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Occurrence of reovirus (ARV) infections in poultry flocks in Poland in 2010–2017

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Abstract

Introduction: Avian reovirus (ARV) infections in poultry populations are reported worldwide. The reovirus belongs to the genus *Orthoreovirus*, family *Reoviridae*. The aim of the study was to evaluate the incidence of ARV infections in the poultry population based on diagnostic tests performed in 2010–2017. **Material and Methods:** Samples of the liver and spleen were collected from sick birds suspected of ARV infection and sent for diagnostics. Isolation was performed in 5–7-day-old SPF chicken embryos infected into the yolk sac with homogenates of internal organs of sick birds. Four primer pairs were used to detect the σNS, σC, σA, and μA ARV RNA gene fragments. A nested PCR was used for the detection of the σNS and σC genes. **Results:** In 2010–2017, ARV infection was found in birds from 81 flocks of broiler chickens and/or layers, 8 flocks of slaughter turkeys, and in 4 hatchery embryos at 17–20 days of incubation. The primers used in RT-PCR and nested PCR did not allow effective detection of ARV RNA in all virus-positive samples. **Conclusion:** The problem of ARV infections in the poultry population in Poland still persist. The primers used for various ARV segments in RT-PCR and nested PCR did not allow effective detection of RNA in the visceral organs of sick birds. The presented results confirm the necessity of using classical diagnostic methods (isolation in chicken embryos, AGID).

Keywords: poultry, avian reovirus**,** isolation, RT-PCR.

Introduction

For many years in the poultry population in Poland, as in many other countries, there has been a significant spread of infections with avian reovirus (ARV) (4). The virus belongs to the genus *Orthoreovirus*, family *Reoviridae* (14).

Its genome is double-stranded, segmented RNA (dsRNA) consisting of 10–12 segments coding for structural and non-structural viral proteins. The genetic material is surrounded by a capsid with twenty-sided symmetry consisting of eight structural polypeptides. The genome structure can be a source of variation within strains in the form of mutations, within individual segments, and in the reassortments within entire segments, especially in the situation of simultaneous infection of birds with different ARV strains (4, 7, 18).

Another feature of this virus is significant resistance to physical and chemical factors and this significantly hampers its elimination from the environment (4, 8).

Infections with reoviruses are described in chickens, turkeys, geese, ducks, guinea fowls, Japanese quails, pigeons, and in many species of wild and exotic birds (14).

Currently, due to the observed molecular differences between ARV occurring in various species of poultry, species-specific name reovirus types are being introduced, namely TRV for turkey, DRV for duck, and GRV for goose, while ARV now refers to reovirus of chickens (4).

Most ARV strains do not show pathogenic properties and they are frequently found in chickens without clinical symptoms. The pathogenic strains are isolated from a variety of tissues and organs in

chickens affected by assorted disease conditions including arthritis, malabsorption syndrome, pericarditis and myocarditis, hepatitis, atrophy of the bursa Fabricii and thymus, acute and chronic respiratory diseases, reovirosis, immunosuppression, and gastrointestinal lesions (4, 10, 11).

The course of infection in birds depends on many factors: the age of the birds and their sensitivity, pathogenicity of the strain, infectious dose, route of infection, presence of maternal antibodies, or conditions of poultry husbandry. The most sensitive are young birds in the first days of life without maternal antibodies, but birds of all ages can be infected (14). Infections are spread both horizontally through direct contact or contaminated feed, water, or equipment and vertically through hatching eggs laid by infected layers, so when hatched, chicks become a source of infection very early, in the hatchery (9, 13, 14). ARV infections are regularly found in many flocks, most often among broiler chickens and turkeys. Economic losses due to ARV are significant in many cases despite the use of prophylactic vaccinations.

For several years, laboratory diagnostics based on the RT-PCR method proved to be unreliable due to the appearance of false-negative results in samples from which ARV was isolated in chicken embryos or cell cultures and which may have been associated with the high variability of ARV strains (1, 8, 11).

A significant spread of ARV infections in poultry being suspected, studies were undertaken to evaluate the occurrence of these infections in the poultry population in Poland based on diagnostic tests carried out in 2010–2017.

