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Generation of temperature-sensitive human metapneumovirus strains that provide protective immunity in hamsters

Sander Herfst, Miranda de Graaf, Eefje J. A. Schrauwen, Leo Sprong, Karim Hussain, Bernadette G. van den Hoogen, Albert D. M. E. Osterhaus and Ron A. M. Fouchier

Department of Virology, Erasmus Medical Center, Rotterdam, The Netherlands

Correspondence

Ron A. M. Fouchier

r.fouchier@erasmusmc.nl

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 titres 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 titres were reduced by 10000-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 in The Netherlands (van den Hoogen *et al.*, 2001). Based on sequence information and genome organization, HMPV was classified as the first mammalian member of the family *Paramyxoviridae*, 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 (van den Hoogen *et al.*, 2003; Williams *et al.*, 2006). 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 (van den Hoogen *et al.*, 2004), 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 (Skiadopoulos *et al.*, 2006; Tang *et al.*, 2005).

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 (Herfst & Fouchier, 2008), including subunit vaccines (Cseke *et al.*, 2007; Herfst *et al.*, 2007), a T-cell epitope vaccine (Herd *et al.*, 2006), live-attenuated vaccines (LAVs) (Biacchesi *et al.*, 2005; Pham *et al.*, 2005; Tang *et al.*, 2005) and formalin-inactivated (FI) HMPV (de Swart *et al.*, 2007; Hamelin *et al.*, 2007; Yim *et al.*, 2007). Immunization with FI-HMPV primed for hypersensitivity responses upon challenge infection (de Swart *et al.*, 2007; Hamelin *et al.*, 2007; Yim *et al.*, 2007), 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 virus (Fulginiti *et al.*, 1967; Kim *et al.*, 1969; Polack, 2007). For LAVs, no enhanced disease has been observed in studies performed in naïve animals with HRSV or HMPV. In addition, live-attenuated measles virus vaccines have 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 (Biacchesi *et al.*, 2004; Herfst *et al.*, 2004) 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, and a human/bovine parainfluenza virus type 3 (H/BPIV3) expressing the F protein of HMPV (Biacchesi *et al.*, 2005; Pham *et al.*, 2005; Tang *et al.*, 2005). 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 titres in the upper respiratory tract (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) supplemented with 10% fetal calf serum (Greiner Bio-One), 100 IU penicillin ml⁻¹, 100 µg streptomycin ml⁻¹ and 2 mM glutamine. Subclone 83 of Vero cells (WHO) was selected for virus passaging at low temperatures and subclone 118 (Kuiken *et al.*, 2004), 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% BSA fraction V (Invitrogen), 100 IU penicillin ml⁻¹, 100 µg streptomycin ml⁻¹, 2 mM glutamine and 3.75 µg trypsin ml⁻¹ 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 (van den Hoogen *et al.*, 2004) (lineage B, passage 3 at 37 °C) was serially passaged in Vero-83 cells at decreasing temperatures. Virus was cultured at 34, 31, 28 and 25 °C for 3, 3, 2 and 2 passages, respectively. When the temperature was decreased further to 22 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 post-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) according to the instructions from the manufacturer. RNA was subsequently used in 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 (wt) virus to identify nucleotide substitutions. All primer sequences are available upon request.

Sequence comparison of cp-HRSV and cp-HMPV.

Genome sequences of HRSV strains containing mutations responsible for temperature sensitivity *in vitro* and attenuation *in vivo* (Firestone *et al.*, 1996; Juhasz *et al.*, 1997, 1999;

