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Pathogenicity of the avian *Escherichia coli* isolates from pericarditis and femoral head necrosis lesions of the colibacillosis in experimentally infected chicks

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ABSTRACT

The aim of the present study was to evaluate the relationship between different combinations of seven (*ompT, hlyF, iss, iutA, iroN, tsh* and *cvaC*) Colicin V (ColV) plasmid associated virulence genes (VGs) and pathogenicity of avian *E. coli* isolates. After detection of the different patterns of VGs among 290 *E. coli* isolates from pericarditis and femoral head necrosis lesions of colibacillosis and the 70 isolates from feces of apparently healthy birds (AFEC), the day-old chick lethality test was conducted. Distribution of genetic patterns was different between the APECs and AFECs isolates, so that 67.3% of the APECs typed were represented by two specific genetic patterns ($ompT^+/hlyF^+/iss^+/iutA^+/iroN^+/tsh^+/cvaC^+$; $ompT^+/hlyF^+/iss^+/iutA^+/iroN^+/tsh^+/cvaC^-$). Furthermore, both the APECs and AFECs isolates with different genetic patterns were lethal for day-old chicks. The ColV plasmid or some of its VGs can provide helpful characteristics to describe APECs. Furthermore, extraintestinal environments may supply requirements for the pathogenicity of the avian *E. coli* isolates harboring different genetic background. However, further studies are needed to confirm the definite link between avian *E. coli* isolates pathogenicity and their genotype.

Key words: APEC, Colibacillosis, Colicin V Plasmid, Pathogenicity, Virulence gene

Colibacillosis is one of the most common bacterial infections that is responsible for significant losses to the poultry industry worldwide (Johnson *et al.* 2008, Schouler *et al.* 2012). Avian pathogenic *Escherichia coli* (APEC) strains, a divergent group of bacteria with different phenotype and genotype, are the etiologic agents of avian colibacillosis which appears as an initial colisepticaemia that may be followed by death or localized infection in various organs and tissues of the body (Ozaki *et al.* 2018). APECs, like other extraintestinal pathogenic *E. coli* (ExPEC) strains, have acquired virulence genes (VGs) which provide their extraintestinal life and pathogenicity (Dziva and Stevens 2008). The colicin V (ColV) plasmid harbors many of the genes associated with APEC virulence, including hlyF, a putative hemolysin; ompT, an outer membrane protease; iss, the increased serum survival gene involved in complement resistance; tsh, a temperature-sensitive hemagglutinin; the ColV operon, encoding ColV production; and several iron-related systems (Johnson *et al.* 2006). There are various studies that indicate the significant difference in ColV plasmid-carried VGs prevalence between APECs and *E. coli* strains isolated from the feces of apparently healthy birds (AFECs) (Schouler *et al.* 2012, Mohsenifard *et al.* 2016, Magray *et al.* 2018). Therefore, it has been suggested that some ColV plasmid-associated VGs may be used for differentiation and identification of the APECs (Rodriguez-Siek *et al.* 2005). However, rarely an APEC isolate contains all of the VGs despite their high genetic diversity (Schouler *et al.* 2012). Therefore, a reliable individual method for clear identification and characterization of the APECs has not been reported, so far. The objective of the present study was to determine the presence of different combinations of ColV plasmid-associated VGs among avian *E. coli* isolates and its relationship with experimental pathogenicity for chicks.

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MATERIALS AND METHODS

Genetic patterns: The present study included 220 *E. coli* isolates from pericarditis and femoral head necrosis lesions of the chickens exhibiting clinical and necropsy findings of colibacillosis and 70 isolates from the intestinal content of apparently healthy birds. Isolates were harvested from various poultry farms in south of Iran with sub-tropical climate, and characterized and genotyped by Avian Diseases Research Center laboratory, School of Veterinary Medicine, Shiraz University. Statistical analysis was performed to detect the presence and frequency of different combinations of seven ColV plasmid-associated VGs (ompT, hlyF, iss,

iutA, iroN, tsh, and cvaC) among the avian isolates.

