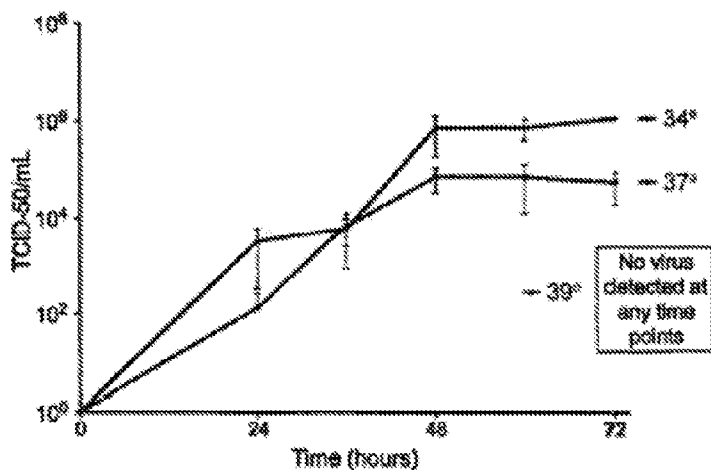




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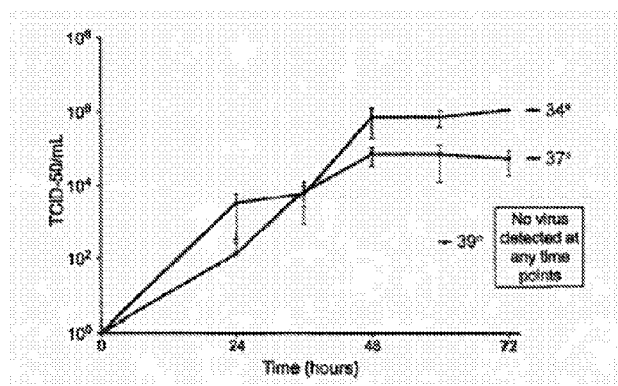
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FIGURE 1



(57) Abstract: Provided herein are attenuated influenza viruses and methods of making attenuated influenza viruses.

ATTENUATED INFLUENZA VACCINES AND USES THEREOF

BACKGROUND

5 Influenza is a serious public health issue marked by mild to serious illness and, in some cases, even death. Current live attenuated influenza vaccines (LAIV) are not sufficiently attenuated for administration to children under the age of 2, pregnant women, persons with compromised immunity, or persons at high risk for complications from influenza. However, these groups of people are at high risk for complications from influenza.

SUMMARY

10 Provided herein is a modified influenza A virus comprising a PB1 polymerase with one or more mutations in amino acids 310 to 325. Further provided is a recombinant nucleic acid encoding a PB1 polymerase of an influenza A virus, wherein the nucleic acid encodes a PB1 polymerase with one or more mutations in amino acids 310 to 325. Also provided are populations of cells comprising any of the influenza A viruses described herein or comprising any of the nucleic acids that encodes the PB1 polymerases described herein. The polymerase mutation results in a temperature sensitive virus, wherein the virus has reduced growth from about 37°C to about 39°C (i.e., at body temperature). This reduced growth potential is advantageous for improving the safety of the virus when used to induce an immune response.

15 Further provided is a method for eliciting an immune response against an influenza virus in a subject, comprising administering an effective dose of a modified influenza A virus described herein and a pharmaceutically acceptable carrier.

20 Also provided is a method for treating or preventing an influenza infection in a subject, comprising administering to a subject with an influenza infection or susceptible to an

influenza infection an effective dose of a modified influenza A virus described herein, and a pharmaceutically acceptable carrier.

Also provided is a method of producing an influenza A virus described herein, comprising (a) transfecting a population of host cells with one or more vectors comprising i) nucleic acid sequences encoding the internal genome segments of an influenza A virus and; ii) a nucleic acid encoding a PB1 polymerase with one or more mutations in amino acids 310 to 325; (b) culturing the host cells; and (c) recovering the modified influenza A virus.

Further provided is a method for producing an influenza immunogen comprising (a) infecting a population of cells with any of the influenza A viruses described herein; (b) culturing the cells; (c) harvesting the virus from the culture of step (b); and (d) preparing an immunogen with the harvested virus.

DESCRIPTION OF THE DRAWINGS

Figure 1 shows the identification of a PB2 single gene replacement virus with temperature sensitivity at 37°C. MDCK cells were infected at a Multiplicity of Infection (MOI) of 0.01 for 1 h with a single gene replacement virus with PB2 from the cold passaged A/AnnArbor/6/60, and all other genes from a seasonal strain A/Korea/82. Cells were washed once with Dulbecco's phosphate-buffered saline (PBS) with magnesium and calcium (Invitrogen), and then cultured at 34°, 37° or 39°C in DMEM containing 0.15% bovine serum albumin (BSA) and tosylsulfonylphenylalanyl chloromethyl ketone (TPCK)-trypsin at 1 µg/ml. At the indicated time points, 10% of the culture supernatant was harvested and replaced, and viral titers were determined through TCID-50 measurements.

Figure 2 shows that the PB1 319Q mutation significantly reduces functional activity of the human influenza A virus RNA polymerase at 37°C. The polymerase activity of the indicated polymerases was characterized in human HEK-293FT cells by quantifying luciferase activity in the clarified cell lysates of cells transfected with PB1-, PB2-, PA-, and NP-protein expression plasmids along with a reporter plasmid expressing an influenza virus-like RNA construct for firefly luciferase. The cells were incubated at the indicated temperatures. All assays utilized the same NP plasmid. Depicted is the ratio of firefly to renilla luminescence. Data are averaged over a minimum of three independent experiments. Error bars represent one standard error of the mean. All plasmids used in this mini-genome assay were identical, except for the PB1 plasmid, which encoded a Q at residue 319 (as indicated). These plasmids were created from viral stocks through cloning the consensus sequence from viral growth curves into the mammalian pCAGGS expression vector.

Figure 3 shows that the PB1 L319Q mutation reduces functional activity of an avian influenza A virus RNA polymerase at 37°C. The polymerase activity of each viral polymerase was characterized in human HEK-293FT cells by quantifying luciferase activity in the clarified cell lysates of cells transfected with PB1-, PB2-, PA-, and NP-protein expression plasmids along with a reporter plasmid expressing an influenza virus-like RNA construct for firefly luciferase. The cells were incubated at the indicated temperatures. Depicted is the ratio of firefly to renilla luminescence. Data are averaged over a minimum of 3 independent experiments. Error bars represent one standard error of the mean. In this experiment, all polymerase gene segments were derived from avian influenza viruses. The PA and PB2 segments were derived from A/California/04/09 H1N1, and the PB1 and NP segments were derived from A/Chicken/Nanchang/3 H3N2. Plasmids differed only at the indicated residues: (1) PB2 encoding either 265S or 265N [wild-type]; (2) encoding either 319Q or 319L [wild-type].

Figure 4 shows the effects of a 319Q mutation in PB1, in combination with three mutations present in LAIV PB1.

Figure 5 shows the effects of a 319Q mutation in PB1, in combination with four mutations present in LAIV PB1.

Figure 6 shows the stability of the mutation at position 319 of PB1.

DESCRIPTION

Provided herein is a modified influenza A virus comprising a PB1 polymerase with one or more mutations in amino acids 310 to 325. Amino acids 310 to 325 of a PB1 polymerase are set forth herein as NENQNPRMFLAMITYI (SEQ ID NO: 1).

As used throughout, any influenza A virus can be modified to comprise a PB1 polymerase with one or more mutations in amino acids 310 to 325. For example, the influenza A virus can be selected from the group consisting of an H2N2 virus, an H3N2 virus, an H1N1 virus, an H9N2 virus and an H5N1 virus. Optionally, the influenza A virus can be selected from the group consisting of A/Ann Arbor/6/60, A/California/04/2009, A/Wisconsin/22/2011 and A/Quail/Hong Kong/G1/97. The influenza A virus can also be an avian influenza A virus. These include, but are not limited to, A/Chicken/Nanchang/3-120/01 H3N2, A/Hong Kong/485/1997(H5N1), A/Anhui/1/2013 (H7N9) and A/Shanghai/1/2013 (H7N9).

Resassortant influenza A viruses comprising one or more genomic segments from one or more influenza A viruses are also contemplated. More specifically, the virus includes

genetic and/or polypeptide components derived from more than one parental viral strain or source. For example, a 7:1 reassortant includes 7 viral genomic segments (or gene segments) derived from a first parental virus and a single complementary viral genomic segment, e.g., encoding hemagglutinin or neuraminidase, from a second parental virus. A 6:2 reassortant includes 6 genomic segments, most commonly the 6 internal genes from a first parental virus, and two complementary segments, e.g., hemagglutinin and neuraminidase, from a different parental virus. Optionally, reassortant viruses are produced by introducing vectors including the six internal genes of a viral strain selected for its favorable properties regarding vaccine production, in combination with the genome segments encoding the surface antigens (HA and NA) of a selected, e.g., pathogenic strain. For example, the HA segment can be selected from an H1, H3 or B strain, as is routinely performed for vaccine production. Similarly, the HA segment can be selected from other pathogenic strains such as an H2 strain (e.g., H2N2), an H5 strain (e.g., H5N1), an H7 strain (e.g., H7N7) or an H9 strain (H9N2). In certain modified viruses, the internal gene segments are derived from the influenza A/Ann Arbor/6/60 strain.

