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Original article

Occurrence of reovirus infection in Muscovy ducks (*Cairina moschata*) in south western Poland

G. Woźniakowski¹, E. Samorek-Salamonowicz¹, A. Gaweł²

¹ Department of Poultry Viral Diseases, National Veterinary Research Institute,
Al. Partyzantów 57, 24-100 Puławy, Poland

² Department of Epizootiology and Clinic of Bird and Exotic Animals,
Wrocław University of Environmental and Life Sciences, Grunwald Square 45, 50-366 Wrocław, Poland

Abstract

During the summer 2012 an incidence of high mortality, above 44 percent, in two flocks of Muscovy ducklings in Poland was noted. The clinical signs included considerable weight loss and inability to walk.

During the post-mortem evaluations dehydration and enteritis, gouty kidneys as well as hemorrhagic liver and spleen lesions were found. The laboratory diagnosis included agar gel precipitation assay (AGP) as well as polymerase chain reaction (PCR) or reverse transcription PCR for the presence of goose parvovirus (GPV), duck circovirus (DuCV), duck reovirus (DRV) and avian reovirus (ARV). Interestingly, the examinations performed by AGP showed partial reactivity of liver homogenates from Muscovy ducklings with chicken S1133 antiserum. The presence of duck reovirus RNA was also detected by real-time RT-PCR targeting the chicken reovirus sigma NS fragment, while the sequencing showed major similarity to chicken S1133, 1733, GX/2010/1 and TARV-MN2 reovirus strains. The virus sequence was also related to a previously isolated TH11 strain from Muscovy ducks in China.

Further study is needed in order to explain the particular epidemiology of the reovirus infection of Muscovy ducklings.

Key words: Muscovy ducklings, avian reovirus, sigma NS sequence analysis

Introduction

Reoviruses isolated from poultry and waterfowl are termed avian reoviruses (ARVs) and belong to the *Orthoreovirus* genus (Simmons et al. 1972, Gouvea et al. 1982). The ARV virions are non-enveloped with

icosahedral symmetry. The capsids contain segmented double-stranded RNA (dsRNA) which consists of three classes: large (L1-L3), medium (M1-M3) and small (S1-S4) segments. Eight of the translated segments encode three categories of structural proteins: λ encoded by L genes, μ encoded by M genes and

σ encoded by S genes. The ARV genome also encodes non-structural proteins – μ NS encoded by M3 and σ NS encoded by S4 genes. Other non-structural proteins are represented by p17 and p10 genes which are encoded by the S1 segment (Gouvea et al. 1982). Avian reoviruses infect poultry and waterfowl. So far the described cases of infection include chickens (Olson et al. 1972), turkeys (Palya et al. 2003), geese (Simmons et al. 1972), ducks (Malikson et al. 1981) and other species. The clinical symptoms caused by ARV in chickens include respiratory or enteric disease, viral arthritis or runting-stunting syndrome (RSS) (Simmons et al. 1972). The lesions are described as inclusion body hepatitis, necrotic foci in liver, splenomegaly and hydropericardium. One of the most important roles of reoviral infection is associated with immunosuppression of affected birds. Morbidity in affected flocks of geese and Muscovy ducks may reach from 10 to 30%, with mortality from 15% to 30% (Chen et al. 2012). The most frequent reports on the occurrence of pathogenic strains of ARV originate from China due to the massive production of Muscovy ducklings and goslings. Nowadays, a considerable increase in farming of Muscovy and Pekin ducks is observed in Poland. During August 2012 in one broiler farm of Muscovy ducklings from the central area of Poland the occurrence of reovirus infection was reported. The clinical signs included weight loss and inability to walk. During post-mortem examinations dehydration, enteritis, gouty kidneys as well as hemorrhagic liver and spleen lesions were found. The ducklings were examined for the detection of goose parvovirus (GPV), duck circovirus (DuCV), duck reovirus (DRV) and avian reovirus (ARV). This study describes the isolation and molecular identification of reovirus infecting Muscovy ducklings.

