Distribution of virulence associated genes in isolated *Escherichia coli* from avian colibacillosis

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Abstract:

BACKGROUND: Colibacillosis is one of the most prevalent diseases in the world that causes multimillion-dollar annual losses. **OBJECTIVES:** In order to evaluate molecular epidemiology of some virulence associated factors in *Escherichia coli*, isolated from poultry, the presence of *iut A*, *iss*, *hly F*, *omp T*, *iro N*, *afa*, *sfa (S)* and *pap G (II)* were investigated by multiplex PCR assay. **METHODS:** Two hundred thirty four *Escherichia coli* isolated from avian colibacillosis (APEC) and fifty four fecal *E. coli* isolates from the feces of apparently healthy birds (AFEC) were investigated for presence of some virulence associated genes by two panel of multiplex PCR. Statistical analysis was performed using χ^2 test. the p-value was ≤0.05. **RESULTS:** Among 234 *E. coli* strains associated with colibacillosis and 54 AFEC strains, 85% of isolates were positive for at least one of the virulence gene. The three most prevalent genes in *E. coli* isolated from colibacillosis were *hly F* (77.3%), *omp T*(73%) and *iss* (68.2%). *Iut A*, *iro N* and *pap G (II)* were detected in 157 (67.4%), 152 (65.2%) and 41(17.6%) respectively. None of isolates harbored *sfa (s)* and *afa* genes. Several combination patterns of virulence genes were detected. Combination of *hly F*, *omp T* (70.8%) was the most prevalent pattern. **CONCLUSIONS:** the prevalence of *iss*, *hly F*, *omp T*, *iro N* genes in APEC isolates was significantly more than AFEC strains and probably these genes play an important role in the pathogenesis of APEC strains.

Introduction

Although *Escherichia coli* are comensal bacteria of intestine of human and animals, some specific strains possess certain virulent factors are able to cause diseases in human, animals and birds (Rodriguez-siek et al., 2005; Delicato et al., 2003). Avian pathogenic *Escherichia coli* (APEC) are a sub set of diverse group of bacteria which called extra intestinal pathogenic *E. coli* (EXPEC) (Ghanbarpour et al., 2010). APEC strains are responsible for some diseases in poultry including colibacillosis, swollen head syndrome, yolk sac infection, omphalitis and coligranuloma (Saif et al., 2008).

One of the most important results of avian colibacillosis is high morbidity and mortality in birds and multimillion dollar annual losses in world of poultry industry. Protection of poultry from predisposing conditions as a management approach had not positive effect in controlling avian colibacillosis (Johnson et al., 2008). Furthermore there is, considerable concern that APEC isolates are becoming more resistant to antimicrobial agents (Johnson et al., 2004; Maynard et al., 2004; Yang et al., 2004; Johnson et al., 2005; Johnson et al., 2008).

In the other hand, some evidences show that

APEC strains play important role as a food born pathogen and poultry products can be a suite source of EXPEC including strains causing human diseases. Therefore, control of colibacillosis will be a serious problem in future and also is beneficial for both human and animal health (Johson et al., 2008).

Accordingly, the vaccine base control of colibacillosis is desirable (Johson et al., 2008) but vaccines that designed to prevent avian colibacillosis have met with mixed results (Arp, L. H. 1982; Bolin, C. A., and A. E. Jensen. 1987; Deb, J. R., and E. G. Harry. 1978; Kwaga et al., 1994; Foley et al., 2000; Kariyawasam et al., 2004; Johnson et al., 2008)

Thus exploitation of common APEC markers as the target of vaccines or for diagnostic tools can yield measures with general applicability to control of colibacillosis. Despite the numerous studies on APEC there are no specific traits to define the APEC pathotype, although a variety of virulent factors like fimbrial and afimbrial adhesions, invasive factors, iron acquisition systems, serum resistance mechanisms, antiphagocytic activity, toxins and some virulent gene regulators are identified that promote diseases in poultry (Dziva and Stevens, 2008).

In Iran like other countries with large poultry industries, commercial flocks are affected by colibacillosis. But information on the virulence traits of *E. coli* isolated from colibacillosis lesions is insufficient. To get more information about the distribution of some genes associated with virulence in Iran, the presence of *iut A, iss, hly F, omp T, iro N, pap G (II), afa* and *sfa (S)* in *E. coli* isolated from poultry colibacillosis by two panels of multiplex PCR was investigated.