Whitehead *et al.*, 1999) were aligned with the full-length sequence of HMPV NL/1/99 using BioEdit software (Hall, 1999). 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 wt recombinant HMPV NL/1/00 and NL/1/99 (genetic lineage A and B, respectively) has been described previously (Herfst *et al.*, 2004). 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 QuikChange multi site-directed mutagenesis kit (Stratagene) according to the 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 cm² flasks containing confluent Vero-118 cells were inoculated at 37 °C for 2 h with wt or mutant HMPV at an m.o.i. 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 fresh medium, and incubation at 32, 37, 38, 39 or 40 °C. Every day, 0.5 ml of the supernatant was collected and replaced by fresh medium. To determine viral titres, supernatants were subjected to plaque assays as described previously (Herfst *et al.*, 2004), with the exception that cells were incubated at 32 °C. Wild-type NL/1/99 virus and the viruses containing cp-HMPV mutations were incubated for 6 days, whereas the virus harbouring 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). Groups of 12 female hamsters, 5–7 week old, were inoculated intranasally with 10⁶ TCID₅₀ of NL/1/99 or LAV in a 100 µl volume. Four days post-infection (p.i.), lungs and nasal turbinates (NT) were collected from six animals per group, snap-frozen immediately and stored at –80 °C until further processing. From the other animals, blood samples were collected by orbital puncture at 21 days p.i. Blood samples were stored overnight at room temperature and centrifuged for 15 min at 1200 *g*; serum was collected and stored at –20 °C.

For the immunization and challenge experiment, animals were immunized by virus inoculation as described above, with 10⁶ TCID₅₀ of LAV or NL/1/99, or PBS as control. At 21 days p.i., animals were challenged intranasally with 10⁷ TCID₅₀ 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 anaesthesia 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 (PRVN) assay.

Virus-neutralizing (VN) antibody titres were determined in serum samples by a PRVN assay as described previously (de Graaf *et al.*, 2007). In brief, serum samples were diluted and incubated for 60 min at 37 °C with approximately 50 p.f.u. of NL/1/00 or NL/1/99, expressing the enhanced green fluorescent protein. Subsequently, the virus–serum mixtures were added to Vero-118 cells in 24-well plates and incubated at 37 °C. After 2 h, 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). VN antibody titres were expressed as the dilution resulting in 50% reduction of the number of plaques, calculated according to the method of Reed & Muench (1938). 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) in infection medium. 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, 2000 *g*) with 100 µl 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 medium. After 3–4 days, 100 µl fresh infection medium 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 (van den Hoogen *et al.*, 2001). Titres expressed as TCID₅₀ were calculated as described by Reed & Muench (1938). Titres were calculated per gram tissue, with a detection limit of 10^{1.6} and 10^{1.2} TCID₅₀ 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 decreasing temperatures until a temperature of 25 °C was reached. When the temperature was further decreased to 22 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, at this point 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 nt changes, resulting in 17 aa 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 virus by passage 35, but this mutation was still included in further studies. Mutations were found throughout the viral genome in all genes, except the genes encoding the nucleoprotein 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 has been assigned to single 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 positions 521 (Juhász *et al.*, 1997), 1169 (Juhász *et al.*, 1999) and 1321 (Whitehead *et al.*, 1999) of the L gene and in the gene start (GS) of M2 (Firestone *et al.*, 1996) were identified, for which the HMPV genome was identical to the wt 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 nt 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 25 °C also replicated very poorly, to low virus titres. Therefore, we next attempted to rescue recombinant viruses that contained only a selection of the cp-mutations, 8 and 11, 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 even 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, 37, 38, 39 or 40 °C. Plaque assays were performed to determine the viral titres in the supernatants of samples that were collected daily. Wild-type HMPV was able to replicate at all temperatures, with the highest virus titre obtained at 37 °C. At 40 °C, the virus titre was reduced more than 100-fold compared with 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 titres as compared with the wt 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 titres were reached compared with wt HMPV. Mutant HMPV_{M11} also displayed optimal virus growth at a temperature of 32 °C. Peak titres were reached later for HMPV_{M11}, but virus titres at 6 days p.i. at 32 °C were higher as compared with HMPV_{M8} (Fig. 1c). This virus did not replicate at 39 or 40 °C, indicating that this virus was ts. 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 with wt NL/1/99. The replication kinetics of HMPV_{M2} was most similar to that of the wt 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 titre at 37 °C, but at 32 °C the peak titre was comparable to wt HMPV. At 38 °C, no virus was detected until 4 days p.i., and at 39 and 40 °C the virus did not replicate at all. Thus, HMPV_{HRSV3} appeared to be ts *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 TCID₅₀ HMPV_{M11}, HMPV_{HRSV3} or wt NL/1/99 (12 animals per group), after which virus titres in the lungs and NT were compared at 4 days p.i. (six animals per group), and VN antibody titres were determined at 21 days p.i. (six animals per group). In the NT of animals inoculated with wt HMPV, virus titres up to 10^7 TCID₅₀ (g NT)⁻¹ were detected (Fig. 2a). In the animals inoculated with each of the candidate LAVs, however, mean virus titres ranged from 10^2 to 10^4 TCID₅₀ (g NT)⁻¹, indicating that virus replication was reduced by approximately 10000-fold in the URT. In the lungs of animals inoculated with wt HMPV, the mean virus titre was $10^{2.2}$ TCID₅₀ (g lung)⁻¹, while in the animals inoculated with HMPV_{M11} or HMPV_{HRSV3} virus titres were below the detection limit of $10^{1.2}$ TCID₅₀, with the exception of a single animal in the HMPV_{M11}-inoculated group ($10^{1.3}$ TCID₅₀). Thus, both ts viruses appeared to be highly attenuated *in vivo* and virus replication was restricted to the URT, where virus titres were reduced by approximately 10000-fold compared with wt HMPV.

