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THE CAUSATIVE AGENTS OF COLIBACILLOSIS IN POULTRY: CARRIERS OF GENES ASSOCIATED WITH EXTRAINTESTINAL AND INTESTINAL PATHOGENIC *Escherichia coli*

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Abstract

The expansion and intensification of poultry farming increases the risk of spreading colibacillosis among poultry, so there is an urgent need to monitor avian pathogenic *Escherichia coli* (APEC), study their genetic diversity and identify strains that pose a threat to human health. Determination of virulence-associated genes and the degree of specific adhesion may be useful for a comprehensive assessment of the epidemic and epizootic significance of *E. coli* strains isolated from livestock. In this study, an extended molecular analysis of *E. coli* strains isolated from poultry during outbreaks of colibacillosis was performed with the objective to genotypically characterize the isolated *E. coli* strains and to evaluate the relationship between genes encoding adhesins and specific adhesion to erythrocytes. It was shown for the first time that the strains were characterized by a high potential for pathogenicity and could be carriers of genes for several pathotypes at once, while the genes of intestinal pathogenic *E. coli* (IPEC) were detected often than others. A positive adhesive profile for a number of genes correlated positively with the activity of strain adhesion to chicken (*Gallus gallus* L.) and human erythrocytes. In the study 28 non-clonal *E. coli* strains, as determined by ERIC-PCR, isolated from various organs (except the intestine) of Ross 308 cross broilers (*Gallus gallus* L.) with generalized colibacillosis in 2016-2018 were characterized. Polymerase chain reaction (PCR) was used to detect virulence-associated genes characteristic of four different *E. coli* pathotypes, the APEC, extraintestinal pathogenic (ExPEC), intestinal pathogenic *E. coli* (IPEC: Enteropathogenic *E. coli* EPEC, Enterotoxigenic *E. coli* ETEC, Enterohemorrhagic *E. coli* EHEC, Enteroaggregative *E. coli* EaggEC), and uropathogenic *E. coli* (UPEC). Previously published protocols were used for all types of PCRs and amplifications were performed in the DNA Engine Dyad Thermal Cycler (Bio-Rad, USA). Band visualization and data documentation were performed using the Gel-Doc XR gel documentation system (Bio-Rad, USA). Formalinized human erythrocytes of the type 0(I) Rh(+) and avian erythrocytes were used as cell substrates for the determination of bacterial adhesion to erythrocytes. To evaluate the bacterial adhesion properties the adhesion index was calculated as the average number of bacteria bound to an erythrocyte in the adhesion assay. The obtained results showed that the characterized strains possessed a high pathogenic potential, as they carried genes associated with APEC, ExPEC as well as IPEC. The presence of APEC-specific marker genes identified most of the strains as APEC. However, potential for human pathogenicity was also found among the analyzed strains. As the IPEC-associated genes were found more frequently than ExPEC-associated genes, the *E. coli* strains studied were more similar to strains causing acute intestinal infections in humans, particularly due to the fact that they carried genes encoding toxins characteristic of IPEC (with the exception of genes for Shiga-like toxins and enterohemolysins). Based on cluster analysis of genetic profiles, the strains studied could be classified into three groups: (i) pathogenic to birds and humans, characterized by the presence of 2-6 genes associated with APEC and 2-6 genes associated with ExPEC or IPEC (24 strains), (ii) pathogenic to birds and nonpathogenic to humans, characterized by the presence of 2-6 genes associated with APEC and 0-1 gene associated with ExPEC or IPEC (2 strains), and (iii) nonpathogenic, characterized by the possession of none or one gene from each pathotype, APEC, ExPEC, IPEC (2

strains). It was found that 75 % of the first group, pathogenic to birds and humans, carried not only a high number of virulence-associated genes, but also pathogenicity island SHI-2, as well as genes for extended-spectrum beta-lactamases and class 1 integrons. Specific adhesion of *E. coli* strains was more pronounced on chicken erythrocytes than on human ones. Statistical analysis revealed several positive correlations between the chicken and human erythrocytes adhesion profiles and a number of genes encoding adhesins. The high adhesion activity of the bacteria, regardless of the type of erythrocyte, also correlated with longer survival in host blood serum (genotype *iss*+) and the possibility of erythrocyte lysis (genotype *hlyF*+). The obtained data on the molecular and adhesive properties of causative agents of colibacillosis in birds allow us to assess their zoonotic potential and epizootic significance and can also serve as the basis for improving the monitoring system for colibacillosis in poultry farms.

Keywords: avian pathogenic *Escherichia coli*, APEC, ExPEC, IPEC, virulence-associated genes, zoonotic potential

Aavian pathogenic *Escherichia coli* (APEC) is an animal pathotype of *Escherichia* found in the gut microbiota of some bird species that causes extraintestinal infections in immunocompromised individuals [1]. APEC strains become the main cause of colibacillosis, a syndrome associated with aerosacculitis, pericarditis, and often sepsis in poultry [2, 3]. Outbreaks of the disease in poultry enterprises lead to a reduction in egg production by 2-3% and mortality of livestock up to 30% which entails economic losses [4]. Experts estimate that at any given time, at least 30% of the individuals of all commercial herds in the United States have colibacillosis [5)]. In Russia, colibacillosis accounts for 60 to 88% of all poultry infections [6].

