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Complete genome sequence of avian paramyxovirus type 3 reveals an unusually long trailer region

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Summary

The complete genome sequence was determined for prototype parakeet/Netherlands/449/75 strain of avian paramyxovirus (APMV) serotype 3. The genome is 16,272 nucleotides (nt) in length, consisting of six non-overlapping genes in the order of 3'-N-P/V/W-M-F-HN-L-5', with intergenic regions of 31–63 nt. APMV-3 genome follows the “rule of six” and is the largest among the avian paramyxoviruses reported to date, with a trailer region of 707 nt, the longest in the family *Paramyxoviridae*. The cleavage site of F protein, A-R-P-R-G-R↓L, does not conform to the preferred cleavage site of the ubiquitous cellular protease furin. Therefore, exogenous protease was needed for replication *in vitro*. Alignment and phylogenetic analysis of the predicted amino acid sequences of strain Netherlands proteins with the cognate proteins of viruses of all of the five genera of family *Paramyxoviridae* showed that APMV-3 strain Netherlands is more closely related to APMV-1 than APMV-6.

Keywords

APMV; Avian paramyxovirus; *Paramyxoviridae*; *Paramyxovirinae*; Genome sequence

Introduction

The family *Paramyxoviridae* includes viruses of many species of avian, terrestrial and aquatic animals isolated worldwide (Lamb and Parks, 2007; Nylund et al., 2007). Members of this family are characterized by pleomorphic enveloped particles that contain a single-stranded, negative sense RNA genome (Lamb and Parks, 2007). The family is divided into two subfamilies, *Paramyxovirinae* and *Pneumovirinae*. Subfamily *Paramyxovirinae* is further divided into five genera: *Rubulavirus* (including mumps virus, human parainfluenza viruses [HPIV] 2 and 4, simian virus type 5 [SV5] and Tioman virus [TiV]), *Respirovirus* (including Sendai virus [SeV] and HPIV-1 and 3), *Henipavirus* (comprising Hendra virus [HeV] and Nipah virus [NiV]), *Morbillivirus* (including measles [MeV] and canine distemper [CDV] viruses), and *Avulavirus* (comprising avian paramyxovirus [APMV] serotypes 1 to 9). Subfamily *Pneumovirinae* is divided into two genera: *Pneumovirus* (comprising human

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respiratory syncytial virus [HRSV] and its animal counterparts including bovine respiratory syncytial virus [BRSV]), and *Metapneumovirus* (comprising human metapneumovirus [HMPV] and avian metapneumovirus [AMPV]) (Lamb et al., 2005; Mayo and Pringle, 1998; Pringle, 1998; Rima et al., 1995).

The genomes of members of family *Paramyxoviridae* range in length from 15–19 kb and contain six to ten genes in a linear array (Lamb and Parks, 2007). Transcription begins at a single promoter at the 3' leader end and the genes are copied into individual mRNAs by a start-stop-restart mechanism guided by conserved gene-start and gene-end transcription signals that border the individual genes (Lamb and Parks, 2007). Genome replication involves the synthesis of a complete positive-sense copy of the genome that is called the antigenome and serves as a template for producing progeny genomes. All members of family *Paramyxoviridae* encode a nucleocapsid protein (N), a phosphoprotein (P), a matrix protein (M), a fusion protein (F), an attachment protein called the hemagglutinin (H) or haemagglutinin-neuraminidase (HN) or glycoprotein (G), and a large polymerase protein (L).

Many paramyxoviruses encode additional proteins that play important roles in viral morphogenesis, RNA synthesis, and pathogenesis (Lamb and Parks, 2007). For example, most members of subfamily *Paramyxovirinae* encode additional proteins from the P gene by RNA editing. This involves the introduction of one or more G residues into a subset of the P transcripts by polymerase stuttering at a specific editing sequence motif. This shifts the reading frame to access alternative ORFs in one or both of the alternative frames, resulting in the expression of proteins in which the upstream domain is P-derived and the downstream domain is derived from the alternative frame. In one such protein, called V, the downstream domain contains a cysteine-rich motif that is highly conserved across the subfamily. The V protein has been implicated in the regulation of viral RNA synthesis (Horikami et al., 1996; Lin et al., 2005) and in counteracting host antiviral responses (Goodbourn et al., 2000). All members of subfamily *Paramyxovirinae* follow the “rule of six”, whereby efficient RNA replication depends on the genome length being an even multiple of six (Calain and Roux, 1993; Kolakofsky et al., 1998; Samal and Collins, 1996).

The APMVs have been classified into nine different serotypes based on haemagglutination inhibition (HI) and neuraminidase inhibition (NI) assays (Alexander and Collins, 1984). Newcastle disease virus (NDV) belongs to serotype 1. It is the most extensively-characterized APMV and is a major pathogen of poultry worldwide (Alexander, 1980). APMV-2 has been isolated from chickens, turkeys and wild birds, and is responsible for causing decreases in egg production and fertility in turkeys (Lipkind et al., 1979; Tumova et al., 1979). The pathogenicity of APMV 4–9 is generally unknown except for a few isolated reports (Alexander, 1982). APMV-3 was first isolated from a turkey in 1968 in Wisconsin. Since then, many APMV-3 strains have been isolated from turkeys in different parts of the world including Canada, the United States (Tumova et al., 1979) and England (Macpherson et al., 1983). The APMV-3 strain parakeet/Netherlands/449/75, isolated from parakeets in the Netherlands, is the prototype for the entire serotype (Alexander and Chettle, 1978). APMV-3 was also isolated from non-domesticated species such as *Psittaciformes* and *Passeriformes* (Alexander, 1980). APMV-3 has been associated with encephalitis and high mortality in caged birds, and with respiratory disease in turkeys (Tumova et al., 1979). The virus causes acute pancreatitis and CNS symptoms in psittacine and *Passeriformes* birds (Beck et al., 2003). APMV-3 also infects chickens at an early age, with evidence of stunting growth that may be more marked in broiler chicken breeds (Alexander and Collins, 1982). In terms of pathogenicity, APMV-3 probably is second in importance only to NDV.

