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Sequence analysis of the N, P, M and F genes of Canadian human metapneumovirus strains☆

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Abstract

The complete nucleotide sequences of the nucleoprotein (N), phosphoprotein (P), matrix protein (M), and fusion protein (F) genes of 15 Canadian human metapneumovirus (hMPV) isolates were determined. Phylogenetic analysis revealed two distinct genetic clusters, or groups for each gene with additional sequence variability within the individual groups. Comparison of the deduced amino acid sequences for the N, M and F genes of the different isolates revealed that all three genes were well conserved with 94.1–97.6% identity between the two distinct clusters The P gene showed more diversity with 81.6–85.7% amino acid identity for isolates between the two clusters, and 94.6–100% for isolates within the same cluster.
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1. Introduction

Respiratory virus infections account for significant morbidity and mortality. Viruses most frequently associated with respiratory tract infections include rhinoviruses, coronaviruses, influenza viruses, parainfluenza viruses, respiratory syncytial viruses and adenoviruses. However, the causative agents for a significant portion of respiratory infections are still unknown. Recently, van den Hoogen et al. reported the isolation of a new paramyxovirus in the Netherlands that was tentatively assigned to the *Metapneumovirus* genus of the Pneumovirinae subfamily (van den Hoogen et al., 2001). This hMPV has been associated with respiratory illnesses ranging from upper respiratory tract disease to severe bronchiolitis and pneumonia similar to those caused by human respiratory syncytial virus (HRSV) infection. hMPV is currently being isolated from patients with respiratory disease in several countries suggesting that hMPV may be present throughout the world (Pelletier et al., 2002; Peret et al., 2002; Stockon et al., 2002). Partial sequence analysis demonstrated sequence similarity between the Dutch and Canadian isolates and the presence of two potential genetic clusters. Phylogenetic

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Table 1
Primers used for amplification and sequencing

Gene	Forward primers	Reverse primers
N	55-GCGCGGATCCATGTCTCTTCAAGGGATT-72	1237-GCGCGGATCCAACTCATAATCATTTTGACT-1219
P	1263-GCGCTGATCAATGTCATTCCCTGAAGGA-1280	2145-GCGCTGATCAAACATAATTAACTGGTAAAT-2127
M	2180-GCGCGGATCCATGGAGTCCTACCTAGTA-2197	2942-GCGCGGATCCAATCTGGACTTCAAGACATA-2924
F	3067-GCGCAGATCTATGTCTTGGAAAGTGGTG-3084	4684-GCGCAGATCTAAACTATGCGGTATAAAA-4668
Junction		
N-P	927-GGTGMGAGAAATGGGYCCTG-946	1339-GATTTYTGGAAAGCTTCTGC-1320
P-M	948-AAGCAGCAGAAATGATGG-965	2372-GTGATGCAGCATACAGAG-2355
M-F	2785-GATCATGACYATGAACAATCC-2805	3176-CTGAGRTATCCYTCAGTTA-3158
F-M2	3385-GCAATAGCACTCGGTGTTGCAA-3405	4782-CTTCATATTTGCAKGGAGCC-4763

Nucleotide sequences and positions were derived from the NDL00-1 isolates (AF371337). Added restriction sites and unspecific sequences are underlined.

analysis of hMPV and the other members of the Pneumovirinae revealed that hMPV is more closely related to avian pneumovirus (van den Hoogen et al., 2001). To gain a better understanding of the molecular biology of hMPV, we report the complete sequence of N, P, M and F genes and intervening regions of multiple recent isolates of this virus from Canada.

2. Materials and methods

2.1. Specimens and patients

Fifteen specimens were collected from patients with acute respiratory illnesses from 1997 to 2000 in Quebec City, Quebec, Canada. The specimens were inoculated onto LLC-MK2 cells and serially blind passaged (Peret et al., 2002). Following viral isolation, the virus was cultured at 37 °C in Eagle's MEM supplemented with penicillin (100 U/ml), streptomycin (100 μg/ml), 30 μg/ml L-glutamine (Life Technologies) and 0.02% TPKC trypsin (Sigma). The virus isolates were cultured for a maximum of 21 days with weekly media change. The cells usually displayed cytopathic effects at 8–14 post inoculation, characterized by focal rounding and cell destruction without apparent syncytia formation. The specimens were negative when tested by indirect immunofluorescent assays for influenza virus A and B, parainfluenza viruses 1, 2 and 3, adenovirus and respiratory syncytial virus (Bartels; Chemicon). These isolates were also negative for influenza A and B, parainfluenza 1, 2, 3, 4 and HRSV by an in-house RT-PCR.

2.2. Primer sequences

Primers used for amplification and sequencing were based on the published N (AF371365), P (AF371366), M and F (AF371367) hMPV sequences (van den Hoogen et al., 2001, 2002). The primer pairs used to amplify each gene and gene junction are described in Table 1.

2.3. RT-PCR and sequence analysis

Viral RNA was extracted from 100 μl of tissue culture fluid using the RNeasy Mini Kit (QIAGEN). Viral RNA was amplified in a one step RT-PCR reaction (QIAGEN) following the manufacturer's recommendations. Briefly, 2–10 μl of RNA was added to the RT-PCR mixture containing 2 μl QIAGEN OneStep RT-PCR enzyme mix, 10 μl of 5X QIAGEN OneStep RT-PCR buffer, 400 μM dNTP, 0.6 μM of each primer and 10 μl of Q-solution in a final volume of 50 μl. The thermocycler conditions used were: 50 °C for 30 min for reverse transcription, 95 °C for 15 min for the activation of the HotStart DNA polymerase; then 35 cycles of 94 °C for 1 min, 50 °C for 1 min, 72 °C for 2 min, followed by an extension of 7 min at 72 °C. The PCR products were purified using QIAquick PCR purification kit (QIAGEN) and cloned in vector pCR2.1 (Invitrogen) according to the manufacturer's instructions. Two independent purified PCR amplicons for each gene were either sequenced directly, or sequenced using M13-specific primers after cloning in pCR2.1, on an ABI 377 Sequencer, using a fluorescent dye-terminator kit (Applied Biosystems). The DNA sequences were assembled and analyzed with SEQMAN, EDITSEQ and MEGALIGN programs in Lasergene (DNASTAR, Madison, WI). Phylogenetic trees were generated by Neighbor-Joining method using the MEGA program (Kumar et al., 2001).

2.4. Nucleotide sequence database accession numbers

The nucleotide sequence database accession numbers of the pneumovirus strains used in this paper are: HRSV-A (all ORFs:M11486); HRSV-B (all ORF-

s:AF013254); BRSV (all ORFs:AF092942); APV-A (N:U39295, P:U22110, M:X58639, F:Y14294); APV-B (N:U39296, P:AF325443, M:U37586, F:Y14293); APV-C (N:AF176590, P:AF176591, M: AF072682; F:AF187152); PVM (N:D10331, P:U092942, M:U66893; F:D11128); hMPV Netherlands isolate 00-1 (NLD00-1) (N: AF371365, P: AF371366, M and F:AF371367). The hMPV sequences described in this paper have been deposited in GeneBank under accession numbers AY145242–AY145301.

