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Original Article

Phylogenetic relationship and virulence gene profiles of avian pathogenic and uropathogenic *Escherichia coli* isolated from avian colibacillosis and human urinary tract infections (UTIs)

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Abstract

Background: There is evidence representing the possible relationship between avian pathogenic *Escherichia coli* (APEC) and other extraintestinal pathogenic *E. coli* (ExPEC) strains such as human uropathogenic isolates. **Aims:** The present study was conducted to evaluate virulence and phylogenetic relationship between a total of 70 APEC and UPEC isolates (35 APEC and 35 UPEC isolates) obtained from the north of Iran which is one of the core areas of the country's poultry industry. **Methods:** Polymerase chain reaction (PCR) and random amplified polymorphism DNA (RAPD) analyses were conducted using specific primers, and data was analyzed using BioNumerics and SPSS softwares. **Results:** The most prevalent gene was *fliC* (70.6%) followed by *fimH* (67.1%), but APEC and UPEC isolates showed inordinate and obvious differences in the presence of some virulence genes such as *fliC*, *hlyD*, and *sfa1* and predominant phylogenetic groups in DNA fingerprinting methods. **Conclusion:** The results showed obvious differences existed between isolates of APEC and UPEC in terms of phylogenetics and pattern of virulence gene; however, despite having virulence genes such as *papC*, *ibeA*, and *iss*, APEC isolates can have a high potential for causing disease in humans and may generate dangerous outbreaks in communities with low levels of hygiene in public and the poultry industry.

Key words: Avian pathogenic *E. coli*, RAPD technique, Uropathogenic *E. coli*, Virulence factors

Introduction

Based on the clinical symptoms and host specificity, there are four pathotypes of extraintestinal pathogenic *Escherichia coli* (ExPEC): avian pathogenic *E. coli* (APEC), uropathogenic *E. coli* (UPEC), neonatal meningitis *E. coli* (NMEC), and sepsis-associated *E. coli* (SePEC) (Jørgensen *et al*., 2019). Among these pathotypes, UPEC and APEC strains have shown extensive properties of virulence factors that contribute to their potency to dominate defense mechanisms in human or animal hosts. These virulence factors consist of various fimbrial adhesions, toxin and flagellar proteins, aerobactin iron-acquisition systems, and outer membrane proteins which are encoded by various genes located on transposable genetic elements or chromosomes (Subedi *et al*., 2018; Dadi *et al*., 2020). Although Shiga toxin-producing *E. coli* (STEC) strains were considered as the only zoonotic pathotype (Jafari *et al*., 2012), it has been shown that APEC and UPEC isolates of *E. coli* have comparable virulence genes and presumably pertain to the similar phylogenetic groups, demonstrating the zoonotic origin for ExPEC (Sarowska *et al*., 2019). In addition, recent studies have shown that poultry-originated ExPEC strains can cause a relatively wide range of extraintestinal infections in animal models (Stromberg *et al*., 2017; Mellata *et al*., 2018). During the last few decades, the poultry industry has become one of the major commercial activities in Iran, a country where the incidence of zoonosis infectious diseases between humans and animals is relatively high (Golshani and Buozari, 2017; Mirsalehian and Dalvand, 2018). Few studies have been conducted to distinguish the relationship between virulence factors and genotypic characteristics of APEC and UPEC isolates (Zhao *et al*., 2009; Bakhshi *et al*., 2020). In this study, we aimed to investigate the presence of nine different virulence-associated genes (*tosA*, *fliC*, *cnf*, *fimH*, *papC*, *sfa*, *ibeA*, *Iss*, and *hlyD*). We also examined *E. coli* isolates to determine and analyze the phylogenetic relationships of APECs and UPECs using the random amplified polymorphism DNA-polymerase chain reaction (RAPD-

PCR) technique.