According to the current classification, the APEC population is included in the group of extraintestinal pathogenic *E. coli* (ExPEC), which also includes uropathogenic *E. coli* (UPEC) associated with neonatal meningitis-causing *E. coli*, (NMEC), sepsis-associated *E. coli (*SEPEC) and other pathotypes [2, 5]. The use of molecular approaches to identify *Escherichia* virulence factors has significantly expanded knowledge of the pathogenetic mechanisms of APEC infection [7]. According to the hypothesis proposed by L.K. Nolan et al. [3], the APEC pathotype is due to the presence of specific marker genes. The presence of a minimal set of genetic determinants allows defining a strain in this group, i.e., *ompT* (outer membrane protease), *iutA* (aerobactin receptor), *iss* (serum survival factor), *iroN* (enterochelin receptor) [3, 5]. In addition, various virulence genes encoding adhesins, toxins, defense factors, iron production systems, as well as autotransporters and the IbeA protein are involved in the development of colibacillosis, which determines many manifestations (forms) of avian infections resulting from the expression of various combinations of virulence determinants [6].

V.G. Maturana et al. [8] concluded that APEC strains do not represent a homogeneous group, but, depending on the combination of pathogenicity determinants, are divided into subgroups-subpathotypes, each of which is associated with a specific infectious syndrome. Studies by L. Mageiros et al. [9] showed that strains of the APEC pathotype arise from ubiquitous commensal intestinal bacteria, including through horizontal transfer of genes expressing pathogenicity factors, allowing divergent clones to infect poultry. Most APECs have been found to contain a highly conserved cluster of plasmid-linked virulence genes found in relatively few *E. coli* faecal isolates from healthy birds (AFEC) [10].

Recent studies have suggested that APEC strains may pose a risk to human health [11, 12]. On the one hand, the possibility of APEC transmission through food products, including poultry meat, has been described [13], on the other hand, the revealed DNA sequence homology between APEC and other ExPEC pathotypes shows that they are closely related phylogenetically [10]. For example, in extraintestinal strains of *E. coli* pathogenic for humans, the iss gene was detected in the genome, which expresses a factor responsible for the survival of bacteria in blood serum. The gene is located on the large virulence plasmid ColV, typical of

avian *E. coli* strains, indicating possible plasmid transfer and hence virulence gene exchange between human and avian *E. coli* strains [10, 14]. The results obtained by K.E. Rodriguez-Siek et al. [15] and T.J. Johnson et al. [16, 17] confirm the close relationship between APEC and UPEC/MNEC cultures. The emergence and spread of hybrid and heteropathogenic strains of *E. coli* carrying, respectively, patterns of ExPEC genes and representatives of intestinal pathogenic *E. coli* (intestinal pathogenic *E. coli*, IPEC) or two or more IPEC pathotypes was noted [18, 19]. The presence of similar virulence-associated genes found in IPEC/ExPEC and APEC strains confirms that the latter can either act as zooanthroponotic pathogens themselves or serve as a reservoir of virulence determinants for *E. coli* that cause infections in humans [12, 20].

Various biological systems, including experimental infection, are used to establish a relationship between the presence of certain APEC pathogenicity factors and their manifestation in host biotopes [21, 22]. The role of pColV plasmid in avian and possibly human virulence has been confirmed: transconjugants (commensal strain with pAPEC-O2-ColV) caused death of chick embryos and urinary tract infection in mice, and also grew well in human urine [23], nevertheless APEC's ability to cause disease in humans has not been conclusively proven.

In this work, based on the results of a comprehensive molecular screening of *E. coli* strains isolated from birds during outbreaks of colibacillosis for the presence of genes of three *Escherichia* pathotypes (APEC, UPEC, IPEC), it was shown for the first time that the strains were characterized by a high pathogenic potential and could be carriers of genes all pathotypes at once, while IPEC determinants were more common than others. It has been established for the first time that a positive adhesive profile for a number of genes positively correlates with the level of strain adhesion to chicken and human erythrocytes.

The aim of the work is to give a genotypic characterization of *Escherichia coli* strains isolated from poultry with colibacillosis, as well as to evaluate the relationship between the adhesive genotype and specific adhesion to erythrocytes.

Materials and methods. We used 28 *E. coli* strains with a unique genotype according to ERIC-PCR, isolated in 2016-2018 from different organs (excluding the intestines) of broiler chickens (*Gallus gallus* L.) of the Ross 308 cross with generalized colibacillosis [24]. The strains were deposited in the Ex culture collection of the Department of Biology, Faculty of Biotechnology, University of Ljubljana (Univerza v Ljubljani, Slovenia).

