Published in final edited form as:

Virus Res. 2013 January ; 171(1): 103–110. doi:10.1016/j.virusres.2012.10.031.

Evaluation of the genetic diversity of avian paramyxovirus type 4

Baibaswata Nayak^{1,§}, Shreeraj Nayak¹, Anandan Paldurai¹, Sachin Kumar¹, Roberta De Nardi², Calogero Terregino², Peter L Collins³, and Siba K Samal^{1,*}

¹Virginia-Maryland Regional College of Veterinary Medicine, University of Maryland, College Park, Maryland, USA

²OIE/FAO and National Reference Laboratory for Newcastle Disease and Avian Influenza, Istituto Zooprofilattico Sperimentale delle Venezie, Viale dell'Università 10, Legnaro (PD), Italy

³Laboratory of Infectious Diseases, National Institute of Allergy and Infectious Diseases, National Institutes of Health, Bethesda, USA

Abstract

Avian paramyxoviruses (APMV) belong to the genus *Avulavirus* in the family *Paramyxoviridae* and include at least nine serotypes, APMV-1 to -9, as well as two additional provisional serotypes. Newcastle disease virus (NDV), which comprises APMV-1, is the most extensively studied APMV because it is an important poultry pathogen. A moderate level of antigenic and genetic diversity is recognized for APMV-1 isolates, but our knowledge of the antigenic and genetic diversity of the other APMV serotypes is limited. APMV-4 is frequently isolated from waterfowl around the world. To date complete genome sequences of APMV-4 are available for only strains, which were isolated from ducks in Hong Kong, Korea and Belgium over a period of 37 years. We have carried out genome sequencing from the nucleocapsid (N) gene-end signal to the polymerase (L) gene-start signal of five APMV-4 strains recently isolated from Italy. Each of the eight APMV-4 strains has the same F protein cleavage site, DIQPR↓F. They also share a high level of nucleotide and amino acid sequence identity: for example, the F and HN glycoproteins have greater than 97% sequence identity between the various strains. Thus, comparison of these eight strains of APMV-4 did not provide evidence of substantial diversity, in contrast to similar studies with APMV-2, -3, and -6, in which the F and HN glycoproteins exhibited up to 20-30% amino acid sequence variation within a subgroup. Reciprocal cross-HI assay using post infection chicken sera also failed to detect significant antigenic variation among the available APMV-4 strains.

Introduction

Viruses of the family *Paramyxoviridae* have been isolated from many species of avian, terrestrial and aquatic animals worldwide (Lamb and Parks, 2007). Most paramyxoviruses that have been isolated from avian species are placed in the genus *Avulavirus* of the subfamily *Paramyxovirinae*, and are called avian paramyxoviruses (APMV). (The only exceptions are the avian metapneumoviruses, which are placed in the genus *Metapneumovirus* of the subfamily *Pneumovirinae* due to extensive sequence divergence and differences in genomic organization.) The APMVs that comprise genus *Avulavirus* have

*Correspondence address: Siba K. Samal, Virginia-Maryland Regional College of Veterinary Medicine, University of Maryland, College Park, MD, 20742, Phone: (301) 314-6813, Fax: (301) 314-6855, ssamal@umd.edu.

§Present address: Department of Gastroenterology and Human Nutrition, All India Institute of Medical Sciences, New Delhi, India

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

been divided into nine serotypes (APMV-1 to -9) on the basis of antigenic differences revealed by hemagglutination inhibition (HI) and neuraminidase inhibition (NI) assays (Alexander, 2000). Recently, two more APMV serotypes (APMV-10 and APMV-11) have been suggested (Briand et al., 2012; Miller et al., 2010). APMV-1, which includes all strains of Newcastle disease virus (NDV), has a wide host range and is able to infect over 240 species of birds (Kaleta and Baldauf, 1988). In contrast, the host ranges of the other APMV serotypes are poorly defined. APMV-2, -3 and -7 have been isolated from captive caged birds and domestic poultry including chickens and turkeys; APMV-4, -6, -8 and -9 appear to be more restricted to water fowl including ducks and geese; and APMV-5 has been isolated from wild birds and mostly from budgerigars (*Melopsittacus undulatus*).

The disease potential of APMV serotypes can vary considerably. APMV-1 (NDV) is relatively well-characterized because of its agricultural importance. Depending on the strain, manifestations of NDV infection can range from subclinical to severe disease causing high mortality in chickens (Beard and Hanson, 1984). The other APMVs are relatively poorly characterized. APMV-2 has been associated with mild respiratory disease and drop in egg production, and infertility in turkeys (Bankowski et al., 1981; Lipkind et al., 1979). APMV-3 has been associated with encephalitis and high mortality in caged birds, respiratory diseases in turkeys and stunted growth in young chickens (Alexander et al., 1983; Tumova et al., 1979). Experimental infection of chickens with APMV-4 resulted in mild interstitial pneumonia and catarrhal tracheitis (Alexander et al., 1983). APMV-5 strains are responsible for depression, dyspnoea, diarrhea, torticollis, and acute fatal enteritis in immature budgerigars, leading to very high mortality (Nerome et al., 1978). APMV-6 was found to cause mild respiratory disease and drop in egg production in turkeys, but was avirulent in chickens (Alexander and Senne, 2008; Alexander et al., 1983; Chang et al., 2001). APMV-7 infection in turkeys caused respiratory disease, mild multifocal nodular lymphocytic air sacculitis, and decreased egg production (Saif et al., 1997). APMV-8 and -9 are not associated with any disease so far and mostly isolated from healthy waterfowl.

The APMV genome is a non-segmented, negative-sense, single-stranded RNA that, for the APMVs sequenced to date, ranges in length from 14,904-17,262 nucleotides. For most of the APMVs, the genome is transcribed into six mRNAs that separately encode a nucleocapsid protein (N), a phosphoprotein (P), a matrix protein (M), a fusion glycoprotein (F), a hemagglutinin-neuraminidase glycoprotein (HN), and a large polymerase protein (L). APMV-6 differs from the other serotypes in having an additional, seventh mRNA that encodes a small hydrophobic (SH) protein (Samal, 2011). RNA editing during transcription of the P gene causes frame-shifts that result in two additional proteins, W and V, which contain N-terminal domains derived from P and C-terminal domains derived from internal ORFs. The APMV gene order is: 3'-N-P-M-F-(SH)-HN-L. Viral transcription begins at 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 (GS) and gene-end (GE) transcription signals that flank the individual genes (Lamb and Parks, 2007). For the APMVs and other members of the subfamily *Paramyxovirinae*, the genome nucleotide length is an even multiple of six, known as 'rule of six' which is required for efficient RNA replication and the precise packaging of the polynucleotide in the nucleocapsid.

