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Bacteriological and Molecular Studies on Virulence Encoding Genes in *Escherichia coli* Isolated from Diseased Ducks

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ABSTRACT

Avian colibacillosis is considered a major bacterial disease in the poultry industry worldwide and one of the most common avian diseases that communicable to human. A prevalence study on APEC was carried out on 56 cloacal swabs isolated from 17 weeks old ducks suffering from diarrhea. The isolates were cultured onto MacConkey and EMB agar for isolation and identification of *E. coli* followed by serotyping then PCR for the detection of virulence encoding genes (*iss, fimH, eaeA, tsh, ibeA, stx1* and *stx2*) and their phylogenetic group. The collected data showed that, out of 56 isolates 12 isolates were typical APEC commonly belong to7 different serogroups and having at least two virulence genes. The data demonstrated that, *iss and fimH* encoding genes were present in all the examined serogroups (100%). In contrast *stx1* and *eaeA* were absent in all isolates, while *tsh* and *ibeA* were variable (63.63%, 27.27%), respectively. The presented phylogenetic groups were A and B1 in percentage of 27.27% and 72.72%, respectively. The use of phylogenetic groups makes a revolution in the identification of APEC which enhanced our knowledge for APEC pathogenicity and helping pave the road for the application of the suitable preventive and curative measures in order to reduce the economic drawbacks of avian colibacillosis in large-scale farms.

Keywords: *E. coli, Virulence genes, Phylogenetic grouping, Ducks*

INTRODUCTION

Escherichia coli is distributed normal inhabitant of the gastrointestinal tract of humans, poultry and animals. It is a nonsporulating rod-shaped, Gram-negative, a facultatively anaerobic bacterium. The optimal growth temreature of most E. coli strains is 37°C and some exceptional strains can grow at temperatures up to 49°C (Tenaillon *et al.*, 2010).

Colibacillosis, caused by avian pathogenic E. coli (APEC) is the most common bacterial infection of all ages of commercial ducks with serious economic losses due to the high morbidity and mortality. Infection with APEC is mainly via the respiratory tract and air sacs and is usually secondary to infection by Mycoplasma or a virus. The disease has two main forms, acute and chronic. The acute form is characterized by the congested carcass, congested lungs and small hemorrhages of the heart and air sacs. While, in case of chronic infections the main signs are pericarditis, perihepatitis, enlargement of the liver, airsacculitis and pneumonia (Johnson *et al.*, 2007).

Colibacillosis is one of the most common avian diseases that posed potential hazards to the public health (Kabir *et al.*, 2017). A number of strains possess certain genes making them pathogenic which associated with intestinal (IPEC) and extraintestinal (ExPEC). The intestinal pathogenic groups includes enterotoxigenic *E. coli* (ETEC), enteropathogenic *E. coli* (EPEC), entero-hemorrhagic *E. coli* (EHEC), enteroinvasive *E. coli* (EIEC), enteroaggregative *E. coli* (EAEC), diffusely adherent *E. coli* (DAEC) (Logue *et al*., 2017), while the extra-intestinal *E. coli* it is known that APEC infecting poultry, UPEC infecting humans and animals, and NMEC infecting infants, share common phylogenetic origins (Moulin *et al.*, 2006; Johnson *et al.*, 2006 & Ewers *et al.*, 2007).

The pathogenesis of APEC infections comprises four stages; colonization of the

respiratory tract, crossing of the epithelium and penetration into the mucosa of the respiratory organs (air sacs), survival and multiplication in the bloodstream and in the internal organs, production of deleterious effects on the eukaryotic cells and tissues leading to lesions followed by the clinical signs (Dho-Moulin and Fairbrother, 1999).The serotyping analysis was recommended as a more specific identification approach that aims to classify the pathogenic strains based on their surface antigens, which divided into serogroups and serotypes on basis of their antigenic composition (somatic or O antigens for serogroups and flagella or H antigens for serotypes). Many strains express a third class of antigens (capsular or K antigens) (Compos *et al.*, 2004).

