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(54) Title: BROADLY REACTIVE IMMUNOGENS OF INFLUENZA H3 VIRUS, COMPOSITIONS AND METHODS OF USE
THEREOF

TJ-1 HA

MKTIIALSYILCLVFAQKLP GNDNSTATLCLGHHAVPNGTIVKTITNDQIEVTNATELVQSS
STGGICDSPHQILDGENCTLIDALLGDPQCDGFQNKKWDLFVERSKAYSNCYPYDVPD
YASLRSLVASSGTLEFNNESEFNWGTQNGTSSACKRRSNKSFFSRLNWLTHLKYKYP
ALNVTMPNNEKFDKLYIWGVHHPGTDSDQISLYAQASGRITVSTKRSQQTVIPNIGSRP
RVRDVSSRSIYWTIVKPGDILLINSTGNLIAPRGYFKIRSGKSSIMRSDAPIGKCNSECITP
NGSIPNDKPFQNVNRITYGACPRYVKQNTLKLATGMRNVPEKQTRGIFGAAGFIENGW
EGMVDGWYGFRRHQNSEGIGQAADLKSTQAAINQINGKLNRLIGKTNEKFHQIEKEFSEV
EGRIQDLEKYVEDTKIDLWSYNAELLVALENQHTIDLT DSEMKNLFERTKKQLRENAED
MGNGCFKIYHKCDNACIGSIRNGTYDHDVYRDEALNNRFQIKGVELKSGYKDWILWISF
AISCFLLCVALLGFIMWACQKGNIRCNICI

(57) Abstract: Provided herein are non-naturally occurring, broadly reactive, pan-epitopic antigens derived from H3 influenza virus that are immunogenic and are capable of eliciting a broadly reactive immune response, such as a broadly reactive neutralizing antibody response, against H3 virus following introduction into a subject. Also provided are non-naturally, broadly reactive occurring immuno-
gens, vaccines, virus-like particles (VLPs) and compositions comprising the immunogens and vaccines. Methods of generating an im-
mune response in a subject by administering the immunogens, vaccines, VLPs, or compositions thereof are provided. In particular, the
immunogens comprise the hemagglutinin (HA) protein of H3 influenza virus strains.

BROADLY REACTIVE IMMUNOGENS OF INFLUENZA H3 VIRUS, COMPOSITIONS AND METHODS OF USE THEREOF

CROSS-REFERENCE TO RELATED APPLICATION

This application claims priority to and benefit of U.S. Provisional Application No. 62/697,846, filed on July 13, 2018, the entire contents of which are incorporated by reference herein in their entirety.

BACKGROUND

In 2017, the U.S. Centers for Disease Control and Prevention estimated that the seasonal flu vaccine was only 42% effective. This limited effectiveness was due to a mutation that occurred in the influenza A (H3N2) vaccine strain that causes flu in infected individuals. In addition, cases of flu caused by influenza B viruses have risen in the 2017 to 2018 time period. Given that a bad flu season can kill on the order of 50,000 people in the United States alone, improved immunogens and vaccines that could provide broad protection against viruses, particularly, against influenza A H3 or H3N2 viruses in present and future circulation, are urgently needed.

SUMMARY OF THE DISCLOSURE

As described below, non-naturally occurring, broadly reactive antigens and antigen sequences derived from the Influenza H3 virus (also referred to as “H3 influenza,” “H3 influenza virus,” or “H3 virus,” herein), such as subtype H3N2, are provided. These H3 virus antigens are typically structural proteins or peptides and include, for example, the hemagglutinin (HA) protein, or the HA1 (head) or HA2 (tail or stalk) portions of the HA protein, and are potent immunogens that elicit a broadly reactive immune response against H3 HA protein and, ultimately, against present and future H3 virus strains in a subject. As referred to herein, the H3 virus antigens or antigen sequences that elicit an immune response in a subject are immunogenic antigens or immunogens. These H3 immunogens are termed broadly reactive and pan-epitopic, because they can elicit the production of broadly reactive antibodies that are directed against different subtypes or strains of H3 viruses having both sequence similarity and variability, and a diversity of epitopes (antigenic determinants) in their antigens and sequences thereof, particularly, the HA antigen.

In an aspect, the non-naturally occurring H3 virus antigen amino acid sequences and the antigens (e.g., structural antigens) comprising the sequences described herein contain broadly reactive epitopes that reflect sequence similarities and variabilities of past, present and future H3 antigens. Such antigen sequences and the antigens comprising the sequences are thus called “non-naturally occurring, broadly reactive, pan-epitopic” antigens. The antigens are immunogenic and, when introduced into or administered to a subject, elicit broadly reactive antibodies, such as neutralizing antibodies, against the H3 virus, in particular, H3 antigens, such as HA, or an antibody binding portion thereof, in the subject. In an embodiment, such H3 antigen sequences are amino acid sequences. In an embodiment, the H3 antigen sequences are polynucleotide sequences, for example, polynucleotide sequences that encode the amino acid sequences of the antigens described herein. For ease of reference, a “non-naturally occurring, broadly reactive, pan-epitopic” antigen of H3 virus described herein is referred to as a “broadly reactive antigen.”

The broadly reactive H3 antigens described herein are immunogens as they elicit a broadly reactive immune response in a subject. The immune response is particularly in the form of a neutralizing antibody response, for example, neutralizing antibodies that are specifically directed against the HA antigen of the H3 virus and that neutralize the activity of the HA protein. Accordingly, also provided are immunogens and immunogenic compositions that contain the broadly reactive H3 antigens described herein, including immunogenic compositions, such as vaccines (e.g., polypeptide or polynucleotide products), that induce an immune response directed against H3 virus, such as against the HA protein of H3 virus, in a subject. For ease of reference, a “non-naturally occurring, broadly reactive, pan-epitopic” H3 virus immunogen described herein will be referred to as a “broadly reactive immunogen.”

Also provided are methods of using the immunogens as described herein to induce an immune response against H3 influenza infection, disease, and/or the symptoms thereof in a subject. In a particular embodiment, the H3 virus antigen is the HA, HA1, or HA2 protein of H3 influenza virus, or the H3N2 subtype of influenza virus, or a virus type related thereto, or an antibody binding portion thereof. Methods of using the immunogens to induce an immune response in a subject are also provided.

In an aspect, the H3 HA immunogenic antigen has an amino acid sequence that is at least or equal to 85%, at least or equal to 90%, at least or equal to 91%, at least or equal to 92%, at least or equal to 93%, at least or equal to 94%, at least or equal to 95%, at least or equal to 96%, at least or equal to 97%, at least or equal to 98%, or at least or equal to 99%

identical to an H3 HA polypeptide (or an HA1 or HA2 polypeptide) sequence of one or more of the HA proteins (called “TJ1-9 HA” herein) as set forth in **FIGS. 1A and 1B**.

In an aspect, a broadly reactive H3 antigen sequence that is capable of generating an immune response against present and future H3 influenza virus strains may be generated by a method such as described in co-pending provisional patent application number 62/697,803, filed on July 13, 2018, the contents of which are incorporated herein by reference, and which involves a consideration of the parameters of H3 antigen sequences, e.g., HA antigen sequences, from a time span or range (e.g., a linear time range), such as one or more flu seasons, and geographical location(s) in which the H3 virus was isolated, such as, for example, the Southern or Northern Hemisphere.

Provided in an aspect is a non-naturally occurring, broadly reactive, pan-epitopic antigen of H3 influenza virus (H3 virus) capable of generating an immune response against present and future H3 virus strains; wherein the H3 virus antigen comprises an amino acid sequence that is at least 95% identical to an amino acid sequence of an HA antigen (TJ-1-TJ-9) as set forth in **FIGS. 1A-1C**.

Provided in another aspect is a non-naturally occurring, broadly reactive, pan-epitopic antigen of H3 influenza virus (H3 virus) capable of generating an immune response against present and future H3 virus strains. In an embodiment, the antigen is hemagglutinin (HA), HA1, or HA2, or an antibody binding portion thereof. In an embodiment, the H3 virus antigen comprises an amino acid sequence that is at least 95% identical or at least 98% identical to an amino acid sequence of an HA antigen as set forth in **FIGS. 1A-1C**. In a particular embodiment, the H3 virus antigen comprises an amino acid sequence of an HA antigen as set forth in **FIGS. 1A-1C**.

It will be understood a non-naturally occurring, broadly reactive, pan-epitopic immunogen is provided, which may be referred to interchangeably herein, as a “non-naturally occurring immunogen,” a “broadly reactive immunogen,” or a “pan-epitopic immunogen,” for simplicity.

Provided in another aspect is a virus-like particle (VLP) comprising the H3 virus immunogenic antigen according to the foregoing aspects. In an embodiment, the VLP comprises a polynucleotide encoding the H3 virus antigen.

Provided in another aspect is a non-naturally occurring, pan-epitopic immunogen capable of generating an immune response against present and future H3 influenza (H3) virus strains; wherein the immunogen comprises an amino acid sequence that is at least 95%

identical to an amino acid sequence of an HA antigen (TJ-1-TJ-9) as set forth in FIGS. 1A-1C.

Provided in another aspect is a non-naturally occurring, broadly reactive, pan-epitopic immunogen capable of generating an immune response against present and future H3 influenza virus strains. In an embodiment, the H3 virus antigen, immunogen, or VLP elicits the production of neutralizing antibodies. In an embodiment, the antibodies have hemagglutinin inhibitory activity. In an embodiment, the H3 virus antigen, immunogen, or VLP elicits the production of T-lymphocytes.

Provided in another aspect is a pharmaceutically acceptable composition comprising the H3 virus antigen, immunogen, or VLP of any of the foregoing aspects and delineated embodiments, and a pharmaceutically acceptable carrier, diluent, or excipient. In an embodiment, the composition further comprises an adjuvant.

Provided in another aspect is a vaccine or an immunogenic composition comprising the H3 virus antigen, immunogen, or VLP of any of the foregoing aspects and delineated embodiments.

Provided in another aspect is a method of generating an immune response in a subject, in which the method comprises administering to the subject an effective amount of the immunogen, pharmaceutical composition, vaccine, or VLP of any of the foregoing aspects and delineated embodiments. In an embodiment, the immune response elicited comprises the production of neutralizing antibodies and/or T-lymphocytes.

In an aspect, the broadly reactive H3 immunogen is isolated and/or purified. In another aspect, the broadly reactive H3 immunogen is formulated for administration to a subject in need thereof. In another aspect, the broadly reactive H3 immunogen or a composition containing the immunogen is administered to a subject in need thereof in an effective amount to elicit an immune response in the subject. In an embodiment, the immune response elicits neutralizing antibodies. In an embodiment, the immune response is prophylactic or therapeutic.

In another aspect, a vaccine or an immunogenic composition comprising the broadly reactive H3 immunogen is provided.

In another aspect, virus-like particles (VLPs) comprising the broadly reactive H3 immunogen or a sequence thereof are provided. In an embodiment, the sequence is an amino acid sequence. In an embodiment, the sequence is a polynucleotide sequence which encodes the amino acid sequence.

In another aspect, a method of generating an immune response in a subject is provided, in which the method comprises administering to the subject an effective amount of the H3 broadly reactive immunogen, vaccine, VLP, or composition of any of the above aspects and delineated embodiments. In an embodiment of the method, an adjuvant is
5 concomitantly administered to the subject.

Definitions

Unless defined otherwise, all technical and scientific terms used herein have the meaning commonly understood by a person skilled in the art to which this invention pertains or relates. The following references provide one of skill with a general definition of many of
10 the terms used in this invention: Singleton et al., *Dictionary of Microbiology and Molecular Biology* (2nd ed. 1994); *The Cambridge Dictionary of Science and Technology* (Walker ed., 1988); *The Glossary of Genetics*, 5th Ed., R. Rieger et al. (eds.), Springer Verlag (1991); Benjamin Lewin, *Genes V*, published by Oxford University Press, 1994 (ISBN 0-19-854287-9); Kendrew et al. (eds.); *The Encyclopedia of Molecular Biology*, published by Blackwell
15 Science Ltd., 1994 (ISBN 0-632-02182-9); *Molecular Biology and Biotechnology: a Comprehensive Desk Reference*, Robert A. Meyers (ed.), published by VCH Publishers, Inc., 1995 (ISBN 1-56081-569-8); and Hale & Marham, *The Harper Collins Dictionary of Biology* (1991). As used herein, the following terms have the meanings ascribed to them below, unless specified otherwise.

By “adjuvant” is meant a substance or vehicle that non-specifically enhances the
20 immune response to an antigen. Adjuvants may include a suspension of minerals (e.g., alum, aluminum hydroxide, or phosphate) on which antigen is adsorbed; or water-in-oil emulsion in which antigen solution is emulsified in mineral oil (e.g., Freund's incomplete adjuvant), sometimes with the inclusion of killed mycobacteria (Freund's complete adjuvant) to further
25 enhance antigenicity. Immunostimulatory oligonucleotides (such as those including a CpG motif) can also be used as adjuvants (*see, e.g.*, U.S. Patent Nos. 6,194,388; 6,207,646; 6,214,806; 6,218,371; 6,239,116; 6,339,068; 6,406,705; and 6,429,199). Adjuvants also include biological molecules, such as costimulatory molecules. Exemplary biological adjuvants include, without limitation, interleukin-1 (IL-2), the protein memory T-cell
30 attractant “Regulated on Activation, Normal T Expressed and Secreted” (RANTES), granulocyte-macrophage-colony stimulating factor (GM-CSF), tumor necrosis factor-alpha (TNF- α), interferon-gamma (IFN- γ), granulocyte-colony stimulation factor (G-CSF), lymphocyte function-associated antigen 3 (LFA-3, also called CD58), cluster of

differentiation antigen 72 (CD72), (a negative regulator of B cell responsiveness), peripheral membrane protein, B7-1 (B7-1, also called CD80), peripheral membrane protein, B7-2 (B7-2, also called CD86), the TNF ligand superfamily member 4 ligand (OX40L) or the type 2 transmembrane glycoprotein receptor belonging to the TNF superfamily (4-1BBL)

5 By "administer" is meant giving, supplying, dispensing a composition, agent, therapeutic and the like to a subject, or applying or bringing the composition and the like into contact with the subject. Administering or administration may be accomplished by any of a number of routes, such as, for example, without limitation, topical, oral, subcutaneous, intramuscular, intraperitoneal, intravenous (IV), (injection), intrathecal, intramuscular, 10 dermal, intradermal, intracranial, inhalation, rectal, intravaginal, or intraocular.

By "agent" is meant any small molecule chemical compound, antibody, nucleic acid molecule, peptide, polypeptide, or fragments thereof.

By "alteration" is meant a change (increase or decrease) in the expression levels or activity of a gene or polypeptide as detected by standard art known methods such as those 15 described herein. As used herein, an alteration includes a 5% change in expression levels, a 10% change in expression levels, preferably a 25% change, more preferably a 40% change, and most preferably a 50% or greater change in expression levels. "

By "ameliorate" is meant decrease, reduce, diminish, suppress, attenuate, arrest, or stabilize the development or progression of a disease or pathological condition.

20 By "analog" is meant a molecule that is not identical, but has analogous functional or structural features. For example, a polypeptide analog retains the biological activity of a corresponding naturally-occurring polypeptide, while having certain biochemical modifications that enhance the analog's function relative to a naturally occurring polypeptide. Such biochemical modifications could increase the analog's protease resistance, membrane 25 permeability, or half-life, without altering, for example, ligand binding. An analog may include an unnatural amino acid.

By "antibody" is meant an immunoglobulin (Ig) molecule produced by B lymphoid cells and having a specific amino acid sequence. Antibodies are evoked or elicited in subjects (humans or other animals or mammals) following exposure to a specific antigen 30 (immunogen). A subject capable of generating antibodies/immunoglobulin (i.e., an immune response) directed against a specific antigen/immunogen is said to be immunocompetent. Antibodies are characterized by reacting specifically with (e.g., binding to) an antigen or

immunogen in some demonstrable way, antibody and antigen/immunogen each being defined in terms of the other.

"Eliciting an antibody response" refers to the ability of an antigen, immunogen or other molecule to induce the production of antibodies. Antibodies are of different classes, e.g., IgM, IgG, IgA, IgE, IgD and subtypes or subclasses, e.g., IgG1, IgG2, IgG2a, IgG2b, IgG3, IgG4. An antibody/immunoglobulin response elicited in a subject can neutralize a pathogenic (e.g., infectious or disease-causing) agent by binding to epitopes (antigenic determinants) on the agent and blocking or inhibiting the activity of the agent, and/or by forming a binding complex with the agent that is cleared from the system of the subject, e.g., via the liver.

As used herein, "broadly reactive" means that an immune response is elicited against a viral protein (e.g., a virus antigen, antigen sequence, protein, or protein sequence) in a subject that is sufficient to block, inhibit, impede, neutralize, or prevent infection of a broad range of related influenza viruses (such as most or all influenza viruses within a specific subtype, e.g., viruses related to H3 influenza virus).

By "antigen" is meant a compound, composition, or substance that can stimulate the production of antibodies or a T-cell response in an animal, including compositions that are injected or absorbed into an animal. An antigen reacts with the products of specific humoral or cellular immunity, including those induced by heterologous immunogens. In some embodiments of the disclosed compositions and methods, the antigen is an influenza hemagglutinin (HA) protein. In many cases, an antigen that elicits or stimulates an immune response in a subject is termed an "immunogen."

The term "antigenic drift" refers to a mechanism for variation in organisms or microorganisms such as viruses that involves the accumulation of mutations within the genes that code for antibody-binding sites (also called antigenic determinants or epitopes). This process results in a new strain of virus/virus particles that is not inhibited or blocked as effectively by antibodies that were originally generated against the antigens of virus strains prior to mutation, thus allowing the virus to spread more easily throughout a partially immune population. By way of example, antigenic drift occurs in both influenza A and influenza B viruses.

In the context of a live virus, the term "attenuated" reflects a virus that is attenuated if its ability to infect a cell or subject and/or its ability to produce disease is reduced (for example, diminished, abrogated, or eliminated) compared to the ability of a wild-type virus to

produce disease in the subject. Typically, an attenuated virus retains at least some capacity to elicit an immune response following administration to an immunocompetent subject. In some cases, an attenuated virus is capable of eliciting a protective immune response without causing any signs or symptoms of infection. In some embodiments, the ability of an attenuated virus to cause disease or pathology in a subject is reduced at least about or equal to 5%, or at least about or equal to 10%, or at least about or equal to 25%, at least about or equal to 50%, at least about or equal to 75%, or at least about or equal to 80%, or at least about or equal to 85%, or at least about or equal to 90%, or at least about or equal to 95%, or greater, relative to the ability of a wild-type virus to cause disease or pathology in the subject.

The term “clade” refers to the different categorizations (often called subtypes) of the known influenza viruses, such as, e.g., the influenza A H3N2 virus. Viruses in an H3N2 clade are genetically related, but do not share the exact viral genome. As appreciated by the skilled practitioner, there are many clades and subclades of H3N2 virus subtypes designated in the art. By way of example, one clade is 3C.2a; subclades of this clade include 3C.2a.1, 3C.2a.2, 3C.2a.3 and 3C.2a.4. In addition, there are at least ten different clades of H5N1 virus subtypes designated in the art: clade 0, clade 1, clade 2, clade 3, clade 4, clade 5, clade 6, clade 7, clade 8 and clade 9 (Abdel-Ghaffar et al., *N Engl J Med* 358:261-273, 2008). Clade 2 is further divided into sub-clades (including clade 2.1, clade 2.2, clade 2.3, clade 2.4 and clade 2.5).

A “codon-optimized” nucleic acid (polynucleotide) refers to a nucleic acid sequence that has been altered such that the codons are optimal for expression in a particular system (such as a particular species or group of species). For example, a nucleic acid sequence can be optimized for expression in mammalian cells. Codon optimization does not alter the amino acid sequence of the encoded protein.

