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Research Article
Genetic Variation among Avian Pathogenic *E. coli* Strains Isolated from Broiler Chickens

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Abstract

Background: Avian pathogenic *E. coli* cause serious disease in chickens, which mainly chracterised by airsaculitis, perihepatitis and pericarditis resulting in large economic losses in poultry industry worldwide. **Methodology:** In order to investigate the genetic variation among different *E. coli* strains isolated from chickens, one hundred diseased broiler chickens at Sharkia province, Egypt were examined. Liver and heart blood samples were collected from each bird and subjected to bacteriological examination, where the prevalence of *E. coli* was 60% from the total collected samples. *Escherichia coli* strains were serogrouped. The PCR was used for detection of Shiga-like toxins genes *(stx*1 and *stx*2), attaching and effacing (*eae*A) gene and enterohaemolysin gene *(hly)* in the typable isolated *E. coli* strains. **Results:** The results showed that the isolated strains belonged to 11 serogroups including O1, O2, O26, O44, O55, O78, O111, O119, O125, O127 and O128. Untypable strains were also recovered. The detected virulence genes were *stx*1 in all *E. coli* strains (100%), *stx*2 in 17 strains (47.2%), *eae*A in 12 strains (33.3%) and *hly* only in three strains (8.3%). **Conclusion:** In conclusion, the combination of genotypic and phenotypic analysis of *E. coli* is more valuable as an epidemiological tool for identification of isolates. This study established the presence of *stx*1 and *stx*2 containing *E. coli* in chickens.

Key words: Avian pathogenic *E. coli*, broilers, virulence genes, PCR

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Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

Escherichia coli is one of the major infectious agents that can cause tremendous problems in chickens. Pathogenic *E. coli* strains can be classified into intestinal and extra-intestinal *E. coli* according to their virulence factors and clinical symptoms[1]. Colibacillosis is one of the most serious diseases affecting poultry resulting in severe economic losses due to mortalities, weight loss, carcass condemnations and costs of treatment and preventive measures. In addition, Avian Pathogenic *E. coli* (APEC), the causitive agent of colibacillosis, is proved to be a zoonotic pathogen[2].

Presently, diarrheagenic *E. coli* strains are classified into 6 main pathogenic types according to their virulence factors, including enteropathogenic *E. coli* (EPEC), enterotoxogenic *E. coli* (ETEC) that produce heat labile and heat stable enterotoxins, enteroinvasive *E. coli* (EIEC) that simulates Shigella strains in their ability to invade the intestinal epithelial cells, enterohaemorrhagic *E. coli* (EHEC) that produce Shiga-like toxins, enteroaggregative strain (EAggEC) and Diffusively adherent *E. coli* (DAEC)[3]. Shiga toxin-producing *E. coli* (STEC) is the most important group of foodborne pathogens[4]. The EHEC strains are one of the subsets of Shiga toxin (Stx)-producing *E. coli* (STEC) strains[5,6].

The most dangerous *E. coli* serotype associated with EHEC is O157:H7, which is chracterised by the ability to cause severe disease in humans, such as Hemorrhagic Colitis (HC), Hemolytic Uremic Syndrome (HUS) and Thrombotic Thrombocytopenic Purpura (TTP)[7]. The major virulence factors involved in STEC infection are the powerful Shiga toxins, which are classified into 2 main groups: Stx1 and Stx2. Other important virulence factors of STEC are intimin (encoded by *eae*A gene), a plasmid-encoded enterohaemolysin and, in strains lacking eae, an autoagglutinating adhesin[8].

The aim of this study was to investigate the genetic variation among avian pathogenic *E. coli* strains isolated from chickens. This aim was achieved by isolation and identification of *E. coli*, serogrouping of isolated *E. coli* strains and detection of virulence genes in the isolated *E. coli* strains by using PCR.

MATERIALS AND METHODS

Sampling: A total of 100 samples (liver and heart blood from each bird) from 100 diseased broiler chickens were collected under complete aseptic condition separately in sterile plastic bags and transferred to the laboratory for bacteriological examination.

