Identification and molecular characterization of novel duck reoviruses in central China, 2021: Implications for control programs

Zhifeng Peng[1], Han Zhang[1], Xiaozhan Zhang[1], Zihan Liu[1], Hongxing Qiao[1], Zenghai Jiang[1], Yujin Lv[1], and Chuanzhou Bian[1]

[1]Henan University of Animal Husbandry and Economy

April 19, 2022

Abstract

Novel Duck [reovirus](https://www.sciencedirect.com/topics/immunology-and-microbiology/reoviridae) (NDRV) is an ongoing non-enveloped virus with ten double-stranded RNA (dsRNA) genome segments that belong to the genus *Orthoreovirus*, in the family *Reoviridae*. NDRV-associated spleen swelling and necrosis disease have caused considerable economic losses to the waterfowl industry worldwide. Since 2017, a significant number of NDRV outbreaks have emerged in China. Herein, we describe two cases of duck spleen necrosis disease among ducklings on duck farms in Henan province, central China. Other potential causative agent, including Muscovy duck reovirus (MDRV), Duck hepatitis A virus type 1 (DHAV-1), Duck hepatitis A virus type 3 (DHAV-3), Newcastle disease virus (NDV), and Duck tembusu virus (DTMUV), were excluded by reverse transcription-polymerase chain reaction (RT-PCR), and two NDRV strains, HeNXX-1/2021 and HNJZ-2/2021, were isolated. Sequencing and phylogenetic analysis of the σC genes revealed that both newly identified NDRV isolates were closely related to DRV/SDHZ17/Shandong/2017. The results further showed that Chinese NDRVs had formed two distinct clades, with late 2017 as the turning point, suggesting that Chinese NDRVs have been evolving in different directions. In conclusion, this study provides an insight into the ongoing emerged duck spleen necrosis disease, and a foundation for developing of effective vaccine and control programs.

Identification and molecular characterization of novel duck reoviruses in central China, 2021: Implications for control programs

Zhifeng Peng[1], Han Zhang[1], Xiaozhan Zhang[1], Zihan Liu[1, 2], Hongxing Qiao[1], Zenghai Jiang[1], Yujin Lv[1], Chuanzhou Bian[1]

[1] College of Veterinary Medicine, Henan University of Animal Husbandry and Economy, Zhengzhou 450046, China

[2] College of Animal Science and Veterinary Medicine, Henan Institute of Science and Technology, Xinxiang 453003, China

Correspondence

Chuanzhou Bian, College of Veterinary Medicine, Henan University of Animal Husbandry and Economy, No. 6 Longzihu North road, Zhengzhou 450046, Henan, China.

E-mail:*chuanzhou-bian@126.com.*

Abstract

Novel Duck reovirus (NDRV) is an ongoing non-enveloped virus with ten double-stranded RNA (dsRNA) genome segments that belong to the genus *Orthoreovirus* , in the family *Reoviridae* . NDRV-associated spleen

swelling and necrosis disease have caused considerable economic losses to the waterfowl industry worldwide. Since 2017, a significant number of NDRV outbreaks have emerged in China. Herein, we describe two cases of duck spleen necrosis disease among ducklings on duck farms in Henan province, central China. Other potential causative agent, including Muscovy duck reovirus (MDRV), Duck hepatitis A virus type 1 (DHAV-1), Duck hepatitis A virus type 3 (DHAV-3), Newcastle disease virus (NDV), and Duck tembusu virus (DTMUV), were excluded by reverse transcription-polymerase chain reaction (RT-PCR), and two NDRV strains, HeNXX-1/2021 and HNJZ-2/2021, were isolated. Sequencing and phylogenetic analysis of the σC genes revealed that both newly identified NDRV isolates were closely related to DRV/SDHZ17/Shandong/2017. The results further showed that Chinese NDRVs had formed two distinct clades, with late 2017 as the turning point, suggesting that Chinese NDRVs have been evolving in different directions. In conclusion, this study provides an insight into the ongoing emerged duck spleen necrosis disease, and a foundation for developing of effective vaccine and control programs.

KEYWORDS

Duck spleen necrosis disease, NDRV, Epidemiology, Evolutionary

1 INTRODUCTION

Avian reovirus (ARV) has caused immense economic problems in the chicken, duck, goose, and turkey industries worldwide (Palya et al., 2003, Rosa et al., 2014). Waterfowl reoviruses (WRVs) have been associated with various disease conditions in ducks and geese of different species, including Muscovy duck white spot disease caused by classical Muscovy duck reovirus (MDRV) (Yun et al., 2013, Farkas et al., 2014, Kuntz-Simon et al., 2002, Le Gall-Recul et al., 1999, Gaudry et al., 1972), duck hemorrhagic necrotizing hepatitis, and spleen necrosis disease in ducks caused by the novel Muscovy duck reovirus (N-MDRV) (Yun et al., 2014, Li et al., 2016, Zhang et al., 2019) and the novel Duck reovirus (NDRV) (Liu et al., 2011, Chen et al., 2012b, Yun et al., 2014, Zhu et al., 2015, Wang et al., 2020c, Zhang et al., 2019, Luo et al., 2021), spleen and liver inflammation in geese caused by goose reovirus (GRV) (Wang et al., 2013, Palya et al., 2003, Yun et al., 2013), hemorrhagic necrotic hepatitis caused by the new type of goose reovirus (N-GRV) (Wang et al. 2002; Yun et al., 2012). It has been reported that WRVs can be classified into two genotypes(Wang et al., 2013). Genotype 1 comprises MDRV and GRV. While genotype 2 comprises NDRV, N-MDRV, and N-GRV (Wang et al., 2013).

