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Virulence-Associated Gene Profiles of Avian Pathogenic *Escherichia coli* Isolated From Broilers With Colibacillosis: A Pilot Study in Iran

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Abstract

Background: Avian pathogenic *Escherichia coli* (APEC) causes economic losses in the chicken industry worldwide.

Objective: In this study, virulence-associated gene profiles of APEC isolates were investigated by polymerase chain reaction (PCR).

Materials and Methods: A total of 60 *Escherichia coli* isolates were collected from 60 colibacillosis cases from 30 broiler poultry farms in Alborz, Tehran, and Golestan provinces, Iran. After identification by biochemical tests, DNA was extracted by boiling method and 5 virulence-associated genes including: *iutA, hlyF, iroN, ompT*, and *iss* were detected by 2 multiplex PCR protocols.

Results: Of the 60 APEC isolates, 26 (43.3%) isolates had at least three virulence genes from which 12 (20%) isolates were positive for all 5 virulence genes, whereas 34 (56.6%) carried no investigated virulence genes. Presence of *iutA, hlyF, iroN, ompT*, and *iss* genes in the APEC isolates were 17 (28.3%), 17 (28.3%), 24 (40%), 26 (43.3%), and 23 (38.3%), respectively.

Conclusion: According to the results, four different virulence-associated gene profiles were seen in isolates, from which profile 1 with 12 (20%) isolates was predominant. These findings were in agreement with the previous reports.

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Background

Avian pathogenic *Escherichia coli* (APEC) causes a variety of diseases in poultry, and results in significant economic losses in the chicken industry.[1,2] Pathogenicity of APEC strains relates to the expression of several putative virulence factors including adhesions, toxins, protectins, as well as iron acquisition mechanisms and invasion factors.[1,2] Johnson et al presented pentaplex design for 5 genes carried by plasmids (*iutA, hlyF,* episomal *iss, iroN,* and episomal *ompT*), which are able to discriminate APEC from avian fecal *E. coli* (AFEC) to nearly the same degree as virulence genotyping for 46 genes.[3] In this study, 5 virulence genes, *iroN, ompT, iss, iutA*, and *hlyF* were evaluated. The *iss* gene encodes the ISS protein which increases serum survival and prevents deposition of membrane attack complex of complement system. This gene occurs more frequently in APEC strains than in other strains isolated from apparently healthy birds.[1-7] The *iroN* gene encodes IroN, which acts as siderophore receptor, and *iutA* gene encodes ferric aerobactin receptor, which involves in iron acquisition mechanism. *OmpT* encodes OmpT protease, which has been shown to cleave colicins A-E_1-E_2 and E_3, and hlyF acts as avian hemolysin.[7] There is no report, to the best of our knowledge, on the prevalence of these 5 genes (*iutA, hlyF, iroN, ompT,* and *iss*) in APEC isolates in Iran. The aim of the present study was therefore to determine the virulence gene profiles of avian pathogenic *E. coli* isolated from cases of colibacillosis in poultry farms in Alborz, Tehran, and Golestan provinces, Iran, by multiplex PCR technique.

Materials and Methods

Collection of Samples and Microbiology

A total of 60 *E. coli* isolates were obtained from 60 colibacillosis cases from 30 commercial broiler poultry farms (2 isolates per farm) in Golestan, Alborz, and Tehran provinces, Iran, in 2017. Blood samples from hearts with pericarditis were cultured on the MacConkey agar (Merck, Germany) and incubated overnight at 37°C. The lactose positive colonies were identified by biochemical tests by Enterobacteriaceae diagnostic kit (Iran Daru Co., Iran), which included IND-GLU-H2S-CIT-ORN-LYS-

URE-TBA-OMPG-MR-VP reactions. Isolates that were catalase, indole, and methyl-red positive, and oxidase, citrate, H_2S, oxidase, and VP negative were considered as *E. coli*.

