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Isolation and characterization of an Aves polyomavirus 1 from diseased budgerigars in China

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ABSTRACT

Aves polyomavirus 1 (APV) causes inflammatory disease in psittacine birds, especially in young budgerigar. In this study, an APV virus (SD18 strain) was isolated from a diseased psittacine birds breeding facility. The full genome (4981 bp) of SD18 was determined and analyzed. Phylogenetic analysis of full genome sequences indicated all the APV strains form two groups. The SD18 strain showed close relationship with APV isolated from Poland, however, the other Chinese strains are located in group II, which suggested different genotypes APVs are co-circulating in China. Compared with the consensus sequence of APV full genome, the SD18 strain contains 13 nucleotide mutations, and 2 unique amino acid substitutions (R179M and Q382K) located in VP2/3 and Large T proteins. To explore the pathogenicity of the virus, the SD18 strain was used to challenge 2-week-old budgerigars. All infected birds died no later than 5 days post infection, and virus was detected in multiple organs including brain, heart, ingluvies, liver, and intestine, which indicated that SD18 is fatal and causes systemic infection in young budgerigar. *In vitro* studies showed that SD18 replicated efficiently in CEF cells and reached the highest viral titers at 9 days post infection. Notably, replication of SD18 stimulated IFN-β response in CEF cells and overexpression of the VP4 or VP4Delta proteins significantly inhibited IFN-β promoter activation, which could be the strategy of APV to escape from the host innate immunity.

1. Introduction

Budgerigar fledgling disease is an important disease of psittacine birds (parrots, parakeets) caused by Aves polyomavirus 1, a member of polyomaviridae family (Bernier et al., 1981). Polyomaviruses have been isolated from a wide range of vertebrate hosts, including horses (Renshaw et al., 2012), rodents (Ehlers et al., 2015), birds (Heenemann et al., 2015; Rinder et al., 2018), bats (Tao et al., 2013), humans (Allander et al., 2007; Gheit et al., 2017), and non-human primates (Deuzing et al., 2010). Serological studies have shown that up to 90% of the human population has been exposed to human polyomaviruses, and most infections are asymptomatic (Sroller et al., 2016). In immunocompromised patients, polyomavirus infection could induce clinical signs including Merkel cell carcinoma, kidney disease, and progressive multifocal leukoencephalopathy (Feng et al., 2008). By contrast, bird polyomaviruses are able to cause severe disease in young birds. So far, multiple polyomaviruses have been identified in birds, *e.g.* Aves polyomavirus 1 (APV) (Bozeman et al., 1981), goose hemorrhagic polyomavirus (GHPV) (Feher et al., 2014), and finch polyomavirus (FPyV) *etc.* (Heenemann et al., 2015). New born to a few weeks old birds are susceptible to APV, which could cause inflammatory disease and sudden death in young budgerigar birds with up to 100% mortality, however, the underlying mechanism remains unclear. APV also causes chronic disease in adult psittacine birds, including abdominal distention, feather abnormality, lack of down feathers on the back and abdomen, and filoplumes on the head and neck (Davis et al., 1981).

Polyomaviruses are non-enveloped, icosahedral viruses with a circular, double-stranded DNA genome of approximately 5000 bp (Fajfr et al., 2015). The genome contains an early region and a late region. A non-coding control region (NCCR) located between the two genome regions contains the origin of DNA replication, transcription factor, binding sites, promoters, and enhancers. The early region encodes the

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Table 1
Nucleotide and amino acid mutations in APV and reference strains.

