Published in final edited form as:
Virus Res. 2011 September ; 160(1-2): 200–205. doi:10.1016/j.virusres.2011.06.014.

Genomic Analysis of Four Human Metapneumovirus Prototypes

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SUMMARY

Human metapneumovirus (HMPV) is an important cause of acute respiratory illness in children. We determined the complete genome sequence of four strains of HMPV representing each of the four lineages. These sequences were compared with published HMPV genome sequences. Most genes were conserved between the genetic lineages (79.5%-99.6%), though nucleotide diversity was greater than amino acid diversity, suggesting functional constraints on mutation. However, the SH and G open reading frames were more variable (mean 76.4% and 59.0% aa identity, respectively), with mostly nonsynonymous changes, suggesting selective pressure on the SH and G proteins. Gene-start regions were largely conserved between genes and viruses, while gene-end sequences were conserved between viruses but not between genes. The SH-G and G-L intergenic regions were extremely long (~200 nt) and have no defined function, yet were highly conserved within major groups. These findings highlight broadly conserved regions of the HMPV genome and suggest unidentified biological roles for SH and G.

Keywords

Metapneumovirus, Human; Metapneumovirus, Avian; Paramyxovirus, Genome

1. INTRODUCTION

Human metapneumovirus (HMPV) is a leading cause of respiratory infection in children worldwide(Boivin et al., 2003; Dollner et al., 2004; Ebihara et al., 2004; Esper et al., 2004; Mackay et al., 2006; McAdam et al., 2004; Peiris et al., 2003; van den Hoogen et al., 2003; Williams et al., 2004; Williams et al., 2006). HMPV is also associated with severe disease in immunocompromised hosts and persons with underlying conditions(Englund et al., 2006; Larcher et al., 2005; Madhi et al., 2003; Vicente et al., 2004; Williams et al., 2005a; Williams et al., 2005b). Candidate vaccines for HMPV are under development(Buchholz et al., 2006; Cseke et al., 2007; Herfst et al., 2004; Tang et al., 2005). Therefore, it is important to define genetic diversity of HMPV. The negative sense, single stranded RNA genome encodes eight genes: N, nucleocapsid; P, phosphoprotein; M, matrix; F, fusion; M2; SH, small hydrophobic; G, glycoprotein; and L, polymerase. The M2 gene encodes two different

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reading frames that are transcribed to yield M2-1 and M2-2 proteins, for a total of nine proteins(Buchholz et al., 2005; van den Hoogen et al., 2002). Analysis of three HMPV genomes has been published(Biacchesi et al., 2003; van den Hoogen et al., 2002). We determined the entire genomic sequence of four prototype strains, one in each lineage(van den Hoogen et al., 2004) and compared these to available full genome sequences of HMPV and other *Mononegavirales*.

2. MATERIALS AND METHODS

2.1. HMPV strains

Virus isolates were recovered from specimens collected in the Vanderbilt Vaccine Clinic as described(Williams et al., 2004; Williams et al., 2006). Each virus was passaged 5-7 times from the original clinical specimen in LLC-MK2 cells and then thrice plaque-purified. Nomenclature in this study uses letters representing the geographic site of isolation (*e.g.*, "TN" represents Tennessee) followed by the year of isolation and isolate number. **Table 1** shows the strains, source, and genotypes analyzed.

2.2. RNA extraction, RT-PCR, and sequencing of genes

RNA was isolated from virus infected cell supernatants using an RNeasy Mini kit(Qiagen) and amplified using a One Step RT-PCR kit(Qiagen). Primers were based on previously published HMPV sequences and gradient RT-PCR was performed to identify optimum annealing temperatures. Primers and annealing temperatures are listed in **Supplemental Table 1**. RT-PCR products were gel electrophoresed, excised, and gel purified using a Qiaquick Gel Extraction Kit(Qiagen). The 3' leader and 5' trailer sequences were amplified using a modified RACE method for *Mononegavirales*(Gubala et al., 2008; Li et al., 2005; Tillett et al., 2000).