Materials and Methods

APEC and UPEC isolates

In the present study, a total of 70 *E. coli* isolates that had been previously isolated and stored at -20°C in the microbiology laboratory of the Faculty of Veterinary Medicine, Amol University of Special Modern Technologies, were considered for investigation (data not yet published). The isolates were obtained from avian collibacilosis (n=35) and human urinary tract infections (UTIs) (n=35). The *E. coli* strain ATCC 25992 was used as positive control during cultivation and molecular examinations. Samples were collected during summer 2020 from UTI cases referred to laboratories, and avian colibacillosis suspected cases with clinical symptoms were referred to the veterinary clinics in Amol city, Northern Iran. Sampling was done using sterile cotton swabs from suspected poultry respiratory organs, livers and hearts and human urine culture from UTI samples. Samples were cultured immediately on MacConkey and EMB (Eosin Methylene Blue) agars (HiMedia, India) and incubated for 24 h at 37°C. Suspected colonies were identified by Gram staining and biochemical tests according to standard procedures. The ethical clearance of the study was approved by the Deputy of Research and Technology, Amol University of Special Modern Technologies, based on recommendation of research and publication committee with the number of Ir.ausmt.rec. 1400.02.

DNA purification

DNA extraction from APEC and UPEC isolates was performed using a DNA extraction kit specific to Gram-negative bacteria (CinaClone, Tehran, Iran) according to the manufacturer's guidelines. Spectrophotometry at 260 and 280 nm wavelengths was used to determine the purity and concentration of the DNA (Nanodrop 1000; Thermo Scientific). The extracted and purified DNA samples were stored at -20°C for further use.

Detection of virulence genes

The isolates were investigated for the occurrence of nine putative *E. coli* virulence-associated genes to determine the possible differences among APEC and UPEC isolates (*tosA* related to *in vivo* induced repeat-in-toxin (RTX) family member, *fliC* related to *E. coli* flagellin gene, *cnf1* related to cytotoxic necrotizing factor 1, *fimH* related to type 1 pili, *papC* related to pyelonephritis-associated pili, *sfa1* related to S fimbriae, *ibeA* for virulence factor responsible for neonatal meningitis in humans, *iss* related to serum resistance and *hlyD* for haemolysin). Polymerase chain reaction was performed using specific primers (Table 1) in a final volume of 25 µL (including 12.5 µL PCR master mix, 1 µL (0.4 µM) of both forward and reverse primers and 2 µL of DNA). All components were acquired from Sinaclon Corporation, Iran. The PCR product was then evaluated by electrophoresis in 1.5% agarose gel. A 100 bp DNA marker (Sinaclon, Iran) was used to evaluate the obtained DNA amplicons. Different virulence gene patterns were described based on the presence of corresponding genes.

DNA fingerprinting and phylogenetic analysis

Random amplification of polymorphic DNA-polymerase chain reaction was carried out by random primers (1254) previously described with the 5´-CCG CAG CCA A-3´ sequence (Aslam *et al*., 2004). The thermocycler device (MJ Mini, USA) program for the RAPD method was as follows: initial denaturation at 94°C for 5 min, 34 cycles at a denaturation temperature of 94°C for 1 min, 36°C annealing temperature for 1 min, and extension at 72°C for 1 min. After that, the final extension took place at 72°C for 6 min. The PCR products were visualized using gel electrophoresis in a 3% agarose gel. The images of RAPD reactions were loaded to BioNumerics version 6 (Applied Maths, Kortrijk, Belgium) for analysis. Genetic similarity was calculated using Pearson's correlation. The dendrogram of the isolates was also generated by the Dice correlation coefficient and the un-weighted pair group method with

Table 1: Nucleotides used as primers in PCR to detect virulence genes of *E. coli* isolates