E. coli isolated from clinical cases of colibacillosis were shown to have different combinations of virulence genes (Ewers *et al.*, 2005). However, in some *E. coli* possess virulence genes in combinations not known to be associated with disease and may be considered as potentially pathogenic (Nicholson *et al.*, 2016 and Logue *et al.*, 2017). Recently, the use of phylogenetic groups makes a revolution in the identification of APEC, which enhanced our knowledge for APEC pathogenicity aiming to evaluate the possibility of these *E. coli* strains as predictors for future hazard zoonotic diseased. In addition, the role of phylogenetic type in APEC examines to assess the association between virulence gene carriage and phylogenetic type (Alizade *et al*., 2017). The species is divided into four groups (A, B1, B2, D) (Clermont *et al.*, 2000). The commensal strains belong to groups (A and B1), whilst ExPEC strains frequently belong to phylogroups (B2 and D) and the intestinal pathogenic strains belonging to groups (A, B1 and D). Addition of new subgroups those were the groups A1, B3 (only found in humans) and D2 (Carlos *et al.*, 2010) which were determined by presence and/or absence of the three genetic markers, *chu*A, *yja*A, and *TSP*E4.C2 by a triplex PCR: *chu*A, a gene associated with heme transport in (EHEC); *yja*A, a gene of unknown function and an anonymous DNA fragment called *TSP*E4.C2 that was identified as a putative lipase esterase gene (Gordon *et al*., 2008).

The present study was aimed to isolate and identify the most common serogroups of *E. coli* associated with diarrhea in ducks. In addition, the isolates were further examined for the presence of some virulence encoding genes using specific primer sets.

MATERIALS AND METHODS

Samples and samples preparation

A total of 56 cloacal swabs were collected from 16- and 17-weeks old ducks at Behera Governorate, then transferred immediately under full aseptic conditions for bacteriological isolation and identification.

Isolation and identification of *E. coli*

The samples were cultured on the MacConkey agar plate for 24hrs. The typical colony characteristic of *E. coli* where it appeared as a rose pink colony on MacConkey agar medium, then the suspected colony cultured on EMB media the typical colony gave the characteristic metallic sheen appearance. The isolates were further identified based on the biochemical tests and the serology using O and H antigens according to Kok *et al*., (1996) by using rapid diagnostic *E. coli* antisera sets **(**DENKA SEIKEN Co., Japan) for diagnosis of the Enteropathogenic types. slide agglutination tests were performed with the diagnostic sera to identify the O-antigen.

pathogenicity test

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 also demonstrate wrinkling of the colony surface, *E. coli* colonies that do not bind the dye (CR-) demonstrate a smooth white colonial morphology (Vinal, 1986).

Extraction of bacterial DNA

DNA extraction was carried out from pure strains of *E. coli* cultured on nutrient broth and incubated overnight using The QIAamp DNA Mini Kit Multiplex Polymerase chain reaction (PCR) for the detection of *E.coli.*

Multiplex PCR reaction was performed from the positive isolates. The reaction mixture was made containing 1µl DNA template, 0.5µM of each primer, 25 µL of 2x multiplex master mix (Takara) and the final volume was adjusted to 50 µL with PCR water. To obtain the amplicon 35 cycles of denaturation at 94°C for 30 s, 55°C for 30 s, and annealing at 72°C for 2 min and a final extension at 72°C for 10

min. The amplified products were then resolved by electrophoresis in 2% agarose gel at 100 V for 60 min. Gels were stained with ethidium bromide solution and documentation was done using the Gel Doc system. The primers used are mentioned in Table 1.

PCR amplification of virulence genes and phylogenetic groups

PCR amplification of seven virulence genes and three genes of phylogenetic groups using specific primers for all *E. coli* isolates as showed in table (1). The reaction mixture was made containing 1 µl DNA template, 0.5 µM of each primer, 25 µL of 2x multiplex master mix (Takara) and the final volume was adjusted to 50 µL with PCR water. The isolates were amplified individually for the four genes using specific primers with, 35 cycles of initial denaturation 95°C 3m, denaturation 95°C for 30 sec, annealing at 55°C for 30 sec, extension at 72°C for 1.2 m and a final extension at 72°C for 7m.

Table (1): Oligonucleotide primers sequences

Gene	Primer Sequence 5'-3'	Amplified product	Refrances
stx1	ACACTGGATGATCTCAGTGG CTGAATCCCCCTCCATTATG	614 bp	**Dipineto *et al.*, 2006**
stx2	CCATGACAACGGACAGCAGTT CCTGTCAACTGAGCAGCACTTTG	779 bp	
chuA	GAC GAA CCA ACG GTC AGG AT TGC CGC CAG TAC CAA AGA CA	279 bp	
yjaA	TGA AGT GTC AGG AGA YGC TG ATG RAG AAT GCG TTC CTC AAC	211 bp	**Jeong *et al.*,**
tspE4C2	GAG TAA TGT CGG GGC ATT CA CGC GYC AAC AAA GTA TTR CG	152 bp	
fimH	TGCAGAACGGATAAGCCGTGG GCAGTCACCTGCCCTCCGGTA	508 bp	**Ghanbarpour and Salehi, 2010**
Tsh	GGT GGT GCA CTG GAG TGG AGT CCA GCG TGA TAG TGG	620 bp	**Delicato *et al.*, 2003**
eaeA	ATG CTT AGT GCT GGT TTA GG GCC TTC ATC ATT TCG CTT TC	248 bp	**Bisi-Johnson *et al.*, 2011**
Iss	ATGTTATTTTCTGCCGCTCTG CTATTGTGAGCAATATACCC	266 bp	**Yaguchi *et al.*, 2007**
ibeA	TGGAACCCGCTCGTAATATAC CTGCCTGTTCAAGCATTGCA	342 bp	**Ewers *et al.*, 2007**

Table (16): The prevalence of the virulence genes among isolated *E. coli* strains using PCR.

