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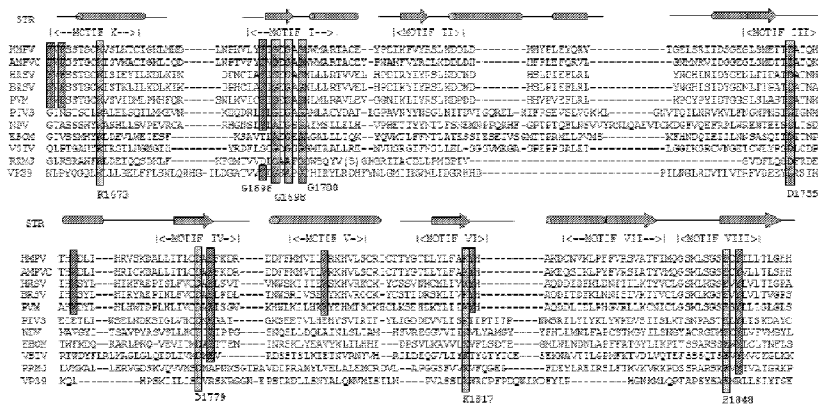


Fig. 1

- (57) Abstract:** The present invention includes methyltransferase (MTase) -defective recombinant viruses as live vaccine candidates for human metapneumovirus (hMPV), human respiratory syncytial virus (hRSV), and human parainfluenza virus type 3 (PIV3). Here the inventors provide the technical description for generating MTase-defective paramyxoviruses useful as immunogens, as well as related materials and methods.

TITLE

Paramyxovirus Immunogens and Related Materials and Methods

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CROSS REFERENCE TO RELATED APPLICATIONS

[0001] This application claims the benefit of United States Provisional Application No. 61/495,119 filed June 9, 2011, the disclosure of which is incorporated herein by reference.

STATEMENT REGARDING FEDERALLY SPONSORED RESEARCH

[0002] This invention was made with U.S. Government financial support under 60026680, awarded by NIH/NIAID R56 and 60024452 awarded by NIH/NIAID R01. The government may have certain rights in this invention.

REFERENCE TO SEQUENCE LISTING SUBMITTED VIA EFS-WEB

[0003] This application is being filed electronically via the USPTO EFS-WEB server, as authorized and set forth in MPEP§1730 II.B.2(a)(A), and this electronic filing includes an electronically submitted sequence (SEQ ID) listing. The entire content of this sequence listing is herein incorporated by reference for all purposes. The sequence listing is identified on the electronically filed .txt file as follows: 604_53068_SeqListing_OSU-2011-146.txt, created on June 11, 2012 and is 19,469 bytes in size.

TECHNICAL FIELD OF THE INVENTION

[0004] The present invention relates to biotechnology, including modified viruses. The invention is in the field of medicine and immunology, including immunogen delivery to living cells and organisms.

BACKGROUND OF THE INVENTION

[0005] Paramyxoviruses are the leading causative agents of acute viral respiratory tract infections. Among the paramyxoviruses, human metapneumovirus (hMPV), human respiratory syncytial virus (RSV), and human parainfluenza virus type 3 (hPIV3) account for more than 70% of acute viral respiratory diseases. All of three viruses cause similar clinical signs and symptoms, ranging from mild respiratory problems to severe coughs, bronchiolitis, and pneumonia. All three viruses cause acute respiratory tract disease in individuals of all ages, especially in infants, children, the elderly, and immunocompromised individuals. In the United States, 60% of infants are infected during their first RSV season, and nearly all children will have been infected with the virus by 2–3 years of age. HMPV is a newly discovered human pathogen, first identified in 2001 in The Netherlands. Soon after its discovery,

hMPV was recognized as a globally prevalent pathogen. Epidemiological studies suggest that 5 to 15% of all respiratory tract infections in infants and young children are caused by hMPV, a proportion second only to that of RSV. PIV3 is the third causative agent of viral respiratory infection in children and infants. All three pathogens are globally prevalent.

[0006] Despite the enormous economic losses and emotional burdens these viruses cause, vaccines and anti-viral drugs are currently not available. For decades, approaches to generate vaccines employing viral proteins or inactivated vaccines have failed either due to a lack of immunogenicity or the potential for causing enhanced pulmonary disease upon natural infection with the same virus.

[0007] Vaccination has been the most effective public health strategy to reduce morbidity and mortality associated with viral infections. The increasing clinical significance of RSV, hMPV and PIV3 infections suggest that there is an urgent need for a safe and efficacious vaccine against these viruses, particularly for the populations at high risk such as infants, children, elderly, and immunocompromised individuals. An effective vaccine would not only prevent acute respiratory tract infection caused by these viruses, but also block transmission routes and thus improve human and public health. In the current inventions, the inventors developed a panel of live attenuated vaccines against RSV, hMPV and PIV3.

[0008] However, development of vaccine against human paramyxoviruses has met serious challenge. With the exception of the influenza virus, there is no FDA approved vaccine for other viruses that cause acute upper and lower respiratory tract infections in human. Generally, inactivated and live attenuated vaccines are the two most common strategies used in vaccines against infectious diseases. For safety, an inactivated vaccine is preferred. However, development of an inactivated vaccine for the paramyxovirus RSV turned out to be a problem. A formalin-inactivated RSV vaccine developed and tested in the 1960s not only failed to induce a protective immune response in human, but led to an enhanced respiratory disease upon natural infection with RSV. Eighty percent of the vaccinated children were hospitalized following natural RSV infection, and two children died. Enhanced respiratory disease following vaccination with inactivated vaccine has been observed in other paramyxoviruses such as PIV-3, hMPV and measles virus. A recent study showed that cotton rats immunized with inactivated hMPV vaccine were protected against infection, but developed increased lung pathology. These observations suggest that inactivated vaccines are not the primary choices for three paramyxoviruses: hMPV, RSV, and PIV3, all of which cause extensive morbidity and mortality in the same population, infants and children.

[0009] In contrast to inactivated vaccines, enhanced lung diseases have not been observed for candidate live attenuated RSV vaccines. Therefore, live attenuated vaccines are the most promising vaccine candidates for hMPV, RSV, and PIV3. However, it has been technically challenging to isolate a virus with low virulence that retains high immunogenicity. In paramyxoviruses, spike proteins (F and G proteins for RSV and hMPV) are major determinants of virulence. Therefore, traditional attenuation

strategies have been focused on engineering mutations in these two glycoproteins. However, F and G proteins are also viral immunogenic antigens that are responsible for immune response. As a consequence, mutations in glycoproteins may impair the immunogenicity of the attenuated live vaccine. Therefore, exploration of new attenuation approaches is urgently needed.

[0010] This invention develops new attenuated viruses as live vaccine candidates for major human paramyxoviruses including hMPV, RSV and PIV3 by targeting viral mRNA cap methyltransferase (MTase). Paramyxoviruses share a common strategy for replication and gene expression. During RNA synthesis, paramyxoviruses yield capped, methylated, and polyadenylated mRNAs. Methylation of the mRNA directly impacts the stability of mRNA and subsequent translation of viral proteins, which in turn affects viral genome replication, virus assembly, and budding. The large (L) polymerase protein catalyzes the mRNA cap MTases. Recombinant virus defective in MTase can be recovered from cloned full-length viral cDNA by a reverse genetics system. Viruses lacking MTase would likely be attenuated without affecting immunogenicity, since the MTase is located in L protein, which is not a neutralizing antibody target. Thus, ablating viral mRNA cap methylation provides a new avenue to rationally attenuate these viruses for development of live attenuated vaccines.

SUMMARY OF THE INVENTION

[0011] The present invention provides recombinant paramyxovirus compositions comprising a nucleic acid molecule which encodes a defective mRNA cap MTase in a paramyxovirus viral genome. Provided are those compositions wherein the composition is defective in mRNA cap MTase gene expression. Also provided are those compositions wherein paramyxovirus viral genome is selected from the group consisting of: a human metapneumovirus (hMPV); human respiratory syncytial virus (hRSV); and human parainfluenza virus type 3 (PIV3).

[0012] The present invention also provides compositions wherein the nucleic acid molecule which encodes a defective mRNA cap MTase carries at least one mutation in the MTase catalytic site. Also provided are those compositions wherein the at least one mutation in the MTase catalytic site is in at least one of the K-D-K-E tetrad sites. Also provided are those compositions wherein the paramyxovirus is human metapneumovirus (hMPV) and the at least one K-D-K-E tetrad site mutation is at least one of rhMPV-K1673A, rhMPV-D1779A, rhMPV-K1817A, rhMPV-E1848A, and rhMPV-E1848Q. Also provided are those compositions wherein the paramyxovirus is human respiratory syncytial virus (hRSV) and the at least one K-D-K-E tetrad site mutation is at least one of rRSV-K1831A, rRSV-D1936A, rRSV-K1973A, rRSV-E2004A. Also provided are those compositions wherein the paramyxovirus is human parainfluenza virus type 3 (PIV3) and the at least one K-D-K-E tetrad site mutation is at least one of rPIV3-K1786A, rPIV3-D1905A, rPIV3-K1941A, rPIV3-E1978A.

