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Characterization of a P18 protein in the S1 segment of the novel duck reovirus genome

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Summary. – Novel duck reovirus (NDRV), the prototype strain of avian orthoreoviruses, continues to circulate among ducks. Analysis of its genome suggested that a putative second open reading frame in the S1 segment encodes a 162-amino acid nonstructural protein with size of 18 kDa, provisionally designated P18. This protein is different from the 17 kDa nonstructural protein encoded in the same open reading frame in other avian orthoreoviruses, which is designated P17 and consists of 146 amino acids. There is no corresponding protein in Muscovy duck reovirus. Antibodies raised to the purified recombinant protein reacted with viral P18 both *in vitro* and *in vivo*. In cells, P18 was located predominantly in the nucleus at 6–12 h post-infection, with negligible levels in the cytoplasm. However, the protein accumulated both in the nucleus and cytoplasm at 24 to 36 h post-infection. Immunohistochemistry indicated that P18 strongly accumulates in spleen tissues of infected ducklings. Collectively, the data provide the direct experimental evidence that P18 is expressed by novel duck reovirus both *in vivo* and *in vitro*.

Keywords: duck reovirus; expression; characterization; novel P18 protein

Introduction

Avian orthoreoviruses and mammalian reoviruses belong to the genus *Orthoreovirus* (the subfamily *Spinareovirinae*, the family *Reoviridae*). The virions are icosahedral and nonenveloped, and form two-layer capsids enclosing 10 dsRNA segments that can be separated by polyacrylamide gel electrophoresis into large (L1–L3), medium (M1–M3), and small (S1–S4) fragments (Benavente *et al*., 2007; Johne *et al*., 2015). The S1 segment is bicistronic in most mammalian reoviruses, and encodes one structural and one nonstructural polypeptide in overlapping open reading frames (Boehme *et al*., 2013). On the other hand, the S1 segment in fusogenic avian reoviruses and Nelson Bay mammalian reovirus (NBV) encodes one structural and two nonstructural proteins in partially overlapping open reading frames (Zhang *et al*., 2015; Kawagishi *et al*., 2016).

In avian orthoreoviruses, the first open reading frame encodes P10, a small, nonstructural transmembrane protein that destabilizes the membrane in host cells and promotes virus entry, while the second open reading frame encodes P17, a 146-amino acid nonstructural, nucleocytoplasmic shuttling protein of size 17 kDa (Hsu *et al*., 2005; Chulu *et al*., 2010; Chi *et al*., 2013; Li *et al*., 2015). The third open reading frame encodes σC protein, an elongated trimeric structural protein that mediates initial virus attachment to cell receptors (Benavente *et al*., 2007; Lin *et al*., 2012).

Avian orthoreoviruses cause a range of diseases in chicken, including viral arthritis/tenosynovitis, respiratory disease, enteric disease, inclusion body hepatitis, hydropericardium, runting stunting syndrome, malab-

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Abbreviations: DAPI = 4',6-diamidino-2-phenylindole; DEFs = duck embryo fibroblasts; NDRV = novel duck reovirus; TF = trigger-factor

sorption syndrome, and sudden death (Bányai *et al*., 2011; Woźniakowski *et al*., 2014). In farmed Muscovy ducks (*Cairina moschata*) in Israel, China, and other countries, 30% and 20% of infections cause illness and death, respectively (Woźniakowski *et al*., 2014; Heffels-Redmann *et al*., 1992). Importantly, in the Muscovy duck reovirus, σC and the nonstructureal protein P10 are encoded by the segment S4 (Shmulevitz *et al*., 2002).

Avian orthoreoviruses are classically nonpathogenic in Peking ducks (*Anas platyrhynchos*) when inoculated subcutaneously. Nevertheless, isolates that cause 40% mortality in ducks of various age and 35–40% mortality in different flocks have been isolated with increasing frequency since 2011. We isolated one such strain, novel duck reovirus TH11, which causes 100% mortality in specific pathogen free-chickens (Chen *et al*., 2012; Zhu *et al*., 2015). Different strains of this virus were detected at different sites in China (Liu *et al*., 2011; Li *et al*., 2016; Zheng *et al*., 2016). The basis for the expanded host range and the increased virulence is unknown.

