OCCURRENCE OF ADENOVIRUS FIELD STRAINS IN BIRDS INFECTED WITH MAREK’S DISEASE VIRUS

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Abstract

The strains of adenoviruses were isolated from 356 birds with clinical form of Marek’s disease and coinfection with adenoviruses. A hexon gene fragment coding loop L1 of adenovirus strains was sequenced and obtained data were analysed with BLAST, Geneious 5.3, and MEGA5 software by comparison with nucleotide sequences of reference strains of fowl adenoviruses (FAdV-1 - FAdV-12), two turkey adenoviruses, and two goose adenovirus strains. On this basis, serotypes of adenovirus strains were determined. Sequences of all adenovirus strains isolated from birds infected with Marek’s disease virus were classified into six serotypes representing four species. Mostly FAdV-7, FAdV2/11, and FAdV-8a serotypes were found.

Key words: chickens, Marek’s disease, adenoviruses, phylogenetic analysis.

International Committee of Taxonomy of Viruses (ICTV) divided Adenoviridae family, due to host species, into four genera: Mastadenovirus, Aviadenovirus, Siadenovirus, and Atadenovirus (10, 18). Recently new genus Ichadenovirus was created (9). Avian adenoviruses were divided into three subgroups. Fowl adenoviruses belonging to subgroup I, subgroups II and III are composed of adenoviruses of turkeys, geese, and falcons. The subgroup I was classified into five species, designated A–E, including 12 serotypes, on the basis of antigenic characteristics and restriction profiles of BamHI and HindIII enzymes (2, 13, 20).

Avian adenoviruses, similar to other adenoviruses, are icosahedral, non-enveloped, dsDNA viruses with capsid 74-80 nm in diameter, constructed of 252 capsomers surrounding a core (2, 8). The main protein of the adenovirus capsid is hexon 93-950 aa long and with mass of 103 kDa, forming 240 trimers. This protein is composed of two conservative fragments P1 and P2, which form a structure of protein and take a role in creation of trimers. Variable regions of hexon protein form loops L1, L2, L3, and L4 (1, 25). Loop L1, flanked by region P1, is a fragment characterised by the highest variability of nucleotide sequence (hyper variable region, HVR) of 130 amino acids long. There are four HVRs in loop L1, two HVRs in L2, and one HVR in loop L4. Type specific antigenic determinants “ε” are coded by HVRs of loops L1 and L2 (25). Hexon protein is responsible for antigenic characteristics of adenoviruses. Nucleotide sequence of loop L1 is specific for each of 12 serotypes of fowl adenoviruses (25-27), whereas, fiber located on penton basis has a CAR adenoviral receptor taking part in virus adhesion to host cell (9).

Every year in Poland, the number of cases of adenovirus infections in birds is increasing. The pathogenic role of the aviadenoviruses is not entirely clear. Adenoviruses can be isolated from both sick birds, and birds without any signs of illness (15, 17). In one case they can cause asymptomatic infections, in another they are an aetiological agent of diseases such as inclusion body hepatitis – IBH (6, 19) or hydropericardium hepatitis syndrome – HHS (14). The viruses have been also isolated from birds with respiratory tract diseases (22), gizzard erosion and ulceration (GEU) (5, 21, 23), and pancreatitis (30). Adenoviruses can be the reason of immunodeficiency in young chicks, that can further lead to suppression of the immunity after the vaccination against Marek’s disease.

Marek’s disease (MD) is the first neoplastic disease in chickens subjected to prophylactic vaccination. During the immunoprophylaxis, vaccines containing attenuated serotype 1 of CVI988/Rispens strain of Marek’s disease virus and apathogenic serotype 3 of FC126 HVT strain are used. Numerous factors can affect prophylactic vaccination of chickens, such as: breeding conditions, feed, or infection with other pathogens. One of these factors may be the infection with adenoviruses, which frequently accompany other viruses and are widely spread among chicken flocks in the country. Yearly, an increase in infections of chickens with suppression of vaccinal immunity is observed.
Vaccinated birds display the clinical signs of Marek’s disease. The reason of vaccine suppression is an increased virulence of MDV. Because of occurrence of adenovirus isolation in such cases, it is possible that adenovirus strains also disturb the effectiveness of vaccination of chickens in Poland.