An understanding of the molecular and pathological characteristics of APMV-3 is of general interest and is important for developing vaccines against this virus. Here, we report the first

complete genome sequence of APMV-3 strain parakeet/Netherlands/449/75. The genome was found to be the longest among the APMVs and has the longest trailer region in family *Paramyxoviridae*. APMV-3 strain Netherlands would be predicted to be avirulent based on its F protein cleavage site and its observed requirement for exogenous protease for replication *in vitro*.

Materials and Methods

Virus and cells

APMV-3 strain parakeet/Netherlands/449/75 was obtained from the National Veterinary Services Laboratory, Ames, Iowa. Nine-day-old specific pathogen free (SPF) embryonated chicken eggs. Chicken embryo fibroblast DF1 cells were used to propagate APMV-3 strain Netherlands. Hemagglutination (HA) titers of virus stocks were determined using 0.5% chicken RBC at room temperature. The ability of the virus to replicate in cell culture was examined in nine different established cell lines, each representing a different species of origin: DF1, chicken embryo fibroblast; QT35, Quail fibrosarcoma; MDBK, Madin Darby Bovine Kidney; MDCK, Madin Darby Canine Kidney; Vero, African green monkey kidney; HEp2, Human Epidermoid carcinoma; PK15, Pig Kidney; CEF, Chicken Embryo Fibroblast; and BHK21, Baby Hamster Kidney. All the cells, except DF1 cells, were grown in Eagle's minimum essential media (MEM) with 10% fetal calf serum (FCS), while DF1 cells were grown in Dulbecco's MEM containing 10% FCS. All the cells were grown in a 37°C incubator with 5% CO₂. Cell monolayers were infected with a 10⁻³ dilution of 2⁹ HA units of egg-grown APMV-3 strain Netherlands and, after 1 hr of adsorption, the viral inoculum was replaced with maintenance media containing 2% FCS. The growth of virus in each cell type was observed with and without the presence of 10% allantoic fluid, or 1–5 µg/ml of acetyl trypsin/ml (Gibco), or 1–5 µg/ml of α-chymotrypsin/ml (Sigma), each of which provided a source of protease for cleavage of the F protein if necessary. The cells were observed daily for cytopathic effects (CPE) and HA titers were recorded every 24 h until the fifth day post-infection. The virus titer in each cell type was quantified by plaque assay in DF 1 cells as previously described (Krishnamurthy et al., 2000). The cells were overlaid with DMEM containing 2% FCS and 0.9% methylcellulose that were further supplemented with 10% allantoic fluid as a source of proteases. The plaques were visualized by staining with 1% crystal violet after 4 days of infection.

Virus RNA isolation and sequence analysis

Total RNA from virus-infected DF1 cells was isolated using TRIZOL reagent, following the manufacturer's instructions (Invitrogen, USA). Viral RNA also was isolated from allantoic fluid collected from virus-infected eggs, using RNeasy kit according to the manufacturer's instruction (QIAGEN, USA).

Most of the APMV-3 genome, except the 3' and 5' termini, was copied and amplified into cDNA using (i) gene-specific primers designed from the known APMV-3 M gene sequence, and (ii) consensus primers designed by using published *Rubulavirus*, *Respirovirus* and *Morbillivirus* genome sequences (Table 1). All primers were synthesized by a commercial provider (IDT, USA). N 787 forward and N 1022 reverse consensus primers were used to amplify a 235 base pair (bp) fragment that served as one starting point for downstream genome walking (Chang et al., 2001). The primer designed from the available M gene sequence (GenBank Accession number [AY137207](#)) was used in conjunction with a consensus sequence designed for the F gene, F 350 reverse, to amplify a 800-bp product and F 842 reverse, to amplify a 1500-bp product that were used as a second starting point for downstream genome walking. The HN and L genes were amplified by using gene-start forward (GS forward) primers and consensus reverse primer (Table 1). Initial 500 nt in HN gene was amplified by consensus

GS forward and consensus HN550 reverse primer (Table 1), further downstream was amplified by HN gene-specific forward primer (HN428F correspond to 7047–7068 in antigenome sequence) and consensus HN1568 reverse primer. Furthermore, upstream of L gene was amplified by gene-specific HN forward primer (HN960F correspond to 7579–7603 in antigenome sequence) and consensus L1460 reverse primer (Table 1). Subsequently, downstream of L gene was amplified by L gene-specific forward and consensus L reverse primers listed in table 1.

