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Molecular characteristics of fowl adenovirus strains detected in broiler chickens on diets without immunostimulant supplements

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Abstract

Introduction: Outbreaks of fowl adenovirus (FAdV) infection in chicken flocks in Poland threaten birds' health and lives and are rising in frequency. The risk of these infections in immunocompromised poultry flocks with developed clinical symptoms was analysed through virus detection in broiler chicks and correlation of cases with the birds' immune strength. **Material and Methods:** Samples were analysed from four broiler farms with chicks from the same hatchery in Silesia, Poland where feeding regimes were different. A normal diet was provided to birds on the control farm; a normal diet and probiotic, prebiotic, vitamin and microelement supplementation was supplied on another farm; a normal diet and antibiotics on the third; and a normal diet and both forms of supplementation were given on the fourth farm. Amplification of the virus DNA in a PCR with hexon gene L1 loop hypervariable region 1–4 primers determined the molecular characteristics of isolates of adenovirus strains obtained from necropsy tissue samples. The amplicon sequences were analysed, the pair-wise distances were determined, the maximum likelihood estimate for the gamma parameter for site rates was produced, Tajima's D neutrality test was run and the relative synonymous codon usage and transition/transversion bias were calculated. **Results:** Two species and two serotypes of fowl adenovirus – MW353018-FAdV-1/A-L-liver and MW353019-FAdV-5/B-I-intestine – were isolated in three-week-old broiler chicks on the control farm. **Conclusion:** Supplementation of broiler chicken flocks with probiotics, prebiotics, vitamins and microelements may have a significant beneficial effect on immunity and can prevent virus infection. The studies provided new information on the molecular characteristics of adenovirus strains isolated from chicks with a low level of immunity.

Keywords: adenovirus infection, antibiotic, chickens, immunity, supplementation.

Introduction

The microbiome in the gastrointestinal tract of broiler chickens has been extensively studied, and plays an important role in the health of the host. Poultry production operators can manipulate the microbiome to improve broilers' immune systems as well as the physiology of the digestive tract (2, 7, 8, 11). The microbiota processes may be affected by different factors: organ functions, chicken age, probiotics, prebiotics and microelements added as feed supplements and administration of antibiotics (4, 12, 13, 27).

Current commercial poultry base diets do not include any *Lactobacillus*, *Bifidobacterium*, *Pediococcus* or *Saccharomyces* probiotics and prebiotics. These are the species that are known to stimulate the immunity and strengthen the intestinal barrier of chickens against pathogenic bacteria (3, 7, 32). A study suggested that the use of a probiotic and prebiotic mix as a supplement three times during the broiler production cycle has a positive effect on the health of the broilers and decreases pathogen load with increasing immunity (32).

Poultry adenoviruses are major pathogens causing diseases or disease syndromes with a significant impact on the health and well-being of birds. It is known that strains with increased pathogenicity are among the variants of the fowl adenovirus (FAdV) type species and may be a source of infections which cause the characteristic clinical changes without the co-infections with other pathogens (5). Because these strains cause

serious concern worldwide, appropriate methods for identifying and differentiating FAdV strains have been developed, which enable a significant understanding of the mechanisms of their pathogenicity. The syndromes caused by adenoviruses in poultry include gizzard erosion and ulceration, the aetiological factor of which is the type species FAdV-1/A, which is of concern because in recent years the number of type 1 infections in Poland and other European Union countries has increased. In most cases, these infections affect broiler chickens aged three to five weeks.

In the context of the diverse research on poultry adenoviruses which has been and is being conducted around the world, the evaluation of pathogenic strains of FAdV type species 1/A and 5/B isolated from chicks from a flock with reduced immunity was the aim of the study. Lowered immune status in chickens is in most cases correlated with bacterial and viral infections (22). Therefore, this research was also undertaken to assess adenovirus infections in chickens from farms on which neither probiotics, prebiotics, vitamins, microelements nor antibiotics were used.

Material and Methods

Study design and farm description. Four Ross 308 broiler farms in the centre of the Silesia voivodeship of Poland were enrolled. The study indicated the same environmental conditions, normal diet feed supplier, water sources, supplying hatchery and husbandry conditions on each studied farm. The broilers on farm AO received antibiotics in drinking water and a normal diet. The chickens on farm P&AO were supplemented with probiotics, prebiotics, vitamins, microelements and antibiotics in their drinking water and ate a normal diet. Farm P chickens' drinking water was dosed with probiotics, prebiotics, vitamins and microelements, and their diet was the normal one; and on farm C chickens were fed the normal diet and given drinking water to which neither antibiotics nor supplements were added. This farm was the control. The broilers were examined on the 1st, 21st and 42nd days of life. The scheme of the supplementation with probiotic supplements and antibiotics was the same as that described by Tomczyk *et al.* (32).

Ethics committee approval. This study was approved by and conducted in accordance with the guidelines of the Local Ethics Committee. All samples were collected under permission in accordance with the institutional guidelines of this committee.