The ARV genome includes ten segments that can be separated into three classes based on their sizes: large (L1, L2, L3), middle (M1, M2, M3), and small (S1, S2, S3, S4). The S1 segment is the only tricistronic gene that encodes P10, P18, and σC proteins (Zhu et al., 2015, Benavente and Martínez-Costas, 2007). The outer capsid proteins σC, σB, and μB of ARV are considerably variable, whereas the inner capsid proteins are relatively conservative (Benavente and Martínez-Costas, 2007). Additionally, σC proteins play an essential role in viral fusion, invasion, neutralizing antibody induction, and pathogenicity (Du et al., 2020, Ma et al., 2012, Shih et al., 2004, Benavente and Martínez-Costas, 2007). Meanwhile, σC is regarded as the most variable protein of all the ARV proteins (Benavente and Martínez-Costas, 2007). σC gene was usually used for epidemiological studies and viral classification of ARVs (Luo et al., 2021, Palomino-Tapia et al., 2018).

In recent years, many NDRV outbreaks emerged in China (Wang et al., 2019, Wang et al., 2020a, Cao et al., 2019, Zhang et al., 2019). However, few studies have performed the evolutionary status analysis on NDRV in central China. In this study, we isolated two NDRV strains from different duck farms in Henan province, central China. To better understand the molecular characteristics of the NDRVs circulating in duck populations, the σC genes of both isolates were cloned, sequenced, and their phylogeny and mutations were analyzed. This study systematically describes the genetic and evolutionary characteristics of the ongoing NDRV strains and highlights that continuous surveillance is needed to develop proper vaccines and reasonable control programs.

2 MATERIALS AND METHODS

2.1 Case presentation

Case 1. In March 2021, acute outbreaks of spleen necrosis disease occurred on many commercial duck farms in Xinxiang City of Henan province, central China. A duck farm with approximately 30% of 10,000 10-day-old ducklings showed sudden onset and severe symptoms, such as listlessness, white diarrhea, anorexia, and lameness. The outbreak started on 3 March 2021, and antibiotic-traditional veterinary drugs combination therapy did not work. Seven dead ducklings were randomly selected and sent to the laboratory for diagnosis.

Case 2. An epidemic characterized by the sudden death of ducklings emerged on another commercial duck farm in Jiaozuo city of Henan province. The duck flock had approximately 11,000 ducklings of 11-day-old. From 12 August 2021 to 22 August 2021, approximately 350 ducklings per day died acutely. The great majority of the diseased ducklings showed listlessness, white diarrhea, and anorexia. The mortality was about 30%. The antibiotic therapy did not work. Eight dead ducklings were selected randomly to send to the laboratory for diagnosis.

2.2 Molecular diagnosis

To identify the causative agent of the disease, the potential viral and bacterial pathogens were examined. The bacteriological culture was performed as described previously (Wang et al., 2020d). The samples from the same duckling were combined, and processed by extracting RNA using the EasyPure Viral RNA Kit (Takara, Shanghai). Subsequently, the RNA samples were used to detect MDRV, DHAV-1, DHAV-3, NDV, DTMUV, and NDRV using RT-PCR protocols as described previously (Yan et al., 2021).

2.3 Virus isolation

The positive samples of liver and spleen from dead ducklings from the same flock were homogenized in phosphate-buffered saline (PBS, pH 7.2), freeze-thawed three times, and centrifuged at 8000× g for 10 min. The supernatants were filtered through a 0.22 μm filter to remove bacteria and other larger particles. Subsequently, 0.2 mL of each of the two supernatants was separately used to propagate in the allantoic cavity of 10-day-old healthy duck embryos in a 37 incubator. If the embryo died at 3-4 days post-inoculation, the allantoic fluid was harvested for another round of inoculation. After three passages in healthy embryonated duck eggs, the allantoic fluids and duck embryos were harvested sterilely and stored at -80°C. The viral RNA extracted from the allantoic fluids were used to detect potential causative agent.

2.4 Gene amplification and sequencing

To analyze the genotype and genetic characteristics of the both newly isolated NDRV strains in this study, the complete σC genes of the NDRV were amplified by using primers as follows: NDRV-S1 forward: 5'-GCTTTTTTCTTCTCTGCCCAT-3' and DRV-S1 reverse: 5'-GATGAATAGCTCTTCTCATCGC-3', which were designed based on the S1 gene of NDRV downloaded from NCBI (https://www.ncbi.nlm.nih.gov/). The RT-PCR products were purified and cloned into a pMD18-T vector (Takara, Shanghai) for sequencing with universal M13 forward and reverse primers by Sangon Biotech in Shanghai.

