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A comparative phylotyping analysis of *E. coli* originating from avian colibacillosis based on the Clermont triplex scheme and *gyrA* gene sequencing

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Abstrac: Avian colibacillosis (AC) is frequently reported from poultry farms in the west of Iran. The aim of the present survey was to evaluate the phylogeny of *Escherichia coli* isolated from AC through the Clermont triplex protocol and *gyrA* gene sequencing. One hundred and fifty fresh carcasses suspected of having died from colibacillosis were sampled. The specimens were examined for *E. coli* isolation and identification. The extracted DNA repertoire of the isolates was subjected to phylogenetic analysis using Clermont triplex PCR. A DNA fragment of 1.8 kbp of the *gyrA* gene was amplified from a representative of each phylogroup or subgroup and the final product was sequenced to depict a phylogenetic tree. In general, among 119 *E. coli* isolates, 31.93%, 6.72%, 34.45%, and 23.52% were clustered in phylogroups A, B1, B2, and D, respectively. The phylogenetic assignment of the isolates based on the phylogenetic tree was completely uniform with the results of the triplex method. The outcome represents the segregation of avian pathogenic *E. coli* isolates to various phylogroups in the region. Moreover, the precise cluster analysis of *E. coli* based on *gyrA* sequencing confirms this method as a robust candidate for phylotyping of the bacterium.

Key words: *Escherichia coli*, poultry, phylogrouping, Clermont triplex PCR, *gyrA* gene sequencing

1. Introduction

Escherichia coli is a common symbiont of the gastrointestinal tract in humans and animals, including poultry. Despite this, avian pathogenic *E. coli* (APEC) strains are implicated in a variety of extraintestinal clinical repercussions, among which is colibacillosis. Considering the great economic burden imposed by the disease, colibacillosis is postulated as a universal threat to the poultry industry (1). Recently, a triplex PCR approach targeting two genes, *chuA* and *yjaA*, and a DNA fragment, TSPE4.C2, was elucidated as a dichotomous key for assignment of an anonymous *E. coli* isolate into one of the four major phylogroups including A, B1, B2, and D (2). Because of the variety of isolates in the ecological niche, lifestyle, propensity to cause disease, and phenotypic and genotypic traits regarding their phylogroup background, phylotyping analysis of APEC strains confers significant information in epizootiological studies (3,4).

DNA gyrase, a vital bacterial enzyme, constitutes the negative supercoiled structure of DNA and encompasses two subunits, gyrA and gyrB. The ubiquitous presence of the *gyrA* gene in all bacteria and the conserved sequence of the gene among species makes it an appropriate marker for phylogenetic analysis (5). Designing of universal primers based on the presence of extensively conserved motifs in the *gyrA* gene sequence has been proposed for studying bacterial identity and diversity (6). Clustering of *E. coli*, *Flexispira*, and *Helicobacter cinaedi* strains is successfully assessed by phylogenetic trees illustrated based on the *gyrA* gene sequencing (7,8). To date, because studies only represent *E. coli* phylogrouping based on the *gyrA* gene sequencing and with the proposal of the authors for further investigations regarding this method (8), the aim of the present research was to identify the phylogenetic background of APEC strains isolated from Avian colibacillosis (AC) in the west of Iran based on the Clermont triplex protocol and *gyrA* gene sequencing.

2. Materials and methods

2.1. Sample collection and *E. coli* isolation

In a cross-sectional study from January to June 2018, a total of 150 specimens were collected from newly dead poultry carcasses suspected of colibacillosis. The sampling was from diverse farms all over the west of Iran. The lungs, liver, spleen, and peritoneal fluid of each bird were collected as a pooled sample. The colibacillosis cases were diagnosed clinically by a trained poultry veterinarian.

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In the laboratory, *E. coli* was recovered from the samples based on initial culture on MacConkey agar (MAC, Quelab, Canada), following by biochemical identification of one pink colony on MAC. The bacteria with green metallic shiny appearance on eosin methylene blue agar (EMB, Quelab, Canada), acid/acid reaction in TSI, and + + - - IMViC reactions were considered as *E. coli* (9).