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

Immunization-challenge experiment

Since both HMPV_{M11} and HMPV_{HRSV3} induced a detectable but low VN 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 TCID₅₀ of HMPV_{M11}, HMPV_{HRSV3}, wt HMPV NL/1/99 or PBS. Three weeks after immunization, animals were challenged with 10^7 TCID₅₀ 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 the challenge infection, lungs, NT and blood samples were collected. In PBS-immunized control hamsters, virus titres upon challenge reached $>10^8$ TCID₅₀ (g tissue)⁻¹ in the NT samples. These virus titres were reduced more than 1000-fold in animals immunized with HMPV_{HRSV3}, and were reduced >10000 -fold in the animals immunized with HMPV_{M11} or wt HMPV (Fig. 4a). In the lungs of PBS-immunized animals, the mean virus titre after the challenge infection was $10^{4.3}$ TCID₅₀ g (lung tissue)⁻¹. Virus was undetectable in lungs of all animals immunized with HMPV_{M11}, HMPV_{HRSV3} or wt HMPV NL/1/99 (Mann–Whitney test, $P<0.05$) (Fig. 4b).

Serum samples of all animals inoculated with wt 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 titres in animals inoculated with these viruses were comparable to wt HMPV-inoculated animals (Fig. 5). As expected, PRVN titres 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 LRT 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 gradual decreasing temperatures, and then using the temperature of 25 °C until passage 35 was reached. After sequencing of this virus at passage 35, 19 nt mutations were found resulting in 17 aa substitutions (Table 1). The mutations at positions 3341 (E93K) and 3365 (S101P) have been described previously (Biacchesi *et al.*, 2006; de Graaf *et al.*, 2007; Schickli *et al.*, 2005). 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 S101P mutation 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 mutations detected by passage 35 failed repeatedly. The parental virus at passage 35 replicated very slowly and to low virus titres. It is possible that the consensus sequence generated on the basis of the virus at passage 35 was derived from a variety of quasispecies 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 virus at passage 35, plaque purification of this virus was not attempted. Rather, we next tested whether viruses with a subset of the mutations of the virus at passage 35 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 eight 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} which contained all the mutations of HMPV_{M8} and the mutation in the P gene was not ts. Also a recombinant virus that contained only the two mutations in the L gene 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 harbouring all three 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 into the HMPV genome. When three of these mutations were introduced into 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 the introduction of L1321 mutation 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 to replication at 39 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 (Skiadopoulos *et al.*, 1999). This amino acid substitution resulted in a virus bearing a ts phenotype with virus replication reduced by 10-fold in the URT, but not the LRT of hamsters. Substitution of this amino acid in two cp-HPIV3 candidates (*rcp45* and *rcp45_L*) induced a 100- to 1000-fold more restricted replication in hamsters than their cp parents (Skiadopoulos *et al.*, 1999).

