Coinfection of novel goose parvovirus–associated virus and duck circovirus in feather sacs of Cherry Valley ducks with feather shedding syndrome

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ABSTRACT Since 2017, an infectious disease, named feather shedding syndrome (**FSS**), has consistently broken out in Cherry Valley ducks in East China. The sick ducks showed the new clinical symptoms of feather shedding and being plucked off with difficulty after slaughter. The high incidence rate of 20 to 70% predominantly happened in ducks of 4 to 5 wk of age, and nearly 40% mortality rate was observed in infected ducks. To explore the possible role of novel goose parvovirus–associated virus (**NGPV**) and duck circovirus (**DuCV**) in this disease, a total of 540 feather sac samples were collected from sick ducks with FSS. The infection rates of NGPV and DuCV in samples were 82.78 and 78.89%, respectively, and the coinfection rate of the 2 viruses was 70.00%. Notably, ducks of 4 to 5 wk of age usually presented obvious and severe FSS in the flocks with high codetection rate of NGPV and DuCV. Furthermore, 9 NGPV strains were isolated from feather sacs and 5 synchronous amino acid mutations were demonstrated in VP3 protein. These results indicated that coinfection of NGPV and DuCV might play an important role in duck FSS disease.

Key words: novel goose parvovirus–related virus (NGPV), duck circovirus (DuCV), feather shedding syndrome (FSS), feather sac, coinfection

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INTRODUCTION

Since 2015, beak atrophy and dwarfism syndrome (**BADS**) had a sudden outbreak in Cherry Valley duck flocks, leading to severe economic losses to waterfowl industry in China (Chen et al., 2015, 2016; Li et al., 2016, 2017; Wang et al., 2016). A novel goose parvovirus (**GPV**)–related virus (**NGPV**) was identified as the causative agent of BADS (Chen et al., 2015, 2016). Goose parvovirus belongs to the *Dependovirus* genus of Parvoviridae family. It can cause high morbidity and high mortality of goslings and young Muscovy ducks (Brown et al., 1995; Jansson et al., 2007; Woźniakowski et al., 2009). As a GPV-derived virus, NGPV has 89.7 to 96.7% nucleotide homology and similar genome structure with GPV (Chen et al., 2015; Ning et al., 2017; Li et al., 2018a). Its genome includes 2 open reading frames (**ORF**), named the left ORF and the right ORF. The right ORF encodes 3 capsid proteins VP1, VP2, and VP3. The *VP2* and *VP3* genes are contained in the *VP1* gene (Zádori et al., 1995). As the most abundant core protein in purified virions, VP3 consisted of 534 amino acids and could induce neutralizing antibodies with high immunogenicity (Le Gall-Reculé and Jestin, 1994; Le Gall-Reculé et al.,1996; Yin et al., 2012).

As a member of the genus *Circovirus* of the family Circoviridae, duck circovirus (**DuCV**) was first found in mulard ducks (Hattermann et al., 2003). Until now, DuCV has been detected in a variety of domestic duck breeds and wild ducks (Chen et al., 2006; Banda et al., 2007; Zhang et al., 2009; Wan et al., 2011; Matczuk et al., 2015). The typical symptoms of DuCV in meat ducks are growth retardation, weight loss, feathering disorders, and poor body condition (Hattermann et al., 2003; Soike et al., 2004; Cha et al., 2014; Hong et al., 2018). DuCV often coinfects with other bacterial and viral pathogens (Hattermann et al., 2003; Ball et al.,

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2004; Soike et al., 2004; Jiang et al., 2008; Zhang et al., 2009). Our previous report suggested that the coinfection of NGPV and DuCV might aggravate the development of the BADS disease in Cherry Valley ducks and mule ducks (Li et al., 2018b).

