

# Effect of goose parvovirus and duck circovirus coinfection in ducks

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## Abstract

**Introduction:** Coinfection of goose parvovirus (GPV) and duck circovirus (DuCV) occurs commonly in field cases of short beak and dwarfism syndrome (SBDS). However, whether there is synergism between the two viruses in replication and pathogenicity remains undetermined. **Material and Methods:** We established a coinfection model of GPV and DuCV in Cherry Valley ducks. Tissue samples were examined histopathologically. The viral loads in tissues were detected by qPCR, and the distribution of the virus in tissues was detected by immunohistochemistry (IHC). **Results:** Coinfection of GPV and DuCV significantly inhibited growth and development of ducks, and caused atrophy and pallor of the immune organs and necrosis of the liver. GPV and DuCV synergistically amplified pathogenicity in coinfecting ducks. In the early stage of infection, viral loads of both pathogens in coinfecting ducks were significantly lower than those in monoinfecting ducks ( $P < 0.05$ ). With the development of the infection process, GPV and DuCV loads in coinfecting ducks were significantly higher than those in monoinfecting ducks ( $P < 0.05$ ). Extended viral distribution in the liver, kidney, duodenum, spleen, and bursa of Fabricius was consistent with the viral load increases in GPV and DuCV coinfecting ducks. **Conclusion:** These results indicate that GPV and DuCV synergistically potentiate their replication and pathogenicity in coinfecting ducks.

**Keywords:** goose parvovirus, duck circovirus, synergism, viral replication, pathogenicity.

## Introduction

Goose parvovirus (GPV) belongs to the *Dependovirus* genus and *Parvoviridae* family, causing Derzsy's disease in Muscovy ducklings (*Cairina moschata*) and goslings (*Anser anser domestica*) with high morbidity and mortality (7, 8). The virus causes anorexia, watery diarrhoea, and stunted growth. Less than one-week-old goslings have a mortality rate of up to 100% after infection (7, 11, 24). In addition, GPV can also cause short beak and dwarfism syndrome (SBDS), first reported in 1971 in France in mule duck flocks (20). The clinical signs of SBDS are growth retardation, tongue protrusion, and short and thick tibias, and it is characterised by a low morbidity and mortality rate. Since 2015, GPV has been reported to cause SBDS in Cherry Valley ducks in China (4, 6, 15, 29).

Duck circovirus (DuCV), a member of the genus *Circovirus* of the *Circoviridae* family, was originally reported in two female 6-week-old Mulard ducks from a German farm (10); both had a feathering disorder and poor body condition (22). DuCV infection may predispose the host to immunosuppression, and disease progression is further intensified by coinfection with other bacterial and viral pathogens (22, 27, 30). For example, one study found that ducks infected with DuCV were more likely to be infected with *Riemerella anatipestifer* and/or *Escherichia coli* and/or duck hepatitis virus I. Little is known regarding the molecular biology and pathogenesis of DuCV because of the lack of a cell culture system for propagating the virus. To definitively characterise diseases associated with DuCV infection, an appropriate animal model is needed.

Epidemiological investigation showed that both the GPV and DuCV present in SBDS are common in duck

flocks in East China (12). Though GPV was demonstrated as the pathogen of SBDS in ducks (20), the disease is rarely reproduced by experimental infection (5, 16, 26). It is unclear whether DuCV synergises with GPV, and affects the replication and pathogenicity of both viruses. In this study, a coinfection model was established, and viral replication and pathogenicity were evaluated in ducks to understand the synergism between GPV and DuCV.