Both HMPV_{M11} and HMPV_{HRSV3} were found to be attenuated in hamsters, with approximately 10000-fold reduction of virus replication in the URT, and no detectable virus in the LRT as compared with wt virus (Fig. 2). In immunized animals, the HMPV-specific antibody titres were slightly lower as compared with animals inoculated with wt virus (Figs 3 and 5). Nevertheless, immunized animals were completely protected from HMPV LRT infection, and virus titres in the URT were reduced to the same extent as seen in hamsters exposed to wt HMPV (Fig. 4). Viral titres in both the lungs and NT of PBS-immunized animals that were challenged with NL/1/00 were approximately 100-fold higher than the titres obtained in NL/1/99-infected animals (Fig. 2). Similar differences in replication between these two viruses have been observed before in cynomolgus macaques (van den Hoogen *et al.*, 2007), but may 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 addressed further.

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 wt codon by preferably 2 or 3 nt, 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 H/BPIV3 expressing the F protein of HMPV that induced protective immunity against both HMPV and HPIV3 infection in African green monkeys (Tang *et al.*, 2005).

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.

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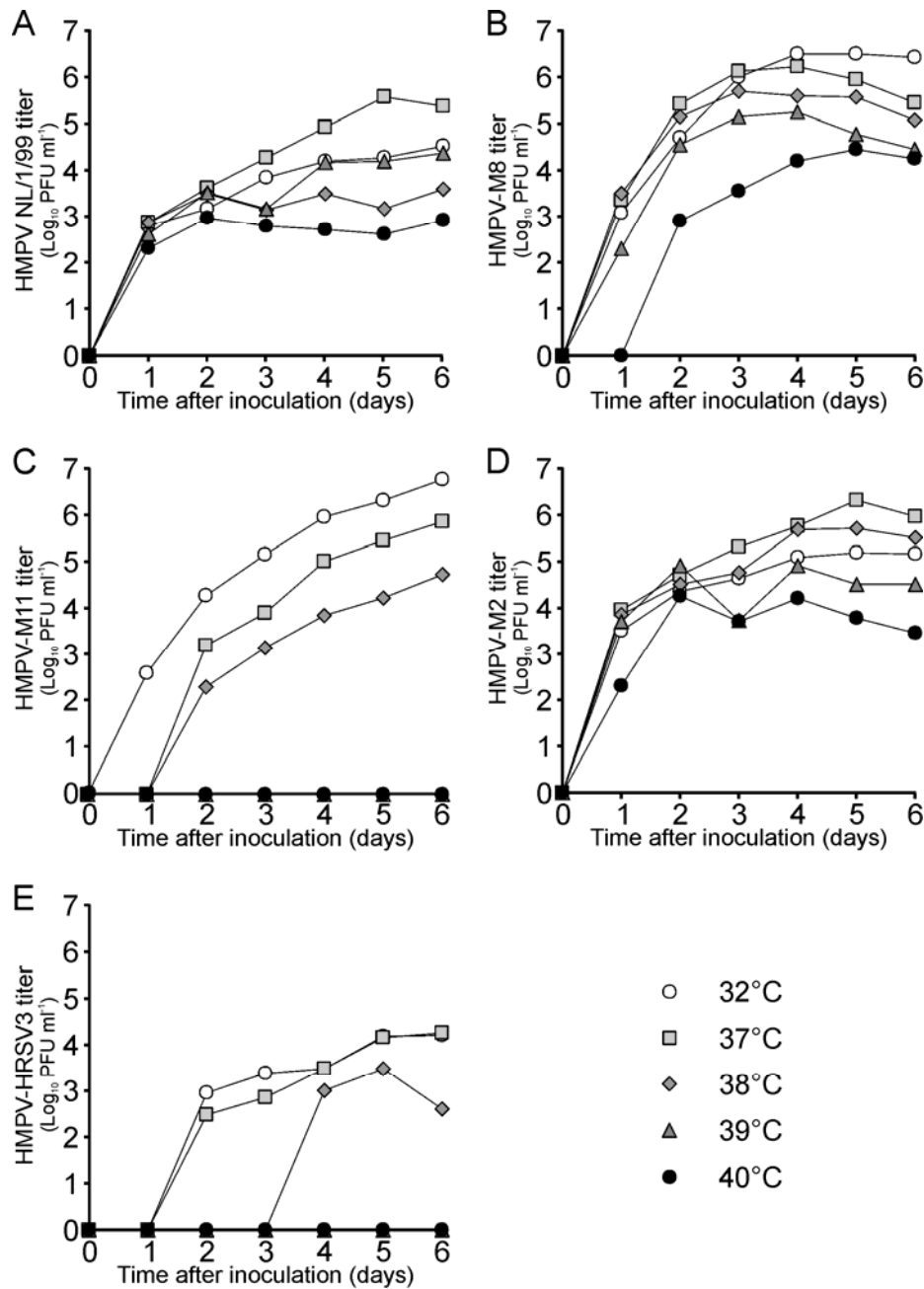