In this disclosure, “comprises,” “comprising,” “containing” and “having” and the like can have the meaning ascribed to them in U.S. Patent law and can mean “includes,” “including,” and the like; “consisting essentially of” or “consists essentially” likewise has the meaning ascribed in U.S. Patent law and the term is open-ended, allowing for the presence of more than that which is recited so long as basic or novel characteristics of that which is recited is not changed by the presence of more than that which is recited, but excludes prior art embodiments.

“Detect” refers to identifying the presence, absence or amount of an analyte, compound, agent, or substance to be detected. By “detectable label” is meant a composition

that, when linked to a molecule of interest, renders the latter detectable, e.g., via spectroscopic, photochemical, biochemical, immunochemical, or chemical means.

Nonlimiting examples of useful detectable labels include radioactive isotopes, magnetic beads, metallic beads, colloidal particles, fluorescent dyes, electron-dense reagents, enzymes
5 (for example, as commonly used in an ELISA), biotin, digoxigenin, or haptens.

By “disease” is meant any condition, disorder, or pathology that damages or interferes with the normal function of a cell, tissue, or organ. Examples of diseases include those caused by H3 virus infection and the symptoms and adverse effects that are caused by infection of the body with the H3 virus. Influenza virus causes flu and its symptoms in
10 infected individuals.

By "effective amount" is meant the amount of an active therapeutic agent, composition, compound, biologic (e.g., a vaccine or therapeutic peptide, polypeptide, or polynucleotide) required to ameliorate, reduce, improve, abrogate, diminish, or eliminate the symptoms and/or effects of a disease, condition, or pathology relative to an untreated patient.

The effective amount of an immunogen or a composition comprising an immunogen, as used to practice the methods of therapeutic treatment of disease, condition, or pathology caused by the H3 virus, varies depending upon the manner of administration, the age, body weight, and general health of the subject. Ultimately, the attending physician or veterinarian will decide the appropriate amount and dosage regimen. Such amount is referred to as an "effective"
15 amount.
20

A “therapeutically effective amount” refers to a quantity of a specified agent sufficient to achieve a desired effect in a subject being treated with that agent. For example, this may be the amount of an H3 influenza virus immunogen or vaccine useful for eliciting an immune response in a subject and/or for preventing infection by H3 influenza virus. Ideally,
25 in the context of the present disclosure, a therapeutically effective amount of an influenza vaccine or an anti-influenza immunogenic composition is an amount sufficient to increase resistance to, prevent, ameliorate, reduce, and/or treat infection caused by influenza virus in a subject without causing a substantial cytotoxic effect in the subject. The effective amount of an influenza vaccine of immunogenic composition useful for increasing resistance to,
30 preventing, ameliorating, reducing, and/or treating infection in a subject depends on, for example, the subject being treated, the manner of administration of the therapeutic composition and other factors, as noted *supra*.

By "fragment" is meant a portion of a polypeptide or nucleic acid molecule. This portion contains, preferably, at least 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, or 90% of the entire length of the reference nucleic acid molecule or polypeptide. A fragment may contain 10, 20, 30, 40, 50, 60, 70, 80, 90, or 100, 200, 300, 400, 500, 600, 700, 800, 900, or 1000 nucleotides or amino acids. A portion or fragment of a polypeptide may be a peptide. In the case of an antibody or immunoglobulin fragment, the fragment typically binds to the target antigen.

By "fusion protein" is meant a protein generated by expression of a nucleic acid (polynucleotide) sequence engineered from nucleic acid sequences encoding at least a portion of two different (heterologous) proteins or peptides. To create a fusion protein, the nucleic acid sequences must be in the same reading frame and contain no internal stop codons. For example, a fusion protein includes an H3 influenza HA protein fused to a heterologous protein.

By "genetic vaccine" is meant an immunogenic composition comprising a polynucleotide encoding an antigen."

The terms "geographical location or geographical region" refers to preselected divisions of geographical areas of the earth, for example, by continent or other preselected territory or subdivision (e.g., the Middle East, which spans more than one continent). Examples of different geographical regions include countries (e.g., Turkey, Egypt, Iraq, Azerbaijan, China, United States); continents (e.g., Asia, Europe, North America, South America, Oceania, Africa); recognized geopolitical subdivisions (such as the Middle East); or hemispheres of the world (e.g., Northern, Southern, Eastern, or Western hemispheres).

By "H3 virus polypeptide" is meant an amino acid sequence that is at least 85% identical to an amino acid sequence of an HA antigen as set forth in FIGS. 1A-1C or a fragment thereof capable of inducing an immune response in an immunized subject. In an embodiment, an H3 virus polypeptide comprises or consists of TJ1-9 HA sequences or a fragment thereof.

By "H3 virus polynucleotide" is meant a nucleic acid molecule encoding an H3 virus polypeptide (antigen or antigen protein).

The term "Hemagglutinin (HA)" refers to a surface glycoprotein expressed by an influenza virus. HA mediates binding of the virus particle to a host cell and subsequent entry of the virus into the host cell. The nucleotide and amino acid sequences of numerous influenza HA proteins are known in the art and are publically available, such as those

deposited with GenBank, *see, e.g.*, U.S. Publication No. US 2015/0030628, Table 1). HA (along with neuraminidase (NA)) is one of the two major influenza virus antigenic proteins having antigenic determinants (epitopes) that are recognized and bound by antibodies/immunoglobulins.

5 By way of example, a hemagglutinin (HA) protein of an influenza H3N2 virus is a polypeptide or fragment thereof having at least about or equal to 85%, or at least about or equal to 90%, 95%, 98%, 99%, or greater, amino acid sequence identity to the amino acid sequence of Influenza A virus (A/Hong Kong/1-4/1968(H3N2)) segment 4, complete sequence, Accession Number CY033017, as set forth below:

10 MKTIIALSII FCLALGQDLPGNDNSTATLCLGHHAVPNGTLVKTITDDQIEVTNATEL
VQSSSTGKICNNPHRILDGIDCTLIDALLGDPHCDVFQNETWDLFVERSKAFSNCYPY
DVPDYASLRSLVASSGTLEFITEGFTWTGVTQNGGSNACKRGPGSGFFSRLNWLTKSG
STYPVLNVTMPNNDNFDKLYIWGVHHPSTNQEQTSLYVQASGRVTVSTRRSQQTIIIPN
IGSRPWVRGLSSRISIIYWTIVKPGDVLVINSNGNLIAPRGYFKMRTGKSSIMRSDAPI
15 TCISECITPNGSIPNDKPFQNVNKITYGACPKYVKQNTLKLATGMRNVPEKQTRGLFG
AIAGFIENGWEGMIDGWYGFRHQNSEGTQQAADLKSTQAAIDQINGKLN RVIEKTNEK
FHQIEKEFSEVEGRIQDLEKYVEDTKIDLWSYNAELLVALENQHTIDLTDSEMNKLFE
KTRRQLRENAEDMGNGCFKIYHKCDNACIESIRNGTYDHDVYRDEALNNRFQIKGVE
LKSGYKDWILWISFAISCFLLCVLLGFIMWACQQRGNIRCNICI.

20

In addition, a hemagglutinin (HA) protein of an influenza H3N2 virus is encoded by a polynucleotide or fragment thereof having at least about or equal to 85%, or at least about or equal to 90%, 95%, 98%, 99%, or greater, sequence identity to the polynucleotide sequence as follows:

25 1 attaatcatg aagaccatca ttgctttgag ctacattttc tgtctggctc tcggccaaga
61 ccttccagga aatgacaaca gcacagcaac gctgtgcctg ggacatcatg cggtgccaaa
121 cggaacacta gtgaaaacaa tcacagatga tcagattgaa gtgactaatg ctactgagct
181 agttcagagc tcctcaacgg ggaaaatatg caacaatcct catcgaatcc ttgatggaat
241 agactgcaca ctgatagatg ctctattggg ggaccctcat tgtgatgttt ttcaaaatga
30 301 gacatgggac cttttcgttg aacgcagcaa agctttcagc aactgttacc cttatgatgt
361 gccagattat gcctccctta ggtcactagt tgcctcgtca ggcactctgg agtttatcac
421 tgaggggtttc acttggactg gggtcactca gaatggggga agcaatgctt gcaaaagggg
481 acctggtagc ggttttttca gtagactgaa ctgggtgacc aaatcaggaa gcacatatcc
541 agtgctgaac gtgactatgc caaacaatga caattttgac aaactataca tttgggggggt
35 601 tcaccacccg agcacgaacc aagaacaaac cagcctgtat gttcaagcat caggagagat
661 cacagtctct accaggagaa gccagcaaac tataatcccg aatatcgggt ccagaccctg
721 ggtaaggggt ctgtctagta gaataagcat ctattggaca atagttaagc cgggagacgt
781 actggtaatt aatagtaatg ggaacctaat cgctcctcgg ggttattttca aaatgcgcac
841 tgggaaaagc tcaataatga ggtcagatgc acctattgat acctgtattt ctgaatgcat

901 cactccaaat ggaagcattc ccaatgacaa gccctttcaa aacgtaaaca agatcacata
 961 tggagcatgc cccaagtatg ttaagcaaaa caccctgaag ttggcaacag ggatgcggaa
 1021 tgtaccagag aaacaaacta gaggcctatt cggcgcaata gcaggtttca tagaaaatgg
 1081 ttggggaggga atgatagacg gttggtacgg tttcaggcat caaaattctg agggcacagg
 5 1141 acaagcagca gatcttaaaa gcactcaagc agccatcgac caaatcaatg ggaaattgaa
 1201 cagggtaatc gagaagacga acgagaaatt ccatcaaadc gaaaaggaat tctcagaagt
 1261 agaagggaga attcaggacc tcgagaaata cggtgaagac actaaaatag atctctggtc
 1321 ttacaatgag gagcttcttg tcgctctgga gaatcaacat acaattgacc tgactgactc
 1381 ggaaatgaac aagctgtttg aaaaaacaag gaggcaactg agggaaaatg ctgaagacat
 10 1441 gggcaatggt tgcttcaaaa tataccacaa atgtgacaac gcttgcatag agtcaatcag
 1501 aaatgggact tatgaccatg atgtatacag agacgaagca ttaaacaacc ggtttcagat
 1561 caaaggtggt gaactgaagt ctggatacaa agactggatc ctgtggattt cctttgccat
 1621 atcatgcttt ttgctttgtg ttgttttgct ggggttcac atgtgggcct gccagagagg
 1681 caacattagg tgcaacattt gcatttgagt gtattagtaa

15 "Hybridization" means hydrogen bonding, which may be Watson-Crick, Hoogsteen, or reversed Hoogsteen hydrogen bonding, between complementary nucleobases. For example, in DNA, adenine and thymine, and cytosine and guanine, are, respectively, complementary nucleobases that pair through the formation of hydrogen bonds.

20 By "immunogen" is meant a compound, composition, or substance which is capable, under appropriate conditions, of eliciting or stimulating an immune response, such as the production of antibodies, and/or a T-cell response, in an animal, including compositions that are injected or absorbed into an animal. As used herein, an "immunogenic composition" is a composition comprising an immunogen (such as an H3 HA polypeptide) or a vaccine

25 comprising an H3 HA polypeptide). As will be appreciated by the skilled person in the art, if administered to a subject in need prior to the subject's contracting disease or experiencing full-blown disease, an immunogenic composition can be prophylactic and result in the subject's eliciting an immune response, e.g., a neutralizing antibody and/or cellular immune response, to protect against disease, or to prevent more severe disease or condition, and/or the

30 symptoms thereof. If administered to a subject in need following the subject's contracting disease, an immunogenic composition can be therapeutic and result in the subject's eliciting an immune response, e.g., a neutralizing antibody and/or cellular immune response, to treat the disease, e.g., by reducing, diminishing, abrogating, ameliorating, or eliminating the

35 disease, and/or the symptoms thereof. In an embodiment, the immune response is a B cell response, which results in the production of antibodies, e.g., neutralizing antibodies, directed against the immunogen or immunogenic composition comprising the antigen or antigen sequence. In a manner similar to the foregoing, in some embodiments, an immunogenic

composition or vaccine can be prophylactic. In some embodiments, an immunogenic composition or vaccine can be therapeutic. In an embodiment, the disease is influenza (flu).

The term "immune response" is meant any response mediated by an immunoresponsive cell. In one example of an immune response, leukocytes are recruited to carry out a variety of different specific functions in response to exposure to an antigen (e.g., a foreign entity). Immune responses are multifactorial processes that differ depending on the type of cells involved. Immune responses include cell-mediated responses (e.g., T cell responses), humoral responses (B cell/antibody responses), innate responses and combinations thereof.

By "immunogenic composition" is meant a composition comprising an antigen, antigen sequence, or immunogen, wherein the composition elicits an immune response in an immunized subject.

The term "immunize" (or immunization) refers to rendering a subject protected from a disease, infectious disease, or pathology, or the symptoms thereof, caused by an H3 virus, such as by vaccination.

The term "influenza virus" refers to a segmented negative-strand RNA virus that belongs to the *Orthomyxoviridae* family of viruses. There are three types of Influenza viruses: A, B and C. Influenza A viruses infect a wide variety of birds and mammals, including humans, horses, marine mammals, pigs, ferrets, and chickens. In animals, most influenza A viruses cause mild localized infections of the respiratory and intestinal tract. However, highly pathogenic influenza A strains, such as H3N2, cause systemic infections in poultry in which mortality may reach 100%.

By "inhibitory nucleic acid" is meant a double-stranded RNA, siRNA, shRNA, or antisense RNA, or a portion thereof, or a mimetic thereof, that when administered to a mammalian cell results in a decrease (e.g., by 5%, 10%, 25%, 50%, 75%, or even 90-100%) in the expression of a target gene. Typically, a nucleic acid inhibitor comprises at least a portion of a target nucleic acid molecule, or an ortholog thereof, or comprises at least a portion of the complementary strand of a target nucleic acid molecule. For example, an inhibitory nucleic acid molecule comprises at least a portion of any or all of the nucleic acids delineated herein.

The terms "isolated," "purified," or "biologically pure" refer to material that is free to varying degrees from components which normally accompany it as found in its native state. "Isolate" denotes a degree of separation from original source or surroundings. "Purify"

denotes a degree of separation that is higher than isolation. A "purified" or "biologically pure" protein is sufficiently free of other materials such that any impurities do not materially affect the biological properties of the protein or cause other adverse consequences. That is, a nucleic acid, protein, or peptide is purified if it is substantially free of cellular material, debris, non-relevant viral material, or culture medium when produced by recombinant DNA techniques, or of chemical precursors or other chemicals when chemically synthesized. Purity and homogeneity are typically determined using standard purification methods and analytical chemistry techniques, for example, polyacrylamide gel electrophoresis or high performance liquid chromatography. The term "purified" can denote that a nucleic acid or protein gives rise to essentially one band in an electrophoretic gel. For a protein that can be subjected to modifications, for example, phosphorylation or glycosylation, different modifications may give rise to different isolated proteins, which can be separately purified. The term "isolated" also embraces recombinant nucleic acids, proteins or viruses, as well as chemically synthesized nucleic acids or peptides.

By "isolated polynucleotide" is meant a nucleic acid (e.g., a DNA molecule) that is free of the genes which, in the naturally-occurring genome of the organism from which the nucleic acid molecule of the invention is derived, flank the gene. The term therefore includes, for example, a recombinant DNA that is incorporated into a vector; into an autonomously replicating plasmid or virus; or into the genomic DNA of a prokaryote or eukaryote; or that exists as a separate molecule (for example, a cDNA or a genomic or cDNA fragment produced by PCR or restriction endonuclease digestion) independent of other sequences. In addition, the term includes an RNA molecule that is transcribed from a DNA molecule, as well as a recombinant DNA that is part of a hybrid gene encoding additional polypeptide sequence.

By an "isolated polypeptide" is meant a polypeptide of the invention that has been separated from components that naturally accompany it. Typically, the polypeptide is isolated when it is at least 40%, by weight, at least 50%, by weight, at least 60%, by weight, free from the proteins and naturally-occurring organic molecules with which it is naturally associated. Preferably, an isolated polypeptide preparation is at least 75%, more preferably at least 90%, and most preferably, at least 99%, by weight, free from the proteins and naturally-occurring organic molecules with which it is naturally associated. An isolated polypeptide may be obtained, for example, by extraction from a natural source; by expression of a recombinant nucleic acid encoding such a polypeptide; or by chemically synthesizing the

protein. Purity can be measured by any standard, appropriate method, for example, column chromatography, polyacrylamide gel electrophoresis, or by HPLC analysis. An isolated polypeptide can refer to broadly active virus immunogen polypeptide generated by the methods described herein.

5 By “linker” is meant one or more amino acids that serve as a spacer between two polypeptides or peptides of a fusion protein.

By “marker” is meant any protein or polynucleotide having an alteration in expression level or activity that is associated with a disease, condition, pathology, or disorder.

10 A “Matrix (M1) protein” refers to an influenza virus structural protein found within the viral envelope. M1 is thought to function in assembly and budding of virus following infection of a cell.

The term “Neuraminidase (NA)” refers to an influenza virus membrane glycoprotein. NA is involved in the destruction of the cellular receptor for the viral HA by cleaving terminal sialic acid residues from carbohydrate moieties on the surfaces of infected cells. NA
15 also cleaves sialic acid residues from viral proteins, preventing aggregation of viruses. NA (along with HA) is one of the two major influenza virus antigenic determinants.

As used herein, “obtaining” as in “obtaining an agent” includes synthesizing, isolating, purchasing, or otherwise acquiring the agent.

20 The term “operably linked” refers to nucleic acid sequences as used herein. By way of example, a first nucleic acid sequence is operably linked to a second nucleic acid sequence when the first nucleic acid sequence is placed in a functional relationship with the second nucleic acid sequence. For instance, a promoter is operably linked to a coding sequence if the promoter affects (allows) the transcription or expression of the coding sequence. Generally, operably linked DNA sequences are contiguous and, where necessary to join two protein-
25 coding regions, are in the same reading frame.

An influenza HA protein that is “computationally optimized” generally reflects an HA protein sequence resulting from the comparison of sequences (amino acid sequences) from two or more viruses, such as, for example, sequences of clades of H3 influenza viruses, such as described, for example, in US Patent Application Publication US 2015/0030628. The
30 nucleotide sequence encoding an H3 HA protein generated by the described methods can be optimized for expression in mammalian cells via codon-optimization and RNA optimization (such as to increase RNA stability) using procedures and techniques practiced in the art.

A broadly reactive, pan-epitopic immunogen, such as H3 influenza hemagglutinin (HA) protein, for eliciting an immune response in a subject possesses a collective set of strongly immunogenic epitopes (also called antigenic determinants). An H3 virus HA protein described herein is a “pan-epitopic” H3 immunogen that is suitable for use as a vaccine, which elicits a broadly reactive immune response, e.g., a neutralizing antibody response, against a plurality of H3 virus types which express HA proteins on the viral surface, when introduced into a host subject, in particular, a human subject infected with H3 virus. The immunogenic antigen (or vaccine) is advantageous for providing an anti-H3 virus immunogen (or a vaccine) that elicits a broadly active immune response against H3 influenza virus HA antigens with antigenic variability and similarity, and treats or protects against infection and disease caused by more than one H3 influenza virus subtype.

By “open reading frame (ORF)” is meant a series of nucleotide triplets (codons) that code for amino acids without any termination codons. These sequences are usually translatable into a peptide or polypeptide.

As used herein, an influenza virus "outbreak" refers to a collection of virus isolates from within a geographical location (e.g., within a single country) in a given period of time (e.g., in a year).

