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THE PRESENCE OF *IRON* AND *IUCC* VIRULENCE-ASSOCIATED GENES IN ROMANIAN APEC ISOLATES

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Abstract

Colibacillosis in poultry is relating with the colonisation with so called Avian Pathogen E. coli (APEC) strains. It is already known that usually in the APEC isolates are present at least 13 virulence-associated genes. We selected 12 non-repetitive E. coli isolates from different Romanian poultry outbreaks. Isolates have been evaluated for the presence of the virulence-associated genes, iroN and iucC. The DNA extraction was made using QIAamp cador Pathogen Mini Kit (Qiagen). The amplification protocol was: a cycle of denaturation at 94°C for 5 min followed by 35 cycles of 94°C for 30 s, 63°C for 45 s and 72°C for 105 s, and a cycle of 72°C for 7 min. Both virulence-associated genes were identified in 83.33% (11/12) isolates. In two APEC isolates, only one gene was identified, iroN or iucC, respectively. According to these preliminary results it could be assumed that iroN and iucC genes are independently expressing their virulence.

Keywords: *Colibacillosis, Avian Pathogen E. coli, PCR, molecular biology diagnostic.*

INTRODUCTION

In poultry farms, one of the most frequent pathogens is *E. coli.* The classification of pathogenic *E. coli* by the manifested virulence is: entero-toxigenic, entero-pathogenic and entero-haemorrhagic. APECs are the virulent strains of E. coli in poultry and other avian species.

Colibacillosis produced by APEC in avian remain an issue of main concern due to the economic losses it produces in poultry, by the decrease of the egg production and the increased mortality especially in young broiler in which an important role it has also the growth technology (Barnes et al., 2003, Tudorache et al., 2016, Dou X et al., 2016). The clinical sings and lesions of avian colibacillosis depend on the age bird. In young's there is acute septicaemia with enlarged spleen and liver. In sub-acute form of the disease there are airsacculitis and pericarditis. Other lesions are salpingitis, pneumonia, arthritis.

The virulence of the strains of avian *E. Coli* depends on the virulence genes´ from the nucleus and plasmids (Dozois et al., 2003, Akram et al., 2017). It is already known that the most frequently each APEC isolates own at least 13 virulence-associated genes (Johnson et al., 2008; Ewers et al., 2007). The genetic relatedness between APEC strains has been investigated through molecular studies: it has been found that virulence determinants can be individual or polygenic, depending as frequency in the isolates (Vandekerchove et al., 2005).

The association of different genes with pathogenicity is not fully elucidated: there are many involved genes that make different associations in APECs, rarely repetitive (Delicato et al., 2003, Chakraborty et al., 2015). The literature studies showed that the virulence genes are very rarely present all in the same isolate. This demonstrated that APEC is a heterogeneous group of strains. Using PCR methods for the analysis of pathogenicity genes of APEC, it is possible to detect genes like: *iss, tsh, iucC, cvi, iutA, hlyF, iss, iroN, ompT* (Skyberg JA et al., 2003; Johnson TJ et al., 2008).

In our study it has been looked for two different APEC virulence genes, using PCR method, *iroN* and *iucC*, in order to characterize some

Romanian strains of avian *E. coli*. The genes have been chosen, according with literature data, being frequently identified in APECs from other countries (Li et al., 2008; Rouquet et al., 2009). *IroN* gene it is a salmochelinsiderophore receptor that favours chelation of iron in the host, and *iucC* gene is an *iuc* operon that contributes to iron purchase (Baumler et al., 1998; Runyen Janecky et al., 2003).

MATERIALS AND METHODS

It was investigated a number of 12 strains of *E. coli* for the presence of *iroN* and *iucC* virulence genes. The studied strains originated from three different regions of Romania, namely Transylvania, Muntenia and Moldova (Figure 1). The samples were collected from different counties of the regions before named, as follows: Brasov, respectively Dambovita, Calarasi, and Giurgiu and respectively Vrancea and Iasi. These strains are from avian colibacillosis outbreaks diagnosed in commercial poultry at different ages: 1 day, 7 days, 10 days, 23 weeks, 24 weeks, 25 weeks, 65 weeks and 87 weeks (table 2). The samples originated from different holding category, broiler or layers.

