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First molecular detection and characterization of fowl adenovirus in commercial broilers in Serbia

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ABSTRACT

The present study was conducted to reveal and identify the epidemiological features of the fowl adenovirus species circulating in the Belgrade city area. The research included clinical examination, necropsy, pathohistology, enzyme-linked immunosorbent assay (ELISA), and PCR analysis. Clinically, general weakness, increased conversion, and poor growth were observed. Postmortem examinations showed multiple necrotic foci in the liver, and swollen and pale kidneys. Pathohistological examination of the liver revealed hepatocellular necrosis, vacuolar degeneration of hepatocytes, cholestasis, and hyperemia. Serological analysis of 210 blood sera confirmed seroconversion in 156 (74.28%) samples against FAdVs, and depending on the farm, it ranged from 23.33 to 100%. Using the PCR method, 105 cloacal swabs were analyzed to determine the virus genome found in 31 (29.52%) samples. Hexon gene sequence analysis identified two species: fowl aviadenovirus D (serotype FAdV-2, FAdV-11) and fowl aviadenovirus E (serotype FadV-8b). Those species are closely related to strains circulating in Hungary, Turkey, and other countries. Our findings suggest that FAdV strains are significantly prevalent and could be an emerging pathogen in Serbia.

Key words: broilers; fowl adenovirus; epidemiology; Serbia

Introduction

Fowl adenovirus is widespread and covers different geographical locations worldwide, where the virus appears to infect chickens and various other avian species (SCHACHNER et al., 2018; HESS, 2020). Fowl adenoviruses (FAdVs) cause a variety of diseases in chickens, such as inclusion body hepatitis (IBH), hydropericardium hepatitis syndrome (HHS), and adenoviral gizzard erosion (AGE) (HESS, 2017; CIZMECIGIL et al., 2020). According to the International Committee on Taxonomy of Viruses (ICTV), FAdVs are double-strand DNA viruses that belong to the genus *Aviadenovirus,* within the family *Adenoviridae* (BENKO et al., 2022). They are divided into five species (fowl aviadenovirus A to fowl aviadenovirus E - FAdV-A–FAdV-E) and further

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subdivided into 12 different serotypes (FAdV-1 to -8a, and -8b to -11). Within the same genus of *Aviadenovirus* there are other bird-specific species (Turkey Aviadenovirus B, C, and D, Pigeon Aviadenovirus A and B, Duck Aviadenovirus B, Goose Aviadenovirus A, Psittacine Aviadenovirus B and C, and Falcon Aviadenovirus A) (BENKO et al., 2022). The capsid structural proteins are hexon, penton, and fiber. The most abundant viral surface protein is hexon, thus it is used for serotyping FAdVs. (OJKIC et al., 2008; CHEN et al., 2019).

Adenoviruses can be transmitted vertically and horizontally (HESS, 2020). The vertical pathway is important, as parent birds can be subclinically infected and serve as reservoirs of the virus for progenies. In progenies, the virus can cause severe disease and high economic losses (GRAFL et al., 2012; HESS, 2020).

