

Glycoprotein gene truncation in avian metapneumovirus subtype C isolates from the United States

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Abstract The length of the published glycoprotein (G) gene sequences of avian metapneumovirus subtype-C (aMPV-C) isolated from domestic turkeys and wild birds in the United States (1996–2003) remains controversial. To explore the G gene size variation in aMPV-C by the year of isolation and cell culture passage levels, we examined 21 turkey isolates of aMPV-C at different cell culture passages. The early domestic turkey isolates of aMPV-C (aMPV/CO/1996, aMPV/MN/1a-b, and 2a-b/97) had a G gene of 1,798 nucleotides (nt) that coded for a predicted protein of 585 amino acids (aa) and showed >97% nt similarity with that of aMPV-C isolated from Canada geese. This large G gene got truncated upon serial passages in Vero cell cultures by deletion of 1,015 nt near the end of the open reading frame. The recent domestic turkey isolates of aMPV-C lacked the large G gene but instead had a small G gene of 783 nt, irrespective of cell culture passage levels. In some cultures, both large and small genes were detected, indicating the existence of a mixed population of the virus. Apparently, serial passage of aMPV-C in cell cultures and natural passage in turkeys in the field led to truncation of the G gene, which may be a mechanism of virus evolution for survival in a new host or environment.

Keywords Avian metapneumovirus · Turkey · Glycoprotein · Gene variation · Truncation

Introduction

Avian metapneumovirus (aMPV) is an economically significant pathogen of turkeys with a worldwide distribution [1, 2]. Though the disease caused by aMPV was identified in South Africa in late 1970s [3] and in Europe in 1980s [4, 5], the United States was free of aMPV infection until 1996. An outbreak of upper respiratory tract infection of commercial turkeys in Colorado in May, 1996 resulted in the isolation of aMPV, for the first time in the United States [6]. The aMPV outbreak in Colorado that lasted for 10 months was eliminated by intense biosecurity measures and has not been reported since. In Minnesota, the virus was first identified in 1997 [6] and is still an economic concern to the turkey industry in the state [7]. Now there is serological evidence for the spread of aMPV to neighboring states of North Dakota, South Dakota, Iowa, and Wisconsin [8].

The virus causes an acute upper respiratory tract infection in turkeys [9–11] and is also involved in the etiology of swollen head syndrome in chickens [12, 13], although aMPV has not been detected in chickens in the United States. The disease caused by aMPV in turkeys is characterized by coughing, sneezing, nasal discharge, tracheal rales, foamy conjunctivitis, and swollen sinuses [14]. Uncomplicated cases have low mortality (2–5%) but infections accompanied by concurrent secondary bacterial infections can result in up to 25% mortality [9]. In breeder turkey flocks, there is a transient drop in egg production and paleness of the eggshell [15].

The aMPV belongs to the genus *Metapneumovirus* of the subfamily *Pneumovirinae* of the family *Paramyxoviridae* [16]. It contains a negative-sense, non-segmented,

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single-stranded RNA genome that encodes eight genes, namely, nucleocapsid (N), phosphoprotein (P), matrix (M), small hydrophobic (SH), surface glycoprotein (G), fusion (F), second matrix (M2), and RNA-dependent RNA polymerase (L) genes in the order 3'-le-N-P-M-F-M2-SH-G-L-tr-5' [17, 18]. In contrast to the members of the genus *Pneumovirus* in the subfamily *Pneumovirinae*, aMPV lacks the non-structural (NS1 and NS2) genes [16, 19] and has a smaller L gene than other pneumoviruses [20, 21]. Based on sequence divergence described in the G gene and antigenic differences observed among aMPV strains, the aMPV isolates are classified into four subgroups: A, B, C, and D. The isolates from the United States belong to subgroup C (aMPV-C), while the strains isolated from other parts of the world belong to one of the other three subgroups [22–24]. Interestingly, sequence analyses have shown that aMPV-C isolates from the United States are more closely related to human metapneumovirus (hMPV) than to their avian counterparts [25–28].