Determining whether a strain belongs to a phylogenetic group, sensitivity to antibiotics, and the presence of genes encoding the most common extended-spectrum beta-lactamases (ESBLs) was described by us previously [24]. In the sample ($n = 28$), polymerase chain reaction (PCR) at the end point was used to detect virulence genes characteristic of four conditional groups: genes that ensure the pathogenicity of bacteria of the *E. coli* species occurring in various pathotypes, genes that are pathogenic for birds *E. coli* (APEC), enteric pathogenic *E. coli* (IPEC: EPEC/ETEC/EHEC/EaggEC) and uropathogenic *E. coli* (UPEC).

For all types of PCR, primers and protocols of the authors who proposed them were used. Amplification was performed on a DNA Engine Dyad Thermal Cycler (Bio-Rad, USA). Band visualization and data documentation were performed using the Gel-Doc XR gel documentation system (Bio-Rad, USA).

The adhesion of bacteria to erythrocytes (specific adhesion) was determined by the method of V.I. Brilis et al. [38]. Formalized human erythrocytes of the 0(I) Rh(+) group and avian erythrocytes served as the cell substrate. The cells were preliminarily washed twice in 0.01 M phosphate-buffered medium (PBS) and standardized to a density of 100 million/ml. A suspension of microbial

cells, standardized in PBS to 2.0 according to McFarland, and erythrocytes were mixed in equal amounts (0.1 ml) in Eppendorf tubes, shaken for 20 min at 37 °C, after which smears were prepared on a glass slide. The smears were dried at room temperature, fixed with methanol for 10 min, and stained with 2% methylene blue.

The number of microbial cells attached to one erythrocyte was counted for at least 25 erythrocytes. The adhesive properties of cells were assessed using the microorganisms' adhesion index (MAI): the average number of bacteria attached to one erythrocyte involved in the adhesion process. Microorganisms were considered non-adhesive at MAI ≤ 1.75, low-adhesive at 1.76-2.5, medium-adhesive at 2.51-4.0, highly adhesive at MAI ≥ 4.0.

Statistical data processing was carried out using Microsoft Excel 2013 and Statistica v. 6.0 (StatSoft, Inc., USA). To assess quantitative indicators, the median *Me* and quartiles, Q1-Q3 were calculated. Relationships between traits were identified using Spearman's nonparametric rank correlation coefficient (Rs). Significance of differences between two dependent samples was assessed using the Wilcoxon signed-rank *W*-test, independent samples were compared using the Mann-Whitney *U*-test. The classification of strains was carried out by the method of hierarchical clustering (tree cluster analyses; the measure of distance is the Euclidean distance). Qualitative features were compared using χ^2 (with Yates correction) or Fisher's exact *F*-test. At $p < 0.05$, it was concluded that there was a statistically significant difference between the compared samples.

Results. Among 28 individual *E. coli* strains, carriers of both virulence genes common to all pathotypes and genes characteristic of representatives of the APEC, UPEC, and IPEC groups were found (Table 1).

1. Virulence genes detected in APEC (avian pathogenic *Escherichia coli*) strains isolente from cross Ross 308 broiler chickens (*Gallus gallus* L.) with generalized colibacteriosis (n = 28, 2016-2018)

Gene	Pathotype	Virulence factor and its function	References
fimH	UPEC, NMEC,	Universal fimbrial adhesive	[25]
ompT	SEPEC, APEC	Surface protein with protease activity	[26]
kpsMTII		Capsule formation type 2 gene	[27]
iroN		Receptor protein of the iron uptake and transport system	
traJ		Positive regulator of plasmid conjugative transfer	[28]
hlyF	APEC	Specific avian hemolysin F	[29]
Iss		Factor that increases cell survival in blood serum	[10]
iutA		Adhesin homologue	[30]
yqi		Specific avian adhesin	[21]
beA	EPEC, ETEC,	Invasive protein	[27]
Iha	EHEC, EaggEC	Adhesin	[31]
eaeA		Intimin	
*stx*1		Shigatoxin	
stx2			
estI		Thermostable enterotoxin	
estII			
ehxA		Enterohemolysin	
eltA		Heat-labile enterotoxin	
east1		Enteroaggregative thermostable enterotoxin	
subAB		Cytotoxin subtilase	[32]
hlyA		Alpha hemolysin	[33]
papGII	UPEC	Fimbrial adhesin, binds the Gal-alpha1-4Gal receptor found on	[30]
papGIII		epithelial cells lining the upper urinary tract	
papC		Outer membrane carrier protein involved in the export and assembly of pili subunits across the outer membrane	
sfaDE		Non-fimbrial adhesin	
afa/		Hemagglutinins of uropathogenic Escherichia coli mediate adherence	[27]
draBC		to the upper urinary tract	
upaG		Mediates aggregation, biofilm formation and adhesion for a number of extracellular matrix proteins; mediates adhesion to human T24 bladder epithelial cells	[34]
usp		Uropathogenic specific protein, colicin	[35]

