Emergence and genomic analysis of a novel ostrich-origin GPV-related parvovirus in China

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ABSTRACT In recent years, ostrich disease characterized by paralysis and diarrhea has been circulating in some regions of China, causing huge economic losses to the ostrich breeding industry. In our study, clinical samples from diseased ostriches were collected, and only parvovirus was detected. The virus distribution analysis by histopathology and quantitative real-time PCR assays indicated that the virus had a wide range of tissue tropisms. The full-length genome of the ostrich parvovirus (OsPV) was sequenced and comprehensively analyzed. Interestingly, the phylogenetic and alignment results indicated that the OsPV and the goose parvovirus (GPV) form a separate branch. In contrast to GPV strains, OsPV showed 2 new 14 nucleotide deletions in the inverted terminal repeat (ITR) region. Furthermore, recombination analysis indicated that OsPV was a recombination strain between the vaccine strain SYG61v and the virulent strain B strain, with the major parent of OsPV as the SYG61v strain and the minor parent as the B strain. The 14 nucleotide deletions in the ITR region as well as recombination may be some of the reasons for the cross-species transmission of parvovirus from goose to ostrich. The above data will contribute to a better understanding of the molecular biology of the novel OsPV and help to develop the vaccine candidate strain.

Key words: ostrich, parvovirus, paralysis, recombination, cross-species transmission

INTRODUCTION Parvoviruses are small (20 nm) non-enveloped icosahedral viruses that belong to the Paroviridae family and that infect and cause diseases in many vertebrate hosts (Boftsi et al., 2020). The International Committee on Taxonomy of Viruses (ICTV) classifies the family Paroviridae into 2 subfamilies, Densovirinae and Parovirinae, whose hosts are vertebrates and arthropods, respectively (Wan et al., 2018). Recent studies have revealed that the diversity of some parovirus species has greatly expanded, and some paroviruses have evolved the capacity to expand their host range by biologically adapting to novel hosts (François et al., 2016). In recent years, paroviruses have become widespread in birds, especially waterfowl paroviruses, goose parovirus (GPV), and Muscovy duck parovirus (MDPV). GPVs can infect goslings, Muscovy ducklings, swans, and Anser cygnoides, whereas MDPV has only been discovered in Muscovy ducklings (Liu et al., 2014; Shao et al., 2014; Wan et al., 2015; Wan et al., 2016). Recent studies suggest that a novel duck parovirus (NDPV), identified as a new variant of GPV, can infect ducks and geese (Fan et al., 2017; Ning et al., 2018). Moreover, recombination can also occur between GPV and MDPV, which accelerates the evolution of paroviruses.

Recently, a disease outbreak characterized by paralysis occurred in farmed ostriches in the Hebei Province, China. The incidence rate was approximately 50%, and most of the diseased ostriches were 1 to 3 months of age. Clinical symptoms mainly include paralysis, inability to stand, and a reduced diet. Ostrich diarrhea can also be present with white feces, gradually resulting in weak and thin ostriches. Because the ostriches are paralyzed for a long time, this can affect intestinal peristalsis and cause intestinal blockages, which can eventually lead to death. Antibacterial and antiviral drugs have no effect on treating this disease, and thus far, no effective
solution has been reported. More importantly, the agent of this disease is unknown, causing extensive loss within the ostrich farming industry.

Therefore, to determine the pathogen of this disease, sick ostriches were autopsied and examined in this study. Finally, it was determined that the pathogen causing ostrich paralysis was a new ostrich-derived parvovirus (OsPV), which was named HB-2019 strain. The whole genome of the virus was also sequenced, and phylogenetic tree analysis showed that the virus was correlated to GPV. Recombination analysis revealed that this OsPV HB-2019 strain is a recombination strain between the vaccine SYG61v strain and the virulent B strain, which may result in cross-species transmission.

MATERIALS AND METHODS

Sample Collection

In 2019, an ostrich farm in the Hebei Province suffered from an outbreak of paralysis and diarrhea. Muscles, sciatic nerve, kidneys, spleen, thymus, heart, pancreas, and gut samples were collected from one diseased ostrich and processed for viral detection and isolation.

Clinical Anatomy and Histopathology

The diseased ostriches were dissected, and tissues from their muscle, kidneys, liver, heart, and spleen were collected and examined. All tissues were stored at $-80^\circ$C for further study. Parts of the tissues were fixed in 4% neutral formalin at room temperature, embedded in paraffin, and cut into 5-μm-thick sections. After deparaffinization, the sections were stained with hematoxylin and eosin (H&E). Microscopic lesions were observed under an Olympus microscope (Olympus, Tokyo, Japan).