Lethality test: Unvaccinated day-old commercial chicks (225) were divided into 15 groups and triple repeats and inoculated with APEC and AFEC isolates with more abundant genetic patterns detected according to the day-old chick lethality test (Dho and Lafont 1984). One group received normal saline as negative control. Isolates with a specific genetic pattern were designated as pathogenic when at least one chick died (Dozois *et al.* 2000). Lethality score was calculated (from 0 to 5) according to the number of chicks died within 4 days after inoculation (de Brito *et al.* 2003).

Statistical analysis: The prevalence of genetic patterns among APECs and AFECs isolates was analyzed using Chi-square test. One-way ANOVA was used for comparison of the lethality scores between the groups. P values of < 0.05 were considered significant.

RESULTS AND DISCUSSION

Genetic patterns: By detection of different combinations of seven ColV plasmid associated VGs, day-old chick lethality test was conducted to evaluate the pathogenicity of the APEC isolates from pericarditis and femoral head necrosis lesions of collibacilosis and AFEC isolates.

Twenty third different genetic patterns were observed among the 290 isolates of APEC and AFEC examined (Fig. 1). The genetic patterns 1 and 5 were the most frequent VG combinations identified among the APEC and AFEC isolates, respectively. The frequency of some genetic patterns varied between APECs and AFECs isolates (Table 1).

Many molecular methods detecting various VGs have already been used for identification of APECs. However, the presence of any individual VGs cannot be a perfect molecular marker to distinguish APECs. Therefore, it seems that simultaneous detection of a combination of VGs may be a more efficient approach. In 2005, some genetic patterns were shown to be more frequent among APECs compared to the AFECs, but most of the isolates belonged to those genetic patterns were not APEC (Vandekerchove *et al.* 2005). Schouler *et al.* (2012) characterized four different patterns of VGs among 352 APECs and 108 AFECs isolates, each including more than 90% of pathogenic isolates. Furthermore, 70.2% (247/352) of the APEC isolates contained one of the four genetic patterns detected. Therefore, they could identify 70.2% of the APEC isolates using four different genetic patterns. Although, the frequency of these patterns among AFEC isolates was not reported (Schouler *et al.* 2012). In the present study, more than 96% of the isolates with genetic patterns 1 ($ompT^+$/$hlyF^+$/iss^+/$iutA^+$/$iroN^+$/tsh^+/$cvaC^+$) and 4 ($ompT^+$/$hlyF^+$/iss^+/$iutA^+$/$iroN^+$/tsh^+/cvaC-) were pathogenic (Table 2). Of the 220 APEC isolates tested, 148 isolates had genetic pattern 1 or 4. In contrast, the genetic patterns 1 and 4 were presented only in 5.8% (4/70) of the AFEC isolates. So, 67.3% of the APEC isolates were identified based on the two different genetic patterns with a 1.4% (4/290) error margin. If the genetic pattern 3 was taken into account, 74%

Table 1. Prevalence (%) of the genetic patterns among the *E. coli* isolates

Genetic pattern	APEC isolates	AFEC isolates	Total isolates
1[a]	40.5	2.9	31.1
2	5.7	5.7	5.7
3	6.7	4.3	6.1
4[a]	26.7	2.9	20.7
5[a]	3.8	18.6	7.5
6[a]	0.5	7.1	2.1
7	0.5	0	0.35
8	1.9	0	1.4
9	1.4	0	1.1
10	2.4	0	1.8
11	7.1	10	7.9
12	0.5	0	0.35
13	1	0	0.7
14	1.4	0	1.1
15[a]	0	17.1	4.3
16[a]	0	18.6	4.6
17	0	1.4	0.35
18	0	1.4	0.35
19	0	1.4	0.35
20[a]	0	4.3	1.1
21	0	1.4	0.35
22	0	1.4	0.35
23	0	1.4	0.35

[a]indicates the presence of significant difference between APEC and AFEC isolates.