N o t e. UPEC — uropathogenic *E. coli*, NMEC — neonatal meningitis-causing *E. coli*, SEPEC — sepsis-associated *E. coli*, APEC — avian pathogenic *E. coli*, EPEC — enteropathogenic *E. coli*, ETEC — enterotoxigenic *E. coli,* EHEC — enterohemorrhagic *E. coli*, EaggEC — enteroaggregative *E. coli*, ExPEC — extraintestinal pathogenic *E. coli.*

The studied *Escherichia* strains had a high overall virulence potential. The *fimH* gene encoding fimbrial adhesin was carried by 92.8% of the strains, the capsular formation gene *kpmsT* by 82.1%, the ompT gene for outer membrane protein with protease activity by 71.4%, and the iron uptake and transport system gene *iroN* by 67.8%. In almost half of the strains (46.4%), we identified all of the listed genes, in 32.1% three genes, in 14.3% two genes, one strain carried one gene and none of the listed genes. Separately, it should be noted that 53.6% of the strains had the gene for the positive conjugation regulator *traJ*.

Among the genes that most often characterize the APEC pathotype, the most common gene was the specific avian hemolysin gene *hlyF* (82.1%), the next was the avian adhesin Yqi (the *yqi* gene, 60.7%), *iss* was carried by 57.1% of strains, *iutA* by 42.8%. A quarter of the strains had all four genes, 21.4% had three genes, 25.0% had two genes, 21.4% had one gene, and only two strains had none of the genes from this group. The presence of specific marker genes made it possible to identify most *Escherichia* strains as APEC.

The present sample of strains lacked genes for Shiga-like toxin (*stx1/2*), genes for the main adhesion factor EHEC intimin (*eaeA*) and enterohemolysin (*exhA*). At the same time, 75% of the cultures were carriers of the *subAB* gene encoding the cytotoxin subtilase which is characteristic of Shiga toxin-producing *E. coli* strains. Carriers of other enterotoxin genes from the ETEC group were widely represented. The genes for thermostable enterotoxins *estI/II* were detected in 42.8 and 82.1% of strains, respectively, more than half of the strains (60.7%) carried the gene for enteroaggregative thermostable enterotoxin (*eastI*), thermolabile enterotoxin (*eltI*) was found in 14.3% cultures. In addition, 75% of APECs had the iha adhesin gene, which is one of the pathogenicity factors of diarrheagenic *E. coli*. Nineteen strains (67.8%) carried four or more of the listed genes, six strains (24.1%) carried from one to three genes, three strains did not have the genes of this group.

Of the group of genes most characteristic of uropathogenic *E. coli* strains, only *upaG* (67.8%) and *usp* (7.1%) were found.

When comparing the prevalence of the analyzed marker genes in avian strains, it turned out that the pathogenicity genes common to all *E. coli* pathotypes were more common in the sample than the APEC (*W*-test: $p = 0.029$) or UPEC (*W*-test: $p < 0.01$) genes. .01); APEC were more common than UPEC (*W*-test: $p < 0.01$); IPEC genes had a similar frequency of occurrence compared to genes common to all groups, but significantly exceeded the frequency of APEC and UPEC (*W*-test: $p < 0.01$) (Fig. 1).

According to the results of a cluster analysis of the presence of pathogenicity genes, three conditional groups of strains were identified (Fig. 2): pathogenic for birds and humans (the presence of 2-6 genes associated with APEC and 2-6 genes associated with ExPEC or IPEC) (24 strain); pathogenic for birds and not pathogenic for humans (presence of 2-6 genes associated with APEC, and 0-1 gene associated with ExPEC or IPEC) (2 strains); non-pathogenic (0-1 gene from any group, APEC, ExPEC, IPEC) (2 strains). Strains with phylogroup B1 in 85.7% were assigned to the first group. Based on the data obtained earlier [24], the group of strains isolated as pathogenic for birds and humans was characterized by a high frequency of occurrence not only of virulence genes, but also of ESBL genes, such as CTX (57.1% of strains) and TEM (71.4 % strains). In addition, 42.8% of the representatives of this group had the *traJ* gene (60% of the total frequency of occurrence of the gene in the sample) and 28.5% had segments of class 1 integrons (75% of the total frequency of occurrence in the sample).