Adenovirus reference strain. The reference strain represented the type species FAdV-1/A and was provided by Charles River (Shrewsbury, MA, USA). It was used as a positive control in PCR studies.

Chicken embryo fibroblast (CEF) cell cultures. Cultures of CEF were prepared from 9–11 day-old specific pathogen–free chicken embryos (Lohmann, Ankum, Germany) according to a standard protocol for research conducted in the Department of Poultry Diseases at the Polish National Veterinary Research Institute. Modified Eagle's medium, 10% foetal bovine serum and 1% antibiotic mix (Antibiotic-Antimycotic; Gibco, Thermo-Fisher, Paisley, U.K.) were used. A monolayer of CEF culture was obtained after 24 h incubation at 37°C in an atmosphere of 5% CO_2.

Chicken samples. Samples from internal organs of n = 60 birds per flock were taken from each of the four broiler farms. Liver, spleen, gizzard and caecal tonsil samples were collected aseptically during necropsy examinations. Twenty chickens were examined per flock on day 1, day 21 and day 42 of life. Homogenates prepared from internal organs as a 1:1 dilution in modified Eagle's medium with an addition of 1% antibiotic mixture (Antibiotic-Antimycotic, Gibco) were passed through a 0.45 µm syringe filter (Minisart; Sartorius, Göttingen, Germany). Samples were preserved at −20°C for the next step of the studies.

Virus replication in CEF cultures and FAdV DNA amplification. Homogenates obtained from internal organs of experimental birds were used for CEF inoculation. Prepared CEF cultures were incubated at 37°C for 7 d in a 5% CO_2 atmosphere. The cytopathic effect characteristic for FAdV infection was observed and images of it were captured in daily microscopic examinations. The third and final passage of the examined strains was used for the analysis in the next step of the studies. DNA was isolated from the CEF cultures infected with the examined strains using a QIAamp Mini Kit (Qiagen, Hilden, Germany) as described in the manufacturer's instructions. The adenovirus FAdV-1 reference strain was the positive control. The negative control was DNA isolated from uninfected CEF cultures which had been stored at −20°C.

The amplification of the HVR1–4 hypervariable regions of the L1 loop of the hexon gene of FAdV was carried out in a PCR as described by Niczyporuk *et al.* (17). The reaction was performed in duplicate with a 25 µL volume of final reaction mix.

Sequencing and analysis of the amino acid sequences. The PCR amplicons were purified using NucleoSpin Extract II (Macherey-Nagel, Hoerdt, France) and Sanger sequenced by Genomed (Warsaw, Poland). Molecular analysis of the examined strain sequences was performed by the alignment of the nucleotide sequences of the amplified fragments originating from the hexon gene of two adenovirus field strains. The aligned sequences were compared with the twelve fowl adenovirus reference sequences obtained from the GenBank database sing the Basic Local Alignment Search Tool (BLASTn) from the National Centre for Biotechnology Information (NCBI) and MEGA version 11 software (31). Phylogenetic analyses were carried out and evolutionary associations of the nucleotides (nt) and amino acids (aa) sequences after the translation process were inferred and then the neighbour-joining method (23, 30) with 1,000 bootstrap replications was used. The nt and aa sequence

similarities were calculated using the MegAlign module of the Clustal W algorithm included in MEGA 11.

The evolutionary distances in the phylogenetic tree were calculated by the p-distance method. The molecular studies included two adenovirus sequences from the field strains and twelve adenovirus reference sequences. All ambiguous, absent or incorrect nt and aa positions in the constructed alignments were removed from each sequence pair before analyses. The phylogeny was analysed in MEGA 11.

A pair-wise distance method was applied to compute the phylogenetic distance between a given group of different adenovirus type species. Analysis of distance estimation was determined with the maximum-composite-likelihood method with substitutions including the transition/transversion rate. The composite likelihood was the sum of related log likelihoods.

Strain sequences. Twelve reference sequences were selected from the NCBI GenBank database for use in the analysis. These sequences' accession numbers were AF339915 (in the case of the 2/D sequence), FJ360747 (the 11/D sequence), AF339921 (6/E), AF339918 (8a/E), AF339922 (7/E), AF339924 (10/C), AF339919 (5/B), KT862811 (8b/E), AF339916 (3/D), AF339923 (9/D), AF339917 (4/C) and MK050972 (1/A).

Tajima's D neutrality test. This test was applied to the two field adenovirus nucleotide sequences and twelve nucleotide sequences of adenovirus reference strains.

Results

Farm characterisation and isolation of the strains. Fowl adenovirus strains were isolated from tissue specimens of 20 three-week-old Ross 308 broiler chickens from farm C that received neither probiotics, prebiotics, vitamins, microelements nor antibiotics. The virus strains were detected in the liver and intestines. No strains were detected in any tissue samples from farm P, where chickens received probiotics and prebiotics, farm P&AO where birds took probiotics, prebiotics and antibiotics, or farm AO where only antibiotics were administered (Table 1).