Samples were collected from cases exhibiting perihepatitis, pericarditis and airsacculitis. Liver and heart blood samples were collected based on clinical signs and pathogenomic lesions observed during post mortem examination of broiler chickens at Reference Laboratory for Veterinary Quality Control on Poultry Production in Sharkia province.

Isolation and identification of *E. coli*: For enrichment, one gram of each collected sample was aseptically added to 9 mL of buffered peptone water and incubated at 37°C for 24 h. A Loopful from the incubated broth was streaked onto MacConkey s agar plates and incubated at 37°C for 24 h. Lactose fermenting colonies were picked up and streaked onto EMB agar and incubated at 37°C for 24 h. Metallic green sheen colored colonies on EMB were sub cultured on Nutrient agar slant and incubated at 37°C for 24 h for storage at 4°C in the refrigerator for further studies and characterization. Suspected *E. coli* isolates were identified morphologically by Gram stain, motility test and biochemically according to Quinn *et al.*[9].

Serotyping of *E. coli* isolates: The somatic (O) antigen was determined by slide agglutination test according to Edwards and Ewing[10], while Flagellar (H) antigen serotyping was carried out using tube method according to Davies and Wray[11]. Serotyping of the isolated *E. coli* strains was carried out at The Serology Unit in Animal Health Research Institute in Dokki, Cairo, Egypt.

In vitro pathogenicity testing

Congo red test: Various serotypes were tested for pathogenicity based on Congo red dye binding test as described by Berkhoff and Vinal[12]. Trypticase soya agar was supplemented with 0.003% Congo red dye (Sigma) and 0.15% bile salts. Each isolate was streaked on a separate plate and incubated at 37°C for 24 h. After 24 h incubation, plates were left at room temperature for 48 h to facilitate annotation of results. Invasive *E. coli* were identified by their ability to take up Congo red dye. Appearance of red colonies was recorded as a positive reaction. Negative colonies did not bind the dye and remained white or grey.

Hemolysis production test: *Escherichia coli* strains were propagated on tryptose blood agar (Difco) plates supplemented with 5% defibrinated washed sheep blood and incubated at 37°C for 24 h. Then plates were examined for "greening" or clearing of the agar around areas of bacterial growth as an indication of alpha or beta hemolytic activity Livezey and Zusi[13].

PCR detection of *stx* 1, *stx*2, *eae*A and *hly* genes in the isolated *E. coli* strains: Thirty sex typable *E. coli* strains were subjected to PCR for detection of *stx*1, *stx*2, *eae*A and *hly* genes.

Extraction of DNA: *Escherichia coli* strains were cultured in brain heart infusion broth (LAB M-LAB 49); at 37°C for 18-24 h. DNA was extracted using QIAamp DNA Mini Kit (Catalogue No. 51304).

Polymerase chain reaction: Primers used in PCR were shown in Table 1.

DNA samples were tested in 50 µL. Reaction volume in a 0.2 mL PCR tube, containing PCR buffer (50 mM KCl, 10 mM tris-HCl, 1 mM $MgCl_2$) each dNTPS (Deoxy nucleotide triphosphate) 200 µM each (dATP, dGTP, dCTP and dTTP), Two primer pairs each at 50 picomol/reaction and 0.5 of taq DNA polymerase. Thermal cycling in a programmable heating block (Coy vorporation, Grasslake, Michan, USA) was done[14].

PCR recycling conditions:

- The *stx*1 gene according to Sahilah *et al.*[15]: Initial denaturation at 94°C for 7 min, 35 cycles (denaturation at 94°C for 30 sec, annealing at 51°C for 30 sec, extension at 72°C for 30 sec), final extension 72°C for 7 min

Table 1: Oligonucleotide primers sequences

Target gene	Primers sequences	Amplified segment (bp)	Reference
*stx*1	CAGTTAATGTGGTGGCGAAG CTGTCACAGTAACAACCGT	180	Sahilah *et al.*[15]
*stx*2	CCATGACAACGGACAGCAGTT CCTGTCAACTGAGCAGCACTTTG	779	Dipineto *et al.*[16]
hly	AACAAGGATAAGCACTGTTCTGGCT ACCATATAAGCGGTCATTCCCGTCA	1177	Piva *et al.*[8]
*eae*A	ATGCTTAGTGCTGGTTTAGG GCCTTCATCATTTCGCTTTC	248	Bisi-Johnson *et al.*[17]