Materials and Methods

Bacterial strains: Two hundred thirty four *Escherichia coli* isolated from avian colibacillosis (APEC) and fifty four fecal *E. coli* isolates from the feces of apparently healthy birds (AFEC) were used in this study. The APEC isolates were collected from various diagnostic veterinary laboratories in Tehran, Semnan, Garmsar and some cities in the north of Iran.

To confirm the *E. coli* strains, standard biochemical tests were used. All isolates were stored in the Brain Heart Infusion (BHI) broth (Difco) with 30% glycerol at -80ºC.

Virulence genotyping (Polymerase chain reaction): one set of pentaplex PCR for *iut A, iss, hly F, omp T* and *iro N* and one set of triplex PCR for *pap G (II), sfa s* and *afa* carrired out. Description and primer sequences used in this study is summarized in Table 1. To generate template DNA, individual colonies from EMB agar (Difco) was inoculated in 3mL of Luria-bertani broth (Difco) at 37ºC overnight. 30 µL of culture was added to 270 µL of TE buffer (10mM Tris-Hcl, 1mM EDTA [pH= 8.0]) and boiled for 10 min, supernatant was used as template.

For pentaplex PCR amplification reactions were performed in a 25 µL reaction mixture including 0.25 mM deoxynucleoside triphosphate (Takara Bio. Inc [Shiga Japan]) 0.3µM of each primer and 1U Extaq DNA polymerase (Takara Bio. Inc [Shiga Japan]). This enzyme includes $Mgcl_2$. All reactions were performed using Verity Thermal cycler (Applied Biosystem) with the following conditions: 94ºC for 2min, 25 cycles 63ºC for 30 s, 68ºC for 3min and a final cycle of 72ºC for 10min (Johnson et al., 2008).

As positive control, strains confirmed by microarray assay were used. After doing a few PCR amplifications, we found some strains which possess five interesting virulence genes, one of which was selected. After sequencing, it was used as positive control. Deionized distilled water was used as negative control.

To amplify *pap G (II), sfa S* and *afa*, reactions were performed in a 25µL reaction mixture including 0.25 mM dNTP (Takara Bio. Inc [Shiga Japan]), 0.3 µM of forward and reverse primers and 1.25 U Extaq (Takara Bio. Inc [shiga Japan]), with the following conditions: 2min at 95ºC to activate Taq polymerase, 25 cycles of 30s at 94ºC, 30s at 63ºC and 3min at 68ºC, with a final cycle of 10min at 72ºC, followed by a hold at 4ºC (Rodriguez-siek et al.,2005). To analyze amplification products, horizontal gel electrophoresis on 3% agarose was performed and the size of amplicons was determined by comparison with the 100 bp DNA ladder (Takara Bio. Inc [Shiga Japan]). AH-2 and C81 were used as positive control for *pap G (II)* and *afa*. For *sfa* S we used confirmed strain by microarray as described above.

Statistical analysis: Statistical analysis was performed using χ^2 test. Significance was accepted when the p-value was ≤0.05.

Table 1. Description and primer sequences.

Gene	Product size (bp)	primer Sequence	Description
afa	594	F 5 -GGCAGAGGGCCGGCAACAGGC- 3 R 5 -CCCGTAACGCGCCAGCATCTC- 3	Afimbrial adhesion : responsible for biosynthesis of adhesins and invasins (28)
sfa S	244	F 5 -GTGGATACGACGATTACTGTG- 3 R 5 -CCGCCAGCATTCCCTGTATTC- 3	Mannose resistant adhesion (28)
pap G (II)	190	F 5 -GGGATGAGCGGGCCTTTGAT- 3 R 5 -CGGGCCCCCAAGTAACTCG- 3	Pilus associated with pyelonephritis (28)
iro N	553	F 5 -AATCCGGCAAAGAGACGAACCGCCT- 3 R 5 -GTTCGGGCAACCCCTGCTTTGACTTT- 3	Salmochelin siderophore receptor gene (12)
omp T	496	F 5 -TCATCCCGGAAGCCTCCCTCACTACTAT- 3 R 5 -TAGCGTTTGCTGCACTGGCTTCTGATAC- 3	Episomal outer membrane protease gene (12)
iutA	302	F 5 -GGCTGGACATCATGGGAACTGG- 3 R 5 -CGTCGGGAACGGGTAGAATCG- 3	Episomal outer membrane protease gene (12)
iss	323	F 5 -CAGCAACCCGAACCACTTGATG - 3 R 5 -AGCATTGCCAGAGCGGCAGAA- 3	Episomal increased serum survival gene (12)
hly F	450	F 5 -GGCCACAGTCGTTTAGGGTGCTTACC- 3 R 5-GGCGGTTTAGGCATTCCGATACTCAG- 3	Putative avian hemolysin (12)

