



# Duck circovirus induces a new pathogenetic characteristic, primary sclerosing cholangitis

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## ABSTRACT

Primary sclerosing cholangitis (PSC) is a chronic, cholestatic liver disease of unknown cause. In the study, we found that duck circovirus (DuCV) induces PSC in natural and reproductive cases. PSC in DuCV naturally infected ducks was investigated by PCR and histopathology. A model of PSC was developed in one-day old duck by infection of DuCV. Effects on serum levels of liver enzymes and histology were evaluated, and DuCV tropism for bile duct in liver was analyzed by immunohistochemistry. Pathology observation of natural or reproductive DuCV infected ducks showed that the lesion of liver were characterized by cholangiocytic injuries and progressive fibrous obliteration of the biliary tree associated with lymphocytes infiltration. ALT, AST, ALP, GGT, ALB, TBIL and TP were significantly increased in serum of DuCV infected ducks. DuCV showed higher tropism for epithelial cells of bile duct than other cells in PSC.

## 1. Introduction

Duck circovirus (DuCV), a member of the genus *Circovirus* of the *Circoviridae* family, was first isolated in 6-week-old female Mallard ducks by Hattermann in Germany [1]. The DuCV virion is icosahedral, nonenveloped, and 15–16 nm in diameter [1]. The genome of DuCV is a single-stranded circular DNA of about 1.99 kb. Three major open reading frames (ORFs), ORF1, ORF2 and ORF3, have been recognized for DuCV [1,2]. The ducks infected with DuCV showed feathering disorders, poor body condition, weight loss and severe immunosuppression [1], predisposing affected birds to secondary bacterial, viral, fungal and parasitic infections [3–7]. Histopathologic examination of the bursa of Fabricius demonstrated lymphocyte depletion, necrosis, and histiocytosis [8].

Primary sclerosing cholangitis (PSC) is a chronic, cholestatic liver disease of unknown cause that is characterized by diffuse biliary inflammation and fibrosis of both small and large bile ducts, eventually progressing to biliary cirrhosis, portal hypertension and hepatic failure [9]. PSC commonly presents asymptotically or with non-specific symptoms, such as fatigue, right upper quadrant abdominal pain and pruritus [10]. Diagnosis is established by cholangiography, whereupon band-like stricture, pruned-tree appearance, beaded appearance,

diverticulum-like out pouching and a shaggy appearance are readily visible [11]. Fibrous cholangitis (fibrous obliterative cholangitis, onion-skin lesion) is considered to be histopathological diagnostic of PSC [12]. Patients with PSC usually have concurrent inflammatory bowel disease (IBD) and autoimmune diseases [13–16], and carry a high lifetime risk of gastrointestinal cancer [13,17]. Thus, PSC represents a significant burden on hepatobiliary and oncological services [18]. Several aetiopathogenesis of PSC were studied, such as genetic factors, immune-mediated, toxic effect and pathogens [19,20]. However, at present, the aetiopathogenesis of PSC remains unclear, and a truly conceptual pathogenic model is lacking, and there is no effective medication available.

Here, we found that typical PSC lesion was present in DuCV single-infected ducks. Further, we established a pathology model for PSC in experimental reproduction of DuCV infection. The novel finding suggests that duck with PSC caused by DuCV may be considered for pathogenesis study of PSC.

## 2. Materials and methods

This study was carried out in strict accordance with the recommendations in the Shandong Institutional Animal Care and Use.

**Abbreviations:** PSC, primary sclerosing cholangitis; DuCV, duck circovirus; ALT, alanine aminotransferase; AST, aspartate aminotransferase; ALP, alkaline phosphatase; GGT, gamma-glutamyltransferase; ALB, albumin; TBIL, total bilirubin; TP, total protein

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The protocol was approved by the Committee on the Ethics of Animal Experiments of the Shandong Province (Permit Number: 20160523). All study participants provided written, informed consent.

