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Frz OPERON AND *R4* GENE VIRULENCE PRESENT IN ROMANIAN APEC (AVIAN PATHOGENIC *ESCHERICHIA COLI*) ISOLATES

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Abstract

Escherichia coli (E. coli) infection has a major effect on poultry production, the numerous clinical forms making it the most frequently reported diseases in commercial flocks of chickens but also in turkeys and pigeons. Some Avian pathogenic *E. coli* (APEC) strains, may share virulence factors with human extraintestinal pathogenic. The data presented in this study complete the characterization of the pathotypes of 13 APEC strains, previously isolated and characterized (Gurau M.R. et al., 2018, 2020), with the investigation over the presence of Frz operon and R4 virulence gene, by PCR method. Amplification was performed in two different runs, one for each gene, the temperature protocols being different. The Frz operon was identified in 11 isolates (84.61% or 11/13) and R4 was identified in 4 isolates (30.76% or 4/13). These results, correlated with those previously reported, highlighted that Frz operon and R4 genes are independently expressing their virulence.

Key words*: APEC, ExPEC, PCR, virulence genes.*

INTRODUCTION

In birds, as in humans, *Escherichia coli* is a member of the normal intestinal micro flora but some strains, known as APEC (Avian Pathogenic *Escherichia coli*), occur in bodily sites outside the intestinal lumen and under favorable circumstances, invade various internal organs, inducing bacteremia, respiratory tract infections, septicemia and causing fatal systemic colibacillosis (Someya et al., 2007; Kabir, 2010). Among the intestinal coliforms of chickens, 10-15% belong to pathogenic serotypes (Tabatabaei and Nasirian, 2007).

APEC (Avian pathogenic *Escherichia coli*) belong to a large and diverse group of *E. coli* strains, known as extra-intestinal pathogenic *E. coli* (ExPEC). ExPEC causes a number of systemic diseases that affect nervous system, respiratory system and urinary tract in humans, animals and birds. APEC (ExPEC strains of avian origin) has a phylogenetic relationship with human strains isolated from extra-intestinal tissues, normally sterile, and they share some of the virulence factors of the human strains. This suggests that APEC strains could pose a zoonotic risk (Moulin-Schouleur et al., 2007*)*. The scientific community is also concerned on APEC strains that are becoming an emerging pathogen in relation to food safety. One of the incriminated source of the ExPEC growing incidence in humans are the poultry products (Ewers et al., 2009).

Infections with APEC strains causes in poultry, an acute disease most often systemic, which generates significant losses in world poultry farming (Ewers et al., 2003).

APEC strains are responsible for a considerable number of clinical manifestations at different ages. Avian colibacillosis is widespread in chickens, in all age categories, with the highest prevalence in laying hens (36.73%) (Kabir, 2010). In this context, a better knowledge of phenotype and genotype characteristics of APEC strains circulating in Romanian poultry flocks was requested.

To survive and compete to the variation of the environment, bacteria have developed a complex molecular mechanisms by which they are adapting to environmental stimulus (Roquet et al., 2009). Roquet et al. 2009, identified the close association (75%) between the presence of *Frz* operon with increased virulence of avian *E. coli*.

The *Frz* operon is regulating the genes expression for the protection of the bacterium under dysgonic conditions, as the oxygen restriction or the nutritional deprivation (stationary growth phase), as well as, in promoting the bacterium development in the presence of the bird serum or in the intestinal tract, basically, by regulating the expression of adaptation and virulence genes (Patron et al., 2015).

There were identified five types of lipopolysaccharide core types in *E. coli*, known to play a crucial role in the pathogenesis of bacteraemia, sepsis and shock, marked with R1, R2, R3, R4 and K12 (Amor et al., 2000; Dissanayake et al., 2008).

In clinical, human and avian isolates, the lipopolysaccharide center R1 predominates, while R4 is found in the smallest proportion (Amor et al., 2000; Li et al., 2005). In broilers, Ozaki et al. 2017 identified the gene encoding the center lipopolysaccharide R4 in 45% of APEC strains, being the most common.

The data presented in this study complete the characterization of the 13 APEC strains, previously isolated and characterized (Gurau M.R. et al., 2018, 2020), with the investigation over the presence of *Frz operon* and *R4* virulence factors.

MATERIALS AND METHODS

There were investigated 13 *E. coli* isolates for the presence of *Frz operon* and *R4,* virulence genome determinants. The studied strains belong from farms located in Brasov, Calarasi, Dambovita, Giurgiu, Vrancea and Iasi counties. The age of poultry flocks under study ranged from 1 day to 87 weeks, breeding categories being broiler or laying hens.

The extraction of the DNA was made with the QIAamp cador Pathogen Mini Kit (Qiagen, Dusseldorf, Germany), according with the kit insert (Table 1).

The PCR amplification temperature protocol for the *Frz operon* gene of *E. coli* was: 94°C 5 minutes, 35 cycles with 94°C for 30 seconds, 63°C for 45 seconds and 72°C for 1.5 minutes. The final elongation: 72°C for 7 minutes.