Materials and Methods

Muscovy ducklings

The flock size was 14 thousand birds. The birds were aged from 3 to 5 weeks. The ducklings were allocated for farming with an interval of 2 weeks. The birds were kept on grates and fed with standard fodder mix. The observed clinical signs included weight loss and inability to walk, as well as enteric disorders such as diarrhea. Liver sections were collected from dead ducklings and homogenized as 10% (w/v) suspensions in sterile phosphate-buffered saline (PBS). The homogenates were then centrifuged at $6000 \times g$ for 10 min, and the supernatants were filtered using 0.45 μ m filters. The filtered supernatants were used for the infection of chicken embryo kidney cells (CEKs).

Chicken embryo kidney cells (CEKs)

Cells were prepared from 18-d old SPF chicken embryos (LTZ, Cuxhaven, Germany). The growth medium MEM (Gibco, Paisley, Scotland) supplemented with 10% bovine fetal serum and a 1% antibiotics (Antibiotic-antimycotic, Gibco) was used, while a maintenance medium consisted of MEM with a 1% mixture of antibiotics as above. Infected cells were incubated at 37.8°C in 5% CO₂ until a cytopathic effect (CPE) was observed.

Agar gel precipitation (AGP)

Detection of reovirus antigen in homogenates of liver from Muscovy ducklings was conducted by agar-gel precipitation assay (AGP). The antiserum was specific against S1133 chicken reovirus strain (Charles River laboratories, North Franklin, USA). The serum was inactivated by heating at 56°C for 30 min. The reovirus antigen was tested for precipitation with S1133 reovirus antibodies on microscope slides in a layer of 1.5% agar gel supplemented with 8% NaCl (POCH, Gliwice, Poland). The slides were incubated for 48 h in a humidity chamber at 20°C.

DNA extraction

Total DNA was extracted from infected CEKs using a QIAamp Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's procedure. DNA templates were then frozen and stored at -80°C.

RNA extraction

RNA was extracted from CEKs harvested as described for DNA extraction using the RNAsy Mini Kit (Qiagen, Hilden, Germany) following the manufacturer's instructions. The extracted RNA templates were then frozen and stored at -80°C.

Real-time polymerase chain reaction (PCR)

Detection of GPV and MDPV was conducted by real-time PCR using primers and probe as previously described (Woźniakowski et al. 2012a). The positive control was represented by the GPV 88_v strain of the goose parvovirus (Department of Poultry Viral Diseases at the National Veterinary Research Institute (NVRI) in Puławy, Poland). The MDPV FM standard strain was provided by CEVA-Phylaxia Corporate,

Budapest, Hungary. Negative control was represented by DNA templates extracted from goose circovirus (GCV), avian adenovirus 1 (FAAdV-1) and goose hemorrhagic polyomavirus (GHPV) (Department of Poultry Viral Diseases at NVRI, Poland).

Detection of DuCV was examined using a previously developed and described real-time PCR method (Woźniakowski et al. 2012b). The reference DNA of goose circovirus strain P_1_03 as well as a set of negative controls represented by the DNA of goose hemorrhagic polyomavirus (GHPV) strain 2003, and Muscovy duck parvovirus strain FM (Vilmos Palya, CEVA-Phylaxia, Ceva Sante Animale, Budapest, Hungary) were also used.

Detection of duck reovirus (DRV) was conducted using primers specific for the S2 segment of DRV as previously described (Zhang et al. 2006). Positive control was the DRV strain (Department of Poultry Viral Diseases, NVRI, Poland) while the negative control was extracted from the liver of non-infected 12-d old goose embryos.

