ECOR phylotyping and determination of virulence genes in *Escherichia coli* isolates from pathological conditions of broiler chickens in poultry slaughter-houses of southeast of Iran

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**Abstract**

Avian pathogenic *Escherichia coli* (APEC) are responsible for wide ranges of extra-intestinal diseases in poultry including colibacillosis, cellulitis, coligranuloma and yolk sac infection. Numbers of virulence are considered important in the pathogenicity of these diseases. The aims of the present study were phylogenetic typing and virulence genes detection in *Escherichia coli* isolates from colibacillosis and cellulitis of broiler chickens in poultry slaughterhouses of Shahrbabak region, Kerman, Iran. A total number of eighty three *E. coli* isolates were taken from broiler chickens with colibacillosis and thirty four isolates were taken from carcasses with cellulitis in the industrial slaughterhouses. Biochemically confirmed *E. coli* isolates were subjected to polymerase chain reaction assay to determine phylogenetic groups and presence of *pap C*, *sfa/focDE*, *icuD*, *afaIB*, *crl*, *fimH* and *crl* virulence genes. Colibacillosis isolates were belonged to A (54.21%), B1 (7.22%), B2 (6.03%) and D (32.53%) phylogroups. Whereas, the isolates from cellulitis cases were belonged to three main phylogroups; A (55.88%), B1 (5.88%) and D (38.24%). Statistical analysis showed a specific association between the presence of *crl* virulence gene and phylogroups of A and D in colibacillosis isolates. The results showed that the isolates from both diseases in broiler chickens could be assigned to various phylogenetic groups (mainly A). Also, the virulence genes profile of cellulitis *E. coli* is completely different from that of colibacillosis in this region.

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Introduction

Assessment of the evolutionary origins via phylogenetic analysis has been helpful for determination of the pathogenic and/or non-pathogenic nature of E. coli isolates. The E. coli can be categorized into four recognized phylogenetic groups; A, B1, B2 and D. This classification has been performed using a triplex polymerase chain reaction (PCR) based method defined previously on detection of three genetic sequences called chuA, yjaA and TSPE4.C2.1

Avian pathogenic Escherichia coli (APEC) are responsible for wide ranges of extra-intestinal diseases in poultry including colibacillosis, cellulitis, coligranuloma, yolk sac infection, airsacculitis and omphalitis.2 Cellulitis is characterized by a necrotic dermatitis of thighs and abdomen.3 Avian cellulitis and colibacillosis are considered as the most important economic threats to poultry industry.4

Reportedly, E. coli isolates from avian cellulitis harbor similar virulence factors with other colibacillosis lesions.5 Numbers of virulence factors including adhesins, toxins, anti-host defense factors, iron acquisition systems, autotransporters and the IbeA protein are involved in these pathological conditions.6

Existence of adhesins factors such as S and P fimbriae is considered to be an essential factor in pathogenesis of these isolates due to their abilities to adhere the poultry epithelial cells. The P fimbriae are important factors for the beginning and expansion of human urinary tract infections; however their roles in the pathogenesis of avian isolates have not been completely clarified. Also, curli fimbriae (encoded by curl and csgA genes) facilitate E. coli isolates adherence to fibronectin and laminin, but their roles in the pathogenesis of APEC are not certainly understood.7 Aerobactin production which facilitates iron scavenging by E. coli has been related to pathogenicity of avian E. coli. The iucD gene encoding aerobactin secretion is highly prevalent virulence gene in APEC isolates.8

Phylogenetic typing of broiler chickens with colibacillosis in Alvaz (southwest of Iran) has showed that these isolates are mainly belonged to A phylotype.9 Phylootyping and ColV plasmid-associated virulence genotyping of E. coli isolated from broiler chickens with colibacillosis in Fars province, Iran, were also investigated.10 However, the link between avian E. coli pathogenicity and different phylotypes and genotypes might provide useful characteristics for the better Iranian APEC identification.