DNA Extraction

Overnight cultures in 4 mL ELB broth were centrifuged for 7 minutes at 3500 rpm. The bacterial pellet was re-suspended in 300 µL distilled water and boiled for 15 minutes. The supernatant was used as template DNA after the tubes were centrifuged.[8]

Polymerase Chain Reaction Method

Five sets of primers, previously described by Johnson et al,[3] were used in the present study. The sequences of the primers, the related genes encoding virulence factors, and the thermocycler programs, are summarized in Table 1. Synthesis of the primers were done by CinnaGen, Iran. In 2 different protocols, the primers were used. In the first protocol, *iss, ompT*, and *iroN* primers, and in the second protocol, *iutA* and *hlyF* primers were amplified in 25 µL as total volume, containing 0.5 mM $MgCl_2$, 2.5 µL 10× polymerase chain reaction (PCR) buffer, 250 µM dNTPs, 0.5 U Taq DNA polymerase, and 1 µM (0.2 µL) of each primer. The amplified products were visualized by gel electrophoresis using 12 µL of the final mixture in 1.5% agarose gel in TBE buffer (2.5 mM EDTA, 89 mM boric acid, 89 mM Tris). A 100–1000 bp DNA ladder (Fermentas, Massachusetts, United States) was used to determine the molecular weight of PCR products.

Results

Culture Findings

Escherichia coli was identified by biochemical tests in 100% of the samples that were collected from the heart, liver, air sacs, and lunges of chickens.

Polymerase Chain Reaction Findings

PCR amplicons with molecular sizes of 302, 450, 553, 496, and 323 bp were considered *as iutA, hlyF, iroN, ompT*, and *iss* gene specific segments (Figure 1). Presence of virulence genes were detected for *iutA* in 17 isolates (28.3%), *hlyF* in 26 isolates (28.3%), *iroN* in 24 isolates (40%), *ompT* in 26 isolates (43.3%), and *iss* in 23 isolates (38.3%). No amplification was detected for negative control. In addition, 26 (43.3%) isolates had at least three virulence genes and 12 (20%) isolates were positive for all 5 virulence genes. In addition, the results of PCR assay indicated that 17 (28.3%) isolates carried *iutA* and *hlyF* genes together and 21 (35%) isolates carried *iroN* and *ompT* genes together, whereas, 34 (56.6%) isolates carried no investigated virulence genes. Furthermore, according to the results, four different virulence gene profiles were present in the isolates from which, profile one (*iutA, hlyF, iroN, ompT, iss*) with 12 (20%) isolates was predominant. Distribution of 5 virulence gene profiles of APEC isolates is presented in Table 2.

Discussion

Escherichia coli is present in the environment of poultry as well as in normal microflora of the intestinal tract. However, the strains can act as APEC that possess specific virulence factors and are able to cause avian colibacillosis, resulting in great economic loss. The pathogenesis of *E. coli* is related to the wide range of different virulence genes. In this study, the presence of 5 virulence genes, including *iutA, hlyF, iroN, ompT,* and *iss* were verified by multiplex PCR analysis. The *iss* gene involves in serum survival, *iroN* and *iutA* involve in iron acquisition mechanisms, *ompT* encodes protease which cleaves colicins, and *hlyF* acts as avian hemolysin.[7] The link between APEC virulence and the possession of ColV plasmid was shown in several studies.[4,5] The 93-kb putative virulence region of pAPEC-O_2-COIV was found to contain several genes or operons such as *ompT*, salmochelin operon (iroBCDN), *iss, iutA* and *hlyF*.[5] Therefore, evaluation of some genes in the conserved region of ColV plasmid could be helpful in the differentiation of APEC from commensal strains.[3,5,7] Studies have shown that the *iss* gene,[3-5,7,10,11] *iroN, iutA, ompT* genes,[3,5,7] and *hlyF* gene[3,5] occur more frequently

Table 1. Primer Sets Used in Multiplex PCR for Amplification of APEC Virulence Genes