2.5 Phylogenetic analysis and sequence comparison

The σC genes of both newly identified NDRV isolates were subjected to the online BLAST program (https://blast.ncbi.nlm.nih.gov/Blast.cgi). And sequences sharing more than 95% nucleotide identity were downloaded for further genetic analysis with Clustal Omega. Furthermore, the phylogenetic tree was conducted by MEGA 6.0 software with the neighbor-joining method using 1,000 bootstrap replicates.

3 RESULTS

3.1 Clinical signs and post-mortem examinations

For both NDRV outbreaks, most diseased ducklings were characterized by diarrhea, lethargy, increased eye discharge, loss of appetite, stunted growth, and paralysis. Among the organs collected from the dead ducklings in both cases, the predominant histologic lesions are located in the liver and spleen. The livers

(Figure 1a, 1b, 1c) and spleens (Figure 1d, 1e, 1f) showed swelling, hemorrhage, and irregular necrosis in all dead ducklings. And other organs did not exhibit apparent lesions.

3.2 Molecular diagnosis

To identify the causative agent of the disease, RT-PCR assays were used to detect the potential viral pathogens. The samples from the dead duckling were positive for NDRV, and no corresponding nucleotide fragments were observed for MDRV, DHAV-1, DHAV-3, NDV, and DTMUV (Figure S1). In addition, *Riemerella anatipestifer* was also identified from four dead ducklings in case 2 by bacteria isolation.

3.3 Virus isolation

The homogenates of the positive liver and spleen samples were then inoculated into the allantoic cavity of 10-day-old healthy duck embryos. The inoculated duck embryos' bodies showed varying degrees of hemorrhage and dysplasia (Figure 2b). The allantoic fluids of the duck embryo inoculated with the samples were only positive for NDRV. Eventually, two NDRV strains causing duck spleen necrosis disease were successfully isolated and termed as HNXX-1/2021 and HNJZ-2/2021, respectively.

3.4 Genetic and phylogenetic analysis

The σC genes of both newly identified NDRV isolates were 1568 bp in length. To further investigate the genetic characteristics of both newly isolated NDRV strains, their σC genes and induced amino acid were compared with reference WRV strains. According to the sequence alignment of σC genes, Nucleotide identity between HNXX-1/2021 and HNJZ-2/2021 was 99.1%, which shared 98.4% and 98% identities with DRV/SY/Jiangsu/2018 in nucleotide, and 98.8% and 99.1% in deduced amino acid, respectively. Meanwhile, HNXX-1/2021 and HNJZ-2/2021 shared 98.4% and 98% in nucleotide, 98.5% and 98.8% in deduced amino acid with DRV/SDHZ17/Shangdong/2017, respectively. The nucleotide sequences of the σC gene of HNXX-1/2021 and HNJZ-2/2021 were deposited into GenBank (accession numbers: ON012751 and ON012752).

To further explore the evolutionary characters of the newly identified NDRV strains, the phylogenetic tree of NDRVs was constructed based on the σC genes deduced amino acid sequences (Figure 3). As shown in the phylogenetic tree, HNXX-1/2021 and HNJZ-2/2021 were clustered together with DRV/QR/Hubei/2020, DRV/GX-Y7/Guangxi, DRV/SDLY18/Shandong/2018 and DRV/SY/Jiangsu/2018, Which were NDRV strains emerged in recent years in China.

Remarkably, all the DRVs strains were clustered into two distinct clades. Clade1 comprised the majority of DRVs strains that emerged before 2017, and few strains only emerged in Shandong province after 2017. Clade 2 comprised the majority of the NDRV strains that emerged after 2017, including the newly identified NDRV strains HNXX-1/2021 and HNJZ-2/2021 in this study.

3.5 Amino acid polymorphism analysis

To further explore the characteristics of both newly identified NDRV strains, amino acid polymorphism of the σC proteins was compared with reference strains. The results showed that most of the amino acid mutations in both newly identified NDRV isolates located in the σC protein head domain (Table 1), such as P233S, V253A, and A298V, indicating both isolates were closely related to each other. Meanwhile, some mutations were only found in HNXX-1/2021 & HNJZ-2/2021 (P233S in σC protein head domain), HeNXX-1/2021 (E305G, A328R, and T329Q in σC protein head domain) and HNJZ-2/2021 (A328R and T329Q in the σC protein head domain). Moreover, there was a unique amino acid mutation site (Q158H) in the σC protein shaft domain of both newly identified NDRV strains.