Table 2. Prevalence of each gene in pathogenic and fecal isolates.

AFEC	APEC n= 234		Gene n= 54	
	n	(%)	n	(%)
iut A	157	67.4	32	60.4
iss	159	68.2	13	24.5
hly F	180	77.3	20	37.7
omp T	170	73	20	37.7
iro N	152	65.2	12	22.6
pap G(II)	41	17.6	5	9.4
sfa (s)	0	0	0	0
afa	0	0	0	0

Table 3. Percentage of strains with the given pair of virulence-associated genes among 234 *E. coli* strains isolated from avian colibacillosis.

Genes	*iut A*	*iss*	*hly F*	*omp T*	*iro N*	pap G(II)
iut A	-					
Iss	54.9	-				
hly F	61.8	64.4	-			
omp T	57.1	61.4	70.8	-		
iro N	50.6	59.7	61.8	57.5	-	
pap G(II)	12.9	10.3	12	12	9.9	-

Results

We examined 288 *E. coli* to evaluate the presence of eight virulence associated genes. Prevalence of each gene in pathogen and fecal isolates are summarized in Table 2. The amplicon patterns of virulence associated genes by penta pelex and triplex PCR are shown in Figures 1and 2.

Results of this study showed that, none of the strains harbored *afa* and *sfa S*, only 41 (17.6%) pathogen strains and 5 (9.4%) fecal strains possessed *pap G (II)*. Out of 234 *E.coli* strains in pathogen group 157 (67.4%) isolates were positive for *iut A*, 180 (77.3%) for *hly F*, 159 (68.2%) for *iss*, 152 (65.2%) for *iro N* and 170 (73%) for *omp T*. while, out of 54 fecal isolates, 32 (60.4%) isolates were positive for *iut A*, 20 (37.7%) for *hly F*, 13 (24.5) for *iss*, 12 (22.6%) for *iro N* and 20 (37.7%) for *omp T*. Several significant differences were detected, including in the *iss*, *hly F*, *omp T*, *iro N* and *pap G (II)*. We detected several combinations of the virulence genes. *hly F*, *omp T* was the most prevalent pattern (Table 3).

Discussion

Actually, of the further studies on *Escherichia coli* isolated from poultry, there are no certain traits to define APEC pathotype. Plasmid mediated horizontal gene transfer causes a large extensive diversity within APEC strains (Dziva and Stevens, 2008).

According to the results of this study, 85% of *E. coli* strains associated with colibacillosis possess at least one of the examined virulence genes, whereas 66% of *E. coli* strains isolated from apparently healthy birds were considered positive for at least one

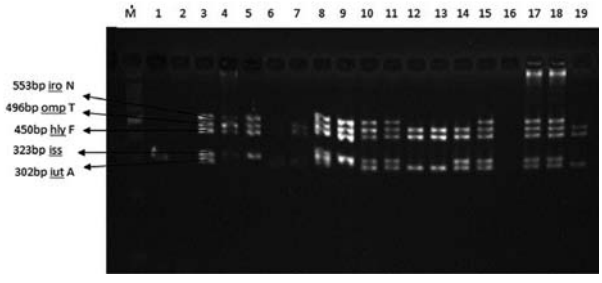

Figure 1. Pentaplex PCR of *Escherichia coli* isolated from avian colibacillosis using primer set; *iut A*: 302 bp, *iss*: 323 bp, *hly F*: 450 bp, *omp T*: 496 bp, *iro N*: 553 bp. Lane M: 100 bp marker. Lane 3: positive control, lane 2: negative control (water). Lanes 1 and 4 to 19: samples.