Detection of chicken reovirus (ARV) was conducted using oligonucleotide primers specific for the sigma NS non-structural gene of ARV. The primers were designed using Primer Express version 2.0.1 (Applied Biosystems, Foster City, California, USA) on the basis of chicken reovirus sequence (Accession number: JN559378). The primer sequences were: ARV F 5'- CCGAGTGGCCCTATTGACTA-3', ARV R 5'- CAGCGACCACTTAGATGCAA-3'. The reactions were conducted in an ABI 7500 real-time system (Applied Biosystems, Foster City, California, USA) in 0.2 ml tubes using a One-step RT-PCR Kit (EurX, Gdansk, Poland). The reaction volume was 25 µL which contained: 12.5 µL of Master Buffer, 0.5 µL (0.2 µM) of ARV F and ARV R primer, 1 µL of Master enzyme mix, 1 µL of RNA template (~50 ng) and deionised water. As the positive control RNA extracted from Nobilis 1133 vaccine (MSD Animal Health, AA Boxmeer, Netherlands) was applied. Reaction conditions were as follows: 50°C/30 min. (reverse transcription), 95°C/15 min. (initial denaturation), then 40 cycles of 95°C/1 min. (exact denaturation) and 60°C/1 min. (primer annealing and signal acquisition). Reaction was followed by melting curve analysis. PCR products were then separated in 2% agarose gels under 120 V for 40 min. For the determination of product length GeneRuler 100 bp ladder molecular marker (Thermo-Scientific, Waltham, Massachusetts, USA) was applied. The images were documented under UV light transilluminator and photographed (GenoSmart, VWR, Germany).

DNA sequencing

Three most visible products obtained by real-time RT-PCR were cut out from the gel and purified according to the procedure of QIAquick Gel Extraction Kit (Qiagen, Hilden, Germany). The amplicons were then sequenced on GS FLX/Titanium sequencer (Roche, Branford, Connecticut, USA by Genomed (Warsaw, Poland). Each product was sequenced in both forward and backward directions then assembled into a single contig in Genious™ Software ver. 6.5.5 (Biomatters Ltd, Auckland, New Zealand).

The sequences were submitted to the GenBank database. Accession numbers are as follows: GenBank: KC18913 (Isolate GAW1), GenBank: KC18914 (Isolate GAW2) and GenBank: KC18915 (Isolate GAW3). The amplicons were aligned and presented as a phylogram using a neighbor-joining algorithm (Tamura et al. 2011) with BOOTSTRAP analysis (1000 repetitions).

Results

In our study 3 and 5 week old Muscovy ducklings were sent for examination to NVRI and showed clinical symptoms specific for reovirus infection. These birds were kept within the same poultry house in two separated groups of age. The mortality during 7 weeks reached 44.5%. The clinical signs included weight loss, inability to walk, and diarrhea. During post-mortem examinations dehydration and enteritis, gouty kidneys as well as hemorrhagic liver and spleen lesions were found.

After three successive passages in CEKs an evident cytopathic effect (CPE) was observed as rounded cells forming focuses and plaques starting from the 56 h.p.i of the 1st passage (Fig. 1). The virus caused total disruption of the cell monolayer after 96 h.p.i. Detection of reovirus antigen in homogenates of liver from 3 and 5 week old Muscovy ducklings with specific S1133 chicken reovirus antibodies showed partial reactivity as this was observed as the precipitation lines between the examined antigen and standard serum (Fig. 2).

To exclude other possible duck infections real-time PCR and RT-PCR methods specific for goose parvovirus (GPV), Muscovy duck parvovirus (MDPV), duck circovirus (DuCV) and duck reovirus (DRV) were conducted. The analysis did not show the presence of any of these infectious agents. Confirming our suspicions, real-time RT-PCR for the detection of avian reovirus (ARV) showed the presence of clear fluorescent curves at the cycle threshold values (C_T) ranging from 14.1 to 16.2 (Fig. 3). After gel

