Metapneumovirus

determinants of host range and replication

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determinanten van gastheerspecificiteit en replicatie

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CHAPTER 1
GENERAL INTRODUCTION

HUMAN METAPNEUMOVIRUS

In June 2001, human metapneumovirus (HMPV) was first reported as a causative agent of respiratory tract disease in children in the Netherlands (238). In the following years HMPV was shown to be circulating worldwide (54). Based on gene constellation and sequence homology with avian metapneumovirus (AMPV), HMPV has been classified as the first human virus within the genus Metapneumovirus, subfamily Pneumovirinae (238) (Figure 1).

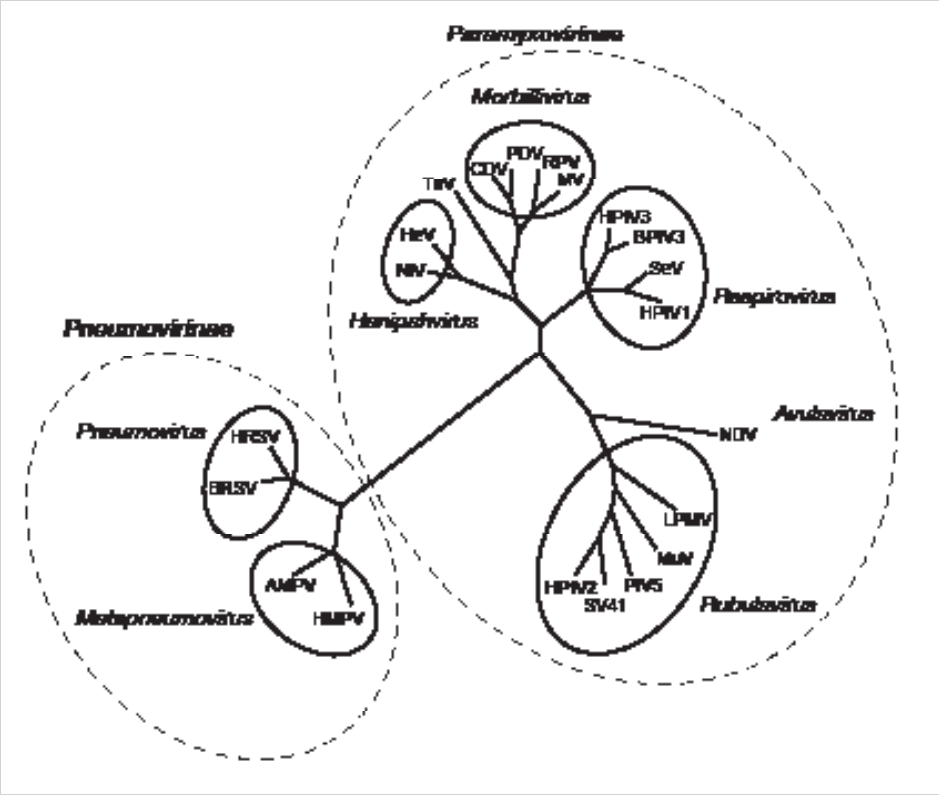

Figure 1
Schematic diagram of members of the family Paramyxoviridae based on phylogenetic analysis of the nucleotide sequences for the polymerase gene of these viruses. Abbreviations: NiV: nipah virus; HeV: hendra virus; TuV: tupaia virus; CDV: canine distemper virus; PDV: phocine distemper virus; RPV: rinderpest virus; MV: measles virus; PIV-3: parainfluenza virus type 3; SeV: sendai virus; PIV-1: parainfluenza virus type 1; NDV: newcastle disease virus; LPMV: la piedad michoacan virus; MuV: mumps virus; PIV-5: parainfluenza virus type 5; SV41: simian virus 41; PIV-2: parainfluenza virus type 2; AMPV: avian metapneumovirus; HMPV: human metapneumovirus; RSV: respiratory syncytial virus; B:bovine; H:human.

METAPNEUMOVIRUS GENOME

The general composition of the metapneumovirus genome is similar to that of pneumoviruses but differs in the number of genes and their order in the genome (Figure 2) (237). Metapneumoviruses do not encode the nonstructural proteins NS1 and NS2, and the small hydrophobic (SH) and attachment (G) protein genes follow the fusion (F), M2.1 and M2.2 protein genes, whereas for pneumoviruses this is the other way around. In evolutionary terms, this difference is very striking since recombination for members of the subfamily Pneumovirinae has been observed in a laboratory setting only (218). The 3′ and 5′ ends of the genome consist of short extragenic regions, referred to as the leader and trailer regions, and approximately the terminal 12 nt of the leader and the terminal 12 nt of the trailer are complementary, most likely due to the fact that each contains the basic elements of the viral promoter (21, 57, 163). The 3′ end of the viral RNA (vRNA) directs both replication and transcription, while the 5′ end of the genome contains signals that direct the replication of the antigenome. Each gene is preceded by a gene start (GS) signal at which the synthesis of mRNA initiates. Subsequently, each gene terminates with a semiconserved gene end (GE) signal that directs polyadenylation and termination of the completed mRNA. The genomes of metapneumoviruses consist of non-segmented negative stranded RNA and are smaller (~13.000 kb) than those of pneumoviruses (~15.000 kb) (70). For members of the subfamily Paramyxovirinae, the nucleotide length of each genome is a multiplicity of six ("rule of six"), reflecting a requirement of the nucleocapsid for RNA binding (e.g., binding units of six nucleotides) (34). This rule does not apply for the subfamily Pneumovirinae (201), although the reasons underlying this discrepancy have not been determined.

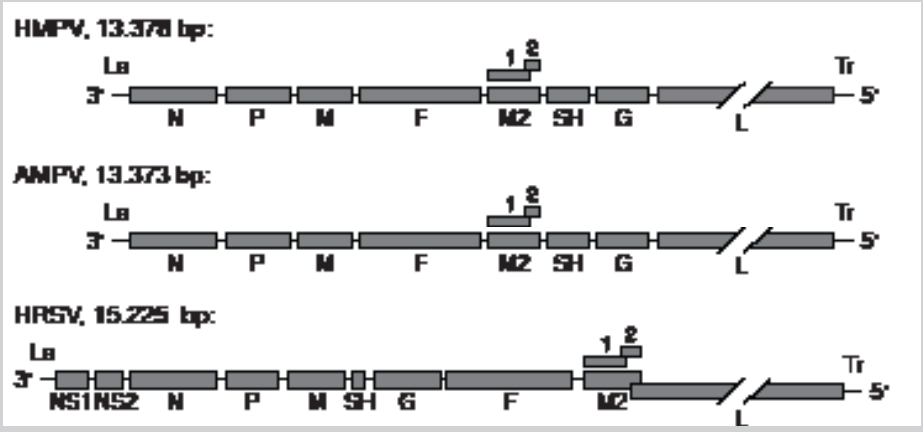

Figure 2
Schematic representation of HMPV, HRSV and AMPV genomes.

VIRION STRUCTURE

Metapneumoviruses are large pleomorphic enveloped virus particles ranging from 150 to 600 nm in diameter (Figure 3) (51, 238). A lipid envelope, which is derived from the host cell surrounds the metapneumovirus virion. The envelope contains three surface glycoproteins; the G protein, responsible for attachment of the virus to the host cell, the F protein required for the fusion of the viral envelope with the host cell membrane, and the SH protein, the function of which is not completely understood. The F protein is a type I membrane protein, with the amino terminus located outside the virion and a short cytoplasmic carboxy-terminus inside. The G and SH proteins are type II membrane proteins, with internal amino and external carboxy-terminal components (43). Whereas all members of the subfamily Pneumovirinae encode a SH protein, within the subfamily Paramyxovirinae only the members of the genus rubulavirus parainfluenza type 5 (PIV-5) and mumps virus (MuV) encode a SH protein (145). The SH protein of PIV-5 and members of the subfamily Pneumovirinae have an external carboxy-terminal (47, 116), while MuV encodes a cytoplasmic carboxy-terminal (225). The

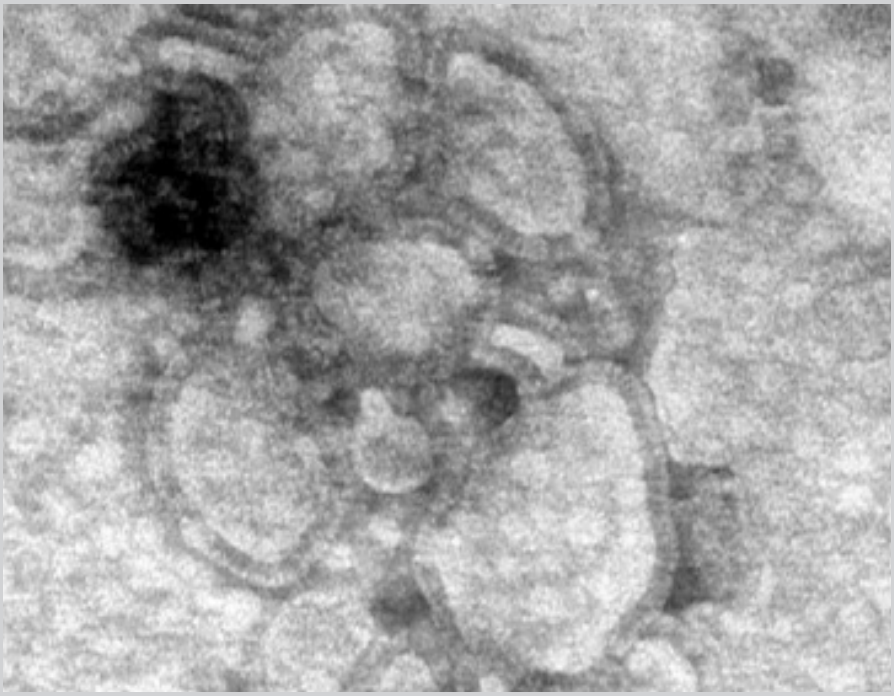

Figure 3
Electron micrograph of HMPV particles. Virus concentrated from infected tertiary monkey kidney (tMK) cell culture supernatants were visualized by negative contrast electron microscopy after potassium tungstat acid (PTA) staining. Magnification, x 92,000. Taken from (238).

SH protein of HMPV is expressed in several different forms in virus infected cells depending on the glycosylation status. There is an unglycosylated form (SH0), an N-glycosylated form (SHg1) and a more extensively glycosylated form (SHg2) (5, 19). The SH protein of HMPV is the largest amongst members of the subfamily Pneumovirinae. RSV from which the SH protein was omitted replicated efficiently in cell culture but was slightly attenuated in the higher upper respiratory tract of mice and chimpanzees (31, 248). The SH proteins of other members of the family Paramyxoviridae have been suggested to act as a viroporin (185), or to have a function in blocking the TNF-a-mediated apoptosis pathway (5, 83, 151, 257). The matrix (M) protein lies internal to the viral envelope and is believed to be important during assembly of the virus particle. The helical nucleocapsid is located within the M-protein layer and contains the genomic RNA encapsidated by the nucleoprotein (N). This complex is associated with the large polymerase (L) protein and the phosphoprotein (P) forming the ribonucleoprotein (RNP) (160). The L protein contains all of the catalytic activities necessary for virus RNA synthesis (95, 108, 173). The M2.1 protein is also thought to be associated with the RNP and for RSV this protein was shown to act as a transcriptional elongation factor (45).

VIRUS EVOLUTION AND EMERGENCE

The basic mechanisms of a virus to evolve and adapt to changes in its environment include mutation, reassortment and recombination. The RNA-dependent RNA polymerase protein is relatively error prone, resulting in mutation rates of 10^{-3} to 10^{-5} substitutions per nucleotide copied (64). These high mutation rates in combination with short generation times and large population sizes result in high substitution rates. For segmented RNA viruses, exchange of genetic material most commonly occurs by reassortment, which involves the exchange of entire gene segments (38, 64). Another way to exchange genetic information is by recombination, which involves the generation of chimeric molecules containing segments of more than one parent virus. For RNA viruses, recombination events occur during RNA replication when the polymerase switches from one template to another, thereby generating a chimeric progeny molecule consisting of elements derived from both parental templates (64). Recombination is common for positive sense RNA viruses (118, 144, 162, 164), but recombination in negative-sense RNA viruses seems to be extremely rare (37), most likely because their RNA is always encapsidated in RNP, thereby limiting template switching.

To be able to replicate in a different host, a foreign virus has to complete the virus infectious cycle of adsorption, fusion, transcription, translation, genome replication, assembly and release from the cell (39). Each time a cross-species transmission occurs, there is a small chance that it will hold. For such a virus, it is crucial that it adapts to replicate in the new species. An alternative model is that rather than adapting to a new host species after emergence, successful emergence will only occur if a virus already possesses the necessary mutations. Upon successful infection of the host, viruses face immune surveillance by the cellular and humoral arms of the host immune system. Viruses are frequently capable to overcome such external selective constraints intended to limit their replication. Immune evasion through antigenic variation of neutralizing epitopes is a major force driving the evolution of HRSV in humans, and is likely important in the response to HMPV infection (64, 258, 267).

VIRUS HETEROGENEITY

On the basis of sequence diversity between the G and F protein genes of a large number of HMPV strains, and differences in virus neutralization titers, two HMPV serotypes were defined, A and B. Each serotype could be divided in two genetic sublineages, named HMPV-A1, -A2, -B1 and -B2 (241). The overall level of amino acid (aa) sequence identity between the main lineages was relatively high and around 86% - 98%, except for the SH and G protein, which share only 57%-62% and 33%-35% aa identity, respectively (Table 1). The only other member of the genus Metapneumovirus is AMPV. Based on sequence divergence in the G protein and antigenic differences between strains, AMPVs have been classified into four subgroups, A through D (9, 77, 130, 208). AMPV-C is more closely related to HMPV than to other AMPVs (91-93, 235, 237, 263). For the N, P, M, F, M2.1, M2.2 and L open reading frames, there is approximately 80% aa sequence identity between AMPV-C and HMPV, although the highly variable HMPV and AMPV-C SH and G proteins only share 27%-31% and 21%-24% aa identity (Table 1) (237).

Table 1

Amino acid sequence identity between the putative N (a), P (b), M (c), F (d), M2.1 (e), M2.2 (f), SH (g), G (h) and L (i) ORFs of the HMPV-A1 (A1), HMPV-A2 (A2), HMPV-B1 (B1), HMPV-B2 (B2) and AMPV-C (C) (nucleotide sequence identity). Sequences are available from Genbank under accession No. AF371337.2 (HMPV-A1), FJ168779 (HMPV-A2), AY525843.1 (HMPV-B1), FJ168779 (HMPV-B2) and AY579780 (AMPV-C).

A

N	A1	A2	B1	B2	C
A1		99 (94)	95 (86)	96 (85)	88 (76)
A2	99 (94)		95 (87)	96 (86)	88 (75)
B1	95 (86)	95 (87)		99 (95)	89 (76)
B2	96 (85)	96 (86)	99 (95)		89 (76)
C	88 (76)	88 (75)	89 (76)	89 (76)	

B

P	A1	A2	B1	B2	C
A1		96 (92)	86 (81)	87 (82)	68 (68)
A2	96 (92)		86 (81)	86 (81)	68 (68)
B1	86 (81)	86 (81)		96 (93)	68 (68)
B2	87 (82)	86 (81)	96 (93)		68 (68)
C	68 (68)	68 (68)	68 (68)	68 (68)	

C

M	A1	A2	B1	B2	C
A1		99 (94)	98 (86)	98 (86)	87 (77)
A2	99 (94)		97 (85)	97 (85)	87 (76)
B1	98 (86)	97 (85)		100 (94)	87 (77)
B2	98 (86)	97 (85)	100 (94)		87 (78)
C	87 (77)	87 (76)	87 (77)	87 (78)	

D

F	A1	A2	B1	B2	C
A1		98 (94)	94 (84)	94 (84)	81 (71)
A2	98 (94)		94 (84)	95 (84)	81 (70)
B1	94 (84)	94 (84)		99 (94)	80 (72)
B2	94 (84)	95 (84)	99 (94)		81 (72)
C	81 (71)	81 (70)	80 (72)	81 (72)	

E

M2.1	A1	A2	B1	B2	C
A1		98 (94)	95 (86)	95 (86)	84 (76)
A2	99 (94)		95 (87)	96 (85)	83 (75)
B1	95 (86)	95 (87)		98 (94)	83 (74)
B2	95 (86)	96 (85)	98 (94)		84 (76)
C	84 (76)	83 (75)	83 (74)	84 (76)	

F

M2.2	A1	A2	B1	B2	C
A1		96 (95)	90 (86)	92 (87)	56 (65)
A2	96 (95)		89 (87)	90 (88)	56 (65)
B1	90 (86)	89 (87)		99 (97)	58 (68)
B2	92 (87)	90 (88)	99 (97)		56 (66)
C	56 (65)	56 (65)	58 (68)	56 (66)	

G

SH	A1	A2	B1	B2	C
A1		84 (88)	57 (68)	58 (67)	31 (46)
A2	84 (88)		61 (69)	62 (69)	27 (48)
B1	57 (68)	61 (69)		81 (87)	29 (45)
B2	58 (67)	62 (69)	81 (87)		29 (46)
C	31 (46)	27 (48)	29 (45)	29 (46)	

H

G	A1	A2	B1	B2	C
A1		65 (76)	33 (54)	33 (60)	21 (49)
A2	65 (76)		35 (58)	36 (55)	23 (44)
B1	33 (54)	35 (58)		65 (77)	23 (49)
B2	33 (60)	36 (55)	65 (77)		24 (48)
C	21 (49)	23 (44)	23 (49)	24 (48)	

I

L	A1	A2	B1	B2	C
A1		99 (94)	94 (84)	94 (84)	80 (73)
A2	99 (94)		94 (84)	94 (84)	80 (73)
B1	94 (84)	94 (84)		99 (95)	80 (73)
B2	94 (84)	94 (84)	99 (95)		81 (73)
C	80 (73)	80 (73)	80 (73)	81 (73)	

METAPNEUMOVIRUS REPLICATION

The replication cycle of members of the subfamily Pneumovirinae is shown in figure 4. The F and the G proteins interact with molecules on the target cell surface (149). For RSV and HMPV it has been suggested that cellular glycosaminoglycans or heparin-like molecules are involved in the binding of the G protein to the cell (80, 99, 100, 142, 231). Subsequently, the virus enters the cell through PH-independent fusion of the cell membrane and the virus envelope (114). The trigger mechanisms that control the viral entry machinery remain to be elucidated. For the F protein to become functionally active, the F0 precursor protein needs to be cleaved by cellular proteases into F1 and F2. The hydrophobic F1 component then promotes the fusion process, which introduces the internal components of the virion into the cytoplasm of the host cell, were the remainder of the infectious cycle takes place. In the cytoplasm, the vRNA is transcribed into two species of positive sense RNA: messenger RNA (mRNA) and copy RNA (cRNA). Unlike the vRNA and cRNA, the mRNA is capped and has a poly(A) tail (145). For HRSV switch from RNA transcription to replication is controlled by the M2.2 protein (13).

The vRNA represents a succession of individual protein-encoding genes. Transcription is initiated at the very 3′ end of the genomes producing approximately 50-nucleotides leader RNA. This is followed by sequential transcription of the individual mRNAs, giving rise to a gradient of transcripts, steadily decreasing toward the template 5′ end. Thus, the gene order roughly reflects the required amount of mRNA products (Figure 5). The cRNA is assembled into an antigenomic RNP that serves as a template for the synthesis of new vRNA. These newly synthesized vRNAs are assembled into genomic RNPs, which are subsequently packaged into virions.

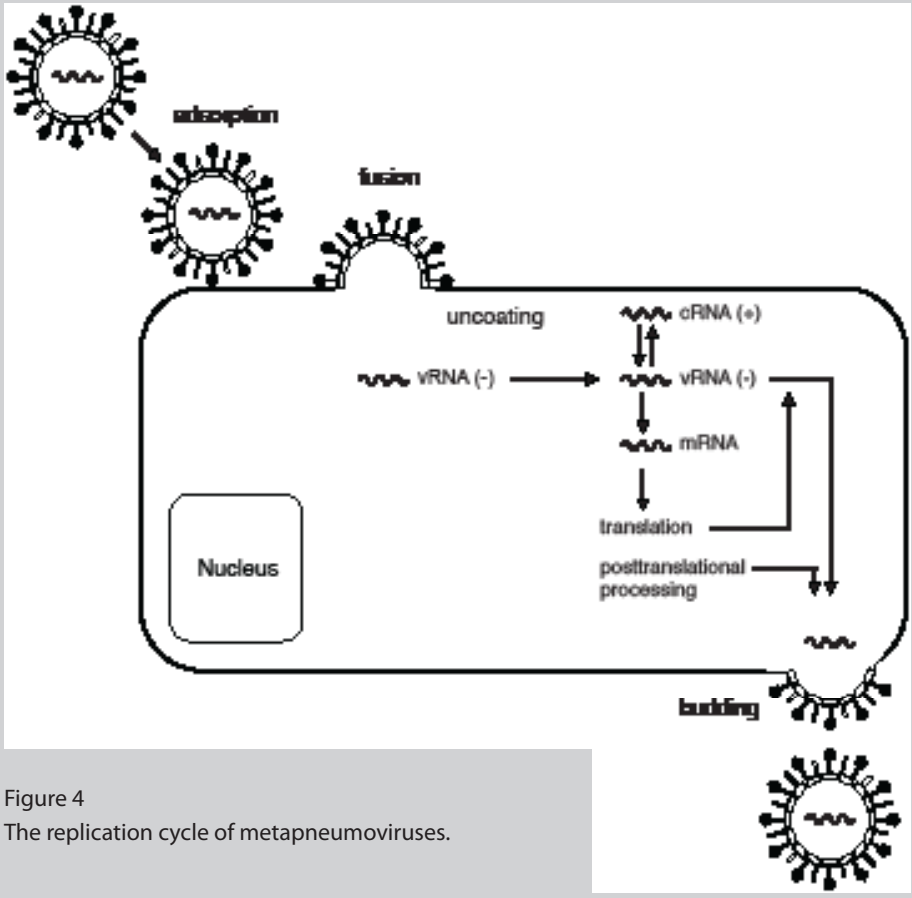

Figure 4
The replication cycle of metapneumoviruses.

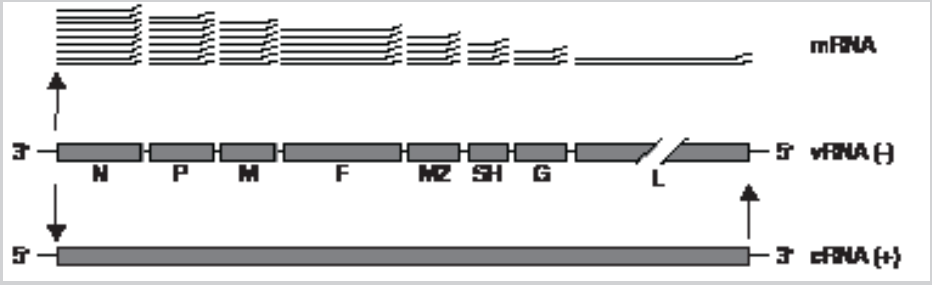

Figure 5
Schematic representation of RNA synthesis of metapneumoviruses.

The accumulation of the newly synthesized M protein may be crucial for initiating the assembly of virions (229). The integral membrane glycoproteins (F, SH and G) are synthesized in the endoplasmatic reticulum and transported to the cell surface by the cellular secretory pathway. It is generally believed that the nucleocapsid complex/M-protein structure, then associates with the cytoplasmic tails of the glycoproteins that are inserted into the cell membrane (70). Virus assembly and budding in polarized cells occur at the apical surface of the cells (199, 265).

METAPNEUMOVIRUS IN HUMANS

HMPV was first identified in the Netherlands (238), and evidence of infection has since been collected worldwide. By the age of five, virtually every individual has experienced at least one HMPV infection (43). Retrospective analysis of serum samples collected from humans in the Netherlands in 1958 revealed that HMPV has been widespread in the population for at least 50 years, and thus is a newly identified rather than newly emerging virus (238). HMPV has been overlooked previously due to its poor replication in cell cultures in vitro, the limited numbers of cell substrates it can infect, and the dependency on the addition of trypsin for replication in cell culture.

Re-infections in humans occur relatively frequent (71), which is in agreement with the transient protection observed in macaques that were experimentally infected with HMPV (240). HMPV has a seasonal distribution overlapping with HRSV circulation, with most cases reported during the winter and early spring in moderate climate zones (254). Annual circulation of all four genetic lineages of HMPV has been observed worldwide, with reports of a predominating strain changing on a yearly basis in some studies, but not in others (85, 216, 241).

HMPV has been detected in 5-25% of patients hospitalized for respiratory tract illness (RTI) (54, 242, 243, 253). Symptoms of both upper and lower respiratory tract disease have been associated with HMPV in infants, young children, the elderly and immunocompromised (54, 78, 133, 183, 238, 239). In young hospitalized children, the clinical features associated with HMPV infections are very similar to those of HRSV (23, 82, 121, 247). Diagnoses of bronchiolitis, with or without pneumonitis, have been most commonly reported. A substantial portion (up to 50%) of infected children also has concomitant otitis media (OM) (253, 254). It has been hypothesized that the inflammatory reaction to the virus, leading to incomplete or complete blockage of the Eustachian tubes and subsequent invasion by bacteria, is a likely mechanism for virus-induced OM. In children with underlying medical conditions such as prematurity, cardiopulmonary problems, and immunosuppression, HMPV related disease tends to be more severe (22, 220). Several studies have reported the association of HMPV with asthma or wheezing exacerbation in children, whereas other found no association (82, 121, 158, 166, 181, 196, 252, 253). Dual virus infections are frequently identified in the same clinical specimen particularly for HMPV with HRSV (96, 148), and HMPV has been found to co-circulate with SARS coronavirus (148). Molecular mechanisms have been described that enhance the human host's susceptibly to pneumococcal infection by influenza and RSV (103). To date there is no data that suggests that bacterial infection complicating HMPV infection is common. However, the proper studies to investigate this issue have not been conducted.

METAPNEUMOVIRUS IN POULTRY

A disease called turkey rhinotracheitis (TRT) was first described in South Africa in 1978 (32, 33). AMPV was later found in France (89) and in the UK, where the causative agent was isolated and characterized as a pneumovirus (36, 161, 250, 260). At present, AMPV has been found to infect domestic poultry worldwide (49). In 1997, an AMPV was detected in turkeys in Colorado (53) that was antigenically and genetically distinct from European subgroups A, B and D and was designated AMPV-C (9, 77, 130, 208). The outbreak in Colorado lasted for 10 months before eradication, primarily by slaughter and stringent biosecurity measures (172). Around the same time, AMPV-C outbreaks were reported in Minnesota, which is the largest turkey producing state in the USA. In the following years, AMPV-C related disease emerged as a major economic problem for turkey farmers in Minnesota. The most important species susceptible to AMPV are turkeys and chickens, although the virus can also infect other species of birds (172). AMPV RNA has been isolated from wild ducks, geese, sparrows, swallows, starlings, guinea fowls and blue winged teals (11, 12, 209). The USA AMPV-C outbreaks in turkeys displayed a seasonal pattern with approximately 80% of the outbreaks occurring in spring (March to May) and autumn (October to November) corresponding to periods of wild bird migration. Thus wild birds may be involved in the transmission of virus between commercial turkey farms (172). However, in the UK no seasonal pattern of infection was observed. Moreover, no serious AMPV-C outbreaks have been reported in Canada (which neighbours Minnesota) or the U.S states neighboring Minnesota (26).

AMPV causes respiratory disease of turkeys, which is characterized by snicking, ocular and nasal discharge, and swelling of the infraorbital sinuses. Infection with AMPV can also result in a transient drop in egg production (126, 128). In chickens, AMPV is also associated with 'swollen head syndrome'. AMPV infections regularly get complicated with secondary bacterial infections resulting in air sacculitis and pneumonia (125), whereas infections without any secondary infections are mainly confined to the nasal turbinate and trachea (126, 245). Co-infections with Bordetella avium, Pasteurella like organisms, Mycoplasma gallisepticum, Ornithobacterium rhinotracheale, and Escherichia coli, have been found to aggravate disease condition as well as to increase the invasiveness of the virus (52, 125, 169). The mortality due to AMPV infection and concomitant secondary bacterial infections range from 0 to 30% in the USA (2).

REVERSE GENETICS OF NON-SEGMENTED NEGATIVE STRAND VIRUSES

For non-segmented negative strand RNA viruses, Park et al. and Collins et al. were the first to demonstrate that short artificial negative-sense RNA molecules inside a cell could be packaged into virus particles in the presence of helper virus (46, 177). Rabies virus (RV) was the first non-segmented negative strand virus that was recovered solely from cloned cDNA (206). For the recovery of RV, a cDNA clone encoding full-length antigenomic vRNA was generated. This full-length genome was flanked by a T7 RNA polymerase promoter and a hepatitis d virus (HDV) ribozyme sequence next to a T7 RNA polymerase terminator. Following transcription by T7 RNA polymerase, the HDV ribozyme at the 3' end generates a precise 3' end of the genome

by autolytic cleavage. Viral RNA lacks both a 5′ cap and a 3′ poly (A) tail. For the rescue of infectious non-segmented negative strand viruses from cDNA, the generation of correct 3′ and 5′ ends is essential. This full-length cDNA plasmid was co-transfected with expression plasmids encoding the viral N protein and the polymerase proteins (L and P) under the control of a T7 RNA polymerase promoter into cells previously infected with vaccinia virus expressing T7 RNA polymerase (VacT7). After expression of the encoded proteins, N proteins assemble around the antigenomic cRNAs and polymerase proteins then replicate these antigenomic RNPs to form RNPs containing genomic vRNAs. Subsequent transcription, translation and virus assembly lead to release of infectious virus particles.

For successful recovery of non-segmented negative strand RNA viruses from cDNA, the use of positive-sense antigenomic RNA seemed more promising. It was thought that simultaneous presence of naked negative-sense genomic RNA and mRNA would result in hybridization, thereby preventing the assembly of the genome into the RNP. Negative sense non-segmented RNA viruses normally do not encounter this problem since they always keep their genomes in RNP form, which prevents hybridization. For the recovery of pneumoviruses from cDNA it was found that co-expression of M2.1, which was designated a transcriptional elongation factor, was necessary (45). In most reverse genetics systems, the transcription of viral genomes and mRNA is performed by T7 RNA polymerase. T7 RNA polymerase can be provided through different mechanisms; via VacT7, a recombinant fowlpox (25), defective Sindbis virus replicons (159), modified vaccinia virus Ankara (MVA-T7) (222), plasmids (147), or stable cell lines. Minireplicon systems facilitate the development and optimization of reverse genetics systems. Minireplicon systems include a minigenome construct encoding a reporter gene such as chloramphenicol acetyltransferase (CAT) or green fluorescent protein (GFP) flanked by the 5′ and 3′ vRNA ends of the virus of interest, under the control of T7 RNA polymerase. Co-transfection of the minigenome construct with plasmids expressing the polymerase complex proteins into cells expressing T7 RNA polymerase, results in expression of the reporter gene. Reverse genetics systems are currently available for a wide range of negative strand RNA viruses. In addition to their use for the generation of vaccine candidates, reverse genetics facilitates both fundamental and applied virological research.

HMPV VACCINES

The clinical impact of HMPV warrants the development of a vaccine, especially for the paediatric population and possibly for the elderly and immunocompromised individuals. There are currently no vaccines or prophylactic therapies against HMPV available. Vaccine development for RSV has been the goal of multiple studies for the past 40 years. Several strategies have been used, but only inactivated, subunit and live attenuated HRSV vaccines have been evaluated in human clinical trials to date. Vaccine-associated disease enhancement has been described for formalin-inactivated HRSV and measles virus vaccines and was a major complication for HRSV vaccine development (137, 140, 165). It is possible that HMPV vaccine development will encounter similar problems. Recent studies in animal models demonstrated that formalin-inactivated HMPV vaccines have the same propensity to predispose for immune-mediated enhanced disease as inactivated HRSV vaccines (62, 262).

Other possible vaccination strategies for HMPV include live, attenuated vaccines (LAVs), subunit vaccines, DNA vaccines and vectored vaccines. An ideal LAV mimics natural infection without causing disease, but induces a robust immune response, including innate and cell-mediated immunity, as well as local and systemic antibodies. There are several approaches to create such LAVs. Conventional techniques for attenuation of viruses such as passaging at low temperature, mutagenesis or passage in heterologous tissues, followed by selection of the desired phenotype, continue to play a role in the development of LAVs (94). Deletion of one or more genes that are not required for replication in vitro can attenuate the virulence of the virus for its host as well. The Jennerian approach to the development of LAVs involves the use of a virus strain of animal origin to immunize humans against a antigenically related human virus. Non-human mammalian and avian viruses that are well adapted to their natural host often do not replicate efficiently in humans and hence are attenuated. In analogy to this Jennerian approach, an innovative method for creating LAVs is to genetically modify the virus of interest such that its major neutralization and protective antigen genes are left undisturbed while replacing one or more of the other genes with the counterpart(s) from a closely related virus that has a different natural host. Studies have shown that the two major surface proteins of members of the subfamily Pneumovirinae, F and G are the most important antigens for the induction of protective immune response. Therefore, vaccines should contain the F and G proteins (127, 174, 210). The HMPV G protein of HMPV is only weakly immunogenic and protective in animal models, in contrast to that of HRSV (174, 210).

AMPV VACCINES

The economic losses associated with AMPV necessitate the development of a suitable vaccine. Effective attenuated subgroup-specific AMPV vaccines have been produced by repeated passage of the virus in non-turkey cells. It has been reported that 25 passages of subgroup A virus in Vero cells resulted in an attenuated virus that did not induce clinical signs but was protective against virulent virus challenge. In contrast, 98 passages of the virus in turkey tracheal organ cultures did not attenuate the virus (255, 256). In the USA, an AMPV-C strain that was attenuated by 63 serial passages in cell culture and did not induce clinical signs was licensed by the department of Agriculture for vaccination of commercial turkeys (178). However, this vaccine does not induce a strong humoral response, is only partially protective against an experimental challenge with a virulent strain, and under field conditions, appears to reduce the severity of clinical signs but not the number of infected birds (129, 178). Recently, reports have demonstrated the loss of attenuation and extended field persistence of such LAVs (35). Reverse genetics could be used to stabilize mutations responsible for the attenuation of AMPV LAVs, making reversion to the wild-type less likely. Several research groups have attempted to develop other AMPV vaccine types, such as AMPV-fowlpox recombinant (191), virosomes (134), formalin-inactivated (135) and DNA (136) vaccines. None of these approaches resulted in comparable levels of protection to those induced by LAVs.

SCOPE OF THIS THESIS

The aim of the studies described in the present thesis was to generate tools and techniques necessary for HMPV research, and to increase our understanding of determinants of host range and replication of metapneumoviruses. In chapter 2, we describe the recovery of infectious HMPV lineage A and HMPV lineage B solely from cDNA. The reverse genetics system was subsequently used to study various aspects of virus replication, host range and the development of vaccine candidates. In chapter 3, a plaque reduction virus neutralization assay using GFP-expressing viruses was described. This method facilitates the quantification of virus neutralizing antibodies, which are important correlates of protection against HMPV. Reverse genetics was further used for the generation of two LAV candidates described in chapter 4. Passaging HMPV at slowly decreasing temperatures resulted in a cold-passaged (cp) temperature sensitive (ts) strain. Mutations responsible for this phenotype were introduced in a recombinant HMPV by reverse genetics. Moreover, three mutations, which were previously described to result in a ts-phenotype in RSV were introduced in a recombinant HMPV. Both viruses were tested for temperature sensitivity in vitro and for attenuation and protective efficacy in hamsters. In chapters 5-7, the evolutionary histories of AMPV and HMPV, and determinants of host range of these viruses were studied. Using large sets of HMPV and AMPV-C G, F and N sequences and a Bayesian Markov Chain Monte Carlo (MCMC) framework, the evolutionary dynamics of HMPV and AMPV-C were studied (chapter 5). In chapter 6, the specificity and functional interaction of the polymerase complex proteins of human and avian metapneumoviruses both in vitro and in vivo were studied using the reverse genetics and minireplicon systems described in chapter 2. In chapter 7, the contribution of each of the metapneumovirus genes to HMPV and AMPV-C host range was determined using a large set of chimeric recombinant viruses. This study revealed that the F protein is the main determinant of host range. In chapter 8, we investigated the function of the HMPV SH protein using HMPV SH deletion mutants. Chapter 9 is a summarizing discussion, were the results described in chapter 2 to 8 are discussed.

CHAPTER 2
RECOVERY OF HUMAN METAPNEUMOVIRUS GENETIC LINEAGES A AND B FROM CLONED CDNA

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ABSTRACT

Human metapneumovirus (HMPV) is a newly discovered pathogen associated with respiratory tract illness, primarily in young children, immunocompromized individuals and the elderly. The genomic sequence of the prototype HMPV isolate NL/1/00 without the terminal leader and trailer sequences has been reported previously. Here we describe the leader and trailer sequences of two HMPV isolates, NL/1/00 and NL/1/99, representing the two main genetic lineages of HMPV. Minigenome constructs in which the green fluorescent protein or chloramphenicol acetyl transferase genes are flanked by the viral genomic ends derived from both HMPV lineages and transcribed using a T7 RNA polymerase promoter-terminator cassette were generated. Cotransfection of minigenome constructs with plasmids expressing the polymerase complex components L, P, N and M2.1 in 293T or baby hamster kidney cells resulted in expression of the reporter genes. When the minigenome was replaced by a sense or antisense full-length cDNA copy of the NL/1/00 or NL/1/99 viral genomes, recombinant virus was recovered from transfected cells. Viral titers up to $10^{7.2}$ and $10^{5.7}$ 50% tissue culture infective dose/ml were achieved with the sense and antisense plasmids, respectively. The recombinant viruses replicated with similar kinetics to the parental viruses in Vero cells. This reverse genetics system provides an important new tool for applied and fundamental research.

INTRODUCTION

The human metapneumovirus (HMPV) was recently identified in respiratory specimens obtained from children in The Netherlands suffering from respiratory tract illness (RTI) (238). Subsequently HMPV has been detected around the world, in clinical samples collected from children, immunocompromised individuals and elderly individuals suffering from RTI (8, 23, 40, 73, 76, 78, 157, 181, 184, 224, 232, 247). Clinical signs and symptoms associated with HMPV infection are similar to those caused by respiratory syncytial virus (RSV), ranging from mild respiratory problems to severe cough, bronchiolitis and pneumonia, often accompanied by high fever, myalgia and vomiting (22, 76, 78, 175, 181, 183, 224, 241, 243). The genomic organization of HMPV resembles that of avian metapneumovirus (AMPV), and HMPV has therefore been classified as the first mammalian member of the family Paramyxoviridae, subfamily Pneumovirinae, genus Metapneumovirus (237). Two main genetic lineages of HMPV were first described for the Dutch isolates (238) and the presence of these genotypes has been confirmed worldwide (7, 17, 23, 181, 184, 247). Recently, the two main lineages were found to represent two serotypes of HMPV, A and B, each of which can be further divided in two genetic sublineages A1, A2, B1, and B2 (241).

For AMPV, the other member of the metapneumovirus genus, a minigenome system has been established (194), but a recombinant virus rescue system is not yet available. Such reverse genetics systems have been reported for other paramyxoviruses, including RSV, measles virus, parainfluenza viruses, rinderpest virus and canine distemper virus (6, 44, 87, 117, 192). The systems for the recovery of recombinant paramyxovirus use a cDNA plasmid encoding the full-length viral RNA in sense or antisense orientation flanked by sequences to ensure the transcription of genome-length RNA such as a T7 RNA polymerase promoter-terminator cassette and a hepatitis delta virus (HDV) ribozyme sequence. Upon transcription of the full-length HMPV cDNA by T7 RNA polymerase expressed either from plasmids, modified vaccinia virus Ankara or recombinant fowl pox virus and coexpression of the viral polymerase protein complex (N, P, L and M2.1), a recombinant virus can be produced.

Reverse genetics systems provide a powerful tool for fundamental virus research and for the generation of vaccine candidates, including live-attenuated vaccines, because point mutations, deletions and insertions can be engineered to suit specific needs. Foreign genes can be introduced in order to attenuate viruses or to create chimeric vaccines. By using reverse genetics, one can envisage creating chimeric viruses between AMPV and HMPV as potential vaccine candidates in humans and poultry or chimeric vaccines between HMPV and other human respiratory pathogens (RSV, parainfluenza virus) to combat RTI in humans.

Here we describe the sequence analysis of the genomic termini of NL/1/00 and NL/1/99, representative strains for HMPV serotypes A (lineage A1) and B (lineage B1), respectively. We successfully used this information for the design of minigenome replication systems using chloramphenicol acetyl transferase (CAT) or green fluorescent protein (GFP) as reporter genes. Subsequently, the reporter genes of the minireplicons were replaced with full-length cDNA copies of the NL/1/00 (serotype A) or NL/1/99 (serotype B) genomes, and recombinant HMPVs were recovered.

MATERIALS AND METHODS

Cells and viruses

Virus isolates hMPV NL/1/00 and NL/1/99 were propagated in tertiary monkey kidney cells as described previously (238). 293T cells were grown in Dulbecco's modified eagle medium (BioWhittaker, Verviers, Belgium), supplemented with 10% fetal calf serum (FCS, Greiner Bio-One, Alphen aan den Rijn, The Netherlands), nonessential amino acids, 100 IU of penicillin/ml, 100 µg of streptomycin/ml and 2mM glutamine. BSR-T7 cells, a kind gift of Dr. K.K. Conzelmann, were grown in the same media without non-essential amino acids and supplemented with 0.5 mg of G418 (Life Technologies, Breda, The Netherlands)/ml. Vero cells were grown in Iscove's modified Dulbecco's medium (BioWhittaker) supplemented with 10% FCS, 100 IU penicillin/ml, 100 µg streptomycin/ml and 2mM glutamine. For hMPV replication, Vero cells were grown in Dulbecco's modified eagle medium supplemented with 3% FCS, 100 IU penicillin/ml, 100 µg streptomycin/ml, 2mM glutamine and 0.25 mg trypsin/ml. For virus titration or plaque assays, Vero cells were grown in Iscove's modified Dulbecco's medium supplemented with 4% bovine serum albumin fraction V (Invitrogen, Breda, The Netherlands), 100 IU penicillin/ml, 100 µg streptomycin/ml, 2mM glutamine and 3.75 µg trypsin/ml.

293T cells were transfected using the $CaPO_4$ precipitation method (180) and BSR-T7 cells were transfected using Lipofectamine 2000 (Invitrogen), according to the instructions of the manufacturer.

Identification of leader and trailer sequences of hMPV

Viral RNA was isolated from concentrated NL/1/00 and NL/1/99 virus stocks using RNAzolB according to instructions from the manufacturer (Campro Scientific, Veenendaal, The Netherlands). The viral RNA was circularized using T4 RNA ligase (New England Biolabs, Frankfurt am Main, Germany) for 1 h at 37°C and cDNA was synthesized using random hexamer primers (Promega, Leiden, The Netherlands) and RNase H-free superscript II reverse transcriptase (Invitrogen) for 50 min. at 42°C. The ligated junction of the leader and trailer was PCR amplified using primers in the 5′ end of the N open reading frame (ORF) and the 3′ end of the L ORF. The PCR products were sequenced upon cloning in pCR2.1 (Invitrogen).

A second approach was used to identify the terminal nucleotides of the leader of NL/1/00. Viral RNA was isolated using a QIAamp Viral RNA Mini Kit (Qiagen, Valencia, Calif.), according to the instructions from the manufacturer. This viral RNA was polyadenylated by incubation with poly (A) polymerase (Ambion, Austin, Tex.) at 37°C for 1 h and purified using a NucAway spin column (Ambion). The viral RNA was reverse transcribed using a primer complementary to the poly(A) tail region and Superscript I reverse transcriptase (Invitrogen). PCR reactions were carried out and sequenced upon cloning in plasmid pCR2.1 (Invitrogen).

Plasmid construction

(i) Minigenome constructs. An NdeI (nucleotide [nt] 2379)-to-HpaI (nt 136) fragment was removed from plasmid pSP72 (Promega) and replaced by a synthetic T7 promoter extended with two or three G residues, two BsmBI sites, the HDV ribozyme, and a T7 terminator to yield pSP72-P_{T7}-d-T_{T7}. The HMPV genomic leader sequence with the gene start of N, and the genomic trailer sequence with the gene end of L were amplified by PCR and ligated and

were separated by two BsmBI sites. This fragment was ligated in the BsmBI site of pSP72-P_{T7}-d-T_{T7} to yield pSP72-P_{T7}-Tr-Le-d-T_{T7}. The ORFs of GFP or CAT were amplified by PCR using primers with type II restriction sites (BbsI, BsaI or BsmBI) and cloned in the BsmBI sites between the N gene start and L gene end signals of HMPV to yield pSP72-P_{T7}-Tr-CAT-Le-d-T_{T7} and pSP72-P_{T7}-Tr-GFP-Le-d-T_{T7}. The junctions between the elements of the minigenome plasmids are shown in Fig. 1.

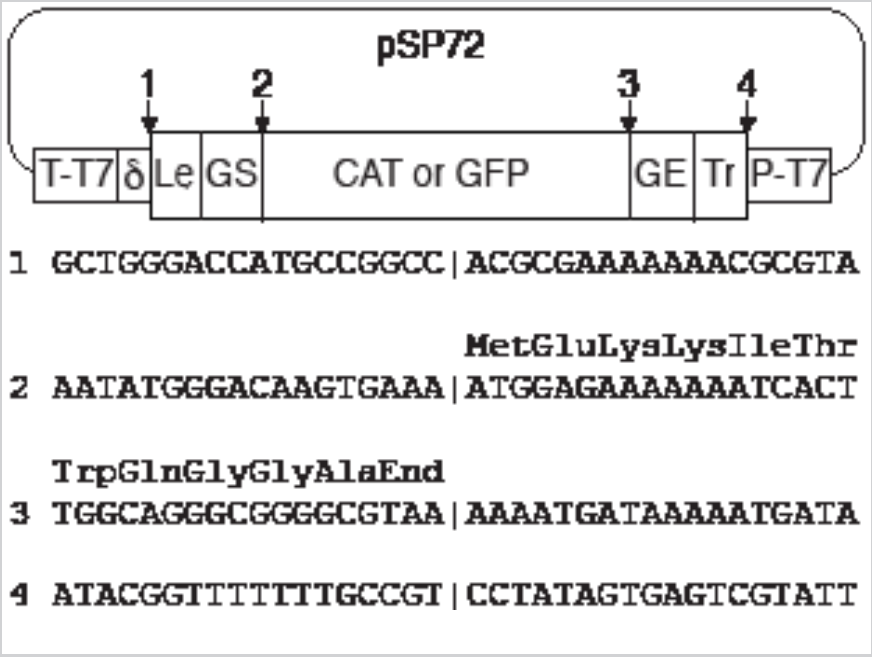

Figure 1

Schematic representation of a minigenome construct. A CAT or GFP reporter gene is flanked by the viral leader sequence (Le) and gene start signal (GS) of the nucleocapsid gene on one end, and the gene end signal (GE) of the polymerase gene and the viral trailer (Tr) on the other end. This minigenome is cloned in the context of a T7 RNA polymerase promoter-terminator (P-T7, T-T7) cassette. The authenticity of the transcribed ends of the negative-sense minigenome are determined by the position of the T7 promoter sequence and a HDV ribozyme sequence (d). The important junctions, depicted with numbers 1-4 are shown in detail below the map, with the translated sequence of the CAT gene as the example.