3. Results

3.1. hMPV isolates

Fifteen isolates obtained from patients suffering from respiratory illnesses were used for the current study. Initial testing of the samples by RT-PCR analysis using primer sets specific for HRSV, parainfluenza virus 1, 2, 3, and 4 as well as Influenza A and B were all negative. Subsequently, the 15 isolates were identified as hMPV using a RT-PCR test based on the fusion (F) protein gene (Peret et al., 2002).

3.2. Phylogenetic analyses

Phylogenetic trees were constructed based on the N, P, M and F ORF sequences of 15 hMPV Canadian isolates, the published full-length hMPV sequence and the A, B and C APV sequences. As shown in Fig. 1, for each of the four ORFs analyzed, all fifteen isolates fell into two distinct genetic clusters or groups that were tentatively named 1 and 2 (Fig. 1). Additional variability was also observed within the individual groups. These results confirm data previously reported for the partial sequences of the N, M, F and L genes of hMPV isolates from the Netherlands and Canada (Peret et al., 2002; van den Hoogen et al., 2001). Partial sequences of the N and F genes of hMPV isolated in Australia have also been reported (Nissen et al., 2002). Phylogenetic analysis of the N and F genes of Australian, Canadian and Dutch isolates also shows the same two distinct genetic clusters (data not shown). Representatives of both clusters 1 and 2 were present in hMPV isolates from the Netherlands and Canada, whereas the Australian isolates all belonged to cluster 1. The hMPV Dutch isolates (NDL00-1) characterized by van den Hoogen et al. clustered with group 1 isolates (van den Hoogen et al., 2002).

3.3. hMPV noncoding sequences

Following the phylogenetic analysis, isolates CAN99-81 (cluster 1) and CAN98-76 (cluster 2) were selected as representatives of each hMPV cluster. Their genomes were sequenced from the gene start codon of the N gene to the one of the M2 gene. The putative gene start signals for the N, P, M and F genes were identified by comparison with those of the hMPV Dutch isolate (NDL00-1) and other pneumoviruses: human and bovine respiratory virus (HRSV, BRSV), avian pneumovirus (APV) and pneumonia virus of the mice (PVM). Nucleotide sequence analysis revealed the presence of a well conserved region located at the beginning of each hMPV gene that potentially serves as gene-start signal (Table 2). However, since these signals were identified from the viral RNA, they remain tentative and should be confirmed by sequencing of the viral mRNA. The same is true for the gene end sequences described below. No sequence variation was observed between the two genetic clusters or the NDL00-1 isolate for N and P putative gene start signals (van den Hoogen et al., 2002). The putative gene start signal of the M gene had one A/C nucleotide change and the F gene one A/G substitution between the two clusters. The CAN99-81 (cluster 1) putative gene start sequence was identical to the one of NDL00-1 (cluster 1). All gene start sequences contained a (GGGACAAG/AU) motif that is identical to the gene start signal characterized for the matching genes of APV subgroups A, B and C (Dar et al., 2001; Li et al., 1996; Ling et al., 1995; Naylor et al., 1998; Randhawa et al., 1996; Seal, 1998; Seal et al., 2000; Yu et al., 1992). In contrast, no consensus sequences could be identified as a common gene termination signal. Comparison with NDL00-1 sequences revealed the presence of a putative gene end signal for each of the genes analyzed (bolded in Table 2). Each putative gene end signal was conserved between the two hMPV genetic clusters and NDL00-1. Further comparison with APV sequences showed that the putative gene end signal for the hMPV N gene ($AGUAAUUA_6$) was identical to the one characterized for APV-C (Dar et al., 2001; van den Hoogen et al., 2002). Similar analysis revealed that the putative gene end sequence for the P gene ($UAGUUUAAUA_5$) contained the same (AGUU) element of transcription as those of APV P genes (Dar et al., 2001; Ling et al., 1995). The putative gene end signals identified for the M ($AGUUAUAUA_5$) and F ($UAGUUAGUUAAUUA_5$) genes of hMPV possess a similar (AGUUA) element of transcription stop signal found for the F genes of APV-A and C, but varies from those found for APV M gene (AGUCA/APV-A, AGUUU/APV-B, UAUUA/APV-C) (Naylor et al., 1998; Randhawa et al., 1996; Seal, 1998; Seal et al., 2000; Yu et al., 1992).

Similarly to other pneumoviruses, the putative gene end and intergenic sequences of the hMPV gene varied in length (Table 2). That region of the P gene was strictly conserved between the two clusters and NDL00-1, whereas those of the N, M and F genes varied. In particular, that region of the M gene showed only 37%

