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 to 7 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 temperature 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), enterohemorrhagic *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 diseases. 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, chuA, yjaA, and TSPE4.C2 by a triplex PCR: chuA, a gene associated with heme transport in (EHEC); yjaA, a gene of unknown function and an anonymous DNA fragment called TSPE4.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
The amplified products were then resolved by electrophoresis in 2% agarose gel at 100 V 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 shown 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>
<thead>
<tr>
<th>Gene</th>
<th>Primer Sequence 5'-3'</th>
<th>Amplified product</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>stx1</td>
<td>ACACTGGATGATCTCAGTGG</td>
<td>614 bp</td>
<td>Dipineto et al., 2006</td>
</tr>
<tr>
<td>stx2</td>
<td>CTGAATCCCCCTCCATTATG</td>
<td>779 bp</td>
<td></td>
</tr>
<tr>
<td>chuA</td>
<td>GAC GAA CCA AGC GTC AGG AT</td>
<td>279 bp</td>
<td></td>
</tr>
<tr>
<td>yjaA</td>
<td>ATG RAG AAT GCG TTC CTC AAC</td>
<td>211 bp</td>
<td>Jeong et al.,</td>
</tr>
<tr>
<td>tspE4C2</td>
<td>GCG GYC AAC AAA GTA TTR CG</td>
<td>152 bp</td>
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</tr>
<tr>
<td>fimH</td>
<td>GCA AGT CCA TGG GGC ATT CA</td>
<td>508 bp</td>
<td>Ghanbapour and Salehi, 2010</td>
</tr>
<tr>
<td>Tsh</td>
<td>AGT CCA GCG TGA TAG TGG</td>
<td>620 bp</td>
<td>Delicato et al., 2003</td>
</tr>
<tr>
<td>eaeA</td>
<td>ATG TCT AGT GCT GGT TTA GG</td>
<td>248 bp</td>
<td>Bisi-Johnson et al., 2011</td>
</tr>
<tr>
<td>Iss</td>
<td>CTATTGTGAGCAATATACC</td>
<td>266 bp</td>
<td>Yaguchi et al., 2007</td>
</tr>
<tr>
<td>ibeA</td>
<td>TGGCCTGTTAATATAC CTCGGTATCAT</td>
<td>342 bp</td>
<td>Ewers et al., 2007</td>
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<p>| Table (16): The prevalence of the virulence genes among isolated <em>E. coli</em> strains using PCR. |
|-----------------|-----------------|----------------|-----------------|-----------------|</p>
<table>
<thead>
<tr>
<th>Genes</th>
<th>eaeA</th>
<th>fimH</th>
<th>tsh</th>
<th>iss</th>
<th>ibeA</th>
<th>Stx1</th>
<th>Stx2</th>
<th>chuA</th>
<th>yjaA</th>
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<tr>
<td>O2</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>-</td>
<td>-</td>
<td>EPEC</td>
</tr>
<tr>
<td>O26</td>
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<td>+</td>
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<td>+</td>
<td>+</td>
<td>ND</td>
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<td>ND</td>
<td>ND</td>
<td>ND</td>
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</tr>
<tr>
<td>O128</td>
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<td>-</td>
<td>-</td>
<td>-</td>
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</tr>
<tr>
<td>O158</td>
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<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
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<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
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</tr>
<tr>
<td>O2</td>
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<td>O78</td>
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<tr>
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<td>+</td>
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<td>-</td>
<td>ND</td>
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<td>O78</td>
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<td>+</td>
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<td>-</td>
<td>ND</td>
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<td>O91</td>
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<td>+</td>
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<td>-</td>
<td>-</td>
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</table>

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(\%)

The stxl 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 inconsistency 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., 2011 and 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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