### 2.1. Samples, virus and animals

Sixty seven sick ducks, including Mulard duck, Maple duck, Cherry Valley duck, Peking duck and Muscovy duck from different regions in China, were collected from September 2015 to December 2016, and identified by PCR with the primer of DuCV. The birds were aged between 5 and 30 weeks, and came from 20 duck flocks. The number of cases per flock ranged from two to four. The primer of DuCV was as follow, forward: CGCCCTTGAAGAGTGCCT; Reverse: CGAGTAACCGTCCCACCA. For DuCV isolation, the livers of infected ducks were used to inoculate embryonated eggs of Cherry Valley ducks at 9 days old (purchased from Rongda Company, Gaotang, China). The virus was passaged in embryonated eggs for five generations. Allantoic fluid was collected at 15 days old for sucrose density gradient centrifugation and tested by PCR for avian influenza virus (H5N1), duck Tembusu virus, duck parvovirus and DuCV. Liver of duck embryo was prepared for ultrathin section. Viral particles and ultrathin sections images were captured on a transmission electronic microscope (TEM) (JEOL, Tokyo, Japan). The PSC in liver was diagnosed by histopathology observation.

### 2.2. Establishment of pathology model of PSC in duck by infection of DuCV

To explore whether the PSC is really associated with DuCV infection, we establish a pathology model of PSC in duck by infection of DuCV. One-day old healthy Cherry Valley ducks were purchased from Rongda Company (Gaotang, China) and tested for DuCV negative. Twenty healthy Cherry Valley ducks of one-day old were infected with 0.2 ml allantoic fluid with DuCV by intraperitoneal injection and were housed in an animal room under SPF conditions. Twenty uninfected Cherry Valley ducks were as control. The weight gain of ducks in each group was measured at interval 3 days. At 7, 14, 21, 28, 35, and 42 days of post infection (dpi), three ducks from each group were euthanatized and examined postmortem for evidence of gross lesions, and immune organ to body weight ratio was measured. Two sets of tissues were prepared from each duck for histopathology examination and immunohistochemistry (IHC).

### 2.3. Histopathology

Tissue samples from the liver, spleen, heart, lung, kidney, proventriculus, brain and bone marrow were removed from the birds and fixed in 10% neutral buffered formalin. The tissues were processed by standard paraffin embedding, sectioned at approximately 4  $\mu$ m, and stained with H&E for observation [21].

### 2.4. Standard biochemical analyses

To test the degree of liver injury, serum biochemical analyses were measured on a Cobas Integra 400 Clinical Analyzer (Roche Diagnostics) at 14, 28 and 42 dpi, including aspartate aminotransferase (AST), alanine aminotransferase (ALT), alkaline phosphatase (ALP), gamma-glutamyltransferase (GGT), total bilirubin (TBIL), albumin (ALB), globulin (GLOB) and total protein (TP). All assays were performed according to the manufacturers' instructions.

### 2.5. Immunohistochemistry (IHC)

To test the tropism of DuCV for biliary epithelial cells, livers fixed with formalin were cut at 4  $\mu$ m thickness and mounted on poly-L-lysine coated slides. The primary antibody was obtained from single factor anit-serum of ducks that infected DuCV. The treatment of sections and IHC protocol were followed as previous description of our lab [21].

**Table 1**

The origin of the 8 cases that single-infected by DuCV.

No.	Species	Year	Age(wks)	Flock size	Region
1	Cherry Valley duck	2015	8	10000	FeiCheng, Shandong
2	Maple duck	2015	12	8000	Heze, Shandong
3	Peking duck	2015	20	5000	Beijing
4	Muscovy duck	2015	25	10000	Guangzhou, Guangdong
5	Mulard duck	2015	5	1000	Taian, Shandong
6	Maple duck	2016	20	10000	Laiwu, Shandong
7	Cherry Valley duck	2016	30	8000	Dangshan, Anhui
8	Peking duck	2016	6	5000	Xuzhou, Jiangshu

Finally, the slides were examined using light microscopy (Olympus).

### 2.6. Statistical analyses

Results are presented as the mean  $\pm$  standard deviation(s). The *t*-test and ONEWAY ANOVA test was performed using SPSS 13.0 statistical software. A P value less than 0.05 was considered statistically significant.