As set forth herein, modifications include, but are not limited to, mutations in the amino acid sequence of a PB1 polymerase. Optionally, the one or more mutations in the PB1 polymerase are non-naturally occurring and are produced by human intervention (e.g., by mutagenesis of cloned DNA sequences), such as induced point mutation, deletion, insertion and substitution mutants. Amino acid sequence mutations typically fall into one or more of three classes: substitutional, insertional or deletional mutations. Insertions include amino and/or carboxyl terminal fusions as well as intrasequence insertions of single or multiple amino acid residues. Insertions ordinarily will be smaller insertions than those of amino or carboxyl terminal fusions, for example, on the order of one to four residues. Deletions are characterized by the removal of one or more amino acid residues from the protein sequence. Typically, no more than from about 2 to about 6 residues are deleted at any one site within the protein molecule. Amino acid substitutions are typically of single residues but can occur at a number of different locations at once, for example in one, two, three, four, five, six, seven or more amino acids of the polypeptide sequence set forth as SEQ ID NO: 1; insertions usually will be on the order of about from 1 to 10 amino acid residues; and deletions will range from about 1 to 10 residues. Deletions or insertions preferably are made in adjacent pairs, i.e., a deletion of 2 residues or insertion of 2 residues. Substitutions, deletions, insertions or any combination thereof may be combined to arrive at a final construct. The mutations must not place the sequence out of reading frame and preferably will not create complementary regions that could produce secondary mRNA structure. Substitutional modifications are

those in which at least one residue has been removed and a different residue inserted in its place. Such substitutions can be made in accordance with the following Table 1 and are referred to as conservative substitutions.

5 TABLE 1: Amino Acid Substitutions

Amino Acid	Substitutions (others are known in the art)
Ala	Ser, Gly, Cys
Arg	Lys, Gln, Met, Ile
Asn	Gln, His, Glu, Asp
Asp	Glu, Asn, Gln
Cys	Ser, Met, Thr
Gln	Asn, Lys, Glu, Asp
Glu	Asp, Asn, Gln
Gly	Pro, Ala
His	Asn, Gln
Ile	Leu, Val, Met
Leu	Ile, Val, Met
Lys	Arg, Gln, Met, Ile
Met	Leu, Ile, Val
Phe	Met, Leu, Tyr, Trp, His
Ser	Thr, Met, Cys
Thr	Ser, Met, Val
Trp	Tyr, Phe
Tyr	Trp, Phe, His
Val	Ile, Leu, Met

Amino acid substitutions are not necessarily conservative as amino acid substitutions that change the side chain length, hydrophobicity or the polarity of a particular amino acid can also be made in order to alter the temperature sensitivity and/or increase the attenuation of virus.

10

In the PB1 polymerases described herein, one or more mutations in amino acids 310 to 325 can be selected from the group consisting of a leucine to glutamine substitution at position 319 (L319Q), an asparagine to valine substitution at position 310 (N310V), an asparagine to valine substitution at position 312 (N312V), a glutamine to leucine substitution

at position 313 (Q313L), an asparagine to valine substitution at position 314 (N314V), a phenylalanine to tyrosine substitution at position 318 (F318Y), a leucine to glutamine substitution at position 319 (L319Q), an alanine to threonine substitution at position 320 (A320T), an isoleucine to glutamine substitution at position 321 (I321Q), a threonine to alanine substitution at position 323 (T323A), a tyrosine to phenylalanine substitution at position 324 (Y324F) and an isoleucine to glutamine substitution at position 325 (I325Q).

It is understood that SEQ ID NO: 1 is an example of amino acids 310 to 325 of a PB1 polymerase. A sequence of amino acids 310 to 325 of any PB1 polymerase that is at least about 80%, 85%, 90%, or 95% identical to SEQ ID NO: 1 can also be modified as set forth herein. Those of skill in the art readily understand how to determine the identity of two polypeptides or nucleic acids. For example, the identity can be calculated after aligning the two sequences so that the identity is at its highest level. Another way of calculating identity can be performed by published algorithms. Optimal alignment of sequences for comparison can be conducted using the algorithm of Smith and Waterman *Adv. Appl. Math.* 2: 482 (1981), by the alignment algorithm of Needleman and Wunsch, *J. Mol. Biol.* 48: 443 (1970), by the search for similarity method of Pearson and Lipman, *Proc. Natl. Acad. Sci. U.S.A.* 85: 2444 (1988), by computerized implementations of these algorithms (GAP, BESTFIT, FASTA, and TFASTA in the Wisconsin Genetics Software Package, Genetics Computer Group, 575 Science Dr., Madison, WI; the BLAST algorithm of Tatusova and Madden *FEMS Microbiol. Lett.* 174: 247-250 (1999) available from the National Center for Biotechnology Information (<http://www.ncbi.nlm.nih.gov/blast/bl2seq/bl2.html>), or by inspection.

The PB1 polymerases of any modified influenza A virus described herein can optionally comprise one or more mutations selected from the group consisting of a lysine to glutamic acid substitution at position 391 (K391E), a glutamic acid to glycine substitution at position 581 (E581G) and an alanine to threonine substitution at position 661 (A661T).

Any of the influenza A viruses described herein, including those with one or more mutations in a PB1 polymerase, as described above, can further comprise a PB2 polymerase comprising an asparagine to serine substitution at position 265 (N265S). Further, any of the influenza A viruses described can further comprise an influenza virus nucleoprotein (NP) comprising an aspartic acid to glycine substitution at position 35 (D35G).

Modifications, including the specific amino acid substitutions disclosed herein, are made by known methods. By way of example, modifications are made by site specific mutagenesis of nucleotides in the DNA encoding the polypeptide, thereby producing DNA

encoding the modification, and thereafter expressing the DNA in recombinant cell culture. Techniques for making substitution mutations at predetermined sites in DNA having a known sequence are well known, for example M13 primer mutagenesis and PCR mutagenesis.

As used throughout, the PB1 polymerase can be any influenza A PB1 polymerase, including but not limited to, a A/Ann Arbor/6/60 H2N2 PB1 polymerase (GenBank Accession No. AY210012.1) (SEQ ID NO: 2), a A/California/04/2009 H1N1 PB1 polymerase (GenBank Accession No. GQ377049.1) (SEQ ID NO: 3), an H3N2 A/Wisconsin/22/2011 PB1 polymerase (GenBank Accession No. KC883051.1) (SEQ ID NO: 4) and a A/Quail/Hong Kong/G1/97 H9N2 and H5N1 PB1 polymerase (GenBank Accession No. AF156421.1) (SEQ ID NO: 5). Optionally, the nucleic acid sequence set forth under GenBank Accession No. AY210012.1 (SEQ ID NO: 6), also known as a nucleic acid sequence that encodes the Master Donor Virus (MDV) PB1 can comprise one or more mutations selected from the group consisting of A99G, A1171G, G1371T, A1742G, G1981A, and C1995T. Optionally, the PB1 nucleic acid sequence from A/Ann Arbor/6/60 comprises A99G, A1171G, G1371T, A1742G, G1981A, and C1995T.

As used throughout, the PB2 polymerase can be any influenza A PB2 polymerase, including but not limited to A/Ann Arbor/6/60 H2N2 PB2 polymerase (GenBank Accession No. AY209938) (SEQ ID NO: 7), A/Quail/Hong Kong/G1/97 H2N2 PB2 polymerase (GenBank Accession No. AF156435) (SEQ ID NO: 8), A/Shanghai/02/2013 H7N9 PB2 polymerase (Gen Bank Accession No. KF021594) (SEQ ID NO: 9) or A/Chicken/Nanchang/3-120/2001 H3N2 PB2 polymerase (Gen Bank Accession No. AY180761) (SEQ ID NO: 10)

Recombinant nucleic acids encoding a PB1 polymerase of an influenza A virus, wherein the nucleic acid encodes a PB1 polymerase with one or more mutations in amino acids 310 to 325, are also provided. For example, a nucleic acid encoding a PB1 polymerase comprising a leucine to glutamine substitution at position 319 (L319Q) is provided herein. Further provided is a nucleic acid encoding a PB1 polymerase comprising a leucine to glutamine substitution at position 319 (L319Q) and one or mutations selected from the group consisting of a lysine to glutamic acid substitution at position 391 (K391E), a glutamic acid to glycine substitution at position 581 (E581G) and an alanine to threonine substitution at position 661 (A661T). Further provided are nucleic acids that encode both PB1 and PB2 polymerases with one or more mutations and compositions comprising nucleic acids that encode PB1 and PB2 polymerases with one or more mutations.

