A study on the presence of some potential virulence genes and quinolone resistance in avian pathogenic *Escherichia coli* isolated from chickens in Northeast of Iran

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Abstract:

BACKGROUND: Avian pathogenic *Escherichia coli* (APEC), which is the causative agent of colibacillosis, harbors several putative virulence genes. An important trait of APEC for both poultry and public health is antibiotic resistance. **OBJECTIVES:** In the present study, some potential virulence genes of APECs isolated from Northeast of Iran and their resistance to the quinolones antibiotics were studied. **METHODS:** The conventional polymerase chain reaction (PCR) was used to determine the presence of four virulence genes, including *iss*, cvi, *iuc*, and *tsh*, in 52 isolates of *E. coli* from avian colibacillosis and 11 isolates from feces of apparently healthy chicken. Disk diffusion method was used to also determine the resistance of all the isolates against nalidixic acid, norofloxacin, ciprofloxacin, and enrofloxacin. **RESULTS:** The presence of *iss* and *tsh* virulence genes in isolates from diseased chickens was significantly higher than isolates from healthy chickens. There was no significant difference between APEC and fecal *E. coli* when it comes to quinolone resistance. However, *cvi* and *iuc* genes were significantly higher in susceptible isolates of *E. coli* from healthy chickens. **CONCLUSIONS:** *iss* and *tsh* genes are more prevalent in APEC isolates than in fecal isolates. There is no association between lack of virulence and resistance to quinolones in *E. coli* isolates from diseased chickens.

Introduction

Escherichia coli is regarded as a normal microflora of the intestinal tract and poultry environment. However, due to the acquisition of virulence factors, some strains of *E. coli* are pathogenic (Vidotto et al., 1990). Colibacillosis is a disease of severe economic importance to the poultry industry worldwide and is characterized by a diverse array of lesions (Vidotto et al., 1997; Dziva and Stevens, 2008). Several valuable virulence genes were described in avian pathogenic *E. coli* (APEC). These genes could express different traits including adhesions, toxins, iron uptake systems, and resistance to the host serum (Delicato et al., 2003).

The temperature-sensitive hemagglutinin (tsh) may act as an adhesion, particularly in the initial stages of colonization of the respiratory tract. Many studies have shown that colV type plasmids carry the *tsh* gene and also frequently carry genes for the aerobactin iron

uptake system (Delicato et al., 2002; Tivendale et al., 2004; Nakazato et al., 2008). The association of *tsh* with lethal APEC isolates suggested that *tsh* may be a virulence factor and/or could be physically linked to some independent virulence determinants (Dozois et al., 2000). The increased serum survival (*iss*) gene encodes a protein that plays a role in serum resistance, and the presence of this gene in pathogenic avian strains has been shown to be highly significant (McPeake et al., 2005). The *iuc*C, a gene encoding a protein involved in aerobactin production and cvi, the colicin V (colV) immunity gene, are involved in the establishment of avian infection (Skyberg et al., 2003; Moon et al., 2006).

Several studies have shown that quinolone resistant *E. coli* strains display reduced virulence (Johnson et al., 2004; Sherwood et al., 1983; Orden et al., 1999). However, the lack of pathogenicity islands does not arise from *gyrA* mutation, but both are consequences of chromosomal characteristics and would be intrinsic bacterial characteristics (Piatti et al., 2008). This study was designed to investigate the presence of four virulence-associated genes in avian *E. coli* isolated from diseased and healthy chickens. This study also aimed at clarifying the association of the incidence of these virulence factors with resistance against quinolones.

Material and Methods

Bacterial isolates: A total of 63 *E. coli* isolates were collected from diseased and healthy chickens in several broiler farms in Khorasan district (Northeast Iran). Fifty two isolates out of 63 were obtained from heart and liver specimens of colobacillosis cases in pure culture. The remaining isolates were obtained from feces of apparently healthy chickens. The identity of *E. coli* isolates was confirmed using standard biochemical tests (Quinn et al., 2002).

All isolates were stored in nutrient broth with 15% glycerol at -20°C. For DNA extraction, isolates were grown on either MacConkey agar (HiMedia, India) or nutrient agar (HiMedia, India) over night at 37°C.

