

Molecular Characterization of Avian Pathogenic *Escherichia coli* in Broilers Bred in Northern Iran

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Abstract: The patterns of existence of eight APEC virulence genes including *astA*, *iss*, *irp2*, *papC*, *iucD*, *tsh*, *vat* and *cva/cvi* were determined among *Escherichia coli* (*E. coli*) strains isolated from broiler chickens. A total of 105 broiler chickens were collected from 10 different cities located in Mazandaran province, Iran, during years 2009-2010. A number of *E. coli* strains were isolated from different organs including heart, liver and air sac and identified by biochemical methods within the family *Enterobacteriaceae*; and the presence of eight virulence-associated genes of Avian Pathogenic *Escherichia coli* (APEC) were tested by multiplex PCR. In this study, 26.7% of the *E. coli* strains tested were determined to have pathogenicity potential. Since the strains carried at least five virulence genes, of which 96.4% included *tsh* and *iss*, 85.7% *astA*, *iucD* and *vat*, 82.1% *papC*, 53.5% *irp2* and 14.2% *cva/cvi*. The results obtained by this study were consistent with those of some other studies regarding the most prevalent virulence genes which were *iss* and *tsh* ($f = 0.964$). However, significant differences in frequency of other virulence genes were observed between our study and those reported by other researchers.

Key words: Avian Pathogenic *E. coli* • Virulence Genes • Broiler Chickens • Multiplex PCR

INTRODUCTION

Escherichia coli is a rod-shaped, Gram-negative bacterium that belongs to the *Enterobacteriaceae* family. It is one of the most prevalent pathogenic agents in avians [1, 2]. *E. coli* is known as one of the most important pathogenic agents causing disease in fowls and mammals in its both primary and secondary presence [3, 4] of which Avian Pathogenic *E. coli* (APEC) strains impose severe problems on aviculture industries [4-6]. The infection with APEC exacerbates following infection with primary factors such as viral infections like Newcastle disease and Mycoplasmosis, being exposed to the cold weather during breeding and lack of an efficient air conditioning system during the second half of breeding. It leads to the development of some diseases like septicemia, chronic respiratory disease, egg yolk

infection, Eustachian tube edema, peritonitis, chronic coetaneous infection, osteomyelitis and swollen head syndrome [4, 7, 8].

APEC is recognized by its virulence genes that enable it to live an extra-intestinal life [2, 4, 5, 7]. The pathogenicity of the strain is caused by presence of at least five virulence genes [4]. The bacterial adhesion to the intestinal epithelial cells is considered to be an important step in the establishment of colibacillosis [8]. The adhesion factor is P fimbria that is coded by *papC* operon in bacterial chromosome. This factor contributes in prevention of APEC strain to be phagocytosed. Some studies have shown that *papC* operon is located in bacterial colonization in respiratory epithelium which directly affects the intensity of infection [1, 7, 11]. *Iss* gene contributes in increasing of APEC survival in serum and is described as an important characteristic of severe

colibacillosis in broiler chickens [4]. *Iss* gene was first found in human *E. coli* strains, however it has also been found in APEC strains isolated from chickens suffering from systemic colibacillosis [7, 12, 13]. This gene is located on the CoIV plasmid [3, 4]. and its product plays an important role in inhibition of the complement system. In addition to *Iss* gene, 078 polysaccharide and K1 capsule are two other virulence factors that enhance the survival of bacterium in serum [4, 7, 8]. *Irp2*, another virulence gene found in *E. coli* (colibacillosis) isolated from humans and birds, was first found in *Yersinia*. Ewers *et al.*[9]. demonstrated that pathogenic APEC is correlated with the aerobactin iron uptake system. In a low iron medium, APEC strain can only grow inside the host body [3, 4, 7, 14].

