Scientific Works. Series C. Veterinary Medicine. Vol. LXVI (2), 2020
ISSN 2065-1295; ISSN 2343-9394 (CD-ROM); ISSN 2067-3663 (Online); ISSN-L 2065-1295

IUC D AND *PAP C* VIRULENCE-ASSOCIATED GENE PRESENT IN ROMANIAN AVIAN PATHOGEN *ESCHERICHIA COLI* ISOLATES

Maria Rodica GURĂU, Hasan Majid HAMEED, Dragoș COBZARIU, Doina DANEȘ

University of Agronomic Sciences and Veterinary Medicine of Bucharest, 59 Marasti Blvd, District 1, Bucharest, Romania

Corresponding author email: otelea_maria@yahoo.com

Abstract

Avian colibacillosis produced by Escherichia coli, a common infection facing poultry flocks, affects poultry industry by the broad spectrum of induced entities: systemic acute infection - colisepticemia, localised infection as omphalitis, cellulitis, enteritis, salpingitis/peritonitis, cronic disease - choligranulomatosis, the chronic respiratory disease and many more. The E. coli infection is causing large losses for the poultry industry, impairing the performances: decreased egg production, with drown of the carcasses at slaughterhouse and the increased mortality rate, are recorded in the affected flocks. E. coli strains inducing poultry diseases are a specific group of strains, called Avian Pathogen E. coli (APEC) strains: these strains randomly share more virulence-associated genes. 13 E. coli isolates from different Romanian poultry outbreaks/flocks have been investigated for the presence of Iuc D and Pap C genes. The Pap C gene is responsible for adhesin assembly and its frequency is associated with the cystitis strain like Santo E. proves in his article from 2006. Gene Iuc D of the plasmid encodes a membrane-bound enzyme synthesizing N6-hydroxylysine, the first product of the aerobactin biosynthesis pathway. The DNA extraction was made using QIAamp cador Pathogen Mini Kit (Qiagen). The amplification protocol was: a cycle of denaturation at 94°C for 30 s followed by 25 cycles of 94°C for 30 s, 58°C for 30 s and 68°C for 3 min, and a cycle of 72°C for 10 min. The Iuc D gene was present in 100% (13/13) isolates. Pap C gene was present in 23.07% (3/13) isolates. *Considering these preliminary results, it can say that Iuc D and Pap C genes are independently expressing their virulence and further research should be performed to establish the pathogenicity of each of the two genes together with other pathogenic genes.*

Key words*: PCR, gene. Iuc D, Pap C.*

INTRODUCTION

Escherichia coli is frequently found in poultry flocks and the strains of *E. coli* are classified in entero-toxigenic, entero-pathogenic and entero-haemorrhagic.

The *E. coli* infection - Avian Pathogen *Escherichia coli* (APEC) strains - is causing large losses for the poultry industry, impairing the performances: decreased egg production, withdrawn of the carcasses at slaughterhouse and the increased mortality rate, are recorded in the affected flocks (Barnes et al., 2003; Dou et al., 2016). The clinical signs in young are: septicaemia, enlarged spleen and liver. Airsacculitis, pericarditis are present in sub-acute form of the disease. It is already known that the virulence factors of the APEC strains are coded by genes located into the nucleus and plasmids of the bacterial cell (Akram et al., 2017; Dozois et al., 2003).

The APEC strains harbor more virulence-associated. The pathogenicity of an APEC strain relates with the multitude of virulence factors it encodes (Vandekerchove et al., 2005). There are different associations of virulence genes, rarely repetitive in APECs strains (Delicato et al., 2003; Chakraborty et al., 2015). APECs are a heterogeneous group of strains. The virulence factors that an APEC strain posses, could be assessed by PCR and thus can be established its role into the outbreak.

In our study 13 *E. coli* isolates from different Romanian poultry farms/outbreaks have been evaluated for the presence of *Iuc D* and *Pap C* genes. The genes were frequently identified in APEC strains from other countries (Rouquet et al., 2009; Li et al., 2008).

MATERIALS AND METHODS

13 *E. coli* isolates were investigated for the presence of *Iuc* D and *Pap* C genes.

