Pathogenicity of Pekin duck- and goose-origin paroviruses in Pekin ducklings

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Goose parovirus (GPV) usually affects goslings and Muscovy ducks but not Pekin ducks. Earlier works showed that a variant GPV can cause short beak and dwarfism syndrome (SBDS) in Pekin ducks. Here, we investigated the pathogenicity of a variant GPV of Pekin duck-origin (JS1) and a classical GPV of goose-origin (H) in Pekin ducklings. Following intramuscular infection at two days of age, both JS1 and H strains influenced weight gain and development of beaks and bones of wings and legs, and caused microscopic lesions of internal organs of ducks. However, the clinical signs typical of SBDS could only be replicated with the JS1 isolate. The findings suggest that both variant and classical GPVs are pathogenic for Pekin ducklings, while the former is more virulent than the latter. Using a quantitative real-time PCR assay, high levels of viral load were detected from blood, internal organs, leg muscles, and ileac contents in JS1- and H-infected ducks from 6 h to 35 days postinfection (DPI). Using a GPV VP3-based ELISA, antibodies in sera of JS1- and H-infected ducks were detectable at 1 DPI and then persistently rose during the subsequent five weeks. These results suggest that both variant and classical GPVs can infect Pekin ducklings. The present work contributes to the understanding of pathogenicity of GPV to Pekin ducks and may provide clues to pathogenesis of GPV-related SBDS.

1. Introduction

Goose parovirus (GPV) was originally recognized as the causative agent of Derzsy’s disease, affecting both goslings (Anser anser domestica) and Muscovy ducks (Cairina moschata) (Fang, 1962; Derzsy, 1967; Gough, 1991; Glávits et al., 2005). In general, Pekin ducks (Anas platyrhynchos) are believed to be refractory to GPV infection (Hoekstra et al., 1973; Gough et al., 1981; Gough, 1991). Since 2014, GPV has been noted to cause short beak and dwarfism syndrome (SBDS) in Pekin ducks (strain Cherry Valley) in China (H. Chen et al., 2015; Li et al., 2016; S. Chen et al., 2016; Yu et al., 2016; Ning et al., 2017), which resembles SBDS occurred in mule ducks (intergeneric cross of Pekin and Muscovy ducks) in France in 1971/1972 and in Poland in 1995 (Villatte, 1989; Palya et al., 2009; Woźniakowski et al., 2012). Phylogenetic analysis of partial VP1 and VP3 sequences revealed that GPV strains from SBDS in mule and Pekin ducks belong to a distinct lineage of GPV, namely West-European lineage (Palya et al., 2009; Ning et al., 2017). Studies undertaken in Hungary have shown that SBDS in mule ducks can be reproduced with a GPV of mule duck-origin following infection at one day and 2 weeks of age, but not with a GPV isolate from Derzsy’s disease (Palya et al., 2009). The findings suggest that the GPV isolate from SBDS (designated variant) is distinct from the classical GPV strain from Derzsy’s disease in terms of pathogenicity to mule ducks.

It has been shown previously that changes of amino acids at the receptor binding site of the capsid protein can influence host range and pathogenicity of paroviruses (P. Wu et al., 2000, 2006; Govindasamy et al., 2003; López-Bueno et al., 2006; Z. Wu et al., 2006; Kailasan et al., 2015). Thus, the occurrence of SBDS in Pekin ducklings seems to be attributed to alteration of host ranges of GPV caused by variation of the virus. However, compared with classical GPV strains, the capsid proteins of Chinese variant GPV strains contain only two common substitutions, which are located in positions distantly from the potential receptor binding sites (Ning et al., 2017). Based on the recent description of natural and experimental hosts of GPV (Palya, 2013), the embryo-adapted GPV given by parenteral route can infect young and adult Pekin ducks. Therefore, we consider it likely that the GPV-caused SBDS might not be attributed to host range switch of the virus from goslings to Pekin ducklings (Ning et al., 2017). To search for clues to pathogenesis of the GPV-related SBDS, we investigate the pathogenicity of a variant GPV of Pekin duck-origin (JS1) and a classical GPV of goose-origin (H) and their capacity to replicate in 2-day-old Pekin ducklings.
2. Materials and methods

