



## ARTICLE

# Molecular Analysis of Pathogenicity Differences of Avian Paramyxovirus 1 Genotypes VI and VII in Chickens

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### ARTICLE INFO

#### Article history

Received: 29 April 2019

Accepted: 12 May 2019

Published Online: 1 June 2019

#### Keywords:

Avian avulavirus-1

Genotype

Pathogenicity

Chicken

Molecular determinant

### ABSTRACT

Background: Genotypes VI and VII of (APMV-1) have different host range and pathogenicity in pigeons and chickens. However, the molecular determinants of these differences are still unclear. Methods: Here, we aligned the DNA sequences of 56 genotype VI and 33 genotype VII APMV-1 strains. Sequence alignment results revealed that there are 17 amino acids sites differed between APMV-1 strains of these two genotypes. We then constructed a plasmid based on the full-length genome of rSG10 APMV-1 strain, which belongs to genotype VII but was mutated with these 17 VI-genotype-specific amino acids, and rescued as rSG10-17 strain. The restriction digestion and ligation and overlapping PCR methods were used in the construction of plasmids with amino acids mutation. This virus was evaluated for its virulence and growth characteristics. Results and conclusion: The results indicated that the virulence and the growth characteristics have no obvious difference between the rSG10-17 virus and its parental strain rSG10. The simultaneous mutation of 17 genotype-specific amino acids did not affect the virulence of APMV-1 in chickens. Further analysis of these amino acids is required by taking into consideration of the functions of encoded proteins.

## 1. Introduction

Newcastle disease (ND) is a highly infectious viral disease in the poultry industry caused by Avian avulavirus-1 (APMV-1), which is taxonomically classified in the genus *Orthoavulavirus* in the family of *Paramyxoviridae*.<sup>[1]</sup> APMV-1 is an enveloped virus with a non-segmented, negative-sense, single-stranded RNA genome of approximately 15 kb. The viral RNA encodes six structural proteins, including nucleoprotein (N), phosphoprotein (P), matrix (M), fusion (F), hemagglutinin-neuraminidase (HN), and large protein (L), and two

nonstructural proteins, V and W.<sup>[2]</sup>

Since it was first discovered in Newcastle-on-Tyne in 1926, there has been 18 genotypes and caused four panzootics.<sup>[3, 4]</sup> Pigeon paramyxovirus type 1 (PPMV-1), which is belonged to genotype VI, was responsible for the third panzootics.<sup>[5]</sup> PPMV-1 isolates first appeared in the late 1970s in the Middle East, and mainly infects birds in the family of Columbidae, which contains species of domestic and wild doves and pigeons.<sup>[6, 7]</sup> This panzootic peaked during the early 1980s. Although the reported cases have decreased since the panzoonosis, there is still occurrence in China and other countries.<sup>[8, 9]</sup> In general, the disease

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symptoms include a series of nervous disorders. Pathogenicity tests and monoclonal antibody binding studies have shown that PPMV-1 is a variant form of classical APMV-1. <sup>[10]</sup> Even though strains of PPMV-1 cause morbidity in pigeons, most of them are considered mesogenic or lentogenic according to the intracerebral pathogenicity index (ICPI) in chicken and they are unable to cause disease even for chicks. <sup>[11,12]</sup>

The genotype VII strains were responsible for the fourth panzootics. <sup>[13]</sup> And genotype VII APMV-1 has become the predominant strains circulating in the world since it was first isolated in 2000s. <sup>[14,15]</sup> Viruses of genotype VII have a wide range of host, and most birds are reportedly infected by the virus. <sup>[16]</sup> Almost all genotype VII APMV-1 strains are velogenic and result in more mortality in poultry. <sup>[17,18]</sup> Many studies also showed that strains of genotype VII APMV-1 induced more innate immune response and cell death in lymphoid tissues compared with virulent strains of other genotypes. <sup>[19-21]</sup>

The complete genome of genotype VI and genotype VII strains is 15,192 nt, which could encode six structural proteins. Although the F protein of those viruses is associated with virulent APMV-1 strains, the pathogenicity in chickens is different obviously. However, the molecular determinants of pathogenicity differences are still unclear. In this study, we wanted to find amino acids which could influence the pathogenicity of genotype VI and genotype VII strains in chickens. 17 specific amino acids were identified between genotype VI viruses group and genotype VII viruses group. Then we used reverse genetics to study the functions of these amino acids, to identify the molecular determinant(s) of the different pathogenicities of these two APMV-1 genotypes in chickens.