The strains came from the Romanian counties Brasov, Calarasi, Dambovita, Giurgiu, Vrancea

and Iasi, belonging to the three historical regions, Transylvania, Muntenia and Moldova. The age of the poultry flocks where these strains has been isolated ranged (1 day, 10 days, 7 days, 23 weeks, 24 weeks, 25 weeks, 65 weeks and 87 weeks), the flocks being broiler or layers. The DNA extraction was performed with the QIAamp cador Pathogen Mini Kit (Qiagen, Dusseldorf, Germany). The lysis of the samples was carried at the room temperature with proteinase K and the VXL buffer to inactivate the nucleases. The purification of the DNA was done with the ACB buffer that strengthened the binding conditions, and then the solution was transferred to the column in order to be purified. The elution of nucleic acids was made by adding the AVE buffer into the column and by centrifugation it was eluted (Qiagen).

The PCR amplification protocol were: 94°C 3 minutes, 25 cycles with 94°C for 30 seconds, 58°C for 30 seconds and 68°C for 3 minutes. The final elongation: 72°C for 10 minutes.

The mix for the reaction was made in a volume of 25 µl with 2 µl DNA template, 1µl dNTPs 10 mM, RNase free water 18.7 µl, 0.4 µl of Taq platinum polymerase (5 U/µl) (Invitrogen®, Itapevi, São Paulo, Brazil), 2.5 µl of PCR buffer (50 mM KCl, 10 mM Tris-HCl pH 8.0), $MgCl_2$ (1.5 mM) 2 µl and 0.1 µl of primers forward and reverse for each of the two tested genes (10 pmol) (Table 2). It was carried one reaction for the two genes because the annealing temperature of primers is the same for the *Iuc D* and *Pap C* genes.

The primers anneling temperature was before optimized as the two tested genes to have the same temperature.

The sequence primers for *Iuc D* and *Pap C* genes, are described in the Table 1. The amplicons were visualized by electrophoresis in 1.5% agarose, at 90 V, 1.5 A, for 35 min.

Table 1. Sequence of primers-forward and reverse – used for amplification of *Iuc D* and *Pap C* genes fragments and expected size

Primers name	Sequence	Size (bp)
iucD F	ACAAAAAGTTCTATCGCTTCC	714
iucD R	CCTGATCCAGATGATGCTC	
Pap C F	TGATATCACGCAGTCAGTAGC	501
Pap C R	CCGGCCATATTCACATAA	

Table 2. The reagents and the quantities of the reaction mix for the *Iuc* D and *Pap* C genes

Reaction mix	
Reagents	**µl/sample**
RNase free water	18.7 µl
10 X PCR buffer (50 mM KCl, 10 mM Tris-HCl pH 8.0)	2.5 µl
$MgCl_2$ (50 mM)	2 µl
dNTP solution (10 mM - Promega, USA)	1 µl
P_A (100 pmol)	0.1 µl
P_B (100 pmol)	0.1 µl
Pc(100 pmol)	0.1 µl
Pd(100 pmol)	0.1 µl
Taq platinum polymerase (5 U/µl) (Invitrogen®, Itapevi, São Paulo, Brazil)	0.4 µl
Total	25 µl

RESULTS AND DISCUSSION

The predicted size for the PCR amplicons to be visualized in electrophoresis for the *Iuc D* is 714 bp, and for the *Pap C* is 501 bp (Ewers et al., 2005).

The *Iuc D* gene was present in all investigated strains (13/13) isolates. *Pap C* gene was present in 23.07% (3/13) isolates.

The strain no. 8 isolated from broiler, 7 days old, from Calarasi County was positive to *Pap C* gene, also the strain no. 12, broiler 7 day old from Giurgiu was positive to *Pap C* gene. The strain no. 13 from Iasi, positive to *Pap C* gene, was also from broiler, 11 day old. All these 3 samples (8, 12, 13), positive for *Pap C*, show in electrophoresis a product of 501 bp (Figure 1), as expected for the size of *Pap C*.

As for the *Iuc D* gene, the results shows that all the 13 strains were positive, as seen in electrophoresis, were the amplicon at 714 bp was present in all 13 samples (Figure 1).

The isolate no. 1 was from broiler 7 day old, from Vrancea, no. 2 from Dambovita, 23 weeks, layer, no. 3 strain *E. coli* was from Iasi, 25 weeks, layer also. The strain no. 4 was layer, 87 weeks, from Brasov, no. 5 strain from Calarasi, broiler 10 day old, no. 6 from Dambovita, 24 weeks, layer, and the strain no. 7 also from Dambovita, broiler 1 day old. The strain no. 9 from Brasov, 65 weeks, layer, no. 10 from Vrancea 11 days, broiler, no. 11 Iasi 11 days, broiler.

