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# Genotypic and Phenotypic Characteristics of the Phylogenetic Groups of *Escherichia Coli* Isolates From Ostriches in Iran

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## ABSTRACT

Increased antibiotic use in the ostrich industry could lead to the emergence of virulent antibiotic-resistant bacterial strains transmissible to human. This study investigated the genotypic and phenotypic characteristics of the phylogenetic groups of *Escherichia coli (E. coli)* isolates from ostrich and reveal their health risk potential. One hundred twenty-nine confirmed presumptive commensal (44) and suspected pathogenic (85) *E. coli* isolates from ostrich flocks in Mashhad, Northeast Iran, were phylo-typed by the Clermont quadruplex polymerase chain reaction. The phylogenetic profile of the isolates was comparatively investigated based on antimicrobial susceptibility, resistance, and virulence gene profiles. Results indicated that both groups of presumptive commensal and pathogenic isolates were mostly distributed within phylogroups A (with proportions 31.81% and 32.94%, respectively) and B1 (with proportions 36.36% and 31.76%, respectively). Multi-drug resistance was highest within the phylogroup B2 ($p \geq 0.05$). The phylogroup B1, typically known for commensal strains, unlike B2, showed the most negligible proportions of isolates which were devoid of resistance genes ($p \geq 0.05$) and virulence genes ($p \geq 0.05$). The findings of this study expanded the horizon of the genotypic and phenotypic characteristics of the phylogenetic groups of *E. coli* isolates from ostrich. Moreover, we indicated a complicated inconsistency between both characteristics. Therefore, more comprehensive and comparative studies on *E. coli* isolates from ostrich and human are favoured in future research.

## Keywords

*Antimicrobial susceptibility, Clermont quadruplex PCR, MDR, Resistance genes, Virulence genes*

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## Abbreviations

*E. coli: Escherichia coli*
PCR: Polymerase Chain Reaction
MDR: Multi-drug resistance
APEC: Avian pathogenic *E. coli*
ExPEC: Extra-intestinal pathogenic *E. coli*



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## Introduction

The presence of antimicrobial residues and the emergence of resistant bacterial pathogens in food and the environment have complicated the strategies for appropriate treatment, raising serious public health concerns. In this sense, the improper or extensive use of antimicrobials in food-producing animals, particularly poultry, for improving growth and health probably plays a significant part. This practice imposes a selection pressure, leading to resistant bacterial strains transmissible to human [1-5]. The emergence and dissemination of diversified phylogenetic groups of antibiotic-resistant *E. coli* strains is a global health concern. Furthermore, *E. coli* strains provide accurate findings on antimicrobial resistance status because of their presence in the environment and as commensal flora in humans and animals. Therefore, monitoring the phylogenetic distribution of *E. coli* strains could benefit the design of preventative and therapeutic strategies with economic significance [6-8]. The phylogenetic background, indicating the ecological distribution, evolutionary history, and virulence of pathogens, could be affected by geographical region, sampling area, site of infection, antibiotic resistance, and host response. Environmental, social, and dietary conditions are also considered to cause phylogenic heterogeneity [9]. Most extra-intestinal pathogenic *E. coli* strains belong to the previously described phylogroups. On the other hand, newly described phylogroups mostly include intestinal pathogenic *E. coli* strains. As a result, the phylotype-related traits necessitate a reliable detection of *E. coli* phylogroups and also the investigation of probable impacts of virulence genes, MDR characteristics, and their cross-talk regarding each phylogroup [7, 10]. Finding the relationship between pathogenic traits and the phylogeny of E. coli is a complicated phenomenon due to distinctive interplays [11]. However, some previous studies indicated that commensal *E. coli* strains are within phylogroups A and B1, while extra-intestinal pathogenic *E. coli* strains belong to phylogroups D and B2. Moreover, E. coli strains within phylogroup B2, which have the highest susceptibility to antimicrobials, have previously exhibited promoted virulence capacity compared to commensal groups [12].

The APEC strain belongs to the ExPEC category [13]. Phylogenic backgrounds and virulence genes within the ExPEC strains from both human and avian sources are identical [5]. Therefore, monitoring the ExPEC strains in poultry is crucial from the perspective of public health [14]. Comprehensive research has demonstrated that APEC and human ExPEC strains have multiple common traits, encompassing serogroups, virulence factors, and sequence types. In addition, the APEC strains might function as a reservoir for the virulence genes of ExPEC in humans. Therefore, for the effective surveillance and control of avian colibacillosis, it is vital to identify the phylogeny, lineage, and virulence of APEC strains that commonly infect poultry flocks. Early identification of these strains using phylogenetic analyses could beneficially provide the needed preventive measures [15, 16].