4 DISCUSSION

ARV, MDRV, and NDRV are classified into the genus *Orthoreovirus* , members of the family *Reoviridae*(Benavente and Martínez-Costas, 2007, Chen et al., 2012b). ARV was first isolated from the respiratory tract of chickens suffering from chronic respiratory diseases in 1954 (Fahey and Crawley, 1954). MDRV was

isolated from Muscovy ducklings for the first time in 1972 (Gaudry et al., 1972). In 2005, a novel duck reovirus disease was discovered in duck flocks in China, characterized by irregular hemorrhage and liver necrosis. The pathogen of this disease was isolated in 2011 and named NDRV distinguished from the classical MDRV (Chen et al., 2012a). Since then, NDRV has become the predominant strain of reovirus that emerged in duck flocks in China (Ma et al., 2012, Li et al., 2016, Zhu et al., 2015, Wang et al., 2019, Luo et al., 2021, Wang et al., 2020c). Previous studies have demonstrated that NDRV has a broader host spectrum than MDRV (Cao et al., 2019, Zhang et al., 2019, Luo et al., 2021), and different pathogenicity (Wang et al., 2020a, Zheng et al., 2016, Luo et al., 2021, Wang et al., 2020c, Farkas et al., 2018). In recent years, the diseases caused by NDRVs broke out in waterfowl flocks worldwide, have led to significant economic losses to the waterfowl industry.

As the cell attachment protein of ARVs, σC proteins play an essential role in viral fusion, invasion, and pathogenicity (Du et al., 2020, Ma et al., 2012, Shih et al., 2004, Benavente and Martínez-Costas, 2007). Additionally, the σC protein possesses the highest sequence variability among ARVs (Benavente and Martínez-Costas, 2007). The 18-aa deletion in the σC protein significantly enhanced the virulence of reovirus (Zheng et al., 2016). In this study, two NDRV isolates were identified, and the σC genes were amplified and sequenced. Phylogenetic analysis based on the σC genes deduced amino acid sequences demonstrated that Chinese NDRVs had formed two distinct clades, with late 2017 as the turning point, suggesting that Chinese NDRVs have been evolving in different directions. More importantly, NDRV strains within clade 2 have become predominant in duck flocks in China, and emerged in the major duck production regions in China, such as Shandong, Henan, Jiangsu, Hubei, and Guangxi. Remarkably, four amino acid mutation sites located in the head domain, and one amino acid mutation site located in the shaft domain were discovered in both σC proteins of the newly identified NDRV strains, which might have changed the virulence and led to differences in the epidemic.

Previous studies have demonstrated that the genetic diversity of NDRV circulating in China is complex (Yun et al., 2013, Zhu et al., 2015, Cao et al., 2019, Zhang et al., 2019, Wang et al., 2020c). Since using the attenuated MDRV vaccine in 2013, MDRV infection in waterfowl has significantly reduced in China in recent years. However, NDRV and N-MDRV emerged and spread widely in waterfowl in China (Luo et al., 2021, Ji et al., 2020, Cao et al., 2019, Wang et al., 2019, Zhang et al., 2019), which caused significant economic losses in China. The complexity of epidemic strains undoubtedly facilitated virus mutation and recombination(Wang et al., 2020c, Luo et al., 2021), and complicated the genetic diversity of DNRV. To date, no effective NDRV vaccine is available, the complex genetic diversity of NDRV hampers the development of an effective vaccine. Though diverse vaccines against NDRV infection are under development, none is commercially available. The complex genetic diversity of NDRV poses more challenges to vaccine development, including poor cross-protection and the lack of markers for sero-surveillance.

With African swine fever outbreak in China in 2018, China's swine industry has suffered devastating destruction (Wang et al., 2018). After that, the waterfowl industry developed considerably in China. China has the largest waterfowl population worldwide. However, small-scale farms with poor biosecurity produce more than 70% of waterfowl in China, resulting in a higher risk of spreading NDRV. Furthermore, NDRV can spread vertically, as well as horizontally (Wang et al., 2020b). Therefore, NDRV infection is much more challenging to prevent and control in duck production.

In conclusion, two NDRV strains were isolated from duck farms during NDRV outbreaks in central China. Phylogenetic analysis revealed that Chinese NDRVs had formed two distinct clades. The both newly identified NDRV strains, which belong to clade 2, are current predominant strains. This study highlights the importance of continuous surveillance and evaluation of the epidemiology of NDRV in ducks. It improves the understanding of the genetic heterogeneity of NDRV in China, providing a foundation for developing effective prevention and control strategies for this persistent disease.

ACKNOWLEDGEMENTS

We are very grateful to Mr. Changyu Ma (Henan Xurui duck industry Co. Ltd) for kindly providing the

healthy duck embryos and the epidemic information. This work was supported by the grants from Key Scientific Research Foundation of the Higher Education Institutions of Henan Province (19B230010), Scientific Innovation Fund of Henan University of Animal Husbandry and Economy (XKYCXJJ2020011), Youth Science Foundations of Henan University of Animal Husbandry and Economy (2021HNUAHEDF004) and Scientific Innovation Team Project of Henan University of Animal Husbandry and Economy (2018KYTD13).

CONFLICT OF INTEREST

The authors declare no commercial or financial conflict of interest.