Genes	*eaeA*	*fimH*	*tsh*	*iss*	*ibeA*	*Stx1*	*Stx2*	*chu*A	*yja*A	*tsp*E4.C2	Strain characterization
O2	-	+	+	+	-	ND	ND	-	-	-	EPEC
O26	-	+	+	+	+	ND	ND	-	-	+	EHEC
O128	-	+	+	+	-	-	-	-	-	+	ETEC
O158	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	EPEC
O2	-	+	-	+	-	ND	ND	-	-	+	EPEC
O78	-	+	-	+	-	ND	ND	-	-	-	EPEC
O91	-	+	+	+	-	-	+	-	-	+	EHEC
O91	-	+	+	+	+	-	-	-	-	+	EHEC
	-	+	+	+	+	-	-	-	-	-	EPEC
O78	-	+	-	+	-	ND	ND	-	-	+	EPEC
O78	-	+	+	+	-	ND	ND	-	-	+	EPEC
O91	-	+	-	+	-	-	-	-	-	+	EHEC
Total Positive	0	11	7	11	4	0	1	0	0	8	

Positive: + Negative: - Not done: ND

Results and Discussion

The presented data in table 2 showed that, E. coli was recovered from 12/56 cloacal samples collected from 16 and 17 weeks of diseased ducks with diarrhea (reddish, whitish and yellowish diarrhea) in a percentage of 21.52%. This outcome is higher compared to that previously obtained by (Amin *et al.,* 2013) and similar to obtained by (Shaaban, 2015), this result is lower compared to that obtained by (Moemen *et al.,* 2014 and Saviolli *et al.,* 2016) who isolated E. coli form 92.6% and 86.8% of the examined samples, respectively. The confirmed strains belonged to 7 different serogroups (O91, O113, O78, O2, O158, O128 and O26). The predominant serogroups in duck isolated from cloacal swabs were O78, O91 and O2 in a percentage of (25%, 25% and 16.67%), respectively. These outcomes are consistent with the findings previously obtained by (Huja *et al.,* 2015) who reported that serotype O78 was the major cause of avian colisepticemia. In addition (Ewers *et al.,* 2007; Asway *et al.,* 2008; Aaron *et al.,* 2012 and Nolan *et al.,* 2013) noticed that (O2 and O78) serogroups were the predominantly isolated serogroups. Also, O113:H4 has identified herein a similar finding to that previously showed by (Monaghan *et al.,* 2011 and Feng *et al.,* 2017) who recognized O113:H4 as a major public health concern. Moreover, O26 was successfully identified from the cloacal swabs collected from 17-weeks-old diseased ducks; this outcome is similar to that obtained by (Paddock *et al.,* 2012; Amin *et al.,* 2013 and shaaban, 2015). In contrast, this result is inconsistent with the findings of (Torky *et al.,* 1995 and Marwah *et al.,*2010) who recorded these serogroups are not common in chickens and ducks but may be transmitted from other animals to chickens raised near to these animals. Also, O158 and O128:H2 were successfully identified as similar to (shaaban., 2015). O91: H21 was successfully recovered from the cloacal swabs of diseased ducks and this serotype is considered as (EHEC). These results agreed with (Neher *et al.,* 2016). Different serotypes were previously reported in different studies which similar to our results O91, O78 and O2, and serotypes O113, O128, O26, O91 were previously obtained in the study carried out by (Cordoni *et al.,* 2016 and Son *et al.,* 2014) respectively. On the other hand (Beutin *et al.,* 1993) isolated O91: H- and O128: H2 from animal species.

In the present study, Examination of the pathogenicity of the confirmed isolates using congo red pathogenicity test showed that nearly 72% of the isolates were positive. This outcome is agreed with (El-ashker, 2006) who reported that (70%) of E. coli isolated from poultry were Congo red positive and disagreed with (Hassan ,2009) who reported that Congo red positive rate of.(%١٠٠)

The *stx1* gene not amplified in all examined serogroups. These outcomes are consistent with the findings previously obtained by (Shaaban., 2015) and inconsistent with the findings of (Farooq *et al.,* 2009 and Johura *et al.,* 2017). Also, *stx2* gene was not amplified in serogroups (O113, O128 and two different isolates of O91). These outcomes are consistent with the findings previously obtained by (Shimaa, 2013 and Shaaban, 2015). The *stx2* was successfully amplified in one isolate of serogroup O91. This result is similar to (Farooq *et al.,* 2009; Son *et al.,*2014; Yousef *et al.,* 2015; Neher *et al.,* 2016 and Johura *et al.,* 2017) .

