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Case Report

INCLUSION BODY HEPATITIS ASSOCIATED WITH FOWL ADENOVIRUS TYPE 8b IN BROILER FLOCK IN SLOVENIA – A CASE REPORT

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Summary: Inclusion body hepatitis (IBH) is an acute disease in chickens caused by fowl adenoviruses (FAdVs). Among twelve known FAdV types (FAdV-1 to 8a, and FAdV-8b to 11), classified into five different species (A-E), all of them were already involved in naturally occurring cases. The disease is mainly distributed in areas with intensive poultry industry. In Slovenia the etiological agent of IBH has not been confirmed to date.

An outbreak of acute mortality affected a broiler flock of 12,000 animals. In two waves of elevated mortality rate, a total of 264 (2.2%) chickens were found dead in one week time. Affected birds showed ruffled feathers, depression, watery droppings and some of them limping. The most common pathological lesions seen on necropsy were pale, swollen and friable livers with subcapsular hemorrhages. On histological examination, acute hepatitis characterized by necrosis and hepatocytes, with large basophilic intranuclear inclusion bodies, were observed. The histological results were characteristic for IBH caused by adenovirus infection. The causative agent was identified as fowl adenovirus (FAdV) type 8b, a member of the Fowl adenovirus E species, based on PCR results of partial sequence of adenoviral polymerase and hexon gene. The confirmed type in our case is one of the most common causative agents involved in IBH. In addition, infectious bursal disease virus and infectious bronchitis virus were detected in the same flock.

Key words: inclusion body hepatitis outbreak; fowl adenovirus 8b; broilers; Slovenia

Introduction

Fowl adenoviruses (FAdVs) are a very heterogeneous group of viruses. Twelve types (formerly serotypes), named FAdV-1 to 8a, and FAdV-8b to 11, are classified into five different species (A-E) (1). They are believed to be ubiquitous in poultry farms (2). Not all FAdVs are considered to be pathogenic for chickens but every type has already been recovered from naturally occurring cases of inclusion body hepatitis (IBH) (3, 4).

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IBH was first described in the USA in 1963 and then rapidly spread over the world (5). Its importance in the poultry industry has been increasing in recent years (2). A sudden onset of increased mortality may reach 10% in 3-4 days and usually returns to normal after 5 days from the onset of clinical signs. If there are secondary bacterial infections ongoing contemporarily, mortality can reach 30% and can continue for several weeks (5, 6, 7, 8, 9). The severity of the disease may also depend on some other predisposing factors that enhance the pathogenic potential of FAdV infection, such as a poor environment and management (9, 10). It has been proven that the initial involvement of immunosuppressive agents, including infectious bursal disease virus

(IBDV) and chicken anemia virus (CAV) or some mycotoxins, such as aflatoxins, is needed for IBH onset (10, 11, 12, 13, 14).

On the other hand, different FAdV types, called highly pathogenic FAdVs, have been considered to be the primary pathogen in IBH. Among them, FAdV-8 and 9 are the most commonly detected ones (3, 9, 15, 16, 17) and seem to predominate in some areas (3, 17).

The present paper is the first report of IBH with detailed clinical and laboratory findings in an outbreak in commercial broilers in Slovenia.

Material and methods

Case history

Acute mortality was observed in a broiler flock of 12,000 animals. The birds were housed as day-old chickens and were vaccinated against Newcastle disease (ND) on day 6 (Pestikal La Sota SPF, Veterina, CRO), IBD on day 13 (Nobilis Gumboro 228E, Intervet/Schering-Plough, USA) and infectious bronchitis on day 16 (Nobilis IB 4-91, Intervet/Schering-Plough, USA). At the age of 17 days, 146 chickens (1.22%) were found dead at random throughout the house. The flock was treated with amoxicillin (Paracilin, Intervet/Schering-Plough, USA) for 5 days. In the six days following the first onset, 118 chickens (0.98%) died. Affected birds showed ruffled feathers, depression, watery droppings and limping.