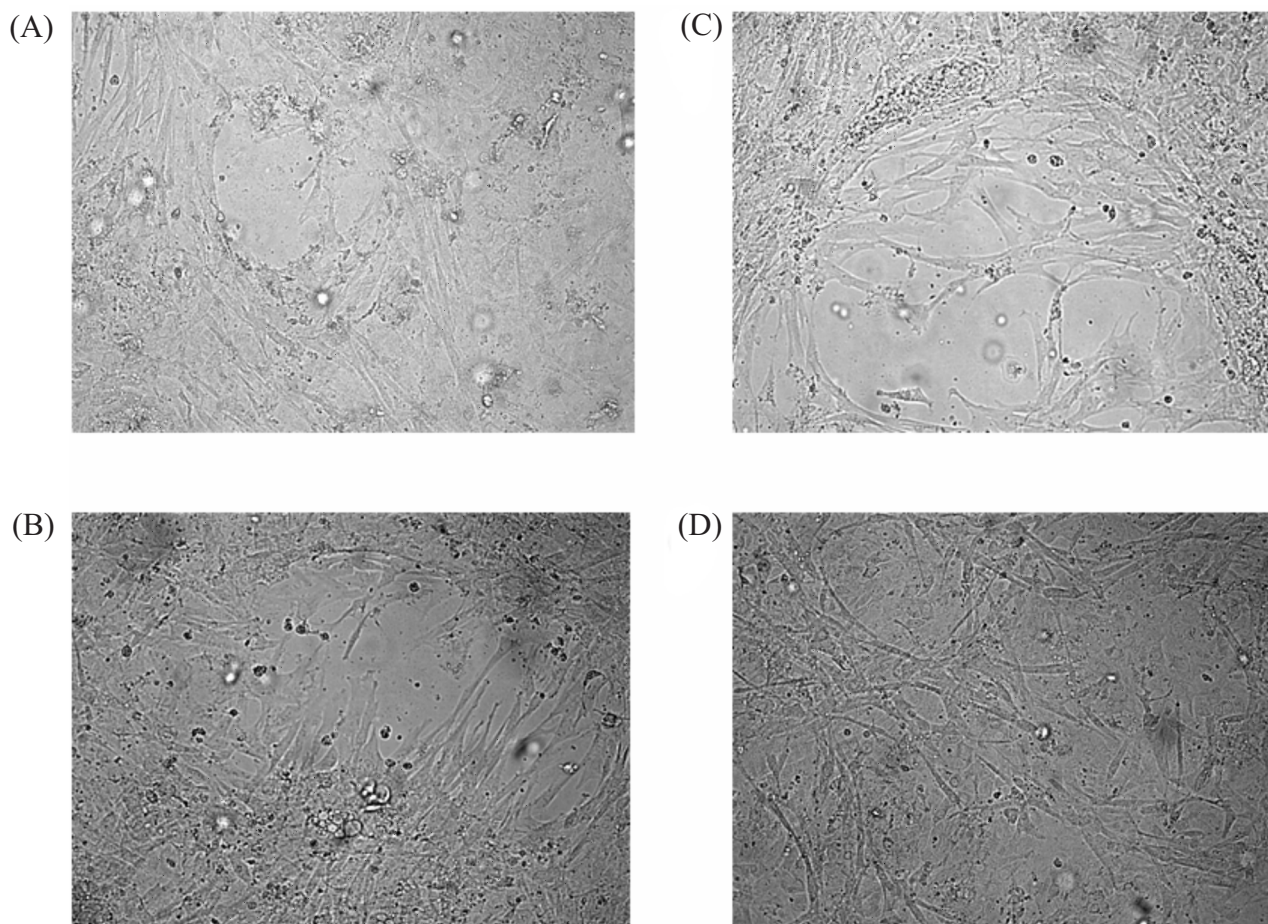


Fig. 1. Isolation of duck reovirus in chicken embryo kidney cells (CEKs). Pictures taken at 72 h.p.i. of the 1st virus passage. Visible cell foci and disruption of cell monolayer (A, B, C). Negative control (D) – mock-infected (CEKs). Mag. x 200 (Axio Observer D1, Zeiss, Jena, Germany).