## Material and Methods

**Viruses and animals.** Strains of DuCV and GPV were isolated from the Cherry Valley ducks in 2016 in in Tai'an City and Heze City, Shandong Province, China. A 0.1 mL volume of DuCV virus suspension diluted to  $10^{-1}$ – $10^{-6}$  was inoculated into ducklings, and the median infective dose ( $ID_{50}$ ) was measured as  $10^{4.37}/0.1$  mL by PCR. A 0.1 mL volume of GPV virus suspension, diluted to  $10^{-1}$ – $10^{-9}$ , was inoculated into duck embryo fibroblasts and the median tissue culture infective dose ( $TCID_{50}$ ) was measured as  $10^{5.75}/0.1$  mL by immunofluorescence assay (IFA). Cherry Valley ducklings bred for meat were purchased from a commercial hatchery in Tai'an City.

**Coinfection experiment design.** Sixty 2-day-old ducklings were divided into four equal groups. The ducks were inoculated intraperitoneally as summarised in Table 1. The control group received no treatment. At 10, 20, and 30 days post infection (dpi), three ducks were selected randomly from each group and euthanised, and tissues were sampled. Body weights were measured every five days after infection.

**Table 1.** Experimental design

Group	Number of ducks	DuCV ( $10^{4.37} ID_{50}/0.1$ mL)	GPV ( $10^{5.75} TCID_{50}/0.1$ mL)
Control	15	—	—
DuCV	15	0.1 mL	—
GPV	15	—	0.1 mL
GPV+DuCV	15	0.1 mL	0.1 mL

**Histopathological examination.** The tissues obtained at necropsy (liver, kidney, duodenum, spleen, thymus, bursa of Fabricius, and bone marrow) were fixed in 10% neutral formalin for 48 h at room temperature, embedded in paraffin, and cut into 4- $\mu$ m-thick sections. After deparaffinisation, the sections were stained with haematoxylin and eosin and examined using light microscopy (Olympus, Tokyo, Japan).

**Quantitative real-time PCR (qPCR).** GPV and DuCV loads in the heart, liver, lung, kidneys, duodenum, spleen, thymus, bursa, and bone marrow were quantified using a qPCR assay. Two primer pairs were designed coding for the GPV *vp3* gene and DuCV *cap* gene. The expected lengths of the amplified products were 164 bp and 212 bp, respectively. The

primers were as follows: GPVF, 5'-TCGGCTACG GAAGG-3'; GPVR, 5'-AGCATCTGACTAGGGGAAG-3'; DuCVF, 5'-CGAACATCGCCCATCTCA-3'; DuCVR, 5'-AAGGCTACGAATCGCAAG-3'. Cloning of the PCR product into pMD18-T EasyVector (TaKaRa Bio, Kusatsu, Japan) was carried out to obtain the recombinant plasmid. Ten-fold serial dilutions of the vector construct were used to generate a standard curve for the qPCR assay. The TransStart Top Green qPCR SuperMix (Transgen Biotech, Beijing, China) was used according to the manufacturer's recommendations, under the following procedure: 95°C for 30 s, 35 cycles at 95°C for 5 s and 60°C for 34 s, and extension at 95°C for 15 s. Each experiment was repeated three times.

**Immunohistochemistry (IHC).** Paraffin sections were dewaxed and antigen repaired. After incubation with endogenous peroxidase blocking agent at 37°C for 1 h, sections were incubated with a primary polyclonal anti-GPV or anti-DuCV antibody at 4°C overnight, followed by incubation with a biotin-labelled secondary antibody at 37°C for 30 min. Biotin–streptavidin HRP detection systems (Zsgb-Bio, Beijing, China) were used. The sections were stained with DAB, and finally the nuclei were stained with haematoxylin. Recombinant fusion proteins of DuCV CAP and GPV VP3 were expressed in *Escherichia coli* (Tiangen Biotech, Beijing, China), and rabbits were immunised with the protein three times to obtain antiserum. The purified antiserum was subsequently used as the primary antibody. The negative control was serum collected from virus-free rabbits. The results were analysed by Image J (<https://imagej.net/Welcome>).

**Statistical analysis.** The statistical significance of data was calculated with one factor analysis of variance (ANOVA) between the experimental groups. Differences were considered as significant at  $P < 0.05$ .