The aim of this study was the identification and molecular characterisation of adenovirus strains isolated from birds with clinical form of Marek’s disease.

**Material and Methods**

**Birds.** The birds were sent to our Department for diagnostic examinations between 2009 and 2011 from different regions of Poland. 356 chickens from 32 different flocks of broilers and egg layers between 2 and 33 weeks of age were examined. They had many dysfunctions and disorders, like neurological signs, disturbances in the alimentary and urinary tracts, and dysfunctions in respiratory system. During the post-mortem examinations, the pathological changes were observed in the liver, spleen, intestines, and gizzards. In some cases, the liver and spleen were enlarged with small necrotic focuses. In these birds the simultaneous infections with adenoviruses and Marek’s disease virus were confirmed.

**Samples for the examinations.** Samples of the liver, intestines, kidneys, and gizzard from the birds were collected and stored at -20°C

**Chicken embryo kidney cell cultures (CEK).** CEK were prepared from 18-19-day-old SPF chicken embryos (Lohman, Germany) according to the standard procedure. Eagle’s growth medium (MEM) was used with addition of 10% of bovine serum and 0.1% of antibiotic mixture (Antibiotic–Antimycotic, Gibco). Maintaining medium consisted of MEM with 0.1% of antibiotic-antimycotic mixture. A monolayer of CEK culture was received after 24 h incubation at 37.5°C.

**Reference strains.** The reference aviadenovirus strains belonging to the serotype FAdV (1-12) were acquired as a lyophilisate from a commercial company (Charles River Laboratory, USA). The strains were replicated in CEK cultures. When the CPE effect was complete, the cells and the supernatant were transferred to the tubes.

**DNA isolation.** Whole cell DNA was isolated directly from the liver, intestine, kidney, and gizzard cells. The isolation was performed using a DNA Mini Kit (Qiagen, Germany) according to manufacturer’s procedure. Isolates of DNA were preserved at -20°C for the next step of the study.

**PCR positive control.** Whole cell DNA as positive control was obtained from the reference strain CELO FAdV serotype 1 and propagated in CEK cell cultures. The negative control was the whole cell DNA isolated from non-infected CEK cells.

**Primers.** The specific oligonucleotide primers were designed using the GeneBank database and Primer3 programme and were used for PCR amplification of the loop L1 of the hexon gene of all 12 adenovirus serotypes from the genus Aviadenovirus. The primers were synthesised in the Institute of Biochemistry and Biophysics PAN in Warsaw. The sequence of nucleotide primers were: FAdV F JSN (sense primer): 5’AATGTCACNACCGARAAGGC 3’ and FAdV R JSN’ (antisense primer): 5’CDBGCTRACATGACTGTGTAA 3’. The expected product had predicted size of 830 bp.

**PCR.** The amplification procedure was performed in a final volume of 25 μl containing 2.5 μl 10x PCR buffer, 1 μl of dNTP (at 10 nM concentration), 1.5 μl of primer FAdV JN-F, 1.5 μl of primer FAdV JNR (each primer in 10 μM stock), 1.0 μl of polymerase finish enzyme (5U/μL), 4 μl of O Solution, and 2.0 μl of DNA sample (positive control in control samples, negative control in negative samples, DNA samples obtained from the liver, intestines, kidneys, and gizzard), and 11.5 μl of sterile water. After pre-denaturation at 95°C for 5 min, the denaturation was performed at 94°C for 45 s, the primers annealing was at 55°C for 1 min, the product elongation was at 72°C for 2 min, and the final elongation at 72°C for 10 min. Thirty-five replication cycles were performed. The reaction of amplification was conducted in basic gradient thermocycler (Biometra, Germany).

**Analysis PCR product.** After the amplification, the electrophoresis was carried out in 2% agarose gel with 1 μg/mL of ethidium bromide. The electrophoresis process was conducted in a tris borate EDTA buffer, pH 8.2, (150 V and 80 mA) for 50 min in Mini Sub-Cell (Biorad). After the electrophoresis, the size of amplification products was compared with the DNA Mass Ruller 1.031 bp (Fermatas). The results were visualised using a transilluminator UV, then photographed, and analysed. The results were positive if the received DNA product had predicted size of 830 bp for the pair of nucleotide primers.