Clinical manifestation. The chickens from farm AO showed clinical signs connected with liver and intestine damage with mortality during fattening. The most typical clinical signs of infection caused by adenoviruses were seen on farm C. The foremost sign was depression and apathy, and additionally ruffled feathers, a drooping head and a crouched position were evident in infected chickens. In some cases nervous signs of infection and digestive tract lesions were observed. In necropsies, anaemic combs and wattles and reduced weight gain were also noted.

Anatomopathological changes. The necropsy showed petechiae and ecchymosis in the skeletal muscles, and hepatomegaly with a pale brownish-to yellowish colour and fragile tissue consistency were observed. Splenomegaly was also indicated.

Molecular adenovirus serotype identification. Two FAdV type species – FAdV1/A and FAdV-5/B – were detected in the clinical samples of examined birds. The hexon gene L1 loop HVR1–4 regions of the examined strains were submitted to GenBank with the accession numbers MW353018 of type 1/A and MW353019 of 5/B.

Pair-wise distances. The pair wise distance of the examined nt sequences was indicated as 1.285. Pair-wise distances are presented in Table 2.

Estimated pair-wise distances and the related substitution parameters are presented in Table 3.

Codon usage. The codon usage in the HVR1–4 region of the sequences of the hexon gene of the two field and twelve reference sequences were studied. Cytosine was the most frequent nucleotide amongst adenovirus type species, comprising between 24.1% and 32.3% of nucleotides. Cytosine appeared most often in the first position of the codon in every sequence, and the percentage range was 15.6%–36.9%. It appeared in the second position of the codon between 30.5% and 34.3% of instances, whereas in reference strain sequences it did so between 29.7% and 33.4%. Guanine was more frequent in the third position of the codon in each reference strain sequence (36.1%–48.0%) than in field sequences (25.7%–27.6%). Results for adenovirus sequences of field type/species comparison to other reference strain sequences are presented in Table 4 as codon locations on the $1^{st}+2^{nd}+3^{rd}+$ non-coding regions.

Relative synonymous codon usage. The relative synonymous codon usage values indicated the measure of gene expression level (16, 17, 21). The investigation of the synonymous codon usage in the examined region revealed differences in this region depending on the strain and adenovirus type species. The relative synonymous codon usage (RSCU) was estimated as the ratio of the indicated frequency of examined codons to the frequency expected in the synonymous codons for the same amino acids. This parameter is given following the codon frequency in Table 5. The average codon is equal to 1,303.

Maximum likelihood estimate of gamma parameter for site rates. The estimated value of the shape parameter for the gamma distribution was 1.3314. All substitution patterns in examined regions and rates of substitution were calculated with the Tamura–Nei model (+G) (29). The model's results are presented in Table 6. Each entry is the probability of substitution (r) from one base (row) to another (column). Rates of different substitutions which are transitional were indicated in bold. The substitutions are indicated in italics. Relative values named as instantaneous (r) should be considered as simplicity sum of (r) values that were made equal to 100. A gamma distribution was used to model phylogenetic rate differences among sites (5 categories (+G)). The mean evolutionary rates were 0.16, 0.44, 0.77, 1.23 and 2.49 substitutions per site. The nucleotide frequencies were 20.89% for A, 24.74% for T/U, 26.03% for C and 28.34% for G. For calculating maximum likelihood

(ML) values, a tree topology was constructed. The maximum log likelihood for the analysis was estimated as −4,809.387. A total of 500 positions were included in the final round are presented in Table 6. The table facilitated the maximum-likelihood estimation of the parameters and the variance-covariance matrix in examined fowl adenovirus strain sequences presented different type species.

Table. 1. The presence of FAdV strains in the examined Ross 308 broiler chicken flocks

Flock from farm	Age		
	1 day old	21 days old	42 days old
AO	not detected	not detected	not detected
P	not detected	not detected	not detected
AO&P	not detected	not detected	not detected
C	not detected	FAdVs 1/A and 5/B	not detected

AO – flock given antibiotics in drinking water; P – flock given probiotic and prebiotic additives with vitamins and microelements in drinking water; AO&P – flock given antibiotics and probiotic and prebiotic additives with vitamins and microelements in drinking water; C – flock of controls given neither antibiotics nor probiotics, prebiotics vitamins or microelements; FAdVs – fowl adenoviruses