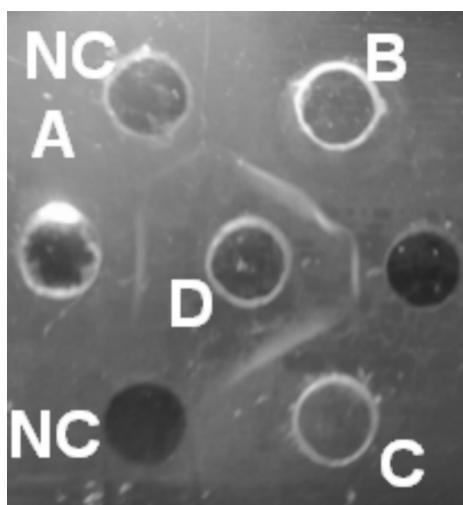


Fig. 2. Detection of reovirus antigen in homogenates of liver from 3- and 5-week Muscovy ducklings with specific S1133 chicken reovirus antibodies. (A) Positive control S1133 reovirus antigen Charles River laboratories, North Franklin, USA), (B) – liver homogenate from 3-week old ducklings, (C) – liver homogenate from 5-weeks old ducklings, (D) -S1133 reovirus antiserum (Charles River laboratories, North Franklin, USA), NC – negative controls.

electrophoresis of real-time PCR visible bands about 522 bp long were observed in RNA samples extracted from the liver of 3 and 5 week old ducklings. Melting curve analysis indicated a common temperature for all obtained amplicons and was 83.33°C. The products were sequenced and compared with 10 sequences of reference duck and chicken reoviruses accessible in the GenBank database (Fig. 4). Comparison revealed that the isolated reovirus strain has a distinct sigma NS gene sequence. The most similar sequences were found in cluster of avian reovirus group including S1133, 1733, TARV-MN2 and GX/2010/1 strains as well as Muscovy duck reovirus strains (S14, C4, S12, J18 and NP03). The virus was also similar to a previously described TH11 strain. The BOOTSTRAP coefficient value ranged from 53 to 100, indicating the reliability of the obtained branches and nodes of the phylogram.

Discussion

The occurrence of novel viruses in poultry and waterfowl husbandry remains one of the most serious

