

NEW ATTENUATED VIRUS STRAIN AND USE THEREOF AS A VACCINE

FIELD OF THE INVENTION

5 The present invention relates to virus strains genetically modified from a new human *metapneumovirus* strain.

The present invention relates to these modified strains, of which the virulence is attenuated, for the use thereof in the prophylactic or therapeutic treatment of infections with viruses of the *Pneumoviridae* family, as well as vaccine compositions comprising said modified virus strains.

INTRODUCTION

10 Acute infections of the lower respiratory tracts are one of the major causes of morbidity and mortality on the global scale. In children less than 5 years old in particular, they represent up to more than 15 % of mortality, depending on the countries evaluated, and are thus the second cause of mortality according to the World Health Organisation.

15 The majority of acute infections of the lower respiratory tracts, associated with variable pathologies ranging from a simple cold to serious pneumonia, are notably caused by viruses, such as those belonging to the following families:

- (i) virus of the *Orthomyxoviridae* family (notably comprising Influenza viruses);
- (ii) virus of the *Paramyxoviridae* family;
- (iii) virus of the *Adenoviridae* family;
- 20 (iv) virus of the *Picornaviridae* family;
- (v) virus of the *Coronaviridae* family and
- (vi) virus of the *Pneumoviridae* family, notably comprising the human syncytial respiratory virus and the human metapneumovirus virus.

25 The human syncytial respiratory virus (hRSV) and the human metapneumovirus (hMPV) are viruses responsible for acute infections of the respiratory tracts such as bronchiolitis, bronchitis or pneumonias. They mainly affect populations at risk, which are young children less than 5 years old, the elderly and immuno-depressed persons.

30 The hRSV is the main etiological agent of bronchiolitis and pneumonias in infants less than 1 year old, with an increased incidence below 6 months old. In adults, infection with hRSV is rare and benign, except in elderly subjects. Transmission essentially takes place by respiratory route, the virus replicating in the respiratory tract.

The hRSV was isolated in 1957. It is a virus with single-stranded RNA of negative polarity, of “enveloped” type, with helical symmetry capsid. It belongs to the family *Pneumoviridae* and to the genus *Orthopneumovirus*.

5 The hMPV is prevalent in bronchiolitis and pneumonias in infants, and particularly severely affects children between 1 and 3 years old.

The main symptoms of infection by hMPV in children comprise rhinorrhoea, coughing, respiratory distress or instead fever. hMPV may also cause infections of the upper respiratory tracts, being able to be associated with ear infections, whereas non-respiratory symptoms, such as diarrhoea, vomiting and the occurrence of erythema, are rarer. hMPV preferentially
10 targets the ciliated cells of the human respiratory tree.

An infection by hMPV induces histo-pathological modifications in the lungs of the host organism, and generates in particular the following physiological effects:

- Damage at the level of the respiratory epithelium;
- Excessive production of mucus, and
- 15 - Acute inflammation, notably at the level of the interstitial pulmonary tissues, linked to an important secretion of pro-inflammatory cytokines and chemokines.

In respiratory pathologies in adults, hMPV has been identified in 5 to 10% of adults or older persons having an acute infection of the respiratory tracts, and in 3 to 5% of adults having an exacerbation of a chronic pulmonary pathology or a pneumonia acquired in community.

20 The hMPV was isolated and described for the first time in 2001 in the Netherlands (van den Hoogen et al., 2001). The following year, several strains of hMPV were isolated in patients located in North America (Peret et al., 2002). hMPV are viruses with negative single-stranded RNA, belonging to the family *Pneumoviridae* and to the genus *Metapneumovirus*.

The average age of children hospitalised due to complications of an infection by hMPV is 6 to
25 12 months, i.e. later than that caused by hRSV, which mainly occurs between 0 and 3 months. However, the epidemiology of hMPV has numerous common points with that of hRSV:

- it is present on all continents;
- its annual distribution is mainly in winter and spring, overlapping that of hRSV;
- its transmission probably also takes place by salivary droplets.

No prophylactic or therapeutic modality that is efficient and specific against infection by the viruses hRSV and hMPV exists today on the market, although active research is underway (Mazur et al., 2018).

For treatment, ribavirin, not exempt from undesirable effects, or intravenous immunoglobulins, very expensive, may be used occasionally in serious cases of infections by hMPV or hRSV. Other types of treatments are currently being developed such as the use of fusion inhibitor peptides, sulphated glycosaminoglycans, interfering RNAs and certain immunomodulators.

The usual and widely favoured clinical approach today consists in treating especially the symptoms of the infection, while placing patients under respiratory assistance (administration of oxygen or mechanical ventilation) and by administering to them bronchodilators, corticosteroids and/or antibiotics for preventing or treating bacterial superinfections.

Regarding the vaccination, the populations particularly affected by hMPV being infants, young children and the elderly, it is crucial to have rapidly safe and effective vaccines, in order to reduce severe respiratory attacks which have a dramatic impact in these age ranges.

The development of a vaccine against hRSV and hMPV thus represents not only a major health challenge, but also a real socio-economic issue with the objective of reducing the high cost of treatments and hospitalisations associated with these infections, and of decreasing the use of antibiotics in the context of bacterial superinfections, and thus limiting the emergence of resistances.

PRIOR ART

Tables 1, 2 and 3 below list the different approaches under development for obtaining living attenuated vaccines from wild hMPV strains.

Virus strains are considered as being attenuated *in vitro* when they exhibit decreased replicative capacity compared to the wild type (WT) virus, and/or when these virus strains lead to the more restricted formation of infectious outbreaks, notably of syncytia (adjacent cells fusing following viral infection). *In vivo*, attenuated virus strains replicate at a lower maximum load and/or induce less severe pathology (in terms of weight loss or inflammatory profile or histo-pathological damage) than the wild virus strain.

Table 1. List of living vaccine candidates attenuated on the basis of a strain of hMPV virus having one or more mutations, developed or under development

Name of the virus	Description	Attenuation <i>in vitro</i>	Attenuation <i>in vivo</i>	Protective neutralising antibodies	Reference
<i>cp</i> -HMPV M11 (B1/NL/1/99)	Insertion of 11 mutations aa among the 17 induced by passages on Vero cells, temperature decreasing down to 25°C.	✓ Vero > 39°C	✓ H	✓ H	(Herfst, of Graaf et al. 2008)
<i>cp</i> -HMPV HRSV3 (B1/NL/1/99)	4 mutations <i>cp</i> (<i>cold passaging</i>) of hRSV	✓ Vero > 37°C	✓ H	✓ H	(Herfst, of Graaf et al. 2008)
rhMPV-R329K rhMPV-D331A (A1/NL/1/00)	Mutations in the domain of link to the integrin of the F protein	✓ LLC-MK2	✓ CR	✓ CR	(Wei, Zhang et al. 2014)
HMPV M2 (A1/NL/1/00)	Deletion of the N-glycosylation site (aa 172) of the F protein	✓ Vero.	✓ M ✓ M SCID	✓ M	(Yu, Li et al. 2012) (Liu, Shu et al. 2013)
HMPV-MTase (A1/NL/1/00)	Mutations of the methyltransferase site of L polymerase G1696A, G1700A, and D1755A	✓ LLC-MK2	✓ CR	✓ CR	(Zhang, Wei et al. 2014)

The term “Vero” designates an African green monkey kidney cell line, widely used in cell culture for testing various viruses.

The term “LLC-MK2” designates a cell line derived from rhesus monkey kidney cells, used for tests of infection by various viruses.

The symbol ✓ indicates that the cells used are liable to viral infection.

The following abbreviations are used for the *in vivo* study models: M for Mouse; H for Hamster; CR for Cotton Rat; CM for Cynomolgus Macaque; AGM for African Green Monkey; Ch for Chimpanzee; Rh for Rhesus Monkey; and SCID for Severe Combined ImmunoDeficiency.

Table 2. List of attenuated living vaccine candidates developed or under development, comprising a hMPV virus strain having complete deletion of at least one gene

Name of the virus	Description	Attenuation <i>in vitro</i>	Attenuation <i>in vivo</i>	Protective neutralising antibodies	References
HMPV-ΔSH (A2/CAN97-83)	Deletion of the SH gene	Ø LLC-MK2	Ø H ✓ Ch	✓ H ✓ Ch	(Biacchesi, Skiadopoulos et al. 2004) (Biacchesi, Pham et al. 2005)

HMPV-ΔG (A2/CAN97-83)	Deletion of the G gene	Ø LLC-MK2	✓ H at 3 days Ø H > 3 days ✓ Ch	✓ H ✓ Ch	(Biacchesi, Skiadopoulos et al. 2004) (Biacchesi, Pham et al. 2005)
HMPV-ΔSH/ΔG (A2/CAN97-83)	Deletion of the SH and G genes	Ø LLC-MK2	✓ H at 3 days. Ø H > 3 days	✓ H	(Biacchesi, Skiadopoulos et al. 2004)
HMPV- ΔM2-2 (A2/CAN97-83)	Mutations codon initiation + codon stop	Ø Vero	✓ H ✓ Ch	✓ H ✓ Ch	(Buchholz, Biacchesi et al. 2005) (Biacchesi, Pham et al. 2005) (Schickli, Kaur et al. 2008)
HMPV- ΔM2-1 (A2/CAN97-83)	Mutation codon stop	Ø Vero	Ø H	Ø H	(Buchholz, Biacchesi et al. 2005)
HMPV- ΔM2-1/ ΔM2-2 (A2/CAN97-83)	Deletion of the complete M2 gene	Ø Vero	Ø H	Ø H	(Buchholz, Biacchesi et al. 2005)

Δ: total deletion of the gene. Ø = no attenuation or more replicative.

All the attenuated virus strains tested are derived from the wild strain CAN97-83 of the sub-group A2.

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Table 3. List of attenuated living vaccine candidates developed or under development, based on a chimeric hMPV strain

Name of the virus	Description	Attenuation <i>in vitro</i>	Attenuation <i>in vivo</i>	Protective neutralising antibodies	Clinical test	Ref.
HMPV-Pa (A2/CAN97-83)	hMPV virus genetic background (SH stabilised) Exchange of the P gene with the homologue aMPV C	Ø Vero	✓ H ✓ AGM	✓ H ✓ AGM	Phase I NCT01255410	(Pham, Biacchesi et al. 2005) (Karron, San Mateo et al. 2017)
HMPV Na (A2/CAN97-83)	hMPV virus genetic background (SH stabilised) Exchange of the N gene with the homologue aMPV C	Ø Vero	✓ H at 3 days. Ø H > 3 days ✓ AGM	✓ H ✓ AGM		(Pham, Biacchesi et al. 2005)

b/hPIV3/ hMPV F2 (A1/NL/1/00)	Bovine PIV-3 genetic background Exchange of the F and HN genes with their homologue hPIV-3 Addition F hMPV in 2 nd position on the genome	-	Ø H Ø AGM ✓ Rh seronegative	✓ H ✓ AGM (hMPV/hPIV)		(Tang, Schickli et al. 2003) (Tang, Mahmood et al. 2005)
SeV-MPV-Ft (A2/ CAN00- 16)	SeV genetic background Addition F gene hMPV truncated for its TM domain	-	✓ CR	✓ CR		(Russell, Jones et al. 2017)

The following abbreviations are used: TM = transmembrane domain; PIV = Parainfluenza Virus; SeV = Sendai Virus.

