

**INCLUSION BODY HEPATITIS (IBH) OUTBREAK ASSOCIATED WITH FOWL ADENOVIRUS
TYPE 8b IN BROILERS**

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The causative agent of inclusion body hepatitis (IBH) was identified as fowl adenovirus (FAdV) type 8b, a member of the Fowl adenovirus E species, based on PCR results of adenoviral polymerase and the hexon gene in an outbreak of acute mortality that affected a broiler flock of 12,000 animals. In two waves of elevated mortality rate, a total of 264 chickens were found dead. Affected birds showed ruffled feathers, depression, watery droppings and limping. The most common pathological lesions seen on necropsy were pale, swollen and friable livers. On histological examination, acute hepatitis characterized by necrosis of hepatocytes, with large basophilic intranuclear inclusion bodies, were observed. In addition, infectious bursal disease virus and infectious bronchitis virus were detected in the same flock.

Key words: broilers, FAdV8b, IBH outbreak, Slovenia

INTRODUCTION

Fowl adenoviruses (FAdV) 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) (Benkő *et al.*, 2005). They are believed to be ubiquitous in poultry farms (McFerran and Smyth 2000). Not all FAdV are considered to be pathogenic for chickens but every type has already been recovered from naturally occurring cases of inclusion body hepatitis (IBH) (Gomis *et al.*, 2006; McConnell and Fitzgerald, 2008).

IBH was first described in the USA in 1963 and then rapidly spread over the world (Howell *et al.*, 1970). Its importance in the poultry industry has been increasing in recent years (McFerran and Smyth, 2000). 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 (Howell *et al.*, 1970; Macpherson *et al.*, 1974; McFerran *et al.*, 1976; Baar and Scott, 1988; Ojkić *et al.*, 2008b). 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 (Toro *et al.*, 2000; Ojkić *et al.*, 2008b). 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 (Fadly *et al.*, 1975; Rosenberg *et al.*, 1975; Toro *et al.*, 2000; Toro *et al.*, 2002; Shivachandra *et al.*, 2003).

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 (Christensen and Saifuddin 1989; Saifuddin *et al.*, 1992; Gomis *et al.*, 2006; Ojkić *et al.*, 2008a; Ojkić *et al.*, 2008b) and seem to predominate in some areas (Gomis *et al.*, 2006; Ojkić *et al.*, 2008a).

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. RNA was extracted from the 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 the 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.* (2004). 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 (Meulemans *et al.*, 2001).

Detection of CAV

For detection of CAV in the liver and spleen tissue, primers that target the highly conserved gene encoding VP2 were used, as described by Noteborn *et al.* (1992).

Detection of IBDV

For detection of IBDV from cloacal swabs, a reverse transcription (RT)-PCR method was applied, as described previously (Barlič-Maganja *et al.*, 2002), using primers specific to the genome fragment that codes for the hypervariable region of VP2 (Cao *et al.*, 1998).

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.* (1998).

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.nlm.nih.gov>). Multiple protein alignments were made using the ClustalW program (Thompson *et al.*, 1994). The alignments were reverted back to the saved DNA-sequence and edited using BioEdit (Hall, 1999). Phylogenetic calculations were performed using the Phylip package (Felsenstein, 1989) online (Mobyle@pasteur: <http://mobyle.pasteur.fr>) by Dnadist with the

Kimura two parameters model (Kimura, 1980). The Fitch program was used by the Fitch–Margoliash method with global rearrangements for phylogenetic tree reconstruction. The trees were visualized using Mega (Tamura *et al.*, 2007).

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 (Cowen, 1988). 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 DISCUSSION

Necropsy and histopathology results revealed pathological changes characteristic of IBH (Howell *et al.*, 1970; Macpherson *et al.*, 1974; McFerran 2000). The predominating gross lesions 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 (Figure 1). 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 (Figure 2).

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 the liver and spleen samples were found to be 100% identical on the nucleotide level. According to the phylogenetic tree, the newly detected virus could be classified as FAdV type 8b, a member of the Fowl adenovirus E species (Figure 3). Based on literature data, FAdV-E type 8b is one of the most common causative agents involved in IBH (Christensen and Saifuddin, 1989; Ojkić *et al.*, 2008a; Ojkić *et al.*, 2008b). 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.

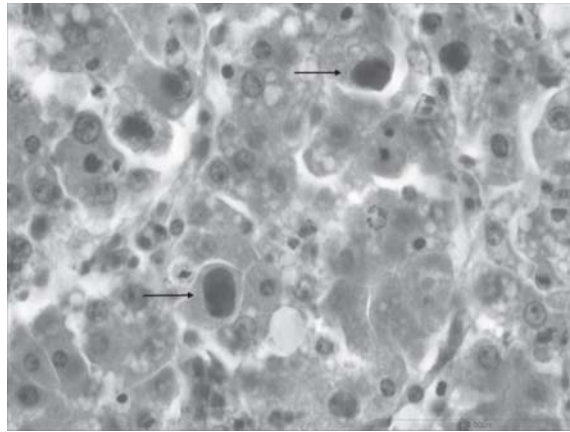


Figure 1. Hepatocytes with large basophilic intranuclear inclusion bodies (arrows) (400×)

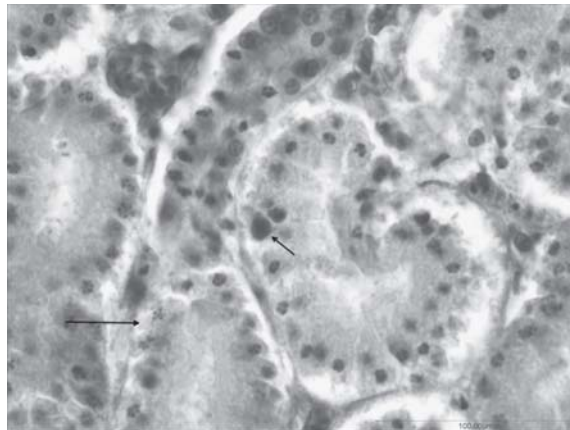


Figure 2. Large intranuclear basophilic inclusion body (short arrow) and karyorrhexis (long arrow) in the tubular cells of the kidney (400×)

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. The presence of FAdV-8b was detected by PCR from the liver tissue of the embryos.