By analyzing the TH11 genome, we found that a putative second open reading frame in the S1 segment encodes a 162-amino acid nonstructural protein of 18 kDa, provisionally designated P18. This protein is different from the 17 kDa nonstructural protein encoded in the same open reading frame in other avian orthoreoviruses, which is designated P17, consisting of 146 amino acids. There is no corresponding protein in Muscovy duck reovirus. Although P18 has some homology to P17 of other avian reoviruses (Zhu *et al*., 2015), it is not even clear whether it is expressed *in vitro* and *in vivo*. We have now expressed P18 recombinantly from a pCold vector, raised antibodies to the purified recombinant protein, and provide direct experimental evidence of P18 expression by novel duck reovirus *in vitro* and *in vivo*.

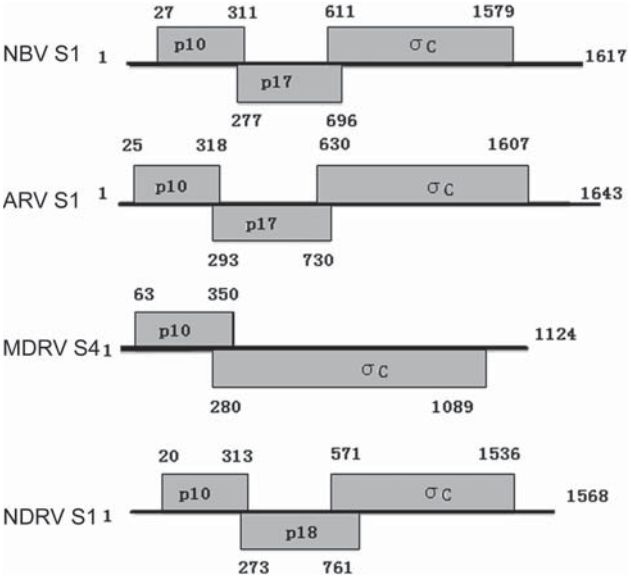

Fig. 1

Genetic structure of the S1 segment in the genome of Nelson Bay mammalian reovirus (NBV), avian orthoreovirus (ARV), Muscovy duck reovirus (MDRV), and NDRV

The organization and arrangement of P10, P17/P18, and σC cistrons are illustrated in rectangles where present. Numbers refer to nucleotide positions of the genome segment, and to the first and last positions of open reading frames excluding stop codons.

Materials and Methods

Strains, cells, and plasmids. Novel duck reovirus TH11 was isolated in 2011 and is archived in our laboratory. DEFs were obtained from American Type Culture Collection, and grown in Dulbecco's modified Eagle's medium (Hyclone, Beijing, China) supplemented with 10% heat-inactivated fetal bovine serum (Invitrogen, Carlsbad, CA) at 37°C in a humidified atmosphere with 5% CO_2. *Escherichia coli* DH5α and BL21 (DE3) competent cells were purchased from Beijing TransGen Biotech Co., Ltd. (Beijing, China), while the expression vector pCold-TF was purchased from TaKaRa (Dalian, China).

Plasmid construction for production of recombinant P18. We have obtained the full-length TH11 genome, and the putative P18 sequence was predicted using ExPASy, Protean (http://www.expasy.org/), and BLASTN (https://blast.ncbi.nlm.nih.gov/); the putative coding sequence is illustrated in Fig. 1. Total RNA was isolated from TH11 using Trizol (Sangon, Shanghai, China) according to the manufacturer's instructions, and reverse-transcribed using AMV RNA PCR Kit (TaKaRa, Dalian, China). The P18 cDNA was then amplified using primers 5'-CGGGGTACCATGTCGCTCCTGCCAA-3' and 5'-CCGGAATTCTCAGTTGTTGATTGTAGA-3', which contain *Kpn*I and *Eco*RI sites to enable insertion into pCold-TF.