ETHICS STATEMENT

The authors confirm that the ethical policies of the journal, as noted on the journal's author guidelines page, have been adhered to. No ethical approval was required in this communication, since this study did not involve any experimental protocol animals. All the animal samples used

were collected from dead ducklings.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

REFERENCES

Benavente, J. and J. Martínez-Costas, 2007: Avian reovirus: structure and biology. *Virus research,* 123, 105-119.

Cao, Y., M. Sun, J. Wang, X. Hu, W. He and J. Su, 2019: Phenotypic and genetic characterisation of an emerging reovirus from Pekin ducks in China. *Scientific reports,* 9, 7784.

Chen, S. Y., S. L. Chen, F. Q. Lin, S. Wang, B. Jiang, X. X. Cheng, X. L. Zhu and Z. L. Li, 2012a: The isolation and identification of novel duck reovirus. *Chinese journal of virology,* 28, 224-230.

Chen, Z., Y. Zhu, C. Li and G. Liu, 2012b: Outbreak-associated novel duck Reovirus, China, 2011.*Emerging infectious diseases,* 18, 1209-1211.

Du, X., M. Ding, Q. Wu, C. H. Li, H. Guo, G. Liu and Z. Chen, 2020: Characterization of a P18 protein in the S1 segment of the novel duck reovirus genome. *Acta Virol,* 64, 59-66.

Fahey, J. E. and J. F. Crawley, 1954: Studies On Chronic Respiratory Disease Of Chickens II. Isolation of A Virus. *Canadian journal of comparative medicine and veterinary science,* 18, 13-21.

Farkas, S. L., E. Dandár, S. Marton, E. Fehér, M. Oldal, F. Jakab, T. Mató, V. Palya and K. Bányai, 2014: Detection of shared genes among Asian and European waterfowl reoviruses in the whole genome constellations. *Infection, Genetics and Evolution,* 28, 55-57.

Farkas, S. L., R. Varga-Kugler, S. Marton, G. Lengyel, V. Palya and K. Bányai, 2018: Genomic sequence and phylogenetic analyses of two novel orthoreovirus strains isolated from Pekin ducks in 2014 in Germany. *Virus Res,* 257, 57-62.

Gaudry, D., J. M. Charles and J. Tektoff, 1972: A new disease expressing itself by a viral pericarditis in Barbary ducks. *Comptes rendus hebdomadaires des seances de l'Academie des sciences. Serie D: Sciences naturelles,* 274, 2916-2919.

Ji, J., Q. Chen, C. Sui, Z. Yu and X. Xu, 2020: Novel genotype definition and genome characteristics of duck circovirus in central and Eastern China. *Transbound Emerg Dis.,*67, 2993-3004.

Kuntz-Simon, G., G. Le Gall-Reculé, C. de Boisséson and V. Jestin, 2002: Muscovy duck reovirus σC protein is atypically encoded by the smallest genome segment. *Journal of General Virology,* 83, 1189-1200.

Le Gall-Recul, G., M. Cherbonnel, C. Arnauld, P. Blanchard, A. Jestin and V. Jestin, 1999: Molecular characterization and expression of the S3 gene of muscovy duck reovirus strain 89026. *The Journal of general virology,* 80 (Pt 1), 195-203.

Li, N., T. Hong, Y. Wang, Y. Wang, K. Yu, Y. Cai, S. Liu, L. Wei and T. Chai, 2016: The pathogenicity of novel duck reovirus in Cherry Valley ducks. *Veterinary microbiology,*192, 181-185.

Liu, Q., G. Zhang, Y. Huang, G. Ren, L. Chen, J. Gao, D. Zhang, B. Han, W. Su, J. Zhao, X. Hu and J. Su, 2011: Isolation and characterization of a reovirus causing spleen necrosis in Pekin ducklings. *Veterinary microbiology,* 148, 200-206.

Luo, D., R. Liu, L. Weng, K. Li, X. Qi, Y. Gao, C. Liu, Y. Zhang, H. Cui, Q. Pan, L. Gao and X. Wang, 2021: Genomic sequences and pathogenic characteristics of two variant duck reoviruses associated with spleen necrosis. *Infection, genetics and evolution : journal of molecular epidemiology and evolutionary genetics in infectious diseases,* 92, 104847.

Ma, G., D. Wang, J. Shi, T. Jiang, Y. Yuan and D. Zhang, 2012: Complete genomic sequence of a reovirus isolate from Pekin ducklings in China. *Journal of virology,* 86, 13137.

Palomino-Tapia, V., D. Mitevski, T. Inglis, F. van der Meer and M. F. Abdul-Careem, 2018: Molecular characterization of emerging avian reovirus variants isolated from viral arthritis cases in Western Canada 2012-2017 based on partial sigma (σ)C gene. *Virology,* 522, 138-146.