The aim of the present study was phylogenetic typing and virulence genes detection in Escherichia coli isolates from pathological conditions of broiler chickens in poultry slaughterhouses of Shahrbabak region, Kerman, Iran.

Materials and Methods

Sample collection and E. coli isolation. A total number of 83 E. coli isolates were taken from broiler chickens with colibacillosis between November 2014 to April 2015 and thirty four E. coli isolates were taken from broiler carcasses with cellulitis between November 2014 to September 2015. The samples were taken from an industrial poultry slaughterhouse in Kerman province (southeast of Iran). For the initial enrichment, the samples were inoculated into 3 mL buffered peptone water (Merck, Darmstadt, Germany) and incubated at 37 °C for 5 to 6 hr. Subsequently, the enriched samples were streaked on MacConkey agar (Merck) and incubated at 37 °C overnight. Biochemical confirmations were performed on suspected colonies using indole, methyl red, Voges-Proskauer and citrate tests11 and finally confirmed E. coli isolates were subjected to PCR assays.

PCR assay for virulence genes detection. The DNA extractions of overnight cultures of E. coli isolates and reference isolates were prepared by lysis method.12 The isolates were examined through PCR for the presence of curli fimbriae (curl), P fimbriae (papC), S fimbriae (sfa/focDE), aerobactin (iucD), afimbrial adhesin Afa (afaIbC) and F1 fimbriae (fimH) encoding genes.6,13,14 Reference isolates used as positive controls for specific virulence genes were included 1404 (f17A), 239KH89 (iucD and afaE-VIII), A30 (afaIbC) and J96 (sfa/focDE, papC, fimH and curl). Laboratory non-pathogenic E. coli isolate MG1655 was used as a negative control.

Phylotyping analysis. Phylogenetic groups were identified by multiplex PCR based on the presence or absence of chuA, yjaA and TSPE4.C2 sequences in each E. coli isolates.1 The E. coli isolates were segregated in four distinct phylogenetic groups; A, B1, B2 and D. Four E. coli isolates from the Escherichia coli reference (ECOR) collection were used as controls for phylogenetic determination; ECOR58 (B1 group), ECOR62 (B2 group), ECOR50 (D group) and E. coli isolate MG1655 as a positive control for phylogenetic ECOR group A. The primers used for virulence genes amplification as well as phylogenetic groups are shown in Table 1. All the reference isolates were provided from the bacterial collection of Microbiology Department of École Nationale Vétérinaire de Toulouse, Toulouse, France.

Statistical analysis. The data were analyzed using SPSS (version 17.0; SPSS Inc, Chicago, USA) and p values were calculated using Chi-square and Fisher’s exact tests to find any significant relationship. The p values less than 0.05 were considered statistically significant.

Results

In total, phylogenetic analysis of 117 E. coli isolates from pathological conditions of broiler chickens showed that these isolates were divided into four phylogenetic groups as follows: 64 isolates (54.70%) into A, 40 isolates (34.18%) into D, eight isolates (6.83%) into B1 and five
isolates (4.29%) into B2 phylogroups. Virulence genotyping of E. coli isolates showed that none of the isolates harbored the \textit{afaI} B-C, \textit{afaE} \textit{VIII}, \textit{hly} and \textit{f17A} genes. The \textit{Crl}, \textit{fimH}, \textit{papC} and \textit{sfa/foc} were the most prevalent patterns of the isolates (40/117, 34.18%) and most of these isolates (27/40, 67.50%) were belonged to \textit{A} phylogenetic group. Seventeen isolates (17/117, 14.52%) didn’t have any virulence genes. Statistical analysis showed a specific association between the presence of \textit{crl} gene and phylogroups of \textit{A} and \textit{D} (p < 0.05). In addition, a specific association between \textit{crl} gene and \textit{fimH} \textit{sfa/foc} was found (p < 0.05). Details of detected patterns of virulence genes in relation to the phylogenetic subgroups/groups of E. coli were shown in Table 2.