**Sequencing and phylogenetic analysis.** After the reaction of amplification, the PCR products were prepared with a NucleoSpin Extract II (Marcherey-Nagel, France) and then sequenced using the GS FLX/Titanium sequencer (Roche, US) in a commercial company GENOMED (Poland). The phylogenetic analysis was performed by comparison between the nucleotide sequences of the amplified fragments of field adenovirus strains FAdV hexon gene and the aviadenovirus reference sequences from GeneBank database identified according to the general standard classifications. Additionally, the serotype consensus sequences were prepared from sequences with high homology with reference strains. In dendrogram, the sequences of the adenovirus reference strains, which occur in different bird species (turkey, goose, falcon, and pigeon) were used. The analyses were performed using the computer programmes MEGA5, Geneious 5.3, and BLAST. On this basis, the calculations of the phylogenetic tree and the relationship between the examined adenovirus strains were created.

**Results**

The presence of adenoviruses genetic material was confirmed in internal organs of 32 birds (8.98%) out of 356 birds with confirmed Marek’s disease. The
serotype FAdV-7 was determined in 13 chickens, serotype FAdV-2/11 in seven samples, serotype FAdV-8a in six samples, serotype FAdV-4 in three samples, serotype FAdV-5 in two samples, and serotype FAdV-5/8 in one chicken. All results are shown in Table 1.

The oldest bird in which adenovirus infection was determined was 33-week-old hen. From this bird two field adenovirus strains, described as 119/10j, isolated from the intestines and classified as serotype FAdV-7 (species E), and strain 119/10w isolated from the liver tumour and classified as serotype FAdV-2/11 (species D), were obtained. During the study, the sequences of the region L1 of hexon gene, which encodes characteristic variable fragments HVRs responsible for specific epitopes of hexon protein in all adenovirus serotypes, were compared (Table 2).

For the phylogenetic analyses, the ~830 nucleotide fragment, corresponding to the section of 18589–19175 of serotype FAdV-1 (Accession No. U46933) was used. The phylogenetic distance was determined by the neighbour-joining (NJ) method. The dendrogram of consensus sequences performed using MEGA5 software was presented in Fig. 1, whereas combination of adenovirus sequences similarity with the reference strains was shown in Table 2. Analysis of DNA derived sequences allowed to determine the species and serotypes of isolates.

All the adenovirus field strains isolated from birds with Marek’s disease virus infection and subjected to phylogenetic analyses were divided into five different branches. One of the groups was formed by seven strains classified as adenovirus species D. The similarity of the examined sequences of this group with the reference sequences of strain FAdV-11 C2B (Accession No AF508959) and strain 2 ATCC VR-827 P7-A (Accession No. AF339915), which were classified by ICTV as species D, was 92.1%. Sequences in this group were too high to allow exact serotype differentiation.

The separate branch of the dendrogram was formed by the reference strains representing species B, strain 340 (Accession No. AF508952) and FAdV-5 strain TR22 (Accession No. AF508953). The similarity of these strains was 96.1%. Another separate branch was created by one strain with the sequence closely homologous to the reference FAdV-5 strain ATCC VR-830 T8-A (Accession No. AF339919). Interestingly, the reference strain 746, representing the serotype FAdV-8b, showed simultaneously a high similarity to these strains. The homology in this group was 97.3% (Table 2 and Fig. 1).

Thirteen strains created a separate branch with sequences of high similarity (89.4%) to reference strains in European and ICTV classification, serotypes FAdV-7 YR36 (Accession No. AF508955) and B-3A (ATCC VR-832, Accession No. AF339922).

The group of six strains, which have been classified as serotype FAdV-8a together with reference strain FAdV- 8a TR59 (Accession No. EU979374) created next branch and had 90.3% homology.

Strains 31/10z, 64/10j and 62/10z created separate branch with the reference FAdV-4 strain KR5 (Accession No. EU 979370) representing species C in ICTV classification with high similarity (96.1%).