Fig. 1. Replication kinetics in Vero cell cultures of wt HMPV and recombinant viruses in which cp-mutations were introduced. Vero cells infected at an m.o.i. of 0.1 with HMPV NL/1/99 (a), HMPV_{M8} (b), HMPV_{M11} (c), HMPV_{M2} (d) or HMPV_{HRSV3} (e) were washed and incubated for 6–8 days at 32 (○), 37 (□), 38 (◇), 39 (▲) or 40 °C (●). Samples were collected daily and virus titres were determined by plaque assay.

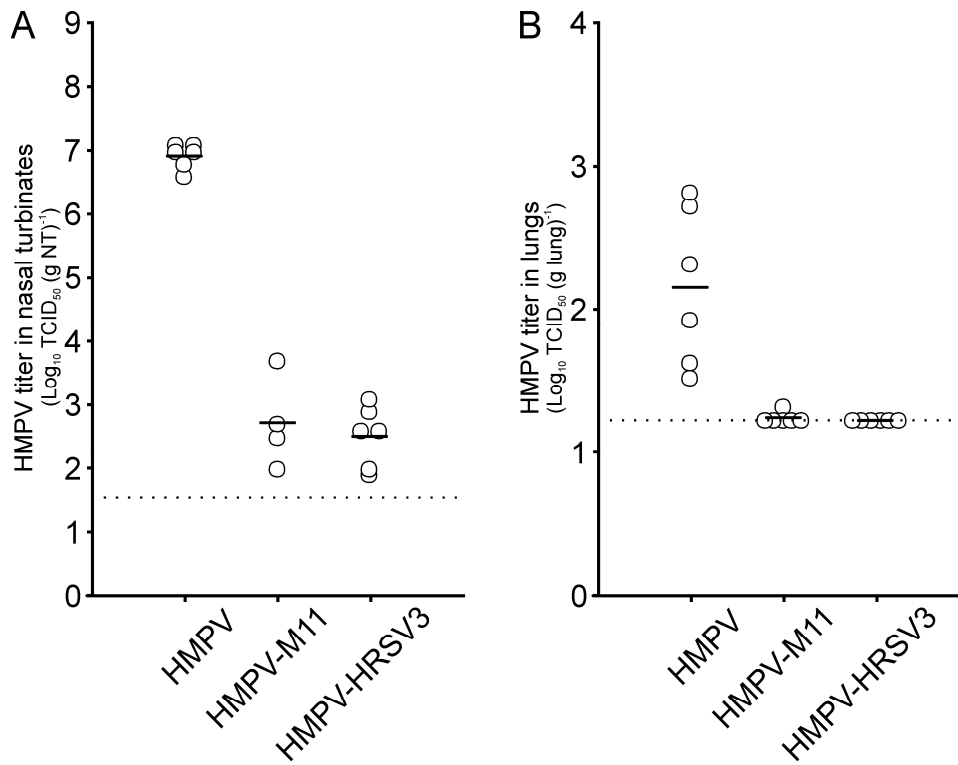


Fig. 2. Infectious virus titres in NT (a) and lungs (b) of Syrian golden hamsters inoculated with 10^6 TCID₅₀ of HMPV NL/1/99, HMPV_{M11} or HMPV_{HRSV3}. NT and