Position Virus	609	1512	2474	2558	2663	2744	2906	2945	3242	3443	3643	3725	4972
AB453159,APV1,Japan	–	–	–	–	–	–	–	–	–	–	–	–	–
AB453160,APV2,Japan	–	–	–	–	–	–	–	–	–	–	–	–	–
AB453162,APV4,Japan	–	–	–	–	–	–	–	–	–	–	–	–	–
AB453162,APV5,Japan	–	–	–	–	–	–	–	–	–	–	–	–	–
FJ385773, China	–	–	–	–	–	–	–	–	–	–	–	–	–
AB453165,APV7,Japan AF118150, USA	–	–	C	A	C	A	T	A		–	–	–	G
	–	–	C	A	G	A		A	G	–	–	–	G
KT203762,PL830X,Poland	C	–	C	A	C	A	T	A	G	–	–	–	G
KT203764,PL1025B,Poland	C	–	C	A	C	A	T	A	G	–	–	–	G
SD18	C	T(M)	C	A	C	A	T	A	G	C	T(K)	G	G
Consensus	T	G(R)	T	G	A	G	C	C	A	T	G(Q)	A	A
Location	VP4	VP2/VP3	VP1	VP1	VP1	VP1	VP1	NCR	L-T	L-T	L-T	LT	ST

Dashes indicate identical nucleotides with consensus sequence; parenthesis indicates amino acid residue; NCR, non-coding region; LT, Large T; ST, Small T.

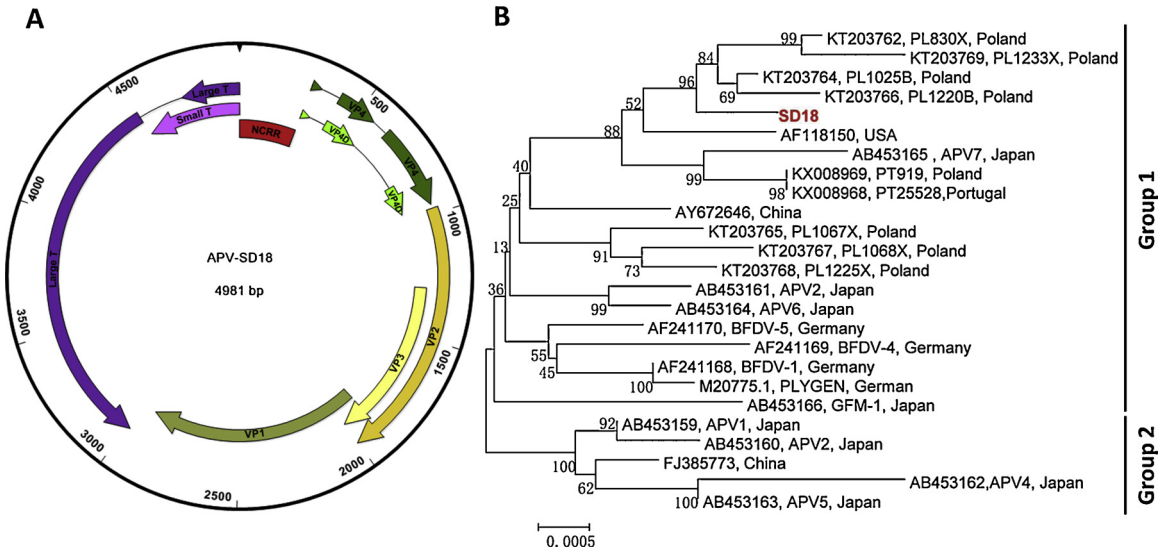

Fig. 1. Genome organization of APV SD18 strain (A), phylogenetic trees of the full genomes of APVs (B). Early proteins are in purple; late proteins are in green, yellow, and orange; the NCCR is in red; ORFs encoding Large T antigen and Small T antigen; VP1, VP2, VP3, VP4, and VP4Delta are marked by arrows. The phylogenetic tree was generated by the neighbor-joining method and bootstrapped with 1000 replicates using the MEGA6 software version 6.05. The APV SD18 isolate is highlighted in red. The scale bar represents the distance unit between sequence pairs (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

Large T and Small T antigens, and the late region encodes the structural proteins VP1, VP2, and VP3. Two novel proteins VP4 and VP4Delta, formerly called agnoprotein 1a and agnoprotein 1b, are only presented in bird polyomavirus, but not in the mammalian polyomavirus (Johne et al., 2007). VP4 and VP4Delta encode 176 and 112 amino acids (AA), respectively.