A seasonal pattern in the occurrence of aMPV-C has been observed in Minnesota with a high rate of incidence in spring (April to May) and autumn (October to December) [29]. One of the reasons for the seasonality of aMPV outbreaks in Minnesota was speculated to be the migratory pattern of wild birds that are involved in the transmission of the virus [29]. Recently aMPV-C was isolated from asymptomatic wild Canada geese in Minnesota which was shown to be avirulent but immunoprotective in domestic turkeys [30]. Genomic analysis of the goose aMPV-C demonstrated a larger G gene compared to that of domestic turkey isolates [30].

The G gene of aMPV has been of much scientific curiosity owing to its role in host cell attachment and possible role in virulence and immunity. Extensive sequence divergence of G gene was observed among European subtypes of aMPV [24]. The G gene of aMPV-C from the United States was independently sequenced by different research groups but the reports on its size and characteristics were often contradictory to each other [30–32]. A G gene size of 783 nt or 1321 nt or 1798 nt was reported by different researchers [30–32]. The reasons for these differences in the G gene in different isolates and the possible consequence of this on the evolution of the virus are not well studied. We speculated that the presence of a large G gene in aMPV-C isolated from wild birds (Canada geese) and domestic turkeys in 1996–1997 when they first appeared in the United States, and a small G gene in those isolated from domestic turkeys after 1997, could be due to selective pressure. The general hypothesis was that aMPV-C in domestic turkeys might have originated from wild birds and that the virus might have lost part of the G gene upon passage in the turkey population. In the present study we investigated the nature of the G gene of different aMPV-C isolates from domestic turkeys and compared that

with the published G gene of aMPV-C from Canada goose in an attempt to answer the question that whether wild bird aMPV was a potential progenitor of the virus that is presently circulating in the domestic turkeys in the United States. The specific objective of the present study was to analyze the G gene of aMPVs isolated from domestic turkeys from the United States from 1996 to 2003 to examine any variation in the gene with respect to the time of isolation from turkeys and passage in cell cultures.

Materials and methods

Viruses

Twenty-one isolates of aMPV-C isolated over a period of 7 years from 1996 to 2003 from the United States (1 from Colorado and 20 from Minnesota) were used in this study (Table 1). For convenience, in the text, viruses were designated as aMPV/CO for the Colorado isolate and as aMPV/MN followed by a number for those isolated from Minnesota. All these viruses were isolated from domestic turkeys following a natural outbreak of aMPV disease in commercial turkey flocks.

In addition to the above-mentioned isolates, two vaccine strains of aMPV-C were also used in the study. The vaccine

Table 1 Different isolates of aMPV/C from naturally infected turkeys used for the analysis of glycoprotein gene

No.	Source	County	Designated name
1	Turbinat	Weld	aMPV/Colorado/Turkey/1996
2	Turbinat	Becker	aMPV/Minnesota/Turkey/1a/1997
3	Tracheal Swab	Becker	aMPV/Minnesota/Turkey/1b/1997
4	Turbinat	Meeker	aMPV/Minnesota/Turkey/2a/1997
5	Tracheal Swab	Meeker	aMPV/Minnesota/Turkey/2b/1997
6	Turbinat	Steele	aMPV/Minnesota/Turkey/4a/1999
7	Choanal Swab	Steele	aMPV/Minnesota/Turkey/4b/1999
8	Choanal Swab	Steele	aMPV/Minnesota/Turkey/4c/1999
9	Choanal Swab	Rice	aMPV/Minnesota/Turkey/5/1999
10	Turbinat	Steele	aMPV/Minnesota/Turkey/6/1999
11	Choanal Swab	Meeker	aMPV/Minnesota/Turkey/7/1999
12	Turbinat	Meeker	aMPV/Minnesota/Turkey/8/1999
13	Turbinat	Meeker	aMPV/Minnesota/Turkey/9/1999
14	Turbinat	Kandiyohi	aMPV/Minnesota/Turkey/10/1999
15	Turbinat	Stearns	aMPV/Minnesota/Turkey/11/1999
16	Turbinat	Meeker	aMPV/Minnesota/Turkey/13/2000
17	Turbinat	Meeker	aMPV/Minnesota/Turkey/14/2001
18	Turbinat	Ottertail	aMPV/Minnesota/Turkey/16/2000
19	Turbinat	Kandiyohi	aMPV/Minnesota/Turkey/17/2000
20	Turbinat	Kandiyohi	aMPV/Minnesota/Turkey/18/2000
21	Choanal Swab	Rice	aMPV/Minnesota/Turkey/19/2003