Since 2017, sick ducks with BADS showed new symptoms of feather shedding and residual feathers being plucked off with difficulty after slaughter in Cherry Valley ducks in East China. The incidence rate of the feather shedding syndrome (**FSS**) disease is 20 to 70%, and the mortality rate of the sick ducks is about 40%. Until now, there is no report of the causes leading to the new FSS in sick ducks. In this study, we collected 540 duck feather sac samples of sick ducks with typical FSS from 27 Cherry Valley duck flocks in East China and investigated the coinfection of NGPV and DuCV. Our study revealed the high coinfection rate of the 2 viruses in ducks with FSS and the NGPV strains isolated from sick ducks with FSS being clustered into a new genotype with 5 specific amino acid mutation points in the VP3 protein, which might lay a foundation for clarifying the causes of the FSS disease in Cherry Valley ducks.

MATERIALS AND METHODS

Clinical Samples Collection

Since 2017, large-scale outbreaks of BADS had occurred in Cherry Valley duck flocks in eastern China, and a new FSS disease was observed in the sick ducks. To better understand the causes of this new disease outbreak, from November 2018 to April 2019, a total of 540 feather sac samples were collected from sick ducks with typical FSS of 27 Cherry Valley duck flocks in Shandong, Jiangsu, and Anhui provinces of East China. The animal experiments were carried out in accordance with the guidelines issued by the Animal Care and Use Committee of Shandong Agricultural University (Approval Number: # SDAUA-2018-045). DNA was extracted from approximately 0.1 g of each feather sac sample using a DNeasy tissue kit (Qiagen, Hilden, Germany) following the manufacturer's recommendations. After that, the DuCV and NGPV viruses were detected, and the *VP3* gene of NGPV was amplified.

Semi-nested PCR Detection of NGPV

In our previous study, a semi-nested PCR assay was developed to specifically detect NGPV (Li et al., 2017). The primers are as follows: NGPV-1F (5′-AGA CTT ATC AACAAC CAT (C)T-3′, position 3278-3296) and NGPV-1R (5′-TCA CTT ATT CCT GCTGTA G-3′, position 4038-4056). They were designed to amplify a 779-bp fragment; NGPV-2R (5′-CAT CAT CCG TAA AAA CTT GG-3′, position 3406-3425) was designed with NGPV-1F to amplify a 147-bp fragment. The primers NGPV-F1 and NGPV-R1 of the semi-nested PCR assay were universal primers for GPV and NGPV, and NGPV-2R was special for NGPV. The semi-nested PCR assay was performed as per our previous work (Li et al., 2017).

Duplex PCR for DuCV-1 and DuCV-2 Detection

In our previous study, 4 primers DuCV-F (5′-CGG GAA ATG ACG TAG TCG TCA TG-3′, position 640-662); DuCV-R (5′-GGA(C) C(T)TG(A) AAC ATG AGA TGG GC-3′, position 1653-1672); DuCV-1F (5′-GTT CAC TCC G(T)GT TGT GTT GTC C(T)GG-3′, position 1226-1249); and DuCV-2R (5′-GAT AAT GCG ACC(T) GGC GAC G-3′, position 1219-1239) were designed for the detection of DuCV-1 and DuCV-2 (Li et al., 2014). Using the 4 primers, both DuCV-1 and DuCV-2 were detected simultaneously by a duplex semi-nested PCR assay (Li et al., 2014).

Pathogen Analysis

Based on the detection results, the positive rates of NGPV and DuCV in the feather sac samples were statistically analyzed with SPSS software.

Amplification and Phylogenetic Analysis of the VP3 Gene of NGPV

From the coinfection of NGPV and DuCV feather sac samples of 9 duck flocks, 9 NGPV strains (JS1201, JS0401, AH0225, AH0309, SD1108, SD1206, SD0103, SD0227, and SD0312) were isolated from duck embryo fibroblast cells. The corresponding *VP3* gene amplification was performed with the primers P1 (5′-ATG GCA GAG GGA GGA GG-3′, position3006-3022) and P2 (5′-TTA CAG ATT TTG AGT TAG ATA TCT-3′, position4587-4610). The PCR products were purified and cloned into pMD18-T vectors, and the recombinant plasmids were sequenced using Sanger sequencing by commercial service (Sangon Biotech, Shanghai, China). Sequence alignment and homology comparison were made using ClustalW method in MegAlign program (DNASTAR, Madison). The phylogenetic tree of the *VP3* gene of NGPV was conducted using MEGA 6.0 with the neighbor-joining method (Tamura et al., 2013).