Gene	Primer pairs (5′-3′)	PCR Program*	Product Size (bp)	Description	Reference
iutA	GGCTGGACATCATGGGAACTGG CGTCGGGAACGGGTAGAATCG	1	302	Aerobactin siderophore receptor gene	3
hlyF	GGCCACAGTCGTTTAGGGTGCTTACC GGCGGTTTAGGCATTCCGATACTCAG	1	450	Putative avian hemolysin	3
iroN	AATCCGGCAAAGAGACGAACCGCCT GTTCGGGCAACCCCTGCTTTGACTTT	1	553	Salmochelin siderophore receptor gene	3
ompT	TCATCCCGGAAGCCTCCCTCACTACTAT TAGCGTTTGCTGCACTGGCTTCTGATAC	2	496	Episomal outer membrane protease gene	3
iss	CAGCAACCCGAACCACTTGATG AGCATTGCCAGAGCGGCAGAA	2	323	Episomal increased serum survival gene	3

* 1, 30 cycles (94°C, 30 s; 63°C, 30 s; 68°C, 2 min); 2, 35 cycles (94°C, 30 s; 40°C, 30 s; 70°C, 2 min).

Table 2. Virulence-Associated Gene Profiles of APEC Isolates

Profile Number	Virulence Gene Profiles	Number of isolates	Percent
1	*iutA, hlyF, iroN, ompT, iss*	12	20
2	*iutA, hlyF, iroN, ompT*	3	5
3	*iutA, hlyF, ompT, iss*	2	3.3
4	*iroN, ompT, iss*	9	15
Total		26	43.3

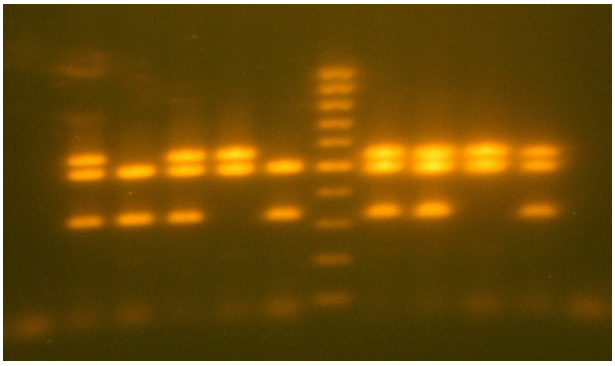

Figure 1. Agarose Gel Electrophoresis (1.5%) of Multiplex PCR Products. Lanes 1 and 12: negative control. Lane 2: positive control for *iutA*, *hlyF*, and *iroN* (302, 450, and 553 bp). Lanes 3 and 6: *iutA*, and *hlyF* positive isolates. Lanes 4, 8, 9, and 11: *iutA*, *hlyF*, and *iroN* positive isolates. Lanes 5 and 10: *hlyF* and *iroN* positive isolates. Lane 7: DNA molecular weight marker (100-1000 bp).