The ostrich farming industry is rapidly expanding for the human consumption of meat, leather, and plumes. This industry plays an integral part in terms of agriculture, economy, and meat production in Iran. Scarce information on ostrich-originated *E. coli* strains, transmissible to humans, necessitates more attention to the potential zoonosis health threats caused by these strains [17, 18]. To the best of our knowledge, this study is the first that comprehensively compared the phylogenetic profile of *E. coli* isolates from ostrich based on their genotypic and phenotypic traits. This study demonstrated some of the genotypic and phenotypic characteristics of the phylogenetic groups of *E. coli* isolates from ostrich, thereby revealing their potential health threats.

## Result

### *Distribution of Phylogenetic Groups Within Isolates and in Relation to the Source of Isolation*

Most isolates were segregated into five phylogenetic groups. However, 11.36% and 4.70% of the presumptive commensal and pathogenic strains were unassignable, respectively. The distribution of both groups of presumptive commensal and pathogenic strains was the highest in the B1 and A phylogroups. Commensal isolates were rarely classified into phylogroups B2 (0%) and C (2.27%). Details on phylogenetic classification results are demonstrated in Figure 1. E. coli strains isolated from each source mostly belonged to the phylogroups A and B1, except that the strains isolated from the lungs were mainly within phylogroups A (42.85%) and E (42.85%). Those isolated from dead-in-shell embryos were mostly within the phylogroups B2 (75%) and E (25%). Moreover, the isolates from the faeces of sick ostriches were abundantly within phylogroups A (53.84%) and Unassignable (23.07%). Isolates from each source, except for the isolates from embryos, yolk sacs, and faeces of sick ostriches, possessed the lowest frequency within the phylogroups B2 and C. The strains within the Unassignable phylogroup were only isolated from faeces (of both healthy and sick birds; $p \geq 0.05$) and yolk sacs ($p \geq 0.05$). The strains within the phylogroup B2 were significantly isolated from dead-in-shell embryos ($p < 0.05$). Details on the phylogenetic groups in relation to the source of isolation are presented in Figure 1.