Target gene	Sequence (5´ to 3´)	Annealing temperature (°C)	PCR product size (bp)	References
tosA	F: GCACAGCATAACGGGAAAAT R: CCAGCATGTTACCACGAATG	52	589	Ochoa *et al*. (2016)
fliC	F: CCAGTCTGCGCTGTCGAG R: CACGTTCACGCCGTTGAAC	54	349	Luna-Pineda *et al*. (2018)
Cnf1	F: TTATATAGTCGTCAAGATGGA R: CACTAAGCTTTACAATATTGA	46	634	Matsuda *et al*. (2010)
fimH	F: ATGAAACGAGTTATTACCCT R: TTATTGATAAACAAAAGTCAC	45	903	Lai *et al*. (2015)
papC	F: GCAACAGCAACGCTGGTTGCATCAT R: AGAGAGAGCCACTCTTATACGGACA	60	337	Johnson and Stell (2000)
Sfa1	F: CTCCGGAGAACTGGGTGCATCTTAC R: CGGAGGAGTAATTACAAACCTGGCA	58	408	Le Bouguenec *et al*. (1992)
ibeA	F: TGAACGTTTCGGTTGTTTTG R: TGTTCAAATCCTGGCTGGAA	51	1556	Germon *et al*. (2005)
Iss	F: GTGGCGAAAACTAGTAAAACAGC R: CGCCTCGGGGTGGATAA	52	760	Rocha *et al*. (2008)
hlyD	F: CTCCGGTACGTGAAAAGGAC R: GCCCTGATTACTGAAGCCTG	52	904	Rodriguez-Siek *et al*. (2005)
1254 (RAPD-PCR)	CCGCAGCCAA	36	Variable	Aslam *et al*. (2004)

F: Forward, R: Reverse, and RAPD-PCR: Random amplified polymorphism DNA-polymerase chain reaction

Table 2: Distribution of virulence genes among APEC and UPEC isolates

Type of isolates	Resistance gene distribution (%)								
	tosA	*fliC*	*Cnf1*	*fimH*	*papC*	*Sfa1*	*ibeA*	*Iss*	*hlyD*
APEC (n=35)	5 (14.2)	18 (51.4)	0 (0)	25 (71.4)	9 (25.7)	0 (0)	3 (8.5)	4 (11.4)	8 (22.8)
UPEC (n=35)	8 (22.8)	35 (100)	4 (11.4)	22 (62.8)	6 (17.1)	15 (42.8)	0 (0)	2 (5.7)	15 (42.8)
Total (n=70)	13 (18.5)	53 (70.6)	4 (5.7)	47 (67.1)	15 (21.4)	15 (21.4)	3 (4.2)	6 (8.5)	23 (32.8)

APEC: Avian pathogenic *E. coli*, and UPEC: Uropathogenic *E. coli*

arithmetic averages (UPGMA: unweighted pair group method with arithmetic mean). A cut-off of 80% was used to determine final groupings.

Statistical analysis

Results of the study were analyzed using SPSS version 22 software (IBM Armonk, North Castle, NY, USA). Statistical analyses were carried out by applying Mann-Whitney, Chi-square and Kolmogorov-Smirnov tests, with a statistical significance of $P<0.05$.

Results

Distribution of virulence-associated genes

Among the nine studied virulence genes, the most prevalent gene was *fliC* (70.6%) followed by *fimH* (67.1%). *FimH* (71.4%) was the most prevalent virulence gene among APEC isolates and *fliC* (100%) was the most prevalent virulence gene among UPEC isolates. Based on the presence of different genes, 35 virulence gene patterns were detected among 70 *E. coli* isolates. The most prevalent pattern was $fimH^{+}/fliC^{+}$ with a frequency of 10/70 (14.2%). Both APEC and UPEC isolates showed 15 specific patterns while five patterns were common among APECs and UPECs. Detailed results of the distribution of the virulence genes and various virulence gene patterns are listed in Tables 2 and 3, respectively.

Phylogenetic study

Figure 1 shows the results of the DNA fingerprinting of APEC and UPEC isolates using RAPD-PCR associated with the distribution of the virulence gene patterns. The fingerprinting technique could type 100% of the isolates and in total, 14 distinct clusters were obtained from the analysis of RAPD-PCR results using BioNumerics software (UPMEGA) named C1-C14 (SID = 0.9056). The most prevalent genotypes were C9 and C3 with the frequency of 11/70 (15.7%) and 10/70 (14.2%), respectively. C4, C5, C6, and C7 clusters (phylogenetic groups) were specific for UPEC isolates and C1, C10, C13, and C14 clusters were specific to APEC isolates. Results of the dispersion of the RAPD associated patterns among APEC and UPEC isolates are listed in Table 4.