2.1. Virus strains

The JS1 strain of variant GPV was originally isolated in 2015 in Jiangsu province of China from a 2-week-old Pekin duckling exhibiting signs typical of SBDS (Ning et al., 2017). The H isolate of classical GPV was kindly provided by Mr. J. Wang, ZhongshengTiaozhan Bioengineering, Tianjin, China. The virus was originally isolated in 2002 from Derzsy’s disease of goslings. For the purpose of this paper, the viruses were passaged three times in embryonated goose eggs. Clarified suspensions of allantoic fluids and embryo bodies harvested from dead goose embryos between 3 and 5 days after inoculation were prepared as described previously (Wang et al., 2013).

2.2. Animal experiments

All procedures involving animals were approved by the Animal Welfare and Ethical Censor Committee at China Agricultural University and Beijing Administration Committee of Laboratory Animals (Approval ID SYXK [Jing] 2015–0028).

Ninety newly hatched Pekin ducklings were derived from a commercial hatchery located in Beijing, where SBDS had never been observed. At day 2, the ducklings were divided into three groups (30 birds/group), and reared in different isolators. The challenged groups were inoculated intramuscularly with the JS1 and H viruses at the dose of 5 × 10^6 ELD₅₀ per bird respectively, and the control group with 0.5 ml of phosphate buffered saline (PBS). The birds were monitored daily for 35 days.

Body weight as well as width and length of beak of birds were measured weekly. At 6 and 12 h (H) as well as 1, 3, 7, 14, 21, and 28 days post infection (DPI), three ducks were selected randomly from each group and blood was collected. Subsequently, the selected ducks were euthanized, and tissues (e.g., heart, liver, and spleen) and ileac contents were sampled. At 35 DPI, the length and width of the tongues of all remaining ducks were measured, and samples were collected from three ducks as above. Ducks in the three groups were examined using X-rays. All samples were subjected to histopathological examination. Serum samples were prepared from blood samples for detection of antibody against GPV.

2.3. Histopathological examination

The organs were fixed in 4% neutral formalin at room temperature for 48 h, embedded in paraffin, and cut into 5-μm-thick sections. After deparaffinization, the sections were stained with haematoxylin and eosin (H & E). Pathological changes were observed under an Olympus microscope (Olympus, Tokyo, Japan).

2.4. DNA extraction

Tissues and ileac contents collected from ducks were processed as 20% suspensions in PBS. DNA was extracted from 200 μl of each suspension prepared from the duck samples and the GPV isolates using a DNA Isolation Kit for Cells and Tissues (Aidlab, Beijing, China) according to the manufacturer’s instructions. For blood samples, DNA was extracted using a DNA Isolation Kit for Blood (Aidlab, Beijing, China) according to the manufacturer’s instructions. Each DNA was eluted in 100 μl elution buffer.

2.5. GPV quantification

GPV loads in duck samples were quantified by using a GPV quantitative real-time PCR (qPCR) assay. Briefly, MUSCLE (http://www.ebi.ac.uk/Tools/msa/muscle/) was used to align genomic sequences of GPVs, including the JS1 (Ning et al., 2017), QH15 (Yu et al., 2016), and SDLCO1 (Chen et al., 2015) isolates of variant GPV and the B (Zadori et al., 1995), YZ09-6 (Wang et al., 2014), 82-0321, 82-0321 V, 06-0329 (Shien et al., 2008), and VG32/1 (Tatár-kis et al., 2004) isolates of classical GPV. Two primer pairs were designed over conserved regions of GPVs, using Primer Premier 5.0 (Premier Biosoft International). The primer pair, 477f (5′-CTTCGGTTGATTGCATTG-3′) and 4673r (5′-GCGACAGGTGCTTATTTTG-3′), encompassed the full-length NS and VP1-coding regions of GPVs and was employed to amplify a PCR product of 4197 base pair (bp) from DNA of the JS1 isolate. The PCR product was then cloned into the pGEM-T Easy Vector (Promega, Madison, USA), resulting in a recombinant plasmid pGEM-JS1. The primer pair, 3010f (5′-CCGAACCTGTGGGAGCATCT-3′) and 3186r (5′-TGTTGATGCTTGCCAGGACC-3′), was designed to amplify a 177 bp fragment from the VP3-coding region of the GPV genome. The specificity of primer pair 3010f and 3186r was evaluated by conventional PCR using pGEM-JS1 and DNAs extracted from the JS1 and H isolates as templates, followed by nucleotide sequence determination and analysis of the amplified fragments (Ning et al., 2007). The reactions and conditions for conventional PCR were the same as previously reported (Chang et al., 2000).

