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(54) Title: RECOMBINANT NDV ANTIGEN AND USES THEREOF

Figure 3A

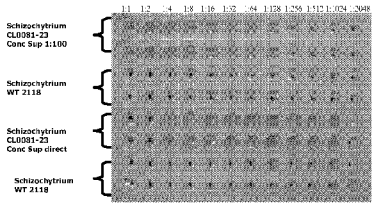
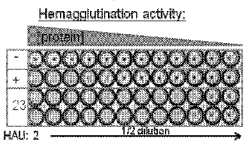


Figure 3B



(57) Abstract: The present invention encompasses NDV vaccines. The vaccine may be a subunit vaccine based on HN of NDV. The NDV HN may be expressed in plants or algae including microalgae. The invention also encompasses recombinant vectors encoding and expressing NDV antigens, epitopes or immunogens which can be used to protect animals against NDV. It encompasses also a vaccination regime compatible with the DIVA strategy, including a prime-boost scheme using viral vector or inactivated vaccines and subunit vaccines.

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RECOMBINANT NDV ANTIGEN AND USES THEREOF

CROSS-REFERENCE TO RELATED APPLICATIONS

[0001] This application claims benefit of US provisional application Serial No. 61/290,297 filed December 28, 2009.

FIELD OF THE INVENTION

[0002] The present invention encompasses pharmaceutical compositions comprising an NDV antigen, in particular pharmaceutical compositions comprising NDV HN antigen.

BACKGROUND OF THE INVENTION

[0003] The virus family *Paramyxoviridae* includes both human (measles, mumps, paraNDV and respiratory syncytial virus) and animal pathogens (Newcastle disease virus and rinderpest virus) that cause significant impact on public health as well as the global economy (Lamb et al., 2007, *Paramyxoviridae*: The viruses and Their Replication, p. 1449-1496). Members of this virus family are defined by having a monopartite, negative sense, single-stranded RNA genome. The *Paramyxoviridae* family consists of two subfamilies namely *Paramyxovirinae* and *Pneumovirinae*. Owing to recent reclassification, the subfamily *Paramyxovirinae* includes five genera, i.e *Morbillivirus, Henipavirus, Rubulavirus, Respirovirus* and *Avulavirus* while *Pneumovirinae* includes *Pneumovirus* and *Metapneumovirus* (Mayo, 2002, Arch Virol 147:1655-63). Avian paramyxoviruses (APMV) are classified in the genus *Avulavirus* and comprise nine antigenically distinct serotypes that have been defined using hemagglutination inhibition (HI) tests (Alexander, 1988, Newcastle disease, p. x, 378 p). Of the nine serotypes, isolates belonging to the APMV-1 subtype can cause a devastating disease in commercial poultry and are classified as velogenic Newcastle disease virus (NDV). Milder forms of NDV are designated as mesogenic and lentogenic isolates, wherein the latter form is mostly asymptomatic in domestic poultry. The genomic RNA of NDV contains genes encoding six proteins: HN (hemagglutinin-neuraminidase), NP (nucleocapsid protein), P (phosphoprotein), M (matrix protein), F (fusion protein), and L (RNA-dependent RNA polymerse).

[0004] Viral vector vaccines represent one of the most rapidly growing areas in vaccine development. Many vaccines in clinical development for major global infectious diseases, HIV, tuberculosis and malaria, are viral vectors. The disadvantage of currently used viral

vectors is the existence of maternally derived antibodies or antibodies acquired due to a past infection.

[0005] Recently, plants and algae have been investigated as a source for the production of therapeutic agents such as vaccines, antibodies, and biopharmaceuticals. These plant and algae expression systems provide several advantages. For example, deriving vaccines from plant or algae expression products can eliminate the risk of contamination with animal pathogens, provide a heat-stable environment, and would avoid injection-related hazards if administered as an edible agent (Thanavala et al., Expert Rev. Vaccines 2006, 5, 249-260). In addition, plants or algae can be grown on a large scale and can utilize existing cultivation, harvest, and storage facilities. Furthermore, there is a lower cost of production and processing to derive therapeutic agents from plants (Giddings et al., Nature Biotech. 2000, 18, 1151-1155) or algae. The F and HN proteins of NDV were expressed in potato plants for developing edible vaccine against NDV (Berinstein A., et al., 2005, Vaccine 23: 5583-6689). WO2004/098533 discloses the expression of the NDV HN antigen and the Avian Influenza Virus HA antigen in tobacco plants. US patent application publication No. US2010/0189731 discloses the expression of Avian Influenza Virus HA antigen in duckweed plants.

[0006] Development of vaccines, antibodies, proteins, and biopharmaceuticals from plants or algae is far from a remedial process, and there are numerous obstacles that are commonly associated with such vaccine production. Limitations to successfully producing plant vaccines include low yield of the bioproduct or expressed antigen (Chargelegue et al., Trends in Plant Science 2001, 6, 495-496), protein instability, inconsistencies in product quality (Schillberg et al., Vaccine 2005, 23, 1764-1769), and insufficient capacity to produce viral-like products of expected size and immunogenicity (Arntzen et al., Vaccine 2005, 23, 1753-1756). In order to address these problems, codon optimization, careful approaches to harvesting and purifying plant or algae products, use of plant parts such as chloroplasts to increase uptake of the material, and improved subcellular targeting are all being considered as potential strategies (Koprowski, Vaccine 2005, 23, 1757-1763).

[0007] Considering the potential effect of animal pathogens, such as NDV on public health and the economy, methods of preventing infection and protecting animals are needed. Moreover, there is a need for an effective vaccine against the pathogens and a suitable method for making the vaccine.

SUMMARY OF THE INVENTION

[0008] Compositions comprising NDV (Newcastle Disease Virus) antigens and fragments and variants thereof are provided. The NDV antigens and fragments and variants thereof possess immunogenic and protective properties. Preferably, the NDV antigens comprise an NDV HN (hemagglutinin-neuraminidase) antigen or fragment or variant thereof. The NDV antigens may be produced in plants or algae.

[0009] The NDV antigens and fragments and variants thereof can be formulated into vaccines and/or pharmaceutical compositions. Such vaccines or compositions can be used to vaccinate an animal and provide protection against at least one form of NDV.

[0010] Methods of the invention include methods for making the NDV antigens and fragments and variants thereof in plants or algae. The methods also include methods of use including administering to an animal an effective amount of NDV antigenic polypeptide(s) and fragments and variants thereof to elicit a protective immunogenic response. After production in plants or algae, the NDV antigenic polypeptides and fragments and variants thereof can be partially or substantially purified for use as a vaccine or composition.

[0011] Kits comprising at least one NDV antigenic polypeptide or fragment or variant thereof and instructions for use are also provided.

BRIEF DESCRIPTION OF THE DRAWINGS

[0012] The following detailed description, given by way of example, but not intended to limit the invention solely to the specific embodiments described, may best be understood in conjunction with the accompanying drawings, in which:

[0013] Figure 1 provides a table showing the SEQ ID NO assigned to the polynucleotide and protein sequence.

[0014] Figure 2 provides DNA and protein sequences of NDV HN genes and the glycosylation sites.

[0015] Figure 3 shows the HA analysis in 96 well plate format.

[0016] Figure 4 shows the SDS-PAGE and Western blot analysis of the expressed NDV HN gene.

[0017] Figure 5 shows the pictures of coomassie stain, periodic acid staining, immunoblot for glycosylation analysis and glycosylation site analysis.

[0018] Figures 6a and 6b provide the protein sequence alignments of NDV HN from different strains and mature protein sequence (without signal peptide).

[0019] Figures 7a provides the sequence alignment of DNA from different NDV strains. Figure 7b shows the DNA sequence alignment between wildtype and codon-optimized

(microalgae-preferred) DNA coding for NDV HN and wildtype and codon-optimized (duckweed-preferred) DNA coding for NDV HN. Figure 7c depicts the plasmid maps for duckweed plant transformation.

[0020] Figure 8 provides the sequence alignment of NDV HN proteins to show the HN linear epitope region.

[0021] Figure 9 provides a table showing the location and presence of glycosylation sites in NDV HN of different strains.

[0022] Figure 10 provides a graphical feature map of the glycosylation sites and the HN linear epitope region of NDV HN CA/02 Protein (SEQ ID NO:3).

[0023] Figure 11 provides peptide sequence analysis of expressed NDV HN (SEQ ID NO:3) in algae.

[0024] Figure 12 provides the HI titer test result and motality test result.

DETAILED DESCRIPTION

[0025] Compositions comprising an NDV (Newcastle Disease Virus) antigen and fragments and variants thereof that elicit an immunogenic response in an animal are provided. The NDV antigen or fragments or variants thereof may be produced in algae. The NDV antigen or fragments or variants may be formulated into vaccines or pharmaceutical compositions and used to elicit or stimulate a protective response in an animal. In one embodiment the NDV antigen is an NDV hemaglutinin-neuraminidase (HN) polypeptide or active fragment or variant thereof.

[0026] It is recognized that the antigenic polypeptides of the invention may be full length polypeptides or active fragments or variants thereof. By "active fragments" or "active variants" is intended that the fragments or variants retain the antigenic nature of the polypeptide. Thus, the present invention encompasses any NDV polypeptide, antigen, epitope or immunogen that elicits an immunogenic response in an animal. The NDV polypeptide, antigen, epitope or immunogen may be any NDV polypeptide, antigen, epitope or immunogen that elicits, induces or stimulates a response in an animal.

[0027] A particular antigenic polypeptide of interest is hemagglutinin-neuraminidase (HN). The glycoprotein, hemagglutinin-neuraminidase (HN) has the transmembrane region located in the amino-terminal region making it a type II integral membrane protein that is involved with viral attachment to cells via sialic acid receptors. The HN protein protrudes from the envelope allowing the virus to contain both hemagglutinin and neuraminidase activities (Yusoff K, Tan WS, 2001, Avian Pathol 30:439-455).

[0028] However, there are different antigens, any of which can be used in the practice of the invention. It is further recognized that precursors of any of these antigens can be used.

[0029] The antigenic polypeptides of the invention are capable of protecting against NDV. That is, they are capable of stimulating an immune response in an animal. By "antigen" or "immunogen" means a substance that induces a specific immune response in a host animal. The antigen may comprise a whole organism, killed, attenuated or live; a portion of an organism; a recombinant vector containing an insert with immunogenic properties; a piece or fragment of DNA capable of inducing an immune response upon presentation to a host animal; a polypeptide, an epitope, a hapten, or any combination thereof. Alternately, the immunogen or antigen may comprise a toxin or antitoxin.

[0030] The terms "protein", "peptide", "polypeptide" and "polypeptide fragment" are used interchangeably herein to refer to polymers of amino acid residues of any length. The polymer can be linear or branched, it may comprise modified amino acids or amino acid analogs, and it may be interrupted by chemical moieties other than amino acids. The terms also encompass an amino acid polymer that has been modified naturally or by intervention; for example disulfide bond formation, glycosylation, lipidation, acetylation, phosphorylation, or any other manipulation or modification, such as conjugation with a labeling or bioactive component.

[0031] The term "immunogenic or antigenic polypeptide" as used herein includes polypeptides that are immunologically active in the sense that once administered to the host, it is able to evoke an immune response of the humoral and/or cellular type directed against the protein. Preferably the protein fragment is such that it has substantially the same immunological activity as the total protein. Thus, a protein fragment according to the invention comprises or consists essentially of or consists of at least one epitope or antigenic determinant. An "immunogenic" protein or polypeptide, as used herein, includes the full-length sequence of the protein, analogs thereof, or immunogenic fragments thereof. By "immunogenic fragment" is meant a fragment of a protein which includes one or more epitopes and thus elicits the immunological response described above. Such fragments can be identified using any number of epitope mapping techniques well known in the art. See, e.g., Epitope Mapping Protocols in Methods in Molecular Biology, Vol. 66 (Glenn E. Morris, Ed., 1996). For example, linear epitopes may be determined by e.g., concurrently synthesizing large numbers of peptides on solid supports, the peptides corresponding to portions of the protein molecule, and reacting the peptides with antibodies while the peptides are still attached to the supports. Such techniques are known in the art and described in, e.g.,

U.S. Pat. No. 4,708,871. Similarly, conformational epitopes are readily identified by determining spatial conformation of amino acids such as by, e.g., x-ray crystallography and 2-dimensional nuclear magnetic resonance. See, e.g., Epitope Mapping Protocols, supra.

[0032] As discussed herein, the invention encompasses active fragments and variants of the antigenic polypeptide. Thus, the term "immunogenic or antigenic polypeptide" further contemplates deletions, additions and substitutions to the sequence, so long as the polypeptide functions to produce an immunological response as defined herein. The term "conservative variation" denotes the replacement of an amino acid residue by another biologically similar residue, or the replacement of a nucleotide in a nucleic acid sequence such that the encoded amino acid residue does not change or is another biologically similar residue. In this regard, particularly preferred substitutions will generally be conservative in nature, i.e., those substitutions that take place within a family of amino acids. For example, amino acids are generally divided into four families: (1) acidic--aspartate and glutamate; (2) basic--lysine, arginine, histidine; (3) non-polar--alanine, valine, leucine, isoleucine, proline, phenylalanine, methionine, tryptophan; and (4) uncharged polar--glycine, asparagine, glutamine, cysteine, serine, threonine, tyrosine. Phenylalanine, tryptophan, and tyrosine are sometimes classified as aromatic amino acids. Examples of conservative variations include the substitution of one hydrophobic residue such as isoleucine, valine, leucine or methionine for another hydrophobic residue, or the substitution of one polar residue for another polar residue, such as the substitution of arginine for lysine, glutamic acid for aspartic acid, or glutamine for asparagine, and the like; or a similar conservative replacement of an amino acid with a structurally related amino acid that will not have a major effect on the biological activity. Proteins having substantially the same amino acid sequence as the reference molecule but possessing minor amino acid substitutions that do not substantially affect the immunogenicity of the protein are, therefore, within the definition of the reference polypeptide. All of the polypeptides produced by these modifications are included herein. The term "conservative variation" also includes the use of a substituted amino acid in place of an unsubstituted parent amino acid provided that antibodies raised to the substituted polypeptide also immunoreact with the unsubstituted polypeptide.

[0033] The term "epitope" refers to the site on an antigen or hapten to which specific B cells and/or T cells respond. The term is also used interchangeably with "antigenic determinant" or "antigenic determinant site". Antibodies that recognize the same epitope can be identified in a simple immunoassay showing the ability of one antibody to block the binding of another antibody to a target antigen.

[0034] An "immunological response" to a composition or vaccine is the development in the host of a cellular and/or antibody-mediated immune response to a composition or vaccine of interest. Usually, an "immunological response" includes but is not limited to one or more of the following effects: the production of antibodies, B cells, helper T cells, and/or cytotoxic T cells, directed specifically to an antigen or antigens included in the composition or vaccine of interest. Preferably, the host will display either a therapeutic or protective immunological response such that resistance to new infection will be enhanced and/or the clinical severity of the disease reduced. Such protection will be demonstrated by either a reduction or lack of symptoms normally displayed by an infected host, a quicker recovery time and/or a lowered viral titer in the infected host.

[0035] By “animal” is intended mammals, birds, and the like. Animal or host as used herein includes mammals and human. The animal may be selected from the group consisting of equine (e.g., horse), canine (e.g., dogs, wolves, foxes, coyotes, jackals), feline (e.g., lions, tigers, domestic cats, wild cats, other big cats, and other felines including cheetahs and lynx), ovine (e.g., sheep), bovine (e.g., cattle), porcine (e.g., pig), avian (e.g., chicken, duck, goose, turkey, quail, pheasant, parrot, finches, hawk, crow, ostrich, emu and cassowary), primate (e.g., prosimian, tarsier, monkey, gibbon, ape), and fish. The term “animal” also includes an individual animal in all stages of development, including embryonic and fetal stages.

[0036] The term “plants” as used herein includes both dicotyledonous (dicot) plants and monocotyledonous (monocot) plant. Dicot plants include, but are not limited to, legumes such as pea, alfalfa and soybean, carrot, celery, tomato, potato, tobacco, pepper, oilseed rape, beet, cabbage, cauliflower, broccoli, lettuce, peanut, and the like. Monocot plants include, but are not limited to, cereals such as wheat, barley, sorghum and millet, rye, triticale, maize, rice or oats, sugarcane, duckweed, grasses, and the like. The term “plant” also include non-flowering plants including, but not limited to, ferns, horsetails, club mosses, mosses, liverworts, hornworts, algae. The terms “algae” and “alga” as used herein include any strain of algae capable of producing a polypeptide or fragment or variant thereof. The algae may include, for example, red, brown, and green algae, gametophytes, and the like. The algae may be microalgae. The microalgae may be *Thraustochytriaceae*, for example, *Schizochytrium, Thraustochytrium, Labyrinthuloides, and Japonochytrium*.