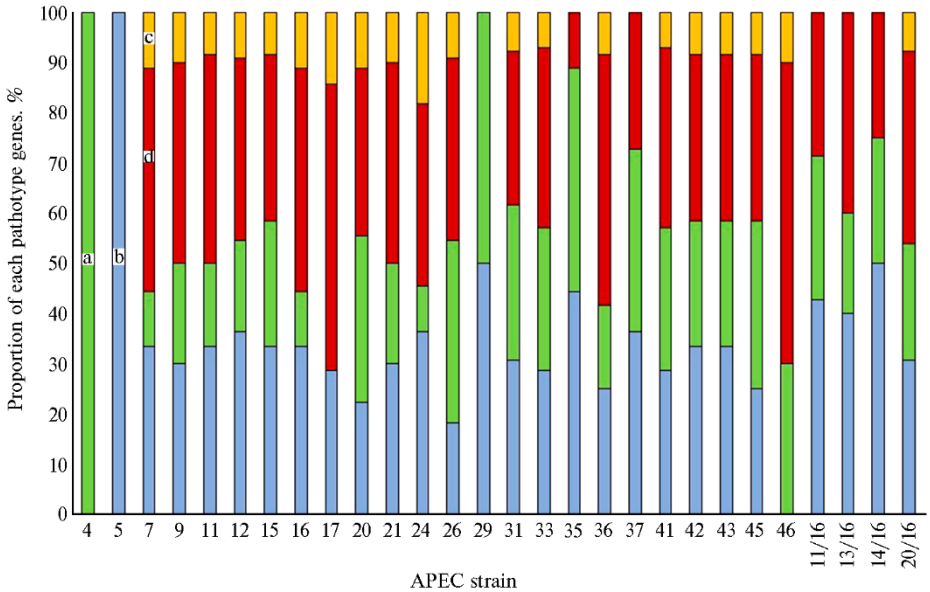

Fig. 1. Ratio of genes common to all pathotypes (b)**, APEC (avian pathogenic *Escherichia coli*)** (a)**, UPEC (uropathogenic *E. coli*)** (c) **and IPEC (intestinal pathogenic *E. coli*)** (d) **in APEC (avian pathogenic *E. coli*) strains isolated from the Ross 308 cross broiler chickens (*Gallus gallus* L.) with generalized colibacillosis** (*n* = 28, 2016-2018)**.**

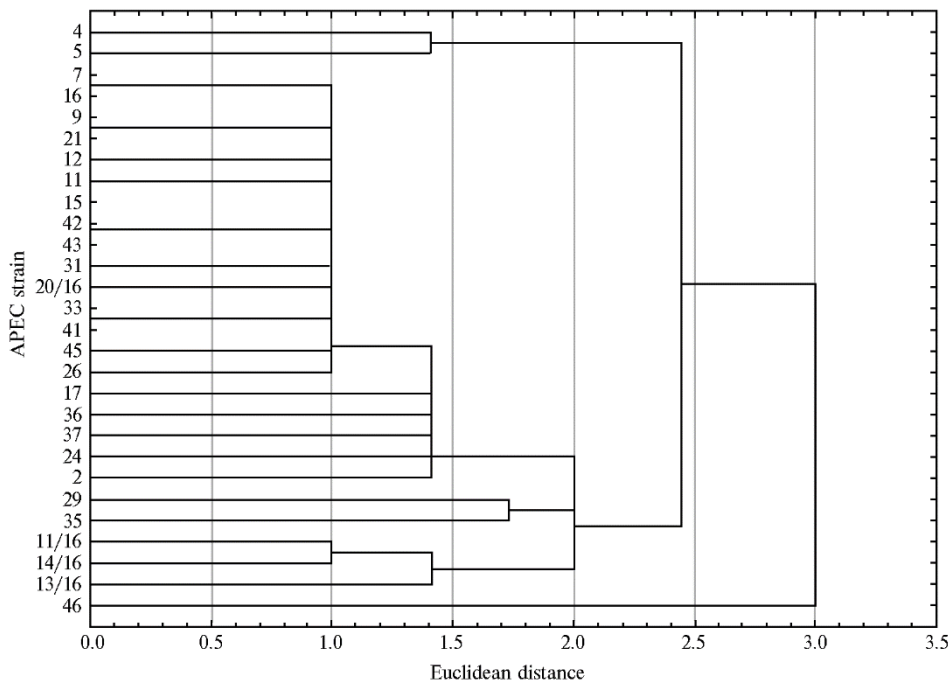

Fig. 2. Results of cluster analysis (Cluster analysis, Statistica v.6.0) **of the distribution of pathogenicity genes among APEC (avian pathogenic *Escherichia coli*) strains isolated from broiler chickens (*Gallus gallus* L.) of the Ross 308 cross with generalized colibacillosis** (*n* = 28, 2016-2018).

To determine the relationship between the presence of genes associated with virulence and antibiotic resistance, cultures were divided into groups with 0-2 and 3-4 genes (for genes of general virulence and APEC markers) and 0-3 and 4-6 genes (for IPEC markers). APEC with 0-2 genes from the group of common virulence were resistant to five or more antibiotics in 42.85% of cases, while strains

with 3-4 genes in 57.14% of cases. For genes characterizing the APEC group, the difference was even more significant: 42.85% resistant to five or more antibiotics among carriers of 0-2 genes and 64.28% among carriers of 3-4 genes. Interestingly, for the genes of the IPEC group, the ratio was reversed. The strains with 0-3 genes were resistant to five or more antibiotics in 66.66% of cases while the strains with a large number of genes only in 47.36%. This trend continued for any combination of the number of genes present and the antibiotics to which the strain developed resistance. It should also be noted that the correlation between these traits was significant only for the genes of the APEC group (Rs = 0.426), while for the IPEC genes it was completely absent (Rs = 0.041). The predominant part of the strains studied by us, according to MAI, was assigned to a low-adhesive group, regardless of the type of erythrocytes used (60.71% of cultures in the test with chicken erythrocytes and 85.71% of cultures with human erythrocytes), while the no correlation between MAI indicators was detected (Rs = 0.046) (Fig. 3).

