

## VIRULENCE GENES AND BIOTYPE PREVALENCE ASSOCIATED WITH AVIAN ESCHERICHIA COLI PATHOGENICITY, IN ALBANIAN POULTRY FARMS

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101 avian pathogenic *Escherichia coli* (APEC) strains from colibacillosis affected poultry were tested for the presence of eight different virulence associated genes. Besides, the APEC strains were biotyped. The association of belonging biotype to the corresponding pathotype was studied basing on the breeding type. The most frequent genes detected by means of PCR-multiplex were *iss*, *Irp2* and *IucD* (43.56%, 41.58% and 48.51%, respectively). Meanwhile their presence was significantly more relevant in the intensive poultry farms strains. The most pathogenic pathotype was considered the one with the combination of seven genes: *astA-iss-irp2-iucD-papC-vat-cva A/B*, with a presence of 5.45% in the intensive farms originating strains and at 0% in the rural type strains. The absence of virulence genes in the pathotype was seen mostly in the rural type strains 43.47% comparing it with the 21.82% in the intensive farms. The most frequent biotypes strains were B 28, B30 and B 31 with 13.86%, 16.83% and 15.84% each. Nevertheless the corresponding presence of these biotypes was more relevant with pathogenic strains originating from intensive breeding poultry farms. No data on the prevalence and the association of poultry *E.coli* biotypes with pathotypes was studied previously for evaluating the real potential pathogenic attitude of the bacterium.

**Key words:** APEC; biotype; pathotype; PCR-multiplex; poultry

### ВИРУЛЕНТНИ ГЕНИ И БИОТИПСКА РАСПРОСТРАНЕНОСТ НА ПТИЧЈИОТ ПАТОГЕН *ESCHERICHIA COLI* ВО ЖИВИНАРСКИТЕ ФАРМИ ВО АЛБАНИЈА

Тестирани се соеви на 101 птичји патоген *Escherichia coli* кај живина заразена со колибактерии поради присуство на осум различни вирулентно поврзани гени. Покрај тоа беа биотипизирани и соевите АПЕС. Поврзаноста на припадноста на биотипот на соодветен патотип беше проучувана врз основа на одгледуваниот тип. Најчестите гени откриени со помош на PCR-мултиплекс беа *iss*, *Irp2* и *IucD* (43, 56%, 41,58% и 48,51%, соодветно). Нивното присуство беше значително повеќе застапено, кај соевите во интензивните живинарски фарми. Како најпатоген беше сметан патотипот со комбинација на седум гени: *astA-iss-irp2-iucD-papC-vat-cva A/B*, присутен како изворен тип со 5,45% во фармите со интензивно производство а со 0% во руралните типови фарми. Отсуството на вирулентни гени во патотипот беше забележано претежно кај руралните видови – 43,47%, во споредба со 21,82% кај видовите во фармите со интензивно производство. Најчестите биотипски соеви беа B 28, B30 и B31 застапени со 13,86%, 16,83% и 15,84% секој од нив. Соодветното присуство на овие биотипови сепак беше повеќе во согласност со патогените видови што потекнуваат од живинарските фарми со интензивно производство. Нема податоци за распространетоста и поврзаноста на живинските биотипови на *E. coli* со патотиповите, што беше проучувано претходно заради оценување на вистинскиот потенцијал на патогеното однесување на испитуваната бактерија.

**Клучни зборови:** АПЕС; биотип; патотип; PCR-мултиплекс; живина

#### INTRODUCTION

Colibacillosis poses a major economic problem to the poultry industry. In spite of the costly

nature of this disease, *E. coli* has poorly described virulence mechanisms. *Escherichia coli* strains cause a number of syndromes in domestic poultry, ultimately leading to disease and death, or to a de-

crease of egg production or condemnation of carcasses (Gross et al., 1991). Among these diseases is a severe systemic syndrome termed colisepticaemia, which is characterized by the presence of *E. coli* in the blood and the colonization of various visceral organs (Barnes et al., 2003).

Several potential virulence factors have been identified on APEC, mainly from a positive correlation between phenotypic characteristics and virulence for poultry. The involvement of these factors in virulence using experimental methods and molecular characterizations is at just the beginning. In fact, the genomic methods have provided worldwide very interesting results and hypothesis for further elucidating study (Dho-Moulin and Fairbrother, 2000).

