**PATHOLOGY AND PHYLOGENETIC ANALYSIS OF SOME VIRUSES IDENTIFIED IN WILDLIFE BIRDS IN CROATIA: PRELIMINARY RESEARCH**

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**Summary**

The article documents the results of pathomorphological and molecular diagnostic testing (polymerase chain reaction, PCR) for adenovirus, astrovirus, circovirus, hepadnavirus and coronavirus determination in a sample of 16 wildlife birds of nine various species (white stork (*Ciconia ciconia*), mute swan (*Cygnus olor)*, Caspian gull ([*Larus cachinnans*](http://en.wikipedia.org/wiki/Larus_argentatus)*,* Pallas 1811*),* mallard duck (*Anas platyrhynchos)*, common buzzard (*Buteo buteo)*, white-tailed eagle (*Haliaeetus albicilla),* domestic pigeon (*Columba livia domestica)*, barn swallow (*Hirundo rustica*) and sparrow (*Passer* sp.). The birds were necropsied at the Laboratory of Pathology, Poultry Centre, Croatian Veterinary Institute, Zagreb, Croatia. Dual infections with astrovirus and circovirus were confirmed in mallard duck having died of the consequences of botulism. The sequence DuCV/M10-448 derived from the mallard duck samples had the greatest nucleotide (99%) and amino acid (100%) similarities with duck circovirus (accession number DQ100076.1) detected in the USA. The PCR positive result of hepadnavirus infection was partly confirmed by histopathologic examination in Caspian gull which suffered from generalized amyloidosis. According to the authors' awareness, this was the very first finding of hepadnavirus in gulls. The circovirus sequence found in the domestic pigeon (PiCV/M10-513) showed a 100% nucleotide and amino acid homology with pigeon circovirus (accession number AF252610.1) identified in Germany. In one of virologically tested mute swans, pathomorphological examination confirmed iron storage disease (ISD), while the rest of the birds died of the consequences of ballistic trauma or gunshot wounds (GSW) and other traumatic lesions (white stork, mute swan, common buzzard, white-tailed eagle, barn swallow and sparrow). Molecular diagnostic test results for the West Nile, avian influenza and Newcastle disease viruses were negative.

**Key words:** wildlife birds, pathomorphology, histopathology, polymerase chain reaction, astrovirus, hepadnavirus, adenovirus, circovirus, coronavirus, Caspian gull ([*Larus cachinnans*](http://en.wikipedia.org/wiki/Larus_argentatus)*,* Pallas 1811*),* mallard duck *(Anas platyrhynchos),* domestic pigeon (*Columba livia domestica),* mute swan (*Cygnus olor)*, iron storage disease (ISD), amyloidosis

**Introduction**

Viruses from the genus *Aviadenovirus,* and *Atadenovirus* from the *Adenoviridae* family are known to infect domestic poultry and are described in several wild bird species, i.e. mallard duck, gulls, psittacines, owls, hawks, pigeons, ostriches and other avian species (McFerran and Smyth, 2000; Harrach and Kaján, 2011). Adenovirus infection has been widely investigated in domestic poultry, while there are not much data available on the infection and outcome of the disease in various wild bird species. Avian astroviruses are small (27-30 nm in size) positive sense RNA viruses, with the genome length of approximately 7 kb. While astrovirus infection has been widely investigated in domestic poultry (enteric disease in chickens and turkeys, and fatal hepatitis in ducklings), little is known about the clinicopathologic manifestations of astrovirus infection in free-living birds. Recently, astroviruses have been identified in guinea fowl, pigeons, domestic geese and several duck species, suggesting that astroviruses are widespread among avian hosts (Schultz-Cherry and Lorne, 2013). Circoviruses are spherical and single-stranded (ssDNA) viruses with a genome of approximately 2 kb. They are currently classified into two genera, *Circovirus* and *Gyrovirus*, both found to infect birds. Generally, circovirus infection results in immunosuppression accompanied by symptoms ranging from anemia and lymphoid atrophy in chickens, feather abnormalities in psittacines, diarrhea, ill-thrift in pigeons, to growth retardation in ducks (Todd, 2004). Par and Robert (2007) report on gull circovirus, goose circovirus, Laughing (Senegal) Dove circovirus, ostrich circovirus and canary circovirus. These authors also mention identification of avian circovirus in a spectrum of avian species encompassing psittacines, Columbidae, passerines (Estrildidae, Fringillidae), Anatidae, Phasianidae and Struthionidae. Recently, new types of circoviruses have been identified in some mammalian species and chickens (Li *et al*., 2010). Avian hepadnaviruses are DNA viruses that have been primarily identified as the causative agents of hepatitis B in ducks. They are hepatotropic viruses with a very narrow host range (Schödel *et al*., 1989). So far, they have been described in a variety of domestic and exotic birds, including domestic ducks and geese, Mandarin duck, Ross’s goose, snow goose, ashy-headed sheldgoose, crane, heron, stork and parrot (Woolcock and Tsai, 2013). Since duck hepatitis B virus (DHBV) has been used as a common model for HBV infection in humans (Mason *et al*., 1980), the pathogenesis and histopathologic changes associated with DHBV infection have been widely investigated. Unlike that, other known avian hepadnaviruses have been characterized mostly upon their genomes. Coronaviruses are enveloped RNA viruses with a positive-strand genome, and have been associated with diseases in several mammalian species and birds. In poultry, coronaviruses cause infectious bronchitis in chickens and enteric disease in turkeys, while they have also been identified in peafowl, teal and racing pigeons (Monceyron Jonassen *et al*., 2005). Hughes *et al*. (2009) detected coronavirus in wildfowl (*Anseriformes*) and waders (*Charadriiformes*), and Chu *et al*. (2011) detected a high prevalence (12.5%) of novel avian coronaviruses in aquatic wild birds (*Anseriformes, Ciconiiformes* and *Pelecaniformes* birds).