Fourteen broiler chickens that died at the age of 23 days were submitted to the Institute of Poultry Health, Veterinary Faculty (VF), University of Ljubljana on the day of death.

The overall production results in the affected flock were comparable to those obtained in other broiler flocks. The mortality was 2.90%. Nonetheless, a higher feed conversion rate (1.94 kg compared to a predicted 1.88 kg) was obtained and the average body weight at the age of 34 days was higher than expected; 1.70 kg compared to 1.50 kg planned.

Gross and histopathological examinations

Pathologic examinations were performed on all birds submitted. Tissue blocks of the liver, kidney, heart and spleen were fixed in 10% buffered formalin, embedded in paraffin, sectioned at 4 µm and stained with hematoxylin and eosin for light microscopy. Histopathology was performed at the Institute of Pathology, Forensic and Administrative Veterinary Pathology, VF.

Virological examinations

For molecular investigations, DNA and RNA were extracted from material taken at necropsy and frozen until investigation. Portions of liver and spleen were separately homogenized in phosphate buffered saline (PBS), prepared as a 10–20% w/v suspension. DNA was extracted by using a QIAamp® DNA Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. The RNA was extracted from trachea and cloacal swab by using QIAamp Viral RNA Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. Before RNA extraction, 2 ml of PBS was added to each swab and vortexed vigorously.

Detection of FAdV

Attempts to detect adenoviruses in liver and spleen tissue were made by using nested polymerase chain reaction (PCR) with degenerate, consensus primers targeting the viral DNA dependent DNA polymerase gene, as described by Wellehan et al. (18). In addition, more specific, degenerate primers hexon A and hexon B that amplify a region of the hexon gene were used for establishing FAdV serotype, as reported previously (19).

Detection of CAV

For detection of CAV in liver and spleen tissue, primers that target the highly conserved gene encoding VP2 were used, as described by Noteborn et al. (20).

Detection of IBDV

For detection of IBDV from cloacal swab, a reverse transcription (RT)-PCR method was applied, as described previously (21), using primers specific to the genome fragment that codes for the hypervariable region of VP2 (22).

Detection of infectious bronchitis virus (IBV)

The presence of the IBV genome in tracheal and cloacal swabs was tested by RT-PCR. Primer pair CK2/CK4 targeting the variable region of S1 gene was used, as described by Keeler et al. (23).

PCR product analysis and typing

PCR products were visualized by electrophoresis on a 1.8% ethidium bromide stained agarose gel. The PCR products were excised and purified with Wizard PCR Preps DNA Purification System

(Promega, Madison, Wi, USA) and sent for sequencing purposes to Macrogen DNA Sequencing Service (South Korea). Sequence analyses were performed by DNAStar (DNAStar Inc., USA) and NCBI BLAST Tools (http://www.ncbi.nl.nih.gov). Multiple protein alignments were made using the ClustalW program. The alignments were reverted back to the saved DNA-sequence and edited using BioEdit. Phylogenetic calculations were performed using the Phylip package online (Mobyle@pasteur: http://mobyle.pasteur.fr) by Dnadist with the Kimura two parameters model. The Fitch program was used by the Fitch–Margoliash method with global rearrangements for phylogenetic tree reconstruction. The trees were visualized using Mega.

Isolation of FAdV

For adenovirus isolation, 8-day-old SPF embryonated chicken eggs (Lohman, Cuxhaven, Germany) were used. Liver homogenate was prepared and inoculated into the egg yolk, as described previously (24). Briefly, liver tissue was homogenized. Ten percent liver solution was made adding the minimum essential medium and penicillin streptomycin solution and centrifuged at 1500×g for 10 minutes. A total of 0.1 ml of the supernatant was used for each inoculation. Inoculated eggs were observed by candling daily. All dead embryos were necropsied. Livers were taken for histopathology and molecular examinations for FAdV detection, as described above.