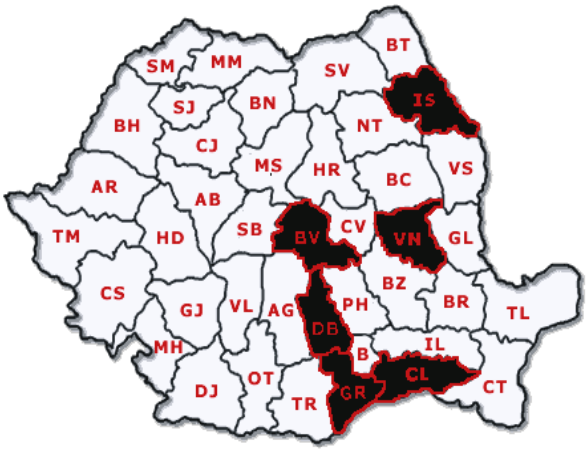

Figure 1. The county distribution of the tested strains; strains were collected from counties marked with black: Vrancea, Dambovita, Iasi, Brasov, Calarasi, and Giurgiu

The *E. coli* strains were cultivated on brain heart infusion (BHI) medium at 37 °C for 24 hours. Then the cultured broth was spreaded into MacConkey agar plates and incubated for 24 hours at 37 °C in order to obtain isolated colonies. The colonies were then stored at -20°C.

DNA extraction was performed using the QIAamp cador Pathogen Mini Kit (Qiagen, Dusseldorf, Germany) in accordance with the manufacturer's instructions. The QIAamp cador Pathogen Mini Kit is designed for the extraction of nucleic acids from different samples such as the bacterial DNA. The samples were lysed under denaturing conditions at room temperature (15–25°C) using the proteinase K and the VXL Buffer (Awwad, E. et al., 2015). Both, the proteinase K and VXL Buffer, did inactivate the nucleases. Once the buffer ACB added, it will be strengthened the binding conditions for the purification of DNA. The lysate is then transferred to column. By centrifugation, nucleic acids are adsorbed by the silica membranes and the contaminants are passing away. Then the silica membranes are subjected to two wash steps in order to remove the remaining contaminants and enzyme inhibitors. By adding the AVE buffer the nucleic acids are eluted. Thus, the eluted nucleic acids can be kept at refrigerator temperature for a limited time or at -80 degrees for a long time (https://www.qiagen.com/us/shop/sample-technologies/dna/genomic-dna/qiaamp-cador-pathogen-mini-kit/#productdetails).

The PCR amplification protocol was carried up as follows: 94°C 5 minutes, 35 cycles with 94°C for 30 seconds, 63°C for 45 seconds and 72°C for 1 minute and 45 seconds. A final elongation step was carried at 72°C for 10 minutes.

The termocycler used was the machine of Applied Biosystems ABI 2720 Thermal Cycler. The mix for the reaction was made in a final volume of 50 µl from: 2 µl DNA template, 2 µl dNTPs 10 mM, RNase free water 35.5 µL, 2 µL of Taq platinum polymerase (5U/µL) (Invitrogen®, Itapevi, São Paulo, Brazil), 5 µL 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 each of the two tested genes (10 pmol), (Tabel 1).

The primers sequence was the one of W.A. van der Westhuizen, 2012. For each virulence gene tested *iroN* and *iucC*, an amplification reaction mix was performed separately. The amplicons were visualized by electrophoresis in 1.5% agarose, subjected to 90V, 1,5A, for 35 min.

Table 1. Sequence of primers - forward and revers - used for amplification of *iroN* and *iucC* gene fragments and expected size

Primer name	Sequence (5´to 3´)	Product size (bp)
IroN F	AAGTCAAAGCAGGGGTTGCCCG	667
IroN.R	GATCGCCGACATTAAGACGCAG	
IucC F	CGCCGTGGCTGGGGTAAG	541
IucC R	CAGCCGGTTCACCAAGTATCACTG	

(W. A. van der Westhuizen and R. R. Bragg, 2012)

Table 2. The *E. coli* strains origin, age and the holding category

Strain	County	Age	Holding category
1	Vrancea	7 days	broiler
2	Dambovita	23 weeks	layer
3	Iasi	25 weeks	layer
4	Brasov	87 weeks	broiler
5	Calarasi	10 days	broiler
6	Dambovita	24 weeks	layer
7	Dambovita	1 day	broiler
8	Calarasi	7 days	broiler
9	Brasov	65 weeks	layer
10	Vrancea	11 days	broiler
11	Iasi	11 days	broiler
12	Giurgiu	7 days	broiler

RESULTS AND DISCUSSION

The *iroN* gene was found in 11 strains from the 12 strains submitted in study, for the presence this virulence gene; the amplification performed with the primers presented before, produced amplicons with the predicted size, identified by electrophoresis, as bands of 667 bp.