Complete genome sequences have been determined for a number of NDV (APMV-1) strains, and antigenic and sequence diversity within APMV-1 have been noted (Samal, 2011). Based on sequence of the F gene, NDV strains have been classified into two major classes (Class I and II). Class II is further divided into 15 genotypes including ten previously established (I-IX, and XI) genotype (Czegledi et al., 2006; Kim et al., 2007) and five (X, XII, XIII, XIV and XV) new genotypes (Diel et al., 2012). Our knowledge on genetic and antigenic diversity of APMV -2 to -9 is more limited. Complete genome sequences have

only recently become available for APMV serotypes 2-9, and the information is limited in some cases to a single prototype strain and in other cases to several strains (Chang et al., 2001; Kumar et al., 2008; Nayak et al., 2008; Paldurai et al., 2009; Samuel et al., 2009; Samuel et al., 2010; Subbiah et al., 2008; Xiao et al., 2010). Phylogenetic analysis of APMV-2 isolates by complete genome sequencing (Subbiah et al., 2010) or by partial HN gene sequencing (Mahmood et al., 2010) revealed a number of subgroups within the serotype. Existence of genetic subgroups among APMV-3 isolates also has been reported based on complete genome sequencing (Kumar et al., 2010) or analysis of partial M gene sequences (Shihmanter et al., 2005). Sequence comparisons of recent APMV-6 isolates with the prototype strain revealed the existence of subgroups within that serotype (Xiao et al., 2010). A somewhat lower amount of genetic diversity among APMV-9 isolates has been reported by phylogenetic analysis of partial F and HN gene sequences (Dundon et al., 2010).

Wild aquatic birds are thought to serve as a reservoir for potentially highly pathogenic strains of NDV and avian influenza virus, and may harbor virus without showing clinical disease signs. This has spurred development of surveillance programs for poultry as well as aquatic and terrestrial wild birds. APMV-4 strains are frequently isolated from waterfowl in many parts of the world during these surveillance programs. However, a genetic analysis of these APMV-4 isolates has not been reported. We previously sequenced the full-length genome of the prototype strain APMV-4/duck/Hong Kong/D3/75 that was isolated in Hong Kong from a duck in the year 1975 (Nayak et al., 2008). In addition two other full-length genome sequences are available for APMV-4 isolated from mallard ducks in Korea (Jeon et al., 2008) and Belgium (Rosseel et al., 2011) in 2006 and 2007, respectively. Recently, one more full-length genome sequence of APMV-4 isolated from Egyptian goose in the year 2010 (Abolnik et al., 2012). Comparison of the full-length genome sequences of the prototype Hong Kong strain and the Korean strain revealed a moderate level of genetic diversity among these strains, raising the possibility that APMV-4 might contain antigenic subgroups (Samal, 2011). In order to further understand the genetic diversity among APMV-4 isolates, we have determined the P, M, F and HN gene sequences of five additional APMV-4 strains isolated from ducks in Italy, and describe comparison with the published sequences of APMV-4 strains from Hong Kong, Korea and Belgium. We also investigated the extent of antigenic diversity among these strains.

2. Materials and Methods

2.1. Virus and cells

APMV-4 field isolates from Italy were kindly provided by Dr. Ilaria Capua (Istituto Zooprofilattico Sperimentale delle Venezie, Padova, Italy). These isolates are APMV-4/Duck/Italy/3670/06 (IT-3670), APMV-4/Mallard/Italy/3936-70/06 (IT-3936), APMV-4/Teal/Italy/4103-27/06 (IT-4103), APMV-4/Mallard/Italy/4284-20/06 (IT-4284), APMV-4/Mallard/Italy/4523-4/06 (IT-4523). APMV-4/duck/Hong Kong/D3/75 (HK) was provided by the National Veterinary Services Laboratory, Ames, Iowa, USA. The viruses were propagated in 9-day-old embryonated specific pathogen free (SPF) chicken eggs. Infected allantoic fluid was harvested 4 days post-inoculation. The titer of each virus was determined by hemagglutination (HA) assay using 1% chicken RBC at room temperature. The chicken embryo fibroblast (DF1) cell line, obtained from the American Type Culture Collection (ATCC, Manassas, VA), was grown in Dulbecco's modified Eagle medium (DMEM) containing 10% fetal bovine serum. The cells were infected with a 10^{-2} dilution of 2 HA units of egg-grown virus, with or without supplementation with 1 μ g/ml trypsin (Invitrogen, USA) in serum-free DMEM. The infected cells were observed daily for 7 days for cytopathic effect (CPE) and HA activity of the cell culture supernatant.

2.2. Sequencing of P, M, F and HN genes

Viral genomic RNA was isolated from purified virus using RNeasy mini kit (QIAGEN, USA) according to the manufacturer's instructions. Virus purification was carried out by discontinuous sucrose gradient (30%) centrifugation of allantoic fluid obtained from embryonated chicken eggs infected with the various strains. Primers for cDNA synthesis and genome sequencing were designed from the published sequence of the APMV-4 prototype strain (APMV-4/duck/Hong Kong/D3/75; Gen Bank accession no. FJ177514). First-strand cDNA was synthesized from viral RNA by Superscript II RT kit (Invitrogen) by using either random hexamers or APMV-4 GS positive-sense primer (5'-CTAGGGTGGGGAAGG-3') according to manufacturer's instructions. PCR amplification of the fragment covering entire P-M-F-HN gene was carried out in two phases. First, F and HN gene was amplified and sequenced using following primer combinations: p 4247 sense (5'-CACTGATCACTGGAAATGTAA-3'), p4365 sense (5'-TCACACCTCCCCGCCACGAAA-3'), p5490 antisense (5'-CCCCACTGAGACACTTGACTAACT-3'), p5340 sense (5'-TCAATAGGGATGGTGGACTTTA-3'), p6239 sense (5'-GTGCATGGTAGGGTGGGG AAGGTA-3'), p6390 antisense (5'-ACTTCTGTTGACTTCTC TTGGTA-3'), p6291 sense (5'-ACCAAGAGAAGTCAACA GAAGTAT-3'), p8223 antisense (5'-ACGTGGGTGC GAGTCAAGGAACAA-3'). Later, P and M gene was amplified and sequenced using following primer combinations: p1331 sense (5'-GACGACTTCCCCTGTCGGGATCCATGCCGG CTCTCTC-3'), p2005 antisense (5'-TTGGGCCTCTTGAGTCTGGCTTTGGCTGC-3'), p1875 sense (5'-GATCATCAGGCCAACGAC-3'), p3160 antisense (5'-ACAGCAGTTC TTTTGGTGGGTTGG-3'), p3043 sense (5'-GGGCTCCCTCATCT TCAGACACG-3'), p4357 antisense (5'-CTTTTGTTTAGACACCCAATCAACT-3'). PCR amplification was carried out by high fidelity Taq polymerase (Invitrogen). The PCR products were purified on agarose gels and were either sequenced directly or cloned into TOPO TA vector (Invitrogen) for sequencing using vector specific M13 Forward or reverse primers. The DNA sequencing was carried out using Big Dye® Terminator v3.1 cycle sequencing kit (Applied Biosystems Inc, USA) in ABI 3130xl genetic analyzer.