The concentration of pGEM-JS1 was measured by Biodropsis BD-1000 ultraviolet spectrophotometry (Beijing Oriental Science and Technology Development, Beijing, China). 10-fold serial dilutions (10^−3–10^−9; corresponding to 2.42 × 10^2–2.42 copies/μl) were used to measure the viral load of the qPCR assay. The reaction mixture (20 μl) contained 2 μl of template, 0.4 μl of each of forward primer 3010f and reverse primer 3186r (10 μM), and 10 μl of AceQ qPCR SYBR Green Master Mix (Vazyme, Nanjing, China). qPCR was conducted using the following conditions: initial annealing at 95 °C for 5 min, followed by 40 cycles of 95 °C for 10 s, 60 °C for 30 s.

The specificity of the qPCR assay was tested in amplification reactions containing DNA/cDNA samples of several frequently occurring pathogens in waterfowl, including GPV isolates JS1 and H, duck enteritis virus (DEV), avian influenza virus (AIV), Newcastle disease virus (NDV), duck hepatitis A virus 1 (DHAV-1), DHAV-3, duck astrovirus 1 (DAsV-1), duck reovirus (DRV), and Tembusu virus (TMUV). The sensitivity for GPV detection was measured by using serial 10-fold dilutions (10^−3–10^−11; corresponding to 2.42 × 10^2–2.42 × 10^−1 copies/μl) of pGEM-JS1 as templates.

For determination of viral load, DNAs extracted from bloods, tissue samples and ileac contents were subjected to qPCR performed as described above. Each DNA sample was tested for three times.

2.6. GPV antibody detection

Antibodies in sera were analysed using a GPV VP3-based enzyme-linked immunosorbent assay (ELISA) as described previously (Zhang et al., 2010). The sera were inactivated at 56 °C for 30 min, diluted 1:100 using 5% skimmed milk, and then used in the assay. The OD values were measured at 450 nm using an ELISA microplate reader. Each sample was tested for three times. A serum sample collected from an infected duck was considered as positive if its OD450 value was more than twice the value obtained from the uninfected control group.

2.7. Statistical analysis

The data was calculated as mean ± standard deviation (SD). For comparison of groups, we used the GraphPad Prism software version 6.01 (GraphPad Software Inc., San Diego, CA, USA). P < 0.05 was considered statistically significant.
3. Results

3.1. SBDS of Pekin ducklings could only be reproduced with GPV JS1

Laboratory infection with JS1 resulted in clinical signs of the disease in the infected ducklings. Early signs included listlessness, weakness, anorexia, and inability to walk, which appeared at 4 DPI. The clinical signs typical of SBDS, including short beak and protruding tongue (Fig. 1A), appeared at 7–14 DPI. By the end of the experiment, 20% of ducks in the JS1-infected group exhibited clinical signs typical of SBDS. No clinical signs of the disease were observed in the H-infected and uninfected control groups (Fig. 1B). No deaths were found in the three groups.

