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Detection of Virulence-Associated Genes of Avian Pathogenic *Escherichia Coli* (APEC) Isolated from Broilers

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Abstract

Escherichia coli is responsible for significant losses in the poultry industry. This study aimed to determine the prevalence, serotypes, the virulence-associated genes and the antimicrobials susceptibility of avian pathogenic *E. coli* (APEC) strains. A total of 1200 samples were collected from 200 birds (60 recently dead, 80 diseased and 60 apparent healthy broilers). Standard disc diffusion method used for determination of antimicrobials susceptibility. PCR used for the detection of virulence genes. Bacteriological examination revealed that E. coli was recovered from 842 samples with overall prevalence of 70.16%. Incidence of *E. coli* from fresh heart blood samples was 75%, from liver 83%, from kidney 64%, from spleen 57%, from small intestine74.5% and from bone marrow was 67.5 %. E. coli isolates belonged to Serotypes O111, O44, O55, O142, O128, O158, O157, O29 and O115. The antimicrobials susceptibility profile of the isolates showed resistance to Ampicillin, Neomycin, Doxycycline and Oxytetracycline, while ciprofloxacin and Erythromycin were effective against the isolates. PCR assay was carried out to detect the presence of phoA, iss and iutA gene, all serovars had the three genes except (O29) not possess iss gene. High prevalence of multidrug resistant avian pathogenic Escherichia coli (APEC) among broilers.

Key words: APEC; Broilers; Egypt

Introduction

Escherichia coli is a normal microflora of the intestinal tract and in the bird's environment, only certain of these strains possessing specific virulence attributes, designated as avian pathogenic *E. coli* (APEC), are able to cause disease. APEC is mostly associated with extra intestinal infections, namely colibacillosis [1-3]. Colibacillosis refers to any localized or systemic infection caused entirely or partly by *E. coli* including colisepticaemia, coligranuloma, chronic respiratory disease (CRD), peritonitis, swollen-head syndrome, Artheritis, synovitis, panophthalmitis, perihepatitis and pericarditis [4].

APEC are mostly associated with infection of extra intestinal tissues in chickens, turkeys, ducks and other avian species with the exception of a possible relationship with the development of enteritis. The most important disease syndrome associated with APEC begins as a respiratory tract infection and may be referred to as aerosacculitis or the air sac disease.

If unchecked, this infection may evolve into a bacteriemia and a generalized infection which manifests as a polyserositis. The respiratory tract complex is most often observed in birds of 4 to 9 weeks of age and may result in extensive economic losses with up to 20 % mortality as well as reduced growth and feed efficiency and an increased condemnation rate at the abattoirs [5].

Virulence of avian strains of *E. coli* is multifactorial and is associated with adherence factors (F1 and P-pili, and curli), the aerobactin iron-sequestering system, serum resistance, cap-

sule production, and temperature sensitive haemagglutination (tsh**)** **[**5]. Although the pathogenic mechanisms of APEC have not yet been fully elucidated, The virulence associated genes such as *fimC, astA, papC, tsh, fyuA, irp2, iucD, iss, hlyE, eaeA, vat, colV* and *stx2F* play important roles individually or in combination in adhesion, ferric transport system, hemolyzation and toxin production of avian pathogenic *E.coli* **[**6]. Multiplex PCR was used to identify traits that predict avian pathogenic *Escherichia coli* (APEC) virulence**.** Five genes carried by plasmids were identified as being the most significantly associated with highly pathogenic APEC strains: *iutA, hlyF, iss, iroN,* and ompT [7].

This study aimed to determine the prevalence, serotypes, antimicrobials susceptibility profile and the virulence-associated genes of avian pathogenic *E. coli* (APEC) strains in broilers farms in Dakahlia Governorate, Egypt.

Material and Methods

Samples collection

The samples collected from 200 broiler chickens (60 recently dead, 80 diseased and 60 apparently healthy chicken) from different private farms Dakahlia Governorate. And these samples include liver, spleen, kidney, fresh heart blood, bone marrow and intestine. All samples were collected in sterile plastic bags and transported directly to the laboratory.

Isolation of the *E. coli* isolates.

The sample was initially inoculated into a non-inhibitory liquid medium to favor the repair and growth of stressed *E. coli*. The internal organs included liver, spleen, kidney, fresh heart blood, bone marrow and intestine were collected and pre-enriched in buffered peptone water as a 1:10 dilution and incubated at 37°C± 1°C for 18 h. Pre-enriched culture was streaked onto Nutrient agar, MacConkey agar, Xylose Lysine Deoxycholate agar (XLD agar) and Eosin methylene blue agar (EMB) and incubated at 37.0 ± 1° C for 24 h for the isolation of *E. coli*.