strain p41 was aMPV/MN 1a serially passaged 41 times in Vero cells and had been used in a controlled exposure program for vaccination in Minnesota beginning in August 1999. The second one was p63, which was aMPV/MN 1a passaged 63 times in Vero cells [33] and is currently a commercially available licensed aMPV-C vaccine (Pneumomune, Biomune Company, Lenexa, KS).

Cells and media

Primary isolation of the virus was done in specific-pathogen-free chicken embryo fibroblasts (CEF). Cells were grown in Minimum Essential medium (MEM, Invitrogen, Carlsbad, CA) supplemented with 10% bovine fetal serum, 2 mM L-glutamine, non-essential amino acids, 100 IU/ml of penicillin G sodium, and 100 µg/ml streptomycin sulfate. The same medium was used for cell maintenance except that the bovine fetal serum was reduced to 3%. Nasal turbinate tissues and/or choanal or tracheal swabs were collected from infected birds. A 20% homogenate of the turbinate was prepared in MEM (Invitrogen) supplemented with penicillin G sodium 100 IU/ml, streptomycin sulfate 100 µg/ml, amphotericin B 25 µg/ml, and gentamicin 150 µg/ml. A 2 ml volume each of the same medium was used for preparing the swab suspension. The turbinate homogenate or swab suspension was then fractionated by centrifugation at $8000 \times g$ for 10 min. An 80–90% confluent monolayer of CEF in a 25 cm² cell culture flask was inoculated with 1 ml of the sample supernatant and incubated at 37°C for 1 h. The cell monolayer was washed with PBS (pH 7.2), fresh medium was added and incubated at 37°C for 3–5 days. Five to seven passages were done in CEF. The end point was determined by aMPV-C M gene RT-PCR [34] and indirect immunofluorescent assay [35]. Following primary isolation, viruses were propagated in Vero cells (ATCC Number: CCL-81) infected at MOI 0.01, under the same conditions described above. Following each cell culture passage, the virus was harvested by three cycles of freezing and thawing. The cell culture suspension was fractionated by centrifugation at $8000 \times g$ for 10 min and the supernatant was aliquoted and stored at –70°C.

RT-PCR

The viral mRNA was extracted from nasal turbinate homogenate, choanal/tracheal swab, CEF or Vero cell culture supernatant using a commercial viral RNA extraction kit (Qiagen, Valencia, CA). RT-PCR was performed using a commercially available one-step RT-PCR kit (Qiagen). A matrix (M) gene-based aMPV RT-PCR was performed for diagnosis and confirmation of samples [34]. The forward primer used was 5'-ACAGTGTGTGAGTTAAAAG-3' (M1) starting from base number 335 of the M gene of

aMPV-C. The reverse primer was 5'-TGACTTCAGGACATATCTC-3' (M2) starting from the M gene base number 754 of aMPV-C (GenBank Accession Number: AF187151).

A set of primers based on the published G gene of aMPV-C isolated from Canada goose was used [30] to examine aMPV-C strains at different CEF and Vero cell passage levels. The forward primer (5'-ACAAGTCAA CATGGAGGTCA-3') was originally derived from the consensus G gene sequences of aMPV-Cs isolated from turkeys and Canada goose (GenBank Accession Numbers: AY579780; NC007652). The reverse primer (5'-GGC AAGAYCCTATTGCATTG-3') was derived from the 5' end of the L gene of aMPV-C (GenBank Accession Numbers: AY579780). These primers were expected to produce a nucleotide sequence size of 1865 bp from aMPV-C of Canada goose origin and 785 bp from aMPV-C of domestic turkey origin [30]. The G gene RT-PCR was performed using a commercially available kit, SuperScript III one-step RT-PCR with platinum taq (Invitrogen). The products of RT-PCR were analyzed by electrophoresis on a 1.2% agarose gel and examined using a trans-illuminator.