2.3. Sequence alignment and phylogenetic analysis

Assembly of sequence contigs and prediction of open reading frames were performed using the SeqMan and EditSeq programs of the Lasergene 8 (DNASTAR) software package. Amino acid and nucleotide sequence identities among APMV-4 isolates were determined by clustalW multiple alignment algorithms of MagAlign program of DNASTAR. Sequence similarity searches also were conducted using the basic length alignment search tool (BLAST) from the National Center for Biotechnology Information (NCBI). Evolutionary relationships among the APMV-4 isolates and other APMV serotypes were evaluated by nucleotide sequence alignment of the region of the genome from the N GE signal to the L GS signal. Alignment gaps that arose due to pair wise sequence comparison between Italian APMV-4 isolates and complete genome sequence of other APMVs were eliminated during phylogenetic analysis. The phylogenetic tree was generated by the neighbor joining method using the Kimura-2 parameter in the MEGA version 4.0 program (Tamura et al., 2007).

2.4. Database accession numbers

The genome sequence of the region encompassing the P-M-F-HN genes of APMV-4 strains IT-3670, IT-3936, IT-4103, IT-4284 and IT-4523 have been deposited in GenBank under accession numbers, JX501910, JX501911, JX501912, JX501913 and JX501914, respectively. Accession numbers for other *Avulavirus* sequences used in this study are given below: APMV-1 (NDV strain LaSota), AF077761; APMV-2, EU338414 (strain Yucaipa), HQ896024 (NK strain), HQ896023 (F8 strain), HM159993 (England/7702), HM159994

(Kenya strain), HM159995 (Bangor); APMV-3, EU403085 (Netherlands strain), EU782025 (Wisconsin strain); APMV-4, FJ177514 (Hong Kong strain), EU877976 (Korea strain) and JN571485 (Belgium strain); APMV-6, GQ406232 (IT4524), EU622637 (Hong Kong strain), EF569970 (FarEast4440), NC_003043, AY029299 (Taiwan strain); APMV-7, FJ231524 (Tennessee strain); APMV-8, FJ215863 (Delaware strain), FJ215863 (Del1053) and FJ215864 (Wakuya strain); APMV-9, EU910942 (New York strain); APMV-10, HM147142 (Penguin/FE324/07); and APMV-11, JQ886184 (Common_snipe/France/100212/2010).

2.5. Immunization of chickens with APMV-4 Italian isolates

Seven groups of three 2-week-old SPF chickens (Charles River Laboratories International, Inc., Wilmington, MA, USA), were infected with the APMV-4 Hong Kong prototype strain or each of the five Italian strains, or were left uninfected as a control. Chickens in each group were infected with a single dose of 200 μ l of each freshly harvested allantoic fluid from APMV-4-infected eggs, given as a 1:4 dilution in PBS through the oculo-nasal route (50 μ l in each eye and nostril). Serum samples were collected on day 0 and day 21 post infection. All infections were carried out in a BSL 2 plus containment facility.

2.6. Serological analysis

Serum antibody responses to APMV-4 infection were evaluated by hemagglutination inhibition (HI) assay using the homologous virus as antigen. For the HI assay, two-fold serial dilutions of immunized chicken sera (50 μ l) were prepared, and each dilution was mixed with 4 HA units of APMV-4. Following 1 h of incubation, 50 μ l of 1 % chicken red blood cells (RBC) was added, the mixture was incubated for 30 min at room temperature, and hemagglutination was scored. Antigenic relationships among the field isolates were assessed by cross-HI assay using the heterologous strains as antigen. Titers were evaluated for significant differences in variation by one-way analysis of variance (ANOVA). Statistical analyses for mean, standard deviation of data, one-way ANOVA were analyzed by using computer software Prism 5.0 (Graph Pad Software Inc., San Diego, CA).

3. Results

3.1. In vitro growth characteristics of APMV-4 isolates from Italy

The replication characteristics of the five APMV-4 Italian strains in 9-day-old embryonated chicken eggs and the chicken embryo fibroblast DF-1 cell line were very similar to those of the three previously isolated APMV-4 strains duck/Hong Kong/D3/75, Korea/YJ/06 and mallard/Belgium/12245/07. Specifically, the strains replicated efficiently in embryonated eggs, yielding titers of up to 2^9 HA units per ml of allantoic fluid at 4 day post-inoculation (dpi). Replication in DF-1 cells was relatively inefficient, yielding a maximum of 2^4 HA units per ml on 4 dpi. The inclusion of exogenous protease, either 10% allantoic fluid or 1 g/ml trypsin, did not affect the efficiency of replication of these viruses in cell culture, indicating a lack of requirement of external proteases for efficient cleavage of the F protein. None of the Italian strains produced syncytia or formed plaques, and instead caused single-cell infections similar to that observed with the three previously isolated APMV-4 strains.

3.2 Determination of partial genome sequences of APMV-4 strains isolated in Italy

Complete genome sequences were available for three previously-isolated APMV-4 strains (Hong Kong, Korea, Belgium strains). In the present study, partial genome sequences of five Italian APMV-4 strains, from the N GE signal to the L GS signal, were determined. The rationale for sequencing this region was that M, F and HN are envelope-associated proteins, and P is the most variable among internal protein genes. The sequence was 6598 nucleotides

in length, accounting for 44% of the complete genome. The length of this sequence was the same for all of the APMV-4 strains.

We previously identified the GS signal of APMV-4 Hong Kong as a 13-nucleotide sequence (3'-UCCCACCCCUUCC-5', negative sense) that was exactly conserved among the various genes with the exception of 3 and 1 nt differences in the N and F genes (UCCACACCCUUCC and UCCCACCCCUCA, differences underlined)(Nayak et al., 2008), respectively. Among the P, M, F and HN genes of the five Italian strains and the previously-available Korean and Belgian strains, these sequences were conserved exactly except that the Hong Kong strain F gene GS signal had 1 nucleotide C→A substitution at the 5' (downstream) end.