Table 3. The PCR-results of the tested isolates for the presence of *Iuc D* and *Pap C* virulence genes

Isolates	Gene *Iuc D*	Gene *Pap C*	County	Age of the originating bird
1	**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**	**-**	Calarasi	10 day, broiler
6	**X**	**-**	Dambovita	24 weeks, layer
7	**X**	**-**	Dambovita	1 day, broiler
8	**X**	**X**	Calarasi	7 days, broiler
9	**X**	**-**	Brasov	65 weeks, layer
10	**X**	**-**	Vrancea	11 days, broiler
11	**X**	**-**	Iasi	11 days, broiler
12	**X**	**X**	Giurgiu	7 day, broiler
13	**X**	**X**	Iasi	11 day, broiler

X = mark the strains containing the gene.

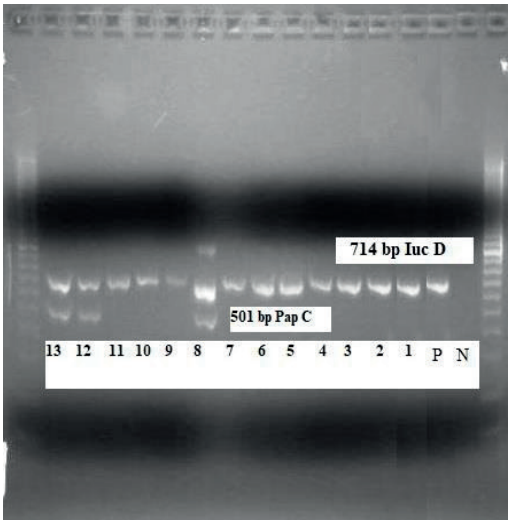

Figure 1. The PCR testing result of the *Iuc D* gene-714 bp; samples from 1 to 13 are positive, and for *Pap C* gene-501 bp, samples 8, 12, 13 are positive; P = positive control; N = negative control

The isolates no. 8, 12 and 13 presented both genes, *Pap C* and *Iuc D*. The pathogenicity of the APEC can be correlated to the association of the genes not only with the presence of them. *E. coli* strains have different pathogenic genes that are not expressed on all APEC strains (van der Westhuizen and Bragg, 2012; Gurau et al., 2018; Kemmett et al., 2013). As in our previous studies on APEC genes, in witch was not found all the virulence genes in all strains, in this fallow up study was found in all strains only one of the two genes *Iuc D* and *Pap C* was found only in 3 strains (Gurau et al., 2018).

If compare our results with the literature data, we can note that the prevalence of the strains containing the *Iuc D* gene was lower than in our study 78.79% (52/66) in Paixao et al. (2016) and 100% (13/13) in our study. In the same study the *Pap C* gene has almost the same prevalence 22.73% (15/66) as our study 23.07% (3/13).

Our results on the APEC strains show a randomised distribution of virulence genes. This is supporting the differences in terms of patogenicity expressed by the different APEC strains. Higher pathogenicity of the APEC determines primary infections while lower pathogenicity causes clinical signs and disease only if the poultry are stressed (Dho-Moulin & Fairbrother, 1999).

CONCLUSIONS

The *Iuc D* gene has a higher prevalence in this study being present in all isolated comparing with the *Pap C* gene which has a much lower prevalence than *Iuc D*. The virulence-associated genes *Iuc D* and *Pap C* were both identified in this study, supporting the higher pathogenicity of these strains, compared to the other strains, containing only the gene *Iuc D*. These results come to confirm other results from the literature, in which, different *E. coli* strains possess different pathogenic genes but not all virulence genes are expressed in all *E. coli* strains.

According to these preliminary results it could be assumed that *Iuc D* and *Pap C* genes are independently expressing their virulence.

ACKNOWLEDGEMENTS

Acknowledgments for the research support to ROMVAC Company S.A.

REFERENCES

Akram, N., Mojtaba H., Azam A., Masoud A., Najmeh H. (2017). Distribution of pathogenicity island markers and virulence factors in new phylogenetic groups of uropathogenic *Escherichia coli* isolates. *Folia Microbiologica*, 1–9.