Expression and purification of recombinant P18. E. coli BL21 (DE3) were transformed with a recombinant pCold-TF plasmid encoding P18 and grown at 37°C in 1 l LB medium containing 100 mg/ml ampicillin. At OD_{600} 0.8, expression was induced with 1 mM IPTG for 24 h at 15°C, 25°C and 37°C, and cells were harvested by centrifugation at 9,000 × g for 10 min at 4°C. Subsequently, pellets were washed three times with PBS, resuspended in lysis buffer, repeatedly frozen at -80°C and thawed at room temperature, lysed by sonication on ice, centrifuged at 4°C for 10 min at 9,000 × g, and the resulting supernatant and pellet were analyzed on 10% SDS-PAGE gels. The extract was firstly purified by a salinity-gradient elution in the following steps. It was eluted three times in an elution buffer (2 M urea, 50 mM sodium phosphate, 0.2 mM EDTA, 100 mM NaCl, and 1% Triton X-100, pH 8.0) and centrifuged at 10,000 × g for 5 min. Then, the protein was eluted twice in a second elution buffer (2 M urea, 0.2 mM EDTA, 50 mM sodium phosphate, 150 mM NaCl and 1% Triton X-100, pH 8.0) and centrifuged at 10,000 g for 5 min.

Finally, the protein was solubilized in 30 ml of 8 M urea buffer (8 M urea, 500 mM NaCl, 20 mM NaH_2PO_4, and 20 mM Na_2HPO_4, pH 7.4). The lysate was then loaded on to a 1 ml nickel-nitrilotriacetic acid column (Qiagen, Hilden, Germany) pre-equilibrated with 2 column volumes of lysis buffer. The column was then washed with 5 column volumes of a buffer containing 20 mM Tris-HCl pH 8.4, 0.5 M NaCl, 10 mM imidazole, and then with 10 column volumes of the same buffer but with 20 mM imidazole. Finally, His-tagged P18 was eluted with 250 mM imidazole in 50 mM sodium phosphate pH 8.4 and 300 mM NaCl, and stored at -80°C for subsequent analysis. Bicinchoninic acid was used to quantify total protein (Thermo Scientific Pierce, UK), and yield was determined in Quantity One (Bio-Rad) based on SDS-PAGE gels stained with Coomassie blue (Gauci *et al*., 2013).

Western blot. Proteins were separated on 10% SDS-PAGE gels in two lanes, one was visualized with Coomassie brilliant blue R250, the other was transferred to a 0.45 μm polyvinylidene fluoride membrane (Bio-rad), which was probed with 1:2,000 murine monoclonal antibody to His tags (Boster, Wuhan, China) and with 1:10,000 goat anti-mouse IgG conjugated to horseradish peroxidase (Boster, Wuhan, China). Finally, the blot was visualized using an ECL detection kit (Thermo Fisher, IL, US), and analyzed in a Bio-Rad gel documentation system.

Preparation of polyclonal antibodies to P18. Purified recombinant P18 was emulsified with an equal volume of complete (for primary immunization) or incomplete (for booster immunization) Freund's adjuvant, and inoculated into three New Zealand white female rabbits approximately 4 months of age. Each rabbit was immunized by multi-point subcutaneous injections of a total dose of 500 μg. Primary immunization was followed by three booster immunizations at approximately 15-day intervals. Rabbits were bled after the final booster, and serum were separated following clotting and centrifugation, purified on a protein A column (Genscript, Nanjing, China), aliquoted, and stored at -80°C. The polyclonal P18 antibodies were characterized by western blot against recombinant P18 separated on SDS-PAGE gels and transferred to a polyvinylidene fluoride membrane (Bio-rad) along with lysates from DEFs infected with NDRV TH11 at multiplicity of infection 0.1. After blocking overnight at 4°C with 5% bovine serum albumin in phosphate-buffered saline (BD), the membrane was probed for 1 h with 1:100 polyclonal P18 antiserum in Tris-buffered saline containing 0.1% Tween-20. Subsequently, the membrane was washed in the same buffer, labeled for 1 h with goat anti-rabbit IgG coupled to horseradish peroxidase (Boster, Wuhan, China) diluted in the same buffer, and visualized using ECL Substrate Kit (Thermo Fisher, IL, US) according to the manufacturer's instructions.