Polymerase chain reaction (PCR) assay: DNA was extracted from all isolates of *E. coli* using genomic DNA extraction kit (Bioneer, Korea). The isolates were examined for *iss*, *tsh*, *iuc*, and *cvi* genes using single PCR assay. Primer sequences are listed in Table 1, on the basis of published sequences for these genes (Skyberg et al., 2003). Primers were synthesized by Bioneer company, South Korea. The PCR assay was carried out in a total volume of 25 µl of the PCR premix mixture (Bioneer company, South Korea) containing Taq DNA polymerase (1U), MgCl2 (1.5 Mm), each of the deoxynucleotide triphosphates (250 µm), KCl (30 Mm) and Tris-Hcl pH=9 (10 mM). Two microliters of the template DNA (200 ng) and 1 µl of each primers (forward and reverse) (25 pmol) were added.

Amplification was performed according to the following conditions: an initial denaturation at 95ºC for 5 min; for 30 cycles: denaturation at 95ºC for 30 s, annealing at 55ºC for 45 s, extension at 72ºC for 1.5 min and a final extension at 72ºC for 7 min. The products were then separated in a 1.5% agarose gel. A 100 bp ladder (Fermentas Inc., USA) was used as a size reference. For establishment of the protocol, four *E. coli* isolates, containing the target genes, were identified by sequencing, and these isolates were used as positive controls. *E. coli* C600 (K12) was used as negative control.

Antimicrobial susceptibility testing: Antimicrobial susceptibility test was carried out for 22 isolates from chicken with colibacillosis and 11 isolates from the feces of healthy birds. The test was done using disk diffusion method on Mueller-Hinton agar (HiMedia, India). The antimicrobial agents used in this study were nalidixic acid, norfloxacin, enrofloxacin, and ciprofloxacin (PadtanTeb, Iran). Results of

the tests were interpreted using the guidelines of Clinical and Laboratory Standards Institute (CLSI) (Quinn et al., 2002).

Statistical analysis: Statistical analysis was performed using the *Chi-square test*. The threshold for significance was a p value <0.05.

Sequence analysis: Four amplicons from four *E. coli* isolates with the genes *iss*, *tsh*, *cvi* and *iuc* were sequenced in one direction by Bioneer company (South Korea). Sequences were examined for identity with published sequence data (Table 1) from National Center for Biotechnology Information (NCBI).

Results

Detection of virulence genes: The detected virulence genes using PCR are shown in Table 2. Amplicons for cvi, *tsh*, *iuc*, and *iss* are easily resolved when compared with a standard 100-bp ladder (Figure 1). Isolates from healthy birds were negative for *iss* gene, but was detected in 38.46% of the diseased isolates. The *tsh* gene was detected in 67.3% of pathogenic isolates, but only 27.3% of fecal isolates were positive for *tsh* gene. The *cvi* gene was detected in 53.84% of pathogenic isolates and was detected in 90.9% of the isolates from healthy birds. Forty-three out of 52 (82.69%) isolates from colibacillosis cases and 10 out of 11 (90.9%) isolates from healthy birds possessed *iuc* sequence. Twelve patterns were found among pathogenic isolates, and 2 of them (*iss*+, cvi+, *iuc*+, *tsh*+ and *iss*-, cvi+, *iuc*+, *tsh*+) being the most prevalent. Only one pattern (*iss*-, cvi+, *iuc*+, *tsh*-) was significantly higher in isolates from healthy chickens (Table 3).

Association of virulence gene with antimicrobial resistance: There was no significant difference on the frequency of virulence genes between susceptible and resistant *E. coli* from diseased chickens against quinolone and fluoroquinolones.

However, *cvi* and *iuc* genes were significantly higher in ciprofloxacin and norfloxacin susceptible isolates as compared to the resistant ones in healthy chickens (Table 4).