Table 2. Pair-wise distance with overall mean distance

	MW353019-FAdV-5/B-I-Intestinum												
MW353018-FAdV-1/A-L-Liver	2.556												
	2.898	2.933											
	2.264	2.499	0.585										
	2.769	2.716	0.495	0.411									
	2.814	2.554	0.565	0.616	0.610								
	2.762	2.753	0.568	0.401	0.413	0.654							
	2.760	2.810	0.611	0.343	0.427	0.655	0.413						
	2.892	2.870	0.515	0.380	0.384	0.623	0.158	0.414					
	2.880	2.591	0.597	0.325	0.428	0.627	0.406	0.049	0.409				
	2.710	2.520	0.558	0.615	0.605	0.047	0.652	0.652	0.621	0.627			
	2.751	2.748	0.568	0.395	0.414	0.676	0.030	0.412	0.152	0.413	0.673		
	2.844	2.837	0.509	0.374	0.367	0.638	0.149	0.408	0.028	0.395	0.632	0.142	
	3.198	2.707	3.204	3.127	3.702	4.099	3.087	3.813	2.972	3.705	4.068	2.893	2.854

Table 3. Maximum composite likelihood estimate of the pattern of nucleotide substitution indicating differences in the nucleotide sequences of the examined strains

	A	T/U	C	G
A	-	*6.58*	*6.92*	**15.17**
T/U	*5.55*	**-**	**10.50**	*7.53*
C	*5.55*	**9.98**	-	*7.53*
G	**11.18**	*6.58*	*6.92*	-

Each entry shows the probability of substitution (r) from one base (row) to another base (column). The sum of r values is made equal to 100. Rates of different transitional substitutions are shown in bold and those of transversional substitutions are shown in italics

Table 4. Nucleotide codon composition of the 2 examined sequences and 12 sequences of adenovirus strains derived from the GenBank database

	T(U)	C	A	G	Total	T-1	C-1	A-1	G-1	Pos 1	T-2	C-2	A-2	G-2	Pos 2	T-3	C-3	A-3	G-3	Pos 3
1/A	24.7	27.5	18.1	29.8	896.0	33	18.4	24.7	24.1	299.0	30	33.4	14.7	22.1	299.0	11	30.5	14.8	43.3	298.0
2/D	26.0	24.9	21.0	28.0	899.0	32	16.7	24.7	27.0	300.0	33	32.7	16.0	18.7	300.0	14	25.4	22.4	38.5	299.0
3/D	25.6	25.3	19.2	30.0	908.0	31	16.5	23.4	29.4	303.3	35	29.7	16.5	18.8	303.1	11	29.8	17.5	41.7	302.0
MW353018-FAdV-1/A-L-Liver	**24.0**	**28.1**	**19.3**	**28.6**	**559.0**	**27**	**36.9**	**12.8**	**23.0**	**187.0**	**14**	**30.5**	**20.3**	**35.3**	**187.0**	**31**	**16.8**	**24.9**	**27.6**	**185.0**
MW353019-FAdV-5/B-I-Intestinum	**22.8**	**32.3**	**22.6**	**22**.2	**517.0**	**17**	**25.0**	**33**.3	**25.0**	**168.0**	**28**	**34.3**	**21.3**	**16.3**	**178.0**	**23**	**37.4**	**13.5**	**25.7**	**171.0**
4/C	23.4	26.1	17.8	32.7	884.0	34	15.6	27.7	28.1	295.0	29	30.5	18.3	21.7	295.0	7	32.3	12.2	48.3	294.0
5/B	25.9	25.7	18.8	29.5	902.0	32	16.6	24.3	26.9	301.0	31	32.6	16.9	19.9	301.0	15	32.3	15.3	41.7	300.0
6/E	27.5	24.1	20.9	27.6	896.0	33	17.4	24.4	25.1	299.0	33	30.8	18.1	18.1	299.0	16	28.0	20.1	39.6	298.0
7/E	25.1	26.1	18.6	30.3	902.0	32	17.9	23.6	26.9	301.0	32	30.2	16.3	21.3	301.0	11	24.2	16.0	42.1	300.0
8a/E	27.0	24.6	21.7	26.7	899.0	33	17.0	24.7	25.7	300.0	32	31.3	18.3	18.3	300.0	16	30.0	22.1	36.1	299.0
9/D	23.6	26.1	17.8	32.5	884.0	34	15.6	22.7	28.1	295.0	29	31.2	18.6	21.4	295.0	9	25.4	11.9	48.0	294.0
10/C	25.9	25.7	18.7	29.6	902.0	32	17.3	23.6	27.2	301.5	31	32.2	16.6	20.3	301.0	15	31.6	16.0	41.3	300.0
11/D	25.1	25.9	17.9	31.1	898.0	31	18.0	23.7	27.0	300.0	32	30.7	16.0	21.7	300.0	12	27.7	14.1	44.6	298.0
8b/E	25.9	25.6	20.3	28.2	898.0	32	17.0	24.7	26.7	300.0	32	33.3	16.0	18.3	300.0	14	29.2	20.1	39.6	298.0
Avg.	25.3	26.0	19.4	29.3	846.0	31	18.3	23.8	26.6	292.1	31	31.6	17.2	20.6	282.8	14	28.2	17.1	40.7	281.1

Pos 1, Pos 2, Pos 3 – number of nucleotides in the sequences tested in the first, second, and third codon positions, respectively. The total number of nucleotides of tested strain sequences and the number of them at the codon positions are indicated by underlining. Sequences obtained from the studies are indicated in bold