As used throughout, the term recombinant means that the material (e.g., a nucleic acid or protein) has been artificially or synthetically (non-naturally) altered by human intervention. It is understood that, when referring to a virus, e.g., an influenza A virus, the virus is recombinant when it is produced by the expression of a recombinant nucleic acid.

5 As used herein, nucleic acid refers to single or multiple stranded molecules which can be DNA or RNA, or any combination thereof, including modifications to those nucleic acids. For example, the nucleic acid can be a cDNA. The nucleic acid may represent a coding strand or its complement, or any combination thereof. The nucleic acid can be directly cloned into an appropriate vector, or if desired, can be modified to facilitate the subsequent
10 cloning steps. Such modification steps are routine, an example of which is the addition of oligonucleotide linkers which contain restriction sites to the termini of the nucleic acid. General methods are set forth in in Sambrook et al. (2001) Molecular Cloning - A Laboratory Manual (3rd ed.) Vol. 1-3, Cold Spring Harbor Laboratory, Cold Spring Harbor Press, NY, (Sambrook).

15 The nucleic acids disclosed herein can be in any vector that can be used for the production of influenza virus in a host cell. The vector can direct the *in vivo* or *in vitro* synthesis of any of the polypeptides described herein, including, but not limited to PB1/and or PB2 polymerases. One or more of the vectors described herein can be part of a multi-vector system used to produce an influenza A virus. The vector is contemplated to have the
20 necessary functional elements that direct and regulate transcription of the inserted nucleic acid. These functional elements include, but are not limited to, a promoter, regions upstream or downstream of the promoter, such as enhancers that may regulate the transcriptional activity of the promoter, an origin of replication, appropriate restriction sites to facilitate cloning of inserts adjacent to the promoter, antibiotic resistance genes or other markers which
25 can serve to select for cells containing the vector or the vector containing the insert, RNA splice junctions, a transcription termination region, or any other region which may serve to facilitate the expression of the inserted gene or hybrid gene (See generally, Sambrook et al. (2001)). The vector, for example, can be a plasmid. The vectors can contain genes conferring hygromycin resistance, ampicillin resistance, gentamicin resistance, neomycin resistance or
30 other genes or phenotypes suitable for use as selectable markers.

As used throughout, a host cell is a cell that contains one or more of the nucleic acids disclosed herein, including any of the nucleic acids in a vector, and supports the replication and/or expression of the nucleic acids, and optionally production of one or more encoded products including a polypeptide and/or a virus. Host cells can be prokaryotic cells, such as

E. coli, or eukaryotic cells, such as yeast, insect, amphibian, avian or mammalian cells, including human cells. Examples of host cells include, but are not limited to, Vero (African green monkey kidney) cells, Per.C6 cells (human embryonic retinal cells), BHK (baby hamster kidney) cells, primary chick kidney (PCK) cells, Madin-Darby Canine Kidney (MDCK) cells, Madin-Darby Bovine Kidney (MDBK) cells, 293 cells (e.g., 293T cells), CEK cells, primary human lung cells, bronchial epithelial cells, COS cells (e.g., COS1, COS7 cells) and any other mammalian or avian cells that can be used to produce or propagate an influenza virus. The term host cell encompasses combinations or mixtures of cells including, but not limited to mixed cultures of different cell types or cell lines.

Any of the modified influenza A viruses described herein can be a live attenuated influenza A virus with reduced growth from about 37°C to about 39°C, as compared to an influenza A virus comprising a PB1 polymerase lacking one or more mutations in amino acids 310 to 325. For example, the modified influenza A virus can have reduced growth at about 37°C, 38°C or 39°C or any temperature in between. Further, the modified influenza A virus can have reduced growth at about 37°C-38°C or at about 38°C-39°C. Optionally, the modified influenza A virus grows at temperatures between about 32°C-34°C and has a reduction in growth at temperatures greater than about 34°C. In this way, the modified influenza A virus can grow, for example, in the upper respiratory tract where temperatures are about 32°C-34°C, and stimulate an immune reaction, without producing symptoms in the lower respiratory tract where temperatures are about 37°C-38°C. Optionally, the modified influenza A virus is attenuated at temperatures between about 32°C-34°C as well as between temperatures of about 37°C to about 39°C. The degree of attenuation does not have to be the same at temperatures between about 32°C-34°C and at temperatures between temperatures of about 37°C to about 39°C, as the reduction in growth at 32°C-34°C can be about the same or less than the reduction in growth at about 37°C to about 39°C. Optionally, the virus exhibits at least about a 100-fold or greater reduction in titer at about 39°C relative to titer at about 34°C.

As used throughout, ranges can be expressed herein as from about one particular value, and/or to about another particular value. When such a range is expressed, another aspect includes from the one particular value and/or to the other particular value. Similarly, when values are expressed as approximations, by use of the antecedent about it will be understood that the particular value forms another aspect. It will be further understood that the endpoints of each of the ranges are significant both in relation to the other endpoint, and independently of the other endpoint. It is also understood that there are a number of values

disclosed herein, and that each value is also herein disclosed as about that particular value in addition to the value itself. For example, if the value 10 is disclosed, then “about 10” is also disclosed. It is also understood that throughout the application data are provided in a number of different formats and that this data represent endpoints and starting points and ranges for
5 any combination of the data points. For example, if a particular data point “10” and a particular data point “15” are disclosed, it is understood that greater than, greater than or equal to, less than, less than or equal to, and equal to 10 and 15 are considered disclosed as well as between 10 and 15. It is also understood that each unit between two particular units are also disclosed. For example, if 10 and 15 are disclosed, then 11, 12, 13, and 14 are also
10 disclosed.

A reduction or a decrease in growth can be a decrease of about 10, 20, 30, 40, 50, 60, 70, 80, 90, 100% or any percentage in between as compared to an influenza A virus comprising a PB1 polymerase lacking one or more mutations in amino acids 310 to 325. Growth indicates viral quantity as indicated by titer, plaque size or morphology, particle
15 density or other measures known to those of skill in the art. A reduction or decrease in growth can also be a reduction or decrease in replication of about 10, 20, 30, 40, 50, 60, 70, 80, 90, 100% or any percentage in between as compared to an influenza A virus comprising a PB1 polymerase lacking one or more mutations in amino acids 310 to 325.

Further provided is an immunogenic composition comprising any of the modified
20 influenza A viruses disclosed herein and a pharmaceutically acceptable carrier to stimulate an immune response against one or more strains of influenza virus. By pharmaceutically acceptable carrier is meant a material that is not biologically or otherwise undesirable, i.e., the material is administered to a subject without causing undesirable biological effects or interacting in a deleterious manner with the other components of the pharmaceutical
25 composition in which it is contained. The carrier is selected to minimize degradation of the active ingredient and to minimize adverse side effects in the subject. One of skill in the art would know how to select a carrier in order to minimize allergic and other undesirable effects, and to suit the particular route of administration. Optionally, the composition can further comprise an adjuvant.

30 Suitable carriers and their formulations are described in *Remington: The Science and Practice of Pharmacy, 21st Edition*, David B. Troy, ed., Lippicott Williams & Wilkins (2005). Examples of pharmaceutically-acceptable carriers include, but are not limited to, sterile water, saline, buffered solutions like Ringer’s solution, glycerol solutions, ethanol, dextrose solutions, allantoic fluid from uninfected chicken eggs (i.e., normal allantoic fluid) or

combinations thereof. The pH of the solution is generally about 5 to about 8 or from about 7 to 7.5. The preparation of such solutions insuring sterility, pH, isotonicity, and stability is effected according to protocols established in the art.

Other carriers include sustained release preparations such as semipermeable matrices of solid hydrophobic polymers containing the immunogenic composition. Matrices are in the form of shaped articles, e.g., films, liposomes, or microparticles. Certain carriers may be more preferable depending upon, for instance, the route of administration and concentration of composition being administered. Carriers are those suitable for administration of the compositions disclosed herein, to humans or other subjects.

Also provided is a method for eliciting an immune response against an influenza virus in a subject comprising administering an effective dose of any of the immunogenic compositions described herein. In the methods disclosed herein, the immune response can be an innate and/or an adaptive immune response. An immune response can be an antibody response against one or more strains of influenza and/or a T cell mediated response.

As used throughout, a subject can be a vertebrate, more specifically a mammal (e.g., a human, horse, cat, dog, cow, pig, sheep, goat, mouse, rabbit, rat, and guinea pig), birds, reptiles, amphibians, fish, and any other animal. The term does not denote a particular age or sex. Thus, adult and newborn subjects, whether male or female, are intended to be covered. As used herein, patient or subject may be used interchangeably and can refer to a subject with or at risk of developing an influenza infection. The term patient or subject includes human and veterinary subjects.