APV was first identified as a pathogen in young budgerigars in the USA, in the 1980s (Davis et al., 1981). Since then, it has been found in many countries and posed a threat to psittacine birds (Davis et al., 1981). In China, psittacine birds are popular companion birds and the population is increasing every year. However, so far there has been no commercial vaccine available for APV, which poses a big threat to psittacine birds. Therefore, continuous surveillance is warranted. In this study, one APV virus was isolated from diseased budgerigars and further characterized.

2. Material and methods

2.1. Sample collection and virus isolation

In 2018, an outbreak of disease was reported in budgerigar breeding farm in Shandong Province, China. The feather, heart, liver, lung, and intestine samples of ten dead birds were collected for the laboratory diagnosis. Viral DNA was extracted from the clinical samples of the ten birds by using QIAamp Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. The extracted DNA was frozen and stored in −70 °C for conventional PCR test.

To isolate the APV virus, chick embryo fibroblasts (CEF) cells were prepared and maintained in Eagle's minimum essential medium supplemented with 10% fetal bovine serum. The liver of the one dead bird was homogenated in PBS and centrifuged at 5000 *g for 10 min*. The supernatant was filtered by passing through 0.22 μm filter. The treated samples were inoculated on CEF cells and incubated for 2 h for virus absorption, and then the supernatant was replaced with DMEM supplemented with 2% fetal bovine serum. The infected cells were observed for cytopathic effect (CPE) daily and the DNA extracted from the infected cell was subjected to PCR.

2.2. PCR and real-time PCR

Two pairs of primers targeting VP1 gene of APV and Rep gene of psittacine beak and feather disease virus (PBFDV) were designed for diagnosis in our previous studies (Ma et al., 2019a, b).To detect Newcastle disease virus (NDV) and avian influenza virus (AIV) infection,