Bacteriological examinations

Routine bacteriology was performed on liver samples. Samples were cultured aerobically at 37°C on 5% sheep's blood and Drigalski agar plates. Cultures were considered negative if no growth was detected after a 48-hour incubation period.

Serological examinations

For serological investigation, 20 blood samples were taken on the 36th day of age. Antibodies against IBV, CAV and IBDV were tested by enzyme-linked immunosorbent assays (ELISAs) (IDEXX, Westbrook, ME), according to the manufacturer's instructions.

Results and disscusion

Necropsy and histopathology results revealed pathological changes characteristic for IBH, as described previously (2, 5, 6). Predominating gross lesions seen were pale, swollen and friable livers, kidneys with subcapsular petechial hemorrhages and pale myocardium. Occasionally, mild tracheitis and catarrhal enteritis were noticed. All examined birds were in good body condition. Microscopic examination revealed acute hepatitis, with randomly distributed multifocal areas of acute necrosis as well as numerous disseminated hepatocytes with large basophilic intranuclear inclusion bodies scattered among necrotic hepatocytes (Figure2). Multiple subcapsular hemorrhages, multifocal groups of hepatocytes with lipid degeneration, and cholestasis were also present. Similar large intranuclear basophilic inclusion bodies as in the liver and karyorrhexis were found in the red pulp cells of the spleen and in the tubular cells of the kidneys but were less frequent than in the liver (Figure3).

Livers and spleens gave a positive PCR result for FAdVs. The PCR targeting the adenoviral DNA dependent DNA polymerase gene resulted in 321-bp-long products, the PCR targeting the hexon gene in 817-bp-long products. The determined partial hexon gene sequences from liver and spleen samples were found to be 100% identical on the nucleotide level (results not shown). According to the phylogenetic tree, the newly detected virus could be classified as FAdV type 8b, a member of the Fowl adenovirus E species (Figure1). Based on literature data, FAdV-E type 8b is one of the most common causative agents involved in IBH (9, 15, 17). The nucleotide sequences described in the present paper were submitted to GenBank and assigned accession numbers JF766220 for DNA polymerase gene and JF766221 for hexon gene, respectively.

Officially accepted adenovirus species names are given in italics. The topology of the tree was tested by bootstrapping. Bootstrap values are given for 1000 datasets if over 750. 1407: the adenovirus strain studied in this paper; FaAdV-1: falcon adenovirus 1; FAdV-1–11: fowl adenovirus 1–11; TAdV-1: turkey adenovirus 1. Accession numbers in the NCBI GenBank and strain names if applicable: 1407: JF766221; FaAdV-1: AY683541; FAdV-1: AC 000014, CELO; FAdV-2: AF508946, SR48; FAdV-3: AF508949, 75; FAdV-4: AF508950, 506; FAdV-5: AF508953, 340; FAdV-6: AF508954.2, CR119; FAdV-7: AF508955, YR36; FAdV-8a: AF508957, 58; FAdV-8b: AF508958.2, 764; FAdV-9: AC 000013, A-2A; FAdV-10: U26221; FAdV-11: EU979378, UF71; TAdV-1: GU936707, D90/2.