Material and Methods

SPF chicken embryos. Embryonated eggs aged between five and seven days derived from SPF eggs (Valo, Lohman Tierzucht, Germany) were used to isolate reoviruses (5). The eggs were incubated at 37ºC in 55% relative humidity.

Material for research. Birds with clinical symptoms from 120 poultry flocks and embryos which died during 17–20 days of incubation in poultry hatcheries located throughout Poland were sent for diagnostics for reovirus infections in 2010–2017. During the post-mortem examination, the occurrence of pathological changes in organs was evaluated and samples of the liver, spleen, and other visceral organs were collected. The organ samples were homogenised and resuspended in Eagle's Minimum Essential Medium (MEM, Gibco, UK) with the addition of 1% antibiotic mixture (Antibiotic-Antimycotic, Gibco, UK) to obtain a 20% (w/v) suspension. Then the biological materials were frozen and thawed three times and centrifuged (4ºC, 3,000 × g/min for 5 min), and the supernatant was collected. Prepared samples were stored at below −20ºC for further testing.

Isolation of reoviruses. It was carried out in five-to-seven-day-old SPF chicken embryos, which were infected into the yolk sac (YS). Samples were diluted 10^{-1} in PBS and then filtered through syringe filters with 450 μm pore diameter (Minisart, Sartorius, Germany). The embryos were inoculated with the prepared biological material at 0.2 mL/embryo, using five embryos for each dose, and three uninfected embryos remained as the control group. All embryos were incubated at 37.8ºC in 55%–56% relative humidity for seven days and were observed daily. Embryos which died in the first 24 h after infection were rejected as showing nonspecific reactions. The remaining embryos were frozen and thawed after seven days of incubation, sectioned, and pathological changes on embryos and embryonic membranes were evaluated. During the study, membranes, embryonic fluids, and internal organs (liver, spleen, and intestines) were collected and homogenates as above were prepared. These samples were used to determine the presence of a group-specific ARV antigen using AGID.

Agar gel immunodiffusion test (AGID). The reaction was performed with a micromethod on glass slides covered with 1.5% agar gel with the addition of 8% NaCl (16). Samples obtained from infected embryos were tested against standard anti-ARV serum. Standard commercial ARV antigen was used as a positive control (Charles River Laboratories, USA). The results were evaluated after 24–48 h incubation in a humid chamber at room temperature. The appearance of white precipitation lines between wells containing test samples and anti-ARV serum was considered a positive result.

Standard viral strains. The standard vaccine strain S1133 and the strain from our own collection derived from a case of chicken reovirosis caused by an enteric reovirus strain (ERS), were used for the study after propagation in CEF cell culture. The total cellular RNA of these strains was used as a positive control. The negative control was total cellular RNA isolated from the internal organs of uninfected SPF embryos.

RNA samples. Total cellular RNA was isolated from homogenates prepared from organ samples of sick birds and/or infected chicken embryos using the QIAamp Viral RNA Mini Kit (Qiagen, Germany). RNase inhibitor (Thermo Scientific, Lithuania) in 1 μL volume was added to the obtained samples. RNA samples were stored at −20ºC for further testing.

Primers (oligonucleotides). Different pairs of oligonucleotide primers specific to several segments of the ARV genome (S4, S1, S2, M1) were used. Previously published sequences of primers specific to the following fragments were used: the genes encoding the σNS viral protein (15), σC protein (6), and σA protein (17). The primers for RT-PCR for detection of σNS I μA genes and internal primers for nested PCR detecting σC were designed in Primer3Plus software (Andreas Untergasser, Germany) based on the

complementary sequence region of standard ARV strains in the GenBank database (Table 1).