Barnes, H.J., Glisson J.R., Fadly A.M., McDougald L.R., Swayne D. (2003). Colibacillosis. In Saif Y.M., Barnes H.J., Glisson J. R., Fadly A. M., McDougald

L.R., Swayne D. E. (Eds.), *Diseases of Poultry* 11th edn. Ames, Iowa State, University Press, 631–652.

Chakraborty, A., Saralaya V., Adhikari P., Shenoy S., Baliga S., Hegde A. (2015).Characterization of Escherichia coli phylogenetic groups associated with extraintestinal infections in South Indian population, *Ann Med Health Sci* Res 5(4), 241–246.

Delicato, E.R., de Brito B.G., Gaziri L.C.J., Vidotto M.C. (2003). Virulence-associated genes in Escherichia coli isolates from poultry with colibacillosis. *Veterinary Microbiology*, vol. 94, 97–103.

Dho-Moulin, M., Fairbrother J.M. (1999). Avian pathogenic *Escherichia coli* (APEC). *Veterinary Research*, 30, 299–316.

Dou, X., Gong J., Han X., Xu M., Shen H., Zhang D., Zhuang L., Liu J., Zou J. (2016). Characterization of avian pathogenic *Escherichia coli* isolated in eastern China Gene, *Gene*, 576(2), 244–248.

Dozois, C.M., Daigle F., Curtiss R. (2003). Identification of pathogen-specific and conserved genes expressed in vivo by an avian pathogenic *Escherichia coli* strain. *Proceedings of the National Academy of Sciences U.S.A.*, 100, 247–252.

Ewers, C., Janssen T., Kiessling S., Philipp H.C., Wieler L.H. (2005). Rapid detection of virulence-associated genes in avian pathogenic *Escherichia coli* by multiplex polymerase chain reaction. *Avian Diseases* 49, 269–273.

Gurău, M.R., Hasan Majid H., Popp M.C., Campeanu M.V., Daneș D. (2018). *Agriculture for Life, Life for Agriculture*, conference, Sciendo, 1(1), 536–541.

Kemmett, K., Humphrey T., Rushton S., Close A., Wigley P., Williams N. J. (2013). A longitudinal study simultaneously exploring the carriage of APEC virulence associated genes and the molecular epidemiology of faecal and systemic *E. coli* in commercial broiler chickens. *PloS One.* 8,1–10.

Li, G., Ewers C., Laturnus C., Diehl I., Alt K., Dai J., Anta ˜o E.M., Schnetz K., Wieler L.H. (2008). Characterization of ayjjQ mutant of avian pathogenic *Escherichia coli* (APEC). *Microbiology*, 154.

Paixao, A. C., Ferreira A. C., Fontes M., Themudo P., Albuquerque T., Soares M. C., Fevereiro M., Martins L., Corrˆea de S ´ M. I. (2016). Detection of virulence-associated genes in pathogenic and commensal avia *Escherichia coli* isolates. *Poultry Science Association Inc*, 1646–1652.

QIAamp cador Pathogen Mini Kit, manufacturer's instructions, https://www.qiagen.com/us/shop/sample-technologies/dna/genomic-dna/qiaamp-cador-pathogen-mini-kit/#productdetails.

Rouquet, G., Porcheron G., Barra C., Re ´pe ´rant M., Chanteloup N.K., Schouler C., Gilot P. (2009). A metabolic operon in extraintestinal pathogenic *Escherichia coli* promotes fitness under stressful conditions and invasion of eukaryotic cells. *Journal of Bacteriology*, 191, 4427–4440.

Santo, E., Macedo, C., Marin, J.M. (2006). Virulence factors of uropathogenic *Escherichia coli* from a university hospital in Ribeirão Preto, São Paulo, Brazil. *Rev. Inst. Med. trop. S. Paulo*, 48,185–188.

Vandekerchove, D., Vandemaele F., Adriaensen C., Zaleska M., Hernalsteens J.P., De Baets L., Butaye P., Van Immerseel F., Wattiau P., Laevens H., Mast J., Goddeeris B., Pasmans F. (2005). Virulence-associated traits in avian *Escherichia coli*: comparison between isolates from colibacillosis-affected and clinically healthy layer flocks. *Veterinary Microbiology*, 108, 75–87.

van der Westhuizen, W. A. and R. R. Bragg (2012). Multiplex polymerase chain reaction for screening avian pathogenic *Escherichia coli* for virulence genes. *Avian Pathology*, 41(1), 33–40