Sequence analysis of aMPV G gene

The RT-PCR products were gel purified using a Gel extraction kit (Qiagen, Valencia, CA) and sequenced directly with the G gene-specific primers derived from the consensus of the published G gene sequences of the goose and turkey isolates [30, 36]. The purified DNA products were also subcloned into a TA cloning vector pCR4-TOPO (Invitrogen) and transformed in One Shot *Escherichia coli* cells (Invitrogen). Transformed *E. coli* were plated on LB agar containing 100 µg/ml of ampicillin and 50 µg/ml of kanamycin and incubated at 37°C overnight. Three randomly selected colonies were grown overnight in LB medium containing 100 µg/ml of ampicillin and 50 µg/ml of kanamycin. Plasmid extraction was performed using commercially available QIAprep Spin Miniprep kit (Qiagen). The prepared plasmids were sequenced with T7 forward and SP6 reverse primers. Sequencing was done at the BioMedical Genomics Center DNA Sequencing and Analysis Facility at the University of Minnesota (Saint Paul, MN). Each clone was sequenced thrice. The DNA sequences obtained were analyzed using BLAST search (NCBI data bank) to compare the sequences with the data bank. The sequences were analyzed using DNA STAR and MEGA version 3.1 [37]. Sequence comparison was performed by multiple alignment using CLUSTALW of MEGA 3.1.

RT-PCR and G gene sequencing were independently repeated in the Southeast Poultry Research Laboratories of the United States Department of Agriculture for confirmation and repeatability of the results.

Results

Identification of two major populations of the G gene

The M gene RT-PCR confirmed all the 21 isolates tested as aMPV-C. The glycoprotein gene of aMPV-C showed extensive variations in the size of the RT-PCR products on agarose gels with respect to the different isolates and their cell culture passages tested. Of the 21 isolates of aMPV-C tested (Table 1) at CEF passage level 5 or 6 (only for aMPV/CO), 5 of them had a large G gene segment of 1.8-kbp size. The large G gene products were obtained for aMPV/CO, aMPV/MN 1a, aMPV/MN 1b, aMPV/MN 2a, and aMPV/MN 2b. All the other 16 isolates (No. 6–21; Table 1) tested at CEF passage level 5 had a small G gene product of about 0.8 kbp in size.

The low CEF passage isolates of aMPV/CO (CEF p6), aMPV/MN 1a (CEF p5), aMPV/MN 2a (CEF p5), and the most recent isolate, aMPV/MN 19, were further propagated in Vero cells. The aMPV/CO at passage level CEF p6 lost the large G gene after 7 passages in Vero cells (Vero p7) but instead had a small G gene product of 0.8 kbp in size (Table 2). Similarly, aMPV/MN 1a and aMPV/MN 2a at

passage level CEF p5 showed a truncation in their G gene after 7 and 13 passages in Vero cells, respectively (Table 2). On the other hand, aMPV/MN 19 (CEF p5 Vero 7) showed a small G gene similar to its low CEF passage (aMPV/MN 19 CEF p5) (Table 2).

A few of the randomly selected virus isolates aMPV/MN 4a, aMPV/MN 7, aMPV/MN 9, and aMPV/MN 19 tested at lower passage level CEF p2 also produced small G gene product of size similar to their CEF p5 strains (data not shown). The RT-PCR products of the vaccine strains p41 and p61 also lacked the large G gene, but instead had a small G gene product of size about 0.8 kbp (data not shown).