Table 5. Analysis of the number of successive codons and relative synonymous codon usage

Codon	Count	RSCU	Codon	Count	RSCU	Codon	Count	RSCU	Codon	Count	RSCU
UUU(F)	18	0.8	UCU(S)	21.9	0.84	UAU(Y)	9.6	0.63	UGU(C)	13.7	0.72
UUC(F)	26.9	1.2	UCC(S)	35.4	1.36	UAC(Y)	20.8	1.37	UGC(C)	24.4	1.28
UUA(L)	11.4	0.64	CA(S)	17.7	0.68	UAA(*)	12.5	0.84	UGA(*)	15.4	1.03
UUG(L)	29.2	1.63	UCG(S)	42.3	1.63	UAG(*)	16.8	1.13	UGG(W)	20.2	1.00
CUU(L)	10.9	0.61	CCU(P)	17.5	0.69	CAU(H)	11.3	0.99	CGU(R)	17.4	0.79
CUC(L)	23.6	1.32	CCC(P)	28.9	1.15	CAC(H)	11.4	1.01	CGC(R)	29.4	1.33
CUA(L)	7.8	0.43	CCA(P)	17.4	0.69	CAA(Q)	11.9	0.88	CGA(R)	18.2	0.83
CUG(L)	24.6	1.38	CCG(P)	37.1	1.47	CAG(Q)	15.1	1.12	CGG(R)	27.1	1.23
AUU(I)	11.3	0.86	ACU(T)	12.2	0.57	AAU(N)	9.9	0.65	AGU(S)	14.1	0.54
AUC(I)	19.8	1.51	ACC(T)	25.9	1.2	AAC(N)	20.6	1.35	AGC(S)	24.6	0.95
AUA(I)	8.2	0.63	ACA(T)	15.3	0.71	AAA(K)	17.9	0.94	AGA(R)	16.5	0.75
AUG(M)	22.1	1	ACG(T)	32.9	1.52	AAG(K)	20.1	1.06	AGG(R)	23.4	1.06
GUU(V)	15.5	0.73	GCU(A)	21.5	0.83	GAU(D)	11.4	0.68	GGU(G)	20.0	0.81
GUC(V)	25.6	1.21	GCC(A)	31.3	1.21	GAC(D)	21.9	1.32	GGC(G)	26.9	1.09
GUA(V)	14.4	0.68	GCA(A)	16.3	0.63	GAA(E)	19.6	0.94	GGA(G)	26.5	1.07
GUG(V)	29	1.37	GCG(A)	34.6	1.34	GAG(E)	22.1	1.06	GGG(G)	15.2	1.02

The average number of codons was 1,303. All examined frequencies are averages over all taxa and shown as percentages. RSCU – relative synonymous codon usage

Table 6. Maximum likelihood indicated in 24 different nucleotide sequences of the examined strains, with their substitutions indicated

Nucleotide sequence	Parameters	Substitution									
		BIC	*AICc*	*InL*	*(+I)*	*(+G)*	*R*	*f(A)*	*f(T)*	*f(C)*	*f(G)*
T92+G+I	29	9,817.521	9,619.014	−4,780.382	0.00	0.43	1.25	0.228	0.228	0.272	0.272
HKY+G+I	31	9,828.001	9,615.822	−4,776.768	0.00	0.41	1.29	0.209	0.247	0.260	0.283
TN93+G+I	32	9,830.888	9,611.875	−4,773.785	0.00	0.41	1.33	0.209	0.247	0.260	0.283
K2+G+I	28	9,853.291	9,661.621	−4,802.694	0.00	0.45	1.20	0.250	0.250	0.250	0.250
GTR+G+I	35	9,853.608	9,614.092	−4,771.865	0.00	0.42	1.33	0.209	0.247	0.260	0.283
T92+G	28	9,875.288	9,683.618	−4,813.693	n/a	1.33	1.01	0.228	0.228	0.272	0.272
KHY+G	30	9,887.801	9,682.458	−4,811.096	n/a	1.32	1.02	0.209	0.247	0.260	0.283
T93+G	31	9,893.237	9,681.058	−4,809.387	n/a	1.33	1.02	0.209	0.247	0.260	0.283
JC+G+I	27	9,902.143	9,717.311	−4,831.547	0.00	0.50	0.50	0.250	0.250	0.250	0.250
K2+G	27	9,905.582	9,720.750	−4,833.267	n/a	1.37	0.98	0.250	0.250	0.250	0.250
CTR+G	34	9,909.610	9,676.927	−4,804.292	n/a	1.32	1.02	0.209	0.247	0.260	0.283
JC+G	26	9,945.931	9,767.937	−4,857.868	n/a	1.41	0.50	0.250	0.250	0.250	0.250
T92	27	10,086.086	9,901.254	−4,923.519	n/a	n/a	0.98	0.228	0.228	0.272	0.272
T92+I	28	10,090.277	9,898.607	−4,921.187	0.01	n/a	1.02	0.228	0.228	0.272	0.272
HKY	29	10,103.858	9,904.644	−4,923.197	n/a	n/a	0.50	0.209	0.247	0.260	0.283
K2	26	10,104.355	9,925.864	−4,936.831	n/a	n/a	0.85	0.250	0.250	0.250	0.250
HKI+I	30	10,107.250	9,899.012	−4,919.373	0.02	n/a	0.87	0.209	0.247	0.260	0.283
TN93	30	10,108.001	9,901.711	−4,920.722	n/a	n/a	0.86	0.209	0.247	0.260	0.283
K2+I	27	10,111.923	9,922.418	−4,934.100	0.01	n/a	0.86	0.250	0.250	0.250	0.250
TN93+I	31	10,113.216	9,895.822	−4,916.768	0.02	n/a	0.87	0.209	0.247	0.260	0.283
GTR	33	10,111.923	9,886.075	−4,909.876	n/a	n/a	0.87	0.290	0.247	0.260	0.283
GTR+I	34	10,113.216	9,880.533	−4,906.095	0.02	n/a	0.87	0.209	0.247	0.260	0.283
JC	25	10,130.574	9,959.419	−4,954.616	n/a	n/a	0.50	0.250	0.250	0.250	0.250
JC+I	26	10,136.144	9,958.150	−4,952.975	0.01	n/a	0.50	0.250	0.250	0.250	0.250