Palya, V., R. Glávits, M. Dobos-Kovács, E. Ivanics, E. Nagy, K. Bányai, G. Reuter, G. Szucs, A. Dán and M. Benko, 2003: Reovirus identified as cause of disease in young geese. *Avian pathology : journal of the W.V.P.A,* 32, 129-138.

Rosa, A. C. G., H. L. Ferreira, D. E. Gomes, C. V. Táparo and T. C. Cardoso, 2014: Isolation and molecular characterization of Brazilian turkey reovirus from immunosuppressed young poults. *Archives of Virology,* 159, 1453-1457.

Shih, W. L., H. W. Hsu, M. H. Liao, L. H. Lee and H. J. Liu, 2004: Avian reovirus σC protein induces apoptosis in cultured cells. *Virology,* 321, 65-74.

Wang, D., J. Shi, Y. Yuan, L. Zheng and D. Zhang, 2013: Complete sequence of a reovirus associated with necrotic focus formation in the liver and spleen of Muscovy ducklings. *Veterinary microbiology,* 166, 109-122.

Wang, H., B. Gao and H. Chen, 2019: Isolation and characterization of a variant duck orthoreovirus causing spleen necrosis in Peking ducks, China. *Transbound Emerg Dis.* ,66, 2033-2044.

Wang, H., B. Gao, X. Liu, S. Zhang, Y. Diao and Y. Tang, 2020a: Pathogenicity of a variant duck orthoreovirus strain in Cherry Valley Ducklings. *Veterinary microbiology,* 242, 108546.

Wang, H., Y. Wang, B. Gao, S. Zhang, Y. Diao and Y. Tang, 2020b: Evidence of vertical transmission of novel duck orthoreovirus in ducks. *Veterinary microbiology,* 251, 108861.

Wang, S., F. Lin, X. Cheng, J. Wang, X. Zhu, S. Xiao, M. Zheng, M. Huang, S. Chen and S. Chen, 2020c: The genomic constellation of a novel duck reovirus strain associated with hemorrhagic necrotizing hepatitis and splenitis in Muscovy ducklings in Fujian, China. *Mol Cell Probes,* 53, 101604.

Wang, T., Y. Sun and H. J. Qiu, 2018: African swine fever: an unprecedented disaster and challenge to China.*Infectious diseases of poverty,* 7, 111.

Wang, W., J. Liang, M. Shi, G. Chen, Y. Huang, Y. Zhang, Z. Zhao, M. Wang, M. Li, M. Mo, T. Wei, T. Huang, X. He and P. Wei, 2020d: The diagnosis and successful replication of a clinical case of Duck Spleen Necrosis Disease: An experimental co-infection of an emerging unique reovirus and Salmonella indiana reveals the roles of each of the pathogens. *Veterinary microbiology,* 246, 108723.

Yan, H., G. Xu, Y. Zhu, Z. Xie, R. Zhang and S. Jiang, 2021: Isolation and characterization of a naturally attenuated novel duck reovirus strain as a live vaccine candidate. *Veterinary microbiology,* 261, 109214.

Yun, T., B. Yu, Z. Ni, W. Ye, L. Chen, J. Hua and C. Zhang, 2013: Isolation and genomic characterization of a classical Muscovy duck reovirus isolated in Zhejiang, China.*Infection, genetics and evolution : journal of molecular epidemiology and evolutionary genetics in infectious diseases,* 20, 444-453.

Yun, T., B. Yu, Z. Ni, W. Ye, L. Chen, J. Hua and C. Zhang, 2014: Genomic characteristics of a novel reovirus from Muscovy duckling in China. *Veterinary microbiology,*168, 261-271.

Zhang, X., J. Shao, X. Li, M. Mei, J. Guo, W. Li, W. Huang, S. Chi, S. Yuan, Z. Li and S. Huang, 2019: Molecular characterization of two novel reoviruses isolated from Muscovy ducklings in Guangdong, China. *BMC veterinary research,* 15, 143.

Zheng, X., D. Wang, K. Ning, T. Liang, M. Wang, M. Jiang and D. Zhang, 2016: A duck reovirus variant with a unique deletion in the sigma C gene exhibiting high pathogenicity in Pekin ducklings. *Virus Res,* 215, 37-41.

Zhu, Y. Q., C. F. Li, Z. L. Bi, Z. Y. Chen, C. C. Meng, G. J. Wang, C. Ding and G. Q. Liu, 2015: Molecular characterization of a novel reovirus isolated from Pekin ducklings in China. *Archives of Virology,* 160, 365-369.

