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Genomic characteristics of a novel reovirus from Muscovy duckling in China

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ABSTRACT

A new reovirus was isolated from a sick Muscovy duckling with hemorrhagic-necrotic lesions in the liver in Zhejiang, China in 2000 and was tentatively denoted a new type of Muscovy duck reovirus (N-MDRV ZJ00M). This reovirus was propagated in a chicken fibroblast cell line (DF-1) with obvious cytopathic effects. The reovirus's genome was 23,419 bp in length with an approximately 50% G+C content and 10 dsRNA segments encoding 12 proteins. The length of the genomic segments was similar to those of avian reoviruses (ARVs), which range from 3959 nt (L1) to 1191 nt (S4) in size. All of the segments have the conserved terminal sequences 5′-GCUUUUU. . .UUCAUC-3′, and all of the genome segments, with the exception of S1, apparently encoded one single primary translation product. The genome analysis revealed that the S1 segment of N-MDRV is a tricistronic gene that encodes the overlapping ORFs for p10, p18, and σC. This finding is similar to that found for ARVs but distinct from that found for classical MDRV and GRV, which have a bicistronic S4 segment that encodes p10 and σC and do not encode p18. The amino acid (aa) alignments of the putative proteins encoded by the main ORF in each segment revealed a high similarity (14.1–100%) to the counterpart proteins encoded by other ARV species from the avian orthoreoviruses (e.g., ARV, classical MDRV and N-MDRV) in the Orthoreovirus genus, particularly with N-MDRV (94.6–100%). The phylogenetic analysis of the nucleotide sequences of all 10 genome segments revealed that N-MDRV ZJ00M is distinct from all other described reovirus species groups but is a separated from the ARV (including MDRV and GRV) species within orthoreovirus species group II and grouped into the classical MDRV and GRV genogroup with the N-MDRV isolates. The MDRV genogroup can be further divided into two genotype clusters. The morphological and pathological analyses and the genetic characterization of N-MDRV ZJ00M suggest that it belongs to genotype 2 (N-MDRV). In addition, the RT-PCR assays of DRV diseased duckling and gosling samples collected from different regions of China during 2000–2013 indicate that N-MDRV is currently the prevalent genotype in China.

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1. Introduction

Avian reoviruses (ARVs) are members of the Orthoreovirus genus in the family Reoviridae. They are non-enveloped viruses that replicate in the cytoplasm of infected cells and contain a fragmented double-stranded RNA genome enclosed within a double protein capsid shell with a diameter of 70–80 nm. The genomic segments can be separated on the basis of their electrophoretic mobility into three size classes: large (L1–L3), medium (M1–M3),

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and small (S1–S4) (Nick et al., 1975; Spandidos and Graham, 1976; Gouvea and Schnitzer, 1982; Benavente and Martínez-Costas, 2007). All avian reovirus (ARV) encoded proteins include at least 10 structural proteins (λA, λB, λC, μA, μB, σC, σA, and σB) and four nonstructural proteins (μNS, p10, p17, and σNS).

Avian reoviruses have been associated with different diseases in a variety of domestic and wild birds, including chicken (Olson and Weiss, 1972), goose (Palya et al., 2003; Yun et al., 2012), turkey (Simmons et al., 1972), Muscovy duck (Gaudry et al., 1972), Pekin duck (Jones and Guneratne, 1984), pigeon (Vindevogel et al., 1982), quail (Ritter et al., 1986), psittacine birds (Conzo et al., 2001), and several other wild bird species (Heffels-Redmann et al., 1992; Kuntz-Simom et al., 2002). Birds are most susceptible at a young age (Rosenberger et al., 1989).

Classical Muscovy duck reovirus (MDRV) is the etiological agent of a disease first described in South Africa in 1950 (Kaschula, 1950) and then isolated in France in 1972 (Gaudry et al., 1972). MDRV mainly infects ducklings between 2 and 4 weeks of age; their resulting morbidity is high, and their rate of mortality ranges from 10 to 50%. In addition, recovered Muscovy ducks are markedly stunted in growth. The disease is characterized by general weakness, diarrhea, serofibrinous pericarditis, swollen liver and spleen, and covered small white necrotic foci (Gaudry et al., 1972; Malkinson et al., 1981; Pascucci et al., 1984; Marius-Jestin et al., 1988).