RESULTS

Coinfection of NGPV With DuCV

A total of 540 feather sacs were tested by the aforementioned GPV, NGPV, and DuCV detection methods (Li et al., 2014, 2017). NGPV and DuCV mixed infection was found in each flock (27 of 27), whereas GPV was not detected. Of 540 feather sacs tested, the infection of NGPV and DuCV was detected in 447 samples (82.78%) and in 426 samples (78.89%), respectively, and the coinfection of the 2 viruses was detected in 378 samples (70.00%) (Table 1). Meanwhile, of the 540 feather sacs tested, the infection of DuCV-1 and

Table 1. Detection of novel goose parvovirus–related virus (NGPV) and duck circovirus (DuCV) in 3 provinces by semi-nested PCR and duplex PCR.

Province	Flocks	Number tested of feather sac	Positive number of NGPV	Positive number of DuCV			Coinfection number of NGPV and DuCV		
				DuCV-1	DuCV-2	DuCV-1 and DuCV-2	NGPV and DuCV-1	NGPV and DuCV-2	NGPV, DuCV-1, and DuCV-2
Shandong	1	20	16	8	7	0	8	7	0
	2	20	18	6	6	1	5	6	1
	3	20	17	7	8	1	6	7	0
	4	20	14	7	9	0	6	7	0
	5	20	16	8	8	1	8	8	0
	6	20	14	3	8	0	3	7	0
	7	20	20	8	7	2	6	6	1
	8	20	15	6	8	0	5	7	0
	9	20	12	5	9	0	5	8	0
	10	20	18	4	5	1	4	5	1
	11	20	16	5	10	1	5	9	1
	12	20	16	8	8	2	7	6	1
	13	20	15	10	7	0	9	5	0
	14	20	19	9	8	0	9	6	0
	15	20	16	7	7	1	6	7	1
Jiangsu	16	20	18	10	4	0	8	4	0
	17	20	17	9	6	0	7	4	0
	18	20	15	11	5	1	10	3	1
	19	20	19	9	5	1	8	4	1
	20	20	20	16	5	1	14	5	1
	21	20	17	12	4	0	10	3	0
Anhui	22	20	19	12	5	3	12	3	3
	23	20	18	18	4	1	18	4	1
	24	20	16	10	3	1	10	3	1
	25	20	14	15	3	2	14	3	2
	26	20	17	8	3	0	7	3	0
	27	20	15	8	3	2	8	2	2
Total	27	540	447	239	165	22	218	142	18

DuCV-2 were reported in 239 (44.26%) and 165(30.56%), respectively, whereas the coinfection of the 2 genotypes of DuCV was reported only in 22 samples (4.07%). Of the 447 NGPV-positive samples, DuCV was reported to be positive in 378 samples (84.56%), and DuCV-1 and DuCV-2 were reported to be positive in 218 (48.77%) and 142 (31.77%) samples, respectively. Only 18 (4.02%) ducks were simultaneously infected with NGPV, DuCV-1, and DuCV-2.

Comparison of NGPV and DuCV Infection in Different Age Ducks

In this study, the feather sac samples were collected from 2- to 6-week-old sick ducks with FSS in different duck flocks from 3 provinces of East China (Table 2). The positive rates of NGPV and DuCV were 78.33 to 86.11% and 60.00 to 86.67% in sick ducks of different ages, respectively. The coinfection rates of NGPV and DuCV in 4- to 5-week-old ducks (76.67–80%) were significantly higher than others (48.33–63.33%), which was consistent with the higher incidence and more obvious FSS in 4- to 5-week-old ducks. It suggested that the appearance of new clinical symptoms in sick ducks might be related to the coinfection of NGPV and DuCV.