Purified RT-PCR products were cloned in pGEM-T Easy vector(Promega) and sequenced (both strands) in the Vanderbilt DNA Sequencing Core on an ABI3700xl. Individual genes were sequenced at least 3 times to resolve discrepancies. At least two separate RT-PCR reactions were cloned and sequenced in both directions for each fragment. In a few cases (discussed under Results), sequencing of multiple clones revealed a mixed population despite triple plaque purification.

Assemblylign(MacVector 11) was used to align overlapping sequence fragments to generate a linear genome. Gene-start/end sequences were determined by identification of putative open reading frames (ORF) and by comparison to published HMPV sequences. Gene-end sequences were defined as the stop codon and subsequent poly-A region, gene-start as the conserved metapneumovirus GGG through the start codon, and the intergenic region as the nucleotides between the gene-end sequence of one gene and the gene-start of the next. Published full-length HMPV genome sequences were obtained from GenBank. The ClustalW algorithm in MacVector 11(MacVector) was used to align and compare sequences.

3. RESULTS

The length of the HMPV genome ranged from 13,281 - 13,387 nt. **Table 2** shows the length of coding and non-coding regions, with predicted amino acid length of ORFs. We aligned individual genes and calculated the percent nt and aa identity between and within major groups (**Table 3**).

3.1. Sequence of the N gene

The N gene was the most conserved at the nt and amino acid level. The three regions shared by all *Mononegavirales*(Barr et al., 1991) were virtually identical within the four subgroups. Many residues identified as critical for RNA binding and tertiary folding in the RSV N crystal structure were conserved in HMPV(Tawar et al., 2009). The N-P intergenic region was 2 nt long in each of the viruses. (**Supplemental Table 2**).

3.2. Sequence of the P gene

The P gene was moderately conserved at the nt level; however, the aa conservation was higher, with most nt changes synonymous. A central region proposed to form α-helical coiled-coils and mediate paramyxovirus P homo-oligomerization was largely conserved in HMPV, encompassing residues 180-236(Dar et al., 2001; Ling et al., 1995; Llorente et al., 2006; Llorente et al., 2008). None of the HMPV strains encoded any cysteine residues in P, a feature shared by AMPV-C and RSV(Bastien et al., 2003; Dar et al., 2001). Further, alternate reading frames contained multiple stop codons, suggesting that like RSV but unlike pneumonia virus of mice (PVM), HMPV P does not encode any alternate proteins(Barr et al., 1991). A sequence of basic residues thought to mediate RNA binding was not conserved(Lin et al., 1997). However, there were two other highly basic conserved regions from 25-34 and 113-125 in HMPV P that may serve a similar function. A substantial number of prolines were found in the N-terminal region and there were numerous conserved glutamines in the C-terminal region, similar to AMPV and RSV P(Dar et al., 2001; Ling et al., 1995). The P-M intergenic region was highly conserved.

3.3. Sequence of the M gene

The M gene was highly conserved, with greater aa than nt identity. A hexameric motif at residues 14-19 (YTAAVQ) present in all pneumoviruses(Easton and Chambers, 1997) was conserved in all HMPV strains. Prolines and cysteines were completely conserved between all HMPV strains and AMPV-C, and hydrophobic sequences shown to mediate membrane interaction were conserved among all HMPV strains(Bellini et al., 1986; Money et al., 2009; Yu et al., 1992). Several groups have described potential alternate ORFs overlapping the M genes of AMPV-A(51 aa), bRSV(52 aa), hRSV(75 aa), PVM(46 aa), and HMPV NL00-1(54 aa)(Easton and Chambers, 1997; Samal and Zamora, 1991; Satake and Venkatesan, 1984; van den Hoogen et al., 2002; Yu et al., 1992). We examined all HMPV sequences for potential alternate ORFs. The 54 aa sequence described in the A1 strain NL00-1 was conserved in TN96-12. However, no alternate ORF >25 aa were present in A2 or B1 HMPV subgroups. All HMPV B2 viruses contained a conserved potential alternate ORF of 62 aa. While we confirmed the presence of the potential alternate ORF in AMPV-A, there were no alternate ORFs in AMPV-B or AMPV-C (not shown). The reported RSV potential alternate ORF was not conserved among RSV Long, B1, and 9320 strains (not shown). No evidence for the synthesis of these proteins has been reported. The M-F intergenic region was mostly conserved.