According to the methods taught herein, the subject is administered an effective amount of the agent, e.g., an immunogenic composition comprising a modified influenza A virus. The terms effective amount and effective dosage are used interchangeably. The term effective amount is defined as any amount necessary to produce a desired physiologic response (i.e., an immune response). Effective amounts and schedules for administering the agent may be determined empirically, and making such determinations is within the skill in the art. The dosage ranges for administration are those large enough to produce the desired effect (e.g., eliciting an immune response to the antigen of interest, i.e. influenza A). The dosage should not be so large as to cause substantial adverse side effects, such as unwanted cross-reactions, anaphylactic reactions, and the like. Generally, the dosage will vary with the age, condition, sex, type of disease, the extent of the disease or disorder, route of administration, or whether other drugs are included in the regimen, and can be determined by one of skill in the art. The dosage can be adjusted by the individual physician in the event of

any contraindications. Dosages can vary, and the agent can be administered in one or more dose administrations daily, for one or several days, including a prime and boost paradigm.

The compositions are administered via any of several routes of administration, including, but not limited to, orally, parenterally, intravenously, intramuscularly, 5 subcutaneously, transdermally, nebulization/inhalation, or by installation via bronchoscopy. Optionally, the composition is administered by oral inhalation, nasal inhalation, or intranasal mucosal administration. Administration of the compositions by inhalant can be through the nose or mouth via delivery by spraying or droplet mechanism, for example, in the form of an aerosol. A form of administration that results in an immune response can be used by one of 10 skill in the art to optimize the response.

In any of the methods described herein, the immunogenic compositions can be used alone or in combination with one or more therapeutic agents such as, for example, antiviral compounds for the treatment of influenza. These include, but are not limited to, amantadine, rimantadine, ribavirin, zanamavir (Relenza®) and oseltamavir (Tamiflu®).

15 Further provided is a method of treating or preventing an influenza infection in a subject, comprising administering to a subject with an influenza infection or susceptible to an influenza infection an effective dose of any of the immunogenic compositions described herein.

For purposes of vaccines, the subject may be healthy and without higher risk than the 20 general public. A subject at risk of developing an influenza infection, however, can be predisposed to contracting an infection (e.g., persons over 65, persons with asthma or other chronic respiratory disease, young children, pregnant women, persons with a hereditary predisposition, persons with a compromised immune system or by being in an environment that facilitates the passage of an influenza infection). A subject currently with an infection 25 has one or more than one symptom of the infection. These symptoms include, but are not limited, fever, sore throat, cough, muscle aches, headache, fatigue, vomiting and diarrhea. The subject currently with an influenza infection may have been diagnosed with an influenza infection.

The methods and compositions as described herein are useful for both prophylactic 30 and therapeutic treatment. For prophylactic use, a therapeutically effective amount of the compositions described herein are administered to a subject prior to onset (e.g., before obvious signs of infection) or during early onset (e.g., upon initial signs and symptoms of infection). Prophylactic administration can occur for several days to years prior to the manifestation of symptoms of the infection. Prophylactic administration can be used, for

example, in the preventative treatment of subjects diagnosed with a predisposition to influenza infection. Therapeutic treatment involves administering to a subject a therapeutically effective amount of the agents described herein after diagnosis or development of infection.

5 As used herein the terms treatment, treat, or treating refers to a method of reducing one or more of the effects of the infection or one or more symptoms of the infection by eliciting an immune response in the subject. Thus in the disclosed method, treatment can refer to a 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90%, or 100% reduction in the severity of an established infection or a symptom of the infection. For example, a method for
10 treating an infection is considered to be a treatment if there is a 10% reduction in one or more symptoms of the infection in a subject as compared to a control. Thus the reduction can be a 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90%, 100%, or any percent reduction in between 10% and 100% as compared to native or control levels. It is understood that treatment does not necessarily refer to a cure or complete ablation of the infection or disease
15 or symptoms of the infection or disease.

As used herein, the terms prevent, preventing, and prevention of an infection, refers to an action, for example, administration of a therapeutic agent (e.g., a composition disclosed herein), that occurs before or at about the same time a subject begins to show one or more symptoms of the infection, which inhibits or delays onset or exacerbation or delays
20 recurrence of one or more symptoms of the infection. As used herein, references to decreasing, reducing, or inhibiting include a change of 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90% or greater as compared to a control level. For example, the disclosed methods are considered to be a prevention if there is about a 10% reduction in onset, exacerbation or recurrence of infection, or symptoms of infection in a subject exposed to an
25 infection when compared to control subjects exposed to an infection that did not receive a composition for decreasing infection. Thus, the reduction in onset, exacerbation or recurrence of infection can be about a 10, 20, 30, 40, 50, 60, 70, 80, 90, 100%, or any amount of reduction in between as compared to control subjects. For example, and not to be limiting, if about 10%> of the subjects in a population do not become infected as compared to subjects
30 that did not receive preventive treatment, this is considered prevention.

Further provided is a method of producing the influenza A viruses disclosed herein comprising (a) transfecting a population of host cells with one or more vectors comprising (i) nucleic acid sequences encoding the internal genome segments of an influenza A virus and; (ii) a nucleic acid encoding a PB1 polymerase with one or more mutations in amino acids 310

to 325; (b) culturing the host cells; and c) recovering the modified influenza A virus.

Methods for producing influenza virus are known to those of skill in the art. In the production methods described herein, plasmids incorporating the internal genes of an influenza master virus strain, (i.e., PB1, PB2, PA, NP, N13, M1, BM2, NS1 and NS2) are transfected into suitable host cells in combination with hemagglutinin and neuraminidase segments. See, for example, U.S. Patent No. 8,354,114. Optionally, the hemagglutinin and neuraminidase segments can be from a strain predicted to cause significant local or global influenza infection. Typically, the master strain is selected on the basis of desirable properties relevant to vaccine administration. For example, for vaccine production, e.g., for production of a live attenuated vaccine, the master donor virus strain can be selected for an attenuated phenotype, cold adaptation and/or temperature sensitivity. For example, Influenza A strain ca A/Ann Arbor/6/60 can be the master donor virus (see, for example, Chan et al., Virology 380: 304-311(2008)). Following replication of the reassortant virus in cell culture at appropriate temperatures for efficient recovery, for example, at temperatures equal to or less than about 35°C, such as from about 32°C to 35°C, from about 32°C to about 34°C, or from about 32°C to about 33°C, the reassortant virus is recovered. Optionally, the recovered virus can be inactivated using a denaturing agent such as formaldehyde or β -propiolactone. Optionally, in the production methods provided herein, the viruses can be further amplified in chicken eggs.

Further provided is a method for producing an influenza vaccine comprising (a) infecting a population of cells with any of the viruses described herein; (b) culturing the cells; (c) harvesting the virus from the culture of step (b); and (d) preparing a vaccine with the harvested virus.

Once the virus is harvested from a cell culture, the virus can be formulated and administered as a composition, according to known methods, as an immunogenic composition to induce an immune response in an animal, e.g., a mammal. Optionally, the immunogenic composition can be formulated as an inactivated vaccine. Methods are well-known in the art for determining whether such inactivated vaccines have maintained similar antigenicity to that of the clinical isolate or a high growth strain derived therefrom. As set forth above, an immunogenic composition can be administered via all the routes conventionally used or recommended for an immunogenic composition. The immunogenic composition can be formulated as an injectable or sprayable liquid, or as a formulation which has been freeze-dried or dried by atomization or air-dried, etc. The immunogenic composition can also be formulated for administration via syringe or by means of a needle-free injector for

intramuscular, subcutaneous or intradermal injection. The immunogenic composition can also be administered by means of a nebulizer capable of delivering a dry powder or a liquid or aerosolized spray to the mucous membranes.

A complete immunogenic composition can be concentrated by ultrafiltration and then purified by zonal centrifugation or by chromatography. Optionally, it can be inactivated before or after purification using formalin or 13-propiolactone, for example.

Disclosed are materials, compositions, and components that can be used for, can be used in conjunction with, can be used in preparation for, or are products of the disclosed methods and compositions. These and other materials are disclosed herein, and it is understood that when combinations, subsets, interactions, groups, etc. of these materials are disclosed that while specific reference of each various individual and collective combinations and permutations of these compounds may not be explicitly disclosed, each is specifically contemplated and described herein. For example, if a method is disclosed and discussed and a number of modifications that can be made to a number of molecules including the method are discussed, each and every combination and permutation of the method, and the modifications that are possible are specifically contemplated unless specifically indicated to the contrary. Likewise, any subset or combination of these is also specifically contemplated and disclosed. This concept applies to all aspects of this disclosure including, but not limited to, steps in methods using the disclosed compositions. Thus, if there are a variety of additional steps that can be performed, it is understood that each of these additional steps can be performed with any specific method steps or combination of method steps of the disclosed methods, and that each such combination or subset of combinations is specifically contemplated and should be considered disclosed.