Novel Goose Parvovirus–Associated Virus VP3 Gene Sequence Analysis and Phylogenetic Analysis

The *VP3* gene length of the 9 NGPV strains isolated in this study was 1605 bases, and the sequences were submitted to GenBank to obtain accession numbers (Table 3). Based on the complete nucleotide sequence of *VP3* gene, the 9 NGPV strains in this study shared 92.5 to 98.0% identity with classical GPV, 80.9 to 91.0% identity with MDPV, 99.3 to 100% identity with each other, and 98.8 to 99.2% identity with other NGPV strains. Phylogenetic analysis of the complete

Table 2. Infection of novel goose parvovirus–related virus (NGPV) and duck circovirus (DuCV) in ducks of different ages.

Ages (wk)	Number tested	Positive number (rate) of NGPV	Positive number (rate) of DuCV	Coinfection number (rate)
2	60	49 (81.67%)	36 (60.00%)	29 (48.33%)
3	120	94 (78.33%)	89(74.17%)	75 (62.50%)
4	180	155 (86.11%)	156 (86.67%)	144 (80.00%)
5	120	101 (84.17%)	103 (85.83%)	92 (76.67%)
6	60	48 (80.00%)	42 (70.00%)	38 (63.33%)
Total	540	447 (82.78%)	426 (78.89%)	378 (70.00%)

Table 3. The 9 novel goose parvovirus–related virus (NGPV) strains isolated from feather sac samples of Cherry Valley ducks with the feathers shedding syndrome in this study.

Virus	Province	Isolation time	Tissue	Host	Age (day)	*VP3* gene size (bp)	GenBank accession no.
AH0225	Anhui	Feb., 2019	feather sac	Cherry Valley duck	27	1,605	MN153039
AH0309	Anhui	Mar., 2019	feather sac	Cherry Valley duck	27	1,605	MN172359
JS0401	Jiangsu	Apr., 2019	feather sac	Cherry Valley duck	30	1,605	MN172360
JS1201	Jiangsu	Dec. 2018	feather sac	Cherry Valley duck	25	1,605	MN172361
SD0103	Shandong	Jan., 2019	feather sac	Cherry Valley duck	32	1,605	MN172362
SD0227	Shandong	Fed., 2019	feather sac	Cherry Valley duck	29	1,605	MN172363
SD0312	Shandong	Mar., 2019	feather sac	Cherry Valley duck	24	1,605	MN172364
SD1108	Shandong	Nov., 2018	feather sac	Cherry Valley duck	31	1,605	MN172365
SD1206	Shandong	Dec., 2018	feather sac	Cherry Valley duck	19	1,605	MN172366

amino acid sequence of VP3 protein indicated that classical GPV, MDPV, and NGPV were classified into 3 different groups. All the NGPV strains clustered into 2 genotypes: genotype 1 included the previous 9 strains isolated from the sick ducks with BADS but no FSS, and the 9 NGPV strains isolated from the sick ducks with FSS in this study formed an independent branch within the NGPV group (Figure 1). Compared with other NGPV strains, the 9 NGPV strains isolated in this study have 5 special amino acid mutation points, "A" at position 63, "S" at position 300, "A" at position 322, "S" at position 336, and "H" at position 462 in VP3 protein (Figure 2).

DISCUSSION

DuCV is a nonenveloped single-stranded closed-closed DNA virus (Hattermann et al., 2003). Its genome contains 3 major ORF, ORF1 and ORF2 encoding the viral replication-associated protein and immunogenic capsid protein, whereas ORF3 encoding the ORF3 protein with apoptotic activity (Hattermann et al., 2003; Xiang et al., 2012). Based on the complete genome and *Cap* gene analysis, the virus can be divided into 2 genotypes (DuCV-1 and DuCV-2) (Jiang et al., 2008; Zhang et al., 2012; Wen et al., 2014). DuCV can spread horizontally and vertically (Liu et al., 2010; Li et al., 2014).