The results of the phylogenetic analysis have shown that, adenovirus strains, which were isolated from the birds with Marek’s disease virus infection, were clearly divided into separate groups. The strains classified as serotypes FAdV-7 and FAdV-8A are closely related and formed cluster of adenoviruses from species E. Similar to them are species B of adenoviruses in next cluster. The obtained results are in accordance with ICTV classification of the adenoviruses into five different fowl adenovirus species A-E.

During the study, five different reference sequences of hexon gene were used: reference strain of falcin adenovirus 1 (Accession No. DQ460220), goose adenovirus 4 P29 strain (Accession No. JF510462), goose adenovirus 5 D1036/08 strain (Accession No. JQ178217), pigeon adenovirus 1 IDA4 strain (Accession No. FN824512), and two reference strains of turkey adenoviruses: D90/2 (Accession No. GU936707.2) and TAV-2 (Accession No. GU936708.1). Strains formed separate branches, which indicates the differences in nucleotide sequences in the hexon genes. Different branches were created by the fowl adenovirus reference strains of serotypes FAdV-3 and FAdV-9, falcin adenovirus 1, and goose adenovirus 4.

Next, a comparison of sequences of all examined strains was made. Similarity between these sequences was 62.4%.

**Discussion**

The analysis of the avian adenovirus fragment L1 of the hexon gene allowed to classify strains derived from clinical cases of Marek’s disease, and to determine their relationship with the reference strains of avian adenoviruses. Because of the three different classification systems, which are not identical in the serotype numeration, comparison between adenovirus strains originating from different continents can be very difficult. It is remarkable that serotype FAdV-11 in different classifications is classified as fowl adenovirus species C, D, or E (7, 12). In the ICTV classification, serotype FAdV-12 does not exist, and serotype FAdV-8 is divided into two serotypes: FAdV-8a and FAdV-8b (7, 10, 12). These classifications are based on the RFLP method and whole genome sequencing of serotype FAdV-1 CELO adenoviruses (17). In this paper, ICTV classification was used.

The L1 fragment of the hexon gene is a domain of the highest variability and is responsible for determination of the antigenic properties of the virus (2, 4, 25). Therefore, this region is very useful for the studies on the phylogenetic typing and genotyping of the adenoviruses, and it was a target for the primers we designed.

Emry et al. (3) in their study pointed out the possibility of cross-reaction between serotype FAdV-4 and serotype FAdV-10, which was confirmed by the AFLP method and suggested to include both serotypes in serotype FAdV-4.
Fig. 1. Phylogenetic tree based on nucleotide sequences of hexon gene – loop L1 of isolated strains of adenoviruses. References strains: fowl adenoviruses and two strains of goose, two strains of turkey, and one strain of pigeon and falcon.
### Table 1
Occurrence of the different strains of adenovirus in internal organs of birds

<table>
<thead>
<tr>
<th>Internal organs</th>
<th>Number of strains</th>
<th>Species (ICTV)*</th>
<th>Serotype</th>
</tr>
</thead>
<tbody>
<tr>
<td>liver</td>
<td>9</td>
<td>E</td>
<td>FAdV-7</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>B</td>
<td>FAdV-8a</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>D</td>
<td>FAdV-2/11</td>
</tr>
<tr>
<td>intestine</td>
<td>4</td>
<td>E</td>
<td>FAdV-7</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>D</td>
<td>FAdV-2/11</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>E</td>
<td>FAdV-8a</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>B</td>
<td>FAdV-5</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>C</td>
<td>FAdV-4</td>
</tr>
<tr>
<td>gizzard</td>
<td>2</td>
<td>C</td>
<td>FAdV-4</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>B</td>
<td>FAdV-2/11</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>E</td>
<td>FAdV-5/8</td>
</tr>
<tr>
<td>kidney</td>
<td>1</td>
<td>D</td>
<td>FAdV-2/11</td>
</tr>
</tbody>
</table>