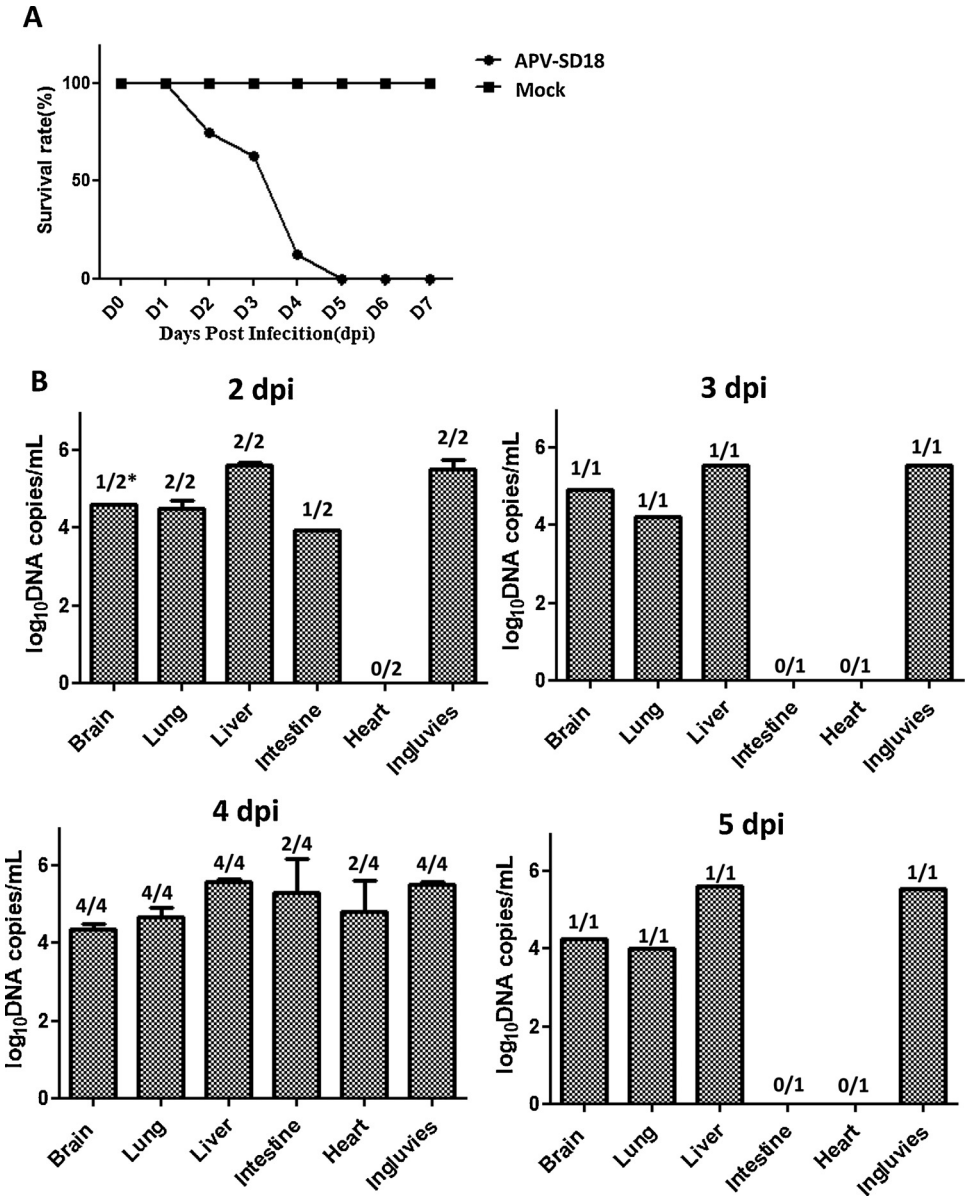

Fig. 2. Survival rate and replication of the APV in infected birds. A: Survival rate of birds infected with APV SD18 strain. B: DNA copies of APV in different tissues of infected birds. *: one out of two infected birds is positive for APV, and the DNA copy values were shown as Mean ± SEM.

lung samples were collected for RNA isolation, and the RNA was reverse transcribed to cDNA for NDV and AIV detection by RT-PCR as described previously (Farkas et al., 2007). The PCR was conducted in a 25 μL volume containing 12.5 μL 2 × PCR Master Mix (New England Biolabs, MA, USA), 1.5 μL forward primer (10 μM), 1.5 μL reverse primer (10 μM), 2 μL of DNA template, and PCR-grade water. The PCR conditions were: 94 °C for 3 min, 30 cycles at 94 °C for 30 s, 53 °C for 30 s, 72 °C for 2 min, with a final extension at 72 °C for 10 min. The PCR products were analyzed by gel electrophoresis with 1.5% agarose gel, and observed under UV light. A quantitative real-time PCR assay was used to test the APV titer as described previously (Ma et al., 2018).

2.3. Sequencing and genetic analysis of full genome of the isolated virus

To sequence the full genome of the isolated APV, two pairs of primers covering the full genome of APV were designed, APV-Genome-1F: CCTTCGGTCTCACGGAATTCCTCC, APV-Genome-1R: AATTGTCGTTG TAGTGTTTGT; APV-Genome-2F: GTGTTACAGCTGTGCCAGGATACC, APV-Genome-2R: AGTCCCAAGTATGGATTTCCGCATTC. The amplified PCR products were sequenced by the GENEWIZ Company (Hangzhou, China), and then the full genome was assembled using DNASTAR software (Lasergene 7.0, USA).

To characterize the genetic features of the isolated APV SD18 strain, the homology between the isolated APV and reference strains were compared by using DNASTAR software, and the phylogenetic trees of full genome were conducted using neighbor joining method by MEGA6.0 (Saitou and Nei, 1987) with 1,000 bootstrap replicates. The reference sequences used for genetic comparison were obtained from GenBank database. There is a total of 24 full genome sequences of APVs deposited in GenBank, including nine strains isolated from Poland, eight strains reported in Japan, three strains from Germany, two strains from China, and three strains from Portugal and the USA.

To study the mutation of the APV SD18 strains, based on the phylogenetic tree of full genome, five reference APV strains located in group I or group II were selected and compared (Table 1).