DuCV infection causes growth retardation and feathering disorders in ducks (Hattermann et al., 2003; Soike et al., 2004; Cha et al., 2014; Hong et al., 2018). Beak abnormality and growth retardation are the common symptoms in parrots infected by beak and feather disease virus and geese infected by goose circovirus (McOrist et al., 1984; Soike et al., 1999). In this study, DuCV was detected in 426 (78.89%) of

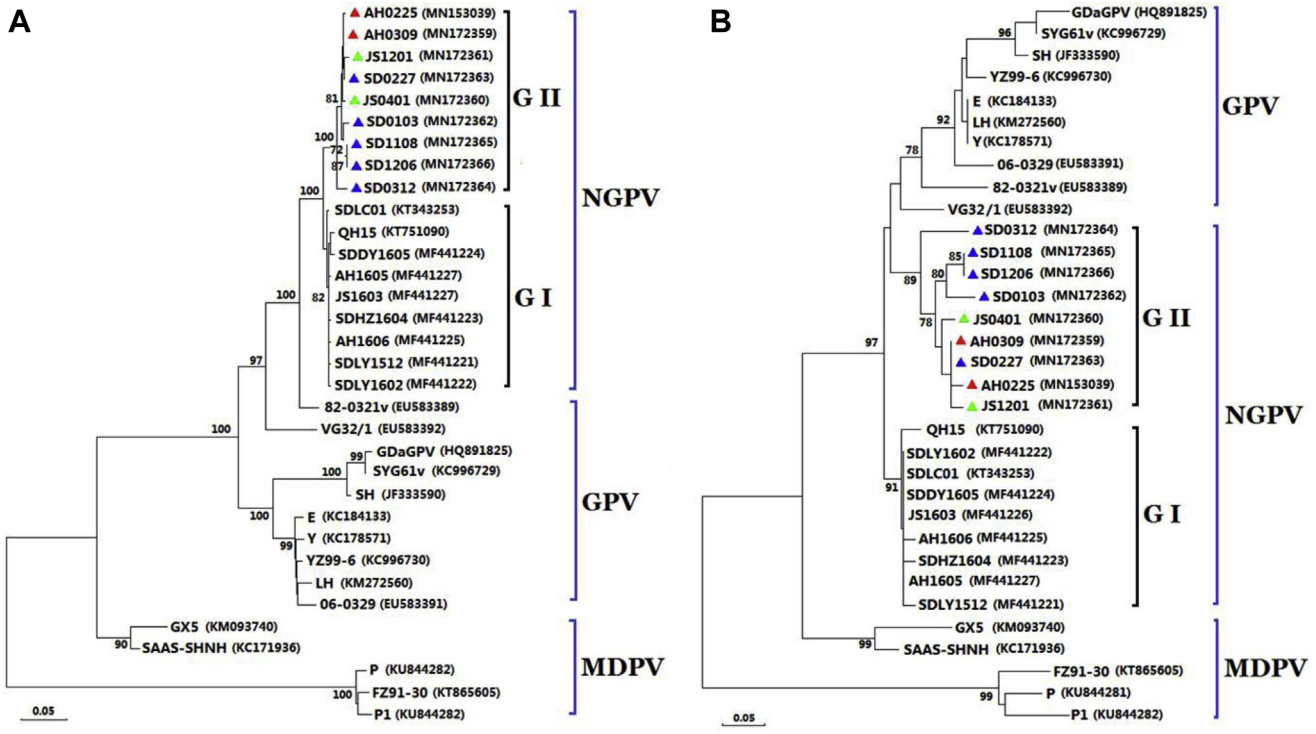

Figure 1. Phylogenetic (neighbor-joining) trees based on VP3 nucleotide sequences (A) and amino acid sequences (B) of goose parvovirus (GPV), novel goose parvovirus–related virus (NGPV), and Muscovy duck parvovirus (MDPV). The values at the forks indicate the percentage of trees in which this grouping occurred after bootstrapping the data (1000 replicates; shown only when >70%). The scale bar shows the number of substitutions per base. Isolates in this study were marked with different color triangles. Abbreviations: G I = genotype 1; G II = genotype 2.