[0013] The present invention also provides compositions wherein the nucleic acid molecule which encodes a defective mRNA cap MTase carries at least one mutation in the SAM binding site. Also provided are those compositions according to claim 9, wherein the at least one mutation in the SAM binding site is in the GxGxG...D/E/W site. Also provided are those compositions wherein the paramyxovirus is human metapneumovirus (hMPV) and the at least one mutation in the GxGxG...D/E/W site is at least one of rhMPV-G1696A, rhMPV-G1698A, rhMPV-G1700A, and rhMPV-D1755A. Also provided are those compositions wherein the paramyxovirus is human respiratory syncytial virus (hRSV) and the at least one mutation in the GxGxG...D/E/W site is at least one of rRSV-G1853A, rRSV-G1855A, rRSV-G1857A, and rRSV-D1912A. Also provided are those compositions wherein the paramyxovirus is human parainfluenza virus type 3 (PIV3) and the at least one mutation in the GxGxG...D/E/W site is at least one of rPIV3-G1808A, rPIV3-G1810A, rPIV3-G1812A, and rPIV3-W1880A. Also provided are those compositions wherein the nucleic acid molecule which encodes a defective mRNA cap MTase carries at least one mutation in the MTase catalytic site and the SAM binding site.

[0014] The present invention also provides compositions wherein the at least one mutation in the MTase catalytic site is in at least one of the K-D-K-E tetrad sites and the at least one mutation in the SAM binding site is in the GxGxG...D/E/W site. Also provided are those compositions wherein the paramyxovirus is human metapneumovirus (hMPV) and the at least one K-D-K-E tetrad site mutation is at least one of rhMPV-K1673A, rhMPV-D1779A, rhMPV-K1817A, rhMPV-E1848A, and rhMPV-E1848Q and the at least one mutation in the GxGxG...D/E/W site is at least one of rhMPV-G1696A, rhMPV-G1698A, rhMPV-G1700A, and rhMPV-D1755A. Also provided are those compositions wherein the paramyxovirus is human respiratory syncytial virus (hRSV) and the at least one K-D-K-E tetrad site mutation is at least one of rRSV-K1831A, rRSV-D1936A, rRSV-K1973A, rRSV-E2004A and the at least one mutation in the GxGxG...D/E/W site is at least one of rRSV-G1853A, rRSV-G1855A, rRSV-G1857A, and rRSV-D1912A. Also provided are those compositions wherein the paramyxovirus is human parainfluenza virus type 3 (PIV3) and the at least one K-D-K-E tetrad site mutation is at least one of rPIV3-K1786A, rPIV3-D1905A, rPIV3-K1941A, rPIV3-E1978A and the at least one mutation in the GxGxG...D/E/W site is at least one of rPIV3-G1808A, rPIV3-G1810A, rPIV3-G1812A, and rPIV3-W1880A.

[0015] Also provided are those compositions which are mammalian immunogens.

[0016] Also provided are those compositions which are human immunogens.

[0017] Also provided are methods of eliciting an immune response in a mammal comprising administering to a mammal a recombinant paramyxovirus composition herein. Also provided are methods wherein the composition is administered orally. Also provided are those compositions wherein

the composition is administered intranasally.

[0018] Also provided are methods of preparing a pharmaceutical composition for passive immunization of an individual in need of immunization comprising mixing a paramyxovirus composition herein with a suitable excipient or carrier, thereby forming a pharmaceutical composition. Also provided are pharmaceutical compositions formulated for oral administration. Also provided are compositions wherein the pharmaceutical composition is formulated for intranasal administration. Also provided are pharmaceutical compositions wherein the paramyxovirus virulence is attenuated or eliminated in any mammal susceptible to paramyxovirus.

BRIEF DESCRIPTIONS OF THE FIGURES

[0019] **Figure 1. Sequence alignment of conserved domain VI of L proteins of human paramyxoviruses and modeling with two known 2'-O MTase structures, VP39 (SEQ ID NO: 11) and RRMJ (SEQ ID NO: 10).**

[0020] STR: structure of RRMJ and VP39. Predicted or known alpha-helical regions are shown as cylinders and the β -sheet regions as arrows. The conserved motifs (X and I to VIII) correspond to the SAM-dependent MTase superfamily are indicated. The predicted MTase active site (K-D-K-E tetrad) is shown by yellow boxes. The predicted SAM binding site (GxGxG---D/E/W) is shown by grey boxes. The conserved aromatic amino acid residues are shown by red boxes. Representative members of *Paramyxoviridae* (HMPV, human metapneumovirus (SEQ ID NO: 1); AMPVC, avian metapneumovirus type C (SEQ ID NO: 2); HRSV, human respiratory syncytial virus (SEQ ID NO: 3); BRSV, bovine respiratory syncytial virus (SEQ ID NO: 4); PVM, pneumonia virus of hamsters (SEQ ID NO: 5); PIV3, human parainfluenza virus type 3 (SEQ ID NO: 6); NDV, Newcastle disease virus (SEQ ID NO: 7)), *Filoviridae* (EBOM, Ebola virus (SEQ ID NO: 8)), *Rhabdoviridae* (VSIV, vesicular stomatitis virus Indiana serotype (SEQ ID NO: 9)) are shown.

[0021] **Figure 2. Examination of the function of hMPV L protein by a minigenome assay.**

[0022] A minigenome system was established to analyze the function of L protein carrying mutations in catalytic site or SAM binding site. To establish this system, the full-length genomic cDNA of hMPV in the plasmid (phMPV) was replaced by green fluorescent protein (GFP) flanked hMPV trailer and gene end sequences on one side and hMPV gene start and leader sequences on the other side, followed by the HDV ribozyme, and a T7 terminator, to yield phMPV-GFP. To achieve maximum level of minigenome replication, vaccinia vTF-7 was used as the source of T7 polymerase. Briefly, BHK cells were infected by vaccinia vTF-7 at a MOI of 10, followed by transfection of phMPV-GFP together with support plasmids (pN, pP, pL and pM2-1) using a standard protocol recommended by Invitrogen. Two days later, GFP expression was observed by fluorescence microscopy. Each amino acid residue in the

MTase catalytic site and SAM binding site was substituted into alanine in the L gene of hMPV (pL) by site-directed mutagenesis. All plasmids were sequenced to confirm the presence of the designed mutation. The effect of each L gene mutation on GFP expression was analyzed by the minigenome assay as described above.

[0023] **Figure 3. Recovery of recombinant hMPV from full-length genomic cDNA clones.**

[0024] A schematic hMPV genome comprising a leader region (Le); eight genes that encode the viral N, P, M, F, M2, SH, G and L proteins, and a trailer region (Tr), is shown. Recombinant hMPV was recovered by transfection of plasmids encoding the full-length hMPV genome (phMPV), pM2, pL, pP, and pN into BSRT7 cells which stably expressing T7 RNA polymerase. Six days post-transfection, the cells were subjected to three freeze-thaw steps and the supernatant was used to infect Vero-E6 cells (ATCC). TPCK-typsin (0.5µg/ml) was added to cells at day 2 post-infection since hMPV requires typsin to grow. Cytopathic effects (CPE) were observed after 5 day post-infection. Viruses were further amplified in Vero-E6 cells. Each amino acid residue in the MTase catalytic site and SAM binding site was substituted into alanine in the L gene of hMPV genome by site-directed mutagenesis. All plasmids were sequenced to confirm the presence of the designed mutation. Recombinant hMPVs carrying mutations in MTase catalytic and SAM binding site were recovered by an identical procedure as described above. Vero E6 cells were infected by the recovered hMPV and viral replication and protein synthesis was determined.

[0025] **Figure 4: Summary of recombinant viruses carrying mutations in either MTase catalytic site or SAM binding site.**

[0026] Recombinant hMPV, RSV and PIV3 carrying mutations in either MTase catalytic site or SAM binding site were recovered by the procedure described in Figure 2. Viral RNA was extracted from each recombinant virus. The entire *L* gene of each recombinant virus was amplified by reverse transcription-PCR (RT-PCR), and sequence analysis confirmed the presence of the desired mutation.

[0027] **Figure 5: Characterization of recombinant MTase-defective hMPV by immunostaining.**

[0028] Recombinant hMPV forms plaques visualized by immunostaining. Vero E6 cells were infected with the indicated virus and overlaid with 2% methyl cellulose. After 5 days, the methyl cellulose was removed, and cells were incubated with a monoclonal antibody against hMPV N protein (Santa Cruz Biotechnology, Inc.), followed by incubation with horseradish peroxidase-labeled rabbit anti-guinea pig antibodies (Invitrogen). After incubation with AEC substrate chromogen (Invitrogen), viral plaques were visualized under the microscope.

[0029] **Figure 6: Characterization of recombinant MTase-defective hMPV by agarose overlay plaque assay.**

[0030] Recombinant hMPV forms small rounded plaques visualized by crystal violet staining. Vero-

E6 cells were infected with indicated virus. After 1 h infection, the cells were overlaid with 2 ml of cell culture medium containing 0.5% agarose and 5% FBS, and incubated for 6 days. The plates were fixed by 10% formaldehyde, followed by staining the crystal violet.

[0031] **Figure 7: MTase-defective recombinant hMPV has a delayed cytopathic effect (CPE) in Vero cells.**

[0032] Recombinant MTase-defective rhMPV-G1696A was shown as an example. Vero E6 cells were infected with wild type rhMPV or rhMPV-G1696A at a MOI of 1, and the cytopathic effect (CPE) was observed at day 0, 3 and 5 post-infection by light microscopy.

[0033] **Figure 8: Single step growth curves of MTase-defective rhMPV.**

Confluent Vero E6 cells were infected with individual viruses at an MOI of 1. After 1 h of incubation, the inoculum was removed, the cells were washed with DMEM, and fresh medium (containing 2% fetal bovine serum) was added, followed by incubation at 37°C. Samples of supernatant were harvested at the indicated intervals over a 48-h time period, and the virus titer was determined by plaque assay using immunostaining. Titers are the average of three independent experiments.