(ii) Polymerase constructs. The N, P and M2.1 ORFs of NL/1/00 and NL/1/99 were amplified by PCR using primers spanning the start and stop codons and flanked by NcoI and XhoI sites, respectively, and were cloned in the multiple cloning site of pCITE (Novagen) to yield plasmids pCITE-N, pCITE-P and pCITE-M2.1. Constructs encoding the L gene of NL/1/00 or NL/1/99 were assembled from overlapping PCR fragments using restriction sites in the L gene (Fig. 2) and were cloned in pCITE. The restriction sites used were NcoI (introduced at nt 7180 before the start codon of L), SwaI (nt 8806), EcoRI (nt 10998), and SmaI (introduced after the PacI site at nt 13283 in the trailer) for NL/1/00, and BamH1 (nt 7127), NsiI (nt 9565), KpnI (nt 11753) and AccI (nt 13276 in trailer) for NL/1/99.

(iii) Full-length cDNA vectors. The minigenome plasmids (pSP72-P_{T7}-Tr-Le-d-T_{T7}) of NL/1/00 and NL/1/99 were used to construct full-length cDNA vectors. The cDNA encoding the genome of NL/1/00 was assembled from overlapping PCR fragments using restriction sites MluI (nt 12), ApaLI (nt 3293), StuI (4480), SalI (6649), ClaI (8678), EcoRI (nt 10998), and PacI (nt 13283) (Fig. 2). The cDNA encoding the genome of NL/1/99 was assembled from overlapping PCR fragments using restriction sites MluI (nt 12), SacI (nt 2034), XbaI (nt 2979), BamHI (nt 5500 and 7127), NsiI (nt 9565), KpnI (nt 11753), and AccI (nt 13276) (Fig. 2). In the genome of both NL/1/00 and NL/1/99, a genetic marker, an AflII restriction enzyme site, was introduced at amino acid (aa) position 8 in the N ORF of the HMPV genome using a QuickChange site-directed mutagenesis kit (Stratagene). Also, for NL/1/99, a negative-sense full-length clone was generated with the extra AflII restriction enzyme site, to compare the functionality of rescue with positive- or negative-sense plasmids.

All plasmid inserts were sequenced to ensure the absence of mutations. All primer sequences used for plasmid construction are available upon request.

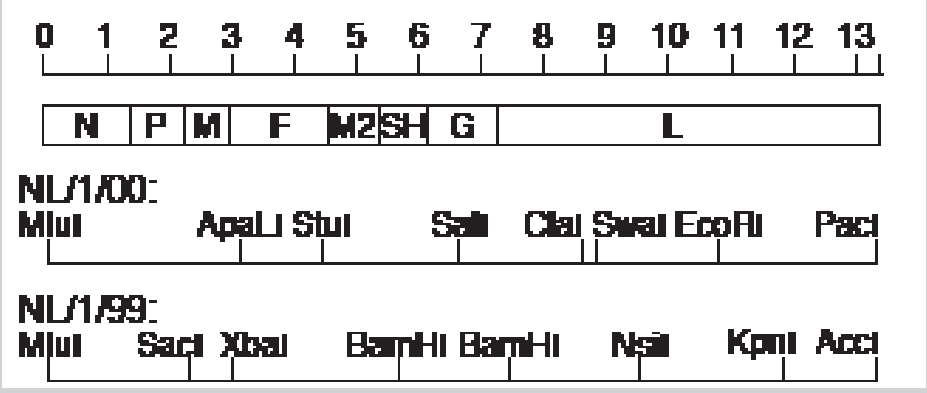

Figure 2
Restriction maps of HMPV NL/1/00 and NL/1/99. The genomic organization of HMPV is shown, roughly drawn to scale. The restriction sites that were used to generate the full-length cDNA constructs and the polymerase expression plasmids are shown for both virus isolates.

Minigenome assays

Minigenome plasmids were cotransfected with plasmid pAR3126, expressing a T7 RNA polymerase, and pCITE-L, pCITE-N, pCITE-P and pCITE-M2.1 in six-well plates containing 3 x 10^5 to 5 x 10^5 293T cells per well (180). Transfections were done overnight and medium was refreshed the next day. In a second approach transfections were done using BSR-T7 cells, a baby hamster kidney cell line stably expressing T7 RNA polymerase (29). Minigenomes were cotransfected with pCITE-L, pCITE-N, pCITE-P and pCITE-M2.1 in six-well plates when cell monolayers were 80 to 95% confluent. Cells were analyzed 3 days after transfection using a CAT enzyme-linked immunosorbent assay (Roche Diagnostics, Almere, The Netherlands) according to the instructions from the manufacturer or a flow cytometer equipped with an argon laser emitting at 488 nm (Becton Dickinson, Erembodegem, Belgium).

Recovery of recombinant HMPV

Both 293T cells and BSR-T7 cells were used for rescue of recombinant HMPV. 293T cells were transfected overnight using the $CaPO_4$ method with the four pCITE polymerase complex plasmids, pAR3126 and the full-length HMPV cDNA plasmid. BSR-T7 cells were transfected for 5 hours with Lipofectamine 2000 (Invitrogen) and the same plasmids without pAR3126. After transfection, the media was replaced with fresh media supplemented with trypsin and incubated for 2 to 4 days. Cells were either used to prepare lysates or for direct cocultivation with Vero cells. To prepare lysates, cells were scraped with a rubber cell scraper, pooled with the supernatant, and subjected to one -70°C freeze-thaw cycle. The virus preparation was cleared from cellular debris by centrifugation and used to inoculate 60 to 70% confluent Vero cells in 100-mm dishes. After 3 or 4 days, half of the medium was replaced with fresh medium, and after 6 or 7 days, the supernatant was collected and the infected Vero cells were immunostained using a guinea pig polyclonal antiserum raised against HMPV and a fluorescein isothiocyanate-labeled rabbit anti-guinea pig serum (DakoCytomation, Heverlee, Belgium). The cells were analyzed using a flow cytometer, and the supernatant was used for virus titrations, reverse transcription (RT)-PCR and restriction digests to confirm rescue of recombinant virus.

RT-PCR to identify genetic marker

In both NL/1/00 and NL/1/99, a silent mutation was introduced in the codon that encodes leucine at aa position 8 in the N ORF of the full-length genome. At this position, CCTAAG (NL/1/99) or CCTGAG (NL/1/00) were changed to CTTAAG in order to introduce an AflII restriction site. After harvesting the rescued virus and RNA isolation with the High Pure RNA Isolation Kit (Roche Diagnostics), RT-PCR was performed with specific primers to amplify the region in which the mutation was introduced. After amplification, a control digestion was done with AflII to confirm that the recovered virus was recombinant in nature.

Virus titration

Titrations were performed with 10-fold serial dilutions in 96-well plates (Greiner Bio-One). Briefly, confluent monolayers of Vero cells were spin-inoculated (15 min, 2000 x g) with 100 µl of 10-fold serial dilutions of each sample and incubated at 37°C. Fresh medium (100 µl) was added to each well after 3 days. Seven days after inoculation, infected wells were identified by immunofluorescence assays with HMPV specific polyclonal antiserum raised in guinea pigs.

Titers expressed as 50% tissue culture infectious dose ($TCID_{50}$) were calculated as described by Reed and Muench (198).

Plaque assay

Twenty-four-well plates containing 95% confluent monolayers of Vero cells were inoculated with 10-fold serial virus dilutions for 1 h at 37°C, after which the media was replaced by 0.5 ml of fresh media and 0.5 ml 2% methyl cellulose (MSD, Haarlem, The Netherlands) and cells were incubated at 37°C for 4 days. Methyl cellulose overlays were removed and cells were fixed with 100% methanol. Blocking was performed for 1 h at 37°C with 5% (wt/vol) nonfat dry milk in phosphate-buffered saline, and cells were subsequently incubated with HMPV specific polyclonal antiserum for 1 h at 37°C, followed by incubation with horseradish peroxidase-labeled rabbit anti-guinea pig antibodies (DakoCytomation). Positive plaques were counted after incubation with AEC substrate chromogen (DakoCytomation) to determine viral titers.

Growth curve

To generate growth curves, 25-cm^2 flasks containing confluent Vero cells were inoculated at 37°C for 2 h with wild-type and recombinant HMPV virus strains at a multiplicity of infection of 0.1. After adsorption of the virus to the cells, the inoculum was removed and cells were washed 2 times with media before addition of 7 ml of fresh media and incubation at 37°C. Every 2 days, 1 ml of the supernatant was collected and replaced by fresh media. Plaque assays or end-point titrations were performed to determine viral titers in PFU per milliliter or $TCID_{50}$ per milliliter, respectively. There is a general correspondence between viral titers determined by these two methods, but the end-point titrations yielded approximately10-fold higher titers; thus, 10 $TCID_{50}$/ml corresponds with approximately 1 PFU/ml.

Nucleotide sequence accession numbers

The updated and new sequences discussed here were submitted to GenBank under accession no. AF371337 and AY525843, respectively.

Table 1

Determination of leader sequences of HMPV NL/1/99 and NL/1/00 obtained by RT-PCR following ligation of viral RNA and 3' RACE[a].

Leader sequence positive strand	NL/1/99 RT-PCR circles (n=26)	NL/1/00 RT-PCR circles (n=35)	NL/1/00 pA-tailing & 3'RACE (n=79)
ACGCGAAAAAAACGCG	0 (0)	0 (0)	15 (19)
CGCGAAAAAAACGCG	0 (0)	0 (0)	6 (8)
GCGAAAAAAACGCG	15 (58)	30 (85)	5 (6)
CGAAAAAAACGCG	6 (23)	2 (6)	23 (29)
GAAAAAAACGCG	2 (8)	1 (3)	0 (0)
AAAAAAACGCG	2 (8)	0 (0)	0 (0)
Larger deletion	1 (4)	2 (6)	30 (38)

[a] All data given as number (%)

RESULTS

Identification of leader and trailer sequences

In order to determine the authentic nucleotide sequences of the HMPV genomic RNA leader and trailer, viral RNA isolated from concentrated virus stocks was circularized using T4 RNA ligase. cDNA was synthesized from this RNA, and the region containing the ligated junction of the genomic termini was amplified by PCR and cloned in plasmid pCR2.1. For NL/1/00 and NL/1/99, 26 and 35 clones were sequenced, respectively, and the position of the junction was determined after comparing all sequences. All but 2 clones showed the same trailer cDNA sequence 5'-TACGGTTTTTTTGCCGT-3'. In the 2 deviant clones, the terminal T nucleotide was missing. The majority of the leader cDNA sequences was 5'-GCGAAAAAAACGCGTAT-3', two nucleotides shorter than we had anticipated based on the leader sequences published for AMPV and RSV (163, 194). All other clones displayed three, four, five, or even longer nucleotide deletions at the leader end (Table 1). We next performed 3' rapid amplification of cDNA ends (RACE) analysis as an alternative strategy to determine the 3' end of the HMPV genome. To this end, fresh viral genomic RNA of NL/1/00 was isolated, which was polyadenylated and subsequently reverse transcribed using a primer complementary to the poly(A) tail region. The cDNA was amplified by PCR and cloned in plasmid pCR2.1. Sequence analysis revealed that 15 out of 79 clones (19%) contained the following HMPV leader sequences 5' ACGCGAAAAAAACGCGTAT 3', which included the AC terminal dinucleotide observed for other paramyxovirus leader termini, but were lacking in the constructs derived upon RT-PCR of ligated viral RNA. The other 81 % of the sequences revealed the absence of 3'- proximal nucleotides. From these data, we conclude that the correct leader cDNA sequence starts with AC and that the shorter leader sequences resulted from nuclease activity present in the virus preparations. It is of interest to note that the HMPV leader and trailer sequences are less complementary than the termini of related paramyxoviruses (Fig. 3).

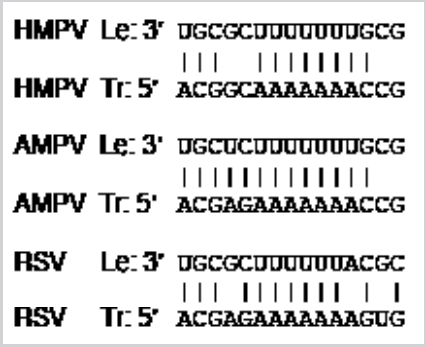

Figure 3

Complementarity of leader and trailer sequences of HMPV, AMPV, and RSV. Viral leader (Le) and trailer (Tr) sequences were aligned, and the complementarity is indicated with connection lines, showing that the terminal nucleotides of HMPV were less complementary than those of RSV and AMPV. The 3' terminal U residue in the AMPV genome has not been detected experimentally, but was added in analogy to other paramyxoviruses (194).

HMPV minigenome assays

Minigenome assays were performed to test whether the sequences of the genomic termini were correct and the plasmids encoding the viral polymerase complex components were functional. For this purpose, we used minigenome reporter constructs containing GFP or CAT ORFs in antisense orientation and flanked by the genomic termini of HMPV. A T7 phage RNA polymerase promoter was added upstream of the last genomic HMPV nucleotide of the trailer, and a HDV ribozyme sequence was linked to the HMPV leader to ensure authentic ends of the viral RNAs (Fig. 1). The minigenome constructs were cotransfected overnight in 293T-cells with pAR3126 expressing T7 RNA polymerase and pCITE plasmids containing the N, P, L and M2.1 ORFs. In a first experiment 1.5 µg of minigenome, 1.5 µg of T7 RNA polymerase and 1.8 µg of polymerase expression plasmids (0.6 µg of N, 0.6 µg of P, 0.3 µg of L, 0.3 µg of M2.1) were cotransfected, yielding 1.3 ng of CAT/ml after 24 hours and 11 ng of CAT/ml after 48 hours (data not shown). Constructs with and without the terminal AC dinucleotide in the leader were both functional, but the construct without the terminal AC nucleotides gave rise to lower (32% reduced) levels of CAT expression (data not shown). Subsequently, we optimized the amounts of total plasmid DNA for transfection and the relative amounts of each individual plasmid using CAT-expression as the readout. The optimal amounts of plasmids were 1 µg of minigenome, 1.5 µg of pAR3126, 0.8 µg of pCITE-N, 0.4 µg of pCITE-P, 0.4 µg of pCITE-M2.1 and 0.4 µg of pCITE-L. By using these optimized amounts, we achieved levels of CAT expression between 20 and 80 ng/ml for both the NL/1/00 and NL/1/99 minigenome systems. When the vector expressing the M2.1 protein is excluded from the experiments, the reporter gene is expressed at lower levels, indicating that the M2.1 protein is dispensable for minireplicon replication, whereas each of the other components is indispensable (Fig. 4). In BSR-T7 cells transfected with 1.5 µg of minigenome, 1.2 µg of pCITE-N, 0.6 µg of pCITE-P, 0.6 µg of pCITE-M2.1, and 0.6 µg of pCITE-L, CAT expression levels up to 140 ng/ml were obtained 48 hours after transfection (data not shown).

We next tested the GFP minigenome constructs in 293T cells using the optimal amounts of plasmids determined with the CAT minigenome constructs. Forty-eight hours after cotransfection, up to 13% of the cells revealed high fluorescence by flow cytometry when all constructs were transfected, whereas such GFP expression was absent when the pCITE expression vectors were omitted (data not shown).

In BSR-T7 cells transfected with the GFP minigenome and pCITE expression vectors, 67.4% of the cells were positive by flow cytometry and 1.1% by fluorescence microscopy. This difference in the proportion of GFP-positive cells likely reflected the detection limits of the two assays; only the proportion of cells expressing very high levels of GFP as determined by flow cytometry can be detected under a normal fluorescence microscope.

Assembly of hMPV cDNA and recovery of recombinant hMPV

Full-length HMPV cDNA clones were generated for both NL/1/00 and NL/1/99 by assembling DNA fragments of up to 3 kb that were generated by RT-PCR. This was done by using unique restriction enzyme sites present in the viral genome (Fig. 2). The full-length genomes were cloned in the minigenome vector (Fig. 1), by using restriction sites present in the leader and trailer. In order to demonstrate that the virus recovered by reverse genetics was derived from

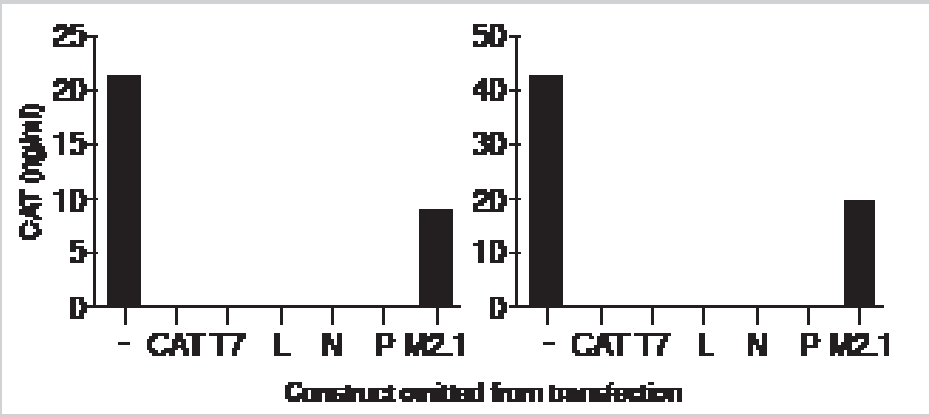

Figure 4

CAT expression in minigenome replication assays in 293T cells. 293T cells were transfected with a T7 RNA polymerase expressing plasmid, plasmids pCITE-L, pCITE-N, pCITE-P, and pCITE-M2.1 and the minigenome vector of either NL/1/00 (left panel) or NL/1/99 (right panel). Each of the individual plasmids was omitted from the transfection mixture to test the requirement of each individual component. Cell lysates were harvested 48 h after transfection and levels of CAT were determined by enzyme-linked immunosorbent assay.

plasmid DNA, a restriction enzyme site (AflII) was added at aa position 8 in the N ORF of the full-length cDNA construct. The NL/1/00 cDNA construct contained three nucleotide substitutions and a five nucleotide insertion compared to the GenBank sequence (AF371337). Upon subsequent sequence analysis of the original virus stock, the three nucleotide substitutions were found to be incorrect in the Genbank sequence entry (nt positions 3838, 8350, and 12218 in the GenBank sequence, were changed to A, T and T, respectively). A five-nucleotide insertion, TAAAA, was found in the intergenic region between F and M at position 4709 as compared to the GenBank sequence. This nucleotide insertion was absent in the virus stock from which the Genbank sequence was derived, but present in all other passages of virus stocks of NL/1/00. The plasmid encoding the full-length genome of NL/1/99 did not show any mutations compared to the wild-type sequence (accession no. AY525843).

Various approaches were followed for virus rescue from plasmids and were found to be successful. Initially, recombinant virus was rescued from 293T cells transfected overnight with 5 µg of the full-length cDNA plasmid, 1.5 µg of pAR3126, 0.8 µg of pCITE-N, 0.4 µg of pCITE-L, 0.4 µg of pCITE-P and 0.4 µg of pCITE-M2.1. Recombinant virus was also rescued from 293T cells infected with fowlpox-T7 and subsequently transfected with plasmids. However, the most efficient virus rescue was observed in BSR-T7 cells transfected for 5 h with 3 µg of the full length cDNA plasmid, 0.3 µg of pCITE-N, 0.3 µg of pCITE-P, 0.15 µg of pCITE-L and 0.24 µg of pCITE-M2. The cells and supernatant were harvested 48 hours after transfection, subjected to one freeze-thaw cycle, and used to inoculate Vero cells. Cytopathic effects were observed after 6 days in culture, at which time the majority of cells expressed HMPV proteins

as determined by staining with an HMPV polyclonal antiserum and flow cytometry (data not shown). Virus titers in the supernatants collected at day 6 ranged from $10^{5.7}$ to $10^{7.2}$ $TCID_{50}$/ml.

The replication kinetics of the wild-type and recombinant HMPV strains are shown in Fig. 5A, revealing that they were indistinguishable. Both wild-type and recombinant NL/1/00 appeared to replicate less efficiently than NL/1/99 in Vero cells. This difference in replication kinetics was observed in all assays that we performed with the NL/1/00 and NL/1/99 viruses. Probably the Vero cell line used in these experiments is more permissive for NL/1/99 than for NL/1/00.

For NL/1/99, recombinant viruses were rescued using plasmids in which the cDNA genome was either cloned in the plus or minus sense orientation. Virus rescue from positive-sense cDNA resulted in virus titers higher than those for negative-sense cDNA (10^7 and $10^{5.7}$ $TCID_{50}$, respectively). Virus rescue in the presence and absence of the pCITE vector expressing M2.1 was equally efficient (data not shown).

The AflII restriction site in the NL/1/99 genome did not affect virus replication in Vero cells (Fig. 5A) or the efficiency of virus rescue (cDNA constructs with and without AflII sites gave rise to virus titers of 10^7 and $10^{7.2}$, respectively). From these virus stocks, RNA was isolated and subjected to RT-PCR analysis and AflII restriction digestion, confirming that both NL/1/00 and NL/1/99 were recovered from plasmid DNA (Fig. 5B).

DISCUSSION

Here we describe the recovery of recombinant HMPV solely from cloned cDNAs. We selected two virus isolates, NL/1/00 and NL/1/99 representing each of the two main genetic lineages or serotypes of HMPV (238). These isolates were selected because they were found to replicate efficiently in tertiary monkey kidney cells and Vero cells.

The extreme termini of the viral genomes of NL/1/00 and NL/1/99 were determined by circularization of viral RNA with T4 RNA ligase, RT-PCR amplification and sequencing. Whereas the trailer sequences were of the expected length, the leader sequences were two or more nucleotides shorter than expected from the leader sequence of RSV (163) according to this approach. A 3'RACE method was used as an alternative strategy to determine the cDNA leader sequence of HMPV, revealing the presence of an additional AC dinucleotide in a proportion of the sequenced clones. In analogy with other paramyxoviruses, we assumed that the leader sequence of HMPV is 3'-UGCGCUUUUUUUGCG-5', and that the truncated versions of this leader sequence that were detected were due to the presence of nuclease activity in the virus preparations. It is interesting to note that Randhawa et al. observed truncation of the leader sequence of AMPV with a single nucleotide in their experiments, but when a hypothetical U residue was added to their minigenome constructs in analogy with other paramyxoviruses, this minigenome was replicated efficiently (194). The leader and trailer of HMPV appear to be less complementary as compared to those of RSV and AMPV (Fig. 3); only three of the five terminal residues of HMPV are complementary, compared to five of five and four of five for AMPV and RSV, respectively. With the completion of the full-

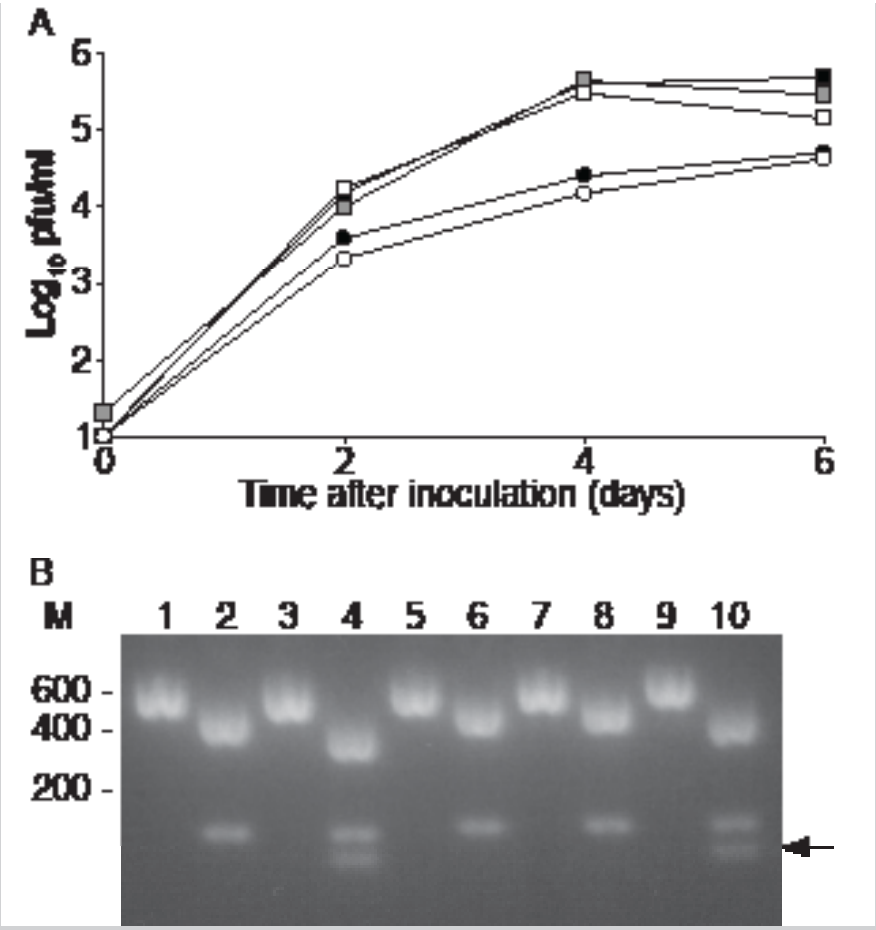

Figure 5

Replication kinetics of wild-type and recombinant HMPV in Vero cells and verification of the presence of recombinant genomes. Vero cells, infected at a multiplicity of infection of 0.1 with wild-type and recombinant HMPV NL/1/00 (circles) and NL/1/99 (squares), were washed and incubated for 6 days. Samples were collected every two days, and virus titers were determined by plaque assay (A). Closed symbols represent wild-type virus, open symbols recombinant virus with an additional AflII site and the gray symbol a NL/1/99 recombinant without the additional AflII site. The presence of the AflII site was confirmed by RT-PCR of viral RNA and AflII digestion (B). Lanes 1, 3, 5, 7, and 9, undigested DNA; lanes 2, 4, 6, 8, and 10, AflII-digested DNA. Lanes 1 and 2; wild-type NL/1/00, lane 3 and 4; recombinant NL/1/00, lane 5 and 6; wild-type NL/1/99, lane 7 and 8; recombinant NL/1/99 without AflII site, lane 9 and 10; recombinant NL/1/99 with the AflII site. When the wild-type 603 bp N fragment is digested with AflII, fragments of 469 and 134 are generated. Due to the introduction of the additional AflII site, the recombinant 469 bp fragment is further digested to yield 402 and 67 bp fragments (the latter fragment is indicated with an arrowhead).

length genomic sequences described here and those described recently by Biacchesi et al., full-length genomic sequences for each of the two main genetic lineages that represent HMPV serotypes A and B and each of the sublineages (A1, A2, B1, and B2) are now available from public databases (17, 241).

The sequences of the genomic termini were used to generate minigenome constructs, containing CAT or GFP as reporter genes. To increase transcription from the T7 promoter, either two or three G residues were placed between the T7 promoter and the end of the trailer sequence (179). Neither the addition of these two or three G residues at the trailer end nor the absence or presence of the terminal UG residues in the leader significantly affected CAT expression from minigenome vectors, suggesting that the authenticity of the sequence was not critical in minigenome assays. In minigenome assays, the N, P and L proteins were found to be indispensable, whereas the M2.1 protein was not. For RSV, the M2.1 protein was shown to enhance the processivity of the viral polymerase, which is important for the efficient synthesis of full-length mRNA (44) and the read-through of intergenic regions (79, 106). The HMPV M2.1 protein may have the same function, but this cannot be concluded yet from our experiments. Although we were able to rescue virus in the absence of the M2.1 expression vector, it was shown for RSV that the simple expedient of omitting an expression plasmid is invalid for evaluating recovery requirements (42). Thus, the functional analysis of M2.1 requires further study.

When the CAT ORF of the minigenome construct was replaced by a full-length cDNA copy of the NL/1/00 or NL/1/99 genome, we could recover infectious virus from transfected cells. An additional AflII restriction site introduced in the N gene was used to confirm that the rescued viruses were recombinants. The AflII restriction site did not affect the efficiency of virus replication in vitro, since all recombinant viruses produced replicated in Vero cells with similar kinetics as their wild-type counterparts. Although transfections in both 293T cells and BSR-T7 cells resulted in the rescue of recombinant virus, the BSR-T7 cells yielded higher virus titers shortly after transfection, perhaps due to higher expression of T7 RNA polymerase in all cells. When we used a fowlpox virus expressing the T7 RNA polymerase gene as an alternative way for T7 delivery in 293T cells, we also successfully recovered recombinant HMPV NL/1/00. Since the efficiency of virus rescue using fowlpox-T7 was not significantly higher than rescue using plasmid-derived T7 RNA polymerase, the latter system was preferred since only plasmid DNAs are used without the need for infectious viruses. We were able to rescue recombinant NL/1/99 by using both positive- and negative-sense full-length cDNA plasmids, revealing that the efficiency with negative-sense cDNA resulted in lower titers than for positive-sense cDNA ($10^{5.7}$ and $10^{7.2}$ $TCID_{50}$ respectively). This is in agreement with the general belief that for rescue of negative-sense nonsegmented viruses, the simultaneous presence of viral mRNAs and naked negative-sense genomic RNA will result in hybridization, preventing the assembly of the genome into the RNP.

A plethora of live-attenuated viruses and chimeric viruses have been generated using reverse genetics (48, 69, 167, 205, 213, 227), and the same can now be envisaged for HMPV. For instance, recombinant HMPV strains harboring the surface glycoproteins of both serotype A and B isolates of HMPV can be generated that may induce a broad antibody response

in infected hosts. Chimeric live-attenuated vaccines based on the HMPV genome, in which genes of RSV and/or parainfluenza viruses are inserted, may be useful as multivalent vaccine candidates. Recently, a chimeric bovine/human parainfluenza virus type 3 vector expressing the HMPV F protein was described and was found to induce protective HMPV antibody titers in a hamster model (227). In addition to its use for the generation of vaccine candidates, the reverse genetics system described here will be useful for fundamental and applied metapneumovirus research.

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CHAPTER 3
AN IMPROVED PLAQUE REDUCTION VIRUS NEUTRALIZATION ASSAY FOR HUMAN METAPNEUMOVIRUS

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ABSTRACT

Virus-neutralizing antibodies against human metapneumovirus (HMPV) have been shown to be important indicators for protection in experimental animal models. An improved plaque reduction virus neutralization assay to detect HMPV-specific neutralizing antibodies was designed using two prototype recombinant HMPV strains expressing green fluorescent protein (GFP). These prototypes represented each of the main antigenic variants of HMPV, because antigenic variability could have implications for vaccine development. The utility of mutations in the F gene resulting in trypsin-independent replication was also tested. Although these mutant HMPV strains could replicate in the absence of trypsin, bigger plaque size was achieved with the addition of trypsin. Insertion of the GFP gene in the genome of HMPV did not affect replication of the virus in vitro. Plaques could be detected by measuring expression of GFP after 5 days by automated scanning. Ferret, hamster, and macaque sera positive for HMPV were compared in a conventional virus neutralization assay and the plaque reduction virus neutralization assay. The results obtained with the two assays were in agreement but the improved plaque reduction virus neutralization assay was faster, more suitable for high throughput testing, and 10-fold more sensitive than the conventional virus neutralisation assay.

INTRODUCTION

Human Metapneumovirus (HMPV) is an enveloped non-segmented negative sense RNA virus and a member of the genus Metapneumovirus in the Pneumovirinae subfamily of Paramyxoviridae (238). Prospective and retrospective studies have shown that HMPV is an important cause of respiratory tract infections in infants, young children, frail elderly, and immunocompromised individuals (54, 78, 133, 183, 239). Currently no vaccine against HMPV is available. However, reverse genetics systems have been developed for HMPV (18, 110), providing an important new tool for the generation of vaccine candidates.

It has been shown that a neutralizing antibody response could be evoked in animals using viral vectors expressing HMPV fusion glycoprotein (F) and that these animals were subsequently protected against challenge infection with homologous or heterologous HMPV strains (211, 226). Animals could also be protected against infection by passive transfer of neutralizing monoclonal antibodies specific for the F protein (236). Fast and reliable virus neutralization assays are indispensable for vaccine development, and applied and fundamental research to monitor the humoral immune response to virus infections. In the past, virus-neutralizing titers have been determined by conventional virus neutralization assays (238) or plaque reduction virus neutralization assays (17, 18, 155). In these assays, virus infected cells were visualized by immunostaining. It has recently been shown that immunostaining was not necessary when an HMPV strain expressing the GFP ORF was used (20).

The HMPV strains that circulate around the globe are genetically and antigenically variable. Two main genetic lineages, A and B have been described, each of which can be further divided in at least two genetic sublineages, A1, A2, B1, and B2 (241). The genetic variability is most pronounced for two viral surface glycoproteins, the attachment protein (G) and the small hydrophobic (SH) protein, while the F gene is relatively conserved. Using post-infection ferret antisera, the two main lineages of HMPV were found to fulfil the classical criteria for two distinct serotypes (241). However, the relevance of these findings for virus epidemiology in humans remains unknown. It has become clear that in animal model systems the conserved F protein can induce antibody responses that are sufficiently broad to protect against both homologous and heterologous virus infections (211, 226). Nevertheless, it will be important to take the antigenic variability of HMPV into account when evaluating promising vaccine candidates.

The HMPV F protein is synthesized as an inactive precursor (F0) that is cleaved by host proteases into the functional subunits F1 and F2. It has been shown that when serine at position 101 in the cleavage sequence of the F protein is changed to proline it is no longer dependent on trypsin for its cleavage in vitro (202). When the full genome sequences of HMPV strains NL/1/00 (a prototype for the A1 lineage) and NL/1/99 (a prototype for the B1 lineage) were determined, and the full- length cDNA constructs were generated for recombinant virus rescue (110), differences were observed in the F cleavage sequence. Whereas NL/1/00 had proline at position 101, the NL/1/99 virus had serine, indicating that the first was not dependent on trypsin in vitro whereas Nl/1/99 was trypsin-dependent.

In this report, an efficient assay to detect virus-neutralizing antibodies is described for HMPV. The assay was designed to make use of two recombinant viruses representing the main HMPV lineages, each expressing a GFP gene from the third position in the virus genome. Using site-directed mutagenesis the NL/1/99 virus was modified to become relatively independent of trypsin, like the NL/1/00 virus. The plaque reduction virus neutralization assay described here is faster and more sensitive than classical virus neutralization assays, and will be a valuable tool for analysis of humoral immune responses in vaccine studies and for serological studies in naturally infected hosts.

MATERIALS AND METHODS

Cells, media and viruses

All assays were performed in Vero-118 cells; this cell-line was shown to be equally susceptible and sensitive to all known genetic lineages of HMPV (143). Vero-118 cells were cultured in Iscove's modified Eagle medium (BioWhittaker) supplemented with 10% fetal calf serum (FCS, Greiner Bio-One, Alphen aan den Rijn, The Netherlands), 100 IU of penicillin/ml, 100 µg of streptomycin/ml, and 2 mM glutamine as described previously. For HMPV rescue, Vero-118 cells and BSR-T7 cells were co-cultured in Dulbecco's medium supplemented with 3% FCS, 100 IU of penicillin/ml, 100 µg of streptomycin/ml, 2 mM glutamine, and 0,25 mg of trypsin/ml. For virus titration or plaque reduction assays, Vero-118 cells were grown in Iscove's modified Dulbecco's medium supplemented with 4% bovine serum albumin fraction V (Invitrogen, Breda, the Netherlands), 100 IU of penicillin, 2 mM glutamine, and 3.75 µg of trypsin. BSR-T7 (a kind gift of K. K. Conzelmann) cells were grown in Dulbecco's modified eagle medium (BioWhittaker, Verviers, Belgium) supplemented with 10% FCS, nonessential amino acids, 100 IU of Penicillin/ml, 100 µg of streptomycin/ml, 2 mM glutamine and supplemented with 0.5 mg of G418 (Life Technologies, Breda, The Netherlands).

Serum samples

Six ferret, six Syrian golden hamster and six macaque sera from previous HMPV vaccination and infection experiments were used. Polyclonal antisera specific for each of the genetic lineages of HMPV were raised in ferrets as described before (241). Syrian golden hamsters were immunized twice, with a 3-week interval, with soluble F proteins of NL/1/00 or NL/1/99 adjuvanted with Specol or iscom matrix. Sera were collected 3 weeks after the second immunization by orbital puncture under anesthesia with inhaled isoflurane. Macaques were infected 3 times with $1x10^6$ to $1x10^7$ $TCID_{50}$ NL/1/99 or NL/1/00 with 4-6-week intervals. Samples collected at different time points, representing low and high HMPV-specific antibody titers from this data set were used. All serum samples were stored at -20°C and were incubated at 56°C for 30 min prior to testing.

Construction of full-length HMPV cDNA plasmids, expressing the GFP gene

Construction of the full-length HMPV cDNA plasmids for both NL/1/99 and NL/1/00 were described previously (110). A fragment of the phosphoprotein (P) ORF including its gene end signal and part of the matrix protein (M) ORF including its gene start signal of HMPV NL/1/00were

cloned in pCR4TOPO (invitrogen). Two BbsI sites were introduced to allow the directional cloning of the GFP ORF flanked by the gene start and the gene end signals of the P gene. The entire P-GFP-M fragment was swapped into the full-length NL/1/00 cDNA plasmid using restriction enzymes ApaI and PmiI. The resulting full-length HMPV cDNA plasmid with GFP flanked by the gene start and gene end signals of P on the third position of the viral genome was designated NL/1/00-gfp.

To construct a full-length cDNA vector of NL/1/99 with GFP at position 3 of the viral genome, both a fragment of the P ORF including its gene end signal and a fragment of the M ORF including its gene start signal were cloned in pSP72 (Promega, Madison, USA) using XhoI and BglII. Two BfuAI sites were introduced to allow the directional cloning of the GFP ORF flanked by the gene start and gene end signals of the P gene in between the P and M ORFs. This insert was then swapped into the full-length NL/1/99 cDNA plasmid using SacI and SnaBI, resulting in a full-length NL/1/99 cDNA vector with GFP flanked by the gene start and gene end signals of the P gene on the third position. This plasmid was designated NL/1/99-gfp.

HMPV NL/1/00 has proline on position 101 in the cleavage site of the F gene and is trypsin-independent. NL/1/99 has serine on position 101 and is trypsin-dependent, but it could be easier to handle if it was trypsin-independent. To construct a trypsin-independent version of NL/1/99-gfp, T3365C that encodes S101P was introduced into NL/1/99-gfp using a QuikChange,Multi Site-Directed Mutagenesis Kit (Stratagene). The integrity of all constructs was confirmed by nucleotide sequencing.

Recovery of recombinant HMPV

Recovery of recombinant HMPV containing the GFP gene was performed as described previously (Herfst et al., 2004). Briefly BSR-T7 cells were transfected for 5 h with 5 µg full-length HMPV cDNA plasmid, 2 µg pCITE-N, 2 µg pCITE-P, 1 µg pCITE-L and 1µg pCITE-M2.1 using Lipofectamine 2000 (Invitrogen). After transfection the medium was replaced with fresh medium supplemented with trypsin. Three days after transfection, the BSR-T7 cells were scraped and co-cultured with Vero-118 cells for 8 days. Recovered viruses were propagated on Vero-118 cells and virus titers were determined as described previously (110).

Plaque reduction virus neutralization assay

Four-fold serial dilutions starting at 1:4 of ferret, hamster or macaque sera were prepared in 96-well plates (Greiner Bio-One). Each serum dilution was mixed with an equal volume of virus (50-60 pfu per well) and incubated for 1 hr at 37°C. Following this incubation the virus serum mixture was transferred to Vero-118 cell monolayers in 24-well plates. After 2 h of incubation at 37°C the virus serum mixture was removed and the cell monolayers were overlaid with one ml of a 2% methyl cellulose – infection medium (1:1) + trypsin (3,75 µg/ml). After 5 days, plaques were visible and were scanned on a Typhoon 9410 variable mode imager (GE Healthcare, Diegem, Belgium). This data was analysed with ImageQuant TL Colony counter & Image feature measurement (Amersham Biosciences, Freiburg, Germany). Neutralizing antibody titers were expressed as the reciprocal of the highest serum dilution that resulted in 50% reduction in plaque numbers as compared to virus only control (depicted as means of duplicate measurements).

Conventional virus neutralization assay

The conventional virus neutralization assay was performed as previously described (241) with minor adjustments. Two-fold serial serum dilutions starting at 1:8 were incubated with 30 $TCID_{50}$ of virus for 1 hr at 37°C. Virus serum mixtures were transferred to Vero-118 monolayers in 96-well plates and centrifuged at 2000 x g for 10 min. After 2 hr of incubation at 37°, the plates were washed and 100 µl of infection medium plus trypsin (3,75 µg/ml) was added to each well. After 7 days the plates were analyzed on a Typhoon 9410 variable mode imager. The virus neutralization titer was defined as the reciprocal of the highest serum dilution at which no positive GFP signal was obtained (depicted as means of triplicate measurements). Each experiment included virus titrations of the working solution of the virus, using two fold dilutions.

RESULTS

Replication characteristics of the recombinant HMPV viruses carrying a GFP gene

The GFP gene was inserted between the P and M genes of the NL/1/00 and NL/1/99 viruses using reverse genetics. GFP was expressed in the virus-infected cells and could be easily detected by the use of a blue-excitation filter under a fluorescent microscope (data not shown). The replication kinetics of the GFP expressing viruses were compared with their recombinant wild-type counterparts. Vero-118 cells were infected at a multiplicity of infection (MOI) of 0.1 with the recombinant viruses after which supernatant samples were collected daily and virus titers were determined by plaque assay (Fig. 1). Plaque numbers were determined by immunostaining with a guinea-pig antiserum and manual counting, since the wild-type viruses did not express GFP. Plaque numbers of HMPV-gfp viruses were also analyzed on a Typhoon 9410 variable mode imager, which resulted in similar numbers of plaques as obtained with immunostaining and manual counting (data not shown). The insertion of a GFP gene did not impair virus growth in Vero-118 cells.

Plaque size of recombinant viruses in the presence or absence of trypsin

It was shown that a serine to proline change at position 101 in F is responsible for the trypsin-independent cleavage of the NL/1/00 F protein (202). A NL/1/99-gfp virus with the same S101P mutation was generated via reverse genetics in order to determine the influence of the S101P amino acid substitution on trypsin requirement of NL/1/99. First, Vero-118 cells were inoculated with NL/1/99-gfp and NL/1/99-S101P-gfp at a MOI of 0.1 and cultured in the presence and absence of trypsin. Inoculation with NL/1/99-gfp in the absence of trypsin resulted in single infected cells as determined by immunofluorescence but no plaques were produced (Fig. 2A). In contrast, inoculation with NL/1/99-S101P-gfp did result in plaque production. In the presence of trypsin both viruses formed plaques, which were comparable in size. When NL/1/00-gfp or NL/1/99-S101P-gfp were grown in the presence and absence of trypsin, the addition of trypsin still resulted in a six-fold increase of plaque size (Fig.2B and C, right panels). Plaque reduction virus neutralization assays were subsequently performed in the presence of trypsin, because this resulted in larger plaques despite less dependence on trypsin.

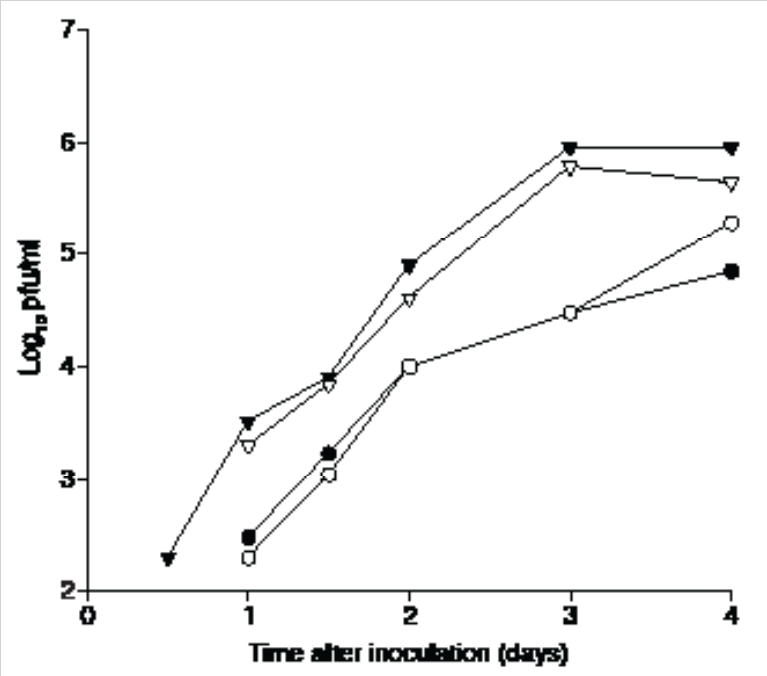

Figure 1
Replication kinetics of recombinant HMPV NL/1/00 and NL/1/99 with and without a GFP gene in Vero-118 cells. Vero-118 cells, infected at a MOI of 0.1 with HMPV NL/1/00 (circles) and NL/1/99 (triangles) were washed and incubated. Supernatants were collected daily and virus titers were determined by plaque assay. Closed symbols represent wild-type recombinant virus and open symbols represent GFP-expressing viruses.

Comparative analysis of virus neutralizing antibody titers determined by conventional virus neutralization assay and plaque reduction virus neutralization assay

Virus neutralizing antibody titers of ferret, hamster, and macaque sera were determined using two different methods: conventional virus neutralization assay using NL/1/00-gfp and plaque reduction virus neutralization assay (Fig. 3.). For the plaque reduction virus neutralization assay, sera were diluted and incubated with a standard virus inoculum, followed by infection of Vero-118 cell monolayers. After 5 days plaques were clearly visible by fluorescence microscopy and plates were scanned using the Typhoon 9410 variable mode imager. Neutralizing antibody titers were expressed as the reciprocal of the highest serum dilution that reduced the number of plaques by 50%. For the conventional virus neutralization assay, the same sera and viruses were used.