A total of 231 wildlife birds of various species (pigeons /*Columbidae/*, pheasants and grouse */Phasianidae/,* birds of prey (eagles /e.g., white-tailed eagle - *Haliaeetus albicilla,* golden eagle - *Aquila chrysaetos;* hawks /Sparrowhawk - *Accipiter nisus/*, vultures /e.g., griffon vulture *- Gyps fulvus/*; owls (Ural Owl - *Strix uralensis* /*Strigidae*/), buzzard (e.g., common buzzard - *Buteo buteo* [(*Accipitridae*](http://en.wikipedia.org/wiki/Accipitridae)); wading birds (e.g., white stork /*Ciconiidae/);* swans and ducks *(*e.g., mute swan - *Cygnus olor* and mallard duck - *Anas platyrhynchos/Anatidae/);* gulls *(*e.g.,Caspian gull - [*Larus cachinnans*](http://en.wikipedia.org/wiki/Larus_argentatus)*,* Pallas 1811, European herring gull - [*Larus argentatus*](http://en.wikipedia.org/wiki/Larus_argentatus)*,* Black-headed gull - *Chroicocephalus ridibundus /Laridae/);* passerine birds (e.g., great tit - *Parus major* /*Paridae*/, Euroasian tree sparrow - *Passer montanus (Passeridae/,* barn swallow - *Hirundo rustica /Hirundinidae/;* Sylvia Communis /*Sylviidae*/, etc.); woodpeckers (great spotted woodpecker – *Dendrocopos maior /Picidae/*; blackbird (common blackbird - *Turdus Merula /Turdidae*/, etc.) were routinely necropsied at the Laboratory of Pathology, Poultry Centre, Croatian Veterinary Institute, Zagreb, Croatia during the 2010-2014 period.

Since there are not much available data on adenovirus, astrovirus, circovirus, hepadnavirus and coronavirus infections, outcome and clinicopathologic manifestations of the infection in free-living birds, preliminary pathomorphology and molecular biology investigations of the adenovirus, astrovirus, circovirus, hepadnavirus and coronavirus presence were carried out in a randomly chosen sample of 16 wildlife birds of nine various bird species (white stork (*Ciconia ciconia*), mute swan (*Cygnus olor)*, Caspian gull ([*Larus cachinnans*](http://en.wikipedia.org/wiki/Larus_argentatus)*,* Pallas 1811)*,* mallard duck (*Anas platyrhynchos)*, common buzzard (*Buteo buteo)*, white-tailed eagle (*Haliaeetus albicilla),* domestic pigeon (*Columbia livia domestica)*, barn swallow (*Hirundo rustica*) and sparrow (*Passer* sp.).