in APEC strains than in other strains isolated from apparently healthy birds. There is a strong correlation between serum resistance and virulence.[12] Several studies have indicated that *E. coli* can escape from the bactericidal actions of complement system due to serum resistance; this may lead to avian colisepticemia.[13] The *iss* gene is an important gene present in APEC which is responsible for serum resistance.[3,14] Prevalence of *iss* gene was reported in 72%-82% of APEC isolates in several studies.[3,5,7,10,11,14,15] The prevalence was 58.5% in China,[16] 57% and 63% in Hungary,[17,18] 41.5% in Korea,[19] and 38.5% in Brazil.[9] In this study, it was shown that *iss* gene was present in 38.3% of APEC isolated from avian colibacillosis. The *iroN* gene encodes IroN, which acts as siderophore receptor. It is suggested that the iron acquisition system is involved in the virulence of APEC.[19] Among the APEC genes, redundancy in iron-acquisition genes may be an indicator of the role of iron in the pathogenesis of avian colibacillosis.[1,7] The frequency of *ompT* and *iroN* in APEC isolates was reported to be 70.5% and 88.2% by Rodriguez-Siek et al, and 67.2% and 85.5% by Johnson et al, respectively.[3,5,7] In our study, the prevalence of these genes was 43.3% and 40% in APEC isolates. In two studies by Johanson et al, the frequency of *hlyF* in APEC cases was reported 78.2% and 81.7%, while in the current study, 17 (28.3%) of the isolates carried *hlyF*.[3,7] According to the studies, although these genes (*iutA, hlyF, iroN, ompT,* and *iss*) may play an important role in the pathogenicity of APEC isolates, differences in the prevalence rates of these genes were reported.[20] Since the outbreak of disease is dependent on host, pathogen, and environmental factors, any changes in one of these factors can possibly affect the incidence of disease. None of these 5 virulence genes were found in 34 (56.6%) of the isolates; it seems these are opportunistic *E. coli* the lack of which and probably the existence of predisposing factors such as inconvenient management and environmental factors contributed them to cause disease with typical signs and lesions. Virulence factors are multifactorial phenomena; for example, several structural factors including a smooth lipopolysaccharide layer or particular lipopolysaccharide type, K1 capsule or other capsule types, and certain outer membrane proteins including TraT, ISS, and OmpA contribute to the resistance of APEC to the complement system.[1] Thus, certain pathogenic characteristics, like serum resistance, are presented by more than one gene, and the absence of a certain gene does not necessarily lead to the absence of the phenotypic characteristic.[21] Delicato et al suggested that several types of these genes and yet other undetected virulence genes should be searched in APEC, and not any single gene could be used as a target for the differentiation of APEC from AFEC.[9] Furthermore, according to the results, 12 (20%) isolates carried the 5 genes, while 34 (56.6%) isolates carried none of these genes. These results are in agreement with the previous reports from Johnson et al and Barnes et al who emphasized the existence of these virulence genes in conserved region of ColV plasmid, and showed the presence of this plasmid in APEC isolates.[1,3,5,22]

In attempt to identify common traits of the Iranian APECs, some studies have attained interesting findings. Mohsenifard et al[23] phylotyped 290 *E. coli* isolates and investigated the presence of seven virulence genes (*ompT*, *hly*, *iss*, *iutA*, *iron*, *tsh*, and *cvaC*) in ColV plasmid by multiplex PCR. They found that most of the APEC isolates fell into the phylogenetic group A and subgroup A1. Furthermore, ColV plasmid, carrying virulence genes, was significantly higher in APEC compared to the AFEC. They concluded that having several virulence genes in ColV plasmid and belonging to the phylogenetic group A might provide useful characteristics for the better identification of Iranian APECs. Asadi et al[24] phylotyped 83 *E. coli* isolates from poultry with colibacillosis and 34 isolates from carcasses with cellulitis and showed that colibacillosis isolates belonged to A (54.21%), B1 (7.22%), B2 (6.03%), and D (32.53%) phylogroups. Whereas, the isolates from cellulitis cases belonged to three main phylogroups: A (55.88%), B1 (5.88%), and D (38.24%). Moreover, they showed that based on the statistical analysis, there was a specific association between the presence of *crl* virulence gene and phylogroups of A and D in the colibacillosis isolates. Hasani et al[25] searched three virulence genes in 71 isolates and found that 53.5%, 35.2%,

and 49.3% carried *irp2* (the iron-repressible protein), *papC* (pyelonephritis-associated pili), and *tsh* (temperature-sensitive hemagglutinin) genes, respectively. They also showed high rate of antibiotic resistance in these isolates. These authors in another study conducted phylogenic typing of 70 *E.coli* isolates and reported that 50% isolates were classified as type A, 45% as type D, 2.8% as type B1, and 2.8% as type B2, and concluded that possibly this type of *E. coli* could acquire virulence genes from pathogenic types.[26]

Conclusion

Samples of the current study were collected from different broiler flocks; this may have contributed to the differences in the results. Generally, it is concluded that APEC can cause multifactorial diseases, including those caused by host-pathogen interactions. Thus, biological and molecular characterization related to appropriate animal models are needed to well understand the pathogenicity of the APEC.

Authors' Contributions

Conception and design of the study was done by PHK, HP. SM participated in data collection and sampling. HP implemented the experiments and analyzed the results. GA handled drafting and revision of manuscript. Final approval of the manuscript was done by all authors.

Ethical Approval

Our pilot study was anonymous. The consent and agreement form was not applicable in our study.

Conflict of Interest Disclosures

The authors declare that they have no conflict of interests.

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