## 2. Methods

### 2.1 Cells, Virus and Animals

BSR T7/5 cells expressing the T7 RNA polymerase were cultured in Dulbecco's modified eagle medium (DMEM; Gibco, Grand Island, NY, USA) containing 10% fetal bovine serum (FBS; Gibco) and G418 (1 mg/ml) (Invitrogen, CA, USA). The recombinant strain rSG10 was generated in our laboratory, which belongs to genotype VII, with an MDT of 45 h and ICPI value of 1.79. <sup>[22]</sup> The SPF chicken embryos and chickens used for the pathogenicity test were from Beijing Merial Vital Laboratory Animal Technology Co., Ltd, China. All of the birds used in this experiment were cared for in accordance with established guidelines, and the protocols were performed with the approval of the Animal Welfare and Ethical Censor Committee of China Agricultural University (CAU approval number 1901-01).

### 2.2 Phylogenetic and Sequence Analyses

The complete APMV-1 genomic sequences were obtained from NCBI, including 58 strains of genotype VI and 33 strains of genotype VII, and other reference strains known as APMV-1 genotype. A phylogenetic tree was constructed by the MEGA 4.0 (Molecular Evolutionary Genetics Analysis) with the neighbor-joining method (1000 replicates for bootstrapping). Nucleotide sequence editing, analysis, deduction of amino acids, and alignments were performed in the MegAlign program (v4.00) with the Clustal W multiple alignment algorithm in the Lasergene package.

### 2.3 Reverse Genetic Constructs

The construction of rSG10 has been described previously. <sup>[22]</sup> To investigate the function of the 17 genotype-specific amino acids in viral pathogenicity, the pOK-rSG10 full-length cDNA was mutated with overlapping PCR, and designated pOK-rSG10-17. The restriction digestion and ligation were used in the construction of plasmids and the enzymes sites were as same as rSG10. Because the APMV-1 rSG10 strain full-length antigenomic cDNA is made up of six fragments, we mutated each identified amino acid belonged genotype VII into genotype VI in the relevant fragment and then cloned those fragments into the pOK12 vector at seven restriction sites. The mutations were confirmed with DNA sequencing.

The method of rescue infectious APMV-1 was described previously. <sup>[23]</sup> Briefly, BSR T7/5 cells were cultured in six-well plates of 90% confluence and then transfected with 10 µg of DNA, containing a mixture of pOK-rSG10, pCI-NP, pCI-P and pCI-L, using Lipofectamine 2000 (Invitrogen). At 6 h post-transfection, the cells were washed with phosphate-buffered saline (PBS) and maintained in 2% (v/v) FBS DMEM. 72 hours later, the cells were harvested and SPF chicken embryos were used to recover the viruses. After incubation for 96 hours, the allantoic fluid was harvested and the rescued virus named rSG10-17 was collected with an HA test. The mutated amino acids were confirmed with DNA sequencing.

### 2.4 MDT and ICPI of the Virus

The pathogenicity of rSG10-17 was determined with an Mean Death Time (MDT) assay in 9-11 days old SPF chicken embryos and an Intracerebral Pathogenicity Index (ICPI) assay in day-old SPF chicks as described previously. <sup>[24]</sup> Briefly, for the MDT assay, the allantoic cavities of five 9 days old embryos were inoculated with serial 10-fold dilutions of allantoic fluid. The time at which each embryo was first observed dead was recorded. MDT was

calculated as the mean time (hours) required for the minimum lethal dose of the virus that killed all the inoculated embryos. For the ICPI assay, ten of day-old SPF chicks were inoculated with 50 $\mu$ l of a 1:10 dilution of fresh allantoic fluid via the intracerebral route. The clinical symptoms and mortality of the birds were monitored for every 12 h for 8 days. At each observation, the birds were scored as follows: 0 for normal, 1 for sick, and 2 for dead. The ICPI was the mean of the scores per bird per observation point over the observation period.

## 2.5 Viral Growth Kinetics

The growth kinetics of rSG10 and rSG10-17 were determined under multicycle growth conditions in DF-1 cells. The cells in duplicate wells of six-well plates were infected at a multiplicity of infection (MOI) of 0.01. After 1 h adsorption, the cells were washed once with pure DMEM and then incubated in 5% (v/v) CO<sub>2</sub> incubator at 37 °C. The supernatants were harvested at 12-h intervals for 72 h, and the viral titers were determined by TCID<sub>50</sub>.