Phylogeny analysis indicated that eighty three E. coli isolates from heart blood samples of broiler chickens with colibacillosis (septicemic isolates) were belonged to four main groups; \textit{A} (54.21%), \textit{B1} (7.22%), \textit{B2} (6.03%) and \textit{D} (32.53%). Phylogenetic subgroups classification revealed that the predominant phylogroup in colibacillosis E. coli was \textit{A0} (38.50%), whereas 51 isolates (61.44 %) fell into six phylogenetic subgroups as follows: 13 isolates (15.60%) into \textit{A1}, six isolates (7.20%) into \textit{B1}, four isolates (4.81%) into \textit{B2-2}, one isolates (1.20%) into \textit{B2-3}, 26 isolates (31.32%) into \textit{D1} and one isolate (1.20%) into \textit{D2}.

Multiplex and simplex PCR assays showed that out of 83 E. coli isolates from colibacillosis cases, 62 isolates (74.69%), 57 isolates (68.67%) and 53 isolates (63.85%) were positive for \textit{crl}, \textit{fimH} and \textit{sfa/foc} genes, respectively. These three genes were the most three prevalent detected virulence genes. Among 62 \textit{crl}-positive isolates, 40 (64.51%) isolates were in combination with \textit{F1}, \textit{S} and \textit{P} fimbriae encoding genes (Fig. 1). The \textit{fimH} gene as the second most prevalent adhesion gene was found in 57 (68.67%) isolates. All of the\textit{fimH}-positive isolates had one of the other examined genes. The \textit{iucD} gene was found in 2.56% of isolates (Fig. 2). Eight different combination patterns of the virulence genes were detected among colibacillosis isolates (Table 2).

The E. coli isolates from carcasses with cellulitis were belonged to three main phylogenetic groups; \textit{A} (55.88%), \textit{B1} (5.88%) and \textit{D} (38.24%). The predominant phylogroup in E. coli isolates was \textit{A0} (50.00%) and the rest of isolates (17/34) were divided into \textit{A1} (5.88%), \textit{B1} (5.88%), \textit{D1} (11.76%) and \textit{D2} (26.48%).

Table 1. Primers used to detection of virulence genes and phylogenetic groups.

| Target | Sequence (5'-3') | Size (bp) | Ref. |
|--------|-----------------|----------|-----|
| \textit{iucD} | TACCGGATTGTCATATGCAGACCCG | 602 | 13 |
| \textit{hly} | ACCATATAAGCGTGTTCTCGCGACAGAG | 1,177 | 13 |
| \textit{sfa/foc} | CCAGAGAGAATTACAAACCTGGCA | 410 | 13 |
| \textit{afaE} | GCTGGGCAGCAAACTGATACTCTC | 750 | 13 |
| \textit{papC} | AATATCTTCCTCCAGTCCGGAGAAG | 328 | 13 |
| \textit{afa} | ATGAGATGCGTTCCTCAAC | 250 | 18 |
| \textit{FimH} | GCGAGGAGTAATTACAAACCTGGCA | 508 | 6 |
| \textit{ChuA} | TGGCAAGCAGGAAATGCAGTA | 279 | 1 |
| \textit{VjaA} | TGGCCGACGACGCAGACGA | 211 | 1 |
| \textit{TspE4C2} | TACCGGATTGTCATATGCAGACCCG | 152 | 1 |

Table 2. Patterns of detected virulence genes in relation to the phylogenetic subgroups/groups of E. coli isolates from colibacillosis and cellulitis cases of broiler chickens.