## 3. Results

### 3.1. DuCV infected ducks showed various grads PSC lesion in liver

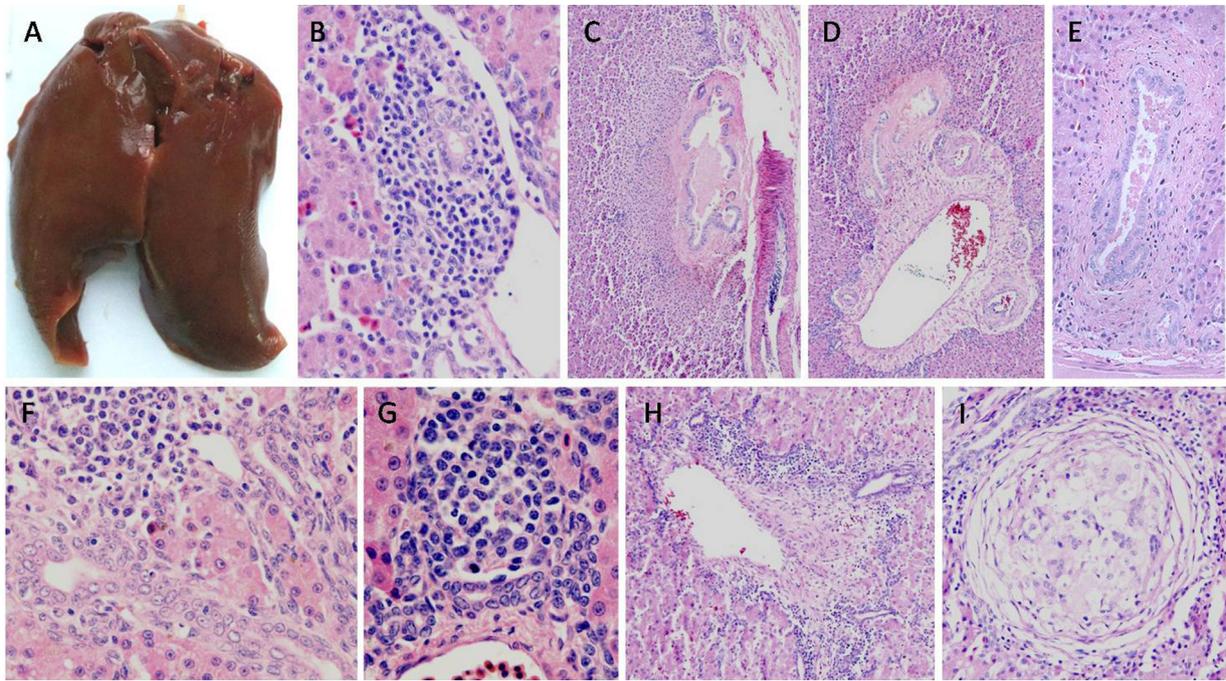
Eight of sixty seven cases from five regions were diagnosed as single DuCV infection (Table 1). Eight cases that infected DuCV showed various grades of PSC lesion in liver (Fig. 1). Among them, there were three cases showing degeneration or necrosis of epithelial cells of bile duct and lymphocytes infiltration (Fig. 1B, F, G); four cases showing fibrous obliterative cholangitis and surrounding hepatocytes necrosis (Fig. 1C–E and H); one case showing onion-skin-like lesion of bile duct (Fig. 1I). It indicated that PSC associated with DuCV infection rather than species or regions.