Detection of the Potential Pathogen Using Polymerase Chain Reaction

The tissue samples were homogenized in Dulbecco’s Modified Eagle Medium (DMEM), freeze-thawed three times, and centrifuged at 12,000 × g for 5 min. The supernatants were collected for DNA extraction using a TransGen BiotechEasyPure Viral DNA/RNA Kit according to the manufacturer’s instructions. As the causing agent of paralysis in the ostriches is unknown, a variety of suspicious pathogens were detected, including avian influenza virus (AIV), avian paramyxovirus-1 (APMV-1), avian encephalomyelitis virus (AEV), avian orthoreovirus (ARV), GPV, and Mycoplasma synoviae (MS). The primers for the different agents used in this study are listed in Table 1. Amplification bands were sequenced to verify the results.

Viral Isolation

GPV was found to be the most homologous to OsPV HB-2019, and the virus was further isolated from 10-day-old goose embryos. The tissue samples were ground and homogenized in physiological saline, freeze-thawed 3 times, and centrifuged at 12,000 × g for 15 min at 4°C. The supernatants were filtered using 0.22-μm sterile filters to remove contaminants and inoculated into the chorioallantoic cavity of 10-day-old goose embryonated eggs (300 μL/egg). The inoculated embryonated eggs were checked daily for survival. The allantoic fluid and

### Table 1. The primers and probe used in this study.

| Primers       | Nucleotide sequence(5′ → 3′)                  | Length of products (bp) |
|---------------|---------------------------------------------|-------------------------|
| AIV-Forward   | CGTACAGCCGCTTTGTCCAGAATGC                   | 428                     |
| AIV-Reverse   | GTCCCTCTTGCCTGACCCATC                      | 268                     |
| AEV-Forward   | AGAGGGTTTTTGTTGACGGGTTACA                  | 268                     |
| AEV-Reverse   | CAGTGGCGTGGTAGGAAAAGGGATC                  | 268                     |
| APMV-1-Forward| TGATGTGGAGGTAGACCCAGAAG                   | 183                     |
| APMV-1-Reverse| CTGAGGCCGACCAAGAGTGAGT                    | 183                     |
| DRV-Forward   | TCGCCTATTTGACGGCCTACT                     | 801                     |
| DRV-Reverse   | GAGGTTGTGATAACCCAGGAGG                    | 801                     |
| MS-Forward    | GTGGGGGACAAAGGATTAG                       | 611                     |
| MS-Reverse    | GTAGACCGCGGTTGTTGACAA                     | 611                     |
| GPV-Forward   | AATGACTGAGCGGCGGTCTGC              | 659                     |
| GPV-Reverse   | GCGACGGTCGTCCTGCTTTTAATTGA               | 659                     |
| ITR-1-Forward | TCACCCCCTCCTGCTGTGCTTGT                   | 188                     |
| ITR-1-Reverse | GCATTGCAGCCGTCACACTA                    | 188                     |
| ITR-2-Forward | GCACTGCCGCGGCAGCCCAA                    | 795                     |
| ITR-2-Reverse | GTATGCTGAGTTCAGGCTTCT                  | 795                     |
| T2-Forward    | GGGAGGACAGAAAGAGAGAC                  | 1,437                   |
| T2-Reverse    | TTTTCTGACATCATCAATGT                   | 1,417                   |
| T3-Forward    | TGTTCTTACATCATGTCCTC                     | 1,417                   |
| T3-Reverse    | GCCGCCAGGGCATAGACAT                   | 1,417                   |
| T4-Forward    | CATCACCAAGGACCACCA                     | 1,088                   |
| T4-Reverse    | AAGCTCCAAAGAACATCAA                     | 1,088                   |
| ITR-3-Forward | ACACATCACTAGCTTCCACGAGT                | 712                     |
| ITR-3-Reverse | GCATTGCGCGGTTGCAACCAA              | 712                     |
| OsPV-Forward  | CAAATTCCATCCTTCCCGGTTACCT             | 86                      |
| OsPV-Reverse  | TCTGACAGGCTCGTTGATC                  | 86                      |
| OsPV-Probe    | CTGCAACATACCCACCCACGCGGTTCT           | 86                      |
embryo bodies were harvested 6 days after inoculation and stored at −80°C for further testing.