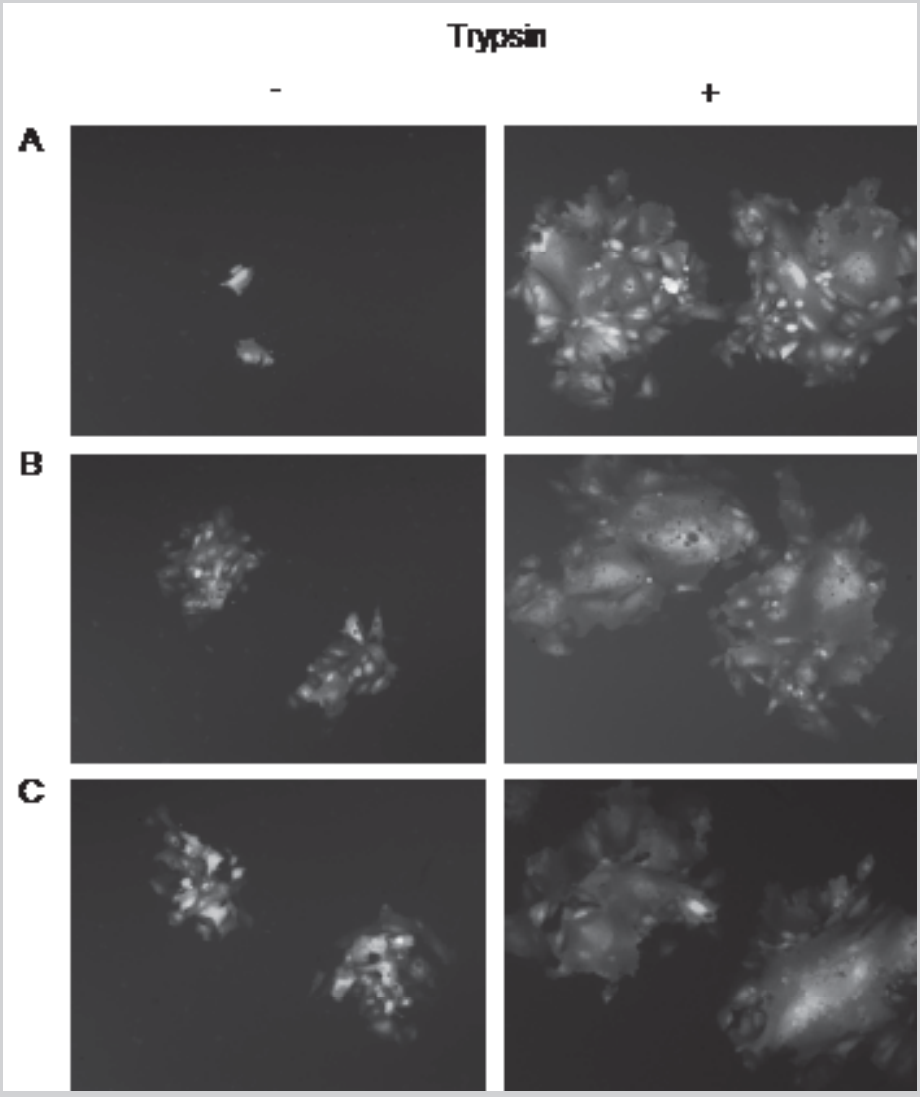

Figure 2
Fluorescence images of plaques produced by GFP-expressing HMPV. Plaques produced with NL/1/99-gfp (A), NL/1/99S101P-gfp (B), and NL/1/00-gfp (C) viruses in the presence (right) or absence (left) of trypsin are shown.

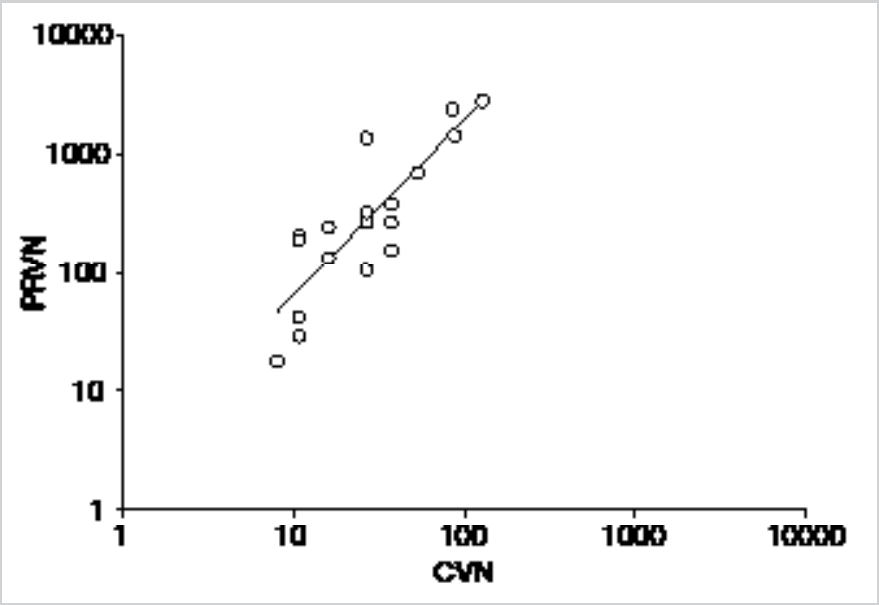

Figure 3
Correlation of the virus-neutralizing antibody titers in ferret, hamster and macaque serum samples detected by conventional virus neutralization assay (CVN; x-axis) or by plaque reduction virus neutralization assay (PRVN; y-axis).

The results obtained on the same sera with two assays showed good correlation (R^2=0.72). However, the plaque reduction virus neutralization assay was more sensitive than the conventional virus neutralization; neutralizing antibody titers were 10-fold higher and titers of sera with neutralizing activity below the detection limit in the conventional virus neutralization assay could be assessed in the plaque reduction assay.

DISCUSSION

Since its initial discovery in 2001 (238) several studies have described the development of vaccines against HMPV (56, 210, 226). Now that there are several potential vaccine candidates, reliable and rapid methods to detect virus neutralizing antibodies are required. Such tests would also be useful for serological investigations on the virus-induced immune response in naturally infected hosts. The aim of this study was to set up a faster and less laborious method to measure HMPV neutralizing antibodies in sera than the conventional virus neutralization assays. The method described here was able to detect both low and high neutralizing antibodies in sera of three different hosts. The results obtained with the same

sera with two assays showed good agreement (R^2= 0.72) although the plaque reduction virus neutralization assay yielded higher titers. Since the plaque reduction virus neutralization assay yielded higher titers, neutralizing antibodies could be detected that were below detection limit in the conventional virus neutralization assay. Similar results were found when virus neutralization assay and plaque reduction virus neutralization assays were performed with NL/1/99-S101P-gfp (data not shown).

For viruses that do not cause extensive cytopathic effects, the plaques routinely need to be visualized using immunostaining. Using GFP-expressing viruses time-consuming staining protocols with costly antibodies could be prevented. Plaque counting with the Typhoon 9410 variable mode imager saved time and removed subjectivity associated with manual counting, making it more applicable for large screens. An additional advantage was that the plaque reduction virus neutralization assay could be read out after 5 days instead of the 7 days used for the conventional virus neutralization assay. The influence of a S101P mutation on the trypsin dependence of NL/1/99 was tested using a NL/1/99-S101P recombinant. Plaque assays showed that the S101P substitution does make NL/1/99 independent of trypsin, but that the addition of trypsin still resulted in larger plaques. It has recently been shown that when the cleavage site of HMPV F was replaced with one derived from avian metapneumovirus subgroup A it was completely independent of trypsin (16). However, the addition of trypsin is a small effort as it increases the plaque size considerably, making the assay easier to read out and thus more reliable.

The antigenic variability of HMPV could prove to have implications for future vaccine strategies, and vaccine candidates thus need to be evaluated taking this antigenic variability into account. The two GFP-expressing viruses described here represent the two main lineages of HMPV. This provides the opportunity to not only quantitatively detect virus-neutralizing antibodies, but also to test to which extend they are cross-neutralizing, giving an indication about possible cross-protection. This is an advantage to a previous described microneutralization assay using only one lineage of HMPV expressing GFP (20).

Here a plaque reduction virus neutralization assay using GFP-expressing viruses is described that is more sensitive and faster than the conventional virus neutralization assay. Furthermore it is more suitable for large-scale screening and takes the antigenic variability of HMPV into account. This improved method facilitates the quantification of neutralizing antibodies and will be useful as a tool in vaccine research and other applications.

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GENERATION OF TEMPERATURE-SENSITIVE HUMAN METAPNEUMOVIRUS STRAINS THAT PROVIDE PROTECTIVE IMMUNITY IN HAMSTERS

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ABSTRACT

Human metapneumovirus (HMPV) causes acute respiratory tract illness primarily in young children, immunocompromised individuals and the elderly. Vaccines would be desirable to prevent severe illnesses in these risk groups. Here, we describe the generation and evaluation of cold-passage (cp) temperature-sensitive (ts) HMPV strains as vaccine candidates. Repeated passage of HMPV at low temperatures in Vero cells resulted in the accumulation of mutations in the viral genome. Introduction of these mutations in a recombinant HMPV by reverse genetics resulted in a ts-phenotype, judged on the decreased shut-off temperature for virus replication in vitro. As an alternative approach, three previously described cp-respiratory syncytial virus (cp-HRSV) mutations were introduced in a recombinant HMPV, which also resulted in a low shut-off temperature in vitro. Replication of these ts-viruses containing either the cp-HMPV or cp-HRSV mutations was reduced in the upper respiratory tract (URT) and undetectable in the lower respiratory tract (LRT) of hamsters. Nevertheless, high titers of HMPV-specific antibodies were induced by both ts-viruses. Upon immunization with the ts-viruses, the LRT of hamsters were completely protected against challenge infection with a heterologous HMPV strain, and URT viral titers were reduced by 10 000-fold. In conclusion, we provide proof-of-principle for two candidate live-attenuated HMPV vaccines that induce cross-protective immunity to prevent infection of the LRT in Syrian golden hamsters. Further mapping of the molecular determinants of attenuation of HMPV should be the subject of future studies.

INTRODUCTION

The human metapneumovirus (HMPV) was first isolated from respiratory specimens obtained from children hospitalized for acute respiratory tract illness (RTI) in The Netherlands (238). Based on sequence information and genome organization, HMPV was classified as the first mammalian member of the Paramyxoviridae family, subfamily Pneumovirinae, genus Metapneumovirus. Clinical manifestations of HMPV infections are similar to those caused by the closely related respiratory syncytial virus (HRSV), ranging from mild respiratory illness to bronchiolitis and pneumonia (239, 254). Phylogenetic analysis of a large number of HMPV isolates revealed the existence of two main genetic virus lineages, which were found to be antigenically distinct in virus-neutralization assays with ferret sera (241). Whereas two surface glycoproteins, the attachment protein (G) and the small hydrophobic protein (SH) are highly variable among virus isolates, the fusion protein (F) is highly conserved, and antibodies induced against F correlate with protection in animal models (210, 226).

A variety of vaccination strategies may be required to prevent HMPV respiratory tract infections in different risk groups, such as young children, individuals with underlying disease, and the elderly. Several vaccination strategies have been explored since the discovery of HMPV (113), including subunit vaccines (56, 112), a T-cell epitope vaccine (109), live attenuated vaccines (LAVs) (15, 186, 226) and formalin-inactivated (FI-) HMPV (62, 102, 262). Immunization with FI-HMPV primed for hypersensitivity responses upon challenge infection (62, 102, 262), suggesting that classical inactivated vaccines for HMPV may predispose for enhanced disease when used in immunologically naïve recipients, similar to what was previously described for HRSV and measles (84, 140, 187). For LAVs, no enhanced disease has been observed in studies performed in naïve animals with HRSV and HMPV. In addition, live attenuated measles virus vaccines have also not been associated with vaccine-mediated enhanced disease, either in humans or in animal models. LAVs may be useful to prime or boost HMPV-specific immune responses since such viruses have the advantage of mimicking a natural infection, and thus could provide protection against subsequent infections without inducing enhanced disease. Recently developed reverse genetics systems for HMPV (18, 110) facilitate the modification of viral genomes and thus provide a powerful tool to design LAVs. Several LAVs for HMPV have recently been described, including HMPV deletion mutants, chimeric viruses based on HMPV and avian metapneumovirus (AMPV), and a human/bovine parainfluenza virus type 3 (B/HPIV3) expressing the F protein of HMPV (15, 186, 226). Here, classical methods of virus adaptation to replication at low temperatures (cold-passage, cp) were used to attenuate HMPV, and the associated sequence changes in the viral genome were identified. Recombinant viruses containing HMPV or HRSV cp-mutations were generated by reverse genetics. These recombinant viruses were found to be temperature-sensitive (ts) in vitro, attenuated for replication in hamsters, yet highly immunogenic in this animal model. Hamsters immunized with ts-HMPV strains were protected against heterologous virus infection in the lower respiratory tract (LRT), and had significantly reduced virus titers in the URT. Thus, cp/ts-HMPV strains represent promising LAV candidates to protect against HMPV infections.

METHODS

Cells

Vero cells were grown in Iscove's Modified Dulbecco's Medium (IMDM, BioWhittaker, Verviers, Belgium) supplemented with 10% fetal calf serum (FCS, Greiner Bio-One, Alphen aan den Rijn, The Netherlands), 100 IU ml^{-1} penicillin, 100 µg ml^{-1} streptomycin and 2 mM glutamine. Subclone 83 of WHO Vero cells was selected for virus passaging at low temperatures, and subclone 118 (143), which was selected for being permissive for the four genetic HMPV lineages, was used for all other experiments. To produce purified and concentrated virus stocks, virus strains were grown in infection medium consisting of IMDM supplemented with 4% bovine serum albumin fraction V (Invitrogen, Breda, The Netherlands), 100 IU ml^{-1} penicillin, 100 µg ml^{-1} streptomycin, 2 mM glutamine and 3.75 µg ml^{-1} trypsin until 70–90% of the cells displayed cytopathic effects. After one freeze-thaw cycle, cell-free supernatants were purified and concentrated using a 30–60% (w/w) sucrose gradient.

Cold-passaging of virus

HMPV isolate NL/1/99 (241) (lineage B, passage 3 at 37°C) was serially passaged in Vero-83 cells at decreasing temperatures. Virus was cultured at 34°C, 31°C, 28°C and 25°C for 3, 3, 2 and 2 passages respectively. When the temperature was decreased further to 22°C or 20°C, virus replication was impaired too much, and passaging was thus continued at 25°C until passage 35 was reached. Cultures were harvested from every passage approximately 7 days after inoculation and stored in 25% sucrose at -80°C.

Sequence analysis

Viral RNA was isolated from virus stocks of cp-NL/1/99 passage 35, and intermediate passages 14, 23 and 29, using the High Pure RNA Isolation Kit (Roche Diagnostics, Almere, The Netherlands) according to instructions from the manufacturer. RNA was subsequently used in reverse transcriptase polymerase chain reaction (RT-PCR) assays using primer sets designed on the basis of the full-length genome sequence of NL/1/99 (GenBank accession no. AY525843). Both strands of the overlapping PCR-fragments were sequenced without prior cloning, to minimize amplification and sequencing errors. The nucleotide sequence of the cp-NL/1/99 genome was compared with the genome of the wild-type virus to identify nucleotide substitutions. All primer sequences are available upon request.

Sequence comparison of cold-passaged HRSV and HMPV

Genome sequences of HRSV strains containing mutations responsible for temperature sensitivity in vitro and attenuation in vivo (81, 131, 132, 249) were aligned with the full-length sequence of HMPV NL/1/99 using BioEdit software (98). Regions containing known ts-mutations in the HRSV genome were compared with their counterparts of HMPV, to determine whether HRSV ts-mutations could be introduced in homologous sites, conserved in the HMPV genome.

Recombinant viruses

The construction of wild-type recombinant HMPV NL/1/00 and NL/1/99 (genetic lineage A and B respectively) has been described previously (110). Mutations that were found in cp-NL/1/99, or identified upon sequence comparison of ts-HRSV and HMPV, were generated in the context

of NL/1/99 using the QuickChange multi site-directed mutagenesis kit (Stratagene, Leusden, The Netherlands) according to instructions of the manufacturer. All primer sequences used for mutagenesis are available upon request.

Virus growth at different temperatures

To generate virus growth curves, 25 cm^2 flasks containing confluent Vero-118 cells were inoculated at 37ºC for 2 hours with wild-type or mutant HMPV at a multiplicity of infection (MOI) of 0.1. After adsorption of the virus to the cells, the inoculum was removed and cells were washed 2 times with media before addition of 7 ml of fresh media, and incubation at 32ºC, 37ºC, 38ºC, 39ºC or 40ºC. Every day, 0.5 ml of the supernatant was collected and replaced by fresh media. To determine viral titers, supernatants were subjected to plaque assays as described previously, with the exception that cells were incubated at 32ºC. Wild-type NL/1/99 virus and the viruses containing cp-HMPV mutations were incubated for 6 days, whereas the virus harboring the cp-HRSV mutations was incubated for 8 days, since only very small plaques were observed after 6 days.

Hamster experiments

The replication kinetics and immunogenicity of the recombinant candidate LAVs were studied in Syrian golden hamsters (Mesocricetus auratus; Charles River, Sulzfeld, Germany). Groups of 12 female hamsters, 5-7 week old, were inoculated intranasally with 10^6 50% tissue-culture infectious dosis ($TCID_{50}$) of NL/1/99 or LAV in a 100 µl volume. Four days post infection (dpi), lungs and nasal turbinates (NT) were collected from six animals per group, snap-frozen immediately and stored at –80ºC until further processing. From the other animals, blood samples were collected by orbital puncture at 21 dpi. Blood samples were stored overnight at room temperature and centrifuged 15 min at 1200 x g; serum was collected and stored at –20ºC.

For the immunization and challenge experiment, animals were immunized by virus inoculation as described above, with 10^6 $TCID_{50}$ of LAV or NL/1/99, or PBS as control. At 21 dpi, animals were challenged intranasally with 10^7 $TCID_{50}$ of NL/1/00 virus. Four days after heterologous challenge infection, lungs, NT and blood samples were collected for further processing.

All intranasal inoculations, orbital punctures and euthanasia were performed under anesthesia with inhaled isoflurane. All animal studies were approved by an independent Animal Ethics Committee and the Dutch authority for working with genetically modified organisms, and were carried out in accordance with animal experimentation guidelines.

Plaque reduction virus neutralization assay

Virus neutralizing (VN) antibody titers were determined in serum samples by a plaque reduction virus neutralization (PRVN) assay as described previously (59). In brief, serum samples were diluted and incubated for 60 minutes at 37°C with approximately 50 plaque forming units (pfu) of NL/1/00 or NL/1/99, expressing the enhanced green fluorescent protein (eGFP). Subsequently, the virus-serum mixtures were added to Vero-118 cells in 24 well plates, and incubated at 37°C. After two hours, the supernatants were replaced by a mixture of equal amounts of infection medium and 2% methyl cellulose. Six days later, fluorescent plaques were counted using a Typhoon 9410 Variable Mode Imager (GE Healthcare, Diegem,

Belgium). VN antibody titers were expressed as the dilution resulting in 50% reduction of the number of plaques, calculated according to the method of Reed and Muench (198). Per assay, each serum was tested in duplicate against HMPV NL/1/00 and NL/1/99.

Virus titrations

Tissues from the inoculated hamsters were homogenized using a polytron homogenizer (Kinematica AG, Littau-Lucerne, Switzerland) in infection media. After removal of tissue debris by centrifugation, supernatants were used for virus titration in Vero-118 cells. Titrations were performed with 10-fold serial dilutions in 96-well plates (Greiner Bio-One). Confluent monolayers of Vero-118 cells were spin-inoculated (15 min., 2.000 x g) with 100 µl of 10-fold serial dilutions of each sample and incubated at 37°C. Two hours after the spin-inoculation, the inoculum was replaced with fresh infection media. After 3 - 4 days, 100 µl of fresh infection media was added to each well. Seven days after inoculation, infected wells were identified by immunofluorescence assays with HMPV-specific polyclonal antiserum raised in guinea pigs, as described previously (238). Titers expressed as $TCID_{50}$ were calculated as described by Reed and Muench (198). Titers were calculated per gram tissue, with a detection limit of $10^{1.6}$ and $10^{1.2}$ $TCID_{50}$ per gram of tissue for the NT and lungs, respectively.

RESULTS

Sequence analysis of cp-NL/1/99

HMPV isolate NL/1/99 was serially passaged in Vero-83 cells at slowly decreasing temperatures until a temperature of 25°C was reached. When the temperature was further decreased to 22°C or 20°C, virus replication was severely impaired and virus yield was negligible. Therefore, passaging was continued at 25°C until passage 35 was reached, when no changes in replication kinetics had been observed for 5 passages. Viral RNA of cp-NL/1/99 obtained after 35 passages was subjected to RT-PCR, followed by direct sequencing. Analysis of the full viral genome sequence and comparison with the original NL/1/99 genome revealed the presence of 19 nucleotide changes, resulting in 17 amino acid substitutions (Table 1). Analysis of virus genome sequences after fewer passages (passage 14, 23, and 29) indicated the gradual accumulation of these mutations (data not shown). One mutation that was found in the L gene after 29 passages had disappeared in the passage 35 virus, but this mutation was also included in further studies. Mutations were found throughout the viral genome in all genes, except the genes encoding the nucleoprotein (N) and the SH protein (Table 1).

Sequence comparison of cp-HRSV and HMPV

For HRSV, numerous mutations that accumulated in the viral genome after cold-passaging have been identified. After extensive studies, the ts-phenotype of cp-HRSV could be assigned to single mutations, or combinations of mutations. To explore the possibility of introducing these known cp/ts-mutations of HRSV into the HMPV genome, sequences of HRSV genes containing known cp/ts-mutations were aligned with their counterparts of HMPV NL/1/99. Most mutations could not be introduced in HMPV, because of a lack of similarity between the genes of HRSV and HMPV. However, four mutations at position 521 (132), 1169 (131) and 1321 (249) of the L gene and in the gene start (GS) of M2 (81) were identified, for which the

Table 1
Nucleotide and amino acid substitutions found after 35 rounds of cold passaging of HMPV NL/1/99 in Vero cells.

Position*	Gene	nt (wt)	nt (cp)	aa‡	aa (wt)	aa (cp)	$HMPV_{M19}$§	$HMPV_{M8}$	$HMPV_{M11}$	$HMPV_{M2}$
1458	P	GAA	GTA	66	Glu	Val	X		X	
2203	M	TAT	CAT	9	Tyr	His	X			
2291	M	TTA	TCA	38	Leu	Ser	X			
2333	M	CTA	CCA	52	Leu	Pro	X			
2514	M	GTT	GTG	112	Val	Val	X			
2572	M	TCA	CCA	132	Ser	Pro	X	X	X	
2614	M	CTA	TTA	146	Leu	Leu	X	X	X	
3341	F	GAG	AAG	93	Glu	Lys	X			
3365	F	TCA	CCA	101	Ser	Pro	X	X	X	
3903	F	GAT	GGT	280	Asp	Gly	X	X	X	
4476	F	CAG	CGG	471	Gln	Arg	X	X	X	
4658	F	AAC	TAC	532	Asn	Tyr	X			
4676	F	CAT	TAT	538	His	Tyr	X			
5255	M2	AGC	ATC	187	Ser	Ile	X	X	X	
6609	G	ACA	CCA	139	Thr	Pro	X	X	X	
6685	G	CAA	CCA	164	Gln	Pro	X			
7826†	L	GGA	AGA	235	Gly	Arg	X		X	X
8090	L	AAC	GAC	323	Asn	Asp	X		X	X
11480	L	TTC	CTC	1453	Phe	Leu	X	X	X	

*Position is specified as the nucleotide position numbered from the 3'-end of negative sense RNA (GenBank accession no. AY525843). †Transient mutation in passage 29 at 25ºC. nt = nucleotide, wt = wild-type, cp = cold-passaged, aa = amino acid, P = phosphoprotein, M = matrix protein, F = fusion protein, M2 = putative 22K protein, G = attachment protein, L = large polymerase protein. ‡Position is specified as the amino acid number in the indicated HMPV ORF. §$HMPV_{M19}$ indicates that this virus contains 19 mutations found after cold passaging. X indicates the presence of this mutation in the recombinant virus. Nucleotide changes in each codon are underlined.

HMPV genome was identical to the wild-type HRSV sequence (Table 2). Thus, these cp/ts-mutations of HRSV could be introduced easily in the genome of HMPV NL/1/99.

Construction of recombinant HMPV cp-NL/1/99

Wild-type recombinant HMPV NL/1/99 was used as a backbone for the introduction of mutations as listed in tables 1 and 2. Three different viruses containing all mutations or subsets of cp-HMPV mutations were constructed. These viruses containing 19, 8 or 11 nucleotide substitutions were named $HMPV_{M19}$, $HMPV_{M8}$ and $HMPV_{M11}$ respectively, based on the number of mutations that were introduced (Table 1). Mutant virus $HMPV_{M19}$ could not be rescued by reverse genetics after three attempts. The parental virus obtained after 35 passages at 25ºC also replicated very poorly, to low virus titers. Therefore, we next attempted

Table 2
Nucleotide substitutions in cp-HRSV that were introduced in recombinant HMPV/NL/1/99.

Position*	Position†	Gene	Virus origin	nt (wt)	nt (cp)	aa (wt)	aa (cp)	Ref.
521	456	L	cpts530	TTC (HRSV) TTT (HMPV)	TT<u>A</u>	Phe	Leu	128
1169	1094	L	cpts530/1009	ATG	<u>G</u>TG	Met	Val	127
1321	1246	L	cpts530/1030	TAT	<u>A</u>AT	Tyr	Asn	240
--	--	GS-M2	cpts248/404	AATA	AA<u>C</u>A	--	--	74

*Position is specified as the amino acid number of the L gene of HRSV (GenBank accession no. U63644). †Position is specified as the amino acid number of the L gene of HMPV. nt = nucleotide, wt = wild-type, cp = cold-passaged, aa = amino acid, L = large polymerase protein, GS-M2 = gene-start sequence of the M2 gene. Nucleotides changes in each codon or nucleotide sequence are underlined.

to rescue recombinant viruses that contained only a selection of the cp-mutations, 8 and 11 respectively, which were generated as cloning intermediates during the cloning of $HMPV_{M19}$.

Upon introduction of the four cp-HRSV mutations in the NL/1/99 backbone, no virus could be recovered after three attempts. Therefore, four viruses containing each possible combination of three mutations were generated, thus omitting one of the mutations (Table 2). Only the virus in which the L1321 mutation was omitted (named $HMPV_{HRSV3}$ hereafter) could be rescued.

Temperature-sensitivity

To study the possible ts-phenotype of recombinant viruses, virus growth curves were generated in Vero cells at 32°C, 37°C, 38°C, 39°C or 40°C. Plaque assays were performed to determine the viral titers in the supernatants of samples that were collected daily. Wild-type HMPV was able to replicate at all temperatures, with the highest virus titer obtained at 37°C. At 40°C, the virus titer was more than 100-fold reduced compared to the optimal temperature of 37°C (Fig. 1a). $HMPV_{M8}$ which was an intermediate virus in the cloning procedure of $HMPV_{M19}$, also replicated at all temperatures, but with higher titers as compared to wild-type HMPV, and an optimal replication temperature of 32°C (Fig. 1b). Even at 40°C, $HMPV_{M8}$ displayed faster replication kinetics in Vero cells and at all temperatures higher peak virus titers were reached compared to wild-type HMPV. Mutant $HMPV_{M11}$ also displayed optimal virus growth at a temperature of 32°C. Peak titers were reached later for $HMPV_{M11}$, but virus titers at 6 dpi at 32°C were higher as compared to $HMPV_{M8}$ (Fig. 1c). This virus did not replicate at 39°C and 40°C, indicating that this virus was temperature-sensitive. The only differences between $HMPV_{M11}$ and $HMPV_{M8}$ were two mutations in the L gene and one mutation in the P gene (Table 1). $HMPV_{M9}$ that was also generated as a cloning intermediate, containing all mutations of $HMPV_{M8}$ and the mutation in the P gene turned out to be insensitive to higher temperatures (data not shown). Therefore, $HMPV_{M2}$ was constructed containing only two L mutations (nt 7826 and 8090, Table 1) as compared to wild-type NL/1/99. The replication kinetics of $HMPV_{M2}$

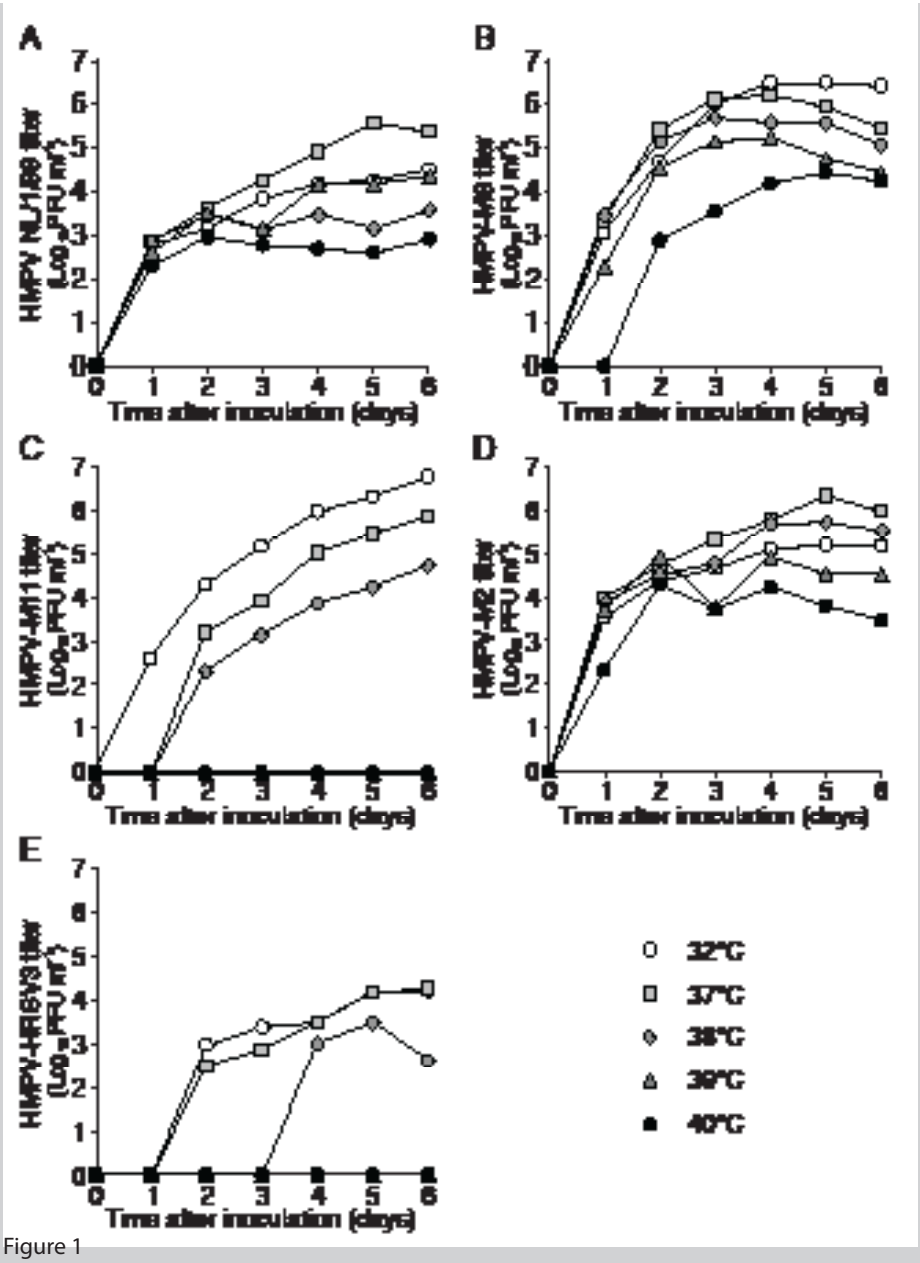

Figure 1

Replication kinetics in Vero cell cultures of wild-type HMPV and recombinant viruses in which cp-mutations were introduced. Vero cells, infected at a m.o.i. of 0.1 with HMPV NL/1/99 (panel A), $HMPV_{M8}$ (panel B), $HMPV_{M11}$ (panel C), $HMPV_{M2}$ (panel D) or $HMPV_{HRSV3}$ (panel E) were washed and incubated for 6 to 8 days at 32°C (open circle), 37°C (square), 38°C (diamant), 39°C (triangle) or 40°C (closed circle). Shading of symbols varies from white to black with increasing temperature. Samples were collected daily, and virus titers determined by plaque assay.

was most similar to that of the wild-type NL/1/99 virus (compare Fig. 1a and 1d), suggesting that these two L mutations alone are not ts- or Vero-cell adaptive mutations.

The only viable NL/1/99 with cp-HRSV mutations, $HMPV_{HRSV3}$, replicated slowly and to a 10-fold lower peak titer at 37°C, but at 32°C the peak titer was comparable to wild-type HMPV. At 38°C, no virus was detected until 4 dpi, and at 39°C and 40°C the virus did not replicate at all. Thus, $HMPV_{HRSV3}$ appeared to be temperature-sensitive in vitro (Fig. 1e).

Replication kinetics and immunogenicity in hamsters

For the two viruses with a ts-phenotype in vitro, $HMPV_{M11}$ and $HMPV_{HRSV3}$, we tested the level of attenuation in hamsters. Syrian golden hamsters were inoculated with 10^6 $TCID_{50}$ $HMPV_{M11}$, $HMPV_{HRSV3}$, or wild-type NL/1/99 (12 animals per group), after which virus titers in the lungs and NT were compared at four dpi (6 animals per group), and virus neutralizing antibody titers were determined at 21 dpi (6 animals per group). In the NT of animals inoculated with wild-type HMPV, virus titers up to 10^7 $TCID_{50}$ $gram^{-1}$ NT were detected (Fig. 2a). In the animals

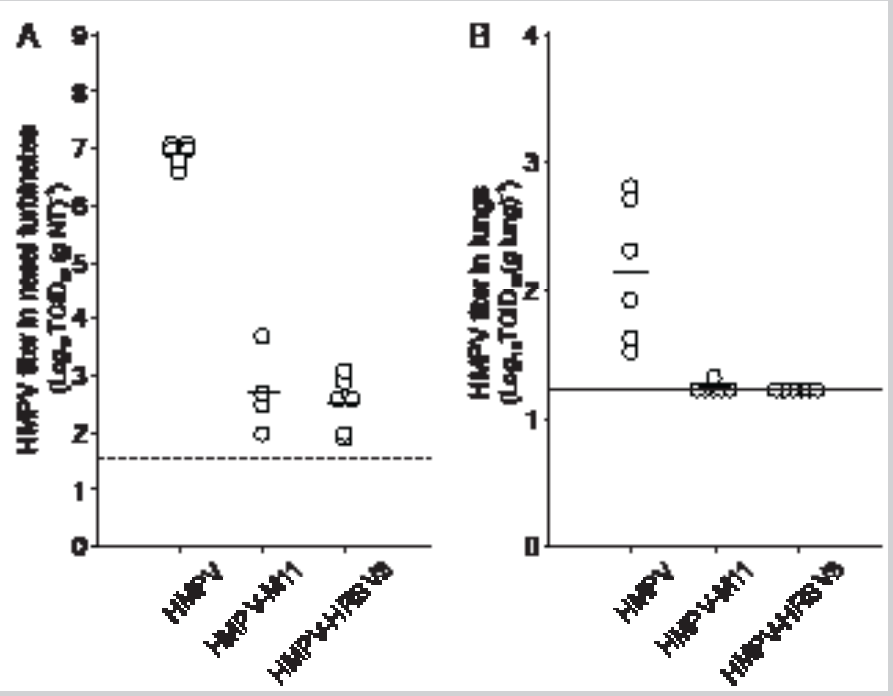

Figure 2

Infectious virus titers in (A) nasal turbinates and (B) lungs of Syrian golden hamsters inoculated with 10^6 $TCID_{50}$ of NL/1/99, $HMPV_{M11}$ or $HMPV_{HRSV3}$. Nasal turbinates and lungs were collected at 4 dpi. Virus in tissues was quantified by serial dilution in Vero-118 monolayers. The solid lines represent the geometric mean titer (GMT), the lower limit of detection is indicated with the dotted line.

inoculated with each of the candidate LAVs however, mean virus titers ranged from 10^2 to 10^4 $TCID_{50}$ $gram^{-1}$ NT, indicating that virus replication was ~10 000-fold reduced in the URT. In the lungs of animals inoculated with wild-type HMPV, the mean virus titer was $10^{2.2}$ $TCID_{50}$ $gram^{-1}$ lung, while in the animals inoculated with $HMPV_{M11}$ or $HMPV_{HRSV3}$ virus titers were below the detection limit of $10^{1.2}$ $TCID_{50}$, with the exception of a single animal in the $HMPV_{M11}$ inoculated group ($10^{1.3}$ $TCID_{50}$). Thus, both ts viruses appeared to be highly attenuated in vivo and virus replication was restricted to the URT, where virus titers were ~10 000-fold reduced compared to wild-type HMPV.

From the remaining six animals of each group, serum samples were collected and subjected to a PRVN assay to determine virus neutralizing antibody titers against HMPV NL/1/99, induced by the candidate LAVs (Fig. 3). The PRVN titers in the wild-type HMPV inoculated animals were slightly higher than those observed in the $HMPV_{M11}$ or $HMPV_{HRSV3}$ inoculated animals (mean VN antibody titers of 90, 25, and 28 respectively, not significantly different, Mann-Whitney test).

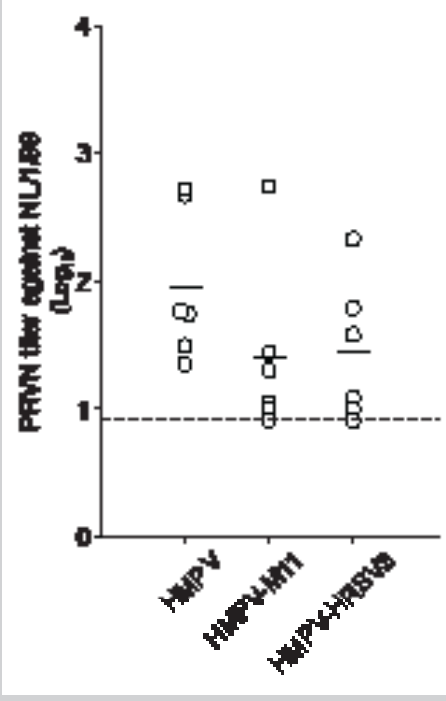

Figure 3
50% Plaque reduction virus neutralization (PRVN) titers measured against NL/1/99, after immunization with NL/1/99, $HMPV_{M11}$ or $HMPV_{HRSV3}$. Blood samples were collected by orbital puncture at 21 dpi. Titers were calculated according to the method of Reed and Muench. The solid lines represent the GMT, the lower limit of detection is indicated with the dotted line.

Immunization-challenge experiment

Since both $HMPV_{M11}$ and $HMPV_{HRSV3}$ induced a detectable but low virus neutralizing antibody response, we investigated whether these viruses had induced sufficient protective immunity to prevent subsequent HMPV infection. Groups of six animals were immunized with 10^6 $TCID_{50}$ of $HMPV_{M11}$, $HMPV_{HRSV3}$, wild-type HMPV NL/1/99 or PBS. Three weeks after immunization, animals were challenged with 10^7 $TCID_{50}$ of the heterologous HMPV strain NL/1/00, to evaluate whether the induced immune response was robust enough to provide cross-protection against heterologous infection. Four days after challenge infection, lungs, NT and blood samples were collected. In PBS-immunized control hamsters, virus titers upon challenge reached $>10^8$ $TCID_{50}$ $gram^{-1}$ tissue in the NT samples. These virus titers were more than 1000-fold reduced in animals immunized with $HMPV_{HRSV3}$, and >10 000-fold reduced in the animals immunized with $HMPV_{M11}$ or wild-type HMPV (Fig. 4a). In the lungs of PBS-immunized animals, the mean

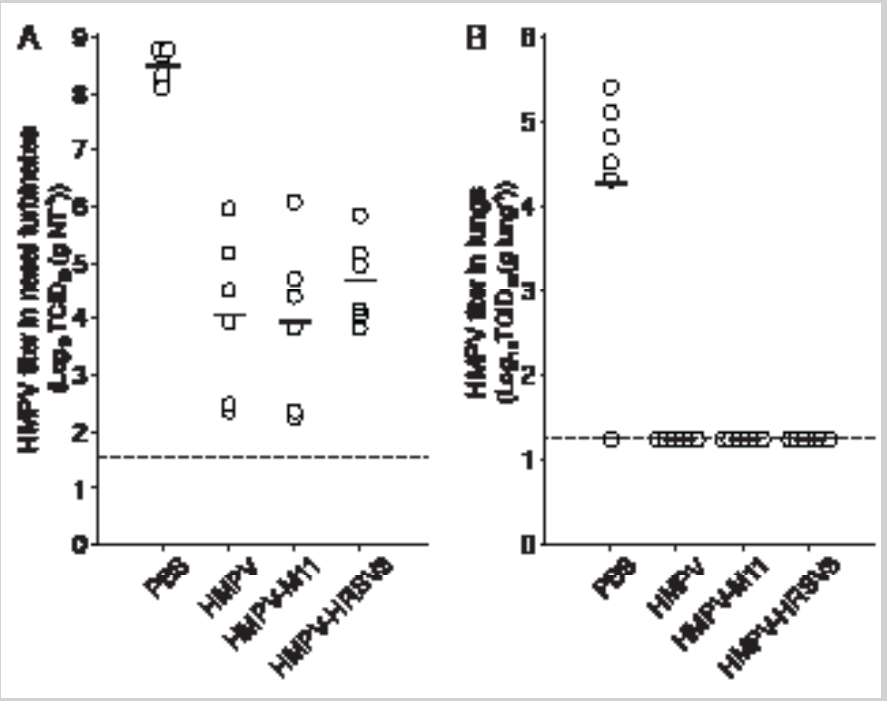

Figure 4

Infectious virus titers in (A) nasal turbinates and (B) lungs of Syrian golden hamsters. Animals were immunized with PBS, NL/1/99, $HMPV_{M11}$ or $HMPV_{HRSV3}$. Three weeks after immunization, animals were challenged with 10^7 $TCID_{50}$ of the heterologous virus HMPV NL/1/00. Animals were euthanized at 4 dpi. Virus present in tissues was quantified by serial dilution in Vero-118 monolayers. The solid lines represent the GMT, the lower limit of detection is indicated with the dotted line.

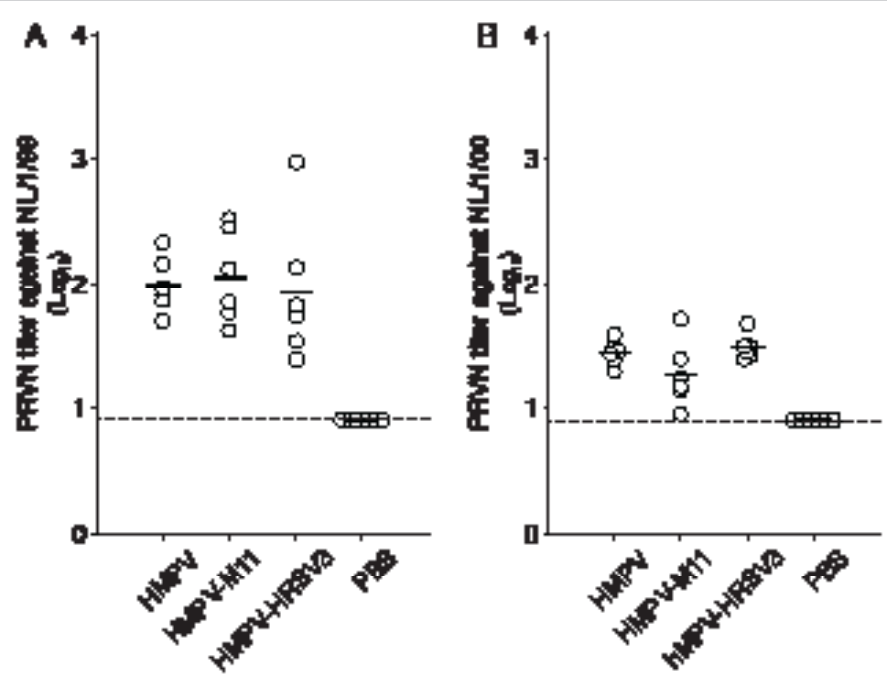

Figure 5
50% Plaque reduction virus neutralization (PRVN) titers measured against NL/1/99 (A) or NL/1/00 (B), after immunization with NL/1/99, $HMPV_{M11}$ or $HMPV_{HRSV3}$ or PBS and subsequent challenge with NL/1/00. Blood samples were collected by orbital puncture 4 days post challenge. Titers were calculated according to the method of Reed and Muench. The solid lines represent the GMT, the lower limit of detection is indicated with the dotted line.

virus titer after challenge infection was $10^{4.3}$ $TCID_{50}$ $gram^{-1}$ lung tissue. Virus was undetectable in lungs of all animals immunized with $HMPV_{M11}$, $HMPV_{HRSV3}$, and wild-type HMPV NL/1/99 (Mann-Whitney test, $P<0,05$) (Fig. 4b). Serum samples of all animals inoculated with wild-type NL/1/99 or one of the ts-HMPV candidates based on NL/1/99, were tested in a PRVN assay against both NL/1/99 (lineage B) and NL/1/00 (lineage A) virus. Although $HMPV_{M11}$ and $HMPV_{HRSV3}$ are highly attenuated, PRVN titers in animals inoculated with these viruses were comparable to wild-type HMPV inoculated animals (Fig. 5). As expected, PRVN titers against homologous virus were higher than against heterologous virus. Thus, we conclude that $HMPV_{M11}$ and $HMPV_{HRSV3}$ are attenuated in hamsters, yet induce an HMPV-specific immune response that is sufficient to provide protective immunity to prevent HMPV lower respiratory tract infections.

DISCUSSION

The clinical impact of HMPV warrants the development of vaccines to prevent serious respiratory tract disease in young children, immunocompromised individuals and the elderly. Here, a classical method for obtaining LAVs, by passaging virus at low temperatures, was explored for HMPV. HMPV isolate NL/1/99 was passaged at gradually decreasing temperatures, until passage 35 at a temperature of 25ºC was reached. After sequencing of this passage 35 virus, 19 nucleotide mutations were found resulting in 17 amino acid substitutions (Table 1). The mutations at nt position 3341 (E93K) and 3365 (S101P) have been described previously (16, 59, 202). The S101P mutation is located in the putative cleavage site motif of the F protein and viruses containing this mutation did not require trypsin for growth in tissue culture. Moreover, this trypsin-independent cleavage of the HMPV F protein containing the 101P was enhanced by the amino acid substitution E93K. Thus, repeated passaging of HMPV NL/1/99 in Vero cells resulted in the introduction of mutations that render the virus to be relatively independent of trypsin.

Initial attempts to rescue a recombinant virus with all 19 passage-35 mutations failed repeatedly. The parental passage-35 virus replicated very slowly, and to low virus titers. It is possible that the consensus sequence generated on the basis of the passage-35 virus was derived from a variety of quasi-species in the culture supernatant, which were replication-deficient upon clonal passage. Since HMPV generally replicates poorly in in vitro cell cultures, which was even more severe for the passage-35 virus, plaque purification of this virus was not attempted. Rather, we next tested whether viruses with a subset of the mutations of the passage-35 virus could be rescued. A virus with 11 of the 19 mutations, $HMPV_{M11}$ turned out to have a ts-phenotype in vitro (Fig. 1c). Ten of these 11 mutations were non-silent, and were located in the P, M, F, M2, G, and L genes. To our knowledge, none of these mutations has been observed in cp/ts-HRSV. A recombinant virus with 8 of the 11 cp-mutations, $HMPV_{M8}$, did not display a ts-phenotype in vitro. The only differences between $HMPV_{M11}$ and $HMPV_{M8}$ were two mutations in the L gene (nt 7826 and 8090), and one mutation in the P gene (nt 1458, Table 1). $HMPV_{M9}$, containing all mutations of $HMPV_{M8}$ and the mutation in the P gene was not temperature sensitive. A recombinant virus containing only the two mutations in L also did not display a ts-phenotype. Therefore it seems likely that one or both of the L mutations in combination with one or more other mutations is responsible for the ts-phenotype of $HMPV_{M11}$. Further studies are needed to map the phenotype of all cp-mutations, and especially the phenotype of HMPV harboring all 3 mutations that are different between $HMPV_{M8}$ and $HMPV_{M11}$ should be evaluated.