The strain isolated from broilers aged 11 days, breed in Vrancea County (strain no. 10) was negative for the presence of *ironN* virulence gene: his electrophoretic pattern did not present any band (Figure 2 and Table 3).

Looking for the *iucC* gene, the results were as follows: strain 1 isolated from 7 days old broiler, breed in Vrancea County, and was negative; no amplicon resulted. Meanwhile, the all remaining 11 strains no. 2, 3, 4, 5, 6, 7, 8, 9, 10, 11 and 12 were positive for the presence of *iucC* gene.

The electrophoretic pattern of amplification product, in each of these strains, was a product at the expected size of 541 bp (Figure 2, Figure 3 and Table 3).

Each of virulence-associated genes, *iroN* and *iucC* were identified in 83.33% (10/12) isolates (Figure 5). In two APEC isolates, it was identified only one gene, either *iroN* or *iucC*.

In the strain 1 was identified only *iroN* gene, and in the strain 10 was identified only *iucC* gene.

These two strains - isolated from the same county, from the same breeding category, aged under two weeks – did not presented both virulence genes, despite being isolated from outbreaks: this suggests that for younger poultry, even strains missing some virulence genes could get disease and losses.

IroN or *IucC* genes were not identified in 8.34 % isolates (1/12) (Figure 5).

These results could lead to a superficial conclusion if we correlate pathogenicity of an APEC with the number of virulence genes that it possesses: the result of this characterization supports the multi-genic determinism of pathogenicity of APEC strains, relayed not only by the presence of genes but also by their association.

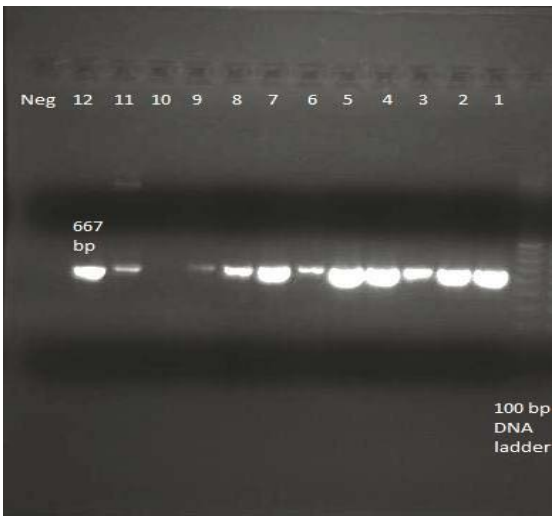

Figure 2**.** The PCR testing result of the *iroN* gene; 100 bp DNA ladder, samples from 1 to 12 and a negative control represented by free RNase water

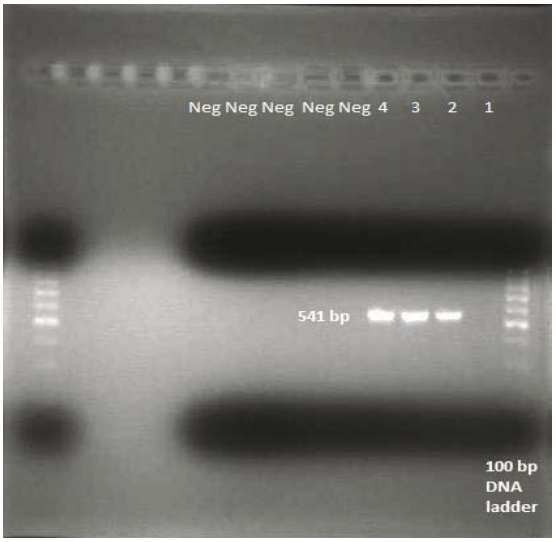

Figure 3. The PCR testing result of the *iucC* gene; 100 bp DNA ladder, samples from 1 to 4 and 5 negative controls represented by free RNase water. Sample caption

The results of our research are consistent with those in the literature, in that, different *E. coli* strains possess different pathogenic genes. Not all virulence genes are expressed on all *E. coli* strains (W. A. van der Westhuizen and R. R. Bragg, 2012).