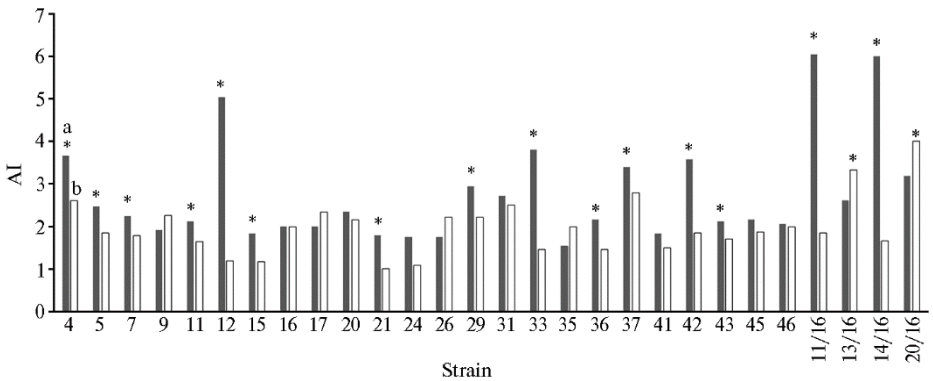

Fig. 3. Microbial adhesion index (MAI) **for APEC (avian pathogenic *Escherichia coli*) strains isolated from the Ross 308 cross broiler chickens (*Gallus gallus* L.) with generalized colibacillosis in the tests with chicken** (a) **and human** (b) **erythrocytes** (*n* = 28, 2016-2018).
* Differences between variants with different types of erythrocytes are statistically significant at p ≤ 0,05 (*W*-test).

For most of the APEC strains, the adhesive activity in relation to chicken and human erythrocytes was significantly different. Cells of 15 strains (53.57%) adhered better to chicken erythrocytes, two strains (7.14%) to human erythrocytes, and in 11 cultures (39.29%) adhesion indices did not differ. The average MAI index Me(Q1-Q3) for avian and human erythrocytes was 2.21(1.96-3.25) and 1.87(1.61-2.24), respectively, and was lower in the latter case (p = 0.0057). In the group of strains pathogenic for birds and humans according to the genotype, the degree of adhesion to avian erythrocytes, as in the general sample, was still significantly higher than to human erythrocytes (*W*-test: p = 0.007), which was determined by the greater tropism of APEC to bird erythrocytes.

When analyzing the relationship between the number of virulence genes and the degree of adhesion to two types of erythrocytes, dependences similar to those for antibiotic resistance were obtained. If in the groups of virulence genes common to all pathotypes and the APEC genes, the adhesion indices were approximately the same in strains with different numbers of detected genes, then within the IPEC gene group, the degree of adhesion to both types of erythrocytes was higher in strains with a smaller number of genetic determinants (Table 2). Separately, indicators of the adhesive activity of bacterial cells with *iss*$^-$ and *iss*$^+$ as well as *hlyF*$^-$ and *hlyF*$^+$ genotypes, which give strains with a positive genotype an advantage of survival during systemic coli infection and damage to erythrocytes, were analyzed. A tendency to an increase in MAI indices in *iss*$^+$ strains was revealed, 2.88(1.85-3.40) vs 2.14(2.00-2.47) and 2.07(1.67-2.23) vs 1 .83(1.47-

2.27) upon adhesion to human and avian erythrocytes, respectively. Similarly, the MAI indices for *hlyF*+ strains were higher in both models, while this difference turned out to be statistically significant with chicken erythrocytes, 2.35(1.93-2.27) vs 2.00(2.00-2.07) at $p \leq 0.01$.

2. The degree of specific adhesion of APEC (avian pathogenic *Escherichia coli*) strains isolated from the Ross 308 cross broiler chickens (*Gallus gallus* L.) of with generalized colibacillosis, depending on the genotype (n = 28, 2016-2018)

Group	AI	
	0-2 genes	3-4 genes
Genes found in various *E. coli* pathotypes	2.35 (2.00-2.54)	2.16 (2.00-3.410)
	2.23 (2.07-2.47)*	1.79 (1.47-2.00)
Genes of the APEC group (avian pathogenic E. coli)	2.20 (2.00-3.40)	2.24 (2.00-3.13)
	1.82 (1.51-2.20)	2.00 (1.75-2.23)
	0-3 genes	4-6 re genes
Genes of the IPEC (intestinal pathogenic E. coli) group	2.94 (2.47-3.66)*	2.00 (1.46-2.98)
	2.15 (1.86-2.60)*	1.79 (1.47-2.11)

N o t e. AI — adhesion index. - AIs for chicken erythrocytes are above the line, AIs for human erythrocytes.are below the line.
* Differences between the strains with different numbers of genes are statistically significant at $p \leq 0.05$ (*U*-test).