### 3.2. Isolation of DuCV

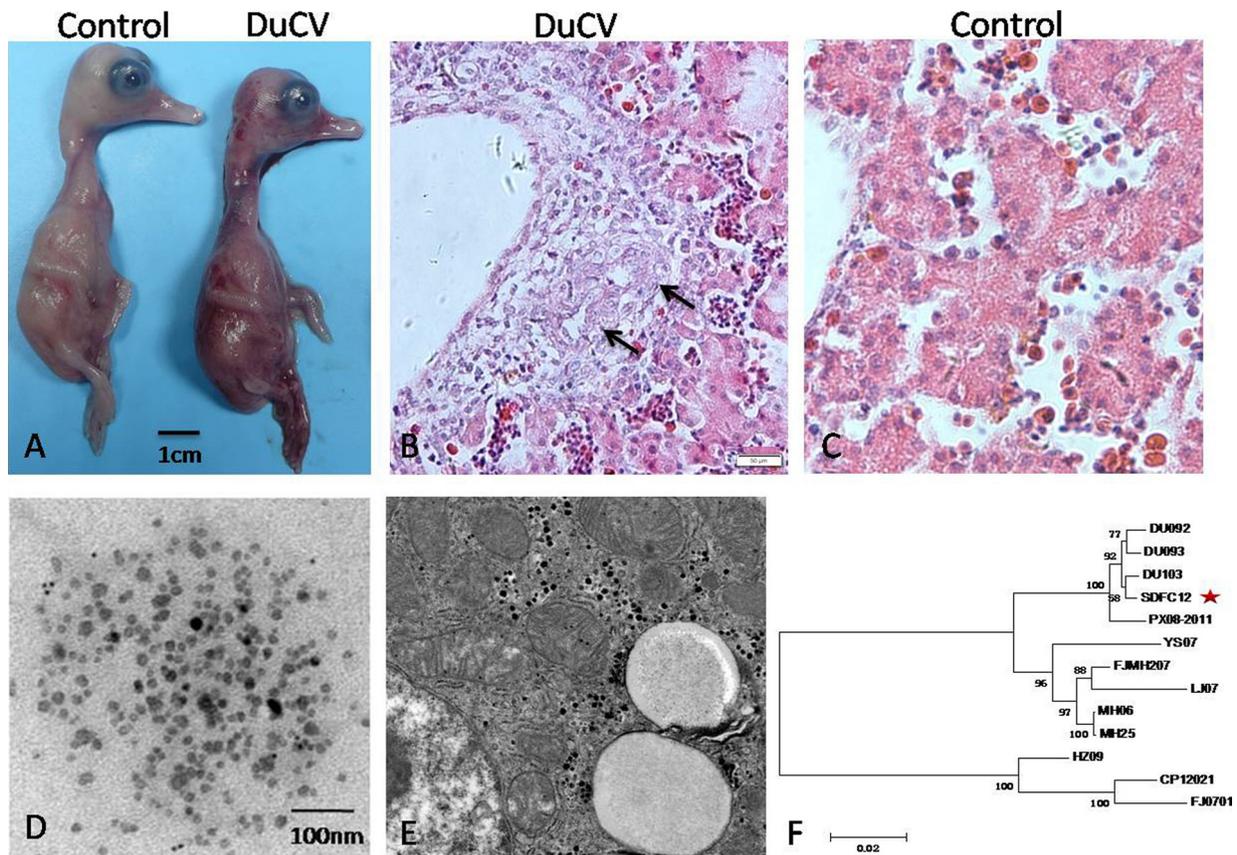
A strain named SDFC12 of DuCV was isolated from a case that came from Feicheng. The isolate of DuCV caused hemorrhage in embryo body (Fig. 2A). The liver injury of duck embryos caused by DuCV was characterized by degeneration and necrosis of epithelial cells of bile duct and its surrounding hepatocytes (Fig. 2B, C). The TEM observation of DuCV particles showed that the virus is 16–20 nm in size and presents in plasma (Fig. 2D, E). The whole genome of SDFC12 that amplified by inverse PCR (iPCR) was 1993bp (Deposit in GenBank: KY328304.1). A phylogenetic tree analysis (Fig. 2F) indicates that SDFC12 is more closely related to DU103 (GenBank accession number: HM162352.1), an isolate from northern China, than the strains isolated from other areas of China.

### 3.3. The dynamic pathology progress of PSC in experimental infection of DuCV

All infected ducks did not show any clinical symptoms. However, the weight gain of infected group was extremely significant lower than normal control group from 6 dpi (Fig. 3). The lymphoid organ atrophy and the decreased value of immune organ/body weight indicated the significant immunosuppression in DuCV infected ducks (Table 2). Histopathology showed early cholangiography, portal tract inflammation with lymphocytes, progressing to obliterative concentric fibrosis and bile duct destruction (Fig. 4). Initially (7–14dpi), epithelial cells of septal bile ducts and interlobular bile ducts showed vacuolar degeneration, and then developed to necrosis. At 21 to 28dpi, bile ducts showed



**Fig. 1.** Various grades of PSC lesion present in liver from DuCV infected duck. There were three cases showing degeneration and necrosis of epithelial cells of bile duct and lymphocytes infiltration (B, F, G); four cases showing fibrous oblitative cholangitis and surrounding hepatocytes necrosis (C, D, E and H); one case showing onion-skin-like lesion of bile duct (I).