Statistical analysis

The prevalence of *fliC*, *hlyD*, and *sfa1* among UPEC isolates was significantly higher than APECs ($P<0.05$). Although the presence of the *ibeA* and *cnf* genes was observed in APEC and UPEC, respectively, there was no significant relationship between these genes and a specific pathotype ($P>0.05$). No significant relationship was found between a specific cluster and a specific virulence gene. There was no significant correlation between a specific RAPD cluster and any pattern of virulence genes present. In addition to the specificity of some clusters for each pathotype (Table 4), the C3 phylogenetic group had a significant relationship with the APEC pathotype ($P<0.05$). Finally, there was a significant relationship between UPEC isolates and virulence gene patterns with five or four virulence genes ($P<0.05$).

Discussion

In recent decades, the poultry industry in developing countries has been growing quickly (Nkukwana, 2018; Wahyono and Utami, 2018). Some infectious diseases of birds such as colibacillosis caused by APEC strains of *E. coli* during breeding periods have caused problems in these communities, not only in terms of development and increase of antibiotic resistance, but also due to the

Table 3: Virulence gene profiles of the APEC and UPEC isolates

Virulence gene profiles	Number of isolates		
	APEC	UPEC	Total
fimH	4	0	4
fliC	1	0	0
papC	2	0	2
hlyD	1	0	1
fimH / hlyD	3	0	3
fimH / iss	2	0	2
fliC / fimH	6	4	10
fliC / papC	1	2	3
fliC / sfa1	0	3	3
fliC / hlyD	2	2	4
fliC / ibeA	1	0	1
papC / fimH	1	0	1
papC / ibeA	1	0	0
papC / hlyD	1	0	1
fliC / fimH / ibeA	1	0	1
fliC / cnf / sfa1	0	1	1
fliC / sfa1 / fimH	0	2	2
fliC / sfa1 / hlyD	0	1	1
fliC / papC / fimH	0	1	1
tosA / fliC / hlyD	0	2	2
tosA / fliC / fimH	3	1	4
tosA / papC / fimH	1	0	1
fliC / fimH / hlyD	1	3	4
fliC / fimH / iss	1	0	1
fliC / cnf / hlyD	0	1	1
papC / fimH / iss	1	0	1
fliC / cnf / fimH / iss	0	1	1
tosA / fliC / sfa1 / fimH	0	2	2
tosA / fliC / papC / fimH	1	0	1
fliC / sfa1 / fimH / hlyD	0	3	3
fliC / papC / fimH / hlyD	0	1	1
fliC / sfa1 / papC / hlyD	0	1	1
tosA / fliC / cnf / sfa1 / fimH	0	1	1
tosA / fliC / sfa1 / fimH / hlyD	0	1	1
tosA / fliC / papC / fimH / hlyD	0	1	1

APEC: Avian pathogenic *E. coli*, and UPEC: Uropathogenic *E. coli*

Table 4: Distribution of the RAPD fingerprinting patterns

Pathotype/No.	Frequency of RAPD analysis patterns (%)													
	C1	C2	C3	C4	C5	C6	C7	C8	C9	C10	C11	C12	C13	C14
APEC/35	7 (20)	3 (8.5)	8 (22.8)	0 (0)	0 (0)	0 (0)	0 (0)	2 (5.7)	5 (14.2)	3 (8.5)	1 (2.8)	3 (8.5)	2 (5.7)	1 (2.8)
UPEC/35	0 (0)	6 (17.1)	2 (5.7)	9 (25.7)	1 (2.8)	3 (8.5)	1 (2.8)	5 (14.2)	6 (17.1)	0 (0)	1 (2.8)	1 (2.8)	0 (0)	0 (0)
Total/70	7 (10)	9 (12.8)	10 (14.2)	9 (12.8)	1 (1.4)	3 (4.2)	1 (1.4)	7 (10)	11 (15.7)	3 (4.2)	2 (2.8)	4 (5.7)	2 (2.8)	1 (1.4)

RAPD: Random amplified polymorphic DNA, APEC: Avian pathogenic *E. coli*, UPEC: Uropathogenic *E. coli*, and C: Cluster