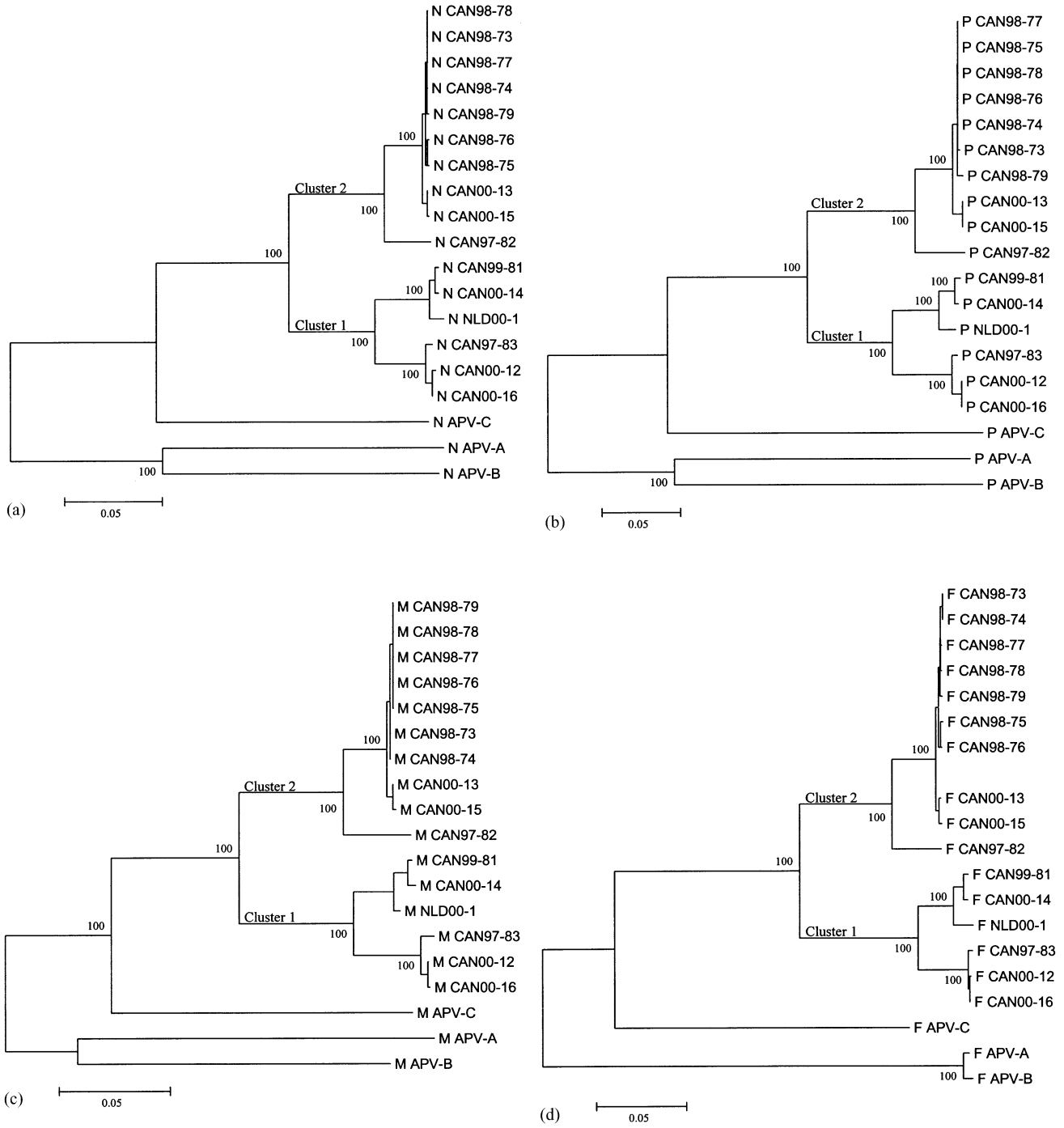

Fig. 1. Phylogenic analysis of hMPV Canadian isolates. The N (a), P (b), M (c) and F (d) ORFs were amplified by PCR and sequenced. The corresponding gene sequences from APV subgroups and hMPV from the Netherlands (NLD00-1) were also analyzed. Bootstrap proportions were plotted at the main internal branches of the phylogram to show support values. Phylogenetic analysis was performed using the Neighbor-Joining method of the MEGA program. The year in which the isolate was collected is indicated by the first two numbers in the isolate name.

identity between the cluster 1 and 2, and cluster 1 sequence had three additional nucleotides. It was suggested that this region contained part of a potential secondary ORF of 33 aa residues that starts at nt 2281 (van den Hoogen et al., 2002). The corresponding region of the F gene varied greatly in length (20–80 nucleotides long) between clusters. The NDL00-1 putative gene end and intergenic sequences were more closely related with CAN99-81, but were not identical. Four nucleotide changes were found in those M noncoding sequences (nt: 2947-G/A, 2976-U/C, 2990-U/C, 3053-U/C) of NDL00-1. The F putative gene end and intergenic sequences of NDL00-1 contained two nucleotides substitution (4712-A/T, 4717-T/A) and had a 21 nucleotides

Table 2
HMPV noncoding regions

Gene		Putative gene start signal	Putative gene end and intergenic sequences
N	CAN99-81	42-GGGACAAGUGAAA……..//……	1235-**AGUAAUUAAAAAA**GU-1249
	CAN98-76	42-GGGACAAGUGAAA……..//……	1235-**AGUAAUUAAAAAA**CU-1249
P	CAN99-81	1250-GGGACAAGUCAAA……..//……	2145-**UAGUUUAAUAAAAA**UAAACAAU-2166
	CAN98-76	1250-GGGACAAGUCAAA……..//……	2145-**UAGUUUAAUAAAAA**UAAACAAU-2166
M	CAN99-81	2167-GGGACAAGUAAAA……..//…	2942-UAACAGCCAAGCACCUUGGCCAAGAGCUACUAACUCUA UCUCAUAGAUUAUAAAGUCACCAUUCU**AGUUAUAUAAA AA**UCAAGUUAGAACAAGAAUUAAAUCAAUCAAGAAU-3053
	CAN98-76	2167-GGGACAAGUCAAA……..//……	2942-UAAAAAUAACUGUCCUAAUCAAUAAUUGCUUAUAUAAU CUUAAAGAUCAAUGAGCUUAUUAUUAU**AGUUAUAUAAA AAA**UUUAGAACUAGGAAGGUAUUAAUAGAAAGC-3050
F	CAN99-81	3054-GGGACAAAUAAAA……../……..	**UAGUUAAUUAAAA**AUAAAGUAAAUUAAAUUAAAA 4684-UAAAAUAAAAUUAAAAUUAAAAUAAAAUAAAAAUAAAA AUUU-4759
	CAN98-76	3051-GGGACAAGUAAAA……..//……	4681-**UAGUUAGUUAAUUAAAAA**AU-4696

Noncoding sequences of hMPV CAN99-81 (cluster 1) and CAN98-76 (cluster 2) are shown in the positive sense. Positions are giving for the first nucleotide of the putative gene start sequences and the first and last nucleotide of the putative gene end and intergenic sequences. Transcriptional stop codons are underlined and the putative gene end signals are bolded.

deletion (4724-4744) in comparison to CAN99-81 sequences.

3.4. Sequence of the N gene

Sequence alignment with other members of the *pneumovirus* genus revealed that the N gene coding region was 1185 bases in length. The deduced N protein was 394 aa in length with an estimated molecular mass of 43.5 kDa (Fig. 2). The N protein of hMPV is similar in size to the ones of other pneumoviruses (391–394 residues), which is relatively smaller than those reported for the other members of the Paramyxoviridae family (489–583 residues) (Barr et al., 1991; Collins et al., 1985; Curran and Kolakofsky, 1999; Dar et al., 2001; Spriggs and Collins, 1986). The N gene of isolates within the same cluster were highly conserved with nucleotide sequence identity of 93.8–100%, whereas sequence identity between the two different groups was 85.3–86.1% (Table 3). The aa identity was higher both within and between groups, 98.3–100% and 94.2–95.9%, respectively. The N protein of NDL00-1 was identical to the one of CAN99-81 and therefore showed similar level of aa identity with the respective hMPV cluster. The levels of aa identity observed between the two hMPV clusters are comparable to the ones observed between the two subgroups of HRSV (96%) (Johnson and Collins, 1989). The N proteins of APVs are not highly conserved, with 69–89% aa identity between the different subgroups (Dar et al., 2001; Li et al., 1996). When compared to the other pneumovirus members, the hMPV N gene showed 69–89% aa identity with APV members, 42–44% with RSV members and 45% with PVM (Barr et al., 1991; Buchholz et al., 1999; Dar et al., 2001; Karron et al., 1997; Li et al., 1996; Venkatesan et al., 1983) (Table 4). The N genes of all non-segmented, negative-stranded RNA viruses have three relatively conserved domains with homology in sequence and predicted secondary structure (Barr et al., 1991; Li et al., 1996; Dar et al., 2001). Sequence comparison with APV N genes allowed us to identify the position of these domains at residues 161–173, 251–263 and 279–326 (Li et al., 1996) (Fig. 2). These three domains were highly conserved between the two hMPV clusters and only one conservative aa substitution (aa 164) was found with their closest pneumovirus relative (APV-C). Most of the aa changes (66.6%) in the N genes of the two hMPV clusters were located within the first 136 aa of the protein. However the majority of these (62.5%) were conservative substitutions. Two of the remaining changes were found in the central part of the protein, and the last two in the last 38 aa.