To verify the above results, two different sets of primers were also used for RT-PCR amplification of the G region from selected aMPV isolates. One pair of primers annealed to the end of the SH gene (forward primer) and the beginning of the L gene (reverse primer) about 270 nts downstream of the L gene start signal. The second pair of primers targeted to the SH and G intergenic region (forward primer) and the beginning of the L gene open reading frame (reverse primer). Both pairs of primers produced similar results as to what was observed with the G gene-specific primers (data not shown).

Table 2 Glycoprotein gene sizes of different isolates of avian metapneumovirus subtype C at different cell culture passage levels

No.	Virus designation	Passage level CEF/Vero	Size of glycoprotein gene RT-PCR product (kbp)
1a	aMPV/Colorado/Turkey/1996	CEFp6	1.8
1b	aMPV/Colorado/Turkey/1996	CEFp6; Verop7	0.8
2a	aMPV/Minnesota/Turkey/1a/1997	CEFp5	1.8
2b	aMPV/Minnesota/Turkey/1a/1997	CEFp5; Verop7	0.8
3	aMPV/Minnesota/Turkey/1b/1997	CEFp5	1.8
4a	aMPV/Minnesota/Turkey/2a/1997	CEFp5	1.8
4b	aMPV/Minnesota/Turkey/2a/1997	CEFp5; Verop13	0.8
5	aMPV/Minnesota/Turkey/2b/1997	CEFp5	1.8
6	aMPV/Minnesota/Turkey/4a/1999	CEFp5	0.8
7	aMPV/Minnesota/Turkey/4b/1999	CEFp5	0.8
8	aMPV/Minnesota/Turkey/4c/1999	CEFp5	0.8
9	aMPV/Minnesota/Turkey/5/1999	CEFp5	0.8
10	aMPV/Minnesota/Turkey/6/1999	CEFp5	0.8
11	aMPV/Minnesota/Turkey/7/1999	CEFp5	0.8
12	aMPV/Minnesota/Turkey/8/1999	CEFp5	0.8
13	aMPV/Minnesota/Turkey/9/1999	CEFp5	0.8
14	aMPV/Minnesota/Turkey/10/1999	CEFp5	0.8
15	aMPV/Minnesota/Turkey/11/1999	CEFp5	0.8
16	aMPV/Minnesota/Turkey/13/2000	CEFp5	0.8
17	aMPV/Minnesota/Turkey/14/2001	CEFp5	0.8
18	aMPV/Minnesota/Turkey/16/2000	CEFp5	0.8
19	aMPV/Minnesota/Turkey/17/2000	CEFp5	0.8
20	aMPV/Minnesota/Turkey/18/2000	CEFp5	0.8
21a	aMPV/Minnesota/Turkey/19/2003	CEFp5	0.8
21b	aMPV/Minnesota/Turkey/19/2003	CEFp5; Verop7	0.8

Discovery of a truncation in the G gene of recent turkey isolates and cell culture highly adapted variants

Sequencing of the large G gene from low passage aMPV/CO, aMPV/MN 1a, and aMPV/MN 2a revealed a size of 1798 nt that had a predicted amino acid sequence of 585 aa in size (Fig. 1). These sequences showed more than 97% nucleotide similarity and above 90% predicted amino acid identity with the previously published G gene sequences of the same isolates (GenBank Accession numbers AY590692 (aMPV/CO), AY590690 (aMPV/MN 1a), and AY590693 (aMPV/MN 2a) [32]; and also the Canada goose isolate of aMPV-C (GenBank Accession number NC007652) [30]. Two of the small G gene products, aMPV/MN 1a (CEF p5 Vero p7) and aMPV/MN 2a (CEF p5 Vero p13) were cloned and sequenced, whereas the rest were sequenced directly from the RT-PCR product. The small G gene sequence analysis revealed that the gene appeared to be a truncated product of large G gene with a deletion of 1015-nt-long segment from the large G gene (underlined area of Fig. 1). Analysis of the small G gene of high passage aMPV/MN 1a and aMPV/MN 2a showed a gene size of 783 nt and more than 96% nucleotide similarity with the first 752 nucleotides of their respective large G genes from the low passage (Fig. 2). Sequence analysis also showed that clones derived from the same purified small fragment of high passage aMPV/MN 1a and aMPV/MN 2a also appeared to contain a different length of the G gene. Some of the clones had the same length of G as the published small G gene (783 nts), but others contained a truncated G with different lengths of nucleotide deletions (38–325 nts) internally or at the C terminus when compared with the published small G. The small G gene sequenced directly from the RT-PCR products also showed a high level of nucleotide sequence similarity to the 5' end of that of large G gene sequences of aMPV/CO (CEF p6), aMPV/MN 1a (CEF p5), and aMPV/MN 2a (CEF p5).