Table 2: Prevalence of detected serotypes based on total No. of *E. coli* strains

Types of pathogenic *E. coli*	Serotypes	No. of isolates	Prevalence of serotypes (%)
EPEC	O1 : H7	3	5.0
	O2 : H6	2	3.33
	O44 : H18	2	3.33
	O55 : H7	3	5.0
	O78	7	11.66
	O119 : H4	2	3.33
ETEC	O125 :H21	2	3.33
	O127 : H6	3	5.0
	O128 : H2	3	5.0
EHEC	O26 : H11	4	6.67
	O111 : H4	5	8.33
Untypeable		24	40
Total		60	100

- The *stx*2 gene according to Dipineto *et al.*[16]: Initial denaturation at 94°C for 10 min, 35 cycles (denaturation at 94°C for 1 min, annealing at 58°C for 1 min, extension at 72°C for 1 min), final extension 72°C for 10 min
- The *hly* gene according to Piva *et al.*[8]: Initial denaturation at 94°C for 15 min, 35 cycles (denaturation at 94°C for 1 min, annealing at 60°C for 1 min, extension at 72°C for 15 min), final extension 72°C for 12 min
- The *eae*A gene according to Bisi-Johnson *et al.*[17]: Initial denaturation at 94°C for 7 min, 40 cycles (denaturation at 94°C for 30 sec, annealing at 51°C for 30 sec, extension at 72°C for 30 sec), final extension 72°C for 7 min

c-Screening of PCR products: Ten microliter of amplified PCR product was analyzed by electrophoresis on a 2% agarose gel stained with 0.5 µg of ethedium bromide/mL. Electrophoresis was carried out in 1X TAE buffer at 80 volt for 1 h[14,18]. Gels were visualized under UV transilluminator (UVP, UK) and photographed.

RESULTS

Prevalence and serogruoping of *E. coli*: In the present study, the prevalence of *E. coli* was (60%), the isolated *E. coli* strains were typed serologically into 11 different 'O' groups; O78 (11.66%), O111: H4 (8.33%), O26: H11 (6.67%), O1: H7, O55: H7, O127: H6 and O128: H2 (5% for each), O2: H6, O44: H18, O119: H4 and O125: H21 (3.33% for each) and 24 *E. coli* strains were untypable (40%) as shown in Table 2.

***In vitro* pathogenicity testing**

Congo red test: *In vitro* pathogenicity testing (Congo red binding assay) indicates that (91.7%) (n = 55 strains) of the isolated *E. coli* strains were positive and only (8.3%) (n = 5) were negative.

Hemolysis on blood agar: Regarding to hemolysis production on blood agar; all the isolated strains were non hemolytic.

PCR detection of *stx* 1, *stx*2, *eae*A and *hly* genes: As ahown in Table 3 and Fig. 1-4, 36 *E. coli* strains were subjected to PCR for detection of *stx*1, *stx*2, *hly* and *eae*A genes. The PCR results revealed that:

***stx*1 gene :** All *E. coli* strains (100%) were positive to *stx*1 gene

***stx*2 gene :** The 17 strains (47.2%) were positiv for *stx*2 gene including; eight EPEC strains [2 (O2: H6), 2 (O44: H18), 2 (O55: H7) and 2(O119: H4)]; four