The mix for the reaction of *Frz operon* gene was made in a volume of 50 µl total from which 2 µl DNA template, 2µl dNTPs 10 mM, RNase free water 35.5 µL, 2 µL of Taq platinum polymerase (5U/µL) (Invitrogen®, Itapevi, São Paulo, Brazil), 5µL of PCR buffer (50 mM KCl, 10mM Tris–HCl pH 8.0), $MgCl_2$ (1.5 mM) 1.5 µL and 1 µL of primers forward and reverse for *Frz operon* gene (10 pmol) (Table 2).

Table 1. Nucleic acid extraction protocol (QIAamp cador Pathogen Mini Kit)

REAGENT	**µl/sample**	**No. samples**	**Total**
Proteinase K	20 µl		
Sample	200 µl		
Buffer VXL	100 µl		
Pipetting / vortex mixing			
Incubate for 15 minutes at room temperature			
Spin centrifuge for liquid collection			
Buffer ACB	350 µl		
Pipetting / vortex mixing			
Spin centrifuge for liquid collection			
Transfer of samples to purification colonitis			
Centrifuge at 8000 – 10.000 rpm for 1 minute. Replacement manifold tube.			
Buffer AW 1	600 µl		
Centrifuge at 8000 – 10.000 rpm for 1 minute.			
Eluted remove			
Buffer AW2	600 µl		
Centrifuge at 8000 – 10.000 rpm for 1 minute. Replacement manifold tube.			
Eluted remove			
Centrifuge at maximum speed for 2 minutes. Introduction of colonitis into the collection tube.			
Buffer AVE	50 µl		
Incubation for 1 minute at room temperature.			
Centrifuge at maximum speed for 1 minute.			
Storage the elute at 1-2°C until the amplification step.			

Table 2. The reagents and the quantities of the reaction mix for the *Frz operon* gene

Reaction mix	
Reagents	**µl /sample**
RNase free water	35.5 µL
PCR buffer (50 mM KCl, 10mM Tris–HCl pH 8.0)	5 µL
$MgCl_2$ (1.5 mM)	1.5 µL
dNTP solution (10 mM)	2 µL
P Frz F (10 pmol)	1 µL
P Frz R (10 pmol)	1 µL
Taq platinum polymerase (5 U/µL) (Invitrogen®, Itapevi, São Paulo, Brazil)	2 µL
DNA template	2 µL
Total	50 µL

The sequence primers for *Frz operon* gene, are described in the Table 3. The amplicons were visualized by electrophoresis in 1.5% agarose , at 90V, 1,5A, for 35 min.

Table 3. Sequence of primers-forward and reverse –used for amplification of *Frz operon* gene and expected size

Primers name	Sequence	Size (bp)
P Frz F	GAGTCCTGGCTTGCGCCGTT	843
P Frz R	CCGCTCCATCGCAGCCTGAA	

(Van der Westhuizen and Braag, 2012)

The PCR amplification temperature protocol for the *R4* gene of *E. coli* was: 94°C 4 minutes, 35 cycles with 94°C for 20 seconds, 50°C for 30 seconds and 72°C for 3 minute. The final elongation: 72°C for 7 minutes.

The mix for the reaction of *R4 gene* was made in a volume of 27 µl from which 2 µl DNA template, 1 µl dNTPs 10 mM, RNase free water 19.1 µL, 0.4 µL of Taq platinum polymerase (5U/µL) (Invitrogen®, Itapevi, São Paulo, Brazil), 2.5 µL of PCR buffer (50 mM KCl, 10mM Tris–HCl pH 8.0), $MgCl_2$ (1.5 mM) 1.5 µL and 0.25 µL of primers forward and reverse for *R4* gene (10 pmol) (Table 4).

Table 4. The reagents and the quantities of the reaction mix for the *R4* gene

Reaction mix	
Reagents	**µl /sample**
RNase free water	19.1 µL
PCR buffer (50 mM KCl, 10mM Tris–HCl pH 8.0)	2.5 µL
$MgCl_2$ (1.5 mM)	1.5 µL
dNTP solution (10 mM)	1 µL
P_A (10 pmol)	0.25 µL
P_B (10 pmol)	0.25 µL
Taq platinum polymerase (5 U/µL) (Invitrogen®, Itapevi, São Paulo, Brazil)	0.4 µL
DNA template	2 µL
Total	27 µL

The sequence primers for *R4* gene, are described in the Table 5. The amplicons were visualized by electrophoresis in 1.5% agarose, at 90V, 1.5A, for 35 min.

Table 5. Sequence of primers-forward and reverse –used for amplification of *R4* gene and expected size

Primers name	Sequence	Size (bp)
P_A	TGCCATACTTTATTCATCA	699
P_B	TGGAATGATGTGGCGTTAT	

(Amor et al., 2000)

RESULTS AND DISCUSSIONS

The *Frz operon* is rarely found in non - pathogenic strains of avian origin (5%), and his presence in the ExPEC strain increases with the increasing of the virulence in 1-day-old chicks (Roquet et al., 2009).