Tables

Table 1 Amino acid polymorphism of the σC protein among HNXX-1/2021, HNJZ-2/2021 and other WRVs strains

Amino acid position	WRVs strains	WRVs strains	WRVs strains	WRVs strains	WRVs strains	WRVs strains	Location
	Clade 1 within genotype2 HNXX-1/2021	Clade 1 within genotype2 HNJZ-2/2021	Clade 1 within genotype2 Other		Clade 2 within genotype2 Isolated after 2017	Clade 2 within genotype2 Isolated before 2017	
88	N	N	N/D		N	D	/
93	T[a]	T[a]	T[a]		S	S	/
132	A	A	S		A	S/A/T[e]	σC_shaft domain
138	R	R	R		R	R/Q	σC_shaft domain
158	H[b]	H[b]	Q		Q	Q/R[e]	σC_shaft domain
233	S[b]	S[b]	P		P	P	σC_head domain
253	A[a]	A[a]	V/A[a]		V	V	σC_head domain
298	V	V	V/A		V	A/T[c]	σC_head domain
305	G[c]	E	E		E	E	σC_head domain
328	R[c]	G[d]	A		A	A	σC_head domain

Amino acid position	WRVs strains	WRVs strains	WRVs strains	WRVs strains	WRVs strains	WRVs strains	Location
329	Q[c]	K[d]	T		T	T	σC_head domain

Note:[a] Specific amino acid mutation only found in strains in clade 1.

[b] Specific amino acid mutation only found in HNXX-1/2021 and HNJZ-2/2021.

[c] Specific amino acid mutation only found in HNXX-1/2021.

[e] Tiny minority amino acids existed in the WRVs strains.

Figure legends

Figure 1

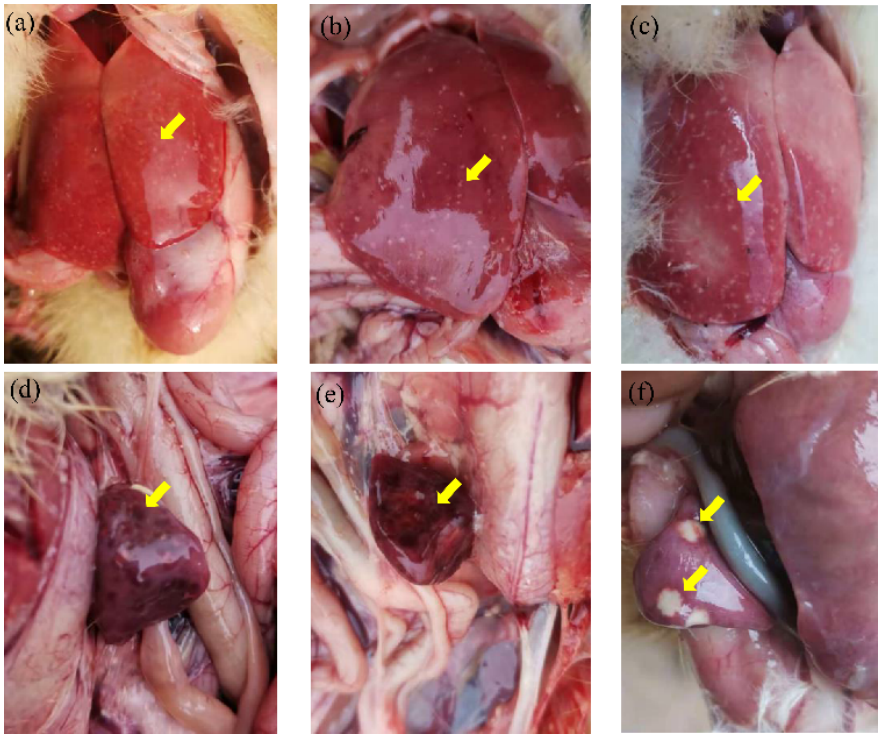

Figure 1 The pathological changes of the infected ducklings from the commercial duck farms in Henan province, China. a-c showed the liver pathological changes, the livers showed hemorrhage and necrosis; d-f showed the spleen pathological changes, the spleens showed different degrees of congestion and necrosis.

Figure 2

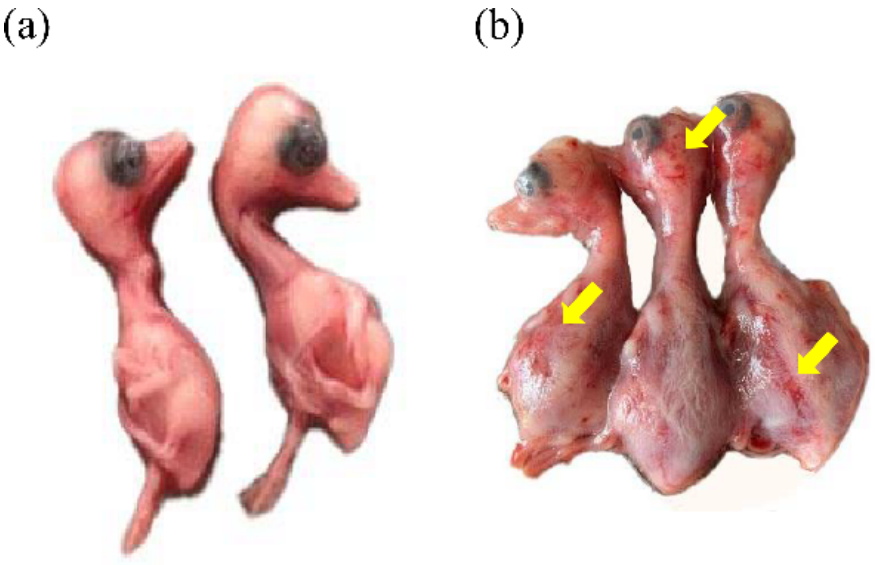

Figure 2 The pathogenicity of NDRV to duck embryos. (a) The duck embryos inoculated with PBS as the control. (b) The duck embryos inoculated with NDRV isolates in this study showed haemorrhage and oedema.