The term “pharmaceutically acceptable vehicle” refers to conventional carriers (vehicles) and excipients that are physiologically and pharmaceutically acceptable for use, particularly in mammalian, e.g., human, subjects. Such pharmaceutically acceptable vehicles are known to the skilled practitioner in the pertinent art and can be readily found in *Remington's Pharmaceutical Sciences*, by E. W. Martin, Mack Publishing Co., Easton, Pa., 15th Edition (1975) and its updated editions, which describes compositions and formulations suitable for pharmaceutical delivery of one or more therapeutic compositions, such as one or more influenza vaccines, and additional pharmaceutical agents. In general, the nature of a pharmaceutically acceptable carrier depends on the particular mode of administration being employed. For instance, parenteral formulations usually comprise injectable fluids/liquids that include pharmaceutically and physiologically acceptable fluids such as water, physiological saline, balanced salt solutions, aqueous dextrose, glycerol or the like as a vehicle. For solid compositions (for example, powder, pill, tablet, or capsule forms), conventional non-toxic solid carriers may include, for example, pharmaceutical grades of mannitol, lactose, starch, or magnesium stearate, which typically stabilize and/or increase the half-life of a composition or drug. In addition to biologically-neutral carriers, pharmaceutical

compositions to be administered can contain minor amounts of non-toxic auxiliary substances, such as wetting or emulsifying agents, preservatives, and pH buffering agents and the like, for example sodium acetate or sorbitan monolaurate.

By "plasmid" is meant a circular nucleic acid molecule capable of autonomous replication in a host cell.

By "polypeptide" (or protein) is meant a polymer in which the monomers comprise amino acid residues that are joined together through amide bonds. When the amino acids are alpha-amino acids, either the L-optical isomer or the D-optical isomer can be used. The terms "polypeptide" or "protein" as used herein are intended to encompass any amino acid sequence and include modified sequences such as glycoproteins. The term "polypeptide" is specifically intended to cover naturally occurring proteins, as well as those which are recombinantly or synthetically produced. The term "residue" or "amino acid residue" also refers to an amino acid that is incorporated into a protein, polypeptide, or peptide.

Conservative amino acid substitutions are those substitutions that, when made, least interfere with the properties of the original protein, that is, the structure and especially the function of the protein is conserved and is not significantly changed by such substitutions. Examples of conservative amino acid substitutions are known in the art, e.g., as set forth in, for example, U.S. Publication No. 2015/0030628. Conservative substitutions generally maintain (a) the structure of the polypeptide backbone in the area of the substitution, for example, as a sheet or helical conformation; (b) the charge or hydrophobicity of the molecule at the target site; and/or (c) the bulk of the side chain

The substitutions that are generally expected to produce the greatest changes in protein properties are non-conservative, for instance, changes in which (a) a hydrophilic residue, for example, seryl or threonyl, is substituted for (or by) a hydrophobic residue, for example, leucyl, isoleucyl, phenylalanyl, valyl or alanyl; (b) a cysteine or proline is substituted for (or by) any other residue; (c) a residue having an electropositive side chain, for example, lysyl, arginyl, or histadyl, is substituted for (or by) an electronegative residue, for example, glutamyl or aspartyl; or (d) a residue having a bulky side chain, for example, phenylalanine, is substituted for (or by) one not having a side chain, for example, glycine.

"Primer set" means a set of oligonucleotides that may be used, for example, for PCR. A primer set would consist of at least 2, 4, 6, 8, 10, 12, 14, 16, 18, 20, 30, 40, 50, 60, 80, 100, 200, 250, 300, 400, 500, 600, or more primers.

By "promoter" is meant an array of nucleic acid control sequences, which direct transcription of a nucleic acid. A promoter includes necessary nucleic acid sequences near the start site of transcription. A promoter also optionally includes distal enhancer or repressor sequence elements. A "constitutive promoter" is a promoter that is continuously
5 active and is not subject to regulation by external signals or molecules. In contrast, the activity of an "inducible promoter" is regulated by an external signal or molecule (for example, a transcription factor). By way of example, a promoter may be a CMV promoter.

As will be appreciated by the skilled practitioner in the art, the term "purified" does not require absolute purity; rather, it is intended as a relative term. Thus, for example, a
10 purified peptide, protein, virus, or other active compound is one that is isolated in whole or in part from naturally associated proteins and other contaminants. In certain embodiments, the term "substantially purified" refers to a peptide, protein, virus or other active compound that has been isolated from a cell, cell culture medium, or other crude preparation and subjected to routine methods, such as fractionation, chromatography, or electrophoresis, to remove
15 various components of the initial preparation, such as proteins, cellular debris, and other components.

A "recombinant" nucleic acid, protein or virus is one that has a sequence that is not naturally occurring or that has a sequence that is made by an artificial combination of two otherwise separated segments of sequence. Such an artificial combination is often
20 accomplished by chemical synthesis or by the artificial manipulation of isolated segments of nucleic acids, for example, by genetic engineering techniques. A "non-naturally occurring" nucleic acid, protein or virus is one that may be made via recombinant technology, artificial manipulation, or genetic or molecular biological engineering procedures and techniques, such as those commonly practiced in the art.

By "reduces" is meant a negative alteration of at least 5%, 10%, 25%, 30%, 40%,
25 50%, 75%, 80%, 85%, 90%, 95%, 98%, or 100%.

By "reference" is meant a standard or control condition.

A "reference sequence" is a defined sequence used as a basis for sequence comparison. A reference sequence may be a subset of or the entirety of a specified sequence;
30 for example, a segment of a full-length cDNA or gene sequence, or the complete cDNA or gene sequence. For polypeptides, the length of the reference polypeptide sequence will generally be at least about 16 amino acids, preferably at least about 20 amino acids, more preferably at least about 25 amino acids, and even more preferably about 35 amino acids,

about 50 amino acids, or about 100 amino acids. For nucleic acids, the length of the reference nucleic acid sequence will generally be at least about 50 nucleotides, preferably at least about 60 nucleotides, more preferably at least about 75 nucleotides, and even more preferably about 100 nucleotides or about 300 nucleotides or any integer thereabout or therebetween.

By "specifically binds" is meant a compound or antibody that recognizes and binds a polypeptide, such as a virus polypeptide, peptide, or vaccine product, but which does not substantially recognize and bind other molecules in a sample, for example, a biological sample, which naturally includes a polypeptide, such as a virus polypeptide or peptide.

Nucleic acid molecules useful in the methods described herein include any nucleic acid molecule that encodes a polypeptide as described, or a fragment thereof. Such nucleic acid molecules need not be 100% identical with an endogenous nucleic acid sequence, but will typically exhibit substantial identity. Polynucleotides having "substantial identity" to an endogenous sequence are typically capable of hybridizing with at least one strand of a double-stranded nucleic acid molecule. By "hybridize" is meant pairing to form a double-stranded molecule between complementary polynucleotide sequences (e.g., a gene), or portions thereof, under various conditions of stringency. (*See, e.g.,* Wahl, G. M. and S. L. Berger, (1987), *Methods Enzymol.*, 152:399; Kimmel, A. R. ,(1987), *Methods Enzymol.* 152:507).

By way of example, stringent salt concentration will ordinarily be less than about 750 mM NaCl and 75 mM trisodium citrate, preferably less than about 500 mM NaCl and 50 mM trisodium citrate, and more preferably less than about 250 mM NaCl and 25 mM trisodium citrate. Low stringency hybridization can be obtained in the absence of organic solvent, e.g., formamide, while high stringency hybridization can be obtained in the presence of at least about 35% formamide, and more preferably at least about 50% formamide. Stringent temperature conditions will ordinarily include temperatures of at least about 30°C, more preferably of at least about 37°C, and most preferably of at least about 42°C. Varying additional parameters, such as hybridization time, the concentration of detergent, e.g., sodium dodecyl sulfate (SDS), and the inclusion or exclusion of carrier DNA, are well known to those skilled in the art. Various levels of stringency are accomplished by combining these various conditions as needed. In a preferred embodiment, hybridization will occur at 30°C in 750 mM NaCl, 75 mM trisodium citrate, and 1% SDS. In a more preferred embodiment, hybridization will occur at 37°C in 500 mM NaCl, 50 mM trisodium citrate, 1% SDS, 35%

formamide, and 100 µg/ml denatured salmon sperm DNA (ssDNA). In a most preferred embodiment, hybridization will occur at 42°C in 250 mM NaCl, 25 mM trisodium citrate, 1% SDS, 50% formamide, and 200 µg/ml ssDNA. Useful variations on these conditions will be readily apparent to those skilled in the art.

5 For most applications, washing steps that follow hybridization will also vary in stringency. Wash stringency conditions can be defined by salt concentration and by temperature. As above, wash stringency can be increased by decreasing salt concentration or by increasing temperature. For example, stringent salt concentration for the wash steps will preferably be less than about 30 mM NaCl and 3 mM trisodium citrate, and most preferably
10 less than about 15 mM NaCl and 1.5 mM trisodium citrate. Stringent temperature conditions for the wash steps will ordinarily include a temperature of at least about 25°C, more preferably of at least about 42°C, and even more preferably of at least about 68°C. In a preferred embodiment, wash steps will occur at 25°C in 30 mM NaCl, 3 mM trisodium citrate, and 0.1% SDS. In a more preferred embodiment, wash steps will occur at 42 C in 15
15 mM NaCl, 1.5 mM trisodium citrate, and 0.1% SDS. In a more preferred embodiment, wash steps will occur at 68°C in 15 mM NaCl, 1.5 mM trisodium citrate, and 0.1% SDS.

Additional variations on these conditions will be readily apparent to those skilled in the art.

Hybridization techniques are well known to those skilled in the art and are described, for example, in Benton and Davis (*Science* 196:180, 1977); Grunstein and Hogness (*Proc. Natl. Acad. Sci., USA* 72:3961, 1975); Ausubel et al. (*Current Protocols in Molecular Biology*,
20 Wiley Interscience, New York, 2001); Berger and Kimmel (*Guide to Molecular Cloning Techniques*, 1987, Academic Press, New York); and Sambrook et al., *Molecular Cloning: A Laboratory Manual*, Cold Spring Harbor Laboratory Press, New York.

By "substantially identical" is meant a polypeptide or nucleic acid molecule
25 exhibiting at least 50% identity to a reference amino acid sequence (for example, any one of the amino acid sequences described herein) or nucleic acid sequence (for example, any one of the nucleic acid sequences described herein). Preferably, such a sequence is at least 60%, or at least 80% or 85%, or at least or equal to 90%, 95% or even 99% identical at the amino acid level or nucleic acid to the sequence used for comparison.

30 "Sequence identity" refers to the similarity between amino acid or nucleic acid sequences that is expressed in terms of the similarity between the sequences. Sequence identity is frequently measured in terms of percentage identity (or similarity or homology); the higher the percentage, the more similar the sequences are. Homologs or variants of a

given gene or protein will possess a relatively high degree of sequence identity when aligned using standard methods. Sequence identity is typically measured using sequence analysis software (for example, Sequence Analysis Software Package of the Genetics Computer Group, University of Wisconsin Biotechnology Center, 1710 University Avenue, Madison, Wis. 53705, BLAST, BESTFIT, GAP, or PILEUP/PRETTYBOX programs). Such software matches identical or similar sequences by assigning degrees of homology to various substitutions, deletions, and/or other modifications. Conservative substitutions typically include substitutions within the following groups: glycine, alanine; valine, isoleucine, leucine; aspartic acid, glutamic acid, asparagine, glutamine; serine, threonine; lysine, arginine; and phenylalanine, tyrosine. In an exemplary approach to determining the degree of identity, a BLAST program may be used, with a probability score between e^{-3} and e^{-100} indicating a closely related sequence. In addition, other programs and alignment algorithms are described in, for example, Smith and Waterman, 1981, *Adv. Appl. Math.* 2:482; Needleman and Wunsch, 1970, *J. Mol. Biol.* 48:443; Pearson and Lipman, 1988, *Proc. Natl. Acad. Sci. U.S.A.* 85:2444; Higgins and Sharp, 1988, *Gene* 73:237-244; Higgins and Sharp, 1989, *CABIOS* 5:151-153; Corpet et al., 1988, *Nucleic Acids Research* 16:10881-10890; Pearson and Lipman, 1988, *Proc. Natl. Acad. Sci. U.S.A.* 85:2444; and Altschul et al., 1994, *Nature Genet.* 6:119-129. The NCBI Basic Local Alignment Search Tool (BLAST™) (Altschul et al. 1990, *J. Mol. Biol.* 215:403-410) is readily available from several sources, including the National Center for Biotechnology Information (NCBI, Bethesda, Md.) and on the Internet, for use in connection with the sequence analysis programs blastp, blastn, blastx, tblastn and tblastx.

By "subject" is meant an animal, e.g., a mammal, including, but not limited to, a human, a non-human primate, or a non-human mammal, such as a bovine, equine, canine, ovine, or feline mammal, or a sheep, goat, llama, camel, or a rodent (rat, mouse), gerbil, or hamster. In a nonlimiting example, a subject is one who is infected with an H3 virus, or who is at risk of infection by such virus, or who is susceptible to such infection. In particular aspects as described herein, the subject is a human subject, such as a patient.

Ranges provided herein are understood to be shorthand for all of the values within the range, inclusive of the first and last stated values. For example, a range of 1 to 50 is understood to include any number, combination of numbers, or sub-range from the group consisting 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25,

26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50, or greater, consecutively, such as to 100 or greater.

As used herein, the terms “treat,” “treating,” “treatment,” and the like refer to reducing, diminishing, decreasing, abrogating, ameliorating, or eliminating, a disease, condition,
5 disorder, or pathology, and/or symptoms associated therewith. While not intending to be limiting, “treating” typically relates to a therapeutic intervention that occurs after a disease, condition, disorder, or pathology, and/or symptoms associated therewith, have begun to develop so as to reduce the severity of the disease, etc., and the associated signs and symptoms. It will be appreciated that, although not precluded, treating a disorder or
10 condition does not require that the disease, condition, disorder, pathology, or the symptoms associated therewith, be completely eliminated.

As used herein, the terms “prevent,” “preventing,” “prevention,” “prophylactic treatment” and the like, refer to inhibiting or blocking a disease state, or the full development of a disease in a subject, or reducing the probability of developing a disease, disorder or
15 condition in a subject, who does not have, but is at risk of developing, or is susceptible to developing, a disease, disorder, or condition.

As referred to herein, a “transformed” cell is a cell into which a nucleic acid molecule or polynucleotide sequence has been introduced by molecular biology techniques. As used herein, the term “transformation” encompasses all techniques by which a nucleic acid
20 molecule or polynucleotide may be introduced into such a cell, including transfection with viral vectors, transformation with plasmid vectors, and introduction of naked nucleic acid (DNA or RNA) by electroporation, lipofection, and particle gun acceleration.

By “vaccine” is meant a preparation of immunogenic material (e.g., protein or nucleic acid; vaccine) capable of stimulating (eliciting) an immune response, administered to a
25 subject to treat a disease, condition, or pathology, or to prevent a disease, condition, or pathology, such as an infectious disease (caused by H3 virus infection, for example). The immunogenic material may include, for example, attenuated or killed microorganisms (such as attenuated viruses), or antigenic proteins, peptides or DNA derived from such microorganisms. Vaccines may elicit a prophylactic (preventative) immune response in the
30 subject; they may also elicit a therapeutic immune response in a subject. As mentioned above, methods of vaccine administration vary according to the vaccine, and can include routes or means, such as inoculation (intravenous or subcutaneous injection), ingestion, inhalation, or other forms of administration. Inoculations can be delivered by any

of a number of routes, including parenteral, such as intravenous, subcutaneous or intramuscular. Vaccines may also be administered with an adjuvant to boost the immune response.

As used herein, a “vector” refers to a nucleic acid (polynucleotide) molecule into which foreign nucleic acid can be inserted without disrupting the ability of the vector to replicate in and/or integrate into a host cell. A vector can include nucleic acid sequences that permit it to replicate in a host cell, such as an origin of replication. An insertional vector is capable of inserting itself into a host nucleic acid. A vector can also include one or more selectable marker genes and other genetic elements. An expression vector is a vector that contains the necessary regulatory sequences to allow transcription and translation of inserted gene or genes in a host cell. In some embodiments of the present disclosure, the vector encodes an influenza HA, NA or M1 protein. In some embodiments, the vector is the pTR600 expression vector (U.S. Patent Application Publication No. 2002/0106798; Ross et al., 2000, *Nat Immunol.* 1(2):102-103; and Green et al., 2001, *Vaccine* 20:242-248).

By “virus-like particle (VLP)” is meant virus particles made up of one or more viral structural proteins, but lacking the viral genome. Because VLPs lack a viral genome, they are non-infectious and yield safer and potentially more-economical vaccines and vaccine products. In addition, VLPs can often be produced by heterologous expression and can be easily purified. Most VLPs comprise at least a viral core protein that drives budding and release of particles from a host cell. One example of such a core protein is influenza M1. In some embodiments herein, an H3 influenza VLP comprises the HA, NA and M1 proteins. As described herein, H3 influenza VLPs can be produced by transfection of host cells with plasmids encoding the H3 HA, NA and M1 proteins. After incubation of the transfected cells for an appropriate time to allow for protein expression (such as for approximately 72 hours), VLPs can be isolated from cell culture supernatants. By way of example, a protocol for purifying or isolating influenza VLPs from cell supernatants involves low speed centrifugation (to remove cell debris), vacuum filtration and ultracentrifugation of the VLPs through 20% glycerol. A virus-like particle may also include a subviral particle (SVP), which is typically smaller in size than a virus and constitutes a particle without a virus capsid or genome.

Unless specifically stated or obvious from context, as used herein, the term “or” is understood to be inclusive. Unless specifically stated or obvious from context, as used herein, the terms “a”, “an”, and “the” are understood to be singular or plural. Similarly, the

word "or" is intended to include "and" unless the context clearly indicates otherwise. Hence "comprising A or B" means including A, or B, or A and B. It is further to be understood that all base sizes or amino acid sizes, and all molecular weight or molecular mass values, given for nucleic acids or polypeptides are approximate, and are provided for description.

5 Unless specifically stated or obvious from context, as used herein, the term "about" is understood as within a range of normal tolerance in the art, for example within 2 standard deviations of the mean. About may be understood as being within 10%, 9%, 8%, 7%, 6%, 5%, 4%, 3%, 2%, 1%, 0.5%, 0.1%, 0.05%, or 0.01% of the stated value. Unless otherwise clear from context, all numerical values provided herein are modified by the term about.

10 The recitation of a listing of chemical groups in any definition of a variable herein includes definitions of that variable as any single group or combination of listed groups. The recitation of an embodiment for a variable or aspect herein includes that embodiment as any single embodiment or in combination with any other embodiments or portions thereof.

Any compositions or methods provided herein can be combined with one or more of
15 any of the other compositions and methods provided herein.

BRIEF DESCRIPTION OF THE DRAWINGS

FIGS. 1A-1C show the amino acid sequences of nine representative HA polypeptides (proteins) of the H3 influenza A virus strain, referred to TJ1-9 HA herein, that are broadly reactive immunogens that elicit an immune response against H3 virus and H3 virus HA
20 protein. Nucleic acid sequences encoding these polypeptides can be used to generate virus-like particles (VLPs) containing the H3 protein antigens, which are used as immunogens/vaccines to generate neutralizing antibodies in immunized subjects. As shown in **FIGS. 1A-1C**, the full-length TJ1-9 HA polypeptides are 566 amino acids in length.

FIGS. 2A-2C present graphs showing hemagglutination inhibition (HAI) titers of
25 serum antibodies generated against different H3 virus HA protein-derived VLPs used as immunogens (vaccines), following incubation of the sera (e.g., mouse sera) with different strains of H3 virus (Historical Strain) in an HAI assay. **FIG. 2A** shows graphs of hemagglutination inhibition (HAI) titers of serum antibodies generated against VLPs produced from the TJ-2, TJ-3, TJ-5, TJ-6, TJ-7, TJ-8 and TJ-9 HA sequences ("TJ" VLPs) as
30 described herein (presented in **FIGS. 1A-1C**) and used as immunogens (vaccines). The antibodies produced against the several TJ VLPs were broadly reactive against different historical strains of H3 viruses (x-axis). The HAI assay was carried out using serum obtained

from immunized mice bled on Day 77, 0.75% guinea pig red blood cells and 20 nM Oseltamivir (Tamiflu). **FIG. 2B** shows the results of HAI assays using VLPs containing other H3 HA sequences (e.g., Bris/07, Perth/09, Vic/11, Tx/12, Switz/13, HK/14). **FIG. 2C** shows the results of HAI assays using a PBS control.