EXAMPLES

The current live attenuated influenza vaccine (LAIV) is recommended as the primary vaccination strategy for healthy subjects aged 2 to 49 years, because of its greater efficacy and ease-of-use than the traditional inactivated influenza vaccine in this age group. However, the current LAIV is not recommended for pregnant women, children under 2, persons with a compromised immune system, (for example, persons with HIV/AIDS), or persons at high risk for complications from influenza.

The current LAIV vaccine was originally derived through cold adaptation, and subsequent work determined that the attenuating gene segments correspond to the viral

polymerase (PB1, PB2, PA) and nucleoprotein (NP). Introduction of the attenuating PB2 segment into the genetic background of a seasonal influenza virus background resulted in temperature sensitivity and attenuation, which could be overcome by serial passage of virus at elevated temperatures.

5 These phenotypic revertant viruses were analyzed with the goal of understanding the molecular mechanism underlying the attenuation of LAIV. Methods for isolating and characterizing mutant viruses, including characterization of temperature-sensitivity are described in Treanor et al. ("Evaluation of the genetic stability of temperature-sensitive PB2 gene mutation of the influenza A/Ann Arbor/6/60 cold-adapted vaccine virus," *J. Virol* 10 68(12): 7684-8 (1994).

 A mutation that results in a leucine to glutamine substitution at position 319 of PB1 was made using the methods described herein. The polymerase activity of the mutant was assayed using a minigenome assay described in Bussey et al. ("PA residues in the 2009 H1N1 pandemic influenza virus enhance avian influenza virus polymerase activity in mammalian 15 cells," *J. Virol.* 85(14): 7020-8 (2011)). It was found that a mutation in the PB1 gene (at residue 319) was sufficient to reverse the temperature sensitive phenotype of the viral RNA polymerase, conferred by the LAIV PB2 gene segment. This was unexpected, since previous studies have identified such reversion mutations only in the PA gene. Follow up studies revealed that the 319 mutation was also sufficient to reverse the temperature-sensitivity of the RNA polymerase from two 20 avian strains in the H5N1 and H9N2 lineage, thus showing that the PB1 319 residue has broad relevance in determining the temperature sensitivity of the virus polymerase. Collectively, these studies revealed a novel molecular determinant of the temperature sensitivity and attenuation of the influenza A virus RNA polymerase.

 The following study was performed by constructing and characterizing mutant viruses 25 as set forth in Treanor et al. Viruses were characterized for temperature sensitivity in the following manner: confluent 6 well plates of MDCK cells were infected at an MOI of 0.01 with the *ts* single gene replacement virus and incubated at 34, 37 and 39 °C for 72 hours. Every 12 hours a sample of the culture supernatant was harvested and replaced with fresh media. These samples were clarified by centrifugation and stored at -80 °C. The samples 30 were then analyzed for viral titer through TCID-50 analysis, as described in Bussey et al. The virus used in this analysis possessed the PB2 segment of cold adapted temperature sensitive and attenuated A/Ann Arbor/6/60 (Genbank ID: AY209938.1) in the background of A/Korea/1982 (see Treanor et al.).

As set forth above, the PB2 segment of a seasonal human influenza A virus strain (A/Korea/82 H3N2) was replaced with the PB2 segment from the cold passaged isolate of A/AnnArbor/6/60. The resulting single gene replacement virus is temperature sensitive (ts) for growth at elevated temperatures. This virus stock was subjected to serial passage at increasing temperatures, in order to identify phenotypically revertant single gene replacement viruses. The ts PB2 single gene replacement virus was subjected to plaque purification and individual plaques were analyzed for their temperature dependent growth properties. The plaque purified viruses were expected to have growth at 34°C and 37°C, but not at 39°C (see Figure 1). Surprisingly, a virus that had reduced growth at 37°C as well as at 39°C was purified.

All components of the viral polymerase were cloned into a mammalian expression vector from the viral RNA and then analyzed. Surprisingly this system revealed a significant decrease in polymerase activity at 37°C. A number of residues were found to be unique as compared to conserved influenza sequences and their importance was examined through mutation to the conserved residue by site directed mutagenesis. A residue of interest resided in PB1, at amino acid 319, and was the substitution of a polar glutamine for a nonpolar leucine. The PB1 L319Q mutation dramatically reduces functional activity of human influenza A virus RNA polymerase at 37°C (see Figure 2).

The impact of this mutation on other influenza A viruses (IAV) was then examined. In these experiments an avian IAV polymerase complex, the polymerase complex from the low-pathogenicity virus, A/Chicken/Nanchang/3-120/01 H3N2, was used. Introduction of the L319Q mutation in PB1 into this polymerase also significantly reduced the functional activity of this avian influenza A virus RNA polymerase at 37°C (Fig. 3). It was also found that a PB1 with a L319Q mutation synergizes with three mutations (K391E, E581G and A661T) found in the LAIV (Figure 4). The polymerase activity was assayed using the minigenome assay described in Bussey et al.

Additional experiments were conducted to further characterize the temperature sensitivity of a modified vaccine strain virus. Viruses were created through site directed mutagenesis of the PR8 bidirectional plasmids described in Martinez-Sobrido et al. ("Generation of Recombinant Influenza Virus from Plasmid DNA," J. Vis. Exp. 42: 2057 (2010)). PR8 live attenuated influenza virus (LAIV) possesses 4 amino acid mutations. These mutations are N265S in PB2, K391E in PB1, E581G in PB1 and A661T in PB1. PR8 LAIV + PB1 319Q possesses the mutation PB1 L319Q in addition to the 4 mutations present in PR8 LAIV. All plasmids were sequenced to confirm successful site directed mutagenesis

and all rescued viruses were sequenced to confirm retention of only the desired mutations. Both viruses were assayed for temperature-sensitive growth via plaque assay, as described in Bussey et al. When the four mutations of LAIV (N265S in PB2, K391E in PB1, E581G in PB1 and A661T in PB1) were added to PR8 no virus was detected by plaque assay at 39°C.

5 However, when PB1 319Q was added in addition to the four mutations of LAIV, no virus growth occurred at 37 C as well (Figure 5).

Experiments were also conducted to characterize the stability of the L319Q mutation. The stability of a glutamine at residue 319Q of PB1 was analyzed by inserting this mutation singly in the background of a wild type virus to determine whether this mutation is stable.

10 These viruses were constructed via site-directed mutagenesis of the PR8 bidirectional plasmids described in Martinez-Sobrido et al. PB1 319Q possesses glutamine instead of the wildtype leucine at residue 319 of PB1. All plasmids were sequenced to confirm successful site-directed mutagenesis and all rescued viruses were sequenced to confirm retention of only the desired mutations. The viruses were then passaged an additional three times at 30 C, 15 33°C, 37°C and 39°C. The PB1 gene was then sequenced in its entirety as described in Zhou et al ("Single-reaction genomic amplification accelerates sequencing and vaccine production for classical and Swine origin human influenza A viruses," *J. Virol.* 19: 10309-13 (2009)). After 1 passage the virus showed uniform stability. After 2 subsequent passages at 30°C, 33°C, 37°C and 39°C, all viruses retained glutamine at this position. This shows that this mutation is stable at various 20 temperatures in influenza A viruses (Figure 6).

Thus, a novel and unexpected mutation in PB1(L319Q), that increases the temperature sensitivity of influenza A viruses was identified. Therefore, this and other mutations can be used to make live attenuated influenza viruses. These mutations can also be used to further attenuate existing live attenuated influenza viruses (LAIV), thereby increasing 25 their safety, and allowing for its use in populations in which the vaccine is presently contraindicated.

SEQUENCES

SEQ ID NO: 1

30 NENQNPRMFLAMITYI

SEQ ID NO:2

MDVNPTLLFLKVPAQNAISTTFPYTGDPPYSHGTGTGYTMDTVNRTHQYSEKGKWT
TNTETGAHQQLNPIDGPLPEDNEPSGYAQTDCVLEAMAFLEESHGPGIFENSCLETMEVI

QQTRVDKLTQGRQTYDWTLNRNQPAATALANTIEVFRSNGLTANESGRLIDFLKDVI
 ESMDKEEMEITTHFQRKRRVRDNMTKKMVTQRTIGKKKQRLNKRSLRALTLNTM
 TKDAERGKLRRAIATPGMQIRGFVYFVETLARSICEKLEQSGLPVGGNEKKAKLAN
 VVRKMMTNSQDTELSFTITGDNTKWNENQNPRMFLAMITYITRNQPEWFRNVLSIAP
 5 IMFSNKMARLGKGYMFKSKSMKLRTQIPAEMLASIDLKYFNSTRKKIEKIRPLLIDG
 TVSLSPGMMMGMFNMMLSTVLGVSILNLGQKKYTKTTYWWDGLQSSDDFALIVNAP
 NHEGIQAGVDRFYRTCKLVGINMSKKKSYINRTGTFFTSFFYRYGFVANFSMELPSF
 GVSIGINESADMSIGVTVIKNNMINNDLGPATAQLALQLFIKDYRYTYRCHRGDT
 QIQTRRSFELKKLWEQTRSKAGLLVSDGGPNLYNIRNLHIPEVCLKWELMDDEDYQGR
 10 LCNPLNPFVSHKEIESVNNAVVMMPAHGPAKSMEYDAVATTHSWIPKRNRSILNTSQR
 GILEDEQMYQKCCNLFEKFFPSSSYRRPVGISSMVEAMVSRARIDARIDFESGRIKKEE
 FAEIMKICSTIEELRRQK