Table 2. Prevalence (%) of the APECs and AFECs with different genetic patterns

	APEC		AFEC	
Genetic patterns	No. of isolates	% of Pattern	No. of isolates	% of Pattern
1	89	97.8	2	2.2
2	13	76.5	4	23.5
3	15	83.3	3	16.7
4	59	96.7	2	3.3
5	8	38.1	13	61.9
6	1	16.7	5	83.3
7	1	100	0	0
8	4	100	0	0
9	3	100	0	0
10	5	100	0	0
11	16	69.6	7	30.4
12	1	100	0	0
13	2	100	0	0
14	3	100	0	0
15	0	0	12	100
16	0	0	13	100
17	0	0	1	100
18	0	0	1	100
19	0	0	1	100
20	0	0	3	100
21	0	0	1	100
22	0	0	1	100
23	0	0	1	100

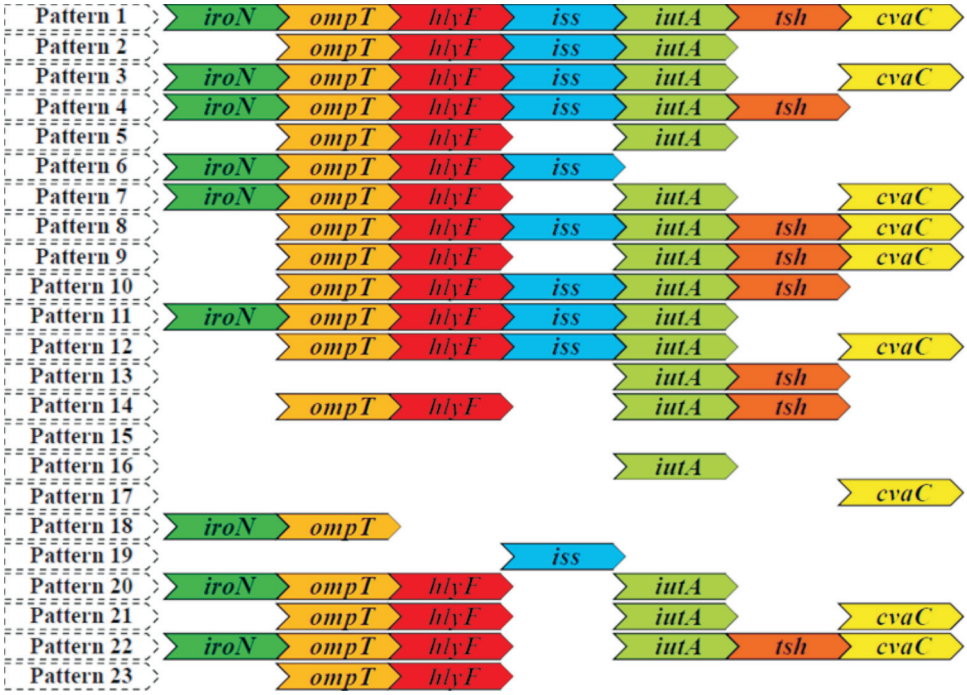

Fig. 1. Gene content of the genetic patterns observed among *E. coli* isolates.

of the APEC isolates could be identified and error margin increased by 4%.

Lethality test: Based on the lethality test, APEC isolates with genetic patterns 1 to 5 and 11, and AFEC isolates with genetic patterns 2 and 4 were pathogenic for chicks. Lethality score was 5 for both APECs and AFECs originated isolates (Table 3). On necropsy, pathological lesions were evident consistent with colisepticemia. The most characteristic lesions were liver, spleen, lungs and muscles congestion and pericarditis. In some cases, cardiac ecchymosis, omphalitis and hemorrhage in bursa of Fabricius were also seen (Fig. 2). *E. coli* was reisolated from the lesions of the chicks died during lethality test. There was no significant difference in lethality scores between APEC and AFEC isolates.

In agreement with previous studies (Ewers *et al.* 2009, Olsen *et al.* 2016, Schouler *et al.* 2012), the result of the present study suggest that some AFECs appear to harbour the same ColV plasmid-associated VGs as APEC and have

Table 3. Pathogenicity of the different genetic pattern observed among *E. coli* isolates.

Group	Patterns	Lethality	LS[a]
APEC	1	+	5
	2	+	5
	3	+	5
	4	+	5
	5	+	5
	11	+	5
AFEC	1	-	0
	2	+	5
	3	-	0
	4	+	5
	5	-	0
	11	-	0
	15	-	0
	16	-	0

+, of five chicks inoculated subcutaneously, one or more died; -, of five chicks inoculated subcutaneously, no chicken died. [a]lethality score was calculated according to the number of birds died within 4 days.