| Phylogenetic group | Phylogenetic subgroup | A | B1 | B2-2 | B2-3 | D | Total No. (%) |
|-------------------|----------------------|---|----|------|------|---|----------------|
| \textit{crl} \textit{fimH} \textit{papC} \textit{sfa/foc} | Colibacillosis | 23 | 4 | 3 | - | - | 10 | 40 (34.18) |
| \textit{crl} \textit{fimH} \textit{papC} | Colibacillosis | 3 | 3 | - | - | - | 1 | 7 (5.98) |
| \textit{crl} \textit{fimH} \textit{iucD} | Colibacillosis | 1 | 2 | - | - | - | - | 3 (2.56) |
| \textit{crl} \textit{fimH} \textit{papC} | Colibacillosis | 3 | - | 1 | 1 | - | - | 5 (4.27) |
| \textit{fimH} \textit{papC} | Colibacillosis | - | 3 | 1 | 2 | - | 1 | 7 (5.98) |
| \textit{fimH} \textit{sfa/foc} | Colibacillosis | - | - | - | - | - | 1 | 1 (0.85) |
| \textit{papC} \textit{sfa/foc} | Colibacillosis | 1 | - | - | - | - | - | 1 (0.85) |
| \textit{fimH} | Cellulitis | 16 | - | 2 | - | - | 3 | 21 (17.90) |
| \textit{crl} | Cellulitis | - | 2 | - | - | - | 4 | 3 (7.69) |
| \textit{papC} | Cellulitis | 1 | - | - | - | - | - | 1 (0.85) |
| Not detected | - | - | - | - | - | 14 | 3 | 17 (14.52) |
| Total phylogroup | - | - | - | - | - | 49 | 15 | 8 | 4 | 1 | 30 | 10 | 117 |
| Total phylogroup | - | - | - | - | - | 64 | 8 | 5 | - | - | 40 | - | 117 |

APEC: Avian pathogenic \textit{Escherichia coli}. 

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34(34.18) APEC: Avian pathogenic \textit{Escherichia coli}.
The prevalence of fimH gene among thirty four E. coli isolates from carcasses with cellulitis was (61.76%), (Fig. 3). The frequency of crl and papC genes was 26.74% and 2.95%, respectively. These isolates were negative for the other examined genes (Table 2).

Discussion

Genotyping of APEC and evolutionary findings of the adhesive capacity of E. coli isolates isolated from avian colibacillosis and cellulitis showed that these isolates can be categorized into different phylogenetic groups.5,10 In the present study, phylogenetic analysis of 117 E. coli from colibacillosis and cellulitis showed that most of the isolates harboring virulence genes were belonged to A phylogroup. Another study on phylogenetic groups in E. coli isolates isolated from septicemic broiler and layer cases has shown that the APEC isolates were belonged to A (71.00%), B1 (4.10%), B2 (7.90%) and D (18.70%) phylo-groups.15 The data about phylogenetic groups of APEC especially isolates from colibacillosis and cellulitis are limited. It has been reported that the isolates from these pathological conditions are mostly belonged to A phylogroup and in the lesser amount to D group which is in accordance with our study.16

Although previous reports have found that APEC isolates can cause serious infections (both localized and systemic infections) in birds, the pathophysiology of these infections has not been elucidated.6 It seems that important stages in the pathogenicity of APEC isolates are fimbrial and putative virulence factors and the expression of these factors can be associated with resistance to host immune response. Previous studies have found that virulence profile and molecular characteristics of E. coli isolates from avian colibacillosis and avian cellulitis are similar,17 but in the present study, the detected gene patterns in colibacillosis cases had higher diversity than cellulitis E. coli.

Several studies have showed sequence homology between specific DNA regions of APEC and human extra-intestinal pathogenic E. coli (ExPEC).18 Moreover, the presence of similar virulence genes in APEC and ExPEC isolates proposed that APEC isolates can act as zoonotic pathogens and reservoirs of virulence causing human infections.18 It has been reported that the isolate can be considered as ExPEC if contains two or more of the following virulence genes; pap (P fimbriae), sfa/foc (S/F1C

Fig. 1. PCR results for crl, fimH and sfa/foc genes detected from colibacillosis isolates. Lane L: Ladder 1kb; Lane 1: Positive control for crl gene (E. coli J96); Lane 2: Positive isolate for crl gene; Lane 3: Positive control for sfa/foc DE gene (E. coli J96); Lane 4: Positive isolate for sfa/foc DE gene; Lane 5: Positive control for fimH gene (E. coli J96); Lane 6: Positive isolate for fimH gene.