The *eaeA* gene was not amplified in all serogroups. This outcome came in accordance with that previously obtained by (Olsen and Christensen, 2011, and Shimaa, 2013) who found *eaeA* gene not detected in all APEC isolates, also our result is similar to (Feng *et al.,* 2017) who detected O113:H4 not produce the intimin protein, meanwhile these outcomes inconsistence with previously obtained by (Farooq *et al.,* 2009; Yousef *et al.,* 2015; Son *et al.,*2014; Neher *et al.,* 2016 and Bai *et al.,* 2016) who found *eaeA* gene detected in APEC isolates .

The *fimH* gene was successfully amplified in all examined serogroups giving a positive PCR product of 508 bp. These outcomes came in accordance with the finding previously recorded by (Maciel *et al.,* 2016; Saviolli *et al.,*2016 and Alizade *et al.,*2017). Also, the tsh gene was successfully amplified in examined E. coli serogroups. These outcomes go in parallel with those obtained (Qabajah and Yaquoub, 2010). In contrast, it higher compared to that previously obtained by (Shaaban., 2015) who found *tsh* in four strains in ducks .

The successfully amplified of *iss* (increase serum survival) in all examined serogroups was consistent with those finding previously by (Johnson *et al.,* 2008; Qabajah and Yaqoub, 2010; Jeong *et al.,* 2011and Shaaban 2015) who stated that iss gene is the most important and widely distributed virulence marker of APEC. The *ibeA* (invasion of brain endothelium protein A) is a virulence factor found in newborn meningitis and avian pathogenic strains. The *ibeA* gene was successfully amplified in (O113, O26 and one isolates of O91). This outcome is higher compared to that previously obtained by (Wang *et al.,* 2010) who found *ibeA* gene in a percentage of (10.6%) of APEC isolates and was not found in healthy ducks. In addition, these outcomes go in parallel with those previously obtained by (Saviolli *et al.,* 2016) who detected *ibeA* in APEC. The highest amount of E. coli strains (72.72%) belong to group B1, followed by group A (27.27%). These outcomes are consistent with the findings previously obtained by (Walk *et al.,* 2007, Carlos *et al.,* 2010, Logue *et al.,* 2017 and Alizade *et al.,* 2017) and lower compared to that obtained by (Carlos *et al.,* 2010) who found that group B1 was (100%) among all the analyzed hosts followed by group A was (83.3%). Of note, the majority of E. coli strains that are able to persist in the environment belong to the B1 phylogenetic group (Walk *et al.,* 2007).The successfully amplified of *iss* (increase serum survival) in all examined serogroups was consistent with those finding previously by (Johnson *et al*., 2008; Qabajah and Yaqoub, 2010; Jeong *et al*., 2011and Shaaban 2015) who stated that *iss* gene is the most important and widely distributed virulence marker of APEC. The *ibeA* (invasion of brain endothelium protein A) is a virulence factor found in newborn meningitis and avian pathogenic strains. The *ibeA* gene was successfully amplified in (O113, O26 and one isolates of O91). This outcome is higher compared to that previously obtained by (Wang *et al.,* 2010) who found *ibeA* gene in a percentage of (10.6%) of APEC isolates and was not found in healthy ducks. In addition, these outcomes go in parallel with those previously obtained by (Saviolli *et al.,* 2016) who detected *ibeA* in APEC. The highest amount of *E. coli* strains (72.72%) belong to group B1, followed by group A (27.27%). These outcomes are consistent with the findings previously obtained by (Walk *et al.,* 2007, Carlos *et al*., 2010, Logue *et al*., 2017 and Alizade *et al*., 2017) and lower compared to that obtained by (Carlos *et al*., 2010) who found that group B1 was (100%) among all the analyzed hosts followed by group A was (83.3%). Of note, the majority of *E. coli* strains that are able to persist in the environment belong to the B1 phylogenetic group (Walk *et al.,* 2007).

CONCLUSION

The better understanding of the pathogenesis of *E. coli* induced colibacillosis in ducks will potentially minimize the economic drawbacks of such this infection. The introducing of modern techniques such as PCR significantly improved our knowledge about E. coli induced colibacillosis in ducks.

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