## 3. Results

### 3.1 Phylogenetic and Sequence Analyses

To reconfirm the genotypes of the selected viruses, a phylogenetic tree was constructed based on the nucleotide sequences of the F genes of the 91 viruses and other reference strains (Figure 1). The phylogenetic tree showed that 58 strains clustered in the genotype VI group and 33 strains clustered in the genotype VII group. Therefore, the selected viruses could be used for further study. We set the 58 genotype VI viruses as one group and the 33 genotype VII viruses as another group, and analyzed the similarities of the complete genomes and the coding sequences of each gene between the two groups. The results showed that the complete genomes of the two genotypes shared 86.4%–91.7% identity (Table 1). For the contractual proteins, the NP and L genes were relatively conserved between two genotypes, with amino acid identities of 91.7%–98% and 94.2%–97.2%, respectively. The P gene was the most variable, with amino acid identities of 78.8%–89.9% among this two groups. Therefore, from an evolutionary perspective, the NP and L proteins are relatively well conserved and the P protein is the least conserved.

### 3.2 Genotype-specific Amino Acid Analysis

To analyze the molecular determinant(s) of the difference in pathogenicity of APMV-1 genotypes VI and VII, we aligned the amino acid sequences of every structural protein of the 91 APMV-1 strains. We identified 17 consensus

amino acids that are conserved among the genotype VI viruses, which were also completely conserved among the genotype VII strains but differed between the two genotypes (Table 2). Therefore, these genotype-specific amino acids might be the common characters shared by the viruses of a single genotype that contribute to the differences between the two genotypes.

Of the identified 17 genotype-specific amino acids, one site located in each M, F, and HN proteins coding region, while the NP and P proteins each contain two sites. Surprisingly, the L protein contains 10 sites of these amino acids. To exclude the high number of conserved sites in L protein was due to its relatively large scale, we divided the number of genotype-specific amino acids in each protein by the total number of amino acids of that protein. The ratio was represented as the proportion of genotype-specific amino acids in each protein (Table 3). We found that the viral replication complex contains more genotype-specific amino acids than the M, HN, and F proteins, although the NP and L proteins are relatively conserved between the two viral genotypes. The L protein also has a high ratio of conserved genotype-specific amino acids, which indicates it is not attributed to the length of the protein.

### 3.3 Recovery of Strain rSG10-17

To study the role of the 17 genotype-specific amino acids, the cDNA clone pOK-rSG10-17 encoding the antigenome of strain SG10, with mutations at the 17 genotype-specific amino acid sites was constructed with reverse transcription PCR from the genomic RNA. The cDNA sequence analysis confirmed the intended amino acid mutations. According to the methods, the strain rSG10-17 was rescued and detected with a hemagglutination (HA) test. Then strain rSG10-17 was amplified by RT-PCR and confirmed the correct mutation sites and a lack of adventitious mutations.

### 3.4 Mean Death Time (MDT) and ICPI Test of rSG10-17

To determine the contributions of the genotype-specific amino acids to the pathogenicity of APMV-1 in chickens, the virulence of the rSG10-17 viral strain was evaluated by determining its MDT and its ICPI. The MDT value for rSG10-17 was 48 h, and its ICPI was 1.92. The results indicated that the virulence and the growth characteristics have no obvious difference between the rSG10-17 virus and its parental strain rSG10.

### 3.5 Growth Characteristics of rSG10-17 and rSG10

To further compare the properties of strains rSG10-17

and its parental rSG10, the kinetics and final viral titers under multistep growth conditions in DF-1 cells were analyzed. The kinetics and replication of rSG10-17 were very similar to those of rSG10, which meant that rSG10-17 retained the replicate characteristics of the parental virus (Figure 2).

#### 4. Discussion

Researchers have reported that genotype VI and VII of APMV-1 have different pathogenicities in chickens.<sup>[2,12,13]</sup> However, the molecular determinants of this difference are still unclear. In this study, we aligned 91 APMV-1 strains and identified 17 genotype-specific amino acids. Then we used a reverse genetics method to investigate the functions of these amino acids. The results indicated that these mutations do not influence the virulence of the virus. However, these amino acids located in different functional proteins regions, thus we need to further analyze of the effects of these individual amino acids by taking the protein function into consideration.