## Results

**Body weights and gross lesions.** The body weights were measured every five days post infection to evaluate the effects of GPV and DuCV infection on ducks. Both viruses affected the ducks causing retardation of growth and poorer body weight gain such that the birds attained only 60% of that of virus-free ducks. The rate of gain decreased significantly from 10 dpi onwards (Fig. 1).

The pathological changes in infected ducks were determined by gross and necropsy examinations. At necropsy (10, 20, 30 dpi), severe thymic atrophy, a mild to moderately atrophied spleen and bursa of Fabricius, pale bone marrow and spleen, and hepatic necrosis were the common lesions in infected ducks. When the size and colour of the tissues were compared, it was found that the lesions became more severe over time, and the severity of the disease caused by coinfection with GPV and DuCV was more serious than with GPV or DuCV infection alone (Fig. 2).

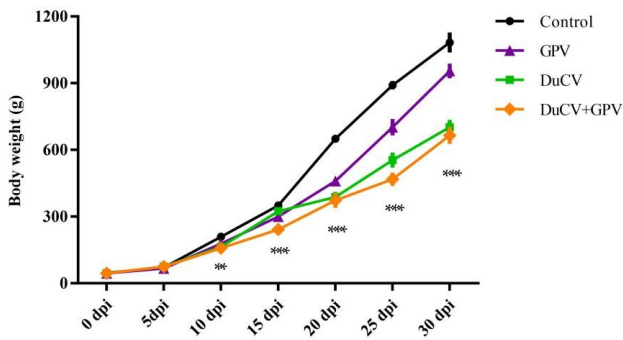


Fig. 1. Weight gain of infected ducks. \*\*P < 0.01 and \*\*\*P < 0.001

**Histopathological changes.** The histopathological results are shown in Fig. 3. The most important lesions were seen in the liver, kidneys, bursa of Fabricius, bone marrow, spleen, thymus, and duodenum. In the liver, focal vacuolar degeneration, necrosis of hepatocytes and infiltration of lymphocytes occurred in the GPV-infected group. Hepatic vacuolar degeneration and inflammatory foci were observed in the DuCV-infected group. In the coinfecting group, hepatic vacuolar degeneration was more severe, with large areas of

hepatocyte disintegration and loss of structure accompanied by haemorrhages (Fig. 3A). In the kidneys, renal tubular epithelial cells showed mild-to-moderate vacuolar degeneration in the GPV-infected and DuCV infected groups, while in the coinfecting group, renal tubular epithelial cells showed severe degeneration, necrosis, exfoliation, and a large extent of heterophilic granulocytes infiltration (Fig. 3A). In the duodenum, villous exfoliation and lymphocytic infiltration were noted in the GPV-infected group, while no obvious lesions were found in the DuCV-infected group. Villous necrosis and exfoliation, haemorrhages, glandular tube epithelial cell atrophy, and lymphocytic infiltration were observed in the coinfecting group (Fig. 3A). Lesions in the immune organs (the spleen, thymus, bursa of Fabricius, and bone marrow) were more severe in the coinfecting group than in the monoinfected groups, including lymphocyte depletion, haemorrhage, and infiltration of heterophilic granulocytes. In addition, the number of histiocytes was decreased in the bone marrow, thymus, and spleen and thymic corpuscles were disintegrated (Fig. 3B).