The sequence of the 3' terminus was determined from cDNA prepared by rapid amplification of cDNA ends (RACE) (Li et al., 2005; Troutt et al., 1992). Genomic RNA obtained from partial purified virus was ligated to adopter1 (5' GGTTTTGCGGTAAAGGTGGAAGAGAAG 3'), using T4 RNA ligase according to the manufacturer's instructions (Invitrogen). The ligated RNA was purified and reverse-transcribed, using adopter2 (5' CCAAACGCCATTTCACCTTCTCTC 3') primer according to the manufacturer's instructions (Invitrogen). PCR was carried out with adopter 2 and a gene-specific reverse primer (complementary to nucleotides 450 to 472 in the antigenome sequence), using recombinant Taq polymerase (Invitrogen).

The sequence of the 5' end of the genome was determined from cDNA obtained by 5' RACE (Invitrogen). Briefly, the downstream end of the L gene and adjoining trailer region were reverse-transcribed using a gene-specific forward primer L6760F representing nucleotides 15411–15433 in the antigenome sequence. The resulting cDNAs were column-purified and tailed with dCTP or, in a parallel reaction, with dGTP, using terminal deoxynucleotide transferase (TdT, Invitrogen). The cDNAs were then amplified in separate reactions, using an L gene-specific forward primer L7308F representing nucleotides 15959–15981 in the antigenome sequence, and an anchored G primer (provided in the kit) in the case of product tailed with dC, or with oligo dC in the case of product tailed with dG.

PCR-amplified products and plasmid DNAs were sequenced by using BigDye terminator v 3.1 matrix standard kit (Applied Biosystem) and 3130xl genetic analyzer data collection software v3.0 (Applied Biosystem). All sequencing was done in a 3130xl genetic analyzer or 3100 Avant genetic analyzer, using the manufacturer's instructions. The entire genome was sequenced at least three times, and at least once from uncloned PCR product, to ensure a consensus sequence.

Sequence and phylogenetic tree analysis

Sequence similarity searches were conducted using the basic length alignment search tool (BLAST) from the National Center for Biotechnology Information (NCBI). DNA pair-wise alignment was done using MagAlign (clustalW) in a Lasergene6 software package. To construct phylogenetic trees and determine divergence among different genus, amino acid sequences were aligned using clustalW multiple alignment algorithm. Modified data sets were generated by bootstrapping samples using a Seqboot program, and modified data were analyzed by Protodist and Fitch program (Phylip 3.67).

Database accession numbers

The complete genome sequence of APMV-3 strain Netherlands has been submitted in GenBank under accession number **EU403085**. Different sequences from GenBank were used for designing primers and comparing the APMV-3 genome. These include **NC_003043** for APMV-6, **NC_002617** for NDV, **NC_003443** for hPIV2, **NC_006430** for SV5, **NC_004074** for Tioman virus, **NC_001552** for Sendai virus, **NC_001796** for hPIV3, **NC_003461** for hPIV1, **AY988601** for Nipah virus, **NC_001906** for Hendra virus **NC_001498** for Measles virus, **NC_001921** for Canine distemper virus and **NC_007454** for J virus.

Results

In vitro growth characteristics of APMV-3 strain Netherlands

APMV-3 strain Netherlands produced a titer of 2^8 – 2^{10} HA units in nine-day-old embryonated SPF chickens eggs four days post inoculation. It was necessary to include 10% allantoic fluid for replication of the virus in cell culture, indicating a requirement of external proteases for efficient cleavage of the F protein. Eight out of nine cell lines were found to support growth of APMV-3, as determined by observable CPE and HA activity of the infected cell culture supernatant (data not shown). In each of these cell lines, it was necessary to include 10% allantoic fluid for replication of the virus in cell culture, indicating a requirement of external proteases for efficient cleavage of the F protein. The virus failed to grow in MDCK cells even after three serial passages. The virus grew most efficiently in MDBK cells, followed by chicken embryo fibroblast, BHK21 and Vero cells. The peak HA titers of the different cell lines tested were: MDBK, 2^7 ; BHK21, 2^6 ; CEF, 2^6 ; Vero, 2^5 ; PK15, 2^3 ; DF1, 2^3 ; QT35, 2^2 ; and HEp2, 2^2 . Viral CPE involved rounding, detachment and syncytia formation. APMV-3 was able to produce distinct plaques under methylcellulose overlay in DF1 cells in the presence of 10% allantoic fluid. The growth of APMV-3 in different cell lines was evident after the addition of allantoic fluid which was not substituted by the addition of either trypsin or chymotrypsin in the medium.

Examination of partially-purified virus under an electron microscope showed that the particles were enveloped, pleomorphic but mostly spherical in shape, with a size ranging from 150–250 nm (data not shown).

Determination of the complete genome sequence of APMV-3 strain Netherlands

The genome of APMV-3 strain Netherlands consists of 16,272 nt (GenBank accession number [EU403085](#)). The genome organization is very similar to those of other members of family *Paramyxoviridae*. The genome encodes for six open reading frames (ORF) corresponding to putative proteins N, P, M, F, HN and L, from the 3' to 5' end of the genome. In addition, there is evidence of a cysteine-rich V ORF in the P coding region. 84.1% of the genome codes for protein, which is less than the average coding percentage (92%) of other members of subfamily *Paramyxovirinae* (Miller et al., 2003). The length, position and characteristics of the six genes and their intergenic sequences (IGS) are summarized in Table 2 and Table 3 and described in detail below.