The APEC pathotype is considered relatively new in the classification of *E. coli*, and despite the active study of its representatives throughout the world, questions about the autonomy of this ecological group and its zoonotic potential remain open. Each of the described extra- and intra-intestinal pathotypes is a group of serotypes united by certain virulence factors. However, it should be noted that due to the plasticity of the *E. coli* genome, *Escherichia* subpathotypes cannot be conclusively identified, as some strains combine the main virulence characteristics of different groups and are considered potentially more virulent hybrid variants [18]. Phylogenetic analysis has shown that APECs have significant genetic similarity to dominant human ExPEC pathogens and, in addition, can be a source of ColV-localized genes or even whole plasmids for other ExPEC strains [10]. L. Zhao et al. [37] reported that the various UPEC and APEC genes had a similar tendency to be expressed in a mouse-avian cross-infection experiment. Through modeling of neonatal meningitis in rats, it has been shown that some strains of APEC are capable of causing meningitis in mammals, and possibly humans, and strains of NMEC cause colisepticemia in birds. These data support the hypothesis that APECs have zoonotic potential [38]. Currently, studies of *Escherichia* biodiversity are aimed, on the one hand, at searching for phylogenetic relationships between APEC representatives and strains of other pathotypes, and on the other hand, at assessing their potential pathogenicity (including epidemic danger) for humans. Considering that in our study all *E. coli* strains were isolated from parenchymal organs (spleen, liver, and kidneys), lungs, and internal part of the bones of a dead bird, they were regarded by us as the APEC pathotype and further genotyped.

It is known that there is not a single ExPEC pathogenicity factor associated exclusively with a specific disease or macroorganism, while the ability of opportunistic Escherichia to cause an infectious process in various biotopes of immunocompetent hosts is mediated by the presence of certain virulence determinants. According to J.R. Johnson et al. [39], a strain can be considered ExPEC if it contains two or more of the following virulence genes, the pap (P-pilus), *sfa/foc* (S/F1C-pilus), *afa/dra* (Dr, binding adhesins), *iutA* (aerobactin receptor) and *kpsM* II (group 2 capsule synthesis). The distribution structure of the ExPEC genes (see Table 2) made it possible to associate *E. coli* strains isolated during poultry colibacillosis with this group, except for two cultures. To classify a strain as belonging to the APEC group according to T.J. Johnson et al. [5], the presence of at least two marker genes in its genome is necessary. Twenty-five (89.3%) cultures

were assigned strictly to the APEC pathotype. Likewise R.R. Spurbeck et al. [40] proposed a set of four genes to identify ExPEC strains with uropathogenic potential. In our study, from the group of genes most characteristic of uropathogenic *E. coli* strains, only *upaG* and *usp* were revealed which are often found in the APEC genome [41, 42].

Comparison of the genetic and phenotypic characteristics of APEC and IPEC remains an area of extensive study in terms of identifying heteropathogenic and hybrid *Escherichia* strains [18]. In Russia, a new strain of *E. coli* serotype O101:H33 has been identified that exhibits properties and carries genes that are simultaneously characteristic of enterohemorrhagic and enterotoxigenic strains of *E. coli*, i,e., *stx2a*, eae_{γ}, *ehxA* and *est1* [19]. Hybrid strains phylogenetically located between Shiga toxin-producing *E. coli* and UPEC have also been described. They have been shown to possess pathogenicity factors characteristic of both pathogroups and are capable of causing both diarrhea and urinary tract infection [43]. Few works are devoted to comparison of the genotypes of avian pathogens and causative agents of acute intestinal infections in humans [44]. In our study, the presented sample of strains lacked the Shiga-like toxin (*stx1/2*) genes; nevertheless, 75% of the cultures were carriers of the *subAB* gene encoding the cytotoxin subtilase, which is characteristic of Shiga-toxin-producing *E. coli* strains — STEC [45]. STECs are known to synthesize two different types of cytotoxins, the StxI/II itself and cytotoxin subtylase (SubAB) which are structurally similar and consist of a single A subunit and a B subunit pentamer. Cytotoxin subtilase causes various cellular effects, including inhibition of protein synthesis, suppression of nuclear factor-kappa B activation, apoptotic cell death, and stress granule formation [46]. Intraperitoneal administration of purified subtylase cytotoxin to mice resulted in extensive microvascular thrombosis, as well as necrosis of the brain, kidneys, and liver, and was fatal for animals. Oral infection of animals with *E. coli* K-12 strain with cloned *subA* and *subB* genes caused dramatic weight loss in mice. These data suggest that subtilase cytotoxin may contribute to the pathogenesis of human diseases and become a novel toxic virulence marker in animal-derived *E. coli*.