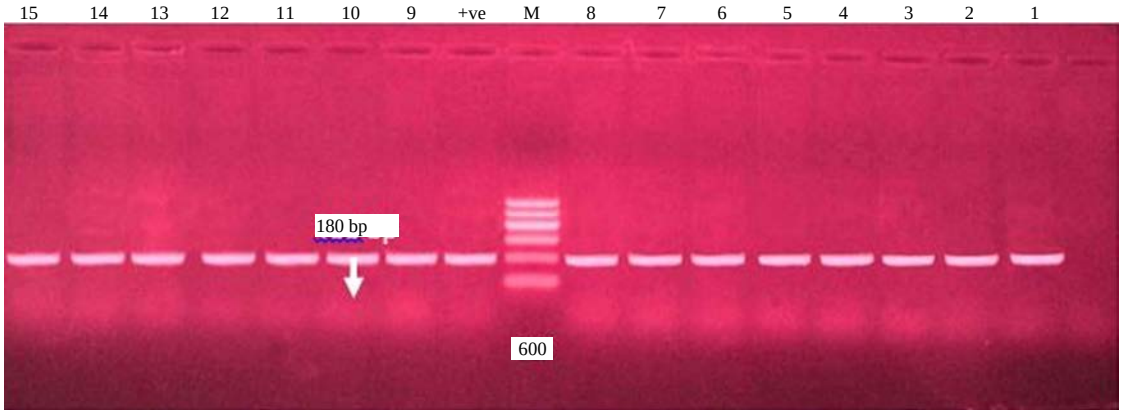

Fig. 1: Electrophoretic pattern of the PCR products of *stx*1 gene, Agarose gel electrophoresis of the PCR products of *stx*1 gene (180 bp). Lane M: 100 bp DNA Ladder (100-600 bp), lanes (1-15) positive *E. coli* strains, positive control (lane +ve) and negative control (lane -ve)

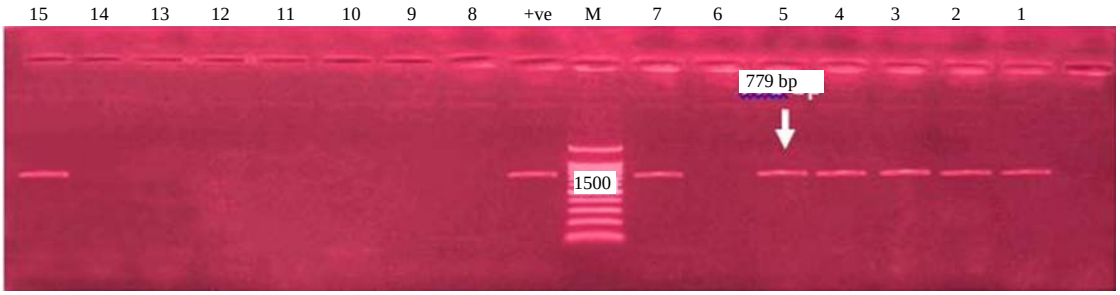

Fig. 2: Electrophoretic pattern of the PCR products of *stx*2 gene, Agarose gel electrophoresis of the PCR products of s*tx*2 gene (779 bp). Lane M: DNA marker (100-1500 bp), lanes (1, 2, 3, 4, 5, 7, 15) +ve *E. coli* strains, lanes (6, 8, 9, 10, 11, 12, 13, 14) –ve *E. coli* strains, positive control (lane +ve) and lane: negative control

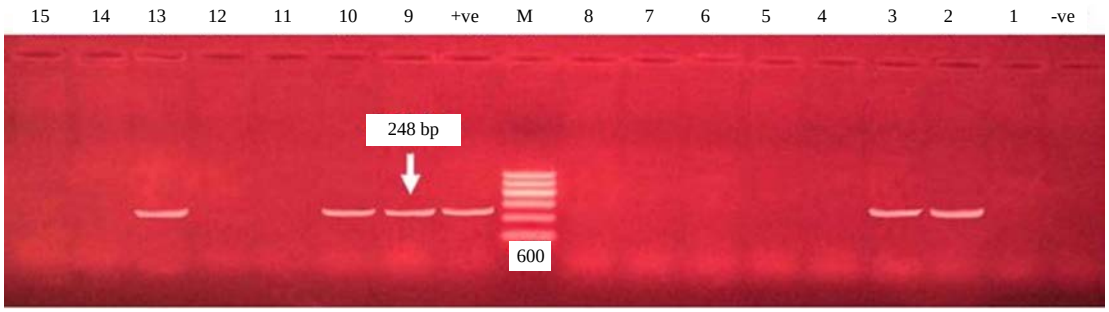

Fig. 3: Electrophoretic pattern of the PCR products of *eae*A gene, Agarose gel electrophoresis of the PCR products of *eae*A gene (248 bp). Lane M: 100 bp DNA Ladder (100-600 bp), lanes (2, 3, 9, 10, 13): +ve *E. coli* strains, lanes (1, 4, 5, 6, 7, 8, 11, 12, 14, 15): -ve *E. coli* strains, positive control (lane +ve) and negative control (lane -ve)