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DETAILED DESCRIPTION OF THE INVENTION

The H3 influenza virus routinely spreads in humans and causes seasonal flu epidemics. The H3 virus typically causes severe flu disease and adapts to evade being eradicated by constantly changing its surface proteins, such as the HA protein. H3 influenza A virus was found to be a dominant strain in the U.S. and worldwide, e.g., in Australia and the United Kingdom, in the flu season that extended from the year 2017 into 2018. The H3 strain was particularly problematic to treat because of its unusually high rate of mutation and an inability to generate vaccines that were effective against the relatively rapid changes that occurred in its HA surface protein, such as during production of a vaccine against this strain.

Featured herein are synthetic (non-naturally occurring), immunogenic antigens, e.g., protein and glycoprotein antigens, derived from the influenza (“flu”) hemagglutinin (HA) protein of the H3 strain of influenza A virus, that elicit a potent, broadly reactive and long-lasting immune response in a subject, particularly, a human subject. Such immunogenic antigens are also referred to as “immunogens” herein.

Provided are immunogens that protect against disease caused by the influenza H3 strain, or seasonal influenza H3 strains, spanning several years, including drifted strains not yet in existence. In an embodiment, fully synthetic protein antigens are featured, such as influenza H3 virus HA protein antigens. Such H3 HA antigens are synthetic proteins not found in nature, yet they retain all of the functions of a natural H3 HA viral protein and are immunogenic, i.e., they can elicit an immune response, in particular, a broadly active immune response in the form of neutralizing antibodies and/or reactive T lymphocytes, following administration or delivery to, or introduction into, a subject. Also provided are immunogenic compositions, e.g., vaccines, comprising the synthetic H3 virus protein antigens, or nucleic acids encoding the antigens.

An H3 HA amino acid sequence and a protein antigen having such sequence are particularly for use as an immunogen, or in an immunogenic composition, e.g., a vaccine, that elicits a broadly reactive immune response in a subject, particularly a human subject, to whom the composition, or vaccine, is administered. The H3 virus immunogens comprise

antigenic determinants that represent different “antigenic spaces” that are derived from the sequences of many H3 virus strains analyzed based on seasonal periods of time (either overlapping or non-overlapping seasonal time periods). Such overlapping or non-overlapping seasonal time periods may encompass different intervals of time, for example, 5 months, 6 months, 7 months, eight months, nine months, 10 months, 11 months, 1 year, 2 years, 3 years, 4 years, 5 years, 6 years, 7 years, 8 years, 10 years or more, including time intervals therebetween.

The H3 virus antigens described herein embrace seasonal, pan-epitopic, broadly reactive antigens of H3 influenza virus and subtypes thereof, especially antigens containing sequences based on H3 drift variants, wherein the antigens are designed to generate a broadly active immune response, particularly in the form of neutralizing antibodies, in a subject, particularly a human subject. Such antigens are beneficial as immunogens, which elicit an immune response (e.g., production of neutralizing antibodies) against the H3 virus where multiple strains of H3 co-circulate at one time. The broadly reactive H3 immunogenic antigens can be derived from H3 virus that frequently mutates parts of its genome to escape immune pressure, and as a consequence, evades immune surveillance in a subject whose immune system is not primed or stimulated to generate antibodies against antigenic epitopes (determinants) on the H3 antigens following infection. Thus, the synthetic H3 antigens, e.g., H3 HA antigen, comprise amino acid (or polynucleotide) sequences that will elicit greater numbers of neutralizing antibodies against potential H3 drift variants within and across multiple seasons compared with wild-type antigen sequences.

An H3 HA immunogenic protein, or immunogen, as described herein can be employed in an immunogenic composition or as a vaccine that may afford protection against many H3 virus strains over several years. The broadly reactive H3 influenza immunogens and vaccines described herein are advantageous in that they are designed to provide broader and longer-lasting protection against several seasonal H3 flu strains (or clades) prevalent in different geographical locations. Provided by the immunogens and their sequences as described herein is a universal and broad-spectrum H3 flu vaccine that may alleviate the need for a seasonal flu vaccine (immunogenic composition) against the H3 strain and subtypes of influenza virus that is administered annually.

The immunogenic H3 virus HA antigens described herein may be used in immunogenic compositions (e.g., influenza vaccines) that are capable of affording protective immunity against H3 influenza infection and disease in a subject. The protective immunity is

provided in the subject through the elicitation of potent, broadly reactive, anti-H3 HA specific antibody responses that protect the subject against drifted, seasonal H3 influenza virus strains and pandemic H3 influenza virus strains. The immunogenic compositions and vaccines provide an advantage over prior and traditional immunogenic compositions and vaccines directed against H3 virus, which typically depend on the selection of candidate vaccine viruses by public health authorities following analysis of data collected through active surveillance of influenza viruses circulating each year.

Influenza virus

Influenza viruses are segmented negative-strand RNA viruses that belong to the *Orthomyxoviridae* family. There are three types of Influenza viruses: A, B and C. Influenza A viruses infect a wide variety of birds and mammals, including humans, horses, marine mammals, pigs, ferrets, and chickens. In animals, most influenza A viruses cause mild localized infections of the respiratory and intestinal tract. However, highly pathogenic influenza A strains, such as H3, cause systemic infections in poultry in which mortality may reach 100%. Animals infected with influenza A often act as a reservoir for the influenza viruses and certain subtypes have been shown to cross the species barrier to humans in whom they can cause severe disease and devastating flu outbreaks that can lead to death of the infected human subjects.

Influenza A viruses can be classified into subtypes based on allelic variations in antigenic regions of two genes that encode surface glycoproteins, namely, hemagglutinin (HA) and neuraminidase (NA) which are required for viral attachment and cellular release. Currently, sixteen subtypes of HA (H1-H16) and nine NA (N1-N9) antigenic variants are known for influenza A virus. Previously, only three subtypes were known to circulate in humans (H1N1, H1N2, and H3N2). However, in recent years, for example, the pathogenic H5N1 subtype of avian influenza A has been reported to cross the species barrier and infect humans as documented in Hong Kong in 1997 and 2003, leading to the death of several patients.

In humans, the avian influenza virus infects cells of the respiratory tract as well as the intestinal tract, liver, spleen, kidneys and other organs. Symptoms of avian influenza infection include fever, respiratory difficulties, including shortness of breath and cough, lymphopenia, diarrhea and difficulties regulating blood sugar levels. In contrast to seasonal influenza, the group most at risk is healthy adults which make up the bulk of the population.

Due to the high pathogenicity of certain avian influenza A subtypes, particularly H3, and their demonstrated ability to cross over to infect humans, there is a significant economic and public health risk associated with these viral strains, including a real epidemic and pandemic threat.

5 The influenza A virus genome encodes nine structural proteins and one nonstructural (NS1) protein with regulatory functions. The influenza virus segmented genome contains eight negative-sense RNA (nsRNA) gene segments (PB2, PB1, PA, NP, M, NS, HA and NA) that encode at least ten polypeptides, including RNA-directed RNA polymerase proteins (PB2, PB 1 and PA), nucleoprotein (NP), neuraminidase (NA), hemagglutinin, e.g., subunits
10 HA1, frequently referred to as the “head” subunit; and HA2, frequently referred to as the “tail” or “stalk” subunit; the matrix proteins (M1 and M2); and the non-structural proteins (NS1 and NS2) (*See, e.g.,* Krug et al., 1989, In: *The Influenza Viruses*, R. M. Krug, ed., Plenum Press, N.Y., pp. 89 152).

 The ability of influenza virus, e.g., H3, to cause widespread disease is due to its
15 ability to evade the immune system by undergoing antigenic change, which is believed to occur when a host is infected simultaneously with both an animal influenza virus and a human influenza virus. During mutation and reassortment in the host, the virus may incorporate an HA and/or NA surface protein gene from another virus into its genome, thereby producing a new influenza subtype and evading the immune system.

20 Because of antigenic variation (drift) in the circulating strains of H3 influenza virus, in particular, in the HA and NA proteins of the virus, the efficacy of vaccines against H3 influenza virus has frequently been less than optimal and sub-par. The methods described herein provide broadly reactive, pan-epitopic HA or NA antigens of H3 influenza virus that generate a broadly reactive immune response, particularly, in the form of neutralizing
25 antibodies that bind to the H3 viral antigens and neutralize the activity of the virus (e.g., its ability to infect cells), to treat H3 influenza and its symptoms more effectively.

Influenza Virus Hemagglutinin (HA) and Neuraminidase (NA) Proteins

 HA is a viral surface glycoprotein that generally comprises approximately 560 amino
30 acids (e.g., 566 amino acids) and represents 25% of the total virus protein. As described herein, HA is a protein antigen that is highly useful as an immunogen against the H3 virus because it contains a diverse repertoire of epitopes against which antibodies are generated in a subject or host that encounters the H3 HA antigen during infection.

HA is responsible for adhesion of the viral particle to, and its penetration into, a host cell, particularly, in the respiratory epithelium, in the early stages of infection. Cleavage of the virus HA0 precursor into the HA1 and HA2 sub-fragments is a necessary step in order for the virus to infect a cell. Thus, cleavage is required in order to convert new virus particles in a host cell into virions capable of infecting new cells. Cleavage is known to occur during transport of the integral HA0 membrane protein from the endoplasmic reticulum of the infected cell to the plasma membrane. In the course of transport, HA undergoes a series of co- and post-translational modifications, including proteolytic cleavage of the precursor HA into the amino-terminal fragment HA1 ("head") and the carboxy terminal HA2 ("tail" or "stalk"). One of the primary difficulties in growing H3 influenza strains in primary tissue culture or established cell lines arises from the requirement for proteolytic cleavage activation of the influenza hemagglutinin in the host cell.

Although it is known that an uncleaved HA can mediate attachment of the virus to its neuraminic acid-containing receptors on a cell surface, it is not capable of the next step in the infectious cycle, which is fusion. It has been reported that exposure of the hydrophobic amino terminus of HA2 by cleavage is required so that it can be inserted into the target cell, thereby forming a bridge between the virus and the target cell membranes. This process is followed by fusion of the two membranes and entry of the virus into the target cell.

Proteolytic activation of HA involves cleavage at an arginine residue by a trypsin-like endoprotease, which is often an intracellular enzyme that is calcium-dependent and has a neutral pH optimum. Since the activating proteases are cellular enzymes, the infected cell type determines whether the HA is cleaved. The HA of the mammalian influenza viruses and the nonpathogenic avian influenza viruses are susceptible to proteolytic cleavage only in a restricted number of cell types. There are also differences in host range resulting from differences in hemagglutinin cleavability which are correlated with the pathogenic properties of the virus.

Neuraminidase (NA) is a second membrane glycoprotein of the influenza viruses. The presence of viral NA has been shown to be important for generating a multi-faceted protective immune response against an infecting virus. For most influenza A viruses, NA is 413 amino acid in length, and is encoded by a gene of 1413 nucleotides. Nine different NA subtypes have been identified in influenza viruses (N1, N2, N3, N4, N5, N6, N7, N8 and N9), all of which have been found among wild birds. NA is involved in the destruction of the cellular receptor for the viral HA by cleaving terminal neuraminic acid (also called sialic

acid) residues from carbohydrate moieties on the surfaces of infected cells. NA also cleaves sialic acid residues from viral proteins, preventing aggregation of viruses. Using this mechanism, it is hypothesized that NA facilitates the release of viral progeny by preventing newly formed viral particles from accumulating along the cell membrane, as well as by promoting transportation of the virus through the mucus present on the mucosal surface. NA is an important antigenic determinant that is subject to antigenic variation.

In addition to the surface proteins HA and NA, H3 influenza virus comprises six additional internal genes, which give rise to eight different proteins, including polymerase genes PB1, PB2 and PA, matrix proteins M1 and M2, nucleoprotein (NP), and non-structural proteins NS1 and NS2 (*See, e.g.,* Horimoto et al., 2001, *Clin Microbiol Rev.* 14(1):129-149).

In order to be packaged into progeny virions, H3 viral RNA is transported from the nucleus as a ribonucleoprotein (RNP) complex composed of the three influenza virus polymerase proteins, the nucleoprotein (NP), and the viral RNA, in association with the influenza virus matrix 1 (M1) protein and nuclear export protein (Marsh et al., 2008, *J Virol*, 82:2295-2304). The M1 protein that lies within the envelope is thought to function in assembly and budding. A limited number of M2 proteins are integrated into the virions (Zebedee, 1988, *J. Virol.* 62:2762-2772). These M2 proteins form tetramers having H⁺ ion channel activity, and when activated by the low pH in endosomes, acidify the inside of the virion, thus facilitating its uncoating (Pinto et al., 1992, *Cell* 69:517-528). Amantadine is an anti-influenza drug that prevents viral infection by interfering with M2 ion channel activity, thus inhibiting virus uncoating.

NS1, a nonstructural protein, has multiple functions, including regulation of splicing and nuclear export of cellular mRNAs as well as stimulation of translation. The major function of NS1 seems to be to counteract the interferon activity of the host, since an NS1 knockout virus was viable, although it grew less efficiently than the parent virus in interferon-nondefective cells (Garcia-Sastre, 1998, *Virology* 252:324-330).

The NS2 nonstructural protein has been detected in virus particles (Richardson et al., 1991, *Arch. Virol.* 116:69-80; Yasuda et al., 1993, *Virology* 196:249-255). The average number of NS2 proteins in a virus particle was estimated to be 130-200 molecules. An *in vitro* binding assay has demonstrated direct protein-protein contact between M1 and NS2. NS2-M1 complexes have also been detected by immunoprecipitation in virus-infected cell lysates. The NS2 protein is thought to play a role in the export of the RNP from the nucleus through interaction with M1 protein (Ward et al., 1995, *Arch. Virol.* 140:2067-2073).

Broadly Reactive Influenza Proteins and Virus-Like Particles (VLPs)

Provided are non-naturally occurring, broadly reactive, pan-epitopic H3 influenza HA immunogenic polypeptides (immunogens) and influenza virus-like particles (VLPs) comprising an H3 HA immunogen containing diverse epitopes (antigenic determinants) that endow the HA antigen with the ability to generate a broadly active immune response against influenza and its symptoms, either prophylactic or therapeutic, following administration and delivery to a susceptible subject. By way of example, representative H3 HA immunogenic antigen sequences generated by the practice of methods described herein are presented in **FIGS. 1A-1C** herein. In particular examples, the broadly reactive, pan-epitopic H3 HA polypeptides are administered as part of a VLP.

It will be understood that the H3 influenza virus immunogens and sequences described and provided herein are non-naturally occurring, broadly reactive and pan-epitopic, whether or not these characteristics and features are explicitly stated. It will also be appreciated that the H3 antigen proteins, e.g., HA, HA1, or HA2, as described herein and used as immunogens are non-naturally occurring or synthetic antigens that elicit an immune response, e.g., neutralizing antibodies, in a subject.

The broadly reactive and immunogenic H3 antigen sequences that are capable of generating an immune response against H3 influenza virus strains, including present and future H3 virus, may be generated by a method such as described in co-pending provisional patent application number 62/697,803, filed on July 13, 2018, the contents of which are incorporated herein by reference, and which involves a consideration of the parameters of H3 antigen sequences, e.g., HA antigen sequences, from a time span or range (e.g., a linear time range), such as one or more flu seasons, and geographical location(s) in which the H3 virus was isolated, such as, for example, the Southern or Northern Hemisphere.

In an embodiment, the H3 influenza VLPs include the viral HA proteins. In embodiments, the VLPs may include the HA1 and/or the HA2 proteins. It will be appreciated that in some cases, H3 influenza virus VLPs may include the viral NA and M1 proteins. The production of influenza VLPs has been described in the art and is within the skill and expertise of one of ordinary skill in the art. Briefly, and as described, influenza VLPs can be produced by transfection of host cells with one or more plasmids containing polynucleotide sequences that encode the HA, NA and M1 proteins. After incubation of the

transfected cells for an appropriate time to allow for protein expression (such as for approximately 72 hours), H3 VLPs can be isolated from cell culture supernatants. H3 influenza VLPs can be purified from cell supernatants using procedures practiced in the art, for example, VLPs can be isolated by low speed centrifugation (to remove cell debris), vacuum
5 filtration and ultracentrifugation through 20% glycerol.

The influenza VLPs can be used as immunogenic compositions or influenza vaccines to elicit an immune response against H3 influenza viruses. In particular, the component, broadly reactive, pan-epitopic H3 influenza HA polypeptides of the immunogenic compositions or vaccines (or VLPs) contain antigenic (pan-epitopic) determinants that are
10 broadly reactive and serve to elicit an immune response in a subject (e.g., the production of neutralizing antibodies and/or activated T-cells) that can treat an H3 virus-infected subject (e.g., neutralize the infecting virus) and/or protect a subject against full-blown virus infection or the signs and symptoms thereof.

In an embodiment, the antigen sequence of a broadly reactive and immunogenic H3
15 influenza antigen as described herein, such as an H3 HA antigen, contains a diverse repertoire of epitopic determinants that can reflect antigenic drift and sequence variability in the H3 virus's antigenic proteins, for example, over seasons (time) and in different geographic locations. In particular, an H3 virus HA antigen as described herein can comprise an amino acid sequence that contains antigenic determinants (epitopes) derived from sequence diverse
20 influenza virus strains, including drift variants, against which broadly reactive neutralizing antibodies can be raised, especially when the antigen is used as an immunogenic product, (an immunogen), e.g., an antiviral vaccine, that is introduced into a subject.

In an aspect, the H3 viral antigen amino acid sequences provide a composite, immunogenic antigen sequence, which includes epitopic determinants ultimately derivable
25 from both past and more recent seasons of virus infection or disease, and/or from viruses in different geographical locales, and/or from different subtypes or clades of H3 viruses, i.e., a "pan-epitopic" antigen that elicits a broadly reactive immune response when used as an immunogen, a vaccine, or a VLP. In an embodiment, the immunogenic H3 virus HA antigen sequences encompass epitopes that result from antigenic changes in the sequences of H3 HA
30 surface antigens that arise from point mutations during viral replication, giving rise to new H3 influenza variants. As a result, the administration to a subject of an H3 immunogen as described herein can elicit a broadly reactive immune response in the subject that is directed against epitopes reflecting such antigenic changes.

Because the broadly reactive H3 HA antigens and the sequences thereof as described herein and used as an immunogen or immunogenic composition, such as a vaccine, elicit a broadly reactive immune response in an immunocompetent subject, they provides a superior vaccine that captures the antigenic epitopes of many different H3 influenza isolates (subtypes or strains), against which broadly active immune responses (e.g., broadly active neutralizing antibodies) are generated. It is noted that the terms “broadly active” and “broadly reactive” are used synonymously herein.

In an embodiment, the H3 virus antigen as described herein is a polypeptide or peptide antigen of H3 virus which currently causes disease or infection and its symptoms, such as seasonal H3 influenza, and which is native to certain geographical locales. In another embodiment, the H3 virus antigen is a polypeptide or peptide antigen which will, in future, cause disease and symptoms of H3 infection. In an embodiment, the H3 virus antigen is a polynucleotide sequence. In an embodiment, the H3 virus antigen is a polynucleotide sequence that encodes a polypeptide or peptide antigen as described herein. By way of example, representative broadly reactive H3 virus HA immunogens are shown in **FIGS. 1A-1C**.

In another embodiment, the H3 immunogen sequence described herein is expressed in a cell as a polypeptide, protein, or peptide. In an embodiment, the H3 immunogen is isolated and/or purified. In an embodiment, the immunogen is formulated for administration to a subject in need. In an embodiment, the immunogen is administered to a subject in need thereof in an effective amount to elicit an immune response in the subject. In an embodiment, the immune response elicits neutralizing antibodies. In an embodiment, the immune response is prophylactic or therapeutic.

