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Complete genome sequences of avian paramyxovirus type 8 strains goose/Delaware/1053/76 and pintail/Wakuya/20/78

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ABSTRACT

Complete consensus genome sequences were determined for avian paramyxovirus type 8 (APMV-8) strains goose/Delaware/1053/76 (prototype strain) and pintail/Wakuya/20/78. The genome of each strain is 15,342 nucleotides (nt) long, which follows the "rule of six". The genome consists of six genes in the order of 3′-N-P/V/W-M-F-HN-L-5′. The genes are flanked on either side by conserved transcription start and stop signals, and have intergenic regions ranging from 1 to 30 nt. The genome contains a 55 nt leader region at the 3′-end and a 171 nt trailer region at the 5′-end. Comparison of sequences of strains Delaware and Wakuya showed nucleotide identity of 96.8% at the genome level and amino acid identities of 99.3%, 96.5%, 98.6%, 99.4%, 98.6% and 99.1% for the predicted N, P, M, F, HN and L proteins, respectively. Both strains grew in embryonated chicken eggs and in primary chicken embryo kidney cells, and 293T cells. Both strains contained only a single basic residue at the cleavage activation site of the F protein and their efficiency of replication *in vitro* depended on and was augmented by, the presence of exogenous protease in most cell lines. Sequence alignment and phylogenic analysis of the predicted amino acid sequence of APMV-8 strain Delaware proteins with the cognate proteins of other available APMV serotypes showed that APMV-8 is more closely related to APMV-2 and -6 than to APMV-1, -3 and -4.

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1. Introduction

Paramyxoviruses are single-stranded, negative-sense, RNA viruses that are large (150–300 nm), enveloped and pleomorphic. Paramyxoviruses have been isolated from and implicated in many disease conditions across ecologically diverse species of animals, fish, birds, and humans (Lamb and Parks, 2007; Nylund et al., 2008). These viruses are grouped under the order *Mononegavirales* in the family *Paramyxoviridae*, which includes two subfamilies, *Paramyxovirinae* and *Pneumovirinae*. The subfamily *Paramyxovirinae* is further divided into five genera: *Morbillivirus* (includes measles [MeV] and canine distemper [CDV] viruses), *Rubulavirus* (mumps virus and human parainfluenza virus [HPIV-2]), *Respirovirus* (Sendai virus [SeV] and HPIV-1), *Henipavirus* (Hendra virus [HeV] and Nipah virus [NiV]) and *Avulavirus* (Newcastle disease virus (NDV) and other avian paramyxoviruses [APMVs]). The subfamily *Pneumovirinae* comprises two genera: *Pneumovirus* (human and bovine respiratory syncytial virus [HRSV and BRSV]), and *Metapneumovirus* (human and avian metapneumoviruses [HMPV and AMPV]) (Lamb et al., 2005; Mayo and Pringle, 1998).

The genomes of all paramyxoviruses range from 15 to 19 kb and contain 6–10 genes that code for up to 12 different proteins (Lamb and Parks, 2007). Efficient genome replication for the members of subfamily *Paramyxovirinae* depends on the total genome length being an even multiple of six, known as 'rule of six' (Calain and Roux, 1993; Kolakofsky et al., 1998). The genome termini consist of extragenic regions, called 3′-leader and 5′-trailer, which contain conserved promoter sequences involved in RNA replication and genome transcription. Each gene starts with a conserved gene start (GS) sequence and ends with a conserved gene end (GE) sequence. Transcription begins at the 3′-leader region and proceeds in a sequential manner by a start–stop mechanism using GS and GE signals (Lamb and Parks, 2007). Between the gene boundaries, there are non-coding intergenic sequences (IGS).