MDRV shares common properties with avian reovirus, such as syncytium formation in cell culture and inability to hamagglutinate (Malkinson et al., 1981). However, several notable differences exist between MDRV and ARV, including different antigenicity by cross-neutralization tests (Heffels-Redmann et al., 1992), host species differences (chicken and Muscovy duck), pathogenic properties (Marius-Jestin et al., 1988), protein profiles (Heffels-Redmann et al., 1992), electropherotypes, and genomic coding assignments (Kuntz-Simom et al., 2002). For example, the classical MDRV minor outer capsid protein σC is encoded by S4 (Kuntz-Simom et al., 2002) and not by S1, as is usually found in ARV.

In China, classical MDRV infection has been reported since 1997 (Wu et al., 2001). The virus isolates share identical properties in pathology, culture, and genome (Kuntz-Simom et al., 2002; Zhang et al., 2007; Bányai et al., 2005; Wang et al., 2013). Since 2002, a new infectious disease emerged among Muscovy ducks and geese in Southeast China. The disease is characterized mainly by hemorrhagic-necrotic lesions in the liver and spleen of the sick birds and is tentatively designated hemorrhagic-necrotic hepatitis (Liu et al., 2011). Recently, the causative agent of the disease was isolated and identified; its pathogenicity, growth properties, and genome sequences classify it as a novel duck reovirus (NDRV) (Chen et al., 2012; Yun et al., 2012; Ma et al., 2012; Wang et al., 2012).

In this study, a novel duck reovirus strain, named N-MDRV ZJ00M, was isolated from a diseased Muscovy duckling in Zhejiang province of China in 2000. Its whole genome was cloned, sequenced, and analyzed. The genome of N-MDRV ZJ00M exhibited distinct molecular characteristics compared with ARV and classical MDRV. The study revealed that N-MDRV has existed in China at least since the 2000s and provided additional insights into the reassortment and evolutionary relationship within intra- and interspecies of *Orthoreovirus* species groups II.

2. Materials and methods

2.1. Virus isolation and virological characterization

Liver samples of the dead Muscovy duckling with hemorrhagic-necrotic lesions were collected from a duck farm in Zhejiang Province and processed for virus isolation using embryonated SPF chicken eggs. Briefly, the liver samples were homogenized in PBS (pH 7.2) containing antibiotics (10,000 units/ml penicillin and 10,000 mg/ml streptomycin) to obtain a 20% suspension (w/v). The suspension was centrifuged at 12,000 × *g* for 10 min and then inoculated on the chorioallantoic membrane of 10-day-old chicken embryos (0.2 ml/embryo). The embryonic viability was monitored daily for 7 days. For cell culture passage, the allantoic fluid was inoculated into chicken embryo fibroblast (DF-1) cells (1.0 ml of a 1:10 dilution in medium) and incubated at 37 °C for 1 h for virus adsorption. The inoculums were then removed, and fresh medium containing 1% FBS was added. The cells were incubated for an additional 48–72 h at 37 °C/5% CO_2 and checked daily for cytopathic effects (CPE). The cells were freeze-thawed three times, the cellular debris was removed through low-speed centrifugation, and the supernatant fluid was stored at −70 °C for the following experiments. The isolated virus was cultured for at least three passages for amplification and sequencing.

The virus titers were determined by plaque assay on DF-1 cells (Igarahi et al., 1981; Okuno et al., 1984). Briefly, monolayer cultures of DF-1 cells (1×10^5/well) grown in six-well plates were incubated with 10-fold serial dilutions of the virus for 1 h at 37 °C. The infected cells were then overlaid with 2 ml of DMEM containing 1.5% methyl cellulose and 2% fetal bovine serum and incubated at 37 °C under a 5% CO_2 atmosphere for 72 h. The cells were fixed with 1 ml of 10% formaldehyde for 30 min, washed with PBS (pH 7.2), and stained with methylene blue tetrahydrate solution to visualize the plaques, and the visualized plaques were counted.

The virions were purified by differential centrifugation. First, the virus suspension (crude extract) was centrifuged at 10,000 × *g* for 30 min at 4 °C to remove the cellular debris. Second, the resultant supernatant was precipitated with 50% saturated ammonium sulfate at 4 °C. The precipitate was collected by centrifugation at 10,000 × *g* for 20 min and suspended in a buffer consisting of 0.02 M Tris (pH 7.0), 0.001 M EDTA, and 0.15 M NaCl. This virus buffer was then ultracentrifuged for 3 h at 130,000 × *g* in a Beckman SW70 rotor at 4 °C on a 40% sucrose cushion (W/V, prepared with PBS), and the virus pellet was resuspended in 50–100 μl of cold DEPC H_2O and stored at −80 °C until use.

The viral morphology was determined through transmission electron microscopy as described previously (Hoshino et al., 2007). Briefly, the cells were fixed in 2.5% glutaraldehyde and 1% osmic acid for 2 h on ice, and

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