5 The international application WO 2005/014626 relates to several strains of hMPV, designated by the following denominations: CAN 97-83, CAN 98-75 and HMPV 00-1. This application describes a strain of recombinant hMPV, designated CAN 97-83, genetically modified to attenuate its virulence. The modifications proposed notably relate to the total deletion of the genes encoding for the G and/or SH proteins. The modified virus strains may be used in human therapy, for preventing or treating infections with pneumoviruses. The administration of the wild
10 strain or of these attenuated strains to hamsters makes it possible to protect them against later infection by a CAN 97-83 hMPV. However, WO 2005/014626 makes no mention of complete protective properties, in particular no weight monitoring of the treated animals is carried out. The same experimental results are described in the scientific articles cited in table 2.

15 With the same attenuated strains, *in vivo* results were next obtained on a monkey animal model; the recombinant virus strains deleted of SH, G and M2-2 genes continue to replicate in the respiratory tracts of the monkeys; and furthermore induce the production of neutralising antibodies just like the CAN97-83 wild virus strain.

Other isolated hMPV strains have been described in the patent US 8,841,433, the use thereof for the preparation of vaccines also being proposed.

20 Vaccines based on the use of attenuated living virus strains have numerous advantages.

On the one hand, vaccines produced from attenuated living viruses have the advantage of inducing a strong immune response from a virus of which the capacity to multiply is

considerably reduced, thus making it possible to avoid the occurrence of the pathology while mimicking the natural infection. During the development of these attenuated strains, it is important to find the ideal balance between attenuation and immunogenicity.

On the other hand, these vaccines may be administered by intra-nasal route, and thus mimic the natural entry route of wild viruses, thus inducing an immune response quite similar to the physiological response to an infection.

In addition, this vaccination strategy does not generate an exaggerated inflammatory reaction, as may be the case with inactivated vaccines. Finally, the addition of adjuvant is generally not necessary.

It is thus the optimal vaccinal strategy for prevention in populations at risk such as young children and infants.

DESCRIPTION OF THE INVENTION

The subject-matter of the invention is an attenuated virus strain derived from a particular clinical strain rC-85473 of human *metapneumovirus*, comprising the genome sequence represented by sequence SEQ ID NO. 1, said attenuated strain comprising one or more genetic modifications of said sequence SEQ ID NO.1 attenuating the virulence of said strain.

The attenuated virus strains according to the invention are notably recombinant strains modified by inactivation or deletion of at least one gene encoding for one of the G and SH accessory proteins, to generate recombinant viruses designated Δ G rC-85473 and Δ SH rC-85473, respectively.

More specifically, the subject-matter of the present invention is an attenuated virus strain derived from a clinical strain of human *metapneumovirus*, comprising the genome sequence represented by sequence SEQ ID NO. 1, said attenuated strain comprising at least one genetic modification selected from: inactivation of the gene encoding for the accessory G protein, and inactivation of the gene encoding for the accessory SH protein.

These strains were characterised with respect to their infectivity and their viral replication in *in vitro* (LLC-MK2 cell line) and *ex vivo* (reconstituted human respiratory epitheliums, cultured at the air-liquid interface) cell models, as well as with respect to viral pathogenesis and their property of vaccinal protection in a murine infection model by hMPV.

The results presented in the experimental section highlight the specific properties of these virus strains Δ G rC-85473 and Δ SH rC-85473, in comparison with those of virus strains modified in

a similar manner, also deleted of G or SH genes, but derived from another clinical strain of hMPV (CAN98-75).

The viruses Δ G or Δ SH rC-85473 have in particular:

- (i) an attenuated viral character in a murine model, unlike the recombinant virus Δ SH CAN98-75 which is very virulent for its part;
- (ii) while conserving their replication capacity in binding cell systems (reference line LLC-MK2) and in suspension, unlike the recombinant viruses CAN98-75 which are much less productive;
- (iii) the viruses Δ G or Δ SH C-85473 advantageously have significant protective properties: 100% survival of infected mice, little weight loss, induction of a neutralising antibody response against the homologous virus strain (C-85473, serotype A) and at least against one heterologous strain (CAN98-75, serotype B), as well as reduced pulmonary pathogenesis in the infected animals, vis-à-vis an infectious challenge by a wild hMPV (strain rC-85473), which causes 50% lethality in the non-immunised control groups.

This attenuated virus strain may be used *in vivo*, *ex vivo* and *in vitro* as an expression vector of at least one exogenous gene, in particular a gene encoding for a viral antigen and, above all, viral antigens derived from the human respiratory syncytial virus (hRSV) and in particular its F fusion protein.

The invention also relates to this attenuated virus strain for the use thereof as a medicine, and more particularly for preventing and/or treating infections by viruses of the *Pneumoviridae* family.

Finally, the invention also relates to a vaccine composition comprising, in a pharmaceutically acceptable vehicle, at least one attenuated virus strain such as defined above, and optionally an adjuvant.

DESCRIPTION OF THE FIGURES

Figure 1. Recombinant hMPVs generated from the clinical strains C-85473 and CAN98-75.

[Fig. 1A] Schematic representation of the genome of the recombinant viruses generated by reverse genetics from the clinical strain C-85473, and in which has been cloned the GFP (Green Fluorescent Protein) coding sequence at its end 3'. The virus rC-85473 contains the genome of the wild strain C-85473 expressing in addition the GFP gene. The genomes of the

recombinant viruses derived from the strain C-85473 and deleted of SH (Δ SH-rC-85473) or G (Δ G-rC-85473) genes are represented below. The genetically modified viruses are functional and replicative, such as indicated by the images of LLC-MK2 cells infected with a Multiplicity of Infection (MOI) of 0.01 by these viruses, and observed by fluorescence microscopy at three days post-infection.

[Fig. 1B] Schematic representation of the genome of the recombinant viruses generated by reverse genetics from the clinical strain CAN98-75, and in which has been cloned the GFP (Green Fluorescent Protein) coding sequence. The virus rCAN98-75 contains the genome of the wild strain CAN98-75 expressing in addition the GFP gene at its end 3'. The genomes of the recombinant viruses derived from the strain CAN98-75 and deleted of SH (Δ SH-rCAN98-75) or G (Δ G-rCAN98-75) genes are represented below. The genetically modified viruses are functional and replicative, such as indicated by the images of LLC-MK2 cells infected with 0.01 MOI by these viruses and observed with fluorescence microscopy at three days post-infection.

Figure 2. *In vitro* replicative capacities of the virus strains rC-85473 and rCAN98-75 and strains derived therefrom.

[Fig. 2A] Viral load evaluated by TCID₅₀/ml in the cell supernatants collected each day for 7 days after infection of LLC-MK2 cells by the recombinant viruses rCAN98-75 (black circles), Δ SH-rCAN98-75 (black squares) and Δ G-rCAN98-75 (black triangles) with a multiplicity of infection (MOI) of 0.01.

[Fig. 2B] Viral load evaluated by TCID₅₀/ml in the cell supernatants collected each day for 7 days after infection of LLC-MK2 cells by the recombinant viruses rC-85473 (black circles), Δ SH-rC-85473 (black squares) and Δ G-rC-85473 (black triangles) with a multiplicity of infection of 0.01.

** $p < 0.01$ and ***, $p < 0.001$ (Two-way ANOVA statistical tests).

Figure 3: *In vivo* characterisation of the recombinant hMPVs rCAN98-75 and rC-85473 by infection of BALB/c mice: weight curves, and pulmonary viral loads at 5 days post-infection.

[Fig. 3A] BALB/c mice were infected by intranasal instillation with OptiMem culture medium ("mock" negative control, black triangles) or 2.5×10^5 FFU of the recombinant viruses rCAN98-75 (black circles), Δ SH-rCAN98-75 (grey squares) and Δ G-rCAN98-75 (grey triangles). The weight and survival of the mice was monitored daily for 14 days.

[Fig. 3B] 5 days after infection, 5 mice per group (except negative control group) underwent euthanasia for a measurement of pulmonary infectious viral loads of TCID₅₀/gramme of lung.

[Fig. 3C] BALB/c mice were infected by intranasal instillation with OptiMem culture medium (“mock” negative control, black diamonds) or 5x10⁵ TCID₅₀ of the recombinant viruses rC-85473 (black circles), ΔSH-rC-85473 (grey squares) and ΔG-rC-85473 (grey triangles). The weight and the survival of the mice was monitored daily for 14 days.

[Fig. 3D] 5 days after infection, 5 mice by group (except negative control group) underwent euthanasia for a measurement of pulmonary infectious viral loads of TCID₅₀/gramme of lung.

Statistical tests: Two-way ANOVA tests to compare each virus with the “mock” condition. *, p < 0.05, ** p < 0.01 and ***, p < 0.001.

Figure 4: *In vivo* protective properties of the recombinant hMPVs ΔG-rC-85473 and ΔSH-rC-85473 in a BALB/c murine infectious challenge model.

[Fig. 4A] Diagram of the immunisation protocol (top) followed by the infectious challenge protocol (bottom). The lethal dose corresponds to 1x10⁶ TCID₅₀.

[Fig. 4B] Infectious challenge of mice immunised with the virus strains rC-85473 (black circles), ΔSH-rC-85473 (grey squares) or ΔG-rC-85473 (grey triangles), or non-immunised (“mock”, black triangles)

21 days after immunisation, the mice were infected by intranasal instillation with a lethal dose (1x10⁶ TCID₅₀) of the wild virus rC-85473. Their weight was monitored daily for 14 days.

[Fig. 4C] Post-challenge survival curves of the mice of the different groups of immunised mice. The “mock” non-immunised mice have only 50% survival; those immunised by the recombinant viruses rC-85473, ΔSH-rC-85473 or ΔG-rC-85473 have 100% survival.

[Fig. 4D] 5 days after the infectious challenge (i.e. at day 26 post-immunisation), 4 mice of each group underwent euthanasia to measure their pulmonary infection viral loads of TCID₅₀/g of lung. “nd” signifies non-detectable.