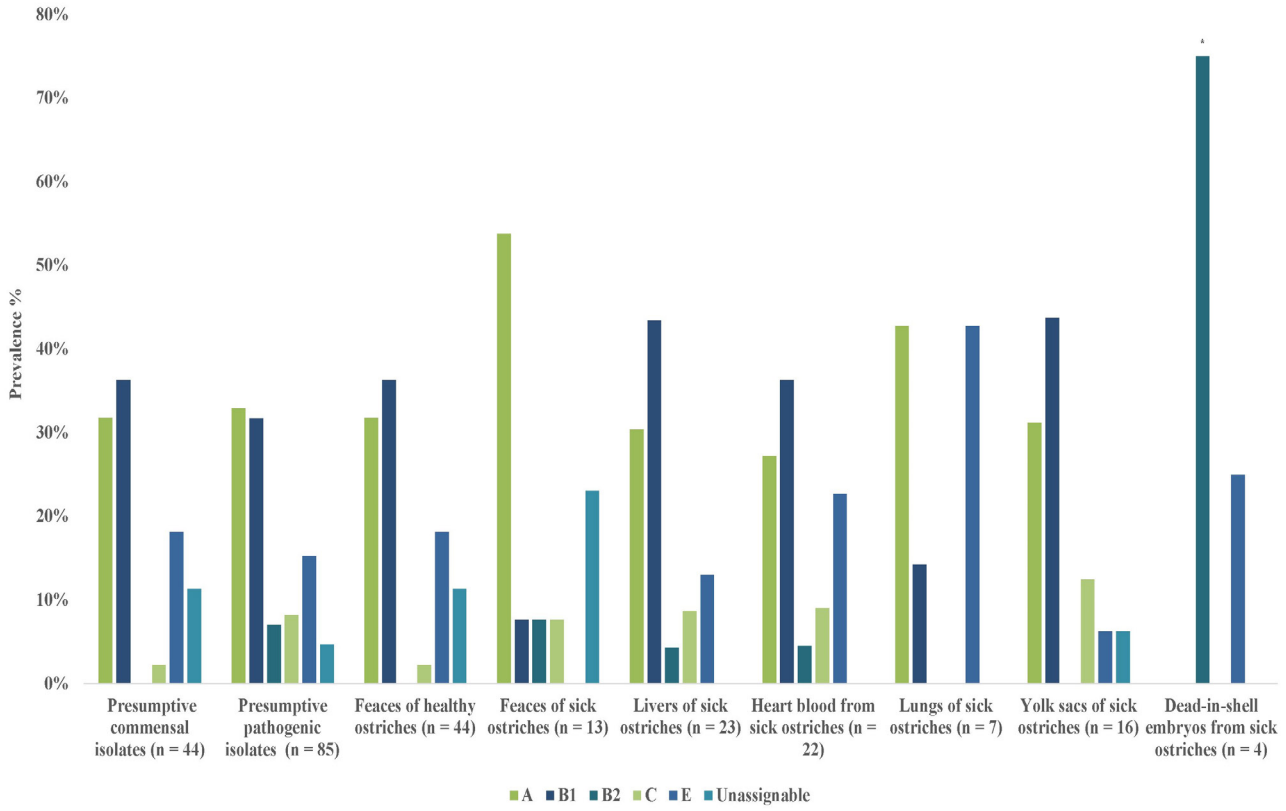


**Figure1.**
Distribution of phylogroups within the presumptive commensal and pathogenic *E. coli* isolates from ostrich and in relation to their source of isolation. Against categorical variables, *P-value* is ≥ 0.05 for each phylogroup, excluding one indicated by * (*P-value* < 0.05).

### *Antimicrobial Resistance Profile of Isolates and Phylogenetic Groups*

Comparing the resistance of the pathogenic and commensal groups of isolates against four antimicrobials revealed a higher proportion of resistance for the earlier group against tetracycline ($p \geq 0.05$); however, both groups of isolates showed a comparable proportion of resistance against the other two tested antimicrobials, lincomycin ($p \geq 0.05$) and ceftriaxone ($p \geq 0.05$). The distribution of antibiotic resistance within both groups of presumptive commensal and pathogenic isolates was highest against lincomycin and tetracycline, respectively, and lowest against ceftriaxone (Figure 2). Isolates from all phylogenetic groups harboured 100% resistance proportion against lincomycin. The isolates of the phylogroup C were entirely susceptible to streptomycin, trimethoprim + sulfamethoxazole, and doxycycline. Isolates of the phylogroup C also recorded no resistance against enrofloxacin and amoxicillin, similar to unassignable isolates. Isolates of C and B2 phylogroups showed complete susceptibility to florfenicol. While full susceptibility was recorded against ceftriaxone for isolates within most phylogenetic groups, the isolates of C and E phylogroups showed 12.5% and 9.52% resistance to this antimicrobial, respectively ($p \geq 0.05$). Resistance to gentamicin was also low within the isolates of all phylogroups, and only the isolates within phylogroups A, C, and E indicated a negligible resistance (4.76%, 12.5%, and 4.76%, respectively) ($p \geq 0.05$). Details on antimicrobial resistance frequency in relation to phylogeny are demonstrated in Figure 3a. Results also revealed that the highest total MDR proportion belonged to the phylogroup B2 (33%) ($p \geq 0.05$) compared to the MDR observed within the phylogroups A (10%), B1 (21%), C (0%), E (24%), and Unassignable (0%). More details on MDR frequency in relation to phylogeny are demonstrated in Figure 3b.

### *Antimicrobial Resistance Gene and Virulence Gene Profiles of Phylogenetic Groups*

Within phylogroups B2 and C, sul1 and tet(A) genes were undetectable. In addition, sul1 gene was absent within the Unassignable phylogroup ($p \geq 0.05$). Phylogroups B2 and C included the isolates devoid of blaTEM and qnrA gene, respectively. More than half of the isolates within the phylogroup B2 (66.66%) lacked any resistance genes, which was the most abundant but was not significantly different ($p \geq 0.05$) compared to the corresponding rate of the phylogroup B1 (30.23%). Details on antimicrobial resistance gene frequency in relation to phylogeny are demonstrated in Figure 4a. Within phylogroups C and Unassignable, astA and Irp2 genes were absent. Within the Unassignable phylogroup, iucD gene was