**Whole Genome Amplification of OsPV HB-2019 Strain and Sequencing**

The sequencing results of the detected bands were input into the NCBI Basic Local Alignment Search Tool (BLAST) to screen for the sequences with the highest homology, and 6 pairs of primers were designed (Table 1). The genomes were amplified using PrimeStar HS DNA polymerase (TaKaRa Biotechnology, Dalian, People’s Republic of China). Amplified DNA fragments were visualized via electrophoresis on a 1% agarose gel. Polymerase chain reaction (PCR) products were purified and cloned into pEasy-Blunt vector and sequenced by Sangon Biotech.

**qPCR**

To further detect the distribution and content of the virus in different tissues, 3 g of each sample collected from the diseased ostriches were used for homogenization. The supernatants were collected for DNA extraction, according to the manufacturer’s instructions. The primers and probes are listed in Table 1 and were synthesized by Sangon Biotech. The reaction was carried out in a LightCycler PCR (Roche), and the optimal reaction was carried out as follows: 95 °C for 30 s, followed by 35 cycles of 95 °C for 5 s and 60 °C for 35 s (Wang et al., 2017b).

**Phylogenetic and Recombination Analysis**

For the phylogenetic analysis, the whole genome sequences of GPV, MDPV, and novel-goose parovirus (N-GPV) were retrieved from the NCBI nucleotide database as reference sequences. Detailed information and GenBank numbers of these viruses are shown in Table 2. The phylogenetic tree was constructed by MEGA 5.0 software, using the neighbor-joining method with 1,000 bootstrap replicates. The generated tree was annotated by ITOL (https://itol.embl.de/) (Yin et al., 2021). Multiple sequence alignments were analyzed using DNASTAR software to determine sequence homology. To detect probable recombination events, the OsPV HB-2019 strain was analyzed using the Recombination Detection Program v.4.43 (RDP4) and SimPlot version 3.5.1. Different methods implemented in RDP4 were used to detect recombinant events under default settings for different detection programs. The results of the recombination analysis using RDP4 are shown in Supplementary Table 1. If the recombination event was supported by at least 3 methods with a P-value of <10^{-6} or a recombination score above 0.6, the recombination event was considered as true and analyzed via Simplot (Wang et al., 2017a). Simplot analysis was conducted by setting the window width and step size to 200 bp and 20 bp, respectively.

**Statistical Analysis**

Statistical analyses were performed using GraphPad Prism 6 (GraphPad, La Jolla, CA). Statistical significance was assessed using a Student’s t test. Differences were considered to be statistically significant at P < 0.05.

**RESULTS**

**Clinical Syndrome and Histopathologic Changes**

The morbidity of ostrich farms in Hebei Province is approximately 50% (17/30), and all the sick ostriches were 1 to 3 months of age. The classic symptoms exhibited by the diseased ostriches included paralysis and diarrhea (Figure 1A-a). Bleeding spots were also detected in multiple viscera, especially in the intestines, muscle, and thymus. In addition, the knee joint exhibited prominent effusion and swelling, as shown in Figure 1A-b. HE staining results of different tissue sections showed that a massive infiltration of hemocytes was present in the leg muscle, and muscle fibers in the muscular layer exhibited differing degrees of swelling and degeneration. The renal tubules also showed degeneration and necrosis with massive lymphocytic infiltration in the interstitium. Hemorrhagic tissue was also observed in the thymus and bursa (Figure 1B).

**Detection of the OsPV and Complete Genome Sequencing**

To determine the causative pathogen of the outbreak in the studied ostrich farm in the Hebei Province, PCR was used to detect the potential pathogens causing paralysis and diarrhea. The results showed that the samples were parovirus-positive, but negative for AIV, APMV-1, AEV, ARV, and MS (Figure 2A). The newly detected viral strain was named OsPV HB-2019. Six PCR products were obtained which covering the full length of the viral genome. The nucleotide sequence of the complete OsPV HB-2019 genome has been deposited in GenBank (accession No. MT010852).

**Virus Isolation and Propagation**

The goose embryos were inoculated with the supernatant of the tissue homogenate and propagated for five passages, and no obvious changes or deaths occurred in infected goose embryos on the fifth passage until 6 dpi. The allantoic fluids and embryo bodies were harvested for the PCR assay. PCR results showed that viral DNA was present in the allantoic fluid after 5 passages (Supplementary materials 1). However, the viral load was extremely low, which may be related to the presence of maternal antibodies in non-SPF goose embryos.
Different tissues from the sick ostriches were homogenized, and qPCR was used to detect the distribution of the virus. The results showed that OsPV HB-2019 is widely distributed in different organs. As shown in Figure 3A, viral DNA copies were all above $10^6$ in the muscles, sciatic nerve, kidneys, spleen, thymus, heart, and pancreas. Moreover, the viral load in the gut was also high, especially in the duodenum, jejunum, ileum, and cecum (Figure 3B).
Genomic Characterization and Homology Analysis