In an embodiment, a non-naturally occurring H3 virus immunogen (immunogen sequence), e.g., a vaccine, is provided that elicits a broadly reactive immune response in a subject following introduction, administration, or delivery of the immunogen to the subject. The route of introduction, administration, or delivery is not limited and may include, for example, intravenous, subcutaneous, intramuscular, oral, etc. routes. The vaccine may be therapeutic (e.g., administered to a subject following a symptom of disease (flu) caused by H3 virus or prophylactic (protective), (e.g., administered to a subject prior to the subject having or expressing a symptom of disease (flu), or full-blown disease, caused by H3 virus).

In an embodiment, the final amino acid sequence of the antigen, e.g., HA, is reverse translated and optimized for expression in mammalian cells. As will be appreciated by the

skilled practitioner in the art, optimization of the nucleic acid sequence includes optimization of the codons for expression of a sequence in mammalian cells and RNA optimization (such as RNA stability).

In an embodiment, an isolated nucleic acid molecule (polynucleotide) comprising a nucleotide sequence encoding a polypeptide or peptide antigen, such as an H3 influenza HA polypeptide (or HA1 or HA2 polypeptide), is provided. In certain embodiments, the nucleotide sequence encoding the H3 HA polypeptide is at least 94%, at least 95%, at least 96%, at least 97%, at least 98% or at least 99% identical to a polynucleotide encoding an HA polypeptide (or HA1 or HA2 polypeptide) sequence shown in **FIGS. 1A-1C**.

In other embodiments, the nucleotide sequence encoding an H3 influenza HA polypeptide (or HA1 or HA2 polypeptide) that is at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, or at least 99% identical to a polynucleotide encoding an H3 HA polypeptide (or HA1 or HA2 polypeptide) sequence shown in **FIGS. 1A-1C** lacks the start codon encoding an N-terminal methionine.

Vectors containing a nucleotide sequence encoding a non-naturally occurring, broadly reactive polypeptide or peptide antigen, such as an H3 influenza HA polypeptide, (or HA1 or HA2 polypeptide), are provided. In some embodiments, the vectors comprise a nucleotide sequence encoding the polypeptide or peptide antigen, such as an influenza H3 HA polypeptide antigen, that is at least 94%, at least 95%, at least 96%, at least 97%, at least 98% or at least 99% identical to a polynucleotide encoding an H3 HA polypeptide (or HA1 or HA2 polypeptide) sequence shown in **FIGS. 1A-1C**. In some embodiments, the vector further includes a promoter operably linked to the nucleotide sequence encoding the H3 HA polypeptide (or HA1 or HA2 polypeptide). In a particular embodiment, the promoter is a cytomegalovirus (CMV) promoter. In some embodiments, the nucleotide sequence of the vector is at least 85%, at least 90%, at least 95%, at least 98% or at least 99% identical to a polynucleotide encoding an H3 HA polypeptide (or HA1 or HA2 polypeptide) sequence shown in **FIGS. 1A-1C**. In particular embodiments, the nucleotide sequence of the vector comprises the polynucleotide encoding an H3 HA polypeptide (or HA1 or HA2 polypeptide) sequence shown in **FIGS. 1A-1C**. In embodiments, the vector is a prokaryotic or eukaryotic vector. In an embodiment, the vector is an expression vector, such as a eukaryotic (e.g., mammalian) expression vector. In another embodiment, the vector is a plasmid (prokaryotic or bacterial) vector. In another embodiment, the vector is a viral vector.

The vectors used to express an H3 virus antigen, e.g., an H3 viral protein, such as the HA protein, as described herein may be any suitable expression vectors known and used in the art. The vectors can be, for example, mammalian expression vectors or viral vectors. In some embodiments, the vector is the pTR600 expression vector (U.S. Patent Application
5 Publication No. 2002/0106798, herein incorporated by reference; Ross et al., 2000, *Nat Immunol.* 1(2):102-103; and Green et al., 2001, *Vaccine* 20:242-248).

Provided are H3 influenza virus-derived, non-naturally occurring polypeptide antigens, e.g., H3 influenza HA polypeptide antigens, or HA1 or HA2 polypeptide antigens, produced by transfecting a host cell with an expression vector as known and used in the art
10 under conditions sufficient to allow for expression of the polypeptide, e.g., an H3 HA, HA1, or HA2 polypeptide, in the cell. Isolated cells containing the vectors are also provided.

Also provided are non-naturally occurring, broadly reactive, pan-epitopic H3 antigen polypeptides as described herein, such as pan-epitopic, broadly reactive H3 influenza HA polypeptides. In certain embodiments, the amino acid sequence of the polypeptide is at least
15 95% to 99% (inclusive) identical to the amino acid sequence of an HA, HA1, or HA2 polypeptide as shown in **FIGS. 1A-1C**. In particular embodiments, the amino acid sequence of the H3 influenza HA, HA1, or HA2 polypeptide that is at least 95% to 99% (inclusive) identical to the amino acid sequence of an HA, HA1, or HA2 polypeptide shown in **FIGS 1A-1C** lacks the N-terminal methionine residue. In a particular embodiment, the amino acid
20 sequence of the H3 influenza HA polypeptide is at least 95% to 99% (inclusive) identical to amino acids 1-566 of the H3 HA polypeptides shown in **FIGS 1A-1C**.

In some embodiments, fusion proteins comprising the broadly reactive, pan-epitopic H3 virus antigen polypeptides described herein, e.g., without limitation, the H3 influenza HA polypeptides disclosed herein, are also provided. In some embodiments, the H3 influenza
25 HA polypeptide can be fused to any heterologous amino acid sequence to form the fusion protein. By way of example, HA1 and HA2 polypeptides may be generated independently and then fused together to produce an H3 HA polypeptide antigen, e.g., comprising 566 amino acids.

Also provided are virus-like particles (VLPs), in particular, H3 influenza VLPs,
30 containing a pan-epitopic, broadly reactive protein antigen, e.g., H3 influenza HA, HA1, or HA2 protein, as described herein. In certain embodiments, the HA protein of the VLP is at least or equal to 94%, at least or equal to 95%, at least or equal to 96%, at least or equal to 97%, at least or equal to 98%, at least or equal to 99% or 100% identical to the H3 HA

proteins as shown in **FIGS 1A-1C**. The virus or influenza VLPs can further include any additional viral or influenza proteins necessary to form the virus particle. In certain embodiments, the virus or influenza VLPs further include influenza neuraminidase (NA) protein, influenza matrix (M1) protein, or both.

Also provided is an H3 influenza VLP containing an H3 influenza HA, HA1, or HA2 polypeptide as described herein, produced by transfecting a host cell with a vector containing a polynucleotide encoding the H3 HA, HA1, or HA2 polypeptide. Also provided in an embodiment is an H3 influenza VLP containing an H3 influenza HA polypeptide, or HA1 or HA2 polypeptide, as described herein, produced by transfecting a host cell with a vector encoding the H3 HA, HA1, or HA2 polypeptide, a vector encoding an influenza NA protein and a vector encoding an influenza M1 protein, under conditions sufficient to allow for expression of the H3 HA, NA and M1 proteins. Such VLPs comprising the sequences as presented in **FIGS 1A-1C** and used as immunogens generate antibodies having high hemagglutinin inhibition (HAI) titers against different strains of H3 influenza virus, as observed in **FIGS. 2A-2C**.

Collections of plasmids (vectors) are also contemplated. In certain embodiments, the collection of plasmids includes a plasmid encoding an influenza H3 NA, a plasmid encoding an H3 influenza MA, and a plasmid encoding a broadly reactive H3 HA protein as described herein. In some embodiments, the nucleotide sequence encoding an H3 influenza HA protein of the HA-encoding plasmid is at least 94%, at least 95%, at least 96%, at least 97%, at least 98% or at least 99% identical to a polynucleotide encoding an H3 HA amino acid sequence as shown in **FIGS 1A-1C**. In some embodiments, the nucleotide sequence encoding a codon-optimized H3 influenza HA protein of the HA-encoding plasmid is at least 94%, at least 95%, at least 96%, at least 97%, at least 98% or at least 99% identical to a polynucleotide encoding an H3 HA amino acid sequence as shown in **FIGS. 1A-1C**.

In the context of the present disclosure, "broadly reactive" or "broadly active" means that the H3 protein (e.g., an H3 HA protein sequence) is immunogenic and contains a diversity of epitopes (antigenic determinants; pan-epitopic) that elicit in a subject an immune response (e.g., neutralizing antibodies directed against the diversity of H3 virus HA epitopes, frequently accompanied by a T-cell response) sufficient to treat disease or infection, and/or to inhibit, neutralize, or prevent infection, caused by most or all H3 influenza viruses within a specific subtype, or by related virus strains. In embodiments, the broadly reactive, H3 virus-derived antigen protein, e.g., HA protein, is capable of eliciting a protective immune response

against most or all known H3 influenza virus isolates, such as about 80%, about 85%, about 90%, about 95%, or about 96%-99% of the known H3 influenza virus isolates.

Compositions and Pharmaceutical Compositions for Administration

Compositions comprising a broadly reactive, pan-epitopic H3 influenza HA protein, or a fusion protein or VLP comprising such a broadly reactive H3 influenza HA protein as described herein are provided. In some embodiments, the compositions further comprise a pharmaceutically acceptable carrier, excipient, or vehicle. In some embodiments, an adjuvant (a pharmacological or immunological agent that modifies or boosts an immune response, e.g. to produce more antibodies that are longer-lasting) is also employed. For example, without limitation, the adjuvant can be an inorganic compound, such as alum, aluminum hydroxide, or aluminum phosphate; mineral or paraffin oil; squalene; detergents such as Quil A; plant saponins; Freund's complete or incomplete adjuvant, a biological adjuvant (e.g., cytokines such as IL-1, IL-2, or IL-12); bacterial products such as killed *Bordetella pertussis*, or toxoids; or immunostimulatory oligonucleotides (such as CpG oligonucleotides).

Compositions and preparations (e.g., physiologically or pharmaceutically acceptable compositions) containing the non-naturally occurring, broadly reactive, pan-epitopic H3 influenza HA polypeptides and H3 influenza virus-like particles (VLPs) for parenteral administration include, without limitation, sterile aqueous or non-aqueous solutions, suspensions, and emulsions. Nonlimiting examples of non-aqueous solvents include propylene glycol, polyethylene glycol, vegetable oils, such as olive oil and canola oil, and injectable organic esters, such as ethyl oleate. Aqueous carriers include water, alcoholic/aqueous solutions, emulsions or suspensions, including saline and buffered media. Parenteral vehicles include, for example, sodium chloride solution, Ringer's dextrose, dextrose and sodium chloride, lactated Ringer's, or fixed oils. Intravenous vehicles include, for example, fluid and nutrient replenishers, electrolyte replenishers (such as those based on Ringer's dextrose), and the like. Preservatives and other additives may also be present in such compositions and preparations, such as, for example, antimicrobials, antioxidants, chelating agents, colorants, stabilizers, inert gases and the like.

Some of the compositions may potentially be administered as a pharmaceutically acceptable acid- or base-addition salt, formed by reaction with inorganic acids, such as hydrochloric acid, hydrobromic acid, perchloric acid, nitric acid, thiocyanic acid, sulfuric acid, and phosphoric acid, and organic acids, such as formic acid, acetic acid, propionic acid, glycolic acid, lactic acid, pyruvic acid, oxalic acid, malonic acid, succinic acid, maleic acid,

and fumaric acid, or by reaction with an inorganic base such as sodium hydroxide, ammonium hydroxide, potassium hydroxide, and organic bases such as mono-, di-, tri-alkyl and aryl amines and substituted ethanolamines.

5 Provided herein are pharmaceutical compositions which include a therapeutically effective amount of a non-naturally occurring, broadly reactive, pan-epitopic, H3 virus protein antigen, or H3 influenza VLPs, alone, or in combination with a pharmaceutically acceptable carrier. Pharmaceutically acceptable carriers include, but are not limited to, saline, buffered saline, dextrose, water, glycerol, ethanol, and combinations thereof. The carrier and composition can be sterile, and the formulation suits the mode of administration.

10 The composition can also contain minor amounts of wetting or emulsifying agents, or pH buffering agents. The composition can be a liquid or aqueous solution, suspension, emulsion, dispersion, tablet, pill, capsule, powder, or sustained release formulation. A liquid or aqueous composition can be lyophilized and reconstituted with a solution or buffer prior to use. The composition can be formulated as a suppository, with traditional binders and

15 carriers such as triglycerides. Oral formulations can include standard carriers, such as pharmaceutical grades of mannitol, lactose, starch, magnesium stearate, sodium saccharine, cellulose, and magnesium carbonate. Any of the commonly known pharmaceutical carriers, such as sterile saline solution or sesame oil, can be used. The medium can also contain conventional pharmaceutical adjunct materials such as, for example, pharmaceutically

20 acceptable salts to adjust the osmotic pressure, buffers, preservatives and the like. Other media that can be used in the compositions and administration methods as described are normal saline and sesame oil.

Methods of Treatment, Administration and Delivery

25 Methods of treating a disease or infection, or symptoms thereof, caused by H3 influenza virus are provided. The methods comprise administering a therapeutically effective amount of a broadly reactive, pan-epitopic immunogen as described herein or a pharmaceutical composition comprising the immunogen, or a vaccine (e.g., a VLP vaccine) as described herein to a subject (e.g., a mammal), in particular, a human subject). One

30 embodiment involves a method of treating a subject suffering from, or at risk of or susceptible to disease or infection, or a symptom thereof, caused by H3 influenza virus. The method includes administering to the subject (e.g., a mammalian subject), an amount or a therapeutic amount of an immunogenic composition or a vaccine comprising a non-naturally

occurring, broadly reactive, pan-epitopic, H3 virus antigen polypeptide, such as HA polypeptide, or HA polypeptide VLPs, sufficient to treat the disease, infection, or symptoms thereof, caused by H3 influenza virus under conditions in which the disease, infection, and/or the symptoms thereof are treated.

5 In an embodiment, the methods herein include administering to the subject (including a human subject identified as in need of such treatment) an effective amount of a non-naturally occurring, broadly reactive, pan-epitopic, H3 virus antigen polypeptide, such as H3 virus HA polypeptide, or a vaccine, or a composition as described herein to produce such effect. The treatment methods are suitably administered to subjects, particularly humans,
10 suffering from, having, susceptible to, or at risk of having a disease, disorder, infection, or symptom thereof, namely, flu or influenza. Identifying a subject in need of such treatment can be based on the judgment of the subject or of a health care professional and can be subjective (e.g. opinion) or objective (e.g. measurable by a test or diagnostic method). Briefly, the determination of those subjects who are in need of treatment or who are "at risk"
15 or "susceptible" can be made by any objective or subjective determination by a diagnostic test (e.g., genetic test, enzyme or protein marker assay), marker analysis, family history, and the like, including an opinion of the subject or a health care provider. The non-naturally occurring, broadly reactive, pan-epitopic H3 immunogens, such as H3 HA polypeptide immunogens and vaccines as described herein, may also be used in the treatment of any other
20 disorders in which infection or disease caused by H3 influenza virus may be implicated. A subject undergoing treatment can be a non-human mammal, such as a veterinary subject, or a human subject (also referred to as a "patient").

In addition, prophylactic methods of preventing or protecting against a disease or infection, or symptoms thereof, caused by H3 influenza virus are provided. Such methods
25 comprise administering a therapeutically effective amount of a pharmaceutical composition comprising an H3 immunogenic composition or vaccine (e.g., an H3 VLP vaccine) as described herein to a subject (e.g., a mammal such as a human), in particular, prior to infection of the subject or prior to onset of the disease, such as H3 virus-associated disease.

In another embodiment, a method of monitoring the progress of an H3 virus infection
30 or disease caused by H3 virus, or monitoring treatment of the H3 infection or disease is provided. The method includes determining a level of a diagnostic marker or biomarker (e.g., an H3 virus protein, such as H3 HA), or a diagnostic measurement (e.g., screening assay or detection assay) in a subject suffering from or susceptible to infection, disease or symptoms

thereof associated with H3 influenza virus, in which the subject has been administered an amount (e.g., a therapeutic amount) of a non-naturally occurring, broadly reactive, pan-epitopic H3 virus HA protein as described herein, or a vaccine as described herein, sufficient to treat the infection, disease, or symptoms thereof. The level or amount of the marker or biomarker (e.g., protein) determined in the method can be compared to known levels of the marker or biomarker in samples from healthy, normal controls; in a pre-infection or pre-disease sample of the subject; or in other afflicted/infected/diseased patients to establish the treated subject's disease status. For monitoring, a second level or amount of the marker or biomarker in a sample obtained from the subject is determined at a time point later than the determination of the first level or amount, and the two marker or biomarker levels or amounts can be compared to monitor the course of disease or infection, or the efficacy of the therapy/treatment. In certain embodiments, a pre-treatment level of the marker or biomarker in the subject (e.g., in a sample obtained from the subject) is determined prior to beginning treatment as described; this pre-treatment level of marker or biomarker can then be compared to the level of the marker or biomarker in the subject after the treatment commences and/or during the course of treatment to determine the efficacy of (monitor the efficacy of) the disease treatment.

The non-naturally occurring, broadly reactive, pan-epitopic, H3 virus antigen polypeptides, such as H3 virus HA polypeptides as described, and VLPs comprising H3 HA polypeptides, or compositions thereof, can be administered to a subject by any of the routes normally used for introducing a recombinant protein, composition containing the recombinant protein, or recombinant virus into a subject. Routes and methods of administration include, without limitation, intradermal, intramuscular, intraperitoneal, intrathecal, parenteral, such as intravenous (IV) or subcutaneous (SC), vaginal, rectal, intranasal, inhalation, intraocular, intracranial, or oral. Parenteral administration, such as subcutaneous, intravenous or intramuscular administration, is generally achieved by injection (immunization). Injectables can be prepared in conventional forms and formulations, either as liquid solutions or suspensions, solid forms (e.g., lyophilized forms) suitable for solution or suspension in liquid prior to injection, or as emulsions. Injection solutions and suspensions can be prepared from sterile powders, granules, and tablets. Administration can be systemic or local.

The non-naturally occurring, broadly reactive, pan-epitopic, H3 virus polypeptides, such as H3 virus HA polypeptides as described, and VLPs comprising H3 HA polypeptides,

or compositions thereof, can be administered in any suitable manner, such as with pharmaceutically acceptable carriers as described *supra*. Pharmaceutically acceptable carriers are determined in part by the particular immunogen or composition being administered, as well as by the particular method used to administer the composition.

- 5 Accordingly, a pharmaceutical composition comprising the non-naturally occurring, broadly reactive, pan-epitopic, H3 virus antigen polypeptides, such as H3 virus HA polypeptides, and VLPs comprising H3 HA polypeptides, or compositions thereof, can be prepared using a wide variety of suitable and physiologically and pharmaceutically acceptable formulations.

Administration of the broadly reactive, pan-epitopic, H3 virus antigen polypeptides, such as H3 virus HA polypeptides, and VLPs comprising HA polypeptides, or compositions thereof, can be accomplished by single or multiple doses. The dose administered to a subject should be sufficient to induce a beneficial therapeutic response in a subject over time, such as to inhibit, block, reduce, ameliorate, protect against, or prevent disease or infection by H3 influenza virus. The dose required will vary from subject to subject depending on the species, age, weight and general condition of the subject, by the severity of the infection being treated, by the particular composition being used and by the mode of administration. An appropriate dose can be determined by a person skilled in the art, such as a clinician or medical practitioner, using only routine experimentation.

Further provided is a method of eliciting an immune response to H3 influenza virus in a subject by administering to the subject a non-naturally occurring, broadly reactive, pan-epitopic, H3 influenza HA protein disclosed herein, fusion proteins containing the H3 influenza HA protein, VLPs containing the influenza HA protein, or compositions thereof as described herein. In some embodiments, the H3 HA protein, HA fusion protein or VLP can be administered using any suitable route of administration, such as, for example, by intramuscular injection. In some embodiments, the H3 HA protein, fusion protein, or VLP is administered as a composition comprising a pharmaceutically acceptable carrier. In some embodiments the composition comprises an adjuvant selected from, for example, alum, Freund's complete or incomplete adjuvant, a biological adjuvant or immunostimulatory oligonucleotides (such as CpG oligonucleotides). In other embodiments, the composition may be administered in combination with another therapeutic agent or molecule.