Comparing the results of our study with literature data, we observe that the percentage of strains containing both pathogenicity genes *IroN* and *iucC*, 83.33%, was higher in our study than in the other study, 28.57%(W. A. van der Westhuizen and R. R. Bragg, 2012), representing a number of 10 strains from 35 strains tested (10/35). The strains that had only the *iucC* gene in other studies represented 28.57% (10/35) compared to the current research respectively 91.66 % (11/12). The strains that had only the *iroN* gene represented 54.28% in other studies compared to the present studies 91.66 % (11/12).

At the same time, the number of strains that did not contain any virulence gene *IroN* and/or *iucC* represented 17.14 % (6/35) in other studies, compared to the current study that was 0% (W. A. van der Westhuizen and R. R. Bragg, 2012). This means that all 12 tested strains were pathogenic.

Also in studies like Lixiang Zhao, 2009, APEC isolates originating from Jiangsu province and Anhui province in eastern China, with *iroN* and *iucC* occurred in ≥ 71% of the isolates. This means that the number of APEC strains with high virulence gene containing *iroN* and *iucC* gene are different depending on the geographical area where they occurred.

The strain number 10, collected from Vrancea County has only the *iucC* gene and the strain number 1, collected also from Vrancea County but from other holding, has only the *iroN* gene. Analysing the results of APEC strains, we must note that not all APEC have the same virulence. APEC with high pathogenicity goes to primary infections. Meanwhile strains with lower pathogenicity causes disease only when poultry are subject to stress such as the coexistence of other diseases or stressful environmental factors or age related (Dho-Moulin and Fairbrother, 1999).

Table 3. The results of the positive and negative strains for the presence of *E. coli iroN* and *iucC* gene virulence

STRAINS	GENE *iroN*	GENE *iucC*
1	X	-
2	X	X
3	X	X
4	X	X
5	X	X
6	X	X
7	X	X
8	X	X
9	X	X
10	-	X
11	X	X
12	X	X

X= mark the strains that containing the gene.

Figure 4. The PCR testing result of the *iucC* gene; 100 bp DNA ladder, samples from 5 to 12 and a negative control represented by free RNase water. The entire samples are positive for the presence of *iucC*.

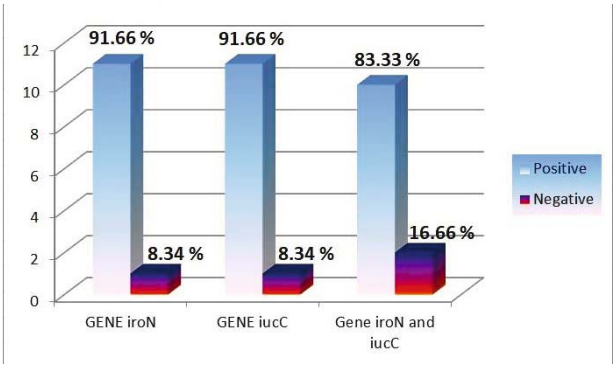

Figure 5. Percentage distribution of *IroN* and *iucC* genes on the 12 strains tested; both virulence - genes IroN and iucC was in 83.33 % of the tested strains meaning 10 strains from 12

CONCLUSIONS

Virulence-associated genes *iroN* or *iucC* were identified in 83.33% (11/12) isolates.

In two APEC isolates, only one gene was identified, *iroN* or *iucC*, 8.34 % from the total isolates (1/12), which means that these strains have less pathogenicity than the other strains that contain both genes.

These results come to confirm other results from the literature, in which, different *E. coli* strains possess different pathogenic genes and not all virulence genes are expressed in all E. coli strains.

APEC with high pathogenicity goes to primary infections and strains with lower pathogenicity causes disease only when poultry are subject to stress such as the coexistence of other diseases or stressful environmental factors.

According to these preliminary results it could be assumed that *iroN* and *iucC* genes are independently expressing their virulence.

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