* ICTV - International Committee of Taxonomy of Viruses

### Table 2
Homology of isolated strains of adenoviruses with the reference strains.

<table>
<thead>
<tr>
<th>Serotype</th>
<th>Reference strains *</th>
<th>Species (ICTV)**</th>
<th>Homology (%)</th>
<th>Number of examined strains</th>
</tr>
</thead>
<tbody>
<tr>
<td>FAdV-2/11</td>
<td>2 ATCC VR-827 P7-A, 11 C2B</td>
<td>D</td>
<td>92.1%</td>
<td>7</td>
</tr>
<tr>
<td>FAdV-4</td>
<td>4 KR5</td>
<td>C</td>
<td>96.1%</td>
<td>3</td>
</tr>
<tr>
<td>FAdV-5</td>
<td>5340, 5 TR22</td>
<td>B</td>
<td>94.0%</td>
<td>2</td>
</tr>
<tr>
<td>FAdV-5/8</td>
<td>5 ATCC VR-830 T8-A</td>
<td>B</td>
<td>97.3%</td>
<td>1</td>
</tr>
<tr>
<td>FAdV-7</td>
<td>7 ATCC VR-832 B-3A</td>
<td>E</td>
<td>89.4%</td>
<td>13</td>
</tr>
<tr>
<td>FAdV-8A</td>
<td>8a TR59</td>
<td>E</td>
<td>90.3%</td>
<td>6</td>
</tr>
</tbody>
</table>

* sequence of the reference strains derived from the database of the GeneBank
** ICTV - International Committee of Taxonomy of Viruses

In such case, L1 sequencing can be a method of choice. Unfortunately, during our study we did not isolate the serotype FAdV-10. Still it is difficult to differentiate between all of serotype FAdV4 isolates and FAdV-10 reference strain used in comparison. Evaluation of the variability of the adenovirus strains and their phylogenesis can be useful for future investigation and prediction of cross-reactivity between serotypes (3).

It is possible that one bird can be infected by two different adenovirus serotypes (17). This situation occurred in the case of isolates 119/10j and 119/10wz from 33-week-old hen having a mixture of two serotypes FAdV-7 and FAdV-2/11. The obtained results indicate that this is a mixture of genomes of two different serotypes and it can affect the sequencing process, which is difficult or impossible to detect. This situation can be confirmed by the results DNA sequencing of the mixture of two serotypes FAdV-1 and FAdV-5 obtained from one bird (19).

During the study, the presence of adenovirus strains in birds was confirmed in 32 birds out of 356 examined, in which Marek’s disease was identified. The phylogenetic analyses of adenovirus field strains, which were isolated from birds with Marek’s disease virus infection allowed us to determine the presence of six serotypes representing four different species of adenoviruses in poultry. Mostly strains of the serotype FAdV-7 and serotype FAdV-2/11 were determined. Only one strain of the serotype FAdV-5/8 was isolated. Fowl adenovirus species E and D were the most common, whereas species A, B, and C were rarely found. The fact that the adenovirus infections were accompanied by Marek’s disease may indicate the possibility of influence of adenoviruses on the efficiency of the vaccinations or accidental of adenoviral infection in the examined birds.

In our study, the estimated homology of nucleotide sequences between strains belonging to the same species was 89.4%-93.7% (Table 1). However, the similarity of nucleotide sequence of all examined strains was 62.4%. Raue *et. al.* (24) determined the level of the similarity between serotype FAdV-1 and serotype FAdV-10 hexon protein to be 76%. The adenovirus strains, which were isolated from birds with Marek’s disease, represent four species and six serotypes. Regardless of a high intraspecies homology between the examined strains, we can indicate the clear differences between the species. It is possible that birds can be infected with different serotypes of adenoviruses during...
the Mareks disease virus infection, which gives similar effect to the host organism. The significant differences between poultry and goose adenoviruses and other avian adenoviruses can indicate a low probability of simultaneous infections with the adenoviruses other than fowl adenoviruses.

The pathogenic role of adenoviruses is not well defined. They can exploit opportunities presented when the health of the bird is compromised by coinfection with other pathogens. Results of our study indicate that the adenovirus infections commonly exist as a accompanying infections in birds with MDV. This is the first study on this subject performed in Poland.

Study on the occurrence of adenoviruses and simultaneous infections in poultry flocks will be proceeded.

References