[0034] **Figure 9. Analysis of viral protein synthesis in virus-infected cells.**

[0035] Confluent Vero E6 cells were infected with either rhMPV or MTase-defective rhMPV at a MOI of 1. After 48 h postinfection, cells were washed with methionine- and cysteine-free (M^-C^-) medium and incubated with fresh M^-C^- medium supplemented with actinomycin D (15 μ g/ml). After 1 h of incubation, the medium was replaced with M^-C^- medium supplemented with EasyTag ^{35} S-Express (4 μ Ci/ml; Perkin-Elmer, Wellesley, MA). After 24 h of incubation, cytoplasmic extracts were prepared and analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) as described previously. Labeled proteins were detected either by autoradiography or by using a phosphorimager.

[0036] **Figure 10A – Figure 10B. Pathogenicity of MTase-defective hMPV in a hamster model**

[0037] (A) Diagram of proposed animal experiments. (B) Two-week-old female hamsters (Charles River laboratories, Wilmington, MA) were inoculated intranasally with three different doses (6.48×10^6 , 1.0×10^5 , 1.0×10^4 PFU) of the wild type hMPV or MTase-defective hMPV. In one group, hamsters were inoculated with cell culture medium (DMEM) and served as uninfected controls. After inoculation, the animals were evaluated on a daily basis for mortality, weight loss, and the presence of any respiratory symptoms of hMPV. At day 4 post-infection, five hamsters from each group were sacrificed, and their lungs and nasal turbinates were removed for pathogenicity studies as follows. (i) **Virus titer in lung.** One lung from each animal was weighed and homologized in 1 ml of phosphate-buffered saline (PBS). Viral titer was determined by plaque assay and viral RNA was quantified by real-time reverse-transcriptase polymerase chain reaction (RT-PCR). (ii) **Virus titer in nasal turbinate.** Nasal turbinate from each hamster was removed, weighed, and virus titer was determined by plaque assay. (iii) **Pulmonary histopathology.** One

lung from each hamster was inflated with 10% buffered formalin, embedded in paraffin, sectioned, and stained with hematoxylin-eosin. Histopathological changes were scored include the extent of inflammation (focal or diffuse), the pattern of inflammation (peribronchilolar, perivascular, interstitial, alveolar), and the nature of the cells making up the infiltrate (neutrophils, eosinophils, lymphocytes, macrophages). Deparaffinized sections were also stained with polyclonal antiserum to determine the distribution of viral antigen. Five animals per cohort was used in these experiments.

[0038] **Figure 11. Immunogenicity of MTase-defective hMPV in a hamster model**

Two-week-old female hamsters were inoculated intranasally with two different doses (10^5 and 10^4 PFU) of the MTase-defective hMPV strains. As the controls, hamsters were inoculated with PBS. Serum samples were collected at days 7, 14, 21, and 28 post-inoculation for the detection of humoral immune response. At day 28 post-inoculation, hamsters were sacrificed, and spleen samples were isolated for the detection of cellular immune response. The hamsters were also challenged with 10^6 PFU of the wild-type hMPV. After the challenge, each animal was evaluated on a daily basis for weight loss and the presence of any respiratory symptoms. At day 4 post-challenge, all the animals were sacrificed and lung samples were collected for virus detection and pathological examination. The immunogenicity of the MTase-defective hMPV was evaluated as the following: (i) **humoral immunity** was determined by virus-serum neutralization assay using an end-point dilution plaque reduction assay. (ii) **Cellular immunity** was determined by a T cell proliferation assay. (iii) **Viral clearance in the lungs**. Lung samples were homogenized in PBS. Viral titer was determined by plaque assay and viral RNA was quantified by real-time RT-PCR. (iv) **Evaluation of the protection efficacy after challenge**. The protection was evaluated with respect to weight loss, respiratory symptoms, and pulmonary histopathology as described above.

DETAILED DESCRIPTION

[0039] The present invention provides methyltransferase (MTase)-defective recombinant viruses as live vaccine candidates for hMPV, RSV and PIV3. Messenger RNA (mRNA) modification is the essential issue in paramyxovirus gene expression and replication. During viral RNA synthesis, paramyxoviruses produce capped, methylated, and polyadenylated mRNAs. Methylation of the mRNA directly impacts the subsequent translation of viral proteins, which in turn affects viral genome replication, virus assembly, and budding. Viruses lacking MTase would likely be attenuated without affecting immunogenicity, since the MTase is located in L protein, which is not a neutralizing antibody target. Therefore, MTase is a novel and new target for the development of a stable and efficacious live vaccine. It is known that viral large RNA polymerase (L) protein of paramyxovirus contains mRNA cap MTase activity. L protein is a 230-250-KDa multifunctional protein consisting of 2005-2200 amino acids. Amino acid sequence alignments of the L protein of paramyxoviruses identified six conserved domains

numbered I to VI. The MTase activity is located in the conserved domain VI of the L protein. Using a reverse genetics system, the inventors have successfully generated a panel of recombinant hMPV, RSV and PIV3 that are defective in MTase. These recombinant viruses were attenuated in cell culture as well as in hamster models. More importantly, these attenuated viruses elicited high level of neutralizing antibody and cellular immune response in hamsters, and protected hamsters from challenge of virulent viruses. Taken together, these MTase-defective viruses are excellent candidates for live attenuated vaccine for RSV, hMPV and PIV3.

[0040] **MTase-defective viruses as live vaccine candidates for hMPV.**

[0041] Specifically, provided are MTase-defective hMPV carrying mutations in MTase catalytic site (rhMPV-K1673A, rhMPV-D1779A, rhMPV-K1817A, rhMPV-E1848A,) and SAM binding site (rhMPV-G1696A, rhMPV-G1698A, rhMPV-G1700A, and rhMPV-D1755A). All MTase-defective hMPV were attenuated in cell culture as well as in animal, and remained excellent immunogenicity. Therefore, MTase-defective hMPVs are excellent live vaccine candidates.

[0042] **MTase-defective viruses as live vaccine candidates for RSV.**

[0043] Specifically, provided are MTase-defective RSV carrying mutations in MTase catalytic site (rRSV-K1831A, rRSV-D1936A, rRSV-K1973A, rRSV-E2004A,) and SAM binding site (rRSV-G1853A, rRSV-G1855A, rRSV-G1857A, and rRSV-D1912A). All MTase-defective RSV were attenuated in cell culture as well as in animal, and remained excellent immunogenicity. Therefore, MTase-defective RSVs are excellent live vaccine candidates.

[0044] **MTase-defective viruses as live vaccine candidates for PIV3.**

[0045] Specifically, provided are MTase-defective PIV3 carrying mutations in MTase catalytic site (rPIV3-K1786A, rPIV3-D1905A, rPIV3-K1941A, rPIV3-E1978A,) and SAM binding site (rPIV3-G1808A, rPIV3-G1810A, rPIV3-G1812A, and rPIV3-W1880A). All MTase-defective PIV3 were attenuated in cell culture as well as in animal, and remained excellent immunogenicity. Therefore, all MTase-defective PIV3 are excellent live vaccine candidates.

[0046] **EXAMPLES**

[0047] **Example 1: Identification of critical amino acid residues that are essential for mRNA cap MTase.**

[0048] To develop MTase-defective hMPV as live vaccine candidates, the inventors characterized critical amino acid residues that are essential for mRNA cap MTase. The SAM-dependent MTase superfamily contains six motifs involved in either SAM binding (motifs I, III, IV) or in the catalytic reaction (motifs IV, VI, VIII, X) (Fig. 1). Sequence alignment and structural modeling with crystal structure-solved ribose 2'-O MTases, vaccinia virus VP39 and *E.coli* RRMJ, identified these motifs in the conserved domain VI of paramyxovirus L proteins.

[0049] **1.1. Putative catalytic site of the hMPV MTases.** The SAM-dependent MTase superfamily contains a K-D-K-E tetrad that functions as the catalytic residues of the MTase. Structural modeling and amino acid sequence alignments indicate that residues K1673, D1779, K1817, and E1848 of the hMPV L protein correspond to the catalytic K-D-K-E tetrad (Fig. 1). In RSV, these amino acids are K1831, D1936, K1973, and E2004 (Fig.1). In PIV3, these amino acids are K1786, D1905, K1941, and E1978 (Fig.1).

[0050] **1.2. Putative SAM binding site of the hMPV MTases.** In methylation reactions, a G-rich motif and an acidic residue (D/E/W) are involved in binding the methyl donor, SAM. Indeed, this GxGxG....D/E/W motif is conserved in all paramyxoviruses. Sequence alignments indicate that the SAM binding site residues of hMPV L protein include G1696, G1698, G1700 and D1755 (Fig. 1). In RSV these amino acids are G1853, G1855, G1857, and D1912 (Fig.1). In PIV3, these amino acids are G1808, G1810, G1812, and W1880 (Fig.1).