Also provided is a method of immunizing a subject against infection or disease or the symptoms thereof caused by the H3 influenza virus, in which the method involves administering to the subject VLPs containing a non-naturally occurring, pan-epitopic, broadly

reactive H3 influenza HA protein as described herein, or administering an immunogenic composition thereof. In some embodiments of the method, the composition further comprises a pharmaceutically acceptable carrier and/or an adjuvant. For example, the adjuvant can be alum, Freund's complete or incomplete adjuvant, a biological adjuvant or immunostimulatory oligonucleotides (such as CpG oligonucleotides). In an embodiment, the H3 VLPs (or compositions thereof) are administered intramuscularly.

In some embodiments of the methods of eliciting an immune response or immunizing a subject against virus infection or disease caused by or associated with H3 influenza virus, the subject is administered at least 1 µg of the VLPs containing a non-naturally occurring, broadly reactive, pan-epitopic H3 virus HA protein, such as at least 5 µg, at least 10 µg, at least 15 µg, at least 20 µg, at least 25 µg, at least 30 µg, at least 40 µg or at least 50 µg of the VLPs containing the non-naturally occurring, broadly reactive, pan-epitopic H3 virus HA protein, for example about 1 to about 50 µg or about 1 to about 25 µg of the VLPs containing the H3 HA protein. In particular examples, the subject is administered about 5 to about 20 µg of the VLPs, or about 10 to about 15 µg of the VLPs. In a specific, yet nonlimiting example, the subject is administered about 15 µg of the VLPs. However, one of skill in the art is capable of determining a therapeutically effective amount of VLPs (for example, an amount that provides a therapeutic effect or protection against H3 influenza virus infection) suitable for administering to a subject in need of treatment or protection from virus infection.

It is expected that the administration of VLPs comprising a non-naturally occurring, broadly reactive, pan-epitopic H3 HA protein as described herein will elicit high titers of neutralizing antibodies directed against the diverse repertoire of epitopic determinants on the H3 HA protein immunogen, as well as protective levels of H3 HA-inhibiting (HAI) antibodies that are directed against a number of representative H3 isolates and will provide complete protection against lethal challenge with H3 virus and/or related H3 virus types. The VLPs containing a non-naturally occurring, broadly reactive, pan-epitopic H3 influenza HA protein as described herein elicit a broader immune response (e.g., elicit neutralizing antibodies directed against a broader range of H3 virus isolates compared to the immune response elicited by a polyvalent H3 influenza virus vaccine.

Adjuvants and Combination Therapies

The H3 virus immunogens or immunogenic compositions containing an H3 protein antigen (e.g., an H3 HA antigen), or containing H3 virus VLPs as described herein, can be

administered alone or in combination with other therapeutic agents to enhance antigenicity or immunogenicity, i.e., to increase an immune response, such as the elicitation of specific antibodies, in a subject. For example, the H3 influenza VLPs can be administered with an adjuvant, such as alum, Freund's incomplete adjuvant, Freund's complete adjuvant, biological
5 adjuvant, or immunostimulatory oligonucleotides (such as CpG oligonucleotides).

One or more cytokines, such as interleukin-1 (IL-2), interleukin-6 (IL-6), interleukin-12 (IL-12), the protein memory T-cell attractant "Regulated on Activation, Normal T Expressed and Secreted" (RANTES), granulocyte-macrophage-colony stimulating factor (GM-CSF), tumor necrosis factor-alpha (TNF- α), or interferon-gamma (IFN- γ); one or more
10 growth factors, such as GM-CSF or granulocyte-colony stimulation factor (G-CSF); one or more molecules such as the TNF ligand superfamily member 4 ligand (OX40L) or the type 2 transmembrane glycoprotein receptor belonging to the TNF superfamily (4-1BBL), or combinations of these molecules, can be used as biological adjuvants, if desired or warranted (*see, e.g.,* Salgaller et al., 1998, *J. Surg. Oncol.* 68(2):122-38; Lotze et al., 2000, *Cancer J. Sci. Am.* 6(Suppl 1):S61-6; Cao et al., 1998, *Stem Cells* 16(Suppl 1):251-60; Kuiper et al.,
15 2000, *Adv. Exp. Med. Biol.* 465:381-90). These molecules can be administered systemically (or locally) to a subject.

Several ways of inducing cellular responses, both *in vitro* and *in vivo*, are known and practiced in the art. Lipids have been identified as agents capable of assisting in priming
20 cytotoxic lymphocytes (CTL) *in vivo* against various antigens. For example, palmitic acid residues can be attached to the alpha and epsilon amino groups of a lysine residue and then linked (for example, via one or more linking residues, such as glycine, glycine-glycine, serine, serine-serine, or the like) to an immunogenic peptide (U.S. Patent No. 5,662,907). The lipidated peptide can then be injected directly in a micellar form, incorporated in a
25 liposome, or emulsified in an adjuvant. As another example, *E. coli* lipoproteins, such as tripalmitoyl-S-glycerylcysteinylserine can be used to prime tumor-specific CTL when covalently attached to an appropriate peptide (*see, e.g.,* Deres et al., 1989, *Nature* 342:561). Moreover, the induction of neutralizing antibodies can also be primed with the same molecule conjugated to a peptide which displays an appropriate epitope, and two
30 compositions can be combined to elicit both humoral and cell-mediated responses where such a combination is deemed desirable.

While treatment methods may involve the administration of VLPs containing a non-naturally occurring, broadly reactive, pan-epitopic H3 HA immunogenic protein as described

herein, one skilled in the art will appreciate that the non-naturally occurring, broadly reactive, pan-epitopic H3 influenza HA protein itself (in the absence of a viral particle), as a component of a pharmaceutically acceptable composition, or as a fusion protein, can be administered to a subject in need thereof to elicit an immune response in the subject.

5

Kits

Also provided are kits containing a non-naturally occurring, broadly reactive, pan-epitopic H3 virus immunogen as described, or a vaccine, or a pharmaceutically acceptable composition containing the immunogen and a pharmaceutically acceptable carrier, diluent, or excipient, for administering to a subject, for example. The immunogen may be in the form of an H3 virus protein (polypeptide) or a polynucleotide (a polynucleotide encoding an H3 virus polypeptide, e.g., an H3 HA protein), as described herein. Kits containing one or more of the plasmids, or a collection of plasmids as described herein, are also provided. As will be appreciated by the skilled practitioner in the art, such a kit may contain one or more containers that house the immunogen, vaccine, or composition, diluents or excipients, as necessary, and instructions for use.

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Recombinant Polypeptide Expression

The practice of the present invention employs, unless otherwise indicated, conventional techniques of molecular biology (including recombinant techniques), microbiology, cell biology, biochemistry and immunology, which are well within the purview of the skilled artisan. Such techniques are explained fully in the literature, such as, “Molecular Cloning: A Laboratory Manual”, second edition (Sambrook, 1989); “Oligonucleotide Synthesis” (Gait, 1984); “Animal Cell Culture” (Freshney, 1987); “Methods in Enzymology” “Handbook of Experimental Immunology” (Weir, 1996); “Gene Transfer Vectors for Mammalian Cells” (Miller and Calos, 1987); “Current Protocols in Molecular Biology” (Ausubel, 1987); “PCR: The Polymerase Chain Reaction”, (Mullis, 1994); “Current Protocols in Immunology” (Coligan, 1991). These techniques are applicable to the production of the polynucleotides and polypeptides of the invention, and, as such, may be considered in making and practicing the invention. Particularly useful techniques for particular embodiments will be discussed in the sections that follow.

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EXAMPLES

The following examples are provided to illustrate particular features and/or embodiments. The examples should not be construed to limit the disclosure to the particular features or embodiments described.

Example 1

Hemagglutination-Inhibition (HAI) Assay

A hemagglutination inhibition (HAI) assay was used to assess functional antibodies to the HA protein that are able to inhibit agglutination of guinea pig, horse, or turkey erythrocytes (red blood cells (RBCs)).

Sera used in the assay contained antibodies generated following immunization of animals (mice) with VLPs containing H3 virus HA antigen sequences such as described herein, e.g., TJ2, TJ-3, TJ5-9 (**FIGS. 1A-1C**), other H3 virus HA sequences (e.g., Bris/07, Perth/09, Vic/11, Tx/12, Switz/13, HK/14), wild-type H3 HA sequences, and/or PBS control. For immunization, 5 mice were used per group. Each animal received 3 μ g of the HA antigen/VLP and squalene adjuvant (AF03) (MF Klucker, 2012, *J. Pharm. Sci.*, 101(12):4490-4500) per dose. A homologous prime, boost, boost immunization/administration regimen was employed. Mice were bled on day 77 following a boost with the immunogen on day 56, as presented in Table 1 below.

Table 1

8/18/17	9/15/17	9/29/17	10/13/17	10/27/17	11/2/17
Prime D0	Boost D28	Bleed D42	Boost D56	Bleed D70	Bleed D77
TJ-2	TJ-2		TJ-2		
TJ-3	TJ-3		TJ-3		
TJ-5	TJ-5		TJ-5		
TJ-6	TJ-6		TJ-6		
TJ-7	TJ-7		TJ-7		
TJ-8	TJ-8		TJ-8		
TJ-9	TJ-9		TJ-9		
Bris/07	Bris/07		Bris/07		
Perth/09	Perth/09		Perth/09		
Vic/11	Vic/11		Vic/11		
Tx/12	Tx/12		Tx/12		
Switz/13	Switz/13		Switz/13		
HK/14	HK/14		HK/14		

The protocols were adapted from the WHO laboratory influenza surveillance manual (Gillim-Ross and Subbarao, 2006, *Clin Microbiol Rev* 19(4):614-636) and use the host-species that is frequently used to characterize contemporary H3N2 strains that have preferential binding to alpha (2, 6) linked sialic acid receptors. Turkey or guinea pig erythrocytes were used to compare whether there was a difference in HAI depending on the type of erythrocyte that was used.

To inactivate nonspecific inhibitors, sera were treated with receptor-destroying enzyme (RDE) (Denka Seiken, Co., Japan) prior to being tested. (Bright et al., 2005, *Lancet* 366(9492):1175-1181; Bright et al., 2003, *Virology* 308(2):270-278; Bright et al., 2006, *JAMA* 295(8):891-894; Mitchell et al., 2004, *Vaccine* 21(9-10):902-914; Ross et al., 2000, *Nat Immunol* 1(2):127-131). Briefly, three parts of RDE was added to one part of sera and incubated overnight at 37°C. RDE was inactivated by incubation at 56°C for approximately 30 minutes (~30 min.). RDE-treated sera were diluted in a series of two-fold serial dilutions in v-bottom microtiter plates. An equal volume of each virus, e.g., H3N2 virus, adjusted to approximately 8 hemagglutination units (HAU)/50 µl, was added to each well. The plates were covered and incubated at room temperature for 20 minutes, followed by the addition of 0.75% or 0.8% guinea pig erythrocytes (Lampire Biologicals, Pipersville, PA, USA) in

phosphate buffered saline (PBS). Red blood cells (erythrocytes) were stored at 4°C and used within 72 hours of preparation.

The plates were mixed by agitation and covered, and the RBCs were allowed to settle for 1 hour at room temperature. The HAI titer was determined by the reciprocal dilution of the last well that contained non-agglutinated RBCs. Positive and negative serum controls were included for each plate. All mice were negative ($\text{HAI} \leq 1:10$) for preexisting antibodies to currently circulating human influenza viruses prior to vaccination. Seroconversion was defined as HAI titer $>1:40$, and seroconversion was defined as a 4-fold increase in titer compared to baseline, as per the WHO and European Committee for Medicinal Products to evaluate influenza vaccines. A more stringent threshold of $>1:80$ was often examined. Because mice are naïve and seronegative at the time of vaccination, seroconversion and seroprotection rates are interchangeable in the experiments.

FIGS. 2A-2C show hemagglutination inhibition antibody titers of antibodies generated against VLPs comprising the H3 virus HA immunogens (immunogen sequences) using the immunization/administration regimen as described above and herein (**FIG. 2A**), compared with VLPs produced using different H3 virus HA sequences (**FIGS. 2B**) or PBS (**FIG. 2C**).

Example 2

Virus-Like Particle (Vaccine) preparation

Mammalian 293T cells were transfected with each of three mammalian expression plasmids expressing either the influenza neuraminidase (A/mallard/Alberta/24/01, H7N3), the HIV p55 Gag sequences, or one of the broadly reactive HA expression plasmids (e.g., containing sequence encoding the HA immunogens shown in **FIGS. 1A-1C**), using previously described methods (*see, e.g.*, US Patent Application Publication US 2015/0030628). Following 72 hours of incubation at 37°C, supernatants from transiently transfected cells were collected, centrifuged to remove cellular debris, and filtered through a 0.22µm pore membrane. Mammalian virus-like particles (VLPs) were purified and sedimented by ultracentrifugation on a 20% glycerol cushion at 135,000 x g for 4 hours at 4°C. VLPs were resuspended in phosphate buffered saline (PBS) and total protein concentration was assessed using a conventional bicinchoninic acid assay (BCA). The hemagglutination activity of each preparation of VLPs was determined by adding an equal volume of turkey or guinea pig red blood cells (RBCs) to a V-bottom 96-well plate and incubating with serially diluted volumes

of VLPs for a 30-minute incubation at room temperature (RT). The highest dilution of VLP with full agglutination of RBCs was considered the endpoint HA titer.

Example 3

Determination of HA content by Enzyme Linked Immunosorbent Assay (ELISA)

5 A high-affinity, 96-well, flat-bottom ELISA plate was coated with 5-10 µg of total protein of VLP and serial dilutions of a recombinant H3 antigen (3006_H3_Vc, Protein Sciences, Meriden, CT) in ELISA carbonate buffer (50 mM carbonate buffer, pH 9.5) were added to the wells. The plate was incubated overnight at 4°C on a rocker. The next morning, the plates were washed in PBS with 0.05% Tween-20 (PBST), and non-specific epitopes were
10 blocked with 1% bovine serum albumin (BSA) in PBST solution for 1 hour at RT. The buffer was removed, and stalk-specific Group 2 antibody CR8020 (Tharakaraman, K. et al., 2014, *Cell Host & Microbe*, Vol. 15, pp. 644-651; Ekiert, D.C. et al., 2012, *Science*, 333(6044):843-850; Creative Biolabs, Shirley, NY) was added to plate, followed by a 1 hour incubation at 37°C. The plates were washed and then were probed with goat anti-human IgG horseradish-
15 peroxidase-conjugated secondary antibody (2040-05, Southern Biotech, Birmingham, AL) for 1 hour at 37°C.

The plates were washed. Freshly prepared o-phenylenediamine dihydrochloride (OPD) (P8287, Sigma, City, State, USA) substrate in citrate buffer (P4922, Sigma) was then added to wells, followed by the addition of 1N H₂SO₄ stopping reagent. The plates were read
20 at 492 nm absorbance using a microplate reader (Powerwave XS, Biotek, Winooski, VT). Background signal was subtracted from negative wells. Linear regression standard curve analysis was performed using the known concentrations of recombinant standard antigen to estimate the HA content in lots of VLPs.

25 Example 4

Mouse and ferret studies

Mouse studies

BALB/c mice (*Mus musculus*, females, 6 to 8 weeks of age) were purchased from Jackson Laboratory (Bar Harbor, ME, USA), housed in microisolator units and allowed free
30 access to food and water. The animals were cared for under University of Georgia Research Animal Resources guidelines for laboratory animals. All procedures were reviewed and approved by the Institutional Animal Care and Use Committee (IACUC). Mice (5 mice per group) were vaccinated with purified virus-like particles (VLPs), (3.0 µg/mouse), based upon

HA content from the ELISA quantification, and VLP immunogens (vaccines) were delivered to the animals via intramuscular injection at week 0. Animals were boosted with the same immunogen (vaccine) at the same dose at weeks 4 and 8. Vaccines at each dose were formulated with an emulsified squalene-in-water adjuvant (Sanofi Pasteur, Lyon, France).

- 5 The final concentration after mixing 1:1 with VLPs was 2.5% squalene. Twenty-eight days after each vaccination, blood samples were collected via the submandibular cheek, and the samples were transferred to a microcentrifuge tube. The tubes were centrifuged at 10,000 rpm for 10 minutes. Serum samples were removed and frozen at $-20^{\circ}\text{C} \pm 5^{\circ}\text{C}$.

10 Ferret studies

Fitch ferrets (*Mustela putorius furo*, female, 6-12-months of age), influenza naive and de-scented, were purchased from Marshall Farms (Sayre, Pa., USA). Ferrets were pair-housed in stainless steel cages (Shor-line, Kansas City, Kans., USA) containing Sani-chips Laboratory Animal Bedding (P.J. Murphy Forest Products, Montville, N.J., USA). Ferrets
15 were provided with Teklad Global Ferret Diet (Harlan Teklad, Madison, Wis., USA) and fresh water *ad libitum*.

The purified VLPs were diluted in PBS, pH 7.2, to achieve final concentration. Ferrets (n=3) were vaccinated with 15 μg of purified VLPs, based upon HA content as determined by densitometry assay, via intramuscular injection in the quadriceps muscle in a
20 volume of 0.25 ml at week 0, and then were boosted with the same dose at week 3. Vaccines were stored at -80°C prior to use and formulated with IMJECT® alum adjuvant (IMJECT® Alum; Pierce Biotechnology, Rockford, IL USA) or with the above-described emulsified squalene-in-water adjuvant immediately prior to use. Animals were monitored for adverse events including weight loss, temperature, loss of activity, nasal discharge, sneezing and
25 diarrhea weekly during the vaccination regimen. Prior to vaccination, animals were confirmed by HAI assay to be seronegative for circulating influenza A (e.g., H1N1) and influenza B viruses. Fourteen to twenty-one days after each vaccination, blood was collected from anesthetized ferrets via the anterior vena cava and transferred to a microfuge tube. The tubes were centrifuged; serum was removed and frozen at $-20 \pm 5^{\circ}\text{C}$.

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Other Embodiments

From the foregoing description, it will be apparent that variations and modifications may be made to the invention described herein to adopt it to various usages and conditions. Such embodiments are also within the scope of the following claims.

5 The recitation of a listing of elements in any definition of a variable herein includes definitions of that variable as any single element or combination (or subcombination) of listed elements. The recitation of an embodiment herein includes that embodiment as any single embodiment or in combination with any other embodiments or portions thereof.

10 All patents and publications mentioned in this specification are herein incorporated by reference to the same extent as if each independent patent and publication was specifically and individually indicated to be incorporated by reference.

What is claimed is:

1. A non-naturally occurring, broadly reactive, pan-epitopic antigen of H3 influenza virus (H3 virus) capable of generating an immune response against present and future H3 virus strains; wherein the H3 virus antigen comprises an amino acid sequence that is at least 95% identical to an amino acid sequence of an HA antigen (TJ-1-TJ-9) as set forth in FIGS. 1A-1C.
5
2. The H3 virus antigen of claim 1, wherein the antigen is hemagglutinin (HA), HA1, or HA2, or an antibody binding portion thereof.
10
3. The H3 virus antigen of claim 1 or claim 2, which comprises an amino acid sequence that is at least 98% identical to an amino acid sequence of an HA antigen as set forth in FIGS. 1A-1C.
15
4. The H3 virus antigen of any one of claims 1-3, which comprises an amino acid sequence of an HA antigen as set forth in FIGS. 1A-1C.
5. The H3 virus antigen of any one of claims 1-3, which consists of an amino acid sequence of an HA antigen as set forth in FIGS. 1A-1C.
20
6. A virus-like particle (VLP) comprising the H3 virus antigen of any one of claims 1-5.
7. The VLP of claim 6, which comprises a polynucleotide encoding the H3 virus antigen.
25
8. A non-naturally occurring, pan-epitopic immunogen capable of generating an immune response against present and future H3 influenza (H3) virus strains; wherein the immunogen comprises an amino acid sequence that is at least 95% identical to an amino acid sequence of an HA antigen (TJ-1-TJ-9) as set forth in FIGS. 1A-1C.
30
9. The H3 virus antigen, immunogen, or VLP of any one of claims 1-8, wherein the immune response comprises the production of neutralizing antibodies.