[0037] Unless otherwise explained, all technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to which this disclosure belongs. The singular terms “a”, “an”, and “the” include plural referents unless

context clearly indicates otherwise. Similarly, the word "or" is intended to include "and" unless the context clearly indicates otherwise.

[0038] It is noted that in this disclosure and particularly in the claims and/or paragraphs, terms such as "comprises", "comprised", "comprising" and the like can have the meaning attributed to it in U.S. Patent law; e.g., they can mean "includes", "included", "including", and the like; and that terms such as "consisting essentially of" and "consists essentially of" have the meaning ascribed to them in U.S. Patent law, e.g., they allow for elements not explicitly recited, but exclude elements that are found in the prior art or that affect a basic or novel characteristic of the invention.

Compositions

[0039] The present invention relates to an NDV vaccine or composition which may comprise an effective amount of a recombinant NDV polypeptide or antigen and a pharmaceutically or veterinarily acceptable carrier, excipient, or vehicle. The NDV polypeptide, antigen, epitope or immunogen may be any NDV polypeptide, antigen, epitope or immunogen that elicits, induces or stimulates a response in an animal. In one embodiment, the NDV polypeptide, antigen, epitope or immunogen is a hemagglutinin-neuramidase (HN), RNA polymerase, Fusion protein (F), matrix protein, phosphoprotein and a nucleoprotein. In another embodiment, the NDV antigen may be a hemagglutinin-neuramidase (HN).

[0040] The invention is based, in part, on Applicants' surprising discovery that a recombinant NDV HN gene expressed in a plant or algal protein expression system was highly immunogenic and protected animals against challenge from homologous and heterologous NDV strains.

[0041] The present invention relates to an NDV vaccine or composition which may comprise an effective amount of a recombinant NDV HN polypeptide or antigen and a pharmaceutically or veterinarily acceptable carrier, excipient, or vehicle. In one embodiment, the recombinant NDV HN antigen is expressed in algae. In yet another embodiment, the algae are selected from *Schizochytrium*. In one embodiment, the recombinant NDV HN antigen may be expressed in a *Schizochytrium* protein expression system, as described, for example, in US 7,001,772, US 2008/0022422, US 2006/0275904, US 2006/0286650.

[0042] In an embodiment, the subject matter disclosed herein is directed to a composition comprising a recombinant NDV HN polypeptide or antigen produced by a duckweed expression system and plant material from duckweed, including the genus *Lemna,* and a pharmaceutical or veterinarily acceptable carrier, excipient or vehicle. In another

embodiment, the subject matter disclosed herein is directed to an optionally aglycosylated protein produced by a duckweed expression system comprising an NDV HN polypeptide or antigen. The recombinant NDV HN polypeptide or antigen may be expressed in a *Lemna minor* protein expression system, such as Biolex's LEX system^{SM}.

[0043] In one embodiment, the pharmaceutically or veterinarily acceptable carrier, excipient, or vehicle may be a water-in-oil emulsion. In another embodiment, the water-in-oil emulsion may be a water/oil/water (W/O/W) triple emulsion. In yet another embodiment, the pharmaceutically or veterinarily acceptable carrier, excipient, or vehicle may be an oil-in-water emulsion.

[0044] In an embodiment, the composition or vaccine comprises a recombinant vector and a pharmaceutical or veterinary acceptable excipient, carrier or vehicle. The recombinant vector is plant or algae expression vector which may comprise a polynucleotide encoding an NDV polypeptide, antigen, epitope or immunogen. In one embodiment, the NDV polypeptide, antigen, epitope or immunogen may be derived from an avian infected with NDV or an avian NDV strain.

[0045] In an embodiment, the NDV polypeptide or antigen or fragment or variant thereof comprises an NDV HN polypeptide or fragment or variant thereof. In an aspect of this embodiment, the HN polypeptide or fragment or variant thereof is a recombinant polypeptide produced by an NDV HN gene. In another aspect of this embodiment, the NDV HN gene has at least 70% identity to the sequence as set forth in SEQ ID NO: 1, 2, 4, 6, 8, 12, 14, 16, 18, 22 or 23. In another aspect of this embodiment, the NDV HN polypeptide or fragment or variant thereof has at least 80% identity to the sequence as set forth in SEQ ID NO: 3, 5, 7, 9, 10, 11, 13, 15, 17, 19, 20, 21, or 28. In another aspect of this embodiment, the HN polypeptide or fragment or variant thereof comprises an HN linear epitope region. In another aspect of this embodiment, the epitope region has at least 80% identity to the sequence as set forth in SEQ ID NO: 10, 11, or 28.

[0046] In an embodiment, the NDV antigen is partially purified; or, in another embodiment, the NDV antigen is substantially purified. In yet another embodiment, the NDV antigen is present in the microalgae harvested in whole. In yet another embodiment, the NDV antigen is present in the low-speed supernatant.

[0047] Synthetic antigens are also included within the definition, for example, polyepitopes, flanking epitopes, and other recombinant or synthetically derived antigens. See, e.g., Bergmann et al., 1993; Bergmann et al., 1996; Suhrbier, 1997; Gardner et al., 1998. Immunogenic fragments, for purposes of the present invention, will usually include at least

about 3 amino acids, at least about 5 amino acids, at least about 10-15 amino acids, or about 15-25 amino acids or more amino acids, of the molecule. There is no critical upper limit to the length of the fragment, which could comprise nearly the full-length of the protein sequence, or even a fusion protein comprising at least one epitope of the protein.

[0048] Accordingly, a minimum structure of a polynucleotide expressing an epitope is that it comprises or consists essentially of or consists of nucleotides encoding an epitope or antigenic determinant of an NDV polypeptide. A polynucleotide encoding a fragment of an NDV polypeptide may comprise or consist essentially of or consist of a minimum of 15 nucleotides, about 30-45 nucleotides, about 45-75, or at least 57, 87 or 150 consecutive or contiguous nucleotides of the sequence encoding the polypeptide. Epitope determination procedures, such as, generating overlapping peptide libraries (Hemmer et al., 1998), Pepscan (Geysen et al., 1984; Geysen et al., 1985; Van der Zee R. et al., 1989; Geysen, 1990; Multipin.RTM. Peptide Synthesis Kits de Chiron) and algorithms (De Groot et al., 1999; PCT/US2004/022605) can be used in the practice of the invention.

[0049] Glycosylation of a protein may have multiple effects on the immunogenicity of a protein. In the case of NDV, it appears glycosylation is required for the proper folding of the protein and conformational epitope formation (McGinnes, L. W., and T. G. Morrison. 1995, Virology 212:398-410.). According to McGinnes, *et al.*, glycosylation of the HN protein at glycosylation sites 433 and 481 (see Figures 8-10) are required for protein binding activity and conformational epitope formation. Proper glycosylation of the HN protein may be required for the protein function and the immune response of the host to the composition of the invention.

[0050] Conformational epitopes and a primary linear epitope of the HN protein are described in Gotoh, BT, et al., 1988, Virology 163:174-82, Iorio, R. M., J. B. et al., 1986, J Gen Virol 67:1393-403, Iorio, RM, et al.,1989, Virus Res 13:245-61. It appears that variations in the linear epitope may be the cause of vaccine evasion by emerging strains (Cho, SH, et al., 2008, J Clin Microbiol 46:1541-4). Figure 8 provides an alignment of the HN proteins from four NDV strains to show the level of variation in the linear epitope region.

[0051] The term "nucleic acid" and "polynucleotide" refers to RNA or DNA that is linear or branched, single or double stranded, or a hybrid thereof. The term also encompasses RNA/DNA hybrids. The following are non-limiting examples of polynucleotides: a gene or gene fragment, exons, introns, mRNA, tRNA, rRNA, ribozymes, cDNA, recombinant polynucleotides, branched polynucleotides, plasmids, vectors, isolated DNA of any sequence, isolated RNA of any sequence, nucleic acid probes and primers. A polynucleotide may

comprise modified nucleotides, such as methylated nucleotides and nucleotide analogs, uracyl, other sugars and linking groups such as fluororibose and thiolate, and nucleotide branches. The sequence of nucleotides may be further modified after polymerization, such as by conjugation, with a labeling component. Other types of modifications included in this definition are caps, substitution of one or more of the naturally occurring nucleotides with an analog, and introduction of means for attaching the polynucleotide to proteins, metal ions, labeling components, other polynucleotides or solid support. The polynucleotides can be obtained by chemical synthesis or derived from a microorganism.

[0052] The term "gene" is used broadly to refer to any segment of polynucleotide associated with a biological function. Thus, genes include introns and exons as in genomic sequence, or just the coding sequences as in cDNAs and/or the regulatory sequences required for their expression. For example, gene also refers to a nucleic acid fragment that expresses mRNA or functional RNA, or encodes a specific protein, and which includes regulatory sequences.

[0053] The invention further comprises a complementary strand to a polynucleotide encoding an NDV antigen, epitope or immunogen. The complementary strand can be polymeric and of any length, and can contain deoxyribonucleotides, ribonucleotides, and analogs in any combination.

[0054] An "isolated" biological component (such as a nucleic acid or protein or organelle) refers to a component that has been substantially separated or purified away from other biological components in the cell of the organism in which the component naturally occurs, for instance, other chromosomal and extra-chromosomal DNA and RNA, proteins, and organelles. Nucleic acids and proteins that have been "isolated" include nucleic acids and proteins purified by standard purification methods. The term also embraces nucleic acids and proteins prepared by recombinant technology as well as chemical synthesis.

[0055] The term "purified" as used herein does not require absolute purity; rather, it is intended as a relative term. Thus, for example, a partially purified polypeptide preparation is one in which the polypeptide is more enriched than the polypeptide is in its natural environment. That is the polypeptide is separated from cellular components. By "substantially purified" is intended that such that at least 60%, at least 70%, at least 80%, at least 90%, at least 95%, or at least 98%, or more of the cellular components or materials have been removed. Likewise, a polypeptide may be partially purified. By "partially purified" is intended that less than 60% of the cellular components or material is removed. The same

applies to polynucleotides. The polypeptides disclosed herein can be purified by any of the means known in the art.

[0056] In one aspect, the present invention provides NDV HN polypeptides. In another aspect, the present invention provides an NDV HN polypeptide having a sequence as set forth in SEQ ID NO: 3, 5, 7, 9, 10, 11, 13, 15, 17, 19, 20, 21, or 28 and variant or fragment thereof.

[0057] Moreover, homologs of NDV HN polypeptides are intended to be within the scope of the present invention. As used herein, the term "homologs" includes orthologs, analogs and paralogs. The tem "anologs" refers to two polynucleotides or polypeptides that have the same or similar function, but that have evolved separately in unrelated organisms. The term "orthologs" refers to two polynucleotides or polypeptides from different species, but that have evolved from a common ancestral gene by speciation. Normally, orthologs encode polypeptides having the same or similar functions. The term "paralogs" refers to two polynucleotides or polypeptides that are related by duplication within a genome. Paralogs usually have different functions, but these functions may be related. Analogs, orthologs, and paralogs of a wild-type NDV polypeptide can differ from the wild-type NDV polypeptide by post-translational modifications, by amino acid sequence differences, or by both. In particular, homologs of the invention will generally exhibit at least 80-85%, 85-90%, 90-95%, or 95%, 96%, 97%, 98% , 99% sequence identity, with all or part of the wild-type NDV polypeptide or polynucleotide sequences, and will exhibit a similar function.

[0058] In another aspect, the present invention provides a polypeptide having at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, at least 95%, 96%, 97%, 98% or 99% sequence identity to an NDV HN polypeptide having a sequence as set forth in SEQ ID NO: 3, 5, 7, 9, 10, 11, 13, 15, 17, 19, 20, 21, or 28. In yet another aspect, the present invention provides an NDV HN polypeptide comprising an immunogenic fragment having a sequence as set forth in SEQ ID NO:10, SEQ ID NO:11, or SEQ ID NO:28, and wherein the polypeptide has at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, at least 95%, 96%, 97%, 98% or 99% sequence identity to a polypeptide having a sequence as set forth in SEQ ID NO: 3, 5, 7, 9, 15, 17, 19, or 20.

[0059] In yet another aspect, the present invention provides fragments and variants of the NDV HN polypeptides identified above (SEQ ID NO: 3, 5, 7, 9, 10, 11, 13, 15, 17, 19, 20, 21, or 28) which may readily be prepared by one of skill in the art using well-known molecular biology techniques.

[0060] Variants are homologous polypeptides having an amino acid sequence at least 75%, 80%, 85%, 90%, 95%, 96%, 97%, 98%, or 99% identity to the amino acid sequence as set forth in SEQ ID NO: 3, 5, 7, 9, 10, 11, 13, 15, 17, 19, 20, 21, or 28.

[0061] Variants include allelic variants. The term "allelic variant" refers to a polynucleotide or a polypeptide containing polymorphisms that lead to changes in the amino acid sequences of a protein and that exist within a natural population (e.g., a virus species or variety). Such natural allelic variations can typically result in 1- 5% variance in a polynucleotide or a polypeptide. Allelic variants can be identified by sequencing the nucleic acid sequence of interest in a number of different species, which can be readily carried out by using hybridization probes to identify the same genetic locus in those species. Any and all such nucleic acid variations and resulting amino acid polymorphisms or variations that are the result of natural allelic variation and that do not alter the functional activity of gene of interest, are intended to be within the scope of the invention.

[0062] As used herein, the term "derivative" or “variant” refers to a polypeptide, or a nucleic acid encoding a polypeptide, that has one or more conservative amino acid variations or other minor modifications such that (1) the corresponding polypeptide has substantially equivalent function when compared to the wild type polypeptide or (2) an antibody raised against the polypeptide is immunoreactive with the wild-type polypeptide. These variants or derivatives include polypeptides having minor modifications of the NDV polypeptide primary amino acid sequences that may result in peptides which have substantially equivalent activity as compared to the unmodified counterpart polypeptide. Such modifications may be deliberate, as by site-directed mutagenesis, or may be spontaneous. The term “variant” further contemplates deletions, additions and substitutions to the sequence, so long as the polypeptide functions to produce an immunological response as defined herein.

[0063] The term "conservative variation" denotes the replacement of an amino acid residue by another biologically similar residue, or the replacement of a nucleotide in a nucleic acid sequence such that the encoded amino acid residue does not change or is another biologically similar residue. In this regard, particularly preferred substitutions will generally be conservative in nature, as described above.

[0064] An immunogenic fragment of an NDV HN polypeptide includes at least 8, 10, 13, 14, 15, or 20 consecutive amino acids, at least 21 amino acids, at least 23 amino acids, at least 25 amino acids, or at least 30 amino acids of an NDV HN polypeptide having a sequence as set forth in SEQ ID NO: 3, 5, 7, 9, 10, 11, 13, 15, 17, 19, 20, 21, or 28, or variants thereof. In another embodiment, a fragment of an NDV HN polypeptide includes a

specific antigenic epitope found on a full-length NDV HN polypeptide. An immunogenic fragment may comprise a fragment containing the NDV HN linear epitope region. In one embodiment, the immunogenic fragment comprises the polypeptide having a sequence as set forth in SEQ ID NO:10, SEQ ID NO:11, or SEQ ID NO:28.

[0065] In another aspect, the present invention provides a polynucleotide encoding an NDV HN polypeptide, such as a polynucleotide encoding an NDV HN polypeptide having a sequence as set forth in SEQ ID NO: 3, 5, 7, 9, 10, 11, 13, 15, 17, 19, 20, 21, or 28. In yet another aspect, the present invention provides a polynucleotide encoding an NDV HN polypeptide having at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, at least 95%, 96%, 97%, 98% or 99% sequence identity to a polypeptide having a sequence as set forth in SEQ ID NO: 3, 5, 7, 9, 10, 11, 13, 15, 17, 19, 20, 21, or 28, or a conservative variant, an allelic variant, a homolog or an immunogenic fragment comprising at least eight or at east ten consecutive amino acids of one of these polypeptides, or a combination of these polypeptides. In yet another aspect, the present invention provides a polynucleotide encoding an NDV HN polypeptide comprising an immunogenic fragment having a sequence as set forth in SEQ ID NO:10, SEQ ID NO:11, or SEQ ID NO:28, and wherein the polypeptide has at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, at least 95%, 96%, 97%, 98% or 99% sequence identity to a polypeptide having a sequence as set forth in SEQ ID NO: 3, 5, 7, 9, 15, 17, 19, or 20.

[0066] In another aspect, the present invention provides a polynucleotide having a nucleotide sequence as set forth in SEQ ID NO: 1, 2, 4, 6, 8, 12, 14, 16, 18, 22, or 23, or a variant thereof. In yet another aspect, the present invention provides a polynucleotide having at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, at least 95%, at least 95%, 96%, 97%, 98% or 99% sequence identity to one of a polynucleotide having a sequence as set forth in SEQ ID NO: 1, 2, 4, 6, 8, 12, 14, 16, 18, 22, or 23, or a variant thereof.