Nucleotide sequence	Parameters	Substitution											
		r(AT)	*r(AC)*	*r(AG)*	*r(TA)*	*r(TC)*	*r(TG)*	*r(CA)*	*r(CT)*	*r(CG)*	*r(GA)*	*r(GT)*	*r(GC)*
T92+G+I	29	0.050	0.060	0.152	0.050	0.152	0.060	0.050	0.127	0.060	0.127	0.050	0.060
HKY+G+I	31	0.054	0.056	0.160	0.045	0.147	0.061	0.045	0.140	0.061	0.118	0.054	0.056
TN93+G+I	32	0.053	0.055	0.202	0.044	0.115	0.600	0.044	0.110	0.060	0.149	0.053	0.055
K2+G+I	28	0.057	0.057	0.136	0.057	0.136	0.057	0.057	0.136	0.057	0.136	0.057	0.057
GTR+G+I	35	0.055	0.043	0.201	0.047	0.116	0.049	0.034	0.111	0.080	0.148	0.043	0.073
T92+G	28	0.057	0.068	0.137	0.057	0.137	0.068	0.057	0.115	0.068	0.115	0.057	0.068
KHY+G	30	0.061	0.064	0.144	0.051	0.132	0.070	0.051	0.126	0.070	0.106	0.061	0.064
T93+G	31	0.061	0.064	0.168	0.051	0.112	0.070	0.051	0.106	0.070	0.124	0.061	0.064
JC+G+I	27	0.083	0.083	0.083	0.083	0.083	0.083	0.083	0.083	0.083	0.083	0.083	0.083
K2+G	27	0.063	0.063	0.124	0.063	0.124	0.063	0.063	0.124	0.063	0.124	0.063	0.063
CTR+G	34	0.052	0.050	0.168	0.044	0.112	0.061	0.040	0.107	0.098	0.124	0.053	0.090
JC+G	26	0.083	0.083	0.083	0.083	0.083	0.083	0.083	0.083	0.083	0.083	0.083	0.083
T92	27	0.061	0.073	0.126	0.061	0.126	0.073	0.061	0.106	0.073	0.106	0.061	0.073
T92+I	28	0.061	0.073	0.127	0.061	0.127	0.073	0.061	0.106	0.073	0.106	0.061	0.073
HKY	29	0.066	0.069	0.132	0.056	0.121	0.076	0.056	0.115	0.076	0.097	0.066	0.069
K2	26	0.067	0.067	0.115	0.067	0.115	0.067	0.057	0.115	0.067	0.115	0.067	0.067
HKI+I	30	0.066	0.069	0.133	0.056	0.122	0.075	0.056	0.116	0.075	0.098	0.066	0.069
TN93	30	0.066	0.069	0.151	0.056	0.105	0.076	0.056	0.100	0.076	0.111	0.066	0.069
K2+I	27	0.067	0.067	0.115	0.083	0.115	0.067	0.067	0.115	0.067	0.115	0.067	0.067
TN93+I	31	0.066	0.069	0.152	0.056	0.104	0.075	0.056	0.099	0.075	0.112	0.066	0.069
GTR	33	0.048	0.052	0.153	0.041	0.106	0.070	0.042	0.101	0.110	0.113	0.061	0.101
GTR+I	34	0.048	0.053	0.154	0.041	0.106	0.069	0.043	0.101	0.110	0.113	0.060	0.102
JC	25	0.083	0.083	0.083	0.083	0.083	0.083	0.083	0.083	0.083	0.083	0.083	0.083
JC+I	26	0.083	0.083	0.083	0.083	0.083	0.083	0.083	0.083	0.083	0.083	0.083	0.083