Due to the well fare concerns for the birds involved and the high economic loss, infections caused by *E. coli* are of great importance, and disease control has been complicated over the past years by the increasing of the antimicrobial resistance frequency (Vandemaele et al., 2002). This has lead the research concentrating on the role of specific *E. coli* genes play in the outbreaks of the disease, by targeting and identifying antigens for a future vaccine developing. A large number of genes encoding virulence factors have been identified (Janben et al., 2001). In fact, the possession of some virulence factors as *increased serum survival system* or *ColV* plasmid which encodes for further several potential virulence factors (e.g. bacterial antibiotic resistance, *iss*, *tsh* and other iron chelators) may render a microorganism in posses of these factors more adaptable and improve its survival in the hosts organisms using host's resources for replication. The association of more than one virulence gene in the same bacterial strain may provide a specific pathogenic attitude and may lead to an increased virulence.

In this study, multiplex-PCR protocol was developed for characterization of the *E. coli* capable of causing colibacillosis, which is an effort to facilitate the study of this pathogen behaviour in poultry organisms.

## MATERIAL AND METHODS

### *Bacterial strains*

One hundred and one *E. coli* strains from poultry (55 of laying hens and 46 of broilers) of

different types of breeding (55 of industrial and 46 of rural farms) were investigated. All the bacterial strains were isolated from inner organs of poultry died by being affected form colibacillosis. This fact was presumed by findings of typical preceding lesions lice septicaemia, respiratory infections, polyserositis and peritonitis (Gross, 1994). All the strains were isolated from liver of the affected birds. The sampling was performed in the period between December 2007 and December 2008, in Albanian wide territory. In addition to these strains, reference and positive control strains were used for multiplex-PCR incompliance. Negative control isolates were also used.

Test and control organisms were plated on MacConkey agar (OXOID) and incubated overnight at 37°C. Isolates were considered positive for lactose fermentation and susceptible for *E. coli* colonies if pink colonies were observed (Ewers et al., 2005).

All bacterial strains were stored at -80°C Brucella Broth Infusion with 20% of glycerol till use.

### *Multiplex polymerase chain reaction*

*Avian pathogenic Escherichia coli* isolates were analyzed by polymerase chain reaction (PCR) for the presence of following categories of virulence genes: enteroaggregative toxin (*astA*), increased serum survival protein (*iss*), iron-repressible protein (*irp2*), aerobactin (*iucD*), a temperature-sensitive hemagglutinin (*tsh*), P-fibrin (*papC*), vacuolating autotransporter toxin (*vat*) and factors associated with the complement resistance such as colicin V plasmid operon (*cva A/B*).

Isolates, from witch DNA was to be used as a target for the PCR amplification, were grown overnight in Luria-Bertani broth at 37°C and the DNA was released from whole microorganisms by boiling them for 10 minutes. After centrifugation 2 µl of the supernatant was taken as template DNA and was added to the reaction mixture (25 µl) containing 0.1 µl of each primer pair, sense and antisense (MWG, Biotech, Germany) in a 10 pmol concentration, 0.5 µl of 10 mM of the four deoxynucleoside triphosphates (Sigma-Aldrich, Germany), 2.5 µl of 10 times PCR buffer, 1 µl of 50 mM magnesium chloride, and 1 unit of Taq-Polymerase (Rapidozym, Germany).