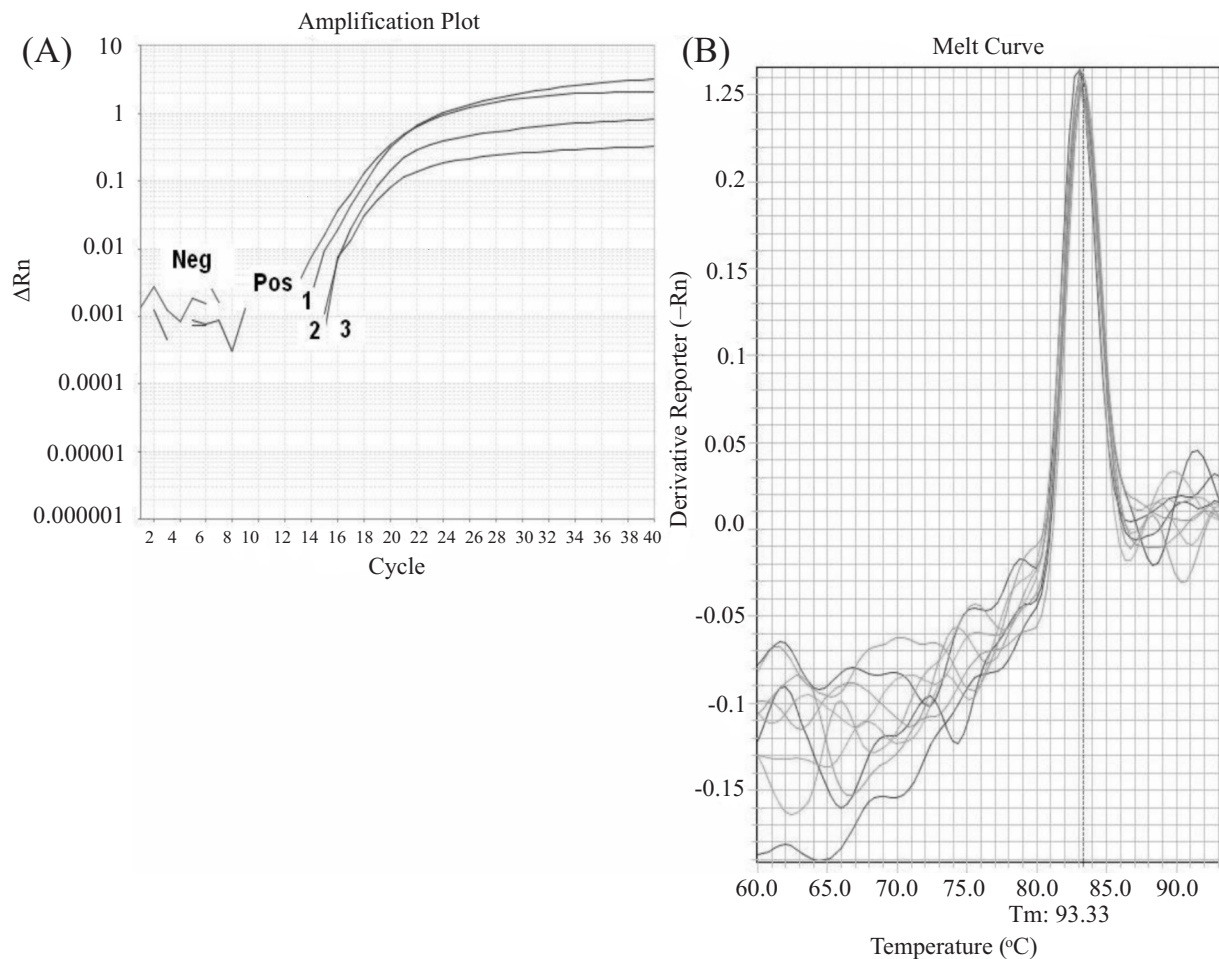


Fig. 3. Detection of sigma NS reovirus gene by real-time RT-PCR. (A) fluorescent curves of ARV positive samples, (B) melting curve analysis of real-time PCR products. Descriptions : Pos – S1133 strain, 1- GAW/1/2012, 2-GAW/2/2012, 3-GAW/3/2012, common melting temperature 83.33°C.

epizootic and economic problems. In the case of avian influenza (AIV) ducks are natural recombination vessels (Zhao et al. 2012) which cause the emergence of more virulent and transmissible AIV strains. Meanwhile, new poultry viruses may also originate from recombination between Marek's disease virus (MDV) and reticuloendotheliosis virus (REV) (Su et al. 2012). A previous report on the occurrence of a novel duck reovirus strain from China shows the emergence of a duck reovirus causing 40% mortality in birds of various ages (Chen et al. 2012). In our study we observed similar mortality and clinical symptoms specific for reovirus infection in a flock of Muscovy ducklings. Among the clinical signs was an inability to walk indicating possible viral arthritis caused by reovirus infection. Due to suspicion of chicken reovirus infection an attempt was made to isolate the virus in chicken embryo kidney cells (CEKs). Meanwhile, Liu et al. (2011) conducted experimental reovirus infection in Pekin ducks but failed to re-isolate the virus, as was achieved in our study. Chen et al. (2012) successfully

isolated reovirus from experimentally infected ducks but all birds died at 48-72 h.p.i. They also applied virus isolation in duck embryo fibroblasts (DEF). Since we suspected that our aetiological agent was more related to chicken reovirus we decided to perform virus isolation in CEKs. In order to confirm the reovirus presence an AGP was conducted and a clear reaction between S1133 chicken reovirus antibodies and the examined antigen was observed. However, in order to exclude other possible viral infections a set of real-time PCR tests were conducted but we were unable to find GPV, MDPV, DuCV or DRV in the investigated material. The final part of our study for the detection of avian reovirus (ARV) showed the presence of its RNA. The conducted sequence analysis also confirmed a partial identity with ARV as we indicated by comparison with 10 sequences of reference duck and chicken reoviruses accessible in GenBank. According to the previously conducted study by Chen et al. (2012), the duck reovirus isolated from outbreaks in China fell into the group of duck reoviruses.