The 3' leader sequence of APMV-3 consists of 55 nt, a length that is conserved among almost all the members of subfamily *Paramyxovirinae* (Krishnamurthy and Samal, 1998). The similarity of APMV-3 leader sequence to that of NDV and APMV-6 indicated similar promoter recognition patterns for both viruses (Fig 1a).

Surprisingly, the length of the APMV-3 5' trailer sequence is 707 nt, which is the longest among the members of family *Paramyxoviridae* sequenced to date. Also, the nucleotide assignment at the third position from the 3' leader (3'UGAU) and 5' trailer (ACUA 5') termini was different from those found in other APMVs and other members of subfamily *Paramyxovirinae*. Otherwise, the sequence of the genomic termini of APMV-3 strain Netherlands are closely related to those of APMV-1 and -6, but are different from those of the rubulaviruses, consistent with the classification of avian paramyxoviruses in the genus *Avulavirus* (Fig 1b). The sequences of the 3'leader and 5'trailer termini showed a high degree of complementarity (87.5 percent complementarity for the first 16 nt), suggestive of conserved elements in the 3' promoter regions of the genome and antigenome (Fig 1c). The gene-start sequence of APMV-3 strain Netherlands is 3'UCCUCGCCUU and is conserved exactly among the six genes (Table 3). The gene-end signal is slightly less well-conserved: the consensus

gene-end sequence is 3'AAUUA(U)₆, but among three of the six signals there is a total of four nt differences involving positions 2, 3, 4, and 6 (Table 3). The intergenic sequences of APMV-3 range in length from 31 to 63 nt.

The nucleocapsid (N) gene

The APMV-3 N gene is 1604 nt long and encodes an N protein of 457 amino acid. It contains a highly-conserved sequence motif, 322-FAPGNYSLLYSYAMG-336 (F-X4-Y-X3- α -S- α -AMG, where X is any amino acid and α is any aromatic amino acid), that corresponds to a motif previously identified in the central region of the N protein of other members of *Paramyxovirinae* and which has been implicated in the self assembly of N with RNA or with other N monomers (Lamb and Parks, 2007; Yu et al., 1998). The APMV-3 N protein has 39–41% amino acid sequence identity with that of APMV-1, 38.3% with APMV-6, 32–34% with *Rubulavirus*, 21–22% with *Respirovirus*, 26% with *Henipavirus*, 24% with *Morbillivirus* and 25.4% with J virus.

The phosphoprotein (P) gene

The APMV-3 P gene is 1378 nt long. The unedited version of the P mRNA encodes a P protein of 386 amino acids. The predicted P protein contains an abundance of phosphorylation sites, with 19 serine and 6 threonine residues identified as potential phosphorylation sites using NetPhos 2.0 software. The APMV-3 P protein has 22.8% amino acid sequence identity with that of APMV-1, 19% with APMV-6, 18%–21% with *Rubulavirus*, 9–12% with *Respirovirus*, 13–15% with *Henipavirus*, 12% with *Morbillivirus* and 11.4% with J virus.

The P gene contains a putative editing site, 5'-TTTTTAAAGGGGG (mRNA sense), at positions 362–374 in the P gene (positions 2076–2088 in the complete genome sequence), which showed a high degree of sequence identity with the editing sites in other members of subfamily *Paramyxovirinae* (Fig 2). The insertion of a single G residue (mRNA sense) at the editing site would shift the reading frame to encode a 248-amino acid V protein in which the N-terminal 112 amino acids were derived from P, and the downstream 136 amino acids include seven cysteine residues and one tryptophan residue that are highly conserved within *Paramyxovirinae* (Fig 3). Alternatively, the insertion of two G residues would shift the reading frame to access an internal ORF that is open for only 14 additional amino acids, yielding a 125-amino acid W protein.

The matrix protein (M) gene

The M gene of APMV-3 is 1478 nt long and encodes a 396-amino-acid M protein. The protein is highly basic with an isoelectric point of 9.3, which may be important for ionic interaction with acidic N protein (Lamb and Parks, 2007). The bipartite cluster, which comprises clusters of basic amino acids—either 'single cluster', containing three to five basic amino acids, or 'bipartite' sequence, containing two interdependent clusters of such amino acids separated by an intervening sequence, is clearly seen in the positions 257–272, which was similar to that described previously (Coleman and Peeples, 1993). The M protein of APMV-3 contains a putative nuclear localization sequence (NLS) at position 371–383 aminoacids, which was previously found in the C terminal of NDV M protein (Coleman and Peeples, 1993; Shihmanter et al., 2005). . The APMV-3 M protein has 26.3% amino acid sequence identity with that of APMV-1, 25.8% with APMV-6, 22%–24% with *Rubulavirus*, 15–18% with *Respirovirus*, 13–14% with *Henipavirus*, 13–15% with *Morbillivirus* and 15.9% with J virus.

The fusion protein (F) gene

The APMV-3 F gene is 1922 nt long and encodes for a 543-amino-acid F protein. The protein is predicted to be a type I transmembrane protein, similar to the F proteins of other members

of family *Paramyxoviridae*. It has a predicted signal anchor of residues 1–19 and a predicted transmembrane anchor of residues 491–513. The putative cleavage site of the APMV-3 F protein is A-R-P-R-G-R↓L (corresponding to 101–107 amino-acid position) (Fig 4).