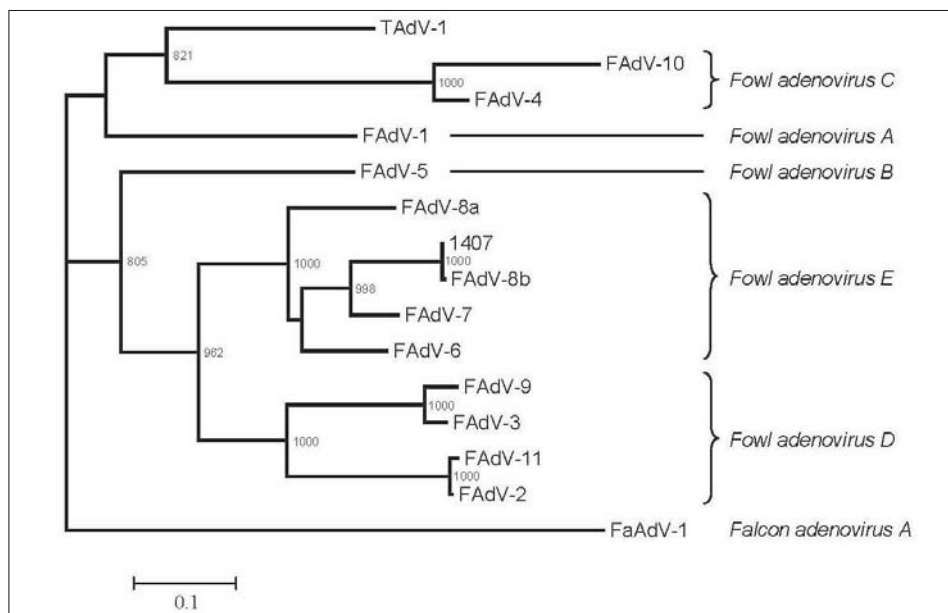


Figure 3. Phylogenetic tree showing the distance matrix analysis of partial hexon gene DNA sequences from aviadenoviruses. Officially accepted adenovirus species names are given in italics. The topology of the tree was tested by bootstrapping. Bootstrap values are given for 1000 datasets if over 750. 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

The severity of AdV-caused IBH can vary in broiler chickens. In general, it is believed that for the development of the clinical form of the 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 (Toro *et al.*, 2000; Toro *et al.*, 2002; Shivachandra *et al.*, 2003). On the other hand, many cases of IBH with highly pathogenic AdV types as primary pathogens have

also been described (Christensen and Saifuddin 1989; Gomis *et al.*, 2006; Ojkić *et al.*, 2008a; Ojkić *et al.*, 2008b).

In our case, in addition to FAdV-8b, the presence of IBDV but not CAV was detected. 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 of age revealed an antibody response to IBDV (Table 1) originating from vaccination or/and from field infection. Since Zorman Rojs *et al.* (2011) 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 (Fadly *et al.*, 1975; Rosenberg *et al.*, 1975; Toro *et al.*, 2000).

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	-	

In addition, the presence of IBV in the trachea was confirmed. 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 IBV infection. Presumably it did not have a significant influence on the course of IBH in our case. Interestingly, Ojkić *et al.*, (2008b) found that co-infections with other viruses (IBDV, IBV and 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.*

(2008) demonstrated by experimental infection that FAdV-4 caused a depletion of B- and T-lymphocytes in lymphoid organs in SPF chickens. The question of whether immunosuppressive interactions of FAdVs with the host organism are enough to cause clinical manifestation of different diseases, remains unanswered.

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**POJAVA HEPATITISA SA INKLUZIJAMA (IBH) ZAJEDNO SA ADENOVIRUSOM
ŽIVINE TIP 8b KOD BROJLERA**

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SADRŽAJ

Uzročni agens hepatitsa sa inkluzivnim telašcima (IBH) je identifikovan kao živinski adenovirus (FAdV) tip 8b, i član je grupe adenovirusa živine tipa E. Ovo je zaključeno na osnovu PCR analize adenovirusne polimeraze i genskog heksona, posle izbijanja bolesti praćene akutnim mortalitetom u jatu od 12 000 ptica. U dva talasa povećanog mortaliteta, ukupno su uginule 264 jedinke. Obolele ptice su bile depresivne imale su nakostrešeno perje, vodenkasti izmet i hramale su. Najčešće patološke promene su obuhvatale bledu, otečenu i trošnu jetru. Na histopatološkim preparatima jetre, zapažene su promene u tipu akutnog hepatitisa sa nekrozom i velikim bazofilnim intranuklearnim inkluzijama. Dodatno su, u ovom istom jatu, detektovani infektivni burzitis i infektivni bronhitis.