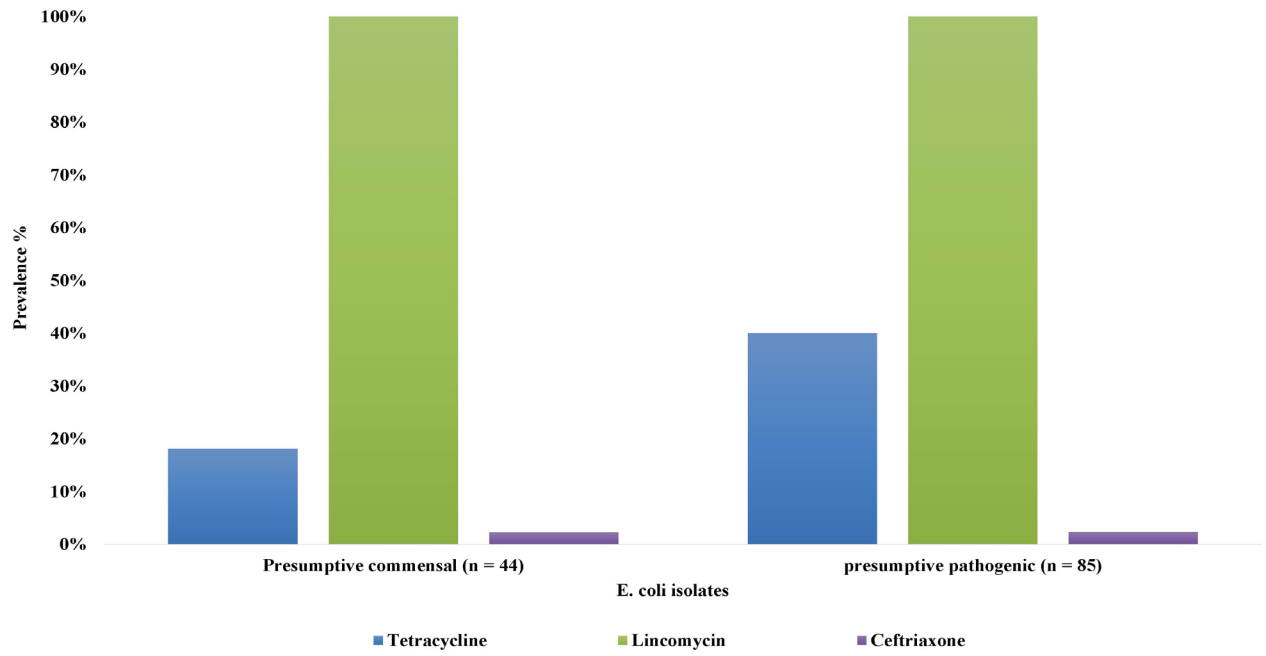


**Figure 2.**
Distribution of antibiotic resistance against three different antimicrobials within presumptive commensal and pathogenic *E. coli* isolates from ostrich. *P-value* is ≥ 0.05 for each antimicrobial between categorical variables.