The deduced amino acid sequence contains five putative N-linked glycosylation sites at positions 68 and 81 in the F2 subunit, and at positions 356, 435 and 485 in the F1 subunit, predicted using EXPASY software. The amino-acid sequence of APMV-3 contained heptad repeats located between position 105 to 189 similar to those of other fusion glycoproteins (Chambers et al., 1990). The F protein sequence of APMV-3 also contained an ALGVAT fusion peptide motif (amino acid positions 116–121 in F protein), which is conserved among other family members (Lamb and Parks, 2007). The APMV-3 F protein has 31.3% amino acid sequence identity with that of APMV-1, 30.6% with APMV-6, 29% with APMV-2, 24%–29% with *Rubulavirus*, 21–23% with *Respirovirus*, 21–23% with *Henipavirus*, 20–22% with *Morbillivirus* and 23.9% with J virus.

The hemagglutinin-neuraminidase protein (HN) gene

The HN gene is 1974 nt long and encodes a polypeptide of 577 amino acids. The HN protein is a type II integral membrane protein that spans the membrane once and has a predicted hydrophobic signal anchor domain spanning residues 27–46. Seven potential N-linked glycosylation sites were identified at positions 32, 57, 122, 312, 325, 383 and 502, using the Expasy, NetNGlyc program. The HN protein contains a high amount of acidic amino acids with an isoelectric point (pI) of 7.83. The HN proteins of subfamily *Paramyxovirinae* and the HA proteins of influenza viruses contain a conserved sequence, N-R-K-S-K-S, which is thought to contribute to the sialic acid binding site (Varghese et al., 1983). The amino acid sequence of APMV-3 HN protein contains a similar motif, N-R-K-S-C-S (237–243 amino-acid position in HN protein). The APMV-3 HN protein has 34% amino acid sequence identity with that of APMV-1, 31% with APMV-6, 31% with APMV-2, 40% with APMV-4, 18%–33% with *Rubulavirus*, 21–22% with *Respirovirus*, 16–17% with *Henipavirus*, 10–11% with *Morbillivirus* and 23% with J virus.

The large polymerase protein (L) gene

The L gene is 6913 nt long and encodes for 2198 amino-acids L protein. Alignment of L gene sequence of APMV-3 with other members of *Paramyxoviridae* demonstrated six linear conserved domains, which have been described earlier for these family members and represent catalytic domains (Poch et al., 1989). The conserved QGDNQ sequence that is found within domain III involved in transcription activity (Schnell and Conzelmann, 1995) was also found in L protein of APMV-3 at amino-acid positions 744–748. The APMV-3 L protein has 33% amino acid sequence identity with that of APMV-1, 36% with APMV-6, 32–34% with *Rubulavirus*, 25–26% with *Respirovirus*, 25% with *Henipavirus*, 25–26% with *Morbillivirus* and 24.7% with J virus.

Phylogenetic analysis

Phylogenetic trees were generated from amino acid sequence alignments of N, P, M, F, HN and L proteins of APMV-3 strain Netherlands with the cognate proteins of representative viruses of all five genera of family *Paramyxoviridae* (Fig 5). Based on N, P, M, F, HN and L protein phylogenetic trees, APMV-3 was phylogenetically closely related to NDV. Phylogenetic analysis of HN protein showed that APMV-3 was more closely related to that of APMV-4. All six trees clearly indicated the close genetic relationship between APMV-3 and APMV-1.

Discussion

The genome of APMV-3 is 16,272 nt long, which is larger than the other two APMV serotypes sequenced to date, APMV-1 (15,186 nt, 15,192 nt and 15,198 nt) (Czegledi et al., 2006; Krishnamurthy and Samal, 1998; Ujvari, 2006) and APMV-6 (16,232 nt) (Chang et al., 2001). The APMV-3 genome length is also larger than the typical genome size (approximately 15,500 nt) of other members of family *Paramyxoviridae*, but is smaller than the 18,234 nt genome of Hendra virus (Wang et al., 2000) and the 18,954 nt genome of J virus, the largest genome reported to date for *Paramyxoviridae* (Jack et al., 2005). The genome length of APMV-3 was consistent with the “rule of six” that is a characteristic of the subfamily *Paramyxovirinae* (Calain and Roux, 1993). The genome contains six genes (N, P, M, F, HN and L) that are also found in all members of the subfamily. The amino acid sequence relatedness of APMV-3 proteins with the corresponding proteins of APMV-1 and APMV-6 clearly places this virus in the genus *Avulavirus*, consistent with the International Committee on Taxonomy of Viruses statement that amino acid sequence relationships are the main criteria for grouping viruses into genera within the family *Paramyxoviridae* (Lamb et al., 2005). APMV-3 has a 55 nt 3' leader region, which is exactly conserved in length with all other known members of *Paramyxoviridae* (Lamb and Parks, 2007), and its sequence is generally conserved within subfamily *Paramyxovirinae*. For members of subfamily *Paramyxovirinae*, the first 12–13 nt genomic termini are genus-specific and are used in classification of new isolates.