All members of the family *Paramyxoviridae* studied to date encode a major nucleocapsid protein (N) that binds the entire length of the genomic and antigenomic RNAs, a nucleocapsid-associated polymerase co-factor called phosphoprotein (P), a major polymerase protein (L) that contains catalytic domains, a matrix protein (M) that lines the inner surface of the virus envelope and is involved in viral morphogenesis, a fusion glycoprotein (F) that is a surface antigen that mediates viral penetration and syncytium formation and whose functional activity depends on host protease cleavage into F1 and F2 subunits, and a major glycoprotein (G)

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or hemagglutinin-neuraminidase (HN) glycoprotein that is a second surface antigen and mediates attachment (Lamb and Parks, 2007). Most members of subfamily *Paramyxovirinae* engage in RNA editing, whereby an "editing" motif, located midway along the P gene, directs the non-templated insertion of one or more guanine nucleotides into a proportion of P transcripts during mRNA synthesis. P gene editing shifts the reading frame to access one or more internal open reading frames (ORFs), resulting in mRNAs that encode chimeric proteins in which the N-terminal domain is that of the P protein and the C-terminal is derived from the alternative ORF. For viruses of *Respirovirus*, *Avulavirus*, *Morbillivirus*, and *Henipavirus*, the addition of one G results in expression of the V protein, which contains a C-terminal domain with a highly conserved cysteine-rich sequence. The addition of two G residues results in expression of the W protein. However, for members of *Rubulavirus*, it is the unedited mRNA that encodes V, while an mRNA with an insertion of two G residues encodes P (Lamb and Parks, 2007).

All paramyxoviruses that have been isolated from domestic and wild birds are grouped under the genus *Avulavirus* except for avian metapneumovirus (AMPV), which is classified under genus *Metapneumovirus* (Lamb and Parks, 2007). APMVs under the genus *Avulavirus* are divided into nine serotypes (APMV-1–9) based on hemagglutination inhibition (HI) and neuraminidase inhibition (NI) tests (Alexander, 2003). APMV-1 or Newcastle disease virus (NDV) is economically the most important viral disease of poultry and is the most studied member of this group. Very little information is available about the molecular and biological characteristics and pathogenicity of APMV-2 through -9. APMV-2, -3, -6 and -7 have been associated with disease in domestic poultry (Zhang et al., 2006, 2007; Redmann et al., 1991; Alexander and Collins, 1982; Bankowski et al., 1981; Tumova et al., 1979) and APMV-5 has been implicated in a severe pulmonary disease of budgerigars (Nerome et al., 1978). The pathogenicity of the remaining APMV serotypes is not known. Recently, the complete genome sequences of APMV-2 (Subbiah et al., 2008), APMV-3 (Kumar et al., 2008), APMV-4 (Nayak et al., 2008; Jeon et al., 2008) and APMV-6 (Chang et al., 2001) were determined, which has improved our understanding of the members of genus *Avulavirus*. However, the complete genome sequences of APMV-5, -7, -8 and -9 have not yet been determined.

APMV-8 strain goose/Delaware/1053/76 was first isolated in 1976 (Cloud and Rosenberger, 1980) from a feral Canadian goose (*Branta canadensis*) in the Atlantic Flyway in the U.S. APMV-8 strain pintail/Wakuya/20/78 was isolated from a feral pintail duck (*Anas acuta*) in Wakuya, Japan in 1978 (Yamane et al., 1982). Both these strains were later identified as representative strains of a new APMV serotype and designated APMV-8 by HI assay, structural protein profile and double immuno-diffusion tests (Alexander et al., 1983). Strain Delaware is considered to be the prototype strain of APMV-8. Since then, many APMV-8 strains have been isolated from geese and pintail ducks in wild bird population. However, very little is known about the molecular characteristics and pathogenicity of APMV-8.

In this paper, we describe the growth characteristics and complete consensus genome sequences of APMV-8 strains Delaware and Wakuya, and their phylogenetic relationship with the members of family *Paramyxoviridae.*