Indirect immunofluorescence. Duck embryonic fibroblasts were cultured on sterile glass coverslips and infected with novel duck reovirus at multiplicity of infection 0.1 after reaching 80% confluence. Cells were then fixed in 4% paraformaldehyde at different time points post-infection, washed three times with phosphate-buffered saline, permeabilized with 0.05% Triton X-100 for 5 min, and blocked for 1 h with 1% BSA in phosphate-buffered saline. Specimens were then probed for 2 h at 37°C with polyclonal antibodies to P18 diluted in the same buffer, washed multiple times with phosphate-buffered saline, and labeled for 1 h with goat anti-rabbit IgG conjugated to fluorescein isothiocyanate. Finally, specimens were washed with phosphate-buffered saline, stained with DAPI, and imaged on a Zeiss Axiovert 200 fluorescence microscope using the associated software (Zeiss, Germany).

Immunohistochemistry. Following local and national guidelines, five 3-day ducklings at Shanghai Veterinary Research Institute were inoculated intracerebrally with 10^3 TCID50 of novel duck reovirus. Litters inoculated with tissue culture media were used as control. Ducklings were euthanized 7 days post-infection or earlier, if advanced illness was observed. Spleens were collected, fixed in formalin, embedded in paraffin, sectioned at 4–6 μm, probed with polyclonal antibodies to P18, and visualized with EnVision (DAKO).

Results

Expression and purification of P18

E. coli BL21 (DE3) were transformed with a recombinant plasmid encoding P18 fused to His-tagged trigger factor, and induced with 0.5–1 mM IPTG at various temperatures. Protein accumulation was the highest after induction with 1 mM IPTG for 24 h at 15°C, as observed on 10% SDS-PAGE gels (Fig. 2a). Quantitative analysis suggested that the fusion protein was 93.5% pure, and that 1 l of cell culture produced 126.23 mg (Table 1).

The fusion protein was further purified on a Ni-NTA column and again analyzed by SDS-PAGE (Fig. 2b), from which the molecular weight was estimated to be 70 kDa as expected, since P18 is 18 kDa and His-tagged trigger factor is 52 kDa. Western blot with a monoclonal antibody to His

Table 1. Purification of recombinant P18 from a 1 L – culture of BL21(DE3) *E. coli*

Step	Volume (ml)	Concentration (mg/ml)	Total protein (mg)	Purity (%)	Total P18 protein (mg)	Yield (%)
Crude	40	15.7	628	34.5	216.66	100
Ni-NTA	100	1.35	135	93.5	126.23	58.25

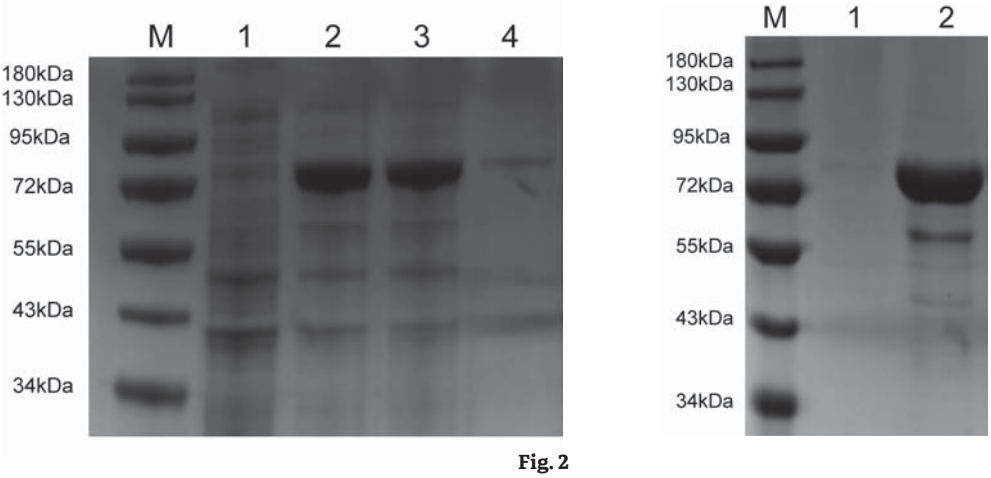

Fig. 2

Expression and purification of recombinant P18 using a pCold-TF vector
(a) Protein gel demonstrating expression of P18 in *E. coli*. Lane 1, uninduced control; lane 2, overnight induction at 15°C; lanes 3 and 4, supernatant (3) and pellet (4) from cells induced overnight at 15°C; M, marker. **(b)** Purification of recombinant protein on nickel-nitrilotriacetic acid. Lane 1, negative control; lane 2, purified protein; M, marker.