[0051] **Example 2: Examination of the effect of mutations to catalytic site and SAM binding site on gene expression by a minigenome system.**

[0052] The inventors used the minigenome assay to determine whether L protein is functional in replication and RNA synthesis in cells. If a mutant L is functional in the minigenome assay, the inventors may be able to recover recombinant hMPV carrying this mutation by reverse genetics. Briefly, a minigenome plasmid phMPV-GFP containing green fluorescent protein (GFP) flanked hMPV trailer and gene end sequences on one side and hMPV gene start and leader sequences on the other side was constructed. BHK cells were infected by vaccinia vTF-7 at a MOI of 10, followed by transfection of phMPV-GFP together with support plasmids expressing hMPV proteins (pN, pP, pL and pM2-1). Two days later, GFP expression was observed by fluorescence microscopy. As shown in Fig.2, a strong GFP signal was observed when minigenome was transfected with wild type hMPV L (pL). As a negative control, no GFP expression was observed when pL was omitted from transfection. However, mutations in MTase catalytic site (pL-K1673A, pL-D1779A, pL-K1817A, and pL-E1848A) and SAM binding site (pL-G1696A, pL-G1698A, pL-G1700A, and pL-D1755A) had a diminished GFP expression level. This result demonstrated that mutations in catalytic site and SAM binding site of hMPV L protein were functional but had a diminished replication and/or gene expression. This result also suggests that we may be able to recover viable recombinant hMPV carrying these mutations since they are not lethal in replication and gene expression.

[0053] **Example 3. Recovery of MTase-defective paramyxoviruses from full-length cDNA clones.**

[0054] **The inventors have successfully generated a panel of recombinant hMPV that are defective in mRNA cap MTase.** The putative MTase catalytic K-D-K-E tetrad and potential SAM binding site GxGxG...D/E/W motif was individually mutated to alanine in the hMPV infectious clone.

The mutations in MTase catalytic site were named K1673A, D1779A, K1817A, and E1848A. The mutations in SAM binding site were named G1696A, G1698A, G1700A and D1755A. Using the reverse genetics technique (Fig.3), recombinant hMPV viruses carrying these mutations were recovered from an infectious cDNA clones. Briefly, recombinant hMPV was recovered by co-transfection of plasmid encoding full-length genomic cDNA of hMPV (phMPV) and support plasmids encoding viral N (pN), P (pP), L (pL) and M2-1 (pM2-1) proteins into BHK.SR19T7pac cells stably expressing T7 RNA polymerase. Six days post-transfection, the cells were subjected to three freeze-thaw steps and the supernatant was used to infect Vero-E6 cells (ATCC). TPCK-typsin (0.5µg/ml) was added to cells at day 2 post-infection since hMPV requires typsin to grow. Cytopathic effects (CPE) were observed after 5 day post-infection. Viruses were further amplified in Vero-E6 cells. Recombinant hMPV (rhMPV) carrying mutations in MTase catalytic site were named rhMPV-K1673A, rhMPV-D1779A, rhMPV-K1817A, and rhMPV-E1848A. Recombinant hMPV carrying mutations in SAM binding site were named rhMPV-G1696A, rhMPV-G1698A, rhMPV-G1700A and rhMPV-D1755A. All recombinant viruses were sequenced to confirm the presence of the designated amino acid changes in the L gene. These recombinant MTase-defective hMPVs were summarized in Fig.4.

[0055] **Using similar approaches, the inventors recovered a panel of MTase-defective RSV.** Specifically, these MTase-defective RSVs carrying mutations in MTase catalytic site (rRSV-K1831A, rRSV-D1936A, rRSV-K1973A, rRSV-E2004A,) and SAM binding site (rRSV-G1853A, rRSV-G1855A, rRSV-G1857A, and rRSV-D1912A). All recombinant viruses were sequenced to confirm the presence of the designated amino acid changes in the L gene. These recombinant MTase-defective RSVs were summarized in Fig.4.

[0056] **Using similar approaches, the inventors recovered a panel of MTase-defective PIV3.** Specifically, these MTase-defective PIV3 viruses carrying mutations in MTase catalytic site (rPIV3-K1786A, rPIV3-D1905A, rPIV3-K1941A, rPIV3-E1978A,) and SAM binding site (rPIV3-G1808A, rPIV3-G1810A, rPIV3-G1812A, and rPIV3-W1880A). All recombinant viruses were sequenced to confirm the presence of the designated amino acid changes in the L gene. These recombinant MTase-defective PIV3 were summarized in Fig.4.

[0057] **Example 4. MTase-defective hMPVs were attenuated in cell culture.**

[0058] The attenuation of MTase-defective hMPV in cell culture was determined by evaluation of the size of viral plaque, single-step virus growth curve, viral RNA synthesis, and viral protein synthesis. The inventors found that all the MTase-defective viruses carrying mutations either in MTase catalytic site (rhMPV-K1673A, rhMPV-D1779A, rhMPV-K1817A, and rhMPV-E1848A) or SAM binding site (rhMPV-G1696A, rhMPV-G1698A, rhMPV-G1700A and rhMPV-D1755A) were attenuated in cell culture. Specifically, all MTase-defective hMPV formed significantly smaller plaque size, had a delayed

viral replication and single step growth curve, and had significantly less protein synthesis as compared to wild type hMPV.

[0059] Fig.5 showed the viral plaque size by immunostaining assay. Briefly, Vero E6 cells were infected with the indicated virus and overlaid with 2% methyl cellulose. After 5 days, the methyl cellulose was removed, and cells were incubated with a monoclonal antibody against hMPV N protein (Santa Cruz Biotechnology, Inc.), followed by incubation with horseradish peroxidase-labeled rabbit anti-guinea pig antibodies (Invitrogen). After incubation with AEC substrate chromogen (Invitrogen), viral plaques were visualized under the microscope. As shown in Fig.5, wild type hMPV formed big plaques after 5 days post-infection. However, recombinant MTase-defective hMPV formed significantly smaller plaques as compared to wild type hMPV. This result suggested that MTase-defective hMPV had a defect in cell-to-cell spread, replication or gene expression.

[0060] Fig.6 showed viral plaque size by agarose overlay plaque assay. Briefly, Vero-E6 cells were infected with indicated virus. After 1 h infection, the cells were overlaid with 2 ml of cell culture medium containing 0.5% agarose and 5% FBS, and incubated for 6 days. The plates were fixed by 10% formaldehyde, followed by staining the crystal violet. As shown in Fig.6, wild type hMPV forms rounded plaques visualized by crystal violet staining. However, MTase-defective hMPV formed smaller plaques as compared to wild type hMPV. Again, this data suggest that MTase-defective hMPV had a defect in cell-to-cell spread, replication or gene expression.

[0061] Fig.7 showed cytopathic effect (CPE) of MTase-defective hMPV in Vero-E6 cells. Briefly, confluent Vero E6 cells were infected with individual viruses at an MOI of 1. CPE was monitored every day after infection. As shown in Fig.7, wild type hMPV exhibited extensive CPE after 3 days post-infection and cells were killed at day 5 post-infection. However, MTase-defective hMPV had a significant delayed CPE. CPE was observed until day 8-10 days post-infection. Cells were not killed until day 14 post-infection. This results demonstrated MTase-defective hMPV had a defect in viral growth.

[0062] Fig.8 showed a single-step growth curve of MTase-defective hMPV in cell culture. Briefly, confluent Vero E6 cells were infected with individual viruses at an MOI of 1. After 1 h of incubation, the inoculum was removed, the cells were washed with DMEM, and fresh medium (containing 2% fetal bovine serum) was added, followed by incubation at 37°C. Samples of supernatant were harvested at the indicated intervals over a 48-h time period, and the virus titer was determined by plaque assay using immunostaining. As shown in Fig.8, wild type hMPV grew to high titer at 5 days post-infection and remained high titer after 5-10 days post-infection. After 10 days, virus titer gradually decreased. However, MTase-defective hMPV (rhMPV-G1696A and rhMPV-D1755A) had significant defects in viral growth curve. MTase-defective hMPV replicated to peak titer around 8-10 days post-infection and viral titer gradually decreased after 12 days post-infection. These results demonstrated that MTase-

defective hMPV had significant defects in viral replication.

[0063] Fig.9 showed viral protein synthesis of MTase-defective hMPV in Vero cells. Briefly, confluent Vero E6 cells were infected with either wild type rhMPV or MTase-defective rhMPV at a MOI of 1. After 48 h postinfection, cells were metabolically labeled with [35S] methionine. After 24 h of incubation, cytoplasmic extracts were prepared and analyzed by SDS-PAGE. Labeled proteins were detected by using a phosphorimager. As shown in Fig. 9, viral N, P and M proteins were detected in wild type hMPV. However, the abundance of viral proteins significantly diminished for MTase-defective hMPV. Only 20-30% and 60-70% of viral proteins were detected for recombinant rhMPV-G1696A and rhMPV-D1755A respectively. This result demonstrated that MTase-defective hMPV had defects in viral protein synthesis.

[0064] Taken together, MTase-defective hMPV was attenuated in cell culture as judged by viral plaque size, replication, growth curve and gene expression.

[0065] **Example 5. MTase-defective RSVs were attenuated in cell culture.**

[0066] Using the techniques of the previous examples, the inventors found that certain MTase-defective RSVs carrying mutations in MTase catalytic site (rRSV-K1831A, rRSV-D1936A, rRSV-K1973A, rRSV-E2004A,) and SAM binding site (rRSV-G1853A, rRSV-G1855A, rRSV-G1857A, and rRSV-D1912A) were attenuated in cell culture.

[0067] **Example 6. MTase-defective PIV3s were attenuated in cell culture.**

[0068] Using similar approaches, the inventors found that MTase-defective PIV3 viruses carrying mutations in MTase catalytic site (rPIV3-K1786A, rPIV3-D1905A, rPIV3-K1941A, rPIV3-E1978A,) and SAM binding site (rPIV3-G1808A, rPIV3-G1810A, rPIV3-G1812A, and rPIV3-W1880A) were attenuated in cell culture.