We previously identified the GE signal of APMV-4 Hong Kong strain as a 12-nt sequence (3'-AAAUAUUUUU-5', negative-sense) that was exactly conserved among the various genes with the exception of 2, 1, and 1 nt differences in the P, M, and L genes (AAAAUAAGUUUU, AGAUUAUUUUU, AAUAUAUUUUU), respectively(Nayak et al., 2008). Among the P, M, F and HN genes of the five Italian strains and the previously-available Korean and Belgian strains, these sequences were conserved exactly except that of M gene. The conserved M gene end signal for Italian and Belgian strain is UUAGUAAUCGUUU whereas, GE signal for Hong Kong (UAGUUAAGUUUUU) and Korean strain (UUCGUCAAUCGUUU) differ from both Italian and Belgian strain.

We previously showed that the lengths of the IGS of APMV-4 Hong Kong between the N/P, P/M, M/F, F/HN, and HN/L genes are 9 nt, 34 nt, 13 nt, 37 nt and 42 nt, respectively (Fig. 1A). These lengths were exactly conserved among the APMV-4 strains from Italy, Hong Kong, Korea and Belgium. However, a number of nucleotide substitutions were observed, as shown in Fig.1B.

3.3 The phosphoprotein (P) gene

The P genes of each of the five APMV-4 Italian strains is 1364 nt in length and encodes a P protein of 393 aa, which is same as Hong Kong, Korean and Belgium strains. There are 10 serine, 6 threonine and one tyrosine residues that are conserved among all of the APMV-4 strains as potential phosphorylation sites. The P gene nucleotide and deduced amino acid sequences had 97.2-100% and 97.5-100% identity within the Italian isolates, 97.5-98.2% and 97.5-98% identity between the Korean and Italian strains, 96.6-98.8% and 96.2-98.7% identity between the Belgium and Italian strains, and 89.2-90% and 92.1-93.1% identity between the Hong Kong and Italian strains, respectively. All of the APMV-4 strains have the same conserved editing site 5'-AAAGGGGGG-3' (mRNA sense) at positions 442-450 nt of the P gene, where addition of a single non-templated G residue would yield a predicted V protein. The C-terminal domain of the V protein has 7 conserved invariantly spaced cysteine residues and one histidine residue among all the isolates (Nayak et al., 2008). The V protein of the Italian strains had greater amino acid sequence identity with the Korean strain (96-97.3%) and the Belgium strain (96-98.2%) than with the Hong Kong strain (92.4-93.8%).

3.4 The matrix protein (M) gene

The M gene of each of the Italian APMV-4 strains is 1293 nt long. The Italian, Korean and Belgium strains encode a M protein of 370 aa whereas the M gene of the Hong Kong strain encodes a M protein of 369 aa due to longer 3' (downstream) untranslated region. The potential late domain motif (FPLI) and nuclear localization signal motif at 246 to 270 aa position are conserved among all strains except the Belgium strain (FPLV). The M gene nucleotide and deduced amino acid sequences had 96.7-99.8% and 98.4-99.8% identity

among Italian isolates, 96.8-97.8% and 98.7-98.9% identity between the Korean and Italian strains, 96.8-98.9% and 98.7-99.5% identity between the Belgium and Italian strains, and 91.1-91.8% and 97.8-98.1% identity between the Hong Kong and Italian strains, respectively.

3.5 The fusion protein (F) gene

The F gene of each of the Italian APMV-4 strains is 1891 nt long and encodes a protein of 566 amino acids. The major sequence features of the F protein, including the proteolytic cleavage site (DIPQR↓F), N-glycosylation sites, cysteine residues, and heptad repeat motifs (HRA and HRB) are conserved among all of the Italian, Korean, Belgium and Hong Kong strains. However, amino acid sequence alignment of F protein revealed more variation in the N-terminal signal sequence region. The F gene nucleotide and deduced amino acid sequences had 97.6-99.8% and 98.6-99.8% identity among the Italian isolates, 97.9-98.5% and 98.8-99.3% identity between the Korean and Italian strains, 97.5-99.4% and 98.6-99.8% identity between the Belgium and Italian strains, and 91.8-92.4% and 97.2-97.9% identity between the Hong Kong and Italian strains, respectively.

3.6 The hemagglutinin-neuraminidase (HN) gene

The HN gene is 1914 nt long for all of the APMV-4 strains. The Belgium and Italian isolates encode HN proteins of 565 amino acids, but both the Korean and Hong Kong strains encode HN proteins of 569 amino acids due to shift of the initiation codon due to point mutations. Critical sequence features like potential glycosylation sites, a putative sialic acid binding motif (NRKSCS), neuraminidase active residues, and cysteine residues are conserved among all of the APMV-4 strains. The HN gene nucleotide and deduced amino acid sequences had 96.9-99.6% and 98.8-99.5% identity among the Italian isolates, 97.5-98.5% and 99.1-99.5% identity between the Korean and Italian strains, 98.5-99.1% and 98.6-99.3% identity between the Belgium and Italian strains, and 90.5-90.9% and 97.7-98.4% identity between the Hong Kong and Italian strains, respectively.

3.7 Genetic relatedness and phylogenetic analysis

The phylogenetic relationships among the eight APMV-4 strains, and between the eight APMV-4 strains and the other APMV serotypes, was investigated by comparing the nucleotide sequences of the region from N GE signal to the L GS signal covering entire P-M-F-HN genes. The resulting phylogenetic tree is depicted in Figure 2 and 3. The phylogenetic analysis clearly indicates that all Italian isolates belongs to APMV-4 and cluster together with Korean, Belgium and Hong Kong APMV-4 strains (Fig 2). Within the genus *Avulavirus*, the various APMV serotypes segregated into two clusters, one containing APMV-1, -3, -4, and -9, and the other containing APMV-2, -5, -6, -7, -8, -10 and -11 (Fig 2). The eight strains fell into three distinct lineages within the APMV-4 serotype (Fig 3). The Hong Kong strain was relatively more distinct, whereas three of the Italian strains segregated with the previously-isolated Belgium strain, and the other two Italian strains segregated with the previously-isolated Korean strain (Fig 3). Phylogenetic analysis based on the nucleotide sequences of individual genes and the deduced amino acid sequences of individual proteins also yielded the same three-lineage branching pattern (data not shown).