Table 1. The sequence primers for detection of ARV

Primer	Sequence 5'–3'	Product size (pz)
ARVσNS-F	CCCACTTTCCATTCCTTTCA	967
ARVσNS-R	AGTATTTGTGAGTACGATTG	
ARVM1-F	CTTGCTTCCTTCTCGTCGTT	400
ARVM1-R	GCGTGCAGTAAGGACACAAT	
ARVnestedσC-F	CAGCGAAGAGAGGTCGTCA	960
ARVnestedσC-R	CACGGTCAAGGAACGAATG	

RT-PCR. RT-PCR was carried out in 25 µL of reaction mixture that contained: 5 µL of 5× One-step RT-PCR buffer (containing 2.5 mM of Mg_2 in the final volume of the reaction mixture), 1 µL of dNTP mix (10 mM), 0.5 µL each of the F and R primers (0.2 µM starter concentration), 1 µL of RT-PCR Enzyme Mix, 5.0 µL of Q-solution (Qiagen, Germany), and 10 µL of free RNA water. RNA of the samples tested was added to the prepared reaction mixture in 2 µL volume. The reaction conditions were determined on a temperature gradient (Biometra, Germany). Reverse transcription was performed at 50°C for 30 min and initial denaturation at 95°C for 15 min. The PCR involved 40 cycles: denaturation at 94°C for 45 s; annealing at 56.8°C for ARVσNS, 58°C for ARVσC, 53°C for ARVσA, and 63°C for ARVM1; and elongation of DNA at 72°C for 1 min. The final extension chain elongation was performed at 72°C for 10 min.

Nested PCR. The RT-PCR products from the first amplification reaction step were used as a nested PCR template. In this reaction, specific internal primers for the σNS gene (15) and σC ARV (Table1) were used. The reaction was carried out in a volume of 25 µL, which contained: 2.5 µL of 10 × Pol Buffer C, 0.5 µL of $MgCL_2$, 1.0 µL of dNTPmix (5 mM of each dNTP), 0.5 µL of Starter F (10 mM), 0.5 µL of Starter R (10 mM), 0.5 µL of Perpetual Taq 5 U/µL polymerase (EURx, Poland), 17.5 µL of double-sterile water, and 2.0 µL of PCR product from the first reaction. The reaction mixture was pre-denatured at 95ºC for 5 min, then the reaction was carried out for 35 cycles: denaturation for 30 s at 94ºC; annealing at 60ºC for 30 s for primers of the σNS gene (15), and 52ºC for 1 min for σC primers; and elongation of DNA at 72ºC for 1 min. Final DNA elongation was at 72ºC for 10 min.

Electrophoresis of RT-PCR and nested PCR products. The obtained RT-PCR and nested PCR products were electrophoresed in a 2% agarose gel with GelRed Nucleic Acid Gel Stain, 10,000 × in DMSO (biotium) in a MiniGel apparatus (Biorad, U.K.) for 1 h at 120 V. After electrophoresis, the gel was evaluated under UV light in a gel documentation apparatus (VWR Genosmart, Germany). In RT-PCR the result was considered positive when base pair lengths were present appropriate for the used primers: 967 bp for the σNS gene, 1,088 bp for the σC gene, 598 bp for the σA gene, and 400 bp for the µA gene. The size of the expected product in the nested PCR reaction was 507 bp for the σNS gene and 960 bp for the σC gene.

Results

Table 2 contains data on the number of flocks examined in individual years, divided into flocks of layers, turkeys, and dead embryos. During this examination period, the suspicion of ARV infection was recorded in 99 flocks of broiler chickens and/or commercial layers, 16 flocks of slaughter turkeys, and in 6 hatcheries where vertical infection with ARV was suspected. The examined birds were of different ages: broiler chickens of 1–45 days old, layers of 8–12 weeks old, and turkeys of 4–16 weeks old.

Table 2. Flocks of birds suspected of ARV infection in 2010–2017

Year	Number of flocks suspected of ARV infection	Layer/broiler flocks		Turkey flocks		Hatchery/dead embryos	
		Number of examined flocks	Number of infected flocks	Number of examined flocks	Number of infected flocks	Number of examined flocks	Number of infected flocks
2010	11	8	8	3	3	-	-
2011	6	6	5	-	-	-	-
2012	11	9	8	-	-	2	1
2013	11	10	10	1	0	-	-
2014	8	8	6	-	-	-	-
2015	9	8	8	-	-	1	0
2016	29	23	17	5	3	1	1
2017	35	27	19	7	2	2	2
Total	120	99	81	16	8	6	4

The data obtained from the veterinarians directing the birds to the examination indicated that the most common sign to suspect ARV infection was a significant inhibition of growth, diversity of birds in the flock, drop in production rates, locomotor disturbances, arthritis, increased mortality, and a higher proportion of birds lost due to poor condition. All these resulted in economic losses in farming. The dead embryos were examined due to increased mortality during hatching on the premises and health problems in flocks of broiler chickens from these hatcheries.