[0069] **Example 7. Genetic stability of MTase-defective hMPV in cell culture.**

[0070] All MTase-defective were passed 10 times in Vero-E6 cells. At each passage, the L gene for each virus was sequenced to confirm the presence of the designed mutation. No additional mutation was found. These data indicated that MTase-defective hMPV is genetically stable in cell culture.

[0071] **Example 8. MTase-defective hMPV were attenuated in animal models.**

[0072] To determine whether MTase-defective hMPVs are attenuated in animal, all recombinant viruses were inoculated into two-week-old specific-pathogen-free female hamsters (Charles River laboratories, Wilmington, MA). After inoculation, the animals were evaluated on a daily basis for mortality, weight loss, and the presence of any respiratory symptoms of hMPV. At day 4 post-infection, five hamsters from each group were sacrificed, and their lungs were removed for pathogenicity studies as follows. (i) **Lung virus titer.** One lung from each animal were weighed and homologized in 1 ml of phosphate-buffered saline (PBS). Viral titer was determined by plaque assay. (ii) **Pulmonary**

histopathology. One lung from each hamster was inflated with 10% buffered formalin, embedded in paraffin, sectioned, and stained with hematoxylin-eosin. Histopathological changes were scored include the extent of inflammation (focal or diffuse), the pattern of inflammation (peribronchilolar, perivascular, interstitial, alveolar), and the nature of the cells making up the infiltrate (neutrophils, eosinophils, lymphocytes, macrophages). Deparaffinized sections were also stained with polyclonal antiserum to determine the distribution of viral antigen. Five animals per cohort were used in these experiments. The present results demonstrated that wild type hMPV was virulent to hamster (Fig. 10B). Specifically, hamsters inoculated with wild type hMPV exhibited mild clinical signs of respiratory tract infection, including ruffled fur, tendency to huddle, heavy breathing, and body weight losses. Wild type hMPV caused moderate to severe damage lung as confirmed by pulmonary histopathology. In contrast, all MTase-defective hMPV were highly attenuated in hamsters. Specifically, hamsters inoculated with MTase-defective hMPV did not exhibit any clinical sign for respiratory tract infection or weight loss. In addition, MTase-defective hMPV did not cause or only caused mild pulmonary histopathological changes (Fig. 10B). Taken together, these results demonstrated that MTase-defective hMPV were highly attenuated in hamsters and may be good live vaccine candidates for hMPV.

[0073] **Example 9. MTase-defective RSVs were attenuated in animal models.**

[0074] Using similar approaches, the inventors found that MTase-defective RSVs carrying mutations in MTase catalytic site (rRSV-K1831A, rRSV-D1936A, rRSV-K1973A, rRSV-E2004A,) and SAM binding site (rRSV-G1853A, rRSV-G1855A, rRSV-G1857A, and rRSV-D1912A) were attenuated in animals.

[0075] **Example 10. MTase-defective PIV3s were attenuated in animal models.**

[0076] Using similar approaches, the inventors found that MTase-defective PIV3 viruses carrying mutations in MTase catalytic site (rPIV3-K1786A, rPIV3-D1905A, rPIV3-K1941A, rPIV3-E1978A,) and SAM binding site (rPIV3-G1808A, rPIV3-G1810A, rPIV3-G1812A, and rPIV3-W1880A) were attenuated in animals.

[0077] **Example 11. MTase-defective hMPVs as immunogens.**

[0078] To determine whether MTase-defective hMPV can be used as live vaccine candidates, the inventors examined the immunogenicity of these recombinant viruses. All MTase-defective hMPV were inoculated intranasally into two-week-old female hamsters (10 hamsters per group). Serum samples were collected at days 7, 14, 21, and 28 post-inoculation for the detection of humoral immune response. At day 28 post-inoculation, 5 hamsters from each group were sacrificed, and spleen samples were isolated for the detection of cellular immune response. The remaining 5 animals were challenged with 10^6 PFU of the wild-type hMPV. After the challenge, each animal was evaluated on a daily basis for weight loss and the presence of any respiratory symptoms. At day 4 post-challenge, all the animals were sacrificed and lung

samples were collected for virus detection and pathological examination. The immunogenicity of the MTase-defective hMPV was evaluated as the following: (i) humoral immunity was determined by virus-serum neutralization assay using an end-point dilution plaque reduction assay. (ii) Cellular immunity was determined by a T cell proliferation assay. (iii) Viral clearance in the lungs. Lung samples were homogenized in PBS. Viral titer was determined by plaque assay. (iv) Evaluation of the protection efficacy after challenge. The protection was evaluated with respect to weight loss, respiratory symptoms, and pulmonary histopathology as described above. The present results demonstrated that all MTase-defective hMPV elicited high level of neutralizing antibody and T cell immune response in hamsters, and protected hamsters from virulent challenge (Fig. 11). Specifically, severe lung damage was observed in PBS challenge control. However, no or only mild lung damage was observed in hamsters that were immunized by MTase-defective hMPV. Protection rate for each MTase-defective hMPV was 100%. These results demonstrated that MTase-defective hMPVs have excellent immunogenicity. Thus, MTase-defective hMPVs are excellent live vaccine candidates.

[0079] **Example 12. MTase-defective RSVs as immunogens.**

[0080] Using similar approaches, the inventors found that MTase-defective RSVs carrying mutations in MTase catalytic site (rRSV-K1831A, rRSV-D1936A, rRSV-K1973A, rRSV-E2004A,) and SAM binding site (rRSV-G1853A, rRSV-G1855A, rRSV-G1857A, and rRSV-D1912A) retained excellent immunogenicity.

[0081] **Example 13. MTase-defective PIV3s as immunogens.**

[0082] Using similar approaches, the inventors found that MTase-defective PIV3 viruses carrying mutations in MTase catalytic site (rPIV3-K1786A, rPIV3-D1905A, rPIV3-K1941A, rPIV3-E1978A,) and SAM binding site (rPIV3-G1808A, rPIV3-G1810A, rPIV3-G1812A, and rPIV3-W1880A) retained excellent immunogenicity.

CLAIMS

What is claimed is:

1. A recombinant paramyxovirus composition comprising a nucleic acid molecule which encodes a defective mRNA cap MTase in large (L) polymerase gene in a paramyxovirus viral genome.
2. The recombinant paramyxovirus composition of claim 1, wherein the composition is defective in mRNA cap MTase gene expression.
3. The recombinant paramyxovirus composition according to claim 1, wherein paramyxovirus viral genome is selected from the group consisting of: a human metapneumovirus (hMPV); human respiratory syncytial virus (hRSV); and human parainfluenza virus type 3 (PIV3).
4. The recombinant paramyxovirus composition of claim 1, wherein the nucleic acid molecule which encodes a defective mRNA cap MTase carries at least one mutation in the MTase catalytic site.
5. The recombinant paramyxovirus composition according to claim 4, wherein the at least one mutation in the MTase catalytic site is in at least one of the K-D-K-E tetrad sites.
6. The recombinant paramyxovirus composition according to claim 5, wherein the paramyxovirus is human metapneumovirus (hMPV) and the at least one K-D-K-E tetrad site mutation is at least one of rhMPV-K1673A, rhMPV-D1779A, rhMPV-K1817A, rhMPV-E1848A, and rhMPV-E1848Q.
7. The recombinant paramyxovirus composition according to claim 5, wherein the paramyxovirus is human respiratory syncytial virus (hRSV) and the at least one K-D-K-E tetrad site mutation is at least one of rRSV-K1831A, rRSV-D1936A, rRSV-K1973A, rRSV-E2004A.
8. The recombinant paramyxovirus composition according to claim 5, wherein the paramyxovirus is human parainfluenza virus type 3 (PIV3) and the at least one K-D-K-E tetrad site mutation is at least one of rPIV3-K1786A, rPIV3-D1905A, rPIV3-K1941A, rPIV3-E1978A.
9. The recombinant paramyxovirus composition of claim 1, wherein the nucleic acid molecule which encodes a defective mRNA cap MTase carries at least one mutation in the SAM binding site.
10. The recombinant paramyxovirus composition according to claim 9, wherein the at least one mutation in the SAM binding site is in the GxGxG...D/E/W site.