For HRSV, this classical approach of generating attenuated cp-viruses has resulted in several candidate LAVs that have been tested extensively in animal models and even in human volunteers. Sequence comparison of HMPV with different cp-HRSV strains resulted in the identification of four cp-HRSV mutations that could be introduced in the HMPV genome. When three of these mutations were introduced in the HMPV genome, omitting the L1321 mutation (Table 2), virus could be rescued ($HMPV_{HRSV3}$). Upon introduction of the HRSV L1321 mutation in the HMPV genome, recombinant HMPV could not be rescued. It is possible that

introduction of L1321 in HMPV yields a stronger ts-phenotype if other, potentially compensatory, mutations are present. Virus replication curves generated at different temperatures revealed that $HMPV_{HRSV3}$ was restricted for replication at 39°C and 40°C, indicating that this virus had a ts-phenotype (Fig. 1e). The L521 phenylalanine (Phe) to leucine mutation, present in $HMPV_{HRSV3}$, has previously been mutagenized at the analogous Phe at amino acid position 456 of recombinant HPIV3 (214). This amino acid substitution resulted in a virus bearing a ts phenotype with virus replication 10-fold reduced in the upper, but not the lower respiratory tract of hamsters. Substitution of this amino acid in two cp-HPIV3 candidates (rcp45 and rcp45L) induced a 100- to 1000-fold more restricted replication in hamsters than their cp parents (214).

Both $HMPV_{M11}$ and $HMPV_{HRSV3}$ were found to be attenuated in hamsters, with ~10 000-fold reduction of virus replication in the URT, and no detectable virus in the LRT as compared to wild-type virus (Fig. 2). In immunized animals, the HMPV-specific antibody titers were slightly lower as compared to animals inoculated with wild-type virus (Fig. 3 and Fig. 5). Nevertheless, immunized animals were completely protected from HMPV LRT infection, and virus titers in the URT were reduced to the same extent as seen in hamsters exposed to wild-type HMPV (Fig. 4). Viral titers in both the lungs and NT of PBS-immunized animals that were challenged with NL/1/00 were approximately 100-fold higher than the titers obtained in NL/1/99-infected animals (Fig. 2). Similar differences in replication between these two viruses have been observed before in cynomolgus macaques (240), but may here also be caused by differences in inoculum size, 106 versus 107 for NL/1/99 and NL/1/00, respectively.

Altogether, it seems that the balance between the level of attenuation and the level of induction of specific immune responses for both $HMPV_{M11}$ and $HMPV_{HRSV3}$ in hamsters is appropriate for these viruses to serve as vaccines. The results of this study justify subsequent studies in non-human primates, in which the attenuation, stability, immunogenicity and safety issues can be further addressed.

Future studies should determine the contribution to the ts-phenotype of each individual mutation that was introduced in $HMPV_{M11}$ and $HMPV_{HRSV3}$. During passaging at lower temperatures, both ts-mutations, mutations associated with adaptation to Vero cells, and mutations without apparent phenotype changes may be observed, and it would be good to discriminate amongst these. The S101P and E93K mutations in the F protein may be good examples of cell culture adaptation mutations. When the mutations responsible for the attenuated phenotype have been defined, amino acid point mutations should be generated using codons that differ from the wild-type codon by preferably two or three nucleotides, in order to reduce the frequency of reversion.

In addition to the use of LAVs for the induction of protective immunity against the attenuated virus itself, attenuated candidate HMPV may be used as a vector to induce immunity against a second or even a third viral respiratory pathogen, such as HRSV or HPIV3. A similar approach has already been conducted with a chimeric human/bovine parainfluenza virus type 3 (B/HPIV3) expressing the F protein of HMPV, that induced protective immunity against both HMPV and HPIV3 infection in African green monkeys (226).

Our results demonstrate that immunization of Syrian golden hamsters with attenuated recombinant viruses containing cp-HMPV or cp-HRSV mutations induced a good antibody response, and provided complete protection against LRT infection with a heterologous strain of HMPV. The high degree of attenuation and the high level of immunogenicity suggest that $HMPV_{M11}$ and $HMPV_{HRSV3}$ represent excellent candidate LAVs for further exploration to prime the HMPV-specific immune response in non-human primates, and perhaps humans.

ACKNOWLEDGEMENTS

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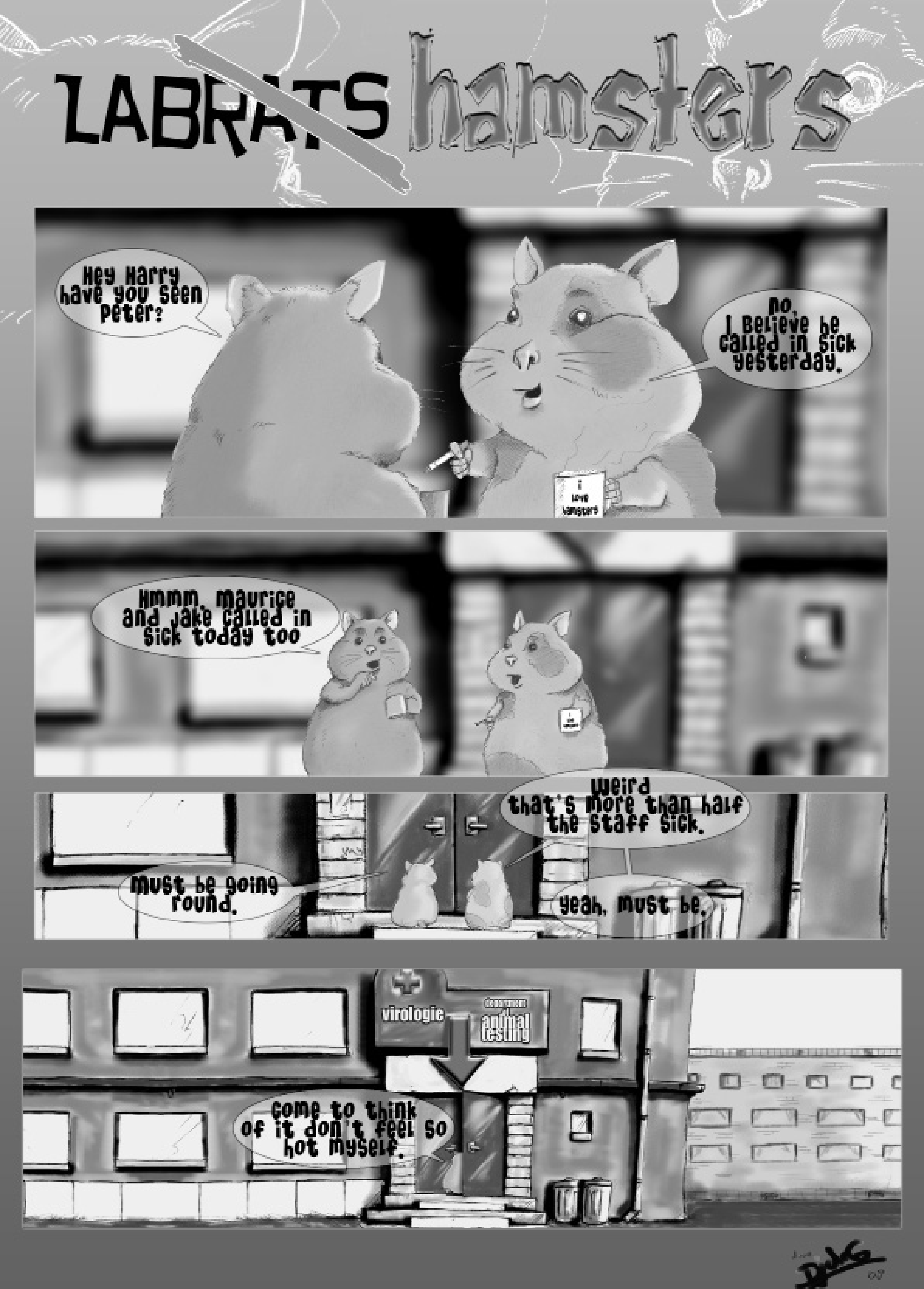
LABRATS hamsters
Hey Harry have you seen Peter?
No, I believe he called in sick yesterday.
I love hamsters
Hmmm, Maurice and Jake called in sick today too
Weird that's more than half the staff sick.
Must be going round.
Yeah, must be.
virologie
department of animal testing
Come to think of it don't feel so hot myself.

CHAPTER 5
EVOLUTIONARY DYNAMICS OF HUMAN AND AVIAN METAPNEUMOVIRUSES

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ABSTRACT

Human (HMPV) and avian (AMPV) metapneumoviruses are closely related viruses that cause respiratory tract illnesses in humans and birds, respectively. Although HMPV was first discovered in 2001, retrospective studies have shown that HMPV has been circulating in humans for at least 50 years. AMPV was first isolated in the 1970s, and can be classified in four subgroups, A-D. AMPV subgroup C is more closely related to HMPV than to any other AMPV subgroup, suggesting that HMPV has emerged from AMPV-C upon zoonosis. Presently, at least four genetic lineages of HMPV circulate in human populations – A1, A2, B1 and B2 – of which lineages A and B are antigenically distinct. We used a Bayesian Markov Chain Monte Carlo (MCMC) framework to determine the evolutionary and epidemiological dynamics of HMPV and AMPV-C. The rates of nucleotide substitution, relative genetic diversity and time to the most recent common ancestor (TMRCA) were estimated using large sets of sequences of the nucleoprotein, the fusion protein and attachment protein genes. The sampled genetic diversity of HMPV was found to have arisen within the last 119-133 years, with consistent results across all three genes, while the TMRCA for HMPV and AMPV-C was estimated to have existed around 200 years ago. The relative genetic diversity observed in the four HMPV lineages was low, most likely reflecting continual population bottlenecks, with only limited evidence for positive selection.

INTRODUCTION

Human metapneumovirus (HMPV) is a respiratory pathogen that was first described in 2001 (238). HMPV causes respiratory illnesses ranging from mild upper respiratory symptoms to severe lower respiratory tract disease (238, 239, 253). HMPV is an enveloped, non-segmented, negative-strand RNA virus and was classified as a member of the family Paramyxoviridae, the subfamily Pneumovirinae, the genus Metapneumovirus. HPMV seroprevalence in the human population reaches 100% by the age of five (238). Experimental HMPV infections in macaques have been shown to induce transient protection (240), which is in agreement with relatively frequent occurrences of reinfection in humans (71). HMPV can be divided in two main genetic lineages (A and B), each consisting of at least two sublineages – A1, A2, B1 and B2 (241). Annual circulation of all four lineages has been observed worldwide, with reports of a predominating strain changing on a yearly basis in some studies, but not in others (85, 216, 241).

The only other member of the genus metapneumovirus is avian metapneumovirus (AMPV). AMPV has been found to infect domestic poultry worldwide, causing acute respiratory infections (50). AMPVs have been classified into four subgroups, A through D (9, 77, 130, 208). AMPV subgroups A and B were first detected in South Africa in 1978 and were subsequently found in Europe, Israel and Asia (49). AMPV subgroup D was first described in France (9). Finally, AMPV subgroup C was first detected in the USA in 1996 and is more closely related to HMPV than to any other AMPV subgroup (91-93, 235, 237, 263).

The genome organizations of HMPV and AMPV are similar; from the 3′ to 5′ ends the genomes encode nucleoprotein (N), phosphoprotein (P), matrix protein (M), fusion protein (F), M2-1 and M2-2, small hydrophobic protein (SH), attachment protein (G), and the large polymerase protein (L). Overall, HMPV and AMPV-C share 80% amino acid identity, with N being most conserved and SH and G the most variable proteins (17, 237, 238). The viral RNA is encapsidated by the N protein, which, together with the P and L proteins, form the ribonucleoprotein complex required for genome replication. With analogy to respiratory syncytial virus (RSV), M2-1 is a transcriptional elongation factor that enhances synthesis of readthrough mRNAs (45, 79, 106), although for HMPV its function is not completely understood as M2-1 is not essential for virus viability (28, 110). HMPV has three surface glycoproteins; SH, G, and F. The F protein is synthesized as an inactive precursor (F0) that is cleaved by host proteases into the functional subunits F1 and F2. The F protein of HMPV is highly immunogenic and protective, whereas G and SH are not (112, 210).

The close genetic relationship between HMPV and AMPV-C is not reflected in host range. To date, no AMPV-C infections have been reported in humans and it has been shown that HMPV does not replicate in turkeys (238). However, using both chimeric viruses and minireplicons systems it has been shown that the ribonucleoprotein complex proteins of HMPV and AMPV-C are functionally interchangeable (58, 90, 186). Spill-over events of pathogens from birds to humans are not uncommon (197). Since HMPV is most closely related to AMPV-C, it is plausible that an AMPV-C-like virus was the ancestor of HMPV. Retrospective analysis of serum samples collected from humans in 1958 revealed that HMPV has been widespread in the human population for at least 50 years. Thus, any cross-species transmission event must

have taken place before 1958 (238). Herein we test this hypothesis using sequence data from the N, G and F genes of HMPV and AMPV-C collected over a 25-year sampling period. In addition, to understand the evolutionary dynamics of HMPV in more detail we estimated rates of nucleotide substitution, times to common ancestry, and relative genetic diversity of all HMPV lineages using a Bayesian coalescent approach, as well as gene- and site-specific selection pressures.

MATERIALS AND METHODS

Data preparation

All gene sequence data sets were compiled from Genbank. Two additional nearly complete HMPV genome sequences were submitted to Genbank (nos FJ168778 and FJ168779). The data for the G, F, and N genes were divided into subsets comprising the individual sublineages of HMPV, AMPV-C or combinations thereof. For the G gene, sequence alignments were made for HMPV-A1 (ORF nt position 1-616, n=19), HMPV-A2 (nt 1-616, n=29), HMPV-B1 (nt 1-634, n=14), HMPV-B2 (nt 1-619, n=14) and for all HMPV lineages (nt 1-442, n=76). For the F gene, sequence alignments were compiled for HMPV-A1 (n=22), HMPV-A2 (n=30), HMPV-B1 (n=25) HMPV-B2 (n=30) and AMPV-C (n=12) using ORF nt positions 781-1221. For the N gene, sequence alignments were made for HMPV (complete ORF, n=21) and AMPV-C (complete ORF, n=15). All sequence alignments were created using both MUSCLE (74) and ClustalW software running within the BioEdit (version 7.0.1) (98) program, and adjusted manually where necessary. Sequence alignments are available from the authors on request.

Estimating evolutionary dynamics

Overall rates of evolutionary change (nucleotide substitutions per site, per year), the time to the most recent common ancestor (TMRCA) in years and relative genetic diversity (expressed as $N_e t$, where N_e is the effective population size and t the generation), were estimated using the BEAST program (http://beast.bio.ed.ac.uk/). This program employs a Bayesian Markov Chain Monte Carlo (MCMC) approach, utilizing the number and temporal distribution of genetic differences among viruses sampled at different times (65-67). To estimate the substitution rates with as much accuracy as possible, we compared a variety of models of demographic history, namely constant population size, exponential population growth, and Bayesian skyline reconstruction, and with both strict (constant) and relaxed (variable) molecular clocks, and using a variety of prior values for the substitution rate. As similar rate/date estimates were obtained under all demographic models, we only report those from the Bayesian skyline model as this employs the least constrained coalescent prior. Similarly, a preliminary analysis of a subset of the data collected here (data not shown) revealed that the uncorrelated lognormal relaxed clock model gave the best estimation, although rate/date estimates were again highly congruent across all clock models suggesting that they are robust. Because the sequences analyzed here were very closely related, sampled over a relatively narrow time-span and exhibited little multiple substitution at single nucleotide sites, we used the simple HKY85 model of nucleotide substitution in each case as more complex models sometimes failed to converge (data not shown). All MCMC chains were run for a sufficient length of time to ensure convergence (allowing a 10% burn-in) and uncertainty in parameter estimates is

reported as values of the 95% highest probability density (HPD). Finally, the MCMC samples were summarized to infer the maximum clade credibility (MCC) trees of each dataset using TreeAnnotator v1.4.7(66), with posterior probability values shown for the major branches. The MCC trees were depicted with estimated branch lengths in time (years).

Analysis of selection pressures

We estimated overall (i.e. gene-specific) and site-specific pressures in alldata sets using the Datamonkey web interface of the HY-PHY package (www.datamonkey.org) (189). This method quantifies selection pressures as the ratio of non-synonymous (d_N) to synonymous substitutions (d_S) per site, using the random effects likelihood (REL) method for data sets smaller than 40 sequences, the fixed effects likelihood (FEL) method for the data sets up to 100 sequences, and the single likelihood ancestor counting (SLAC) for the data sets containing more than 100 sequences and the mean d_N/d_S (w) values. These were run with three different nucleotide substitution models – HKY85, TrN93 and REV – although for most alignments the TrN93 model was the best-fitting model of nucleotide substitution.

For an additional analysis of selection pressures we used the CODEML program (261) to estimate the maximum likelihood w value for all branches of each phylogeny (the 'one-ratio' model), and compared this to w values estimated separately for the external (w_e) and internal (w_i) branches using the 'two-ratio' model. Input phylogenetic trees for this analysis were estimated under the general time reversible (GTR)+I+G4 substitution model, using PAUP* (223), and employing tree bissection-reconnection (TBR) branch-swapping.

RESULTS

Evolutionary dynamics of metapneumoviruses

Rates of nucleotide substitution and TMRCAs for the G, N and F genes of HMPV and AMPV were estimated using the BEAST program. For the G gene, separate datasets for all four sublineages (HMPV-A1, -A2, -B1 and -B2) were analyzed both individually and as part of combined datasets containing all sequences of HMPV-A, HMPV-B or HMPV. No dataset containing both HMPV and AMPV-C sequences was made, since it was not possible to ensure proper alignment in this case. For the F gene similar datasets were compiled with an additional dataset containing all HMPV and AMPV-C sequences. Because of limited sequence availability, no N gene dataset was made for the individual HMPV lineages. MCC trees were also inferred for each metapneumovirus dataset and plotted in (mean) evolutionary time (Fig 1). The mean rate of nucleotide substitution for the G gene of HMPV was 3.5×10^{-3} (HPD, $2.3\text{-}4.8 \times 10^{-3}$) nucleotide substitutions per site, per year. Somewhat lower rates were found for the F and N genes, at 9.0×10^{-4} (HPD, $6.6\text{-}11.5 \times 10^{-4}$) and 8.5×10^{-4} (HPD, $2.0\text{-}15.0 \times 10^{-4}$) nucleotide substitutions per site, per year, respectively. Because of the small size of some of the individual sublineage datasets, and hence the large sampling error, we fixed their substitution rates – using the values obtained from the complete datasets determined above – to obtain a better estimate of TMRCAs. Accordingly, the mean TMRCA for HMPV-A1, A2, B1 and B2 were estimated to be 14 (HPD, 11-20), 26 (HPD, 25-28), 13 (HPD, 10-16), and 18 (HPD, 12-24), years, respectively, based on the G gene, and 12 (HPD, 6-20), 28 (HPD, 24-

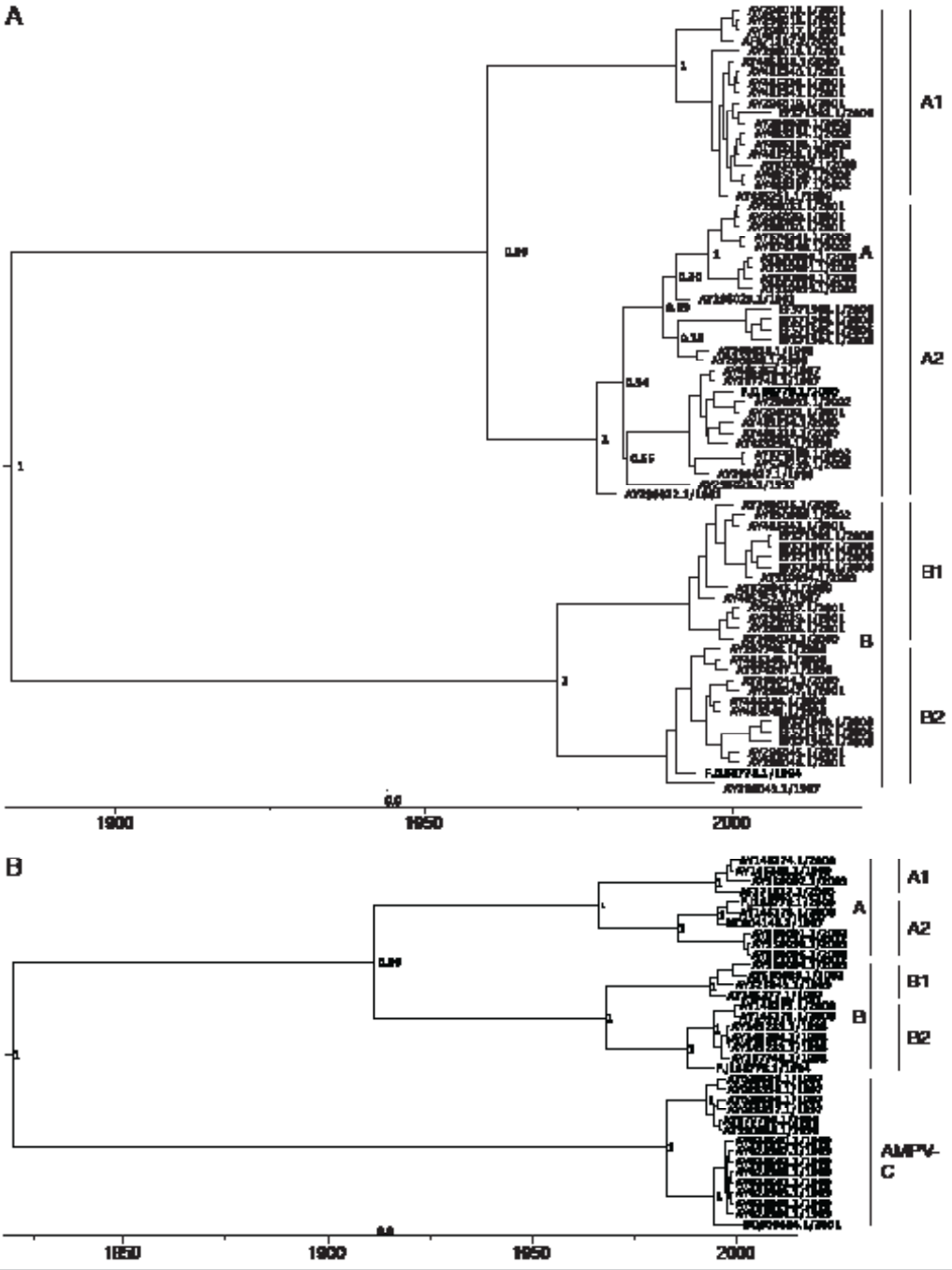
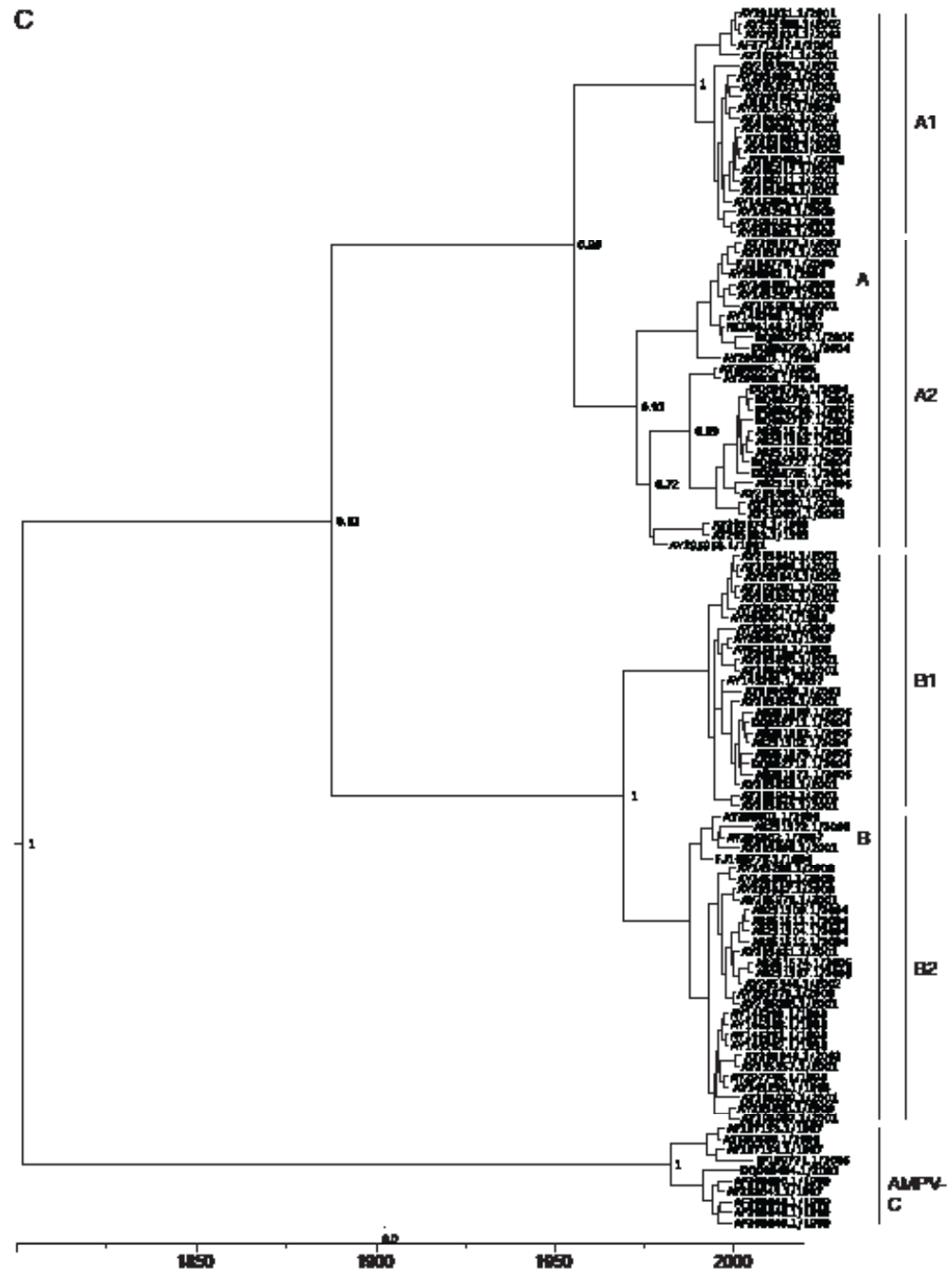


Figure 1

Maximum Clade Credibility (MCC) trees from the Bayesian analysis of the HMPV G (a) the HMPV and AMPV-C N (b) and F (c) sequences. The posterior probabilities of the branches are depicted in each case. Note that the mean TMRCAs on the MCC trees can differ from those reported in the text, although all are contained with the same 95% HPD values. The scale bar represents time in years.


C
A1
A2
A
B1
B2
B
AMPV-C
1850
1900
1950
2000

34), 13 (HPD, 8-20) and 18 (HPD, 12-24) years based on the F gene data sets. The estimated TMRCA of the two main lineages HMPV-A and HMPV-B are strikingly similar, at 41 (HPD, 24-61) and 34 (HPD, 20-51) years for the F gene, and 41 (HPD, 32-51) and 37 (HPD, 20-26) years for the G gene. The TMRCA for HMPV as a whole (i.e. all lineages) was estimated to be 122 (HPD, 54-204) years for the G, 119 (HPD, 79-166) for the F, and 133 (HPD, 40-264) for the N gene. Thus, the genetic diversity within the four sublineages is of extremely recent origin, with a number of lineage diversification events occurring at approximately the same time. Lastly, the substitution rates found for the HMPV/AMPV-C F and the N genes were 9.3×10^{-4} (HPD, 6.0×10^{-4}-1.3×10^{-3}) and 1.1×10^{-3} (HPD, 5.3×10^{-4}-1.6×10^{-3}) nucleotide substitutions per site, per year, respectively. Under these rates, the TMRCA of HMPV and AMPV-C was estimated to be 208 (HPD, 86-346) and 180 (HPD, 89-284) years for the F and N genes respectively.

Standing genetic diversity

The demographic history of the HMPV data set was inferred using the bayesian skyline plot (Figure 2). Notably, the relative genetic diversity ($N_e t$) in all HMPV lineages was very low, ranging from only 3.3 to 96.8. In addition, the Bayesian skyline plot also showed that there was an increase and subsequent decrease in genetic diversity between ~1996 and ~2001 (although the 95% HPD interval is wide), a pattern that was seen for all lineages and in both the G and F genes.

Selection pressures

Our analysis of selection pressures through computation of the ratio of non-synonymous (d_N) to synonymous (d_S) substitutions per site d_N/d_S (w) revealed limited positive selection and abundant negative selection for the G (mean w=0.524) and F proteins (mean w=0.075) (Table 1). It should be noted that for the HMPV set the G sequences were shortened to ensure proper alignment. Neither positive nor negative selection could be detected for the N protein (mean w =0.038). Overall, this analysis revealed that the mean w is roughly 10-fold higher for the G than for the F and N genes, compatible with the localized action of immune selection on the former. Positively selected sites in the F protein were only found for the A1 (aa 34) and A2 (aa 34 and 50) sets. Both sites are located in the extracellular domain of F. Positively selected sites for the G protein were found for the A1 (aa 82, 125, 156, and 158), A2 (aa 146 and 151), B2 (aa 85) and HMPV (aa, 85, 86, 93, 111, 112, 113, 140, and 142) sets and were all located in the extracellular domain. By using the program NetOglyc (version 3.1) it was predicted that all but one (aa 86) of the positively selected sites found in the G protein corresponded to potential O glycosylated sites in one or more strains of that data set (105).

To obtain a more precise picture of selection pressures we also estimated w separately for the internal (w_i) and external (w_e) branches of each phylogeny using the CODEML program (Table 2). In case of the F and N genes the w values were higher on the tips compared with the internal branches of the trees, resulting in an increased w_e/w_i value, and strongly suggesting that most of the non-synonymous mutations in these proteins are transiently deleterious (so that they are purged by purifying selection before they can reach high frequencies). However, values of both w_i and w_e values were high in the case of the G gene, resulting in a low w_e/w_i value, and again suggestive of immune selection.

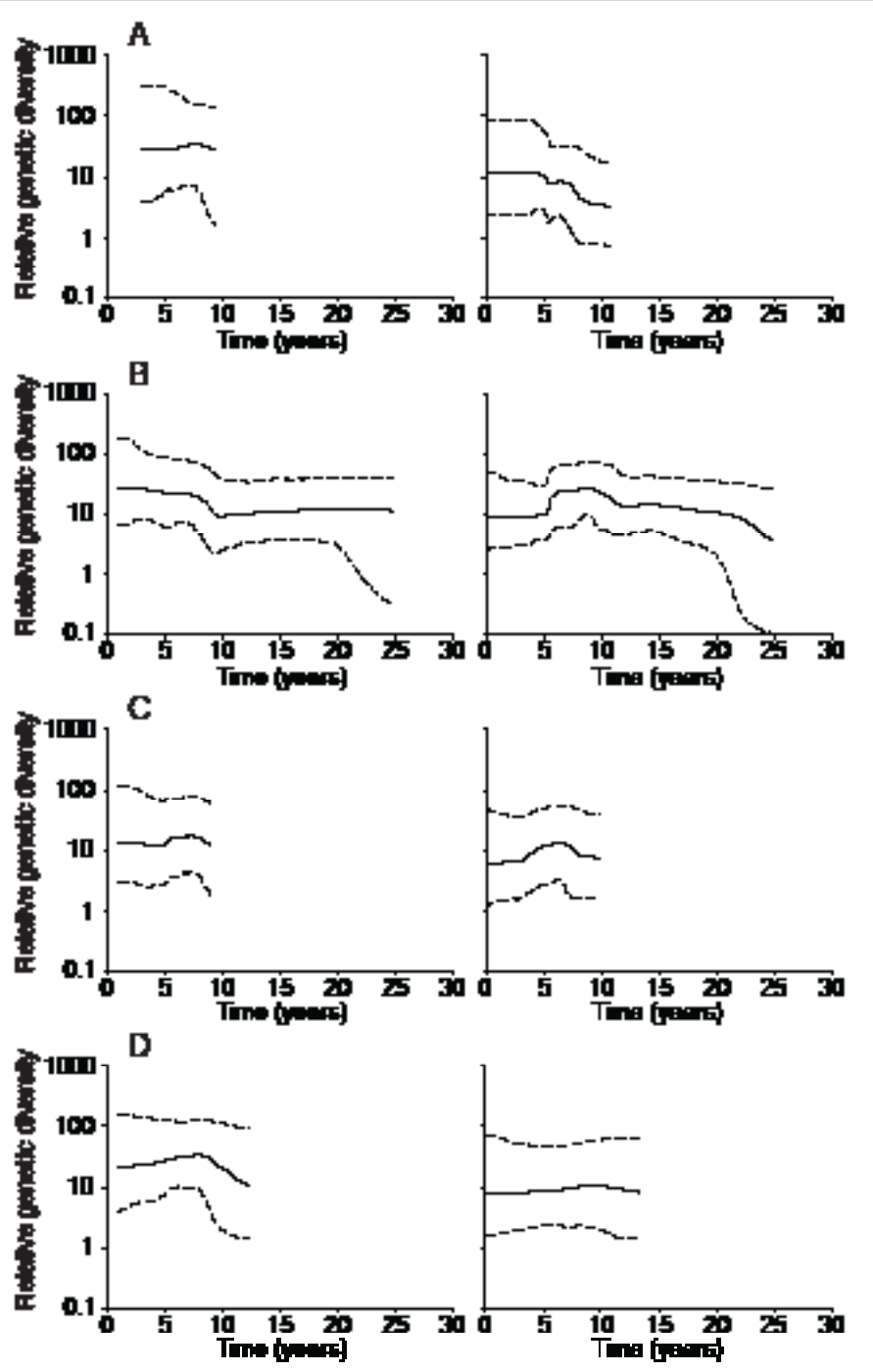
Table 1
Comparison of mean values of w (d_N/d_S) of the G, F and N gene between the different HMPV lineages.

	Gene	w	Positively selected sites[a]	Negatively selected sites[a]	Method
HMPV-A1	**G**	**0.544**	**4 (aa 82, 125, 156, 158)**	**2**	**REL**
HMPV-A1	**F**	**0.041**	**1 (aa 294)**	**1**	**REL**
HMPV-A2	**G**	**0.518**	**2 (aa 146, 151)**	**3**	**REL**
HMPV-A2	**F**	**0.166**	**2 (aa 294, 310)**	**1**	**REL**
HMPV-B1	**G**	**0.495**	**0**	**37**	**REL**
HMPV-B1	**F**	**0.108**	**0**	**0**	**REL**
HMPV-B2	**G**	**0.693**	**1 (aa 85)**	**0**	**REL**
HMPV-B2	**F**	**0.100**	**0**	**27**	**REL**
HMPV	**G**	**0.524**	**8 (aa 85, 86, 93, 111,112, 113, 140, 142)**	**28**	**FEL**
HMPV	**F**	**0.075**	**0**	**30**	**SLAC**
HMPV	**N**	**0.038**	**0**	**0**	**REL**

[a]p< 0.1

Table 2
The mean ratio w (d_N/d_S) for all branches in the tree, and the separate w values estimated for the external (w_e) and internal (w_i) branches of the phylogenies of the HMPV G, F and N genes.

Virus (gene)	Number of sequences	Sequence length (codons)	w (all branches)	w_i (internal branches)	w_e (external branches)
HMPV N	**20**	**394**	**0.027**	**0.021**	**0.060**
HMPV F	**107**	**147**	**0.042**	**0.015**	**0.151**
HMPV G	**76**	**147**	**0.422**	**0.422**	**0.421**
HMPV A G	**48**	**147**	**0.399**	**0.433**	**0.358**
HMPV B G	**28**	**147**	**0.497**	**0.476**	**0.523**


A
B
C
D
Relative genetic diversity
1000
100
10
1
0.1
0
5
10
15
20
25
30
Time (years)

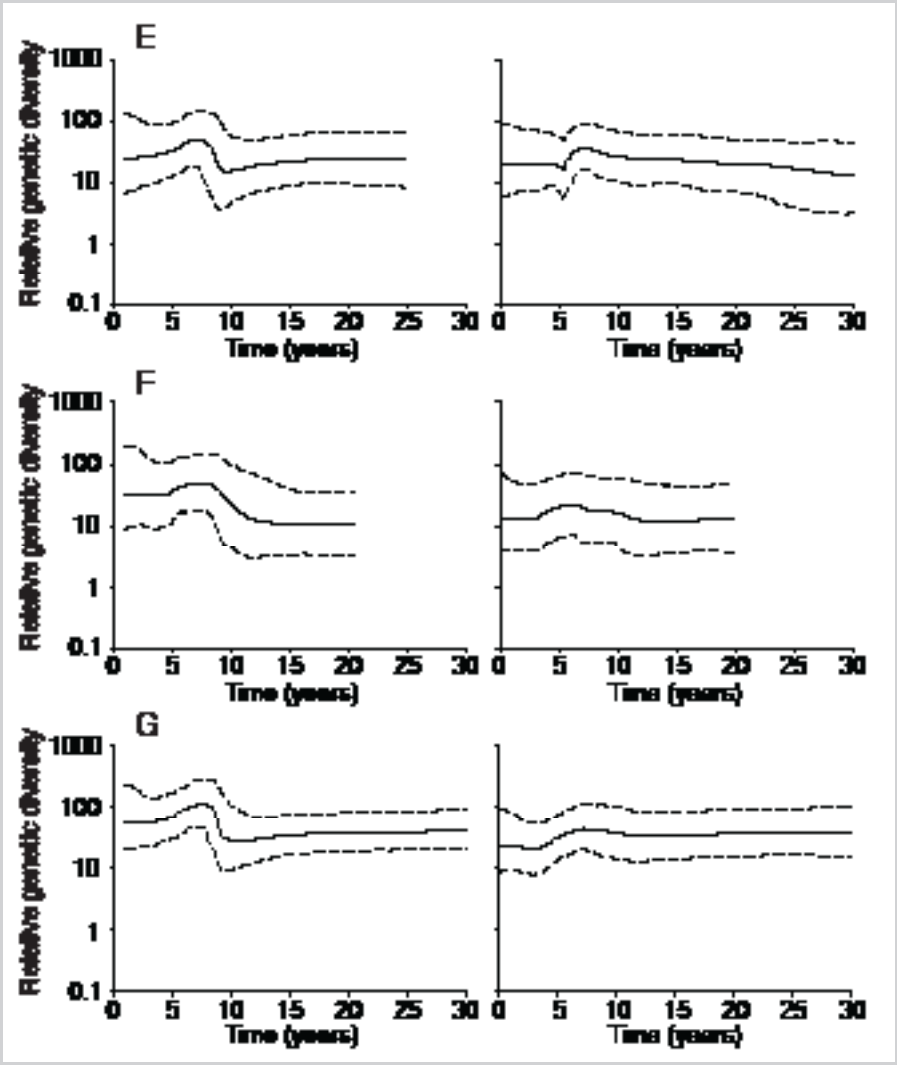


Figure 2

Bayesian skyline plots of the changing levels of relative genetic diversity ($N_e t$) of HMPV shown on a log scale against time backwards since the most recent (t_0=2006). Datasets containing HMPV-A1 (a), HMPV-A2 (b), HMPV-B1 (c), HMPV-B2 (d), HMPV-A1 and -A2 (e), HMPV-B1 and -B2 (f) HMPV (g) sequences from the F gene (left panels) and G (right panels) were used. The thick line represents the median estimate, and the dashed lines show the 95% HPD limits.

DISCUSSION

In the present study we explored the evolutionary dynamics of HMPV and AMPV through analyses of a large set of HMPV and AMPV-C G, F and N gene sequences. For HMPV our results were highly consistent across all three data sets indicating that the admittedly sparse sampling did not introduce major biases. In particular, both the highly variable G sequences and the less variable F and N sequences gave similar demographical signatures and times to common ancestry, although the nucleotide substitution rates were higher for the G gene. The results for the G gene were comparable to those previously described (176). Overall, evolutionary rates were high and of the level expected of RNA viruses (104, 122). In addition, the genetic diversity found within the different sublineages of HMPV was of a very recent origin, and HMPV as a whole was characterized by low levels of standing genetic variation (as reflected in estimates of $N_e t$). Such limited genetic diversity, which is also characteristic of human influenza A virus (193), as well as other paramyxoviruses (188) is most likely due to repeated (annual) population bottlenecks, which periodically purge genetic variation.

Overall, the Bayesian skyline plots for HMPV suggest that the population dynamics of this virus are rather complex, with both increases and decreases in population size, and again expected for a virus where there are distinct peaks and troughs in prevalence. The general trend for all four lineages was an increase and subsequent decrease in population size between ~1996 and ~2001. Unfortunately the resolution of these Bayesian skyline plots was not sufficient to allow detailed analyses of the population dynamics in each viral lineage, or their precise cause. However, for the future it will be interesting to determine whether the increases and decreases in genetic diversity are in-phase or out-of-phase, as demonstrated in some other RNA viruses (1). At present it is not possible to compare these demographics to epidemiological studies published, as none cover a comparable period of time and include data of viruses isolated from patients and healthy individuals, including children as well as adults. Our analysis also provides new insights into the timescale of HMPV evolution. Although HMPV had already been shown to be widely spread in the human population in 1958, our coalescent analysis reveals that the TMRCA of HMPV existed approximately 70 years before this, so that HMPV was circulating in human populations long before its detection. Since HMPV and AMPV-C are highly similar in terms of protein composition and sequence identity it has been speculated that HMPV is the result of a cross-species transmission event from birds to humans (238). Further, since there is a wider variety of AMPV subgroups compared with HMPV subtypes it seems most likely that the direction of transmission is from birds to humans. As the TMRCA for HMPV and AMPV-C was calculated to be around 200 years ago, we suggest that the cross-species transmission event from birds to humans occurred at about this time.

Despite its capacity to cause human disease, we found few positively selected sites for the F and G gene and none for the N gene of HMPV. Indeed, negatively selected sites were abundant for both the F and G. The positively selected sites found for the F protein were located in the extracellular domain of the F1 chain in the cysteine-rich region. Although the location of the antigenic sites of the F protein of HMPV have yet to be identified, some neutralizing monoclonal antibodies specific for the F protein have been described previously (236). The epitopes of these monoclonal antibodies do not correspond to the location of the

positively selected sites described here. For RSV, most antigenic sites are located in the cysteine-rich region (153). However, alignment of HMPV and RSV F sequences showed that the positively selected sites did not correspond to any of these antigenic sites. All positively selected sites found for the G protein were located in the extracellular domain, and all but one corresponded to potential O-linked glycosylated sites in one or more HMPV strains. For RSV there is a strong association between the positively selected sites and neutralizing epitopes (267). However, the G protein of HMPV is only weakly immunogenic and protective in animal models in contrast to RSV (174, 210). The mean w was approximately 10-fold higher for the G gene than for the N and F genes. In addition, separate estimates of the w ratio on internal versus external branches revealed a major difference between the G, F and N genes, with a far greater we in the former protein. Hence, the N and F genes are evidently more constrained than the G gene, so that mutations in these genes are more likely to be deleterious, and resulting in an elevation in the number of (young) non-synonymous mutations towards the tips of the trees. In contrast, a greater proportion of the mutations occurring in the G gene are likely to be either neutral or positively selected (although this latter process is difficult to detect using the analytical methods employed here), resulting in a higher level of non-synonymous diversity and lower w_e/w_i values. Similar results have been observed in the G gene of bovine respiratory syncytial virus (190, 268).

In conclusion, we were able to elucidate the evolutionary history and dynamics of metapneumoviruses by using dated genomic HMPV and AMPV sequences. Metapneumoviruses have high substitution rates like most RNA viruses due to high mutation rates, short generation times and large population sizes. Although such high mutation rates are not necessarily adaptive, and most often result in deleterious mutations, as reflected in the purifying selection on the F and N genes, they may occasionally facilitate viral emergence in novel hosts.

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SPECIFICITY AND FUNCTIONAL INTERACTION OF THE POLYMERASE COMPLEX PROTEINS OF HUMAN AND AVIAN METAPNEUMOVIRUSES

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ABSTRACT

Human metapneumovirus (HMPV) and avian metapneumovirus (AMPV) have a similar genome organization and protein composition, but a different host range. AMPV subgroup C is more closely related to HMPV than other AMPVs. To investigate the specificity and functional interaction of the polymerase complex proteins of human and avian metapneumoviruses, a minireplicon system was generated for AMPV-C and used in combination with minireplicon systems for HMPV lineages A1 and B1. Viral RNA-like molecules representing HMPV-A1 and -B1, AMPV-A and -C and human respiratory syncytial virus were replicated efficiently by polymerase complexes of HMPV-A1 and -B1 and AMPV-C but not by polymerase complexes of bovine parainfluenza virus type 3. Upon exchange of HMPV and AMPV-C polymerase complex components, all chimeric polymerase complexes were functional; exchange between HMPVs did not result in altered polymerase activity, whereas exchange between HMPVs and AMPV-C did. Recombinant HMPV-B1 viruses in which polymerase genes were exchanged with those of HMPV-A1 replicated with normal kinetics in vitro, whilst replacement with AMPV-C genes resulted in moderate differences in virus replication. In hamsters, recombinant HMPV-B1 viruses in which individual polymerase genes were exchanged with those of AMPV-C were attenuated, irrespective of the results obtained with minireplicon systems or in vitro replication assays. This study provides insight in the specificity and functional interaction of polymerase complex proteins of human and avian metapneumoviruses, but neither minireplicon systems nor in vitro replication kinetics were found to be predictive for attenuation in permissive animals.

INTRODUCTION

Human metapneumovirus (HMPV) is an enveloped, non-segmented, negative-strand RNA virus that causes respiratory tract illnesses primarily in infants, young children, the frail elderly, and immunocompromised individuals (54, 78, 133, 183, 238, 239). HMPV is a member of the family Paramyxoviridae, subfamily Pneumovirinae, genus Metapneumovirus, and can be divided in two main genetic lineages (A and B) each consisting of two sublineages, A1 and A2 and B1 and B2 (241). The only other member of the genus Metapneumovirus is avian metapneumovirus (AMPV). AMPV has been found to infect domestic poultry worldwide, causing acute respiratory infections (50). AMPVs have been classified into four subgroups, A-D (9, 77, 130, 208). AMPV-C was first detected in the USA and is more closely related to HMPV than the other AMPV subgroups (91-93, 235, 237, 263). Human respiratory syncytial virus (HRSV) is the only other member of the subfamily Pneumovirinae that infects humans. In comparison with HRSV, metapneumoviruses lack the non-structural proteins NS1 and NS2, and the order of genes between the matrix (M) and large polymerase (L) is different: 3′ -le-N-P-M-F-M2-SH-G-L-tr- 5′ for HMPV and AMPV; 3′ -le-NS1-NS2-N-P-M-SH-G-F-M2-L-tr- 5′ for HRSV.