During the necropsy pathological changes were found in internal organs in the form of enlargement and congestion of the liver and spleen, often with white necrotic foci in their parenchyma. There was also observed enteritis and oedema in the area of hock joints with the accretion of a straw-coloured fluid in the joint space. Embryos inoculated with homogenates from the liver and spleen died within 3 and 7 days after the infection. No dead embryos were noticed in the case of injection of five suspected materials. The flocks with confirmed reovirus infections showed 20%–30% mortality of embryos inoculated.

During embryopathological examination, congestion of the embryonic membranes, congestion and oedema in the area of the back of the head and neck and congestion, enlargement, and, less frequently, small white spots in the liver parenchyma were observed. Pathological changes of varying intensity were also observed in embryos which survived the infection.

In the samples taken from the embryonic membranes, fluids, and internal organs, the presence of a group-specific ARV antigen against the monovalent anti-ARV serum was determined by the AGID. Based on these tests, the presence of ARV infection was found in chickens and layers from 81 flocks and in slaughter turkeys from 8 flocks. As can be seen from the data in Table 2, the number of cases of suspected ARV infection has increased significantly in the last two years. While in 2010–2015 only a few flocks were examined, in 2016 and 2017 their numbers were 23 and 27. During this period, ARV infection was confirmed in 17 (2016) and 19 (2017) bird flocks.

The most frequent ARV infections were observed in broiler chickens aged 2–6 weeks (24 cases), slaughter turkeys aged 7–9 weeks (5 cases), laying hens aged 8–16 weeks (5 cases), and embryos from 3 hatcheries which died on the 17^{th}–20^{th} days of incubation.

Due to necessity of conducting diagnostic tests with classical methods and a high demand for ARV infection diagnostics, an attempt was made to optimise RT-PCR using different primers. Four different primer pairs were used, which were conservative for fragments of selected segments of the ARV genome. The conditions for performing RT-PCR were determined on the basis of RNA of the reference strain S1133 and 218 (ERS) isolated from broiler chickens in cases of reovirosis in 2001. The examination of 36 ARV strains isolated from sick birds in the last two years showed that the most positive reactions were found when conservative primers were used for fragments of genes encoding the σNS protein (14 tested RNA samples) and σC protein (10 tested RNA samples). The other two pairs of starters for σA (segment S2) and μA genes (segment M1) detected the ARV genetic material in eight and six ARV RNA samples tested, respectively. These results did not provide fully effective detection of ARV in biological material obtained from sick birds compared to results obtained by classical methods.

In order to increase the level of detection of the ARV genetic material, the products of the first stage PCR amplification (RT-PCR) were used as a matrix in the second stage (nested PCR). The nested PCR conditions were used for the σNS and σC gene fragments. Fig. 1 shows the exemplary results of the nested PCR. The use of the second stage of the nested PCR for the σNS gene allowed the additional detection of ARV genetic material in 15 samples. In contrast, the use of primers for the σC gene fragment confirmed the presence of ARV genetic material in addition in three samples.

Discussion

The presented research was based on the diagnostic needs of veterinarians who take care for poultry flocks in different regions of Poland. In recent years, the number of clinical cases in poultry suggestive of reovirus infections has increased. Data from anamnesis and pathological changes observed in the liver, spleen, small intestines, and joints, and bird differentiation confirmed the possibility of ARV infection in the majority of sick birds. This information is also validated by observations published by other authors (1, 5, 11, 14).

The pathogenicity of viral strains occurring in poultry varies widely, and besides non-pathogenic strains, there are pathogenic strains causing significant losses in poultry farming, especially in flocks of broiler chickens (14, 18).

The largest losses caused by ARV were recorded in Poland at the turn of the 20^{th} century. At that time, the occurrence of reovirosis in the broiler chicken flocks also touched birds with high levels of ARV antibodies. The course of the disease was acute and was associated with significant mortality and large economic losses (10). Only the introduction of an inactivated vaccine based on strains (ERS) isolated from diseased birds proved to be an effective method of solving this problem. Such vaccinations based on currently occurring ARV strains were also used in the USA (4, 11).