Identification of *E. coli* isolates:

Microscopic examination

Suspected purified colonies were smeared, fixed and stained with Gram's according to [8].

Biochemical Identification

Purified isolates were examined by oxidase, urea hydrolysis, H_2S production on TSI, lysine decarboxylation, indole, methyl red test; Voges-Proskauer, citrate utilization, motility test and Analytical profile index 20 E (API 20 E)[8].

Serological identification:

The preliminarily identified isolates as *E. coli* were subjected to serological identification according to [9]. for determination of (O) antigen using slide agglutination test.

Antimicrobials susceptibility testing:

Determination of the susceptibility of the isolated strains to antibiotic discs was adopted using the disc diffusion technique according to Clinical and Laboratory Standards Institute (CLSI) instructions [10].

Detection of virulence genes in *E. coli* isolates using PCR:

DNA was extracted using QIAamp DNA Mini Kit according to the instructions of the manufacturer. Detection of virulence genes was performed by PCR. Primer sequences and PCR conditions used for the study listed in Table (1)**.** PCR performed in T3 Thermal cycler (Biometra). PCR products were separated and visualized by gel electrophoresis in 1.5% agarose in Tris–acetate–EDTA (TAE) buffer at 100 V. And Gel Pilot 100 bp ladder (QIAGEN, USA) was included in each agarose run, accordingly the amplified product.

Table 1. The primers sequence of virulence genes

Primer	Sequence	Amplified product	Reference
phoA	CGATTCTGGAAATGGCAAAAG	720 bp	Hu *et al.* 2011
	CGTGATCAGCGGTGACTATGAC		
iss	ATGTTATTTTCTGCCGCTCTG	266 bp	Yaguchi *et al.* 2007
	CTATTGTGAGCAATATACCC		
iutA	GGCTGGACATGGGAACTGG	300 bp	
	CGTCGGGAACGGGTAGAATCG		

Results

The prevalence of *E. coli* in examined broilers.

Morphologically *E. coli* isolates were G-ve rods appeared as pink colonies when cultured on MacConkey media, yellow on XLD and green metallic colonies on EMB medium. Biochemically, all *E. coli* suspected isolates were lactose fermenting colonies, positive indole, methyl red, and Catalase. Meanwhile all isolates were negative oxidase, urea hydrolysis, citrate utilization, Voges-Proskauer and didn't produce H_2S. The prevalence of suspected *E. coli* isolates from dead chickens was (56 / 60; 92%), followed by diseased chickens was (86 /80; 85%) and from apparently healthy chickens was (42 / 60, 70%) as shown in Table (2).

Table 2. The prevalence of E. coli in examined broilers:

case	No. of birds	Positive cases	
		No.	%
Apparently healthy chickens	60	42	70%
Diseased chickens	80	68	85%
Dead chickens	60	56	92%
Total	200	166	83%

The recovery rate of *E. coli* from internal organs

As shown in Table (3), the highest incidence of *E. coli* was re-

covered from liver (166 / 200; 83 %), followed by fresh heart blood (150 / 200; 75 %), small intestine (149 / 200; 74.5 %), bone marrow (135 / 200, 67.5 %), kidney (128 / 200, 64 %) and the lowest incidence was recovered from spleen (114/ 200, 57 %).

Table 3. Recovery rate of E. coli isolates from internal organs.

Case / Organs	Apparently healthy chickens (n = 60)	Diseased chickens (n = 80)	Dead chickens (n = 60)	Total (n = 200)
Fresh heart blood	35	62	53	150 (75%)
Liver	42	68	56	166 (83%)
Kidney	25	57	46	128 (64%)
Small intestine	33	63	53	149 (74.5%)
Spleen	17	48	49	114 (57%)
Bone marrow	25	60	50	135(67.5%)

E. coli serotyping

Results of serotyping of 166 *E. coli* isolates as shown in **Table (4);** revealed the high incidence of serotypes O_{29} and O_{115} serotypes (19.9%), followed by O_{157} serotypes (15%), then O_{142}, O_{128} andO_{158}2 serotypes (9.6%) and serotype O_{111}, O_{44} and O55 (5.4%).