SEQ ID NO: 3

15 MDVNPTLLFLKIPAQNAISTTFPYTGDPPYSHGTGTGYTMDTVN
 RTHQYSEKGWTTNTETGAPQLNPIDGPLPEDNEPSGYAQTDCVLEAMAFLEESHPG
 IFENSCLETMEVVQQTRVDKLTQGRQTYDWTLNRNQPAATALANTIEVFRSNGLT
 NESGRLIDFLKDVMESEMNKEEIEITTHFQRKRRVRDNMTKKMVTQRTIGKKKQRLN
 KRGYLIRALTLNTMTKDAERGKLRRAIATPGMQIRGFVYFVETLARSICEKLEQSG
 20 PVGGN
 EKKAKLANVVRKMMTNSQDTEISFTITGDNTKWNENQNPRMFLAMITYITRNQPEW
 FRNILSMAPIMFSNKMARLGKGYMFESKRMKIRTQIPAEMLASIDLKYFNSTRKKIE
 KIRPLLIDGTASLSPGMMMGMFNMMLSTVLGVSILNLGQKKYTKTIYWWDGLQSSDD
 FALIVNAPNHEGIQAGVDRFYRTCKLVGINMSKKKSYINKTGTFFTSFFYRYGFVAN
 25 FSMELPSFGVSGVNESADMSIGVTVIKNNMINNDLGPATAQMALQLFIKDYRYTYR
 HRGDTQIQTRRSFELKKLWDQTQSKVGLLVSDGGPNLYNIRNLHIPEVCLKWELMD
 DDYRGRLCNPLNPFVSHKEIDSVNAVVMMPAHGPAKSMEYDAVATTHSWIPKRNRS
 ILNTSQRGILEDEQMYQKCCNLFEKFFPSSSYRRPVGISSMVEAMVSRARIDARVDFE
 SGRIKKEEFSEIMKICSTIEELRRQK

30

SEQ ID NO: 4

MDVNPTLLFLKVPAQNAISTTFPYTGDPPYSHGTGTGYTMDTVN
 RTHQYSERGWTTNTETGAPQLNPIDGPLPEDNEPSGYAQTDCVLEAMAFLEESHPG
 IFENSCLETMEAVQQTRVDKLTQGRQTYDWTLNRNQPAATALANTIEVFRSNGLT
 35 NESGRLIDFLKDVMESEMDKEEMEITTHFQRKRRVRDNMTKKMVTQRTIGKKKQRV
 NKRGYLIRALTLNTMTKDAERGKLRRAIATPGMQIRGFVYFVETLARSICEKLEQ
 GLPVGGNEKKAKLANVVRKMMTNSQDTELSFTITGDNTKWNENQNPRMFLAMITYI
 TKNQPEWFRNILSIAPIMFSNKMARLGKGYMFESKKMKLRTQIPAEMLASIDLKYFN
 ESTRKKIEKIRPLLIDGTASLSPGMMMGMFNMMLSTVLGVSILNLGQKKYTKTTYWW
 40 DGLQSSDDFALIVNAPNHEGIQAGVDRFYRTCKLVGINMSKKKSYINKTGTFFTSFF
 YRYGFVANFSME
 LPSFGVSGINESADMSIGVTVIKNNMINNDLGPATAQMALQLFIKDYRYTYRCHRGD
 TQIQTRRSFEIKKLWDQTQSRAGLLVSDGGPNLYNIRNLHIPEVCLKWELMDENYRG
 RLCNPLNPFVSHKEIESVNNAVVMMPAHGPAKSMEYDAVATTHSWIPKRNRSILNTSQ

20

1321 ctccaatcct ctgatgactt cgccctcata gtgaatgcac caaatcatga gggaatacaa
 1381 gcaggggtgg atagattcta cagaacctgc aagctagtcg gaatcaatat gagcaaaaag
 1441 aagtctaca taaataggac agggacattt gaattcaca gcttttcta tcgctatgga
 1501 ttgtagcca attttagcat ggagctgccc agctttggag tgtctggaat taatgaatcg
 5 1561 gctgatatga gcattggggg aacagtata aagaacaaca tgataaaca tgacctggg
 1621 ccagcaacag cccaactggc tctcaacta ttcataaag actacagata tacgtaccgg
 1681 tgccacagag gagacacaca aattcagaca aggagatcat tcgagctaaa gaagctgtgg
 1741 gagcaaaccc gctcaaaggc aggacttttg gtttcggatg gaggacaaa cttatacaat
 1801 atccggaatc tccacattcc agaagtctgc ttgaagtggg agctaattga tgaagactat
 10 1861 caggggaggg ttgtaatcc cctgaatcca ttgtcagtc ataaggagat tgagtctgta
 1921 aacaatgctg tggtaatgcc agctcaggt ccagccaaga gcatggaata tgatgctgtt
 1981 gctactacac actcctggat ccctaagagg aaccgctcca ttctaacac aagccaaagg
 2041 ggaattcttg aagatgaaca gatgtatcag aagtgtgca atctattcga gaaattcttc
 2101 cctagcagtt cgtacaggag accagttgga attccagca tgggtggagc catggtgtct
 15 2161 agggcccgga ttgatgcacg gattgacttc gagtctggac ggattaagaa agaggagttc
 2221 gctgagatca tgaagatctg ttccaccatt gaagagctca gacggcaaaa atag

SEQ ID NO: 7

MERIKELRNLMQSRTREILTKTTVDHMAIIKKYTSGRQEKNPS
 20 LRMKWMAMKYPITADKRITEMIPERNEQGQTLWSKMSDAGSDRVMVSPLAVTW
 WNRNGPMTSTVHYPKIYKTYFEKVERLKHGTFGPVHFRNQVKIRRRVDINPGHADL
 SAKEAQDVIMEVVPNEVGARILTSESQLTITKEKKEELQDCKISPLMVAYMLERELV
 RKTRFLPVAGGTSSVYIEVLHLTQGTCWEQMYTPGGEVRNDDVDQSLIIARNIVRR
 AAVSADP
 25 LASLLEMCHSTQIGGTRMVDILRQNPTEEQAVEICKAAMGLRISSSFSGGFTFKRTS
 GSSVKREEEVLTLGNLQTLKIRVHEGYEFTMVGKRATILRKATRRLIQLIVSGRDEQ
 SIAEAIIVAMVFSQEDCMIAVRGDLNFVNRRANQRLNPMHQLLRHFQKDAKVLFFQN
 WGIEHIDNVMGMIGVLPDMTPSTEMSMRGVRVSKMGVDEYSSAERVVVSIDRFLRV
 RDQR
 30 GNVLLSPEEVSETQGTEKLTITYSSSMMWEINGPESVLVNTYQWIIRNWETVKIQWS
 QNPTMLYNKMEFEPFQSLVPKAIKRGQYSGFVRTLFQQMRDVLGTFDTTQIIKLLPFA
 AAPPKQSRMQFSSLTVNVRGSGMRILVRGNSPIFNYNKTTKRLTILGKDAGTLTEDP
 DEGTSGVESAVLRGFLILGKEDRRYGPALSINELSNLAKGEKANVLIGQGDVVLVMK
 RKRN
 35 SSILTDSQTATKRIRMAIN

SEQ ID NO: 8

IKELWDLMSQSRTREILTKTTVDHMAIIKKYTSGRQEKNPALRM
 KWMAMKYPITADKRIMEMIPERNEQGQTLWSKTNDAGSDRVMESPLAVTWNR
 40 NGPTTSTVHYPKVYKTYFEKVERLKHGTFGPVHFRNQVKIRRRVDMNPGHADLSAK
 EAQDVIMEVVPNEVGARILTSESQLTITKEKREELKNCNIAPLMVAYMLERELVRKT
 RFLPVA
 GGTSSVYIEVLHLTQGTCWEQMYTPGGEVRNDDVDQSLIIAVGNIVRRATVSADPLA
 SLLEMCHSTQIGGVRMVDILKQNPTEEQAVDICKAAMGLKISSSFSGGFTFKRTKGS