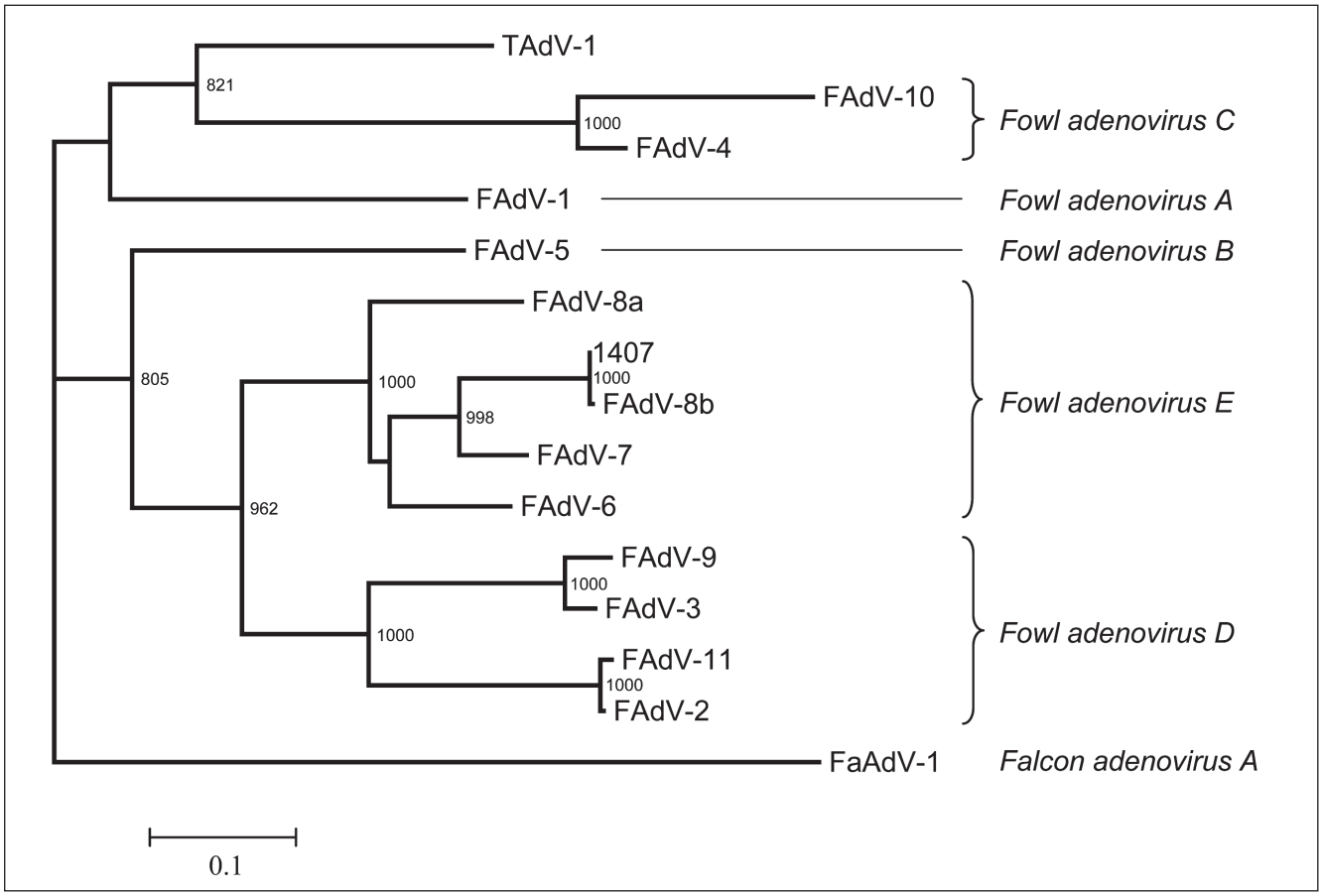

Figure1: Phylogenetic tree showing the distance matrix analysis of partial hexon gene DNA sequences from aviadenoviruses

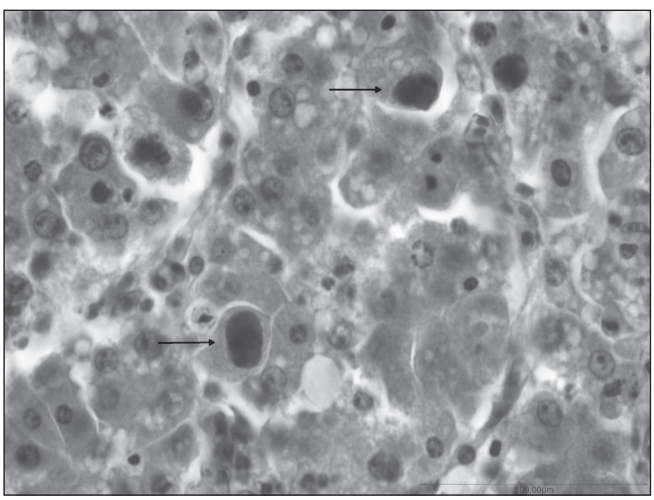

Figure2: Hepatocytes with large basophilic intranuclear inclusion bodies (arrows) (400×)