3.8 Serological relationship among APMV-4 strains

The serological relationship among five Italian APMV-4 strains and prototype Hong Kong strain was evaluated by cross-HI assay using convalescent antisera raised by a single infection of chickens through oculo-nasal route, mimicking natural infection. Even though chickens are not considered natural host to APMV-4, sero-conversion to all APMV-4 strains was observed. However, homologous HI titer was low indicating that chicken is not a highly

permissive host for APMV-4. Antigenic cross reactivity was observed among isolates by HI assay. There was some evidence of antigenic differences. Specifically, reactivity against the Hong Kong strain (the most divergent based on the phylogenetic analysis) was somewhat higher with sera raised against the Hong Kong strain versus the seven other strains. However, in general, the reactivity patterns were not suggestive of antigenic subgroups. For example, even though the IT-4284 and IT-4103 strains represent two different lineages, antisera against each of the eight strains reacted most efficiently with IT-4284 followed by IT-4103.

4. Discussion

The APMVs in the genus *Avulavirus* are divided up into nine established serotypes (1-9) and two provisional new serotypes (10, 11), and are isolated from a wide variety of avian species around the world. Wild ducks and waterfowl are considered reservoirs for many avian viruses and play an important role in epidemiology of these viruses due to their long distance travel during periods of migration. APMV-4 strains have been isolated from wild mallard, gadwall, ringed teal, and wood ducks in the United States (Alexander et al., 1979a; Alexander et al., 1979b; Gough and Alexander, 1984; Webster et al., 1976) and also from chickens, ducks, and geese in Hong Kong during avian influenza surveillance programs (Alexander et al., 1979b). Since then, APMV-4 has been frequently isolated from waterfowl but very little is known about the epidemiology and disease potential of APMV-4 in chickens and ducks (Stanislawek et al., 2002; Tumova et al., 1989; Yamane et al., 1982).

APMV-4 has been reported to cause an increase in white-shelled eggs (Alexander, 2000). However, experimental infection of chickens and ducks did not produce any clinical disease (Kim et al., 2012; Nayak et al., 2012). APMV-4 replicated slightly better in ducks than chickens (Kim et al., 2012). Although APMV-4 was first isolated in 1975, complete genome sequences are available for only three strains (Jeon et al., 2008; Nayak et al., 2008; Rosseel et al., 2011), and sequence comparison among the APMV-4 strains has never been conducted. In this study, we analyzed five APMV-4 isolates from Italy obtained from a variety of waterfowl including duck, mallard, and teal. The genetic heterogeneity was analyzed by partial genome sequence of these strains from N GE signal to L GS signal, representing 44% of the genome. The genetic relatedness of these Italian APMV-4 isolates was compared with the previously isolated Hong Kong, Korean and Belgium APMV-4 strains. Antigenic relatedness among APMV-4 strain was studied by cross HI test using sera raised in chickens.

The region of the genome from the N GE signal to the L GS signal was chosen for sequence determination because it contains the envelope protein gene (M, F and HN) and the internal protein gene P. In avian paramyxoviruses, sequence variation among strains within a serotype is greatest in these genes. Furthermore, the F and HN proteins are the major neutralizing antigens of APMV (Kumar et al., 2011).

The P gene is the most variable among paramyxovirus internal protein genes and contains the editing site for production of V proteins. The P gene encodes the P and V proteins that are important for viral replication and pathogenesis. The P protein is part of viral replication complex (Parks, 1994) and essential for viral RNA synthesis (Curran, 1996). The V protein produced by mRNA editing of P gene is involved in host-interferon antagonism (Park et al., 2003). Although the P protein is the most variable paramyxovirus protein among those found the APMVs, the lowest level of amino acid sequence identity among these eight strains was 92.1%, and the length of the protein was invariant. These recent isolates carry same conserved mRNA editing site for V mRNA transcription like the Hong Kong strain.

The M protein plays a central role in the morphogenesis and budding of paramyxovirus. The late domain of M protein is involved in protein-protein interactions and recruits host factors at viral assembly sites for virus release. The proposed late domain (FPLI) was conserved for all strains except the Belgian APMV-4 strain (FPLV). Late domain motif of APMV-3 has FPLI like other APMV-4 whereas, APMV-8 has FPLV motif like Belgium strain (Xiao et al., 2009). Similar motifs are present in the M proteins of other APMV, namely: YPLI for APMV-2; FPIV for APMV-5; FPLV for APMV-6; FPII for APMV-7; FPIV for **APMV-9**. The nuclear localization signal in the M protein was conserved among all the APMV-4 strains. In general, the M protein was highly conserved among the eight strains: its length was invariant, and the lowest level of sequence identity was 97.8%

The F protein cleavage site sequence of APMV-4 (DIPQR↓F) contains a single basic amino acid at -1 position and was conserved at positions 116-121 in all eight APMV-4 strains. This resembles the cleavage site sequence of avirulent NDV strains. But, unlike avirulent NDV strains, APMV-4 replicates in vitro without added protease and its replication is not augmented by added proteases (Nayak et al., 2008)(present study). The F protein also was highly conserved among the eight APMV-4 strains: its length was invariant, and the lowest level of sequence identity was 97.2%. The most divergent region in the F protein was its N-terminal signal sequence that mediates translocation of the nascent F0 protein into the lumen of the endoplasmic reticulum (Martoglio and Dobberstein, 1998). The predicted cleavage site for the signal peptidase was predicted to be between residues 24-25 except for the Korean and IT-3670 strains, where the prediction was between residues 21-22 and 17-18, respectively. Similar variation was recently observed among Italian APMV-6 field isolates, which are also isolated from ducks (Xiao et al., 2010).

Two different amino acid sequence lengths for the HN protein were observed among the eight APMV-4 isolates. Both the Hong Kong and Korea strains have HN protein lengths of 569 aa, whereas the Belgian and all of the Italian APMV-4 strains have HN proteins of 565 aa due to a shift in the position of the HN start codon. Similarly, different amino acid lengths HN proteins were observed among APMV-1 viruses (Gould et al., 2003) and APMV-2 viruses (Mahmood et al., 2010) due to change in position of either the stop or start codon. It has been shown for APMV-1 viruses that this variation in length is not linked to pathogenicity (Romer-Oberdorfer et al., 2003), but its effect on virus replication and host range is yet to be determined. Apart from the length differences, the HN protein was highly conserved among the eight APMV-4 viruses, with the lowest level of sequence identity being 97.7%.