The syndrome of Inclusion Body Hepatitis (IBH) has been associated with several serotypes of fowl adenoviruses: species FAdV D (serotypes -2, -3, -9, and -11) and species FAdV E (serotypes -6, -7, -8a, and -8b), which show their relatively close molecular relationship and similar features of pathogenesis (OJKIC et al., 2008; SCHACHNER et al., 2018; MOHAMED and EL-SABAGH, 2023). There are reports about outbreaks in many countries, and an increasing number of published articles have been recorded focusing on field outbreaks, especially in the last few years (SCHACHNER et al., 2021; MOHAMED and EL-SABAGH, 2023). According to the literature data, the manifestation of IBH is related to concurrent infections with immunosuppressive pathogens, such as chicken anemia virus (CAV), infectious bursal disease virus (IBDV), reovirus infections, and Marek's Disease virus (MD) (HESS, 2020; NICZYPORUK et al., 2021). An increased number of IBH cases were also connected with mycotoxins in feed, as well as a climate variation (HESS, 2017; SCHACHNER et al., 2018). However, certain findings suggested that some FAdV serotypes were pathogenic enough and had the primary role in the IBH occurrence without predisposing factors, which was attributed to the diverse pathogenicity of FAdV (MIRZAZADEH et al., 2021). IBH syndrome usually affects young birds up to five weeks of age, but it has been reported in birds as young as seven days old, and 20 weeks old (HESS, 2017; SCHACHNER et al., 2018). Confirmed infection at an early age is usually associated with vertical transmission from the parent flocks (GRAFL et al., 2013). It has been reported that fowl adenovirus induced immunosuppression and compromised the immunological capabilities of broilers (SCHACHNER et al., 2018; ZHAO et al., 2020). It is noteworthy that FAdVs potentially reduce humoral response during regular vaccination programs, even in subclinically infected birds (NIU et al., 2017; MALETIĆ et al., 2023). Clinically, the disease is characterized by a sudden onset of mortality, which varies from 2% to 30% (OJKIC et al., 2008; ZHAO et al., 2020). The pathogenicity of FAdV and clinical signs depend on the age of the chickens, and the route and timing of infection (ZHAO et al., 2020). Affected chickens show lethargy, huddling with ruffled feathers, and inappetence (HESS, 2017). The pathomorphological changes include a pale, swollen, and friable liver, with pale necrotic foci, a color-changed pancreas with petechial hemorrhages, swollen and pale kidneys, and a mottled spleen (GRAFL et al., 2012; ZADRAVEC et al., 2013; CIZMECIGIL et al., 2020; NICZYPORUK et al., 2021).

There is no conclusive evidence about the occurrence of FAdV infections in the population of broiler chickens in Serbia, so we found it essential to study its fundamental role in clinically overt situations.

This study aimed to investigate the seroprevalence, pathological lesions, and molecular characterization of different FAdV strains confirmed in affected broiler flocks in Serbia.

Materials and methods

Birds and sample collection. During 2021, lower productivity and health disorders were reported from seven commercial broiler flocks with a size of 10,000 to 25,000 chickens in different parts of the Belgrade city area (Central Serbia). Birds were of two different proveniences and originated from two breeder farms. Also, the farms were using feed from different feed mills. The first visit was undertaken

between three and four weeks of age of chicken. During the visit, in order to take as many samples as possible and test all the farms, 105 cloacal swabs were collected (15 samples per farm) from apparently healthy birds for potential detection and molecular identification of FAdV. A second visit was undertaken at the age of slaughter (42 days old) when the birds were regularly sampled to assess antibody response following vaccination against the Newcastle disease virus, and at the same time checked for serological analysis against FAdV. The blood samples for serological analysis were collected from the wing veins of 30 broilers per farm. Carcasses of 25 recently dead birds were taken for necropsy.

Detection and molecular identification of FAdV. Cloacal swabs were immersed in 1 ml of sterile PBS and thoroughly vortexed. The suspensions were centrifuged for 10 min at 4,000 rpm, and the decanted supernatants were used for DNA extraction (IndiSpinPathogen Kit, Indical, Germany). PCR was completed using the commercial HotStarTaq Master Mix Kit (Qiagen, Germany). The reaction mix was composed of 2 µl template DNA, 10 µl 1x HotStar Master Mix, 0.6 µl of each primer (10 µM), as previously published by MEULEMANS et al. (2001), and 6.8 µl RNase-free water. Amplification of the partial hexon gene was accomplished using a Mastercycler, Eppendorf (Germany), with the temperature profile as follows: initial denaturation at 95°C for 15 min, 40 cycles of denaturation at 94°C for 20 s min, annealing at 62°C for 20 s, and elongation at 72°C for 30 s, and single step of final extension at 72°C for 10 min. PCR products were analyzed in a 2% agarose gel stained by ethidium bromide, and visualized under UV light after electrophorese at 60 V for 1 hour. PCR products showing specific amplification of 897 bp were purified using a GeneJET PCR Purification Kit (ThermoFisher Scientific), and sequenced in Macrogen Europe, The Netherlands, using the Sanger method. The consensus sequence was constructed using the Staden package 2003 and submitted to NCBI GenBank under accession numbers OM858813-OM858818. Phylogenetic analysis was performed using Mega X, the Neighbor-Joining method, and the bootstrap test (1000).