3.4. Sequence of the F gene

The F gene was highly conserved with mostly synonymous changes leading to greater nt than aa diversity, as previously described(de Graaf et al., 2008b; Miller et al., 2007; van den Hoogen et al., 2004; Yang et al., 2009). The F-M2 intergenic regions were quite different with regard to length between the viruses.

3.5. Sequence of the M2 gene

The M2 gene contains two overlapping ORFs designated M2-1 and M2-2 with the latter starting at nt position 512 of M2-1. Both ORFs were highly conserved, with greater aa

identity than nt identity. The HMPV M2-1 protein contained four conserved cysteines that are absolutely conserved among other pneumoviruses(Ahmadian et al., 1999; Collins et al., 1996; Cuesta et al., 2000). The first three cysteines are found in a putative zinc finger domain, and serine/threonine residues shown to be phosphorylated and to contribute to RNA binding for RSV M2-1 were relatively conserved(Cuesta et al., 2000). The M2-2 protein contained 3 conserved cysteine residues. Like RSV M2-2(Bermingham and Collins, 1999; Collins et al., 1990), HMPV M2-2 is involved in RNA replication and its deletion leads to attenuation in rodents(Buchholz et al., 2005; Kitagawa et al.; Schickli et al., 2008). HMPV M2-2 shares minimal sequence identity with PVM and RSV M2-2 (not shown), but three cysteines are absolutely conserved between HMPV and AMPV-A, -B, and -C. HMPV M2-2 shared 56% aa identity with AMPV-C. The M2-SH intergenic sequence was short and generally conserved.

3.6. Sequence of the SH gene

The SH gene was less conserved, with greater aa than nt diversity. SH protein length was variable due to point mutations creating stop codons near the C-terminal end of the gene. Thus, while the length of all other A subgroup strains was 183 aa, the length of TN/94-49 was 181 aa; repeated sequencing of multiple TN/94-49 clones confirmed this CAA→TAA mutation. A point T→C mutation at position 532 in the TN/99-419 nt sequence changed the stop codon TAA (present in 982-42) to CAA. We sequenced 6 different clones and only 1/6 had the stop codon TAA at position 532. The resulting TN/99-419 nt sequence encoded an SH protein that was 36 aa residues longer than that of TN/98-242. We also re-sequenced TN/98-242 several times and in this case, only 1/7 sequences had a CAA codon at position 532, the remainder having the stop codon TAA.

The HMPV SH protein contained up to 10 cysteines, though only five of these were absolutely conserved; nine of these were conserved in AMPV-C SH. In contrast, AMPV-A and -B contain 15 conserved cysteines, while RSV SH contains only one cysteine(Olmsted and Collins, 1989). Alternate start codons used in RSV SH(Olmsted and Collins, 1989) were not present in any HMPV strain. A hydrophobic putative transmembrane domain from aa ~30-50 was conserved. The SH-G intergenic region was the longest intergenic region, varying from 203-213 nt.

3.7. Sequence of the G gene

G was the most variable HMPV gene, differing in length and sequence among subgroups. The cytoplasmic tail was approximately 32 residues in length and conserved, as was the transmembrane domain (aa 33-51). The single cysteine residue present in the intracellular domain was conserved. The majority of the diversity was located in the extracellular domain, with greater sequence diversity between major subgroups than within subgroups. In contrast to all other genes except SH, aa identity was lower than nt identity. RSV G protein has alternate start codons C-terminal to the transmembrane domain that result in soluble RSV G(Bukreyev et al., 2008; Olmsted et al., 1989; Roberts et al., 1994); analogous alternate start codons are present in all HMPV subgroups except A2. The G-L intergenic nt sequences were even longer than the SH-G intergenic.