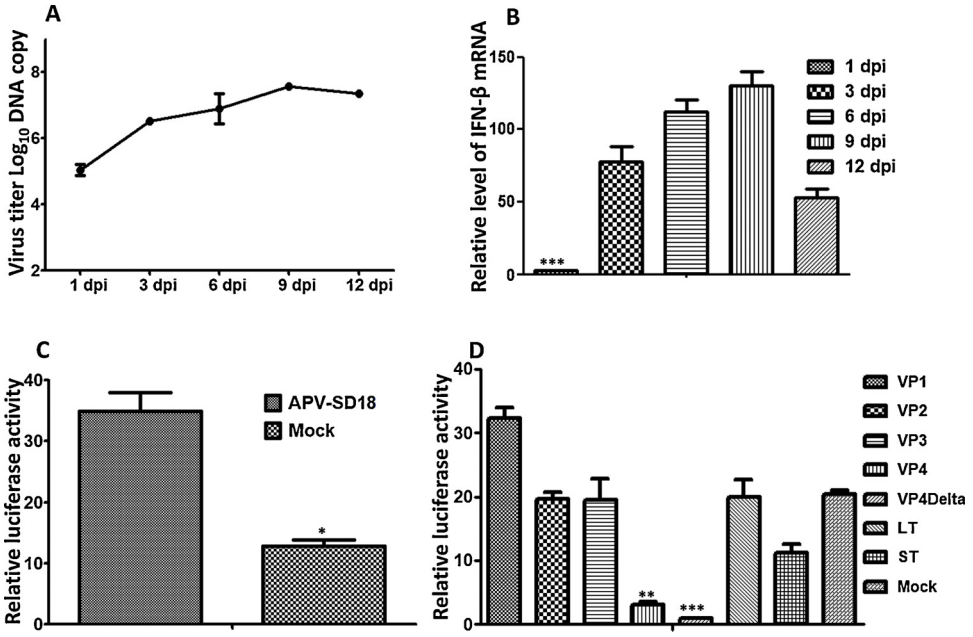

Fig. 3. Growth kinetics of APV-SD18 and mRNA level of IFN-β on infected CEF cell, effects of different proteins and the APV infection on IFN-β promoter activation on CEF cells. A: Growth kinetics of APV-SD18 on CEF cells. B: mRNA level of IFN-β on infected CEF cell. C: The APV infection induced IFN-β promoter activation. D: Effects of different proteins of APV on dsRNA-induced IFN-β promoter activation.

2.4. Bird experiment

APV is an important pathogen for the psittacine birds, however, there is limited information on the replication and pathogenesis of APV in birds. In this study, the virulence and replication of SD18 in young budgerigars were evaluated. A total of 16 2-week-old budgerigars were obtained from a budgerigar breeding facility in Shanghai. Feather samples were collected from all the birds for APV and PBFDV detection by PCR. The results showed that all the 16 birds are free of APV and PBFDV infection. To exclude the Newcastle disease virus (NDV) and avian influenza virus (AIV) infection, oral swabs were collected for NDV and AIV detection by RT-PCR as described previously (Farkas et al., 2007). The RT-PCR results showed that all the 16 birds are free of AIV and NDV infection. Eight budgerigars were inoculated orally with the SD18 of 2×10^7 DNA copies, and the remaining eight birds were inoculated with PBS as a control group. All the birds were observed for clinical signs and survival rate for 7 days. All the dead birds were necropsied, and ingluvies, heart, liver, lung, brain, and intestine tissues were collected and tested by real-time PCR.