Serological analysis. Serology analysis was performed using a commercial antibody test, an indirect ELISA test for avian adenovirus (Fowl Adenovirus 1 Antibody test kit, BioChek Netherlands), according to the manufacturer's instructions. The ELISA test detects group-specific antibodies.

Pathology examination. After the necropsy of 25 broiler chickens, the samples of liver tissue were subjected to histopathological examination and fixed in 10% buffered formalin, routinely processed and embedded in paraffin blocks. Paraffin sections about five µm thick were stained using the hematoxylin-eosin (HE) method.

Results

Out of 105 cloacal swab samples, 31 samples (29.52%) tested positive for the FAdV genome by PCR (Table 1). The amplified fragments of the hexon gene were 870 bp in length (Fig. 1).

Nucleotide sequences of good quality were obtained for six FAdVs strains that were detected from farms 1, 2, 3, 4, 5, and 7, respectively. By analyzing nucleotide sequences by subjecting them to BLAST searches against reference genomes in the NCBI, it was confirmed that the fowl adenoviruses detected belonged to serotype 2 and serotype 8b. OM858813-OM858817 matched with high scores with fowl adenovirus D and OM858818 with fowl adenovirus E (Fig. 2).

Serological testing of 210 blood samples confirmed seropositive samples for fowl adenovirus on all farms. The seropositivity rate differed within farms, with a range of 23.33% - 100%, and the average seropositivity at the farm level was 74.28% (Table 1). According to the analysis of good quality sequences, on six farms the exact FAdV species were detected: on farms labeled as 1, 2, 3, 4, and 5 species of fowl adenovirus D were detected, and on the farm labeled as 7 fowl adenovirus E species was detected (Table 1).

In the necropsied chickens, degenerative and necrotic changes in the liver were observed. The liver was pale, friable, and enlarged, with focal or diffuse areas of necrosis. Also, multifocal subcapsular liver hemorrhages were observed.

Fig. 1. Agar gels electrophoresis of PCR

On the right side are the numbers of bp, and above the numbers of FAdV positive samples, from lines 1 – 31 (The length of bands is 870 bp of the hexon gene)