lungs were collected at 4 days p.i. Virus in tissues was quantified by serial dilution in Vero-118 monolayers. The solid lines represent the geometric mean titre (GMT), the lower limit of detection is indicated with a dotted line.

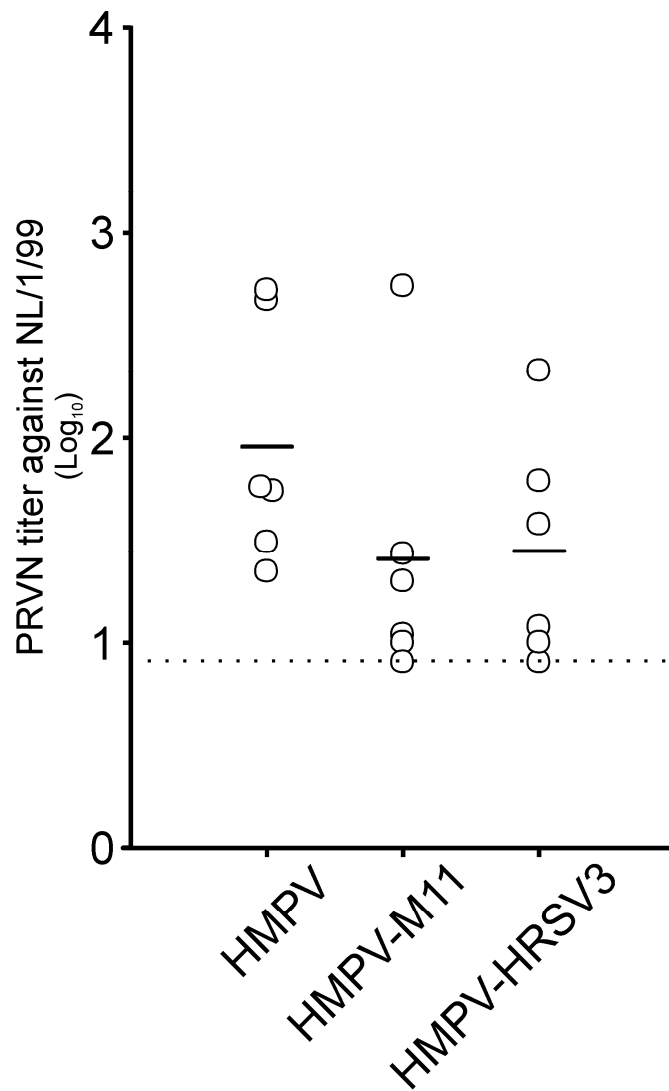


Fig. 3. PRVN titres (50%) measured against NL/1/99, after immunization with HMPV NL/1/99, HMPV_{M11} or HMPV_{HRSV3}. Blood samples were collected by orbital puncture at 21 days p.i. Titres were calculated according to the method of Reed & Muench (1938). The solid lines represent the GMT, the lower limit of detection is indicated with a dotted line.