2.5. Type I IFN response in CEF infected by APV

Type I IFN, as an innate immune system, is the first line of defense against pathogen infection. Many DNA virus infections stimulate type I IFN response in infected cells, and the viruses have evolved a variety of responses to inhibit host type I IFN response (Ghosh et al., 2019; Lin et al., 2013). To explore whether APV infection induce type I IFN response in infected cells, CEF cells were infected with SD18. The cells were collected at different time points post infection, then mRNA levels of IFN-β were tested by relative quantitative real-time PCR as described previously (Niu et al., 2019). Briefly, total RNA was extracted from the cells and reverse transcribed to cDNA using a cDNA synthesis kit (Vazyme, China). The qRT-PCR targeting IFN-β and β-actin was performed according to the manufacturer's instructions using ChamQTM SYBR® qPCR Master Mix (Vazyme, China). The relative expression levels for the tested mRNA were determined with normalization of β-actin as an internal reference using the comparative Ct ($2^{-\Delta\Delta CT}$) method.

To confirm Type I IFN response in CEF infected with APV, luciferase reporter assay was conducted as described previously (Liu et al., 2018). Briefly, CEF cells cultured in 24-well-plate were transfected by chicken IFN-β luciferase reporter plasmid (pGL-IFN-β-Luc 0.4 μg/well) and pRL-TK (0.07 μg/well). 24 h post transfection, the cells were infected with SD18, and the mock cells were only transfected by plasmids. Twenty-four hours later, all cells were lysed, and the luciferase activity was carried out using dual-luciferase reporter assay kit (Promega, USA).

2.6. IFN antagonism assay

Both of DNA and RNA viruses have evolved different strategies to antagonize host type I IFN response, such as NS1 protein of influenza virus inhibits IFN-β response (Marc, 2014). NP1 protein of porcine bocavirus inhibits interferon signaling pathway by interfering with IRF9 DNA-binding activity (Zhang et al., 2015). To further determine whether the viral proteins of APV antagonize IFN-β response, the VP1, VP2, VP3, VP4, VP4Delta, Large T, and Small T genes were cloned into pCDNA3.0 vector, and a FLAG-tag was added to the N-terminal of those proteins. CEF cells were co-transfected with individual recombinant plasmid (VP1, VP2, VP3, VP4, VP4Delta, Large T, and Small T expression plasmids, 0.4 μg/well), pGL-IFN-β-Luc (0.4 μg/well), pRL-TK (0.07 μg/well), and the IFN-β inducer poly(I:C) (0.2 μg/well). Twenty-four hours post transfection, the cells were lysed and subjected to dual-luciferase reporter assay (Promega, USA).

2.7. Ethics statement and statistical analysis

The animal study was conducted in accordance to the guidelines of the Animal Care and Use Committee of Shanghai Jiao Tong University. All data were analyzed using analysis of variance (two-way ANOVA) in GraphPad Prism version 5.0 (GraphPad software Inc. La Jolla, CA); a P-value of 0.05 or less was considered significant.

3. Results

3.1. Diagnosis and virus isolation

In this diseased farm, about 100 younger budgerigar birds with age of one to 20 days old showed clinical signs, *e.g.* inappetence, lethargy, diarrhea, and sudden death with 70% mortality. During necropsy, subcutaneous hemorrhage, hepatomegaly, and pneumorrhagia were observed. The tissue samples of ten dead birds were collected for PCR diagnosis. The results showed that all the clinical samples are APV positive, but no PBFDV, AIV, or NDV was detected. It indicated that the

dead birds were infected with the APV. To further confirm the results, the VP1 PCR products amplified from the clinical samples were sequenced and analyzed. The results showed that the VP1 sequences showed 100% identity and shared 99% identity with reference sequences from strains APV7 (AB453165) and WF-GM01 (GU452537) (Katoh et al., 2009; Zhuang et al., 2012).

To isolate the APV virus, the liver sample of one dead bird was processed and inoculated on CEF cells. After 10 days post infection (dpi), the infected cell was collected and subjected to PCR test. The PCR result indicated that one APV (named as SD18) was isolated from the liver sample. CPE characterized by swelling of the nuclei and gradual detachment of the cells was observed in CEF infected by APV at about 10 days post inoculation.