The *Frz operon* was detected in 8.9% of *E. coli* strains isolated from birds with colibacillosis in Zimbabwe (Mbanga and Nyararai, 2015). The frz_{orf4} is located chromosomal and belongs to the *Frz operon*. The prevalence of this operon, upon the studis, range from 53.4% of 352 APEC strains to 16.7% of 108 AFEC strains in a screening conducted by Schouler et al., 2012. In the present study, the presence of the F*rz operon* was detected in 10 (76.92%) of the 13 pathogenic avian *E. coli* strains (Table 6, Figure 1).

Table 6. The PCR-results of the tested isolates for the presence of *Frz operon* and *R4* virulence genes

Isolates	*Frz operon*	*R4*	County	Age of bird
1	**X**	**X**	Vrancea	broiler 7 day
2	**X**	**-**	Dambovita	23 weeks, layer
3	**X**	**-**	Iasi	25 weeks, layer
4	**X**	**-**	Brasov	87 weeks, layer
5	**X**	**X**	Calarasi	10 day, broiler
6	**X**	**-**	Dambovita	24 weeks, layer
7	**-**	**-**	Dambovita	1 day, broiler
8	**-**	**-**	Calarasi	7 days, broiler
9	**-**	**-**	Brasov	65 weeks, layer
10	**X**	**X**	Vrancea	11 days, broiler
11	**X**	**-**	Iasi	11 days, broiler
12	**X**	**-**	Giurgiu	7 day, broiler
13	**X**	**X**	Iasi	11 day, broiler

X = mark the strains containing the gene.

Figure 1. PCR results, *Frz gene* (843 bp). Lines 1, 2- negative control, line 3 - positive control, lines 4-7 *E. coli* strains; line 8: 100 bp DNA ladder (*Bio-Rad*)

Thus, we can note that in our study the presence of the *Frz operon* is higher (76.92%) than in the screening conducted by Schouler et al. (2012) (53.4%, 16.7%), but it should also be taken into account that in this study were tested a much smaller number of strains of *E. coli.*

In broilers, Ozaki et al. (2017) identified the gene encoding *R4* core chemoype in 45% of pathogenic *E. coli* strains, this one being the most frequent core type. In a more detailed study, Dissanayake et al. (2008) identified the core lipopolysaccharide *R4* in 13% of clinical isolates and in 4% of commensal isolates of *E. coli.* But he also noticed that *E. coli* strains with R4 core type register the lesser frequency into the group of the commensal strains, the R4 is randomly associated with other virulence genes and belong to the groups of birds` pathogens, being significantly associated with APEC strains..

In the present study, the R4 core type was detected in 4 (30.76%) from the 13 pathogenic APEC strains, sugesting that the prevalence of this determinant in the Romanian strains is higher than in Dussanayake's study (2008) (Table 6, Figure 2).

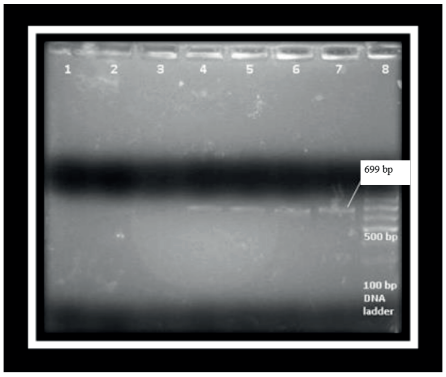

Figure 2. PCR results, *R4* gene (699 bp) Lines 1, 2 - negative control, lines 3-6 *E. coli* strains, line 7 positive control, line 8 100 bp DNA ladder (*Bio-Rad*)

The 4 APECs that house R4 are strains isolated from broilers: strains 1 from county Vrancea, broiler 7 day, strain 5 from Calarasi county, 10 day broiler, strain 10 also from Vrancea county, 11 day broiler and strains no. 13 from Iasi, 11 day broiler (Table 6). These are being in accordance with Ozaki`s study in which, R4 coretype was also identified in broilers`strains, in 45% of APEC strains (Ozaki et al., 2017).

Strains no. 7, 8 and 9 did not present any of the two studied genomic determinants, neither the *Frz operon,* nor R4. But these strains, 7, 8 and 9, according to our previously presented studies, posses other pathogenicity determinants of *E. coli*, namely the IucD, IucC and IronN genes.

CONCLUSIONS

The *Frz opreron* has a higher prevalence in our study (76.92%) than those reported by others.

We detected the R4 core type in 4 (30.76%) from 13 the APEC strains, isolated from broilers, this meaning a higher prevalence than in study of Dussanayake et al. (2008), but a lower prevalence than reported by Ozaki et al. (2017), in which R4 type was found in broilers too, but in 45% of the APECs strains.

These results came to reconfirm other results from the literature, in which, the different *E. coli* strains posses different pathogenicity genes but not all the virulence genes are present in all *E. coli* strains. According to this study and our previous ones it could be assumed that APEC pathogenicity genes are expressing their virulence in different association, without a pre-seted pattern.

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