[0067] The polynucleotides of the disclosure include sequences that are degenerate as a result of the genetic code, e.g., optimized codon usage for a specific host. As used herein, "optimized" refers to a polynucleotide that is genetically engineered to increase its expression in a given species. To provide optimized polynucleotides coding for NDV polypeptides, the DNA sequence of the NDV protein gene can be modified to 1) comprise codons preferred by highly expressed genes in a particular species; 2) comprise an A+T or G+C content in nucleotide base composition to that substantially found in said species; 3) form an initiation sequence of said species; or 4) eliminate sequences that cause destabilization, inappropriate polyadenylation, degradation and termination of RNA, or that form secondary structure

hairpins or RNA splice sites. Increased expression of NDV protein in said species can be achieved by utilizing the distribution frequency of codon usage in eukaryotes and prokaryotes, or in a particular species. The term "frequency of preferred codon usage" refers to the preference exhibited by a specific host cell in usage of nucleotide codons to specify a given amino acid. There are 20 natural amino acids, most of which are specified by more than one codon. Therefore, all degenerate nucleotide sequences are included in the disclosure as long as the amino acid sequence of the NDV HN polypeptide encoded by the nucleotide sequence is functionally unchanged.

[0068] The sequence identity between two amino acid sequences may be established by the NCBI (National Center for Biotechnology Information) pairwise blast and the blosum62 matrix, using the standard parameters (see, e.g., the BLAST or BLASTX algorithm available on the "National Center for Biotechnology Information" (NCBI, Bethesda, Md., USA) server).

[0069] The "identity" with respect to sequences can refer to the number of positions with identical nucleotides or amino acids divided by the number of nucleotides or amino acids in the shorter of the two sequences wherein alignment of the two sequences can be determined in accordance with the Wilbur and Lipman algorithm (Wilbur and Lipman), for instance, using a window size of 20 nucleotides, a word length of 4 nucleotides, and a gap penalty of 4, and computer-assisted analysis and interpretation of the sequence data including alignment can be conveniently performed using commercially available programs (e.g., Intelligenetics™ Suite, Intelligenetics Inc. CA). When RNA sequences are said to be similar, or have a degree of sequence identity or homology with DNA sequences, thymine (T) in the DNA sequence is considered equal to uracil (U) in the RNA sequence. Thus, RNA sequences are within the scope of the invention and can be derived from DNA sequences, by thymine (T) in the DNA sequence being considered equal to uracil (U) in RNA sequences.

[0070] The sequence identity or sequence similarity of two amino acid sequences, or the sequence identity between two nucleotide sequences can be determined using Vector NTI software package (Invitrogen, 1600 Faraday Ave., Carlsbad, CA).

[0071] Hybridization reactions can be performed under conditions of different stringency. Conditions that increase stringency of a hybridization reaction are well known. See for example, "Molecular Cloning: A Laboratory Manual", second edition (Sambrook et al., 1989).

[0072] The invention further encompasses the NDV polynucleotides contained in a vector molecule or an expression vector and operably linked to a promoter element.

[0073] A "vector" refers to a recombinant DNA or RNA plasmid or virus that comprises a heterologous polynucleotide to be delivered to a target cell, either *in vitro* or *in vivo*. The heterologous polynucleotide may comprise a sequence of interest for purposes of prevention or therapy, and may optionally be in the form of an expression cassette. As used herein, a vector needs not be capable of replication in the ultimate target cell or subject. The term includes cloning vectors and viral vectors.

[0074] The term "recombinant" means a polynucleotide with semisynthetic, or synthetic origin which either does not occur in nature or is linked to another polynucleotide in an arrangement not found in nature.

[0075] "Heterologous" means derived from a genetically distinct entity from the rest of the entity to which it is being compared. For example, a polynucleotide may be placed by genetic engineering techniques into a plasmid or vector derived from a different source, and is a heterologous polynucleotide. A promoter removed from its native coding sequence and operatively linked to a coding sequence other than the native sequence is a heterologous promoter.

[0076] The polynucleotides of the invention may comprise additional sequences, such as additional encoding sequences within the same transcription unit, controlling elements such as promoters, ribosome binding sites, 5'UTR, 3'UTR, transcription terminators, polyadenylation sites, additional transcription units under control of the same or a different promoter, sequences that permit cloning, expression, homologous recombination, and transformation of a host cell, and any such construct as may be desirable to provide embodiments of this invention.

[0077] Elements for the expression of an NDV polypeptide, antigen, epitope or immunogen are advantageously present in an inventive vector. In minimum manner, this comprises an initiation codon (ATG), a stop codon and a promoter, and optionally also a polyadenylation sequence for certain vectors such as plasmid and certain viral vectors, e.g., viral vectors other than poxviruses. When the polynucleotide encodes a polypeptide fragment, e.g. an NDV peptide, advantageously, in the vector, an ATG is placed at 5' of the reading frame and a stop codon is placed at 3'. Other elements for controlling expression may be present, such as enhancer sequences, stabilizing sequences, such as intron and signal sequences permitting the secretion of the protein.

[0078] The present invention also relates to compositions comprising vectors, such as expression vectors. The compositions can comprise one or more vectors, e.g., expression vectors, such as *in vivo* expression vectors, comprising and expressing one or more NDV

polypeptides, antigens, epitopes or immunogens. In one embodiment, the vector comprises a polynucleotide coding for and/or expressing an NDV polypeptide, antigen, epitope or immunogen, in a pharmaceutically or veterinarily acceptable carrier, excipient or vehicle.

[0079] According to another embodiment of the invention, the expression vector is a plasmid vector, in particular an *in vivo* expression vector. In a specific, non-limiting example, the pVR1020 or 1012 plasmid (VICAL Inc.; Luke et al., 1997; Hartikka et al., 1996, see, e.g., U.S. Patent Nos. 5,846,946 and 6,451,769) can be utilized as a vector for the insertion of a polynucleotide sequence. The pVR1020 plasmid is derived from pVR1012 and contains the human tPA signal sequence. In one embodiment the human tPA signal comprises from amino acid M(1) to amino acid S(23) of GenBank accession number HUMTPA14. In another specific, non-limiting example, the plasmid utilized as a vector for the insertion of a polynucleotide sequence can contain the signal peptide sequence of equine IGF1 from amino acid M(24) to amino acid A(48) of GenBank accession number U28070. Additional information on DNA plasmids which may be consulted or employed in the practice are found, for example, in U.S. Patent Nos. 6,852,705; 6,818,628; 6,586,412; 6,576,243; 6,558,674; 6,464,984; 6,451,770; 6,376,473 and 6,221,362.

[0080] The term plasmid covers any DNA transcription unit comprising a polynucleotide according to the invention and the elements necessary for its *in vivo* expression in a cell or cells of the desired host or target; and, in this regard, it is noted that a supercoiled or non-supercoiled, circular plasmid, as well as a linear form, are intended to be within the scope of the invention.

[0081] Each plasmid comprises a polynucleotide encoding an NDV polypeptide, antigen, epitope or immunogen operably linked to a promoter or under the control of a promoter or dependent upon a promoter. In general, it is advantageous to employ a strong promoter functional in eukaryotic cells. The strong promoter may be, but not limited to, the immediate early cytomegalovirus promoter (CMV-IE) of human or murine origin, or optionally having another origin such as the rat or guinea pig.

[0082] In more general terms, the promoter has a viral, a plant, or a cellular origin. A strong viral promoter other than CMV-IE that may be usefully employed in the practice of the invention is the early/late promoter of the SV40 virus or the LTR promoter of the Rous sarcoma virus. A strong cellular promoter that may be usefully employed in the practice of the invention is the promoter of a gene of the cytoskeleton, such as e.g. the desmin promoter (Kwissa et al., 2000), or the actin promoter (Miyazaki et al., 1989).

[0083] The plasmids may comprise other expression control elements. It is particularly advantageous to incorporate stabilizing sequence(s), e.g., intron sequence(s), for example, maize alcohol dehydrogenase intron (maize ADHI intron), the first intron of the hCMV-IE (WO1989/01036), the intron II of the rabbit β-globin gene (van Ooyen et al., 1979). In another embodiment, the plasmids may comprise 3' UTR. The 3' UTR may be, but not limited to, agrobacterium nopaline synthase (Nos) 3' UTR. The plasmids may further comprise the OrfC terminator (also known as the PFA3 terminator).

[0084] As to the polyadenylation signal (polyA) for the plasmids and viral vectors other than poxviruses, use can be made of the poly(A) signal of the bovine growth hormone (bGH) gene (see U.S. 5,122,458), or the poly(A) signal of the rabbit β-globin gene or the poly(A) signal of the SV40 virus.

[0085] A "host cell" denotes a prokaryotic or eukaryotic cell that has been genetically altered, or is capable of being genetically altered by administration of an exogenous polynucleotide, such as a recombinant plasmid or vector. When referring to genetically altered cells, the term refers both to the originally altered cell and to the progeny thereof.

[0086] In one embodiment, the recombinant NDV HN antigen is expressed in a transgenic plant. In another embodiment, the recombinant NDV HN antigen is expressed in transgenic algae. In yet another embodiment, the transgenic algae are *Schizochytrium*. Details of the algae protein expression system may be found, for example, in US 7,001,772, US 2008/0022422. The NDV HN polypeptide or antigen in the embodiments may be any polypeptide disclosed herein, or a polypeptide encoded by any polynucleotide disclosed herein.

Methods for Expressing NDV polypeptides in Microalgae or Duckweed

[0087] In some embodiments of the invention, NDV HN polypeptides, or fragments or variants thereof, are expressed in microalgae or duckweed. These methods comprise the use of expression cassettes that are introduced into algae or plants using any suitable transformation method known in the art. Polynucleotides within these expression cassettes can be modified for enhanced expression of the antigenic NDV HN polypeptide, or fragment or variant thereof, in microalgae or duckweed, as follows.

Cassettes for Microalgae or duckweed Expression of Antigenic NDV Polypeptides

[0088] Transgenic microalgae or duckweed expressing an NDV HN polypeptide, or fragment or variant thereof are obtained by transformation of microalgae or duckweed with

an expression cassette comprising a polynucleotide encoding the NDV HN polypeptide, or fragment or variant thereof. In this manner, a polynucleotide encoding the NDV HN polypeptide, or fragment or variant thereof, is constructed within an expression cassette and introduced into microalgae or duckweed by any suitable transformation method known in the art.

[0089] In some embodiments, the microalgae or duckweed that are transformed with an expression cassette comprising a polynucleotide encoding the NDV HN polypeptide, or fragment or variant thereof, have also been transformed with an expression cassette that provides for expression of another heterologous polypeptide of interest, for example, another NDV polypeptide, fragment, or variant thereof. The expression cassette providing for expression of another heterologous polypeptide of interest can be provided on the same polynucleotide (for example, on the same transformation vector) for introduction into microalgae or duckweed, or on a different polynucleotide (for example, on different transformation vectors) for introduction into the microalgae or duckweed at the same time or at different times, by the same or by different methods of introduction, for example, by the same or different transformation methods.

[0090] The expression cassettes for use in transformation of microalgae or duckweed comprise expression control elements that at least comprise a transcriptional initiation region (e.g., a promoter) operably linked to the polynucleotide of interest, i.e., a polynucleotide encoding an NDV HN polypeptide, fragment, or variant thereof. "Operably linked" as used herein in reference to nucleotide sequences refers to multiple nucleotide sequences that are placed in a functional relationship with each other. Generally, operably linked DNA sequences are contiguous and, where necessary to join two protein coding regions, in reading frame. Such an expression cassette is provided with a plurality of restriction sites for insertion of the polynucleotide or polynucleotides of interest (e.g., one polynucleotide of interest, two polynucleotides of interest, etc.) to be under the transcriptional regulation of the promoter and other expression control elements. In particular embodiments of the invention, the polynucleotide to be transferred contains two or more expression cassettes, each of which contains at least one polynucleotide of interest.

[0091] By "expression control element" is intended a regulatory region of DNA, usually comprising a TATA box, capable of directing RNA polymerase II, or in some embodiments, RNA polymerase III, to initiate RNA synthesis at the appropriate transcription initiation site for a particular coding sequence. An expression control element may additionally comprise other recognition sequences generally positioned upstream or 5' to the TATA box, which

influence (e.g., enhance) the transcription initiation rate. Furthermore, an expression control element may additionally comprise sequences generally positioned downstream or 3' to the TATA box, which influence (e.g., enhance) the transcription initiation rate.

[0092] The transcriptional initiation region (e.g., a promoter) may be native or homologous or foreign or heterologous to the microalgal host or duckweed plant, or could be the natural sequence or a synthetic sequence. By foreign, it is intended that the transcriptional initiation region is not found in the wild-type microalgal host or duckweed plant into which the transcriptional initiation region is introduced. By "functional promoter" is intended the promoter, when operably linked to a sequence encoding an NDV HN polypeptide, or fragment or variant thereof, is capable of driving expression (i.e., transcription and translation) of the encoded polypeptide, fragment, or variant. The promoters can be selected based on the desired outcome. Thus the expression cassettes of the invention can comprise constitutive, inducible, tissue-preferred, or other promoters for expression in microalgae or duckweed.

[0093] Any suitable promoter known in the art can be employed in the expression cassettes according to the present invention, including bacterial, yeast, fungal, insect, mammalian, algae, and plant promoters. For example, plant or algae promoters, including microalgae promoters, may be used. Exemplary promoters include, but are not limited to, the Cauliflower Mosaic Virus 35S promoter, the opine synthetase promoters (e.g., nos, mas, ocs, etc.), the ubiquitin promoter, the actin promoter, the ribulose bisphosphate (RubP) carboxylase small subunit promoter, and the alcohol dehydrogenase promoter. U.S. patent No. 7,001,772 disclosed nucleic acid and amino acid sequences for an acetolactate synthase, an acetolactate synthase promoter and terminator region, an α-tubulin promoter, a promoter from a *Thraustochytriales* polyketide synthase (PKS) system, and a fatty acid desaturase promoter, each from a *Thraustochytriales* microorganism. U.S. patent application publications US2006/0275904 and US2006/0286650 disclose the sequences for promoters and terminators for each of *Schizochytrium* actin, elongation factor 1 alpha and glyceraldehyde 3-phosphate dehydrogenase as well as their use in vectors for expressing genes in *Schizochytrium* host cells. The microalgae RubP carboxylase small subunit promoter is known in the art (Silverthorne *et al.* (1990) *Plant Mol. Biol.* 15:49). Other promoters from viruses that infect plants or algae are also suitable including, but not limited to, promoters isolated from Dasheen mosaic virus, Chlorella virus (e.g., the Chlorella virus adenine methyltransferase promoter; Mitra *et al.* (1994) *Plant Mol. Biol.* 26:85), tomato spotted wilt virus, tobacco rattle virus, tobacco necrosis virus, tobacco ring spot virus, tomato

ring spot virus, cucumber mosaic virus, peanut stump virus, alfalfa mosaic virus, sugarcane baciliform badnavirus and the like.

[0094] Expression control elements, including promoters, can be chosen to give a desired level of regulation. For example, in some instances, it may be advantageous to use a promoter that confers constitutive expression (e.g, the mannopine synthase promoter from *Agrobacterium tumefaciens*). Alternatively, in other situations, it may be advantageous to use promoters that are activated in response to specific environmental stimuli (e.g., heat shock gene promoters, drought-inducible gene promoters, pathogen-inducible gene promoters, wound-inducible gene promoters, and light/dark-inducible gene promoters) or plant growth regulators (e.g., promoters from genes induced by abscissic acid, auxins, cytokinins, and gibberellic acid). As a further alternative, promoters can be chosen that give tissue-specific expression (e.g., root, leaf, and floral-specific promoters).

[0095] The overall strength of a given promoter can be influenced by the combination and spatial organization of cis-acting nucleotide sequences such as upstream activating sequences. For example, activating nucleotide sequences derived from the *Agrobacterium tumefaciens* octopine synthase gene can enhance transcription from the *Agrobacterium tumefaciens* mannopine synthase promoter (see U.S. Patent 5,955,646). In the present invention, the expression cassette can contain activating nucleotide sequences inserted upstream of the promoter sequence to enhance the expression of the antigenic NDV polypeptide of interest, or fragment or variant thereof. In one embodiment, the expression cassette includes three upstream activating sequences derived from the *Agrobacterium tumefaciens* octopine synthase gene operably linked to a promoter derived from an Agrobacterium tumefaciens mannopine synthase gene (see U.S Patent 5,955,646).