The viral genome of all members of the subfamily Pneumovirinae is of antisense polarity and is assembled into a ribonucleoprotein complex (RNP). This RNP contains the viral genomic RNA (vRNA) encapsidated by the nucleocapsid protein (N), the phosphoprotein (P) and the large polymerase protein (L). By analogy with other paramyxoviruses, the L protein is responsible for the main catalytic activities of the viral polymerase complex (95, 108, 173). The assembly and polymerase co-factor P and the L protein form the minimal complex needed for viral polymerase activity (160). HRSV RNA synthesis involves an additional viral protein, the M2.1 protein, a transcriptional elongation factor that enhances the synthesis of readthrough mRNAs (45, 79, 106). For HMPV, the function of M2.1 is not completely understood, as recombinant HMPV can be recovered in the absence of M2.1 and viruses from which the M2.1 gene is deleted grow efficiently in vitro but not in vivo (28, 110). The 3′ (leader) and 5′ (trailer) ends contain the viral promoters necessary for replication and transcription. Transcription of paramyxoviruses is further directed by gene start (GS) and gene end (GE) sequences flanking each of the open reading frames (ORFs) in the viral genome. Transcription of the viral genome results in a gradient of transcripts, steadily decreasing toward the 5′ end of the genome. Thus, the gene order roughly reflects the relative amount of gene products required for efficient virus replication (Lamb & Parks, 2007).

A tool frequently used for the analysis of cis- and trans-acting elements influencing viral RNA synthesis is minireplicon systems. In such systems, all components of the viral polymerase complex are transfected and the replication and transcription of a synthetic vRNA-like molecule is measured using reporter genes. Exchanging proteins of the polymerase complex or synthetic vRNA-like molecules allows analysis of specificity and functional interactions. For the genera Respirovirus, Henipahvirus, and Pneumovirus of the family Paramyxoviridae it was shown that polymerase complexes provided by expression plasmids or co-infection, can replicate vRNA-like molecules of other viruses belonging to the same genus (101, 182, 264). vRNA-like molecules of the genus morbillivirus are efficiently replicated by polymerase

complex proteins of other morbilliviruses, but not or less efficiently by polymerase complexes consisting of proteins of two different morbilliviruses (3, 27). For pneumoviruses it has been shown that vRNA-like molecules based on AMPV-A can be replicated by the polymerase complex proteins of HRSV (159). For metapneumoviruses, it has been shown that polymerase complexes consisting of both human and avian metapneumovirus components are able to rescue virus from cDNA (90).

Chimeric viruses in which polymerase genes are exchanged between two related viruses are frequently used to generate attenuated vaccine strains (4, 90, 186, 212). We hypothesized that minireplicon assays in which polymerase complex components of human and avian metapneumoviruses are exchanged could provide a rational basis for the design of live, attenuated metapneumovirus vaccine strains. To this end, an AMPV-C minireplicon system was generated and used in combination with minireplicon systems for HMPV-A1 and -B1. Each of these sets of metapneumovirus polymerase complex proteins was able to replicate synthetic vRNA-like molecules of HMPV-A1 and -B1, AMPV-A and -C and HRSV, but not bovine parainfluenza virus type 3 (BPIV-3). To test the functional interaction of polymerase complex proteins of HMPV-A1 and -B1 and AMPV-C, vRNA-like molecules were co-transfected with different combinations of N, P, L and M2.1 expression plasmids, revealing that chimeric polymerase complexes were functional but with different efficiencies. Subsequently, several chimeric viruses were created that contained polymerase complex genes of HMPV-A1 and -B1 or HMPV-B1 and AMPV-C. Most of these chimeric viruses replicated with similar efficiency to the wild-type viruses in vitro. A subset of these viruses was tested for attenuation in Syrian golden hamsters and was found to replicate to lower titers than the wild-type viruses. This study provides insight in the specificity and functional interaction of polymerase complex proteins of human and avian metapneumoviruses.

METHODS

Cells, media and viruses

Vero-118 cells (143) were cultured in Iscove's modified Dulbecco's medium (IMDM; BioWhittaker, Verviers, Belgium) supplemented with 10% Fetal Calf serum (FCS, Greiner Bio-One, Alphen aan den Rijn, The netherlands), 100 IU of penicillin ml^{-1}, 100 µg of streptomycin ml^{-1}, and 2 mM glutamine as described previously. Baby hamster kidney cells stably expressing T7 RNA polymerase (BSR-T7) (a kind gift of dr K. Conzelmann, (29)) were grown in Dulbecco's modified Eagle medium (DMEM; BioWhittaker, Verviers, Belgium) supplemented with 10% FCS, nonessential amino acids, 100 IU of Penicillin ml^{-1}, 100 µg of streptomycin ml^{-1}, 2 mM glutamine and 0.5 mg of G418 (Invitrogen, Breda, The Netherlands) ml^{-1}. For HMPV rescue, Vero-118 cells and BSR-T7 cells were co-cultured in DMEM supplemented with 3% FCS, 100 IU of penicillin ml^{-1}, 100 µg of streptomycin ml^{-1}, 2 mM glutamine, and 0.25 mg trypsin ml^{-1}. For virus propagation and titration of HMPV-A1 and -B1, all chimeric viruses, and AMPV-C (Colorado strain, Intervet, Boxmeer, The Netherlands), Vero-118 cells were grown in IMDM supplemented with 4% bovine serum albumin fraction V (Invitrogen), 100 IU of penicillin ml^{-1}, 2 mM glutamine, and 3.75 µg of trypsin ml^{-1}.

Plasmids

The minireplicon systems of HMPV-A1 and -B1 have been described previously (110). The minireplicon system for AMPV-C was constructed using the same vectors, with primers designed on the basis of the published sequence of AMPV-C (Gene bank accession no. AY57978). For construction of the AMPV-C vRNA-like molecule, the leader and the GS of N and the trailer and GE of L were amplified by PCR and ligated, separated by two BsmBI sites. This fragment was ligated in a plasmid containing T7 RNA polymerase promoter (P_{T7}) and terminator (T_{T7}) sequences and a hepatitis delta ribozyme (pSP72-P_{T7}-d-T_{T7}, (110)) to yield pSP72-P_{T7}-Tr-Le-d-T_{T7}. The ORF of the chloramphenicol acetyltransferase (CAT) reporter gene was amplified by PCR and cloned in the BsmBI sites between the GS of N and GE of L to yield pSP72-P_{T7}-Tr-CAT-Le-d-T_{T7}. For the construction of plasmids expressing the polymerase complex proteins, N, P, and M2.1, ORFs of AMPV-C were amplified by PCR using primers spanning the start and stop codons and flanked by NcoI and XhoI sites, respectively, and were cloned in the multiple cloning site of pCITE (Novagen, Amsterdam, The Netherlands) to yield plasmids pCITE-N, pCITE-P, and pCITE-M2.1. Constructs encoding the L gene of AMPV-C were assembled from overlapping PCR fragments using restriction sites in the L gene and were cloned in pCITE. The restriction sites used were NcoI (introduced at nt 6935 before the start codon of L), ScaI (nt 8557), NdeI (nt 9770), and BclI (nt 11535) and XhoI (introduced at nt 13135 after the trailer). The minireplicon system of AMPV-A was a kind gift of dr A. Easton (170, 194). The minireplicon systems of BPIV-3 and HRSV (123) were a kind gift of dr R. Tang (MedImmune Vaccines.Inc., Mountain View, CA, USA)

The full-length HMPV cDNA plasmids for HMPV-A1 and -B1 have been described previously (110). For the construction of the full-length chimeric HMPV-B1 cDNA plasmids containing the N, N and P, P, M2.1 or L of HMPV-A1 or the N, P or L of AMPV-C cDNA, fragments of HMPV-B1 were amplified by PCR and cloned in pCR4TOPO (Invitrogen). All fragment were cloned such that type II restriction sites replaced the N, P, M2.1 or L ORFs and their GS and GE sequences. The N and P ORFs of AMPV-C and the N, N and P, P and M2.1 ORFs of HMPV-A1 were amplified by PCR using primers spanning GS and GE flanked by type II restriction sites. The L ORF and GS and GE of HMPV-A1 was assembled from overlapping PCR fragments using unique restriction sites in the L ORF and type II sites flanking GS and GE. For the construction of full-length chimeric HMPV-B1 cDNA plasmid containing the L of AMPV-C, fragments of HMPV-B1 were amplified by PCR and cloned in pBluescript SK^+ (Stratagene). The L ORF of AMPV-C was assembled from overlapping PCR fragments using unique restriction sites in the L ORF, and type II sites flanking GS and GE were introduced. Using unique restriction sites, the fragments containing the desired ORF were swapped back into the full-length HMPV-B1 cDNA plasmids. All plasmid inserts were sequenced to ensure the absence of undesired mutations. All primer sequences are available upon request.

Minigenome assays

BSR-T7 cells grown to 80-95% confluence in six-well plates were transfected with 1µg of the vector expressing the vRNA-like molecule, 1 µg pCITE-N, 0.5 µg pCITE-P, 0.5 µg pCITE-L, 0.5 µg pCITE-M2.1 and 0.4 µg of pTS27, a vector expressing b-galactosidase under the control of a cytomegalovirus immediate-early promoter (a kind gift of dr. M. Malim). Cells were analyzed 3 days after transfection by using enzyme-linked immunosorbent assays (ELISA) for CAT and

b-galactosidase (Roche Diagnostics, Almere, the Netherlands) according to the instructions of the manufacturer. All transfections were done in triplo and CAT values were standardized to 10 ng b-galactosidase to control for transfection efficiency and sample processing.

Recovery of recombinant virus

Recovery of recombinant HMPV was performed as described previously (Herfst et al., 2004). Briefly, BSR-T7 cells were transfected for 5 h with 5 µg full-length HMPV cDNA plasmid, 2 µg pCITE-N, 2 mg pCITE-P, 1 µg pCITE-L and 1 µg pCITE-M2.1 using Lipofectamine 2000 (Invitrogen). The HMPV-B1 polymerase expression plasmid set was used for the recovery of all chimeric HMPV-B1/HMPV-A1 and HMPV-B1/AMPV-C viruses. After transfection, the medium was replaced with fresh medium supplemented with trypsin. Three days after transfection, the BSR-T7 cells were scraped and co-cultured with Vero-118 cells for 8 days.

Virus titrations

Viruses were propagated in Vero-118 cells and virus titers were determined as described previously (110). Confluent monolayers of Vero-118 cells in 96-well plates (Greiner Bio-One) were spin-inoculated (15 min., 2000 x g) with 100 µl of 10-fold serial dilutions of each sample and incubated at 37°C. After 2 h and again after 3-4 days, the inoculum was replaced with fresh infection medium. Seven days after inoculation, infected cells were identified by immunofluorescence assays with HMPV-specific polyclonal antiserum raised in guinea pigs, as described previously (238). Titers expressed as 50% tissue culture infectious dose ($TCID_{50}$) were calculated as described by Reed and Muench (198).

Growth curves

Growth curves were generated as described previously (110). Flasks (25 cm^2) containing confluent Vero-118 cells were inoculated for 2 h at 37°C with HMPV-A1 or -B1, AMPV-C or one of the chimeric virus strains at a multiplicity of infection (MOI) of 0.1. After adsorption of the virus to the cells, the inoculum was removed and cells were washed twice with medium before the addition of 7 ml of fresh medium and incubation at 37°C. Every day, 0.5 ml of supernatant was collected and replaced with fresh medium. Plaque assays were performed to determine viral titers.

Plaque assays

Plaque assays were performed as described previously (110), with minor adjustments. Twenty-four-well plates containing 95% confluent monolayers of Vero-118 cells were inoculated with 10-fold serial virus dilutions for 1 h at 37 °C, after which the medium was replaced by 0.5 ml of fresh medium and 0.5 ml of 2% methylcellulose (MSD, Haarlem, the Netherlands) and cells were incubated at 37°C for 4 days. Methylcellulose overlays were removed and cells were fixed with 80% acetone. Cells were incubated with HMPV-specific polyclonal antiserum for 1 h at 37°C, followed by incubation with horseradish peroxidase-labeled rabbit anti-guinea pig antibodies (DakoCytomation, Heverlee, Belgium). Plaques were quantified after incubation with a freshly prepared solution of 3-amino-9-ethylcarbazole substrate chromogen (Sigma-Aldrich, Buchs, Switzerland) to determine viral titers.

Animal experiments

Six-week-old female Syrian golden hamsters (Mesocricetus auratus) (Harlan Sprague Dawley

Inq., Horst, The Netherlands) were inoculated intranasally with 10^6 $TCID_{50}$ of virus in 100 µl, diluted in PBS. Four days after inoculation, lungs and nasal turbinates (NT) were collected, snap-frozen immediately and stored at –80ºC until further processing. All intranasal inoculations and euthanasia were performed under anesthesia with inhaled isoflurane. All animal studies were approved by an independent Animal Ethics Committee and the Dutch authority for working with genetically modified organisms, and were carried out in accordance with animal experimentation guidelines. Tissues from the inoculated hamsters were homogenized using a Polytron homogenizer (Kinematica AG, Littau-Lucerne, Switzerland) in infection medium. After removal of tissue debris by centrifugation, supernatants were used for virus titration in Vero-118 cells. Titers were calculated as $TCID_{50}$ (g tissue)$^{-1}$, with a detection limit of $10^{1.6}$ and $10^{1.2}$ $TCID_{50}$ (g tissue)$^{-1}$ for NT and lung samples, respectively.

RESULTS

Replication of paramyxovirus vRNA-like molecules by heterologous polymerase complexes

To determine whether the polymerase complexes of different members of the family Paramyxoviridae recognize heterologous templates, vRNA-like molecules containing a CAT ORF in antisense orientation flanked by the genomic termini of HMPV-A1 and -B1, AMPV-A and -C, HRSV and BPIV-3 were used. Each of these plasmids was co-transfected in BSR-T7 cells with four plasmids expressing the N, P, L, and M2.1 proteins of HMPV-A1 or -B1, or AMPV-C. For the BPIV-3 system, the M2.1 expression plasmid was omitted as the virus does not need M2.1 for efficient replication and transcription (68). As expected, upon co-transfection of plasmids expressing the N, P, L and M2.1 protein together with their homologous vRNA-like molecules, the reporter gene CAT was expressed efficiently (Fig. 1). Polymerase complex proteins of HMPV-A1 and -B1 and AMPV-C could replicate the vRNA-like molecules of HMPV-A1 and -B1, AMPV-A and -C, and HRSV, but not BPIV-3. Conversely, the BPIV-3 polymerase complex only replicated the homologous vRNA-like molecule. The metapneumovirus polymerase complexes revealed little substrate specificity, as they replicated heterologous metapneumovirus vRNA-like molecules with similar efficiency to homologous molecules. vRNA-like molecules based on the HRSV genome were replicated less efficiently than the metapneumovirus vRNA-like molecules by the HMPV polymerase complexes.

Replication of metapneumovirus vRNA-like molecules by chimeric polymerase complexes

For morbilliviruses it was found that the vRNA-like molecules can be replicated by heterologous polymerase complexes but not or less efficiently by chimeric polymerase complexes (3, 27). To investigate the functional interaction between polymerase complex proteins of human and avian metapneumoviruses, the N, P, L and M2.1 expression plasmids were exchanged individually between the HMPV-A1 and -B1 and AMPV-C minireplicon systems (Fig. 2). All chimeric HMPV-A1/HMPV-B1 polymerase complexes were functional and replicated vRNA-like molecules with similar efficiency to the homologous complex protein sets (Fig. 2A and C). Chimeric polymerase complexes consisting of HMPV-A1 and AMPV-C or HMPV-B1 and AMPV-C components were functional but differed in their replication efficiency (2B, D – F).

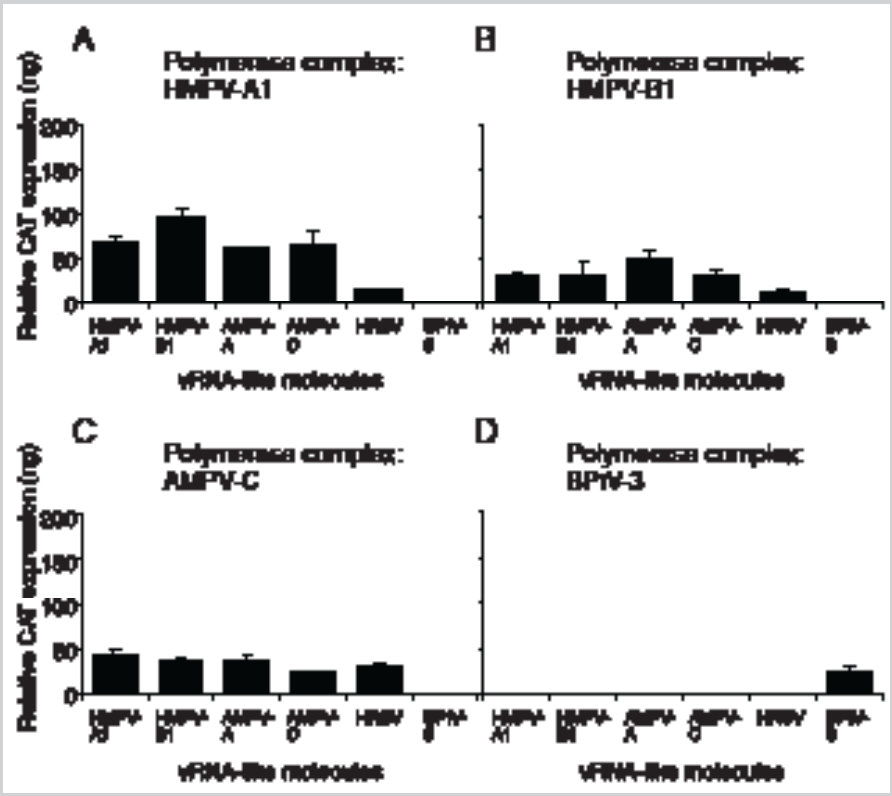

Figure 1
Replication of vRNA-like molecules by polymerase complexes of homologous or heterologous viruses. VRNA-like molecules were co-transfected into BSR-T7 cells with N, P, L and M2.1 expression plasmids and a plasmid expressing b-galactosidase. The means±SD of three independent transfection experiments are given. CAT values are standardized to 10 ng b-galactosidase.

Furthermore, HMPV-A1 and HMPV-B1 polymerase complex proteins appeared to be highly conserved, as they generally caused similar increases and decreases in replication efficiency when exchanged with those of AMPV-C (compare Fig. 2B and 2D or 2E and 2F). Chimeric HMPV-A1 (Fig. 2B) and HMPV-B1 (Fig. 2D) polymerase complexes in which the P protein was substituted with the P protein of AMPV-C were less efficient in the replication of vRNA-like molecules than the wild-type HMPV polymerase complexes. Smaller differences were observed when the N or M2.1 proteins were substituted. Chimeric polymerase complexes in which the HMPV-A1 or -B1 L protein was substituted with the L protein of AMPV-C replicated HMPV-A1 or -B1 vRNA-like molecules with higher efficiency compared with polymerase

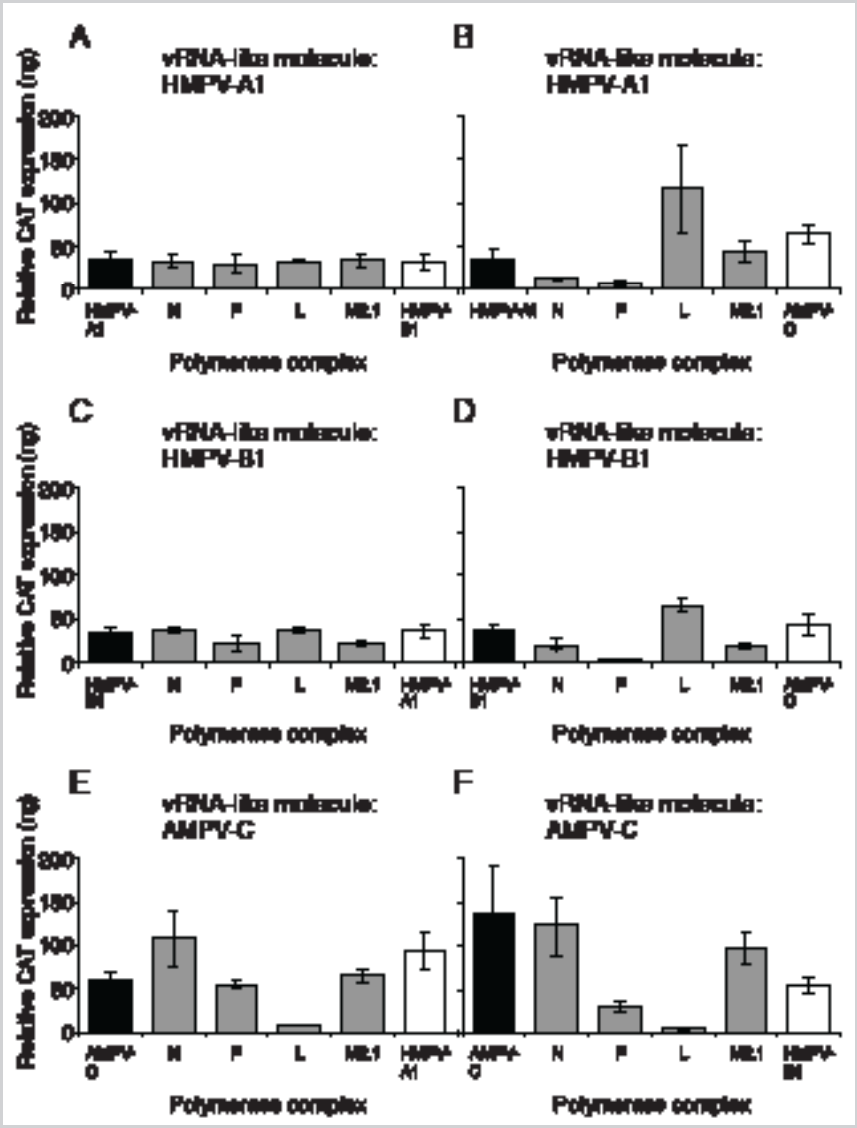

Figure 2

Replication of vRNA-like molecules by chimeric metapneumovirus polymerase complexes. VRNA-like molecules were co-transfected into BSR-T7 cells with their own N, P, L and M2.1 expression plasmids (black bars), chimeric sets of expression plasmids (grey bars), or the heterologous set of expression plasmids (white bars) and a plasmid expressing b-galactosidase. Plasmids supplied from a heterologous virus species are indicated along the x-axis. The means±SD of three independent transfection experiments are given. CAT values are standardized to 10 ng b-galactosidase.

complexes consisting of HMPV-A1 or -B1 or AMPV-C proteins only (Fig. 2B, 2D). In agreement with this observation, chimeric polymerase complexes in which the AMPV-C L protein was substituted with the L protein of HMPV-A1 or -B1 replicated AMPV-C vRNA-like molecules with lower efficiency compared to polymerase complexes consisting of HMPV-A1 or -B1 or AMPV-C proteins only (Fig. 2E, 2F). Substitution of the P protein of AMPV-C with P of HMPV-A1 resulted in similar CAT expression, whereas substitution with P of HMPV-B1 resulted in lower CAT expression. Substitution of the N or M2.1 proteins had less of an impact on replication efficiency. It should be noted that the M2.1 expression plasmid of pneumovirus and metapneumovirus minireplicon systems can be omitted without significant effects on the levels of CAT (44, 45, 110, 170).

Rescue of HMPV-B1 by chimeric polymerase complexes

As chimeric polymerase complexes consisting of human and avian metapneumovirus proteins revealed differences in minireplicon assays, we next tested wether it was possible to rescue recombinant HMPV using these chimeric polymerase complexes. The full-length HMPV-B1 cDNA plasmid was co-transfected into BSR-T7 cells with the N, P, L and M2.1 expression plasmids of HMPV-B1 or AMPV-C or sets in which the HMPV-B1 N, P, L and M2.1 expression plasmids were individually exchanged with those of AMPV-C. It was possible to rescue HMPV-B1 using the HMPV-B1, AMPV-C and all chimeric HMPV-B1/AMPV-C polymerase complexes (data not shown).

Replication characteristics of chimeric HMPV-B1/HMPV-A1 viruses in tissue culture

Minireplicon systems only include the components of the viral polymerase complex necessary for replication and transcription of the viral genome. To investigate the functionality of chimeric polymerase complexes in the context of a complete virus, a panel of chimeric viruses was made. The N, P, N and P, M2.1 and L genes of HMPV-B1 were replaced with those of HMPV-A1, resulting in HMPV-B1/$N_{HMPV-A1}$, HMPV-B1/$P_{HMPV-A1}$, HMPV-B1/$NP_{HMPV-A1}$, HMPV-B1/$M2.1_{HMPV-A1}$ and HMPV-B1/$L_{HMPV-A1}$, respectively. All chimeras could be rescued with efficiencies similar to HMPV-B1 (data not shown). Standard multi-step growth curves were generated to compare the growth of the chimeric viruses with those of the parental viruses HMPV-A1 and -B1 (Fig. 3). No apparent differences in replication kinetics could be observed between wild-type and chimeric HMPV-B1 viruses, indicating that the viruses containing chimeric polymerase complexes are fully functional in vitro, in agreement with the minireplicon assays.

Replication characteristics of chimeric HMPV-B1/AMPV-C viruses in tissue culture

To investigate further the functionality of chimeric HMPV-B1/AMPV-C polymerase complexes, a panel of chimeric viruses was made. The N, P and L genes of HMPV-B1 were replaced with those of AMPV-C, resulting in HMPV-B1/N_{AMPV-C}, HMPV-B1/P_{AMPV-C} and HMPV-B1/L_{AMPV-C}, respectively. All chimeras could be rescued with efficiencies similar to HMPV-B1 (data not shown). Standard multi-step growth curves were generated to compare the growth of the chimeric viruses with those of the parental viruses HMPV-B1 and AMPV-C (Fig. 4). This revealed that AMPV-C replicated faster than its human counterpart HMPV-B1. Furthermore, HMPV-B1/L_{AMPV-C} and HMPV-B1/N_{AMPV-C} grew to similar titers as the backbone virus HMPV-B1. In contrast, the HMPV-B1/P_{AMPV-C} grew to higher titers than HMPV-B1.

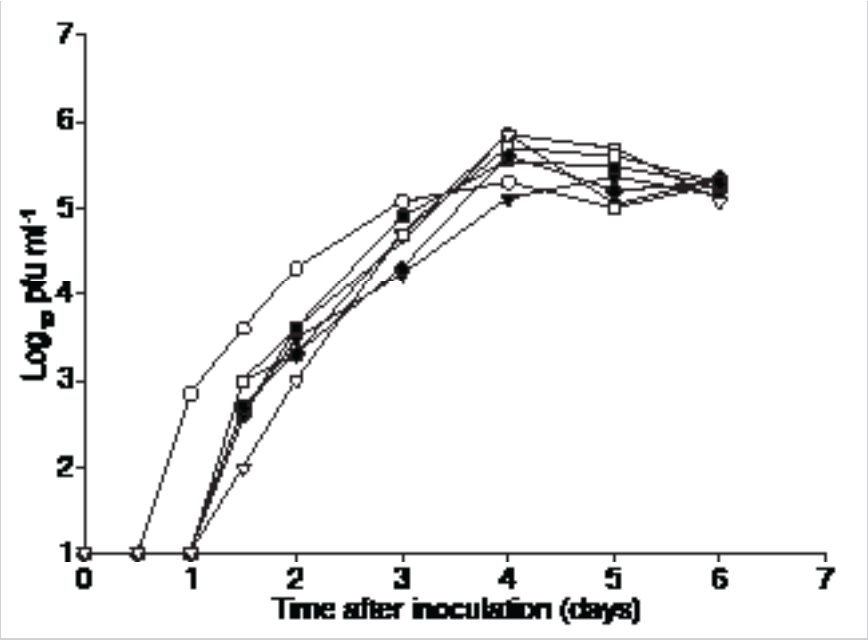

Figure 3

Replication kinetics of chimeric HMPV-B1/HMPV-A1 viruses. Vero-118 cells, inoculated at a MOI of 0.1 with HMPV-B1 (closed circle), HMPV-B1/$N_{HMPV-A1}$ (closed triangle down), HMPV-B1/$P_{HMPV-A1}$ (open triangle down), HMPV-B1/$NP_{HMPV-A1}$ (closed square), HMPV-B1/$M2.1_{HMPV-A1}$ (open square), HMPV-B1/$L_{HMPV-A1}$(closed diamond) and HMPV-A1 (open circle). Supernatants were collected daily and virus titers were determined by plaque assay. The results shown are representative of assays performed at least twice for each virus.

Characterization of chimeric HMPV-B1/AMPV-C viruses in a hamster model

The level of replication of the chimeric HMPV-B1/AMPV-C viruses in the upper and lower respiratory tract was evaluated in Syrian golden hamsters, which represent a permissive small-animal model for HMPV (155). Five groups (n=6) of hamsters were inoculated intranasally with 10^6 $TCID_{50}$ of the parental and chimeric viruses; the lungs and NT were harvested on day 4 post-infection, and the virus titers present in tissue homogenates were determined (Fig. 5). AMPV-C replicated to 100-fold higher titers in the lungs, but 10-fold lower titers in the NT compared to HMPV-B1. The HMPV-B1/N_{AMPV-C} and HMPV-B1/L_{AMPV-C} chimeric viruses did not replicate in the lungs and slightly less efficiently in the NT compared to HMPV-B1. HMPV-B1/P_{AMPV-C} did not replicate in the lungs and resulted in 10.000-fold lower titers in the NT compared with HMPV-B1.

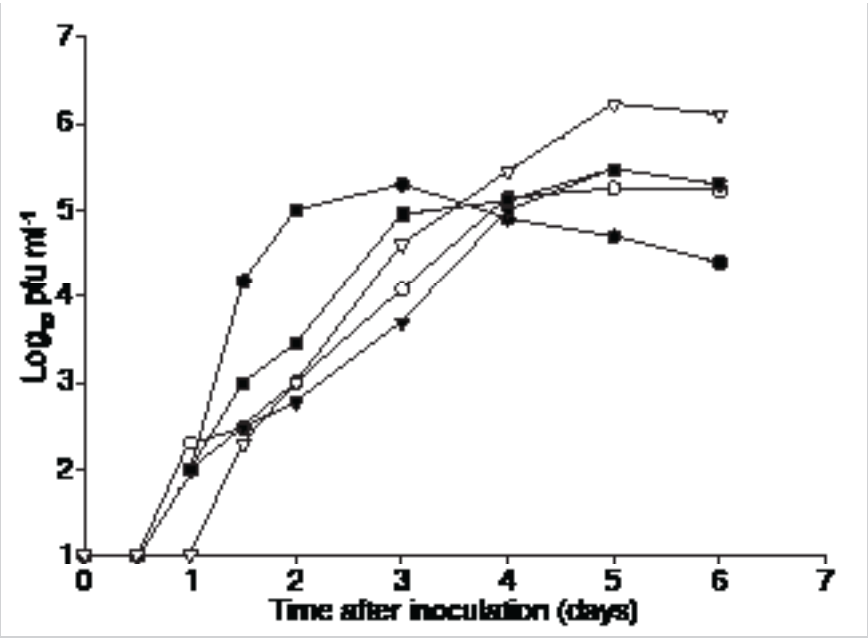

Figure 4
Replication kinetics of chimeric HMPV-B1/AMPV-C viruses. Vero-118 cells, inoculated at a MOI of 0.1 with HMPV-B1 (open circle), HMPV-B1/$_{NAMPV-C}$ (closed square), HMPV-B1/P_{AMPV-C} (open triangle down), HMPV-B1/L_{AMPV-C} (closed triangle down), or AMPV-C (closed circle). Supernatants were collected daily and virus titers were determined by plaque assay. The results shown are representative of assays performed at least twice for each virus.

DISCUSSION

Here, a newly developed minireplicon system for AMPV-C has been described and used to study the specificity and functional interactions of the polymerase complex proteins of human and avian metapneumoviruses. As expected, replication and transcription of metapneumovirus vRNA-like molecules was efficient when the homologous virus polymerase complex proteins were present. Replication and transcription of vRNA-like molecules of HMPV-A1 and -B1, AMPV-A and -C and HRSV also occurred when heterologous polymerase complex proteins derived from HMPV-A1 or -B1 or AMPV-C were provided. However, these polymerase complex proteins were not able to replicate the vRNA-like molecules representing BPIV-3. Thus the cis-acting elements in the genomic termini of HMPV-A1 and -B1, AMPV-A and -C and HRSV are conserved and functionally interchangeable. This is in agreement with the fact that the leader and trailer regions within the subfamily Pneumovirinae display a high degree of sequence conservation, but less so between pneumoviruses and BPIV-3 (Fig. 6). The N, P, L and M2.1 expression plasmids of AMPV-A and HRSV also gave rise to replication and transcription of vRNA-like molecules derived from viruses of the same genus but not other

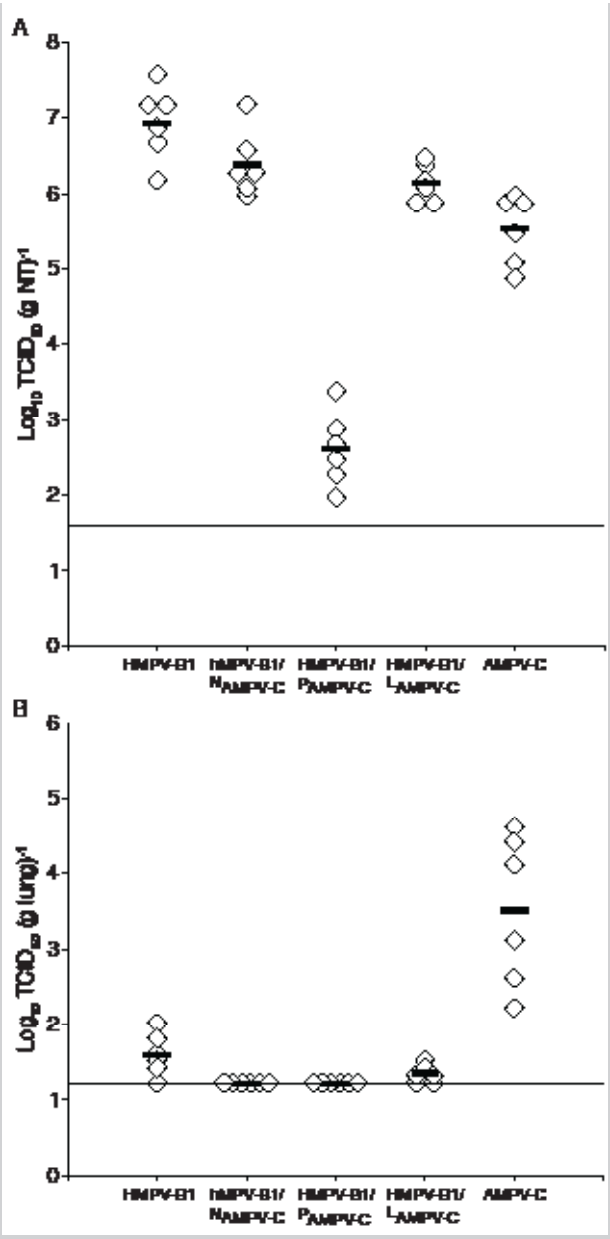

Figure 5

Evaluation of attenuation of HMPV-B1/AMPV-C chimeric viruses in Syrian golden hamsters. Infectious virus titers were determined in NT (A) and lungs (B) of hamsters inoculated with HMPV-B1, HMPV-B1/N_{AMPV-C}, HMPV-B1/P_{AMPV-C}, HMPV-B1/L_{AMPV-C}, and AMPV-C. NT and lungs were collected 4 days after inoculation. The lower limit of detection is indicated by the dotted line.

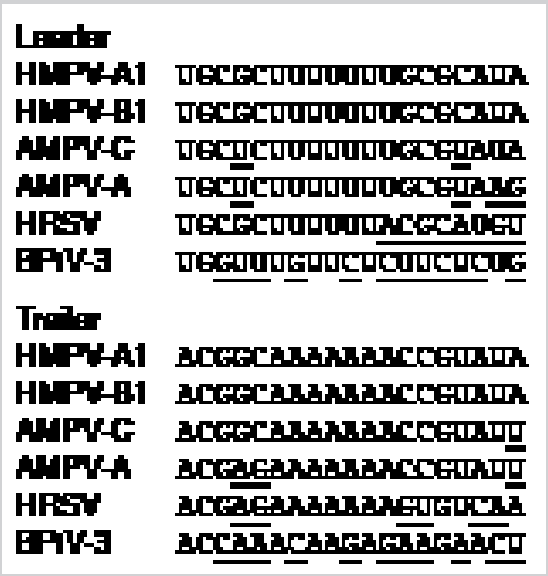

Figure 6
Alignment of HMPV-A1, HMPV-B1, AMPV-C, AMPV-A, RSV, and PIV-3 leader and trailer sequences. Differences in sequence compared with HMPV are underscored.

genera (data not shown). Because the polymerase protein expression vectors for AMPV-A and HRSV were far less efficient than for HMPV-A1 and -B1, AMPV-C and BPIV-3 under our experimental conditions, solid conclusions about specificity and functional interactions could not be made. However, it has been shown previously that HRSV and AMPV-A vRNA-like molecules can be replicated following heterologous or homologous infection with AMPV-A or HRSV or co-transfection with HRSV polymerase protein expression plasmids (159).

Chimeric polymerase complexes of members of the family Paramyxoviridae vary in their ability to replicate vRNA-like molecules or rescue recombinant virus (3, 27, 90). Exchanging polymerase genes between two related viruses with a different host range is a method frequently used for the design of live, attenuated vaccine strains (4, 90, 186, 212). Therefore, it was next tested wether minireplicon assays in which polymerase complex components of human and avian metapneumoviruses are exchanged could provide a rational basis for the design of live, attenuated metapneumovirus vaccine strains. To this end, HMPV-A1, -B1 and AMPV-C, vRNA-like molecules were co-transfected with different combinations of N, P, L and M2.1 expression plasmids to investigate the functional interaction between the polymerase complex proteins. All chimeric polymerase complexes based on HMPV-A1 and -B1 were functional and displayed similar replication and transcription efficiency to the wild-type polymerase complexes. In contrast, when polymerase complex proteins were exchanged between human and avian metapneumoviruses, CAT expression levels varied significantly. The most striking differences in CAT expression were observed when the L or P expression

plasmid was exchanged. When the P proteins of HMPVs were replaced with the P protein of AMPV-C CAT expression was lower than when the homologous P protein was used. When the L proteins of HMPVs were replaced with the L protein of AMPV-C, CAT expression increased compared with the wild-type polymerase complex, and, conversely, when the L protein of AMPV-C was replaced with L of HMPVs CAT expression decreased. Exchange of the N or M2.1 proteins between the HMPVs and AMPV-C in general had a modest effect. These data suggested that chimeric viruses consisting of HMPV-A1 and -B1 genes would replicate similarly to wild-type virus, whilst chimeras based on HMPV and AMPV-C, in particular those where L or P are exchanged, could display differences in virus replication.

Indeed, growth curves of chimeric HMPV-B1 viruses in which the N, P, L or M2.1 gene was replaced with that of HMPV-A1 revealed similar replication kinetics as the wild-type HMPV-B1. However, replication kinetics of HMPV-B1 with the N, P or L genes replaced with those of AMPV-C only showed some differences, and these differences were unexpected; exchange of P resulted in low activity in minigenome assays but high virus production, whereas exchange of L resulted in high activity in minigenome assays but had no effect on virus replication. Thus, there was poor agreement between minigenome assays and virus replication in vitro as measures of functional interactions between metapneumovirus polymerase complex proteins. In an attempt to explain this discrepancy, chimeric HMPV-B1/HMPV-A1 and HMPV-B1/AMPV-C viruses were rescued with reverse genetics and wild-type HMPV-B1 virus was rescued using chimeric polymerase complexes. However, because the resolution of the latter type of assay to read out polymerase activity is low, no differences in virus rescue efficiency were observed (data not shown). It was also tested whether differences in results obtained with minigenome and virus replication assays were due to the choice of cell lines, BSR-T7 and Vero-118, respectively. Minigenome assays performed with 293T and Vero-118 cells revealed similar trends of activity of HMPV-B1/AMPV-C polymerase complexes as those in Fig. 2D, with lower activity of N and P chimeric complexes, and relatively high CAT activity of the L chimeric complex. Moreover, when virus replication assays were performed in BSR-T7 cells, the same cell line used for minigenome assays, there still was no agreement between the minigenome assays and virus replication data (data not shown).

The functionality of chimeric polymerase complexes was next tested in a hamster model, using chimeric viruses that displayed the largest differences in minireplicon assays and virus replication curves; those based on HMPV-B1 and AMPV-C. Each of the three chimeric viruses, HMPV-B1/N_{AMPV-C}, HMPV-B1/P_{AMPV-C} and HMPV-B1/L_{AMPV-C} displayed some level of attenuation in the respiratory tract of inoculated hamsters. The virus that replicated to the highest titers in vitro, HMPV-B1/P_{AMPV-C}, was the most attenuated in hamsters with more than 10.000-fold reduced viral titers in the NT and undetectable virus titers in the lungs. Whilst this could suggest that minireplicon assays are more predictive for attenuation than in vitro replication assays, the opposite was true for the L chimera, which was attenuated in hamster but revealed increased levels of CAT expression in minigenome assays.

Exchanging genes between two related paramyxoviruses with different host range or replication properties has been shown useful for the rational design of live, attenuated vaccine strains (4, 90, 186, 212). Intuitively, the level of attenuation of such chimeric viruses would increase

when the relatedness of the two paramyxoviruses decreases. Our in vitro results suggest that this is indeed the case. Gene exchange between two HMPVs had no discernible effect on polymerase complex activity in vitro. Gene exchange between HMPV and AMPV-C resulted in modest differences in polymerase complex activity, in vitro and in vivo. It is likely that gene exchange between HMPV and AMPV-A, HRSV and other paramyxoviruses such as BPIV-3 would result in higher levels of attenuation with decreasing relatedness of the viruses.

For efficacious live, attenuated vaccine candidates, a proper balance between the level of attenuation and immunogenicity is crucial. In this context, evaluation of vaccine candidates in more than one animal model is needed. Recently, Pham et al evaluated chimeric HMPV/AMPV-C viruses in hamsters and African green monkeys (186). Recombinant HMPV (lineage A2) in which the N or P gene was replaced by that of AMPV-C, was found to grow to high titers in cell culture but were attenuated in hamsters and African Green Monkeys, indicating that such strains are promising HMPV vaccine candidates. Further studies in animal models and humans are required to study the level of attenuation, immunogenicity and stability of such live, attenuated vaccines; exchange of multiple genes rather than just one and/or exchange of genes from more distantly related viruses may be required to design the ideal live, attenuated vaccine.

From these studies, we conclude that neither minireplicon assays nor in vitro replication kinetics can be used as predictive models for attenuation of metapneumoviruses. Whilst minireplicon assays and in vitro replication assays remain crucial tools to study fundamental aspects of virus transcription and replication, animal model systems remain indispensable to evaluate the level of attenuation of e.g. live attenuated vaccine candidates.

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CHAPTER 7
FUSION PROTEIN IS THE MAIN DETERMINANT OF METAPNEUMOVIRUS HOST RANGE

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Submitted

ABSTRACT

Human metapneumovirus (HMPV) and avian metapneumovirus subgroup C (AMPV-C) infect humans and birds, respectively. We here confirmed the difference in host range in turkey poults, and analyzed the contribution of the individual metapneumovirus genes to host range in an in vitro cell culture model. Mammalian Vero-118 cells supported replication of both HMPV and AMPV-C in contrast to avian QT6 cells in which only AMPV-C replicated to high titers. Inoculation of Vero-118 and QT6 cells with recombinant HMPV in which genes were exchanged with those of AMPV-C revealed that the metapneumovirus F protein is the main determinant for cellular host range. Chimeric viruses in which polymerase complex proteins were exchanged between HMPV and AMPV-C replicated less efficiently compared to HMPV in QT6 cells. Using minigenome systems it was shown that exchanging these polymerase proteins resulted in reduced replication and transcription efficiency in QT6 cells. Examination of infected Vero-118 and QT6 cells revealed that viruses containing the F protein of AMPV-C yielded larger syncytia compared to viruses with the HMPV F protein. Cell content mixing assays revealed that the F protein of AMPV-C was more fusogenic compared to the F protein of HMPV, and that the F2 region is responsible for the difference observed between AMPV-C and HMPV F promoted fusion in QT6 and Vero-118 cells. This study provides insight into the determinants of host range and membrane fusion of metapneumoviruses.

INTRODUCTION

The Metapneumovirus genus (family Paramyxoviridae, subfamily Pneumovirinae) consists of two members, human metapneumovirus (HMPV) and avian metapneumovirus (AMPV). HMPV is a major cause of respiratory tract illnesses in humans, especially in young children, the elderly and immunocompromised individuals (54, 78, 238, 239, 254). Clinical symptoms range from mild respiratory problems to severe cough, bronchiolitis and pneumonia (239, 254). AMPV causes respiratory tract illnesses in domestic poultry (50). AMPV infections frequently get complicated with secondary bacterial infections resulting in air sacculitis and pneumonia (125), whereas AMPV infections without any secondary infections are mainly confined to the nasal turbinate and trachea (126, 245). AMPVs have been classified in four subgroups; A through D (9, 77, 130, 208) and AMPV-C is more closely related to HMPV than the other AMPV subgroups (91-93). It has been demonstrated that HMPV has probably originated from birds and that a common ancestor of AMPV-C and HMPV existed around 200 years ago (60).

The metapneumovirus genome is non-segmented and of antisense polarity. Genome replication requires the viral genomic RNA (vRNA) to be encapsidated in a ribonucleoprotein complex (RNP). This RNP comprises of the nucleoprotein (N), which encapsidates the viral genome, the phosphoprotein (P), the large polymerase protein (L), and the putative transcriptional elongation factor (M2.1). These polymerase complex proteins can be functionally exchanged between HMPV and AMPV-C both in minireplicon systems and chimeric viruses (58, 90, 186). Metapneumoviruses encode three membrane glycoproteins, the attachment (G) the small hydrophobic (SH) and the fusion (F) protein. The HMPV and AMPV-C SH and G proteins are type II membrane proteins that are not essential for virus replication in vitro (19, 170). Moreover, recombinant HMPV from which the SH gene was deleted replicated with normal kinetics in hamsters and monkeys (15, 19). In contrast, HMPV from which the G gene was deleted was viable but attenuated (15, 19). Thus, it appears that the F protein by itself can promote attachment and fusion. The F protein is a type I membrane protein and is synthesized as an inactive precursor F0 that is cleaved by host proteases into the functional subunits F1 and F2 (146, 200). The F protein mediates fusion of the virus envelope with the cell membrane during viral entry, and induces syncytium formation in infected cells (114, 207). For bovine respiratory syncytial virus (BRSV) and human respiratory syncytial virus (HRSV), the F protein determines the cellular host range (204).