**Material and Methods**

***Case description and sampling methods***

Routine necropsy procedures of the total of 231 wildlife birds were performed during the 2010-2014 period at the Laboratory of Pathology, Poultry Centre, Croatian Veterinary Institute, Zagreb, and, where indicated, histologic, virologic, bacteriologic and other diagnostic techniques (x-ray examination) were applied. Only the birds examined in 2014 were tested for West Nile virus infection and the birds necropsied during the 2010-2014 period were tested for avian influenza and Newcastle disease virus infections (Laboratory of Virology, Poultry Centre, Croatian Veterinary Institute, Zagreb, Croatia). The organs dissected from 16 birds of nine various species (white stork (*Ciconia ciconia*), mute swan (*Cygnus olor)*, Caspian gull ([*Larus cachinnans*](http://en.wikipedia.org/wiki/Larus_argentatus)*,* Pallas 1811*),* mallard duck (*Anas platyrhynchos)*, common buzzard (*Buteo buteo)*, white-tailed eagle (*Haliaeetus albicilla),* domestic pigeon (*Columba livia domestica)*, barn swallow (*Hirundo rustica*) and sparrow (*Passer* sp.)) were tested using a molecular biology testing procedure for adenovirus, astrovirus, circovirus, hepadnavirus and coronavirus infections (Department of Poultry Diseases with Clinic, Faculty of Veterinary Medicine, University of Zagreb, Croatia).

***Pathomorphology analysis***

During necropsy**,** tissues were taken for subsequent histopathologic, virologic, bacteriologic and other diagnostic examinations. For histopathologic analysis, organ samples were fixed in neutral 10% formalin solution, embedded in paraffin and cut into 4-μm thick sections on a rotary microtome (MICROM HM 325; Zeiss, Austria). After deparaffinization, the sections were stained with hematoxylin and eosin (HE) using Congo-red and Prussian Blue techniques for confirmation of amyloid and of iron. The slices were examined under the light microscope (LEICA DMLB, Germany) and the images were captured with a PIXERA Pro 150ES digital camera.

***Molecular diagnostic testing for adenovirus, astrovirus, circovirus, hepadnavirus and coronavirus determination***

*Molecular diagnosis*

The organs were dissected and collected for molecular diagnostic testing (Table 1). DNA and RNA were extracted in a volume of 50 μL from organ homogenates using High Pure Viral Nucleic Acid Kit according to the manufacturer's instructions (Roche Diagnostics, Mannheim, Germany). DNA was synthesized using SuperScript TM III (Invitrogen, Carlsbad, California, USA). Reverse transcription reaction (42 oCfor 50 min and inactivation for 15 min at 70 oC) was carried out in the Gene AMP PCR Systems 2400 (California, USA). PCR reaction: 1.5 μL of DNA or cDNK, 25 μL of Go Taq Green Master Mix (Promega, Madison, USA) and 0.20 μM forward and reverse primer contained in a total volume of 50 μL. The PCR reaction conditions were adjusted according to the references shown in Table 2. The PCR reaction products were analyzed by 1.5% gel electrophoresis stained with ethidium bromide.

**Table 1.** Alphabetical list, scientific name, year of sampling, laboratory identifier and collected organs of birds tested

**Tablica 1.** Abecedni popis i latinski naziv, godina prikupljanja, laboratorijska oznaka i uzorkovani organi ptica

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
| Bird species | Scientific name | Year | Identifier | Organs |
| White stork | *Ciconia ciconia* | 2013 | M13-69 | Intestine, liver, spleen, kidney |
| Mute swan | *Cygnus olor* | 2011 | M11-794 | Intestine, liver |
| Mute swan | *Cygnus olor* | 2013 | M13-82 | Intestine, kidney |
| Mute swan | *Cygnus olor* | 2014 | M14-01 | Intestine, liver |
| Mallard duck | *Anas platyrhynchos* | 2010 | M10-448 | Intestine, spleen, bursa of Fabricii |
| Domestic pigeon | *Columba livia domestica* | 2010 | M10-513 | Liver, kidney |
| Barn swallow | *Hirundo rustica* | 2012 | M12-906 | Intestine, liver, spleen, kidney |
| Caspian gull | [*Larus cachinnans*](http://en.wikipedia.org/wiki/Larus_argentatus)*,* Pallas 1811 | 2012 | M12-950 | Liver, kidney |
| Common buzzard | *Buteo buteo* | 2011 | M13-54 | Intestine, liver, spleen, kidney |
| Common buzzard | *Buteo buteo* | 2013 | M13-55 | Intestine, liver, spleen, kidney |
| Common buzzard | *Buteo buteo* | 2013 | M13-56 | Intestine, liver, spleen, kidney |
| Common buzzard | *Buteobuteo* | 2013 | M13-57 | Intestine, liver, spleen, kidney |
| Common buzzard | *Buteo buteo* | 2013 | M13-58 | Intestine, liver, spleen, kidney |
| Common buzzard | *Buteo buteo* | 2013 | M13-59 | Intestine, liver, spleen, kidney |
| White-tailed eagle | *Haliaeetus albicilla* | 2011 | M12-792 | Intestine, liver, spleen, kidney |
| Sparrow | *Passer* sp. | 2012 | M 12-907 | Intestine, liver, spleen, kidney |