Table 7. Tajima's neutrality test results

m	*S*	p_s	ø	π	*D*
14	487	0.974000	0.306276	0.431275	1.846440

m – number of sequences; *S* – number of segregating sites; π – nucleotide diversity; *D* – Tajima test statistic; *p*s = number of segregating sites/ total number of sites (*n*); ø = number of segregating sites / total number of sites/a1

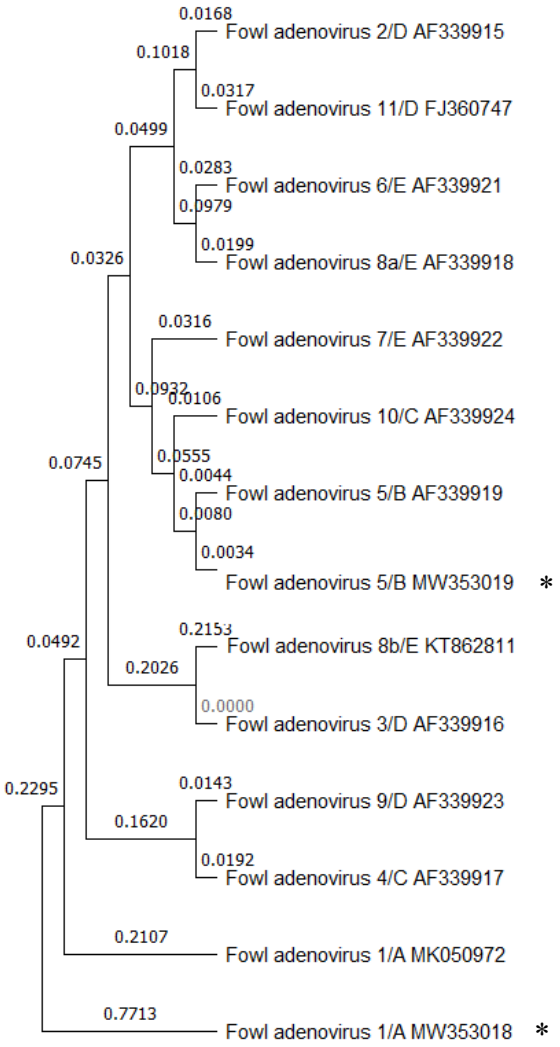

Fig 1. The constructed phylogenetic tree based on nucleotide sequences of Loop L1 region HVR1-4 of the hexon gene of fowl adenoviruses isolate obtained in the present investigation and submitted to GenBank
* – Polish strains of FAdVs

Maximum-likelihood fits of 24 different nucleotide substitution sites. Models with the lowest Bayesian information criterion scores were used to describe the substitution pattern (10). The Akaike information criterion value corrected, maximum-likelihood value, and the number of parameters (including branch lengths) are presented in Table 6. Non-uniformity of calculated evolutionary rates among sites may be modelled by using a gamma distribution (+G) with five rate categories and certain fraction of sites is evolutionary analysis invariable (+I). Applicable estimates of gamma shape parameter and the estimated fraction of invariant sites were indicated and are presented. Assumed or designated values of transcription/transversion bias (R) are shown. In the analysis, the frequencies (f) and rates of base substitutions (r) for each examined nucleotide pair were calculated. Relative instantaneous (r) values could be considered the evaluation. For straightforwardness, the sum of (r) values was made equal to 1 for each examined model. For estimating ML values, a tree topology was calculated. The analysis was based on 14 nucleotide sequences.

Tajima's D neutrality test. The results of Tajima's D neutrality test, the purpose of which was to identify sequences which do not fit the neutral theory model at equilibrium between mutation and genetic drift, are shown in Table 7.

Phylogenetic tree. The evolutionary comparisons on adenovirus sequences were created by using the neighbour-joining method by Saitou and Nei (23). The phylogenetic tree was created with the evaluation of branch length at 6.69858462 and was calculated by bootstrapping with 1,000 replicates. The phylogenetic tree was created with adequate branch lengths in the same units as those of the evolutionary distance used to estimate the phylogenetic tree. The evolutionary distances were calculated using the ML method (30) and are in the points of the number of substitutions per site. Sequence analysis HVR1–4 region of the L1 loop of the hexon gene was performed, and the obtained sequences MW353018-FAdV-1/A and MW353019-FAdV-5/B were found to be closely related. The first of these was closely related to the sequence of fowl adenovirus 1/A, MK050972, having 98% identity with it, and the second was closely related to the type species FAdV-5/B AF339919 with 99.8% identity (Fig. 1).