11. The recombinant paramyxovirus composition according to claim 10, wherein the paramyxovirus is human metapneumovirus (hMPV) and the at least one mutation in the GxGxG....D/E/W site is at least one of rhMPV-G1696A, rhMPV-G1698A, rhMPV-G1700A, and rhMPV-D1755A.
12. The recombinant paramyxovirus composition according to claim 10, wherein the paramyxovirus is human respiratory syncytial virus (hRSV) and the at least one mutation in the GxGxG....D/E/W site is at least one of rRSV-G1853A, rRSV-G1855A, rRSV-G1857A, and rRSV-D1912A.
13. The recombinant paramyxovirus composition according to claim 10, wherein the paramyxovirus is human parainfluenza virus type 3 (PIV3) and the at least one mutation in the GxGxG....D/E/W site is at least one of rPIV3-G1808A, rPIV3-G1810A, rPIV3-G1812A, and rPIV3-W1880A.
14. The recombinant paramyxovirus composition of claim 1, wherein the nucleic acid molecule which encodes a defective mRNA cap MTase carries at least one mutation in the MTase catalytic site and the SAM binding site.
15. The recombinant paramyxovirus composition according to claim 14, wherein the at least one mutation in the MTase catalytic site is in at least one of the K-D-K-E tetrad sites and the at least one mutation in the SAM binding site is in the GxGxG....D/E/W site.
16. The recombinant paramyxovirus composition according to claim 15, wherein the paramyxovirus is human metapneumovirus (hMPV) and the at least one K-D-K-E tetrad site mutation is at least one of rhMPV-K1673A, rhMPV-D1779A, rhMPV-K1817A, rhMPV-E1848A, and rhMPV-E1848Q and the at least one mutation in the GxGxG....D/E/W site is at least one of rhMPV-G1696A, rhMPV-G1698A, rhMPV-G1700A, and rhMPV-D1755A.
17. The recombinant paramyxovirus composition according to claim 15, wherein the paramyxovirus is human respiratory syncytial virus (hRSV) and the at least one K-D-K-E tetrad site mutation is at least one of rRSV-K1831A, rRSV-D1936A, rRSV-K1973A, rRSV-E2004A and the at least one mutation in the GxGxG....D/E/W site is at least one of rRSV-G1853A, rRSV-G1855A, rRSV-G1857A, and rRSV-D1912A.
18. The recombinant paramyxovirus composition according to claim 15, wherein the paramyxovirus is human parainfluenza virus type 3 (PIV3) and the at least one K-D-K-E tetrad site mutation is at least one of rPIV3-K1786A, rPIV3-D1905A, rPIV3-K1941A, rPIV3-E1978A and the at least one mutation in

the GxGxG....D/E/W site is at least one of rPIV3-G1808A, rPIV3-G1810A, rPIV3-G1812A, and rPIV3-W1880A.

19. The recombinant paramyxovirus composition of any of the claims herein, which is a mammalian immunogen.

20. The recombinant paramyxovirus composition of any of the claims herein, which is a human immunogen.

21. A method of eliciting an immune response in a mammal comprising: administering to a mammal a recombinant paramyxovirus composition herein.

22. The method according to claim 21 wherein the composition is administered orally.

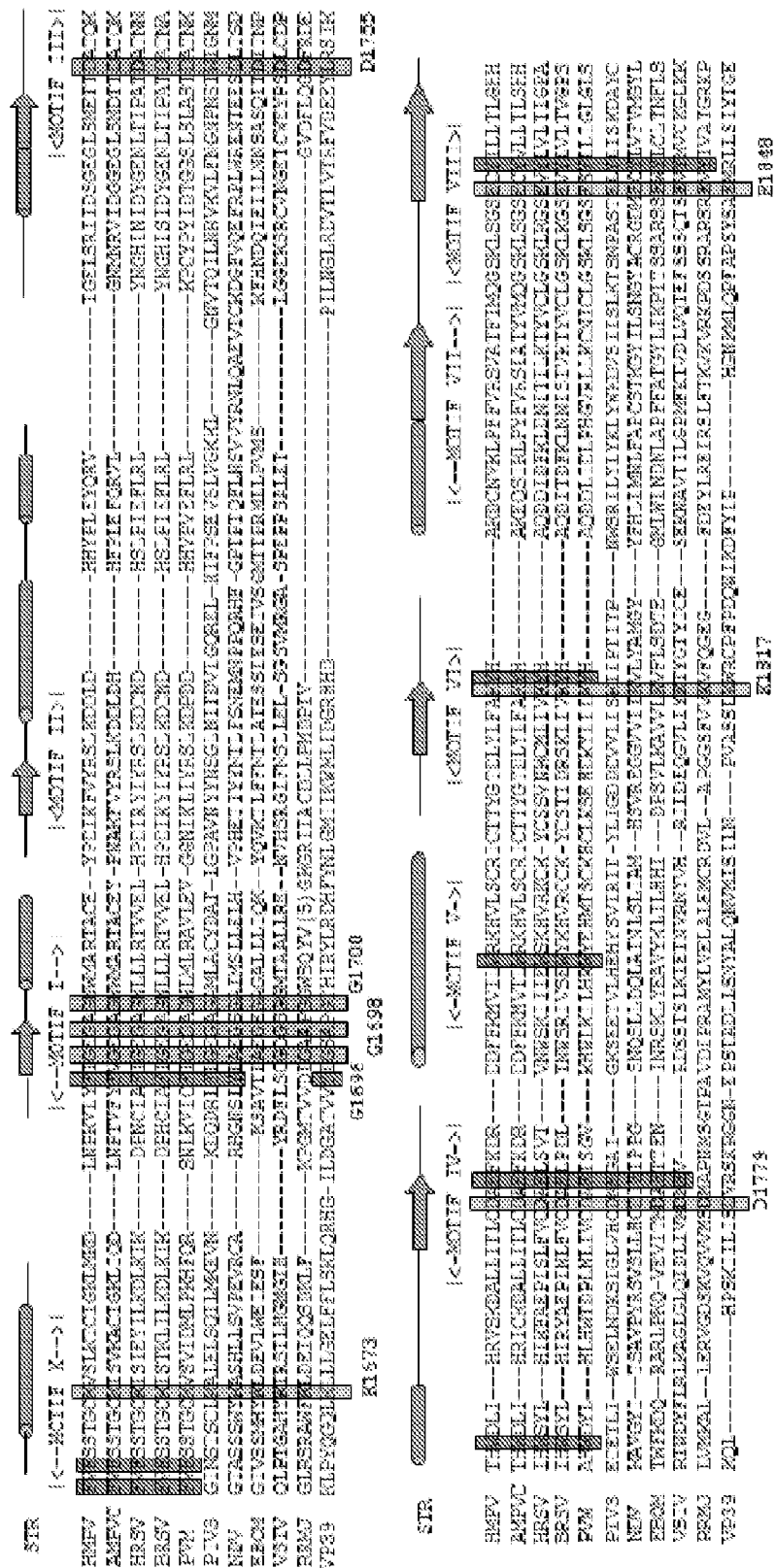
23. The method according to claim 21 wherein the composition is administered intranasally.

24. A method of preparing a pharmaceutical composition for passive immunization of an individual in need of immunization comprising: mixing a paramyxovirus composition herein with a suitable excipient or carrier, thereby forming a pharmaceutical composition.

25. The method according to claim 24 wherein the pharmaceutical composition is formulated for oral administration.

26. The method according to claim 24 wherein the pharmaceutical composition is formulated for intranasal administration.

27. A composition or method of any of the previous claims, wherein the paramyxovirus virulence is attenuated or eliminated in any mammal susceptible to paramyxovirus.