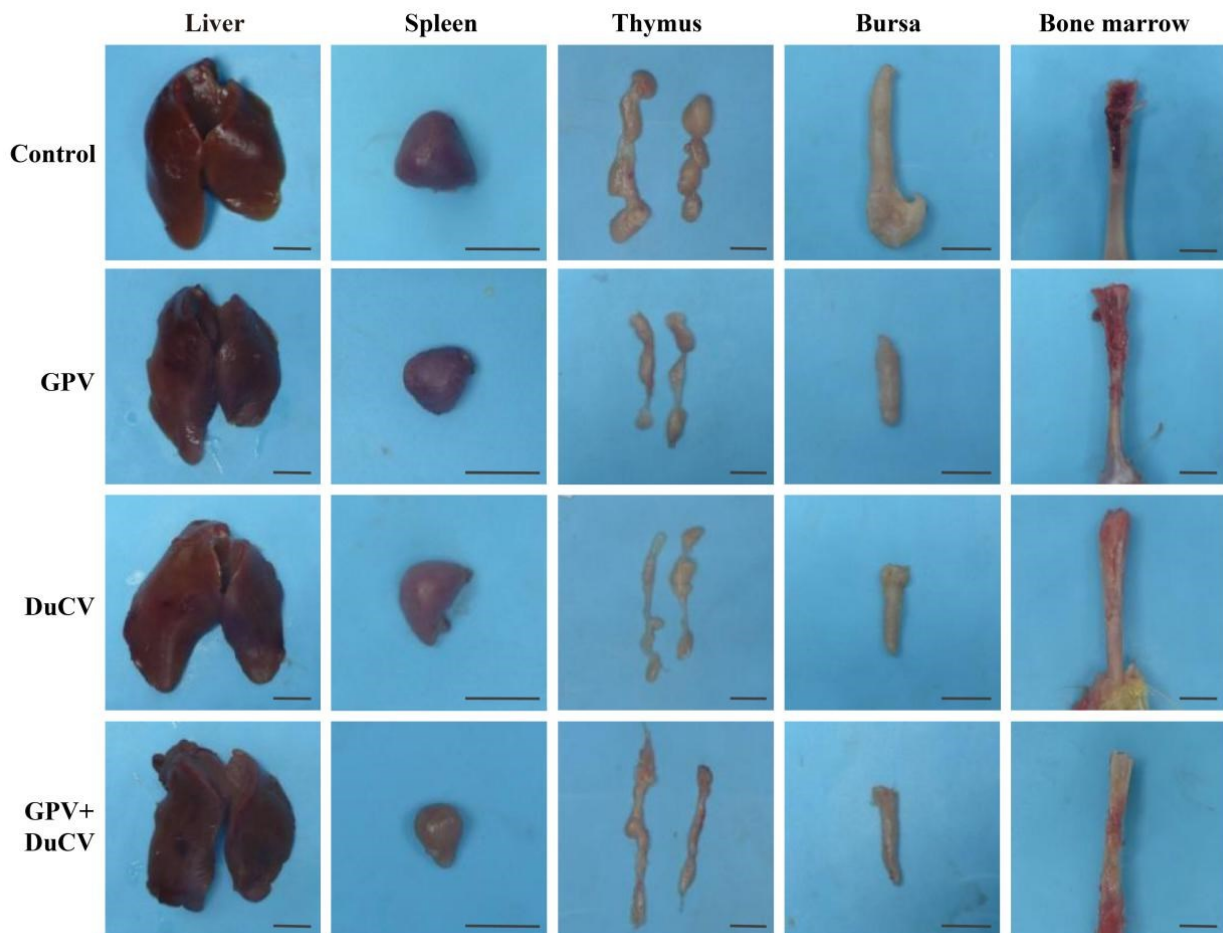