Unexpectedly, however, the third nucleotide of the leader region of APMV-3 differed from that of other members of subfamily *Paramyxovirinae*. It will be of interest to determine whether this difference is consistent in other strains of APMV-3 and might also be found in some of the other serotypes. In most members of family *Paramyxoviridae*, the 5' trailer region is 40–60 nt long (Shioda et al., 1986), although there are a few exceptions where the trailer region is somewhat longer: for example, 155 nt in RSV (Mink et al., 1991) and 159 nt in Mapuera virus (Wang et al., 2007). The trailer region of APMV-3 is unusually long (707 nt), and is the longest trailer among members of family *Paramyxoviridae* sequenced to date. RNA secondary structure analysis using Genebee (www.genebee.msu.su/genebee.html) software provided suggestive evidence of secondary structure in this region (not shown), although it is not clear whether a structure could form with an RNA that is completely encapsidated. Further analysis of the 707 nt trailer region did not reveal any open reading frame, indicating that it is not a part of the L gene which was truncated at some point in the evolution. Whether this unusually long trailer region has any functional significance can ultimately be determined using reverse genetics techniques.

The availability of the first sequence of a representative of APMV-3 described here, together with the recent description of complete sequence of representative of APMV-6 and the availability of complete genome sequences from APMV-1, provides the opportunity to assess the relatedness of these putative serotypes on a genome-wide basis. For example, APMV-3 Netherlands strain shares 41.7% and 42.2% nt sequence identity with representatives of APMV-1 and -6, respectively (a comparison made exclusive of the SH gene of APMV-6 and the long trailer of APMV-3). Amino acid sequence identity between APMV-3 versus -1 and -6 for the N protein was 39–41% and 38.3%, respectively; that involving P was 22.8% and 19%; that involving M was 26.3% and 25.8% respectively; that involving F was 31.3% and 30.6%, respectively; that involving HN was 36% and 31%, respectively (and 41% with HN of APMV-4, the sole reported sequence for that serotype); and that involving L was 33% and 36%, respectively. These values, together with the phylogenetic analysis shown in Fig 5, show that APMV-3 strain Netherlands is more closely related to APMV-1 than APMV-6. This is consistent with the results of cross HI and NI tests, which suggested that the nine putative serotypes segregated into two broad, larger groupings, the first containing APMV-2 and -6, and the second APMV-1, -3, -4, -7, -8, and -9 (Lipkind and Shihmanter, 1986). Furthermore, the pattern of sequence relatedness outlined above also indicates that the overall extent of

divergence among the proposed APMV serotypes is comparable to that among the HPIV serotypes. For comparison, we note the genomes of HPIV-1 and HPIV-3, both of *Respirovirus*, have 60.3% nt identity, and their proteins share the following percent amino acid sequence identities: N, 62; P, 26; M, 63; F, 45; HN, 49%; L, 61 (Newman et al., 2002). HPIV-1 and HPIV-2, representing different genera (*Respirovirus* versus *Rubulavirus*, respectively), share 42.9% nt sequence identity and share the following percent amino acid sequence identities: N, 25; M, 20; F, 24; HN, 28; L, 31 (Newman et al., 2002). Thus, the APMVs appear to be somewhat more divergent than the HPIV-1 and HPIV-3 difference and somewhat less divergent than the HPIV-1 and HPIV-2 difference. This supports the idea that APMV-1 through -9 are indeed true serotypes. This is offered with the caveat that it will be important to examine cross-reactivity and cross-protection following experimental primary infections of the various APMV serotypes. In addition, it will be important to determine complete sequences for representatives of each of the APMV serotypes and to investigate potential diversity within the serotypes. The F protein of members of *Paramyxoviridae* is responsible for virus penetration into a host cell and cell fusion. The putative F protein cleavage site of APMV-3 strain Netherlands (A-R-P-R-G-R↓L) does not conform to the preferred furin cleavage site (R-X-R/K-R↓F), which is an important determinant of virulence for NDV and avian influenza viruses. In addition, the first residue of the F1 subunit is leucine, and thus, matches the assignment in avirulent strains of NDV as well as the single strain of APMV-6 sequenced to date, but differs from virulent strains of NDV, which have phenylalanine at this position. The observation that the F protein of APMV-3 strain Netherlands has characteristics consistent with that of an avirulent NDV strain is consistent with its dependence of exogenous protease for replication *in vitro*. However, it is in sharp contrast to the observed virulence of APMV-3 in birds (Alexander and Collins, 1982). It will be interesting to study further the pathogenicity of this virus in chickens and turkeys. The analysis of additional strains of APMV-3, and the development of a reverse genetics system to study viral biology and pathogenesis, and to create live attenuated strains, will be of applied, as well as basic, interest, and should help develop better vaccines for this poultry pathogen.

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a)

3'	55nt N gene start	
UGAUUUUGUCUUUUAAUUAUGAACAAUCAU-CAGCGGCUAUUUAGGCGAUUAUUA	UCCUCGCCUU	APMV-3
..G.....UCC..UGG...ACGA..CC.C.UA..AU.CUCUUGCAGAAUAU..U-.GC.	CU.C.C.U.C	APMV-6
..G.....C...GCC.CUC.AUGC..UUUC..CU.CC.CGUAUAC..C-.GCG	G.C.AU...	NDV
..G..CCC..C...G.CUACCGU.GCA..AU.CU.CU...CGUUUUUC.A.-UGC.	..AG.....	HPIV-2
..G..CCC.....C..CACC..U-GAG.UU..UA...UC.GG.AGCUG..AUGU.	..AG.....	SV5
..G..CCC.....G..C.UCCU.UAAC.UGC AU.CU.U..C.UGCUC.AU-.GC.	CU.GG.U...	TiV
..G.....UC.C.UC..UGA.CA..C.U.-AUUAUUUAUCU.C.AU.UG.CC..A..	..CA.UU.C	SeV
..G.....UC.C.UC.CUGA.CA..C.U.UAUUUUUAA.U...AUUU-.A..UG.	..AAUU.C	HPIV-3
..G.....UCCC.UU.AUACCUAUGCA..UUUAUUA.UGCAUAAA...U-UG.	..U.GU.C	NiV
..GC.....UCCCCU.UAUACCUAUGCAAAUUUUUUUGACGCAUACAA...U-UG.	..U.GU.C	HeV
..G.....U.CAACCCA.U.CCUAU-CU.GUU..UUA...G.AUAAGAUAUGUG.	..AAGU.C	MeV
..G.C...U.CAACCGAU.CCUAU-CA..UU.AUAA..U..A.AAU..U.UG.	..CA.UUAC	CDV
..G.....U.C..C.CACCCU.AUUA..UGCUU.UUCU.C.UAUUAG..G-GA.	..UU.C	J virus