[0096] The expression cassette thus includes in the 5'-3' direction of transcription, an expression control element comprising a transcriptional and translational initiation region, a polynucleotide of encoding an NDV HN polypeptide (or fragment or variant thereof), and a transcriptional and translational termination region functional in plants or algae. Any suitable termination sequence known in the art may be used in accordance with the present invention. The termination region may be native with the transcriptional initiation region, may be native with the coding sequence of interest, or may be derived from another source. Convenient termination regions are available from the Ti-plasmid *of A. tumefaciens*, such as the octopine synthetase and nopaline synthetase termination regions. See also Guerineau *et al.* (1991) *Mol. Gen. Genet.* 262:141; Proudfoot (1991) *Cell* 64:671; Sanfacon *et al.* (1991) *Genes Dev.* 5:141; Mogen *et al.* (1990) *Plant Cell* 2:1261; Munroe *et al.* (1990) *Gene* 91:151; Ballas *et*

al. (1989) *Nucleic Acids Res.* 17:7891; and Joshi *et al.* (1987) *Nucleic Acids Res.* 15:9627. Additional exemplary termination sequences are the pea RubP carboxylase small subunit termination sequence and the Cauliflower Mosaic Virus 35S termination sequence.

[0097] Generally, the expression cassette will comprise a selectable marker gene for the selection of transformed microalgae cells or tissues. Selectable marker genes include genes encoding antibiotic resistance, such as those encoding neomycin phosphotransferase II (NEO) and hygromycin phosphotransferase (HPT), as well as genes conferring resistance to herbicidal compounds. Herbicide resistance genes generally code for a modified target protein insensitive to the herbicide or for an enzyme that degrades or detoxifies the herbicide in the plant before it can act. See DeBlock *et al.* (1987) *EMBO J.* 6:2513; DeBlock *et al.*(1989) *Plant Physiol.* 91:691; Fromm *et al.* (1990) *BioTechnology* 8:833; Gordon-Kamm *et al.* (1990) *Plant Cell* 2:603. For example, resistance to glyphosphate or sulfonylurea herbicides has been obtained using genes coding for the mutant target enzymes, 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS) and acetolactate synthase (ALS). Resistance to glufosinate ammonium, boromoxynil, and 2,4-dichlorophenoxyacetate (2,4-D) has been obtained by using bacterial genes encoding phosphinothricin acetyltransferase, a nitrilase, or a 2,4-dichlorophenoxyacetate monooxygenase, which detoxify the respective herbicides.

[0098] For purpose of the present invention, selectable marker genes include, but are not limited to, genes encoding neomycin phosphotransferase II (Fraley *et al.* (1986) *CRC Critical Reviews in Plant Science* 4:1); cyanamide hydratase (Maier-Greiner *et al.* (1991) *Proc. Natl. Acad. Sci. USA* 88:4250); acetolactate synthase (ALS, Li, *et al.* (1992) *Plant Physiol.* 100:662-668); aspartate kinase; dihydrodipicolinate synthase (Perl *et al.* (1993) *BioTechnology* 11:715); bar gene (Toki *et al.* (1992) *Plant Physiol.* 100:1503; Meagher *et al.* (1996) *Crop Sci.* 36:1367); tryptophan decarboxylase (Goddijn *et al.* (1993) *Plant Mol. Biol.* 22:907); neomycin phosphotransferase (NEO; Southern *et al.* (1982) *J. Mol. Appl. Gen.* 1:327); hygromycin phosphotransferase (HPT or HYG; Shimizu *et al.* (1986) *Mol. Cell. Biol.* 6:1074); dihydrofolate reductase (DHFR; Kwok *et al.* (1986) *Proc. Natl. Acad. Sci. USA* 83:4552); phosphinothricin acetyltransferase (DeBlock *et al.* (1987) *EMBO J.* 6:2513); 2,2-dichloropropionic acid dehalogenase (Buchanan-Wollatron *et al.* (1989) *J. Cell. Biochem.* 13D:330); acetohydroxyacid synthase (U.S. Pat. No. 4,761,373; Haughn *et al.* (1988) *Mol. Gen. Genet.* 221:266); 5-enolpyruvyl-shikimate-phosphate synthase (aroA; Comai *et al.* (1985) *Nature* 317:741); haloarylnitrilase (WO 87/04181); acetyl-coenzyme A carboxylase (Parker *et al.* (1990) *Plant Physiol.* 92:1220); dihydropteroate synthase (sulI; Guerineau *et al.*

(1990) *Plant Mol. Biol.* 15:127); and 32 kDa photosystem II polypeptide (psbA; Hirschberg *et al.* (1983) *Science* 222:1346 (1983).

[0099] Also included are genes encoding resistance to: gentamycin (*e.g.*, aacC1, Wohlleben *et al.* (1989) *Mol. Gen. Genet.* 217:202-208); chloramphenicol (Herrera-Estrella *et al.* (1983) *EMBO J.* 2:987); methotrexate (Herrera-Estrella *et al.* (1983) *Nature* 303:209; Meijer *et al.* (1991) *Plant Mol. Biol.* 16:807); hygromycin (Waldron *et al.* (1985) *Plant Mol. Biol.* 5:103; Zhijian *et al.* (1995) *Plant Science* 108:219; Meijer *et al.* (1991) *Plant Mol. Bio.* 16:807); streptomycin (Jones *et al.* (1987) *Mol. Gen. Genet.* 210:86); spectinomycin (Bretagne-Sagnard *et al.* (1996) *Transgenic Res.* 5:131); bleomycin (Hille *et al.* (1986) *Plant Mol. Biol.* 7:171); sulfonamide (Guerineau *et al.* (1990) *Plant Mol. Bio.* 15:127); bromoxynil (Stalker *et al.* (1988) *Science* 242:419); 2,4-D (Streber *et al.* (1989) *BioTechnology* 7:811); phosphinothricin (DeBlock *et al.* (1987) EMBO J. 6:2513); spectinomycin (Bretagne-Sagnard and Chupeau, *Transgenic Research* 5:131).

[0100] The bar gene confers herbicide resistance to glufosinate-type herbicides, such as phosphinothricin (PPT) or bialaphos, and the like. As noted above, other selectable markers that could be used in the vector constructs include, but are not limited to, the pat gene, also for bialaphos and phosphinothricin resistance, the ALS gene for imidazolinone resistance, the HPH or HYG gene for hygromycin resistance, the EPSP synthase gene for glyphosate resistance, the Hm1 gene for resistance to the Hc-toxin, and other selective agents used routinely and known to one of ordinary skill in the art. See Yarranton (1992) *Curr. Opin. Biotech.* 3:506; Chistopherson *et al.* (1992) *PNAS USA* 89:6314; Yao *et al.* (1992) *Cell* 71:63; Reznikoff (1992) *Mol. Microbiol.* 6:2419; Barkley *et al.* (1980) *The Operon* 177-220; Hu *et al.* (1987) *Cell* 48:555; Brown *et al.* (1987) *Cell* 49:603; Figge *et al.* (1988) *Cell* 52:713; Deuschle *et al.* (1989) *PNAS USA* 86:5400; Fuerst *et al.* (1989) *PNAS USA* 86:2549; Deuschle *et al.* (1990) *Science* 248:480; Labow *et al.* (1990) *Mol. Cell. Biol.* 10:3343; Zambretti *et al.* (1992) *PNAS USA* 89:3952; Baim *et al.* (1991) *PNAS USA* 88:5072; Wyborski *et al.* (1991) *Nuc. Acids Res.* 19:4647; Hillenand-Wissman (1989) *Topics in Mol. And Struc. Biol.* 10:143; Degenkolb *et al.* (1991) *Antimicrob. Agents Chemother.* 35:1591; Kleinschnidt *et al.* (1988) *Biochemistry* 27:1094; Gatz *et al.* (1992) *Plant J.* 2:397; Gossen *et al.* (1992) *PNAS USA* 89:5547; Oliva *et al.* (1992) *Antimicrob. Agents Chemother.* 36:913; Hlavka *et al.* (1985) *Handbook of Experimental Pharmacology* 78; and Gill *et al.* (1988) *Nature* 334:721.

[0101] The above list of selectable marker genes is not meant to be limiting. Any selectable marker gene can be used in the present invention.

Modification of Nucleotide Sequences for Enhanced Expression in a Microalgal Host or Duckweed Plant

[0102] Where the NDV HN polypeptide or fragment or variant thereof is expressed within microalgae or duckweed, the expressed polynucleotide sequence encoding the NDV HN polypeptide or fragment or variant thereof can be modified to enhance its expression in microalgae. One such modification is the synthesis of the polynucleotide using plant- or algae-preferred codons, particularly microalgae-preferred codons. Methods are available in the art for synthesizing nucleotide sequences with plant- or algae-preferred codons. See, e.g., U.S. Patent Nos. 5,380,831 and 5,436,391; EP 0 359 472; EP 0 385 962; WO 91/16432; Perlak *et al.* (1991) *PNAS USA* 15:3324; Iannacome *et al.* (1997) *Plant Mol. Biol.* 34:485; and Murray *et al.* (1989) *Nucleic Acids. Res.* 17:477. Synthesis can be accomplished using any method known to one of skill in the art. The preferred codons may be determined from the codons of highest frequency in the proteins expressed in microalgae. For example, the frequency of codon usage for microalgae is found in Table A.

Table A ***Schizochytrium sp. ATCC_20888*** **[gbpln]: 3 CDS's (6473 codons)**

fields: [triplet] [frequency: **per thousand**] ([number])

UUU 12.2(79)	UCU 7.0(45)	UAU 1.1(7)	UGU 0.8(5)
UUC 19.9(129)	UCC 23.8(154)	UAC 21.5(139)	UGC 15.3(99)
UUA 0.0(0)	UCA 0.5(3)	UAA 0.5(3)	UGA 0.0(0)
UUG 0.6(4)	UCG 18.8(122)	UAG 0.0(0)	UGG 8.3(54)
CUU 12.7(82)	CCU 11.7(76)	CAU 2.3(15)	CGU 7.1(46)
CUC 61.2(396)	CCC 23.8(154)	CAC 12.8(83)	CGC 42.9(278)
CUA 0.0(0)	CCA 1.5(10)	CAA 2.3(15)	CGA 0.3(2)
CUG 7.4(48)	CCG 16.2(105)	CAG 27.7(179)	CGG 0.8(5)
AUU 13.9(90)	ACU 9.1(59)	AAU 1.9(12)	AGU 1.5(10)
AUC 33.5(217)	ACC 29.2(189)	AAC 32.4(210)	AGC 15.6(101)
AUA 0.0(0)	ACA 1.5(10)	AAA 2.2(14)	AGA 0.2(1)
AUG 27.8(180)	ACG 9.6(62)	AAG 54.5(353)	AGG 0.0(0)
GUU 8.3(54)	GCU 24.4(158)	GAU 13.4(87)	GGU 13.0(84)
GUC 53.0(343)	GCC 86.0(557)	GAC 45.0(291)	GGC 54.5(353)
GUA 0.2(1)	GCA 4.0(26)	GAA 7.3(47)	GGA 3.9(25)
GUG 14.4(93)	GCG 15.9(103)	GAG 62.3(403)	GGG 0.5(3)

[0103] For purposes of the present invention, "microalgae-preferred codons" refers to codons that have a frequency of codon usage in microalgae of greater than 17%. The term "microalgae-preferred codons" as used herein refers to codons that have a frequency of codon usage in the family *Thraustochytriaceae* of greater than 17%. "*Schizochytrium*-preferred codons" as used herein refers to codons that have a frequency of codon usage in *schizochytrium* of greater than 17% where the frequency of codon usage in *schizochytrium* is obtained from the Codon Usage Database (GenBank Release 160.0, June 15, 2007).

[0104] The frequency of codon usage for *Lemna minor* is found in Table B, the frequency of codon usage for *Schizochytrium* is found in Table C.

Table B. ***Lemna minor*** **[gbpln]: 4 CDS's (1597 codons)**

fields: [triplet] [frequency: **per thousand**] ([number])

UUU 17.5(28)	UCU 13.8(22)	UAU 8.8(14)	UGU 5.0(8)
UUC 36.3(58)	UCC 17.5(28)	UAC 15.7(25)	UGC 14.4(23)
UUA 5.6(9)	UCA 14.4(23)	UAA 0.0(0)	UGA 1.9(3)
UUG 13.8(22)	UCG 13.8(22)	UAG 0.6(1)	UGG 16.3(26)
CUU 15.7(25)	CCU 11.9(19)	CAU 6.9(11)	CGU 4.4(7)
CUC 25.7(41)	CCC 15.7(25)	CAC 16.9(27)	CGC 18.2(29)
CUA 5.0(8)	CCA 11.3(18)	CAA 10.0(16)	CGA 6.3(10)
CUG 21.3(34)	CCG 14.4(23)	CAG 22.5(36)	CGG 10.6(17)
AUU 18.8(30)	ACU 9.4(15)	AAU 13.8(22)	AGU 10.0(16)
AUC 19.4(31)	ACC 17.5(28)	AAC 21.9(35)	AGC 15.0(24)
AUA 1.9(3)	ACA 5.0 (8)	AAA 15.7(25)	AGA 20.7(33)
AUG 20.7(33)	ACG 10.0(16)	AAG 35.7(57)	AGG 17.5(28)
GUU 15.0(24)	GCU 25.0(40)	GAU 20.0(32)	GGU 8.1(13)
GUC 25.0(40)	GCC 22.5(36)	GAC 26.3(42)	GGC 21.9(35)
GUA 6.3(10)	GCA 14.4(23)	GAA 26.3(42)	GGA 16.9(27)
GUG 30.7(49)	GCG 18.2(29)	GAG 40.1(64)	GGG 18.2(29)

Table C ***Schizochytrium sp. ATCC_20888*** **[gbpln]: 3 CDS's (6473 codons)**

fields: [triplet] [frequency: **per thousand**] ([number])

UUU 12.2(79)	UCU 7.0(45)	UAU 1.1(7)	UGU 0.8(5)
UUC 19.9(129)	UCC 23.8(154)	UAC 21.5(139)	UGC 15.3(99)
UUA 0.0(0)	UCA 0.5(3)	UAA 0.5(3)	UGA 0.0(0)
UUG 0.6(4)	UCG 18.8(122)	UAG 0.0(0)	UGG 8.3(54)

CUU 12.7(82)	CCU 11.7(76)	CAU 2.3(15)	CGU 7.1(46)
CUC 61.2(396)	CCC 23.8(154)	CAC 12.8(83)	CGC 42.9(278)
CUA 0.0(0)	CCA 1.5(10)	CAA 2.3(15)	CGA 0.3(2)
CUG 7.4(48)	CCG 16.2(105)	CAG 27.7(179)	CGG 0.8(5)
AUU 13.9(90)	ACU 9.1(59)	AAU 1.9(12)	AGU 1.5(10)
AUC 33.5(217)	ACC 29.2(189)	AAC 32.4(210)	AGC 15.6(101)
AUA 0.0(0)	ACA 1.5(10)	AAA 2.2(14)	AGA 0.2(1)
AUG 27.8(180)	ACG 9.6(62)	AAG 54.5(353)	AGG 0.0(0)
GUU 8.3(54)	GCU 24.4(158)	GAU 13.4(87)	GGU 13.0(84)
GUC 53.0(343)	GCC 86.0(557)	GAC 45.0(291)	GGC 54.5(353)
GUA 0.2(1)	GCA 4.0(26)	GAA 7.3(47)	GGA 3.9(25)
GUG 14.4(93)	GCG 15.9(103)	GAG 62.3(403)	GGG 0.5(3)

[0105] For purposes of the present invention, "duckweed-preferred codons" refers to codons that have a frequency of codon usage in duckweed of greater than 17%. "*Lemna*-preferred codons" as used herein refers to codons that have a frequency of codon usage in the genus *Lemna* of greater than 17%. "*Lemna minor*-preferred codons" as used herein refers to codons that have a frequency of codon usage in *Lemna minor* of greater than 17% where the frequency of codon usage in *Lemna minor* is obtained from the Codon Usage Database (GenBank Release 160.0, June 15, 2007).

[0106] It is further recognized that all or any part of the polynucleotide encoding the antigenic NDV polypeptide of interest, or fragment or variant thereof, may be optimized or synthetic. In other words, fully optimized or partially optimized sequences may also be used. For example, 40%, 45%, 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 87%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or 100% of the codons may be microalgae-preferred or duckweed-preferred codons. In one embodiment, between 90% and 96 % of the codons are microalgae-preferred or duckweed-preferred codons. In one embodiment, the NDV polypeptide is an NDV HN polypeptide, for example, the NDV HN polypeptide as set forth in SEQ ID NO:3 or 15, and the expression cassette comprises an optimized coding sequence for this NDV HN polypeptide, wherein the coding sequence comprises microalgae-preferred codons, for example, *Thraustochytriaceae* -preferred or *Schizochytrium* -preferred codons. In one such embodiment, the expression cassette comprises SEQ ID NO:1 or 14, which contains *Schizochytrium* -preferred codons encoding the HN polypeptide set forth in

SEQ ID NO:3 or 15. In another such embodiment, the expression cassette comprises SEQ ID NO:22 or 23, which contains *Schizochytrium*-preferred codons encoding the HN polypeptide set forth in SEQ ID NO: 17, or 20.