3.2. Organization of APV SD18 full genome

The genome of APV SD18 isolate is circular and 4981 bp in length. The full genome sequence was deposited in GenBank (accession number MH643735). The genome structure of the APV SD18 isolate is similar to the Aves polyomaviruses, containing non-coding control region (NCCR, nucleotide positions: 1 to 289 bp), early region, and late region. The early region (4981 to 2987 bp) encodes ORFs for Large T (4981 to 4733, join 4537 to 2987 bp, 599 AA) and Small T antigens (4981 to 4544 bp, 145 AA). The late region of APV isolate encodes five proteins, VP1 (1899 to 2930 bp, 343 AA), VP2 (977 to 2002 bp, 341 AA), VP3 (1295 to 2002 bp, 235AA), VP4 (290 to 331, join 408 to 569, join 634 to 960 bp, 176AA), and VP4Delta proteins (290 to 331, join 408 to 569, join 826 to 960 bp, 112AA) (Fig. 1A). The start codon for VP3 ORF is located within VP2 ORF, and the amino acid sequence of VP3 is identical to the C-terminal (235 AA) of VP2. VP4Delta shared the same amino acid sequence with VP4 except the truncation from 68 to 131 AA (Fig. 1A).

3.3. Genetic analysis of APV SD18 full genome

In phylogenetic trees of full genome, all APV strains fell into two groups, while SD18 showed close relationship with strains from Poland, Portugal, China, Japan, and USA in group I (Fig. 1B). The other strains from Poland, Japan, and China were located in group II of the tree, which indicated that different genotypes of APVs are co-circulating in China, Japan, and Poland. As shown in Table 1, compared with the consensus sequence of all APV reference strains, the APV SD18 strain contains 13 nucleotide mutations, located in VP4 (1 site), VP2/3 (1 site), VP1 (5 sites), NCCR (1 site), Large T antigen (4 sites), and Small T antigen (1 site). Notably, four out of 13 mutations, unique for SD18 strain, are G1512T, C3443T, T3643G, and G3725A that are not presented in the other 24 reference strains. Two out of the four mutations result in unique amino acid substitutions (R179M and Q382K) located in VP2/VP3 and Large T proteins, respectively (Table 1). However, the effects of these mutations on virus replication and pathogenesis of SD18 remain unclear.

Interestingly, the APV reference strains from the group I and group II have different genetic markers as shown in Table 1. Most of the APV reference strains (including SD18) in group I contain 2474C, 2558A, 2663C, 2774A, 2906T, 2945A, and 4972G, while most of strains from group II contain 2474T, 2558G, 2663A, 2774G, 2906C, 2945C, and 4972A. All the nucleotide substitutions are nonsense mutations, and most of these mutations are located in VP1 gene. VP1 is the main neutralizing antigen of virus, and it may mutate under the immune pressure. The result suggested that the APV has been evolving slowly over time, although the DNA viruses are relatively more conservative compared with RNA viruses.

3.4. Bird study

All infected birds showed inappetence and lethargy after inoculation, and sudden death was observed at 2 days post infection. All the infected birds died before 5 days post infection, whilst all the control birds did not show any clinical signs (Fig. 2A). During necropsy, similar to the natural infected cases, hepatomegaly and pneumorrhagia were observed in experimental infected birds. The virus distribution in dead birds was assessed by real-time PCR. The results showed that the virus was detected in multiple organs. At 2, 3, 4, and 5 dpi, the virus was detected in brains, lung, liver, and ingluvies from all the dead birds, virus loads in liver and ingluvies were higher than those of other tissues despite there is no significant difference, while only three intestine and two heart samples were APV DNA positive (Fig. 2B). All the data suggested that APV caused systemic infection in young budgerigars as reported previously (Phalen et al., 1993), and especially replication of virus in brain might induce inflammation and sudden death of infected birds. Overall, the SD18 is fatal to young budgerigar, however, the underlying mechanism needs further studies.

