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Detection of avian reoviruses in wild birds in Poland

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Abstract

Introduction: The purpose of this study was to determine the occurrence of avian reovirus (ARV) infections in wild birds in Poland and attempt to propagate the selected ARV strains in chicken embryo kidney (CEK) cells or chicken SPF embryos. **Material and Methods:** The study included 192 wild birds representing 32 species, collected between 2014 and 2016. A part of the S4 segment encoding the σNS protein of avian reoviruses (ARVs) isolated from different species of wild birds from that period was amplified. **Results:** The presence of ARV was demonstrated in 58 (30.2%) wild birds belonging to nine orders. The isolated strains were propagated in chicken embryos by yolk sac inoculation, and CPE was induced in the infected CEK monolayer. Agar gel precipitation showed that two ARV isolates from rock pigeon and mute swan shared a common group-specific antigen with chicken reovirus S1133. Specific products of predicted size were found in two ARV isolates from the chicken embryo passage and 13 ARVs isolated from CEK cells. **Conclusion:** The study indicates the high prevalence of ARV among wild birds in Poland and its possible transmission to farmed birds.

Keywords: wild birds, avian reovirus, Poland.

Introduction

In many parts of the world avian orthoreovirus (ARV) can be a cause of viral arthritis/tenosynovitis in chickens and turkeys (13, 21). However, the virus has also been isolated from birds showing several other disease conditions such as runting-stunting syndrome, hepatitis, myocarditis, and enteric and respiratory diseases in commercial poultry flocks (1, 22).

ARV infections have been increasingly diagnosed in wild avian species (4). Several reports have described ARV as a cause of serious diseases and mortality in free-living corvid species. Lawson *et al.* (10) presented a case of ARV infection in a wild magpie (*Pica pica*) from England. Recently, a novel orthoreovirus strains have been isolated from partridge (*Perdix perdix*) and brown-eared bulbul (*Hypsipetes amaurotis*) (9, 18). In many cases, association of ARV infection with disease/condition in wild birds has not been established, because no experimental studies have been performed. Little is known about the epizootiology of reoviruses in wild bird populations, but asymptomatic carriers have been implicated as potential sources of infection in poultry. Transmission to other birds inhabiting contaminated environment is possible because infected birds excrete significant amounts of the virus in their droppings (6).

ARVs belong to the genus *Orthoreovirus* in the *Reoviridae* family (15). They are characterised by medium-sized (70–80 nm) icosahedral virion and the double-stranded RNA genome consisting of 10–12 segments (20). The segmented genome encodes eight structural proteins (λA, λB, λC, μA, μB, σA, σB, σC) and four non-structural proteins (μNS, P10, P17, and σNS) (14). The ssRNA-binding nonstructural protein σNS is encoded by ARV S4 genome segment. This protein is present in large ribonucleoprotein complexes in the cytoplasm of infected cells and likely plays a key role in RNA packaging and virus replication (2). The site of primary ARV replication is the epithelium of the small intestine and bursa of Fabricius, after which the virus spreads within 24–48 h to other tissues and organs. The infection is pantropic (19).

The objective of this study was to investigate the occurrence of ARV infections in wild birds in Poland and propagate selected ARV strains in chicken embryo

kidney (CEK) cells or chicken SPF embryos. In Poland, studies on the occurrence of ARV in wild bird population have not been conducted so far due to the low availability of research material. This is the first report describing the detection and isolation of ARV in free-living birds in Poland.

Material and Methods

Birds and samples. A total of 192 dead wild birds obtained from ornithological stations and wild birds rehabilitation centres were used in the investigations between 2014 and 2016. The predominant groups among the tested birds were the *Passeriformes* order represented by 72 birds of different species and *Ciconiiformes* represented by 61 white storks (*Ciconia ciconia*). The remaining birds represented other orders and species (Table 1). During necropsy, sections of the heart, lungs, liver, spleen, gizzard, intestines, and kidneys were collected for molecular biology examinations and virus isolation. The sections were homogenised as 10% (w/v) suspensions in sterile phosphate-buffered saline (PBS) and then centrifuged at 6,000 × g for 10 min. The obtained supernatant was stored at –20°C for further investigations.