[Fig. 4E] 5 days after the infectious challenge (i.e. at day 26 post-immunisation), 4 mice of each group underwent euthanasia to measure their pulmonary infection viral loads by RTqPCR (N gene).

Statistical tests: Two-way ANOVA to compare each virus with the mock condition. *, p < 0.05, ** p < 0.01 and ***, p < 0.001.

Figure 5: *In vitro* cell binding capacities of the recombinant viruses rC-85473 and rCAN98-75 and deleted versions thereof.

[Fig. 5A] Percentage of infected LLC-MK2 cells as a function of binding time, with the virus rCAN98-75 (circles) and the attenuated forms thereof Δ SH-rCAN98-75 (squares) and Δ G-rCAN98-75 (triangles).

[Fig. 5B] Percentage of infected LLC-MK2 cells as a function of binding time, with the virus rC-85473 (circles) and the attenuated forms thereof Δ SH-rC-85473 (squares) and Δ G-rC-85473 (triangles).

Figure 6: *Ex vivo* replicative capacities of the recombinant viruses rC-85473 and rCAN98-75 on a reconstituted human respiratory epithelium 3D model and cultured at the air-liquid interface.

[Fig. 6A] The human respiratory epithelium models in culture were infected with the recombinant viruses rCAN98-75, Δ SH-rCAN98-75 and Δ G-rCAN98-75 (photos of the upper line) or with the recombinant viruses rC-85473 (white), Δ SH-rC-85473 (dark grey) and Δ G-rC-85473 (not determinable) (photos of the lower line).

[Fig. 6B] Quantification of the measured viral progeny (via the quantification of viral genome) at the apical surface of epitheliums infected with the recombinant viruses rCAN98-75 (WT, white), Δ SH-rCAN98-75 (dark grey) and Δ G-rCAN98-75 (light grey) (left of the dotted line) or with the recombinant viruses rC-85473 (WT), Δ SH-rC-85473 and Δ G-rC-85473 (right of the dotted line).

[Fig. 6C] Quantification of the viral genome present within epitheliums infected with the recombinant viruses rCAN98-75 (WT, white), Δ SH-rCAN98-75 and Δ G-rCAN98-75 (left of the dotted line) or with the recombinant viruses rC-85473 (WT, white), Δ SH-rC-85473 and Δ G-rC-85473 (right of the dotted line).

Figure 7. Secretion of cytokines and chemokines induced by infection with the recombinant viruses rC-85473 on a reconstituted human respiratory epithelium 3D model and cultured at the air-liquid interface.

Reconstituted human respiratory epitheliums (MucilAir® HAE, Epithelix) were infected with the recombinant viruses rC-85473, Δ SH-rC-85473 and Δ G-rC-85473 at a MOI of 0.1. After 5 days of infection, the quantity of several cytokines and chemokines is measured by the Luminex technique (BioPlex Pro kit assay, BioRad) from the basal medium of the infected epitheliums:

[Fig. 7A] Chemokines MCP-1, IP-10, RANTES, IL-8, T lymphocyte attracting G-CSF, monocytes/ macrophages, eosinophils and/or neutrophils.

[Fig. 7B] Quantification of pro-inflammatory Cytokines IFN- γ and TNF- α .

[Fig. 7C] Quantification of Cytokine IL-7, stimulating the differentiation of T and B lymphocytes.

5 **[Fig. 7D]** Growth factor FGF, involved in tissue repair.

Statistical tests: One-way ANOVA to compare the viruses Δ SH-rC-85473 and Δ G-rC-85473 with the virus rC-85473. *, $p < 0.05$, ** $p < 0.01$ and ***, $p < 0.001$.

10 **Figure 8. *In vitro* viral-induced secretion of the pro-inflammatory cytokines IL-1 β and TNF- α in a model of immortalised murine macrophages infected by the recombinant hMPVs rC-85473.**

[Fig. 8A] Evaluated viral load in FFU/ml in the cell supernatants collected each day for the 2 days following infection of an immortalised murine macrophage cell line (BMDM line derived from the bone marrow of wild mice), by the recombinant viruses rC-85473 (black), Δ SH-rC-85473 (dark grey) or Δ G-rC-85473 (light grey) at a MOI of 0.1.

15 **[Fig. 8B]** Quantity of the pro-inflammatory cytokine TNF- α evaluated by ELISA test from the cell supernatants collected each day for the 2 days following infection of immortalised murine macrophage, by the recombinant viruses rC-85473 (black), Δ SH-rC-85473 (dark grey) or Δ G-rC-85473 (light grey) at a MOI of 0.1, in comparison with non-infected “mock” immortalised murine macrophages (white).

20 **[Fig. 8C]** Quantity of the pro-inflammatory cytokine IL-1 β evaluated by ELISA test from the cell supernatants collected each day for the 2 days following infection of immortalised murine macrophages by the recombinant viruses rC-85473 (black), Δ SH-rC-85473 (dark grey) or Δ G-rC-85473 (light grey) at a MOI of 0.1, in comparison with non-infected “mock” immortalised murine macrophages (white).

25 Statistical tests: Two-way ANOVA to compare the viruses Δ SH-rC-85473 and Δ G-rC-85473 with the virus rC-85473. *, $p < 0.05$ and ** $p < 0.01$.

30 **Figure 9. Pulmonary inflammation and recruitment of immune cells on the site of the infection *in vivo*, 5 days after infection of BALB/c mice by the recombinant hMPVs rC-85473, Δ SH-rC-85473 and Δ G-rC-85473 (pulmonary inflammation uniquely for Δ G-rC-85473).**

[Fig. 9A] Cumulative pulmonary histopathology score, calculated according to an evaluation of the intensity of the inflammation of the bronchial/endobronchial, peribronchial, perivascular, interstitial, pleural and intra-alveolar tissues of the lungs of BALB/c mice infected by the viruses rC-85473 (black), Δ SH-rC-85473 (dark grey), Δ G-rC-85473 (light grey) or “mock” non-infected, said tissues being collected and fixed with formaldehyde 5 days after viral infection.

[Fig. 9B] Phenotyping of immune cells determined by immuno-labelling (anti-CD45, anti-CD11b, anti-CD170, anti-Ly6C, anti-Ly6G, anti-CD11c, anti-CD115, anti-B220, anti-CD3 ϵ , anti-CD4, anti-CD8a) by flow cytometry, from lysates of lungs of BALB/c mice infected by the viruses rC-85473 and Δ SH-rC-85473, after 5 days of infection. The recruited immune cells are classified into four categories: CD8 $^{+}$ T lymphocytes (CD8 $^{+}$ T cells), CD4 $^{+}$ T lymphocytes (CD4 $^{+}$ T cells), monocytes/macrophages, and neutrophils.

Statistical tests: One-way ANOVA to compare the viruses Δ SH-rC-85473 and Δ G-rC-85473 with the virus rC-85473. ** $p < 0.01$ and ***, $p < 0.001$.

Figure 10. Characterisation of the *in vivo* secondary immune response in a BALB/c murine model immunised by the recombinant hMPVs Δ G-C-85473 and Δ SH-C-85473, following an infectious challenge.

1 and/or 5 days after infectious challenge by the virus WT rC-85473 such as described in Figure 4A, the quantity of several cytokines and chemokines is measured by the Luminex technique (BioPlex Pro kit assay, BioRad) from lung lysates [Fig. 10A-C]; the phenotyping of the immune cells infiltrated into the pulmonary tissue is determined by immuno-labelling (anti-CD45, anti-Ly6G, anti-CD11b, anti-CD170/Siglec-F, anti-Ly6C, anti-CD11c, anti-F4/80, anti-B220, anti-CD3 ϵ , anti-CD4 and anti-CD8a) by flow cytometry, from lung lysates [Fig. 10D-F]; and the cumulative pulmonary histopathology score is calculated according to an evaluation of the intensity of the inflammation of the bronchial/endobronchial, peribronchial, perivascular, interstitial, pleural and intra-alveolar tissues of the lungs of the mice fixed with formaldehyde [Fig. 10G] .

In figures 10A to 10G are represented:

- in black, the group of BALB/c mice having been immunised by the virus rC-85473;
- in dark grey, the group immunised by the virus Δ SH-C-85473;
- in light grey, the group immunised by the virus Δ G-C-85473;
- and, if represented, in white the so-called “mock” non-immunised group.

N = 2 or 3 mice / group, respectively for the histopathological or secretion of cytokines/cell recruitment analyses.

Statistical tests: Two-way ANOVA to compare each group with each other *, $p < 0.05$, ** $p < 0.01$ and ***, $p < 0.001$.

[Fig. 10A] Quantification of the pro-inflammatory cytokines IL-6 and IFN- γ , after 1 and 5 days of infectious challenge.

5 **[Fig. 10B]** Quantification of the anti-inflammatory cytokine IL-10, after 1 and 5 days of infectious challenge.

[Fig. 10C] Quantification of the attracting chemokines G-CSF, MCP-1, MIP-1 α and MIP-1 β and stimulating the activation and the differentiation of immune cells, after 1 and 5 days of infectious challenge.

10 **[Fig. 10D]** Quantification of CD8+ and CD4+ T lymphocytes infiltrated into the pulmonary tissue of the BALB/c mice, after 1 and 5 days of infectious challenge.

[Fig. 10E] Quantification of the B lymphocytes infiltrated into the pulmonary tissue of the BALB/c mice, after 1 and 5 days of infectious challenge.

15 **[Fig. 10F]** Quantification of the macrophages and neutrophils infiltrated into the pulmonary tissue of the BALB/c mice, after 1 and 5 days of infectious challenge.

[Fig. 10G] Cumulative pulmonary histopathology score, after 5 days of infectious challenge.

DETAILED DESCRIPTION OF THE INVENTION

Human *Metapneumovirus* (hMPV) virus strains

20 hMPVs were identified in 2001 as forming part of the *Pneumoviridae* family.

The genomic organisation of the hMPV is analogous to the hRSV. It is sub-divided into several ORFs (Open Reading Frames) encoding for N, P, M, F, M2 (M2-1 and M2-2), SH, G and L proteins. The general genome structure is represented for the viruses rC-8543 and rCAN98-75 in figures 1A and 1B.

25 Member viruses of the *Pneumoviridae* family express at their surface three glycoproteins designated F, G and SH proteins.

30 The F fusion glycoprotein, highly conserved between the different hMPV sub-groups, is involved in the penetration of the virus into the target cells, and the formation of syncytium, that is to say large multi-nucleated cells derived from the fusion of individual cells following their infection by the virus. The F glycoprotein is also considered as being the major antigen of the viruses hRSV and hMPV, making it possible to induce the production of neutralising antibodies.