Table 4. E. coli types

E. coli serotypes	No.	%
O29	33	19.9%
O158	16	9.6%
O128	16	9.6%
O142	16	9.6%
O115	33	19.9%
O157	25	15%
O55	9	5.4%
O44	9	5.4%
O_{111}	9	5.4%

4. antimicrobials susceptibility

The most encountered antimicrobials were Ampicillin, Oxytetracycline, Doxycycline, Neomycin and Gentamycin (65%, 55%, 55%, 55% and 50 % respectively). While lower resistance was to Erythromycin and Ciprofloxacin (25 and 20 % respectively) Table (5).

Table 5. antimicrobials susceptibility of E. coli isolates

Antimicrobial agent	Conc. µg	Susceptible %	Intermediate %	Resistant %
Ampicillin	10	10	25	65
Gentamycin	10	25	25	50
Neomycin	30	10	35	55
Doxycycline	30	40	5	55
Oxytetracycline	30	35	10	55
Erythromycin	15	30	45	25
Colistin	10	50	20	30
Streptomycin	10	35	20	45
Ciprofloxacin	5	15	65	20
Florfenicol	30	55	15	30

Distribution of virulence genes among *E.coli* serotypes

Table 6. showed that the tested *E.coli* sertypes contain the 3 virulence genes (*phoA* , *iss* and *iutA*) except O_{29} which didn't have *iss* gene.

Table 6. Distribution of virulence genes among E.coli serotypes.

serotype	*phoA*	*iss*	*iutA*
O_{142}	+	+	+
O_{29}	+	-	+
O_{115}	+	+	+
O_{158}	+	+	+
O_{128}	+	+	+
O_{157}	+	+	+
O_{55}	+	+	+
O_{44}	+	+	+
O_{111}	+	+	+

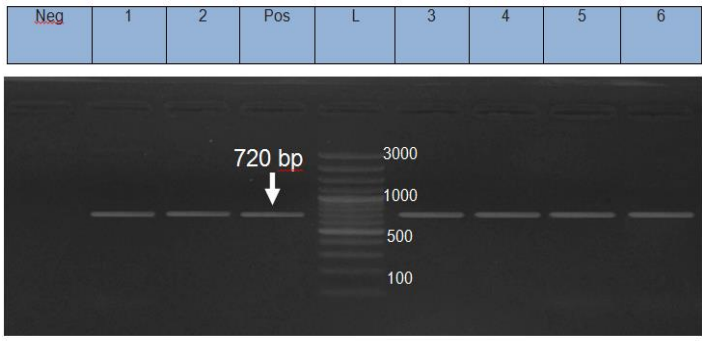

photo (A) Detection of *phoA* gene

L, 100 bp lambda marker; Neg., the negative control; Pos., the positive control. Lane 1,2,3,4,5 and 6 represented positive amplification of *phoA* gene at 720 bp. for *E. coli* isolates recovered Liver, Bone marrow, Kidney, spleen, fresh blood and small intestine.

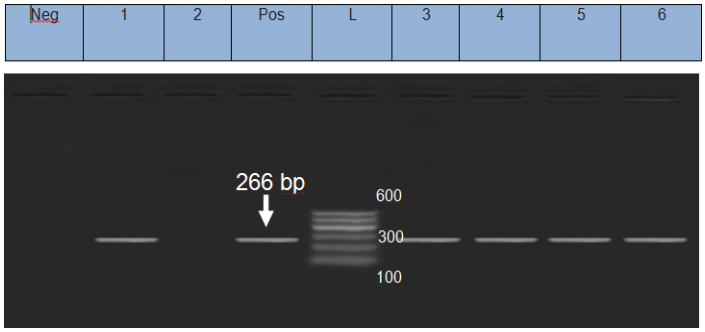

photo (B) Detection of *iss* gene

L, 100 bp lambda marker; Neg., the negative control; Pos., the positive control. Lane 1, 3,4,5 and 6 represented positive amplification of *iss* gene at 266 bp. for *E. coli* isolates recovered Liver, Bone marrow, Kidney, spleen, fresh blood and small intestine. Lane 2 represent *E.coli* serotype O_{29}.

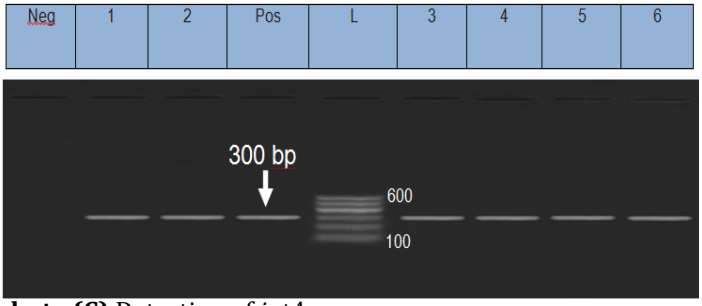

photo (C) Detection of *iutA* gene

L, 100 bp lambda marker; Neg., the negative control; Pos., the positive control. Lane 1,2,3,4,5 and 6 represented positive amplification of *iutA* gene at 300 bp. for *E. coli* isolates recovered Liver, Bone marrow, Kidney, spleen, fresh blood and small intestine.