3.5. Sequence of the P gene

The coding region of the P gene was 885 bases in length, encoding a predicted protein of 294 aa residues with a molecular mass of 32.5 kDa. The size of the hMPV P protein was comparable to those of APVs and PVM (279–295 aa), but was larger than the non-avian pneumovirus (241 aa) (Barr et al., 1994; Buchholz et al., 1999; Dar et al., 2001; Karron et al., 1997; Ling et al., 1995; Satake et al., 1984) (Fig. 3). Within each cluster the nucleotide sequence identity was 91–100% and the identity for the predicted aa sequence was 94.6–100% (Table 3). The P gene was less conserved between clusters with 78.0–79.4% and 81.6–85.7% identity levels for the nucleotide and aa sequences, respectively. The

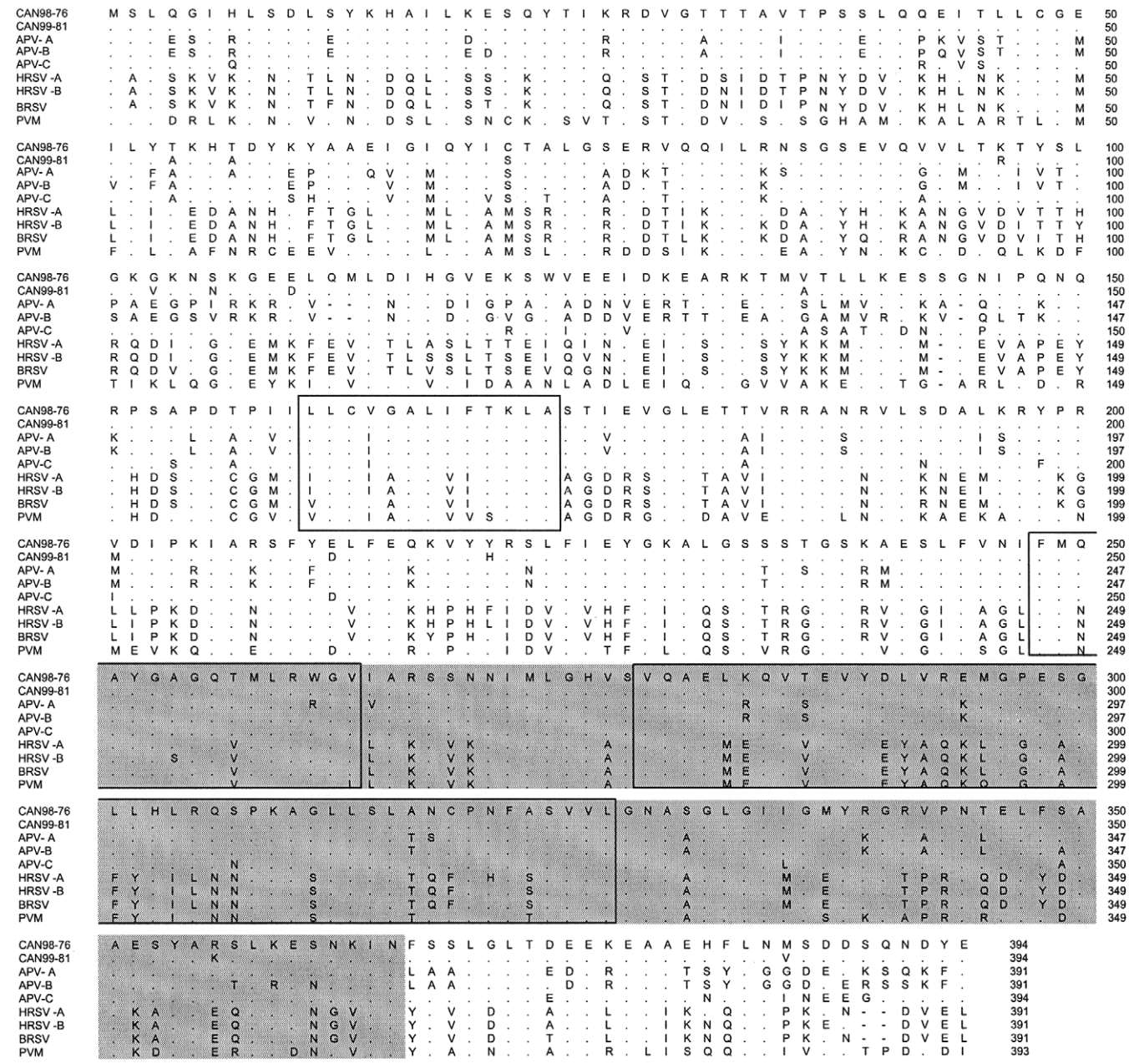

Fig. 2. Alignment of the predicted amino acid sequences of the hMPV N protein of hMPV CAN99-81 (cluster 1) with those of CAN98-76 (cluster 2), APV-A, B and C, HRSV A and B, BRSV and PVM. Only residues that differ from isolate CAN98-76 are shown, identical amino acids are represented by periods, gaps are represented by dashes. A relatively conserved domain is shaded (residues 247–365). Three conserved domains present in all non-segmented, negative stranded viruses at residues 161–173, 251–263 and 279–326 are boxed.

majority of the aa differences (34) between the two clusters were observed at the amino-proximal end of the protein (19–130) (Fig. 3). The central portion of the proteins (aa 131–278) was well conserved, containing only five aa changes, while the remaining changes (five) were found in the last 16 aa of the protein. The majority (65.9%) of the aa substitutions were non-conservative. Six aa changes were found between NDL00-1 and CAN99-81 (S/G-31, L/P-64, I/T-78, A/T-201, T/A-202, I/T280). None of these aa changes were unique to the NDL00-1 sequence. The 201 and 280 threonine residues present in the NDL00-1 sequence were also found in other cluster 1 and 2 Canadian hMPV isolates and the four remainder aa substitutions were identical to cluster 2 sequences.