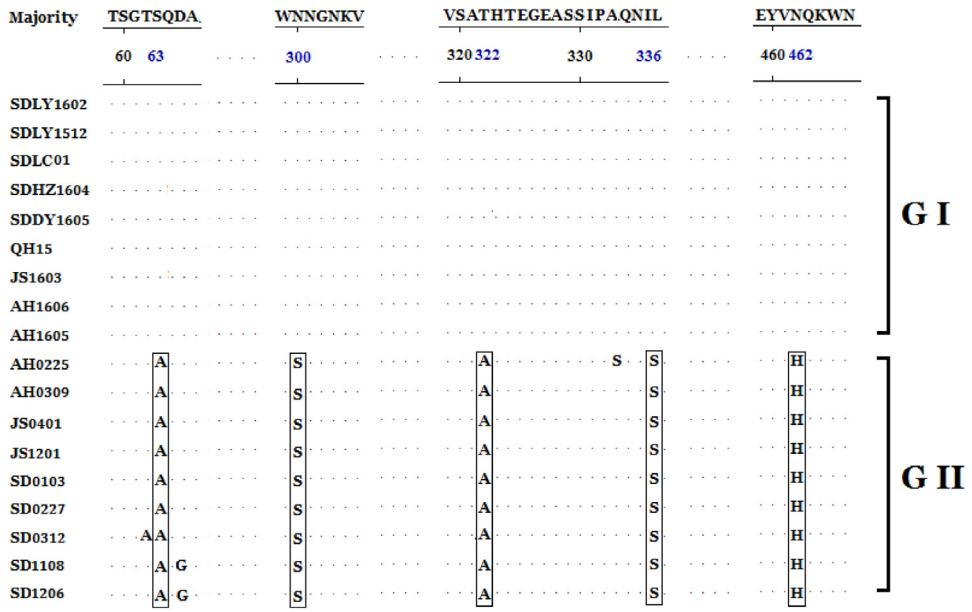

Figure 2. The common 5 specific amino acid mutation points in VP3 protein of the 9 novel goose parvovirus-related virus (NGPV) strains isolated from feather sac samples in this study. The mutated amino acids are marked with black frame. Abbreviations: G I = genotype 1; G II = genotype 2.

540 feather sac samples collected from the sick ducks with obvious FSS (Table 1, Figure 1), which suggested that the FSS might be related to DuCV infection. The ORF3 protein of DuCV-1 induced a higher level of apoptosis than that of DuCV-2, which showed that there were differences in the pathogenicity of the 2 genotypes of DuCV (Wu et al., 2018). In the study, DuCV-1 and DuCV-2 were positive in 239 (44.265) and 165 (30.56%) of 540 feather sac samples, respectively (Table 1).

Cases of coinfection with parvovirus and circovirus have been reported in pigs, canines, and wild carnivores (Sun et al., 2015; Thaiwong et al., 2016; Zaccaria et al., 2016; Anderson et al., 2017). Novel goose parvovirus–associated virus was identified as the causative agent of BADS (Chen et al., 2015, 2016), but only 20% clinically susceptible ducks assumed the typical clinical and histologic changes of BADS by being infected with wild NGPV isolate in laboratories (Ning et al., 2017). Our previous study has shown that the positive rate of coinfection of NGPV and DuCV is very high in ducks with BADS (Li et al., 2018b). In this research, NGPV and DuCV were detected from the feather sac samples of sick ducks with FSS for the first time, and the coinfection rate of the 2 viruses was detected as high as 70.00%. To 4- to 5-week-old ducks, the significantly higher coinfection rate of NGPV and DuCV (76.67–80.00%) was consistent with the higher incidence and more obvious FSS. The results suggested that the appearance of the new FSS disease in ducks might be closely related to the coinfection of NGPV and DuCV.