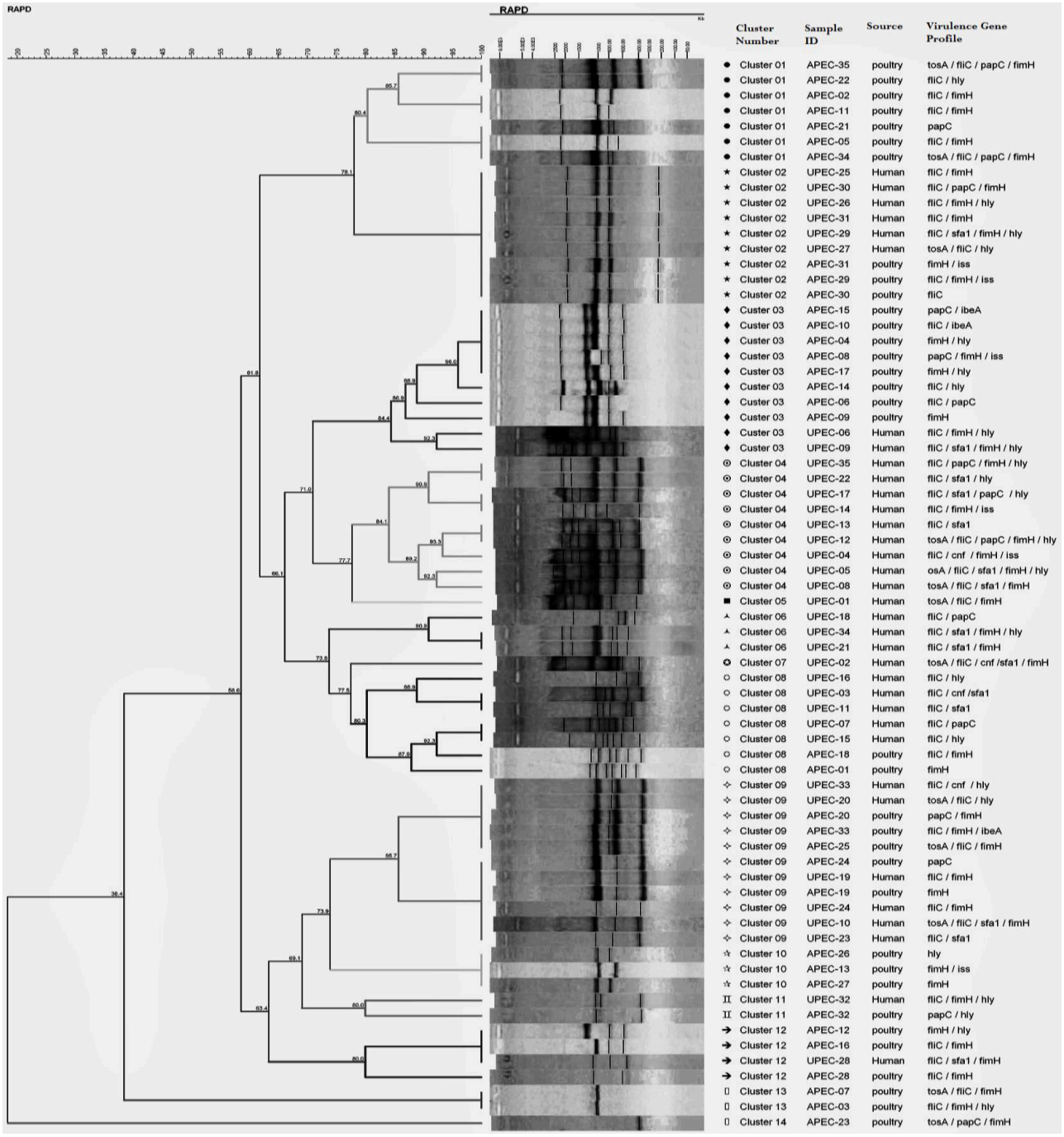

Fig. 1: Dendrogram based on RAPD-PCR fingerprinting of APEC and UPEC isolates, collected from poultry and humans, using the UPGMA analysis. RAPD-PCR assay resulted in 14 different clusters. RAPD-PCR: Random amplified polymorphic DNA-polymerase chain reaction, APEC: Avian pathogenic *E. coli*, UPEC: Uropathogenic *E. coli*, and UPGMA: Unweighted pair group method with arithmetic mean

