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Experimental reproduction of beak atrophy and dwarfism syndrome by infection in cherry valley ducklings with a novel goose parvovirus-related parvovirus

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ABSTRACT

Infection of clinically susceptible ducks, including cherry valley and Muscovy ducks, with a novel goose parvovirus (GPV)-related virus (N-GPV) can result in beak atrophy and dwarfism syndrome (BADS). To obtain new insights into the host range and pathogenic potential of this novel waterfowl parvovirus, cherry valley ducklings ($n=20$) were experimentally infected with N-GPV strain SDLC01. An equal number of ducklings served as uninfected controls. The appearance of clinical signs, histopathological changes, viral shedding, and seroconversion was monitored for 20 days post-infection. Infection status of all ducks was monitored using indirect ELISA, virus neutralization test, nested PCR, clinical indicators, and microscopic examination. Three ducks developed the typical clinical, gross, and histological changes of BADS. By study day 6, the infected ducks had seroconverted to N-GPV. The antibodies raised were neutralizing against the SDLC01 strain in vitro. Here we successfully developed an experimental infection model for studying the pathogenicity and role of N-GPV in BADS.

1. Introduction

In 2015, a sudden outbreak of chronic infectious disease emerged in commercial cherry valley ducks in Shandong, Jiangsu, Henan, Anhui, and Hebei provinces in Northern China. The disease was characterized by short beaks with protruding tongues, growth retardation, and short and thick tibias (Chen et al., 2015a). Infected flocks displayed disease rates of up to 90%. Morbidity rates varied from 10% to 30% which associated with the infected age. The disease was designated as beak atrophy and dwarfism syndrome (BADS), based on the clinical clinical signs. BADS was firstly reported in mule and Muscovy ducks in France and Poland in the early 1970s and later in mule and Tsaiya ducks in Taiwan in the 1990s (Lu et al., 1993; Palya et al., 2009; Samorek-Salamonowicz et al., 1995). The main pathological lesions were consistently observed in affected ducks included swelling and hemorrhage of the thymus, calcification of the tip of the tongue, and degeneration of the liver. Whereas, no other obvious clinical symptoms were observed in other organs or tissues. In most BADS outbreak cases, typical clinical signs usually appeared at 15-day-old ducks, and population of diseased ducks continually growing with the age until the day of slaughter. The feed conversion ratio (FCR) of infected ducks was higher than that of healthy ducks. The beaks, ulna, radius, and tibia of infected ducks were osteosclerotic and fractured easily. Outbreaks of BADS have significantly affected the waterfowl industry and resulted in great economic losses in China.

The novel duck origin parvovirus (N-GPV) was determined to be causative agent of BADS in our previous study and found to be close related with goose parvovirus (Chen et al., 2015c). Similar to other members of the genus *Anseriform dependoparvovirus 1* in the family Parvoviridae, the full-length genomic sequence of N-GPV is approximately 5 kb and contains two major open reading frames (ORF) (Zádori et al., 1995). Although three capsid proteins (VP1, VP2, and VP3) thaencoded by the right ORF t share the same promoter, but have three different stop codons. These three capsid proteins constitute the icosahedral capsid in a ratio of approximately 1:1:8 (Grieger et al., 2007). Compared with the full-length genomes of other classical goose and Muscovy duck parvovirus (MDPV) field isolates, the inverted terminal repeat of N-GPV contains several fragments. These characteristics are similar to those of attenuated GPV vaccine strains.

Classical waterfowl parvoviruses (MDPV and GPV) cause a high mortality rate among goslings and Muscovy ducklings, with gross

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pathological changes including catarrhal enteritis, swollen liver, and fibrinous exudates within the enteric cavity (Glavits et al., 2005; Kisary, 1986; Kisary and Stipkovits, 1975). Almost no N-GPV-infected ducks died in flocks experiencing natural infection. An N-GPV isolate was obtained from a diseased cherry valley duckling with BADS and designated SDLC01. The isolate could be propagated in duck embryo and in duck embryo fibroblast (DEF) cells but not in chicken embryo, goose embryo, chicken embryo fibroblast, or goose embryo fibroblast cells. The pathogenicity of N-GPV was lower than that of classical waterfowl parvoviruses, and the infected host species were different.

Experimental infection of Mule and Tsaiya ducklings with three parvovirus strains through different routes and ages can induce the beak atrophy and growth retardation in infected ducks (Lu et al., 1993; Palya et al., 2009). Classical waterfowl parvovirus strains (GPV and MDPV) and an N-GPV of mule duck origin were used in experimental infections of specific pathogen-free (SPF) animal and mule ducklings, respectively. Only the N-GPV caused atrophic bills and stunting in ducklings. In this study, cherry valley ducklings were experimentally infected with N-GPV strain SDLC01. The appearance of clinical signs, gross and microscopic pathological changes, viral shedding, and seroconverted against N-GPV was monitored.

2. Materials and methods

2.1. Virus strain

A N-GPV strain, SDLC01 (GenBank accession number: T343253), was originally isolated from the liver of the cherry duckling flock with BADS in Shandong province, China. The isolated virus was grown and titrated in 9-day-old embryonated duck eggs through the allantoic cavities route.