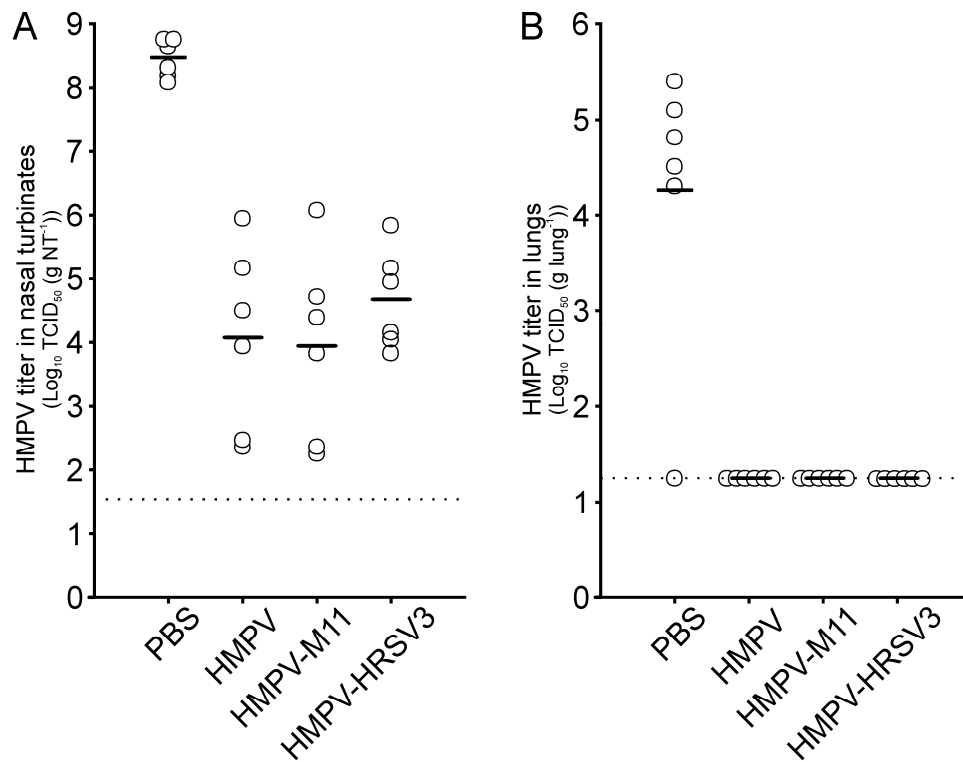


Fig. 4. Infectious virus titres in NT (a) and lungs (b) of Syrian golden hamsters. Animals were immunized with PBS, HMPV NL/1/99, HMPV_{M11} or HMPV_{HRSV3}. Three weeks after immunization, animals were challenged with 10^7 TCID₅₀ of the heterologous virus HMPV NL/1/00. Animals were euthanized at 4 days p.i. 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 a dotted line.