Horizontal gene transfer is an important evolutionary mechanism in bacteria, which determines the complexity and plasticity of their genomes. It is known that opportunistic and pathogenic *E. coli* can originate from commensal strains after the acquisition of virulence-associated genes, which are usually located on the chromosome in certain regions called pathogenicity islands (PAI) [9, 47]. PAI may include genes for type III secretion system proteins, toxins, invasion factors, and iron uptake systems by which PAI can be identified [48]. In our study, we tested two PAI-determining genes, the *eaeA*, encoding the main adhesion factor intimin in the pathogenicity island LEE, locus enterocyte effacement in EPEC and EHEC strains, and *iutA*, encoding the aerobactin receptor in the pathogenicity island SHI-2 (Shigella pathogenicity island 2). The *eaeA* gene, which marks the enterocyte smoothing locus, was not found, while *iutA*, indicating the presence of one of the Shigella pathogenicity islands and designated as SHI-2 PAI, was detected in 42.8% of cultures, which also indicates their potential pathogenicity for humans. For example, 75% of $iutA^{+}$ isolates carried 10 or more virulence genes and were resistant to 5 or more antibiotics, while $iutA^{-}$ met these criteria only in 50.0 and 33.3% of cases. In addition, more than half of all strains had regions of conjugative plasmids, 60% of which were classified as pathogenic for birds and humans, which indicates the possibility of effective spread of pathogenicity determinants through horizontal transfer.

Plasmids pColV have long been associated with *E. coli* virulence, despite the fact that their eponymous trait, ColV bacteriocin production, is not considered a virulence trait [10]. Considering that the *iss*, *ompT*, *hlyF*, *repA* (RepFIB replication

protein) and *traJ* genes, encoding the sequences of putative pAPEC-O2-ColV virulence and transfer regions, were found in our APEC collection at a high frequency, it can be assumed that they were carriers of this plasmid. However, it should be noted that the pathogenicity of APEC does not directly correlate with the presence of other plasmids, as in some *E. coli* pathotypes [49]. For example, putative plasmid genes were widely distributed among both APEC and commensal chicken *E. coli* strains, and in the latter case the average number of plasmid genes per isolate was even higher than among APEC [9].

To identify the pathogenicity of a strain for humans, a quantitative method is used to determine the adhesion of bacteria on erythrocytes [36]. The ability of *E. coli* to attach to and agglutinate RBCs may be determined by mannose-sensitive fimbriae type 1. They are the most common type of bacterial adhesins and are expressed by both commensal and pathogenic strains of *Enterobacteriaceae*. Despite the common primary specificity of these pili for mannose, there is variation in the degree of adhesion between different species, as well as between different isolates of the same species. Thus, FimH of most fecal *E. coli* strains does not provide strong binding to receptors that contain terminal monomannose residues (Man1), however, some FimH variants of uropathogenic *E. coli* have a relatively high ability to bind Man1 due to the presence of functional point mutations at various positions in the FimH molecule [50]. In addition, mannose-resistant adhesins, designated Afa/Dr adhesins, recognizing the Cromer blood group system antigen, complement decay-accelerating factor, or CD55 (complement decay-accelerating factor; DAF) as a receptor, were found in IPEC and UPEC isolates. Interestingly, the AfaE adhesin expressed in E. coli isolates from various animals does not recognize human DAF, while the *afaE8* subtype, first identified in animal *E. coli* isolates, has subsequently been associated with human uropathogenic *E. coli* [51].

Apparently, the natural appearance of various variants of adhesins may reflect the ongoing adaptive molecular evolution of *E. coli* to improve the mechanisms of fixation in biotopes of various hosts. In this regard, we evaluated the adhesive phenotype of APEC using various erythrocytes to prove a possible selective advantage of target cells. The strains we studied are mostky classified as a low-adhesion group, regardless of the type of erythrocytes, while the average MAI when using avian erythrocytes turned out to be higher than that of human ones, which is directly related to the origin of the strains. Nevertheless, in 11 strains, the degree of adhesion did not depend on the type of erythrocytes, and in two cultures it was significantly higher on human than on avian cells. We should also emphasize that the indicators of adhesive activity of bacteria with the iss^+ and $hlyF^+$ genotypes were higher than in the group of strains that do not carry these genes, which gives them an advantage during systemic coli infection.

Thus, the vast majority of *Escherichia coli* strains isolated from the organs of broiler chickens with generalized colibacillosis were characterized as pathogenic for birds and humans, which indicates the potential of APEC as a reservoir of virulence factors for human infectious agents. Their genome simultaneously contained virulence genes characteristic of several pathotypes (with a predominance of APEC/IPEC hybrid pathotypes) while many APEC strains had an affinity with a group of diarrheagenic *Escherichia* in their genetic profile. Being epidemiologically dangerous for humans, they can realize their pathogenic potential to a greater extent due to toxin production genes and genetic determinants associated with general virulence than due to adhesion factors, and without connection with the antibiotic sensitivity profile. The specific adhesion of *E. coli* strains was more pronounced for chicken erythrocytes than for human ones. At the same time, regardless of the type of erythrocytes, high adhesive activity of bacteria correlated with

greater survival in the host's blood serum (*iss*$^{+}$ genotype) and the possibility of erythrocyte lysis (*hlyF*$^{+}$ genotype). The data we obtained on the molecular and adhesive properties of avian colibacillosis pathogens make it possible to assess their zoonotic potential and epizootic significance, and can also serve as the basis for improving the colibacillosis monitoring system in poultry farms.

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