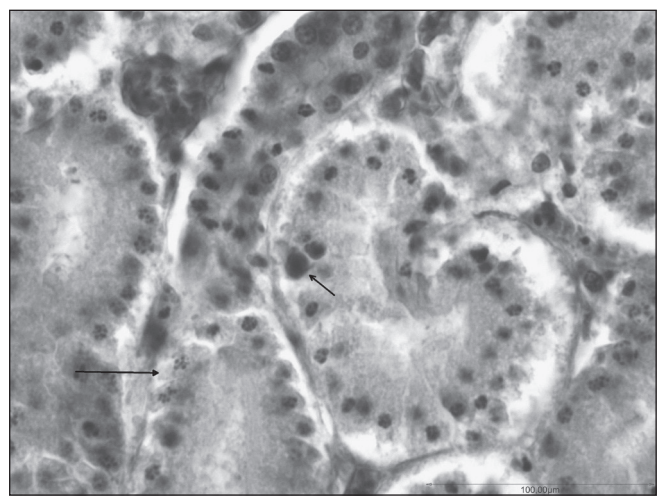

Figure3: Large intranuclear basophilic inclusion body (short arrow) and karyorrhexis (long arrow) in the tubular cells of the kidney (400x)

The infection was also confirmed by virus isolation. All embryos inoculated with liver homogenate of affected chickens died by day 5 post inoculation. On histological examination, acute hepatitis with distortion of the liver plates, multiple necrotic areas and disseminated individual necrotic hepatocytes was diagnosed. Variation of the nuclear size was evident in the hepatocytes of affected embryos but no inclusion bodies were found (results not shown). The presence of FAdV-8b was detected by PCR from the liver tissue of the embryos.

The severity of AdV-caused IBH can vary in broiler chickens. In general, it is believed that for the development of the clinical disease, most FAdVs need some immunosuppressive agents, which may trigger the mechanism. CAV and IBDV have most often been found to be predisposing factors to IBH outbreaks (10, 13, 14). On the other hand, many cases of IBH with highly pathogenic AdV types as primary pathogens have also been described (3, 9, 15, 17).

In our case, in addition to FAdV-8b, the presence of IBDV but not CAV was detected. The RT-PCR performed to test IBDV in cloacal swabs resulted in a product of appropriate size of approximately 630-bp. However, direct sequencing of the PCR product from the VP2 gene failed to give unambiguous results. A concurrent infection with vaccine and field strains of IBDV apparently occurred, resulting in heterogeneous PCR products that could not be sequenced without prior molecular cloning. Serological testing at the age of 36 days revealed an antibody response to IBDV (Table 1) originating from vaccination or/and from field infection. Since Zorman Rojs et al. (25) obtained significantly lower antibody titers (from 102 to 518) detected by the same (IDEXX) ELISA system in non-infected broilers vaccinated with intermediate plus vaccine, field infection is most likely in the present case. Infection with pathogenic strains of IBDV, including some less attenuated vaccine strains, has well-known immunosuppressive effects in chickens and might induce the development of IBH (10, 11, 12).