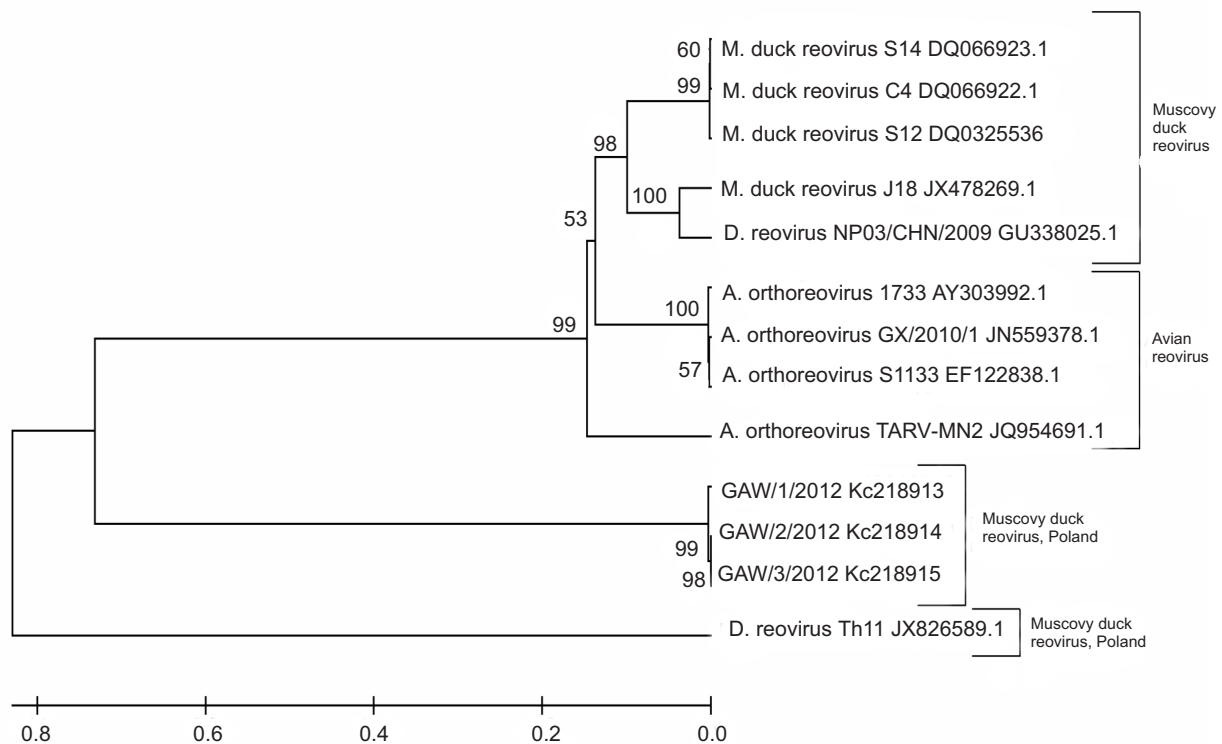


Fig. 4. Phylogenetic relationships between GAW/1/2012, 2-GAW/2/2012 and GAW/3/2012 reovirus strains with avian (ARV) and Muscovy duck (MDRV) orthoreoviruses. Accession numbers (GenBank) of the sequences are indicated on the phylogram. The neighbor-joining tree is based on the fragment of sigma NS gene (522 bp). Numbers at nodes determine the percentage of BOOTSTRAP replicates (1000). Scale bar indicates branch length corresponding 100-nucleotide-state changes.

Similarly to our data, the sequences of the virus were related to the avian orthoreovirus group.

Concluding our study, we have shown the presence of a new pathogenic reovirus of Muscovy ducks. The conducted phylogenetic analysis on the basis of the sigma NS gene has shown that the isolate was distinct from the viruses previously isolated from Muscovy ducks and chickens. Further study is needed to confirm the results obtained by the experimental infection of 1-d old Muscovy ducks as well as 1-d old chickens since the new virus seems to share genetic features of avian and Muscovy duck orthoreoviruses. The sequencing of the whole virus genome might also reveal specific differences in the structure of this virus as was described in previous reports (Zhang et al. 2007, Wang et al. 2012).

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