A large number of studies have been conducted to characterize the antigenic and genetic diversity among isolates of APMV-1, but few studies have been carried out to characterize the antigenic and genetic diversity within each of the other APMV serotypes. Recently, genetic variation was shown among field isolates by complete genome sequencing for APMV-2 (Subbiah et al., 2010), APMV-3 (Kumar et al., 2010) and APMV-6 (Xiao et al., 2010) isolates and by partial genome sequencing of HN gene for APMV-2 (Mahmood et al., 2010), M gene for APMV-3 (Shihmanter et al., 2005) and F and HN gene for APMV-9 isolates (Dundon et al., 2010). For example, for APMV, the F and HN proteins among three different strains had levels of sequence identity as low as 77.6% and 75%, respectively, and had 4- to 8-fold differences in antigenic reactivity (Subbiah et al., 2010). Analysis of two strains of APMV-3 showed that the F and HN proteins had 70.1 % and 72.6% identity between the strains and 2- to 4-fold differences in antibody reactivity (Kumar et al., 2010). Analysis of five strains of APMV-6 showed that the F and HN protein had levels of sequence identity as low as 85.5% and 80.9%, and up to 4- to 8-fold differences in antibody reactivity (Xiao et al., 2010). These results suggest that APMV-2, -3, and -6 each contain at least two antigenic subgroups. In contrast, the high level of amino acid sequence identity

among the eight available APMV-4 strains, particularly in the F and HN major antigen proteins, is not suggestive of significant antigenic heterogeneity. The most divergent strain was the prototype Hong Kong strain, isolated in 1975. Since all of the other strains were isolated in 2006 or 2008, it's possible that this difference reflects progressive sequence changes accumulated over time, perhaps due to immunologic pressure. It will be of interest to sequence additional isolates, old and new, to investigate this issue.

We also carried out antigenic analysis by cross HI assay among the Hong Kong strain and the five Italian isolates. The antisera were raised in chickens by a single infection through the natural oculo-nasal route. However, high titer HI antibodies were not produced against homologous virus indicating that APMV-4 either does not grow well in chickens or does not induce high titer HI antibodies. Similarly, very low HI titers (< 8) were induced by the intranasal inoculation of 1-week-old ducklings and 2-week-old chickens with the APMV-4 isolate from ringed teal (Gough and Alexander, 1984 [ENREF 15](#)). The Hong Kong strain, which appeared to be the most divergent based on phylogenetic analysis, reacted somewhat more efficiently with homologous versus heterologous sera, but in general there was little evidence of antigenic diversity.

In conclusion, we have determined the sequences of P, M, F and HN genes of five APMV-4 strains isolated in Italy. Comparison of sequences of these genes among Italian strains and between APMV-4 strains Hong Kong, Korea and Belgium showed a high degree of sequence identity, including for the amino acid sequences of the major F and HN neutralization antigens. This suggested that the APMV-4 strains isolated to date show extensive sequence and antigenic relatedness. It will be interesting in the future to study antigenic and genetic variation among APMV-4 isolates from different avian species and from additional geographic regions.

Acknowledgments

We thank Drs. Ilaria Capua and Isabella Monne at IZSV, Italy for helpful support and advice. We thank Daniel Rockemann for his excellent technical assistance and Peggy Barrot for proofreading the manuscript. "This research was supported by funding from NIAID contract no. N01A060009 (85% support) and NIAID-NIH Intramural Research Program (15% support). The views expressed herein do not necessarily reflect the official policies of the Department of Health and Human Services; nor does the mention of trade names, commercial practices, or organizations imply endorsement by the U.S. Government."

References

- Abolnik C, de Castro M, Rees J. Full genomic sequence of an African Avian Paramyxovirus Type 4 strain isolated from a wild duck. *Virus genes*. 2012
- Alexander D, Senne D. Avian Paramyxoviruses 2-9. *Diseases of Poultry*. 2008
- Alexander DJ. Newcastle disease and other avian paramyxoviruses. *Rev Sci Tech*. 2000; 19:443–462. [PubMed: 10935273]
- Alexander DJ, Aymard M, Kessler N, Collins MS. Antigenic and structural relationships between avian paramyxoviruses isolated from ducks in Hong Kong and Mississippi, U.S.A. *J Gen Virol*. 1979a; 44:839–842. [PubMed: 93619]
- Alexander DJ, Pattison M, Macpherson I. Avian paramyxoviruses of PMV-3 serotype in British turkeys. *Avian Pathol*. 1983; 12:469–482. [PubMed: 18766806]
- Alexander DJ, Shortridge KF, Collins MS, Chettle NJ. Properties of a newly isolated, serologically distinct avian paramyxovirus. *Arch Virol*. 1979b; 60:105–113. [PubMed: 485856]
- Bankowski RA, Almquist J, Dombrucki J. Effect of paramyxovirus yucaipa on fertility, hatchability, and poult yield of turkeys. *Avian Dis*. 1981; 25:517–520. [PubMed: 7259687]
- Beard, CW.; Hanson, RP. Newcastle disease. In: Hofstad, MS.; Barnes, HJ.; Calnek, BW.; Reid, WM.; Yoder, HW., editors. *Diseases of Poultry*. 8th Edition. Iowa State University Press; Ames: 1984. p. 452-70.