3.8. Sequence of the L gene

The L gene was highly conserved, with aa greater than nt identity. Six conserved blocks identified in all negative-sense single stranded RNA viruses as putative functional domains(Poch et al., 1990; Sidhu et al., 1993) were present and conserved among HMPV strains. These blocks were reasonably conserved between HMPV and the *Pneumovirinae* (53-91% identity, not shown) but less conserved compared to the *Paramyxovirinae* (16-31% identity, not shown). The putative polymerase core motifs (A, B, C, D) of domain III(Poch

et al., 1989) were also conserved in HMPV. The RNA template recognition and phosphodiester bond formation domain represented by the conserved pentapeptide QGDNQ in all negative-strand viruses(Poch et al., 1990; Sidhu et al., 1993) was present in all HMPV strains as NGDNQ. The putative ATP-binding motif K-$(X)_{18}$-GEGAGN-$(X)_{20}$-K in domain VI also was conserved among all HMPV strains(Poch et al., 1990; Sidhu et al., 1993). HMPV L sequences were overall 80% identical to AMPV-C, but only 64% identical to other AMPV and 48% identical to other pneumoviruses.

3.9. Gene-start and gene-end sequences

The gene-start sequence was fairly conserved, with a consensus sequence of GGGAYAARTVRVVATG, similar to AMPV and not unlike the RSV consensus GGGGCAAAT[A/T](Bayon-Auboyer et al., 2000; Biacchesi et al., 2003; Ling et al., 1992). The gene-start was most variable between viruses for the G gene. In contrast, the gene-end sequences were highly variable between different genes, but tended to be conserved between viruses.

4. DISCUSSION

We sequenced the full genomes of four prototype HMPV viruses and analyzed them with eleven published HMPV genomes. Our results confirm the presence of two main genetic groups, A and B, each with two subgroups, that were proposed based on partial gene sequences(van den Hoogen et al., 2004). N, M, F, M2-1, M2-2, and L were broadly conserved. For all of these genes, the amino acid conservation was higher than nt sequence, suggesting functional constraints on diversity. This is not wholly surprising for internal proteins but less expected for the F protein, which is under selective immune pressure. The P protein was less conserved, suggesting that P may be more lenient in its functional and structural constraints. Phylogenetic analysis of each individual gene corresponded to the phylogeny of the genotypes (not shown). Analysis of the aligned genome sequences using Recombination Detection Program software(Martin et al., 2010) did not detect evidence for recombination (not shown).

Major motifs and functional domains identified in other paramyxovirus proteins were present in HMPV proteins, with some notable absences. Like AMPV and RSV, there were no alternate reading frames in HMPV P(Bastien et al., 2003; Dar et al., 2001). Therefore, HMPV, like other *Pneumovirinae* but in contrast to *Paramyxovirinae*, appears not to affect STATs using alternate P gene products(Ramachandran and Horvath, 2009). HMPV lacks the NS1 and NS2 genes encoded by RSV and PVM that modulate host interferons(Spann et al., 2005). Several manuscripts have suggested that HMPV modulates innate immunity, possibly though the G or P proteins, though a clear mechanism has not been identified(Bao et al., 2008; Dinwiddie and Harrod, 2008; Goutagny et al., 2010). The functional domains of HMPV F consistent with its categorization as a class I viral fusion protein have been described(Deffrasnes et al., 2008; Miller et al., 2007; van den Hoogen et al., 2002; Yang et al., 2009). HMPV L encoded conserved domains and motifs analogous to defined *Mononegavirales* polymerase domains; reflecting this conservation, AMPV and HMPV polymerase complex proteins are interchangeable to an extent(de Graaf et al., 2008a).