RNA extraction and RT-PCR. Total cellular RNA was extracted from the homogenates of internal organs, embryonic fluids, and membranes collected from infected chicken SPF embryos and cell culture supernatant using the RNAsy Mini Kit (Qiagen, Germany) according to manufacturer's protocol. The extracted RNA was used for the amplification reaction of a fragment of non-structural sigma NS gene of ARV using previously published primers (23). Amplification was performed in a T-Gradient Biometra Thermocycler (Biometra, Germany) using a One Step RT-PCR kit. The reaction was conducted in a 25 µL reaction mixture containing 2 µL of template RNA, 10 µL of RNase-free water, 4 µL of 5x RT-PCR buffer, 1 µL of dNTP mix (10 mM each dNTP), 1 µL of enzyme mix, 5 µL of 5× Q-solution, and 1 µL of each of the two primers. RT-PCR conditions were as follows: reverse transcription step at 50ºC for 30 min, initial denaturation at 94ºC for 15 min, then 40 cycles at 95ºC for 1 min, 60ºC for 1 min, 72ºC for 1 min, and single cycle at 72ºC for 10 min. RNA extracted from CEK cell culture inoculated with the S1133 strain was used as positive control, whereas non-inoculated CEK cell cultures served as negative control.

PCR product analysis. The ARV S4 segment amplified by RT-PCR was separated in 2% agarose gels in GelRed under 120 V for 40 min. The size of the amplicons was compared with the MassRuler DNA Ladder Mix (Thermo Scientific, USA). The results were visualised using transilluminator UV (GenoSmart, VWR, Germany), then photographed and analysed. The results were considered as positive when the RT-PCR product in RNA samples extracted from internal organs had a predicted size of 522 bp.

Virus isolation in chicken SPF embryos. The supernatants of internal organs homogenates were diluted and filtered through 0.45 µm syringe filters. The filtered suspension was injected into yolk sack of 5-6-day-old specific pathogen free (SPF) chicken embryos (0.2 mL/embryo). The embryos were candled daily for seven days. The amino-allantoic fluids, membranes, livers, and spleens of infected embryos were collected for further study.

Agar gel immunodiffusion test. Serological identification of ARV was performed by agar gel immunodiffusion test (AGID). The antiserum specific for S1133 reference chicken reovirus strain (Charles River Laboratories, USA) was used to detect reovirus antigen in hepatic homogenates, embryonic fluids, and membranes of infected embryos. Immunodiffusion test was performed to identify reoviruses in a layer of 1.5% agar gel supplemented with 8% NaCl. The slides were incubated for 24–48 h in a humidity chamber at room temperature.

Cell cultures. The cultures of chicken embryo kidney (CEK) cells were prepared from 18-19-day-old SPF chicken embryos (Lohman, Germany), according to the standard procedure. The growth medium was Eagle's minimum essential medium (MEM) supplemented with 10% foetal bovine serum and 1% addition of antibiotics (Antibiotic-Antimycotic, Sigma-Aldrich, USA). MEM with the antibiotics was used as the maintenance medium. CEK monolayer was inoculated with homogenates of internal organs centrifuged, diluted 10^{-1}, and filtered through the Millipore 0.45 µm filter. The infected cultures were incubated at 37.5ºC in 5% CO_2 and were observed daily under a microscope to determine a cytopathic effect (CPE). Three passages of the virus were conducted in cell cultures, and then the cells and supernatants were used for further analyses.

Results

All collected birds were necropsied. According to the information obtained from ornithologists, the birds died due to various causes and frequently had different fractures and other injures. Post-mortem examination often revealed swollen liver and spleen with the presence of liver necrosis. The lesions were characteristic of ARV infection. In some birds, gouty kidneys and enteritis were found.