Discussion

There are various reports on the length and characteristics of G gene of aMPV-C isolated from the United States [30–32]. Alvarez et al. [31] reported that the size of G gene of aMPV/CO was about 1321 nt that encoded a single open reading frame (ORF) coding for a protein of 435 aa. Another study indicated that the aMPV/CO G gene had a nucleotide sequence of 783 nt with one ORF coding for a polypeptide of 252 aa [38]. Bennett et al. [30] demonstrated that aMPV-C isolated from Canada goose had a G gene of 1798 nt long that coded for a protein of 585 aa in size. When Bennett et al. [30] analyzed the G gene of aMPV/CO and aMPV/MN 2a, the RT-PCR product was 783 bp in size which was 1015 bp

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GGGACAAGTCAACATGGAGGTCAAGGTAGAGAATGTTGGCAAGTCACAGGA
GCTTAAAGTTAAAGTCAAGAATTTTATAAAAAGGTCTGATTGCAAGAAAAA
CTTTTTGCCTTGATTTTAGGCTAGTTAGCTTTGAACTCACTATGAATATAATG
TGTCTGTCATGTATGTGGAGTCAAATGAGGCCCTAAGTTTATGTAGGATCCAA
GGGACTCCTGCTCCAAGAGATAATAAGAAAAACACAGAAAAACGCAACAAAG
GAAACAACACTCCACACAACGACCACAACAAGGGATCCAGAGGTGAGGGAA
ACAAAAACCACCAAGCCCCAGGCCAATGAAGGAGCAACAAACCAAGCAGG
AACCTCACCACCAAGGGAGACAAACACCAAAACGACAAGAGCAACAACAGAG
GCAGAACTGGAAAAACAAAGCAAGCAACCACAGAGCCAGGCACCAGCACC
CAAAAGCACACCCCGCAAGGCCAAGCAGCAAATCCCCACCACAACACAA
GCAACAGCACAAACCACAAACACCAACAGCCCCAAAAGCAAGCAGCAGCC
AAGAACAGACAGGCAACAACCAAAAAAACCGAAACGGACACCACAACAG
CAAGCAGAGCAAGGAACACCAACAACCCACAGAGACAGCAACAACAACCTC
CCAAAGCAACAACAGAAACAGGCAAGGGCAAAGAGGGGCCAACACAGCAC
ACAACCAAGAACAGCCCCGAGACAACAGCAGCAGAGAGACAACAACCCACAG
CCAAGAAGAACAGCCAGCAGGCCAGGCCAACCAACAAAAATCGAAGAGGAG
GCAGAAACCACCAAAACCAGAAACAACCAAAAAACCCCAACAAGCACAGGC
CCACCAAGACCAACAAGAAGCACACCAAGCAAAACAGCGACAGAAAAACAAC
AAGAGAACCACAACAACAAGAGACCGAACACAGCGAGCAGACAGCAGCAG
ACAACAGACCAGAACAACNGCAGAGCAAGACCAACAAACCCAAACCCAGGGC
CAAACCCACCACAATGGGGCCACCCGCAAAACCACCACCACCCACAGAGCA
CAACACAGACACAACAACAGCACAAAAGGAAAGTCCCAAGGAGGACAAAAA
GACCAGAGACCCAGCAGCAAAACACCAACCGAGCAAGAGGACGCCAGCAAA
GGGAACCGCCCGGCCAACCCCGAGGCGAGCCGAGGCAGACAGGAGAGC
ACCCCGGCAACCACCCCAACGGGGCGCACACAGAATCGGCCGCGGGCAC
AACGGGGGACGACAGCGGGGGCGGAAACCAACAAGACGGAGAAGCGCAGCGG
ACAGACGGCCACGGGGGCGAGCAGCAGCAGCGGAGGCGGGACAGCCAGA
GCGGACGGGCCAGCCGAAGCAGCCGAGCGGGCACGGCAGCAGGGAACA
CTGCACCACCGAACACGAAAGCAGCGGTTCGGGCAGATGCCGACCAGCAG
AGGAAGCCGGGGTTCGGCTCAAGCATCTGGCGAGGGAGACAGTCCCGGGGC
CCTGCAGAGAGAGCGCTCCGGAGCATCCGATGAATCCATGCCTTGGGGACGG
CACTGCACGGACCCGTTCCGGATGGTGGAGGGAATTTGCCGGCTGGTTGTTAT
GATAGTGGAGCAGATGGGGTGGTTTGTCTGTGGAATTTGTGGAGGCAACTCGT
GTTGTTGCGGGCGAGTAGAGTGCACGTGTGGGGGTGGAGCGGGACTACTATC
CTGCTGCTGTGGCTCATACTGGAGTTAGTTCAGAGCAAGTTAGTTAATT
AAAAA