IucD is another gene located on CoIV plasmid that encodes aerobactin. Aerobactin is a kind of siderophore produced by some strains of *E. coli* that absorbs the iron ions bound to transferrins. Siderophore functions through a specific cell surface protein receptor synthesized in low iron condition [3,4,8,14]. The temperature-sensitive hemagglutinin (*tsh*) gene encodes a 140 kDa The temperature-sensitive hemagglutinin (*tsh*) gene encodes a 140 kDa serine protease present in bacterial periplasm and contains two subunits, an extracellular 33 kDa peptide as small subunit and a 106 kDa peptide as large subunit [8, 15-17]. The large subunit plays the most important role in bacterial adhesion to host cell during membrane binding process. This subunit can bind to red blood cells, hemoglobin, fibronectin, extracellular matrix proteins and IV collagen. This gene was first found in APEC strains in 1999 [4, 13, 15]. It helps development of lesions and fibrin precipitation in air sacs, increases colonization at this site and induces lesions and ulcers [10, 13, 16, 17].

Another gene named *vat* encodes a carrier protein that is responsible for vacuolating and transferring of cytotoxin produced by pathogenic strains. It is proposed that this gene is located on pathogenic island. Vacuolating cytotoxin is a toxin floated on the APEC surface with a fatal property similar to that produced by *Helicobacter pylori* [3, 4, 11]. *cva/cvi* is another gene located on the CoIV plasmid. The bacterial colicin produced by these genes inhibits other bacteria to grow in the medium. *astA* gene encodes a heat stable toxin that contributes in bacterial colonization in intestine. This gene is controlled by plasmid. According to the results obtained by studies performed on EAEC (enteroaggregative *E. coli*) strains, this toxin can cause diarrhea in infected animals [4, 9].

In this study, existence of eight APEC virulence genes *astA*, *iss*, *irp2*, *papC*, *iucD*, *tsh*, *vat* and *cva/cvi* was investigated in *E. coli* strains isolated from broiler chickens bred in northern Iran.

MATERIALS AND METHODS

Bacterial Strain: In this study, 105 samples were collected from heart, liver, spleen and air sacs of broiler chickens with colibacillosis symptoms by needle under sterile condition. All samples were obtained through veterinary laboratories located in different cities of Mazandaran province, Iran, including Sari, Qaemshahr, Babol, Babolsar, Neka, Nour, Chalous and Tonekabon during years 2009-2010. They were inoculated on EMB agar plates under sterile conditions and incubated at 35-37°C for approximately 24 h in aerobic conditions. *E. coli* identification was carried out by a group of biochemical tests including Dulcitol, Inositol, Lactose, Manitol, Mannose, Sucrose and Xylose fermentations, IMVIC (Indole, Methyl red, Voges-Proskauer, Citrate) and urea tests, as well as H₂S production, Lysine, motility and gelatin melting methods [18].

DNA Extraction: DNA was extracted by boiling method. A colony grown on EMB agar plate was cultured in nutrient broth medium for 24 h at 35-37°C. 1.5 ml of nutrient broth medium was centrifuged at 1500 rpm for 10 min. The pellet was resolved in 250 µl distilled water (DW) and the solution was frozen at -80°C for 10 min after vortexing, followed by incubation (boiling) at 95 °C for 5 minutes. The freezing-boiling steps were repeated 3 to 4 times. The procedure was followed by centrifugation at 1500 rpm for 10 min. The supernatant containing DNA was poured into a new 1.5 ml microtube and the quantity and quality of DNA were measured by a biophotometer (Eppendorf, Germany) based on its light absorption at 260 and 280 nm.

Multiplex PCR: A multiplex PCR was performed to co-amplify eight *E. coli* virulence associated genes including *astA*, *iss*, *irp2*, *papC*, *iucD*, *tsh*, *vat* and *cva/cvi* based on the conditions given by Ewers *et al.* [9]. The PCR was carried out in a volume of 50 µl including 3.2 µl of each primer pair (50 pmol; Bioneer, Daejeon, South Korea), 33.3 µl of ddH₂O, 0.5 µl of Taq DNA polymerase, 2 µl of 10mM dNTP, 1 µl of 1mM MgCl₂, 5 µl of 10X PCR buffer (Cinnagen, Iran) and 5 µl of template DNA.