In the study, σNS-encoding gene was targeted for diagnostic analysis because it shows less divergence within S class genome of ARV (11). The molecular examination revealed the presence of ARV genetic material in internal organs collected from 58 (30.2%) out of 192 examined wild birds representing different species (Table 1). The presence of ARVs was found in

88 (15.4%) samples of different internal organs. Samples of the lungs (23.6%), spleen (20.6%), and heart (20.4%) gave the highest number of positive results for ARV NS gene (Table 2).

Two homogenates of internal organs from birds with positive result in RT-PCR were used for the infection of chicken SPF embryos. Three days after yolk sack inoculation with kidney homogenate from rock pigeon, one of the five embryos died. The chicken embryos infected with intestine homogenate from white stork survived until the end of the experiment. All embryos exhibited subcutaneous haemorrhages in the muscles and olive green pigmentation of the liver parenchyma.

Table 1. Overview of the samples studied and RT-PCR results

Order	Species – Latin name	Species – English name	Number of birds tested	Number of positive birds	Percentage of positive birds*
Ciconiiformes	*Ciconia ciconia*	white stork	61	32	52.4
Pelecaniformes	*Ardea cinerea*	grey heron	1	1	-
Columbiformes	*Columba liva*	rock pigeon	13	2	15.4
Accipitriformes	*Buteo buteo*	common buzzard	4	1	-
	Aquila chrysaetos	golden eagle	1	1	-
Anseriformes	*Cygnus olor*	mute swan	12	2	16.6
	Anas platyrhynchos	wild duck	3	0	-
Charadriiformes	*Larus argentatus*	European herring gull	1	1	-
	Sterna hirundo	common tern	1	1	-
Strigiformes	*Strix aluco*	tawny owl	3	1	-
Falconiformes	*Falco tinnunculus*	common kestrel	4	0	-
	Falco peregrinus	peregrine falcon	2	0	-
Gruiformes	*Grus grus*	common crane	1	0	-
Piciformes	*Dendrocopos major*	great spotted woodpecker	10	1	10
Apodiformes	*Apus apus*	common swift	2	0	-
Passeriformes	*Corvus corax*	common raven	1	1	-
	Corvus monedula	western jackdaw	14	4	28.6
	Pica pica	Eurasian magpie	3	0	-
	Parus major	great tit	4	4	-
	Fringilla coelebs	common chaffinch	4	4	-
	Sylvia atricapilla	Eurasian blackcap	2	1	-
	Erithacus rubecula	European robin	27	0	-
	Phoenicurus ochruros	black redstart	1	0	-
	Turdus philomelos	song thrush	1	1	-
	Turdus merula	common blackbird	2	0	-
	Regulus regulus	goldcrest	4	0	-
	Periparus ater	coal tit	2	0	-
	Fringilla montifringilla	brambling	1	0	-
	Chloris chloris	European greenfinch	1	0	-
	Cyanistes caeruleus	Eurasian blue tit	4	0	-
	Poecile montanus	willow tit	1	0	-
	Aegithalos caudatus	long-tailed tit	1	0	-
Total: 12	32	32	192	58	30.2

* only if the number of birds tested ≥ 10

Table 2. Samples of internal organs from wild birds tested by RT-PCR

Internal organ	Number of examined samples	Number of positive samples	Percentage of positive samples
heart	113	23	20.4
lungs	55	13	23.6
liver	130	8	6.2
spleen	63	13	20.6
gizzard	81	13	16.0
intestines	78	9	11.5
kidneys	52	9	17.3
Total	572	88	15.4

Table 3. The origin of homogenates used for infection of CEK culture cells, the results of ARV isolation and the RT-PCR test

Isolate of ARV (identity no./year of isolation/internal organ*/sample no.)	Host	CPE	RT-PCR
67/14/j/5	common buzzard	+	+
67/14/n/9	white stork	-	+
67/14/n/10	white stork	+	+
67/14/j/6	rock pigeon	+	+
67/14/n/3	grey heron	+	+
67/14/n/11	white stork	+	+
67/14/w/16	white stork	+	+
116/15/z/3	white stork	+	+
147/15/n/5	white stork	+	+
147/15/n/7	white stork	-	+
147/15/p/3	white stork	+	+
147/15/s/4	white stork	+	+
147/15/z/4	white stork	+	+
22/16/p/1	mute swan	-	-
22/16/sl/1	great tit	-	-
22/16/w/1	common tern	-	-
119/16/s/1	western jackdaw	-	-