ETEC strains [2 (O125: H21) and 2 (O128: H2)]; five EHEC strains [3 (O26: H11) and 2 (O111: H4)]

***eae*A gene:** The 12 strains (33.3%) were positive for *eae*A gene including; six EPEC strains [1 (O1: H7), 2 (O44: H18), 1 (O55: H7), 1(O78) and

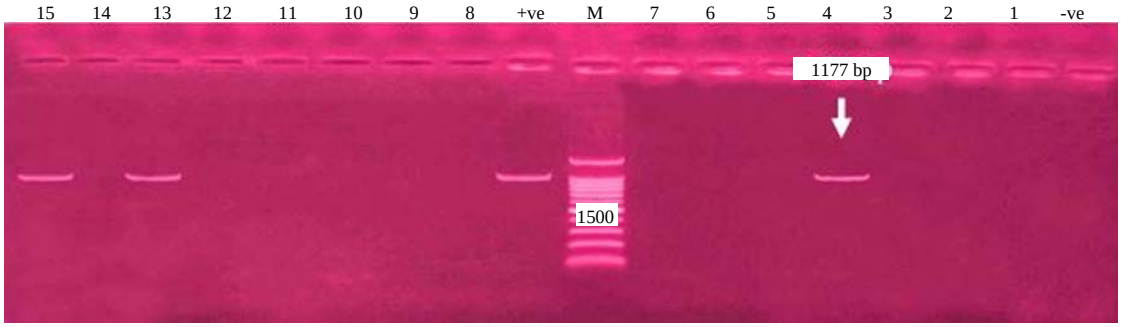

Fig. 4: Electrophoretic pattern of the PCR products of *hly* gene, Agarose gel electrophoresis of the PCR products of *hly* gene (1177 bp). Lane M: DNA marker (100-1500 bp), lanes (4, 13, 15): +ve *E. coli* strains, lanes (1, 2, 3, 5, 6, 7, 8, 9, 10, 11, 12, 14): -ve *E. coli* strains, positive control (lane +ve) and negative control (lane -ve)

Table 3: Prevalence of *stx*1, *stx*2, *eae*A and *hly* genes among the isolated *E. coli* serotypes

Types of pathogenic *E. coli*	Serotypes	No. of isolates	*eae*A	*hly*	*stx*1	*stx*2
EPEC	O1 : H7	3	1/3	-ve	+ve	-ve
	O2 : H6	2	-ve	-ve	+ve	+ve
	O44 : H18	2	+ve	-ve	+ve	+ve
	O55 : H7	3	1/3	-ve	+ve	2/3
	O78	7	1/7	-ve	+ve	-ve
	O119 : H4	2	1/2	-ve	+ve	+ve
ETEC	O125 :H21	2	+ve	-ve	+ve	+ve
	O127 : H6	3	-ve	-ve	+ve	-ve
	O128 : H2	3	-ve	-ve	+ve	2/3
EHEC	O26 : H11	4	1/4	1/4	+ve	3/4
	O111 :H4	5	3/5	2/5	+ve	2/5
Total		36	12 (33.3%)	3 (8.3%)	36 (100%)	17 (47.2%)

1(O119: H4)]; two ETEC [2 O125: H21)]; 4 EHEC strains [1 (O26: H11) and 3 (O111: H4)]

***hly* gene :** Only three strains (8.3%) were positive for *hly* gene including; three EHEC [1 (O26: H11) and 2 (O111: H4)]

DISCUSSION

In the present study,the prevalence of *E. coli* was (60%) as shown in Table 2. Higher rates of *E. coli* also were recorded by Peer *et al.*[19] and Ammar *et al.*[20]. While, lower rates were recorded by Literak *et al.*[21] and Radwan *et al.*[22]. Young aged broiler chickens up to 3 weeks have high affinity to the disease, but older chickens are more resistant. Different predisposing factors may increase the affinity of chickens to colibacillosis, such as viruses affecting the respiratory tract of chickens and bad hygienic mesearues[23].