Table 1: Primer pairs used in Multiplex PCR to co-amplify eight APEC virulence genes and their respective products sizes

Virulence gene	Primer Sequences (5'-3')	Size (bp)
<i>astA</i>	TGCCATCAACACAGTATATCC TCAGGTCGACGTGACGGC	116
<i>iss</i>	ATCACATAGGATTCTGCCGC AGCGGAGTATAGATGCCA	309
<i>irp2</i>	AAGGATTGCTGTTACCGAC AACTCCTGATACAGGTGGC	413
<i>papC</i>	TGATATCACGCAGTCAGT AGCCCGGCCATATTCATAA	501
<i>iucD</i>	ACAAAAAGTTCTATCGCTT CCCCTGATCCAGATGA CTC	714
<i>tsh</i>	ACTATTCTCTGCAGGAAGT CCTTCCGATGTTCTGAACGT	824
<i>vat</i>	TCCTGGGACATAATGGTC AGGTGTCAGAACGGAATTGT	951
<i>cva/cvi</i>	TGGTAGAATGTGCCAGAGC AAGGAGCTGTTGTAGCGAAGCC	1,181

The Cycling Conditions Was Set up as Follows: 94°C for 4 min as initial denaturation, 94 °C for 30 sec, 58°C for 30 sec and 68°C for 3 min for 25 cycles, followed by 72°C for 10 min as final extension. The PCR product was run on 0.8% agarose gel and the bands produced were observed under UV after staining with Ethidium bromide (0.5 µl/ml).

Primers: The primer pairs used in Multiplex PCR were those given by Ewers *et al.*[9]. and are given in Table 1.

RESULTS

The Multiplex PCR results obtained for some APEC and non-APEC strains are shown in Figure 1. Out of the 105 isolated *E. coli* strains, 28 (26.7%) were considered as APEC strains since they contained five or more virulence genes. The other 77 (73.3%) strains contained less than five virulence genes and were classified as non-APEC strains. None of the strains contained all eight virulence-associated genes, while 9 strains included 7 genes, 15 included 6, 4 included 5, 10 included 4, 25 included 3 and 33 included 2 genes. Moreover, 9 strains contained none of the virulence-associated genes examined. The patterns of presence of virulence genes among APEC and non-APEC strains are given in Table 2.

DISCUSSION

APEC strains are characterized by possession at least five virulence genes that enable them to live out of intestine [2, 7]. In this research, the frequency of eight

Table 2: Frequency of different virulence genes among APEC and non-APEC strains examined in this study

Virulence gene	Broiler isolates (n = 105)			
	APEC strains (N = 28)		Non-APEC strains (N = 77)	
	N	f	N	f
<i>astA</i>	24	0.857	53	0.688
<i>iss</i>	27	0.964	50	0.649
<i>irp2</i>	15	0.535	62	0.805
<i>papC</i>	23	0.821	51	0.662
<i>iucD</i>	24	0.857	53	0.688
<i>tsh</i>	27	0.964	50	0.649
<i>vat</i>	24	0.857	53	0.688
<i>cva/cvi</i>	4	0.142	73	0.948

N: Number; f: frequency

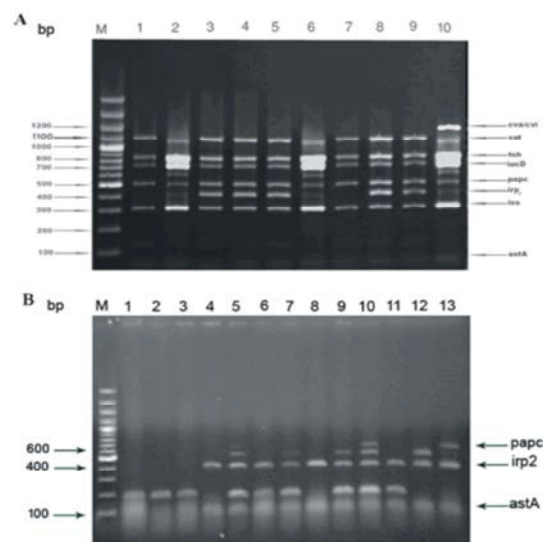


Fig. 1: Multiplex PCR results obtained for some representative APEC (A) and non-APEC strains (B) tested in this study (M: 3000 bp DNA ladder)

virulence genes and their importance in pathogenicity was evaluated in both APEC and non-APEC strains. A multiplex PCR was used to co-amplify all genes tested in a single reaction tube. To the best of our knowledge, this is the first report regarding study of APEC virulence-associated genes by multiplex PCR method in Iran. The frequencies of different virulence genes in APEC strains have been evaluated by different researchers. The presence of these genes was studied on 120 APEC strains by Kwon *et al.* in Korea, of which 100% were carrying *iss* gene, 94% *tsh*, 89% *vat*, 83% *iucD*, 67% *irp2*, 56% *astA*, 16% *cva/cvi* and 11% *papC* genes [4]. Won