Previous studies reported that NGPV isolates share 89.7 to 96.7% identity with classical GPV (Chen et al., 2015, 2016; Ning et al., 2017; Li et al., 2018a). In this study, the 9 NGPV strains shared 92.5 to 98.0% homology with classical GPV. Furthermore, higher homology with 98.8 to 99.2% nucleotide identity in the *VP3* gene was found between feather sac–originated NGPB and other NGPV strains. Phylogenetic analysis showed that the 9 feather sac–originated NGPV strains and other previous NGPV strains were respectively clustered into 2 independent branches (Figure 1). In comparison with previous NGPV strains, 5 amino acid mutations specifically existed in the VP3 protein of feather sac–originated NGPV, posing possible functional and structural changes in the VP3 protein. However, studies with respect to the function and structure of the parvovirus VP3 protein have been barely reported. Thus,we analyzed and modeled the secondary and tertiary structure of NGPV VP3 protein (predictprotein.org and https://swissmodel.expasy.org/). Intriguingly, we found mutations A63S, S300N, A322T, and S336I located in the variable area, but H462N was outside the variable area (Figure 3). Moreover, mutations S300N, A322T, and H462N belonged to part of loops in secondary structure, which was commonly recognized as the combination site of the antigen and antibody (Murre et al., 1994; Stinson et al., 2003). We then constructed tertiary 3-dimensional structure of VP3 protein. Different spatial conformations were described for the 5 amino acid mutations (Figure 4). These intriguing findings trigger important assumptions that the 5 mutations in capsid protein might influence protein function and structure, as well as virus tropism to some extent. However, it is difficult to make any firm conclusions for these assumptions with little information. Further studies are required to facilitate the impact of these amino acids mutations.

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Figure 3. The conservation scale of VP3 protein. The secondary structure was analyzed by predictProtein (predictprotein.org).

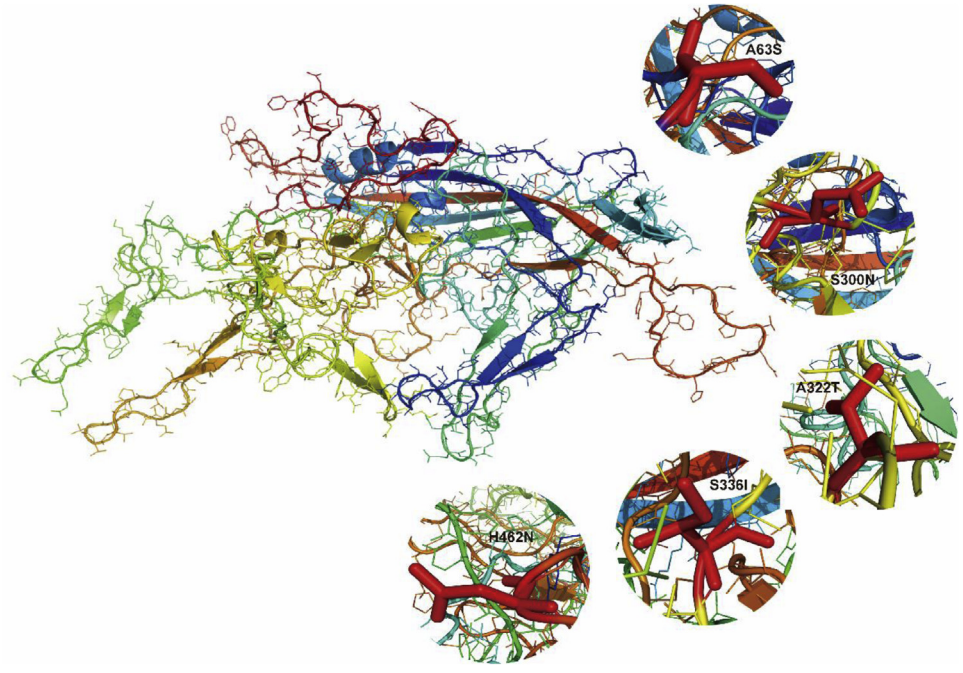

Figure 4. Tertiary structure of novel goose parvovirus-related virus (NGPV) VP3 protein. The model of VP3 protein was analyzed by Swiss-model. The 3D structure was constructed using PyMOL.

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Conflict of Interest Statement: The authors declare that they have no competing interests.

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