The only two genes for which aa identity was lower than nt identity were SH and G. These genes were quite divergent within and between groups. In addition to amino acid changes, another contributor to this divergence was the variable length of the G and SH genes. Mutations in SH have been shown to occur during cell culture(Biacchesi et al., 2007). It is possible that the sequence truncations we observed arose during passage; however, insufficient original clinical specimen remained for sequencing to confirm this. The function of SH is not known; SH-deleted viruses are minimally attenuated in non-human primates but

replication competent in cells and in rodents(Biacchesi et al., 2005; Biacchesi et al., 2004). The ability of the virus to tolerate such variation in SH during culture is unexplained and the biological effect is unknown. Similarly, HMPV G exhibited substantial amino acid diversity, greater than nucleotide variability, suggesting selective pressure. However, HMPV G induces binding but not neutralizing antibodies, and does not provide protection in animal models(Mok et al., 2008; Ryder et al., 2010; Skiadopoulos et al., 2006). A proposed interaction between HMPV G and RIG-I has not been confirmed(Bao et al., 2008). The source of selective pressure, if such exists, and the biological reason for the diversity of G and SH remain unknown.

The non-coding regions in hMPV were mostly conserved and similar to those of other pneumoviruses. In contrast to the other HMPV intergenic regions, the SH-G and G-L intergenic sequences were unusually long. The longest intergenic region in RSV is 56 nucleotides between M-G(Bukreyev et al., 2000); all other paramyxoviruses possess shorter intergenic sequences. Increasing the length of the RSV M-G intergenic sequence up to 160 nt had little effect on growth in cells or in mice(Bukreyev et al., 2000). The error rate of the HMPV polymerase is between 1.1×10^{-3} - 7.1×10^{-4} substitutions per site per year(de Graaf et al., 2008b; Yang et al., 2009). Nonetheless, these intergenic sequences were conserved among the strains described here, isolated between 1994-2003, and in partial sequences as old as 1985 (not shown). Why then are these uniquely long and putatively non-essential intergenic regions maintained and more importantly, the sequence conserved within groups over time? It is possible that these sequences regulate transcription or replication by unknown mechanisms.

In summary, we have sequenced the complete genome of four prototype HMPV strains. Comparison of these genomes to the few published genomes yields insights into this virus. Domains and motifs that are shared with other *Mononegavirales* point to conserved biology; divergent sequences and patterns of variation suggest where HMPV differs from related viruses. The gene order of HMPV is more similar to a Rubulavirus than a pneumovirus, though rather than HN, HMPV possesses a G protein analogous to the G protein of a pneumovirus. HMPV and AMPV may represent an intermediate clade in viral evolution between rubulaviruses and pneumoviruses. The surprising unexplained diversity of some genes (G and SH) and the unexpected conservation of putatively non-functional non-coding regions show that there is much yet to learn about HMPV biology.

Acknowledgments

Financial support: Supported by grants from the National Institute of Allergy and Infectious Diseases, National Institutes of Health AI-085062 and AI-082417 (JVW) and an Infectious Disease Society of America Research Scholarship (R.P.). Sequencing was performed in the VUMC DNA Sequencing Facility.

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Table 1

HMPV genome sequences analyzed.

Virus	Genotype	Source	GenBank accession number
TN/96-12	A1	Nashville, TN	pending
NL00-1	A1	Amsterdam, Netherlands	AF371337
TN/94-49	A2	Nashville, TN	pending
NL00-17	A2	Amsterdam, Netherlands	FJ168779
CAN97-83	A2	Quebec, Canada	AY297749
JPN03-1	A2	Shiga, Japan	AB503857
BJ1887	A2	Beijing, China	DQ843659
GZ01	A2	Guangzhou, China	GQ153651
TN/982-42	B1	Nashville, TN	pending
NL99-1	B1	Amsterdam, Netherlands	AY525843
TN/99-419	B2	Nashville, TN	pending
BJ1816	B2	Beijing, China	DQ843658
TW05-00125	B2	Taiwan	EF535506
CAN98-75	B2	Quebec, Canada	AY297748
NL94-1	B2	Amsterdam, Netherlands	FJ168778

Table 2

Nucleotide length of genes and non-coding regions of the four prototype HMPV strains with predicted amino acid length (within brackets).