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Fig. 1 Nucleotide sequence of the large G gene of aMPV/C (sequence of aMPV/MN 1a; CEF p5 that showed a large G gene of 1798 bp in size). The gene start and stop signals are bolded and italicized. The underlined part of the sequence is the segment that got truncated upon serial passage in Vero cells

less than that of the Goose virus with the same set of primers. Govindarajan et al. [32] reported G gene sequencing of aMPV/CO, aMPV/MN 1a, and aMPV/MN 2a isolates and showed that the G gene was 1798 nt long with a predicted

GGGACAAGTCAACATGGAGGTCAAGGTAGAGAATGTTGGCAAGTCACAGGA
 GCTTAAAGTCAAAGTCAAGAATTTTATAAAAAGGTCTGATTGCAAGAAAAA
 CTTTTGCTTGTATTTAGGGCTAGTCAGCTTTGAACTCACTATGAATATAAT
 GCTGTCTGCATGTATGTGGAGTCAAATGAGGCCCTAAGTTTATGTAGGATCC
 AAGGGACTCTGCTCCAAGGGATAATAAGACAAAACAGAAAACGCAACAA
 AGGAAACAACACTCCACACAACGACCACAACAAGGGATCCAGAGGTGAGGG
 AAACAAAAACCACCAAGCCCAGGCCAATGAAGGAGCAACAAACCCAAGCA
 GGAACCTCACCACCAAGGGAGACAAACACCAAAACGACAAGAGCAACAACAG
 AGGCAGAACTGGAAAAACAAAGCAAACAAACACAGAGCCAGGCACCCAGCA
 CCCAAAAGCACACCCCGCAAGACCAAGCAGCAAATCCCCACCACAACAC
 AAGCAATAGCACAACCTGACAACACCAACAACCCCAAAAGCAAGCACAGCAC
 CCAAGAACAGACAGGCAACAACCAAAAAACCGAAACGGACACCACAACAG
 CAAGCAGAGCAAGGAACACCAACAACCCACAGAGACAGCAACAACAACCTC
 CCAAAGCAACAACAGAAACAGGCAAGAGCAAAGAGGGCCAACACAGCAC
 ACAACCAAGAAGCAGCCGAGACAACAGCAGGAGTTAGTTTAAGAGCAAGT
TAGTTAATAAAAA

Fig. 2 Nucleotide sequence of small G gene of aMPV/C (sequence of aMPV/MN 1a; CEF p5; Vero p7 that showed a small G gene of 783 bp in size). The gene start and stop signals are bolded and italicized

protein size of 585 aa which is in agreement with the results of the present study with regard to the early passages of those isolates.