et al. in Korea showed that out of 118 APEC strains, 94% were carrying *fimC* gene, 57% *coiV*, 55% *tsh*, 50% *clpG* and *iutA*, 47% *iucC* and *iucD*, 41% *iss*, 38% *irp2* and *fyuA*, 37% *vat*, 17% *astA*, 15% *papG*, 14% *papA* and 2% *eaeA* genes, while no strains were carrying *afa8D*, *facA* and *fliA* genes [14]. Another study performed on 118 APEC strains by Moon and his colleagues in Korea showed that 55% of strains were carrying *cvi* and *tsh* genes, 50% *clpG* and *iutA*, 47% *iucC*, 38% *iss*, 15% *papG*, 14% *papA* and 2% *eaeA* genes [19]. Rocha and his colleagues in Brazil also showed that of 61 APEC strains, 73% were carrying *iss* gene, 55% *tsh*, 45% *iutA*, 39% *fliA*, 24% *papC*, 23% *cvaC* and 18% *kpsII* genes [7]. Of the 105 examined *E. coli* strains in our study, 28 were APEC strains since they were carrying five or more virulence genes, while the other 77 samples were non-APEC strains as they had less than five virulence genes. Of 28 APEC strains, 96.4% were carrying *iss* and 96.4% *tsh* genes, 82.1% *papC*, 85.7% *astA*, *vat* and *iucD*, 53.5% *irp2* and 14.2% *cva/cvi* genes. Of 77 non-APEC strains, 94.8% were carrying *cva/cvi*, 80.5% *irp2*, 68.8% *astA*, *iucD* and *vat*, 66.2% *papC* and 64.9% *iss* and *tsh* genes.

Our results showed that *iss* and *tsh* genes were the most frequent virulence genes among the APEC strains ($f = 0.96$) examined while these two genes had the lowest frequency among the non-APEC strains ($f = 0.64$) tested. In contrast, *cva/cvi* gene had the lowest and highest frequency among the APEC ($f = 0.14$) and non-APEC ($f = 0.94$) strains analyzed, respectively. The strains that contained *astA*, *vat*, *iucD* genes had the same frequency among both APEC ($f = 0.85$) and non-APEC strains ($f = 0.68$). Our results are consistent with those obtained by Kwon *et al.* [4] regarding the frequency of *iss* and *tsh* genes as the most common virulence genes carried by the APEC isolates tested; although, different frequencies have been reported for other virulence genes by these two studies. The comparison between results obtained by our research and those obtained by other researchers reveals that presence of *tsh* and *iss* genes can be considered as a key component to distinguish between APEC and non-APEC strains.