Discussion

E. coli is considered a member of the normal microflora of the poultry intestine, but certain strains such as those designated as avian pathogenic *E. coli* (APEC); spread into various internal organs and cause colibacillosis characterized by systematic fatal disease [11]. Typing of isolated bacteria, including *E. coli* could be achieved by Phenotypic and/or genotypic protocols. The phenotypic characteristic method used for identification of *E. coli* includes the morphological and biochemical tests. Most of these techniques are not sufficiently sensitive to distinguish between different strains and they are affected by physiological factors [12]. Therefore, serological protocol was established to differentiate *E. coli* isolates. Regarding the morphological characters used for identification of *E. coli*, depend on that *E. coli* isolates are Gram-negative rods appeared as pink colonies when cultured on MacConkey media, green metallic colonies on EMB medium. Nearly similar results were noted by [13-14].

On the other aspect, results of biochemical tests by using traditional methods revealed that 90% of suspected isolates were biochemical identical to typical *E. coli* features and by using the API20E system for Identification of suspected isolated *E. coli* strains revealed that 100% of suspected isolates were biochemical identical to typical *E. coli* features. These results are similar to those recorded by [15] who used the API 20E system for identification of isolated G-ve bacteria and observed that the API20E system identified about 98,9% of the isolated strains.

Bacteriological study was conducted using randomly organ samples from recently dead, disease and healthy broilers including liver, fresh heart blood, kidney, spleen, small intestine and bone marrow from ten broiler farms located in Dakahlia governorate. In general, investigation of 1200 organ samples collected from recently dead, disease and healthy broilers revealed that *E. coli* isolates was recovered from 842 samples with overall prevalence (70.16%), our result agreed with [16]. who isolated *E.coli* at a percentage of (58%). This study revealed that the *E. coli* isolates were isolated from 842 (70.16%) out of 1200 broiler samples originated from different sources including; Fresh heart blood 150 out of 200(75%), Liver 166 out of 200 (83%), Kidney 128 out of 200(64%), Small intestine 149 out of 200 (74.5%), Spleen114 out of 200 (57%) and bone marrow 135 out of 200 (67.5%). Nearly similar results were recorded by [17]. who isolated 96 *E.coli* from of 165 samples (85%) [17]. isolated *E.coli* from the liver at a percentage of (54.28%). Also, 128 out of 200 examined Kidney samples were *E. coli* positive with an incidence of 64%. While [18]. recorded higher occurrence of *E. coli* in tested poultry kidney samples (96%). Concerning small intestine samples, 149 out of 200 samples of examined small intestine were *E. coli* positive with an incidence of (74.5%). Nearly similar results were recorded by [17].who isolated *E.coli* from the small intestine at a percentage of (81.81%); Meanwhile [19]. reported a lower prevalence for *E. coli* in a percentage 37.5%. Moreover, 114 out of 200 samples of examined spleen were *E. coli* positive with an incidence of (57%) [17] reported a lower percentage 39.13%. Finally, bone marrow samples, 135 out of 200 samples of examined bone marrow were *E. coli* positive with an incidence of (67.5%). While [18] recorded higher occurrence of *E. coli* from tested poultry bone marrow samples (96%).