10. The H3 virus antigen, immunogen, or VLP of any one of claims 1-9, wherein the immune response comprises the production of antibodies have hemagglutinin inhibitory activity.
- 5 11. The H3 virus antigen, immunogen, or VLP of any one of claims 1-10, wherein the immune response further comprises the production of T-lymphocytes.
12. A pharmaceutically acceptable composition comprising the H3 virus antigen, immunogen, or VLP of any one of claims 1-11 and a pharmaceutically acceptable carrier,
10 diluent, or excipient.
13. The pharmaceutically acceptable composition of claim 12, further comprising an adjuvant.
- 15 14. An immunogenic composition or vaccine comprising the H3 virus antigen, immunogen, or VLP of any one of claims 1-11.
15. A pharmaceutically acceptable composition comprising the immunogenic composition or vaccine of claim 14 and a pharmaceutically acceptable carrier, diluent, or
20 excipient.
16. The pharmaceutically acceptable composition of claim 15, further comprising an adjuvant.
- 25 17. A method of generating an immune response in a subject, the method comprising administering to the subject an effective amount of the H3 virus antigen, immunogen, or VLP of any one of claims 1-11.
18. A method of generating an immune response in a subject, the method comprising
30 administering to the subject an effective amount of the pharmaceutical composition of any one of claims 12, 13, 15, or 16.

19. A method of generating an immune response in a subject, the method comprising administering to the subject an effective amount of the immunogenic composition or vaccine of claim 14.

5 20. The method of any one of claims 17-19, wherein the immune response comprises the production of neutralizing antibodies.

21. The method of claim 20, wherein the immune response further comprises the production of T-lymphocytes.

10

22. The method of any one of claims 17-19, wherein an adjuvant is concomitantly administered to the subject.

23. A polynucleotide encoding the H3 virus antigen of any one of claims 1-5.

15

24. A composition comprising the polynucleotide of claim 23 and a pharmaceutically acceptable carrier, diluent, or excipient.

TJ-1 HA

MKTIIALSYILCLVFAQKLPGNDNSTATLCLGHHAVPNGTIVKTITNDQIEVTNATELVQSS
STGGICDSPHQILDGENCTLIDALLGDPQCDGFQNKKWDLFVERSKAYSNCYPYDVPD
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MGNGCFKIYHKCDNACIGSIRNGTYDHDVYRDEALNNRFQIKGVELKSGYKDWILWISF
AISCFLLCVALLGFIMWACQKGNIRCNICI

TJ-2 HA

MKTIIALSYILCLVFAQKLPGNDNSTATLCLGHHAVPNGTIVKTITNDQIEVTNATELVQSS
STGGICDSPHQILDGENCTLIDALLGDPQCDGFQNKKWDLFVERSKAYSNCYPYDVPD
YASLRSLVASSGTLEFNNEFSNWTGVTQNGTSSACKRRSNNSFFSRLNWLTHLKFKYP
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EGMVDGWYGFRRHQNSEGIGQAADLKSTQAAINQINGKLNRLIGKTNEKFHQIEKEFSEV
EGRIQDLEKYVEDTKIDLWSYNAELLVALENQHTIDLTDSMNKLFERTKKQLRENAED
MGNGCFKIYHKCDNACIGSIRNGTYDHDVYRDEALNNRFQIKGVELKSGYKDWILWISF
AISCFLLCVALLGFIMWACQKGNIRCNICI

TJ-3 HA

MKTIIALSYILCLVFAQKLPGNDNSTATLCLGHHAVPNGTIVKTITNDQIEVTNATELVQSS
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EGRIQDLEKYVEDTKIDLWSYNAELLVALENQHTIDLTDSMNKLFERTKKQLRENAED
MGNGCFKIYHKCDNACIGSIRNGTYDHDVYRDEALNNRFQIKGVELKSGYKDWILWISF
AISCFLLCVALLGFIMWACQKGNIRCNICI

TJ-4 HA

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TJ-5 HA (1-566AA)

MKTIIALSYILCLVFAQKLPGNDNSTATLCLGHHAVPNGTIVKTITNDQIEVTNATELVQSS
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NVTMPNNEQFDKLYIWGVHHPGTDKDKQIFLYAQASGRITVSTKRSQQAVIPNIGSRPRV
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MVDGWYGFRHQNSEGRGQAADLKSTQAAIDQINGKLNRLIGKTNEKFHQIEKEFSEVE
GRIQDLEKYVEDTKIDLWSYNAELLVALENQHTIDLTDSMNKLFKTKKQLRENAEDM
GNGCFKIYHKCDNACIGSIRNGTYDHDVYRDEALNNRFQIKGVELKSGYKDWILWISFAI
SCFLLCVALLGFIMWACQKGNIRCNICI

TJ-6 HA (1-566AA)

MKTIIALSYILCLVFAQKLPGNDNSTATLCLGHHAVPNGTIVKTITNDQIEVTNATELVQNS
SIGEICDSPHQILDGENCTLIDALLGDPQCDGFQNKKWDLFVERSKAYSNCYPYDVPDY
ASLRSLVASSGTLEFNNEFNWTGVTQNGTSSACIRRSNNSFFSRLNWLTHLNFKYPAL
NVTMPNNEQFDKLYIWGVHHPGTDKDKQIFLYAQSSGRITVSTKRSQQAVIPNIGSRPRIR
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PNDKPFQNVNRITYGACPRYVKQSTLKLATGMRNVPEKQTRGIFGAIAGFIENGWEGM
VDGWYGFRHQNSEGRGQAADLKSTQAAIDQINGKLNRLIGKTNEKFHQIEKEFSEVEG
RIQDLEKYVEDTKIDLWSYNAELLVALENQHTIDLTDSMNKLFKTKKQLRENAEDMG
NGCFKIYHKCDNACIGSIRNGTYDHDVYRDEALNNRFQIKGVELKSGYKDWILWISFAIS
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TJ-7 HA (1-566AA)

MKTIIALSYILCLVFAQKLPGNDNSTATLCLGHHAVPNGTIVKTITNDQIEVTNATELVQNS
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NIPSRISIIYWTIVKPGDILLINSTGNLIAPRGYFKIRSGKSSIMRSDAPIGKCKSECITPNGSI
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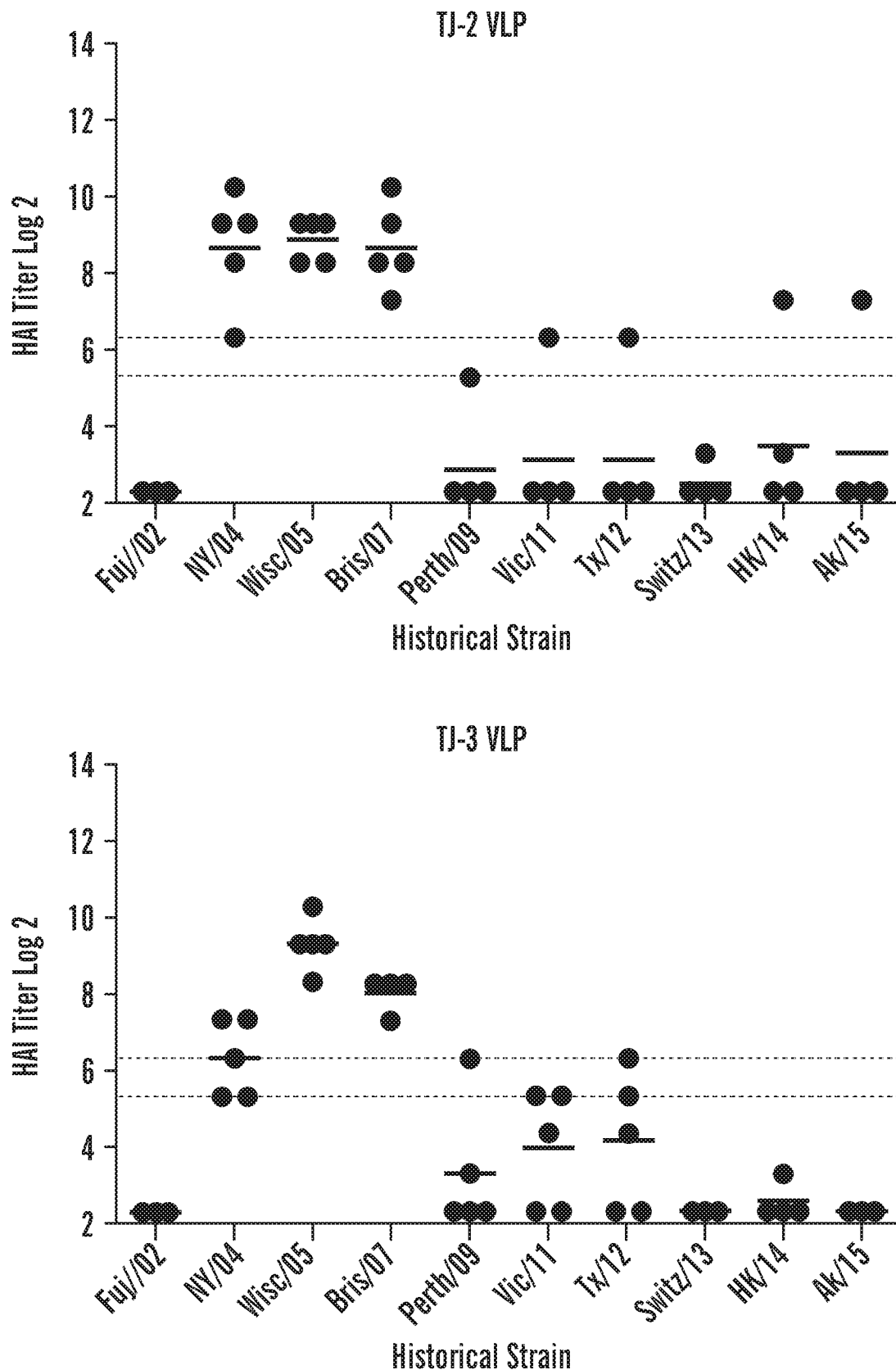
TJ-8 HA (1-566AA)

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MVDGWYGFRHQNSEGRGQAADLKSTQAAIDQINGKLNRLIGKTNEKFHQIEKEFSEVE
GRIQDLEKYVEDTKIDLWSYNAELLVALENQHTIDLTDSEMKNLFEKTKKQLRENAEDM
GNGCFKIYHKCDNACIGSIRNGTYDHDVYRDEALNNRFQIKGVELKSGYKDWILWISFAI
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T-J9 HA (1-566AA)

MKTIIALSYILCLVFAQKLPGNDNSTATLCLGHHAVPNGTIVKTITNDQIEVTNATELVQSS
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NVTMPNNEQFDKLYIWGVHHPGTDKDKQIFLYAQSSGRITVSTKRSQQAVIPNIGSRPRIR
NIPSRISIIYWTIVKPGDILLINSTGNLIAPRGYFKIRSGKSSIMRSDAPIGKCNSECITPNGSI
PNDKPFQNVNRITYGACPRYVKQSTLKLATGMRNVPEKQTRGIFGAIAGFIENGWEGM
VDGWYGFRHQNSEGRGQAADLKSTQAAIDQINGKLNRLIGKTNEKFHQIEKEFSEVEG
RIQDLEKYVEDTKIDLWSYNAELLVALENQHTIDLTDSEMKNLFEKTKKQLRENAEDMG
NGCFKIYHKCDNACIGSIRNGTYDHDVYRDEALNNRFQIKGVELKSGYKDWILWISFAIS
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**FIG. 2A**

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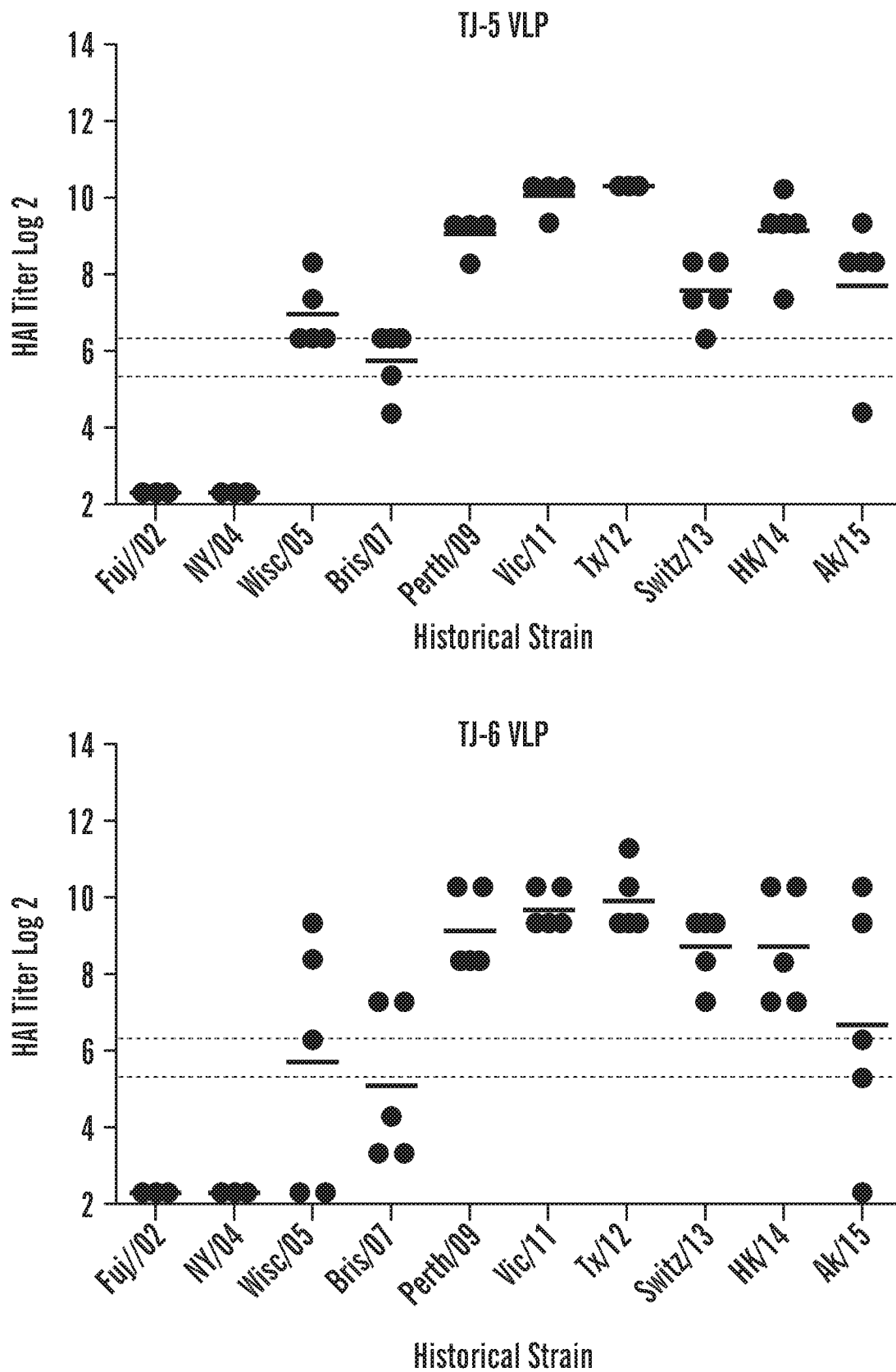
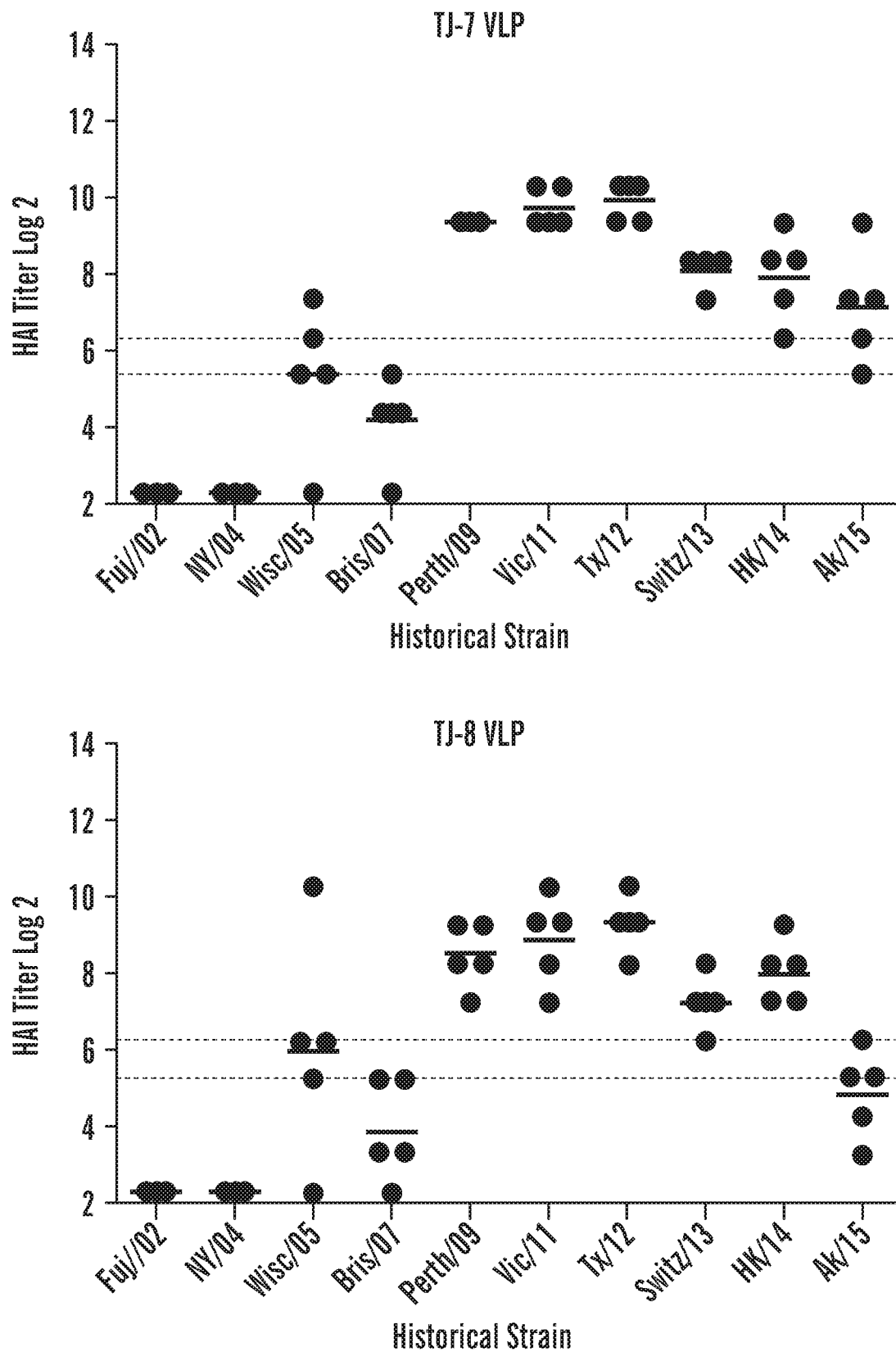