Discussion

A better understanding of the epidemiology of FAdV strains in poultry flocks is essential to limit the epidemic consequences of adenoviruses commonly present on farms worldwide (14–15, 17, 19, 24–26, 28). Adenovirus infections in poultry flocks in Poland are especially associated with inclusion body hepatitis and gizzard erosion and ulceration (16–18). However FAdVs can also be isolated from healthy chickens (1). In Poland during the last decade, many adenovirus type species were isolated, examples being FAdV-1/A (16, 20), FAdV-8a/E (17, 18, 20), FAdV-5/B, FAdV-6/E, FAdV-7/E, FAdV-8b/E (21), FAdV-2/11/D (18–20), FAdV-3/D and FAdV-10/C (15, 18).

The relative synonymous codon usage in the HVR1–4 region of the hexon gene in the sequences of the examined strains indicated that cytosine was the most frequent nucleotide for each adenovirus type species, ranging from 24.1% to 32.3%. Comparison with previous studies on broiler chickens indicated that the codon usage in the examined HVR1–4 regions of the hexon gene from examined sequences confirmed that

cytosine was the most frequent nucleotide sequence in field adenovirus type/species with the values ranging from 29.5% to 31.2% compared with sequences of reference strains which ranged from 25.1% to 29.3%, respectively (20).

The estimates of maximum likelihood of matrix substitution for the nucleotide frequencies were 20.89% for A, 24.74% for T/U, 26.03% for C and 28.34% for G for adenovirus strain sequences obtained from the examined flocks. Similarly, Niczyporuk *et al*. (20) reported the nucleotide frequencies to be 22.98% for A, 23.30% for T/U, 25.43% for C and 28.29% for G for chickens. Performing similar research, Patil *et al.* (21) indicated that codon usage and the differences in codon usage could be the results of natural selection or mutation pressure during the process of correct and efficient translation in the organisms they examined. This same conclusion was reached by Choudhury *et al.* (6) and Kimura *et al.* (9).

The percentage of similarity between reference strains of FAdV types (2/11) belonging to fowl adenovirus D and examined sequences were reported by Niczyporuk *et al.* (19). In studies conducted in Black grouse, fowl adenovirus strains were detected and the heterogenicity of examined strain sequences indicated the percentage of similarity between the reference and examined sequences. It was estimated as 93%, and the virus strains isolated were closely related to the type species FAdV-2/D (19). The pair-wise identity of the examined strain sequences of the hexon gene isolated from broiler flocks was calculated as 90.55% (20).

The adenovirus type species FAdV-5/B was isolated from samples from three-week-old broiler flocks exhibiting clinical signs associated with inclusion body hepatitis. Sequence analysis confirmed its 99% identity with the sequence logged in GenBank under number MT525095 and the reference strain 340 complete genome sequence of FAdV-5. Analysis of the nucleotide codon composition and total number of nucleotides of the tested strain sequence MT525095 at the first, second and third codons carried out by Niczyporuk *et al.* (20) gave data with some similarities to that of the examined strain sequence MW353019 obtained in the present studies. The average values presented for cytosine were the highest for the two compared strain sequences with the accession numbers MT525095 and MW353019 and were calculated as 29.7% and 32.3%, respectively. An identical situation was observed for adenine located in the first position at the first codon, which in both examined sequences was the highest, and the values of 31.3% and 33.3% were obtained, respectively. Also the highest values were noted for cytosine in both examined sequences in the second codon position at 43.1% and 34.3%, respectively. Differences were noted only for the third codon, where the most frequent in MT525095 was guanine with the value of 31.3% and in MW353019 was cytosine with the value of 37.4%.

Farm C had been tested twice before for the presence of adenovirus infection, and the results were negative. The birds came from the same hatchery and the same reproductive flock as birds from the AO, P and P&AO farms. It is probable that the immune status of chickens on the control farm, on which neither antibiotics nor probiotics, prebiotics, vitamins or microelements were applied, was the facilitator of adenovirus infection, which usually appears in flocks with low immune status. On farms where antibiotics, probiotics, prebiotics, vitamins and microelements were used – AO, P and P&AO – no adenovirus infection was recorded. These groups showed high immune status. Infections with other viruses that commonly occur in poultry flocks in the country and appear on farms where the immune status is lowered were also not recorded in these flocks.

Conclusion

When probiotic and prebiotic supplementation was provided to broilers in regular feeding, adenovirus strains were not detected. The chickens in the control group had detectable virus in their organs. The discovery of recombination of two different adenovirus types – FAdV-1/A and FAdV-5/B – was confirmed and new field strains with molecular evolution in the L1 loop HVR1–4 hexon gene region were investigated. These results suggested that the identified adenovirus strains, mainly from the FAdV-B/5 and A/1 species, were genetically more diverse than previously described adenovirus strains in Poland (15–20). Fowl adenovirus epidemiological studies are currently conducted in Poland and can help to prevent the circulation of infections caused by FAdV. The present studies provided new information concerning the base diet and positive effect of probiotics, prebiotics and vitamins on broiler chickens' immunity. Surveillance studies for fowl adenoviruses should be continued in the future.

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