3.5. Type I IFN response in CEF infected with APV

Viral growth kinetics result showed that SD18 replicated efficiently on CEF cells and reached the highest titer at 9 days post infection (Fig. 3A), and mRNA level of IFN-β was significantly upregulated on 3, 6, and 9 days post infection compared with 1 day post infection (Fig. 3B). The mRNA level of IFN-β decreased at 12 days post infection. The IFN-β level positively associated with the SD18 replication on CEF cells, which indicated the APV infection stimulated IFN-β response. Additionally, the luciferase reporter assay result confirmed the APV infection activated IFN-β promoter compared with the mock group (Fig. 3C).

3.6. IFN antagonism assay

The successful replication of the APV on CEF cells suggested the APV has strategy to overcome the host innate immunity response, *e.g.*, type I IFN. To further determine whether viral proteins of APV antagonize IFN-β response, the IFN-β antagonism activity of viral proteins were evaluated. The results indicated VP4 and VP4Delta proteins significantly inhibited the IFN-β promoter activation compared with the empty vector group. However, VP1, VP2, VP3, Large T, and Small T proteins did not suppress the IFN-β response in dual-luciferase reporter assay (Fig. 3D). The results demonstrated that the VP4 and VP4Delta presented only in Aves polyomavirus, but not in mammalian polyomavirus, antagonize host IFN-β response to battle with the host innate immune system.

4. Discussion

The APV distributed geographic globally, including Europe (Bert et al., 2005; Stoll et al., 1993), America (Bernier et al., 1981), and Asia (Katoh et al., 2009; Zhuang et al., 2012). In Japan, a surveillance study showed that 2.7% of the imported psittacine birds (402) were positive for APV. The APV positive birds were imported from South Africa and the USA (Ogawa et al., 2006). In Italy, 0.8% of 877 psittacine birds was positive for APV (Bert et al., 2005). Based on our previous survey on adult psittacine birds, about 60% investigated birds were carrying APV in China (Ma et al., 2019a). Although APV is widespread, there are only 24 full genomic sequences deposited in GenBank, and most of the sequences were from Japan and Poland, as well as two genome sequences from China. In the phylogenetic tree of the full genomes, the APV SD18 isolated in this study was grouped into group I with high identity (99.8%) to strains from Poland Portugal, China, Japan, and USA (Fig. 1), the remaining strains were located in group II. The results showed that different genotypes of APVs are co-circulating worldwide.

Type I IFN is an important pro-inflammatory cytokine produced by host cell during pathogen infection, which induce various immunity molecules with function of antiviral activity and innate immune cell

activation *etc.* Whether the APV infection stimulates IFN response in bird remains largely unclear. Assetta, B. et al. reported that human JC polyomavirus infection induced IFN-β production in human cells (Assetta et al., 2016). And Vivek R. Nerurkar et al. found that IFN-α and IFN-β inhibited JC polyomavirus replication in primary human cells (Co et al., 2007). In this study, our results also showed that APV infection triggered IFN-β response, and the VP4 and VP4Delta proteins antagonized IFN-β activation.

Previous studies showed that VP4 plays an important role in the pathogenesis of Aves polyomavirus and induces cell apoptosis during infection (Johne and Muller, 2001; Johne et al., 2007). Johne et al. reported deletion of both VP4 and VP4Delta abolished Aves polyomavirus infectivity (Johne et al., 2000). Briefly, although the host uses IFN as a weapon to clear the APV virus, the APV also has evolved to defense it with both of VP4 and VP4Delta, which play important roles in the pathogenicity and escape from host innate immunity.

APV is a causative agent of the acute inflammatory disease in psittacine and some non-psittacine birds, and it poses a significant threat to birds. However, the mechanism of the pathogenicity of APV remains unknown. In this study, we reported that APV caused systemic infection in budgerigars, and the VP4 and VP4Delta protein inhibited IFN-β response in CEF cells, which might contribute to the severe disease caused by APV. Moreover, the virus has undergone slow evolution that may change the virus biological features, *e.g.* virulence, host range, and transmission. Therefore, the continuous surveillance of APV is warranted.

Ethical approval

The animal studies were approved by the Animal Care and Use Committee of Shanghai Jiao Tong University.

Declaration of Competing Interest

The authors declare no conflict of interest.

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