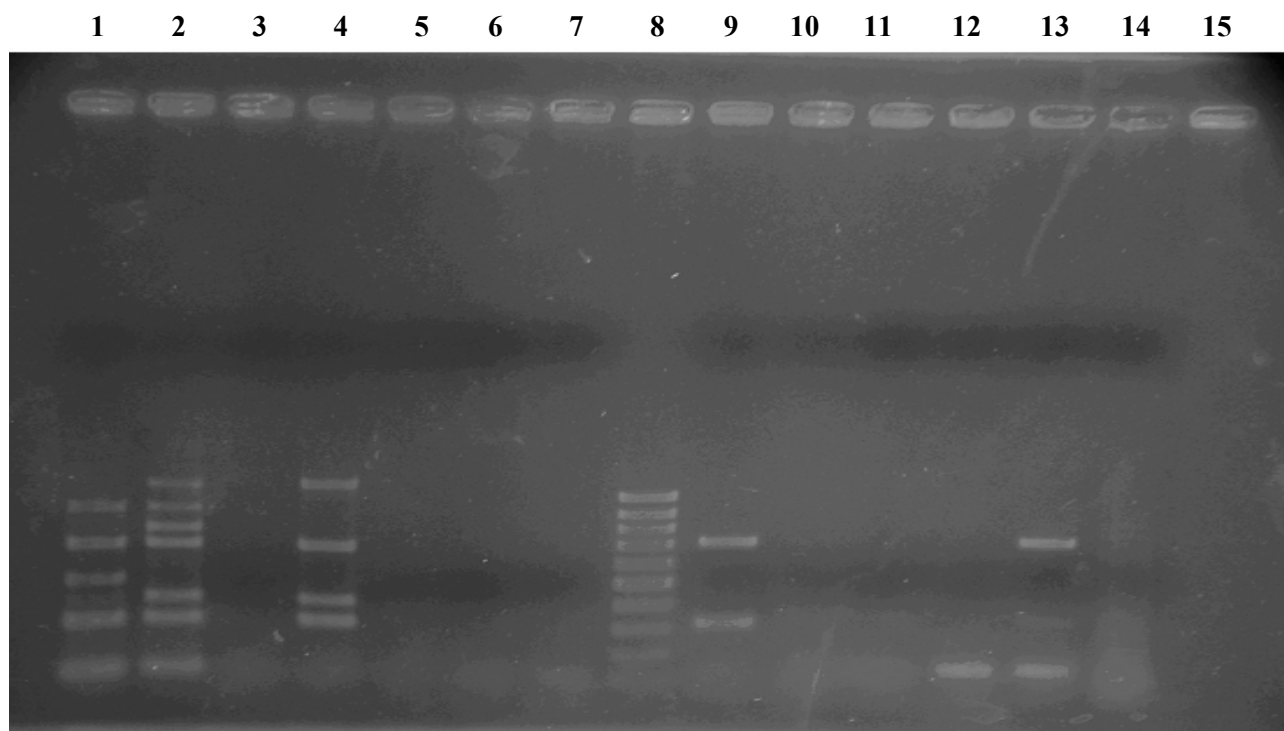
Each reaction mixture was amplified in a 9700 GeneAmp® PCR system (Applied Biosys-

tems, Warrington, United Kingdom) using the following conditions: heat denaturation at 95°C for 15 min; 25 cycles with denaturation at 94°C for 30 s, various annealing at 59°C for 30 s and extension at 68°C for 3 min. This was followed by a final extension step at 72°C for 10 min.

The DNA sequences of oligonucleotide primers, amplification conditions, and sizes of ampli-

fied fragments for the factors studied are listed in Table 2.

The amplification products (2 µl) were analyzed by gel electrophoresis on a 1.5% agarose gel, stained with ethidium bromide. Gels were run for approximately 90 min at 80 v. Products were visualized and photographed at UV exposure (Fig. 1).



**Fig. 1.** Lane 1 and Lane 2: positive control strains for the presence of eight virulence genes. Lane 3, 5, 6, 7, 10, 11, 14 and Lane 15: no presence of any of eight studied genes. Lane 4: pathotype with the presence of *iss*, *papC*, *tsh* and *cva* genes. Lane 8: marker color. Lane 9: positivity for the presence of two genes : *iss* and *tsh*. Lane 12: presence of *astA* gene. Lane 13: presence of gene: *tsh*

### Biotyping

Fermentation activity was tested on phenol red agar base, supplemented with 1% of each selected carbohydrate, in square Integrid Petri dishes (according to Difco Laboratories, Detroit, Mich.). *E. coli* strains were inoculated as spots on the medium and the results were read after 24 and 48 h of incubation, at 37°C. To assess the belonging biotype we have used the simplified biotyping scheme of *Camguilhem and Milon*. The score number was assigned to every positive fermentation reaction as follows: D-raffinose (test score 4), L-ranmose (test score 16), dulcitol (test score 2), sucrose (test score 8), and sorbose (test score 1).

### RESULTS

**Lactose fermentation.** The abilities of APEC isolates to ferment lactose were determined by standard methods. Of the APEC strains, 100% of the isolates were positive to lactose fermentation.

**Biotyping.** A number of 20 different biotypes were revealed (B0, B3, B4, B5, B11, B12, B13, B14, B16, B17, B18, B19, B21, B22, B23, B25, B28, B29, B30 and B31). 73 *E. coli* strains (72.27 %) were positive to rhamnosis fermentation (those with test score 16 and up). The majority (66.34%; 67 to 101 strains) of avian *E. coli* strains were assigned to only four biotypes (B16, B28, B30 and B31). Most of the strains belonged re-

spectively to biotype B30 (16.83%) e B31 (15.84%). The relevant results for the four major biotypes found in this study are presented in the Table 1 by isolates breeding type origin.