2.2. DNA extraction and polymerase chain reactions
The DNA repertoire of each isolate was extracted from the overnight culture of the bacterium in Luria-Bertani broth (Quelab, Canada) through the boiling method (10). The isolates were molecularly confirmed as *E. coli* in a species-specific PCR reaction described elsewhere (11). Consequently, the isolates were assigned to a phylogroup based on the Clermont triplex PCR approach (2). The thermal conditions of the reaction were 5 min at 94 °C for initial denaturation followed by 30 cycles of 30 s at 94 °C, 30 s at 55 °C, and 30 s at 72 °C, with a final extension step of 7 min at 72 °C (2). In the final step, a fragment of the *gyrA* gene of 1.8 kbp was amplified from one *E. coli* strain assigned in each phylogroup/subgroup using the pair of primers and the protocol designed by Shamsi et al. (8). The thermal profile was initiated with a denaturation step at 94 °C for 5 min followed by 35 cycles at 94 °C for 45 s, an annealing step at 60 °C for 45 s, and an extension step at 72 °C for 130 s, with final elongation at 72 °C for 5 min (8). The products generated from *gyrA* gene amplification were subjected to sequencing by SinaClon Company (Tehran, Iran) following the purification of the amplicons from the agarose gel using a PCR purification kit (SinaClon, Iran). The nucleotide sequences' alignment and comparison with some *gyrA* gene sequences retrieved from the GenBank database was conducted using Clustal W Version 2.1 software (12). Phylogenetic analysis was achieved using MEGA 6.0 (13) with the neighbor-joining algorithm (14). Characteristics of the *E. coli* isolates used to illustrate the phylogenetic tree are depicted in Table 1.

3. Results
In total, 119 *E. coli* strains (79.33%) were isolated phenotypically, all of which generated the predicted 662-bp product in a species-specific PCR reaction (Figure 1). Phylogroup assignment of the isolates based on the triplex PCR assay revealed the predominant frequency of phylogroups B2 and A, followed by D and B1. Moreover, both subgroups in phylogroups A, B2, and D were identified with the frequency of 7.5% and 24.37% for A_0 and A_1, 12.60% and 21.84% for $B2_2$ and $B2_3$, and 15.96% and 7.56% for D_1 and D_2, respectively. The phylogroup and subgroup distributions of the *E. coli* isolates are illustrated in Table 2. Figure 2 indicates the phylogenetic profile of *E. coli* phylogroups and/or subgroups. Moreover, four isolates (3.36%) could not be clustered in any phylogroup using the mentioned method and were nominated as untypable.

Table 1. Characteristics of the *E. coli* strains used to illustrate the phylogenetic tree in the present study.

	E. coli strain	GenBank accession number
1	EC11	AGWF01000000
2	O103/RM10042	AP010958
3	KSC1031	NEKK00000000
4	NIVEDI	AP001918
5	WG5	CP024090
6	AS19-RrmA$^-$	CP027430
7	ACN001	CP007442
8	FAM21845	PRJEB21000
9	K-12/MG1655	NC_000913
10	J53	AICK00000000
11	NCTC86	CP019778
12	ME8067	CP028703
13	ML35	CP025747
14	ETEC-2264	CP023349
15	ED1a	CU928162

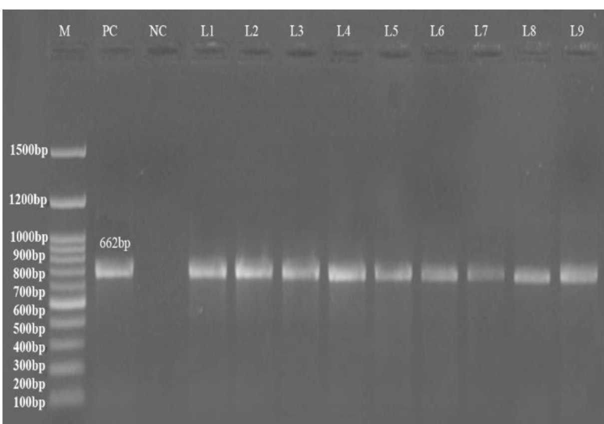

Figure 1. Agarose gel electrophoresis of PCR products with *E. coli* species-specific primers (662 bp). M: 100-bp DNA ladder (CinnaGen, Iran), PC: positive control (*E. coli* O157:H7 ATCC 43895), NC: negative control, Lanes 1–9: field samples.