[0107] Other modifications can also be made to the polynucleotide encoding the antigenic NDV polypeptide of interest, or fragment or variant thereof, to enhance its expression in microalgae. These modifications include, but are not limited to, elimination of sequences encoding spurious polyadenylation signals, exon-intron splice site signals, transposon-like repeats, and other such well characterized sequences that may be deleterious to gene expression. When possible, the polynucleotide encoding the heterologous polypeptide of interest may be modified to avoid predicted hairpin secondary mRNA structures.

[0108] There are known differences between the optimal translation initiation context nucleotide sequences for translation initiation codons in animals and plants. "Translation initiation context nucleotide sequence" as used herein refers to the identity of the three nucleotides directly 5' of the translation initiation codon. "Translation initiation codon" refers to the codon that initiates the translation of the mRNA transcribed from the nucleotide sequence of interest. The composition of these translation initiation context nucleotide sequences can influence the efficiency of translation initiation. See, for example, Lukaszewicz *et al.* (2000) *Plant Science* 154:89-98; and Joshi *et al.* (1997); *Plant Mol. Biol.* 35:993-1001. In the present invention, the translation initiation context nucleotide sequence for the translation initiation codon of the polynucleotide encoding the antigenic NDV polypeptide of interest, or fragment or variant thereof, may be modified to enhance expression in microalgae. In one embodiment, the nucleotide sequence is modified such that the three nucleotides directly upstream of the translation initiation codon are "ACC." In a second embodiment, these nucleotides are "ACA."

[0109] Expression of an antigenic NDV polypeptide in microalgae or duckweed can also be enhanced by the use of 5' leader sequences. Such leader sequences can act to enhance translation. Translation leaders are known in the art and include, but are not limited to, picornavirus leaders, e.g., EMCV leader (Encephalomyocarditis 5' noncoding region; Elroy-Stein *et al.* (1989) *PNAS USA* 86:6126); potyvirus leaders, e.g., TEV leader (Tobacco Etch Virus; Allison *et al.* (1986) *Virology* 154:9); human immunoglobulin heavy-chain binding protein (BiP; Macajak and Sarnow (1991) *Nature* 353:90); untranslated leader from the coat protein mRNA of alfalfa mosaic virus (AMV RNA 4; Jobling and Gehrke (1987) *Nature* 325:622); tobacco mosaic virus leader (TMV; Gallie (1989) *Molecular Biology of RNA*,

23:56); potato etch virus leader (Tomashevskaya *et al.* (1993) *J. Gen. Virol.* 74:2717-2724); Fed-1 5' untranslated region (Dickey (1992) *EMBO J.* 11:2311-2317); RbcS 5' untranslated region (Silverthorne *et al.* (1990) *J. Plant. Mol. Biol.* 15:49-58); and maize chlorotic mottle virus leader (MCMV; Lommel *et al.* (1991) Virology 81:382). See also, Della-Cioppa *et al.* (1987) *Plant Physiology* 84:965. Leader sequence comprising plant intron sequence, including intron sequence from the maize alcohol dehydrogenase 1 (ADH1) gene, the castor bean catalase gene, or the *Arabidopsis* tryptophan pathway gene PAT1 has also been shown to increase translational efficiency in plants (Callis *et al.* (1987) *Genes Dev.* 1:1183-1200; Mascarenhas *et al.* (1990) *Plant Mol. Biol.* 15:913-920).

[0110] In some embodiments of the present invention, nucleotide sequence corresponding to nucleotides 1222-1775 of the maize alcohol dehydrogenase 1 gene (ADH1; GenBank Accession Number X04049) is inserted upstream of the polynucleotide encoding the NDV HN polypeptide, or fragment or variant thereof, to enhance the efficiency of its translation. In another embodiment, the expression cassette contains the leader from the *Lemna gibba* ribulose-bis-phosphate carboxylase small subunit 5B gene (RbcS leader; see Buzby *et al.* (1990) *Plant Cell* 2:805-814).

[0111] It is recognized that any of the expression-enhancing nucleotide sequence modifications described above can be used in the present invention, including any single modification or any possible combination of modifications. The phrase "modified for enhanced expression" in microalgae or duckweed, as used herein, refers to a polynucleotide sequence that contains any one or any combination of these modifications.

Signal Peptide.

[0112] The NDV polypeptide of interest can be normally or advantageously expressed as a secreted protein. Secreted proteins are usually translated from precursor polypeptides that include a "signal peptide" that interacts with a receptor protein on the membrane of the endoplasmic reticulum (ER) to direct the translocation of the growing polypeptide chain across the membrane and into the endoplasmic reticulum for secretion from the cell. This signal peptide may be cleaved from the precursor polypeptide to produce a "mature" polypeptide lacking the signal peptide. The signal peptide may not be cleaved and the entire polypeptide including the signal peptide is secreted from the cell. In an embodiment of the present invention, an NDV HN polypeptide, or fragment or variant thereof, is expressed in microalgae or duckweed from a polynucleotide sequence that is operably linked with a nucleotide sequence encoding a signal peptide that directs secretion of the NDV HN polypeptide, or fragment or variant thereof, into the culture medium. Plant or algae signal

peptides that target protein translocation to the endoplasmic reticulum (for secretion outside of the cell) are known in the art. See, for example, U.S. Patent No. 6,020,169. In the present invention, any plant or algae signal peptide can be used to target the expressed polypeptide to the ER.

[0113] In some embodiments, the signal peptide is the *Arabidopsis thaliana* basic endochitinase signal peptide (amino acids 14-34 of NCBI Protein Accession No. BAA82823), the extensin signal peptide (Stiefel *et al.* (1990) *Plant Cell* 2:785-793), the rice α-amylase signal peptide (amino acids 1-31 of NCBI Protein Accession No. AAA33885; see also GenBank M24286). In another embodiment, the signal peptide corresponds to the signal peptide of a secreted microalgae protein.

[0114] In one embodiment, the signal peptide of the present invention is the NDV HN signal peptide as set forth in SEQ ID NO:13 (encoded by a polynucleotide having the sequence as set forth in SEQ ID NO:12), or SEQ ID NO:21 (encoded by a polynucleotide having the sequence as set forth in SEQ ID NO:27). The NDV HN signal peptide of viral origin showed surprising result in the expression of the NDV protein in microalgae, where it directed the translocation of the polypeptide chain across the membrane and into the endoplasmic reticulum for secretion from the cell.

[0115] Alternatively, a mammalian signal peptide can be used to target the recombinantly produced antigenic NDV polypeptide for secretion from microalgae. It has been demonstrated that plant cells recognize mammalian signal peptides that target the endoplasmic reticulum, and that these signal peptides can direct the secretion of polypeptides not only through the plasma membrane but also through the plant cell wall. See U.S. Patent Nos. 5,202,422 and 5,639,947.

[0116] In one embodiment, the nucleotide sequence encoding the signal peptide is modified for enhanced expression in microalgae, utilizing any modification or combination of modifications disclosed above for the polynucleotide sequence of interest.

[0117] The secreted NDV HN polypeptide, or fragment or variant thereof, can be harvested from the culture medium by any conventional means known in the art, including, but not limited to, chromatography, electrophoresis, dialysis, solvent-solvent extraction, and the like. In so doing, partially or substantially purified antigenic NDV polypeptide, or fragment or variant thereof, can be obtained from the culture medium.

Transformed Microalgae or duckweed

[0118] The present invention provides transformed microalgae or duckweed plant expressing an NDV HN polypeptide, or fragment or variant thereof. The term "microalgae" refers to members of the family *Thraustochytriaceae*. This family currently is divided into four genera: *Schizochytrium, Thraustochytrium, Labyrinthuloides, and Japonochytrium*. Exemplary *Schizochytrium* include, but not limited to, *Schizochytrium aggregatum, Schizochytrium limacinum, Schizochytrium sp.* (S31) (ATCC 20888), *Schizochytrium sp.* (S8) (ATCC 20889), *Schizochytrium sp. (*LC-RM) (ATCC 18915), *Schizochytrium sp.* (SR21) (ATCC 28209) and deposited *Schizochytrium limacinum* strain IFO 32693 (Honda et Yokochi).

[0119] The term "duckweed" refers to members of the family *Lemnaceae*. This family currently is divided into five genera and 38 species of duckweed as follows: genus *Lemna* (*L. aequinoctialis*, *L. disperma*, *L. ecuadoriensis*, *L. gibba*, *L. japonica, L. minor*, *L. miniscula*, *L. obscura*, *L. perpusilla*, *L. tenera*, *L. trisulca*, *L. turionifera*, *L. valdiviana*); genus *Spirodela* (*S. intermedia*, *S. polyrrhiza*, *S. punctata*); genus *Wolffia* (*Wa. angusta*, *Wa. arrhiza*, *Wa. australina*, *Wa. borealis*, *Wa. brasiliensis*, *Wa. columbiana*, *Wa. elongata*, *Wa. globosa*, *Wa. microscopica*, *Wa. neglecta*); genus *Wolfiella* (*Wl. caudata*, *Wl. denticulata*, *Wl. gladiata*, *Wl. hyalina*, *Wl. lingulata*, *Wl. repunda*, *Wl. rotunda*, and *Wl. neotropica*) and genus *Landoltia (L. punctata)*. *Lemna* species can be classified using the taxonomic scheme described by Landolt (1986) *Biosystematic Investigation on the Family of Duckweeds: The family of Lemnaceae—A Monograph Study* (Geobatanischen Institut ETH, Stiftung Rubel, Zurich).

[0120] As used herein, "plant" includes whole plants, plant organs (*e.g.*, fronds (leaves), stems, roots, *etc.*), seeds, plant cells, and progeny of same. Parts of transgenic plants are to be understood within the scope of the invention to comprise, *e.g.*, plant cells, plant protoplasts, plant cell tissue cultures from which plants can be regenerated, tissues, plant calli, embryos as well as flowers, ovules, stems, fruits, leaves, roots, root tips, nodules, and the like originating in transgenic plants or their progeny previously transformed with a polynucleotide of interest and therefore consisting at least in part of transgenic cells.

[0121] The transformed microalgae or duckweed plants of the invention can be obtained by introducing an expression construct comprising a polynucleotide encoding an NDV HN polypeptide, or fragment or variant thereof, into the microalgae or duckweed plant of interest.

[0122] The term "introducing" in the context of a polynucleotide, for example, an expression construct comprising a polynucleotide encoding an antigenic NDV polypeptide, or fragment or variant thereof, is intended to mean presenting to the microalgae or duckweed

plants the polynucleotide in such a manner that the polynucleotide gains access to the interior of a cell of the microalgae or duckweed. Where more than one polynucleotide is to be introduced, these polynucleotides can be assembled as part of a single nucleotide construct, or as separate nucleotide constructs, and can be located on the same or different transformation vectors. Accordingly, these polynucleotides can be introduced into the microalgae or duckweed host cell of interest in a single transformation event, in separate transformation events, or, for example, as part of a breeding protocol. The compositions and methods of the invention do not depend on a particular method for introducing one or more polynucleotides into microalgae, only that the polynucleotide(s) gains access to the interior of at least one cell of the microalgae or duckweed plants. Methods for introducing polynucleotides into plants or algae are known in the art including, but not limited to, transient transformation methods, stable transformation methods, and virus-mediated methods.

[0123] "Transient transformation" in the context of a polynucleotide such as a polynucleotide encoding an NDV HN polypeptide, or fragment or variant thereof, is intended to mean that a polynucleotide is introduced into the microalgae or duckweed and does not integrate into the genome of the microalgae or duckweed.

[0124] By "stably introducing" or "stably introduced" in the context of a polynucleotide (such as a polynucleotide encoding an NDV HN polypeptide, or fragment or variant thereof) introduced into microalgae or duckweed is intended the introduced polynucleotide is stably incorporated into the microalgae or duckweed genome, and thus the microalgae or duckweed plant is stably transformed with the polynucleotide.

[0125] "Stable transformation" or "stably transformed" is intended to mean that a polynucleotide, for example, a polynucleotide encoding an NDV HN polypeptide, or fragment or variant thereof, introduced into microalgae or duckweed plant integrates into the genome of the microalgae or plant and is capable of being inherited by the progeny thereof, more particularly, by the progeny of multiple successive generations. In some embodiments, successive generations include progeny produced vegetatively (i.e., asexual reproduction), for example, with clonal propagation. In other embodiments, successive generations include progeny produced via sexual reproduction.

[0126] An expression construct comprising a polynucleotide encoding an NDV HN polypeptide, or fragment or variant thereof, can be introduced into microalgae or plant of interest using any transformation protocol known to those of skill in art. Suitable methods of introducing nucleotide sequences into microalgae or plant cells or nodules include

microinjection (Crossway *et al.* (1986) *Biotechniques* 4:320-334), electroporation (Riggs *et al.* (1986) *PNAS USA* 83:5602-5606), *Agrobacterium*-mediated transformation (U.S. Patent Nos. 5,563,055 and 5,981,840), direct gene transfer (Paszkowski *et al.* (1984) *EMBO J.* 3:2717-2722), ballistic particle acceleration (see, *e.g.*, U.S. Patent Nos. 4,945,050; 5,879,918; 5,886,244; and 5,932,782); and Tomes *et al.* (1995) "Direct DNA Transfer into Intact Plant Cells via Microprojectile Bombardment," in *Plant Cell, Tissue, and Organ Culture: Fundamental Methods*, ed. Gamborg and Phillips (Springer-Verlag, Berlin); McCabe *et al.* (1988) *Biotechnology* 6:923-926). The cells that have been transformed may be grown into plants or algae in accordance with conventional ways.

[0127] As noted above, stably transformed microalgae or plants can be obtained by any gene transfer method known in the art, such as one of the gene transfer methods disclosed in U.S. Patent No. 6,040,498 or U.S. Patent Application Publication Nos. 2003/0115640, 2003/0033630 or 2002/0088027. Microalgal or plants can be efficiently transformed with an expression cassette containing a nucleic acid sequence as described herein by any one of a number of methods including *Agrobacterium*-mediated gene transfer, ballistic bombardment or electroporation. The *Agrobacterium* used can be *Agrobacterium tumefaciens* or *Agrobacterium rhizogenes*. Stable microalgae or plant transformants can be isolated by transforming the microalgae or plant cells with both the nucleic acid sequence of interest and a gene that confers resistance to a selection agent, followed by culturing the transformed cells in a medium containing the selection agent. *See*, for example, U.S. Patent No. 6,040,498.

[0128] The stably transformed microalgae or plants utilized in these methods should exhibit normal morphology and be fertile by sexual reproduction and/or able to reproduce vegetatively (i.e., asexual reproduction), for example, with clonal propagation. Preferably, transformed microalgae or plants of the present invention contain a single copy of the transferred nucleic acid comprising a polynucleotide encoding an NDV HN polypeptide, or fragment or variant thereof, and the transferred nucleic acid has no notable rearrangements therein. It is recognized that the transformed microalgae of the invention may contain the transferred nucleic acid present in low copy numbers (i.e., no more than twelve copies, no more than eight copies, no more than five copies, alternatively, no more than three copies, as a further alternative, fewer than three copies of the nucleic acid per transformed cell).

[0129] Transformed plants or algae expressing an NDV HN polypeptide, or fragment or variant thereof, can be cultured under suitable conditions for expressing the NDV HN polypeptide, or fragment or variant thereof. The NDV HN polypeptide, or fragment or variant thereof, can then be harvested from the microalgae, the culture medium, or the

microalgae and the culture medium, and, where desired, purified using any conventional isolation and purification method known in the art, including chromatography, electrophoresis, dialysis, solvent-solvent extraction, and the like. The NDV HN polypeptide, or fragment or variant thereof, can then be formulated as a vaccine for therapeutic applications, as described elsewhere herein.

Methods of Preparing an NDV Polypeptide

[0130] As described fully herein, in an embodiment, a method of producing a recombinant NDV HN polypeptide comprises: (a) culturing microalgal or duckweed plants within a microalgal or duckweed culture medium, wherein the microalgal or duckweed plants are stably transformed to express the NDV polypeptide, and wherein the NDV polypeptide is expressed from a nucleotide sequence comprising a coding sequence for said recombinant NDV polypeptide and an operably linked coding sequence for a signal peptide that directs secretion of the NDV polypeptide into the culture medium; and (b) collecting the NDV polypeptide from said culture medium. The term collecting includes but is not limited to harvesting from the culture medium or purifying.