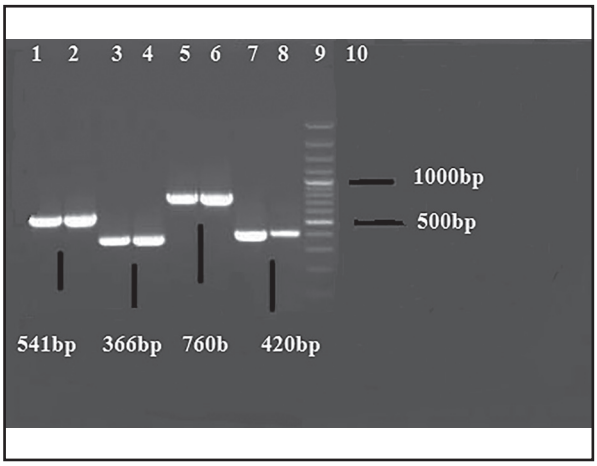

Figure 1. PCR products for *iuc*, cvi, *iss* and *tsh* genes. 9: 100-bp plus DNA ladder. 1, 3, 5 and 7: positive control strains for *iuc*, cvi, *iss* and *tsh* genes, respectively. 2, 4, 6 and 8: positive isolates for *iuc*, cvi, *iss* and *tsh* genes, respectively. 10, negative control.

Sequence analysis: Four amplicons represented the expected sequences with more than 90% identity with published data from NCBI (Table 1).

Discussion

In terms of virulence of *E. coli* strains in poultry, several factors have been reported, including adhesions, iron sequestering systems, capsular and lipopolysaccharide antigens, and toxins (Dho and Lafont, 1984; Penteado et al., 2002; Stordeur et al., 2002; Vidotto et al., 2004; Germon et al., 2005; Knobl et al., 2006; Lymberopoulos et al., 2006; Musa et al., 2009). Rooks can serve as a reservoir of avian pathogenic virulence factors, and potentially transmit *E. coli* over long distances (Kmet et al., 2013). In this study, four virulence genes (tsh, cvi, *iuc*, and *iss*) were detected in *E. coli* isolates from both diseased and healthy chickens. Quinolone resistance was also determined in a few of these isolates. Only *iss*, the increased serum survival gene sequence, was negative in isolates from healthy chickens, but 38.46% of the isolates were detected in diseased chick-

Table1. Sequence and specificity of PCR primers and their product sizes.

Name	Primer Sequence (5´ to 3´)	Gene bank ACC.no.	size (bp)	Reference
iss	F: GTGGCGAAAACTAGTAAAACAGC R: CGCCTCGGGGTGGATAA	FA 042279.1	760	Skyberg et al. 2003
tsh	F: GGGAAATGACCTGAATGCTGG R: CCGCTCATCAGTCAGTACCAC	YA 280856.1	420	Skyberg et al. 2003
iuc	F: CGCCGTGGCTGGGGTAAG R: CAGCCGGTTCACCAAGTATCACTG	X 76100.1	541	Skyberg et al. 2003
cvi	F: GGGCCTCCTACCCTTCACTCTTG R: ACGCCCTGAAGCACCACCAGAA	FA 062858.1	366	Skyberg et al. 2003

Table 2. Distribution of virulence genes between *E. coli* isolates from diseased and healthy chickens.

Virulence genes	*E.coli* isolates from diseased chickens (52)	*E.coli* isolates from healthy chickens (11)	P - value
iss +	20	0	0.013
iss _	32	11	
cvi +	10	28	0.02
cvi _	24	1	
iuc +	43	10	0.50
iuc _	9	1	
tsh +	35	3	0.014
tsh _	17	8	

Table 3. Virulence gene patterns between *E. coli* isolates from diseased and healthy chickens.

Pattern	*iss*	cvi	*iuc*	*tsh*	Isolates from diseased chickens (%)	Isolates from healthy chickens (%)	P-value
1	+	+	+	+	9 (17.3%)	0	0.14
2	-	+	+	-	4 (7.7%)	7 (63.6%)	<0.0001
3	-	+	+	+	9 (17.3%)	2 (18.2)	0.94
4	-	-	+	+	6 (11.5%)	0	0.24
5	-	-	-	+	3 (5.8%)	0	0.42
6	-	-	+	-	8 (15.4%)	1 (9%)	0.59
7	-	+	-	+	2 (3.8%)	1 (9%)	0.46
8	+	+	+	-	3 (5.8%)	0	0.42
9	+	-	+	+	4 (7.7%)	0	0.35
10	+	-	-	-	1 (1.1%)	0	0.65
11	+	-	+	-	1 (1.1%)	0	0.65
12	+	-	-	+	2 (3.8%)	0	0.51

ens. Serum resistance is a characteristic related to the virulence of strains. Majority of the pathogenic strains in 1-day-old chickens that demonstrated the lowest LD50 were resistant to normal rabbit and chicken serum (Vidotto et al., 1990). Horne et al. (2000) found out that the presence of a gene for increased serum survival, the *iss* gene and its protein product, ISS, are potential targets for detection and control of avian colibacillosis.