*Sequence analysis*

The samples showing the expected amplicon length were purified using QI Aquick PCRPurification Kit (Qiagen, Germany) and sequenced in Macrogen Inc. (Amsterdam, the Netherlands). The virus sequences were initially checked against BLAST search database (http:/www.ncbi.nlm.nih.gov/BLAST/). The sequences were assembled and analyzed using Bio Edit (Hall, 1999), Mega 6 (Tamura *et al*., 2013) and Staden (Bonfield *et al*., 1995). Phylogenetic analysis was performed using Mega 6*,* while the best-fit phylogenetic model was selected using Model Test 2 (Darriba *et al*., 2012) (Table 2). The Gen Bank (http://www.ncbi.nlm.nih.gov/genbank/) accession numbers of the circovirus sequences submitted through this study were KP773230 (sequence PiCV/M10-513) and KP773231 (sequence DuCV/M10-448), while the accession numbers of sequences retrieved from the Gen Bank, shown in Figure 1.

**Table 2.** Oligonucleotide sequences, target gene, PCR amplicon size and references of primers used in the study

**Tablica 2.** Sekvence, ciljni gen, veličina proizvoda PCR i referenca početnica korištenih za pretraživanje ptica na navedene viruse

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
| Virus | Primers | Target gene | Amplicon size | Reference |
| Adenovirus | F1: 5'-TNMGNGGNGGNMGNTGYTAYCC-3' | Polymerase | 330 bp | Wellehan *et al*., 2004 |
| R1: 5'-GTDGCRAANSHNCCRTABARNGMRTT-3' |
| F2: 5'-GTNWYGAYATHTGYGGHATGTAYGC-3' |
| R2:5'-CBCDRTTRTGNARNGTRA-3' |
| Astrovirus | F: 5'-GAYTGGACIMGITAYGAYGGIACIATICC-3' | Polymerase | 434 bp | Todd *et al*., 2009 |
| R: 5'-YTTIACCCACATICCRAA-3' |
| Circovirus | F1: 5'-GGIAYICCIAYYTICARGG-3' | Replication gene | 400 bp | Li *et al*., 2010 |
| R1: 5'-AWCCAICCRTARAARTCRTC-3' |
| F2: 5'-GGIAYICCICAYYTICARGGITT-3' |
| R2: 5'-TGYTGYTCRTAICCRTCCCACCA-3' |
| Hepadnavirus | F: 5'-CTCAAGAGATTCCTCAGCC-3' | Polymerase | 906 bp | Walters *et* *al*., 2004 |
| R: 5'-GTCATACCATTCTCCTACT-3' |
| Coronavirus | F: 5'-TCACAYTTWGGATARTCCCA-3' | Replication gene | 250 bp | Stephenson *et al*., 1999 |

***Molecular diagnostic testing for West Nile, avian influenza and Newcastle disease virus determination***

*RNA extraction*

Tissue samples were homogenized in the equal volume of sterile buffered saline. The homogenates were centrifuged at 2000 g for 20 minutes and the resulting supernatant was used for RNA extraction. RNA extraction was done manually from the supernatants using High pure Viral Nucleic Acid Kit (Roche Applied Science, Germany), as described in the kit protocol.

*Reverse transcription and real time PCR*

One-step reverse transcription followed by real time PCR was carried out in LightCycler1.5 thermocycler using LightCycler RNA Master HybProbe (Roche Applied Science, Germany). For the detection of West Nile virus, avian influenza virus, Newcastle disease class I as well as class II viruses, protocols according to Tang *et al*. (2006), Spackman *et al*. (2002), Wise *et* *al*. (2004) and Kim *et al*. (2008) were used, respectively.

# *Bacteriology and mycology analyses*

*Salmonella* spp. *isolation*

Salmonella was isolated from the organs following the instructions for the standard EN ISO 6579:2002 and IOE methods (2008).

*Isolation of the genus Streptococcus*

All the analyzed organs were trans-inoculated onto the blood agar (Columbia agar with the supplementation of 5%-10% of sheep blood), Columbia agar and MacConkey agar. Streptococci did not grow in MacConkey agar (Wages, 2003).