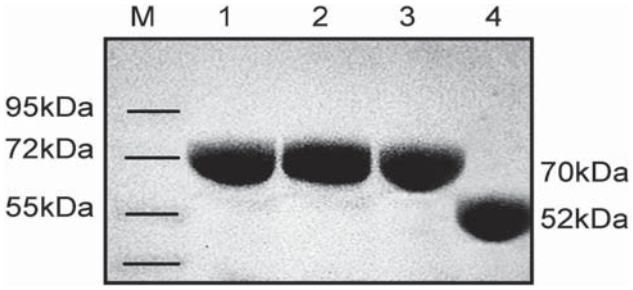

Fig. 3

Western blot for P18 using monoclonal antibodies to His tags
M, protein molecular weight markers; lanes 1 and 2, supernatant (1) and pellet (2) of *E. coli* cells expressing P18; lane 3, purified recombinant P18; lane 4, BL21(DE3) culture, which was transformed with empty pCold vector has a band at about 52 kDa (negative control).

tags (Boster, Wuhan, China) confirmed that the 70 kDa band is P18 fused to His-tagged trigger factor. The negative control, BL21(DE3) culture, which was transformed with empty pCold vector, produced only one band of 52 kDa size (Fig. 3).

Characterization of polyclonal antibodies to P18

Polyclonal antibodies raised against purified recombinant P18 specifically react with the same protein, with a 52 kDa protein in BL21(DE3) culture, which was transformed with empty pCold vector (Fig. 4a), and with an 18 kDa protein in DEFs infected with novel duck reovirus. However, immunoreactivity was not observed in non-infected cells (Fig. 4b).

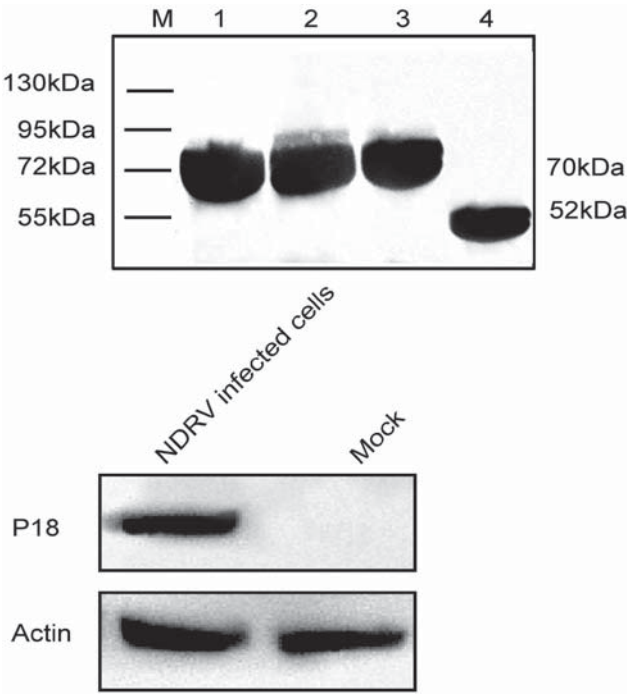

Fig. 4

Western blot using polyclonal antibodies to recombinant P18
(a) Western blot against recombinant P18. M, protein molecular weight markers; lanes 1 and 2, supernatant (1) and pellet (2) of *E. coli* cells expressing P18; lane 3, purified P18 protein; lane 4, BL21(DE3) culture, which was transformed with empty pCold vector has a band at about 52 kDa (negative control). **(b)** Western blot against native protein. Lane 1, duck embryonic fibroblasts 24 h post-infection with novel duck reovirus at multiplicity of infection 0.1; lane 2, non-infected duck embryonic fibroblasts.