The G protein is a type II membrane protein, its C-terminal end being outside of the cell. This protein is non-essential for the assembly of the viral constituents, and for replication *in vitro*. For hRSV, it has been shown that the deletion of the gene encoding this protein attenuated the virulence of the strain during infection of the respiratory tracts of mice.

- 5 The SH protein is also a type II membrane protein, the function of which in the viral cycle is still unknown. In the case of hRSV, the deletion of the gene encoding for the SH protein generates a recombinant virus capable of reproducing *in vitro*, and the virulence of which is attenuated in the upper respiratory tract of mice, but not in the lower part of said tract.

10 Genetic analysis of clinical hMPV strains has made it possible to define two major groups (serotypes A and B) and four “minor” sub-groups (A1, A2, B1 and B2), mainly based on the variability of the sequence of surface binding (G) and fusion (F) glycoproteins. It was next shown that these groups could again be sub-divided into sub-lines such as A2a and A2b (Huck et al., 2006).

15 Among widely studied virus strains may be cited the strain NL 00-1, belonging to the serotype A1; the strain CAN 97-83 belonging to the serotype A2; and the strain CAN 98-75, belonging to the serotype B2.

The present invention is based on a virus strain of human *metapneumovirus*, designated rC-85473, isolated from a patient sample in Canada, notably referenced in the article (Hamelin et al., 2010).

- 20 The use of the F protein of this strain, in combination with the M protein, has been proposed as a vaccinal strategy (Aerts et al., 2015a).

The use of this virus strain rC-85473, inactivated by treatment with formalin, as inactivated living vaccine strain, has been described in the article (Palwithino et al., 2015). A heat inactivated version of the strain rC-85473 has also been tested (Hamelin et al., 2007).

- 25 More recently, the strong fusion capacities of this strain have been studied in detail, on the basis of the protein sequence of its F protein (Dubois et al., 2017).

The sequence of the F gene and the M gene of the strain rC-85473 are referenced in GenBank, respectively with the access numbers KM408076.1 and KM408077.1.

- 30 The complete genome sequence of this virus strain rC-85473, comprising 13394 nucleotides, is here disclosed for the first time: it is represented in the appended list of sequences, under the reference SEQ ID NO. 1.

Table 4 below shows the identity percentage of this strain rC-85473 with three other virus strains of human *metapneumovirus*: CAN 97-83, CAN 98-75 and NL 00-1.

Table 4 – Nucleotide sequence identity percentage for each ORF (the identity of the sequence of amino acids is indicated in brackets) between the virus rC-85473 and other hMPVs

ORF	N	P	M	F	M2-1	M2-2	SH	G	L
CAN97-83 (A2)	94 (99)	91 (95)	93 (99)	94 (98)	94 (96)	94 (94)	90 (85)	81 (68)	94 (98)
CAN98-75 (B2)	86 (96)	81 (85)	85 (97)	84 (94)	85 (94)	85 (88)	70 (61)	56 (39)	84 (93)
NL 00-1 (A1)	98 (99)	97 (98)	98 (99)	97 (99)	98 (98)	98 (97)	97 (96)	93 (89)	98 (99)

With regard to the high sequence homology observed with the strain NL 00-1, it may be concluded that this strain rC-85473 is member of the sub-group A1.

The strain rC-85473 is characterised by considerable fusogenic capacities, enabling it to penetrate into target cells at a high frequency / a high degree of infection. This considerable fusogenic capacity of this strain makes it possible to generate particularly elongated syncytia. Syncytia are giant multinucleated cells. The virus rC-85473 has a high capacity of inducing cell fusion *in vitro*, leading to the formation of these very elongated syncytia, constituted of a very large number of cell nuclei.

It has been shown that 5 amino acids of the HRA functional domain of the F protein, amino acids unique among the other known hMPV strains, are in part responsible for this hyperfusogenic phenotype (Aerts et al., 2015b; Dubois et al., 2017).

According to a preferred embodiment of the present invention, the peptide sequence of the F protein of the attenuated virus strain derived from the strain rC-85473 comprises these 5 amino acids of the HRA functional domain such as described in (Dubois et al., 2017).

According to a preferred embodiment of the present invention, the peptide sequence of the F protein of the attenuated virus strain derived from the strain rC-85473 is not modified, and is thus identical to the peptide sequence of the F protein of the strain rC-85473 such as described in (Dubois et al., 2017).

Reverse genetics technology

Reverse genetics technology makes it possible to create, from a nucleic acid sequence, DNA or RNA, and from appropriate host cells, a functional encapsidated virus.

The recombinant strain rC-85473 of human *metapneumovirus* was thus obtained from a clinical strain designated C-85473, isolated in a patient.

- 5 The first reverse genetics system applied to the hMPV was described in 2004 (Biachesi et al., 2004). This technology is also described in the article (Aerts et al., 2015b).

The principle of this technology, which enables the production of recombinant hMPVs, is based on the use of a hamster kidney cell line (BHK-21) modified to express constitutively the RNA polymerase of the T7 bacteriophage (BHK-T7 or BSR-T7/5 cells).

- 10 The genome elements are distributed in five plasmid elements: A plasmid encoding the antigenome of hMPV and 4 “satellite” plasmids, encoding for the viral proteins of the transcription machinery (L, P, N and M2-1).

- 15 After co-transfection of the antigenomic plasmid hMPV and the four “satellite” plasmids in these cells, an RNA strand corresponding to the viral genomic strand (negative RNA strand), is transcribed by the T7 polymerase from its promoter.

- 20 The four proteins involved in the viral transcription of hMPV are also expressed by the transfected host cells to constitute an active RNA-dependent RNA polymerase (RdRP) complex. This functional viral polymerase thus transcribes the genomic RNA into viral mRNA then replicates it into new molecules of viral genomic RNA, via the transcription of matrix strands.

The translation and the assembly of viral proteins with genomic RNA thus enable the budding of infectious hMPV particles from the cytoplasmic membrane of transfected BHK-T7 cells. Next, amplification of the recombinant viruses is enabled thanks to the addition in co-culture of LLC-MK2 cells (ATCC CCL-7), permissive to infection.

- 25 Thus, from a genome sequence of a particular virus strain, it is possible to produce recombinant viruses thanks to these modified cells, which are commercially available.

Attenuated virus strain derived from the strain rC-85473

- 30 The present invention relates to an attenuated virus strain derived from a human *metapneumovirus* strain comprising the genome sequence represented by sequence SEQ ID

NO. 1, said attenuated strain comprising one or more genetic modifications of said sequence SEQ ID NO. 1 attenuating the virulence of said strain.

In the present application, the terms "a virus" and "a virus strain" are used indiscriminately to designate a particular virus strain, such as identified previously.

5 In the sense of the invention, "derived strain" is taken to mean a recombinant virus strain obtained by the introduction of genetic modifications into the genome of a so-called "original strain" virus strain. The original strain is advantageously a wild strain, for example a clinical isolate.

10 The genetic modifications introduced into the original strain all have the object of attenuating the virulence of said original strain, and not of modifying the identity of its genome.

In particular, these genetic modifications only concern genes encoding for proteins non-essential for the replication of the virus, in other words "accessory proteins", such as SH and G proteins. In this genetically modified attenuated strain, the peptide sequence of the F protein of the original strain rC-85473 is not modified, and thus has the same peptide sequence as the
15 original strain.

The virulence of a virus strain corresponds to the degree of rapidity of multiplication of a virus in a given organism, thus to its invasion rate. "Attenuating the virulence" is thus taken to mean decreasing the invasion rate of a virus in an organism.

20 This attenuation may take the form of a decrease in the replication capacities of the virus strain, and/or a decrease in its capacity to infect target cells, and/or instead a decrease in the pathology induced by the viral infection of the organism.

Thus, "attenuated virus strain" is taken to mean, in the sense of the invention, a recombinant virus, the virulence of which is decreased compared to that of the original virus strain, that is to say less than that of the original virus strain.

25 To measure the virulence of a virus strain, *in vitro*, *ex vivo* or *in vivo* tests may be carried out, such as for example *in vitro* replicative capacity tests (measured by TCID50/ml titration or quantitative PCR), monitoring by microscopic observation of the evolution of *in vitro* and *ex vivo* cytopathic effects, or monitoring of the clinical signs of the pathology, pulmonary histopathological observation and measurement of pulmonary viral loads in an *in vivo* infection
30 model.

To compare the physiological effects of a wild strain and an attenuated strain, it is also possible to measure / determine different physiological parameters modified at the level of the lung and/or other immune systems of a host organism of the virus.

As is described in examples 7, 8 and 9 of the present application, the following viral-induced effects may be evaluated to compare the physiological effects of attenuated virus strains, either compared with each other or with the wild virus strain:

- 5 - The secretion of pro-inflammatory cytokines (by the cells of the epithelium and/or by the macrophages);
- The secretion of chemokines;
- The secretion of growth factors;
- The level of pulmonary inflammation (score calculated by evaluation of the intensity of inflammation of the tissues); and
- 10 - The phenotyping of immune cells recruited on the site of the infection *in vivo*.

These various measurements make it possible to know in a more precise manner the physiological effect of each virus strain, and to choose as a function of these characteristics which would be the most suited for developing an attenuated living virus vaccine.

Genetic modifications introduced into a virus strain to obtain the attenuation thereof

15 The present invention relates to an attenuated virus strain derived from a human *metapneumovirus* strain comprising the genome sequence represented by sequence SEQ ID NO. 1, said attenuated strain comprising one or more genetic modifications of said sequence SEQ ID NO. 1 attenuating the virulence of said strain.

20 “Genetic modifications attenuating the virulence of said strain” is taken to mean genetic modifications relative to genes encoding for proteins non-essential for the replication of the virus, well known to the person skilled in the art. Among these proteins, G and SH proteins, described above, are notably known.

25 In other words, the present application relates to an attenuated virus strain derived from a human *metapneumovirus* strain comprising the genome sequence represented by sequence SEQ ID NO. 1, said attenuated strain having been genetically modified to attenuate its virulence, that is to say that the genetic mutations introduced have been introduced uniquely into genes encoding for proteins non-essential for the replication of the virus.

30 Various genetic modifications making it possible to attenuate the virulence of a virus strain are known to the person skilled in the art. These modifications may be introduced into the genome of the original strain rC-85473.

Genetic modifications designate, in the sense of the invention, all modifications of an original nucleotide sequence such as the deletion of one or more nucleotides, the addition of one or

more nucleotides, and the replacement of one or more nucleotides. These modifications notably comprise all modifications making it possible to shift the genetic reading frame, or to introduce a stop codon into the middle of a coding sequence, or the deletion of all or part of one or more coding sequences.

- 5 Among genetic modifications intended to attenuate the virulence of a virus strain, genetic modifications are notably known making it possible to inactivate or even to delete one or more genes encoding for proteins non-essential for the replication of the virus in culture.