3.2. Strain JS1 had a greater impact on development of Pekin ducklings than strain H

The body weights and beak sizes of ducklings infected with the JS1 and H isolates were measured every week post infection to investigate the impact of the variant and classical GPVs on development of Pekin ducklings (Figs. 1 A and B; 2 A–C). Both JS1 and H viruses caused retardation of growth and influenced the development of beaks of ducklings. While the losses of weight gain (38.22–58.29%) and width (20.63–38.31%) in JS1-infected ducks were much shorter than those (weight loss: 11.77–22.88%; beak length loss: 8.33–13.68%; beak width loss: 3.07–7.73%) in H-infected ducks. There was significant difference in mean body weight and in mean length and width of beaks among the three groups (P < 0.05).

The tongue sizes of Pekin ducklings inoculated with the JS1 and H isolates were examined at 35 DPI (Figs. 1 C; 2 D and E). Significant difference was only observed between the JS1-infected group and the control group in mean length of tongues (P < 0.05). In JS1-infected ducks the loss of tongue length was 4.84%.

Examination under X-rays showed that the development of bones of wings and legs of both JS1- and H-infected ducks was influenced by virus infection, showing decreased bone mineral density with a narrower marrow cavity. Notably, the lengths of ulna, tarsus, radius, tibia, metatarsal, and phalanx of JS1-infected ducks were much shorter than those of the control group. The influence of strain H on development of bone was less when compared with that of strain JS1 (Fig. 3).

3.3. Both JS1 and H strains caused microscopic lesions in internal organs of Pekin ducklings

At necropsy, no apparent lesions were observed in any of the internal organs of the experimentally exposed ducks. However, both JS1 and H strains caused histopathological changes in multiple internal organs, including liver, kidney, lung, brain, bursa of Fabricius, and ileum (Fig. 4). Congestion was observed in central venous and hepatic sinusoid of liver, lung and brain tissues, tubulointerstitium, and lamina propria vessel of ileum. Other consistent lesions included mucosal epithelial detachment of bursa of Fabricius, neuronophagia in the brain, disruption of small intestinal villi, and disappearance of normal tissue structure of ileum.

In ducklings infected with the JS1 isolate other changes were also found, including splenic hemorrhages, disappearance of normal tissue structure of lymph follicle and reduction of lymphocytes in the bursa of Fabricius, exudates and detached lung cells in bronchial lumen, and skeletal muscle fibre degeneration. The ducklings infected with the H isolate presented lymphocytic infiltration in the liver and splitting of renal tubular epithelium from basement membrane (Fig. 4).

3.4. Strains JS1 and H induced similar kinetics of viral load in bloods, tissues and ileac contents of infected Pekin ducklings

The reproductive properties of GPV JS1 and H in Pekin ducklings were compared by detection of viral loads in bloods, internal organs, leg muscles, and ileac contents of infected ducks. GPV quantification involved the use of a qPCR assay. Positive signals were produced from recombinant plasmid pGEM-JS1 and DNAs extracted from the JS1 and H isolates, but not from DNAs/cDNAs prepared from other viruses, demonstrating a well specificity of the qPCR assay. The limit of detection of the GPV qPCR assay was determined to be about 2.42 copies/μl of viral DNA target, reflecting a higher sensitivity of the assay.

At 6 HPI, high levels of viremia were detected in both JS1- and H-infected groups (10³-2.73 ± 0.48 and 10²-8.80 ± 0.14 copies/ml respectively) (Fig. 5A). Relatively high levels of viral load were also detectable in all tested tissues and ileac contents (JS1: 10³-0.01 ± 0.73–10⁶-5.52 ± 0.27 copies/
g; H: 10^{5.61 \pm 0.37} - 10^{8.43 \pm 0.34} copies/g (Fig. 5B–K). Viremia levels in JS1- and H-infected groups peaked at 1 DPI (10^{10.75 \pm 0.22} copies/ml) and 12 HPI (10^{10.29 \pm 0.39} copies/ml) respectively (Fig. 5A). In most cases, the viral loads in tissues and ileac contents peaked at 3 DPI (JS1: 10^{9.12 \pm 0.85} – 10^{10.27 \pm 0.69} copies/g; H: 10^{7.40 \pm 0.17} – 10^{8.08 \pm 0.37} copies/g) (Fig. 5B–K). Only the viral loads in leg muscles in JS1-infected group (10^{9.70 \pm 0.06} copies/g) and in ileac contents in H-infected group (10^{7.39 \pm 0.37} copies/g) peaked at 1 and 7 DPI respectively (Fig. 5J and K). Since then, the viral loads in all organs showed tendency to decline. Nevertheless, relatively high levels of viral load were detected at 35 DPI (Fig. 5A–K).