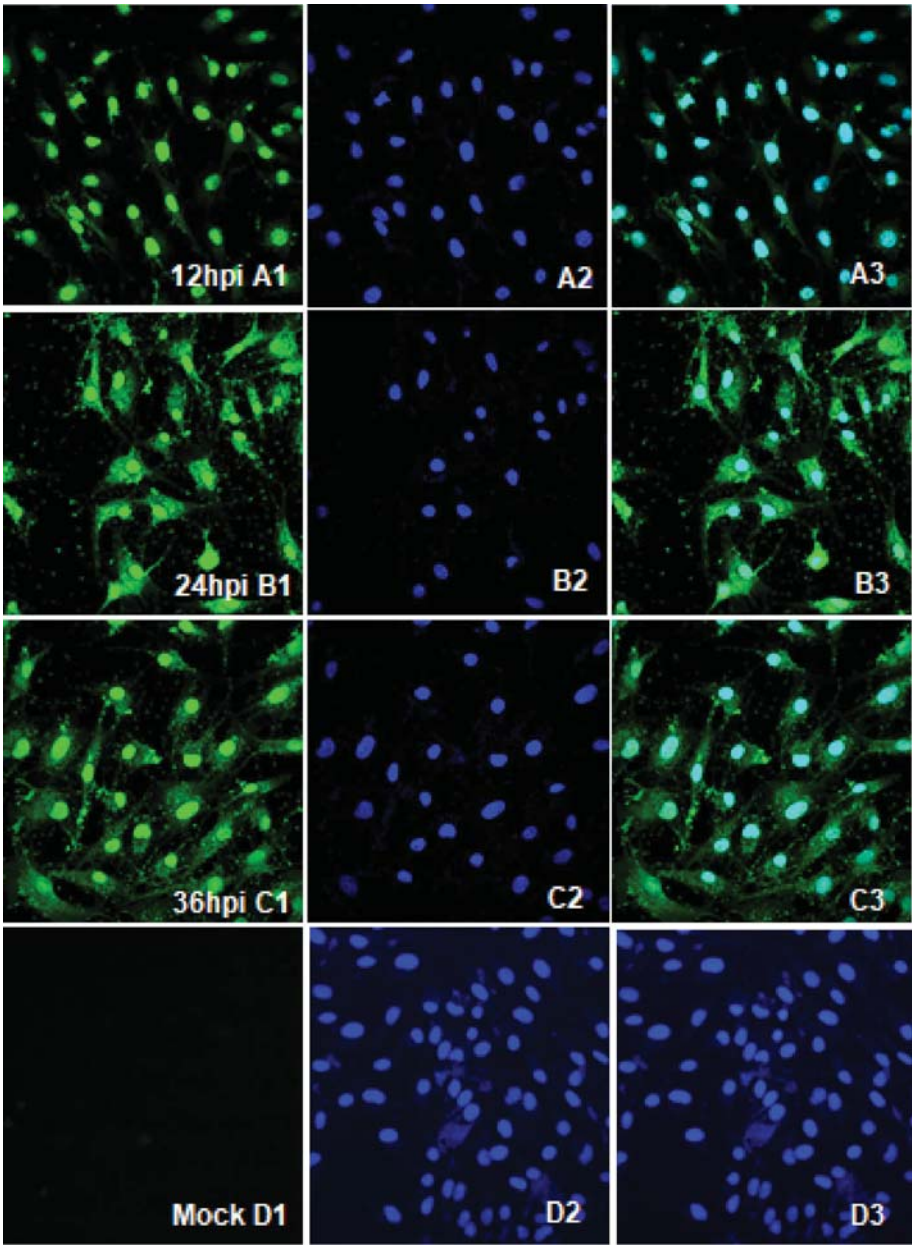

Fig. 5

Detection and localization of P18 in infected cells
Representative immunofluorescence micrographs of duck embryonic fibroblasts infected with novel duck reovirus at multiplicity of infection 0.1 **(a-c),** mock-infected cells **(d),** fixed at indicated time points, and stained with polyclonal antibodies to P18 (A1,B1,C1,D1) and DAPI (A2,B2,C2,D2). Magnification, ×200.