While all APMV-1 viruses belong to a single serotype, the virulence among different strains is varied. The virulence of a virus is determined by its tissue tropism, efficacy of replication and ability to deal with the host immune response. The cleavage sites of the F protein were known as a primary determinant and some other genes have also been shown to contribute to the virulence of APMV-1.<sup>[25,26]</sup>

As is well known, the velogenic and mesogenic APMV-1 viruses contain a polybasic amino acid motif<sup>[112]</sup> (K/R)–R–(Q/K)–(R/K)–R<sup>[116]</sup> and a phenylalanine at the position 117 of F protein, which could be cleaved by furin-like proteases, resulting in systemic infections. However, the lentogenic viruses can only replicate in the respiratory and intestinal tracts.<sup>[27]</sup> The F protein of genotypes VI and VII viruses are associated with virulent APMV-1 strains, but their pathogenicity in chickens is very different. It is reported that besides the proteolytic cleavage site, other regions of the F protein were also influence pathogenicity.<sup>[28]</sup> The F protein is a class I transmembrane protein that is synthesized as a precursor protein F0 and functional domain of the F protein are signal peptide, fusion peptide, three heptad repeats, transmembrane domain.<sup>[29]</sup> Only one of the selected amino acids residues at position 52 (I52 of genotypes VI and V52 of VII viruses) of the F protein, but not in any already known functional domain of the F protein.

There is a correlation between virulence and the efficiency of viral replication. It has been reported for APMV-1 that the replication complex is related with the virulence.<sup>[30]</sup> The APMV-1 replication complex is made up of the NP protein, P protein, and L protein. The NP protein

encapsidates the RNA genome, which acts as the template for viral transcription and replication, forming the nucleocapsid. The P protein and L protein make up the viral polymerase, which transcribes the viral genomic RNA.<sup>[31]</sup> Several studies have demonstrated an increased virulence after the passage of some PPMV-1 isolates in chicken eggs, and they identified the amino acids changed in the L protein during the process.<sup>[32]</sup> Dortmans JC et al. have rescued recombinant viruses, including the viral replication complex of one PPMV-1 virus, using reverse genetics. The researchers' conclusion was that the viral replication complex is related with the virulence of APMV-1.<sup>[33]</sup> In this study, although the NP and L proteins were relatively conserved between genotypes VI and VII viruses, they have 12 genotype-specifically conserved amino acids. And this meant the NP and L proteins may associated with the virulence of PPMV-1 virus as other studies reported. From nucleotide and amino acid sequence similarities, we know that P is the least conserved protein of APMV-1. But the ratio that represented the proportion of selected amino acids of P protein is high. Therefore, we infer that these genotype-specifically conserved amino acids in the replication complex may influence the pathogenicity of the virus, even though, the growth characteristics of rSG10-17 were very similar to those of rSG10 in DF-1 Cells. It is possible that some of these 17 amino acid sites positively affect the pathogenicity of APMV-1 and others may have a negative effect.

HN and M protein also contain genotype-specific amino acids. The HN proteins are multifunctional molecules with three distinct activities: binding activity, neuraminidase activity and fusion-promotion activity.<sup>[34,35]</sup> It is reported that HN protein also affect virulence.<sup>[36]</sup> The genotype-specific amino acid residues at position 390 of HN protein, belonging to the globular head domain. Maybe this genotype-specific amino acids influence the binding activity and neuraminidase activity. The matrix protein (M), plays a crucial role in the viral replication. The genotype-specific amino acid residues at position 193 of M protein. A previous study demonstrated that arginine 36 (R36) of the M protein is a key factor that evolved in the adaptation of PPMV-1 to pigeons.<sup>[37]</sup>

In this study, we only focus on the influence of six structural proteins on the different pathogenicity. We did not study the unique amino acid in V proteins in the manuscript. However, several studies have shown that the nonstructural V protein is also closely associated with the pathogenicity and host range restriction of APMV-1.<sup>[38,39]</sup> So the role of V protein in the different pathogenicity between these two viral genotypes will be evaluated in the future study.

In conclusion, to analyze the molecular basis reasons of the differences in chicken pathogenicity between genotypes VI and VII of APMV-1, we simultaneously mutated all the genotype-specific amino acids in APMV-1 to investigate their influence on the pathogenicity of the virus. Although the ICPI and MDT values of strain rSG10-17 did not differ from those of the parental strain, the effects of each genotype-specific amino acid should be investigated individually in the further study, the function of the relevant protein should also be taken into consideration at the same time.