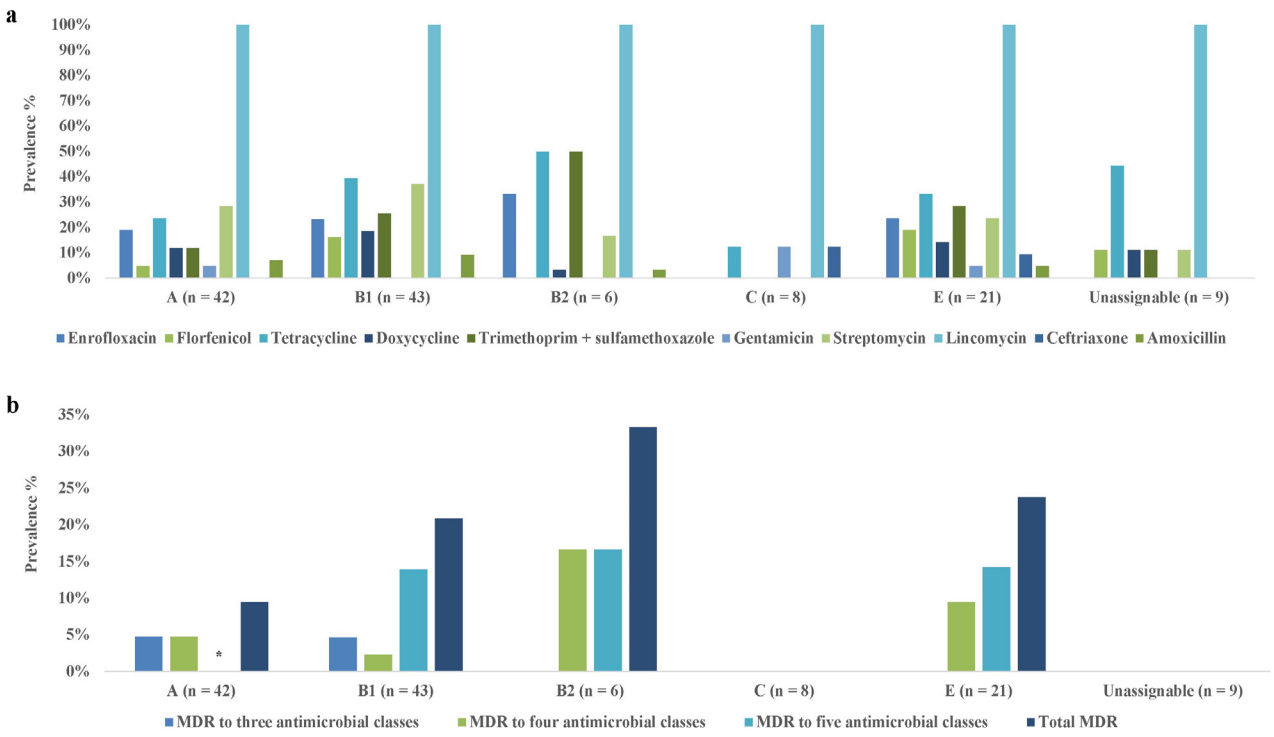


**Figure 3.**
(a) Distribution of antibiotic resistance against 10 different antimicrobials within phylogroups of E. coli isolates from ostrich. *P-value* is ≥ 0.05 for each antimicrobial against categorical variables. (b) Distribution of MDR within phylogroups of *E. coli* isolates from ostrich. Against categorical variables, *P-value* is ≥ 0.05 for each MDR, excluding one indicated by * (*P-value* < 0.05).

also undetectable. Within all phylogroups, excluding B1 ($p < 0.05$), tsh gene was rare. In addition, B1 was the only phylogroup carrying vat gene (2.32%) ($p \geq 0.05$). Only within the phylogroup B2, cvaA/B gene was undetectable ($p \geq 0.05$), whereas this gene was significantly distributed within the phylogroup C ($p < 0.05$). More than half of the isolates of phylogroup B2 (66.66%) did not have any virulence genes, which was the most noticeable but did not have a significant difference ($p \geq 0.05$) with the corresponding proportion of the phylogroup B1 (37.20%). Details on virulence gene frequency in relation to phylogeny are demonstrated in Figure 4b.