The P protein of hMPV showed a significant level of divergence with those of other members of the pneumoviruses (Barr et al., 1994; Buchholz et al., 1999; Dar et al., 2001; Karron et al., 1997; Ling et al., 1995; Satake et al., 1984) (Table 4). The highest levels of identity were

Table 3
Nucleotide and (predicted amino acid) sequence identity levels within and between the two hMPV genetic clusters

hMPV clusters	% of nucleotide and (amino acid) identity			
	N	P	M	F
1/1	93.8–99.8 (99.3–100)	91.0–99.9 (95.0–100)	94.1–99.9 (98.8–100)	94.3–99.9 (98.3–99.8)
2/2	94.5–100 (98.3–100)	93.3–100 (94.6–100)	94.1–100 (99.6–100)	94.4–100 (98.9–100)
1/2	85.3–86.1 (94.2–95.9)	78.0–79.4 (81.6–85.7)	83.5–85.6 (96.9–97.6)	83.0–83.6 (94.1–95.4)

The results represent the range of identity (%) obtained for the comparison of the most divergent to the most conserved sequences within and between each genetic clusters.

Table 4
Amino acid sequence identity (%) between hMPV clusters A and B and the indicated pneumovirus

	N A; B	P A; B	M A; B	F A; B
APV-A	70; 69	55; 55	76; 78	67; 68
APV-B	71; 71	51; 50	77; 76	67; 68
APV-C	89; 88	68; 66	88; 88	81; 82
hRSV-A	43; 43	26; 28	38; 38	32; 32
hRSV-B	43; 42	26; 27	37; 37	33; 30
BRSV	44; 43	28; 28	37; 38	34; 34
PVM	45; 45	23; 25	40; 38	38; 43

observed with the P protein of APVs (50–68%), with the greatest identity shared with APV-C (Dar et al., 2001; Ling et al., 1995). The identity level was only 23–28% with mammalian pneumoviruses (Barr et al., 1994; Buchholz et al., 1999; Karron et al., 1997; Satake et al., 1984). The aa region encompassing residues 171–226 represents a highly conserved domain in pneumovirus P proteins (Ling et al., 1995) (Fig. 3). The amino-terminal portion of this region has a heptad repeat sequence that is frequently implicated in the formation of α-helices and coiled coils (Ling et al., 1995; McLachlan and Karn, 1983). This region of the hMPV P proteins had greater aa identity with both avian and mammalian pneumovirus 80.0–87.5% and 62.5–67.9%, respectively (Fig. 4). Similar to the P proteins of other pneumovirus, the majority (82–85%) of proline residues present in the hMPV P protein were found in the N-terminal half of the protein (Dar et al., 2001; Ling et al., 1995). The large number of charged residues found at the C-terminal portion of hMPV P is another common feature shared in all pneumovirus. As observed for APV-C, HRSV and BRSV, the P protein of hMPV did not contain any cysteine residues. In contrast, the P proteins of APV-A and B have two cysteine residues, and five cysteine residues are found in the PVM protein (Barr et al., 1994; Ling et al., 1995).

3.6. Sequence of the M gene

Sequence alignment analysis showed that the M gene of hMPV was 765 nucleotides in length, encoding a predicted protein of 254 aa residues with a predicted molecular mass of 27.6 kDa (Fig. 4). The M genes of hMPV isolates were highly conserved both within (94.1–100% identity) and between (83.5–85.6% identity) clusters (Table 3). These differences were reflected at the aa level with identity of 96.9–97.6% for members of different clusters and 98.8–100% for isolates belonging to the same cluster. The aa sequences of the M protein of NDL00-1 was identical to the one of CAN99-81 and showed similar levels of aa identity with the two hMPV clusters. The majority of aa substitutions between the two clusters were conservative and distributed throughout the entire protein (Fig. 4). The M protein of hMPV was most closely related to the M proteins of APVs with aa identity ranging from 76–88%, compared to the reduced aa identity 37–40% observed with the remaining pneumoviruses (Table 4) (Buchholz et al., 1999; Easton and Chambers, 1997; Karron et al., 1997; Randhawa et al., 1996; Satake et al., 1984; Seal, 1998; Yu et al., 1992). A short region located at the N-terminal end of the protein (aa 14–19) was conserved for all pneumovirus M proteins, although the biological significance of this region is unknown.

3.7. Sequence of the F gene

The F gene was 1620 bases in length, encoding a protein of 539 aa residues with a predicted molecular mass of 58.4 kDa (Fig. 5). The F gene was well conserved within each group with 94.3–100% and 98.3%–100% for the nucleotide and aa sequences identity, respectively (Table 3). Between the two clusters the identity was 83.0–83.6% and 94.1–95.4% for the nucleotide and aa sequences, respectively. The aa sequence of the F protein of NDL00-1 had 100% identity with the one of CAN99-81. The majority (75.0%) of aa substitutions between the two clusters were conservative. Sequence comparison with avian pneumoviruses showed identity levels of 67–68% with APV subgroup A and B and 81–82% with subgroup C (Table 4) (Naylor et al., 1998; Seal et al., 2000). The F gene of hMPV was less conserved with the mammalian pneumoviruses and showed 30–43% aa identity (Buchholz et al., 1999; Chambers et al., 1992; Collins et al., 1984; Karron et al., 1997; Seal, 1998).

The F proteins of pneumoviruses are synthesized as a precursor F0 that is cleaved by cellular protease into two subunits, F1 and F2. Most pneumoviruses cleavage peptide sequences contain the cleavage motif for the