**Fig. 2.** Isolation and identification of DuCV. (A) The isolate of DuCV caused hemorrhage in embryo body. The liver injury of duck embryos caused by DuCV was characterized by degeneration and necrosis of epithelial cells of bile duct and its surrounding hepatocytes (B), and the control did not show any lesions in liver (C). The TEM observation of DuCV particles showed that the virus is 16–20 nm in size (D) and presents in plasma (E). A phylogenetic tree analysis (F) indicates that the isolate (SDFC12) is more closely related to DU103.

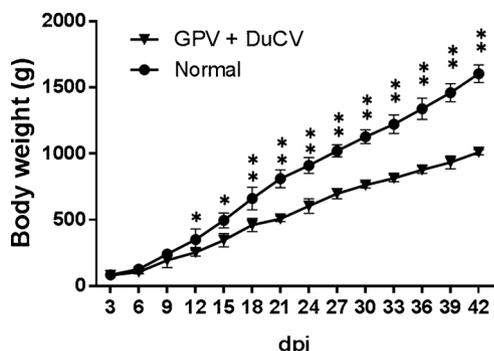


Fig. 3. Comparison of weight gain of DuCV infected group with control group. The weight gain of DuCV infected group was extremely significant ( $P < 0.01$ ) lower than control group from 6dpi.

Table 2  
Effect of DuCV on immune organ in ducks (%).

Days	Immune organ	Control	DuCV
14d	Bursa	0.15 ± 0.03	0.11 ± 0.01 <sup>a</sup>
	Thymus	0.44 ± 0.15	0.17 ± 0.04 <sup>a</sup>
	Spleen	0.10 ± 0.02	0.06 ± 0.02 <sup>a</sup>
21d	Bursa	0.09 ± 0.00	0.04 ± 0.01 <sup>a</sup>
	Thymus	0.27 ± 0.04	0.10 ± 0.03 <sup>a</sup>
	Spleen	0.10 ± 0.00	0.04 ± 0.01 <sup>a</sup>
28d	Bursa	0.15 ± 0.01	0.09 ± 0.01 <sup>a</sup>
	Thymus	0.28 ± 0.01	0.19 ± 0.00 <sup>a</sup>
	Spleen	0.11 ± 0.07	0.07 ± 0.01 <sup>a</sup>
35d	Bursa	0.11 ± 0.01	0.04 ± 0.02 <sup>a</sup>
	Thymus	0.20 ± 0.04	0.11 ± 0.02 <sup>a</sup>
	Spleen	0.04 ± 0.02	0.11 ± 0.07 <sup>a</sup>
42d	Bursa	0.10 ± 0.00	0.07 ± 0.02 <sup>a</sup>
	Thymus	0.14 ± 0.01	0.10 ± 0.02 <sup>a</sup>
	Spleen	0.09 ± 0.01	0.06 ± 0.01 <sup>a</sup>

Note: a: significant difference ( $P < 0.05$ ).

epithelial changes with a few surrounding lymphocytes in hepatic portal area. At 35dpi, the surrounding bile duct presented obliterative, non-suppurative cholangitis with substantial periductal fibrosis. At

42dpi, bile duct destruction or loss, hyaline degeneration of the surrounding connective tissues and necrosis of hepatocytes were present in portal area. In addition, lymphocytes infiltration was present in pro-ventriculus, kidney, heart and lung, and eosinophilic granulocytes were present in spleen, and typical pancreatitis was showed in pancreas (data not shown).

### 3.4. Serum biochemical analyses

In order to evaluate degree of liver injury and PSC associative factors, eight serum biochemical items were tested (Table 3). No significant differences of these items were present between DuCV infected group and normal control group at 14 dpi. At 28 dpi, only ALP of infected group showed significant increasing than those of control group. At 42 dpi, ALP, ALT, GGT and TBIL showed significant increasing, and AST, ALB and TP showed extremely significant increasing, except of GLOB.

### 3.5. DuCV tropism for bile duct

DuCV showed strong tropism for epithelial cell of bile duct. The antigen of DuCV was mainly present in cytoplasm of epithelial cell of bile duct, but not nuclear (Fig. 5). No significant difference was observed at different ages.

## 4. Discussion

PSC is considered a challenging illness that characterized by biliary inflammation and fibrosis of bile duct, and can potentially lead to cirrhosis and endstage liver failure in human [9]. The basic mechanism of PSC pathogenesis is still unclear. At present a truly conceptual pathogenic framework is lacking though several viruses, such as hepatitis C virus [22], cytomegalovirus [23], rotavirus and reovirus [24], were reported association with PSC.