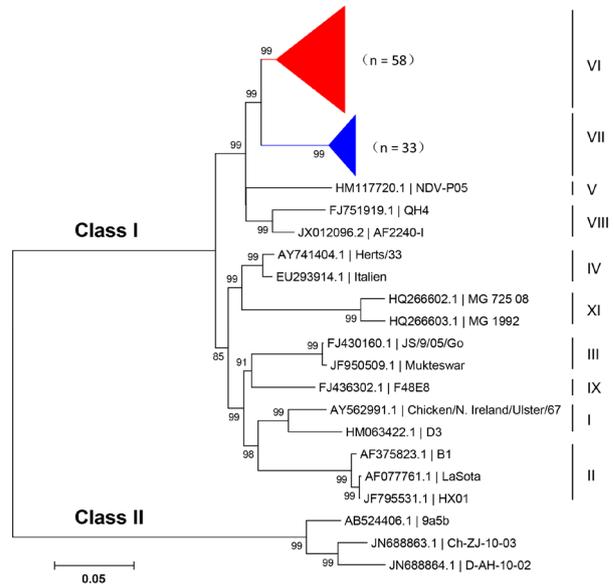
**Acknowledgments**

This study was supported by the Beijing Agriculture Innovation Consortium of Poultry Research System (BAIC04-2019). We thank Ye Zhao, PhD, from China Agricultural University for editing a draft of this manuscript.

**Conflicts of Interest**

The authors declare no conflicts of interest relevant to this study.

**SupplementsFigures**

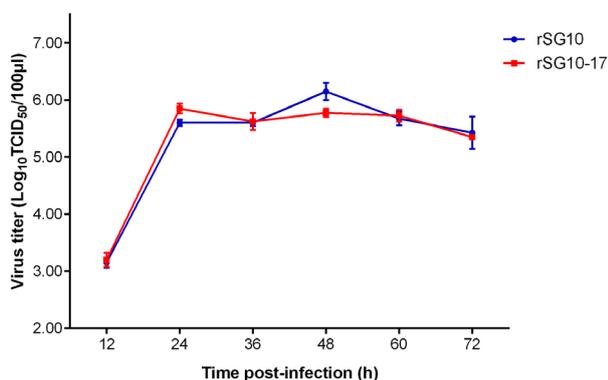


**Figure 1.** The phylogenetic tree based on the nucleotide sequences of the fusion (F) gene of NDV

*Note:* This phylogenetic tree was constructed using the neighbor-joining method with 1000 bootstrap replicates. The red and the blue branches represent viruses of genotypes VI and VII, respectively.

**Table 2.** Seventeen consensus amino acid sites that differ between 58 genotype VI and 33 genotype VII APMV-1 strains

Genotype	Virus	NP <sup>450</sup>	NP <sup>464</sup>	P <sup>162</sup>	P <sup>243</sup>	M <sup>193</sup>	F <sup>52</sup>	HN <sup>1390</sup>	L <sup>329</sup>	L <sup>423</sup>	L <sup>1096</sup>	L <sup>1241</sup>	L <sup>1334</sup>	L <sup>1495</sup>	L <sup>1542</sup>	L <sup>1840</sup>	L <sup>2017</sup>	L <sup>2107</sup>	
		L	S	E	Y	A	I	V	D	K	V	K	K	S	I	F	R	T	
VI	01. AB853926.2	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	
	02. AB853928.2	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	
	03. AY562989.1	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	
	.....	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	
	56. KM374059.1	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	
	57. KM374060.1	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
	58. KM374061.1	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
		F	P	G	H	V	V	I	N	N	I	N	R	N	V	Y	K	F	
VII	01. AF431744.3	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	
	02. DQ485229.1	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	
	03. DQ485230.1	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	
	.....	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	
	31. KC542914.1	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	
	32. KC853019.1	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	
	33. KM885167.1	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	



**Figure 2.** Multistep growth curves for rSG10 and aSG10-17 in DF-1 cells

**Note:** The cells were infected with the indicated APMV-1 viruses at 0.01 MOI. Samples were harvested at 12h intervals for 72 h, and the viral titers were determined with the TCID<sub>50</sub> method in DF-1 cells.

**Table 1.** Nucleotide (nt) and amino acid (aa) sequence identities (%) between 58 genotype VI and 33 genotype VII APMV-1 strains

	Nucleotide sequence identity (%)	Amino acid sequence identity (%)
<b>Genome</b>	86.4-91.7	
<b>NP</b>	87.3-92.7	91.7-98.0
<b>P</b>	82.5-90.2	78.8-89.9
<b>M</b>	86.8-93.6	90.7-97.0
<b>F</b>	86.6-93.2	90.6-96.9
<b>HN</b>	85.6-92.0	89.2-95.3
<b>L</b>	88.6-93.3	94.2-97.2

**Table 3.** Ratios of the number of genotype-specific amino acids to the total amino acids in each APMV-1 protein

Protein	Total amino acids	Selected amino acid sites	No. selected amino acid sites / No. total amino acids (%)
<b>NP</b>	490	2	0.41
<b>P</b>	396	2	0.51
<b>M</b>	365	1	0.27
<b>F</b>	554	1	0.18
<b>HN</b>	571	1	0.18
<b>L</b>	2205	10	0.45

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