotypes	No. of strains	Type of sample	Strain characterization
1	O78	5	Intestinal contents, Spleen, Lungs, Heart(2).	EPEC
2	O8:H21	4	Spleen, Lungs, Heart.	EPEC
3	O1:H7	3	Spleen, Heart(2).	EPEC
4	O145	3	Spleen, Liver, Lungs.	EPEC
5	O117:H7	2	Lungs, Heart.	EPEC
6	O2 : H6	2	Intestinal contents, Liver.	EPEC
7	O166	2	Intestinal swab, Liver.	EPEC
8	O6 : H4	1	Lungs.	EPEC
9	O119: H6	1	Liver.	EPEC
10	O159: H21	1	Lungs.	EPEC
11	O55 : H7	2	Intestinal swab, Lungs.	EHEC
12	O111 : H2	2	Liver	EHEC
13	O26 : H11	1	Lungs.	EHEC
14	O128:H2	2	Lungs, Heart.	ETEC
15	O124	1	Spleen.	EIEC
TOTAL		*32*		

Table (4): The distribution of virulence genes among APEC serotypes

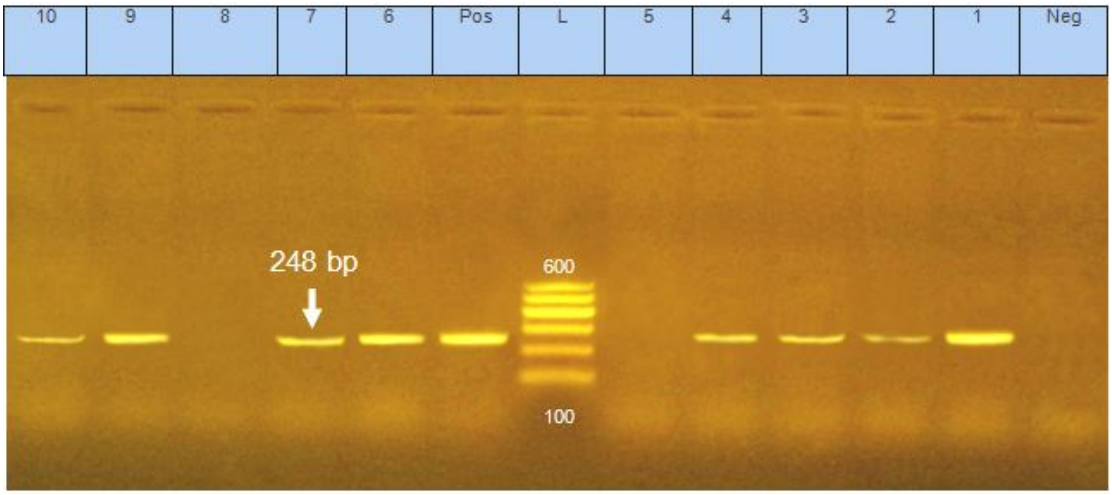
Serotypes	*Strain type*	*Number of isolates*	*Pho* A *gene*	*Stx*1 gene	*Stx*2 *gene*	*eae*A *gene*
O78	EPEC	5	5	0	5(100%)	5(100%)
O8 : H21	EPEC	4	4	0	0	4(100%)
O1 : H7	EPEC	3	3	0	0	3(100%)
O145	EPEC	3	3	0	3(100%)	0
O117::H7	EPEC	2	2	0	0	2(100%)
O2 : H4	EPEC	2	2	0	2(100%)	0
O166	EPEC	2	2	0	2(100%)	2(100%)
O6:H4	EPEC	1	1	0	0	1(100%)
O119:H6	EPEC	1	1	0	0	1(100%)
O159 : H21	EPEC	1	1	0	0	1(100%)
O55:H7	EHEC	2	2	0	2(100%)	0
O111: H2	EHEC	**2**	2	0	2(100%)	2(100%)
O26: H11	EHEC	1	1	0	1(100%)	1(100%)
O128:H2	ETEC	2	2	0	0	2(100%)
O124	EIIEC	1	1	0	1(100%)	1(100%)
Total		32	32	0	16(50%)	26(81.26%)



Photograph(1): Agarose gel electrophoresis showing amplification of 720pb fragment using phoA primer**.** (L) ladder 1000 pb. Lane (1,2,3,4,5,6,7,8,9, 10) positive. Neg (Negative control), Pos (Positive control).



Photograph(2): Agarose gel electrophoresis showing amplification of 614 pb and 779 pb fragment using stx1, 2 primer**s.** (L) ladder 1500 pb. Lane (5,6,7,8,9) positive for stx 2. Neg (Negative control), Pos (Positive control).



Photograph(3): Agarose gel electrophoresis showing amplification of 248 pb fragment eae A primer **.** (L) ladder . Lane (1,2,3,4 ,6,7, 9,10) positive for *eae* A gene. Neg (Negative control), Pos (Positive control).

RESULTS AND DISCUSSION

E. coli is present as the normal microflora in the intestinal tract of poultry, certain strains known as avian pathogenic *E. coli* (APEC) possess specific virulence factors and are able to cause avian colibacillosis. This disease causes great economic losses results from the serious problem occurred in the poultry industry ***(Emery et al., 1992)***.