During investigation of DuCV infection in duck flocks, we found that all DuCV infected cases showed typical PSC in liver. This is first observation of PSC in animal liver. To better understanding the development of PSC caused by DuCV, a DuCV infected duck model was set up. Base on the model data, we demonstrated that DuCV infected duck

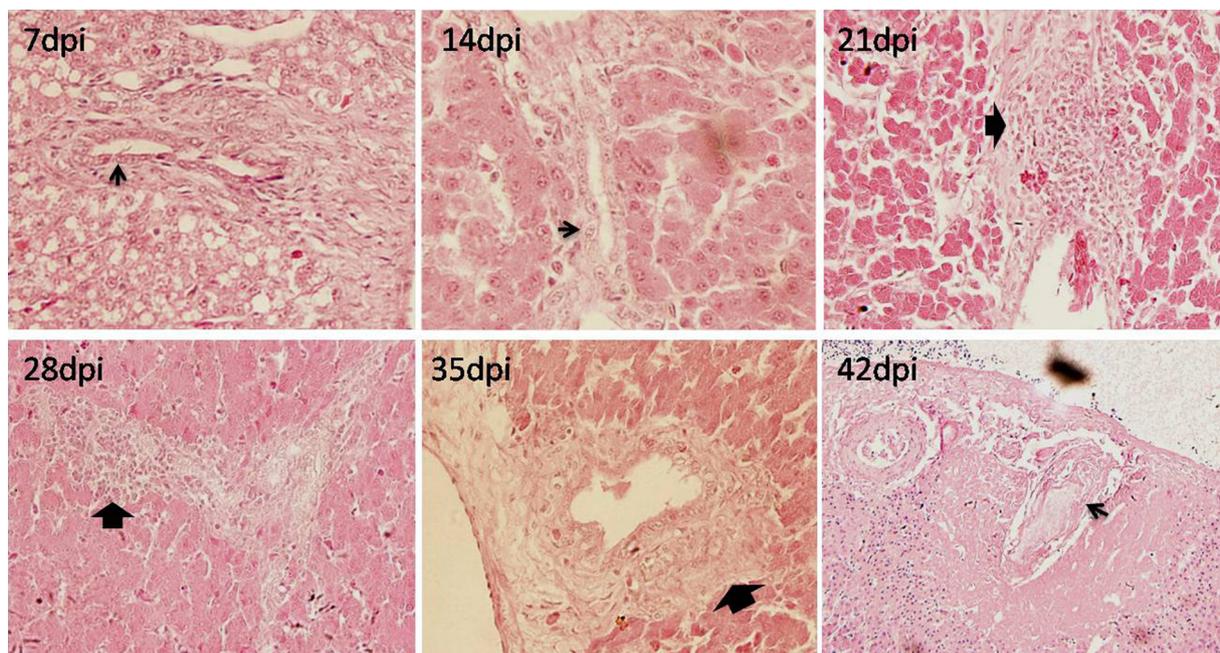
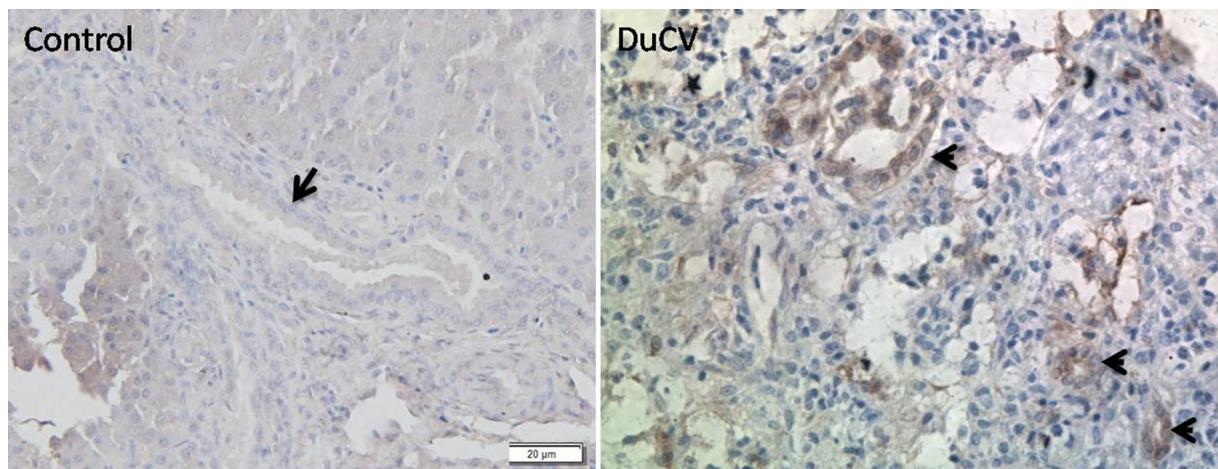