* j – intestine, n – kidney, w – liver, z – gizzard, p – lung, s – heart, sl – spleen

The group-specific antigen of ARV was detected in hepatic homogenates, embryonic fluids, and membranes collected from 10 infected chicken embryos, which showed reactivity with specific S1133 chicken reovirus antibodies. It manifested through the precipitation lines between the examined antigen and standard serum (Fig. 1). To confirm the propagation of ARV in chicken SPF embryos, total RNAs were extracted from hepatic homogenates, embryonic fluids, and membranes collected from infected chicken SPF embryos, and RT-PCR was conducted in order to amplify σNS gene fragment. Specific amplification products of predicted size were found in hepatic homogenate from chicken embryos infected with material from rock pigeon and in embryonic fluids and membranes collected from chicken embryos infected with material from white stork.

The selected homogenates of internal organs from 17 wild birds with positive result in RT-PCR were used for infection of CEK cells (Table 3). Three passages were conducted at 16-h intervals. In the third passage, a CPE in the form of rounded cell focuses starting from the 72 to 120 h.p.i. was observed in 11 cell cultures. In a few cases, the cell culture loosening was observed. CPE indicating the presence of ARV was difficult to identify. The virus did not cause total disruption of the cell monolayer (Fig. 2). To confirm the propagation of ARVs from wild birds in CEK cell cultures, total RNA was extracted from cell culture supernatants, and amplification of σNS gene sequences was conducted. A specific fragment of 522 bp was amplified from 13 isolated strains and the reference strain S1133. RNA extracted from non-infected cells was used as a negative control and no band was observed. In total, 13 ARV strains from 10 white storks, grey heron, common buzzards, and rock pigeon were obtained (Table 3). In the case of two cell cultures infected with material from white storks, no CEK was observed, with simultaneous confirmation of the presence of ARV genetic material in the RT-PCR assay.

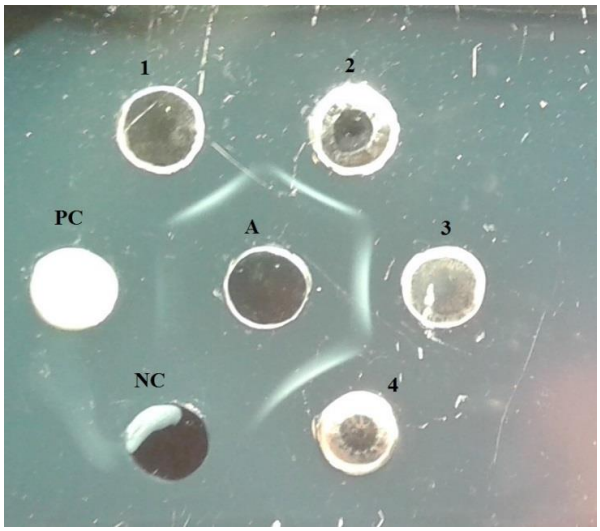

Fig. 1. Antigen-specific group of reovirus in agar gel immunodiffusion (AGID) test. PC – positive control S1133 chicken reovirus antigen (Charles River Laboratories, USA), NC – negative control, A – S1133 reovirus antiserum (Charles River Laboratories, USA), 1 – hepatic homogenate from chicken embryos infected with material from rock pigeon, 2 – embryonic fluids and membranes from chicken embryos infected with material from rock pigeon, 3 – hepatic homogenate from chicken embryos infected with material from white stork, 4 – embryonic fluids and membranes from chicken embryos infected with material from white stork