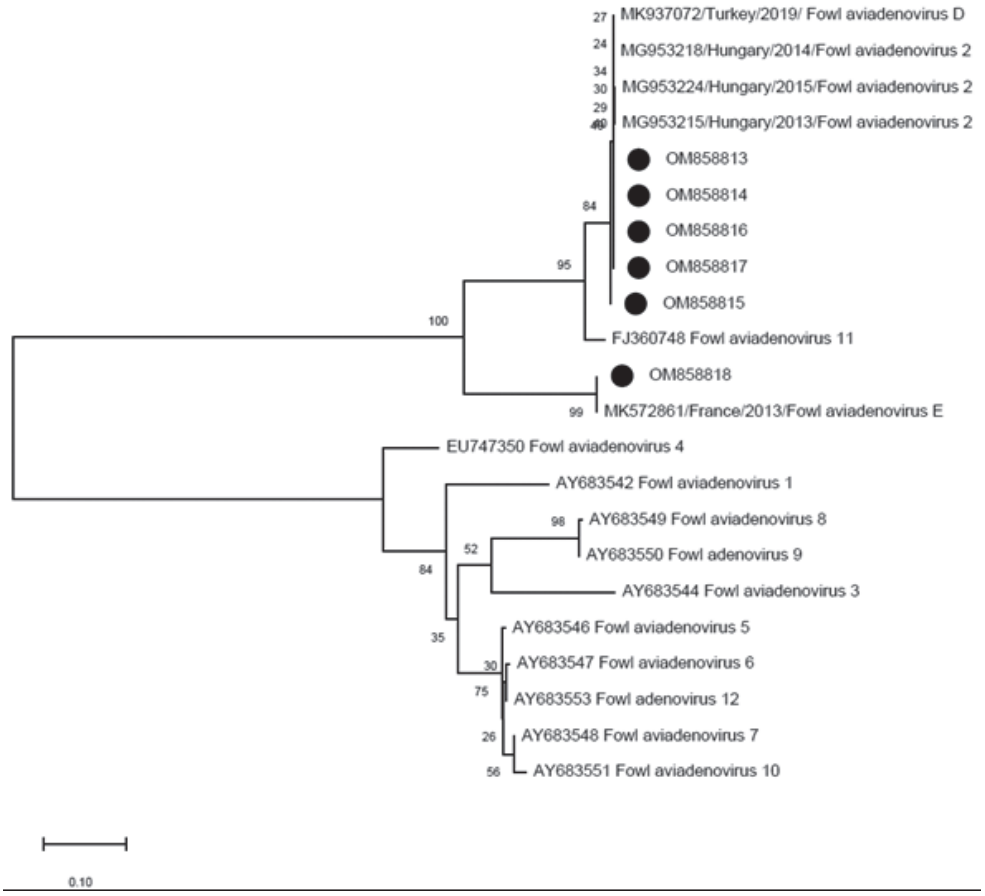

Fig. 2. The phylogenetic tree was inferred using the Neighbor-Joining method

The evolutionary distances were computed using the Maximum Composite Likelihood method and are in the units of the number of base substitutions per site. Partial CDS sequences the length of 870 bp of the hexon gene from this study are indicated by a solid circle. OM858813-OM858817 matched with high scores (99.8%) with fowl adenovirus D and OM858818 (100%) with fowl adenovirus E.

Table 1. Number of cloacal samples per farm and number of positive samples for the FAdV genome with accession numbers of nucleotide sequences and qualification of virus species. The rate of seropositivity within farms

Farms label	Number of cloacal samples	Number of PCR positive cloacal samples	Accession numbers of adenovirus strains	Species FAdV	The rate of seropositivity
1	15	5	OM858813	D	100.00%
2	15	4	OM858814	D	23.33%
3	15	5	OM858816	D	23.33%
4	15	6	OM858817	D	85.00%
5	15	6	OM858815	D	90.00%
6	15	0	-	-	100.00%
7	15	5	OM858818	E	100.00%

The histopathological examinations revealed multifocal cytoplasmic vacuolization of the hepatocytes and hyperemia. Also, depending on the sample, there was a presence of centrilobular or diffuse degeneration, and coagulative necrosis of the hepatocytes, and inflammatory cell infiltration (Fig. 3). Due to impaired bile flow, cholestasis was revealed during histopathological examinations.

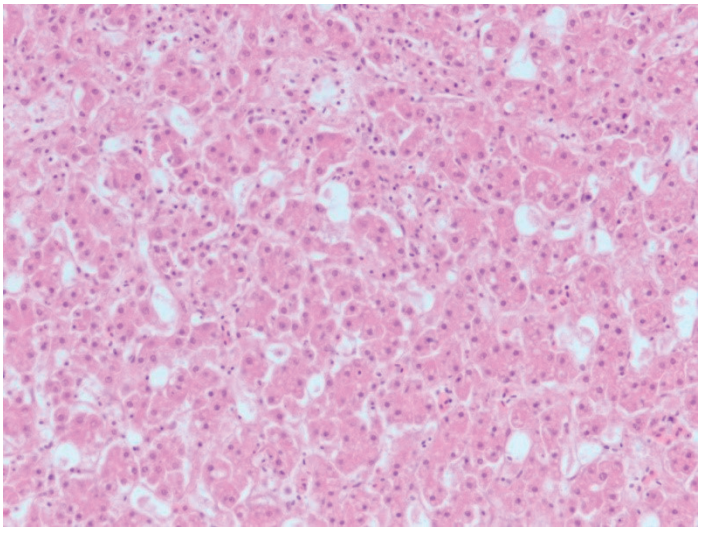

Fig. 3. Chicken liver
Necrosis, degeneration, and inflammatory cell infiltration of the liver (HE, magnification×40)