It was previously suggested that HMPV can infect turkey poults, despite the inability to isolate viable virus from these animals (246). Here we show that AMPV, but not HMPV, can infect and replicate in turkey poults. To determine which genes contribute to this difference in host range, chimeric HMPVs were created that contained individual genes of AMPV-C. These chimeric viruses were tested in Quail fibroblast (QT6) cells, which are permissive for replication of AMPV-C but relatively non-permissive for HMPV. The F protein was found to be primarily responsible for this cellular host-range. Furthermore, all chimeric viruses in which polymerase component proteins were exchanged replicated to lower titers compared to HMPV. This phenomenon was investigated by exchanging polymerase components between HMPV and AMPV-C minireplicon systems, revealing that chimeric HMPV/AMPV-C polymerase

components were not functional in QT6 cells. This study provides insight in the determinants of metapneumovirus host range, specificity and functional interaction of the polymerase proteins and F protein promoted fusion.

MATERIALS AND METHODS

Cells, media and viruses

Subclone 118 of Vero-WHO cells (Vero-118 cells) (143) were cultured in Iscove's Modified Dulbecco's medium (IMDM, BioWhittaker, Verviers, Belgium) supplemented with 10% fetal calf serum (FCS, Greiner Bio-One, Alphen aan den Rijn, The Netherlands), 100 IU of penicillin ml-1, 100 µg of streptomycin/ml, and 2 mM glutamine as described previously (110). Baby hamster kidney cells stably expressing T7 RNA polymerase (BSR-T7), a kind gift of Dr K. Conzelmann (29), were grown in Dulbecco's Modified Eagle medium (DMEM, BioWhittaker) supplemented with 10% FCS, nonessential amino acids, 100 IU of Penicillin/ml, 100 mg of streptomycin/ml, 2 mM glutamine and 0.5 mg/ml of G418 (Invitrogen, Breda, The Netherlands). For HMPV rescue, Vero-118 cells and BSR-T7 cells were co-cultured in DMEM supplemented with 3% FCS, 100 IU of penicillin/ml, 100 µg of streptomycin/ml, 2 mM glutamine, and 0.25 mg trypsin/ml. For virus propagation and titration of HMPV, all chimeric viruses, and AMPV-C (Colorado strain, Intervet, Boxmeer, The Netherlands), Vero-118 cells were grown in IMDM supplemented with 4% bovine serum albumin fraction V (Invitrogen), 100 IU of penicillin, 2 mM glutamine, and 3.75 µg of trypsin/ml. QT6 cells were cultured in Medium 199 (Cambrex Bio Science Walkersville, MD, USA) supplemented with 5% FCS, 1% chicken serum, 5% triptose phosphate broth, 100 IU/ml penicillin, 100 µg/ml streptomyxin, 2 mM glutamine, 0.75 mg/ml sodiumbicarbonate and non-essential amino acids. For virus propagation 0.25 mg trypsin/ml was added. For purification and concentration, viruses were subjected to one freeze-thaw cycle after which cell-free supernatants were purified and concentrated using a 30-60% (w/w) sucrose gradient.

Plasmid construction

The full-length cDNA plasmids for HMPV and the full-length chimeric HMPV cDNA plasmids containing the N, P or L genus of AMPV-C have been described previously (58, 110). For the construction of the full-length chimeric HMPV cDNA plasmids containing the F, G, SH, or F and G genes of AMPV-C, fragments of HMPV were amplified by PCR and cloned into pBluescript SK^+ or pCR4TOPO (Invitrogen). All fragments were cloned such that type II restriction sites replaced the F, G or SH ORFs. The F, G and SH ORFs of AMPV-C were amplified by PCR using primers flanked by type II restriction sites and cloned into the pBluescript SK^+ or pCR4TOPO (Invitrogen) containing the HMPV fragments. Using unique restriction sites, the fragments containing the desired ORF were swapped back into the full-length HMPV cDNA plasmids. All plasmid inserts were sequenced to ensure the absence of undesired mutations. All primer sequences are available upon request.

A pCAGGS plasmid expressing HMPV F has been described previously (now renamed pCAGGS-Fh) (114). For the construction of the AMPV-C F expression plasmid, the AMPV-C F ORF was amplified by PCR and cloned into the multiple cloning site of pCAGGS, using

restriction sites EcoRI and XhoI to yield pCAGGS-Fa. Next chimeric F expression vectors were constructed in which the F1 or F2 region or the C-terminal region of F1 of HMPV and AMPV F were exchanged. For the chimeric F expression vectors in which the F1 and F2 were exchanged, the F1 and F2 regions were amplified separately by PCR using primers flanked by type II restriction sites for regions within the F gene and EcoRI or XhoI at the start of the F2 region and the end of the F1 ORF, respectively. These fragments were combined and cloned into pCAGGS resulting in pCAGGS-F2a-F1h and pCAGGS-F2h-F1a. For the chimeric F expression vectors in which the C-terminal region of F1 was exchanged, the first nt 1-1308 (aa 1 to 436) and nt 1309-1614/1620 (aa 437 to 538/540; the F gene of HMPV and AMPV differ 2 aa in length) of the HMPV and AMPV F ORFs were amplified separately by PCR using primers flanked by type II restriction sites for regions within the F gene and EcoRI or XhoI at the start of the F2 region and the end of the F1 ORF, respectively. These fragments were combined and cloned into pCAGGS resulting in pCAGGS-F2h-F1ha and pCAGGS-F2a-F1ah. All plasmid inserts were sequenced to ensure the absence of undesired mutations. All primer sequences are available upon request.

Animal experiments

Nicholas strain turkey poults (Meleagris gallopavo) were obtained from AMPV antibody-free parents and raised in isolation facilities. As a precaution the turkey poults were given Enrofloxacine 10% (5ml/10 litre water) until they were 11 days of age. At two weeks of age, serum samples were collected and tested for AMPV antibodies using the IDEXX Flockchek ELISA kit (IDEXX Laboratories, Westbrook, USA). At three weeks of age the poults were inoculated with 200 µl 10^5 or 10^7 $TCID_{50}$/ml of purified AMPV-C or HMPV, diluted in PBS (n=6). The poults were inoculated with a 50 µl volume in each conjuctival space and nostril. During the first experiment, swabs were collected from the choanal clefts and nostrils of turkey poults for a period of 10 days, using sterile cotton swabs (Greiner Bio-One), placed in 2 ml transport medium (Hanks balanced salt solution containing, 10% v/v glycerol, 200 U/ml penicillin, 200 µg/ml streptomycin, 100 U/ml polymyxin B sulfate, 250 µl/ml gentamycin (MP Biomedicals, Zoetermeer, The Netherlands), and stored at -80ºC. Ten days post infection (p.i.) serum samples were collected and tested for AMPV antibodies using the IDEXX Flockchek ELISA kit. For the second experiment, at three weeks of age the poults were inoculated with 10^5 $TCID_{50}$/ml of purified AMPV-C or HMPV, diluted in PBS (n=6). The poults were inoculated with 50 µl in each conjuctival space and nostril (total of 200 µl). Three, five and seven days after inoculation, nasal turbinates (NT), trachea and lungs were collected, snap-frozen immediately and stored at -80ºC until further processing. All animal studies were approved by an independent Animal Ethics Committee and the Dutch authority for working with genetically modified organisms, and were carried out in accordance with animal experimentation guidelines.

Virus titrations

Tissues from the inoculated poults were homogenized using a Polytron homogenizer (Kinematica AG, Littau-Lucerne, Switzerland) in infection medium. After removal of tissue debris by centrifugation, supernatants were used for virus titration in Vero-118 cells. Titers were calculated per gram tissue, with a detection limit of $10^{1.8}$, $10^{1.1}$ and $10^{0.7}$ $TCID_{50}$ per gram of tissue for NT, trachea and lung samples respectively. Choanal and nasal swab-material

were centrifuged to remove debris and supernatants were used for virus titration in Vero-118 cells.

Virus titers were determined as described previously (110). Confluent monolayers of Vero-118 cells in 96-well plates (Greiner Bio-One) were spin-inoculated (15 min., 2000 x g) with 100 µl of ten fold serial dilutions of each sample and incubated at 37°C. After 2 hours and again after 3-4 days, the inoculum was replaced with fresh infection media. Seven days after inoculation, infected wells were identified by immunofluorescence assays with HMPV-specific polyclonal antiserum raised in guinea pigs, as described previously (238). Titers expressed as 50% tissue culture infectious dose ($TCID_{50}$) were calculated as described by Reed and Muench (198).

Real-time RT-PCR

Viral RNA was isolated from 200 µl of choanal or nasal swab-material, using the MagnaPure LC system with the MagnaPure LC Total nucleid acid isolation kit (Roche Diagnostics, Almere, The Netherlands), with an elution volume of 50 µl. HMPV genome copies were detected by Taqman real-time PCR as described previously (156, 240).

Replication curves

Replication curves were generated as described previously (110). Twenty-five cm^2 flasks containing confluent Vero-118 or QT6 cells were inoculated for 2 h at 37°C with HMPV, AMPV-C or one of the chimeric viruses at a multiplicity of infection (MOI) of 0.1. After adsorption of the virus to the cells, the inoculum was removed and cells were washed two times with media before addition of 7 ml of fresh media and incubation at 37°C. Every day, 0.5 ml of supernatant was collected and replaced by fresh media. Plaque assays were performed in Vero-118 cells to determine viral titers as described previously (58).

Minigenome assays

QT6 cells grown in 6-wells plates were transfected with 1 µg of the vector expressing the vRNA-like molecule, 0.8 µg pCITE-N, 0.4 µg pCITE-P, 0.4 µg pCITE-L, 0.4 µg pCITE-M2.1, 0.4 µg pTS27 (a constitutive b-galactosidase expression vector, provided kindly by Dr M.H. Malim, King's College London School of Medicine, London, UK) and 1.5 µg pAR3126, a plasmid expressing T7 RNA polymerase (a kind gift of Dr. J. Dunn, Brookhaven National Laboratory, Upton, New York) using Lipofectamine 2000 (Invitrogen). Cells were analysed 3 days after transfection by ELISA for CAT and b-galactosidase (Roche Diagnostics) according to the instructions of the manufacturer. All transfections were carried out in triplicate and CAT values were standardized to 10 ng b-galactosidase to control for transfection efficiency and sample processing.

Cell content mixing assays

Cell content mixing assays in Vero-118 cells were performed as described previously (114). Two wells of a 6-well plate containing Vero-118 cells were each transfected with 2 µg of pCAGGS-F and 0.4 µg of pTS27, using Lipofectamine 2000 (Invitrogen). One well was co-transfected with 2 µg of pLTR-CAT (containing the chloramphenicol acetyl transferase (CAT) gene under control of the HIV-1 LTR) and the other well with 2 µg of pTat (expressing the HIV-1 trans-activator of transcription) (97). One-day post transfection, both cell populations were

harvested using trypsin-EDTA, mixed, and plated in three wells of a 6-well plate. At day three post transfection cell lysates were harvested and CAT and b-gal expression was quantified by ELISA. In this assay, cell content mixing, as a result of F-mediated fusion, resulted in tat-mediated transactivation of the HIV-1 LTR, and hence induction of CAT expression. CAT-expression was normalized based on b-gal expression.

Cell content mixing assays in QT6 cells were performed as described previously (195), with minor adjustments. Two wells of a 6-wells plate containing QT6 cells were each transfected with 2 µg of pCAGGS-F and 0.4 µg of pTS27, using Lipofectamine 2000. One well was co-transfected with 2 µg of pTM1-GFP (containing the green fluorescent protein (GFP) gene under control of the T7 RNA polymerase promoter) (a kind gift of Dr. J. Melero, Centro Nacional de Biologia Fundamental-Instituto de Salud Carlos III, Madrid, Spain) and the other well with 2 µg of pAR3126. One day post transfection, both cell populations were harvested using trypsin-EDTA, mixed, and plated in three wells of a 6-well plate. At day three after transfection, cell lysates were harvested and GFP and b-gal expression was quantified by ELISA (Bioconnect, Huissen, The Netherlands). In this assay, cell contents mixing, as a result of F-mediated fusion, resulted in T7 RNA polymerase-mediated expression of GFP. GFP-expression was normalized based on b-gal expression.

RESULTS

Replication characteristics of HMPV and AMPV-C in turkey poults

We first set up an infection model for HMPV and AMPV-C by inoculating turkeys with different virus doses. Four groups of six turkeys were inoculated with 2.10^4 or 2.10^6 $TCID_{50}$ HMPV or AMPV-C via the conjunctival spaces and nostrils. After inoculation, choanal and nasal swabs were collected daily. No virus was isolated from the nasal or choanal swabs from turkeys inoculated with 2.10^4 or 2.10^6 $TCID_{50}$ HMPV. Moreover, no virus was detected in HMPV inoculated animals by RT-PCR. In contrast, virus was isolated from choanal swabs of turkeys inoculated with 2.10^4 and 2.10^6 $TCID_{50}$ AMPV-C (Figure 1). For the animals inoculated with 2.10^6 $TCID_{50}$ AMPV-C, a clear peak of viral replication could be observed at day 2 p.i. followed by a rapid decrease in virus titers. No virus could be isolated from the nasal swabs. For the animals inoculated with 2.10^4 $TCID_{50}$ AMPV-C the increase and subsequent decrease in viral titers was more gradual compared with animals inoculated with 2.10^6 $TCID_{50}$ AMPV-C. Moreover, the duration of viral shedding was longer, since virus was still isolated from most animals at 7 days p.i, whereas for the animals inoculated with 2.10^6 $TCID_{50}$ AMPV-C only one animal shed virus at day 6 and no virus could be detected at day 7 p.i. Ten days p.i., serum samples from all animals were collected and tested in ELISA. Seroconversion was observed for all AMPV-C inoculated animals but not for HMPV inoculated animals (data not shown).

Although the peak of viral replication was slightly higher in the animals inoculated with 2.10^6 $TCID_{50}$ AMPV-C, the duration of virus shedding was shorter compared with animals inoculated with 2.10^4 $TCID_{50}$. Thus, based on these results subsequent experiments were conducted with 2.10^4 $TCID_{50}$ HMPV and AMPV-C. Next, two groups of 18 turkeys were inoculated with 2.10^4 $TCID_{50}$ HMPV or AMPV-C. Six animals from each group were euthanized at 3, 5 and

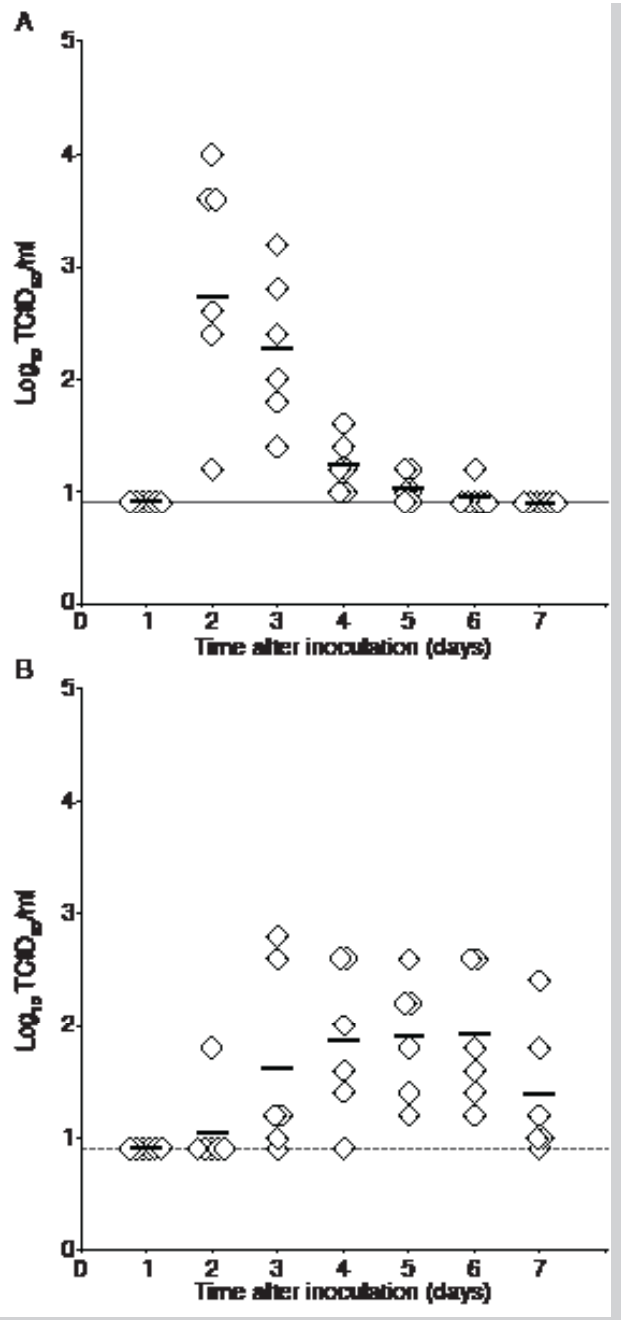

Figure 1

Detection of infectious virus in choanal swabs obtained from turkeys inoculated with 2.10^6 (A) or 2.10^4 (B) $TCID_{50}$ AMPV-C via the conjunctival spaces and nostrils. Virus was quantified by serial dilution in Vero-118 cell monolayers. The lower limit of detection is indicated by the dotted line.

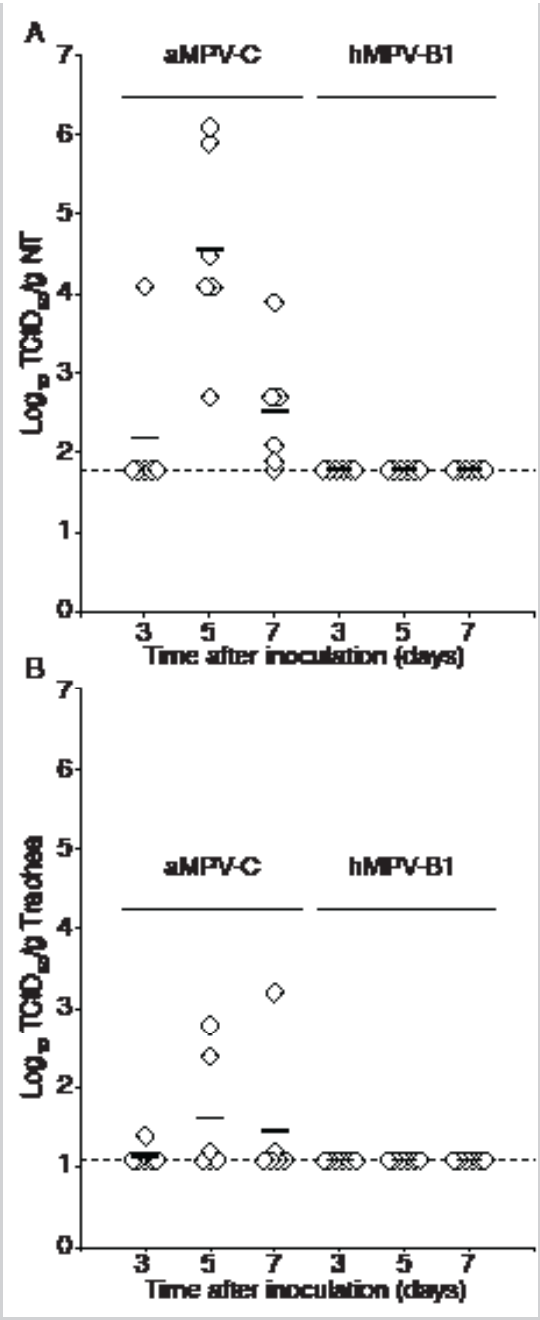

Figure 2

Infectious virus in nasal turbinates (A) and trachea (B) of turkeys inoculated with AMPV-C or HMPV. NT and trachea were collected at day 3, 4 or 7 p.i. Virus was quantified by serial dilution in Vero-118 cell monolayers. The lower limit of detection is indicated by the dotted line.

7 days p.i. and NT, trachea, and lung samples were collected (Figure 2). Virus titers in the NT of AMPV-C inoculated animals were highest at day 5 p.i. and virus could be isolated from all six animals. Virus titers observed at day 3 and 7 p.i. were at least 100-fold lower. In the trachea of AMPV-C inoculated animals, virus titers were higher at day 5 p.i. compared with day 3 and 7 p.i. No virus could be isolated from the lungs at day 3, 5 and 7 p.i. of the AMPV-C inoculated animals. No virus could be isolated from the NT, trachea or lungs at day 3, 5 or 7 p.i. of the HMPV inoculated animals. Based on these results, we concluded that HMPV does not replicate in turkey poults.

Permissiveness of cell lines for HMPV and AMPV-C

In order to set up an in vitro assay to study the host range difference observed in turkeys, several avian and mammalian cell lines were tested. Chicken embryo fibroblasts (CEF), Quail QT6 and QT35 cells supported efficient replication of AMPV-C but to a much lower extend that of HMPV. This difference between AMPV-C and HMPV replication was most pronounced in QT6 cells (Figure 3 and data not shown). In contrast to the avian cell lines, Vero-118 cells supported efficient replication of both HMPV and AMPV-C (Figure 3)(58).

Replication characteristics of chimeric HMPV/AMPV-C viruses in cell culture

To investigate which viral protein was responsible for the host range of HMPV and AMPV-C, a panel of chimeric viruses was made and tested in this in vitro model. The N, P, F, SH, G, F&G and L genes of HMPV were replaced with those of AMPV-C, resulting in HMPV/N_{AMPV-C}, HMPV/P_{AMPV-C}, HMPV/F_{AMPV-C}, HMPV/SH_{AMPV-C}, HMPV/G_{AMPV-C}, HMPV/$F\&G_{AMPV-C}$, and HMPV/L_{AMPV-C}.

First, multi-step replication curves were generated to compare the replication efficiency of the chimeric viruses with those of the parental viruses HMPV and AMPV-C in Vero-118 cells. AMPV-C replicated faster in Vero-118 cells than HMPV. Furthermore, HMPV/N_{AMPV-C}, HMPV/F_{AMPV-C}, HMPV/SH_{AMPV-C}, HMPV/G_{AMPV-C}, HMPV/$F\&G_{AMPV-C}$, and HMPV/L_{AMPV-C} replicated to similar titers as wild-type HMPV. In contrast, HMPV/P_{AMPV-C} replicated to higher titers than the wild-type HMPV as described previously (58, 186).

Next, multi-step replication curves were generated to compare the replication efficiency of the chimeric viruses with those of the parental viruses HMPV and AMPV-C in QT6 cells. AMPV-C replicated efficiently in QT6 cells in contrast to HMPV, which replicated to at least 1000-fold lower titers. HMPV/N_{AMPV-C}, HMPV/P_{AMPV-C}, HMPV/G_{AMPV-C} and HMPV/L_{AMPV-C} did not replicate in QT6 cells. In contrast, HMPV/SH_{AMPV-C} replicated to similar titers as the wild-type virus HMPV. HMPV/F_{AMPV-C} and HMPV/$F\&G_{AMPV-C}$ both replicated at least 10-fold more efficiently than HMPV, but less efficiently than AMPV-C. Since HMPV/G_{AMPV-C} was attenuated in QT6 cells compared to HMPV, these results indicated that metapneumovirus F protein was an important determinant of the cellular host range.

Replication of metapneumovirus vRNA-like molecules by chimeric polymerase complexes

All chimeric HMPV viruses in which polymerase complex proteins were exchanged with those of AMPV-C replicated to similar or higher titers as the wild-type virus in Vero-118, but where attenuated in QT6 cells. To investigate the functional interaction between the polymerase

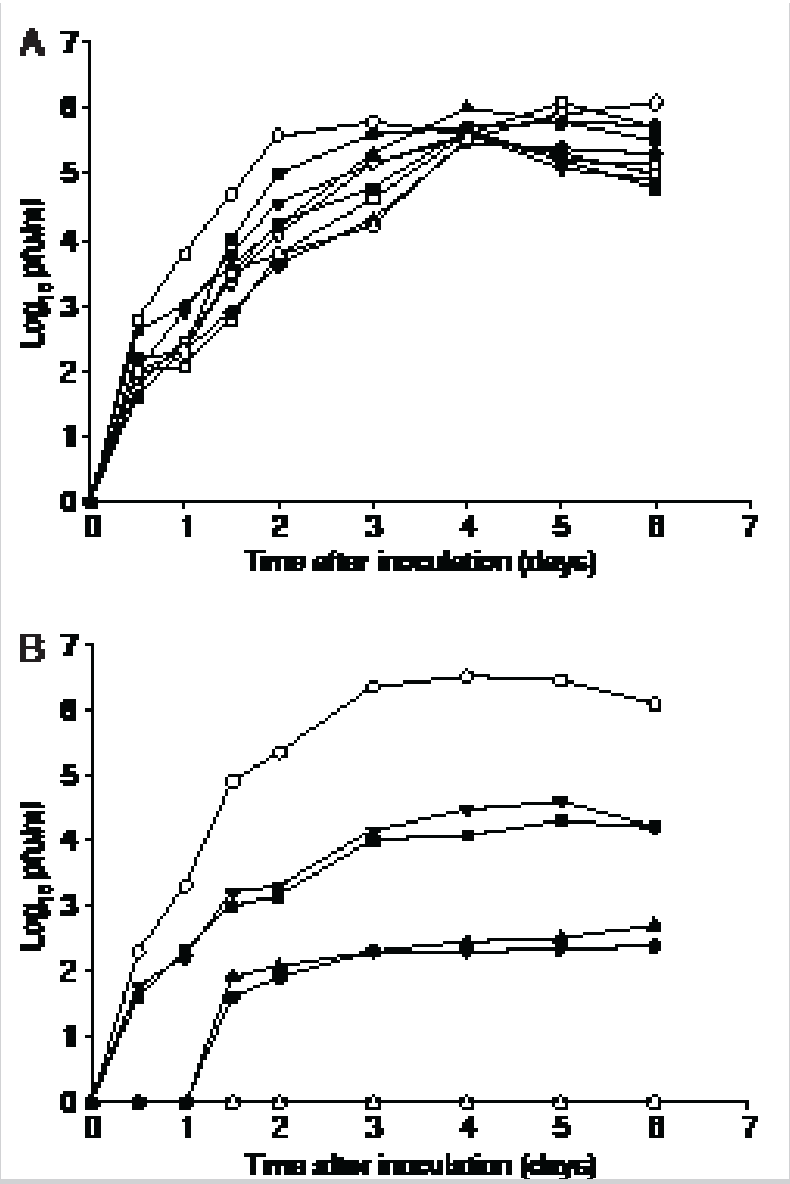

Figure 3

Replication kinetics of chimeric HMPV/AMPV-C viruses. Vero-118 (A) or QT6 (B) cells, inoculated at a MOI of 0.1 with HMPV (closed circle), AMPV-C (open circle), HMPV/N_{AMPV-C} (closed diamond), HMPV/P_{AMPV-C} (open diamond), HMPV/F_{AMPV-C} (closed triangle down), HMPV/SH_{AMPV-C} (closed triangle up), HMPV/G_{AMPV-C} (open triangle down), HMPV/F&G_{AMPV-C} (closed square), or HMPV/L_{AMPV-C} (open square). Supernatants were collected daily and virus titers were determined by plaque assay.

complex proteins of human and avian metapneumoviruses in QT6 cells as the cause of this attenuation, a minireplicon assay was performed. The HMPV and AMPV-C minireplicon systems were both tested and the HMPV N, P, L, and M2.1 expression plasmids were exchanged individually with those of AMPV-C to mimic the gene constellation of the chimeric viruses (Figure 4). The HMPV and AMPV-C minireplicon system were both functional in QT6 cells. However, the HMPV vRNA-like molecules were not replicated at all by the chimeric HMPV/AMPV-C polymerase complexes. This reduction in replication or transcription efficiency observed for the chimeric polymerase complexes could thus be responsible for the impaired replication of the chimeric viruses in QT6 cells.

Cytopathic effect (CPE) in Vero-118 and QT6 cells

Upon examination of Vero-118 and QT6 cells inoculated with HMPV, AMPV-C or chimeric HMPV/AMPV-C viruses, large differences in CPE were observed (Figure 5). HMPV/N_{AMPV-C}, HMPV/P_{AMPV-C}, HMPV/G_{AMPV-C}, HMPV/SH_{AMPV-C} and HMPV/L_{AMPV-C} replicated in Vero-118 cells to high titers with minimal CPE, in contrast to AMPV-C, HMPV/F_{AMPV-C} and HMPV/F&G_{AMPV-C} which induced large syncytia (Figure 5, left panels). Profound CPE was also observed in QT6 cells inoculated with AMPV-C, HMPV/F_{AMPV-C} and HMPV/F&G_{AMPV-C}. HMPV. HMPV/SH_{AMPV-C},

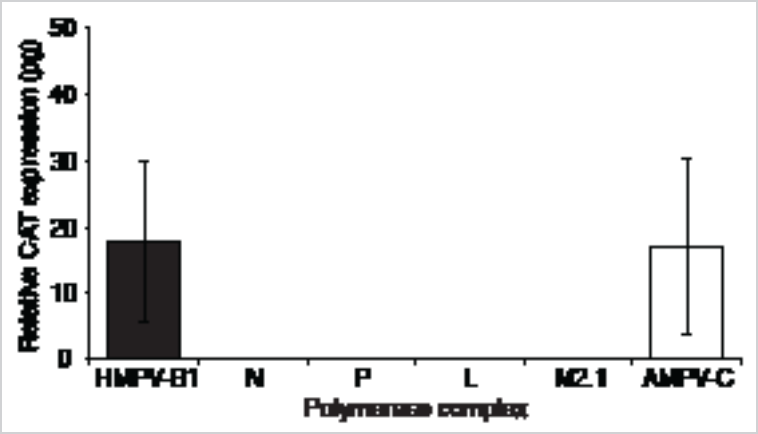

Figure 4

Replication of vRNA-like molecules by chimeric metapneumovirus polymerase complexes. VRNA reporter constructs were co-transfected into QT6 cells with their own N, P, L and M2.1 expression plasmids (HMPV, black bars; AMPV-C, white bars), or the HMPV vRNA reporter construct was co-transfected with mixes of these expression plasmids (grey bars) consisting of 3 HMPV expression plasmids and 1 AMPV-C expression plasmid as indicated. Plasmid expressing T7 RNA polymerase and a plasmid expressing b-galactosidase were co-transfected. The means and standard deviations of three independent transfection experiments are shown. CAT values were standardized to 10 ng b-galactosidase.

which only replicated to low titers in QT6 cells, displayed minimal CPE (Figure 5, right panels). CPE in HMPV/F&G_{AMPV-C} infected cells was not more pronounced compared to cells infected with HMPV/F_{AMPV-C}, suggesting that the F protein by itself was responsible for this difference.

Analysis of HMPV and AMPV-C F promoted cell-cell fusion

To further investigate and quantify the differences observed in CPE between metapneumoviruses expressing the AMPV or HMPV F protein, cell content mixing assays were performed. For this purpose, plasmids expressing the HMPV or AMPV F proteins were constructed (pCAGGS-Fh and pCAGGS-Fa). Furthermore, a set of chimeric F expression plasmids was constructed from which F1, F2 or the C-terminal of F1 were exchanged between HMPV F (Fh) and AMPV-C F (Fa), resulting in pCAGGS-F2a-F1h, pCAGGS-F2h-F1a, pCAGGS-F2h-Fha and pCAGGS-F2a-F1ah (Figure 6A). The chimeric F expression plasmids were tested in a cell content mixing assay in both Vero-118 (Figure 6B) and QT6 (Figure 6C) cells using an LTR-CAT or pT7-GFP based system, respectively. The LTR-CAT yielded higher signals compared to the pT7-GFP based system in Vero-118 cells, but the HIV-1 LTR was less functional in the avian cell line (data not shown).

In Vero-118 cells, higher levels of GFP activity were observed for the AMPV-C F protein than for HMPV F, indicating that AMPV F was more fusogenic than HMPV F in Vero-118 cells. The two chimeric F proteins that contained the F2 region of HMPV (F2h-F1a and F2h-F1ha), yielded similar or lower levels of CAT expression compared with the F protein of HMPV and lower levels compared with the F protein of AMPV-C. In contrast, the two chimeric F proteins that contained the F2 region of AMPV-C (F2a-F1h and F2a-F1ah) resulted in higher levels of CAT expression compared with the F protein of HMPV. The chimeric F protein with the complete F1 region of HMPV F yielded similar levels of CAT expression compared to the F protein of AMPV-C. The chimeric F protein from which only the last part was derived from HMPV F resulted in the highest levels of CAT expression, even higher than observed for wild-type F of AMPV-C

In QT6 cells, higher levels of CAT expression were observed for the AMPV-C F protein than for the HMPV F. Thus, AMPV F was more fusogenic than HMPV F in both Vero-118 and QT6 cells. Furthermore, the chimeric F proteins resulted in similar trends in fusion activity in Vero-118 and QT6 cells. Although in Vero-118 cells F2a-F1ah yielded the highest levels of CAT, F2a-F1h resulted in the highest levels of GFP in QT6 cells. In conclusion, the F2 region was responsible for the increase in fusogenic properties in both QT6 and Vero-118 cells.

DISCUSSION

It was recently suggested that turkey poults can be infected with HMPV (246). This conclusion was based on clinical signs and immunohistochemical findings only, as no seroconversion or recovery of infectious HMPV could be demonstrated (246). Inoculation with HMPV thus resulted in an abortive infection, while AMPV-C replicated efficiently. Here we show clear differences in host-range of HMPV and AMPV-C. An inoculum dose of 2.10^4 or 2.10^6 $TCID_{50}$ of AMPV-C resulted in robust infection of the respiratory tract of turkeys, and seroconversion after 10 days. After inoculation with 2.10^4 $TCID_{50}$, AMPV-C was detected at day 3, 5 and 7

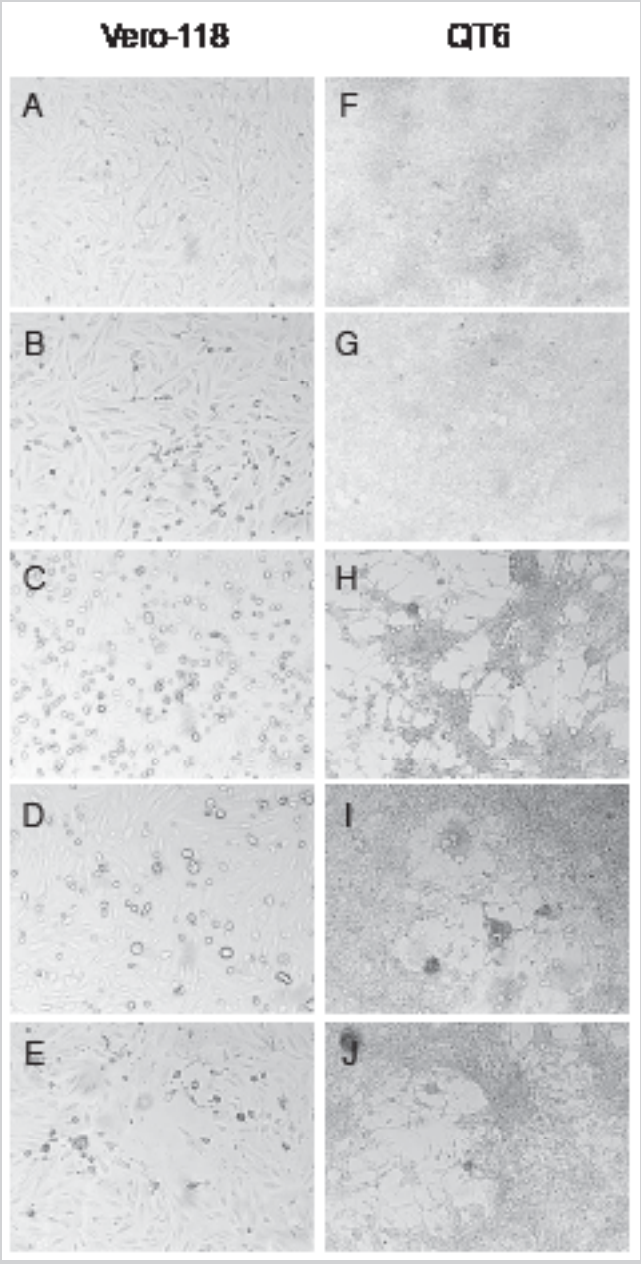

Figure 5
Cytopathic effect in Vero-118 (left panels) or QT6 (right panels), five days post inoculation with mock (A and F), HMPV (B and G), AMPV-C (C and H), HMPV/F_{AMPV-C} (D and I) or HMPV/F&G_{AMPV-C} (E and J) at a MOI of 0.1.

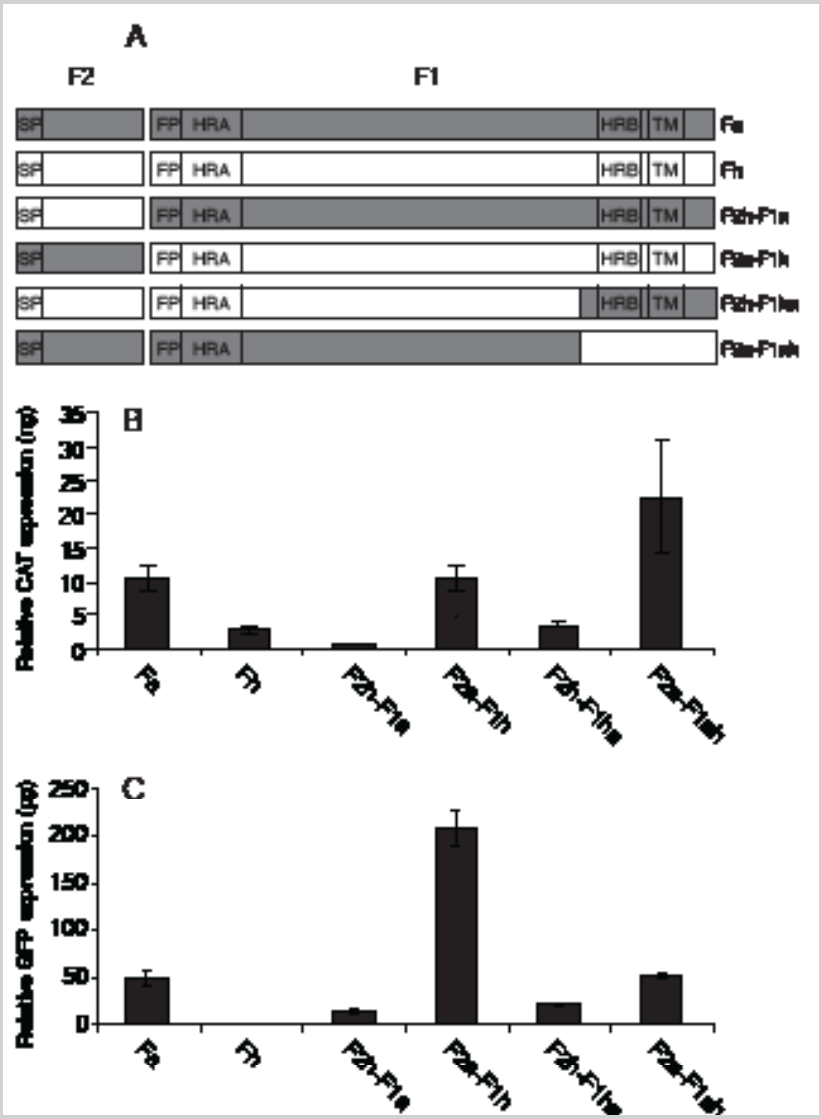

Figure 6

Organization of pCAGGS expressing the wild-type HMPV (white), AMPV-C (gray) or chimeric HMPV/AMPV-C F genes (A). Relevant portions of the F protein are indicated; F1 and F2 fragments; signal peptide (SP); fusion peptide (FP); heptad repeat A (HRA); heptad repeat B (HRB); transmembrane domain (TM). Evaluation of cell-cell fusion mediated by the AMPV-C and HMPV F proteins and chimeras thereof, as measured in cell content mixing assays in Vero-118 cells (B) or QT6 cells (C). The means±SD of two independent transfection experiments are shown. CAT and GFP values were standardized to 10 ng b-galactosidase.

p.i. in the NT and trachea with the peak of infection at day 5 p.i. No virus was isolated from the lungs, in agreement with previous experiments that demonstrated replication in the upper respiratory tract only (126). In sharp contrast, no infectious virus could be recovered from tracheal swaps of turkeys inoculated with an inoculum dose of 2.10^4 or 2.10^6 $TCID_{50}$ HMPV. These results were confirmed by RT PCR, using tests that are more sensitive than virus isolation (156). In addition, after inoculation with 2.10^4 $TCID_{50}$ HMPV no virus was detected in the NT, trachea or lungs at day 3, 5 or 7 days p.i. Therefore, we conclude that HMPV does not replicate in turkeys, in contrast to AMPV-C.

To investigate this difference in host range, an in vitro cell culture model was set up. Mammalian Vero-118 cells supported replication of both HMPV and AMPV-C. In contrast, Avian QT6 cells were highly permissive for AMPV-C replication, but did not result in efficient replication of HMPV. Replication curves in Vero-118 cells of chimeric HMPVs in which the N, P, SH, F, G, F&G and L genes were replaced with those of AMPV-C, revealed similar replication kinetics to the wild-type HMPV with the exception of HMPV/P_{AMPV-C} giving slightly higher virus titers. However, in QT6 cells large differences in replication kinetics were observed. QT6 cells supported efficient replication of AMPV-C but to a much lower extent that of HMPV. Recombinant HMPVs in which the N, P, G and L genes were replaced with those of AMPV-C replicated to lower titers than the wild-type virus. In contrast, the chimeric viruses containing the F protein of AMPV-C replicated more efficiently than HMPV. This indicated that the F protein was the main determinant for cellular host range of metapneumoviruses in vitro. Co-expression of the G protein did not increase replication efficiency, indicating that the difference observed in cellular host range was independent of the G protein. Interestingly, for HRSV and BRSV, similar differences in host range in vivo and in vitro have been described; using primary cell lines it was found that the RSV F protein is the main determinant for cellular host range (204).

The HRSV G protein has an important role in the attachment of viral particles to the host cell and it has been suggested that cellular glycosaminoglycans or heparin-like molecules are involved in this binding (99, 100, 142). The HRSV G protein was not essential for viral viability in vitro but HRSV from which the G gene was omitted was highly attenuated in vivo, as it does not replicate in the upper respiratory tract and at a very low level in the lower respiratory tract of mice (230). Similar to HRSV, HMPV and AMPV-C G proteins are not essential for virus replication in vitro (19, 170). Moreover, HMPV from which the G gene was deleted was attenuated but viable in hamsters and non human primates (15, 19). Thus, it appears that the F protein by itself can promote both attachment and fusion. The fact that chimeric HMPV containing the F protein of AMPV-C replicated to lower titers than AMPV-C could be due a functional restriction of one of the other HMPV proteins in QT6 cells because of host incompatibility.

Chimeric HMPVs containing polymerase complex proteins of AMPV-C did not replicate in QT6 cells. To test if this difference was due to differences in replication and transcription efficiency of the chimeric polymerase complexes, minireplicon assays were performed using similar chimeric polymerasecomplexes. Indeed, chimeric polymerase complexes were dysfunctional with prospect to replicating and transcribing the vRNA-like molecules. In contrast, in

mammalian cell lines, gene exchange between HMPV and AMPV-C resulted only in modest differences in polymerase complex activity both inminireplicon assays and in replication curves using chimeric viruses (58). HMPV did not replicate efficiently in QT6, QT35 (data not shown) and chicken embryo fibroblasts (CEF) (238). In contrast, AMPV-C was more promiscuous in cellular host range. Despite its avian origin, AMPV-C replicated to high titers in mammalian cell-lines such as Vero-118 cells, baby hamster kidney cells (BHK-21), baby grivet monkey kidney (BGM) and African green monkey kidney cells (MA-104)(234). Moreover AMPV-C can infect and replicate in hamsters (58). The fact that HMPV cannot infect birds or cells of avian origin, whereas AMPV-C can infect mammals or cells of mammalian origin, makes it more likely that HMPV is the result of a cross-species transmission event of an AMPV-C like virus from birds to humans, rather than the other way around.

HMPV is a poor inducer of syncytia formation in infected cultures, in contrast to AMPV-C. By comparing chimeric AMPV-C/HMPV viruses in cell cultures, it was found that the F protein caused this difference. To investigate this phenomenon, cell content mixing assays were performed. It was found that the F protein of AMPV-C was more fusogenic in Vero-118 and Q6 cells than HMPV. The region responsible for the difference in fusion was further mapped using chimeric AMPV-C/HMPV F expression plasmids. This analysis revealed that the F2 region of the F protein was responsible for the differences in fusion capacity.

From these studies, we conclude that AMPV-C and HMPV displayed clear differences in their ability to replicate in turkeys, and that the F protein was responsible for difference in cellular host range of HMPV and AMPV-C. The AMPV-C F protein was found to be more fusogenic than F of HMPV, both in avian and mammalian cells. Although the F protein was shown to be the main determinant of cellular host range, this cellular host range was not necessarily related to fusion efficiency itself. The difference in cellular host range and fusion efficiency may be caused by differential capacity to bind to cellular receptors. Future work should be directed to identify the virus receptors on the host cell that are recognized by the F proteins of HMPV and AMPV-C.

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CHAPTER 8
SMALL HYDROPHOBIC PROTEIN OF HUMAN METAPNEUMOVIRUS DOES NOT AFFECT VIRUS REPLICATION AND HOST GENE EXPRESSION IN VITRO

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In preparation

ABSTRACT

Human metapneumovirus (HMPV) encodes a small hydrophobic (SH) protein of unknown function. HMPV from which the SH open reading frame was deleted (HMPVDSH) was viable and displayed similar replication kinetics, cytopathic effect (CPE) and plaque size compared with wild-type HMPV in several cell-lines. In addition, no differences could be observed in infection efficiency or cell-to-cell spreading in human primary bronchial epithelial cells (HPBEC) cultured under air-liquid interphase conditions. Host gene expression was analyzed in A549 cells infected with HMPV or HMPVDSH using microarrays and mass spectrometry (MS) based techniques. No significant differences could be observed in mRNA or protein expression levels. A possible function of SH as apoptosis blocker, as proposed for other members of the family Paramyxoviridae, was rejected based on this analysis. So far, a clear phenotype of HMPV SH deletion mutants in vitro at the virus and host levels is absent.

INTRODUCTION

Since its discovery in 2001, the epidemiology, prevalence, and clinical signs of human metapneumovirus (HMPV) have been studied extensively (133, 238, 239, 253). Based on genetic and antigenic analyses, four sublineages of HMPV (A1, A2, B1 and B2) have been identified (238, 241). Reverse genetics systems are now available for all four sublineages facilitating fundamental and applied research (18, 110).

The non-segmented negative sense genome of HMPV encodes at least 9 putative open reading frames (ORFs); from the 3' to 5' ends: nucleoprotein (N), phosphoprotein (P), matrix protein (M), fusion protein (F), M2-1 and M2-2, small hydrophobic protein (SH), attachment protein (G), and large polymerase protein (L) (237). A possible function has been assigned based on homologies of closely related viruses such as the human respiratory syncytial virus (HRSV) for most of these ORFs. However, several studies have demonstrate that there are functional differences between the ORFs of HRSV and HMPV. For example, HRSV M2.1 was described as a transcriptional elongation factor that is required for virus viability (79), while recombinant HMPV can be recovered in the absence of M2.1 and HMPV M2.1 deletion mutants replicated efficiently in vitro but not in vivo (28, 110). Similarly, in contrast to the HRSV G protein, the HMPV G protein is not essential for replication in vivo (19).