	TN/96-12	TN/94-49	TN/98-242	TN/99-419
Genome length	13, 387	13, 335	13, 294	13, 281
N	1185 (394)	1185 (394)	1185 (394)	1185 (394)
N-P intergenic	19	19	19	19
P	885 (294)	885 (294)	885 (294)	885 (294)
P-M intergenic	32	32	32	32
M	765 (254)	765 (254)	765 (254)	765 (254)
M-F intergenic	122	122	121	121
F	1620 (539)	1620 (539)	1620 (539)	1620 (539)
F-M2 intergenic	75	36	26	26
M2	727	727	727	727
M2-1	564 (187)	564 (187)	564 (187)	564 (187)
M2-2	216 (71)	216 (71)	216 (71)	216 (71)
M2-SH intergenic	30	30	32	30
SH	552 (183)	546 (181)	534 (177)	534 (177)
SH-G intergenic	200	200	190	130
G	711 (236)	690 (229)	730 (241)	711 (236)
G-L intergenic	210	210	184	188
L	6018 (2005)	6018 (2005)	6018 (2005)	6018 (2005)

Table 3

Nucleotide and amino acid identity of HMPV genes within major and minor groups.

Gene	N			P			M			F		
Group	**All**	**A**	**B**	**All**	**A**	**B**	**All**	**A**	**B**	**All**	**A**	**B**
Mean % nt identity	91.1	91.3	91.0	87.8	87.9	87.6	90.3	90.5	90.1	89.8	90.0	89.5
Minimum % nt identity	85.4	85.4	85.9	79.5	79.5	80.3	84.1	84.1	84.1	83.1	83.1	83.1
Maximum % nt identity	99.6	99.6	99.6	99.4	99.3	99.4	99.5	99.2	99.5	99.5	99.5	99.4
Mean % aa identity	98.4	97.9	98.9	92.6	91.1	94.4	98.6	98.0	99.2	97.4	96.7	98.2
Minimum % aa identity	95.2	95.2	98.2	83.0	83.0	90.5	95.7	95.7	98.0	93.7	93.7	97.0
Maximum % aa identity	100.0	100.0	100.0	100.0	99.3	100.0	100.0	100.0	100.0	100.0	99.8	100.0

Gene	M2-1			M2-2			SH			G			L		
Group	**All**	**A**	**B**	**All**	**A**	**B**	**All**	**A**	**B**	**All**	**A**	**B**	**All**	**A**	**B**
Mean % nt identity	90.6	91.0	90.1	91.4	96.6	97.4	76.4	78.4	74.2	71.0	71.7	70.2	90.1	90.4	89.8
Minimum % nt identity	84.2	84.2	84.9	85.6	93.5	94.0	61.1	61.1	61.1	53.5	53.5	53.5	83.8	83.8	84.2
Maximum % nt identity	99.7	99.7	99.3	99.1	99.5	98.4	99.3	99.3	98.2	98.1	98.1	96.9	99.3	99.3	99.3
Mean % aa identity	98.0	97.2	98.8	95.3	94.8	96.1	75.8	74.7	77.0	59.2	55.2	63.7	97.3	96.5	98.1
Minimum % aa identity	94.1	94.1	97.3	87.3	87.3	93.0	53.8	53.8	63.6	28.2	28.2	47.0	93.5	93.5	97.1
Maximum % aa identity	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	96.8	96.8	94.9	100.0	100.0	99.9

Sequences included are the four HMPV genomes included in this report and full-length genome sequences of HMPV available from GenBank (accession numbers AB503857, AF371337, AY297748, AY297749, AY525843, DQ843658, DQ843659, EF535506, FJ168778, FJ168779, and GQ153651).