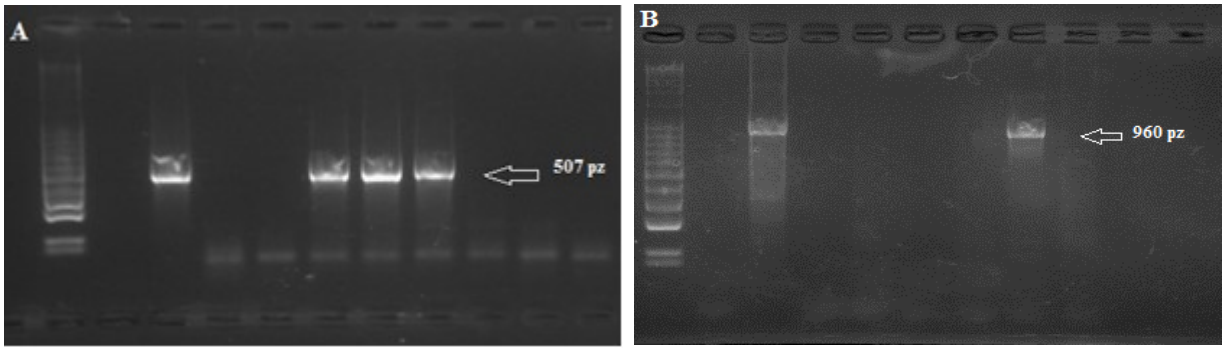

Fig. 1. An example of the electrophoresis of PCR products obtained after the nested PCR with the complementary primers of the σNS gene (A) and the σC gene (B)

Currently used in Poland preventive programmes for reproductive layers most often include four time vaccination: two times live vaccines and two times inactivated vaccine in order to increase the level of antibodies and protect the offspring. However, vaccination is not used in broiler chickens at the moment. The analysed diagnostic tests performed in 2010–2017 confirmed that the most frequent clinical cases of ARV infection relate to flocks of broiler chickens and turkeys and account for approximately 75% of cases (4, 11, 12, 18).

Laboratory diagnostics were performed by isolation of the virus in SPF chicken embryos using yolk sac inoculation after 5–7 days of incubation. Other authors (5, 7) also indicate the efficacy of infection on embryo amniotic membrane (CAM), and infection of primary cultures of chicken embryo fibroblast cells (CEF) and chicken embryo kidney cell (CEK) cultures. However, isolation in cell cultures usually requires several passages, whereas in chicken embryos, ARVs replicate in a large concentration already in the primary material. Most of the tested isolates caused embryo death within 7 days p.i. and induced the formation of pathological changes in extraembryonic membranes and embryos in the form of congestion of the membranes, oedema and congestion around the neck, and changes in the liver. These changes were observed in varying intensity after inoculation with materials obtained from sick birds. These observations coincide with the data in the literature (8, 11, 14). However, in the case of four homogenates, no embryo death was observed, but they induced pathological changes most often in the liver.

Avian reoviruses (ARVs) have group-specific antigens common to all ARVs detected in AGID (8, 14). The presence of a group specific antigen was confirmed in 93 cases.

The characteristic structure and changes in the ARV genome may be the cause of variation of ARV strains. According to data of other authors (2, 3, 11, 14), the smallest variability occurs within small segments of the genome, therefore, primers for fragments of these genes were used in our own research. Because unsatisfactory results were obtained in the RT-PCR, the second nested PCR was performed in which the internal primers for σNS and σC C gene fragments were used. The nested PCR results allowed the detection of ARV genetic material in an additional 15 samples. The use of nested PCR for the σC gene fragment only confirmed the results from the first PCR.

The results of the research have shown that the problem of reovirus infections in poultry has not abated and they contribute to economic losses. The applied primer pairs for different fragments of the ARV genome segments and nested PCR did not allow effective detection of the genetic material of these viruses in the internal organs of sick birds. At the same time, they indicated the occurrence of variability of viruses isolated from affected birds in Poland within various genome segments. The next generation sequential research (NGS) planned for the next years will allow to establish conservative fragments common for the strains occurring in the bird population in Poland, and thus efficient rapid diagnosis of ARV infections will be developed.

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