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REFERENCES

1. Janssen, T., H.C. Philipp, M. Voss, R. Preisinger and L.H. Wieler, 2003. Multiplex PCR is the first technique to allow the specific and sensitive detection of avian pathogenic *Escherichia coli* (APEC). Lohmann Info., 28: 1-5.
2. Barnes, H.J., L. Nolan and J.P. Vaillancourt, 2008. Colibacillosis. In Diseases of Poultry, 12th ed. ISU Press, Ames, IA. pp: 691-732.
3. Ewers, C., T. Janssen, S. Kiessling, S. Philipp and L.H. Wieler, 2004. Molecular epidemiology of avian pathogenic *Escherichia coli* (APEC) isolated from colisepticemia in poultry. Vet. Microbiol, 104: 91-101.
4. Kwon, S.G., S.Y. Cha, E.J. Choi, B. Kim, H.J. Song and H.K. Jang, 2008. Epidemiological prevalence of avian pathogenic *Escherichia coli* differentiated by multiplex PCR from commercial chickens and hatchery in Korea. J. Bacteriol. Virul, 38: 179-188.
5. Wooley, R.E., P.S. Gibbs, T.P. Brown and J.J. Maurer, 2000. Chicken embryo lethality assay for determining the virulence of avian *Escherichia coli* isolates. Avian Dis., 44: 318-324.
6. Ewers, C., E.M. Antoa, I. Dieh, S. Philipp and L.H. Wieler, 2009. Intestine and environment of the chicken as reservoirs for extraintestinal pathogenic *Escherichia coli* strains with zoonotic potential. Appl. Environ. Microbiol, 75: 184-192.
7. Rocha, A.C.G.P., S.L.S. Rocha, C.A.V. Lima-Rosa, G.F. Souza, H.L.S. Moraes, F.O. Salle, L.B. Moraes and C.T.P. Salle, 2008. Genes associated with pathogenicity of avian *Escherichia coli* (APEC) isolated from respiratory case of poultry. Pesq. Vet. Bras, 28: 183-186.
8. Nakazato, G., Amabile de T. Compos, E.G. Stehling, M. Brocchi and W. Dias da Silveira, 2009. Virulence factor of avian pathogenic *Escherichia coli* (APEC). Pesq. Vet. Bras, 29: 479-486.
9. Ewers, C., T. Janssen, S. Kiessling, H.C. Philipp and L.H. Wieler, 2005. Rapid detection of virulence associated genes in avian pathogenic *Escherichia coli* by multiplex polymerase chain reaction. Avian Dis., 49: 269-273.
10. Knobl, T., T.A.T. Gomes, M.A.M. Vieira, F. Ferreira, J.A. Bottino and A.J.P. Ferreira, 2006. Some adhesions of avian pathogenic *Escherichia coli* (APEC) isolated from septicemia poultry in Brazil. Braz. J. Microbiol, 37: 379-384.

11. Gibbs, P.S., J.J. Maurer, L.K. Nolan and R.E. Wooley, 2003. Prediction of chicken embryo lethality with the avian *Escherichia coli* traits complement resistance, colicin V production and presence of the increased serum survival gene cluster (iss). *Avian Dis.*, 47: 370-379.
12. Foley, S.L., S.M. Horne, C.W. Giddings, M. Robinson and L.K. Nolan, 2000. Iss from a virulent avian *Escherichia coli*. *Avian Dis.*, 44: 185-191.
13. Skyberg, J.A., S.M. Horne, C.W. Giddings, R.E. Wooley P.S. Gibbs and L.K. Nolan, 2003. Characterizing avian *Escherichia coli* isolates with multiplex polymerase chain reaction. *Avian Dis.*, 47: 1441-1447.
14. Won, G.Y., B.M. Moon, I.G. Oh, K. Matsuda, A.A. Chaudhari, J. Hur, S.K. Eo, I. Yu, Y.J. Lee, Y.S. Lee, B.S. Kim and J.H. Lee, 2009. Profiles of virulence-associated genes of avian pathogenic *Escherichia coli* isolates from chicken with colibacillosis. *J. Poult. Sci.*, 46: 260-266.
15. Stathopoulos, C., D.L. Provence and R. Curtiss, 1999. Characterization of the avian pathogenic *Escherichia coli* hemagglutinin tsh, a member of the immunoglobulin protease-type family of autotransporters. *Infect. Immun.*, 67: 772-781.
16. Dozois, C.M., M. Dho-Moulin, A. Bree, J.M. Fairbrother, C. Desauters and R. Curtiss, 2000. Relationship between the tsh autotransporter and pathogenicity of avian *Escherichia coli* and localization and analysis of the tsh genetic region. *Infect. Immun.*, 68: 4145-4154.
17. Delicato, E.R., B.G. De Brito, A.P. Konopatzki, L.C. Gaziri and M. Vidotto, 2002. Occurrence of the temperature-sensitive hemagglutinin among avian *Escherichia coli*. *Avian Dis.*, 46(3): 713-716.
18. Quinn, P.J., M.E. Carter, B. Markey and G.R. Carter, eds. 1994. Enterobacteriaceae. In: *Clinical Veterinary Microbiology*. Wolf Publishing, Mosby-Year Book Europe Ltd, London, England. pp: 209-236.
19. Moon, B.M., G.Y. Won, Y.Y. Choi, J.K. Jin, I.J. Oh, J.H. Park, S.K. Eo and J.H. Lee, 2006. Isolation and characteristics of avian pathogenic *Escherichia coli* from birds associated with colibacillosis. *Chulalongkorn Uni. Fac. Vet. Sc.*, pp: 26-29.