*Isolation of the genus Staphylococcus*

All the investigated organs were trans-inoculated onto the blood agar (Columbia agar with the supplementation of 5%-10% of sheep blood), Columbia agar and MacConkey agar. Simultaneously, the organs were trans-inoculated onto mannitol salt agar and onto Baird-Parker agar, these representing selective culture media for Staphylococcus isolation. Staphylococci did not grow in MacConkey agar (Andreasen, 2003).

*Escherichia coli isolation*

At isolating the bacterium *Escherichia coli*, all the analyzed organs were trans-inoculated onto the blood agar (Columbia agar with the supplementation of 5%-10% of sheep blood), Columbia agar and MacConkey agar. Simultaneously, the organs were trans-inoculated onto the TBX agar, a selective medium for *E. coli* isolation (Barnes *et al*., 2003)

*Clostridium* spp. *isolation*

All the analyzed organs were trans-inoculated onto the blood agar (Columbia agar with the supplementation of 5%-10% of sheep blood), and incubated in an anaerobic environment (Barnes, 2003).

*Fungi and molds isolation*

Sabouraud glucose agar was used for the isolation of fungi and molds (Kunkle, 2003).

*Shigella isolation*

All the analyzed organs were trans-inoculated onto the blood agar (Columbia agar with the supplementation of 5%-10% of sheep blood), Columbia agar and Mac Conkey agar, and incubated in an aerobic environment (Naglić *et al*., 2005)

Biochemistry characterization for all the isolated bacteria was performed using API System strips.

**Results**

***Pathomorphology investigation***

**Table 3.** Alphabetical list, scientific name, year of necropsy, laboratory identifier and pathomorphological findings in birds tested for adenovirus, astrovirus, hepadnavirus, coronavirus and circovirus in the present study

**Tablica 3.** Abecedni popis, latinski naziv, godina patoanatomske pretrage, laboratorijska oznaka (virusa) i patomorfološki nalazi ptica pretraživanih na prisutnost adenovirusa, astrovirusa, hepadnavirusa, koronavirusa i cirkovirusa

|  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- |
| Bird  species | Scientific  name | Year | Identifier | Pathoanatomical (PA) diagnosis/findings | Histopathologic (HP)  and/or x-ray analysis  (diagnosis/findings) |
| White stork | *Ciconia ciconia* | 2013 | M13-69 | Gunshot wound (GSW) (frontal part of head) | X-ray findings of lead bullets (no HP investigation) |
| Mute swan | *Cygnus olor* | 2011 | M11-794 | Gunshot wounds (GSW) (left wing and ribs with internal hemorrhages) | X-ray findings of lead bullet particles (no HP investigation) |
| Mute swan | *Cygnus olor* | 2013 | M13-82 | Iron storage disease (ISD) | Iron pigment in hepatocytes (Prussian Blue staining) and focal necroses in the liver |
| Mute swan | *Cygnus olor* | 2014 | M14-01 | Gunshot wounds (GSW) | X-ray analysis  (lead bullet particles) |
| Mallard\* duck | *Anas platyrhynchos* | 2010 | M10-448 | Type C botulism | Adenovirus-like and circovirus inclusions in intestine |
| Domestic\*\* pigeon | *Columba livia domestica* | 2010 | M10-513 | Sepsis | Focal mononuclear cell myocarditis; intra-cytoplasmic inclusions in hepatocytes |
| Barn swallow | *Hirundo rustica* | 2012 | M12-906 | Traumatic wounds (head trauma) | No HP investigation |
| Caspian\*\*\* gull | *[Larus](http://en.wikipedia.org/wiki/Larus_argentatus" \o "Larus argentatus)*  *[cachinnans](http://en.wikipedia.org/wiki/Larus_argentatus" \o "Larus argentatus),*  Pallas 1811 | 2012 | M12-950 | Icterus, hepatomegaly, proventricular hyperplasia | Generalized amyloidosis (Congo-red staining for amyloid) |
| Common buzzard | *Buteo buteo* | 2011 | M13-54 | Traumatic wounds (head trauma) | No HP investigation |
| Common buzzard | *Buteo buteo* | 2013 | M13-55 | Traumatic wounds (wing, leg and ribs) | No HP investigation |
| Common buzzard | *Buteo buteo* | 2013 | M13-56 | Traumatic wounds (head, neck) | No HP investigation |
| Common buzzard | *Buteo buteo* | 2013 | M13-57 | Gunshot wounds (GSW) | X-ray analysis (lead bullet particles) |
| Common buzzard | *Buteo buteo* | 2013 | M13-58 | Traumatic wounds (legs) | No HP investigation |
| Common buzzard | *Buteo buteo* | 2013 | M13-59 | Traumatic wounds (head, neck) | No HP investigation |
| White-tailed eagle | *Haliaeetus*  *albicilla* | 2011 | M12-792 | gunshot wounds (GSW) | X-ray analysis (lead bullet particles) |
| Sparrow | *Passer* sp*.* | 2012 | M 12-907 | Traumatic wounds (head) | No HP investigation |