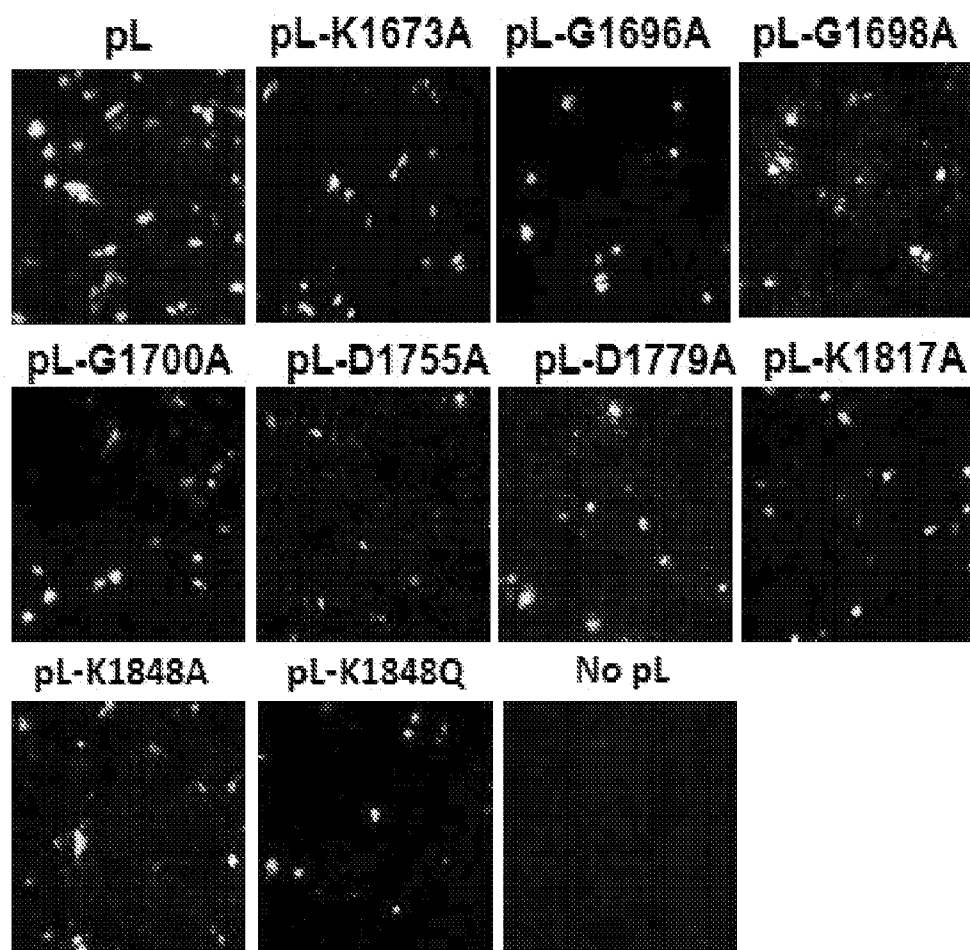


Fig. 2

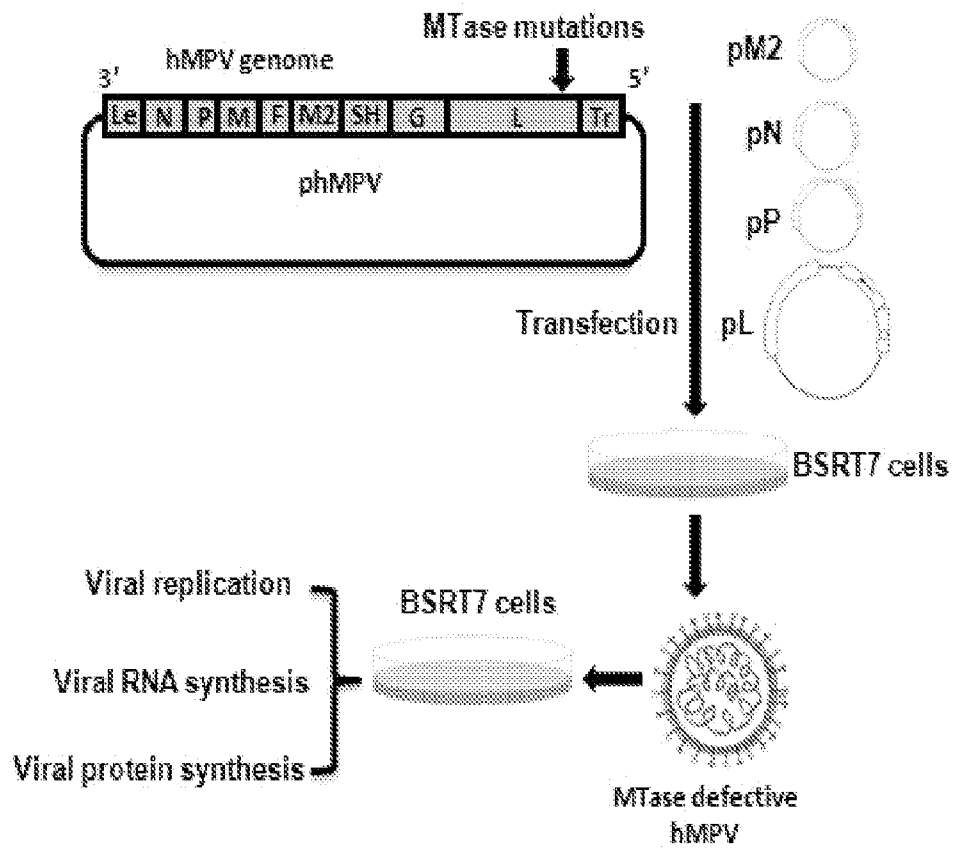


Fig. 3

Virus	MTase catalytic site	MTase SAM binding site
human metapneumovirus (hMPV)	rhMPV-K1673A, rhMPV-D1779A, rhMPV-K1817A, rhMPV-E1848A, and rhMPV-E1848Q. Mutant hMPV carrying two or three amino acid substitutions in catalytic site were also recovered.	rhMPV-G1696A, rhMPV-G1698A, rhMPV-G1700A, and rhMPV-D1755A. Mutant hMPV carrying two or three amino acid substitutions in SAM binding site were also recovered.
human respiratory syncytial virus (hRSV)	rRSV-K1831A, rRSV-D1936A, rRSV-K1973A, rRSV-E2004A Mutant RSV carrying two or three amino acid substitutions in catalytic site were also recovered.	rRSV-G1853A, rRSV-G1855A, rRSV-G1857A, and rRSV-D1912A. Mutant hRSV carrying two or three amino acid substitutions in SAM binding site were also recovered.
human parainfluenza virus type 3 (PIV3)	rPIV3-K1786A, rPIV3-D1905A, rPIV3-K1941A, rPIV3-E1978A Mutant PIV3 carrying two or three amino acid substitutions in catalytic site were also recovered.	rPIV3-G1808A, rPIV3-G1810A, rPIV3-G1812A, and rPIV3-W1880A. Mutant PIV carrying two or three amino acid substitutions in SAM binding site were also recovered.