- Briand FX, Henry A, Massin P, Jestin V. Complete genome sequence of a novel avian paramyxovirus. *J Virol.* 2012; 86:7710. [PubMed: 22733876]
- Chang PC, Hsieh ML, Shien JH, Graham DA, Lee MS, Shieh HK. Complete nucleotide sequence of avian paramyxovirus type 6 isolated from ducks. *J Gen Virol.* 2001; 82:2157–2168. [PubMed: 11514725]
- Curran J. Reexamination of the Sendai virus P protein domains required for RNA synthesis: a possible supplemental role for the P protein. *Virology.* 1996; 221:130–140. [PubMed: 8661421]
- Czegledi A, Ujvari D, Somogyi E, Wehmann E, Werner O, Lomniczi B. Third genome size category of avian paramyxovirus serotype 1 (Newcastle disease virus) and evolutionary implications. *Virus research.* 2006; 120:36–48. [PubMed: 16766077]
- Diel DG, da Silva LH, Liu H, Wang Z, Miller PJ, Afonso CL. Genetic diversity of avian paramyxovirus type 1: Proposal for a unified nomenclature and classification system of Newcastle disease virus genotypes. *Infection, genetics and evolution: journal of molecular epidemiology and evolutionary genetics in infectious diseases.* 2012; 12:1770–1779.
- Dundon WG, Heidari A, De Nardi R, Terregino C, Capua I, Cattoli G. Genetic variation of Italian avian paramyxoviruses serotype 9. *Virus Genes.* 2010; 41:43–46. [PubMed: 20386976]
- Gough RE, Alexander DJ. Avian paramyxovirus type 4 isolated from a ringed teal (*Calonetta leucophrys*). *Vet Rec.* 1984; 115:653. [PubMed: 6098067]
- Gould AR, Hansson E, Selleck K, Kattenbelt JA, Mackenzie M, Della-Porta AJ. Newcastle disease virus fusion and haemagglutinin-neuraminidase gene motifs as markers for viral lineage. *Avian pathology: journal of the W.V.P.A.* 2003; 32:361–373. [PubMed: 17585460]
- Jeon WJ, Lee EK, Kwon JH, Choi KS. Full-length genome sequence of avian paramyxovirus type 4 isolated from a mallard duck. *Virus Genes.* 2008; 37:342–350. [PubMed: 18770019]
- Kaleta, EF.; Baldauf, C. Newcastle disease in free-living and pet birds. In: Alexander, DJ., editor. *Newcastle disease.* Kluwer Academic Publishers; Boston: 1988. p. 197-246.
- Kim LM, King DJ, Curry PE, Suarez DL, Swayne DE, Stallknecht DE, Slemons RD, Pedersen JC, Senne DA, Winker K, Afonso CL. Phylogenetic diversity among low-virulence newcastle disease viruses from waterfowl and shorebirds and comparison of genotype distributions to those of poultry-origin isolates. *J Virol.* 2007; 81:12641–12653. [PubMed: 17855536]
- Kim SH, Xiao S, Shive H, Collins PL, Samal SK. Replication, neurotropism, and pathogenicity of avian paramyxovirus serotypes 1-9 in chickens and ducks. *PLoS One.* 2012; 7:e34927. [PubMed: 22558104]
- Kumar S, Nayak B, Collins PL, Samal SK. Complete genome sequence of avian paramyxovirus type 3 reveals an unusually long trailer region. *Virus Res.* 2008; 137:189–197. [PubMed: 18691616]
- Kumar S, Nayak B, Collins PL, Samal SK. Evaluation of the Newcastle disease virus F and HN proteins in protective immunity using a recombinant avian paramyxovirus type-3 vector in chickens. *J Virol.* 2011
- Kumar S, Nayak B, Samuel AS, Xiao S, Collins PL, Samal SK. Complete genome sequence of avian paramyxovirus-3 strain Wisconsin: evidence for the existence of subgroups within the serotype. *Virus Res.* 2010; 149:78–85. [PubMed: 20079781]
- Lamb, RA.; Parks, GD. Paramyxoviridae: The Viruses and Their Replication. In: Knipe, DM.; H, PM.; Griffin, DE.; Lamb, RA.; Martin, MA.; Roizman, B.; Straus, SE., editors. *Fields Virology.* 5th edition. Lippincott Williams and Wilkins; Philadelphia: 2007. p. 1449-1496.
- Lipkind MA, Weisman Y, Shihmanter E, Shoham D, Aronovici A. The isolation of yucaipa-like paramyxoviruses from epizootics of a respiratory disease in turkey poultry farms in Israel. *Vet Rec.* 1979; 105:577–578. [PubMed: 532076]
- Mahmood S, Alexander DJ, Slomka MJ, Manvell RJ, Hanna A, Fuller CM, Brown IH. Phylogenetic analysis of the nucleotide sequences for the HN gene of 22 avian paramyxovirus type 2 viruses reveals marked heterogeneity. *Avian Pathol.* 2010; 39:453–458. [PubMed: 21154054]
- Martoglio B, Dobberstein B. Signal sequences: more than just greasy peptides. *Trends Cell Biol.* 1998; 8:410–415. [PubMed: 9789330]
- Miller PJ, Afonso CL, Spackman E, Scott MA, Pedersen JC, Senne DA, Brown JD, Fuller CM, Uhart MM, Karesh WB, Brown IH, Alexander DJ, Swayne DE. Evidence for a new avian

- paramyxovirus serotype 10 detected in rockhopper penguins from the Falkland Islands. *J Virol.* 2010; 84:11496–11504. [PubMed: 20702635]
- Nayak B, Dias FM, Kumar S, Paldurai A, Collins PL, Samal SK. Avian paramyxovirus serotypes 2-9 (APMV-2-9) vary in the ability to induce protective immunity in chickens against challenge with virulent Newcastle disease virus (APMV-1). *Vaccine.* 2012; 30:2220–2227. [PubMed: 22222870]
- Nayak B, Kumar S, Collins PL, Samal SK. Molecular characterization and complete genome sequence of avian paramyxovirus type 4 prototype strain duck/Hong Kong/D3/75. *Virol J.* 2008; 5:124. [PubMed: 18937854]
- Nerome K, Nakayama M, Ishida M, Fukumi H. Isolation of a new avian paramyxovirus from budgerigar (*Melopsittacus undulatus*). *J Gen Virol.* 1978; 38:293–301. [PubMed: 627875]
- Paldurai A, Subbiah M, Kumar S, Collins PL, Samal SK. Complete genome sequences of avian paramyxovirus type 8 strains goose/Delaware/1053/76 and pintail/Wakuya/20/78. *Virus Res.* 2009; 142:144–153. [PubMed: 19341613]
- Park MS, Garcia-Sastre A, Cros JF, Basler CF, Palese P. Newcastle disease virus V protein is a determinant of host range restriction. *J Virol.* 2003; 77:9522–9532. [PubMed: 12915566]
- Parks GD. Mapping of a region of the paramyxovirus L protein required for the formation of a stable complex with the viral phosphoprotein P. *J Virol.* 1994; 68:4862–4872. [PubMed: 8035485]
- Romer-Oberdorfer A, Werner O, Veits J, Mebatsion T, Mettenleiter TC. Contribution of the length of the HN protein and the sequence of the F protein cleavage site to Newcastle disease virus pathogenicity. *The Journal of general virology.* 2003; 84:3121–3129. [PubMed: 14573818]
- Rosseel T, Lambrecht B, Vandenbussche F, van den Berg T, Van Borm S. Identification and complete genome sequencing of paramyxoviruses in mallard ducks (*Anas platyrhynchos*) using random access amplification and next generation sequencing technologies. *Virol J.* 2011; 8:463. [PubMed: 21978491]
- Saif YM, Mohan R, Ward L, Senne DA, Panigrahy B, Dearth RN. Natural and experimental infection of turkeys with avian paramyxovirus-7. *Avian Dis.* 1997; 41:326–329. [PubMed: 9201395]
- Samal, SK. Newcastle Disease and related avian paramyxoviruses. In: Samal, SK., editor. *The Biology of Paramyxoviruses*. Caister Academic Press; Norfolk, UK: 2011. p. 69-114.
- Samuel AS, Kumar S, Madhuri S, Collins PL, Samal SK. Complete sequence of the genome of avian paramyxovirus type 9 and comparison with other paramyxoviruses. *Virus Res.* 2009; 142:10–18. [PubMed: 19185593]
- Samuel AS, Paldurai A, Kumar S, Collins PL, Samal SK. Complete genome sequence of avian paramyxovirus (APMV) serotype 5 completes the analysis of nine APMV serotypes and reveals the longest APMV genome. *PLoS One.* 2010; 5:e9269. [PubMed: 20174645]
- Shihmanter E, Panshin A, Lipkind M. Nucleotide sequence of the matrix protein gene of avian paramyxovirus, serotype 3b: evidence on another member of the suggested new genus of the subfamily Paramyxovirinae. *Comp Immunol Microbiol Infect Dis.* 2005; 28:37–51. [PubMed: 15563952]
- Stanislawek WL, Wilks CR, Meers J, Horner GW, Alexander DJ, Manvell RJ, Kattenbelt JA, Gould AR. Avian paramyxoviruses and influenza viruses isolated from mallard ducks (*Anas platyrhynchos*) in New Zealand. *Arch Virol.* 2002; 147:1287–1302. [PubMed: 12111409]
- Subbiah M, Nayak S, Collins PL, Samal SK. Complete genome sequences of avian paramyxovirus serotype 2 (APMV-2) strains Bangor, England and Kenya: evidence for the existence of subgroups within serotype 2. *Virus Res.* 2010; 152:85–95. [PubMed: 20600395]
- Subbiah M, Xiao S, Collins PL, Samal SK. Complete sequence of the genome of avian paramyxovirus type 2 (strain Yucaipa) and comparison with other paramyxoviruses. *Virus Res.* 2008; 137:40–48. [PubMed: 18603323]
- Tumova B, Bachmann PA, Eichhorn W, Pfleger A, Stumpa A. Further evidence of the circulation of PMV-4 and influenza viruses with N2-1957 enzyme in the migratory waterfowls. *Acta Virol.* 1989; 33:573–576. [PubMed: 2576600]
- Tumova B, Robinson JH, Easterday BC. A hitherto unreported paramyxovirus of turkeys. *Res Vet Sci.* 1979; 27:135–140. [PubMed: 523797]