Table 1

*Biotyping results according to poultry breeding type (%)*

Relevant biotype	Industrial type farms originating strains (n = 55)	Rural type farms originating strains (n = 46)	Total of APEC strains (n = 101)
B 28	16.36	18.18%	13.86%
B 30	10.86	15.21	16.83%
B31	26.63	6.5	15.84

**Genotyping.** Molecular investigations displayed that 68.35% of the total of samples carried at least one virulence gene in their genome, which was the most prevalent pathotype of the studied isolates.

The absence of the presence of virulence-associated genes was major in the rural breeding farms (43.47 %) in comparison with the 21.82 % of strains without encoding genes originating from industrial poultry farms. Meanwhile the most frequent pathotype in poultry intensive breeding type was the association of five virulence genes (*Iss-iucD-papC-tsh-vat* or *Iss-irp2-iucD-tsh-cva A/B* or *Iss-irp2-iucD-papC-tsh* or *astA-iss-iucD-papC-vat*) at 21.2% in the total of APEC strains studied.

Table 2

*Percentages of the presence of virulence-associated genes according to the poultry breeding type (%)*

Detected genes	Industrial type farms originating strains (n = 55)	Rural type farms originating strains (n = 46)	Total of APEC strains (n=101)
<i>astA</i>	20	31.61	25.74
<i>Iss</i>	76	28.26	43.56
<i>Irp2</i>	54.55	13.04	41.58
<i>iucD</i>	61.8	17.39	48.51
<i>Tsh</i>	40	4.34	26.73
<i>papC</i>	38.18	2.17	23.76
<i>Vat</i>	20	0	12.87
<i>Cva</i>	21.81	2.17	14.85

The most pathogenic pathotype was considered the one with the combination of seven genes: *astA-iss-irp2-iucD-papC-vat-cva A/B*, with a presence of 5.45% in the intensive farms originating strains and at 0% in the rural type strains.

The detection of virulence-associated genes was significantly more frequent in industrial breeding type farms in comparison with the findings in the rural breeding type poultry farms (see Table 2).

## DISCUSSION

In this study, a relevant correlation between the virulence-associated genes, biotypes to the poultry breeding type, in APEC isolates, was observed.

The results of this study seem to confirm the pathogenic role of some specific *Escherichia coli* pathotypes and for the appearance particularly useful of the application of molecular assays to detect virulence genes, providing so additional information about effective pathogenic attitude of *E.coli* in the poultry organisms.

The detection of the most virulent pathotype with the combination of seven genes: *astA-iss-irp2-iucD-papC-vat-cva A/B*, at a presence of 5.45% in the intensive farms originating strains and at 0% in the rural type strains assumes a higher importance in that the pathogenicity of APEC strains may be enforced by the accumulation and the co-expression of multiple virulence genes. Similarly, some particular combinations of genes may confer powerfully in the pathogenic attributes (Giovanardi, 2005).

Very interesting was the comparison of the belonging pathotypes to the breeding type with the belonging strain's biotype. The biotype B30 was the most prevalent one in this study isolates, followed by biotypes B28 and B31. These biotypes as presented in the Table 1 are mainly frequent in the APEC strains originating from intensive poultry breeding type. This helps the hypothesis that the pathotypes originating from this type of breeding, possessing a major number of genes combinations in its genome and belonging to a higher percentage to specific biotypes can be in possess of highly pathogenic abilities.

Our results support that a wide genomic diversity among avian *pathogenic Escherichia coli*

exists, possibly because of the opportunistic nature of the majority of infections. Also the observation of large variations in contribution of specific pathotypes, the wide variety of virulence genes presence detected, and the large number of revealed biotypes make it difficult to produce a meaningful classification of *E. coli* pathogenic for avian species based on one strain specificity alone.

### CONCLUSIONS

Analyzing the obtained data from our study, we observe a relevant difference in the presence of virulence genes according not only to poultry breeding type, but also to the poultry production type, layers or broilers. There is a strong evidence of the presence of virulence factors mostly in the intensive poultry breeding type. Indeed, the significant differences in gene prevalence seen within the APEC strains, isolated from different poultry breeding suggest that sub-groupings of an *Avian Pathogenic Escherichia Coli* pathotype may exist.

As a previous study on biotyping, avian pathogenic and faecal *Escherichia coli* has not been done before, from our genobiotyping results it comes up clear that the biotypes B28, B30 and B31 have the largest possibility of co-relation with the identified pathotypes. But a conclusion about a specific pathogenic biotype among *E. coli* isolates can not be framed as a large percentage of biotypes coincide also with non-pathogenic *E. coli* isolates.

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