[0131] After production of the recombinant polypeptide in microalgae or plants, any method available in the art may be used for protein purification. The various steps include freeing the protein from the nonprotein or algae or plant material, followed by the purification of the protein of interest from other proteins. The recombinant protein may be a secreted protein that is isolated from the culture medium following its production by the cell and may comprise a signal peptide. Said signal peptide may be cleaved following secretion, to produce a mature protein product. Depending on the vector and host system used for production, resultant recombinant NDV polypeptides of the present invention may either remain within the recombinant cell or be secreted into the fermentation medium or be secreted into a space between two cellular membranes; or be retained on the outer surface of a cell membrane. Initial steps in the purification process include centrifugation, filtration or a combination thereof. After the initial centrifugation at low speed, the low speed supernatant may be used for pharmaceutical composition or vaccine preparation. The low speed supernatant may undergo further purification using various method described below. Proteins secreted within the extracellular space of tissues can be obtained using vacuum or centrifugal extraction. Minimal processing could also involve preparation of crude products. Other methods include maceration and extraction in order to permit the direct use of the extract.

[0132] Recombinant proteins produced by the method of the present invention may be purified using a variety of standard protein purification techniques, such as, but not limited

to, centrifugation, filtration, affinity chromatography, ion exchange chromatography, eletrophoresis, hydrophobic interaction chromatography, gel filtration chromatography, reverse phase chromatography, concanavalin A chromatography, chromatofocusing and differential solubilization. Methods to purify the protein of interest may exploit differences in protein size, physio-chemical properties, and binding affinity. Such methods include chromatography, including procainamide affinity, size exclusion, high pressure liquid, reversed-phase, and anion-exchange chromatography, affinity tags, etc. In particular, immobilized Ni-ion affinity chromatography can be used to purify the expressed protein. See, Favacho *et al.* (2006) Protein expression and purification 46:196-203. See also, Zhou *et al.* (2007) The Protein J 26:29-37; Wang *et al.* (2006) Vaccine 15:2176-2185; and WO/2009/076778. Protectants may be used in the purification process such as osmotica, antioxidants, phenolic oxidation inhibitors, protease inhibitors, and the like.

Methods of Use and Article of Manufacture

[0133] The present invention includes the following method embodiments. In an embodiment, a method of vaccinating an animal comprising administering a composition comprising NDV antigenic polypeptide(s) or fragment or variant thereof and a pharmaceutical or veterinarily acceptable carrier, excipient, or vehicle to an animal is disclosed. In one aspect of this embodiment, the animal is an avian, an equine, a canine, a feline or a porcine.

[0134] In yet another embodiment, the vaccine or composition may be administered to one-day-old or older chickens.

[0135] In one embodiment of the invention, a prime-boost regimen can be employed, which is comprised of at least one primary administration and at least one booster administration using at least one common polypeptide, antigen, epitope or immunogen. Typically the immunological composition or vaccine used in primary administration is different in nature from those used as a booster. However, it is noted that the same composition can be used as the primary administration and the booster administration. This administration protocol is called "prime-boost".

[0136] In the present invention a recombinant viral vector is used to express an NDV coding sequence or fragments thereof encoding an antigenic NDV polypeptide or fragment or variant thereof. Specifically, the viral vector can express an NDV sequence, more specifically an NDV HN gene or fragment thereof that encodes an antigenic polypeptide. Viral vector contemplated herein includes, but not limited to, poxvirus [e.g., vaccinia virus or

attenuated vaccinia virus, avipox virus or attenuated avipox virus (e.g., canarypox, fowlpox, dovepox, pigeonpox, quailpox, ALVAC, TROVAC; see e.g., US 5,505,941, US 5,494,8070), raccoonpox virus, swinepox virus, etc.], adenovirus (e.g., human adenovirus, canine adenovirus), herpesvirus (e.g. canine herpesvirus, herpesvirus of turkey, Marek's disease virus, infectious laryngotracheitis virus, feline herpesvirus, laryngotracheitis virus (ILTV), bovine herpesvirus, swine herpesvirus), baculovirus, retrovirus, etc. In another embodiment, the avipox expression vector may be a canarypox vector, such as, ALVAC. In yet another embodiment, the avipox expression vector may be a fowlpox vector, such as, TROVAC. The NDV polypeptide, antigen, epitope or immunogen may be an NDV HN. For example, the TROVAC vector comprising the NDV HN or F may be vectors as described in US 7,144,578 and US 2008/0188640, the ILTV vector comprising the NDV antigens including HN and F may be vectors as described in US 6,306,400 and US 6,153,199. The NDV polypeptide or antigen of the invention to be expressed in a viral vector is inserted under the control of a specific poxvirus promoter, e.g., the vaccinia promoter 7.5 kDa (Cochran et al., 1985), the vaccinia promoter I3L (Riviere et al., 1992), the vaccinia promoter HA (Shida, 1986), the cowpox promoter ATI (Funahashi et al., 1988), the vaccinia promoter H6 (Taylor et al., 1988b; Guo et al., 1989; Perkus et al., 1989), *inter alia*.

[0137] In another aspect of the prime-boost protocol or regime of the invention, a composition comprising an NDV antigen of the invention is administered followed by the administration of a recombinant viral vector or a plasmid vector that contains and expresses an NDV antigen and/or variants or fragments thereof *in vivo*. Likewise, a prime-boost protocol may comprise the administration of a recombinant viral vector or a plasmid vector followed by the administration of a recombinant NDV antigen of the invention. It is further noted that both the primary and the secondary administrations may comprise the recombinant NDV antigen of the invention. Thus, the recombinant NDV antigen of the invention may be administered in any order with a viral vector or alternatively may be used alone as both the primary and secondary compositions.

[0138] In yet another aspect of the prime-boost protocol of the invention, a composition comprising an NDV antigen of the invention is administered followed by the administration of an inactivated viral composition or vaccine comprising the NDV antigen. Likewise, a prime-boost protocol may comprise the administration of an inactivated viral composition or vaccine followed by the administration of a recombinant NDV antigen of the invention. It is further noted that both the primary and the secondary administrations may comprise the recombinant antigenic polypeptide of the invention. The antigenic polypeptides of the

invention may be administered in any order with an inactivated viral composition or vaccine or alternatively may be used alone as both the primary and secondary compositions.

[0139] A prime-boost regimen comprises at least one prime-administration and at least one boost administration using at least one common polypeptide and/or variants or fragments thereof. The vaccine used in prime-administration may be different in nature from those used as a later booster vaccine. The prime-administration may comprise one or more administrations. Similarly, the boost administration may comprise one or more administrations.

[0140] The dose volume of compositions for target species that are mammals, e.g., the dose volume of avian compositions, based on viral vectors, e.g., non-poxvirus-viral-vector-based compositions, is generally between about 0.1 to about 2.0 ml, between about 0.1 to about 1.0 ml, and between about 0.5 ml to about 1.0 ml.

[0141] The compositions comprising the recombinant antigenic polypeptides of the invention used in the prime-boost protocols are contained in a pharmaceutically or veterinary acceptable vehicle, diluent or excipient. The protocols of the invention protect the animal from NDV and/or prevent disease progression in an infected animal.

[0142] The various administrations are preferably carried out 1 to 6 weeks apart. According to one embodiment, an annual booster is also envisioned. The animals are at least one-day-old at the time of the first administration.

[0143] The efficacy of the vaccines may be tested about 2 to 4 weeks after the last immunization by challenging animals, such as avian, with a virulent strain of NDV. Both homologous and heterologous strains are used for challenge to test the efficacy of the vaccine. The animal may be challenged by spray, intra-nasally, intra-ocularly, intra-tracheally, and/or orally. The challenge viral may be about 10^{5-8} EID_{50} in a volume depending upon the route of administration. For example, if the administration is by spray, a virus suspension is aerosolized to generate about 1 to 100 μm droplets, if the administration is intra-nasal, intra-tracheal or oral, the volume of the challenge virus is about 0.5 ml, 1-2 ml, and 5-10 ml, respectively. Animals may be observed daily for 14 days following challenge for clinical signs, for example, dehydration and pasty vents. In addition, the groups of animals may be euthanized and evaluated for pathological findings of pulmonary and pleural hemorrhage, tracheitis, bronchitis, bronchiolitis, and bronchopneumonia. Orophayngeal swabs may be collected from all animals post challenge for virus isolation. The presence or absence of viral antigens in respiratory tissues may be evaluated by quantitative real time reverse transcriptase polymerase chain reaction (qRRT-PCR). Blood samples may be

collected before and post-challenge and may be analyzed for the presence of NDV-specific antibody.

[0144] It should be understood by one of skill in the art that the disclosure herein is provided by way of example and the present invention is not limited thereto. From the disclosure herein and the knowledge in the art, the skilled artisan can determine the number of administrations, the administration route, and the doses to be used for each injection protocol, without any undue experimentation.

[0145] The present invention contemplates at least one administration to an animal of an efficient amount of the therapeutic composition made according to the invention. The animal may be male, female, pregnant female and newborn. This administration may be via various routes including, but not limited to, intramuscular (IM), intradermal (ID) or subcutaneous (SC) injection or via intranasal or oral administration. The therapeutic composition according to the invention can also be administered by a needleless apparatus (as, for example with a Pigjet, Dermojet, Biojector, Avijet (Merial, GA, USA), Vetjet or Vitajet apparatus (Bioject, Oregon, USA)). Another approach to administering plasmid compositions is to use electroporation (see, e.g. Tollefsen et al., 2002; Tollefsen et al., 2003; Babiuk et al., 2002; PCT Application No. WO99/01158). In another embodiment, the therapeutic composition is delivered to the animal by gene gun or gold particle bombardment.

[0146] In one embodiment, the subject matter disclosed herein provides a vaccination regime and detection method for differentiation between infected and vaccinated animals (DIVA).

[0147] A strategy that allows "differentiation of infected from vaccinated animals" (DIVA), has been put forward as a possible solution for the eventual eradication of virus without involving mass culling of birds and the consequent economic damage, especially in developing countries (Food and Agriculture Organization of the United (FAO) (2004). FAO, OIE & WHO Technical consultation on the Control of NDV. Animal health special report). This strategy has the benefits of vaccination (less virus in the environment) with the ability to identify infected flocks which still allows the implementation of other control measures, including stamping out. At the flock level, a simple approach is to regularly monitor sentinel birds left unvaccinated in each vaccinated flock, but this may cause some management problems, particularly in identifying the sentinels in large flocks. As an alternative, testing for field exposure may be performed on the vaccinated birds. Alternatively the use of vaccines that contains only NDV HN subunit (protein) would allow classical AGID and NP-or matrix-based ELISAs to be used to detect infection in vaccinated birds.

[0148] It is disclosed herein that the use of the vaccine or composition of the present invention allows the detection of NDV infection in a vaccinated animal using available diagnosis test aiming to detect antibody response against NDV proteins other than HA such as agar gel immunodiffusion or NP-based ELISA. It is disclosed herein that the use of the vaccine or composition of the present invention allows the detection of the infection in animals by differentiating between infected and vaccinated animals (DIVA). A method is disclosed herein for diagnosing the infection of NDV in an animal using NP-based immunogenic detection method, such as, NP-based ELISA. In one embodiment, the subject matter disclosed herein is directed to a method of diagnosing NDV infection in an animal, comprising: a) contacting a solid substrate comprising a nucleoprotein (NP) with a sample obtained from the animal; b) contacting the solid substrate with a monoclonal antibody (MAb) against the NP; and c) detecting binding of the MAb to the sample captured by the NP on the solid substrate, wherein the percentage inhibition of test sample relative to the negative control indicates that the subject is infected with NDV, thereby diagnosing NDV infection in the subject.

[0149] Another embodiment of the invention is a kit for performing a method of eliciting or inducing an immunological or protective response against NDV in an animal comprising a recombinant NDV HN immunological composition or vaccine and instructions for performing the method of delivery in an effective amount for eliciting an immune response in the animal.

[0150] In an embodiment, the subject matter disclosed herein is directed to a kit for performing a method of eliciting or inducing an immune response which may comprise any one of the recombinant NDV immunological compositions or vaccines, or inactivated NDV immunological compositions or vaccines, recombinant NDV viral compositions or vaccines, and instructions for performing the method.

[0151] Another embodiment of the invention is a kit for performing a method of inducing an immunological or protective response against NDV in an animal comprising a composition or vaccine comprising an NDV antigen of the invention and a recombinant NDV viral immunological composition or vaccine, and instructions for performing the method of delivery in an effective amount for eliciting an immune response in the animal.

[0152] Another embodiment of the invention is a kit for performing a method of inducing an immunological or protective response against NDV in an animal comprising a composition or vaccine comprising an NDV antigen of the invention and an inactivated NDV

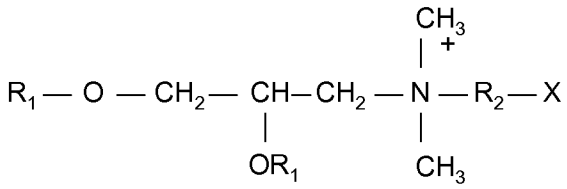
immunological composition or vaccine, and instructions for performing the method of delivery in an effective amount for eliciting an immune response in the animal.

[0153] Yet another aspect of the present invention relates to a kit for prime-boost vaccination according to the present invention as described above. The kit may comprise at least two vials: a first vial containing a vaccine or composition for the prime-vaccination according to the present invention, and a second vial containing a vaccine or composition for the boost-vaccination according to the present invention. The kit may advantageously contain additional first or second vials for additional prime-vaccinations or additional boost-vaccinations.

[0154] In one embodiment, the invention provides for the administration of a therapeutically effective amount of a vaccine or composition for the delivery and expression of an NDV HN antigen or epitope in a target cell. Determination of the therapeutically effective amount is routine experimentation for one of ordinary skill in the art. In one embodiment, the vaccine or composition comprises a recombinant NDV HN polypeptide, antigen or epitope and a pharmaceutically or veterinarily acceptable carrier, vehicle or excipient. In another embodiment, the pharmaceutically or veterinarily acceptable carrier, vehicle or excipient facilitates transfection or infection and/or improves preservation of the vector or protein.

[0155] The pharmaceutically or veterinarily acceptable carriers or vehicles or excipients are well known to the one skilled in the art. For example, a pharmaceutically or veterinarily acceptable carrier or vehicle or excipient can be a 0.9% NaCl (e.g., saline) solution or a phosphate buffer. Other pharmaceutically or veterinarily acceptable carrier or vehicle or excipients that can be used for methods of this invention include, but are not limited to, poly-(L-glutamate) or polyvinylpyrrolidone. The pharmaceutically or veterinarily acceptable carrier or vehicle or excipients may be any compound or combination of compounds facilitating the administration of the vector or recombinant proteins; advantageously, the carrier, vehicle or excipient may facilitate transfection and/or improve preservation of the vector or protein. Doses and dose volumes are herein discussed in the general description and can also be determined by the skilled artisan from this disclosure read in conjunction with the knowledge in the art, without any undue experimentation.

[0156] The cationic lipids containing a quaternary ammonium salt which are, but not exclusively suitable for plasmids, those having the following formula:



[0157] in which R1 is a saturated or unsaturated straight-chain aliphatic radical having 12 to 18 carbon atoms, R2 is another aliphatic radical containing 2 or 3 carbon atoms and X is an amine or hydroxyl group, e.g. the DMRIE. In another embodiment the cationic lipid can be associated with a neutral lipid, e.g. the DOPE.

[0158] Among these cationic lipids, preference is given to DMRIE (N-(2-hydroxyethyl)-N,N-dimethyl-2,3-bis(tetradecyloxy)-1-propane ammonium; WO96/34109), advantageously associated with a neutral lipid, advantageously DOPE (dioleoyl-phosphatidyl-ethanol amine; Behr, 1994), to form DMRIE-DOPE.

[0159] When DOPE is present, the DMRIE:DOPE molar ratio is advantageously about 95: about 5 to about 5: about 95, more advantageously about 1: about 1, e.g., 1:1.

[0160] In another embodiment, pharmaceutically or veterinarily acceptable carrier, excipient, vehicle, or adjuvant may be a water-in-oil emulsion. Examples of suitable water-in-oil emulsions include oil-based water-in-oil vaccinal emulsions which are stable and fluid at 4°C containing: from 6 to 50 v/v % of an antigen-containing aqueous phase, preferably from 12 to 25 v/v %, from 50 to 94 v/v % of an oil phase containing in total or in part a non-metabolizable oil (e.g., mineral oil such as paraffin oil) and/or metabolizable oil (e.g., vegetable oil, or fatty acid, polyol or alcohol esters), from 0.2 to 20 p/v % of surfactants, preferably from 3 to 8 p/v %, the latter being in total or in part, or in a mixture either polyglycerol esters, said polyglycerol esters being preferably polyglycerol (poly)ricinoleates, or polyoxyethylene ricin oils or else hydrogenated polyoxyethylene ricin oils. Examples of surfactants that may be used in a water-in-oil emulsion include ethoxylated sorbitan esters (e.g., polyoxyethylene (20) sorbitan monooleate (TWEEN 80®), available from AppliChem, Inc., Cheshire, CT) and sorbitan esters (e.g., sorbitan monooleate (SPAN 80®), available from Sigma Aldrich, St. Louis, MO). In addition, with respect to a water-in-oil emulsion, see also US 6,919,084. The water-in-oil emulsion may comprise 75% oil phase containing mineral oil and 4% SPAN 80® and 25% aqueous phase containing 0.4% TWEEN 80®. In some embodiments, the antigen-containing aqueous phase comprises a saline solution comprising one or more buffering agents. An example of a suitable buffering solution is phosphate buffered saline. In one embodiment, the water-in-oil emulsion may be a water/oil/water (W/O/W) triple emulsion (U.S. 6,358,500). The WOW triple emultion may

comprise 60% oil phase containing mineral oil and 6% SPAN 80® and 40% aqueous phase containing 1.6% TWEEN 80®. Examples of other suitable emulsions are described in U.S. 7,371,395.