The enveloped HMPV particles potentially contain three virus encoded transmembrane glycoproteins, the F, G and SH proteins. A function for the SH protein cannot readably be assigned for HMPV since its function is largely unclear for other members of the genus Pneumovirinae. The SH protein of mumps virus (MuV) is a type I transmembrane protein, whereas the SH proteins of other paramyxoviruses are type II transmembrane proteins. The HMPV SH protein was found to be expressed as several different forms in virus infected cells depending on the glycosylation status. There was an unglycosylated form (SH0), an N-glycosylated form (SHg1) and a more extensively glycosylated form (SHg2) (5, 19). The SH protein of HMPV is the largest among the members of the family Paramyxoviridae, 183 aa for HMPV-A and 177 aa for HMPV-B compared to avian metapneumovirus subgroup A (AMPV-A) (81 aa), AMPV-B (175 aa), AMPV-C (177 aa), HRSV (64 aa), bovine respiratory syncytial virus (BRSV) (81 aa), MuV (57 aa) and parainfluenza virus type 5 (PIV5) (44 aa). Sequence similarity between HMPV and AMPV-C was found to be low and there is no discernible sequence identity with the SH proteins of other viruses (237, 263).

HRSV from which SH was deleted was previously found to replicate efficiently in cell culture and slightly less efficient in the higher upper respiratory tract of mice and in the lower respiratory tract of chimpanzees (31, 248). In contrast, HMPV SH deletion mutants replicated with an efficiency similar to that of wild-type HMPV in the upper and lower respiratory tract of hamsters (19) and replicated only marginally less efficient in non-human primates (15). The SH proteins of other members of the family Paramyxoviridae have been suggested to act as a viroporin (185), or to have a function in blocking the TNF-a-mediated apoptosis pathway (5, 83, 151, 257). PIV5 from which the SH was deleted (PIV5DSH) was viable and displayed similar replication kinetics and plaque size compared to the wild-type virus, but caused increased cytopathic effect (CPE) in MDBK and L929 cells, via TNF-a-mediated apoptosis (107, 151).

To study the function of the SH protein of HMPV, SH deletion mutants were generated using a wild-type HMPV or HMPV encoding green fluorescent protein (GFP) as backbone (59). These deletion mutants replicated with similar efficiency as the parental viruses in Vero-118 cells and human primary bronchial epithelial cells (HPBEC) cultured under air-liquid interphase conditions. No major differences were observed in host gene or protein expression levels using microarrays and mass spectrometry (MS) based methods upon infection of the A549 lung fibroblast cell line with HMPV or HMPV SH deletion mutants. Based on this study it was concluded that the SH protein of HMPV has no identifiable function in the context of the virus and host cells in vitro.

METHODS

Cells and media

Vero-118 cells (143) were cultured in Iscove's Modified Dulbecco's medium (IMDM, BioWhittaker, Verviers, Belgium) supplemented with 10% Fetal Calf serum (FCS, Greiner Bio-One, Alphen aan den Rijn, The Netherlands), 100 IU of penicillin/ml, 100 µg of streptomycin/ml, and 2 mM glutamine as described previously (110). Baby hamster kidney cells stably expressing T7 RNA polymerase (BSR-T7, a kind gift of dr K. Conzelmann, (29) were grown in Dulbecco's Modified Eagle medium (DMEM, BioWhittaker) supplemented with 10% FCS, nonessential amino acids, 100 IU of Penicillin/ml, 100 µg of streptomycin/ml, 2 mM glutamine and 0.5 mg/ml of G418 (Invitrogen, Breda, The Netherlands). For HMPV rescue, Vero-118 cells and BSR-T7 cells were co-cultured in DMEM supplemented with 3% FCS, 100 IU of penicillin/ml, 100 µg of streptomycin/ml, 2 mM glutamine, and 0.25 mg trypsin/ml. For virus propagation and titration, Vero-118 cells were grown in IMDM supplemented with 4% bovine serum albumin fraction V (Invitrogen, Breda), 100 IU of penicillin, 2 mM glutamine, and 3.75 µg of trypsin/ml. A549 cells were cultured in HAM F12 medium containing 100 IU of penicillin/ml, 100 µg of streptomycin/ml, 2 mM glutamine and 10% FCS (Hyclone, Logan, USA). For virus infection, A549 were grown in HAM F12 medium containing 100 IU of penicillin/ml, 100 µg of streptomycin/ml, 2 mM glutamine and 4% bovine serum albumin fraction V (Invitrogen).

Subcultures of HPBEC were obtained from resected lung tissue as previously described (244). Briefly, HPBEC were grown in keratinocyte serum-free medium (KSFM, Invitrogen) supplemented with 0.2 ng/ml epithelial growth factor (Invitrogen), 25 µg/ml bovine pituitary extract (Invitrogen), 1 mM isoproterenol, 20 U/ml penicillin and 20 µg/ml streptomycin. For air-liquid inferphase cultures, cells were seeded ($1.5x10^5$ cells/cm^2) in DMEM/ bronchial epithelial cell medium (BEGM) 1:1 medium with supplements (Bulletkit; Lonza, Breda, The Netherlands) on 24-well transwell inserts (0.4 mm pore-size, 6 mm diameter; Corning Incorporated, Schiphol-Rijk, The Netherlands) that were precoated with 10 µg/ml bovine fibronectin (Sigma, Zwijndrecht, The Netherlands), 30 µg/ml Purecol (Inamed, Fremont, CA), and 10 µg/ml bovine serum albumin in PBS. When cell layers were confluent the apical medium was removed and basal medium was replaced with DMEM/BEGM supplemented with 10 nM retinoic acid (Sigma, Zwijndrecht, The Netherlands). Cells were allowed to differentiate for at least 2 weeks.

Full-length cDNA vectors

The full-length cDNA plasmids for HMPV-B1 and HMPV-B1 expressing the green fluorescent protein have been described previously (59, 110). For the construction of the full-length HMPV-B1 cDNA plasmids lacking the SH ORF, fragments of HMPV-B1 spanning the gene start (GS) of SH or the gene end (GE) of SH were amplified by PCR by primers flanked by type II restriction sites, and cloned into pBluescript SK^+. Using the type II restriction sites, fragments were cloned such that the GS of SH, was directly ligated to the GE of SH, thus omitting the SH ORF. Using unique restriction sites, the fragment lacking the SH ORF was swapped back into the full-length HMPV-B1 cDNA plasmids. All plasmids were sequenced to ensure the absence of undesired mutations. All primer sequences are available upon request.

Recovery of recombinant virus

Recovery of recombinant HMPV was performed as described previously (110). Briefly, BSR-T7 cells were co-transfected for 5 hours with 5 µg of the full-length HMPV cDNA plasmid, 2 µg pCITE-N, 2 µg pCITE-P, 1 µg pCITE-L and 1 µg pCITE-M2.1 using Lipofectamine 2000 (Invitrogen). After transfection, the media was replaced with fresh media supplemented with trypsin. Three days after transfection, the BSR-T7 cells were scraped and cocultured with Vero-118 cells for 8 days.

Virus titrations

Viruses were propagated in Vero-118 cells and virus titers were determined as described previously (110). Confluent monolayers of Vero-118 cells in 96-well plates (Greiner Bio-One) were spin-inoculated (15 min., 2000 x g) with 100 µl of ten fold serial dilutions of each sample and incubated at 37°C. After 2 hours and again after 3-4 days, the inoculum was replaced with fresh infection media. Seven days after inoculation, infected wells were identified by immunofluorescence assays with HMPV-specific polyclonal antiserum raised in guinea pigs, as described previously (238). Titers expressed as 50% tissue culture infectious dose ($TCID_{50}$) were calculated as described by Reed and Muench (198).

Replication curves

Replication curves were generated as described previously (110). Twenty-five cm^2 flasks (Greiner Bio-One) containing confluent Vero-118 cells were inoculated for 2 h at 37°C with HMPV, HMPV-GFP, HMPVDSH or HMPVDSH-GFP at a multiplicity of infection (MOI) of 0.1. After adsorption of the virus to the cells, the inoculum was removed and cells were washed two times with medium before addition of 7 ml of fresh medium and incubation at 37°C. Every day, 0.5 ml of supernatant was collected and replaced by fresh media. Plaque assays were performed to determine viral titers (110).

Biological materials for RNA isolation and protein purification

A549 cells were seeded at 500.000 cells/well in a 6 wells plate the day before inoculation. These plates were spin-inoculated (15 min., 2000 X g) with HMPV or HMPVDSH and incubated at 37°C. After 2 hours, the inoculum was replaced with fresh infection media. After 24 hours, cells were stained with HMPV-specific polyclonal antiserum and analyzed by FACS. Cells were washed twice with PBS and homogenized in 1 ml TRIzol® reagent (Invitrogen) and stored at -80°C.

RNA isolation, quality control, and Affymetrix microarray hybridization

The TRIzol® homogenates were processed according to the manufacturer's instructions (Invitrogen). Total RNA was isolated and purified using the RNeasy Mini kit (Qiagen, Hilden, Germany): 250 µl of ethanol was added to the upper aqueous phase of the processed TRIzol samples and directly transferred to the RNeasy spin columns for purification. RNA concentrations and OD 260/280 ratios were measured with the NanoDrop® ND-1000 UV-VIS spectrophotometer (NanoDrop Technologies, Wilmington, USA). Assessment of total RNA quality and purity was performed with the RNA 6000 Nano assay on the Agilent 2100 bioanalyzer (Agilent Technologies, Palo Alto, CA, USA). CDNA was synthesized from 2.4 µg total RNA using the One-Cycle Target Labeling kit (Affymetrix, Santa Clara, CA, USA). Biotin-labelled cRNA synthesis, purification and fragmentation were performed according to standard protocols. Fragmented biotinylated cRNA was subsequently hybridised onto Affymetrix Human Genome U133 Plus 2.0 microarray chips, which were scanned with the Affymetrix GeneChip Scanner 7G.

Normalization of Microarray data

All processing of data and statistics were performed in Bioconductor version 2.1, run in R version 2.6.1. The raw intensity values obtained from the scanner (the CEL files) were preprocessed using the expresso function of the affy package (version 1.16.0) (88) and the bioconductor metadata package (hgu133plus2, version 2.0.1). In the expresso function call, parameters were set to use the perfect match (PM) probe intensities only and no background correction. Normalization was performed by the variance stabilization and calibration (VSN) algorithm (package version 3.2.1). In this method, arrays are first made comparable by shifting and scaling of array values to an average reference array and probe expression values are then subjected to a variance stabilizing transformation (119). Finally, the transformed probe values were summarized into one value per probe set by the median polish method that is part of the robust multiarray averaging (RMA) method.

Statistical analysis of Microarray data

Probe set wise comparisons between the experimental conditions were performed by linear models of microarray data (Limma) (version 2.12.0) (217). Correction for multiple testing was achieved by requiring a false discovery rate (FDR) of 0.05, calculated with the Benjamini-Hochberg procedure (10).

Protein purification

Protein samples were processed as follows. After centrifugation of the TRIzol® homogenates and removal of the RNA containing aqueous phase, proteins were isolated from the organic phase. Fifty µl of the organic phase was washed with acetone (-20°C) and precipitated by centrifugation at 10.000 x g three times. Next, 100 µl 0.1% w/v RapiGestTM SF reagent (Walters, Milford, MA, USA), dissolved in 50 mM ammonium bicarbonate, pH 7.0 (Merck, Darmstadt, Germany) was added and sonicated with an Ultrasonic Disruptor Sonifier® II (Branson Ultrasonics, Danbury, Connecticut, USA) at an energy setting of 70%, for a maximum of 5 min at 20°C until the pellet was dissolved. Gold grade trypsin (Promega, Madison, WI, USA) was added to the protein solution at 0.1 g/L and incubated overnight at 37°C. Next 10

µL of 500 mM HCl was added (final concentration: 30-50 mM HCl, pH<2) and incubated for 45 min at 37ºC.

Samples were then processed for proteomics-analyses, performed by a combination of two MS techniques. For peptide quantification, Matrix-assisted laser desorption/ionisation (MALDI) Fourier Transform Ion Cyclotron Resonance Mass Spectrometer (FT-MS) was used, which measures peptide masses with an accuracy better than 1 ppm, with a dynamic range of signal intensities of circa 3-4 orders of magnitude. Identification of the peptides was performed by off-line LC-MALDI-TOF/TOF. Next, the masses of the identified peptides were linked to the MALDI-FT-MS peptide signal intensities to enable fold change analyses of identified proteins (63), as outlined below.

MALDI-FT-MS

MALDI-FT-MS was performed as described previously (168). Samples were spotted onto a 600/384 AnchorChip target plate (Bruker Daltonics, Leipzig, Germany) in duplicate. Each sample (0.5 µl) was mixed on the spot with 0.5 µl of a 2.5-dihydroxybenzoic acid matrix solution (10 mg/ml in 0.1% TFA in water), and then the mixture was allowed to dry at ambient temperatures. The MALDI-FTMS measurements were performed on a Bruker Apex Q intrument with 9.4-tesla magnet (Bruker Daltonics). For each measurement, 200 scans of 10 shots each were accumulated with 60% laser power. Mass spectra were acquired in the mass range of 800-4000 Da. FTMS spectra were processed with a Gaussian filter and two zero fillings.

MALDI-FTMS external and internal calibration

A standard peptide calibration mixture (Bruker Daltonics, Leipzig, Germany), which contains angiotensins I and II, substance P, bombesin, renin substrate, human adrenocorticotropic hormone (ACTH) clip 1-17, ACTH clip 18-39, and somatostatin 28 was used for external calibration. To obtain better mass accuracies, an additional post-acquisition internal calibration step in DataAnalysis version 3.4, build 169 software (Bruker Daltonics) was performed. Ubiquitous actin peptide masses (mass-to-charge (m/z) 1198.70545, 1515.74913, 1790.89186, 2215.06990, and 3183.61423) were used for internal calibration. Peak intensities were retrieved using a peak finding algorithm that determines the highest peak intensity within a 3 ppm window at both sides of each m/z value present above a signal to noise ratio of >4, as described previously (233).

MALDI-TOF/TOF-MS

Trypsin digested samples were run on an Ultimate 3000TM Nano LC System (Dionex, Sunnyvale, California, USA). One ml of the sample was loaded onto the monolith trap column (200-mm inner diameter x 5 cm) with a gradient of eluent A (100% H2O, 0.05% trifluoracetic acid (TFA)) and eluent B (80% acetonitrile (CAN), 20% H2O, 0.04% TFA): 0–5 min, 10% eluent B; 70 min, 50%; 71 min, 90%; 76 min, 90%; 77–100 min, 0% using a flow rate of 1.5 µl/min . Collection was started at 5 min. Fifteen-second fractions of the sample were spotted automatically onto a 384 prespotted AnchorChip plate (Bruker Daltonics) containing a-cyano-4-hydroxycinnamic acid matrix using a robotic system (Probot Micro Fraction Collector, Dionex). To each fraction 1 µl of water was added. Finally, we used a 10 mM $(NH)_4H_2PO_4$ in 0.1% TFA, water solution to wash the prespotted plate for 5 s to remove salts. The plates were subsequently measured by automated MALDI-TOF/TOF (Ultraflex, Bruker Daltonik

GmbH, Leipzig, Germany) using WARP-LC software. This software obtains MS spectra of each individual spot and subsequently performs MS/MS on each peptide. The best spots for performing the MS/MS measurements were determined automatically by the WARP-LC software. A file containing the MS and the MS/MS peak lists was submitted to the MASCOT search engine, version 2.1. (Matrix Science, London, UK) using the MSDB database (release 09-08-2006) allowing 150-ppm parent mass tolerance, 0.5 Dalton fragment tolerance, and one missed trypsin cleavage site.

RESULTS

Replication characteristics of HMPV SH deletion mutants in tissue culture

To investigate the function of the HMPV SH protein, SH deletion mutants were generated by removing the SH ORF, resulting in HMPVDSH. A similar deletion mutant was generated using a recombinant HMPV expressing GFP as backbone virus, resulting in HMPVDSH-gfp. Replication curves were generated to compare the replication kinetics of HMPV SH deletion mutants with those of parental

HMPV (Figure 1). There were no apparent differences in replication kinetics between wild-type HMPV and HMPVDSH confirming that the SH protein is dispensable in vitro in Vero-118 cells. Moreover, HMPV-gfp and HMPVDSH-gfp replicated to similar titers as wild-type HMPV as well. For RSV the deletion of SH did not alter the replication kinetics or production of syncytia but did result in plaques which were 70% larger than plaques produced by wild-type RSV (31). To investigate the effect of the HMPV SH deletion on CPE, Vero-118 cells infected with HMPV and HMPVDSH were photographed after five days post inoculation (Figure 2, left panels). CPE was indistinguishable between HMPV and HMPVDSH infected Vero-118 cells; both viruses yielded focal rounding and detachment of cells. Plaque assays performed with Vero-118 cells inoculated with HMPV or HMPVDSH and overlaid with methylcellulose revealed that plaque sizes were similar (Figure 2, right panels).

Replication characteristics of HMPV and HMPVΔSH in HPBEC

HMPV is known to replicate primarily in ciliated respiratory epithelial cells in macaques (143). Therefore we next investigated the phenotype of HMPV and HMPVDSH in air-liquid interphase cultures of HPBEC, as these cultures have a pseudo stratified epithelial phenotype and consist of both ciliated and mucus producing cells, similar to airway epithelium in vivo (244). Cells were pre-treated with lysophosphatidylcholine (LPC) to enhance infection efficiency, and inoculated with HMPV or HMPVDSH. Every day, fresh medium containing trypsin was added and mucus overlays were washed. HMPV and HMPVDSH both replicated in HPBEC without obvious CPE and no differences were observed in virus spread or the number of virus infected cells between the two viruses (Figure 3A and C). A separate staining specific for ciliated cells was performed, revealing that primarily ciliated cells were infected with HMPV (Figure 3B and D).

mRNA profiling of HMPV and HMPVΔSH infected A549 cells

To identify and characterize differences in host gene expression between HMPV and HMPVDSH infected cells, microarray analysis was performed. To this end, A549 cells inoculated with wild-

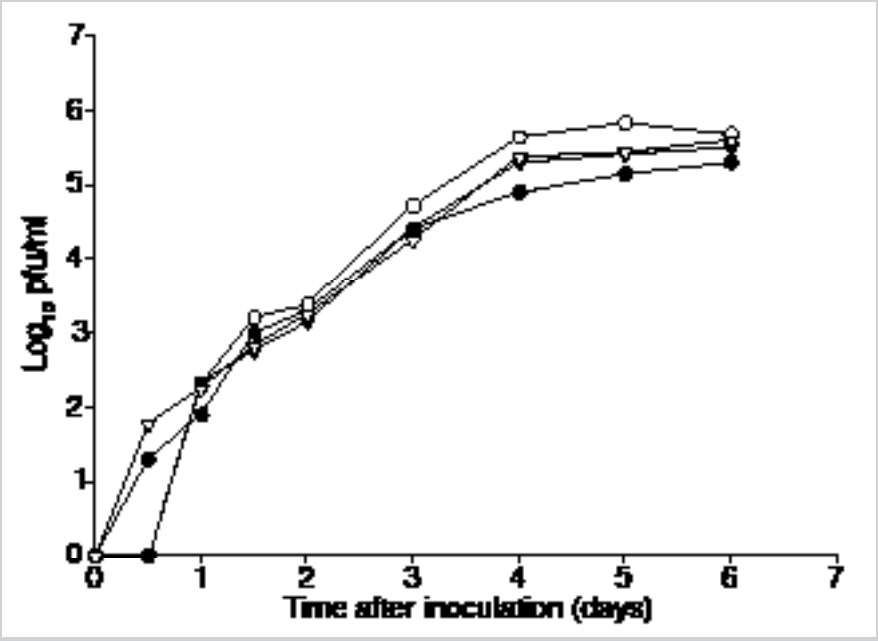

Figure 1
Replication kinetics of HMPV SH deletion mutants. Vero-118 cells were inoculated at a MOI of 0.1 with HMPV (closed circle), HMPVDSH (open circle), HMPV-gfp (closed triangle down) and HMPVDSH-gfp (open triangle down). Supernatants were collected daily and virus titres were determined by plaque assay.

type HMPV or HMPVDSH were harvested twenty-four hours after infection for further analysis. Differentially expressed genes between mock and HMPV infected cells were identified by limma analysis and visualized using Spotfire DecisionSite 9.0 (Spotfire, Somerville, MA). Analysis revealed that of the 54.000 mRNA transcripts represented by probesets on the Affymetrix HG-U133 plus 2 GeneChip, 523 transcripts were differentially expressed in HMPV infected cells compared to mock infected cells at a false discovery rate of smaller than or equal to 0.05 on a $\log_2$ fold change larger than or equal to one (Figure 4). These 523 differentially expressed transcripts represent 366 distinct genes. Differential expression in A549 cells infected with HMPV ranged from a 2-fold reduction to a 6,9-fold ($\log_2$) increase compared to the mock control. The largest fold ($\log_2$) change in upregulation was found for genes that are associated with innate immune responses (Table 1). Down regulated genes were often associated with regulatory roles in cell cycle replication (table 2). Comparing HMPV and HMPVDSH infected cells directly revealed the differential expression of only four transcripts, out of the 54.000 measured. The expression of these four transcripts was slightly upregulated in HMPV infected cells compared to HMPVDSH-infected cells (approximately 1,2 fold, $\log_2$ scale) and related to only two genes: metastasis associated lung adenocarcinoma transcript 1 (MALAT1) and high mobility group AT-hook 2 (HMGA2). The MALAT1 gene encodes a large non-coding RNA.

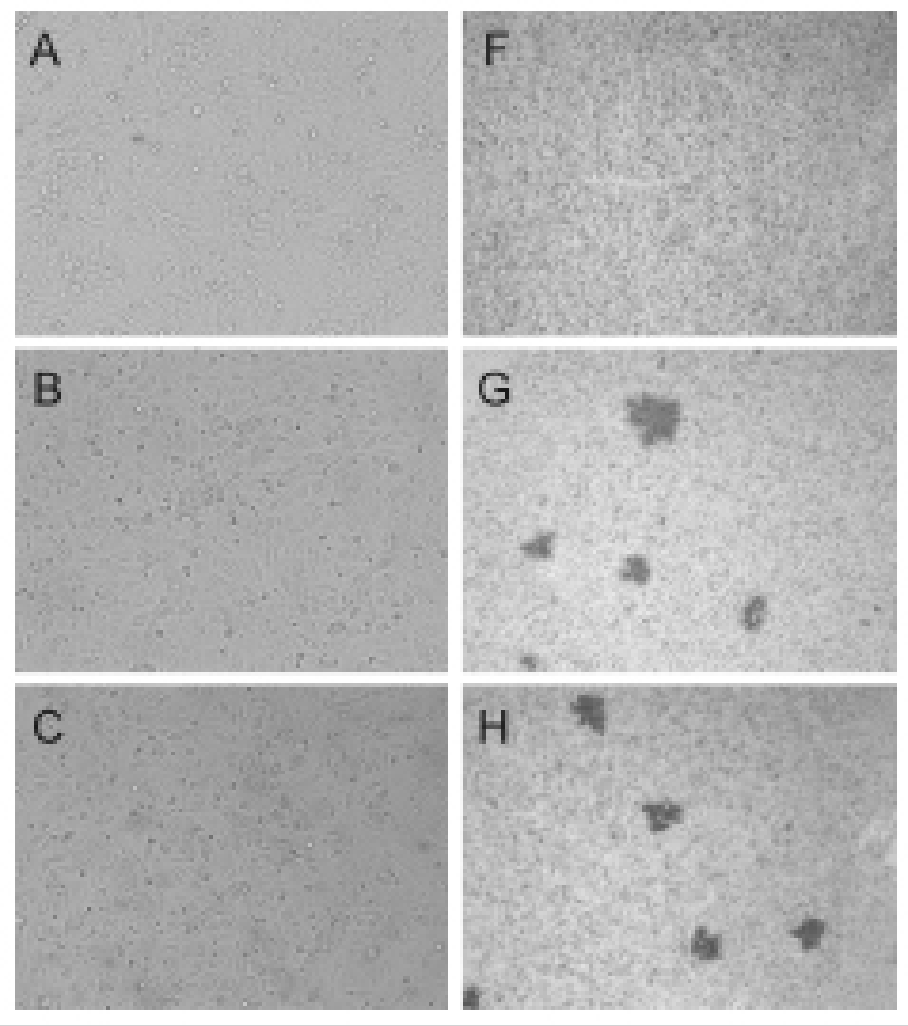

Figure 2

Cytopathic effect (CPE, left panels) or plaques (right panels) in mock (A and D), wild type HMPV (B and E) or HMPVDSH (C and F) inoculated Vero-118 cells. Left panels, Vero-118 cells were inoculated at a MOI of 0.1, were photographed without further treatment 6 days after inoculation. Right panels, Vero-118 cells were inoculated and incubated under a methylcellulose overlay. Plaques were visualized by immunostaining 6 days after inoculation.

Large non-coding RNAs are mRNA-like and perform regulatory functions (75, 120). On the Affymetrix HG-U133 plus 2 GeneChip, MALAT1 is represented by 12 probesets, 3 of which targeted differentially expressed mRNA transcripts. HMGA2 encodes a protein from the non-histone chromosomal high mobility group and may act as a transcriptional regulating factor. HMGA2 is represented by 9 probesetes, only 1 of which targeted differentially expressed mRNA transcripts. Taken together HMPV infection induced a profound perturbation of mRNA expression levels, but the mRNA expression profiles of HMPV and HMPVDSH at 24 hours p.i. were remarkably similar if not identical

Peptide profiling of HMPV and HMPVΔSH infected A549 cells

Samples of A549 cells infected with HMPV and HMPVDSH that were used for the microarray analysis were also analyzed using mass spectrometry-based techniques. For peptide profiling, samples collected at 12, 24 and 36 hours p.i. were analyzed. When applying a signal to noise ratio of 10, approximately 800 mass peaks could be identified in a typical A549 derived peptide profile. On average, these peaks relate to 500 peptides originating from about 400 distinct proteins. When comparing peptide profiles of HMPV infected cells with mock infected cells obtained at the same time point, up to 100 mass peaks differing in signal intensity were observed. In addition to host peptides that were differentially expressed upon infection, several peptides specific for the HMPV N, P and M2.1 proteins could be detected at 12, 24 and 36 p.i. in the HMPV and HMPVDSH infected cells but not in the mock infected cells (data not shown). The HMPV SH protein was not detected, this could be due to low expression levels, the relatively small size of the protein or glycosylation status. When comparing the peptide profiles derived from HMPV and HMPVDSH infected cells directly, not a single host derived peptide was differentially expressed. Thus, the HMPV SH protein did not alter protein expression profiles in infected A549 cells.

DISCUSSION

Despite years of research, not much is known about the function of the SH protein of members of the genus Pneumovirinae. For HRSV the SH protein can be deleted without major effects on virus replication in vitro (19, 31, 124). Similar to HRSVDSH, HMPV SH deletion mutants replicated as efficient as wild-type HMPV in Vero-118 cells an did not display differences in CPE and plaque size. This is in agreement with the results described for HMPV by others using LLC-MK2 cells (19). Replication curves of HMPV and HMPVDSH in cell lines such as MDBK, 293T and A549 cells also did not reveal any differences in replication kinetics or CPE (data not shown). HMPV is known to replicate primarily in ciliated respiratory epithelial cells in macaques (143). For that reason HPBEC might represent a more suitable in vitro cell culture model. HPBEC cultures consist of polarized cells with a pseudostratified mucociliary histology that is very similar to bronchial epithelium in vivo. HPBEC cultures are derived from lung tissue samples and contain a wide variety of cells, including mucus producing cells and ciliated cells. Similar to RSV (265), both HMPV and HMPVDSH were primarily detected in ciliated cells. No differences were observed in cell-to-cell spread, the number of virus infected cells or CPE in these cell cultures.

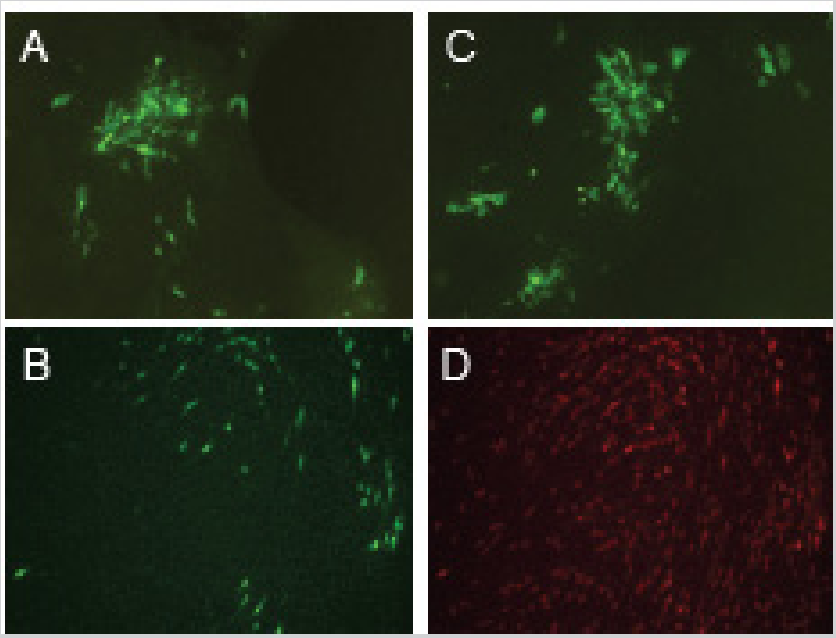

Figure 3
HPBEC cultured at air-liquid interphase were inoculated with wild type HMPV (A, B and D) or HMPVDSH (C) at a MOI of 4. Six days after inoculation, infected cells were visualized by immunostaining with HMPV specific polyclonal anti-serum (A, B and C). Ciliated cells were detected by staining with anti ß-tubulin antibodies (D).

The deletion of the SH protein of PIV5 resulted in an increase of CPE and apoptosis in several cell lines, such as A549, MDBK, HeLaT4 and L929 (107). The PIV5 SH protein was shown to block the TNF-a-mediated apoptosis in L929 cells. Assays with chimeric PIV5DSH expressing the SH proteins of other viruses revealed that the MuV and RSV SH proteins have a similar function in blocking the TNF-a-induced NF-kB activation (83, 257). Infection of Vero-118 with PIV5DSH resulted in a major increase in CPE compared to PIV5 (data not shown). In contrast, Vero-118 or A549 cells infected with HMPV and HMPVDSH did not display this phenotype. Thus, the deletion of HMPV SH does not result in a similar change of phenotype as the deletion of PIV5 SH in vitro.

RNA microarray analysis of A549 cells infected with HMPV or HMPVDSH revealed that out of the 54.000 measured, only 4 transcripts, representing 2 genes, MALAT1 and HMGA2, were differentially expressed and slightly upregulated 24 hours p.i. Both genes were represented by multiple probesets. For MALAT1 and HMGA2 3 out of 12 and 1 out of 9 probe sets were found to react differentially, respectively. HMPV infection pertubated the transcriptome network considerably but the SH protein appeared to be inert in this perspective. Other groups

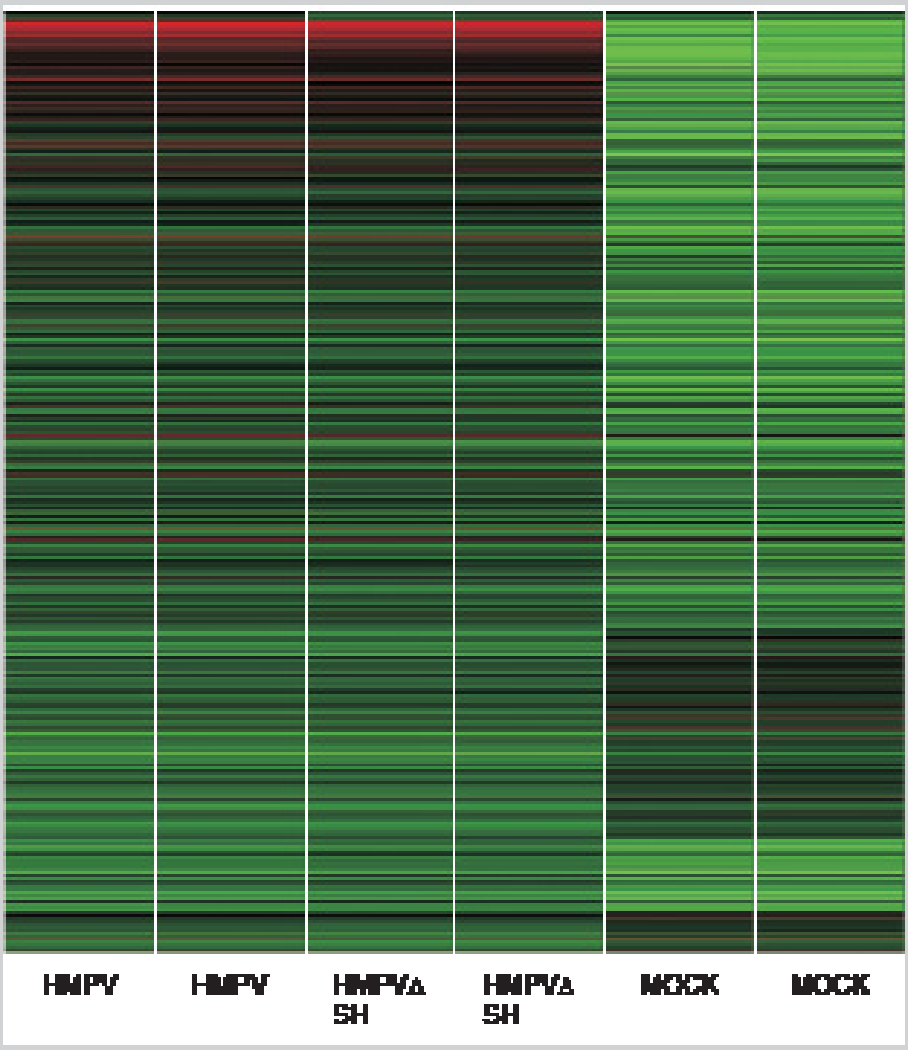

Figure 4
Heat map displaying differential expression of genes following wild-type HMPV, HMPVDSH or mock inoculated A549 cells at 24 hours p.i. in duplicate (Induced red, repressed green values).

Table 1
Up-regulated genes with the highest fold-change (Log_2) in A549 cells infected with type HMPV and HMPVDSH compared with mock 24 hours p.i.

Symbol	Gene	Average fold change (Log_2) HMPV	Average fold change (Log_2) HMPVDSH	No of probes up-regulated
IFIT1	interferon-induced protein with tetratricopeptide repeats 1	6.92	6.70	1 out of 1
IFI27	interferon, alpha-inducible protein 27	6.61	6.58	1 out of 1
IFIT3	interferon-induced protein with tetratricopeptide repeats 3	6.54	6.44	2 out of 2
IFIT2	interferon-induced protein with tetratricopeptide repeats 2	6.35	6.35	1 out of 1
LOC129607	hypothetical protein LOC129607	5.72	5.71	1 out of 1
ISG15	ISG15 ubiquitin-like modifier	5.69	5.52	1 out of 1
MX1	myxovirus (influenza virus) resistance 1, interferon-inducible protein p78 (mouse)	5.68	5.75	1 out of 1
TRIM22	tripartite motif-containing 22	5.67	5.65	1 out of 1
IFITM2	interferon induced transmembrane protein 2 (1-8D)	5.50	5.73	2 out of 2
IFITM1	interferon induced transmembrane protein 1 (9-27)	5.45	5.64	2 out of 2

have described reduced interleukin 6 (IL-6) and IL-8 expression in A549 cells infected with HMPV compared to HMPVDSH, although this difference was only found at 3 and 6 h p.i., and disappeared at 15 h p.i. (5). It was hypothesized that the down regulation of IL-6 and IL-8 was a result of the inhibition of NF-kB dependent transcription by the SH protein, since NF-kB is required for IL-8 and IL-6 expression, thereby suggesting a possible role of the SH protein in blocking of apoptosis (24, 86). However, microarray analysis of HMPV and HMPVDSH infected A549 infected cells did not reveal differential expression of genes encoding IL-6 and IL-8 or other genes involved in the apoptosis pathways at 24 hours p.i., thereby contradicting a possible role of HMPV SH in TNF-a-induced NF-kB activation.

Table 2
Down-regulated genes with the highest fold-change (Log_2) in A549 cells infected with type HMPV and HMPVDSH compared with mock 24 hours p.i.

Symbol	Gene	Average fold change (Log_2) HMPV	Average fold change (Log_2) HMPVDSH	No of probes down-regulated
FAM101B	family with sequence similarity 101, member B	-2.06	-2.08	2 out of 2
KIAA0114	KIAA0114	-1.80	-1.88	1 out of 1
FGB	fibrinogen beta chain	-1.56	-1.26	2 out of 2
TM4SF20	transmembrane 4 L six family member 20	-1.54	-1.31	1 out of 1
FAM101B	family with sequence similarity 101, member B	-1.46	-1.39	2 out of 2
LOC134145	hypothetical protein LOC134145	-1.45	-1.54	1 out of 2
NA	NA	-1.42	-1.54	1 out of 1
TM4SF4	transmembrane 4 L six family member 4	-1.40	-1.08	1 out of 1
FGB	fibrinogen beta chain	-1.37	-1.12	2 out of 2
HSPA2	heat shock 70kDa protein 2	-1.36	-1.04	1 out of 1

Expression levels of mRNA are not always consistent with protein levels and post-translational protein modifications may be altered in virus infected cells (215). Therefore, protein expression profiles of HMPV and HMPVDSH infected A549 cells were generated. Approximately 100 peptides were found to be differentially expressed between HMPV and mock infected cells, some of which represented viral proteins. However, there were no differences between HMPV and HMPVDSH infected A549 cells. The expression of the HMPV SH protein was not detected in HMPV infected A549 cells; this could be due to low expression levels, glycosylation status or its relatively small size, since small proteins render a limited amount of peptides.

From our results, we conclude that function of SH is still not clear. Since HMPV displays a high mutation rates and because SH is present in all primary virus isolates, it seems unlikely that SH has no function in vivo. Frequent frameshift and point mutations in the SH gene have been observed by some groups (14). This phenomenon may be cell type specific, since repeated passage of HMPV-B1 viruses in Vero-118 cells did not result in mutations in the SH ORF (111). This is suggestive of lack of SH function in in vitro models. HMPV SH deletion mutants were not attenuated in hamsters and only slightly attenuated in chimpanzees compared to wild-type HMPV (5, 15, 19). Infection of mice with HMPV SH deletion mutants resulted in enhanced

secretion of proinflammatory mediators compared with wild-type HMPV, although both viruses replicated to similar titers (5). The lack of a clear phenotype could be due to the fact that the animal models used do not mimic the human physiology sufficiently to read out a possible phenotype for the HMPV SH deletion mutants. Therefore, future studies on SH function of metapneumoviruses should focus on the natural hosts, birds and humans for AMPV and HMPV respectively. From the study conducted so far, we conclude that the function of the HMPV SH protein is yet to be discovered.

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CHAPTER 9
SUMMARIZING DISCUSSION

Since its discovery in 2001 (238), the epidemiology, prevalence, and clinical signs of human metapneumovirus (HMPV) have been extensively studied (242, 254). HMPV circulates globally, and shows a seasonal pattern with outbreaks in late winter or early spring in moderate climate zones (254). By the age of 5 years virtually every individual has experienced at least one HMPV infection (43). A wide spectrum of disease symptoms, similar to that of human respiratory syncytial virus (HRSV) is associated with HMPV, ranging from mild upper respiratory tract (URT) to severe respiratory disease requiring hospitalization. Although HMPV infections are found in all age groups, the most important risk groups are infants, immunocompromised individuals and the elderly, similar to what has been reported for HRSV (43). Since its discovery, significant scientific progress has also been made with respect to the basic and molecular virology of HMPV. Below, this progress will be discussed in the context of the work presented in this thesis.

REVERSE GENETICS

The HMPV genome encodes 9 putative open reading frames (ORFs), from the 3' to the 5' end, the nucleoprotein (N), phosphoprotein (P), matrix protein (M) fusion protein (F), M2.1 and M2.2, small hydrophobic protein (SH), attachment protein (G), and the large polymerase protein (L) (237). Initially, a possible function for these ORFs was assigned based on homologies between HMPV and related viruses such as HRSV. Experimental data is still required to formally assign names and functions to the viral genes. Reverse genetics techniques provide a powerful tool for this type of exercise. Before the development of reverse genetics techniques, studies on virus genes and proteins were dependent on random isolation and characterization of virus mutants. In 1997, the first rescue of a non-segmented negative strand virus solely from cDNA was described (206). The development of reverse genetics techniques has advanced research of negative-sense RNA viruses considerably, as it provided further means for applied and fundamental research. HMPV was discovered in 2001 and the reverse genetics systems described in chapter 2, were available within three years (238). This achievement highlights the impact of molecular biology in the virology field in the current era.

For HMPV, two main lineages have been described (A and B), which each can be divided into two sublineages (A1, A2, B1, and B2) (241). Reverse genetics systems were generated for two virus isolates, NL/1/00 and NL/1/99 representing HMPV-A1 and -B1, respectively. First, the complete sequences of NL/1/00 and NL/1/99 including the leader and trailer sequences were determined. To test whether the sequences of the genomic termini were correct and if the plasmids encoding the viral polymerase complex proteins were functional, minireplicon systems were set up for both viruses. The minigenome contained chloramphenicol transferase (CAT) as a reporter gene, flanked by the leader and trailer of HMPV, under the control of T7 RNA polymerase. Upon co-transfection of the minigenome with expression plasmids expressing all viral polymerase complex proteins in baby hamster kidney cells constitutively expressing T7 RNA polymerase (BSR-T7 cells), the reporter gene could be rescued. After optimization of the minireplicon system, the reporter gene was replaced by a full-length cDNA copy of the NL/1/99 or NL/1/00 genome and it was possible to recover infectious virus from

transfected cells. Reverse genetics systems are presently available for all four sublineages of HMPV, facilitating both fundamental and applied research (18).

For efficient rescue of recombinant virus or the reporter gene, the N, P and the L proteins were indispensable, whereas M2.1 was not. The HRSV M2.1 protein functions as a transcriptional antitermination factor and must be provided either by expression of plasmids or by the plasmid encoding the full-length genome for virus recovery (45) (42). In contrast, HMPV from which the M2.1 was deleted (HMPVΔM2.1) could be recovered by reverse genetics although the rescue efficiency was lower compared to that of wild-type HMPV. HMPVΔM2.1 was slightly attenuated in vitro and replication deficient in vivo (28). The deletion of M2.1 did not result in differences in the production of discrete full-length mRNAs or frequency of readthrough mRNAs. Thus, the function of HMPV M2.1 might be different from that of HRSV. Recently a novel role in virus assembly was described for the HRSV M2.1 protein (150). Further studies are necessary to reveal if the HMPV and HRSV M2.1 proteins share this function.

VACCINE DEVELOPMENT

The use of reverse genetics for the generation of vaccine candidates and the evaluation of their immunogenicity is demonstrated in chapter 3 and 4. The clinical impact of HMPV warrants the development of vaccines to prevent serious respiratory tract disease in young children, immunocompromised individuals and the elderly (54, 78, 133, 183, 239). Natural infections of HMPV in humans do not provide life long immunity, and reinfections occur relatively frequently (72). This is in agreement with the fact that experimental HMPV infections in macaques only induced transient protection (240). Ideally, a possible vaccine candidate should thus be more immunogenic and protective than the virus itself. In a ferret model, HMPV-A and HMPV-B were found to represent two different serotypes, further complicating development of HMPV vaccines (241). The existence of two HMPV serotypes is still under debate (15, 211). However, in a suitable nonhuman primate re-infection model (143), it was shown that homologous titers were, in general, higher than heterologous virus neutralization titers, and that heterologous challenge infections were more successful than homologous in primed animals (240). Thus, future vaccine candidates should be evaluated taking into account the genetic and antigenic variation with respect to optimal protection.

The evaluation of vaccine candidates requires a reliable and rapid method to detect virus-neutralizing antibodies, because these are important correlates of protection, in particular for acute respiratory viruses. In addition, such a technique could be used for serological investigations of the virus-induced immune responses in naturally infected hosts. Since antigenic variability could have implications for vaccine development, a plaque reduction virus neutralization assay was designed for both HMPV-A and HMPV-B (chapter 3). Using reverse genetics, HMPVs were generated that expressed GFP from the third position of their genome. Surprisingly, the introduction of an additional gene did not result in altered replication efficiency in vitro. Subsequently, these HMPVs expressing GFP were used to set up a plaque reduction virus neutralization assay that was faster, 10-fold more sensitive and less laborious as compared with conventional virus neutralization assays. In addition, it provided the

opportunity to not only quantitatively detect virus-neutralizing antibodies, but also to test to which extend they are cross-neutralizing against HMPV-A and HMPV-B.

Several possible vaccination strategies to combat HMPV infections have been explored involving subunit vaccines, inactivated virus, cytotoxic T-lymphocyte epitope vaccines and life attenuated vaccines (LAVs)(62, 102, 109, 113). Although studies in rodents and nonhuman primates look promising, and antibodies induced against the F protein correlated with protection in animal models (210, 226), none of the HMPV vaccine candidates has been evaluated in humans yet (113).

Vaccine strategies such as formalin inactivated HMPVs or subunit vaccines may not be suitable for small children as they might result in predisposition for enhanced disease upon natural infection, similar to formalin inactivated HRSV in the 1960s (137, 140). However, F subunit vaccines are promising for boosting HMPV-specific immune responses (56, 112, 115). Therefore, other risk groups, such as the elderly could potentially benefit from such vaccines to boost their preexisting HMPV specific immunity (113).

LAVs are intended to mimic natural infection without causing disease and to induce a broad immune response, including innate and cell-mediated immunity, as well as local and systemic antibodies (94). LAVs can prime and boost the immune system and are considered to be good candidates for immunization of infants without preexisting immunity. The best studied HRSV LAVs are cold passaged temperature sensitive (cpts) mutants. Cpts HRSV mutants were capable of replication to high titers at the permissive temperatures of 32°C in vitro, but were replication incompetent at the human body temperature of 37°C. HRSV cpts LAVs have been tested in adults, children and infants and were promising in preventing natural infections (55, 139, 259).

In chapter 4 we describe the generation of attenuated cold-passaged (cp) HMPV, by repeated passage of HMPV isolate NL/1/99 at gradually decreasing temperatures. Analyses of the full viral genome sequence and comparison with the original NL/1/99 genome revealed 19 nucleotide changes, of which 17 resulted in amino acid substitutions. Mutations were found in the P, M, F, M2, G and L genes. All mutations, or subsets of these, were introduced in HMPV. HMPV containing a subset of 11 mutations ($HMPV_{M11}$) had a temperature sensitive (ts)-phenotype in vitro and replicated to high titers at 32°C. As an additional strategy to acquire HMPV with a ts phenotype three HRSV mutations that are know to cause a ts-phenotype in HRSV were introduced in the HMPV genome and resulted in HMPV with a ts-phenotype ($HMPV_{HRSV}$). Evaluation of $HMPV_{M11}$ and $HMPV_{HRSV}$ in a hamster model revealed that they were highly attenuated. Nevertheless, immunization with these viruses resulted in a good antibody response, and provided protection against heterologous virus infection in the lower respiratory tract (LRT) and significantly reduced viral titers in the URT. Thus, the cpts strains described in chapter 4 represented promising LAV candidates in a rodent model. In a recent study, $HMPV_{M11}$ was further evaluated in cynomolgus macaques. After immunization, vaccine virus could not be isolated from the URT, and although F specific antibodies were induced, the levels of virus-neutralizing antibodies were low (115). The immunization did not result in protection against heterologous infection although the duration of virus shedding was reduced compared to the control animals. Thus, $HMPV_{M11}$ may be too attenuated to induce protective

immunity in nonhuman primates and perhaps therefore also in humans. However, cynomolgus macaques may not be the most permissive model for HMPV and $HMPV_{M11}$. Therefore, $HMPV_{M11}$ requires further evaluation in the more permissive African green monkeys, and eventually in humans (30). In addition, the individual contribution of the identified mutations to the attenuated phenotype should be characterized in further detail. Different combinations of these mutations may yield a candidate that is sufficiently attenuated in humans, yet highly immunogenic.