SVKREEEVLTGNLQTLKIKVHEGYEEFTMVGRRATAILRKATRMIQLIVSGRDEQSI
 AEAIIVAMVFSQEDCMIAVRGDLNFVNANQRLNPMHQLLRHFQKDAKVLFFQNW
 GTEPIDNVMGMIGILPDMTPSTEMSLRGVRVSKMGVDEYSSTERVVVSIDRFLRVRD
 QRGNVLLSPEEVSETQGMEKLTITYSSSMMWEINGPESVLVNTYQWIIRNWETVKIQ
 5 WSQEPMLYNKMEFEPFQSLVPAARSQYSGFVRTLFQQMRDVLGTFDTVQIIKLLP
 FAAAPPEQSRMQFSSLTVNVRGSGMRILVRGNPAFNYNKTTKRLTILGKDAGALTE
 DPDEGTAGVESAVLRGFLILGKEDKRYGPALSINELSNLTKGEKANVLIGQGDVVLV
 MKRKRDSI
 LTDSQTATKRI

10

SEQ ID NO: 9

MERIKELRDLMSQSRTREILTKTTVDHMAIIKKYTSGRQEKNPA
 LRMKWMAMKYPITADKRIMEMIPERNEQGQTLWSKTNDAGSDRVMVSPLAVTW
 WNRNGPTTSTVHYPKVYKTYFEKVERLKHGTFGPVHFRNQVKIRRRVDINPGHADL
 15 SAKEAQDVIMEVVPNEVGARILTSESQLTITKEKKELQDCKIAPLMVAYMLEREL
 VRKTRFL
 PVAGGTSSVYIEVLHLTQGTCWEQMYTPGGEVRNDDVDQSLIIAARNIVRRATVSAD
 PLASLLEMCHSTQIGGIRMVDILRQNPTEEQAVIDICKAAMGLRISSSFSGGFTFKRTS
 GSSVKREEEVLTGNLQTLKIRVHEGYEEFTMVGRRATAILRKATRRLIQLIVSGKDEQ
 20 SIAEAIIVAMVFSQEDCMIAVRGDLNFVNANQRLNPMHQLLRHFQKDAKVLFFQNW
 WG
 IEPIDNVMGMIGILPDMTPSTEMSLRGVRVSKMGVDEYSSTERVVVSIDRFLRVRDQ
 RGNVLLSPEEVSETQGTEKLTITYSSSMMWEINGPESVLVNTYQWIIRNWENVKIQW
 SQDPTMLYNKMEFEPFQSLVPAARGQYSGFVRVLFQQMRDVLGTFDTVQIIKLLPF
 25 AAAPPKQSRMQFSSLTVNVRGSGMRIVVRGNPVPFNYNKATKRLTVLTKDAGALM
 EDPDEG
 TAGVESAVLRGFLILGKEDKRYGPALSINELSNLAKGEKANVLIGQGDVVLVLMKRK
 RDSSILTDSQTATKRIRMAIN

30 SEQ ID NO: 10

SRTREILTKTTVDHMAIIKKYTSGRQEKNPSLRMKWMAMKYPI
 TADKRIMEMIPERNEQGQTLWSKTNDAGSNRVMVSPLAVTWNRNGPTTSTIHYPK
 VYKTYFEKVERLKHGTFGPVHFRNQVKIRRRVDVNPBGHADLSAKEAQDVIMEVVP
 NEVGARILTSESQLAITKEKKEE

35

The embodiments of the invention in which an exclusive property or privilege is claimed are defined as follows:

1. A modified influenza A virus comprising a PB1 polymerase with one or more mutations in amino acids corresponding to amino acid positions 310 to 325 of SEQ ID NO: 2, wherein the PB1 polymerase comprises a leucine to glutamine substitution at an amino acid position corresponding to amino acid position 319 (L319Q) of SEQ ID NO: 2.
2. The virus of claim 1, wherein the PB1 polymerase further comprises one or more mutations comprising a lysine to glutamic acid substitution at an amino acid corresponding to position 391 (K391E) of SEQ ID NO: 2, a glutamic acid to glycine substitution at an amino acid corresponding to position 581 (E581G) of SEQ ID NO: 2, an alanine to threonine substitution at an amino acid corresponding to position 661 (A661T) of SEQ ID NO: 2, or any combination thereof.
3. The virus of claim 1 or 2, wherein the virus further comprises a PB2 polymerase comprising an asparagine to serine substitution at an amino acid corresponding to position 265 (N265S) of SEQ ID NO: 7.
4. The virus of any one of claims 1 to 3, wherein the influenza A virus is an H2N2 virus, an H3N2 virus, an H1N1 virus, an H9N2 virus or an H5N1 virus.
5. The virus of claim 4, wherein the influenza A virus is A/Ann Arbor/6/60 (H2N2).
6. The virus of any one of claims 1 to 5, wherein the virus is a live attenuated influenza A virus with reduced growth from about 37°C to about 39°C, as compared to an influenza A virus comprising a PB1 polymerase lacking one or more mutations in amino acids corresponding to amino acid positions 310 to 325 of SEQ ID NO: 2.

7. An immunogenic composition comprising the virus of any one of claims 1 to 6 and a pharmaceutically acceptable carrier.
8. The immunogenic composition of claim 7, for treating or preventing an influenza infection in a subject.
9. Use of the virus of any one of claims 1 to 6 in the manufacture of a medicament for treating or preventing an influenza infection in a subject.
10. A recombinant nucleic acid encoding a PB1 polymerase of an influenza A virus, wherein the nucleic acid encodes a PB1 polymerase with one or more mutations in amino acids corresponding to amino acid positions 310 to 325 of SEQ ID NO: 2, wherein the PB1 polymerase comprises a leucine to glutamine substitution at an amino acid position corresponding to amino acid position 319 (L319Q) of SEQ ID NO: 2.
11. The nucleic acid of claim 10, wherein the nucleic acid encodes a PB1 polymerase comprising a leucine to glutamine substitution at an amino acid position corresponding to position 319 (L319Q) of SEQ ID NO: 2, and one or mutations comprising a lysine to glutamic acid substitution at an amino acid position corresponding to position 391 (K391E) of SEQ ID NO: 2, a glutamic acid to glycine substitution at an amino acid position corresponding to position 581 (E581G) of SEQ ID NO: 2, an alanine to threonine substitution at an amino acid position corresponding to position 661 (A661T) of SEQ ID NO: 2, or any combination thereof.
12. A vector comprising the nucleic acid of claim 10 or 11.
13. A method of producing the influenza virus of claim 1, comprising:
 - a) transfecting a population of host cells with one or more vectors comprising

- i) nucleic acid sequences encoding the internal genome segments of an influenza A virus and;
 - ii) a nucleic acid encoding a PB1 polymerase with one or more mutations in amino acids corresponding to amino acid positions 310 to 325 of SEQ ID NO: 2, wherein the nucleic acid encoding the PB1 polymerase encodes a PB1 polymerase comprising a leucine to glutamine substitution at an amino acid position corresponding to amino acid position 319 (L319Q) of SEQ ID NO: 2;
- b) culturing the host cells; and
- c) recovering the modified influenza A virus.

14. The method of claim 13, wherein the nucleic acid encoding the PB1 polymerase encodes a PB1 polymerase comprising a leucine to glutamine substitution at an amino acid position corresponding to position 319 (L319Q) of SEQ ID NO: 2, and one or mutations comprising a lysine to glutamic acid substitution at an amino acid position corresponding to position 391 (K391E) of SEQ ID NO: 2, a glutamic acid to glycine substitution at an amino acid position corresponding to position 581 (E581G) of SEQ ID NO: 2, an alanine to threonine substitution at an amino acid position corresponding to position 661 (A661T) of SEQ ID NO: 2, or any combination thereof.

15. The method of claim 13 or 14, further comprising transforming the cells with a nucleic acid encoding a PB2 polymerase comprising a mutation at an amino acid corresponding to amino acid position 265 (N265S) of SEQ ID NO: 7.

16. The method of any one of claims 13 to 15, wherein the influenza A virus is an H2N2 virus, an H3N2 virus, an H1N1 virus, an H9N2 virus or an H5N1 virus.

17. The method of any one of claims 13 to 16, wherein the cells are Vero cells, MDCK cells or CEK cells.

18. A method for producing an influenza immunogen comprising:
 - a) infecting a population of cells with the virus of any one of claims 1 to 6;
 - b) culturing the cells;
 - c) harvesting the virus from the culture of step b); and
 - d) preparing an immunogen with the harvested virus.
19. The method of claim 18, wherein the cell is a mammalian cell or an avian cell.
20. An *in vitro* population of cells comprising the virus of any one of claims 1 to 6.