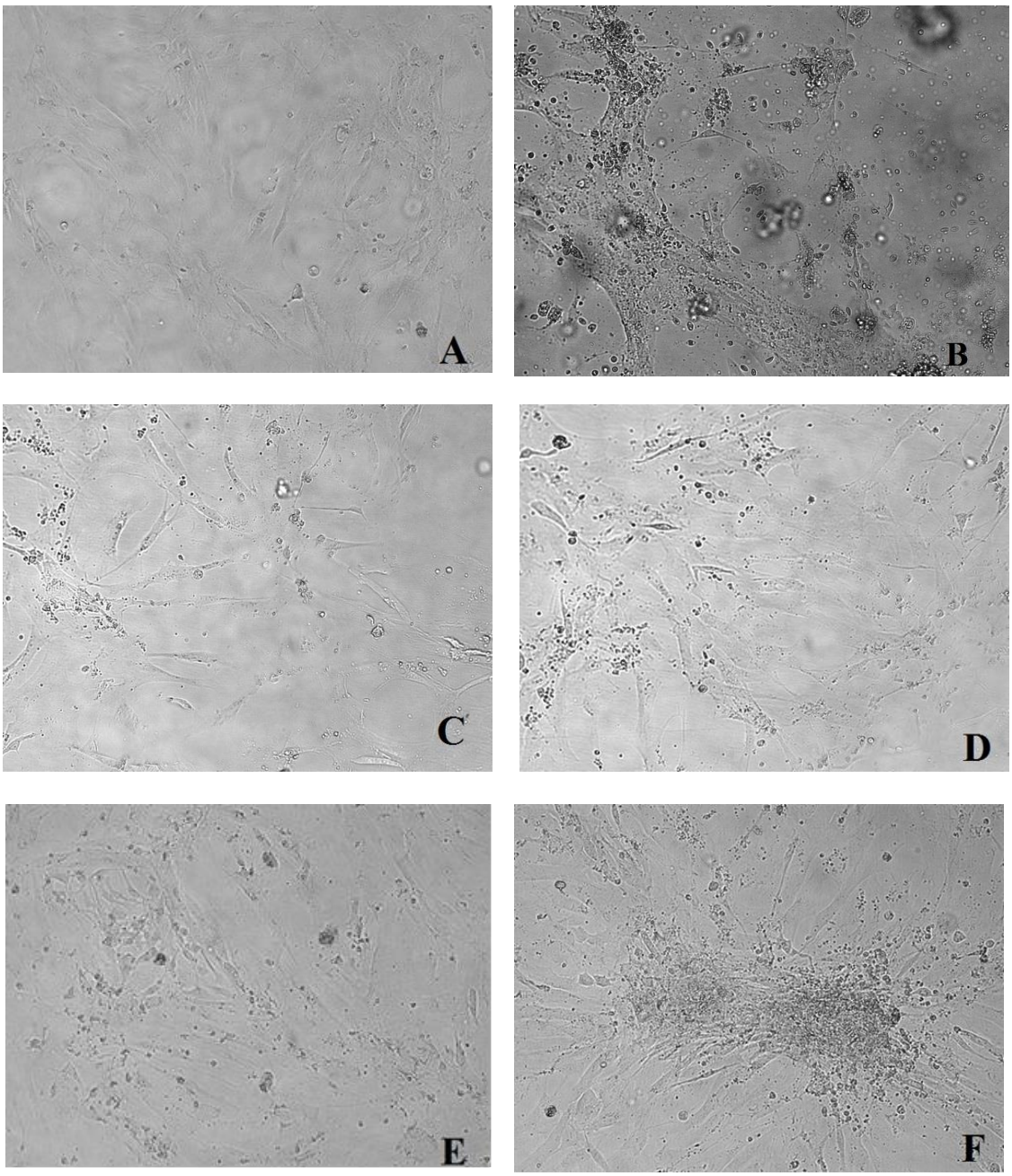

Fig. 2. Changes in chicken embryo kidney (CEK) cell cultures. Pictures taken at 72–120 h p.i. with the 3rd virus passage.
A – negative control – non-infected CEK cells, B – CPE in CEK cells infected with reference strain S1133, C – CPE in CEK cells infected with homogenate of the kidney from grey heron, D – CPE in CEK cells infected with homogenate of the intestine from common kestrel, E – CPE in CEK cells infected with homogenate of the intestine from rock pigeon, F- CPE in CEK cells infected with homogenate of the kidney from white stork. 200× (Axio Observer D1, Zeiss, Germany)