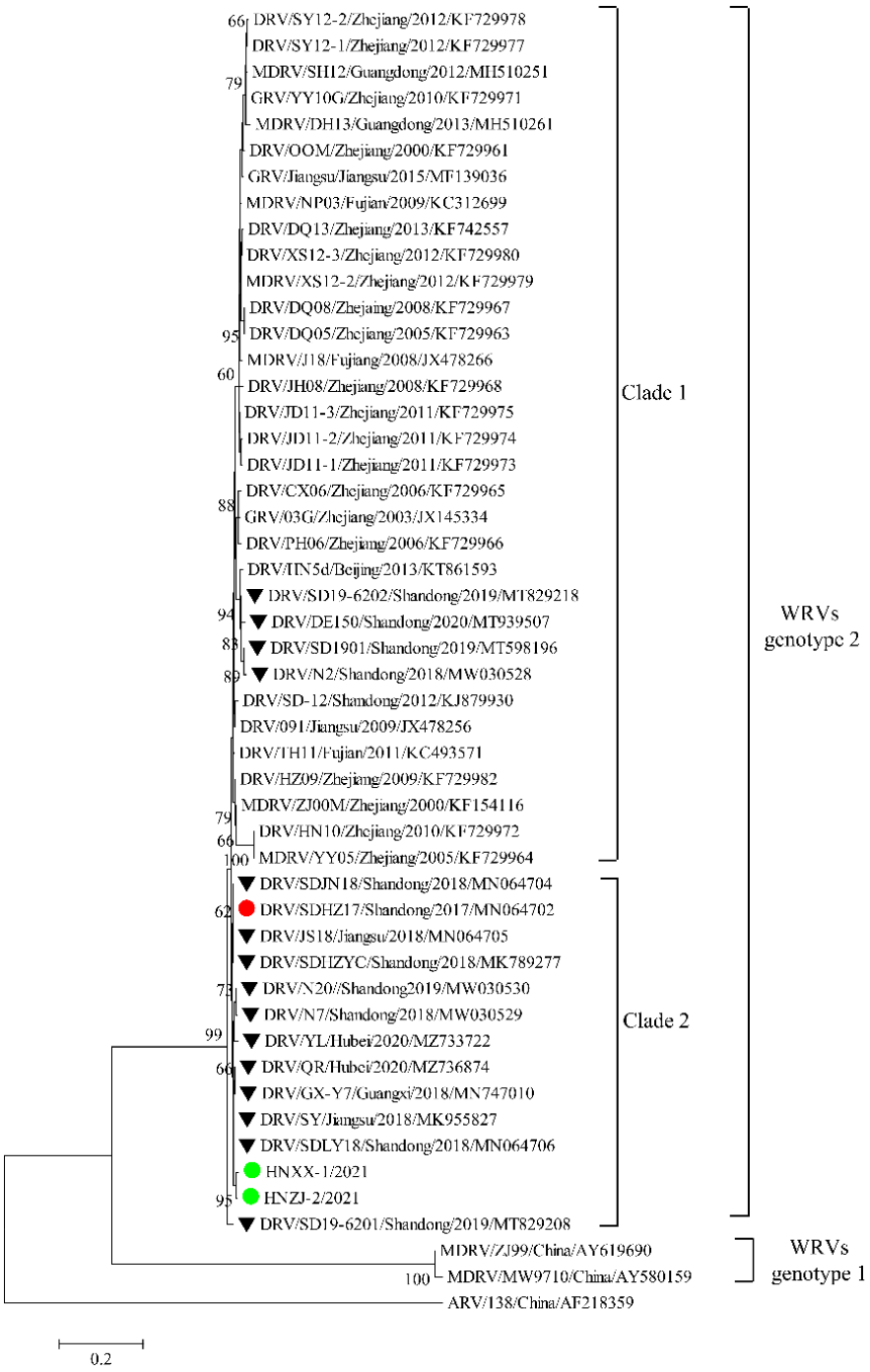

Figure 3

Figure 3. Phylogenetic tree was constructed based on σC protein amino acid sequences of DRVs using a neighbor-joining method in the mega 6.0 software. Numbers at nodes indicated bootstrap percentages obtained after 1000 replicates. The σC protein amino acid sequence of a chicken-origin ARV/138/China strain (accession number AF218359) was included as an outgroup. The green solid circles indicate the HNXX-1/2021 and HNJZ-2/2021 strains described in this study. The solid triangles indicate NDRV strains

emerged after 2017. The province and time in the WRVs name represent the province and time at which the strain was collected, respectively.