Characterization of P18 in cells

Using immunofluorescence confocal microscopy, we analyzed the subcellular localization of viral P18 in cells mock-infected or infected with novel duck reovirus (Fig. 5). These cells were also stained with DAPI to visualize nuclei. In infected cells, P18 was detected predominantly in the nucleus 6 h post-infection, with only background levels in the cytoplasm. Some P18 was then observed in the cytoplasm at 12 h post-infection (Fig. 5a), and throughout the nucleus and cytoplasm at 24 to 36 h post-infection (Fig. 5b,c). Only background staining was observed in mock-infected cells probed with polyclonal P18 antibodies (Fig. 5d).

Characterization of P18 protein in vivo

Immunohistochemistry of spleen tissues indicated no or poor P18 staining in very few splenocytes from uninfected ducklings (Fig. 6a). In contrast, strong cytoplasmic staining was observed in splenocytes from infected ducklings (Fig. 6b).

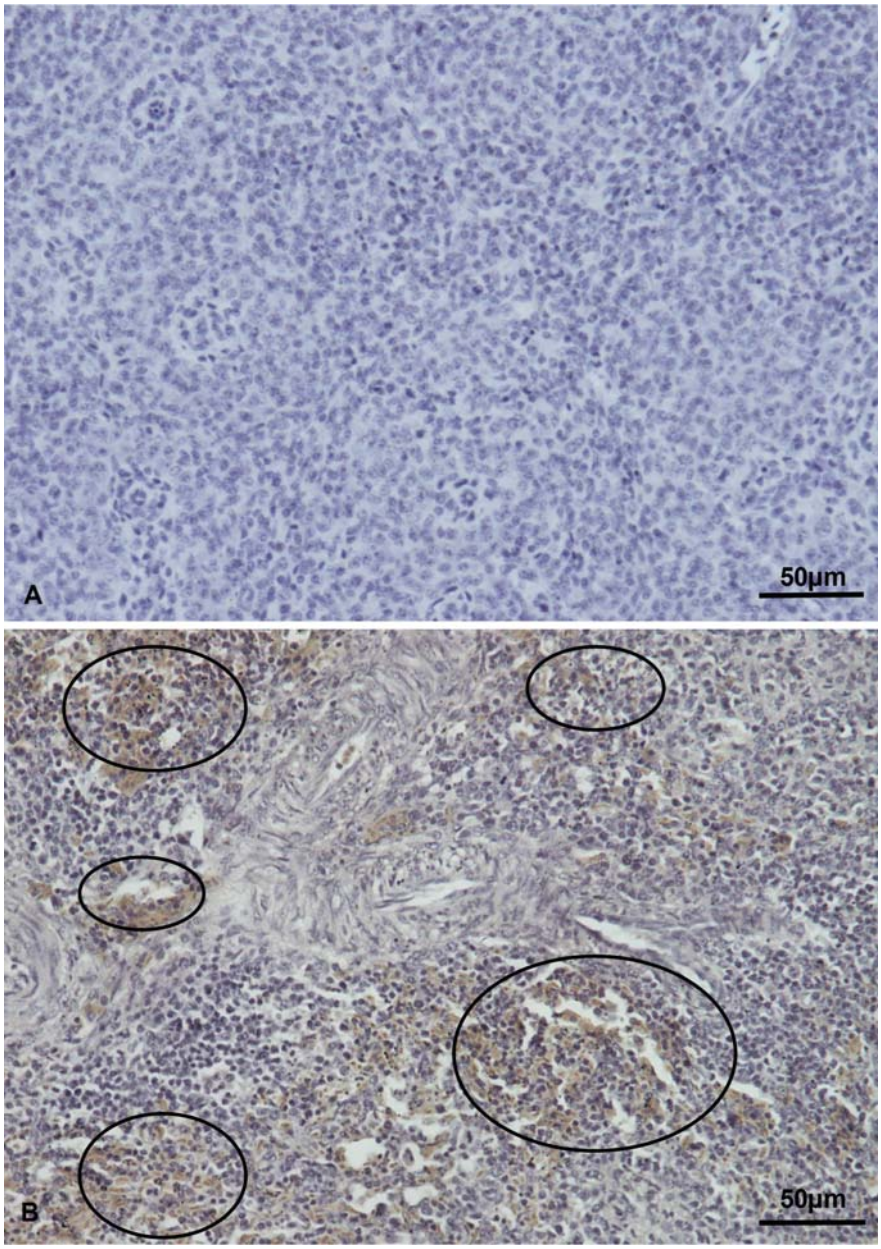

Fig. 6

Immunohistochemistry of P18 in spleen samples

(a) Background staining in non-infected ducklings. Magnification, ×200. **(b)** Strong P18 staining in majority of splenocytes from ducklings infected with novel duck reovirus. Magnification, ×200. Infected cells are marked in black. Scale bar, 50 μm.

Discussion

The first goal of this study was to assess whether the second open reading frame in the S1 segment of the novel duck reovirus genome encodes a protein similar to that in other orthoreoviruses. By comparing this segment to the corresponding segments in avian orthoreovirus and Muscovy duck reovirus, we found that a 162-amino acid nonstructural protein with size 18 kDa is likely encoded. Since previous studies showed that avian orthoreoviruses are nonpathogenic in Peking ducks and that Muscovy duck reovirus infects only Muscovy ducks (Heffels-Redmann *et al*., 1992; Woźniakowski *et al*., 2014), we speculated that P18 determines the host range.

We initially attempted to express P18 from pET or pGEX vectors (data not shown). However, yields were low and sufficient only for limited biochemical studies. Although many factors affect protein yield, including host, growth medium and temperature, fusion partners, etc. (Chen, 2012), all our initial attempts to increase yield were unsuccessful. To overcome this problem, P18 was then cloned into the pCold-TF vector, from which a large quantity of