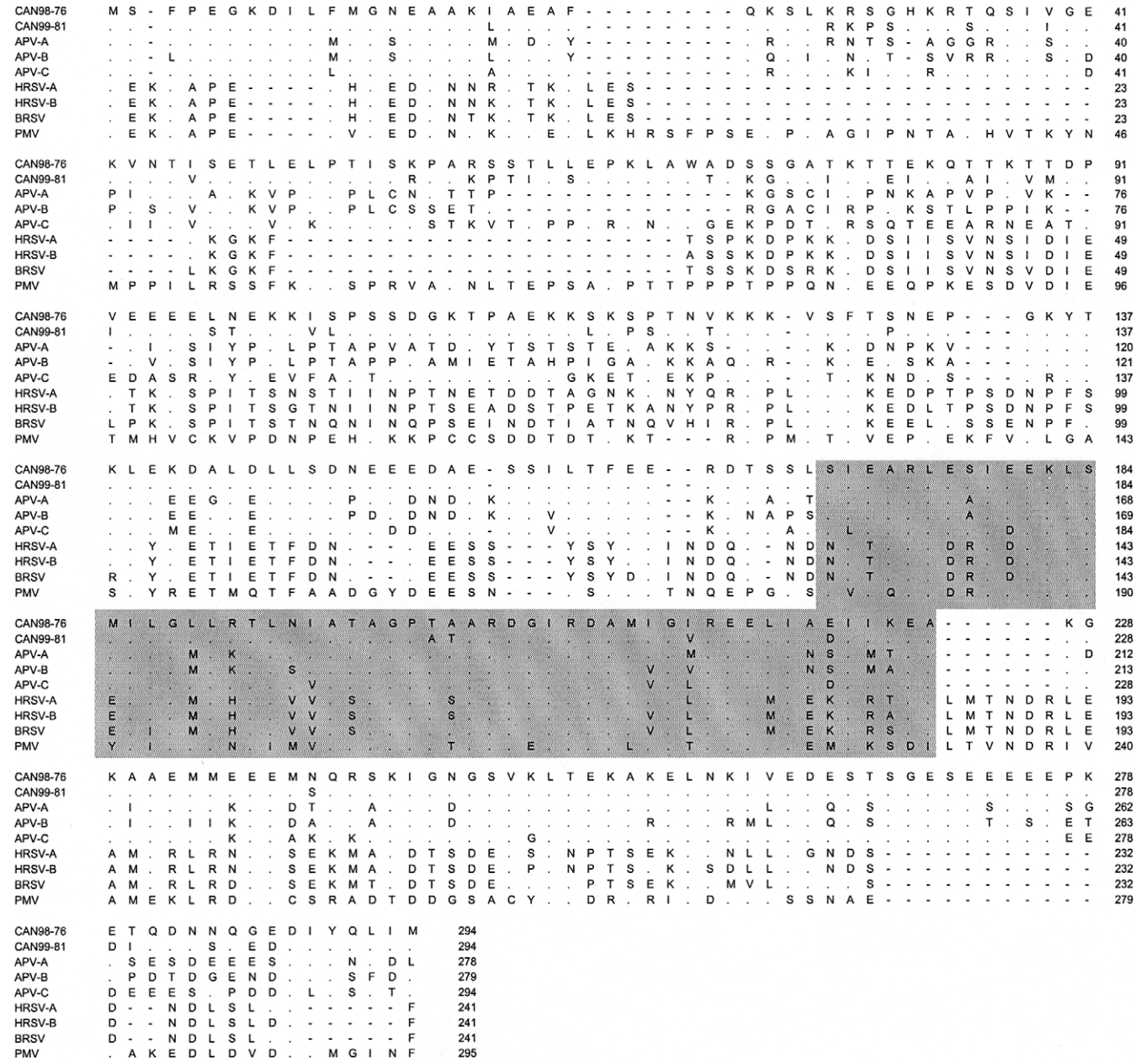

Fig. 3. Alignment of the predicted amino acid sequences of the hMPV P protein of hMPV CAN99-81 (cluster 1) with those of CAN98-76 (cluster 2), APV-A, B and C, HRSV A and B, BRSV and PVM. Only residues that differ from isolate CAN98-76 are shown, identical amino acids are represented by periods, gaps are represented by dashes. A highly conserved domain in pneumovirus P (residues 171–226) is shaded.

cellular furin protease (R/K-X-R/K-R) (Collins et al., 2001). However, the cleavage peptide sequence of APV-C (RKAR) varies from the consensus sequence (Seal et al., 2000). Similarly, sequence analyses revealed that the cleavage peptide sequence of hMPV F protein (RQSR) differs from that of the furin protease motif (Fig. 5). Cleavage of the F0 precursor releases the hydrophobic fusion peptide at the N-terminus of the F1 subunit. The F1 and F2 subunits of the two hMPV lineages showed a similar level of conservation with aa identities of 94% and 97%, respectively. These identity levels were similar to those observed within HRSV subtypes (F1, 98% and F2, 95%) (Naylor et al., 1998). In contrast, greater divergence (83% identity) was observed between subtypes of HRSV and APV (Naylor et al., 1998). Similar to APVs, the carboxy-terminal transmembrane domain and cytoplasmic tail of the two hMPV clusters diverge significantly (16%) (Naylor et al., 1998; Seal et al., 2000). However, this region of the hMPV F protein showed greater aa identity between clusters (84%) than the one observed between the different APV subgroups (32%–39%) (Naylor et al., 1998; Seal et al., 2000). The

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CAN98-76  M E S Y L V D T Y Q G I P Y T A A V Q V D L V E K D L L P A S L T I W F P L F Q A N T P P A V L L D  50
CAN99-81  . . . . . . . . . . . . . . . . . . . . . I . . . . . . . . . . . . . . . . . . . . . . . . . .  50
APV-A     . . . . I I . . . . . V . . . . . . . . . I . . . S N . . T . . V . . . . . . S S . . A P . . . .  50
APV-B     . . . . I I . . . . . V . . . . . . . . . . . . . N N . . K . . V . . . . . . S S . . A P . . . .  50
APV-C     . . . . . . . . . . . V . . . . . . . T . . . . . Q . . . R . . V . . . . . . T . . . . T . . . E  50
HRSV-A    . . T . V N K L H E . S T . . . . . . Y N V L . . . D D . . . . . . . V . M . . S S M . A D L . I K  50
HRSV- B   . . T . V N K L H E . S T . . . . . . Y N V L . . . D D . . . . . . . V . M . . S S V . A D L . I K  50
BRSV      . . T . V N K L H E . S T . . . . . . Y N V I . . . D D . . . . . . . V . M . . S S I S A D L . I K  50
PVM       . . A . . . E M . H . V . . . . . . . L N . . . . H S A N I . . . V . I . M . . T S L . K N S V M .  50

CAN98-76  Q L K T L T I T T L Y A A S Q N G P I L K V N A S A Q G A A M S V L P K K F E V N A T V A L D E Y S  100
CAN99-81  . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . .  100
APV-A     . . . . . S . . . Q . T . . P E . . V . Q . . . A . . . . . . . A . . . . . A . S . A . . . . . .  100
APV-B     . . . . . S . . . Q . T V . P E . . V . Q . . . T . . . . . . . A . . . . . S . S . A A . . . . . .  100
APV-C     . . . . . . . . . . . T . . . . . . . . . . . . . . . . . . . . A . . . S . D . S . S . . . . D . .  100
HRSV-A    E . A N V N . L V K Q I S T P K . . S . R . M I N S R S . V L A Q M . S . . T I C . N . S . . . R .  100
HRSV- B   E . A S I N . L V K Q I S T P K . . S . R . T I N S R S . V L A Q M . S N . I I S . N . S . . . R .  100
BRSV      E . I N V N . L V R Q I S T L K . . S . . I M I N S R S . V L A Q M . S . . T I S . N . S . . . R .  100
PVM       L . H D V . V I C T Q I S T V H . . M I . . D L . S S N . G L A T M . R Q . L I . . I I . . . D W G  100

CAN98-76  K L D F D K L T V C D V K T V Y L T T M K P Y G M V S K F V S S A K S V G K K T H D L I A L C D F M  150
CAN99-81  . . E . . . . . . . E . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . .  150
APV-A     R . E . G T . . . . . R S I . . . . L . . . . . . . . I M T D V R . . . R . . . . . . . . . . I  150
APV-B     . . . . G V . . . . . R A . . . . . L . . . . . . . . I . T N M N T . . R . . . . . . . . . . I  150
APV-C     . . E . . . . . . . E L . A . . . . . . . . . . . . . . . . N . . . A . . . . . . . . . . . . L  150
HRSV-A    . . A Y . V T . P . E I . A C S . . C L . S K N . L T T V K D L T M K T L N P . . . I . . . . E . E  150
HRSV- B   . . A Y . V T . P . E I . A C S . . C L . V K S . L T T V K D L T M K T F N P . . E I . . . . E . E  150
BRSV      . . A Y . I T . P . E I . A C S . . C L . V K N . L T T V K D L T M K T F N P . . E I . . . . E . E  150
PVM       N M . Y E V P V A F . K . S F C V . I L . . K N . L Y T V P . I T - P T N R P . . E . . . V . S . H  150