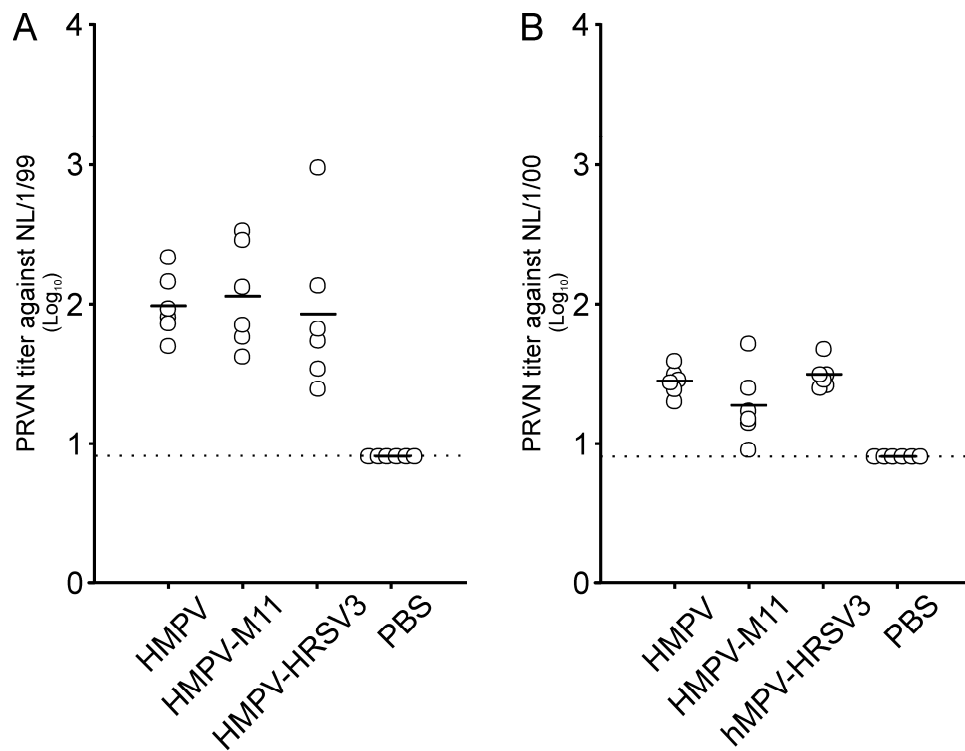


Fig. 5. PRVN titres(50%) measured against HMPV NL/1/99 (a) or HMPV NL/1/00 (b), after immunization with HMPV NL/1/99, HMPV_{M11}, HMPV_{HRSV3} or PBS and subsequent challenge with HMPV NL/1/00. Blood samples were collected by orbital puncture at 4 days post-challenge. Titres were calculated according to the method of Reed & Muench (1938). The solid lines represent the GMT, the lower limit of detection is indicated with a dotted line.

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	<u>G</u> TAA	66	Glu	Val	X	–	X	–
2203	M	TAT	<u>C</u> AT	9	Tyr	His	X	–	–	–
2291	M	TTA	<u>T</u> CA	38	Leu	Ser	X	–	–	–
2333	M	CTA	<u>C</u> CA	52	Leu	Pro	X	–	–	–
2514	M	GTT	<u>G</u> TG	112	Val	Val	X	–	–	–
2572	M	TCA	<u>C</u> CA	132	Ser	Pro	X	X	X	–
2614	M	CTA	<u>T</u> TA	146	Leu	Leu	X	X	X	–
3341	F	GAG	<u>A</u> AG	93	Glu	Lys	X	–	–	–
3365	F	TCA	<u>C</u> CA	101	Ser	Pro	X	X	X	–
3903	F	GAT	<u>G</u> GT	280	Asp	Gly	X	X	X	–
4476	F	CAG	<u>C</u> GG	471	Gln	Arg	X	X	X	–
4658	F	AAC	<u>T</u> AC	532	Asn	Tyr	X	–	–	–
4676	F	CAT	<u>T</u> AT	538	His	Tyr	X	–	–	–
5255	M2	AGC	<u>A</u> TC	187	Ser	Ile	X	X	X	–
6609	G	ACA	<u>C</u> CA	139	Thr	Pro	X	X	X	–
6685	G	CAA	<u>C</u> CA	164	Gln	Pro	X	–	–	–
7826§	L	GGA	<u>A</u> GA	235	Gly	Arg	X	–	X	X
8090	L	AAC	<u>G</u> AC	323	Asn	Asp	X	–	X	X
11480	L	TTC	<u>C</u> TC	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).

†Position is specified as the amino acid number in the indicated HMPV open reading frame. P, Phosphoprotein; M, matrix protein; F, fusion protein; M2, putative 22K protein; G, attachment protein; L, large polymerase protein.

‡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.

§Transient mutation in passage 29 at 25 °C.

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)	Reference
521	456	L	cp/ts 530	TTC (HRSV)	<u>TTA</u>	Phe	Leu	Juhasz <i>et al.</i> (1997)
1169	1094	L	cp/ts 530/1009	ATG	<u>GTG</u>	Met	Val	Juhasz <i>et al.</i> (1999)
1321	1246	L	cp/ts 530/1030	TAT	<u>AAT</u>	Tyr	Asn	Whitehead <i>et al.</i> (1999)
–	–	GS-M2	cp/ts 248/404	AATA	<u>ACA</u>	–	–	Firestone <i>et al.</i> (1996)

*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. L, Large polymerase protein; GS-M2, gene-start sequence of the M2 gene.

‡Nucleotides changes in each codon or nucleotide sequence are underlined.