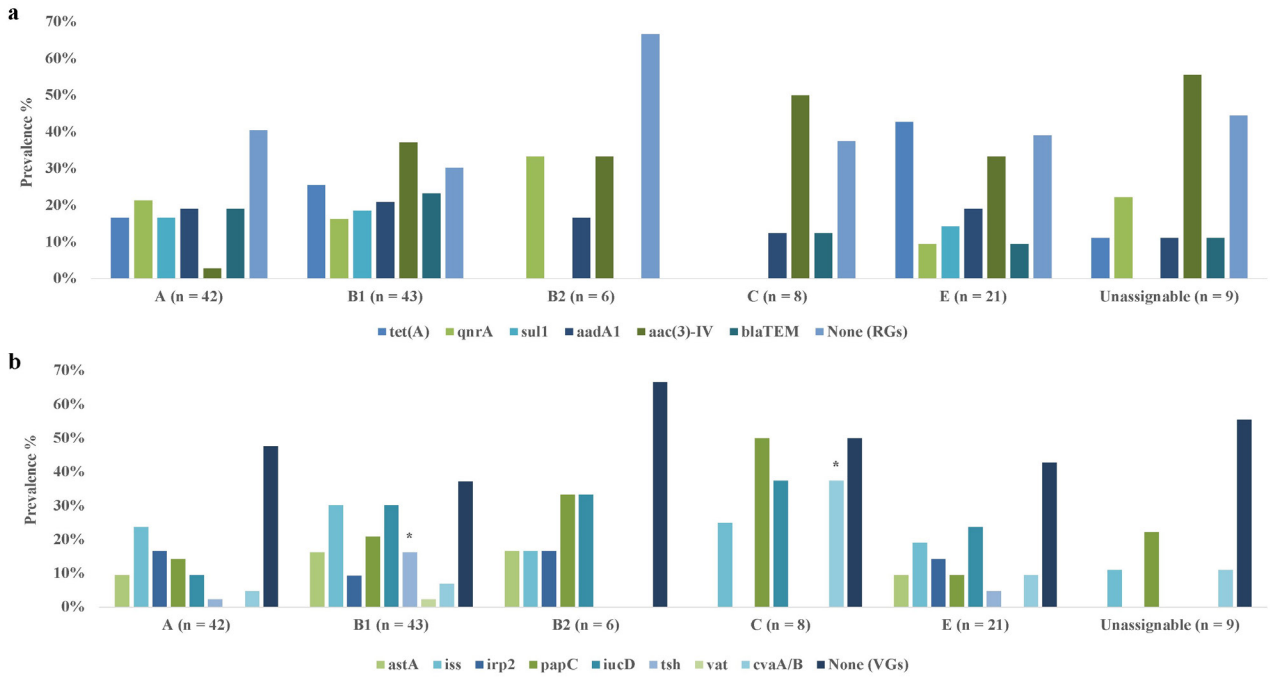


**Figure 4.**
Distribution of antibiotic resistance genes (a) and virulence genes (b) within phylogroups of E. coli isolates from ostrich. tet(A), tetracycline resistance gene; qnrA, quinolone resistance gene; sul1, sulfonamide resistance gene; aadA1, streptomycin resistance gene; aac(3)-IV, aminoglycoside N(3)-acetyltransferase gene; blaTEM, beta-lactamase resistance gene; None (RGs), None resistance genes. *P-value* is ≥ 0.05 for each resistance gene against categorical variables. astA, enteroaggregative toxin gene; iss, increased serum survival protein gene; irp2, iron repressible protein gene; papC, P-fimbriae gene; iucD, aerobactin gene; tsh, temperature-sensitive hemagglutinin gene; vat, vacuolating autotransporter toxin gene; cvaA/B, colicin V plasmid operon gene; None (VGs), None virulence genes. Against categorical variables, P-value is ≥ 0.05 for each virulence gene, excluding two indicated by * (*P-value* < 0.05).

## Discussion

According to the phylotyping results of this study, most isolates were classified into five phylogenetic groups; however, multiple strains were not assignable according to the quadruplex PCR-based method of Clermont et al. [19] (Figure 1). These unassignable strains have also been observed in some previous studies and are probably from extremely rare phylogroups. The loss of specific genes resulting from the plasticity of the genome of *E. coli* or the recombination of isolates from variant phylogroups might also lead to the observation of these unknown strains [19, 23, 24]. Phylogenetic groups B1 and A accounted for most of the phylogenetic profiles of both groups of presumptive commensal and pathogenic isolates with a close distribution proportion (Figure 1). This finding is in line with multiple previous studies on *E. coli* strains originating either from ostrich [25] or other avian species [26-32]. However, some other studies reported different dominant phylogenetic groups within avian-originated *E. coli* strains [7, 13-15, 33-36]. These inconsistencies could be due to the factors causing phylogenic heterogeneity, including geographical region, sampling area, site of infection, antibiotic resistance, host response, as well as environmental, social, and dietary conditions [9]. However, regarding the results of this study, insufficient evidence was obtained to com-

pletely correlate phylogenic heterogeneity to the isolation source of the strains, which justifies the necessity for further studies.