In this study, the recovered *E. coli* strains were typed serologically into 11 different 'O' groups; O78 (11.66%), O111: H4 (8.33%), O26: H11 (6.67%), O1: H7, O55: H7, 127: H6 and O128: H2 (5% for each), O2: H6, O44: H18, O119: H4 and O125: H21 (3.33% for each) and 24 *E. coli* strains were Untypable (40%). High percentage of Untypable strains in APEC was previously recorded by numerous studies in different countries[24,25]. This supports the suggestion that serotyping is not recommended as a specific diagnostic tool for the identification of avian pathogenic *E. coli*[26] and highlights the need for molecular characterization of *E. coli* virulence factors as several authors fail to differentiate between APEC and avian fecal *E. coli* by serogrouping only[26,27]. Untypable strains by O serogrouping are mainly arise due to autoagglutination and an incomplete antisera panel[28].

The results of congo red test revealed that (91.7%) (n = 55 strains) of the isolated *E. coli* strains were positive and only (8.3%) (n = 5) were negative. These results agreed with those obtained by Sharda *et al.*[29] who also reported a clear relationship between the expression of congo red and the pathogenicity in avian *E. coli* and stated that it was due to presence of β–D-glucan in bacterial cell wall. Yoder[30] has reported that Congo red binding did not correlate well with pathogenicity.

The negativity of all the isolates to hemolysis on 5% sheep blood agar is in accordance with Erganiş *et al.*[31] who attributed heavy mortality in chicks due to non-hemolytic strains indicating that avian pathogenic *E. coli* to be independent of hemolytic activity, Sharada *et al.*[32] who reported that avian *E. coli* to be pathogenic needeed not to be hemolytic and Rodriguez *et al.*[33] who reported that none of their isolates from colisepticemic cases was positive for hemolysis on 5% sheep blood agar.

In the present study as shown in Table 3 and Fig. 1-4; 36 serotyped *E. coli* strains were analyzed by PCR for detection of *stx*1, *stx*2, *hly* and *eae*A genes. Regarding to the occurrence of *stx*1 gene, all *E. coli* strains were positive for *stx*1 gene (100%) with specific size of 180 bp, as shown in Fig. 1. These results were similar to those obtained by Momtaz and Jamshidi[34] who showed that 100% of EHEC serogroups were positive for *stx*1.

Regarding to the presence of *stx*2 gene, 17 *E. coli* strains (8 EPEC strains, 4 ETEC strains and 5 EHEC strains) were positive for *stx*2 gene by percentage of (47.2%) and with specific size of 779 bp as shown in Fig. 2. These result were nearly similar to the findings which were recorded by El-Jakee *et al.*[35] who detected *stx*2 in 5 *E. coli* strains (41.67%). The detection of STEC in chicken disagree with the results obtained by Kobayashi *et al.*[36], Schroeder *et al.*[37] and Wani *et al.*[38].

Concerning PCR detection of Intimin gene (*eae*A), as shown in Fig. 3, 12 *E. coli* strains (33.3%) (6 EPEC strains, 2 ETEC and 4 EHEC strains) were positive for intimin gene with specific size of 248 bp as shown in Fig. 3. These findings were nearly agreed with those obtained by El-Jakee *et al.*[35] who detected *eae*A in 5 *E. coli* strains (41.67%).

Regarding PCR amplification of *hly* gene, three non hemolytic EHEC strains (8.3%) were positive for *hly* gene with specific size of 1177 bp as shown in Fig. 4. These results agreed with the findings of Farah *et al.*[39] who detected *hly*A gene in seven non hemolytic strains. The absence of a hemolytic phenotype in the presence of *hly* gene has been identified and mainly attributed to defects in the *hly* genes or defects in the transcriptional activator rfaH[40].

CONCLUSION

The combination of genotypic and phenotypic analysis of *E. coli* is more valuable as an epidemiological tool for identification of isolates. The PCR is a rapid and a specific diagnostic tool used for genetic characterization of Avian pathogenic *E. coli*. *Escherichia coli* strains originating from birds are carrying a low percentage of *eae*A and *hly* genes. This study established the presence of *stx*1 and *stx*2 containing *E. coli* in chickens. Chickens might serve as vectors for transmission of STEC to environment and human.

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