similarities of these strains regarding the presence of virulence factors. Such proximities have increased the hazards and high risks of infections caused by these microorganisms in humans. As a result, various studies have been conducted to find genetic similarities and to investigate the possibility of outbreaks caused by such strains (Kazemnia *et al*., 2014; Bakhshi *et al*., 2020). In this study, the most prevalent virulence pattern and the most prevalent genes among APECs and UPECs were *fimH* and *fliC*, respectively, which is also confirmed by other studies (Xicohtencatl-Cortes *et al*., 2019; Xu *et al*., 2019). In addition, these two *E. coli* pathotypes showed significant differences in virulence gene properties. Noticeably, UPECs exhibited more prevalence of *fliC*, *hlyD*, *sfa*, *cnf* which are associated with motility of the strains, haemolysin toxin, S fimbriae, and necrotizing factor, respectively. These factors are associated with the pathogenicity of UPECs in the human urinary tract and its related damages (Marrs *et al*., 2005; Terlizzi *et al*., 2017). Although the type 1 pilus (fimH) was the most prevalent adhesin-related gene among UPECs, the prominent and exclusive presence of S fimbriae-related gene among these strains is indisputable. On the other hand, the *papC* along with *fimH* played a similar role among APEC isolates. *PapC* is a pyelonephritis pili, so its eminent presence in poultry isolates could indicate the ability of these isolates to bind to human urinary tract epithelium, which could be studied in future studies using cell culture methods. Mortensen *et al*. (2019) showed that UPEC UTI89 and APEC F149H1S2 had a similar potential for causing salpingitis in laying hens in the model used. In their study, no infection differences were observed between the UPEC UTI89 wild type and a mutant strain with a knock-out of the wellknown *fimH* virulence gene. It was shown that *ibeA* gene was responsible for neonatal meningitis in humans, and that the invasion capability of the *ibeA* mutants was expressively reduced by about 30% (Huang and Jong, 2001). Compared to other studies which have shown the role of the *ibeA* gene in the pathogenicity of APEC and suggested a close correlation between APEC and other human ExPEC isolates (Germon *et al*., 2005), the results of the present study showed the occurrence of this virulence gene only among APEC isolates. Based on the results of the present study, it can be expected that the pattern of presence of different virulence genes among APEC and UPEC isolates was mainly dissimilar. A total of 87.5% (35.30) of the obtained virulence gene patterns were specific to one of the pathotypes of APEC and UPEC and only five common patterns were observed (Table 3). Overall, UPEC isolates had a higher number of virulence genes than APEC isolates. As such, the ratio of virulence gene number for UPECs and APECs was 3.05 and 2.05, respectively. It can be generally assumed that UPEC isolates have more virulence and higher pathogenicity potency than APEC isolates.

In genotyping studies, it seems that there are some isolates with the same RAPD-PCR patterns among APEC isolates and UPEC isolates which showed the possible transmission of *E. coli* strains between humans and poultry. Other studies using different genotyping methods confirm this finding (Kazemnia *et al*., 2014; Bakhshi *et al*., 2020). In the present study, typeability power was considered as 100% in the RAPD-PCR method, and isolates with >80% genetic similarity, were considered as the same clusters. Different studies have used various RAPD methods and primers as an available and relatively non-expensive technique to genotype *E. coli* strains obtained from diverse sources including avian colibacillosis and human UTIs (Aslam *et al*., 2004; Afshari *et al*., 2016; Mohammadzadeh *et al*., 2019). According to the genotyping fingerprinting, four clusters were specific to UPEC isolates and four clusters were specific to APEC isolates, whereas six clusters were common between the pathotypes. Mohammadzadeh *et al*. (2019) showed the clonal association and similar antibiotic susceptibility patterns in pathgenicity associated islands-related genotypes. They maintained that closer genetic relationships were found in the RAPD results among UPEC isolates that were categorized in the same genotypes. The results of the current study showed no significant correlation between any particular RAPD cluster and patterns of present virulence genes; nevertheless, RAPD genotyping showed a relatively good resolution and identification power between *E. coli* isolates obtained from two different sources. This shows the important role of this method in identifying the source of infections cause by this microorganism. On the other hand, the presence of some important virulence genes such as *ibeA* and *iss* among poultry isolates indicates the need for extensive and comprehensive future studies of the genetic affinities and proximities of these isolates to human isolates.

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Conflict of interest

The authors have no conflict of interest.

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