**\***dual infections with astrovirus and circovirus (mallard duck /*Anas platyrhynchos/)*

**\*\***circovirus infection (domestic pigeon **/***Columba livia domestica/)*

\*\*\*hepadnavirus infection (Caspian gull /*[Larus cachinnans](http://en.wikipedia.org/wiki/Larus_argentatus" \o "Larus argentatus),* Pallas 1811/)

***Molecular diagnostic testing for adenovirus, astrovirus, circovirus, hepadnavirus and coronavirus determination***

*PCR assays and sequence analysis*

Positive PCR results are shown in Table 4. The circovirus sequence found in pigeon (PiCV/M10-513) showed a 100% nucleotide and amino acid homology with pigeon circovirus (accession number AF252610.1) identified in Germany. The sequence DuCV/M10-448 derived from the mallard duck samples had the greatest nucleotide (99%) and amino acid (100%) similarities with duck circovirus (accession number DQ100076.1) detected in the USA. The sequence shared the greatest nucleotide (99%) and amino acid (100%) similarities with duck circovirus (accession number DQ100076.1) detected in the USA. The phylogenetic analysis results were consistent with those of molecular analysis, and clustered the sequence PiCV/M10-513 within the group of pigeon circoviruses, while the sequence DuCV/M10-448 was grouped together with duck circoviruses (Figure 1).

**Table 4.** Positive PCR results for one or more viruses

**Tablica 4.** Ptice pozitivne na jedan ili više pretraživanih virusa

|  |  |  |  |
| --- | --- | --- | --- |
| Identifier | Host species | Virus | Organ |
| M10-448 | Mallard duck | Astrovirus | Intestine, spleen |
| Circovirus | Intestine, spleen, bursa of Fabricii |
| M12-950 | Caspian gull | Hepadnavirus | Liver |
| M10-513 | Domestic pigeon | Circovirus | Liver, kidney |

PCV-1 (KJ408798.1) pig

PiCV/M10-513 domestic pigeon

CoCV (AF252610.1) pigeon

CoCVPL60 (KF738863.1) pigeon

PiCV/Belgium/11-07574/2011 (JX901127) pigeon

PiCV (DQ915959.1) dove

StCV (DQ172906.1) starling

StCV AM-C (KC846095.1) starling

BFDV (AF080560.1) cockatoo

BFDV (AF311296.1) lovebird

GuCV (DQ845074.1) gull

GuCV (JQ685854.1) gull

RaCV (DQ146997.1) raven

CaCV (DQ339095.1) canary

CaCV (AJ301633.2) canary

FiCV (DQ845075.1) finch

GoCV (AF536934.1) goose

GoCV xs4 (DQ192285.1) goose

SwCV Sw4 (EU056310.1) swan

SwCV H51 (EU056309.1) swan

DuCV FJPT60 (GQ423746.1) Muscovy duck

DuCV (HQ180265.1) Muscovy duck

DuCV (AY228555.1) mulard duck

DuCV D12-KD-028 (KC851821.1) domestic duck

DuCV/M10-448 mallard duck

DuCV 33753-52 (DQ100076.1) Pekin duck

100

100

100

99

100

99

98

80

71

98

72

82

93

90

100

92

0.2

**Figure 1.** Phylogenetic Neighbor-Joining (NJ) tree based on alignments of the partial (417 nt) fragment of 26 circovirus replication gene sequences. The tree was constructed using JukesCantor model. Circovirus sequences identified in this study are highlighted in grey. Porcine circovirus was used as an outgroup. Bootstrap values (>70) were shown next to the branches. The scale on the bottom represents evolutionary distances of the compared sequences.