- Webster RG, Morita M, Pridgen C, Tumova B. Ortho- and paramyxoviruses from migrating feral ducks: characterization of a new group of influenza A viruses. *J Gen Virol.* 1976; 32:217–225. [PubMed: 1018173]
- Xiao S, Paldurai A, Nayak B, Subbiah M, Collins PL, Samal SK. Complete genome sequence of avian paramyxovirus type 7 (strain Tennessee) and comparison with other paramyxoviruses. *Virus Res.* 2009; 145:80–91. [PubMed: 19540277]
- Xiao S, Subbiah M, Kumar S, De Nardi R, Terregino C, Collins PL, Samal SK. Complete genome sequences of avian paramyxovirus serotype 6 prototype strain Hong Kong and a recent novel strain from Italy: evidence for the existence of subgroups within the serotype. *Virus Res.* 2010; 150:61–72. [PubMed: 20206652]
- Yamane N, Arikawa J, Odagiri T, Ishida N. Characterization of avian paramyxoviruses isolated from feral ducks in northern Japan: the presence of three distinct viruses in nature. *Microbiol Immunol.* 1982; 26:557–568. [PubMed: 7132788]

Highlights

- !! We have determined the sequences of P, M, F and HN genes of five APMV-4 strains isolated in Italy, in 2006.
- !! Comparison of sequences of the P, M, F and HN genes among Italian strains with known APMV-4 strains showed a high degree of sequence identity.
- !! Our results suggests that APMV-4 strains isolated to date show extensive sequence and antigenic relatedness.

A. Schematic diagram of APMV-4 genome

B. Intergenic region

	N/P (9 nt)	P/M (34 nt)	M/F (13 nt)
Hong Kong/D3/75	GGCUGGUUG	CUCGAGUAAACUAUACUGGACCAUAGUAUUUA	GGCCUAGGAAUAA
Korea/YJ/06	.A.....A	.UCG..G.....U.G..G.CG..C..	...U.....G.
Belgium/15129/07	.A.....A	.UCG..G.....A.U.G..G.CG..C..	...U.....G.
Italy/3670/06 (6)	.A.....A	.UCG..G.....A.U.G..G.CG..CC.	...U.....CG.
Italy/3936-70/06 (7)	.A.....A	.UCG..G.....A.U.G..GACG..C..	...U.....G.
Italy/4103-27/06 (8)	.A.....A	.UCG..G.....A.U.G..G.CG..C..	...U.....CG.
Italy/4284-20/06 (9)	.A.....A	.UCG..G.....A.U.G..G.CG..C..	...U.....G.
Italy/4523-4/06 (10)	.A.....A	.UCG..G.....A.U.G..G.CG..C..	...U.....G.
	F/HN (37 nt)	HN/L (42 nt)	
Hong Kong/D3/75	GCCAAACUUGACUCCACUCCCCCCCCACACGUACCA	GCCUUUUUACCGGAUCAAAGGUCCUUUCCUACCCUGGUAA	
Korea/YJ/06	.UA..CU..C.....U.....UUUU..A.....	A.U.....G.....UC..C..G..U.....	
Belgium/15129/07	.UA.....C.....U.....U.....A.....	.U..C.....U..C..G..U.....	
Italy/3670/06 (6)	.UA.....C.....U.....UUUU..A.....	A.U.....C.....UC..C..G..U.....	
Italy/3936-70/06 (7)	.UA.....C.....U.....UU..A.....	.U..C.....U..C..G..U.....	
Italy/4103-27/06 (8)	.UA.....C.....U.....UUUU..A.....	A.U.....C.....UC..C..G..U.....	
Italy/4284-20/06 (9)	.UA.....C.....U.....U.....A..U..	U.U..C.....U..C..G..U.....C..	
Italy/4523-4/06 (10)	.UA.....C.....U.....U.....A..U..	U.U..C.....U..C..G..U.....C..	

Figure 1.
Schematic diagram of APMV-4 genome and intergenic region sequence alignment comparison of Italian, Korea and Belgium strains with prototype Hong Kong strain.

Figure.2.

Phylogenetic tree of *Avulavirus* constructed based on genome sequence starting from N gene end to L gene start region. Italian strains clustered together with APMV-4 Hong Kong, Korea and Belgium strains.

Figure.3.

Phylogenetic tree of APMV-4 strains constructed based on genome sequence starting from N gene end to L gene start region. APMV-4 strains formed three distinct genetic groups; where Italian APMV-4 strains clustered together with Korean and Belgium APMV-4 strains and Hong Kong strain is distantly placed within tree.

Figure. 4.

Antigenic analysis of APMV-4 field isolates. Cross hemagglutination inhibition (HI) assay was carried out using chicken sera raised by inoculation with APMV-4 Hong Kong and Italian strains.