For example, attenuated virus strains of human metapneumovirus have been obtained by the inactivation, in particular by the deletion, of genes encoding for accessory SH, G and M2-2
10 proteins.

However, depending on the characteristics of the original virus strain from which the attenuated strains are derived, the functional characteristics of the attenuated virus strains could be very different, the virulence of the strains depending both on the original genome and on the genetic modifications made.

- 15 According to a first aspect of the invention, the attenuated virus strain according to the invention is characterised in that the modifications of the sequence SEQ ID NO.1 comprise the inactivation of the gene encoding for the SH protein.

More particularly, the attenuated virus strain according to the invention is characterised in that the genetic modification of sequence SEQ ID NO.1 attenuating the virulence of said strain
20 consists in an inactivation of the gene encoding for the SH protein.

In the sense of the invention, the inactivation of a gene designates the fact that this gene is modified in such a way that the product of the gene is no longer expressed, or expressed in a non-active form, expressed in such a small amount that the activity of this protein is inexistant. This inactivation of a gene may be carried out by all techniques well known to the person skilled
25 in the art.

In particular, the inactivation of a gene may be obtained by the introduction of a point mutation into the gene, by the partial or total deletion of the coding sequences of the gene, or instead by modification of the gene promoter. These different genetic modifications will be carried out according to any one of the molecular biology techniques well known to the person skilled in
30 the art.

In the sense of the invention, "deletion of a gene" is taken to mean the removal from the genome of the virus strain of a significant part of the coding sequence of this gene, notably:

- When it involves a partial deletion, at least 50%, 60%, 70%, 80%, 90% or 95% of said coding sequence;
- When it involves a complete deletion, 100% of said coding sequence.

According to a preferred embodiment, in the attenuated virus strain according to the invention,
 5 the gene encoding for the SH protein is totally deleted, that is to say that all (100% of) the coding sequence for the SH protein has been removed from the original sequence SEQ ID NO. 1.

In particular, said virus strain comprises the nucleotide sequence such as represented in SEQ ID NO. 2. More specifically, the sequence of said virus strain consists of the nucleotide
 10 sequence such as represented in SEQ ID NO. 2.

As is illustrated in the examples, this attenuated strain designated Δ SH- rC-85473 has the following characteristics:

- *In vitro*, infection and replication on cells in culture are normal (no difference with the characteristics of the original strain);
- 15 - *In vivo*, infection of mice with this recombinant virus Δ SH-rC-85473 only induces very little weight loss, unlike infection with the original strain; survival of the infected mice is 100%, whereas this attenuated virus replicates in the pulmonary tissues;
- *In vivo*, prior immunisation of mice with this attenuated strain protects 100% of the mice challenged by a lethal dose of wild virus rC-85473. This protection is also
 20 illustrated by the absence of detection of infectious virus rC-85473 in the lungs 5 days after the challenge; and by a high micro-neutralisation load against the homologous virus strain (C-85473, of serotype A) and against at least one heterologous strain (CAN98-75, of serotype B) of the serums at D+21 after the infectious challenge;
- 25 - *In vitro*, the capacity of binding to LLC-MK2 cells in culture and of cell infection is much better for the attenuated virus Δ SH- rC-85473 (around 60% of infected cells) in comparison with those of the virus Δ SH-rCAN98-75 (around 40% of infected cells);
- *Ex vivo*, infection of a respiratory epithelium model by this attenuated strain induces
 30 the secretion of cytokines and chemokines in a quantity equivalent to or less than that induced by the wild strain; the inflammatory response is however reduced compared to that induced by the wild strain (figures 7A, 7B);

- *In vitro*, infection of macrophages by this attenuated strain induces the secretion of pro-inflammatory cytokines, but with a different kinetic from that viral-induced by the wild strain (figures 8B, 8C);
- *In vivo*, viral-induced inflammation of the pulmonary tissues is significantly lower following infection by this attenuated strain than with the wild strain (figure 9A); however, the recruitment of immune cells is still ensured (figure 9B).

According to a second aspect of the invention, the attenuated virus strain according to the invention is characterised in that the modifications of sequence SEQ ID NO.1 comprise the inactivation of the gene encoding for the G protein.

- 10 More particularly, the attenuated virus strain according to the invention is characterised in that the genetic modification of sequence SEQ ID NO.1 attenuating the virulence of said strain consists in an inactivation of the gene encoding for the G protein.

According to a preferred embodiment, in the attenuated virus strain according to the invention, the gene encoding for the G protein is totally deleted, that is to say that all (100% of) the coding sequence for the G protein has been removed from the original sequence SEQ ID NO. 1.

In particular, said virus strain comprises the nucleotide sequence such as represented in SEQ ID NO. 3. More specifically, the sequence of said virus strain consists of the nucleotide sequence such as represented in SEQ ID NO. 3.

As is illustrated in the examples, this attenuated strain designated ΔG- rC-85473 has the following characteristics:

- *In vitro*, in the LLC-MK2 cell model, infection and replication on cells in culture are normal (no difference with the characteristics of the original strain);
- *In vivo*, infection of mice infected with this recombinant virus ΔG-rC-85473 only induces very little weight loss; the survival of infected mice is 100%; whereas this virus replicates in the pulmonary tissues;
- *In vivo*, prior immunisation of mice with this attenuated strain protects 100% of the mice challenged by a lethal dose of wild virus rC-85473. This protection is also illustrated by the absence of detection of infectious virus and wild genome rC-85473 in the lungs at D+5 after challenge; and by a high micro-neutralisation load against the homologous virus strain (C-85473, of serotype A) and against at least one heterologous strain (CAN98-75, of serotype B) of the serums at D+21 after infectious challenge;

- *In vitro*, the capacity to bind to LLC-MK2 cells in culture and cell infection is much better for the attenuated virus ΔG- rC-85473 (around 60% of infected cells) compared to those of the virus ΔG-rCAN98-75 (fewer than 20% of infected cells).
- *Ex vivo*, the infection of a respiratory epithelium model by this attenuated strain induces the secretion of cytokines and chemokines in an amount significantly lower than that induced by the wild strain (figure 7);
- *In vitro*, the infection of macrophages by this attenuated strain induces the secretion of TNF-α, in an equivalent manner to the secretion viral-induced by the wild strain; but only induces very slightly the secretion of IL-1β (Figures 8B, 8C);
- *In vivo*, viral-induced inflammation of the pulmonary tissues is significantly lower following infection by this attenuated strain, than with the wild strain or than with the attenuated strain ΔSH- rC-85473 (figure 9A).

According to a third aspect of the invention, the attenuated virus strain according to the invention is characterised in that the modifications of sequence SEQ ID NO.1 comprise the inactivation of two genes encoding for the G protein and the SH protein.

More particularly, the attenuated virus strain according to the invention is characterised in that the genetic modifications of sequence SEQ ID NO.1 attenuating the virulence of said strain consist in an inactivation of the two genes encoding one for the SH protein and the other for the G protein.

According to a particular embodiment, the inactivation of the two genes corresponds to the complete deletion of one or the other or of the two genes encoding for the G and SH proteins.

In addition, it is understood that any other genetic modification making it possible to attenuate the virulence of the strain rC-85437 could be introduced into the genome of said strain, represented by sequence SEQ ID NO.1.

25 Introduction of exogenous genes into the genome of the virus strain rC-85473

According to an aspect of the invention, the nucleotide sequence of the attenuated virus strain according to the invention could be genetically modified by the introduction of at least one exogenous gene.

Thus, the attenuated virus strain according to the invention has a genome sequence that comprises at least one exogenous gene. This exogenous gene could in particular be a gene encoding for a viral antigen.

In the sense of the invention, “viral antigen” is taken to mean a protein element or an element of another nature, expressed by a virus, which the immunological system of an individual recognises as foreign and which causes a response in said individual by the production of specific antibodies and/or the stimulation of a cell immune response.

- 5 Viral antigens could in particular be selected from antigens expressed by at least one influenza virus, or by at least one virus of the *Pneumoviridae* family, such as the hRSV, or by at least one virus of the *Paramyxoviridae* family, such as the parainfluenza virus.

More particularly, said viral antigen could be selected from all or part of the F protein of the hRSV, and all or part of the haemagglutinin of the influenza or parainfluenza virus.

- 10 This attenuated virus strain will make it possible, during its administration to a patient, to generate a multiple immune response, both against the viral antigen expressed and against hMPV.

Such a strain making it possible to obtain a combined immune response against several viruses, following a single administration, is very advantageous.

- 15 In addition, such an attenuated virus strain comprising at least one exogenous gene could be used *in vivo*, *ex vivo* or *in vitro*, as expression vector of at least one exogenous gene in target cells of the human *metapneumovirus*.

‘Target cells of the human *metapneumovirus*’ designates the epithelial cells of the respiratory tract of individuals liable to be infected by this virus. This expression also designates all the cell

20 lines enabling the *in vitro* replication of said virus.

Attenuated virus strain for the use thereof as a medicine

The present invention also relates to an attenuated virus strain such as defined above, for the use thereof as a medicine.

- 25 Indeed, this strain may be used, notably, for treating or preventing viral infections.

The term “treat” designates the fact of combatting infection by a virus in a human or animal organism. Thanks to the administration of at least one composition according to the invention, the level of viral infection (infectious load) in the organism is going to decrease, and preferably the virus is going to disappear completely from the organism. The term “treat” also designates

30 the fact of attenuating the symptoms associated with the viral infection (respiratory syndrome, renal failure, fever, etc.).

In the sense of the invention, the term “prevent” designates the fact of avoiding, or at least decreasing the risk of occurrence of an infection in a human or animal organism. Thanks to the administration of at least one attenuated virus strain according to the invention, the human or animal cells of said organism become less permissive to infection, and are thus best placed not to be infected by said virus.

More specifically, the invention relates to an attenuated virus strain such as defined above, for the use thereof for preventing and/or treating infections by viruses of the *Pneumoviridae* family.

It is understood that this attenuated virus strain will be preferably integrated in a vaccine composition comprising a pharmaceutically acceptable vehicle, suitable for suspending said strain and for the administration thereof.

Said vaccine composition comprises at least one attenuated virus strain according to the invention, making it possible to stimulate in a specific manner the immune system of a human or animal organism.

Thus, this vaccine composition comprises at least one attenuated living virus strain which plays the role of antigen, that is to say of compound inducing a specific immune response in the organism, which will retain the memory thereof.

Such a vaccine composition could be used as a preventive vaccine, that is to say intended to stimulate a specific immune response before infection of an organism by a pathogenic virus.

Such a vaccine composition could also be used as a therapeutic vaccine, that is to say intended to stimulate a specific immune response concomitantly with infection of an organism by said pathogenic virus.