Tivendale et al. (2004) showed that the putative virulence genes *iss* and *tsh*, and the aerobactin operon are on the plasmid pVMO1. They showed that both the aerobactin operon and *iss* were associated with high levels of virulence in APEC, but the possession of either gene was sufficient for intermediate levels of virulence.

Concerning *tsh* gene, some researchers concluded that the presence of *tsh* was not neces-

Table 4. Association of antimicrobial resistance and virulence genesin *E. coli* isolates from diseased and healthy chickens. [a, b] in each row shows significant different ($p<0.05$). [c] and d in each row shows significant different ($p<0.05$).

Pattern	*iss*	*cvi*	*iuc*	*tsh*	Isolates from diseased chickens (%)	Isolates from healthy chickens(%)	P-value
1	+	+	+	+	9 (17.3%)	0	0.14
2	-	+	+	-	4 (7.7%)	7 (63.6%)	<0.0001
3	-	+	+	+	9 (17.3%)	2 (18.2)	0.94
4	-	-	+	+	6 (11.5%)	0	0.24
5	-	-	-	+	3 (5.8%)	0	0.42
6	-	-	+	-	8 (15.4%)	1 (9%)	0.59
7	-	+	-	+	2 (3.8%)	1 (9%)	0.46
8	+	+	+	-	3 (5.8%)	0	0.42
9	+	-	+	+	4 (7.7%)	0	0.35
10	+	-	-	-	1 (1.1%)	0	0.65
11	+	-	+	-	1 (1.1%)	0	0.65
12	+	-	-	+	2 (3.8%)	0	0.51

sary for virulence (Tivendale et al., 2004; Dziva and Stevens, 2008). In contrast, this study showed that the *tsh* gene was significantly higher (67.3%) in the avian pathogenic isolates as compared to the fecal isolates from healthy chickens (27.3%). The results of this study are in agreement with the findings of other studies (Maurer et al., 1998; Dozois et al., 2000; Delicato et al., 2002). The temperature-sensitive hemagglutinin (tsh) is a member of the auto transporter group of proteins and was first identified in APEC strain X7122. The *tsh* occurrence among isolates from diseased chickens was significantly associated with high lethality for chickens (Dozois et al., 2000).

In this study, *E. coli* isolates from healthy chickens had significantly higher incidence of *cvi* gene, when compared with *E. coli* isolates from diseased chickens. The role of colV in pathogenesis of *E. coli* is controversial (Ngeleka et al., 1996). The production of colV, 8 mediated by colV plasmid, is a trait associated with the invasion and pathogenicity of *E. coli*. In general, genes usually found on plasmid pColV (tsh, *iss*, and hlyF) were associated with APEC. On the other hand, extra-intestinal pathogenic *E. coli* strains showed lower frequencies or no statistical difference in the frequencies of genes which are usually found in plasmid pColV (Maluta et al., 2014). However, it has been emphasized that colV activity itself is not necessary for the virulence of *E. coli* (Blanco et al., 1997).

In the present study, there was no significant difference on the incidence of *iuc* gene between *E. coli* isolates from healthy and diseased chickens.

Different gene patterns were considered in pathogenic and fecal isolates. Among 118 APEC isolates tested by Moon et al. (2006), 57.6% of the isolates were positive for cvi, 55% were positive for *tsh*, 47.5% were positive for *iuc*C and 38% were positive for *iss*. They concluded that these virulence genes are associated with avian colibacillosis in Korea. In the present study, among the 57 isolates, 29% of these isolates had cvi, 67% had *tsh*, 83% had *iuc*, and 38% had *iss*. Only one out of twelve gene combination patterns was significantly higher in isolates from healthy chickens than the diseased chickens. Sixty-four percent of the isolates of healthy chicken had this pattern (tsh-, *iss*-, cvi+, and *iuc*+). However, only 8% of the isolates from diseased chicken had the same pattern. In this study, it was shown that there were various gene clusters in avian *E. coli*. The presence of *tsh* and/or *iuc* seems to be an important trait among isolates from coli-

bacillosis. In addition, *iss* virulence gene was found in 38% of the avian pathogenic isolates. But *iss* virulence gene was not found in healthy ones. The presence of *iss* gene may be considered for APEC strains differentiation from the non-pathogenic isolates. However, the absence of this gene cannot be differentiated between pathogenic and non-pathogenic isolates.