E. coli infections in birds cause many clinical symptoms which are characterized by respiratory disease followed by generalized infection that ended by death. Avian pathogenic E. coli (APEC) strains are categorized as extra intestinal pathogenic *E. coli,* that are characterized by presence of virulence factors which enable to live extra intestinal (***Johnson et al., 2006***). The pathogenicity of an *E. coli* strain depends on presence and expression of potential virulence factors (***Won et al., 2009***).

In this study the isolation rate of *E. coli* was relatively high (32 isolates, 16 %). The entire 32 biochemically identified *E. coli* isolates from chicken indicates the role of the organism as potentially important avian pathogen in Egypt (Table 3). These findings are in accordance with those obtained by ***Khalid (1990); Mukhopadhyaya and Mishra (1992) and Sripoernomo et al., (1992) and Yun et al. (1997);.*** From the results presented in this work, it is evident that *E. coli* was isolated from different organs, namely liver, lungs, heart and Spleen. ***Hassanain (1977)*** and ***Mukhopadhyaya and Mishra (1992)*** isolated *E. coli* from liver, lungs, kidneys and yolk materials.

Species of *E. coli* are serologically determined depending on their antigenic composition (somatic or O antigens for serogroups and flagellar or H antigens for serotypes) into 15 serotypes (Table 3). O78, O8, O1, O145, O117, O2, O166, O6, O119, O159, O55, O111, O26, O128 and O124 are the obtained serogroups. Multiple serogroups are associated with disease production especially O1, O2 and O78 among many others ***(Dziva and Stevens, 2008).*** In this study, O78, O8, O1 and O145 are the commonly isolated serogroups ***(Gross, 1994 and Bopp et al., 2005).***

All the obtained isolates were confirmed as *E.coli* by PCR using *pho*A gene (Photograph, 1). Multiplex PCR was used for detection of *stx*1and *stx*2 (Photograph, 2), meanwhile, *eae*A gene was detected by using uniplex PCR (Photograph, 3). Among *E. coli* strains, *stx2* gene was detected in 50% (16/32), while, 81.26% (26/32) of APEC isolates harbored *eae*. *stx1* gene was not detected in all *E.coli* isolates (Table 4).

Some APEC strains are able to produce verotoxins known as Shiga-toxins (*stx*) (***Fantinatti et al. 1994, Blanco et al. 1997***, ***Parreira & Yano 1998, and Farooq et al. 2009***). *stx*- gene was identified amongst *E. coli* strains isolated from chickens with symptoms of septicemia, swollen head syndrome and cellulitis by ***Parreira & Gyles (2002***).

Verotoxigenic *E. coli* (VTEC) are a group of *E. coli* that cause renal failure and colonic disease. The pathogenicity of VTEC comes from deliver strong toxins known as shiga toxins (Stx) to the systemic circulation and virulence factors that allow the microbes to colonize the colon (***Goldwater and Bettelheim, 2012).*** In this study, presence of *stx*2 gene in APEC isolates at a rate of 50% (16/32) was confirmed by ***Parreira & Gyles (2002***) who also used polymerase chain reaction (PCR) amplification and hybridization technique for the detection of Stx genes among

APEC isolates, and reported that 52 of the 97 APEC isolates carried Stx gene. A positive relation among human extraintestinal pathogenic *Escherichia coli* (ExPEC) and APEC have showed by some studies, mainly newborn meningitis-causing *E. coli* (NMEC) and uropathogenic *E. coli* (UPEC), these studies suggested that some avian pathogenic *E. coli* strains could be considered zoonotic agents (***Moulin-Schouleur et al. 2007, Ewers et al. 2007and Johnson et al. 2008).***

Several authors have suggested a strong association between the carriage of *eae* gene and the capability of STEC strains to cause human disease (***Paton, 1998). Morabito et al., (2001)***, found that most of *E. coli* isolates that are positive for *Stx* genes, were encoding for intimin. In this study, 26 (81%) APEC isolates possessed *eae*A gene. A higher percentage of *E. coli* isolates carrying *eae* A gene was recoreded by **Kobayashi(2002)** which was 57% and 40% of chicken and gulls, respectively. However, a lower percentage (2.49 %) of *E. coli* isolates was reported by ***Wani et al. (2004)*** from chicken carrying *eae* A gene. Isolation rates of *eae* A genes among *Escherichia coli* strains have been reported in epidemiological studies from different locations worldwide. The *eae*A gene rate among strains was 60% (***Bi et al, 1999***). Another studies detected the *eae* A gene in 60.9% of *Escherichia coli* strains in poultry at Kenya (***Kariuki et al, 2002).***

In conclusion, at least one virulence gene was detected in APEC isolates. Some avian pathogenic *Escherichia coli* strains are able to produce Shiga-toxins (*stx*) which indicating the hazard of these bacteria to public health.

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الملخص العربي
عوامل الضراوه ومعدل انتشارها للاشرشيا كولاي المعزوله من دجاج التسمين المصاب

جمال يونس . أمل عوض . سماح سمير *

قسم البكتريولوجي و الميكولوجي و الامينولوجي- كلية الطب البيطري- جامعة المنصوره

الايشرشيا كولاي تلعب دور مهم في الوظائف الحيويه بالأمعاء،علي الرغم من ذلك توجد عترات مرضيه تسبب عدد من الأمراض في دجاج التسمين،هذه الأمراض تؤدي إلي الموت وتقلل الإنتاج. و هذه الدراسة تحدد عوامل الضراوه للايشرشيا كولاي المعزولة من الدجاج المصاب والنافق.