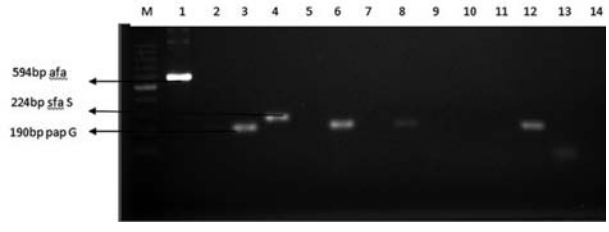

Figure 2. Triplex PCR of *Escherichia coli* isolated from avian colibacillosis using primer set; *pap G (II)*: 190 bp, sfa S: 224 bp, *afa*: 594 bp. Lane M 100 bp marker. Lane 1, 3 and 4: positive controls for *afa*, *pap G (II)* and sfa S. lane 5: negative control (water). Lanes 6 to 14: samples.

gene. Some researchers showed that APEC plasmids possess several virulence associated genes, though some of these have been reported in *E. coli* strains isolated from apparently healthy birds (McPeake et al., 2005).

In this study, *hly F* gene (77.3%) was the most prevalent gene. This prevalence was significantly more than AFEC isolates (37.7%). Johnson et al. (2008) examined genotype, pathogenesis and serogroup of 994 avian *E. coli* isolates. The prevalence of this gene was 78.2%. They reported *hly F* gene can be used as a marker to identify traits that predict avian pathogenic *E. coli*.

Prevalence of *iss* in APEC isolates in this study was 68.2% and it was significantly more than AFEC isolates. Rodriguez-siek et al., (2005) reported that 82.7% of *E.coli* isolates from poultry colibacillosis were positive for *iss*.

Lynne et al., (2006) investigated immune response to recombinant *Escherichia coli* iss protein in chicken. They suggested that *iss* may have potential to protect birds against heterologus E.coli challenge.

Several invasive pathogenic bacteria like *E. coli* have some iron acquisition systems, which can enable them to compete with the host siderophores. These strains are able to grow in a low iron environment (Tokano et al., 2008)

In this study 67.4% and 65.2% of the isolates were positive for *iut A* and *iro N* respectively. Rodriguez-siek et al., (2005) reported 81.2% and 88.2% of E.coli isolates from poultry colibacillosis were positive for *iut A* and *iro N*, respectively. Johnson et al., (2008) presented these two genes as markers to predict APEC pathotype.

According to the results of this study 73% of APEC isolates were considered as *omp T* positive. Johnson et al., (2008) reported *omp T* can be a VF predictor of APEC pathotype. Kanamaru et al., (2003) explained relative prevalence ratio of *omp T* is more frequent in urinary tract infection isolates than in fecal isolates. Although the physiological function of *omp T* is unclear, it has been suggested this enzyme is involved in *E. coli* pathogenesis and it can inactivate antimicrobial peptides (Okuno et al., 2004).

The principal in pathogenesis of *E. coli* is the ability of bacteria to adhere to and colonize the host tissue (Reed and Williams, 1978; Naveh et al., 1984; Gyimah and Panigrahy, 1998; Monroy et al., 2005).

In this study, 17.6% of APEC isolates were positive for *pap G (II)*. Similar to these results, Knöbl et al., (2006) and Stordeur et al., (2002) reported that 17.4% and 23.9% of the *E. coli* isolated from poultry were positive for pap. Ghanbarpour et al., (2010) detected 14.06% *pap G* positive gene in *E. coli* isolated from avian cellulitis in Iran.

Stordeur et al., (2002) reported among *pap* negative strains were F17-positive and /or *afa* - 8 positive strains. Because of the strains that possess F-17 gene cluster were lethal for 1-day old chicks and are also able to induce classical colibacillosis lesion,

p fimbriae may not be essential for pathogenesis. Its function may be substituted by other adhesions (Dziva and Stevens, 2008).