DNA sequences of a fragment of the *gyrA* gene of 1.8 kbp (Figure 3), from a randomly chosen isolate in each phylogroup or subgroup, were used to generate a phylogenetic tree (Figure 4). Also of interest, the six clusters generated in the phylogenetic tree were in accordance with the results obtained by triplex PCR assay (Table 3). Furthermore, two divided clusters regarding phylogroups B2 and D were constituted.

Table 2. Phylogroup and subgroup distributions of the *E. coli* isolated from avian colibacillosis.

Phylogroup	A		B1	B2		D	
Subgroup	A_0	A_1	-	$B2_2$	$B2_3$	D_1	D_2
n	9	29	-	15	26	19	9
(%)	(7.56%)	(24.36%)		(12.60%)	(21.84%)	(15.96%)	(7.56%)
Total n (%)	38 (31.93%)		8 (6.72%)	41 (34.45%)		28 (23.52%)	

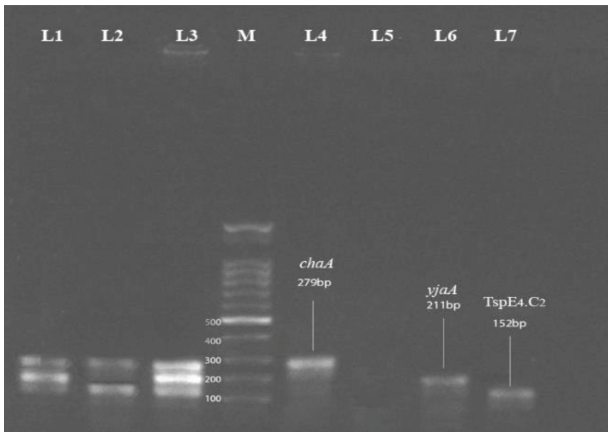

Figure 2. Agarose gel electrophoresis of PCR products representing *E. coli* phylogroups and subgroups. L1: $B2_2$ template ($chuA^+$, $yjaA^+$, $TspE4.c2^-$), L2: D_2 template ($chuA^+$, $yjaA^-$, $TspE4.c2^+$), L3: $B2_3$ template ($chuA^+$, $yjaA^+$, $TspE4.c2^+$), M: 100-bp DNA ladder (CinnaGen, Iran), L4: D_1 template ($chuA^+$, $yjaA^-$, $TspE4.c2^-$), L5: A_0 template ($chuA^-$, $yjaA^-$, $TspE4.c2^-$), L6: A_1 template ($chuA^-$, $yjaA^+$, $TspE4.c2^-$), L7: B1 template ($chuA^-$, $yjaA^-$, $TspE4.c2^+$).

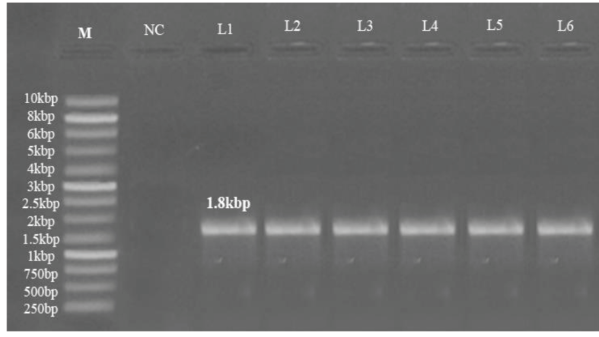

Figure 3. Agarose gel electrophoresis of PCR products with *gyrA* gene primers (1.8 kbp). M: 1-kb XLarge DNA Ladder (GeneDireX, Japan), NC: negative control, L1: positive control (*E. coli* O157:H7 ATCC 43895), Lanes 2–6: field samples.

4. Discussion

Colibacillosis, a devastating disease of poultry, is frequently reported from various districts of Iran (8,15–19), despite the absence of data about the status of the disease in the west of Iran. Phylogenetic analysis of clinically isolated *E. coli* strains is necessary to better understand the epizootiology of the disease in animals (20), in addition to the mechanisms of acquisition of virulence in pathogenic strains (21). Though the derivation of human commensal *E. coli* strains predominantly from phylogroups A and B1 versus the pathogenic strains from phylogroups B2 and D is documented (3), significant differences exist regarding the phylogroup origin of APEC strains. A large number of APEC strains are being accommodated in phylogroups A, D, and B2 (22).