Discussion

To confirm fowl adenovirus infection on as many farms as possible, the cloacal samples were used for further molecular analyses. Cloacal swab samples were also used in the previous studies aimed to confirm the presence of the virus and quantify viral shedding. Depending on the strain that caused the infection, the previous results showed that the highest viral shedding of FAdV-D was 10 d.p.i. (days post-infection) and for FAdV-E 21 d.p.i., and cloacal samples as sample material can be used for virus detection within this period (GUNES et al., 2012). In the present study, we confirmed fowl adenovirus infection on all farms, and identified six FAdV strains. These results show that two species of FAdV are circulating in the Belgrade city area, FAdV species D and FAdV species E. Species D was dominant in our study. It is noteworthy that other researchers from European countries identified a high percentage of FAdV - D and FAdV-E in the total number of IBH cases (SCHACHNER et al., 2018; NICZYPORUK et al., 2021; TSIOURIS et al., 2022; ZADRAVEC et al., 2013). In most cases, the IBH outbreak was associated with serotypes 2 and 11 (FAdV – D) and 8a and 8b (FAdV – E) (SCHACHNER et al., 2018; MOHAMED and EL-SABAGH, 2023).

In our research, we aligned our sequences with those from FAdV species D and E, followed by phylogenetic analysis. According to the phylogenetic analysis with the available sequences from GenBank, the Serbian strains (accession number OM858813-OM858817) showed the highest similarity, of 99.8%, to isolates from Turkey (accession number MK937072), previously described by SAHINDOKUYUCU et al. (2020) which belong to species D, serotype 11 and a similarity of 100% to isolates from Hungary (accession numbers MG953218, MG953224 and MG953215) previously described by Kajan et al. 2019, which belong to species D, serotype 2. The results may indicate the circulation of FAdV serotype 2 in these regions, and we propose additional control measures to lower the FAdV burden. The strain from farm 7 (accession number OM858818) clustered with a similarity of 100% with the strain 13-16424 (accession number MK572861) previously described by SCHACHNER et al. (2019) which belongs to species E, serotype 8b, forming a unique monophyletic group.

During the first visit, the age of chickens was 3 to 4 weeks, when veterinarians noticed an increase in feed conversion and a lack of flock uniformity. Depending on the farm, individual signs of depression, and general uneven growth in the flocks were noticed. No increase in mortality or other clinical disorders was noticed. Enzyme-linked immunosorbent assays (ELISAs) are a suitable tool and the preferred test for detecting adenovirus antibodies in poultry flocks caused by infection or vaccination (MIRZAZADEH et al., 2021). In the present study, serological analysis was used at the age of 42 days to determine the serological status of the flocks. Seropositivity against FAdVs was confirmed on all seven farms but in different percentage ranges. On the farm labeled 6, seroconversion against FAdVs with the ELISA test was high, but in the same flock, the molecular detection was negative. This could be due to the virus elimination over the post-infection period, or that PCR was not able to detect the virus (ARAZI et al., 2020). Also, FAdV was isolated from apparently healthy birds, where birds showed seropositivity, which could be related to the latent form of infection (ADEL et al., 2021; MALETIĆ et al., 2023).

Pathological lesions were recorded in this study following previously published reports by other authors such as ELBESTAWY et al. (2020), MIRZAZADEH et al. (2021), SAHINDOKUYUCU et al. (2020) and NICZYPORUK et al. (2021), who reported cases of outbreaks of IBH in different countries, and their pathomorphological findings indicate specific liver changes. The results of the histopathology examination did not reveal the presence of inclusion bodies in hepatocytes, which is unlike other research. The reason for these findings may be related to the different viral serotypes, or the time that had elapsed after the onset of infection (STEER-COPE et al., 2017; TOROGHI et al., 2022). According to the findings of STEER-COPE et al. (2017) inclusion bodies in hepatocytes were found between 1-4 d.p.i. in 6 of 29 (20.69%) birds that were inoculated with the FAdV-8 serotype,