CAN98-76  D L E K N I P V T I P A F I K S V S I K E S E S A T V E A A I S S E A D Q A L T Q A K I A P Y A G L  200
CAN99-81  . . . . . T . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . .  200
APV-A     . I . . G V . I . . . . Y . . A . . . . D . . . . . . . . . . . G . . . . . I . . . R . . . . . .  200
APV-B     . M . R G . . . . . . Y . . A . . . . D . . . . . . . . . . . G . . . . . I . . . R . . . . . .  200
APV-C     . . . . G V . . . . . Y . . . . . . . . . . . . . . . . . . . G . . . . . I . . . R . . . . . .  200
HRSV-A    N I V T S K K . I . . T Y L R . I . V R N K D L N . L . N I T T T . F K N . I . N . . . I . . S . .  200
HRSV- B   N I M T S K R . I . . T Y L R P I . V . N K D L N S L . N I A T T . F K N . I . N . . . I . . . . .  200
BRSV      N I M T S K R . V . . T . L R . I N V . A K D L D S L . N I A T T . F K N . I . N . . . I . . . . .  200
PVM       N R V T L K S F N . . V . . R A L Y . R Q Q G L D S . . Q . . . . D V . H . I . T . R V . . . . . .  200

CAN98-76  I M I M T M N N P K G I F K K L G A G T Q V I V E L G A Y V Q A E S I S R I C K S W S H Q G T R Y V  250
CAN99-81  . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . K . . . T . . . . . .  250
APV-A     . L . . . . . . . . . . . . . . . . M . . . . . . . . P . . . . . L G K . . . T . N . . R . . . .  250
APV-B     . L L . A . . . . . . . . . R . . . . . . . . . . . . P . . . . . L G K . . . T . N . . R . . . I  250
APV-C     . . . . . . . . . . . . . . . . . . . . V . . . . . . . . . . . . . . . . . . . R N . . . . . .  250
HRSV-A    L L V I . V T D N . . A . . Y I K P Q S . F . . D . . . . L E K . . . Y Y V T T N . K . T A . . F A  250
HRSV- B   V L V I . V T D N . . A . . Y I K P Q S . F . . D . . . . L E K . . . Y Y V T T N . K . T A . . F S  250
BRSV      V L V I . V T D N . . A . . Y I K P Q S . F . . D . . . . L E K . . . Y Y V T T N . K . T A . K F S  250
PVM       T L V I N I T S T . . A . . L . K . . S . I L A . . . P . L T Q V . L H D V I M N . K . T . . S . I  250

CAN98-76  L K S R                254
CAN99-81  . . . .                254
APV-A     . R . .                254
APV-B     . . . .                254
APV-C     . . . .                254
HRSV-A    I . P - - M E D        256
HRSV- B   I . P - - L E D        256
BRSV      I . P - - I E D        256
PVM       . . . S S T S G        258
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Fig. 4. Alignment of the predicted amino acid sequences of the hMPV M protein of hMPV CAN99-81 (cluster 1) with those of CAN98-76 (cluster 2), APV-A, B and C, HRSV A and B, BRSV and PVM. Only residues that differ from isolate CAN98-76 are shown, identical amino acids are represented by periods, gaps are represented by dashes.

remaining portion of the hMPV F1 subunit was more conserved with only 4.2% aa divergence between the two clusters. The two heptad repeats domains (HRA and HRB) necessary for viral fusion of paramyxoviruses (Chambers et al., 1990; Buckland and Wild, 1989; Lamb, 1993; Russell et al., 2001) are also present in the hMPV F gene. There were three potential N-glycosylation sites at aa 57, 172 and 353, two of which (57 and 172) were also present among the F proteins of all APV subgroups (Naylor et al., 1998; Seal et al., 2000). The aa residues at position 102 (Arg), 103 (Phe), 106 (Gly) and 111 (Gly) are structurally important to paramyxovirus F proteins (Horvath and Lamb, 1992) and are present in all pneumoviruses. These aa residues were conserved for the two clusters of hMPV F proteins.

4. Discussion

The phylogenetic analysis of the complete N, P, M and F genes of the Canadian isolates was consistent with previous reports (Peret et al., 2002; van den Hoogen et al., 2001), and showed the presence of two genetic clusters. Comparison of these sequences with the ones reported from the Netherlands and Australia showed that all hMPV isolates reported to date cluster within the same two genetic groups, suggesting that a relatively homogeneous population of hMPV is circulating worldwide (Nissen et al., 2002; van den Hoogen et al., 2001). In fact, hMPV Canadian cluster 1 isolates showed over 96% amino acid identity with the NDL00-1 Dutch isolates for all the viral protein analyzed (van den