b)

APMV-3 5' **ACUAAACAAAA**GUUAUAAUGAUUUUUAAAUCAGUCUUCACCUUUUG
 NDV 5' **ACCAAACAAGA**UUUUGGUGAUGACGAGACUACACUCAAGAAUAAUUG
 APMV-6 5' **ACCAAACAAGGA**AAUCAUAAGCUUUUUGACUAUGAUCCUUGUCGACUU
 SV5 5' ACCAAGGGGAAAACCAAGAUUAAUCCUCUUC-----
 HPIV-2 5' ACCAAGGGGAAAUCAUAUG-----
 TiV 5' ACCAAGGGGAAAUCAUAUAGG-----

c)

Fig. 1.

Alignment of the (a) 3' leader and N gene start sequence. Dots indicate identity with the assignment in APMV-3 (Top line). (b) 5' trailer regions of APMV-3 strain Netherlands with other avian paramyxoviruses and rubulaviruses. Bold letter indicates the similarity of nucleotides among avian paramyxoviruses and underline letters indicates the differences among them (c) Complementary of 3' leader and 5' trailer regions of APMV-3 strain Netherlands. Abbreviations: NDV, Newcastle disease virus (APMV-1); SV5, Simian virus 5; APMV, Avian paramyxovirus; HPIV, Human parainfluenza virus; TiV, Tioman virus; SeV, Sendai virus; NiV, Nipah virus; HeV, Hendra virus; MeV, Measles virus; CDV, canine distemper virus.

APMV-3-	TTAAAGGG	}	<i>Avulavirus</i>
APMV-6-	AAAAAGGG		
NDV-	AAAAAGGG		
SV5-	TAAGAGGG	}	<i>Rubulavirus</i>
HPIV-2-	TAAGAGGG		
TiV	TAAGAGGG		
HPIV-3-	AAAAAGGG	}	<i>Respirovirus</i>
SeV-	AAAAAGGG		
NiV-	AAAAAGGG	}	<i>Henipavirus</i>
HeV-	AAAAAGGG		
CDV-	AAAAAGGG	}	<i>Morbillivirus</i>
MeV-	AAAAAGGG		

Fig. 2.

RNA editing site in P gene of selected members of the family *Paramyxoviridae*. Sequences are shown in positive mRNA sense. Addition of one and two G residues into the stretch of G residues leads to formation of V and W proteins except the members of rubulaviruses where edited transcript codes for P, not V and W.

		☆☆	☆		☆	☆	☆	☆	☆	
APMV-3	184	TWCNPACSPVTAFPKQYKCA	CRQCPRFCDL	CF	215					
APMV-6	194	SWCNPSWSP	PIKAEPKQYPCFCGSFPPTCRLCA	225						
NDV	194	SWCNPSCSPIRAEPRQYSCTCGSCPATCRLCA	225							
HPIV-2	191	EWCNPRCAPVTASARKFTCTCGSCPSICGECE	222							
SV5	188	EWCNPSCSPITAAARRFECTCHQCPVTCSECE	219							
TiV	181	EWCNPICHPI	SQFTYRGTCRCGCCPDVCSLCE	212						
NiV	425	EWCNPACSRITPLPRRQECQCGECPIEC	SHCC	456						
HeV	423	EWCNPVCSRITPQPRKQECYCGECPTECSQCC	454							
CDV	249	KWCNPICTQVNWGIIRAKCVCGECPPTCNECK	280							
JVirus	253	EWCNPQCAPITVTPTQSRCTCGECPKVCARCI	284							

Fig. 3.

Sequence alignment of C terminal end of V proteins of different members of subfamily *Paramyxovirinae*. Stars indicate conserved cysteine and tryptophan residues. Numbers in both side of sequence indicates the amino-acid position.

APMV-3	101	A- <u>R</u> -P- <u>R</u> -G- <u>R</u> -L	107
NDV (Virulent)	111	G- <u>R</u> - <u>R</u> -Q-K- <u>R</u> -F	117
NDV (Avirulent)	111	G-G- <u>R</u> -Q-G- <u>R</u> -L	117
APMV-6	113	P-A-P-E-P- <u>R</u> -L	119
APMV-2	93	D-K-P-A-S- <u>R</u> -F	99
NiV	104	L-V-G-D-V- <u>R</u> -L	110
SV5	97	T- <u>R</u> - <u>R</u> - <u>R</u> - <u>R</u> - <u>R</u> -F	103
HPIV-2	101	K-T- <u>R</u> -Q-K- <u>R</u> -F	107
SeV	111	D-V-P-Q-S- <u>R</u> -F	117
HPIV-3	104	N-P- <u>R</u> -T-K- <u>R</u> -F	110
CDV	219	G- <u>R</u> - <u>R</u> -Q- <u>R</u> - <u>R</u> -F	225
MeV	107	S- <u>R</u> - <u>R</u> -H-K- <u>R</u> -F	113

Fig. 4.