Based on the advantages, including rapidness, inexpensiveness, and lower complexity, of the Clermont triplex PCR scheme (2) over the reference methods of *E. coli* phylotyping, such as multilocus enzyme electrophoresis (23) and ribotyping (24), and an approximate 85%–90% congruity in results (19), it has been frequently applied (15,16,25–27).

The *gyrA* encoded gene is postulated as a pertinent candidate for the genus *Helicobacter* and *E. coli* phylogeny (7,8). Among the different genetic techniques employed for analyzing particular strains, multilocus phylogenetic lineages are introduced as a powerful approach for identification of *E. coli* isolates at strain level. Despite the time-consuming nature and complicated interpretation of the results of sequencing methods, they are recommended because of the higher discriminative potential of these techniques in comparison with rapid genotyping methods (28). *gyrA* gene sequencing is assumed as an initial screening tool for *E. coli* phylogeny (8).

In the present study, in addition to the evaluation of the frequency of the disease in our local area, the phylogroup backgrounds of the isolates were determined based on the Clermont triplex protocol and *gyrA* gene sequencing. The results represented the relatively high prevalence of the disease. A high frequency of AC is also reported from other parts of Iran (8,15–17,24). Besides, 100% consistency was observed between the results of the two *E. coli* phylotyping methods. Therefore, it can be assumed that *gyrA* sequencing may be a proper candidate for *E. coli* phylotyping.