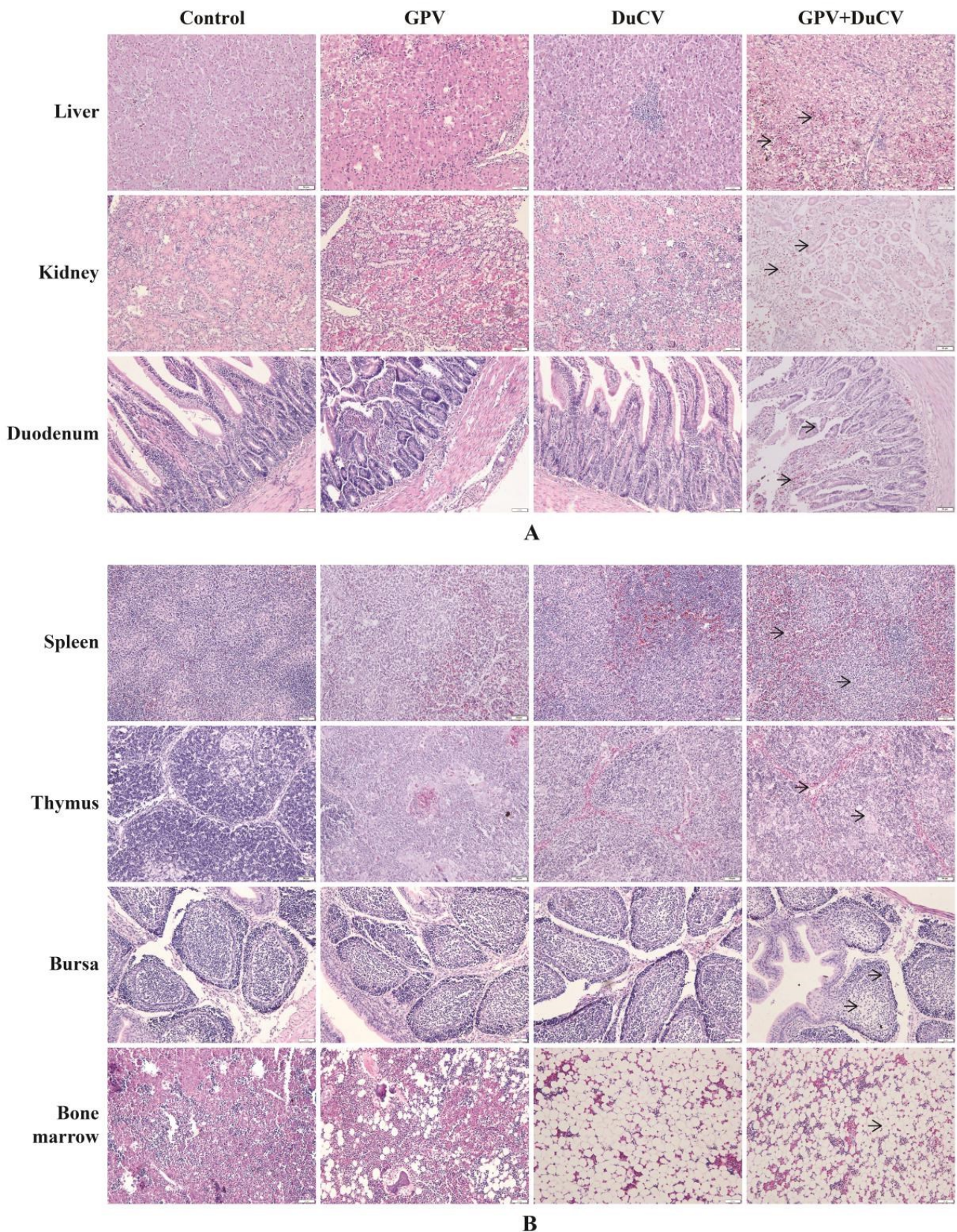