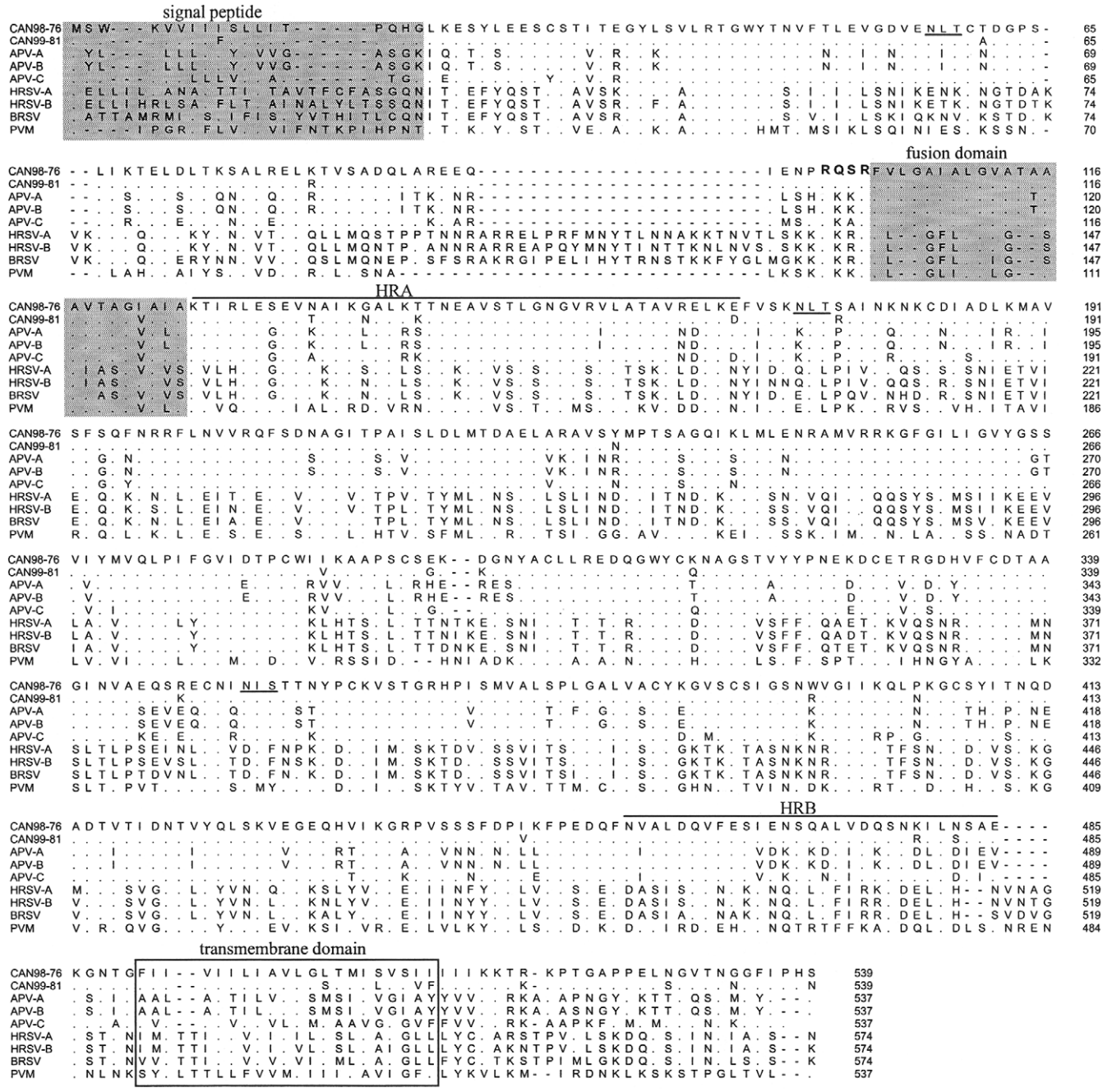

Fig. 5. Alignment of the predicted amino acid sequences of the hMPV F protein of hMPV CAN99-81 (cluster 1) with those of CAN98-76 (cluster 2), APV-A, B and C, HRSV A and B, BRSV and PVM. Only residues that differ from isolate CAN98-76 are shown, identical amino acids are represented by periods, gaps are represented by dashes. The signal peptide and the fusion related domain sequences are shaded. The connecting peptide at the junction of the F1 and F2 subunit is bolded. The transmembrane domain is boxed. The two heptad repeats domains (HRA and HRB) are overlined The putative glycosylation sites present in the hMPV CAN98-76 sequence are underlined.

Hoogen et al., 2002). Our results also show that both hMPV clusters were co-circulating in Canada during 1997, 1999 and 2000. This is comparable to what is observed for the two subgroups of HRSV, which have been co-circulating in most yearly epidemics (Collins et al., 2001). Similar to other pneumoviruses, the genetic diversity observed for the hMPV genes may lead to antigenic variability and these two hMPV genetic clusters may also represent two different antigenic groups. However, distinct lineages of HRSV have been identified within each subgroup, and shown to co-circulate during epidemics and to have broad geographic distribution (Cane and Pringle, 1991, 1995; Coggins et al., 1998; Garcia et al., 1994; Martinez et al., 1999). The

nucleotide identities observed between the two hMPV clusters for each of the genes analyzed are comparable to the ones obtained between members of the two HRSV subgroups (Naylor et al., 1998). However, a greater proportion of silent nucleic acid substitutions are found in the F genes of hMPV clusters leading to an aa identity that is closer to the one observed within HRSV subgroups. At this point, it is still unclear if the differences observed between the hMPV isolates are representative of two distinct hMPV antigenic subgroups. The characterization of the hMPV G genes for which the antigenic diversity is most evident for RSV and APV, as well as serologic studies, are needed to determine the antigenic significance of the genetic diversity observed for hMPV.

Molecular analysis of the N, P, M and F genes provided further support for the assignment of hMPV to the *Metapneumovirus* genus of the Paramyxovirinae subfamily. Analysis of the genomic organization of hMPV revealed several similar features to APVs, especially to the subgroup C (van den Hoogen et al., 2001). They share the same putative gene start sequence (GGGACAAGUGAAA), as well as the same gene end sequences for the N gene (AGUAAUUA$_6$). Although not identical, the gene end sequences of the other genes also show some similarity to APVs. The 3′ noncoding region of the M and F genes varies significantly between the two hMPV clusters. This finding is comparable to the low conservation of some 3′ noncoding sequences of HRSV and APV isolates belonging to different subgroups (Bayon-Auboyer et al., 2000; Seal et al., 2000).

Sequence alignment studies show that the hMPV proteins are more closely related to the subgroup C of APV, with aa identities for the different proteins ranging from 66 to 89% (van den Hoogen et al., 2002). A greater divergence is observed with the non-avian pneumovirus members and the aa identity drops to 23–45%. The N, M and F genes were the most conserved whereas the P genes show greater divergence. Comparison of the predicted aa sequences revealed that the N, M, P and F proteins share most of the common features characterized for these proteins in other pneumoviruses. However, the cleavage peptide sequence for the hMPV F protein (RQSR) varies from the one described for other pneumoviruses, and differs from the furin protease motif (R/K-X-R/K-R) present in all pneumovirus except for APV-C. When compared to hMPV, the cleavage sites of other pneumoviruses contain additional Arg-Lys residues. The substitution of these basic aa in the hMPV cleavage site may play a role in viral pathogenesis, since it may modify the efficiency of cleavage by the host protease. The virulence of Newcastle disease virus (NDV) a member of the *Rubulavirus* genus of the Paramyxovirinae subfamily has been linked to the number of basic residues present at the F protein cleavage site. NDV virulent strains have two pairs of basic residues at the cleavage site, whereas avirulent strains have two basic residues (Collins et al., 1993).

The close relationship observed between hMPV and APV-C suggests that hMPV originated from birds. It is also possible that APV-C could have evolved from human precursor strains of hMPV that infected birds. APV-C has only recently been described infecting commercial poultry (mid-1990s) (Dar et al., 2001; Seal, 1998; Seal et al., 2000). It was speculated that hMPV is an avian virus that is also able to infect humans (van den Hoogen et al., 2001). However, data obtained by van den Hoogen et al., indicate that the human virus is unable to infect turkeys and chickens, but can infect monkeys experimentally (van den Hoogen et al., 2001). The relative importance of hMPV on viral respiratory tract illnesses is still not known, but serological studies demonstrated that by the age of five, practically all children in the Netherlands have been in contact with the virus and that it has been present in the population for at least 50 years (van den Hoogen et al., 2001). These initial studies on the prevalence of hMPV and its possible worldwide distribution strengthen the importance for a better understanding of the newly identified pneumovirus.

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