Comparison of the F protein cleavage site of different members of family *Paramyxoviridae*. Number of polybasic residues (R= Arginine, indicated in bold and underlined) in some of the strains shown represents virulent cleavage site. The number represents the amino acid sequence covering the cleavage site.

M **F**

Fig. 5. Phylogenetic analysis of N, P, M, F, HN and L proteins of members of family *Paramyxoviridae*. The numbers in branches represent the divergence of APMV-3 strain Netherlands with the other members of family *Paramyxoviridae*. The phylogenetic trees were constructed using DNASTAR software package (Lasergene6).

Table 1

Different primers used to amplify APMV-3 genome based on consensus sequences. The primer name represents an individual gene, position from gene-start and forward or reverse from left to right, different unconventional codes represent a mixture of nucleotides (N=A/T/C/G, R= A/G, K= G/T, Y=C/T, S=G/C, W=A/T, D= A/G/T and M=A/C). Sequences used for alignment were taken from published NCBI Genbank NC_003043 for APMV-6, NC_002617 for NDV, NC_003443 for HPIV-2, NC_006430 for SV5, NC_004074 for Tioman virus, NC_001552 for Sendai virus, NC_001796 for HPIV-3, AY988601 for Nipah virus, NC_001906 for Hendra virus NC_001498 for Measles virus, NC_001921 for Canine distemper virus and NC_007454 for J virus.

Position in gene	Primer sequences
GS forward	5'WWWAGGAGCGGAAGG3'
NP787forward	5'AAYRCSGGKMTKRCWSCWTTCTT3'
NP1022reverse	5'GWWSYAYWCCCATKGCWA3'
F350reverse	5'GCNGCNGTNGCNACNCCNARNGC3'
P113forward	5'AGGTTCTGAGCAAGCAAAGTATT3'
P137forward	5'CTCCCTGAACCAAGCACCTCTAC3'
P506reverse	5'ATTGTCTGTGAGTGGGATTCCG3'
P551reverse	5'GGGGTTACTCCACGTTTCGGTTTG3'
F842reverse	5'GAGTTACCTGWATAMCCAWGAKTT3'
HN1568reverse	5'AGTKGWTGWTGWTATGCTGCTT3'
HN550reverse	5'ATNACRTTGTGNGTRTARCACCA3'
L1460reverse	5'ATNGCYTTRTCYTTNARRAA3'
L1917reverse	5'CCARTTNARRCARTAYTTYTGNARRTC3'
L2142reverse	5'ATNSWDATCATNGTCCACAT3'
L4400reverse	5'ACNCKNARRTARTANARYTGRTANGC3'
L5325reverse	5'GCNCCNSWNCCYTCNGCNARRTA3'

Genomic features and protein characteristics of APMV-3 strain Netherlands. The table shows hexamer phasing position along with individual genes, coding and non-coding, and intergenic sequences with their protein profiles.

Genes	Hexamer phasing Position at gene start	mRNA characteristics (nt)				Intergenic Sequence nt	Deduced protein characteristics		
		Total length	5'UTR	ORF	3'UTR		Size(aa)	MW (kDa)	pI
N	2	1604	50	1374	182	55	457	50.9	5.540
P/V(P)	5	1378	42	1161	175	63	386	41.0	5.660
P/V(V)	5	1379	42	747	590	-	248	26.6	4.749
P/V(W)	5	1380	42	378	960	-	125	13.3	4.451
M	5	1478	89	1191	198	31	396	44.1	9.216
F	2	1922	101	1632	189	34	543	59.5	8.318
HN	2	1974	43	1734	197	59	577	63.8	7.830
L	1	6913	25	6597	291	-	2198	248.7	7.010

Table 3

Gene-start, gene-end and intergenic sequences of APMV-3 strain Netherlands. The underline nucleotide shows the difference from conserved gene-end sequence.

Gene	Gene-start	Gene-end	Intergenic sequence
N	UCCUCGCCUU	AAUUAUUUUUU	CCGAAAGCUCGUGUCUGUCUCUAGGACU GUAUUAAGUCGGCCCCCUAAGUUGAA
P	UCCUCGCCUU	AA <u>A</u> UUAUUUUUU	GAGGAUCUAGAUUGUACCUCGUCUGGC UCGAUUACGCCUCCUCAUGUGUUCCCC UUAGUU
M	UCCUCGCCUU	AAUUAUUUUUU	GUAACACUCUUACGUUAAAUCCUGCU UUCA
F	UCCUCGCCUU	A <u>U</u> U <u>A</u> AUUUUUU	GGAGUCCACUGGGUAGGACCGGGUAG GCUAACC
HN	UCCUCGCCUU	AAUUAUUUUUU	AGGAGAUAAUUCUAGGUCUGCUUCCG AUAUAUUCGAUUAUCGAAUCCUACCUU UGCUG
L	UCCUCGCCUU	AAUUA <u>A</u> UUUUUU	