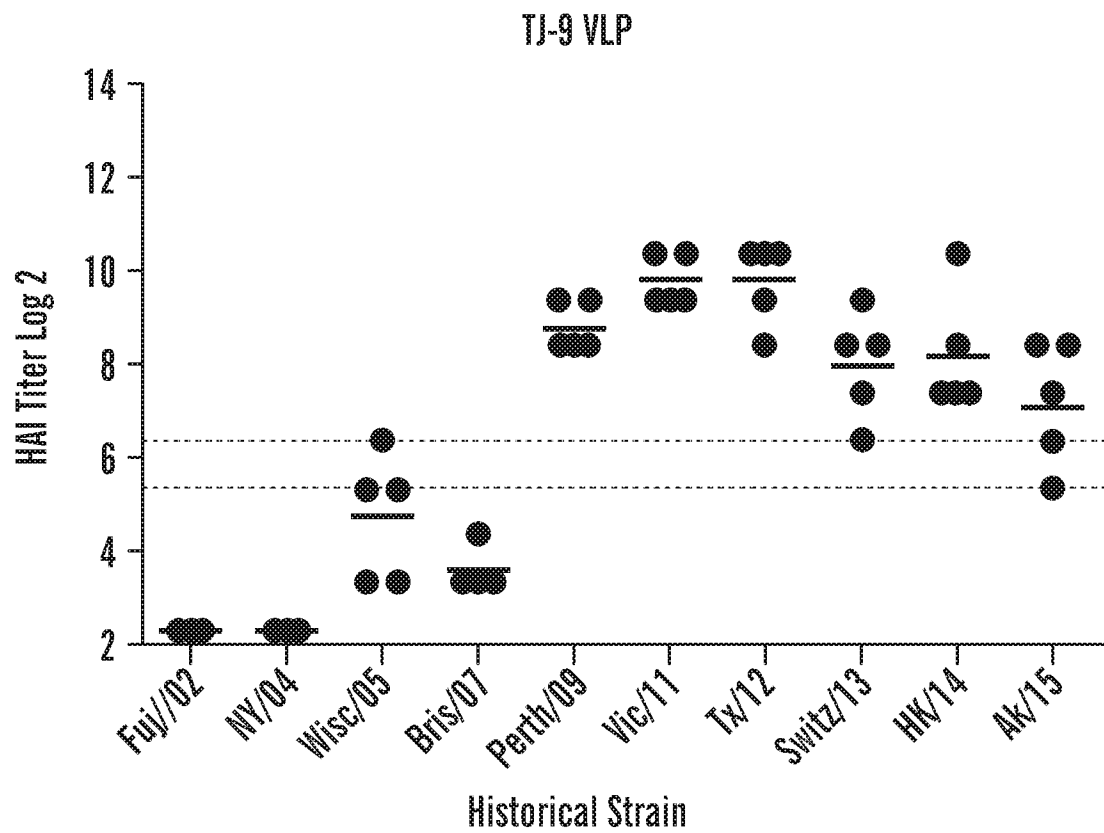
FIG. 2A (cont.)
SUBSTITUTE SHEET (RULE 26)

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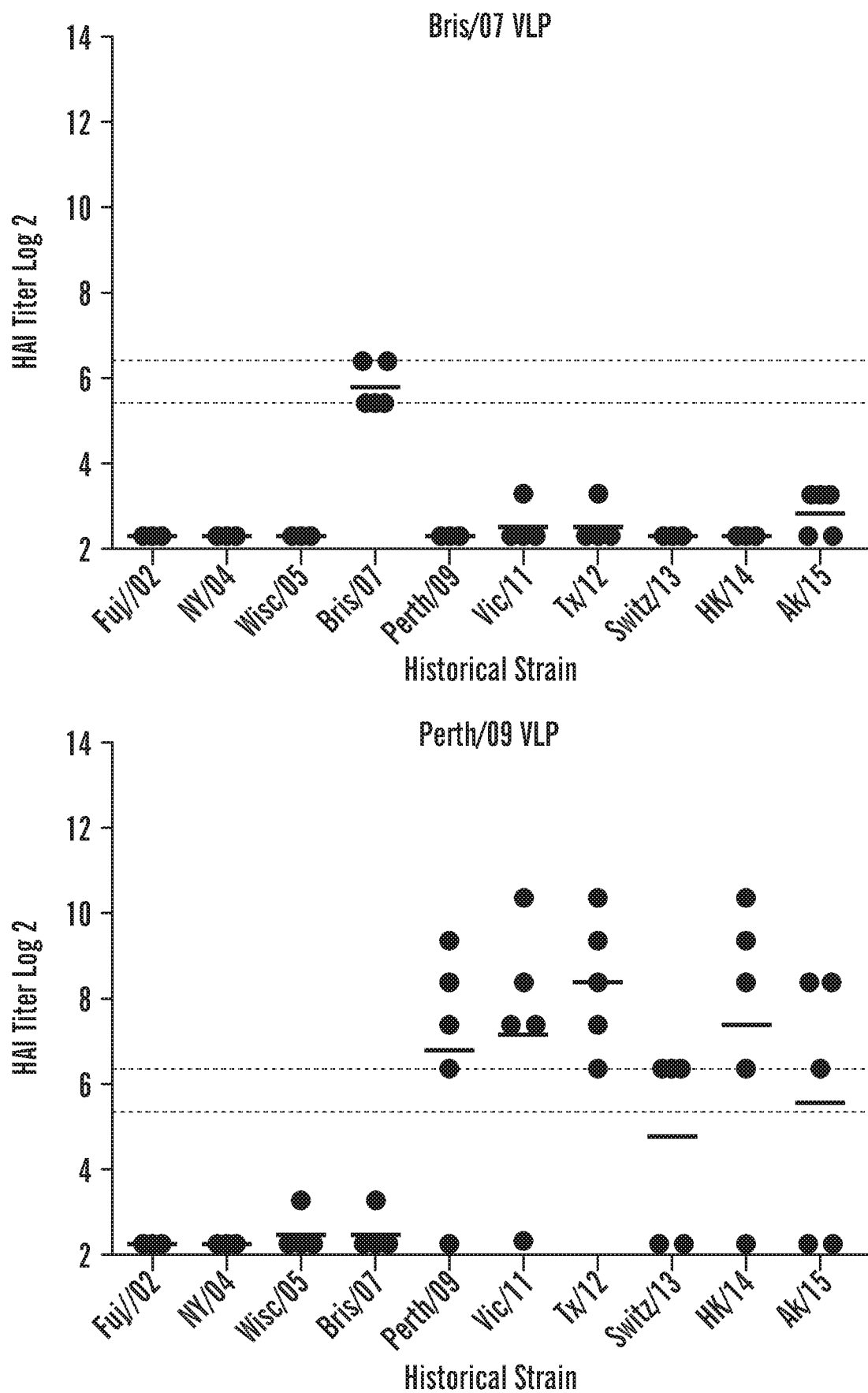
**FIG. 2A (cont.)**

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***FIG. 2A (cont.)***

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**FIG. 2B**

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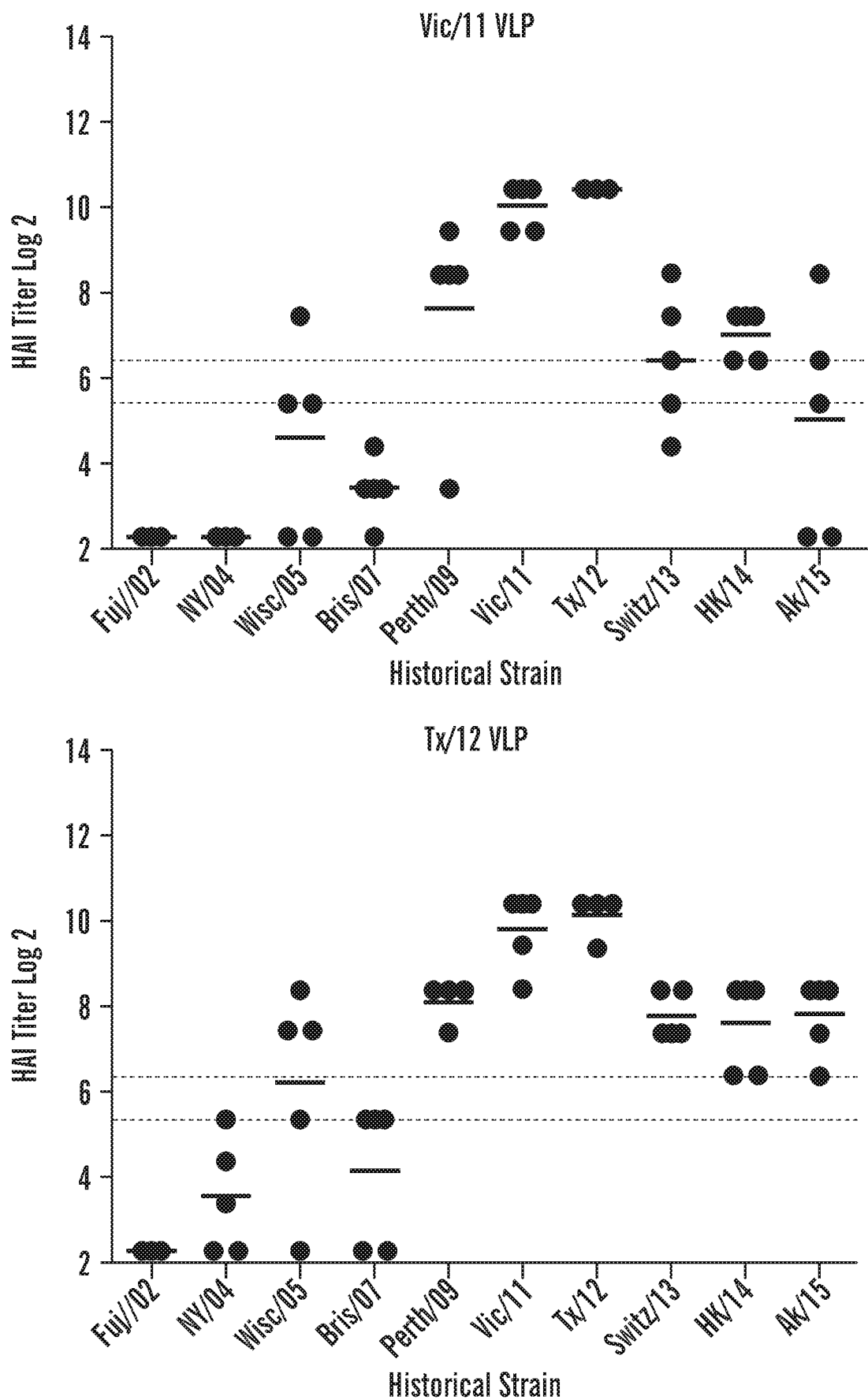


FIG. 2B (cont.)
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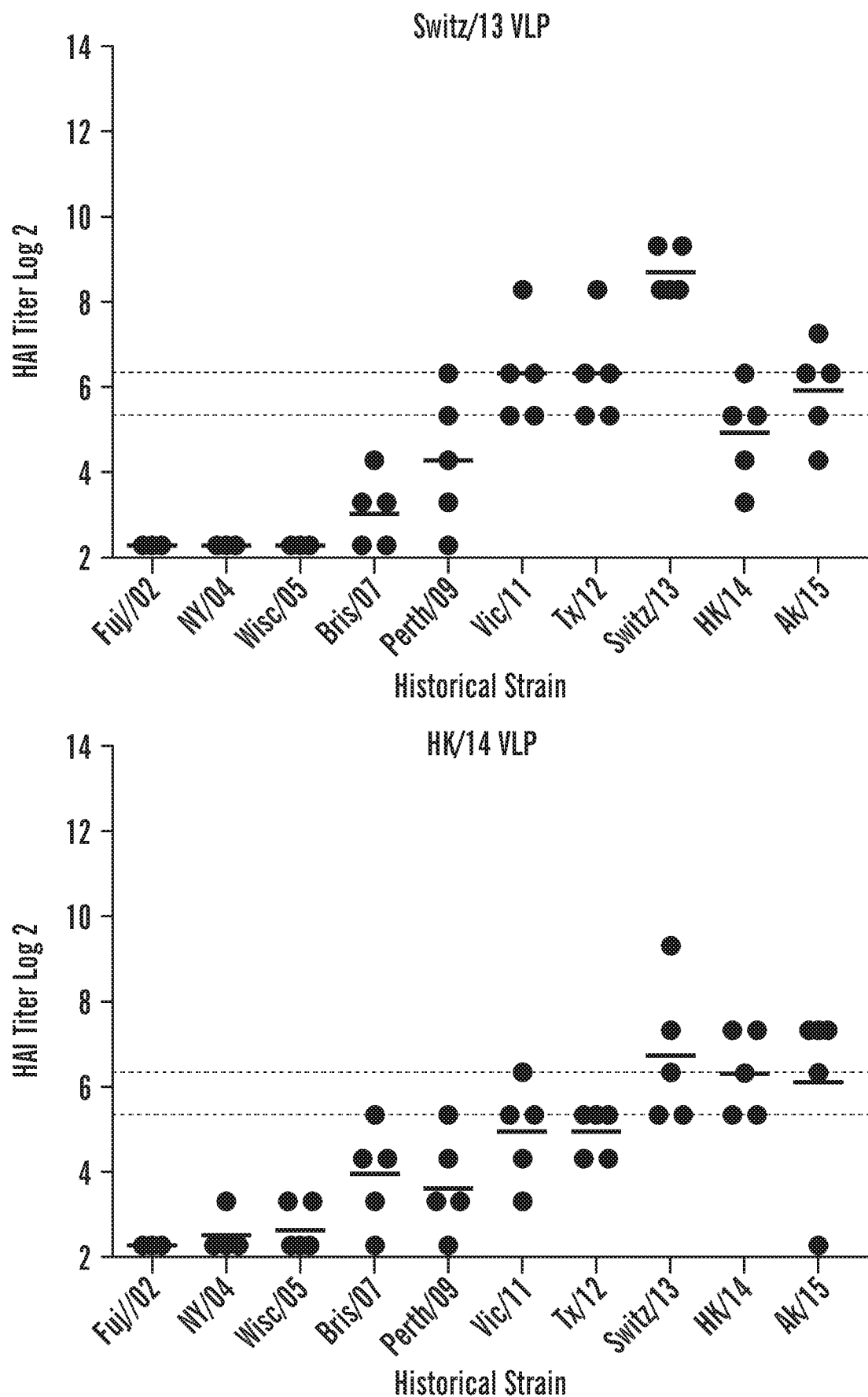
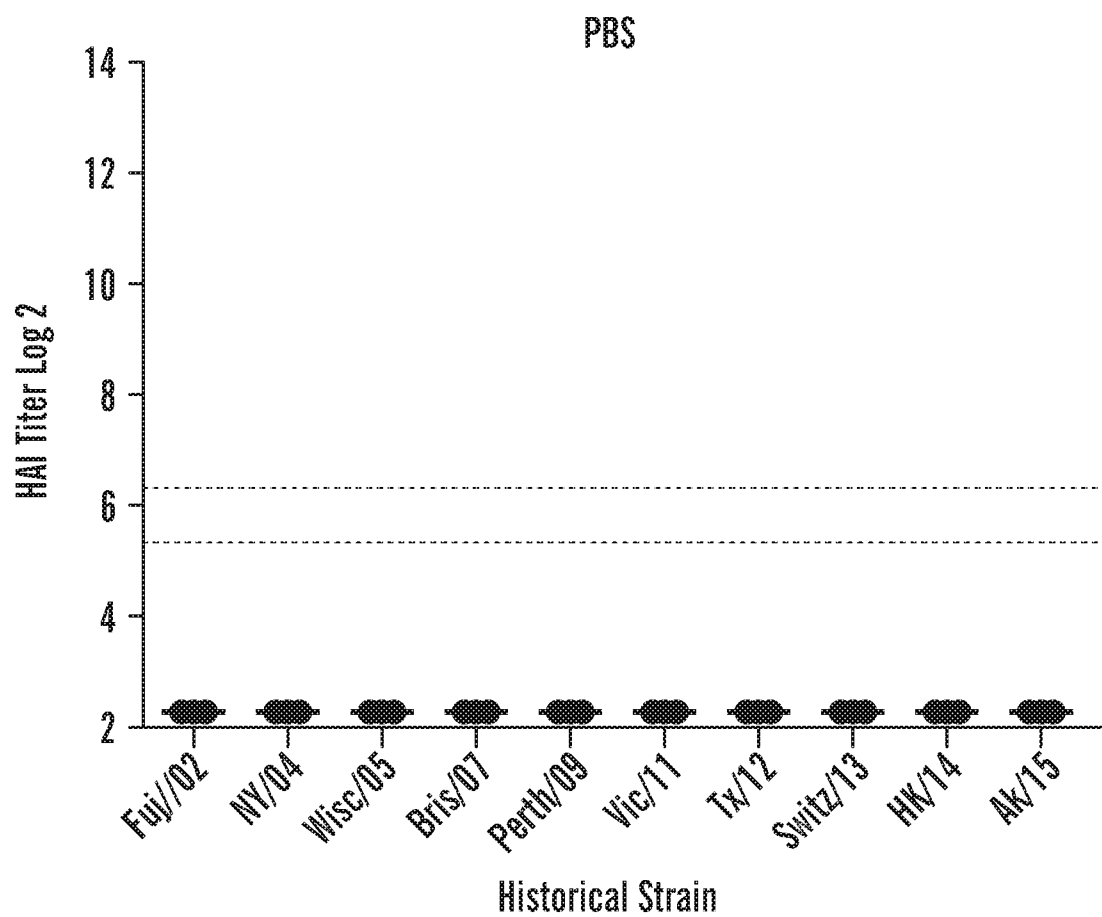


FIG. 2B (cont.)
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**FIG. 2C**

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US2019/041679

A. CLASSIFICATION OF SUBJECT MATTER

IPC(8) - A61K 39/145; C12N 7/00 (2019.01)

CPC - A61K 39/145; A61K 2039/70; C12N 2760/16134 (2019.08)

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)

See Search History document

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

USPC - 424/186.1; 424/210.1 (keyword delimited)

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

See Search History document

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	US 2017/0165353 A1 (THE TRUSTEES OF THE UNIVERSITY OF PENNSYLVANIA) 15 June 2017 (15.06.2017) entire document	1-3, 8
A	US 2017/0049879 A1 (GLAXOSMITHKLINE BIOLOGICALS S.A.) 23 February 2017 (23.02.2017) entire document	1-3, 8
A	US 2010/0166769 A1 (HSIAO et al) 01 July 2010 (01.07.2010) entire document	1-3, 8
A	WO 2018/085488 A1 (IOWA STATE UNIVERSITY RESEARCH FOUNDATION, INC.) 11 May 2018 (11.05.2018) entire document	1-3, 8
A	WO 2017/053374 A1 (OREGON HEALTH & SCIENCE UNIVERSITY) 30 March 2017 (30.03.2017) entire document	1-3, 8
A	WO 2016/207853 A2 (SEQIRUS UK LIMITED) 29 December 2016 (29.12.2016) entire document	1-3, 8

☐ Further documents are listed in the continuation of Box C.☐ See patent family annex.

* 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 October 2019

Date of mailing of the international search report

06 NOV 2019

Name and mailing address of the ISA/US

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

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Authorized officer

Blaine R. Copenheaver

PCT Helpdesk: 571-272-4300

PCT OSP: 571-272-7774

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US2019/041679

Box No. I Nucleotide and/or amino acid sequence(s) (Continuation of item 1.c of the first sheet)

1. With regard to any nucleotide and/or amino acid sequence disclosed in the international application, the international search was carried out on the basis of a sequence listing:

a. ☐ forming part of the international application as filed:

☐ in the form of an Annex C/ST.25 text file.

☐ on paper or in the form of an image file.

b. ☐ furnished together with the international application under PCT Rule 13*ter*.1(a) for the purposes of international search only in the form of an Annex C/ST.25 text file.

c. ☒ furnished subsequent to the international filing date for the purposes of international search only:

☒ in the form of an Annex C/ST.25 text file (Rule 13*ter*.1(a)).

☐ on paper or in the form of an image file (Rule 13*ter*.1(b) and Administrative Instructions, Section 713).

2. ☒ In addition, in the case that more than one version or copy of a sequence listing has been filed or furnished, the required statements that the information in the subsequent or additional copies is identical to that forming part of the application as filed or does not go beyond the application as filed, as appropriate, were furnished.

3. Additional comments:

SEQ ID NOs: 1-11 were searched.

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US2019/041679

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.:
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:

3. ☒ Claims Nos.: 4-7, 9-24
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.