and 4 and 5 d.p.i. in only 1 of 29 (3.44%) birds that were inoculated with the FAdV-11 serotype. It was reported that the severity of lesions is directly associated with the age of the birds and the level of maternally derived antibodies, but also with the pathogenicity of the virus serotype and the immunosuppressive condition of the birds. It was found that the serotype FAdV-8b is more pathogenic than the serotype FAdV-11 (HESS, 2017). FAdV could lead to the degeneration of hepatocytes. Fowl Adenovirus serotype 4 induces liver damage that involves apoptosis and autophagy of hepatocytes, and a severe inflammatory response via over-secretion of cytokines in infected tissue (NIU et al., 2018; ZHAO et al., 2020). According to the findings, it seems that hexon has a significant role in the infection of hepatocytes by binding tightly to coagulation factor X (WANG and ZHAO, 2019).

In the present study, sequence analysis revealed that two FAdV species (D and E) and three serotypes (2, 8b, and 11) were circulating in Serbia. Those results follow the findings of other researchers that species FAdV-D and E, but not all serotypes, could be associated with IBH cases (SCHACHNER et al., 2016; KAJAN et al., 2019; MOHAMED and EL-SABAGH, 2023). The frequency of FAdV species D is higher, which indicates its dominant circulation in Serbia. FAdV-related diseases need more attention in Serbia. It is important to continue investigating the presence of FAdV infections as there is no commercial FAdV vaccine licensed, and it is a major challenge to implement prevention and control strategies.

Conclusions

This is the first report on the seroprevalence, molecular characterization, and pathological lesions caused by FAdV infections in chickens in Serbia. According to our findings, FAdVs could be emerging pathogens in poultry production in the Belgrade city area. The number of confirmed FAdV infections in our study is not representative of the current state of the disease in Serbia, but it gives insights into the distribution and molecular characterization of FAdV on broiler farms in the Belgrade city area. Further studies are needed to examine different poultry categories in other geographic areas in Serbia, to investigate the epidemiological features of FAdV disease, and develop effective control measures and possible vaccine development.

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Author contributions

Ljiljana Spalević: the study's conception and design, sampling and serological analysis, the epidemiological investigation, writing-original draft; Vesna Milićević: supervision and molecular tests and writing review; Branislav Kureljušić: pathological analysis, sampling and writing review; Dimitrije Glišić: molecular analysis and sequence analysis; Dragica Vojinović: serological analysis; Ljubiša Veljović: molecular analysis and sequence analysis; Jelena Maletić: the study's conception and design, supervision, sampling and serological analysis, the epidemiological investigation, writing-original draft.

Declaration of competing interest

The authors declare no conflict of interest related to this article.

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SAŽETAK

Istraživanje je provedeno kako bi se otkrile i identificirale epidemiološke značajke vrste adenovirusa peradi koja cirkulira na području grada Beograda. Istraživanje je uključivalo klinički pregled, nekropsiju, patohistologiju, imunoenzimski test (ELISA) i PCR analizu. Klinički je uočena opća slabost, povećana konverzija hrane i slab rast. Postmortalni pregled je pokazao višestruka nekrotična žarišta u jetri te natečene i blijede bubrege. Patohistološkim pregledom jetre utvrđena je hepatocelularna nekroza, vakuolarna degeneracija hepatocita, kolestaza i hiperemija. Serološkom analizom 210 krvnih seruma potvrđena je serokonverzija u 156 (74,28%) uzoraka protiv FAdV-a, a ovisno o farmi kretala se od 23,33-100%. Metodom PCR-a analizirano je 105 obrisaka kloake za određivanje genoma virusa pronađenog u 31 (29,52%) uzorku. Analiza sekvence gena Hexon identificirala je dvije vrste: aviadenovirus peradi D (serotip FAdV-2, FAdV-11) i aviadenovirus peradi E (serotip FadV-8b). Te su vrste blisko povezane sa sojevima koji cirkuliraju u Mađarskoj, Turskoj i drugim zemljama. Naši nalazi sugeriraju da su FAdV sojevi značajno prevladavajući i da bi mogli biti novi patogen u Srbiji.

Ključne riječi: brojleri; adenovirus peradi; epidemiologija; Srbija