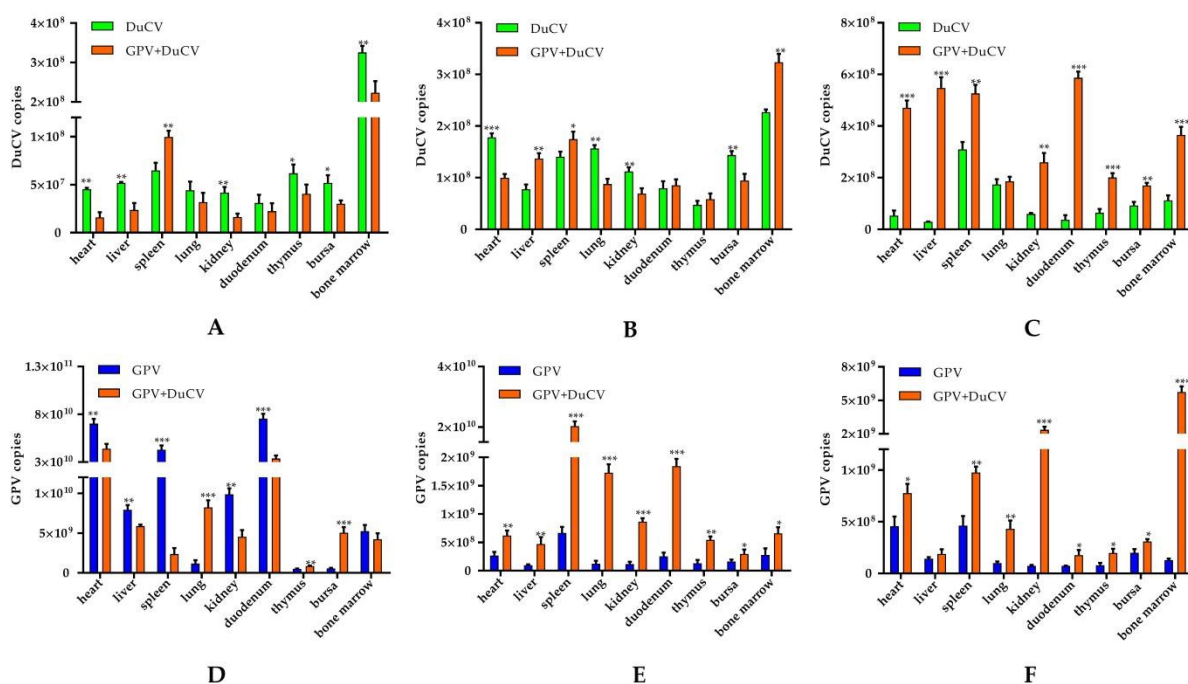
Fig. 2. Gross lesions of infected ducks. They were severe thymic atrophy, a mild to moderately atrophied spleen and bursa of Fabricius, pale bone marrow and spleen, and hepatic necrosis in the GPV and DuCV coinfecting group. Scale bar = 1 cm