The present invention also relates to a method for preventing and/or treating infections by *Pneumoviridae* in humans, comprising the administration, to individuals liable to be infected by such viruses, of at least one attenuated virus strain such as described previously.

The present invention also relates to a method for preventing and/or treating infections by *Pneumoviridae* in humans, comprising the administration, to individuals liable to be infected by such viruses, of at least one vaccine composition comprising at least one attenuated virus strain such as described previously.

According to a specific embodiment of the invention, the infections are infections by human *metapneumoviruses*.

According to another specific embodiment of the invention, the infections are infections by *orthopneumoviruses*, such as the human syncytial respiratory virus (hRSV).

The invention also relates to an attenuated virus strain, the sequence of which comprises at least one exogenous gene encoding for a viral antigen, for the use thereof for preventing and/or treating infections by viruses in which at least one viral antigen is expressed by said attenuated virus strain.

- 5 According to a particular embodiment, an attenuated virus strain according to the invention comprising, as exogenous viral antigen, the F protein of a hRSV, could be used for preventing and/or treating infections by a hRSV.

According to another particular embodiment, an attenuated virus strain according to the invention comprising, as exogenous viral antigen, the haemagglutinin of an influenza virus,
10 could be used for preventing and/or treating infections by an influenza virus.

Pharmaceutical composition comprising at least one attenuated virus strain

The present invention also relates to a vaccine composition comprising, in a pharmaceutically acceptable vehicle, at least one attenuated virus strain according to the invention, and optionally an adjuvant.

- 15 According to the invention, the term “pharmaceutically acceptable vehicle” designates vehicles or excipients, that is to say compounds not having specific action on the infection considered here. These vehicles or excipients are pharmaceutically acceptable, which signifies that they may be administered to an individual or to an animal without risk of significant deleterious effects or prohibitive undesirable effects.

- 20 It is understood that the vaccine composition according to the invention comprises at least one effective amount of the attenuated virus strain. “Effective amount” is taken to mean, in the sense of the invention, a quantity of attenuated virus strain sufficient to trigger an immune reaction in the organism to which it is administered.

The vaccine compositions used according to the present invention are suited for oral,
25 sublingual, inhalation, sub-cutaneous, intramuscular or intravenous administration.

According to a particular embodiment of the invention, the vaccine composition according to the invention is characterised in that it is in a pharmaceutical form intended for administration by inhalation.

- Inhalation designates absorption by the respiratory tracts. It is in particular a method for
30 absorption of compounds for therapeutic purposes, of certain substances in the form of gas, micro-droplets or powders in suspension.

The administration of pharmaceutical or veterinary compositions by inhalation, that is to say by the nasal and/or buccal passageways, is well known to the person skilled in the art.

Two types of administration by inhalation are distinguished:

- administration by insufflation, when the compositions are in the form of powders, and
 - 5 - administration by nebulisation, when the compositions are in the form of aerosols (suspensions) or in the form of solutions, for example pressurised, aqueous solutions.
- The use of a nebuliser or a spray will then be recommended for administering the pharmaceutical or veterinary composition.

10 The pharmaceutical form considered here is thus advantageously selected from: a powder, an aqueous suspension of droplets or a pressurised solution.

The population of individuals to vaccinate is mainly a paediatric population, that is to say constituted of individuals less than 18 years old, and more specifically young children less than 5 years old, and infants. Indeed, viruses of the *Pneumoviridae* family mainly infect these individuals, who have a tendency to have less strong immunity than older individuals.

15 The invention also relates to a vaccine composition such as described above, for the use thereof for preventing and/or treating infections by viruses of the *Pneumoviridae* family

EXAMPLES

20 **Example 1: Recombinant hMPVs generated from the clinical strains C-85473 and CAN98-75. 1A**

The genetic constructions represented in figures 1A and 1B were prepared in order that the viruses are detectable by expression of GFP (Green Fluorescent Protein), and to introduce into the recombinant reference strains rC-85473 and rCAN98-75 the genetic modifications intended to attenuate their virulence:

- 25 - The deletion of the gene encoding for the SH protein (Δ SH);
- The deletion of the gene encoding for the G protein (Δ G).

The complete sequences of these genetic constructions are presented in SEQ ID NO. 4 (GFP rC-85473), SEQ ID NO.5 (GFP Δ SH-rC-85473), SEQ ID NO.6 (GFP Δ G-rC-85473) and SEQ ID NO. 8 (GFP rCAN98-75). The sequences of the virus strains derived from rCAN98-75 and
30 coupled to GFP are not represented but are accessible to the person skilled in the art on the basis of the other genetic constructions presented.

As the photos of LLC-MK2 cells infected with a multiplicity of infection (MOI) of 0.01 show, the viruses being visible thanks to GFP, the following generated recombinant viruses: Δ SH-rCAN98-75, Δ G-rCAN98-75, Δ SH-rC-85473 and Δ G-rC-85473 are functional and replicative. The cells are observed by fluorescence microscopy at three days post-infection.

- 5 The photos suggest that the recombinant viruses rCAN98-75 seem less infectious and less replicative than the recombinant viruses rC-85473: the intensity of the fluorescence is lower, and the syncytia are of more reduced dimensions.

Example 2: *In vitro* replicative capacities of the recombinant viruses rC-85473 and rCAN98-75.

- 10 LLC-MK2 cells were infected separately, with a multiplicity of infection of 0.01, by the following recombinant viruses:

- rCAN98-75, Δ SH-rCAN98-75 and Δ G-rCAN98-75 (figure 2A)
- rC-85473, Δ SH- rC-85473 and Δ G- rC-85473 (figure 2B)

- 15 The cell supernatants were collected each day for 7 days, in triplicate, then their viral loads were evaluated in TCID₅₀/ml. Two-way ANOVA statistical tests were carried out to compare each deleted recombinant virus (Δ SH and Δ G) with the wild recombinant virus.

Figures 2A and 2B show the results obtained: the replicative and production capacities are different as a function of the nature of the original strain, CAN98-75 or C-85473, for the attenuated recombinant viruses.

- 20 The recombinant viruses rC-85473, Δ SH- rC-85473 and Δ G- rC-85473 have replicative and production capacities much better than those of the viruses rCAN98-75, Δ SH-rCAN98-75 and Δ G-rCAN98-75. The viruses Δ SH-rCAN98-75 and Δ G-rCAN98-75 are the least efficient, whereas the viruses Δ SH- rC-85473 and Δ G- rC-85473 have the best production capacities in culture on LLC-MK2 cells.

- 25 In the tables below are represented the average loads of the viral stocks produced and concentrated for each recombinant virus rCAN98-75, Δ SH-rCAN98-75 and Δ G-rCAN98-75 (tableau 5) and rC-85473, Δ SH- rC-85473 and Δ G- rC-85473 (table 6).

- 30 The viral loads are counted in "TCID₅₀" units which represents the final viral dilution at which 50% of the cell tissue is destroyed by the infection, or shows visible cytopathic effects (50% Tissue Culture Infective Dose).

Table 5.

Recombinant viruses	Average load of the concentrated stock (TCID ₅₀ /ml)
rCAN98-75	4.37 E+07
ΔSH –rCAN98-75	2.77 E+07
ΔG –rCAN98-75	8.65 E+06

Table 6.

Recombinant viruses	Average load of the concentrated stock (TCID ₅₀ /ml)
rC-85473	3.92 E+07
ΔSH –rC-85473	1.81 E+08
ΔG –rC-85473	2.04 E+08

These viral loads confirm the preceding results, namely that the attenuated virus strains derived from C-85437 have satisfactory replication capacities, not diminished compared to those of the original strain.

Example 3: *In vivo* characterisation of the recombinant hMPVs rCAN98-75 and rC-85473 on a BALB/c mice viral infection model

BALB/c mice were infected by intranasal instillation with:

- OptiMem culture medium (mock) or
- 2.5x10⁵ Fluorescent Focal Units (FFU) of recombinant viruses expressing GFP: rCAN98-75, ΔSH-rCAN98-75 and ΔG-rCAN98-75, or
- 5x10⁵ TCID₅₀ of viruses not expressing GFP: rC-85473, ΔSH-rC-85473 and ΔG-rC-85473.

The two virus doses used are equivalent (the wild viruses produce equivalent effects in the mice after infection).

The weight and the survival of the mice was monitored daily for 14 days (weight average over 10 mice ± SEM).

In addition, 5 days after the infection, 5 mice by group underwent euthanasia for a measurement of pulmonary infection viral loads in TCID₅₀.

Statistical tests were carried out (two-way ANOVA) to compare each virus with the mock condition. *p < 0.05, ** p < 0.01 and ***, p < 0.001.

Figures 3A and 3B show the results obtained with the viruses rCAN98-75, Δ SH-rCAN98-75 and Δ G-rCAN98-75; figures 3C and 3D show the results obtained with the viruses rC-85473, Δ SH-rC-85473 and Δ G-rC-85473.

These results indicate that the recombinant viruses Δ SH-rC-85473 or Δ G-rC-85473 only induce very little weight loss; in fact these groups of mice behave like the non-infected group of mice.

Furthermore, the pulmonary viral loads (Fig. 3D) are similar to those observed in the group infected by the virus rC-85473, indicating pulmonary replication of the deleted attenuated viruses.

The results are different with the recombinant viruses Δ SH-rCAN98-75 and Δ G-rCAN98-75: the virus Δ SH-rCAN98-75 induces a very great weight loss (Fig. 3A); whereas the virus Δ G-rCAN98-75 only induces a very slight decrease in weight (Fig. 3A), on account of its low capacity for replicating in the lungs (Fig. 3B).

Example 4: *In vivo* protective properties of the recombinant hMPVs Δ G-rC-85473 and Δ SH-rC-85473 in a BALB/c murine model following an infectious challenge by the wild virus rC-85473

The immunisation and infectious challenge protocol is shown in figure 4A.

BALB/c mice were immunised by intranasal instillation with of the OptiMem culture medium ("mock" negative control) or 5×10^5 TCID₅₀ of the recombinant viruses rC-85473, Δ SH-rC-85473 or Δ G-rC-85473.

21 days after immunisation, the mice were infected (infectious challenge) by intranasal instillation of a lethal dose (1×10^6 TCID₅₀) of the wild virus rC-85473.

The weight and survival of the mice was monitored daily for 14 days (weight average over 10 mice $\pm$ SEM, Figure 4B).

The survival of the mice was monitored daily for 14 days post-challenge, for the mice of the different groups (mock with 50% survival and immunised by the recombinant viruses rC-85473, Δ SH-rC-85473 or Δ G-rC-85473, exhibiting 100% survival). The results are shown in Figure 4C.