Other promising LAVs include vectored vaccines, chimeric HMPVs and HMPV deletion mutants. A bovine/human parainfluenza virus type 3 expressing HMPV F (B/HPIV3/HMPVF2) was attenuated in nonhuman primates while providing complete protection of the LRT and a 100-fold reduction in virus titers in the URT upon HMPV challenge (226). Potential LAVs can be created by replacing one or more ORFs with the counterpart(s) from a closely related virus that has a different natural host, while leaving genes encoding neutralization and protective antigens undisturbed (94). A chimeric HMPV in which the P ORF was replaced with that of avian metapneumovirus subgroup C (AMPV-C) was not attenuated in vitro, but was attenuated 100- and 1000- fold in the URT and LRT of African green monkeys and was comparable to wild-type HMPV in immunogenicity and protective efficacy (186). In addition, viruses can be attenuated by the deletion of non-essential genes. Surprisingly, four out of the nine ORFs encoded by HMPV can be deleted without being deleterious to the virus in vitro (15, 28). HMPVs lacking the G or M2.2 ORF were attenuated in rodents and nonhuman primates and immunization resulted in significant protection of the LRT (15).

LAVs must retain the ability to replicate to high titers in in vitro production systems, while undergoing limited replication in human tissues in vivo. At the same time, sufficient virus antigen must be generated upon vaccination to elicit protective immunity. This delicate balance requires careful evaluation in appropriate models and eventually in humans. Small rodents are suitable for first evaluation of attenuation and immunogenicity before evaluation in nonhuman primates. Chimpanzees were the only animal model that exhibited overt symptoms following HMPV infections, but for obvious ethical and economical reasons the chimpanzee model has significant limitations. Other nonhuman primates that are permissive for HMPV infection include African green monkeys and cynomolgus macaques. Although these animals do not display clinical signs upon infection, they will be useful models to study replication, lung pathology and immunogenicity of possible vaccine candidates, before they are evaluated in human trials (203).

The economic losses associated with AMPV-C in the USA warrant the development of a suitable vaccine for this region. However, LAVs based on other subgroups of AMPV cannot be used, since the introduction of a new subgroup of AMPV in the USA is prohibited. Similar to HMPV and HRSV the F protein of AMPV is a major neutralizing and protective antigen (191). Since the metapneumovirus F protein was found to be the main determinant of cellular host range in vitro (chapter 7), HMPV with the F gene of AMPV-C should be tested in turkeys for replication efficiency. If HMPV with the F gene of AMPV-C displays an attenuated phenotype compared to wild-type AMPV-C, it could prove to be useful as a LAV candidate for AMPV-C. Alternatively, a reverse genetics system for AMPV-C has recently become available (90), and

can be used to design LAVs. Similar to the chimeric HMPV/AMPV-C LAV candidate for HMPV, chimeric AMPV-C with the P gene of HMPV can be generated and examined for its vaccine potential in poultry (186).

EVOLUTION OF HUMAN AND AVIAN METAPNEUMOVIRUSES

The genus Metapneumovirus consists of two members, HMPV and AMPV. AMPVs have been classified into four subgroups, A through D (9, 77, 130, 208). Both HMPV and AMPV cause respiratory tract infections, whereas HMPV causes disease in humans, AMPV causes disease in poultry. Surprisingly, AMPV-C was found to be genetically more similar to HMPV than to the other AMPVs (91-93, 235, 237, 263).

In chapter 5, a large set of HMPV and AMPV-C, G, F and N nucleotide sequences were used to investigate the evolutionary dynamics of these viruses. The substitution rates for the G gene were around 3.5×10^{-3} nucleotide substitutions per site per year, somewhat higher than the substitution rates of around 9×10^{-4} nucleotide substitutions per site per year found for the N and F genes. Such high evolutionary rates are not uncommon for RNA viruses (104, 122).

The evolutionary rates of HMPV were high, however the levels of standing genetic variation for HMPV were low. These low levels of standing diversity were most likely due to repeated (annual) population bottlenecks, which periodically purged the genetic variation created by the high evolutionary rates. Such limited genetic diversity was also found for other RNA viruses like mumps virus (MuV), canine distemper virus, and human influenza viruses (188, 193). For dengue virus it has been shown that for three out of the four circulating serotypes, increases and decreases in population size were in-phase, whereas one was out-of-phase, most likely due to cross-protective immunity (1). Since it has been shown that the two main HMPV lineages represent two different serotypes, it will be interesting to establish if increases and decreases in population size of the four lineages are in-phase or out-of-phase. Unfortunately, the sequences currently available were not sufficient for such detailed analysis. In order to achieve a data set allowing for such research there should be more intensive sampling regimes and more detailed information about location and precise sampling dates for the isolates.

It was possible to calculate the times to the most recent common ancestor of HMPV and AMPV-C by a Bayesian coalescent approach (Figure 1). The most recent common ancestor of the currently circulating HMPV-A and HMPV-B lineages was estimated to date back around the year 1964 and 1970, respectively. While the moment of divergence between the two main HMPV lineages was calculated to have occurred around the years 1872-1884. Since the most recent common ancestor of AMPV-C and HMPV was estimated to have existed around 200 years ago, we speculated that HMPV is the result of a cross-species transmission event of an AMPV-C like virus from birds to humans around that time.

Studies on the evolutionary dynamics of HRSV-A and HRSV-B revealed similar rates of nucleotide substitutions per site per year as found for HMPV. The most recent common ancestor of the currently circulating HRSV-A, HRSV-B subgroups was estimated to date back around the year 1940 and 1949, respectively (266, 267). The moment of divergence between

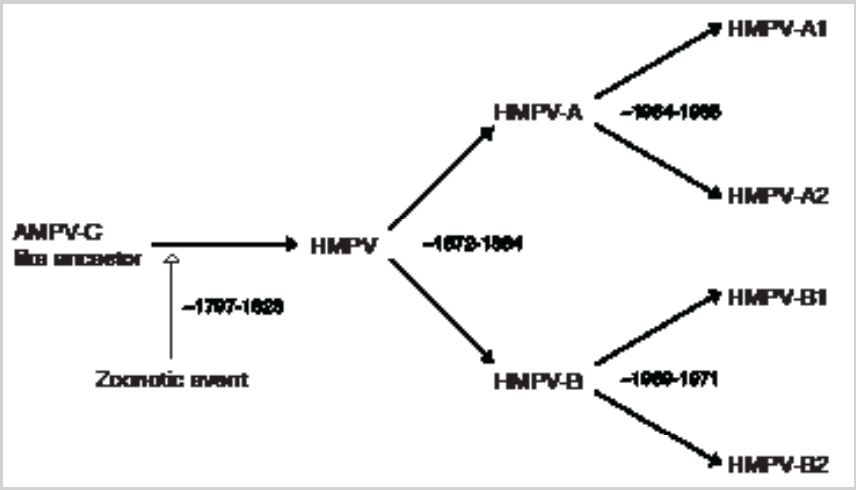


Figure 1
A schematic representation of HMPV evolution through time. The dates of divergence represent the averages calculated for the F, G and N genes of HMPV and AMPV-C.

the two major HRSV subgroups was calculated to have occurred approximately 350 years ago. The moment of divergence between HRSV and its animal counterparts bovine respiratory syncytial virus (BRSV), ovine respiratory syncytial virus, caprine respiratory syncytial virus and pneumonia virus of mice, is not yet known.

Positively selected mutations are beneficial for a virus and result in the dominance of viruses containing these mutations that for example mediate the escape from neutralizing antibodies. A limited number of positively selected sites was found in the HMPV F gene, and none for the N gene. Mutations in the HMPV G gene were likely to be neutral or positively selected. For the G protein of HRSV there was a strong association between neutralizing epitopes and positively selected sites (267). In contrast to HRSV and other paramyxoviruses, the HMPV G protein is not a major neutralization or protective antigen (210). Presently, there is still limited knowledge about the location of the neutralizing epitopes of HMPV F. It is likely that there is a correlation between positive selection and neutralizing epitopes in the HMPV F protein, which is a major neutralization and protective antigen. Monitoring sequence changes in positions that are under positive selection may provide useful information for identifying future dominant epidemic strains.

Metapneumoviruses have high substitution rates, short generation times and large population sizes. Although such high mutation rates are not necessarily adaptive, and most often result in deleterious mutations, they may occasionally facilitate viral emergence in novel hosts. This is a common trait for RNA viruses and is reflected in the fact that most of the emerging viral pathogens are RNA viruses (41). For a foreign virus to cross the species barrier and be able to replicate in a different host, it has to complete the virus infectious cycle of adsorption,

fusion, transcription, translation and replication, and assembly and release from the cell (39). HMPV and AMPV-C are interesting candidates to provide insight into the determinants of host tropism, due to their relatively recent divergence and difference in host range.

HMPV AND AMPV-C REPLICATION

To study the specificity and functional resemblance of the polymerase complex proteins of HMPV and AMPV-C, a newly generated AMPV-C minireplicon system was used in combination with the HMPV minireplicon systems (described in chapters 2 and 6). The cis acting elements in the leader and trailer sequences of HMPV and AMPV-C were found to be conserved and functionally interchangeable within the genus Metapneumovirus. Similar results have been obtained for other genera within the family Paramyxoviridae, where generally, replication efficiency of chimeric polymerase complexes decreases with increasing genetic distance between the viruses from which they were derived (3, 27, 90, 101, 159, 182, 264). When the polymerase complex proteins were individually exchanged between HMPV and AMPV-C, all combinations were functional but differed in activity. Chimeric viruses in which the polymerase complex protein genes of HMPV were exchanged with those of AMPV-C replicated with moderate differences in vitro. The transcription/ replication efficiency of the chimeric polymerase complexes in minireplicon assays did not correlate with the replication efficiency of the chimeric viruses. In hamsters, all chimeric HMPV/AMPV-Cs were attenuated, but the levels of attenuation did not correspond to the minigenome assays or in vitro replication kinetics.

Thus, animal models remain indispensable to determine the level of attenuation as a measure of host adaptation, although minireplicon systems and in vitro replication assays remain important tools to study virus transcription and replication. Evaluation in African green monkeys of a chimeric HMPV in which P protein was replaced with that of AMPV-C revealed that it was a promising LAV candidate (186). If this chimeric virus were insufficiently attenuated in humans, it could be further attenuated by the exchange of additional genes with those of AMPV-C or by the exchange of genes with those of a more distantly related member of the metapneumovirus genus, such as AMPV-A or AMPV-B. Alternatively, if chimeric HMPV/AMPV-Cs prove to be overattenuated, the exchange of genes between HMPV-A and HMPV-B may result in a less attenuated phenotype.

HMPV AND AMPV-C HOST RANGE

Although there is no evidence for recurrent AMPV infections in humans, AMPV-C was able to replicate in a mammalian animal model (chapter 6). Turkeys are the natural host for AMPV-C, and although AMPV-C and HMPV are genetically very similar, HMPV was restricted for replication in turkeys (chapter 7). To determine the contribution of the individual metapneumovirus genes to this specificity, chimeric HMPV/AMPV-C in which the N, P, L, SH, F, G or F& G genes of HMPV were exchanged with the genes of AMPV-C were generated. Replication kinetics of these chimeric viruses in avian and mammalian cell lines revealed that

the F protein is the main determinant of cellular host range. Intriguingly, the RSV F protein is the main determinant of host range as well. For both pneumoviruses and metapneumoviruses, host range was independent of the G protein.

Chimeric HMPV encoding AMPV-C F protein (HMPV-FAMPV-C) and wild-type AMPV-C yielded larger syncytia compared to chimeric viruses encoding the HMPV-F protein and wild-type HMPV. Assays to measure fusion activity revealed that the F protein of AMPV-C was more fusogenic compared to the HMPV F protein and that the F2 region was responsible for this difference. Whereas for most paramyxoviruses fusion mediated by the F protein requires participation of the attachment protein (G, H, or HN, depending on the virus) the members of the subfamily Pneumovirinae are capable of membrane fusion in the absence of the G protein (19, 145, 207, 228). HMPV F protein promoted fusion was suggested to be PH dependent (207). However, subsequent studies revealed that this was a rare strain dependent phenomenon and that the trigger for HMPV F protein to initiate membrane fusion is still unknown (114). Although the F protein is the main determinant of cellular host range, this cellular host range was not necessarily related to fusion efficiency itself. The difference in cellular host range and fusion efficiency may be caused by differential capacity to bind to cellular receptors. Therefore, it will be interesting to determine the cellular receptors for the HMPV and AMPV-C F proteins in future studies.

SMALL HYDROPHOBIC PROTEIN

Only the members of the genus rubulavirus (parainfluenza virus type 5 (PIV-5) and MuV), the genus Metapneumovirus and the genus Pneumovirus of the family Paramyxoviridae encode a SH protein. The SH proteins of PIV5 and members of the subfamily Pneumovirinae are type II membrane proteins (47, 116) in contrast to MuV SH which is a type I membrane protein (225). The SH protein has been suggested to act as a viroporin (185), or to have a function in blocking the TNF-a-mediated apoptosis pathway (5, 83, 151, 257). However, the SH proteins are highly variable and do not necessarily have a common function. Hydrophilicity profiles of AMPV, HMPV, and HRSV SH revealed similarities in composition, although HRSV SH protein appears to be truncated compared with the AMPV and HMPV SH proteins and might therefore not encode a fully functional version of the protein (237). In chapter 8, a potential function of the HMPV SH protein was investigated.

To determine the function of the SH protein HMPV SH deletion mutants (HMPVDSH) were investigated in an in vitro cell culture model (chapter 7). The deletion of the HMPV SH protein did not result in altered replication kinetics, plaque formation or cytopathic effect. Furthermore, analysis of lung epithelial cells infected with HMPV and HMPVDSH did not reveal differential expression of genes or proteins. Thus, surprisingly the deletion of HMPV SH did not result in an altered virus phenotype in vitro nor altered responses of host cells upon infection. Since metapneumoviruses have high evolutionary rates (chapter 4) and the HMPV SH protein was present in all primary virus isolates, it is unlikely that SH has no function in the natural host. Rather, the currently available in vitro model systems appear too limited to investigate such function.

A number of experiments with SH deletion mutants have been performed in cell lines of mice and bovine origin in the past. These cell lines might not be appropriate to study the function of a protein of a human virus considering host range differences, unless it can be shown that similar results can be obtained in cells of human origin or appropriate animal models. HMPVs and HRSV from which the SH proteins were deleted were not attenuated or only marginally attenuated in animal models (5, 15, 19, 31, 248). Infection of mice with HMPVDSH resulted in enhanced secretion of proinflammatory mediators compared to infection with wild-type HMPV, despite the fact that they replicated to similar titers. In seronegative children, the deletion of SH of HRSV did not increase attenuation of a recombinant virus already containing attenuating point mutations (138). In contrast, AMPV-A from which the SH protein was deleted was attenuated in vitro and in turkeys, the natural immunocompetent host for AMPV-A (152). Thus, an effect of deletion of the SH genes of pneumoviruses can be observed in some animal models, but not in all. Ideally, the function of SH of pneumoviruses should be investigated in their natural hosts. As an alternative to studies of HMPVΔSH in humans studies could be performed in nonhuman primates. The deletion of SH only results in minor differences in replication efficiency therefore tissue samples could be analyzed further by RNA microarrays. As an alternative, a reverse genetics systems for AMPV-C has been established and could be used to generate AMPV-C deletion mutants (90). It would be interesting to study AMPV-C SH deletion mutants in turkeys, its natural host.

It has been reported that the HMPV G and SH proteins do not induce neutralizing antibodies and are only weakly immunogenic in rodents (141). However, neutralization of HMPV by antibodies was measured by in vitro assays. Since the HMPV G and SH protein are nonessential for virus viability and efficient replication of HMPV in vitro, it is likely that antibodies directed to the HMPV G and SH proteins are not neutralizing in vitro, this is not necessarily the same in humans, its natural host. There are some indications that the G or the SH protein do induce neutralizing antibodies in vivo. HMPV-A and HMPV-B represent two different serotypes and it is likely that neutralizing antibodies directed against the variable G protein are responsible for this difference, since the F protein is highly conserved between the two serotypes (241). Moreover, the G protein is a major neutralizing antigen for closely related viruses such as HRSV and AMPV (171, 221). HMPV induces only transient immunity in humans and animal models (240), the reason for this waning immunity is not yet known. The HMPV F protein is potentially shielded from neutralizing antibodies by the heavily glycosylated HMPV G and SH proteins. It is also possible that the highly variable HMPV SH and G protein present immunodominant but variable epitopes to the immune system resulting in the generation of non-neutralizing antibodies. Recombinant RSV of which the soluble form of G is deleted has an increased susceptibility to RSV neutralizing antibodies both in vitro and in mice (219). The soluble RSV G protein was hypothesized to mediate evasion of RSV from the host's immune system. Thus, the role of the HMPV SH and G protein in evasion of the host's immune system merit further attention.

In order to identify the function of a non-essential viral protein such as the HMPV SH, G, M2.1 or M2.2 proteins, deletion mutants are frequently used. For such deletion mutants it is important to solely remove the ORF of the protein of interest and not the GS and GE signals, as they can

influence the transcription of downstream genes (171). Deletion of HMPV SH, GS SH and GE SH resulted in attenuation in in vitro replication assays. In contrast the deletion the SH ORF by itself did not result in attenuation (data not shown, chapter 8). Sequential transcription of the individual mRNAs gives rise to a gradient of transcripts, steadily decreasing toward the 5' end of the genome of non-segmented viruses. Thus, the deletion of a gene will influence the transcription efficiency of downstream genes. To ensure that an altered phenotype was not result of altered expression levels of downstream genes, proper controls should be included. The deletion of the SH gene results in an altered CPE phenotype for PIV5. However, this phenotype could be restored to that of wild-type PIV5 by the introduction of a silenced HMPV SH ORF. Thus, the generation of deletion mutants in which a gene is silenced rather than completely deleted or in which a "junk gene" replaces the gene of interest is advisable.

FUTURE PERSPECTIVES

Since its discovery in 2001, much has been learned about HMPV through clinical, epidemiological, applied and fundamental research. The development of reverse genetics systems for HMPV has advanced research considerably. Reverse genetic techniques provided further means to generate highly characterized LAV candidates and to study viral proteins and their properties and will remain important tools for future research. It will be interesting to use HMPV expressing GFP, generated by reverse genetics, to study experimental HMPV infections in animal models, at the cellular, tissue, and whole organism level, as was done recently for another member of the family Paramyxoviridae, measles (61). Moreover, GFP expressing viruses could be used to study HMPV from which non-essential genes are deleted and chimeric HMPV/AMPV-Cs, presuming that the introduction of GFP does not attenuate the virus in vivo or alters tissue tropism by itself.

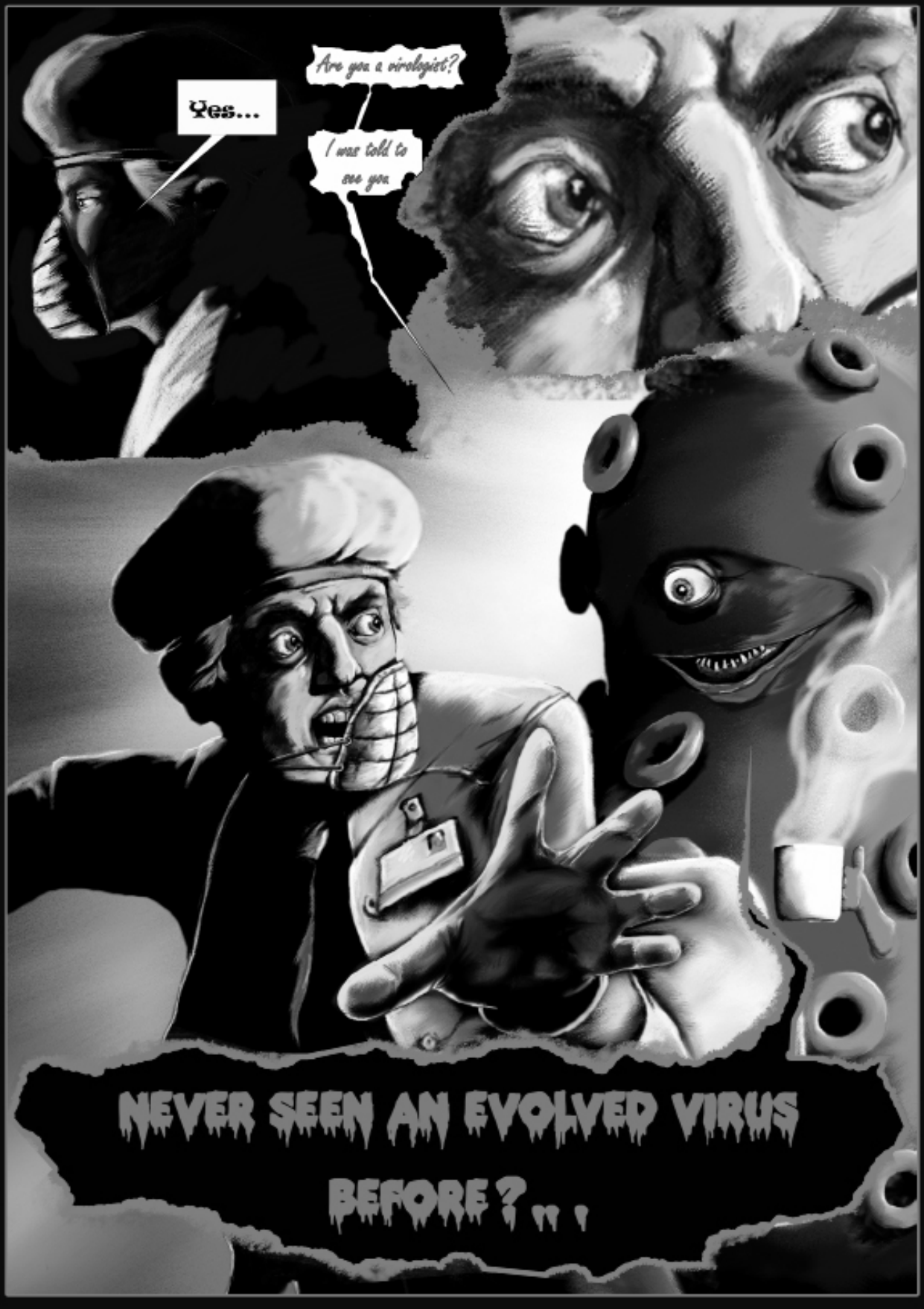
LAVs are promising for the pediatric population since such vaccines can elicit a protective immune response that parallels that induced by natural infections, thus avoiding the induction of an immune response associated with enhanced disease. Several LAV candidates look promising in rodent and nonhuman primate models (113). However, the attenuation of these LAVs still have to be evaluated in seropositive young adults, seronegative children older than 6 months and finally in the target group, in HMPV naïve infants (30). The cpts LAV described in chapter 4 ($HMPV_{M11}$) was promising in rodents but overattenuated in cynomolgus macaques. However, cynomolgus macaques might not represent the most permissive nonhuman primate model for HMPV infection thereby further attenuating $HMPV_{M11}$ infection. Therefore, $HMPV_{M11}$ should be further evaluated either in African green monkeys, which are more permissive, or in seropositive young adults. In addition, more efforts should be dedicated to define the mutations responsible for the attenuated phenotype of the cpts-vaccine candidate. By the introduction of one or a selection of these mutations in the HMPV genome it should be possible to create a cpts vaccine candidate that has the proper balance between level of attenuation and immunogenicity for seronegative subjects, and hopefully for very young infants with or without passively acquired maternal antibodies. By contrast, the elderly have aging immune systems that have been exposed to HMPV before. Therefore, subunit vaccines are most likely

to be useful for immunization of elderly people and immunocompromised individuals to boost their preexisting immunity (113). Clinical studies to determine the true impact of HMPV in elderly individuals are needed to establish whether vaccination would be useful.

Positively selected sites for the HMPV F protein were localized in the extracellular domain of the F1 chain in the cysteine-rich region. For the HRSV G protein is was found that positively selected sites were strongly correlated with neutralizing epitopes (267). Although the generation of several neutralizing monoclonal antibodies have been described (154, 236, 251), surprisingly little is known about the antigenic sites of the HMPV F protein. Since HMPV F protein is the most neutralizing and protective antigen of HMPV (210), an improved understanding will be critical for the development of vaccines and prophylactic antibodies. Therefore, future work should be directed to identify the antigenic sites in the HMPV F protein. The HMPV F protein was identified as the main determinant of cellular host range. However, the cellular host range was not necessarily related to fusion efficiency and might be mediated at the level of receptor binding. Therefore, it will be interesting to determine the cellular receptors for the HMPV and AMPV-C F proteins.

For most of the ORFs encoded by HMPV, the function has been assigned based on homologies of closely related viruses such as HRSV. However, in the seven years after its discovery it has become clear that there are significant differences in protein function between HMPV and other viruses such as HRSV. For example, despite high sequence homology, the HMPV M2.1 protein does not function as a transcription antitermination factor like its HRSV counterpart (28). Similarly, in contrast to the HRSV G protein, the HMPV G protein is not essential for replication in vivo (19). Thus, the functions of these proteins of HMPV might be quite different from those of their HRSV counterparts. Alternatively, our knowledge may be incomplete and functions common to both viruses remain to be identified.

We have proposed that HMPV originated from a cross-species transmission event of an AMPV-C like virus from birds to humans around 200 years ago. Since there is a wider variety of AMPV subgroups as compared to HMPV, we hypothesized that the direction of transmission was from birds to humans. This hypothesis is further supported by the fact that HMPV can only replicate efficiently in cell lines of mammalian origin, while AMPV-C is more promiscuous and can replicate efficiently in cell lines of both avian and mammalian origin. Moreover, HMPV can replicate in mammalian species such as nonhuman primates and rodents but not in avian species, in contrast to AMPV-C, which can replicate both in avian and mammalian species, making AMPV-C a more likely candidate to cross the species barrier than HMPV. To understand metapneumovirus evolution better, it would be important to include AMPV-A, B and D sequences in further research. However, currently the number of sequences available is too limited for such research. Although AMPVs infect poultry worldwide, evidence of AMPV-C infections is mainly confined to the USA. In Europe, turkeys are vaccinated against AMPV-A and B and this vaccine also provides cross-protection against AMPV-C infection (53). Since metapneumovirus infection of wild birds does not result in the same pathogenicity as seen in turkeys (209), it is not unlikely that a wide variety of avian metapneumoviruses is circulating in wild birds in Europe, including AMPV-C. Analyses of AMPVs from wild birds would be an interesting future avenue for metapneumovirus (genetic) research.


Yes...
Are you a virologist?
I was told to see you.
NEVER SEEN AN EVOLVED VIRUS BEFORE?...

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NEDERLANDSE SAMENVATTING

Het humaan metapneumovirus (HMPV) is in 2001 in Nederland ontdekt en behoort tot de orde Mononegavirales, familie Paramyxoviridae, subfamilie Pneumovirinae en genus Metapneumovirus. Het virus is wereldwijd een veroorzaker van luchtweginfecties in de mens. Vooral bij kleine kinderen, bejaarden en mensen met een verzwakt afweersysteem kan het virus een ernstigere infectie veroorzaken. Het klinisch beeld lijkt sterk op dat veroorzaakt door het humaan respiratoir syncytieel Virus (HRSV), een belangrijke veroorzaker van verkoudheid. Zowel HRSV als HMPV-infecties komen vooral in de tweede helft van de winter en de vroege lente voor. Het HMPV-genoom (erfelijk materiaal) bestaat uit acht genen die elk voor één of twee eiwitten coderen. Op basis van genetische verschillen en verschillen in antigene reactiviteit (reactiviteit met antistoffen) kunnen HMPV-isolaten in twee hoofdgroepen worden ingedeeld (A en B) en beide hoofdgroepen op hun beurt elk in twee subgroepen (A1, A2, B1 en B2).

In hoofdstuk 2 wordt de ontwikkeling van een "reverse genetics"-systeem voor HMPV-subgroep A1 en B1 beschreven. Met behulp van deze technologie kunnen mutaties worden aangebracht in het HMPV-genoom om het effect van deze mutaties te bestuderen. Een plasmide dat het volledige HMPV-genoom omvat wordt hiervoor samen met plasmiden die de virale polymerasecomplex-eiwitten tot expressie brengen, in cellen gebracht. Door middel van T7 RNA-polymerase wordt er viraal RNA geproduceerd dat vervolgens wordt ingepakt in een ribonucleoproteincomplex (RNP-complex). Dit RNP bestaat naast het virale RNA uit vier verschillende polymerasecomplex-eiwitten. Door de vermenigvuldiging van het RNP en de productie van virale eiwitten kunnen vervolgens nieuwe virusdeeltjes worden gevormd. Een eenvoudige manier om een reverse genetics systeem te controleren is het vervangen van het virus-genoom door een reportergen zoals chloramphenicol transferase (CAT). Wanneer het reverse genetics systeem werkt en er vermenigvuldiging van het reportergen plaatsvindt, kan CAT-expressie worden uitgelezen.

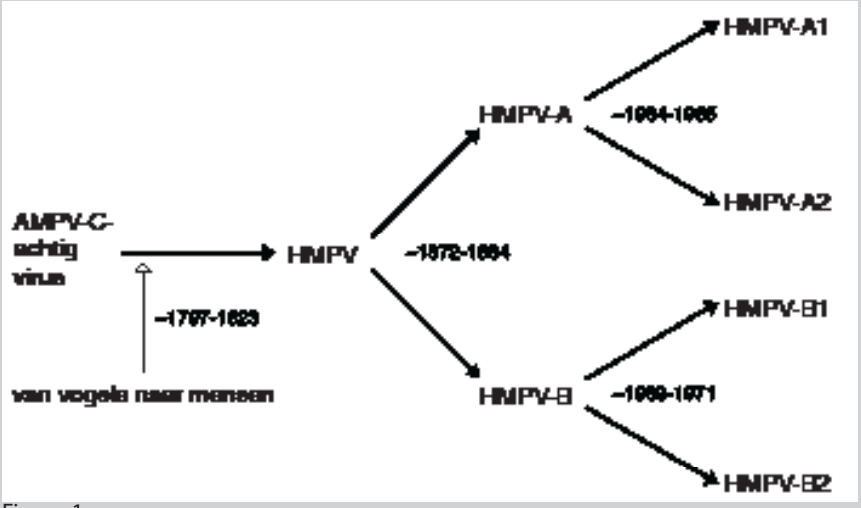
Neutraliserende HMPV-specifieke antistoffen, die door het afweersysteem worden geproduceerd, kunnen, door zich aan een virusdeeltje te binden, voorkomen dat het nog cellen kan infecteren. In hoofdstuk 3 wordt een test waarmee de hoeveelheid neutraliserende HMPV-specifieke antistoffen kan worden gemeten, beschreven. Met behulp van reverse genetics is er een recombinant HMPV gegenereerd dat het groen fluorescerend eiwit (GFP) produceert. Nadat dit virus is gemengd met neutraliserende antilichamen kan aan de hand van de hoeveelheid geneutraliseerd virus worden bepaald hoe hoog de concentratie van neutraliserende HMPV-specifieke antistoffen was. Doordat het HMPV groen licht geeft, kan deze hoeveelheid eenvoudig worden uitgelezen. Deze test was sneller, gevoeliger en gemakkelijker te automatiseren dan conventionele technieken en dus op grotere schaal toe te passen.

In hoofdstuk 4 wordt de ontwikkeling van een temperatuurgevoelig, levend en verzwakt HMPV dat als vaccin kan worden gebruikt, beschreven. Omdat zulke virussen hun vermenigvuldiging aangepast hebben aan lagere temperaturen en daardoor niet meer efficiënt op lichaamstemperatuur kunnen vermenigvuldigen, zijn ze te verzwakt om ziekte te kunnen veroorzaken, maar nog wel in staat om tijdens de infectie een afweerreactie te veroorzaken. Door een HMPV-isolaat herhaaldelijk op lage temperatuur (25^{o}C) te laten vermenigvuldigen,

paste het virus zich aan de lagere temperatuur aan. Na vergelijking van het erfelijk materiaal van dit virus met dat van het originele HMPV bleken er 19 mutaties te zijn opgetreden. Een HMPV met 11 van deze mutaties ($HMPV_{M11}$) bleek temperatuurgevoelig en vermenigvuldigde minder efficiënt dan het originele virus op 37°C in vitro. Voor HRSV zijn er in het verleden al meerdere mutaties beschreven die verantwoordelijk zijn voor een temperatuurgevoelig fenotype. Het introduceren van 3 van deze mutaties in het HMPV-genoom resulteerde in een HMPV ($HMPV_{HRSV}$) dat temperatuurgevoelig was in vitro. $HMPV_{M11}$ en $HMPV_{HRSV}$ vermenigvuldigden zich minder efficiënt in de bovenste en onderste luchtwegen van hamsters, maar infectie met deze virussen resulteerde wel in een hoge productie van HMPV-specifieke antistoffen. Vaccinatie van hamsters met deze virussen leidde tot volledige bescherming van de onderste luchtwegen tegen infectie met HMPV en tot een grote afname van virusproductie in de bovenste luchtwegen. In een latere studie is $HMPV_{M11}$ verder getest in Java-apen. Uit deze studie bleek dat het virus in Java-apen te verzwakt was. Hoewel de vaccinatie wel resulteerde in de productie van HMPV-specifieke antistoffen, was de afweerreactie onvoldoende om bescherming te bieden tegen infectie. Om een verzwakt virus te creëren dat niet te verzwakt is om een goede afweerreactie op te wekken, maar wel voldoende verzwakt is om geen ziekte te veroorzaken, is het belangrijk om de individuele bijdrage van elk van de 11 mutaties vast te stellen. Uiteindelijk zou er zo een virus gemaakt kunnen worden met een combinatie van deze mutaties dat wel het gewenste fenotype heeft.

Het aviair metapneumovirus (AMPV) is een ander lid van het genus Metapneumovirus. Van AMPV bestaan vier subgroepen: A, B, C en D. AMPV-subgroep C is genetisch meer verwant aan HMPV dan aan de andere AMPV-subgroepen. Ondanks de nauwe genetische verwantschap is er een duidelijk verschil in gastheerspecificiteit van HMPV en AMPV-C. HMPV veroorzaakt namelijk luchtweginfecties in mensen en AMPV-C in kalkoenen. Men denkt dat HMPV is ontstaan toen een AMPV van vogels naar de mens is overgesprongen. Door gebruik te maken van de genetische informatie die bekend is voor HMPV en AMPV-C is met behulp van rekenkundige methoden naar de evolutionaire dynamiek van metapneumovirussen gekeken (hoofdstuk 5). Metapneumovirussen hebben net zoals andere RNA-virussen een hoge mutatiesnelheid. Door een hoge mutatie- en vermenigvuldigingssnelheid kunnen deze virussen zich snel aanpassen aan hun omgeving. Mutaties die er bijvoorbeeld toe leiden dat een virus ontsnapt aan het afweersysteem, kunnen resulteren in dominantie in de viruspopulatie. Zulke mutaties zijn positief geselecteerd en werden gevonden in het HMPV fusie (F) eiwit. Het F-eiwit van het metapneumovirus is een belangrijk doelwit voor neutraliserende antistoffen. Verder is berekend dat een gemeenschappelijke voorouder voor alle HMPV A- en HMPV B-virussen respectievelijk 43 en 38 jaar geleden voorkwam en dat de gemeenschappelijke voorouder voor alle HMPV's 125 jaar geleden bestond (Figuur 1). Omdat een gemeenschappelijke voorouder van HMPV en AMPV-C ongeveer 200 jaar geleden bestond, denken we dat rond die tijd een AMPV-C-achtig virus van de vogel naar de mens is overgesprongen.

Voor de vermenigvuldiging van het virale genoom zijn polymerasecomplex-eiwitten nodig. De uitwisseling van deze polymerasecomplex-eiwitten tussen verschillende leden van de subfamilie Pneumovirinae werd beschreven in hoofdstuk 6. Het bleek dat de polymerasecomplex-



Figuur 1
Een schematisch overzicht van HMPV en AMPV-C evolutie.

eiwitten van HMPV nog wel het genoom van het nauw verwante AMPV-C kunnen herkennen en vermenigvuldigen en vice versa, maar minder verwante virussen zoals parainfluenza virus type 3 niet. HMPV's waarvan de polymerasecomplex-genen zijn vervangen door dezelfde genen van AMPV-C, vermenigvuldigden even efficiënt als het wildtype virus in vitro. Edoch, in hamsters vermenigvuldigden deze virussen minder efficiënt in de onderste en bovenste luchtwegen. Omdat deze chimere HMPV/AMPV-C virussen minder efficiënt vermenigvuldigen zouden ze eventueel ook als levende, verzwakte vaccins kunnen worden gebruikt.

In hoofdstuk 7 werd de gastheerspecificiteit van HMPV en AMPV-C onderzocht. Kalkoenen werden geïnfecteerd met verschillende hoeveelheden HMPV en AMPV-C. Hoewel infectie met AMPV-C resulteerde vermenigvuldiging van het virus, kon geen bewijs voor vermenigvuldiging in de met HMPV geinfecteerde dieren worden gevonden. Om te bepalen welke van de virale genen het verschil in gastheerspecificiteit determineert werden er virussen gegenereerd in waarin de genen van HMPV individueel werden uitgewisseld met die van AMPV-C. Alleen virussen met het F-eiwit van AMPV-C konden zich vermenigvuldigen in vogelcellen. Hieruit bleek dat het F-eiwit bepalend was voor de gastheerspecificiteit.

Tijdens de vermenigvuldigingscyclus van metapneumovirussen moet een virusdeeltje zich eerst hechten aan eiwitten op de gastheercel (receptor). Vervolgens fuseren de membranen van het virusdeeltje en de gastheercel. Op deze manier komt het erfelijk materiaal vrij in de gastheercel en kan het virus zich vermenigvuldigen. Het F-eiwit van metapneumovirussen heeft zowel een functie in de hechting van het virusdeeltje aan de cel als in de fusie van de membranen. Het AMPV-C F-eiwit heeft een hogere fusie-efficiëntie dan het HMPV F-eiwit,

maar of fusie-efficiëntie verband houdt met de gastheerspecificiteit is nog niet duidelijk. Omdat het F-eiwit ook belangrijk is voor de aanhechting van het virusdeeltje aan de receptoren van de gastheercel is het thans belangrijk om te bepalen aan welke receptoren de F-eiwitten hechten.

HMPV heeft drie membraaneiwitten: het F-eiwit, het aanhechtingseiwit (G) en een klein hydrofoob (SH) eiwit. Van het SH-eiwit is de functie nog onbekend. In hoofdstuk 8 wordt een studie naar de functie van het SH-eiwit beschreven. Met behulp van reverse genetics werd een virus gegenereerd waaruit het SH-gen is verwijderd (HMPVΔSH). Vervolgens is onderzocht of het verwijderen van het SH-eiwit invloed had op het HMPV-fenotype. Er waren geen verschillen in groeisnelheid, celpathologie of infectieverspreiding in vitro. Omdat uit onderzoek in Java-apen was gebleken dat HMPV voornamelijk longcellen met trilhaartjes infecteert, werden de virussen ook getest in primaire menselijke bronchiale cellen met trilhaartjes. Ook daar werden geen verschillen gevonden. Infectie van menselijke longcellen met HMPV resulteerde wel in grote verschillen in genexpressie en eiwitexpressie van de gastheer, maar de verwijdering van het SH-eiwit had hierop geen invloed. Omdat HMPV een hoge mutatiesnelheid heeft (hoofdstuk 8) en omdat het SH-gen aanwezig is in alle primaire HMPV-isolaten is het onwaarschijnlijk dat het SH-eiwit geen functie heeft in de natuurlijke gastheer.

Samenvattend beschrijft het onderzoek in dit proefschrift de generatie van moleculaire technieken die kunnen worden gebruikt voor fundamenteel en toegepast onderzoek aan HMPV. Verder draagt dit proefschrift bij aan de algehele kennis over de evolutie van metapneumovirussen, gastheerspecificiteit en virusvermenigvuldiging.

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-kuskus-

CURRICULUM VITAE, PUBLICATIES

CURRICULUM VITAE

De auteur van dit proefschrift werd op 29 juni 1979 geboren in Bergen (N.H). In 1997 werd het VWO diploma behaald aan de Bergen scholen gemeenschap aldaar. In datzelfde jaar werd begonnen met de studie biologie aan de Universiteit Utrecht. Tijdens deze studie werden twee stages gelopen. De eerste stage betrof onderzoek naar de hechting van het meningococcal lactoferrin-binding eiwit (LbpB) aan het buitenste membraan van Neisseria meningitidis onder begeleiding van Dr. Hugo-Jan Jansen en Prof. Dr. Jan Tommassen aan de afdeling Moleculaire Microbiologie van de Universiteit Utrecht. De tweede stage betrof het onderzoek naar kruisreagerende paramyxovirus-specifieke T-cellen, en hun mogelijke rol in pathogenese en immunopathogenese, onder begeleiding van Dr. Rik de Swart aan de afdeling Virologie van het Erasmus MC te Rotterdam. Na het behalen van het doctoraaldiploma in 2002, werd begonnen met het promotieonderzoek aan de afdeling Virologie van het Erasmus MC te Rotterdam, onder begeleiding van Prof. Dr. Ron Fouchier en Prof. Dr. Ab Osterhaus, resulterend in dit proefschrift.

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8. Herfst S, Schrauwen EJ, de Graaf M, van Amerongen G, van den Hoogen BG, de Swart RL, Osterhaus AD, Fouchier RA. (2008) Immunogenicity and efficacy of two candidate human metapneumovirus vaccines in cynomolgus macaques. Vaccine 26: 4224-30

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11. De Graaf M, Herfst S, Aarbiou J, Burgers PC, van Ijcken W, Zaaraoui F, Bijl M, Bakker R, Eijkemans R, Schrauwen EJ, Osterhaus AD, Luider TM, Scholte BJ, Andeweg AC, Fouchier RA. Small hydrophobic protein of human metapneumovirus does not affect virus replication and host gene expression in-vitro. In preparation

* S.Herfst. and M. de Graaf contributed equally to the results of this study

PHD PORTFOLIO SUMMARY

Miranda de Graaf
Department of Virology

Research School:	Post-graduate School Molecular Medicine
PhD period:	2002-2008
Promotor(s):	Prof Dr. Ron A.M. Fouchier
	Prof Dr. Ab D.M.E Osterhaus

In-depth courses

2008	Course in phylogeny. International training course in Pathogen Phylogeny, provided by the Post-graduate School Molecular Medicine and the Department of Virology, Erasmus MC.
2004	Course in Virology. One-week international training course in General Virology provided by the Post-graduate School Molecular Medicine and the Department of Virology, Erasmus MC.
2004	Course in Immunology. Two-week international post-doctoral training course in Immunology, provided by the Leiden Institute for Immunology.
2002-present	International seminar series in Virology, Immunology, Cell Biology, and Molecular Medicine, provided by the Post-graduate School Molecular Medicine and the Department of Virology, Erasmus MC
2002-present	Internal and external presentations at the department of Virology twice a week
2003-2004	Journal club presentations at the department of Virology once every two weeks

National and international collaborations

- MedImmune Vaccines Inc, Mountainview, USA. Collaboration on HMPV vaccines and antiviral therapy.
- Prof. Dr. J.A. Melero, Centro Nacional de Microbiología Instituto de Salud Carlos III, Majadahonda, Spain and Prof. A. Easton, Department of Biological Sciences, University of Warwick, UK. Collaboration on HMPV and AMPV within the EU FP5 program "Hammocs".
- Prof. Dr. E.C. Holmes, Center for infectious disease dynamics, Pennsylvania State University, College Park, USA. Collaboration on evolutionary dynamics of HMPV and AMPV.
- Prof. Dr. A. Garcia-Sastre and Dr. L. Martinez, Department Microbiology, Mount Sinai School of Medicine, New York, USA. Collaboration on innate immunity to HMPV.
- Prof. Dr. M. Koopmans, National Institute of Public Health and the Environment, Bilthoven, The Netherlands. Collaboration on evolution of Noroviruses.
- Dr. T.M. Luider, Department Neurology, Erasmus MC Rotterdam. Collaboration within the VIRGO genomics consortium on genomics and proteomics of HMPV-infected cells.

- Dr. B.J. Scholte, Department of Cell Biology, Erasmus MC Rotterdam. Collaboration on primary human bronchial cell culture techniques.

Miscellaneous

- Member of the European Society for Clinical Virology (ESCV).
- Frequent reviewer for Journal of Virology, Journal of General Virology.
- Recipient of an ESCV travel grant for the Third European Congress of Virology, Nürnberg, Germany (€ 500,-)
- Participated in IP generation for HMPV (patent applications NZ527221, AU2004235347, US2005019891, US2004005544, US2003232326, WO2004010935, US2004096451, KR20060022234, CN1813061, US2005142148, US2004005545, US2003232061, WO03072720, EP1351981)
- Supervision of technicians, undergraduate students for short term stays, BSc, and MSc students during the course of my PhD project.
- Member of the student committee of the Erasmus Postgraduate School Molecular Medicine.

International scientific presentations

- Metapneumovirussen, evolutie en gastheerspecificiteit. Biologie en levensloop, Teylers museum, Haarlem, The Netherlands (Oral presentation) (2008)
- Molecular biology and genetics of human and avian metapneumoviruses. Centro Nacional de Microbiología, Instituto de Salud Carlos III, Madrid, Spain. (Oral presentation, on invitation of Prof. J.A. Melero) (2008)
- Molecular biology and genetics of human and avian metapneumoviruses. Department of Molecular and Cellular Biology, CNB-CSIC, Madrid, Spain. (Oral presentation, on invitation of Prof. L. Enjuanes) (2008)
- Specificity and functional interaction of the polymerase complex proteins of human and avian metapneumoviruses. Third European Congress of Virology, Nürnberg, Germany (Poster presentation) (2007)
- Attenuation of HMPV deletion mutants and chimeras. MedImmune Inc collaborative meeting, Gaithersburg MD, USA (Oral presentation) (2004)
- Reverse genetics of human metapneumovirus. Viral vaccine meeting, Barcelona, Spain. (Oral presentation) (2003)
- Reverse genetics of human metapneumovirus. MedImmune Inc collaborative meeting, Rotterdam, The Netherlands (Oral presentation) (2003)
- Human metapneumovirus (hMPV); an important cause of acute respiratory illness.12 Jahrestagung der Deutschen Gesellschaft für Pädiatrische Infektiologie, Mainz, Germany. (Oral presentation) (2002)