In the present study, the *E. coli* isolates of the phylogroup B2 were all from the suspected pathogenic group (Figure 1) and showed the highest MDR (statistically insignificant; $p \geq 0.05$) (Figure 3b). However, these isolates, unlike typically known commensal strains from the phylogroup B1, harboured the lowest virulence and resistance gene capacity (statistically insignificant; $p \geq 0.05$) (Figure 4). Saha et al. [7] have also reported that all APEC phylogroups, including B2, obtained from poultry farms in Bangladesh, showed MDR. Moreover, enhanced virulence capacity, which probably occurs by acquiring virulence factors through horizontal gene transfer, has also been found previously within the non-B2 phylogroups of *E. coli* strains [13, 31, 34, 35]. Among all resistance and virulence genes examined in this study, only two virulence genes were significantly prevalent ($p < 0.05$) within specific phylogroups, tsh within the phylogroup B1 and cvaA/B within the phylogroup C. This observation provides another evidence for the enhanced virulence capacity obtained through horizontal gene transfer within the non-B2 phylogroups of *E. coli* strains. That is because both genes are located on the Colicin V (ColV) plasmid, which is detectable in most APEC strains and transmissible to the non-APEC strains [35]. Furthermore, a part of the inconsistency observed between phenotypic and genotypic traits within the phylogroups B2 and B1 might result from the expression status of virulence and resistance genes, even those not investigated in the present research. In this sense, Amani et al. [1], examining the virulence and resistance gene panels of the isolates, encountered some strains with a specific antimicrobial resistance phenotype lacking the corresponding resistance gene. The authors also found the investigation of the virulence genes insufficient to discriminate pathogenic from commensal strains [1]. Overall, due to discrepancy between the genotypic and phenotypic characteristics of phylogenetic groups of the examined

*E. coli* isolates, further comparative studies on the resistance and virulence properties of the phylogenetic groups of *E. coli* isolates from ostrich and human, especially from larger sample sizes and different geographical locations, are suggested.

In conclusion, the findings of this study provided understanding of the resistance and virulence traits of the phylogenetic groups of *E. coli* isolates from ostrich and indicated that further genotypic and phenotypic analyses on these phylogroups are essential.

## Materials and Methods

### *Sample collection*

From September 2018 to August 2019, a total of 44 presumptive commensal and 85 suspected pathogenic *E. coli* strains were randomly obtained from apparently healthy and sick ostriches, respectively, from six distinct ostrich flocks in Mashhad, Northeast Iran. The presumptive commensal strains were sampled from the fresh faeces of apparently healthy ostriches, and suspected pathogenic strains were sampled from the fresh faeces of sick diarrheic ostriches (n = 13), dead-in-shell embryos (n = 4) from ostriches suspected to colibacillosis, and dead ostrich chicks (n = 68). These chicks were suspected to be infected with *E. coli* through post-mortem examination and samples from their infected organs, including yolk sac (n = 16), lung (n = 7), liver (n = 23), and heart (n = 22), were aseptically taken.

### *Isolation and Detection of E. coli Strains*

All obtained samples were aseptically streaked on MacConkey agar and aerobically incubated at 37°C for 24 h in the laboratory at the Veterinary Teaching Hospital, Ferdowsi University of Mashhad, Iran. The pure bacterial colonies with morphological and Gram staining characteristics similar to *E. coli* underwent biochemical tests (Indol, MR-VP, Simon Citrate, and TSI) and were confirmed as *E. coli* strains. Pure colonies of each sample were stored at -20°C in microtubes containing 2 ml of BHI medium and 15% sterile glycerol until use [37].

### *DNA Extraction*

Following thawing, *E. coli* isolates were cultured on MacConkey agar and were then incubated at 37°C for 24 h. Subsequently, a pure colony of each cultured isolate was suspended in a microtube containing 150 µl sterile distilled water. Extraction of the whole bacterial genome was performed through the rapid boiling method on the mixture [38]. Following the centrifugation of the suspension at 14,000 rpm for 15 min, the supernatant containing the extracted genome as the DNA template was transferred to a new microtube and stored at -20°C for later PCR.

### *Phylogenetic Group Assignment*

The quadruplex PCR method of Clermont et al. [19] was followed entirely for phylotyping. Based on this method, avian *E. coli* strains are classified into one of the eight phylogroups (A, B1, B2, C, D, E, F, or Escherichia cryptic clade I). All PCR amplifications were conducted on a final volume of 20 µl of the mixture, including distilled water, master mix (Ampliqon®, Denmark), DNA tem-

plate, and appropriate primers [19] based on the following procedure: pre-denaturation at 94°C for 4 min, 30 cycles of denaturation at 94°C for 5 sec, annealing at 59°C (quadruplex and phylogroup C) and 57°C (phylogroup E) for 20 sec, and a final extension at 72 °C for 5 min. The control strain was not included in this practice as arpA gene functions as an internal control, meaning that each *E. coli* strain is anticipated to produce at least one PCR product. Amplified products were analyzed through electrophoresis on 1% (w/v) agarose gel. Following staining with ethidium bromide, DNA bands were photographed under UV light, and those with the size of the target gene were considered to possess that gene. Finally, each isolate was classified into a specific phylogenetic group regarding the obtained amplicons.