FIGURE 1

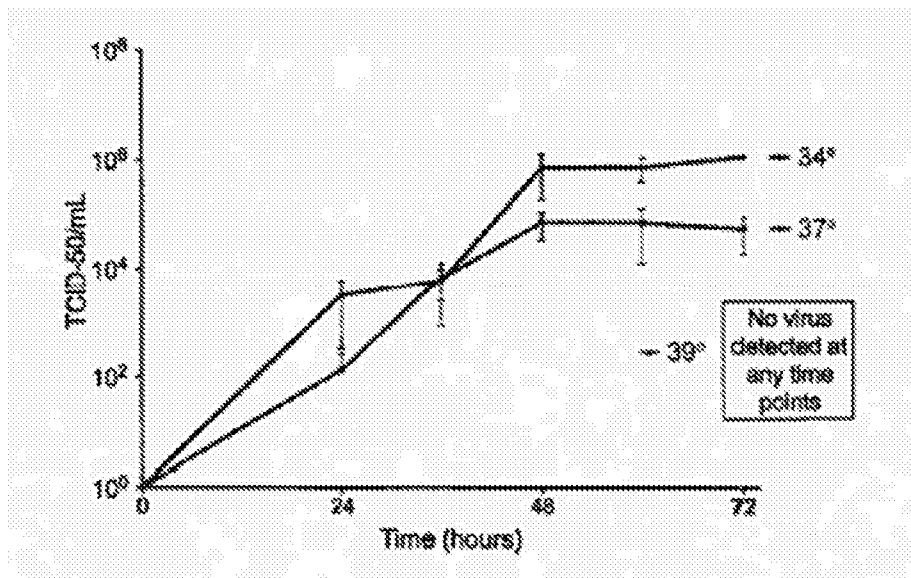


FIGURE 2

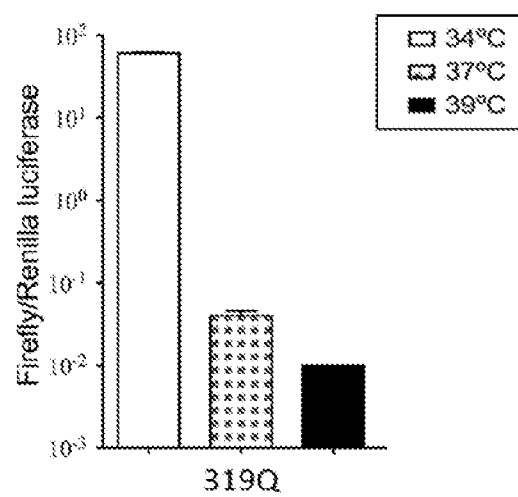


FIGURE 3

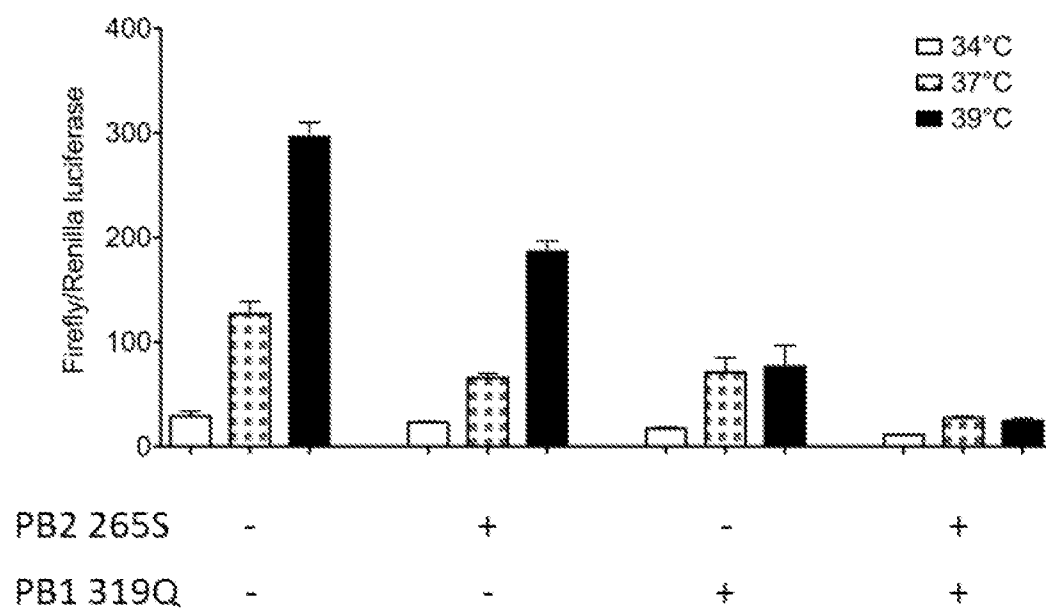


Figure 4

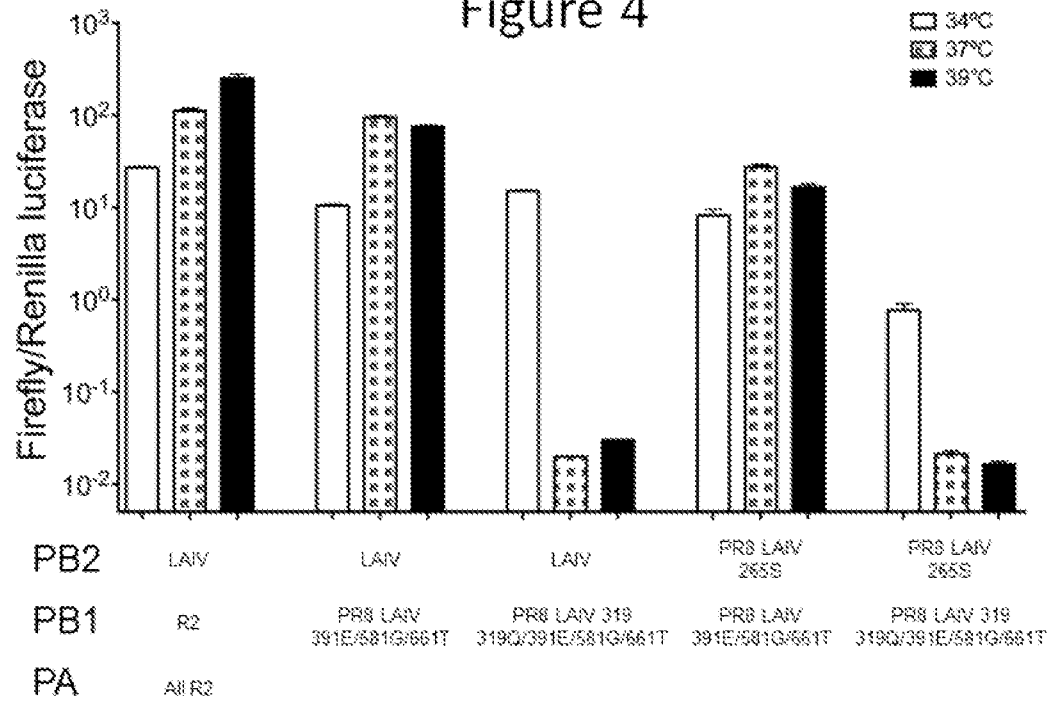


FIGURE 5

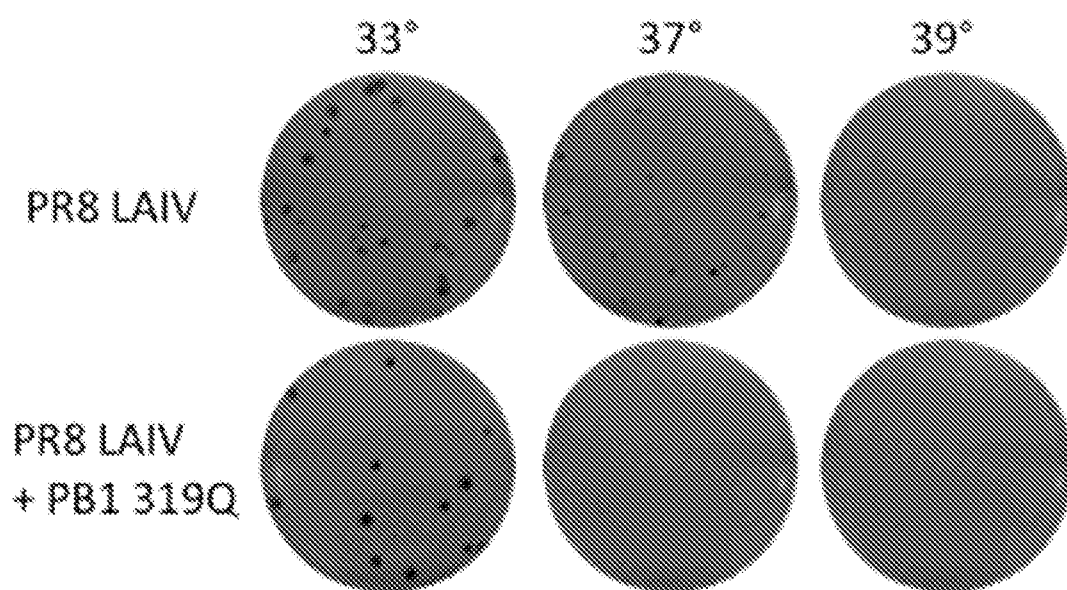


FIGURE 6

WT

PBI 319Q P1

PBI 319Q P3