At 6 and 12 HPI, the levels of viral load detected in H-infected group were higher than those of JS1-infected group. Since 1 DPI, the levels of viral load detected in H-infected group were always lower than those of JS1-infected group. The exceptions were also seen; higher levels of viral load were observed at 7 DPI in spleen and at 28 and 35 DPI in brain of H-infected group. For each sample, significant difference between JS1- and H-infected groups was only observed at partial sampling points (P < 0.05) (Fig. 5A–K). All the ducklings in mock-inoculated control group were negative for GPV during the whole experiment period.

3.5. Strains JS1 and H induced similar kinetics of antibodies in sera of infected Pekin ducklings

The antibody response specific for GPV VP2 protein in sera of JS1- and H-infected Pekin ducklings were detected by using an indirect ELISA (Fig. 6). In both JS1- and H-infected groups GPV antibodies were detectable at 1 DPI (0.37 ± 0.01 and 0.39 ± 0.05 respectively), and then exhibited a tendency to increase. The antibodies increased remarkably from 3 DPI (JS1: 0.55 ± 0.07; H: 0.50 ± 0.03) to 7 DPI (JS1: 0.93 ± 0.05; H: 0.87 ± 0.08), and remained elevated during the subsequent 4 weeks, with the highest titer at 35 DPI (JS1: 1.16 ± 0.05; H: 1.23 ± 0.04). No differences were seen in the antibody levels between the two infected groups (P > 0.05).

4. Discussion

In the present study, we compared clinical signs and pathological lesions of Pekin ducks infected with the JS1 strain from SBDS in Pekin ducklings and the H strain from Derzsy’s disease in goslings. We showed that infection with strain JS1 resulted in significant clinical signs, including those typical of SBDS, supporting the view that variant GPV is the etiological agent of SBDS in Pekin ducks. Our results are in agreement with earlier studies conducted by other workers (H. Chen et al., 2016; S. Chen et al., 2016). The present observations of strain H confirmed previous findings of Hoekstra et al. (1973), who reported that infection of day-old Pekin ducklings did not cause clinical signs of disease. It should be noted that the H strain also influenced weight gain, development of beaks, and of bones of wings and legs of Pekin ducklings, but the impact of strain H is less than those of strain JS1. These findings suggest that the classical GPV is also pathogenic for Pekin ducklings, but its virulence is less than the variant GPV.

Both JS1 and H strains caused microscopic lesions in internal organs including liver, kidney, lung, brain, bursa of Fabricius, and ileum, which confirmed further their pathogenicity to Pekin ducklings. The JS1-infected ducks presented skeletal muscle fibre degeneration, reflecting a greater effect of the variant GPV on the growth of Pekin ducklings when compared with the classical GPV.

We showed that the mean body weight of Pekin ducks 35 days after infection with JS1 was significantly lower than that of uninfected control group, indicating that the SBDS has a significant economic impact on commercial meat-type duck farms. During the course of...
raising and slaughter, the legs and wings of ducks are easily fractured (H. Chen et al., 2016; Yu et al., 2016), which may be attributed to the great influence of variant GPV on the development of bones. It is evident that the virus effect would lead to increase of culling rate, thereby causing more economic losses to commercial meat-type duck farms. The virus effect could also increase the defect rate during the course of slaughter due to easily damaged wings and legs as well as growth retardation. Thus, the SBDS is also of significant economic importance to processing enterprises of meat-type Pekin ducks. Our data, in conjunction with previous evidences (Palya et al., 2009; H. Chen et al., 2015, 2016; Li et al., 2016; S. Chen et al., 2016; Yu et al., 2016; Ning et al., 2017; Xiao et al., 2017), demonstrate that the variant GPV from SBDS is pathogenic for multiple domestic waterfowl, including mule ducks, Pekin ducks, Cherry Valley Pekin ducks, Muscovy ducks, partridge ducks (Anas platyrhynchos), and geese. Therefore, the virus infection has a significant economic impact on waterfowl industry. We noted that the mean body weight of Pekin ducks 35 days after infection with H was also significantly lower than that of uninfected control group. Previously, however, it was difficult to establish linkages between such a loss of weight gain and infection with classical GPV due to its failure to cause clinical signs of disease.