In this study there was no strain carrying sfa S gene. Fimbriae which are able to induce adherence of *E. coli* to endothelial and epithelial cells of cerebral ventriculus and coroid plexus in man (Knöbl et al., 2006), are rarely detected in APEC. Prevalence of this gene has been reported in the range of 0%, 1.8% and 8.8% colisepticemia cases by PCR and/or colony hybridization assay (Dozois et al., 1992; Delicato et al., 2003; Ewers and Wilking, 2007).

The role of this fimbriae in the pathogenesis of avian colibacillosis is not clear. Probably the bacterium with S fimbriae has originated from human and infected chicks due to poor hygiene.

Afa gene cluster encode afimbrial adhesions. These adhesions are expressed by *E. coli* associated with diarrhea and uropathogenic *E. coli*. In this study no *afa* gene was detected. Like this, Ghanbarpour et al., (2010) and Knöbl et al., (2006) reported none of the isolates from cellulitis and omphalitis of chicks in Iran and septicemic poultry in Brazil harbored the *afa* gene.

The results of this study showed that, frequency of investigated virulence associated genes in Iran is similar to several countries. However, the presence of DNA sequences associated with virulence factor does not mean that it is expressed. On the other hand, pathogenesis of these isolates after inoculation to respiratory tract of chick should be evaluated. Thus further studies must be carried out to establish the importance of these virulent factors in pathogenesis of *Escherichia coli* isolated from colibacillosis.

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بررسی میزان شیوع برخی از عوامل حدت اشریشیا کلی های جدا شده از کلی باسیلوز طیور

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چکیده

زمینه مطالعه: کلی باسیلوز یکی از بیماریهای بسیارشایع در صنعت پرورش طیور می باشدکه سالیانه موجب بروز خسارات اقتصادی زیادی می گردد. **هدف:** به منظوربررسی اپیدمیولوژی ملکولی برخی از عوامل مرتبط با حدت اشریشیا کلی های جداشده ازکلی باسیلوز طیور، در این مطالعه هشت ژن *sfa (S)*، *afa, papG (II)*، *iroN*، *ompT*، *hlyF*، *iss*، *iutA* مورد بررسی قرار گرفتند. **روش کار:** در این تحقیق هشت ژن مرتبط با حدت این باکتری در ۲۳۴ سویه جداشده از دستگاه تنفس پرندگان مبتلا به کلی باسیلوز و ۵۴ سویه جداشده ازکلواک پرندگان به ظاهرسالم، با روش واکنش زنجیره ای پلیمراز پنج گانه وسه گانه مورد مطالعه قرارگرفت. نتایج به دست آمده باآزمون آماری مربع کای بررسی شدند. **نتایج:** نتایج حاصل از این مطالعه نشان داد، از میان ۲۳۴ باکتری اشریشیاکلی جدا شده از موارد کلی باسیلوز و ۵۴ سویه جدا شده از مدفوع پرندگان به ظاهرسالم، ۸۵٪ از جدایه‌ها حداقل واجد یکی از عوامل حدت بودند. سه ژن *hlyF*، *ompT* و iss به ترتیب با شیوع ۷۷/۳٪، ۷۳٪ و ۶۸/۲٪، واجد بیشترین فراوانی در موارد جداشده ازکلی باسیلوز بوده و فراوانی ژن های *iutA*، *iroN* و *papG (II)* به ترتیب، ۶۷/۴٪، ۶۵/۲٪ و ۱۷/۶٪ گزارش شد. در هیچ یک از جدایه های مورد بررسی، سویه واجد ژن های *afa* و *sfa (S)* مشاهده نگردید. در این مطالعه الگوهای ژنوتیپی متنوعی مشاهده گردید که بیشترین الگو مربوط به ژنوتیپ *hlyF-ompT* با فراوانی ۷۰/۸٪ بود. **نتیجه‌گیری نهایی:** میزان فراوانی ژن های *iroN*، *ompT*، *hlyF*، *iss* درسویه های مرتبط با APEC به طورمعنی داری بیشتر ازسویه های AFEC بود واحتمالاً این ژن ها در حدت و بیماریزایی اشریشیاهای جداشده ازکلی باسیلوز طیور اهمیت دارند.

واژه‌های کلیدی: عوامل حدت، کلی باسیلوز طیور، واکنش زنجیره ای پلیمراز

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