protein was obtained by induction at low temperature (15°C). This protein was of high quality and thus suitable for biochemical and physiological characterization. Of note, viral proteins produced in *E. coli* have been demonstrated in many studies to be useful as antigens to raise corresponding antibodies that may, in turn, prove useful in diagnosing viral diseases. Thus, antibodies to recombinant P18 may enable selective elimination of birds infected with novel duck reovirus, and thereby control the spread of this disease or even eradicate the virus. Indeed, a similar strategy has been proposed to control other orthoreoviruses.

The recombinant protein we produced is N-terminally fused to His-tagged trigger factor, but eliminating both the His-tag and trigger factor was considered to be time-consuming and costly. Thus, polyclonal antibodies were raised to the intact fusion protein, and then tested against both the recombinant protein and the native protein from cells infected with novel duck reovirus. Results show that the antibodies react with both, implying suitability as reagent to detect novel duck reovirus.

Immunofluorescence confocal microscopy and immunohistochemistry based on P18 polyclonal antibodies further confirmed that these antibodies can be used for early diagnosis of novel duck reovirus infection. Indeed, P18 was detected not only in infected cells, but also in spleen tissues of infected ducklings. In addition, we found that P18 accumulates both in the nucleus and cytoplasm at some point after infection, perhaps suggesting that it is functionally similar to P17, a nucleocytoplasmic shuttling protein in avian orthoreoviruses (Chulu *et al.*, 2010; Chi *et al.*, 2013; Li *et al.*, 2015). We note that the mammalian reovirus S1 segment also expresses σ1s, a 14 kDa nonstructural protein that accumulates in the nuclei of transfected and infected cells (Boehme *et al.*, 2009, 2013). Whether P18 is associated with pathogenesis and virulence remains to be established.

In this study, to overcome a significant obstacle to expression of P18, the low temperature was used to express the trigger-factor fusion protein. We provide the first direct experimental evidence that P18 is expressed by novel duck reovirus both *in vivo* and *in vitro*, and can be used as an early marker of infection. The data also show that polyclonal antibodies raised against recombinant P18 can be used as a reagent to investigate P18 function. Nevertheless, further studies are needed to clarify the role of P18 in virulence and host range.

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