The present study also describes the detection of viral load in tissues and bloods and antibody response following infections of 2-day-old Pekin ducklings. Using a GPV qPCR assay, high levels of viremia were detected in both JS1- and H-infected Pekin ducklings as early as 6 h after leg muscle injection. At that time the virus was detectable in all tested internal organs. These findings demonstrate that both variant and classical GPVs flowed into the blood stream soon after replication in leg muscle tissues, and subsequently the paroviruses spread from bloodstream to whole body, invaded internal organs (e.g., heart, liver, lung, kidney, and brain), and replicated in the tissues.

From the results of the investigation we conclude that in both JS1 and H cases viruses and antibodies coexist in bloods of ducks from 1 to 35 days postinfection. Furthermore, the JS1 and H isolates of GPV induce similar kinetics of viral load in tissues and bloods and of antibody response in sera in Pekin ducklings. The difference between the two paroviruses is that higher levels of viral load were caused by strain H during the early stage (6–12 HPI), and in turn by JS1 from 1 to 35 days. The results support the view that the classical GPV possesses the capacity to replicate rapidly and induce acute viremia in Pekin ducklings, whereas the variation of the virus may improve the capacity to produce higher levels of viral load in Pekin ducklings 1 day after infection.

The present observation confirmed previous findings of Palya et al. (2009), they reported that GPV DNA and antibody were detected from internal organs (liver and spleen) and sera, respectively, of mule ducks infected with the variant and classical GPVs (strains D176/02 and D17/99 respectively) although there are differences in some index. In the investigation of ileal contents taken from ducks infected with strains JS1 and H, GPV DNA was detectable in all samples. This supported the view that birds infected by GPVs may become carriers and excrete virus in their faeces (Palya, 2013; H. Chen et al., 2016). The detection of GPV DNA in ileal contents from 6 HPI and 35 DPI suggested the infected ducks may shed GPVs continually in their faeces.

In summary, our results suggest that both GPVs from SBDS of Pekin ducks and from Derzsy’s disease of goslings are pathogenic for Pekin ducklings, and the former is more virulent than the latter, showing a more dramatic effect on weight gain and development of bones of ducks. The virus from SBDS of Pekin ducks can be recognized as a virulent mutant of goose parovirus. The disparate pathogenic outcomes caused by the two GPVs are likely to be associated with sequence.

Fig. 4. Microscopic lesions in tissues of Pekin ducks 35 days after infection. Panel A, JS1-infected group; panel B, H-infected group; panel C, uninfected control group. The tissue types used for examination are indicated above the pictures. Lesions observed in each tissue were as follows: Liver, congestion in central venous and hepatic sinusoid (triangle) and lymphocytic infiltration (arrow); Spleen, hemorrhage (triangle); Kidney, tubulointerstitial congestion (triangle) and exudates and detached lung cells in bronchial lumen (arrow); Brain, congestion (triangle) and neuronophagia (arrow); Ileum, disruption of small intestinal villi, and disappearance of normal tissue structure (triangle); and Leg muscle, skeletal muscle fibre degeneration (arrow). Bar = 50 μm.

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variation in the GPV genome. Both variant and classical GPVs can infect Pekin ducklings, implying that the occurrence of SBDS in Pekin ducks may not be attributed to changes of host ranges. The present work contributes to the understanding of pathogenicity of GPV to Pekin ducks and may provide clues to pathogenesis of GPV-related SBDS.

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