2. Materials and methods

2.1. Virus and cells

APMV-8 strains goose/Delaware/1053/76 and pintail/Wakuya/20/78 were received from the National Veterinary Services Laboratory, Ames, Iowa, U.S.A. and Central Veterinary Laboratory, Weybridge, Surrey, U.K., respectively. Delaware and Wakuya strains of APMV-8 were grown in the allantoic cavities of 9-day-old embryonated specific pathogen free (SPF) chicken eggs. Infected allantoic fluids were harvested 3 days post-inoculation. The titer of the virus was determined by hemagglutination (HA) assay using 1% chicken red blood cells (RBCs) at room temperature. Replication of the two strains was evaluated in eight established cell lines, namely, chicken embryo fibroblast (DF-1), quail fibrosarcoma (QT-35), African green monkey kidney (Vero), Madin Darby Bovine Kidney (MDBK), Madin Darby Canine Kidney (MDCK), Pig Kidney (PK-15), Bovine Turbinate (BT) and human embryonic kidney (293T) cells, and in two primary cells, namely, chicken embryo fibroblast (CEF) and chicken embryo kidney (CEK) cells. All cells were grown in Dulbecco's minimum essential medium (DMEM) containing 10% fetal bovine serum (FBS) and incubated at 37 °C under 5% CO_2. Each cell type was grown as a monolayer (70–80% confluency) and infected with 10^{-3} dilution of 2^8 HA units of both egg-grown APMV-8 strains, with or without the supplementation of acetyl trypsin (1 μg/ml) (Invitrogen) in serum free medium, as an exogenous source of protease for the cleavage of F protein. The cells were observed daily for 5–7 days for cytopathic effect (CPE) and HA activity of the cell culture supernatant.

2.2. Viral RNA isolation and sequence analysis

The prototype APMV-8 strain Delaware was grown in SPF chicken eggs and the virus from the infectious allantoic fluid was pelleted through 30% sucrose cushion at 25,000 rpm for 2 h in an SW28 rotor and a Beckman XL-80 ultracentrifuge. The pelleted virus was dissolved in 500 μl phosphate buffer saline and used for RNA extraction by Trizol-chloroform method (Invitrogen).

For complete genome sequencing of APMV-8 strain Delaware, RNA was isolated from virions, which were purified on sucrose gradients from allantoic fluid as described above, and was subjected to reverse transcription using random hexamer primers and Superscript II (Invitrogen) reverse transcriptase, using the manufacturer's protocol. The cDNA served as a template for PCR using degenerate consensus primers that were designed for N, HN and L genes by aligning available gene sequences from members of genera *Avulavirus*, *Morbillivirus*, *Respirovirus* and *Rubulavirus*. The N gene primer set N230fwd (5′-WWSGKMKWAG CGGAAATACC-3′) (where N = A/C/G/T, S = G/C, M = A/C, W = A/T, K = G/T) and N630rev (5′-AWNCKNARRWCTCTGGTCTCA-3′) (where R = A/G) yielded a 350 bp length amplicon. The HN gene primer set HN540fwd (5′-AGTKGWTGWTTGCTGGAGGTTCT-3′) and HN770rev (5′-CCARTTNARRCGATWAGGACA-3′) generated an amplicon length of around 200 bp. The L gene primer set L4500fwd (5′-GCNGCNGTNGCNAATTATWTACTT-3′) and L4750rev (5′-CANCKNARRTATCTACCNCTGAT-3′) yielded an amplicon size of about 500 bp. The amplicons of the N, HN and L genes corresponded to regions 247–645, 6832–7026 and 12,349–12,842 nucleotides of the genome sequence of strain Delaware, respectively. A new set of primers was designed from these three virus-specific sequences for determining the remaining sequence of strain Delaware.

The leader sequence of the APMV-8 strain Delaware was determined using 3′-rapid amplification of cDNA ends (3′RACE) (Li et al., 2005; Troutt et al., 1992). Briefly, the genomic RNA was ligated to a 3′-blocked RNA oligo-(5′-GGTTTTGCGGTAAAGGTGGAAGAGAAG-3′-blocked) using T4 RNA ligase according to manufacturer's protocol (Promega). RT-PCR was performed using a DNA complementary oligo-(5′-CCAAAACGCCATTTCCACCTTCTCTTC-3′) with sequence-specific NP190rev primer (5′-TGCTCCTTGCTTCATTGGTGTGG-3′). The resultant PCR product was cloned (see below) and sequenced. The sequence of the trailer region was determined

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