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

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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-...
H2S production on TSI, lysine decarboxylation, indole, methyl
Purified isolates were examined by oxidase, urea hydrolysis,
Biochemical Identification

with Gram’s according to [8].

Suspected purified colonies were smeared, fixed and stained

Microscopic examination

Identification of E. coli isolates:

Microscopic examination

Suspected purified colonies were smeared, fixed and stained

Gram’s according to [8].

Biochemical Identification

Purified isolates were examined by oxidase, urea hydrolysis,
H2S 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

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence</th>
<th>Amplified product</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>phoA</td>
<td>CGATTCTGGAAAATGGGCAAAAG</td>
<td>720 bp</td>
<td>Hu et al. 2011</td>
</tr>
<tr>
<td>iutA</td>
<td>CTATTGTGAGCAATATACCC</td>
<td>266 bp</td>
<td>Yaguchi et al. 2007</td>
</tr>
<tr>
<td>iutF</td>
<td>CGTCGGGAACGGGTAGAATCG</td>
<td>300 bp</td>
<td></td>
</tr>
</tbody>
</table>

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 colo-

gons, positive indole, methyl red, and Catalase. Meanwhile all isolates were negative oxidase, urea hydrolysis, citrate utiliza-
tion, Voges-Proskauer and didn’t produce H2S. 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:

<table>
<thead>
<tr>
<th>Case</th>
<th>No. of birds</th>
<th>Positive cases</th>
</tr>
</thead>
<tbody>
<tr>
<td>Apparantly healthy chickens</td>
<td>60</td>
<td>42</td>
</tr>
<tr>
<td>Diseased chickens</td>
<td>80</td>
<td>68</td>
</tr>
<tr>
<td>Dead chickens</td>
<td>60</td>
<td>56</td>
</tr>
<tr>
<td>Total</td>
<td>200</td>
<td>166</td>
</tr>
</tbody>
</table>

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 %).

4. antimicrobials susceptibility

The most encountered antimicrobials were Ampicillin, Oxy-
tetracycline, Doxycycline, Neomycin and Gentamycin (65%,
55%, 55%, 55% and 50 % respectively). While lower resistan-
tce was to Erythromycin and Ciprofloxacin (25 and 20 % re-
spectively) Table (5).

Table 5. antimicrobials susceptibility of E. coli isolates

<table>
<thead>
<tr>
<th>Antimicrobial agent</th>
<th>Concentration (µg)</th>
<th>Susceptible (%)</th>
<th>Intermediate (%)</th>
<th>Resistant (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ampicillin</td>
<td>10</td>
<td>10</td>
<td>53</td>
<td>150 (75%)</td>
</tr>
<tr>
<td>Gentamycin</td>
<td>10</td>
<td>25</td>
<td>46</td>
<td>128 (64%)</td>
</tr>
<tr>
<td>Neomycin</td>
<td>30</td>
<td>10</td>
<td>35</td>
<td>114 (55%)</td>
</tr>
<tr>
<td>Erythromycin</td>
<td>15</td>
<td>10</td>
<td>45</td>
<td>10 (55%)</td>
</tr>
<tr>
<td>Doxycycline</td>
<td>30</td>
<td>10</td>
<td>35</td>
<td>55</td>
</tr>
<tr>
<td>Ciprofloxacin</td>
<td>5</td>
<td>15</td>
<td>65</td>
<td>20</td>
</tr>
<tr>
<td>Colistin</td>
<td>10</td>
<td>10</td>
<td>50</td>
<td>30</td>
</tr>
<tr>
<td>Streptomycin</td>
<td>10</td>
<td>10</td>
<td>50</td>
<td>30</td>
</tr>
</tbody>
</table>

5. 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 O29 which didn’t
have iss gene.

Table 6. Distribution of virulence genes among E. coli serotypes.
Liver, Bone marrow, Kidney, spleen, fresh blood and small intestine.

**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 represented *E. coli* serotype O2.9.

**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 systemic 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%), Spleen 114 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 O
\[115\], O
\[44\], O
\[55\], O
\[111\], O
\[157\], O
\[29\], O
\[44\] and O
\[11\]. 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 O
\[115\] and O
\[29\]; followed by O
\[157\] then O
\[158\] O
\[128\] and O
\[142\]. and finally the lowest prevalent serotype were O
\[55\] O
\[111\] and O
\[158\]. These results go in hand with those reported by [13] who recorded O
\[115\] is one of the most predominant serogroups from many serotypes recovered from chickens (O
\[29\], O
\[55\], O
\[111\], O
\[157\], O
\[29\], O
\[55\], O
\[111\], O
\[115\], O
\[115\], O
\[115\], O
\[132\] and 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% resistance 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]. the *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 Dakahila Governorate, require the development of hygienic measures in order to avoid loses caused by *colibacillosis*.

**References**


34. Barry LW, Patrick L, Mutants affected in alkaline phosphatase expression: Evidence for multiple positive regula-