Fig. 4

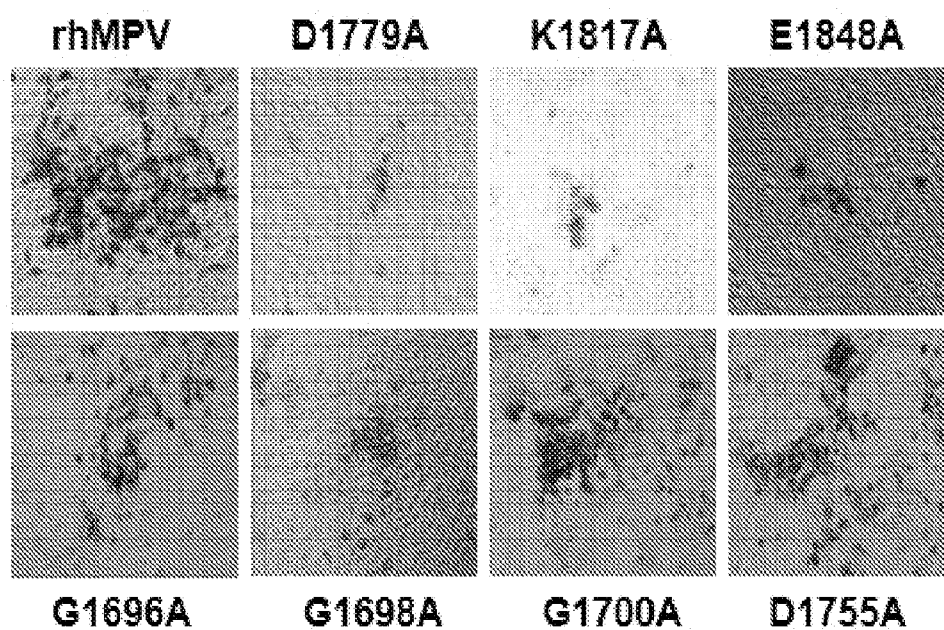


Fig. 5

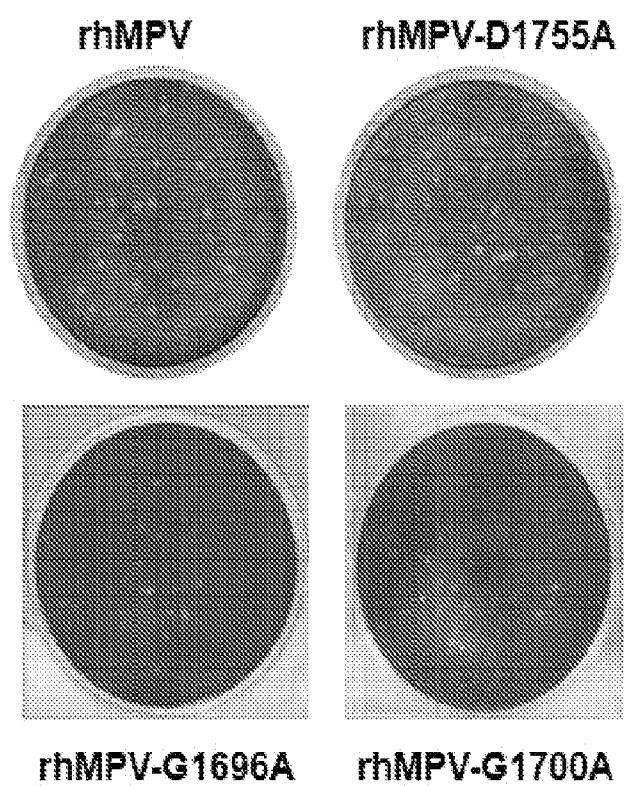


Fig. 6

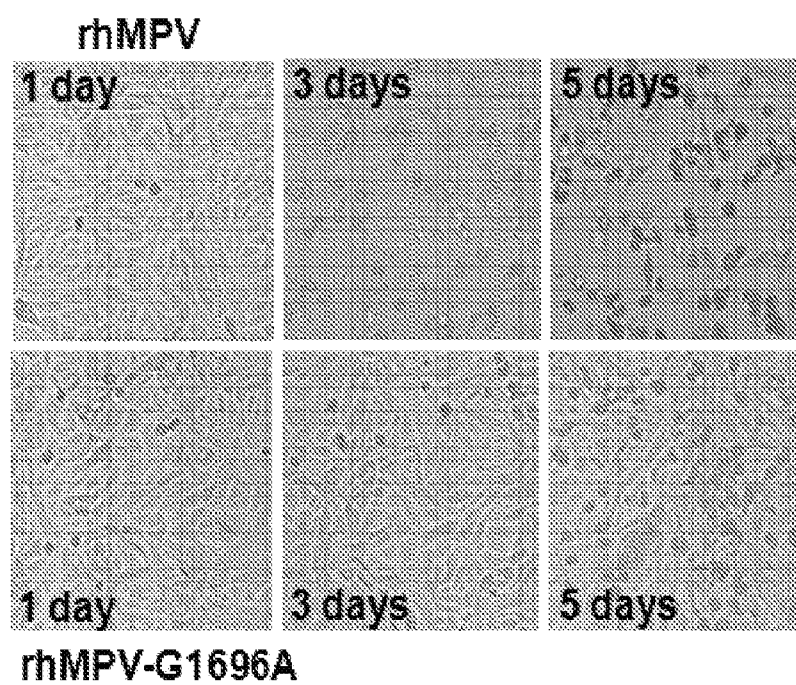


Fig. 7

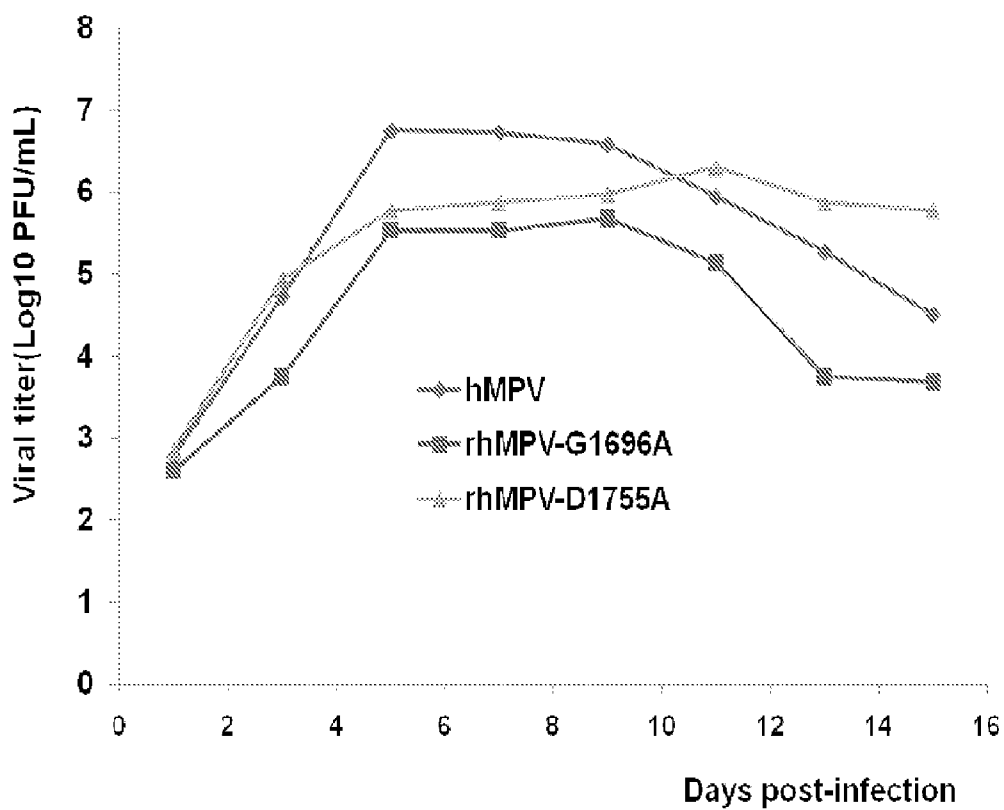


Fig. 8

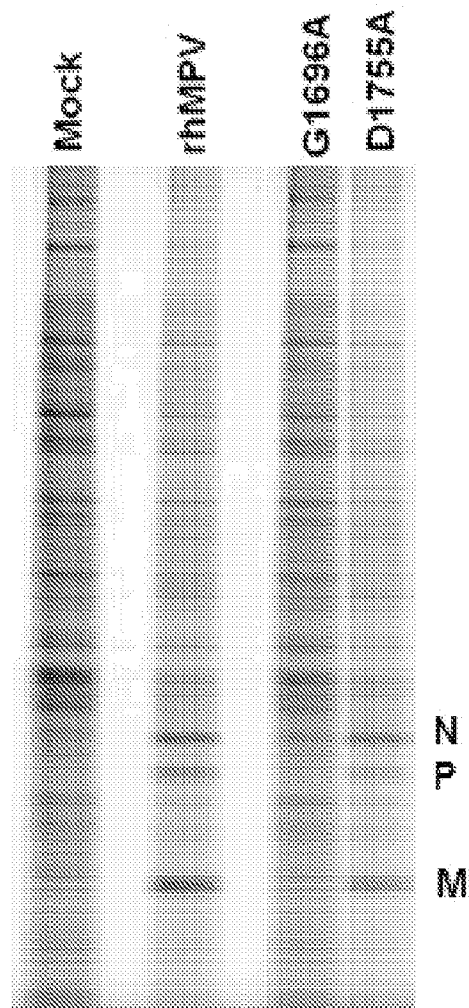


Fig. 9

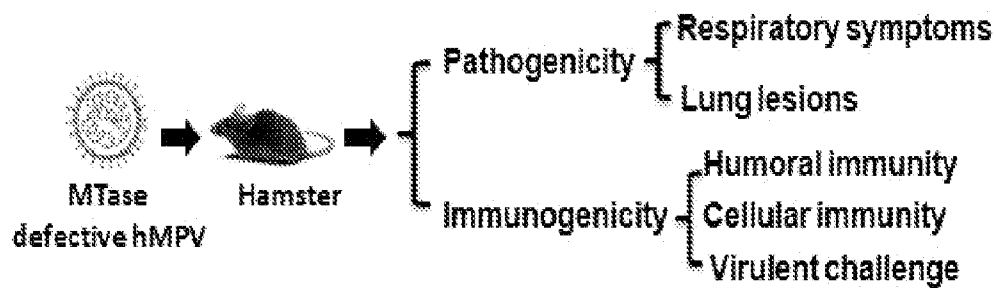


Fig.10A

Virus	Dose (Log10 PFU/hamster)	Number of hamsters	Days post-challenge	% of animals with detectable virus in lungs	Lung histology (severity of damage)
DMEM control	0	5	4	0	No damage
Wild type rhMPV	6.48	5	4	100	Moderate to severe
Wild type rhMPV	5.0	5	4	100	Moderate
Wild type rhMPV	4.0	5	4	100	Moderate
rhMPV-G1696A	6.48	5	4	100	Moderate
rhMPV-G1696A	5.0	5	4	100	Mild
rhMPV-G1696A	4.0	5	4	100	No to Mild
rhMPV-G1700A	6.48	5	4	80	Moderate
rhMPV-G1700A	5.0	5	4	80	Mild
rhMPV-G1700A	4.0	5	4	80	No to Mild
rhMPV-D1755A	6.48	5	4	100	Moderate
rhMPV-D1755A	5.0	5	4	100	Mild
rhMPV-D1755A	4.0	5	4	100	No to Mild

Fig.10B

Virus	Immunization dose (Log10 PFU/hamster)	Number of hamsters	Days post-challenge	Lung histology post-challenge (severity of damage)	Protection rate
PBS normal control	0	5	4	No damage	/
PBS challenge control	0	5	4	Severe damage	0/5
rhMPV-G1696A	5.0	5	4	No damage	5/5
rhMPV-G1696A	4.0	5	4	No damage	5/5
rhMPV-G1700A	5.0	5	4	No damage	5/5
rhMPV-G1700A	4.0	5	4	No to mild damage	5/5
rhMPV-D1755A	5.0	5	4	No damage	5/5
rhMPV-D1755A	4.0	5	4	No to mild damage	5/5

Fig.11

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US 12/41878

A. CLASSIFICATION OF SUBJECT MATTER

IPC(8) - C12N 7/00; A61K 39/155

USPC - 435/235.1; 424/211.1

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
USPC - 435/235.1; 424/211.1

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
DialogClassic files 345,347,348,349,351,371,652,654,2,6,35,65,144,155,240,315,440 - Search terms paramyxovirus,metapneumovirus, hMPV, syncytial,hRSV,parainfluenza,PIV3,methyltransferase,MTase,mutat?,defect?,nonfunct?, cap, mRNA, CL 435/235.1, 424/211.1;
Google Scholar - Search terms MTase, methyltransferase, paramyxovirus, metapneumovirus, MPV, hMP

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	US 2009/0169580 A1 (WHELAN et al.) 2 July 2009 (02.07.2009); esp. paras. [0008], [0029], [0067], [0069], [0070], [0083], [0087]	1 - 19
A	Li, J. et al. "A Conserved Motif in Region V of the Large Polymerase Proteins of Nonsegmented Negative-Sense RNA Viruses That is Essential for mRNA Capping". J. Virol.; January 2008; Vol. 82; pages 775-784.	1 - 19

☐ Further documents are listed in the continuation of Box C.

* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier application or patent but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search

17 August 2012 (17.08.2012)

Date of mailing of the international search report

05 SEP 2012

Name and mailing address of the ISA/US

Mail Stop PCT, Attn: ISA/US, Commissioner for Patents
P.O. Box 1450, Alexandria, Virginia 22313-1450

Facsimile No. 571-273-3201

Authorized officer:

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PCT OSP: 571-272-7774

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US 12/41878

Box No. II Observations where certain claims were found unsearchable (Continuation of item 2 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☒ Claims Nos.: 21-26
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:
Claims 21-26 are not formed according to PCT Article 34(4)(a)(ii), wherein the claims are so unclear that no meaningful opinion can be formed on the novelty, inventive step (non-obviousness), or industrial applicability, of the claimed invention. In particular, claims 21 and 24 are directed to "a recombinant paramyxovirus composition herein" without further indicating the characteristics of the paramyxovirus composition, rendering the claims unclear regarding for what invention protection is being sought. - please see extra sheet -

3. ☒ Claims Nos.: 20, 27
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box No. III Observations where unity of invention is lacking (Continuation of item 3 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. ☐ As all searchable claims could be searched without effort justifying additional fees, this Authority did not invite payment of additional fees.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest and, where applicable, the payment of a protest fee.
- ☐ The additional search fees were accompanied by the applicant's protest but the applicable protest fee was not paid within the time limit specified in the invitation.
- ☐ No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US 12/41878

Continuation of Box II: Observations where certain claims were found unsearchable

Claims 22-23 and 25-26 depend from claims 21 and 22, and are therefore unsearchable for substantially the same reason.