From the above mentioned results, it is obvious that *E .coli* isolates were recovered from poultry farms with higher prevalence from liver samples, followed by Fresh heart blood, Small intestine, Kidney, bone marrow and the lowest prevalence were from spleen. Furthermore, we can conclude that *E. coli* isolates were isolated from different organs at a percentage varied from (57%) to (83%). while the results (39.13%) to (81.81%) recorded by[17]. The results of serotyping clarified the recovery of serotypes $\mathbf{O_{111}}$, $\mathbf{O_{44}}$, $\mathbf{O_{55}}$, $\mathbf{O_{142}}$, $\mathbf{O_{128}}$, $\mathbf{O_{158}}$, $\mathbf{O_{157}}$, $\mathbf{O_{29}}$ and $\mathbf{O_{115}}$. These finding were similar to the results that was recorded from broilers as the following; [20, 21] isolated O_{115} and O_{29}, [22] isolated O_{157} and O_{111}, [23] isolated O_{44}, [13] isolated O_{128} and [24]. isolated O_{55}, O_{111} and O_{158}. From the mentioned data, it was clear that the most prevalent *E. coli* serotype isolates recovered from examined broiler chickens samples were $\mathbf{O_{115}}$ and $\mathbf{O_{29}}$; followed by $\mathbf{O_{157}}$; then $\mathbf{O_{158}}$,$\mathbf{O_{128}}$ and $\mathbf{O_{142}}$; and finally the lowest prevalent serotype were $\mathbf{O_{55}}$, $\mathbf{O_{44}}$ and $\mathbf{O_{111}}$. These results go in hand with those reported by [13] who recorded $\mathbf{O_{115}}$ is one of the most predominant serogroups from many serotypes recovered from chickens ($\mathbf{O_{20}}$, $\mathbf{O_{54}}$, $\mathbf{O_{61}}$, $\mathbf{O_{73}}$, $\mathbf{O_{78}}$, $\mathbf{O_{88}}$, $\mathbf{O_{89}}$, $\mathbf{O_{111}}$, $\mathbf{O_{115}}$, $\mathbf{O_{119}}$, $\mathbf{O_{132}}$ and $\mathbf{O_{153}}$).

Antimicrobials resistance is increasing among many bacterial species and is rapidly becoming a major world health problem [25,26] . Antimicrobials are valuable tools to treat clinical disease and to maintain healthy and productive animals; however the treatment of whole herds and flocks with antimicrobials for disease prevention and growth promotion has become a controversial practice [27,28]. Antimicrobial therapy is one of the primary control measures for reducing morbidity and mortality due to APEC associated avian colibacillosis [29,5,30]. Results of antimicrobials sensitivity of serotyped *E. coli* recovered from broilers showed that the majority of *E. coli* isolates possess resistance to ampicillin (65%). followed by Oxytetracycline, Neomycin and Doxycycline (55%). the results nearly similar to [17] who reported that the highest resistance was to ampicillin and tetracyclines. These results also were confirmed by [31] who proved that the highest rate of resistance was against Oxytetracycline (95%), Doxycycline (88%), Neomycin (81%) and Ampicillin (47%).

The isolates of *E. coli* showed 30% resistence to clostin and florfenicol and 20% to ciprofloxacin, this was nearly similar results was recorded by [31] who found that the resistance to clostin and Florfenicol were (6%) and (27%) respectively and [32] who found that the resistance to ciprofloxacin was 11.8% and [33]. proved that the resistance was higher in case of old compounds than the newer compounds. Finally we concluded that the use of antimicrobials is strongly associated with the prevalence of antimicrobial resistance in *E.coli* isolates in food-producing animals [34].

The present study was directed mainly to recognize some virulence genes, such as *(phoA, iss* and *iutA* genes) which commonly found in *E. coli* isolated from broilers samples by using PCR. Virulence genes of *E. coli* isolates recovered from broiler farms samples are shown in table (6). The choice of these genes due to *iss* and *iutA* were the most significantly associated with highly pathogenic APEC strains as mentioned by [8]. while *phoA* gene is a common gene specific to *E.coli*.

PhoA can be used specifically to detect bacterial genes that code for cell envelope proteins [35] **.**The detection of *phoA* gene showed that all isolates yielded the expected size of 720 bp PCR amplified product for the *phoA* gene. Nearly similar findings were recorded by [36]. Regarding the occurrence of *iss* gene among *E. coli* isolates. The results revealed that all *E. coli* serotypes expressed iss gene except serotype O29. Nearly similar findings were recorded by [37]. who reported that the iss gene was detected significantly more often among colibacillosis isolates. Also, [38] stated that plasmid-related gene was detected in the majority of avian pathogenic *E.coli* (74.8 to 86.7%) [39] recovered the iss gene which encodes a protein of the external membrane inducing resistance to the complement was present in 53 out of the 65isolates at a percentage of 81.5%.

The detection of *iutA* gene showed that all isolates yielded the expected size of 300 bp PCR amplified product for the *iutA* gene. Nearly similar findings were recorded by [37] who reported that the *iutA* gene was detected significantly in all *colibacillosis* isolates.

Conclusion

High prevalence and multidrug resistance of avian pathogenic *Escherichia coli* (APEC) in broilers farms in Dakahlia Governorate, requires the development of hygienic measures in order to avoid loses caused by *colibacillosis*.

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