Fig. 4. The progress of PSC in pathology model induced by DuCV. Early cholangiopathy (7dpi and 14dpi), portal tract inflammation with lymphocytes (21dpi and 28dpi) progressed to obliterative concentric fibrosis (35dpi) and bile duct destruction (42dpi).

**Table 3**  
Serum biochemical analysis.

Items	14dpi		28dpi		42dpi	
	Control	DuCV	Control	DuCV	Control	DuCV
AST(U/L)	55 ± 3.20	61.7 ± 3.84	40 ± 11.86	114 ± 17.10	41.3 ± 7.22	145.3 ± 38.11 <sup>b</sup>
ALP(U/L)	429 ± 80.25	492. ± 203.23	484 ± 46.60	656 ± 35.0 <sup>a</sup>	529.7 ± 53.64	926.7 ± 33.58 <sup>a</sup>
ALT(U/L)	37 ± 1.45	48.7 ± 4.73	43.3 ± 5.67	50.7 ± 4.48	43 ± 6.89	63.7 ± 5.42 <sup>a</sup>
GGT(U/L)	1 ± 0.87	3 ± 0.00	3 ± 0.67	5 ± 0.71	2 ± 0.41	3.7 ± 0.75 <sup>a</sup>
ALB(g/L)	17.2 ± 0.89	18.8 ± 2.65	16.6 ± 0.47	20 ± 1.64	16.5 ± 0.47	27.8 ± 0.22 <sup>b</sup>
GLOB(g/L)	20.7 ± 2.02	23 ± 4.23	19 ± 1.21	25.7 ± 2.00	20.5 ± 1.21	25.3 ± 0.20
TP(g/L)	37.7 ± 2.92	41.7 ± 6.86	36.5 ± 0.86	49 ± 3.53	25.3 ± 0.86	39.6 ± 0.15 <sup>b</sup>
TBIL(μmol/L)	9.1 ± 1.59	11.6 ± 5.42	4.5 ± 1.68	17.2 ± 1.54	7.4 ± 0.98	29.7 ± 0.38 <sup>a</sup>

Note: a: significant difference ( $P < 0.05$ ); b: extremely significant difference ( $P < 0.01$ ).



**Fig. 5.** The tropism of DuCV for epithelial cell of bile duct (14dpi). DuCV has specific tropism for epithelial cell of the bile duct. The positive presented in the cytoplasm of cells.

showed the same pathology progress of PSC with human, as while as serum biochemistry profile (except of GLOB) showed significantly up-regulation, indicating the liver injury. IHC result showed that DuCV had high tropism for epithelial cells of bile duct indicating the inducible role of DuCV for PSC.

In the pathogenic model, DuCV induced significant immunosuppression that characterized by growth retardation, decreasing immune organ/body weight value and lymphocytes deletion in spleen, thymus and bursa. This indicated that PSC may associate with immunosuppression.

Pancreatitis was present at late stage (42dpi) in DuCV infected duck model. Characteristic features include pancreatic enlargement and lymphoplasmacytic infiltrate. This features are similar with autoimmune pancreatitis (AIP) that characterized by intra and extrahepatic biliary stricturin [25]. AIP often may be confused with PSC. Bjornsson et al. have recently suggested that the biliary changes in AIP should be redesignated as 'IgG4-associated cholangitis' [26]. However, in this study, the GLOB in serum did not show any changes. Thus, what the relationship of PSC and pancreatitis need to be further study.

## 5. Conclusion

In this study, we reported the PSC present in DuCV naturally infected duck flocks for the first time, and successfully established a pathology model of PSC that induced by DuCV.

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