In the present study, we examined the G gene sequences of 21 aMPV-C isolates from the United States obtained from 1996 to 2003 and our results indicated extensive variations in the size of the G gene with regard to the year of isolation and passage in cell cultures. Viruses isolated from domestic turkeys in 1996 and 1997, the early years of the first introduction of aMPV in the United States, had a large G gene which got truncated upon serial passage in cell cultures. Viruses isolated from 1999 onward from domestic turkeys lacked this large G gene but instead showed a small G gene. In this context, it is interesting to note that aMPV-C isolated from Canada geese in Minnesota in 2001 also had a large G gene. The large gene segment of aMPV-C isolated from domestic turkeys in 1996 and 1997 was 1798 nt in size which was the same size as the G gene observed in aMPV isolated from Canada geese [30]. The above-mentioned isolates shared a nt similarity of above 97% between each other. Adaptation of 1996 and 1997 isolates from domestic turkeys in cell cultures led to deletion of 1015-nt-long segment from the 1798-nt-long large G gene. On the other hand, aMPV-C isolates obtained from 1999 onward appear to have lost their large G gene and instead have smaller G gene, irrespective of their cell culture passage level. The large G gene size showed in the present study is in agreement with that presented by Govindarajan et al. [32] for turkey

isolates and by Bennett et al. [30] for Canada goose isolates from the United States. Similarly the size of our small G gene fragment was in agreement with the size of aMPV-C G gene published by Toquin et al. [38] and Lwamba et al. [36]. The interesting aspect of the present study is that the results show that all the contradictory reports on the size of G gene were probably true in their respective sense. The present study demonstrated that the large G gene and small G gene are present in different isolates of aMPV-C depending on the year of isolation and level of cell culture passage. The vaccine strains p41 and p63 also showed a small G gene of 783 nt long with a predicted polypeptide of 252 amino acids. The vaccine strain p41 was introduced to Minnesota turkeys in August 1999 as part of a controlled exposure program to protect turkeys against virulent aMPV challenge. In the present study we detected a deletion in the large G gene in aMPVs isolated from April 1999 onward and that excludes the possibilities of a chance introduction of the G gene truncated virus through controlled exposure program.

Our study also suggests the possibility of a mixed population of G gene with varying sizes in aMPV-C. Multiple bands on the gel appeared to persist even after our attempt to optimize the thermocycler conditions. A similar experience with aMPV G gene RT-PCR was also reported elsewhere when genomic RNA was used to amplify the G gene [32]. Differences in the size of PCR products were also observed in the G gene of European subtypes of aMPV and these differences in size were thought to be due to variations in the 3' end of the G mRNAs [24].

Extensive variations in the G gene sequence were observed among the European subtypes and were one of the determinants of aMPV subtype classification [24]. Variations in the size of G gene were also observed in human respiratory syncytial virus where a large portion of the coding sequence of G gene was found to be deleted in a cold-passaged candidate vaccine virus [39]. The ability of viruses to replicate efficiently even after the deletion of G and SH genes was demonstrated in many viruses including aMPV [40], hMPV [41], and RSV [39]. Though considerable divergence in the sequence alignment of G gene of aMPV-C has been reported previously [32], this is the first report of an extensive study of 21 isolates showing a large deletion in the G gene of aMPV-C in those viruses isolated since 1999 and upon serial passage of the early isolates (1996–1997) in cell cultures.

The present study demonstrated that similar to the wild bird (Canada goose) aMPV isolate, early isolates of aMPV-C from domestic turkeys had a large gene which got truncated upon in vitro passage of the virus. Serial passage in cell culture and possibly, natural passage through spread of virus in turkey population, led to the deletion of a large portion of G gene producing a truncated version of the

original gene. The results also suggest a possible role of wild birds in the transmission of aMPV in turkeys. The presence of large and small G gene products may indicate the existence of a mixed population or a quasispecies of the virus as part of a virus survival mechanism in turkeys. The results of this present study warrant further investigation on the role of wild birds in the transmission of aMPV in turkeys in the United States. A detailed study is also indicated to unravel the genetic mechanism of these variations and its possible role in virus evolution.

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