**Fig. 3.** Histological lesions of infected ducks at 30 dpi (200 $\times$ ). A. Lesions of the liver, kidneys, and duodenum. Extensive hepatocellular vacuolar degeneration, necrosis, and lymphocyte infiltration in the liver, severe degeneration, necrosis, exfoliation, and heterophilic granulocyte infiltration in renal tubular epithelial cells, and necrosis and exfoliation, haemorrhage, glandular tube atrophy, and lymphocyte infiltration in the duodenal villi are visible. B. Lesions of immune organs. Lymphocyte loss and haemorrhage in the spleen, infiltrating heterotrophic granulocytes, thymic corpuscle disintegration, loss of lymphocytes in the cortex and medulla of the bursa, and histiocyte loss in the bone marrow are visible





**Fig. 4.** Dynamic changes of GPV and DuCV loads in tissues of experimentally infected ducks. A. DuCV loads at 10 dpi; B. DuCV loads at 20 dpi; C. DuCV loads at 30 dpi; D. GPV loads at 10 dpi; E. GPV loads at 20 dpi; F. GPV loads at 30 dpi. \* $P < 0.05$ , \*\* $P < 0.01$ , and \*\*\* $P < 0.001$

**Viral loads in tissues.** GPV and DuCV loads in tissues (heart, liver, spleen, lung, kidneys, duodenum, thymus, bursa of Fabricius, and bone marrow) at different time points were detected by qPCR to observe the dynamic changes of virus replication *in vivo*. At 10 dpi, DuCV loads in all tissues except the spleen were lower in the coinfecting group than those in the DuCV-infected group ( $P < 0.05$ ) (Fig. 4A). GPV loads in the coinfecting group were lower than those in the GPV-infected group except for the lung, thymus, and bursa of Fabricius ( $P < 0.01$ ) (Fig. 4D). At 20 dpi, DuCV loads had increased in both the DuCV-infected group (except for the thymus and the bursa of Fabricius) and the coinfecting group compared with the loads at 10 dpi ( $P < 0.05$ ), and the DuCV loads in the liver, spleen, and bone marrow of the coinfecting group were significantly higher than the loads in the DuCV-infected group ( $P < 0.05$ ) (Fig. 4B). GPV content in the GPV-infected group and in other tissues except the spleen in the coinfecting group declined from its 10 dpi level ( $P < 0.01$ ). GPV in the coinfecting group was significantly elevated over that in the GPV-infected group ( $P < 0.05$ ) (Fig. 4E). DuCV measured at 30 dpi in the DuCV-infected group was less concentrated except for in the spleen, lung, and thymus ( $P < 0.05$ ), but more concentrated in the coinfecting group ( $P < 0.05$ ) than at 20 dpi. The DuCV burden in the coinfecting group was significantly heavier than in the DuCV-infected group ( $P < 0.01$ ) (Fig. 4C). The spleen, lung, and duodenum 30 dpi levels in the coinfecting group declined from their 20 dpi levels ( $P < 0.001$ ), while they rose in the kidneys and bone marrow ( $P < 0.01$ ). The GPV accumulation in

the GPV-infected group and the heart, liver, thymus, and bursa of Fabricius in the coinfecting group remained at the same level as before. With the exception of the liver, the tissues in the coinfecting group contained significantly more GPV than the GPV-infected group ( $P < 0.05$ ) (Fig. 4F).

In the early stage of infection, the replication of DuCV and GPV was inhibited in the coinfecting group. With the development of the infection process, however, the replication of both viruses was synergistically increased in this group.

**Virus distribution in duck tissues.** The distribution and localisation of GPV and DuCV *in vivo* were detected by IHC, and positive cells were detected in the liver, spleen, kidney, duodenum, and bursa of Fabricius. GPV-positive cells in the bursa of Fabricius were detected in both the cortex and medulla, while DuCV positive cells were mainly distributed in the medulla at 10 dpi, and both the cortex and medulla at the later stage. The distribution and location of the two viruses did not differ in other tissues. In the liver, at 10 dpi and 20 dpi both viruses were located in the hepatocyte nucleus, and at 30 dpi positive signals could be detected in the cytoplasm and hepatocyte nucleus. Positive cells could be detected in renal tubules in the medullary loops of the kidneys. The positive cells in the duodenum were mainly in the intestinal crypt cells in the early stage of infection, and also in the goblet cells of the intestinal villi in the later stage. Splenic lymphocytes also gave positive signals. With time, the number of positive cells of the liver, duodenum, and spleen increased, and the positive cells in coinfecting group

came to exceed those in the monoinfected group. Due to lymphocyte depletion, the number of positive cells in the bursa of Fabricius decreased, but the signal intensity increased. In the kidneys, the number of positive cells in the coinfecting group at 10 dpi and 20 dpi was lower than that in the monoinfected group, but was higher at 30 dpi.

## Discussion

It is reported that GPV causes considerable growth retardation, anorexia, and diarrhoea in ducks (28), while the symptoms of DuCV infection in ducks are characterized as growth retardation and feathering disorders (3, 22). In this study, we demonstrated the synergistic effect of GPV and DuCV on ducks. Compared with the monoinfected group, the pathogenicity of coinfection was significantly enhanced, and coinfection had a more serious impact on the growth and development of ducks, and caused the more serious clinical symptoms and pathological changes. The main lesions were degeneration, necrosis, haemorrhages, lymphocyte infiltration in the liver, kidneys, and duodenum, atrophy of immune organs including the spleen, thymus, bursa of Fabricius, and bone marrow and histiocyte loss in these organs. The characteristic lesions of DuCV and GPV infection appeared, but the lesions of the immune organs were the most severe. The liver and kidneys are the main metabolic organs and therefore the lesions in them are more serious and the viral load is higher.