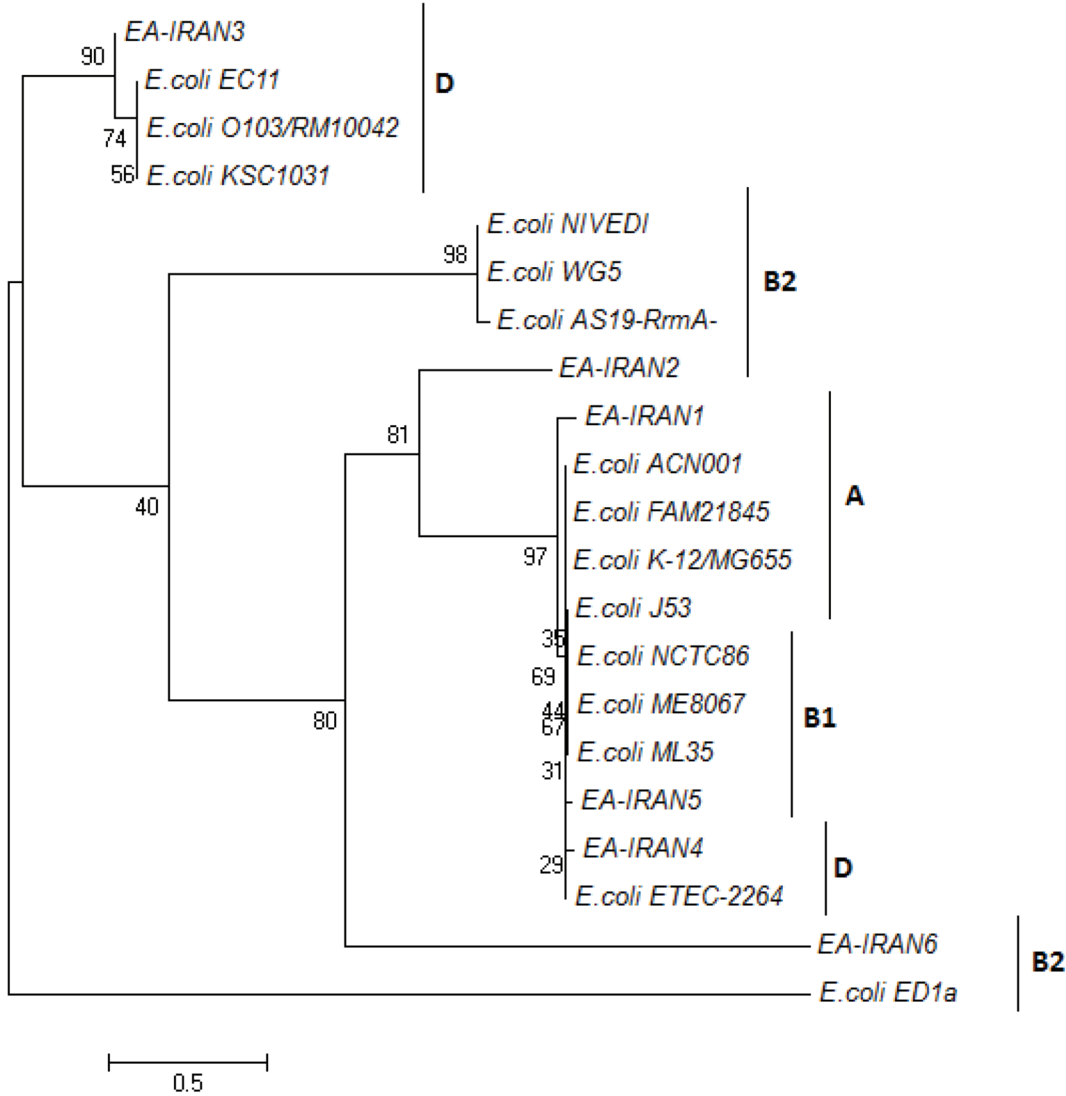

Figure 4. Phylogenetic analysis of *E. coli* isolated from avian colibacillosis based on partial *gyrA* gene sequences constructed using the neighbor joining method with 1000 bootstrap replicates.

Table 3. A comparative clustering of *E. coli* isolated from avian colibacillosis based on triplex PCR scheme and *gyrA* gene sequencing.

E. coli isolate	Phylogroup origin based on triplex PCR assay	Phylogroup origin based on *gyrA* gene sequencing
EA-IRAN1	A	A
EA-IRAN2	$B2_2$	$B2_2$
EA-IRAN3	D_2	D_2
EA-IRAN4	D_1	D_1
EA-IRAN5	B1	B1
EA-IRAN6	$B2_3$	$B2_3$

In the present study, the predominant *E. coli* phylogroups isolated from AC were B2, A, and D. This coincides with previous findings (22). The frequencies of APEC phylogroups in pioneering studies were A (44.7%), D (25.5%), B1 (21.3%), and B2 (8.5%) (15); A (50%), D (45%), B1 (2.8%), and B2 (1.4%) (16); A (36%), B2 (28%), D (20%), and B1 (16%) (17); and D (33.33%), B2 (27.38%), B1 (20.24%), and A (19.05%) (8). The most common APEC phylogroups reported from Mexico were B1 and B2 (both 34.7%) and A (8.6%) (29), versus the predominance of phylogroups A (73%) and D (14%) stated from the south of China (30). In another study, APEC isolates were concentrated in phylogroups A (63%), D (33%), and B2 (4%), with the lack of any isolate in phylogroup B1 (31). The discrepancy in the reported prevalence displays the heterogenicity of the APEC isolates. Various studies explained that some APEC strains may be a derivation of commensal isolates by acquisition of virulence genes through mobile genetic elements (29–31). In the present survey, segregation of *E. coli* isolates into six subgroups concurs with the results revealed by Ghanbarpour et al. (15). Identification of subgroup $B2_3$ contradicts with the idea by which this subgroup is introduced as a good indicator of human fecal contamination (32). The probable explanation for the controversy of phylogroup distribution in various studies may be due to climate and environmental conditions, geographic variation, and body weight of the affected host (33). Further studies are needed to elucidate the virulence of APEC isolates in the region in order to evaluate the potential relevance of the isolates for public health. The frequency of APEC isolates transferring through the food chain to humans in the region is also of value to evaluate. A revised version of the scheme of Clermont et al. (34) was introduced for *E. coli* phylogrouping with the ability to categorize *E. coli* isolates in eight phylogroups. Nowadays, this method is regularly used for classification of human (35) and animal (36) origin *E. coli* isolates. It is highly recommended to use the new Clermont et al. (34) method in future studies.

In conclusion, our results represent valuable information regarding the distribution of colibacillosis and *E. coli* phylogroups in the west of Iran. Moreover, the compatibility of *E. coli* phylogeny through the Clermont triplex assay and *gyrA* sequencing was confirmed. Additional analysis of human and other animal origin *E. coli* strains may better underscore the efficiency of *gyrA* gene sequencing as a trustable candidate for *E. coli* phylogrouping.

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