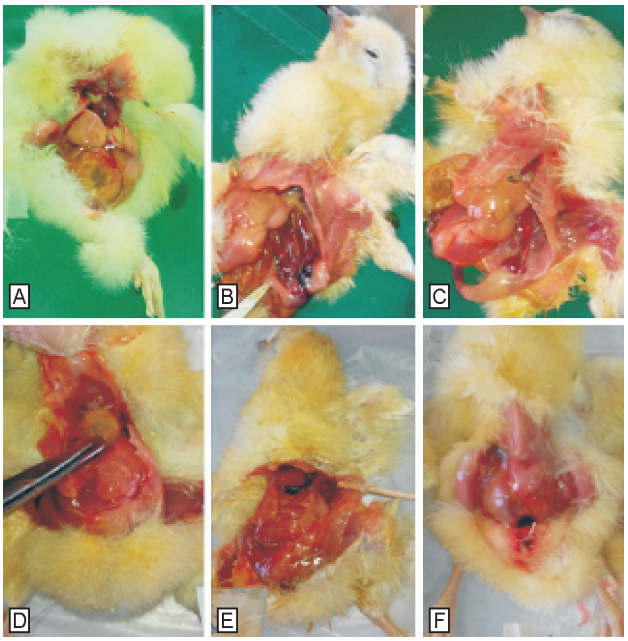

Fig. 2. Necropsy lesions found in the pathogenicity test. **A**. Cardiac ecchymosis, **B**. Congestion of kidneys, **C**. Hemorrhage in bursa of Fabricius, **D**. Pericarditis, **E**. Congestion of lungs, **F**. Omphalitis.

the potential to cause disease. A previous study revealed that intratracheal inoculation of *E. coli* isolates from intestine and environment of chickens caused airsacculitis, pneumonia and pericarditis in five-week-old chickens (Ewers *et al.* 2009). Also, considerable portion (40.8%) of *E. coli* isolated from feces of the healthy chicken were pathogenic for day-old chicks (Schouler *et al.* 2012). Moreover, Olsen *et al.* (2016) examined some APEC and AFEC strains for their potential in induction of salpingitis. Their results revealed that different strains of *E. coli* can cause clinical salpingitis regardless of their origin (Olsen *et al.* 2016). The pathogenicity of some AFEC bacteria is not surprising; since it is well known that intestine of birds can serve as a major source of pathogenic strains of *E. coli* (de Oliveira *et al.* 2015, Ewers *et al.* 2009). These strains contain many virulence factors that enable their extraintestinal adaptation and pathogenicity. In other words, the new extraintestinal environments can provide requirements for expression of VGs or their regulators which are essential for pathogenicity (Kaper *et al.* 2004).

The pathogenicity of isolates with different genetic patterns observed in present study was not correlated with the number of VGs in each pattern, as there were lethal isolates with genetic patterns possessing only 3 VGs and also non-lethal isolates with patterns containing all seven VGs examined. The finding could be related to those observed by de Oliveira *et al.* (2015) who reported that some *E. coli* isolated from colibacillosis cases with no or only one tested VG had higher pathogenicity than those with higher number of VG (de Oliveira *et al.* 2015). This may support the idea that gene count cannot be an ideal approach for discrimination of APECs from AFECs (Mohsenifard *et al.* 2016). Moreover, it may propose that a variety of genes other than those examined in current study may be engaged in the pathogenicity of the isolates and in addition to the presence of the VGs, the necessities for their expression should also be provided. Although, the ColV-associated VGs seem to be required for pathogenicity of avian *E. coli*, colibacillosis is a multi-factorial disease and its occurrence depend on the plasmid and chromosomal virulence factors of pathogen, and immune competence and genetics of the host (Olsen *et al.* 2016).

In conclusion, the difference in frequency of the genetic patterns between APEC and AFEC isolates may indicate that ColV plasmid or some of its VGs may provide helpful characteristics to describe APECs. In addition, the variety of observed genetic patterns with different VG count may suggest that avian *E. coli* isolates are diverse population with different genetic background that employs various tools to deal with the host. However, the link between pathogenicity and genotype of avian *E. coli* needs more future works involving a larger number of VGs and more diverse routes of inoculation to be confirmed.

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