Fig. 2. The PCR result for iucD gene detected from colibacillosis isolates. Lane L: Ladder 1 kb; Lane 1: Negative control (E. coli MG1655); Lane 2: Positive control for iucD gene (E. coli 239KH89); Lane 4, 5 and 7: Positive isolates for iucD gene.

Fig. 3. The PCR result for fimH gene detected from cellulitis isolates. Lane L: Ladder 1 kb; Lane 1: Positive control for fimH gene (E. coli J96); Lane 2 and 3: Positive isolates for fimH gene; Lane 4: Negative control (E. coli MG1655).
fimbriae), afa/dra (binding adhesins), iutA (aerobactin receptor) and kpsM II (group 2 capsule synthesis).³

The prevalence of type 1 fimbrial adhesion gene (fimH) in APEC isolates was 66.66% in this study. The FimH virulence factor is seemed to be an essential unit for protecting the APEC isolates against host immune system,¹² but the exact role of Fim H in the pathogenesis of APEC isolates remains debatable with incompatible results.¹⁰ Other studies have shown higher prevalence of the fimH virulence gene.²⁰

According to the results of the present study, one hundred isolates (100/117, 85.47%) harbored at least one of the examined genes, whereas; the crl gene was the second most prevalent gene (71/117, 60.68%) among APEC isolates. It has been shown that curli-negative mutant isolates have less adherence colonization, invasion and persistence to chicken tissues recommending curli as a virulence factor.²¹ It has been reported that the prevalence of crl gene among APEC isolates was 92.70% in Germany.¹⁶ Therefore, it can be supposed that most of APEC isolates are curliated.²⁰

In the present study, the prevalence of the pap gene (53/83) in colibacillosis E. coli was remarkably higher than that of cellulitis E. coli (1/34). The reason for higher association between pap gene and colibacillosis E. coli has not been yet notified. Accordingly, the pap operon genes in less than 20.00% of the APEC have been identified in broiler chickens previously.²²

None of the studied isolates were positive for afal B-C, afa E-VIII and f17A genes. Previous studies have showed that the Afa adhesions exist on less than 10.00% of APEC isolates suggesting that E. coli isolates expressing afa-B gene are able to reproduce clinical symptoms and lesions of cellulitis and colibacillosis.¹⁶

In this study, fifty three of the isolates were positive for sfa genes among colibacillosis isolates and none of the cellulitis-related isolates were positive for this gene. While, the sfa gene was identified in the range of 0.00%, 1.80% and 8.80% of isolates from APEC by PCR method.¹⁶,²²,²³ Previous study has showed that the S fimbria can be found rarely in APECs and its role in the pathogenicity of them is not completely clarified.²³

In the present study, 3 (3.60%) of E. coli isolates from colibacillosis cases were considered as iucD-positive and none of the isolates from cellulitis cases were positive for iucD gene. These findings are in contrast with the frequencies of aerobactin genes in previous studies ranged from 44.00% to 83.00% in the isolates from coliform cellulitis and colibacillosis.²⁴

In conclusion, E. coli isolates from colibacillosis and cellulitis cases were disseminated in various phylogenetic groups (mainly A). These isolates harbored various fimbrial genes in a relatively high prevalence. On the other hand, the virulence profile of cellulitis E. coli was different from that of colibacillosis cases. However, these aforementioned pathological conditions may be associated with other virulence factors that have not been investigated. More researches are needed to determine the phylogenetic analysis of APEC isolates from other pathological conditions to find the exact relation between the virulence genes and ECOR background of these isolates.

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Conflict of interest

The authors declare that there is no conflict of interest.

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