The complete genomic sequence of OsPV HB-2019 was determined and deposited in the GenBank database under the accession number MT010852. The genomes of OsPV HB-2019 were 5,046 nucleotides (nt) in length, including 2 ITRs at the 3’ and 5’ ends. OsPV HB-2019 shared 92.36–99.80%, 92.81–95.2%, and 85.21–86.36% sequence identity with classic GPV, N-GPV, and MDPV respectively. The NS sequence shared 93.63–99.68%, 96.23–99.36%, and 83.15–83.25% and the VP sequence shared 94.86–99.86%, 94.63–99.41%, and 80.23–90.15% sequence identity with classic GPV, N-GPV, and MDPV, respectively. Besides, the amino acid sequence of NS and VP shared 96.97–99.36%, 96.17–95.2% sequence identity with classic GPV, N-GPV, and MDPV respectively. The NS sequence shared 93.63–99.68%, 96.23–99.36%, and 83.15–83.25% and the VP sequence shared 94.86–99.86%, 94.63–99.41%, and 80.23–90.15% sequence identity with classic GPV, N-GPV, and MDPV, respectively.

Figure 1. Clinical syndrome and histopathologic changes. The diseased ostrich characterized with paralysis (A-a), and the joint exhibiting prominent effusion and swelling (A-b). Microscopic examination of the histopathologic changes in different tissues collected from diseased ostrich (B-a) muscle, (B-b) kidney, (B-c) liver, (B-d) thymus, (B-e) bursa of fabricius, and (B-f) myocardium. The scale bar equal 50 μm.

Figure 2. PCR amplification of different agents and complete genome of ostrich parvovirus. Six potential pathogens were detected with corresponding primers, including MS, GPV, ARV, AEV, APMV-1 and AIV (A). The complete genome of OsPV was divided into six fragments for amplification, and every fragment was cloned to pEasy-Blunt vector for further sequenced (B).
−97.13%, 89.31−90.91% and 95.08−97.95%, 91.39−92.49% identity with classic GPV, N-GPV, and MDPV, respectively (Table 3). Multiple sequence alignment showed that OsPV is characterized by the same deletion as the GPV DY16 strain at the 3′-ITR and 5′-ITR when compared to the GPV virulent B and the vaccine SYG61v strains.

**Phylogenetic Analysis**

Phylogenetic analyses were conducted using the whole genome of OsPV and other parvoviruses, including classic GPV, MDPV, and N-GPV strains available in GenBank. As shown in Figure 4, whole genome phylogenetic analysis resulted in 64 parvovirus strains classified into three major clusters, with OsPVs and GPV RC16 strains (goose parvoviruses isolated in Chongqing in 2016) forming a separate branch. Phylogenetic and alignment analyses showed that OsPV is a branch of the GPV (Figure 4A). There was a greater genetic distance between the OsPVs and SYG61, and an even greater distance between OsPV and N-GPV.

**Recombination Analysis**

Simpplot and RDP software were used to analyze the recombination of OsPV HB-2019, and 2 potential recombination events were detected in strain OsPV HB-2019. Recombination analysis performed with RDP4 showed that the breakpoints were located in VP1 (nt 2,494 and 2,724) and VP1 (nt 3,806 and 4,318; Figure 5A). These breakpoints separated the genome into five regions; three being closely related to virulent B strains of GPV from Europe (Figure 5B), and the remaining two being closely related to vaccine strain SYG61v of GPV (Figure 5C). The results, therefore, show that OsPV HB-2019 is the result of recombination between the virulent B strain and the vaccine strain SYG61v of GPV.