[0161] The immunological compositions and vaccines according to the invention may comprise or consist essentially of one or more pharmaceutically or veterinarily acceptable carriers, excipients, vehicles, or adjuvants. Suitable carriers or adjuvants for use in the practice of the present invention are (1) polymers of acrylic or methacrylic acid, maleic anhydride and alkenyl derivative polymers, (2) immunostimulating sequences (ISS), such as oligodeoxyribonucleotide sequences having one or more non-methylated CpG units (Klinman et al., 1996; WO98/16247), (3) an oil in water emulsion, such as the SPT emulsion described on page 147 of "Vaccine Design, The Subunit and Adjuvant Approach" published by M. Powell, M. Newman, Plenum Press 1995, and the emulsion MF59 described on page 183 of the same work, (4) cation lipids containing a quaternary ammonium salt, e.g., DDA (5) cytokines, (6) aluminum hydroxide or aluminum phosphate, (7) saponin or (8) other adjuvants discussed in any document cited and incorporated by reference into the instant application, or (9) any combinations or mixtures thereof.

[0162] The oil in water emulsion (3), which is especially appropriate for viral vectors, can be based on: light liquid paraffin oil (European pharmacopoeia type), isoprenoid oil such as squalane, squalene, oil resulting from the oligomerization of alkenes, e.g. isobutene or decene, esters of acids or alcohols having a straight-chain alkyl group, such as vegetable oils, ethyl oleate, propylene glycol, di(caprylate/caprate), glycerol tri(caprylate/caprate) and propylene glycol dioleate, or esters of branched, fatty alcohols or acids, especially isostearic acid esters.

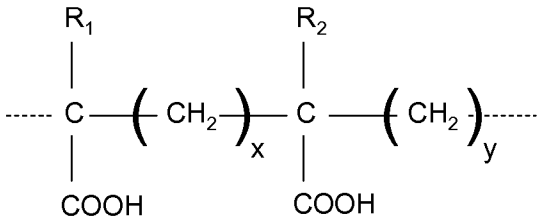
[0163] The oil is used in combination with emulsifiers to form an emulsion. The emulsifiers may be nonionic surfactants, such as: esters of on the one hand sorbitan, mannide (e.g. anhydromannitol oleate), glycerol, polyglycerol or propylene glycol and on the other hand oleic, isostearic, ricinoleic or hydroxystearic acids, said esters being optionally ethoxylated, or polyoxypropylene-polyoxyethylene copolymer blocks, such as Pluronic, e.g., L121.

[0164] Among the type (1) adjuvant polymers, preference is given to polymers of crosslinked acrylic or methacrylic acid, especially crosslinked by polyalkenyl ethers of sugars or polyalcohols. These compounds are known under the name carbomer (Pharmeuropa, vol. 8, no. 2, June 1996). One skilled in the art can also refer to U.S. 2,909,462, which provides such acrylic polymers crosslinked by a polyhydroxyl compound having at least three

hydroxyl groups, preferably no more than eight such groups, the hydrogen atoms of at least three hydroxyl groups being replaced by unsaturated, aliphatic radicals having at least two carbon atoms. The preferred radicals are those containing 2 to 4 carbon atoms, e.g. vinyls, allyls and other ethylenically unsaturated groups. The unsaturated radicals can also contain other substituents, such as methyl. Products sold under the name Carbopol (BF Goodrich, Ohio, USA) are especially suitable. They are crosslinked by allyl saccharose or by allyl pentaerythritol. Among them, reference is made to Carbopol 974P, 934P and 971P.

[0165] As to the maleic anhydride-alkenyl derivative copolymers, preference is given to EMA (Monsanto), which are straight-chain or crosslinked ethylene-maleic anhydride copolymers and they are, for example, crosslinked by divinyl ether. Reference is also made to J. Fields et al., 1960.

[0166] With regard to structure, the acrylic or methacrylic acid polymers and EMA are preferably formed by basic units having the following formula:



in which:

- R1 and R2, which can be the same or different, represent H or CH3
- x = 0 or 1, preferably x = 1
- y = 1 or 2, with x + y = 2.

For EMA, x = 0 and y = 2 and for carbomers x = y = 1.

[0167] These polymers are soluble in water or physiological salt solution (20 g/l NaCl) and the pH can be adjusted to 7.3 to 7.4, e.g., by soda (NaOH), to provide the adjuvant solution in which the expression vector(s) can be incorporated. The polymer concentration in the final immunological or vaccine composition can range between about 0.01 to about 1.5% w/v, about 0.05 to about 1% w/v, and about 0.1 to about 0.4% w/v.

[0168] The cytokine or cytokines (5) can be in protein form in the immunological or vaccine composition, or can be co-expressed in the host with the immunogen or immunogens or epitope(s) thereof. Preference is given to the co-expression of the cytokine or cytokines, either by the same vector as that expressing the immunogen or immunogens or epitope(s) thereof, or by a separate vector thereof.

[0169] The invention comprehends preparing such combination compositions; for instance by admixing the active components, advantageously together and with an adjuvant, carrier, cytokine, and/or diluent.

[0170] Cytokines that may be used in the present invention include, but are not limited to, granulocyte colony stimulating factor (G-CSF), granulocyte/macrophage colony stimulating factor (GM-CSF), interferon α (IFNα), interferon β (IFNβ), interferon γ (IFNγ), interleukin-1α(IL-1α), interleukin-1β (IL-1β), interleukin-2 (IL-2), interleukin-3 (IL-3), interleukin-4 (IL-4), interleukin-5 (IL-5), interleukin-6 (IL-6), interleukin-7 (IL-7), interleukin-8 (IL-8), interleukin-9 (IL-9), interleukin-10 (IL-10), interleukin-11 (IL-11), interleukin-12 (IL-12), tumor necrosis factor α (TNFα), tumor necrosis factor β (TNFβ), and transforming growth factor β (TGFβ). It is understood that cytokines can be co-administered and/or sequentially administered with the immunological or vaccine composition of the present invention. Thus, for instance, the vaccine of the instant invention can also contain an exogenous nucleic acid molecule that expresses *in vivo* a suitable cytokine, e.g., a cytokine matched to this host to be vaccinated or in which an immunological response is to be elicited (for instance, an avian cytokine for preparations to be administered to avians).

[0171] The invention will now be further described by way of the following non-limiting examples.

EXAMPLES

[0172] Without further elaboration, it is believed that one skilled in the art can, using the preceding descriptions, practice the present invention to its fullest extent. The following detailed examples are to be construed as merely illustrative, and not limitations of the preceding disclosure in any way whatsoever. Those skilled in the art will promptly recognize appropriate variations from the procedures both as to reactants and as to reaction conditions and techniques.

[0173] Construction of DNA inserts, plasmids and recombinant microalgal or plant vectors was carried out using the standard molecular biology techniques described by J. Sambrook *et al.* (Molecular Cloning: A Laboratory Manual, 2nd Edition, Cold Spring Harbor Laboratory, Cold Spring Harbor, New York, 1989).

Example 1 Construction of vectors for the NDV HN gene and transformation

[0174] The vector pAB0018 (ATCC deposit no. PTA9616) was digested with *Bam*HI and *Nde*I resulting in two fragments of 838 bp and 9879 bp. The larger fragment was fractionated by standard electrophoretic techniques in an agar gel, and purified using commercial DNA purification kits. This 9879 bp fragment was ligated to a codon-optimized NDV HN gene (SEQ ID NO:1) which had also been previously digested with *Bam*HI and *Nde*I. The ligation was then used to transform commercially supplied strains of competent *E. coli* DH5-α cells (Invitrogen, Carlsbad, CA, USA) using the manufacturer's protocols. These plasmids were then screened by restriction digests or PCR to confirm that the ligation generated the expected plasmid structures. One such plasmid vector resulting from this procedure was verified by Sanger sequencing and designated pCL0081. The specific nucleic acid sequence of NDV HN gene has been optimized for expression in *Schizochytrium* sp. Additionally, the vector pCL0081 contained a selection marker cassette conferring resistance to *Schizochytrium* transformants grown in the presence of sulfometuron methy, a promoter from the *Schizochytrium* elongation factor-1 gene (EF1) to drive expression of the HN transgene, and following the HN transgene, and the OrfC terminator (also known as the PFA3 terminator).

[0175] *Schizochytrium* sp. (ATCC 20888) was used as a host for transformation with the vector pCL0081 using electroporation method. Cells were grown in M50-20 media on a shaker at 200 rpm for 48 hrs at 29°C. The cells were diluted at 1:100 into fresh media and grown overnight. The cells were centrifuged and resuspended in 1 M mannitol, 10mM CaCl2 (pH 5.5) to a final concentration of 2 OD_{600} units. 5 mL of cells were mixed with 0.25 mg/mL Protease XIV (Sigma Chemical) and incubated on a shaker for 4 hrs. The cells were washed twice with 10% ice cold glycerol and resuspended in 500 uL of cold 10% glycerol. 90 uL was aliquoted into a prechilled 0.2 cm gap electro-cuvettes (Biorad 165-2086). 10 ul of DNA (1-5 ug) was added to the cuvette and mixed gently and held on ice. Cells were electroporated at 200 ohms (resistance), 25 uF, 250V (0.1 cm gap) 500V (0.2 cm gap). 0.5mL of media was added immediately to the cuvette. The cells were then transferred to 4.5mL of M50-20 media and incubated for 2-3 hrs at 100 rpm on a shaker. The cells were centrifuged and resuspended in 0.5 mL of media and plated onto 2-5 M2B plates with appropriate selection (if needed) and incubated at 29°C.

Example 2 HA analysis of Algae Transformants

[0176] Cryostocks of transgenic strains of *Schizochytrium* (transformed with pCL0081) were grown in M50-20 (described in US 2008/0022422) to confluency, then propagated in

50mL baffled shake flasks at 27°C, 200rpm for 48 hours in a medium containing (per liter) 13.62g Na_2SO_4, 0.72g K_2SO_4, 0.56g KCl, 2.27g $MgSO_4.7H_2O$, 3g $(NH_4)2SO_4$, 0.19g $CaCl_2.2H_2O$, 3g MSG monohydrate, 21.4g MES, and 0.4g KH_2PO_4. The volume was brought to 900mL with deionized H_2O and the pH was adjusted to 6 before autoclaving for 35min. Filter-sterilized glucose (50g/L), vitamins (2mL/L) and trace metals (2mL/L) were then added to the medium and the volume was adjusted to one liter. The vitamin solution contained 0.16g/L vitamin B12, 9.75g/L thiamine, 3.33g/L Ca-pentothenate. The trace metal solution (pH 2.5) contained 1.00g/L citric acid, 5.15g/L $FeSO_4.7H_2O$, 1.55g/L $MnCl_2.4H_2O$, 1.55g/L $ZnSO_4.7H_2O$, 0.02g/L $CoCl_2.6H_2O$, 0.02g/L $Na_2MoO_4.2H_2O$, 1.035g/L $CuSO_4.5H_2O$, 1.035g/L $NiSO_4.6H_2O$. All reagents were available commercially.

[0177] *Schizochytrium* cultures were transferred to 50mL conical tubes and centrifugated at 3000g for 15min. This low-speed supernatant was used, as is, for hemagglutination activity assay. A portion of the low-speed supernatant was further centrifugated at 100,000g for one hour. The resulting pellet, insoluble fraction containing the HN protein, was resuspended in phosphate buffer saline (PBS) and used for peptide sequence analysis as well as glycosylation analysis.

[0178] The expression of the HN protein by *Schizochytrium* was first evaluated by an activity assay. The functional HN protein displays an hemagglutination activity which was readily detected by a standard hemagglutination activity assay. Briefly, 50uL of doubling dilutions of low speed supernatant in PBS were prepared in a 96-well microtiter plate. Equal volume of an approximate 1% solution of turkey red blood cells (Fitzgerald Industries, Acton, MA, USA) in PBS was then added to each well followed by incubation at room temperature for 30min. The degree of agglutination was then analyzed visually. The hemagglutination activity unit (HAU) is defined as the highest dilution of low-speed supernatant that causes visible hemagglutination in the well. Typical activity was found to be in the order of 512 HAU in transgenic strain "CL0081-23" (Fig. 3B). PBS or the wild-type strain of *Schizochytrium sp.* ATCC 20888, grown and prepared in the same manner as the transgenic strains, was used as a negative control and did not show any hemagglutination activity. An NDV Hemagglutinin (HA) recombinant protein was used as a positive control. Sample titer was scored at the highest dilution before the defined button was observed. HA activity was detected in raw supernatant (Figure 3A). HAU in concentrated supernatant sample is 3200 HAU/50ul. The hemagglutination activity was found to be stable through multiple rounds of freeze/thaw.

Example 3 Expression Analysis of Algae Transformants

[0179] The expression of the HN protein was also verified by immunoblot analysis following standard immunoblotting procedure. The proteins were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) on a 4-12% bis-tris gel (Invitrogen). The proteins were then stained with Coomassie blue (SimplyBlue Safe Stain, Invitrogen) or transferred onto polyvinylidene fluoride membrane and probed for the presence of HN protein with anti- NDV antiserum from chicken in 1:1000 dilution (Charles River laboratories, Wilmington, MA, USA) followed by anti-chicken secondary antibody coupled to alkaline phosphatase in 1:5000 dilution (AP-AffiniPure Goat Anti-Chicken #103-055-155, Jackson ImmunoResearch Laboratories, Inc., West Grove, PA, USA). Membrane was then treated with 5-bromo-4-chloro-3-indoyl-phosphate/nitroblue tetrazolium solution (BCIP/NBT) according to the manufacturer's instructions (KPL, Gaithersburg, MD). An example is presented in Fig. 4 for the transgenic strain "CL0081-23". Coomassie blue-stained gels and corresponding anti-NDV immunoblots are presented in panels B and C. The recombinant HN protein was detected in the low-speed supernatant (panel B) and in the insoluble fraction (panel C). The negative control (-Ctrl) was the wild-type strain of *Schizochytrium sp.* ATCC 20888 or the transgenic strain "AB0018".

Example 4 Glycosylation Analysis

[0180] The presence of glycans on the HN protein was first evaluated by enzymatic treatment. The insoluble fraction of the transgenic strain "CL0081-23" was resuspended in PBS and digested with EndoH or PNGase F according to manufacturer's instructions (New England Biolabs, Ipswich, MA, USA). Removal of glycans was then identified by the expected shift in mobility when separating the proteins on 12% SDS-PAGE stained with Coomassie blue, periodic acid, or immunobloted with anti-NDV antiserum (FIG 5, panel A). The negative control (-Ctrl) of immunoblotting was the transgenic strain "AB0018". The negative control for the enzymatic treatment was the transgenic strain "CL0081-23" incubated without enzymes (non-treated NT). Second, the glycosylation profile of the HN protein produced in *Schizochytrium* was analyzed by matrix-assisted laser-desorption ionization time-of-flight mass spectrometry and NanoSpray ionization-Linear Ion trap Mass spectrometry (Complex Carbohydrate Research Center, Georgia). Coomassie blue-stained gel slices of the proteins of interest were cut into smaller pieces (~1 mm^3) and destained alternately with 40mM Ammonium bicarbonate (AmBic) and 100% acetonitrile until the color turned clear. Destained gel was reswelled in 10 mM DTT in 40mM Ambic at 55°C for

1 hr. The DTT solution was exchanged with 55mM Iodoacetamide (IAM) and incubated in the dark for 45 min. Incubation was followed by washing alternately with 40mM AmBic and 100% acetonitrile twice. Dehydrated gel was reswelled with trypsin solution (trypsin in 40 mM Ambic) on ice for 45 min initially, and protein digestion was carried out at 37°C overnight. The supernatant was transferred into another tube. Peptides and the glycopeptides were extracted from the gel in series with 20% acetonitrile in 5% formic acid, 50% acetonitrile in 5% formic acid and then 80% acetonitrile in 5% formic acid. The sample solutions were dried and combined into one tube. Extracted tryptic digest was passed through a C18 sep-pak cartridge and washed with 5% acetic acid to remove contaminants (salts, SDS, etc.). Peptides and glycopeptides were eluted in series with 20% iso-propanol in 5% acetic acid, 40% iso-propanol in 5% acetic acid and 100% iso-propanol and dried in a speed vacuum concentrator. The dried samples were combined and then reconstituted with 50 mM sodium phosphate buffer (pH 7.5) and heated at 100°C for 5 min to inactivate trypsin. The tryptic digest was incubated with PNGase F at 37°C overnight to release N-glycans. After digestion, the sample was passed through a C18 sep-pak cartridge and the carbohydrate fraction was eluted with 5% acetic acid and dried by lyophilization. Released N-linked oligosaccharides were permethylated based on the method of Anumula and Taylor (Anumula and Taylor, 1992) and profiled by mass spectrometry. Mass spectrometric analysis was performed following the method developed at the Complex Carbohydrates Research Center (Aoki K, Perlman M, Lim JM, Cantu R, Wells L, Tiemeyer M. J Biol Chem. 2007 Mar 23;282(12):9127-42.). Mass analysis was determined by using NSI-LTQ/MS_n. Briefly, permethylated glycans were dissolved in 1mM NaOH in 50% methanol and infused directly into the instrument (LTQ,Thermo Finnigan) at a constant flow rate of 0.4 μL/min. The MS analysis was performed in the positive ion mode. For total ion mapping, automated MS/MS analysis (at 35 collision energy), m/z range from 500 to 2000 was scanned in successive 2.8 mass unit windows that overlapped the preceding window by 2 mass units. Total ion mapping was performed to examine the presence of fragment ions indicative of glycans. All MS/MS data from m/z 500 through m/z 2000 were taken and then the raw data were analyzed manually. The chromatogram and table of species obtained by NSI-total ion mapping are shown in Figure 5 (panels B and C). This chromatogram was processed by the scan filter, neutral loss of m/z 139, which is the one of characteristic neutral loss of high-mannose type glycans. Total ion mapping revealed that this sample contains a series of high-mannose type glycans with long mannose chain.