In the present study, we demonstrated that coinfection of GPV and DuCV synergistically promoted their replication *in vivo*. In viral synergistic interactions, biological traits such as virus replication, cytopathological changes, tissue tropism, host range and the transmission rate of one or both viruses were changed (13). At 10 dpi, the GPV and DuCV monoinfected group had a higher viral load than the coinfecting group in most tissues. At 30 dpi, the DuCV load in the DuCV-infected group decreased, while in the coinfecting group it continued to increase. The viral load of the GPV monoinfected group decreased significantly, while that of the coinfecting group decreased slightly and was higher than that of the monoinfected group at 20 and 30 dpi. At 10 dpi, only the DuCV burden in the spleen and the GPV burden in the thymus, bursa of Fabricius, and lung in the coinfection group were higher than those in the monoinfection group, indicating that the virus first replicates in large quantities in immune organs and induces lesions there. In the coinfecting group, the GPV content in the lungs was consistently high, while that of DuCV was not, suggesting that the GPV and DuCV coinfection caused greater susceptibility to GPV in tissues with high blood content and increased only this virus' replication. At 20 dpi, the highest GPV load was found in the spleens of the coinfecting group. At 30 dpi, the replication of GPV in bone marrow was 43-fold higher than that in the monoinfected group, and the

DuCV content in the duodenum was significantly higher than that seen at 20 dpi. DuCV damaged the bone marrow and spleen, increasing the ease with which GPV infected them. Analogously, GPV damaged the duodenum, facilitating its infection by DuCV. It has been reported that coinfection with porcine circovirus 2 (PCV2) and porcine parvovirus (PPV) may promote PCV2 infection by stimulating immune cells and providing target cells for PCV2 replication (1, 19). GPV and DuCV were mainly distributed in the liver, kidneys, and immune organs, which were consistent with the pathological changes in tissues. The positivity rate and signal intensity were consistent with the change in viral load, which verified the inhibition of viral replication in the initial stage of infection and the subsequent synergism.

DuCV is an immunosuppressive virus that weakens the host's immune response and makes it susceptible to secondary infections. GPV has been widely studied as the pathogen of SBDS in recent years, however, the success rate for laboratory infection is only 20% to 35% at present (5, 16, 26). Addressing this problem, we investigated cases in the field and found a high coinfection rate of GPV and DuCV in ducks, especially in SBDS. Similarly, goose circovirus (GoCV) and GPV were found as coinfections in ornamental ducks and caused high mortality (21). No symptoms of SBDS were found in the ducks in the study due to the different conditions of laboratory infection and natural infection and the short incubation time of DuCV and GPV in ducks, but DuCV and GPV showed a synergistic pathogenic effect.

In the *Circoviridae* family, chicken anaemia virus (CAV), canine circovirus (CaCV), and PCV can all be synergistic with other viruses, which is exemplified by CAV and Marek's disease virus (MDV) (9), PCV and porcine reproductive and respiratory syndrome virus (PRRSV) (2), PCV and PPV (18, 23), and CaCV and canine parvovirus (CPV) (25). There are few studies on coinfection in duck diseases, and no studies on coinfection of DuCV and GPV. The pathogenesis of DuCV infection is unknown, and according to reported studies and data, apoptosis may play an important role in the pathogenesis of circovirus infection (14, 17). In this study, the number of lymphocytes in the spleen and bursa of coinfecting ducks decreased, and the IHC signal strength increased. The increased pathogenicity of coinfection with GPV and DuCV may be due to the induction of immune cell apoptosis by DuCV, which leads to immunosuppression and growth arrest, making the duck more susceptible to GPV and promoting virus replication in anatine tissue, thus aggravating the disease.

Our results suggest that GPV and DuCV can synergistically increase disease severity. Although these findings help to understand the pathogenicity of GPV and DuCV synergism, the molecular mechanism by which this coinfection promotes host gene expression or establishes the signal pathway remains unclear.

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