**Slika 1.** Filogenetsko Neighbor-Joining (NJ) stablo dobiveno analizom nukleotidnih sekvenci replikacijskog gena cirko virusa dužine 417 nukleotida. Korišten je JukesCantorov (JC) model odabran prema analizi BIC (engl. *Bayesian Information Criterion*). U analizu je uvršteno ukupno 26 sekvenci iz roda *Circovirus*. Sekvence dokazane u ovom radu označene su sivom bojom. Cirko virus svinje je korišten kao tzv. vanjska skupina (engl. *outgroup*). Vrijednosti *bootstrap* probe (>70) označene su uz ogranke stabla. Ljestvica na dnu slike označava evolucijsku udaljenost uspoređivanih sekvenci.

***Molecular diagnostic testing for West Nile, avian influenza and Newcastle disease virus determination***

Molecular diagnostic testing for West Nile virus, avian influenza and Newcastle disease virus determinations did not reveal positive results in any of the tested samples.

# *Bacteriology and mycology analyses*

The most severe mixed microbial infections were determined in the domestic pigeon (Columba livia domestica) infected with circovirus (E. coli, Shigella sp., Aspergillus fumigatus, and molds) and in mallard ducks (Anas platyrynchos) infected with astrovirus and circovirus (E. coli, Clostridium sp., Micrococcus sp., Bacillus sp.).

**Discussion**

In 12 out of 16 wildlife birds selected for preliminary virologic testing for the presence of adenovirus, astrovirus, circovirus, hepadnavirus and coronavirus, ballistic and/or traumatic lesions of various origin were confirmed by pathoanatomical and x-ray examinations (Table 3). In the mute swan (*Cygnus olor*) (M 13-82) (Table 3), the gross findings of hepatomegaly with multiple dark foci in gold-brown liver aroused suspicion of iron storage disease (ISD). The histopathology examination confirmed ISD using special histologic staining for iron pigment, Prussian Blue staining. The ISD is one of pigmentary hepatopathies developing due to iron accumulation in hepatocytes. It is predominantly a disease of captive mynahs (*Gracula* sp.), several species of toucans (Ramphastide) and birds of paradise (Paradisaeidae). Infrequently, it can be seen in other bird species. The most susceptible are birds highly efficient at absorbing dietary iron and not down regulating iron absorption sufficiently when fed iron-rich diets. However, in wild birds it is presumed that iron is in a form that is not readily absorbed from the digestive tract (Schmidt *et al*., 2003). Our pathomorphological findings (gold-brown liver with dark spots and the histopathologic findings of iron pigment in hepatocytes and Kupffer's cells) confirmed ISD and not just the excessive liver iron in this swan. Dual infection with astrovirus and circovirus was confirmed in the mallard duck (*Anas platyrhynchos)* died from botulism (*Clostridium botulinum* toxin type C) (Tables 3 and 4). Bacteriologic investigations were positive for *Clostridium* sp. in the gizzard, the most common site of *Clostridium botulinum* because of the anaerobic environment in the gizzard cuticle. Type C botulism can also be caused by the ingestion of a preformed toxin. Outbreaks in waterfowl and other water birds are the result of the consumption of toxin-laden invertebrates (Dohms, 2003). Histopathology findings of the inclusion typical of circovirus infection were shown in one intestinal sample (Table 3). In another duck, however, the adenovirus-like inclusions were confirmed in the liver and intestine. Since one of the most prominent characteristics of circovirus infection is its immunosuppressive effect, it may be possible that simultaneous (adeno)virus infection was present.

According to the authors’ awareness, the hepadnavirus determined in the Caspian gull (*Laruscachinnans*, Pallas 1811) (Table 4) represents the very first finding of this virus in gulls so far. Generalized amyloidosis was confirmed by the special Congo-red staining for amyloid (Table 3), and, according to Zschiesche and Jakob (1989), it represents a common finding in *Laridae*. Avian amlyoidosis can affect a wide variety of birds and is found following a number of diseases, especially chronic infections and inflammation. So, the simultaneous findings of hepadnavirus infection and amyloidosis in the Caspian gull (Tables 3 and 4) may arouse suspicion that generalized amyloidosis was the consequence of chronic hepadnavirus infection. Avian amyloidosis is normally a well-recognized pathologic disorder in birds, especially in the waterfowl (Landman *et al*., 1998).