In addition, the presence of IBV in the trachea was confirmed. Its occurrence in broiler, broiler breeder and layer flocks in Slovenia has been studied extensively lately by Krapež et al. (26). Further molecular investigation showed that strain QX was involved (accession number GU 564331). Tracheitis, found at necropsy in some of the submitted chickens, might have been caused by the IBV infection. Presumably it did not have a significant influence on the course of IBH in our case. Interestingly, Ojkić et al. (9) found that co-infections with other viruses (IBDV, IBV and

Table 1: Summary of virological and serological results in IBH affected broiler flock

Virus	Virological examinations			Serology
	Molecular method (targeted region)	Sample	Result	IDEXX ELISA titer ± SD (Number of ELISA positive birds/ Number of tested birds)
FAdV	Nested PCR (polymerase gene)1	Liver	+	Not done
		Spleen	+	
	PCR (hexon gene)2	Liver	+	
		Spleen	+	
IBDV	RT-PCR (VP-2 region)3	Cloacal swab	+	3783 ± 893 (19/19)
IBV	RT-PCR (S-1 gene)4	Tracheal swab	+	1326 ± 650 (18/19)
		Cloacal swab	-	
CAV	PCR (VP-2 gene)5	Liver	-	1.148 ± 0.103 (0/19)*
		Spleen	-	

[1] Primers designed by Wellehan et al, 2004
[2] Primers designed by Meulemans et al, 2001
[3] Primers designed by Cao et, 1998
[4] Primers designed by Keeler et al, 1998
[5] Primers designed by Noteborn et al, 1992
* Blocking ELISA was used

reovirus) were more frequent in FAdV infections not related to IBH.

Some FAdV types have been described as inducing immunosuppression in chickens without any other predisposing factors. For instance, Schonewille et al. (27) demonstrated by experimental infection that FAdV-4 caused a depletion of B- and T-lymphocytes in lymphoid organs in SPF chickens. Immunosuppressive effect of confirmed FAdV type-8b was not evaluated. Moreover, it is not clear if the presence of IBDV detected in our case was the trigger mechanism for IBH outbreak or isolated AdV type-8b could produce the disease by itself.

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IZBRUH INKLUZIJSKEGA HEPATITISA PRI BROJLERJIH POVZROČEN Z ADENOVIRUSOM (FAdV) TIPA 8b

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Povzetek: Inkluzijski hepatitis je akutna bolezen piščancev, ki jo povzročajo kokošji adenovirusi (angl. fowl adenovirus, FAdV). Poznanih je 12 tipov adenovirusov (FAdV-1 do 8a in FAdV-8b do 11), ki so razvrščeni v 5 vrst (A-E). Vsi navedeni tipi so že bili dokazani v naravno potekajočih primerih inkluzijskega hepatitisa. Bolezen se pojavlja na področjih z razvito intenzivno rejo perutnine, v Sloveniji pa povzročitelj še ni bil dokazan.

V jati 12.000 piščancev brojlerjev je v razmiku tedna dni prišlo do nenadnega povečanja pogina. Poginilo je 264 (2,2 %) živali. Prizadete živali so bile depresivne, apatične, imele so drisko, nekatere izmed njih so šepale. Pri raztelesbi smo ugotovili izrazite spremembe na jetrih, ki so bila blede barve, povečana, krhke konsistence, z vidnimi subkapsularnimi krvavitvami. Histološke preiskave so potrdile akutni hepatitis, z nekrozami in znotrajceličnimi bazofilnimi inkluzijskimi telesi v hepatocitih. Histološki rezultati so bili značilni za adenovirusni inkluzijski hepatitis. Z molekularno metodo verižne reakcije s polimerazo (PCR), ki pomnožuje odsek adenovirusne polimeraze in odsek gena za hekson adenovirusov, smo določili povzročitelja. Dokazani etiološki agens je bil FAdV vrste E, tip 8b, ki je tudi eden izmed najpogosteje opisanih povzročiteljev inkluzijskega hepatitisa. Poleg omenjenega virusa smo v oboleli jati dokazali tudi sočasno prisotnost gumborske bolezni in kužnega bronhitisa.

Ključne besede: izbruh IBH; FAdV8b; brojlerji; Slovenija