Discussion

The epidemiology of ARV infections in wild birds remains unknown, so it was reasonable to conduct a study on their occurrence. The research was performed using molecular methods and classical virus isolation methods, which allowed us to obtain ARV strains for our departmental collection. ARV infections have been increasingly diagnosed in wild avian species. Recent reports have described ARV as a cause of serious disease and/or mortality in free-living corvid species in Great Britain and Finland (5, 10). ARV strain detected in a free-living magpie was genetically related to chicken origin reoviruses, reinforcing the hypothesis on the possible reservoir role of wild birds in reovirus transmission (10). Ogasawara *et al.* (18) isolated a novel orthoreovirus strain from a dead brown-eared bulbul (order *Passeriformes*) in Japan. Scientists from Hungary detected a reovirus strain isolated from partridge and conducted its genomic characterisation. Their results imply that partridges may serve as natural reservoirs of ARVs for domestic poultry (9). In 2011, ARV was detected in black-capped chickadees (*Poecile atricapillus*) and molecular

characterisation showed 89.4%–98.3% nucleotide identity to turkey reoviruses (16). In our study, ARVs were identified in over 30% of the wild birds investigated, highlighting a serious concern for farmed bird flocks. The largest group of birds belonged to *Passeriformes* and *Ciconiiformes* order, which made up 37.5% and 31.8% of the birds tested, respectively. The presence of ARV genetic material was detected in all types of the examined internal organs, which confirms the tropism of the virus to many organs and tissues. ARV infections were found in different species of wild birds, which might indicate their possible transmission to other birds inhabiting Poland.

One of the previous reports described reovirus infection of pheasants in the western part of Turkey, with clinical symptoms manifested as shortness of breath, greenish and watery diarrhoea, and death within a week (17). The material collected in our study did not allow us to observe clinical symptoms, because it was taken from dead birds.

ARV propagation using chicken embryos and CEK cell cultures are useful, although time-consuming, in virological diagnosis. The molecular biology methods targeting specific ARV genes have been shown effective in more rapid detection of ARV infections (3, 8, 24). In the present study, ARVs were propagated in cell culture and chicken SPF embryos in order to confirm the results obtained by RT-PCR. ARVs from wild birds may be propagated on chicken embryos by yolk sac inoculation. They induce CPE in the infected CEK monolayer. ARV strains isolated from wild birds exhibit pathogenicity for chicken embryos, which was confirmed by the embryo pathological changes observed in the form of subcutaneous haemorrhages in the muscle tissue and changes in internal organs characteristic of reovirus infection. Changes in infected CEK cell cultures demonstrated effective replication of the virus. In several cell cultures infected with material from white storks, great tit, western jackdaw, and common tern, no CPE was observed after the third passage. It may be necessary to perform more passages or use a different type of cells for *in vitro* culture. In previous studies, a novel orthoreovirus isolated in BHK (baby hamster kidney-21 cells) from a diseased crow caused a strong CPE, including syncytium formation (5).

ARV can be readily differentiated from other viruses based on the presence of a group-specific antigen in AGID test. This serological method is the most common approach for ARV diagnosis. In our study, the common antigen to chicken reoviruses was detected in ARV isolates from rock pigeon and white stork using AGP method, which confirmed their propagation in chicken embryos. These results are similar to observations of reoviruses isolated from Muscovy ducks (12, 23).

It is possible that there are species-specific reovirus strains, although at least some of them are capable to induce cross-infections among species. Experimental study has shown that reoviruses isolated from wedge-tailed eagle (*Aquila audax*) can be infective for chickens (7). This study requires further efforts to assess the potential pathogenicity of ARV strains isolated from wild birds for domestic poultry.

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