### *Antimicrobial Susceptibility Testing*

Those raw unpublished data on antimicrobial resistance of the isolates against antimicrobials commonly used in ostrich farms, including enrofloxacin, florfenicol, doxycycline, trimethoprim + sulfamethoxazole (sultrim®), gentamicin, streptomycin, and amoxicillin, were kindly provided by Amani et al. [1], who previously tested the isolates for the mentioned antimicrobials. In the current study, the isolates were further tested for three different antimicrobials, including lincomycin (2 µg), tetracycline (30 µg), and ceftriaxone (30 µg). The first two antimicrobials are also used in ostrich farms. Ceftriaxone represents a different class of antimicrobials, namely third-generation cephalosporins which are known as critically important antimicrobial in human medicine [6]. Resistance to CIA is a noticeable concern correlated to the poultry industry [15]. The procedure previously used by Amani et al. [1] was followed entirely for antimicrobial susceptibility testing. Briefly, E. coli isolates, adjusted to 1.5 × 108 CFU/ml with 0.5 McFarland standard turbidity, were cultured on plates containing Mueller-Hinton medium. These plates were then aerobically incubated at 37°C for 18 h. All the relevant antimicrobial disks were provided from PadtanTeb®, Iran. The antimicrobial sensitivity testing was based on the modified Kirby-Bauer disk diffusion method and the guidelines of the Clinical Laboratory Standard Institute [20]. Following measuring the diameter of the zone of inhibition (mm) caused by *E. coli* isolates against each antibiotic, resistance was interpreted as susceptible or non-susceptible. E. coli ATCC 25922 was the quality control strain. Subsequently, resistance against the antimicrobials and the total MDR, which is defined as resistance to at least three antimicrobial classes [3], excluding lincosamides herein, was obtained within the phylogenetic groups.

### *Detection of Antimicrobial Resistance Genes and Virulence Genes*

The antimicrobial resistance genes, including tetracycline resistance gene (tet(A)), quinolone resistance gene (qnrA), sulfonamide resistance gene (sul1), streptomycin resistance gene (aadA1), aminoglycoside N(3)-acetyltransferase gene (aac(3)-IV), and beta-lactamase resistance gene (blaTEM) were detected within the isolates using PCR. These genes are associated with resistance to antimicrobials investigated in the present study and have previously been used in this respect [15, 21]. Furthermore, multiplex PCR was employed to detect the virulence genes, comprising enteroaggregative toxin gene (astA), increased serum survival protein gene (iss), iron repressible protein gene (irp2), P-fimbriae gene (papC), aerobactin gene (iucD), temperature-sensitive hemagglutinin gene (tsh), vacuolating autotransporter toxin gene (vat), and colicin V plasmid operon gene (cvaA/B) within the isolates. Employing these genes has proven to provide the capability of differentiation of APEC from non-APEC isolates [22]. Amani et al. [1], who previously performed both PCR detections for each isolate, kindly provided the raw data to be used for the evaluation of genes distribution within phylogenetic groups.

### *Statistical Analysis*

The Fisher's Exact test was used to compare the proportion of each categorical variable with the cumulative proportion of other categorical variables (a specific phylogroup with different phylogroups pooled together; presumptive commensal with presumptive pathogenic *E. coli* isolates) using GraphPad 2 × 2 contingency table analysis (https://www.graphpad.com/quickcalcs/contingency1). The two-tailed p-value was reported whenever the test was performed. The difference between the proportions was considered significant at a two-tailed *p-value* < 0.05.

## Authors' Contributions

A.G and M. JM conceived and planned the experiments. MS. ED carried out the experiments. MS.ED contributed to sample preparation. A. G and M. JM contributed to the interpretation of the results. E. T the lead in writing the manuscript. All authors provided critical feedback and helped shape the research, analysis and manuscript.

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## Competing Interests

The authors declare no conflict of interest.

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