5 days after the infectious challenge (i.e. at day 26 post-immunisation), 4 mice of each group underwent euthanasia to measure their pulmonary infection viral loads in TCID₅₀ (Figure 4D) and RTqPCR (N gene, Figure 4E).

Statistical tests: Two-way ANOVA to compare each virus with the mock condition. *p < 0.05, ** p < 0.01 and ***, p < 0.001.

These results confirm that the recombinant viruses Δ SH-rC-85473 or Δ G-rC-85473 only induce very little weight loss (the groups of mice behave like the group of non-infected mice) and that, in these experimental conditions, protect 100% of the mice challenged by a lethal dose of wild virus rC-85473. This protection is also illustrated by the absence of detection of infectious virus and wild genome rC-85473 (nd) in the lungs at D+5 after challenge and a high micro-neutralisation load of the serums at D+21 after challenge (> 160) of mice immunised beforehand by the recombinant viruses Δ SH-rC-85473 and Δ G-rC-85473.

Table 7 below shows the results of micro-neutralisation tests, carried out from serums taken from mice of the different groups at D+20 (1 day before the infectious challenge) and at D+42 (21 days after the infectious challenge).

After inactivation for 30 minutes at 56°C and dilution of the serums, they were incubated with 100 TCID₅₀ (that is to say 100 infectious units such as determined according to the TCID₅₀/ml) of virus rC-85473 or virus rCAN98-75 for 2 hours at 37°C. This mix was next applied to the LLC-MK2 cells for a determination of the neutralisation load.

The neutralising antibody load is determined by the greatest dilution of serum at which LLC-MK2 cells, incubated with the serum + virus mix, do not show cytopathic effects, that is to say are considered negative for infection (on the basis of tests carried out in duplicate).

Table 7. Micro-neutralisation test

Serums	Day 20 before infectious challenge	Day 42, 21 days after infectious challenge (strain C-85473)	Day 42, 21 days after infectious challenge (strain CAN98-75, serotype B)
Mock	≤ 5	10	10
rC-85473	5	≥ 160	80
Δ SH rC-85473	5 -10	≥ 160	≥ 160
Δ G rC-85473	5 - 10	≥ 160	80

The figures represent the dilution of the serums tested at which the specific antibody loads efficiently neutralise the viral infection (micro-neutralisation load).

A result of 5 or 10 is representative of a primary immune response (first encounter with the virus). A result greater than or equal to 160 is characteristic of a maximum immune response, and signifies that the organism had already encountered the virus previously and thus was immunised.

- 5 The virus strains derived from the original strain rC-85473 make it possible to obtain a satisfactory neutralising humoral response against hMPV C-85473 (serotype A) and at least against the virus CAN98-75 (serotype B), after prior immunisation by the attenuated strains.

Example 5: *In vitro* cellular binding capacities of the recombinant viruses rC-85473 and rCAN98-75.

- 10 LLC-MK2 cells were infected (t=0) with:

- the virus rCAN98-75 and the attenuated forms thereof Δ SH-rCAN98-75 and Δ G-rCAN98-75 (figure 5A);
- the virus rC-85473 and the attenuated forms thereof Δ SH-rC-85473 and Δ G-rC-85473 (figure 5B);

- 15 The cells were infected on ice, for 0.5, 1, 2 or 3h in order to vary the time of binding the viruses to the cells, before being washed then incubated at 37°C for 24h.

The percentage of cells infected measured at 24h post-infection is representative of the quantity of virus capable of binding to the cells within the allocated time (0.5, 1, 2 or 3h).

- 20 The results indicate different capacities of binding to LLC-MK2 cells in culture (and thus infection capacities) for the recombinant viruses as a function of the origin of their genome CAN98-75 or C-85473.

The results show much better cell binding and cell infection capacity for the attenuated viruses Δ SH- rC-85473 and Δ G-rC-85473 (around 60% of infected cells) in comparison to those of the viruses Δ SH-rCAN98-75 (40%) and Δ G-rCAN98-75 (fewer than 20% of infected cells).

- 25 **Example 6: *Ex vivo* replicative capacities of the recombinant viruses rC-85473 and rCAN98-75 in a reconstituted human respiratory epithelium 3D model and cultured at the air-liquid interface.**

- 30 Reconstituted human respiratory epitheliums (MucilAir® HAE, Epithelix) and maintained in culture at the air-liquid interface according to the instructions of the supplier Epithelix, were used to carry out these *ex vivo* experiments.

The photos shown in figure 6A indicate that the recombinant viruses rCAN98-75 appear less infectious and less replicative than the recombinant viruses rC-85473 (lower intensity of fluorescence and more reduced dimensions of the sites of infection).

The results of quantification of viral genome in the epitheliums (figure 6C) confirm the replicative capacities of the recombinant viruses rC-85473, Δ SH- rC-85473 and Δ G- rC-85473, as well as the recombinant viruses rCAN98-75 and Δ SH-rCAN98-75, unlike the virus Δ G-rCAN98-75 which is very weakly replicative.

The results of quantification of viral genome at the surface of the apical pole of the epitheliums (figure 6B) indicate production of recombinant viruses rCAN98-75, Δ SH-rCAN98-75, Δ G-rCAN98-75, rC-85473 and Δ SH- rC-85473.

On the other hand, no genome of the virus Δ G- rC-85473 is detected, indicating the absence of viral progeny production. A hypothesis is that the virus Δ G-rC-85473 could have lost the capacity to bud at the surface of the cells infected in this complex and pluri-stratified *ex vivo* system.

These differences could be of a nature to further argue in favour of the inventive character of our invention, focusing on the different properties between recombinant viruses of different origins in terms of hMPV virus strain, for the protection of our attenuated living vaccine candidates Δ SH- rC-85473 and Δ G- rC-85473.

Example 7: Secretion of cytokines and chemokines induced by infection with the recombinant viruses rC-85473 on a reconstituted human respiratory epithelium 3D model and cultured at the air-liquid interface.

Reconstituted human respiratory epitheliums (MucilAir® HAE, Epithelix) maintained in culture at the air-liquid interface, according to the instructions of the supplier Epithelix, were used to carry out these *ex vivo* experiments.

The quantification of a selection of cytokines and chemokines secreted in the basal medium of the infected epitheliums reflects the induction of a cell response that differs as a function of the recombinant viruses rC-85473 evaluated (figure 7).

a) the virus Δ G-rC-85473 induces the secretion of a significantly lower quantity of the different cytokines and chemokines tested, in comparison with the wild strain rC-85473, with the exception of the pro-inflammatory cytokine TNF- α . These results are consistent with those shown in figure 6, which show that the infectivity of this virus in reconstituted human respiratory epithelium is significantly reduced compared to the infectivity of the wild strain.

b) the virus Δ SH-rC-85473, even though its capacity for infection and replication in the reconstituted human respiratory epithelium model is increased compared to that of the wild recombinant virus rC-85473 (figure 6), induces the secretion of an equivalent or lower amount of the panel of cytokines and chemokines evaluated compared to that viral-induced by the wild virus.

The levels of secreted chemokines MCP-1, IP-10, RANTES, IL-8, G-CSF and pro-inflammatory cytokines IFN- γ and TNF- α seem to indicate that epithelial cells infected by this virus Δ SH-rC-85473 are able to recruit the T lymphocyte, monocyte/macrophage, eosinophil and/or neutrophil immune cells necessary for putting in place an innate immune response and adaptative response of the host.

In addition, the virus Δ SH-rC-85473 leads to the secretion of IL-7, a cytokine inducing the differentiation of inactive lymphocytes into active T and B lymphocytes, and FGF, a growth factor involved in the repair of organic tissues, in amounts similar to the wild virus rC-85473.

Thus, infection by the virus Δ SH-rC-85473 enables epithelial cells to put in place the primary defence response to the infection, while being associated with a more reduced inflammatory response of the host, in comparison with that induced by the wild virus.

These differences would thus be in favour of the use of recombinant viruses derived from the strain rC-85473 as attenuated living vaccine, and preferentially from the attenuated strain Δ SH-rC-85473.

Example 8: Viral replication and secretion of the pro-inflammatory cytokines IL-1 β and TNF- α *in vitro* of the recombinant hMPVs rC-85473 on an immortalised murine macrophage model.

An immortalised line of mice macrophages was used to evaluate the replication and induction capacity of pro-inflammatory cytokines by the recombinant viruses rC-85473, Δ SH-rC-85473 and Δ G-rC-85473.

Macrophages form part of the first cells of the immune system recruited on the site of infection (in the present case, the respiratory epithelium). Macrophages contribute to the inflammatory response, to the innate response and to the stimulation of the adaptative response of the host.

The macrophages were infected by the recombinant viruses rC-85473, Δ SH-rC-85473 or Δ G-rC-85473 and their cell supernatants were collected each day for 2 days. The results are shown in figure 8.

a) the viral loads measured in FFU/ml demonstrate that the virus Δ SH-rC-85473 has a decreased replicative capacity on the macrophages, compared to the wild recombinant virus, whereas the virus Δ G-rC-85473 shows a viral replication similar to the wild virus (figure 8A). Two-way ANOVA statistical tests were carried out to compare the kinetic of replication of the deleted virus Δ SH-rC-85473 with that of the wild recombinant virus.

b) the secretion of the pro-inflammatory cytokines TNF- α (figure 8B) and IL-1 β (figure 8C) was next measured in the supernatant of the infected macrophages.

The virus Δ SH-rC-85473 leads to a cytokine profile significantly different from that of the wild virus rC-85473 with an earlier secretion peak after 1 day post-infection, in comparison with the wild virus which is associated with a secretion peak at 2 days post-infection, concerning these two cytokines.

The virus Δ G-rC-85473 induces the secretion of cytokine TNF- α at levels comparable to the wild virus, but does not induce the secretion of cytokine IL-1 β during the 2 first days of infection.

These results demonstrate that the virus Δ SH-rC-85473 conserves the capacity to infect murine macrophages, and especially to lead to the secretion of pro-inflammatory cytokines, despite a modified kinetic.

The virus Δ G-rC-85473 efficiently infects murine macrophages, but induces more weakly the secretion of pro-inflammatory cytokines, as has been observed on the reconstituted human respiratory epithelium model.

Example 9: Pulmonary inflammation and recruitment of immune cells on the site of the infection *in vivo* induced after 5 days of infection of BALB/c mice by the recombinant hMPVs rC-85473, Δ G-rC-85473 and Δ SH-rC-85473.

BALB/c mice were infected by intranasal instillation with:

- OptiMem (mock) culture medium

- 5×10^5 TCID₅₀ of the recombinant viruses rC-85473, Δ SH-rC-85473 or Δ G-rC-85473.

After 5 days of infection, 5 mice per group underwent euthanasia and their lungs taken for a histo-pathological analysis and a measurement of the inflammation scores resulting from infection (figure 9A).