**DISCUSSION**

As stated earlier, since 2019 there have been outbreaks of diseases characterized by ostrich paralysis and diarrhea in ostrich farms in many areas of the Hebei Province. In this study, we investigated the pathogen that caused this disease in ostrich farms and detected OsPV in the samples collected from the diseased ostriches. Tests for other viral agents, such as AIV, APMV-1, AEV, ARV, and MS were negative, which is consistent with the findings of a previous study (Yuan et al., 2020). The vaccine generated by inactivating GPV can prevent and treat ostriches to a certain extent, therefore, we speculate that ostrich parvovirus (OsPV) was the pathogen causing ostrich diarrhea and paralysis; however, this needs further investigation. OsPV possesses the highest genome homology to classical GPV; therefore, goose embryos were used for further passaging. Interestingly, OsPV can propagate in goose embryos; however, the viral load was extremely low, which may be related to the non-SPF goose embryos which carrying maternal antibodies. Based on the clinical symptoms, histopathology, and distribution of viruses in various tissues, we know that OsPV has a wide range of tissue tropism. Other waterfowl parvoviruses, such as GPV and MDPV, are mainly distributed in the spleen and intestines after infection. This may be
the main reason ostrich paroviruses cause multiple organ damage in ostriches. In addition, the virus can be detected in ostrich eggs (data not shown), indicating that the virus has the characteristics of vertical transmission.

The whole genome sequence of OsPV HB-2019 consisted of 5,046 nt, and the phylogenetic tree showed that it was closely related to GPV isolates, with 92.36 to 99.80% similarity. The nucleotide sequence similarity was highest with the GPV DY16 strain, a classical virulent GPV strain (Wang et al., 2019). In addition, OsPV HB-2019 had the same deletion as the GPV DY16 strain. According to previous reports, GPV only infects geese and Muscovy ducks, but paroviruses have a wide host range (Li et al., 2018). However, the factors that cause cross-species transmission of this virus are unknown. Interestingly, the genome of OsPV possesses 2 deletions at the ITR compared to the GPV vaccine SYG61v and the virulent B strains (Figure 4B). The ITR not only functions as an origin of genome replication but also contains several transcription factor binding sites, including E-box, activating transcription factor/cyclic AMP-responsive element binding (ATF/CREB), and an upstream stimulatory factor known as MLTF (Yuan et al., 2020). The deletion sequence in the ITR may be one of the reasons why the GPV infects ostriches. Genetic recombination evidently plays an important role in parovirus evolution, and several previous studies have provided evidence of natural genetic recombination in waterfowl paroviruses (Ohshima and Mochizuki, 2009; Zhu et al., 2014; Liu et al., 2020). The resultant recombinant viruses may escape the immunization response elicited by outdated vaccine strains, induce virulence, and eventually lead to cross-species transmission. A previous study confirmed that the recombinant MDPV remains pathogenic to Muscovy
ducks that are older than 19 d and is also associated with high morbidity and mortality rates and intestinal embolism (Chen et al., 2002; Zhu et al., 2014). In addition, different GPV strains can recombine, resulting in a new genotype among GPV strains (Wang et al., 2015). In our study, 2 recombination events were detected using RDP4 and Simplot in the OsPV HB-2019 strain. The VP genes of OsPV from 2,470 nt to 3,839 nt showed the highest similarity with the corresponding gene in the vaccine strain SYG61v, whereas the other regions of the genome showed the highest similarity with those of virulent B strain GPV. Recombination of multiple viruses can cause cross-species transmission, such as circovirus in pigs and porcine deltacoronavirus (Li et al., 2019; Ye et al., 2020). The GPV is a waterfowl virus. Although it has a wide range of host tropism, it is currently mainly limited to waterfowl and other animals. Therefore, how GPV breaks through the interspecies barrier and infects ostriches still remains unclear. Our data revealed that OsPV HB-2019 was a GPV recombination strain, therefore, we speculate that recombination may be one of the main reasons for its cross-species transmission; however, it needs to be further investigated using a reverse genetic system and animal experiments.

In conclusion, parvoviruses, originating from ostriches, causing symptoms of paralysis and diarrhea were detected. This virus is characterized by a wide range of tissue tropism, and the full-length genome of the original ostrich parvovirus, OsPV HB-2019 strain, was sequenced. Phylogenetic tree and sequence alignment results confirmed that OsPV HB-2019 had the highest nucleotide sequence similarity with the GPV DY16 strain, a classical virulent GPV strain. Compared with other waterfowl parvoviruses, two 14 nucleotide deletions were located in the ITR. In addition, the OsPV was a recombination virus, and it was the first to provide evidence of genetic recombination events between GPV, which may be associated with cross-species transmission from goose to ostrich. Therefore, it is necessary to monitor the recombination of parvoviruses from different species, which will contribute to preventing cross-species transmission.

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Ethical statement: Animal experiments in this study were approved by the Animal Welfare and Ethics Committee at Laboratory animal center of HeBei Agriculture University (Approve Number 20200025).

DISCLOSURES

The authors declare no potential conflicts of interest with respect to the research, authorship and publication of this article.
SUPPLEMENTARY MATERIALS

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.psj.2022.101929.

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