In the domestic pigeon (*Columba livia domestica*), circovirus presenting with gross lesions of sepsis and mycotic inflammation (*E. coli, Shigella* sp. and *Aspergillus* sp., and molds, respectively) (Table 3) were determined as well (Table 4). Atypical intracytoplasmic inclusions were found in the liver sample, but no typical circovirus inclusions were seen in immunocompetent organs. Schmidt *et al*. (2003) compared this type of inclusions with atypical adenovirus inclusions. In pigeons infected with circovirus, Abadie *et al*. (2001) also found lesions that could have been related to bacteria, but with no histologic evidence of concomitant viral infection (e.g., adenoviral and/or herpesviral infections).

**Conclusions**

In conclusion, although this study was performed on a limited amount of samples, the findings of astrovirus, circovirus and hepadnavirus suggest that these viruses are widespread among avian hosts. Based on the molecular and phylogenetic analyses of circovirus sequences identified in the pigeon and in the mallard duck, it may be concluded that the same types of viruses circulate and can be found in different geographical regions. However, due to the possible limitations of PCR (e.g., sensitivity of primers), different levels of virus amount in the samples and the stage of autolysis of the collected organs, some viruses could have remained undetected using the current procedures. For that reason, further research combining molecular methods and histopathologic examination is needed to get deeper insight into the free-living bird diseases and pathology.

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**Bolesti i filogenetska analiza nekih virusa identificiranih u slobodno živućih ptica u Hrvatskoj: Početno istraživanje**

**Sažetak**

U radu su prikazani rezultati patološkomorfološke pretrage i pretrage metodama molekularne biologije (lančana reakcija polimerazom /PCR/) za dokaz adenovirusa, astrovirusa, cirkovirusa, hepadnavirusa i koronavirusa u devet različitih vrsta sveukupno 16 slobodnoživućih ptica (bijela roda (*Ciconia ciconia*), crvenokljuni labud (*Cygnus olor)*, galeb klaukavac ([*Larus cachinnans*](http://en.wikipedia.org/wiki/Larus_argentatus)*,* Pallas 1811*),* divlja patka (*Anas platyrhynchos)*, obični škanjac (*Buteo buteo)*, orao štekavac (*Haliaeetus albicilla),* domaći golub (*Columba livia domestica)*, lastavica pokućarka (*Hirundo rustica*) i vrabac (*Passer* sp.)). Ptice su razuđene u Laboratoriju za patologiju Centra za peradarstvo, Hrvatski veterinarski institut, Zagreb. U divlje patke (*Anas platyrhynchos)* uginule od posljedica botulizma (C toksin *Clostridium botulinum*) dokazani su cirkovirus i astrovirus. Cirkovirus nukleotidnog slijeda DuCV/M10-448 dokazan u divlje patke pokazao je najveću nukleotidnu (99%) i amino kiselinsku sličnost (100%) s pačjim cirkovirusom (pristupni broj DQ100076.1) utvrđenim u SAD. Pozitivni rezultat pretrage metodom PCR za dokaz hepadnavirusa u galeba klaukavca ([*Larus cachinnans*](http://en.wikipedia.org/wiki/Larus_argentatus)*,* Pallas 1811)uginulog od posljedica amiloidozepotvrđen je djelomice i patološkohistološkim nalazom. Prema saznanju autora, ovo je ujedno prvi nalaz hepadnavirusa u galebova. Cirkovirus utvrđen u domaćeg goluba (PiCV/M10-513) pokazao je 100%-tnu nukleotidnu i amnokiselinsku homolognost s golubljim cirkovirusom (pristupni broj AF252610.1) dokazanim u Njemačkoj. U jednog crvenokljunog labuda patološkohistološkom pretragom utvrđena je bolest odlaganja željeza (engl. *iron storage disease* /ISD/), dok su ostale ptice uginule pretežito od posljedica nastrijelnih rana i drugih vrsta traumatskih ozljeda (bijela roda, crvenokljuni labud, obični škanjac, orao štekavac, lastavica pokućarka, vrabac). Metodama dijagnostike molekularne biologije nisu izdvojeni virusi groznice Zapadnog Nila, influence ptica i newcastleske bolesti.

**Ključne riječi:** slobodno živuće ptice, patološka morfologija, patološka histologija, lančana rekacija polimerazom (PCR), astrovirus, hepadnavirus, adenovirus, cirkovirus, koronavirus, galeb klaukavac ([*Larus cachinnans*](http://en.wikipedia.org/wiki/Larus_argentatus)*,* Pallas 1811*)*, divlja patka *(Anas platyrhynchos),* domaći golub (*Columba livia domestica*)*,* crvenokljuni labud (*Cygnus olor),* bolest odlaganja željeza, amiloidoza