Statistical tests were carried out (two-way ANOVA) to compare each condition with each other: *p < 0.05, ** p < 0.01 and ***, p < 0.001.

Figure 9A shows that the wild virus rC-85473 induces a strong pulmonary inflammation in the infected mice, whereas the viruses Δ SH-rC-85473 and Δ G-rC-85473 lead to an inflammation that is significantly lower than the wild virus, in adequation with the reduced severity of the pathology with the attenuated strains demonstrated previously in figure 3.

- 5 Furthermore, at 5 days post-infection, 5 mice per group underwent euthanasia and their lungs taken for the absolute quantification of the CD4+, CD8+ lymphocyte, neutrophil and macrophage immune cells infiltrated into the pulmonary tissue following infection (figure 9B).

Statistical tests were carried out (one-way ANOVA) to compare the condition Δ SH-rC-85473 with the condition rC-85473: ** $p < 0.01$.

- 10 Figure 9B demonstrates that the different populations of immune cells (CD4+ T, CD8+ T, neutrophils and macrophages) are recruited on the site of the infection after 5 days of inoculation of the viruses rC-85473 and Δ SH-rC-85473, but in lower quantity for the attenuated strain, notably as regards CD4+ lymphocytes.

- 15 Thus, it would seem that the virus Δ SH-rC-85473 leads to reduced pulmonary inflammation, in coherence with the attenuation of the pathology viral-induced in a murine model (figure 3) and the decrease in the secretion of cytokines by the infected epithelial cells (figure 7), while ensuring efficient recruitment of the different immune cells on the site of the infection.

These different results (figures 7 to 9) show the different properties between wild recombinant rC-85473 and deleted viruses with regard to their post-infection physiological effects.

- 20 The attenuated strain Δ SH-rC-85473 seems particularly promising for the development of an attenuated living vaccine, with regard to its properties from the point of view of (i) the secretion of inflammatory cytokines and chemokines by epithelial and macrophagic cells, (ii) the induction of pulmonary inflammation *in vivo* and (iii) the infiltration *in vivo* of immune cells into the infected organ. Indeed, infection of a host by this attenuated strain is associated both with an efficient
25 primary immune response (equivalent to that observed with the wild virus) and with a reduced inflammatory response.

- 30 **Example 10: Secretion of cytokines/chemokines, recruitment of immune cells on the site of the infection *in vivo* and pulmonary inflammation in a BALB/c murine model immunised by the recombinant hMPVs r Δ G-C-85473 and Δ SH-C-85473, following an infectious challenge.**

Following a viral infection, a complex inflammatory and immune response is put in place on the site of the infection thanks to the secretion of cytokines and chemokines by infected epithelial cells (see figure 7) and resident immune cells (figure 8). Firstly, the effector cells of the innate

response (non-specific) are recruited and activated then, secondly, the effector cells of the adaptive response (specific). When the infected individual has been immunised or vaccinated beforehand, the adaptative response is all the more rapid, important and specific the more efficient the memory response. It is the characterisation of this immune response, called secondary, which is described in this example.

BALB/c mice were immunised by intranasal instillation with OptiMem culture medium ("mock" negative control) or 5×10^5 TCID₅₀ of the recombinant viruses rC-85473, Δ SH-C-85473 or Δ G-C-85473.

21 days after immunisation, the mice were infected (infectious challenge) by intranasal instillation of a lethal dose (1×10^6 TCID₅₀) of the wild virus rC-85473. The immunisation and infectious challenge protocol is shown in figure 4A.

1 or 5 days after the infectious challenge (i.e. at days 22 or 26 post-immunisation respectively), 3 mice of each group underwent euthanasia to quantify a selection of cytokines and chemokines secreted in the pulmonary tissue: the results are shown in figures 10A, 10B and 10C. These results demonstrate the induction of a local immune response that differs as a function of the recombinant viruses rC-85473 used for the immunisation of the BALB/c mice:

- The groups immunised by the viruses Δ G-C-85473 and Δ SH-C-85473 show a stronger secretion of the pro-inflammatory cytokine IL-6 and the chemokine MCP-1 than the group immunised by the virus WT rC-85473, rapidly 1 day after the infectious challenge.
- In addition, the group immunised by the virus Δ SH-C-85473 also shows a significant increase in the secretion of the anti-inflammatory cytokine IL-10 and the chemokines G-CSF and MIP-1 β , compared to the group immunised by the virus WT and/or by the virus Δ G-C-85473

After 5 days, all the cytokines/chemokines tested were in sharp decrease compared to the levels measured on day 1 post-challenge, which demonstrates a resorption of the cytokine and inflammatory response induced by the challenge.

These results suggest that immunisation by the attenuated viruses Δ G-C-85473, and more particularly Δ SH-C-85473, leads to the local putting in place of a cytokine response different to that induced by immunisation by the corresponding non-attenuated virus WT.

To evaluate the recruitment of immunity cells on the site of the infection, the amount of main populations of immune cells that infiltrate into the pulmonary tissue to combat against the infection was measured; the results are shown in figures 10D, 10E and 10F.

Thus, the recruitment of T lymphocytes (CD8+ cytotoxic and CD4+ helpers) and B lymphocytes is significantly increased following immunisation with the virus Δ SH-C-85473, compared to the group immunised by the virus WT rC-85473, and compared to the group immunised by the virus Δ G-C-85473 (significant difference uniquely for B lymphocytes, figure 10E), 5 days after the infectious challenge.

The populations of recruited macrophages and neutrophils do not show significant differences between the different viruses tested (figure 10F).

The group of mice immunised by the virus Δ G-C-85473 shows a cell recruitment profile similar to that of the group immunised by the virus WT, which suggests that the virus Δ G-C-85473 is as efficient for the induction of immune response as a wild virus (WT). Conversely, when the mice were immunised beforehand with the virus Δ SH-C-85473, the adaptive immune response in response to the infectious challenge is more important, while remaining balanced, whereas the innate response does not seem to be affected, which suggests that the virus Δ SH-C-85473 would not induce a more efficient memory immune response.

Finally, 5 days after the challenge, 2 mice per group underwent euthanasia to measure the pulmonary inflammation scores resulting from the challenge of each immunised and “mock” non-immunised group; the results are shown in figure 10G.

As expected with the use of a lethal inoculum of virus WT rC-85473, which corresponds to 50% mortality of the population [see figure 4C], the cumulative inflammation score of the mock-immunised group is very high. As previously, the inflammation scores of the groups immunised by the virus WT rC-85473 or Δ G-C-85473 are very comparable with each other, and slightly decreased compared to the mock-immunised even though the mice of these groups do not demonstrate any mortality [Figure 4C].

The results of figure 10 show that mice immunised by the virus Δ SH-C-85473 put in place a very reduced inflammatory response following the infectious challenge, which suggests that pulmonary attacks would be reduced in this group. Similarly, the pulmonary functionalities would be conserved during infection.

Thus, the attenuated virus Δ SH-C-85473 could enable better pulmonary recruitment of immune cells, in quantity and in quality, in response to a secondary infection, and thus contribute to limiting physiopathology and pulmonary inflammation of the infected host. This more efficient response of the host could also reflect a better memory immune response against the hMPV virus.

In conclusion, this attenuated virus Δ SH-C-85473 has the following characteristics:

(i) it induces greater quantities of pro-inflammatory, anti-inflammatory cytokines and chemokines than the other viruses tested;

(ii) this enabling a more important, but balanced, recruitment of immune cells (CD8+/CD4+ T lymphocytes and B lymphocytes) on the site of the infection,

5 (iii) while generating limited pulmonary inflammation

Table 8. Summary of sequences presented in the sequence listing

	Description
SEQ ID NO. 1	Complete genome sequence of the wild strain of hMPV C-85473
SEQ ID NO. 2	Complete sequence of the recombinant hMPV Δ SH-rC-85473
SEQ ID NO. 3	Complete sequence of the recombinant hMPV Δ G-rC-85473
SEQ ID NO. 4	Complete sequence of the wild recombinant hMPV v rC-85473 coupled to the GFP sequence
SEQ ID NO. 5	Complete sequence of the recombinant hMPV Δ SH-rC-85473 coupled to the GFP sequence
SEQ ID NO. 6	Complete sequence of the recombinant hMPV Δ G-rC-85473 coupled to the GFP sequence
SEQ ID NO. 7	Complete sequence of the wild genome HMPV CAN98-75
SEQ ID NO. 8	Complete sequence of the wild recombinant hMPV CAN98-75 coupled to the GFP sequence

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Patents

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CLAIMS

1. Attenuated virus strain derived from a human *metapneumovirus* strain comprising the genome sequence represented by sequence SEQ ID NO. 1, said attenuated strain comprising one or more genetic modifications of said sequence SEQ ID NO. 1 attenuating the virulence of said strain.
2. Attenuated virus strain according to claim 1, characterised in that the modifications of sequence SEQ ID NO.1 comprise the inactivation of the gene encoding for the SH protein.
3. Attenuated virus strain according to claim 2, characterised in that said strain comprises sequence SEQ ID NO.2.
4. Attenuated virus strain according to claim 1, characterised in that the modifications of sequence SEQ ID NO.1 comprise the inactivation of the gene encoding for the G protein.
5. Attenuated virus strain according to claim 4, characterised in that said strain comprises sequence SEQ ID NO.3.
6. Attenuated virus strain according to claim 1, characterised in that the modifications of sequence SEQ ID NO.1 comprise the inactivation of the two genes encoding for the G protein and the SH protein.
7. Attenuated virus strain according to one of claims 1 to 6, of which the sequence comprises at least one exogenous gene, in particular a gene encoding for a viral antigen.
8. *In vitro* use of an attenuated virus strain according to claim 7, as expression vector of at least one exogenous gene in target cells of human *metapneumovirus*.
9. Attenuated virus strain according to one of claims 1 to 7, for use as a medicament.
10. Attenuated virus strain according to one of claims 1 to 7, for use in preventing and/or treating infections by viruses of the *Pneumoviridae* family.
11. Attenuated virus strain for the use according to claim 10, for preventing and/or treating infections by human *metapneumoviruses*.

12. Attenuated virus strain for the use according to claim 10, for preventing and/or treating infections by *orthopneumoviruses*, such as the human syncytial respiratory virus.

13. Attenuated virus strain according to claim 7 for use in preventing and/or treating infections by viruses whose at least one viral antigen is expressed by said attenuated virus strain.

14. Vaccine composition comprising, in a pharmaceutically acceptable vehicle, at least one attenuated virus strain according to one of claims 1 to 7, and optionally an adjuvant.

15. Vaccine composition according to claim 14, characterised in that it is in a pharmaceutical form intended for administration by inhalation.