The titer of SDLC01 was calculated at 10^6 EID_{50}/0.3 mL for intramuscular challenge in the present study. Due to the SDLC01 strain was not propagated in SPF chicken embryos the viral fluid was strictly tested for sterility and the presence of duck extraneous agents by PCR assays (including avian influenza virus, duck plague virus, Tembusu virus, duck reovirus, duck hepatitis virus, duck circovirus, and avian poxvirus).

2.2. Animals and study design

This study was approved by the Animal Care and Use Committee of Shandong Agricultural University (permit number: 20150622) and performed in accordance with the "Guidelines for Experimental Animals" of the Ministry of Science and Technology (Beijing, China). Forty 1-day-old Cherry Valley ducklings were purchased from the commercial hatchery of Yike Company Limited in Xintai County. Ducklings were kept in SPF animal isolators that were ventilated under negative pressure without any immunizations. Serum samples and cloacal swabs were collected from ducklings prior to challenge to confirm that these animals were free of DPV antigen. The presence of antibody was determined using PCR and an indirect ELISA (iELISA) method.

Forty ducklings were randomly divided into two groups ($n = 20$/group) and housed in separate isolators. Twenty ducklings were challenged with N-GPV by intramuscular inoculation. Preliminary experiments revealed that an appropriate inoculum for use in ducklings in this study was an infectious dose of approximately 10^6 EID_{50} of the N-GPV SDLC01 strain. The other 20 ducklings were inoculated with 0.2 mL of sterile phosphate-buffered saline (PBS, pH 7.2) which served as negative control group. The body weights of five ducklings from each group were measured daily until 20 days post-infection (dpi). All ducks were monitored daily for the occurrence of clinical signs including growth stunt, short beak, short and gross tibia, and diarrhea. The ducklings were euthanized at the end of the experiment (20 dpi). For welfare reasons, ducks were euthanized with intravenous pentobarbital sodium (New Asia pharmaceutical, Shanghai, China) at the fixed time points or when they displayed moderate clinical signs of infection.

2.3. Samples collection

Oral and cloacal swabs, which were collected daily from each duck in each of the groups, were used for PCR analysis. The swabs were immediately placed in 0.5 mL PBS (pH 7.2) and extensively vortexed. The swab fluids were frozen-thawed three times, sterilized by passage through a 0.22 μm filter and stored at −20 °C for further DNA extraction and virus isolation. Blood samples were collected via the cervical vein from all the ducks at 3, 6, 9, 12, 15, and 20 dpi. Blood samples was incubated at 37 °C for 30 min and then centrifuged at 6000 rpm for 10 min. The isolated serum samples were inactivated at 56 °C for 30 min and stored at −80 °C prior to N-GPV-specific antibody detection using an iELISA based on recombinant N-GPV VP3 protein. At necropsy, samples were collected from liver, thymus, spleen, kidney, lung, intestine, brain, pancreas, and glandular stomach. A portion of each sample was homogenized, frozen, thawed, and sterilized for DNA analyses and virus isolation. A separate set of samples from each tissue was fixed in 10% neutral buffered formalin and embedded in paraffin wax, then cut into 4 μm thick sections. Sections were stained with hematoxylin and eosin for microscopic examination.

2.4. Serological assays

Before serological testing, serum samples from the two groups were inactivated at 56 °C for 30 min. Duck parvovirus antibodies were detected using ELISA and virus neutralization (VN) tests. Inactivated N-GPV (SDLC01) was used in an SN test, while recombinant DPV VP3 protein was used as the coated antigen in the iELISA. Sera diluted in 1:10 serum diluent (PBS with 0.05% Tween 20 and 1% skim milk) were used in the iELISA. The positive/negative cutoff values were calculated using the positive/negative sample mean + 3 standard deviations (mean + 3 SDs). The VN test was performed in 960-well plates using DEFs. Serial two-fold dilutions of the sera were mixed with an equal volume containing 100 $TCID_{50}$'s of isolate SDLC01/0.1 mL, and the mixture was incubated for 45 min at 37 °C. After that, the mixtures were incubated with DEF cells cultured in 96-well plates for 4 days. An immunofluorescence assay for N-GPV was performed on incubated DEF cells using mouse antiserum against duck parvovirus as the primary antibody. The 50% neutralization endpoint for each serum sample was calculated using the method of Reed and Muench (Haggett and Gunawardena, 1964). Each sample was tested in triplicate to get the reliable result.

2.5. Virological assays

Total DNA was extracted from the swabs and the tissues using the phenol chloroform method. A nested PCR was performed to

Table 1
Primers used to amplify VP3 gene of N-GPV isolate.

Primers	Nucleotide sequence (5′-3′)	Length of products (bp)
VP3- F1	ACACGACCACTCATTTGGAG	677
VP3- R1	AAACTTGGATTGTTGAGG	
VP3- F2	CTGACCATTACCCAGTAGT	299
VP3- R2	AATTGCTTTGTAGATGTGA	