Quinolones have high *in vitro* activity against Gram-negative bacteria, such as *E. coli*, but the number of quinolone resistant *E. coli* have increased in animals since their introduction in late 1980s (Guler et al., 2008). The association between virulence characteristics of *E. coli* and quinolone resistance is a complex phenomenon. In this study, quinolone-susceptible (ciprofloxacin and norfloxacin) isolates were shown to have significantly higher virulence genes (*cvi* and *iuc*) than quinolone-resistant isolates in healthy chickens. This conclusion could be attributed to the explanation that *cvi* and *iuc* are not located on the plasmid, which mediated antimicrobial resistance. On the other hand, it may be related to over-usage of these antibacterial agents as prophylaxis in healthy birds. For the presence of virulence genes between quinolone-susceptible and quinolone-resistant isolates in diseased chickens, no significant difference was observed.

In contrast to the results of the present study, Johnson et al. (2004) reported that multi-drug-resistant *E. coli* isolates appear to be significantly less virulent than susceptible isolates. Non-enterotoxigenic *E. coli* (ETEC) isolates were shown to be more resistant to antimicrobial agents, such as ampicillin, chloramphenicol, chlortetracycline and polymyxin B, than ETEC isolates (Sherwood et al., 1983). Strains producing some virulence factors of eae, K99, and necrotoxin were shown to be more susceptible to quinolones than not expressing these factors (Orden et al., 1999). However, it seems that the association of antimicrobial resistance with virulence may also depend on the types of virulence factors, antimicrobial agents, and status of the host. Further studies are needed to clarify the association of virulence factors of *E. coli* isolates and resistance against antimicrobial agents.

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بررسی وجود تعدادی از ژن‌های بالقوه حدت‌زا و مقاومت به کینولون‌ها در سویه‌های بیماریزای اشریشیا کلی جدا شده از طیور در شمال شرق ایران

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چکیده

زمینه مطالعه: اشریشیا کلی بیماریزای طیور (APEC)، عامل مسبب کلی باسیلوز بوده که دارای بسیاری از ژن‌های بالقوه حدت‌زا می‌باشد. **هدف:** در این مطالعه تعدادی از ژن‌های بالقوه حدت‌زا در سویه‌های APEC و مقاومت آنها به کینولون‌ها مورد بررسی قرار گرفتند. **روش کار:** وجود چهار ژن حدت شامل *iuc*، *cvi*، *iss* و *tsh* در ۵۲ جدایه اشریشیا کلی از کلی باسیلوز طیور و ۱۱ جدایه از مدفوع طیور به ظاهر سالم با روش قراردادی PCR مورد بررسی قرار گرفت. مقاومت تمام جدایه‌ها در برابرنالیدیکسیک اسید، نوروفلوکساسین، سیپروفلوکساسین و انروفلوکساسین با روش انتشار از دیسک ارزیابی گردید. **نتایج:** وجود ژن‌های *iss* و *tsh* در جدایه‌های طیور بیمار به طور معنی‌داری نسبت به جدایه‌های طیور سالم بیشتربود. تفاوت معنی‌داری بین سویه‌های APEC و اشریشیا کلی مدفوعی در مقاومت به کینولون‌ها وجود نداشت. ولی، ژن‌های *cvi* و *iuc* به طور معنی‌داری در جدایه‌های حساس به کینولون در طیورسالم، بیشتر بود. **نتیجه‌گیری نهایی:** ژن‌های *tsh* و *iss* در بیماریزایی سویه‌های اشریشیا کلی اهمیت دارند. ارتباطی بین عدم وجود ژن حدت و مقاومت در برابر کینولون‌ها در جدایه‌های طیور بیمار مشاهده نشد.

واژه‌های کلیدی: اشریشیا کلی طیور، کلی باسیلوز، مقاومت به کینولون، عوامل حدت

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