Example 5 Peptide Analysis of NDV HN

[0181] The insoluble fraction was separated by SDS-PAGE and stained with Coomassie blue or transferred to PVDF and immunoblotted with anti-NDV antiserum from chicken, as described above. The band corresponding to the cross-reaction in immunoblot was excised from the coomassie stained gel and submitted for peptide sequence analysis. The procedure consisted washing/destaining the bands of interest in 50% ethanol, 5% acetic acid. The gel pieces were then dehydrated in acetonitrile, dried in a Speed-vac, and digested with trypsin by adding 5μL of 10ng/μL trypsin in 50mM ammonium bicarbonate and incubating overnight digestion at room temperature. The peptides that were formed were extracted from the polyacrylamide in two aliquots of 30μL 50% acetonitrile with 5% formic acid. These extracts were combined and evaporated to <10μL in Speed-vac and then resuspended in 1% acetic acid to make up a final volume of approximately 30μL for LC-MS analysis. The LC-MS system was a Finnigan LTQ linear ion trap mass spectrometer system. The HPLC column was a self-packed 9 cm x 75μm id Phenomenex Jupiter C18 reversed-phase capillary chromatography column. Then μL volumes of the extract were injected and the peptides eluted from the colum by an acetonitrile/0.1% formic acid gradient at a flow rate of 0.25μL/min were introduced into the source of the mass spectrometer on-line. The microelectrospray ion source was operated at 2.5kV. The digest was analyzed using a selective reaction (SRM) experiments in which the mass spectrometer fragments a series of m/z ratios over the entire course of the LC experiment. The fragmentation pattern of the peptides of interest was then used to produce chromatograms. The peak areas for each peptide was determined and normalized to an internal standard. The internal standards used in this analysis are proteins that have an unchanging abundance between the samples being studied. The final comparison between the two systems is determined by comparing the normalized peak ratios for each protein. The collision induced dissociation spectra were then searched against the ncbi database. The HN protein was identified by a total of 32 peptides covering 68% of the protein sequence. The results with the specific peptides that were sequenced are shown in Fig. 11.

Example 6 Vaccination of Chickens

[0182] Challenge studies were conducted in specific pathogen free (SPF) chickens vaccinated at 3 to 4 weeks of age with *Schizochytrium* expressed NDV HN protein in an adjuvant. Twelve chickens were assigned to each vaccine group. A Group vaccinated with *Schizochytrium* wild type material in the same adjuvant was included as a negative control

group. Three groups of chickens were tested with one shot scheme at three dosage levels (100 HAU, 1000 HAU, and 10000 HAU). The water-in-oil emulsions of the *Schizochytrium* culture medium was given by the intramuscular route in the leg (0.5 ml per site x 2). On day 27, blood samples were collected for hemagglutination inhibition test, and chickens were then challenged intramuscularly with Newcastle Disease Virus GB Texas strain at $10^{4.0}$ EID_{50} per chicken (Figure 12). The chickens were observed daily to ensure health status of the chickens being challenged. After challenge, the chickens were observed daily for fourteen days for severe clinical signs of NDV, such as but not limited to, extreme nervousness, respiratory distress, nervous signs or death. The mortality data shown in Figure 12 indicate that vaccination with plant derived NDV HN elicits increased protection of 33% at a dose level of 100 HAU, and increased protection of 100% at dose levels of 1000 HAU and 9333 HAU over control.

Example 7 Expression, characterization, immunogenicity and efficacy of NDV HN protein produced in duckweed

[0183] *Lemna minor* protein expression system was used to express NDV HN polypeptide (SEQ ID NO:17, NDV strain YZCQ/Liaoning/08). The *L. minor* optimized HN gene (SEQ ID NO:22 and 23) was cloned into a modified *A. tumefaciens* binary vector (Gasdaska, J., et al., Bioprocessing J. 3, 50–56, 2003). Several vector constructs were made. The constructs contain Super Promoter, 5' leader from *Lemna gibba* RBCS SSU1, and the Nopaline synthase (Nos) terminator. Construct MerH01 contains codon-optimized NDV HN gene with its native signal sequence (signal anchor as shown in the plasmid maps). Construct MerH02 contains codon-optimized NDV HN gene with its native signal sequence and KDEL ER retention sequence. Construct MerH03 contains codon-optimized NDV HN gene (encoding mature HN protein) with the native NDV HN signal sequence replaced with alpha amylase signal sequence. Construct MerH04 contains codon-optimized NDV HN gene with its native signal sequence replaced with alpha amylase signal sequence, and KDEL ER retention sequence. The plasmid maps of the four constructs are shown in Figure 7c. The constructs were transformed to *A. tumefaciens* C58Z707 (Hepburn, A.G. et al., J. Gen. Microbiol. 131, 2961–2969, 1985). Using the *A. tumefaciens* C58Z707 transformed with plant transformation vector constructs, transgenic plants representing individual clonal lines were generated from rapidly growing *L. minor* nodules as described in Yamamoto, Y. et al., In Vitro Cell. Dev. Biol. 37, 349–353 (2001).

[0184] After the transgenic lines are generated, they are screened for expression of NDV HN in the media and the tissue. The plants are grown for two weeks in small research vessels and the resulting media and tissue are collected for analysis. For the tissue analysis, frozen tissue is homogenized, centrifuged and the supernatant is removed for standard hemagglutination assay. The highest lines from the initial screening are being scaled up to provide approximately 1 kg of biomass for further characterization, such as hemagglutination assay, hemagglutination inhibition assay (HI), SDS-PAGE, Western Blot, and immunolocalization.

[0185] Crude plant extract is prepared from transgenic *Lemna* line for evaluation of immunogenicity and efficacy in specific pathogen free (SPF) chickens at 3-4 weeks of age. Twelve chickens are assigned to each vaccine group vaccinated with the composition comprising the recombinant NDV HN polypeptide and an adjuvant. A group vaccinated with *Lemna* wild type material in the same adjuvant is included as a negative control. Other groups of chickens are tested with one shot scheme at different dosage levels. On day 21, blood samples are collected for hemagglutination inhibition test, and chickens are then challenged with different Newcastle Disease Virus strains. After the challenge, the chickens are observed daily for fourteen days for severe clinical signs of NDV, such as but not limited to, extreme nervousness, respiratory distress, nervous signs or death. The composition containing the recombinant NDV HN polypeptide shows efficacy in treating, protecting, and preventing NDV infection and disease.

[0186] All documents cited or referenced in the application cited documents, and all documents cited or referenced herein ("herein cited documents"), and all documents cited or referenced in herein cited documents, together with any manufacturer's instructions, descriptions, product specifications, and product sheets for any products mentioned herein or in any document incorporated by reference herein, are hereby incorporated herein by reference, and may be employed in the practice of the invention.

[0187] Having thus described in detail preferred embodiments of the present invention, it is to be understood that the invention defined by the above paragraphs is not to be limited to particular details set forth in the above description as many apparent variations thereof are possible without departing from the spirit or scope of the present invention.

WHAT IS CLAIMED IS:

1. A composition comprising an NDV (Newcastle Disease Virus) HN (Hemagglutinin-Neuraminidase) polypeptide or antigen and a pharmaceutical or veterinarily acceptable carrier, excipient, vehicle or adjuvant.

2. The composition of claim 1, wherein the NDV HN polypeptide or antigen comprises an immunogenic fragment comprising the NDV HN linear epitope region.

3. The composition of claim 1 or 2, wherein the NDV HN polypeptide or antigen is expressed in microalgae or plants.

4. The composition of any one of claims 1-3, wherein the NDV HN polypeptide or antigen is partially purified.

5. The composition of any one of claims 1-3, wherein the NDV HN polypeptide or antigen is substantially purified.

6. The composition of any one of claims 1-5, wherein the NDV HN polypeptide or antigen has at least 80% sequence identity to the sequence as set forth in SEQ ID NO: 3, 5, 7, 9, 15, 17, 19, or 20.

7. The composition of any one of claims 1-6, wherein the NDV polypeptide or antigen is encoded by a polynucleotide having at least 70% sequence identity to the sequence as set forth in SEQ ID NO: 1, 2, 4, 6, 8, 14, 16, 18, 22, or 23.

8. The composition of any one of claims 1-7, wherein the pharmaceutical or veterinarily acceptable carrier, excipient, vehicle, or adjuvant is a water-in-oil emulsion or an oil-in-water emulsion.

9. A method of vaccinating an animal susceptible to NDV comprising at least one administration of the composition according to any one of claims 1-8.

10. The method of claim 9, wherein the method comprises a prime-boost administration regime.

11. The method of claim 10, wherein the prime-boost regime comprises a prime-administration of a composition according to any one of claims 1-8, and a boost administration of a composition comprising, in a pharmaceutically or veterinary acceptable vehicle or excipient, a recombinant viral vector containing a polynucleotide for expressing, *in vivo*, the NDV HN polypeptide, variant or fragment thereof, to protect the subject from NDV infection and/or to prevent disease progression in infected subject.

12. The method of claim 10, wherein the prime-boost regime comprises a prime-administration of a composition comprising, in a pharmaceutically or veterinary acceptable vehicle, diluent or excipient, a recombinant viral vector containing a polynucleotide for

expressing, *in vivo*, the NDV HN polypeptide, a variant or fragment thereof, and a boost administration of a composition according to any one of claims 1-8 to protect the subject from NDV infection and/or to prevent disease progression in infected subject.

13. The method of claim 10, wherein the prime-boost regime comprises a prime-administration of a composition according to any one of claims 1-8, and a boost administration of an inactivated viral composition or vaccine comprising the NDV antigen.

14. The method of claim 10, wherein the prime-boost regime comprises a prime-administration of an inactivated viral composition or vaccine comprising the NDV antigen and a boost administration of a composition according to any one of claims 1-8 to protect the subject from NDV and/or to prevent disease progression in infected subject.

15. The method of any one of claims 9-14, wherein the animal is avian, equine, canine, feline or porcine.

16. A substantially purified NDV polypeptide or antigen expressed in microalgae or duckweed plants, wherein the polypeptide comprises an amino acid sequence having at least 80% sequence identity to a polypeptide having the sequence as set forth in SEQ ID NO: 3, 5, 7, 9, 15, 17, 19, or 20.

17. Microalgal culture or duckweed plant stably transformed with a gene for expressing an NDV HN polypeptide or fragment or variant thereof.

18. The microalgal culture or duckweed plant of claim 17, wherein the NDV HN polypeptide or fragment or variant thereof has at least 80% sequence identity to the sequence as set forth in SEQ ID NO:3, 5, 7, 9, 15, 17, 19, or 20.

19. A method of producing an NDV HN polypeptide, comprising: (a) culturing microalgal culture or duckweed plant within a microalgal or duckweed culture medium, wherein the microalgal culture or duckweed plant is stably transformed to express the NDV HN polypeptide, and wherein the NDV HN polypeptide is expressed from a nucleotide sequence comprising a coding sequence for the NDV HN polypeptide and an operably linked coding sequence for a signal peptide that directs secretion of the polypeptide into said culture medium; and (b) collecting the NDV HN polypeptide from the culture medium.

20.The method of claim 19, wherein the signal peptide has a sequence as set forth in SEQ ID NO:13, or 21.

21. A plasmid comprising a DNA fragment encoding a signal peptide having a sequence as set forth in SEQ ID NO:13, or 21.

22. The plasmid of claim 21, wherein the DNA fragment is operably linked to a polynucleotide encoding an NDV HN polypeptide.

23. The plasmid of any one of claims 21-22, wherein the plasmid is for plant or algae transformation.

Figure 1

SEQ ID NO:	Type	Description
1	DNA	NDV HN codon-optimized DNA
2	DNA	NDV HN wild type DNA (EF520717) (gamefowl/US(CA)/212676/2002 strain)
3	Protein	NDV HN protein (ABS84265) (gamefowl/US(CA)/212676/2002 strain)
4	DNA	NDV HN DNA (M21409) (Texas GB strain)
5	Protein	NDV HN protein (P12553) (Texas GB strain)
6	DNA	NDV HN DNA (M24709) (LaSota strain)
7	Protein	NDV HN protein (AAA46659) (LaSota strain)
8	DNA	NDV HN DNA (AY288999) (MEX/96 strain)
9	Protein	NDV HN protein (AAQ54638) (MEX/96 strain)
10	Protein	NDV HN Linear Epitope Region 1
11	Protein	NDV HN Linear Epitope Region 2
12	DNA	NDV HN signal sequence
13	Protein	NDV HN signal peptide (from SEQ ID NO:3, ABS84265)
14	DNA	NDV HN DNA encoding mature protein (without signal sequence, 79bp -1716bp of SEQ ID NO:1) (codon-optimized)
15	Protein	NDV HN mature protein (without signal peptide, 27aa-571aa of SEQ ID NO:3) (EF520717) (gamefowl/US(CA)/212676/2002 strain)
16	DNA	NDV HN DNA (FJ608369) (NDV strain chicken YZCQ/Liaoning/08)
17	Protein	NDV HN protein(ACM67348) (NDV strain chicken YZCQ/Liaoning/08)
18	DNA	NDV HN DNA (NDV strain ZJ1)
19	Protein	NDV HN protein (AAL18936) (NDV strain ZJ1)
20	Protein	NDV HN mature protein (without signal peptide, 47aa-571aa of SEQ ID NO:17, ACM67348)
21	Protein	NDV HN signal peptide (from SEQ ID NO:17, ACM67348)
22	DNA	NDV HN codon-optimized DNA encoding ACM67348
23	DNA	NDV HN codon-optimized DNA encoding mature protein (without signal sequence, 139 bp -1713 bp of SEQ ID NO:22, NDV strain chicken YZCQ/Liaoning/08)
24	Protein	ER retention sequence (KDEL)
25	DNA	alpha amylase signal sequence
26	protein	alpha amylase signal peptide
27	DNA	NDV HN signal sequence, codon-optimized (duckweed-preferred codon optimization) (1-138 bp of SEQ ID NO:22)
28	protein	NDV HN Linear Epitope Region 3

Figure 2a (1/2)

HN Seqeunces of LaSota, CA/02 and TX/GB strains of NDV. Glycosylation sites are underlined.

NDV HN CA/02 (ABS84265)(SEQ ID NO:3)

```
  1 MDRVVSRVVL ENEEREAKNT WRLVFRVAVL SLIVMTLAIS VAALVYSMEA STPNDLAGIS
 61 TVISRAEDRV TSLLNSNQDV VDRVYKQVAL ESPLALLNTE SIIMNAITSL SYQINGAANS
121 SGCGAPVHDP DYIGGVGKEL IVDDTSDATS FYPSAYQEHL NFIPAPTTGS GCTRIPSFDM
181 SATHYCYTHN VILSGCRDHS HSHQYLALGV LRTSATGRVF FSTLRSINLD DTQNRKSCSV
241 SATPLGCDML CSKVTETEEE DYKSVTPTSM VHGRLGFDGQ YHEKDLDVTV LFKDWVANYP
301 GVGGGSLIDD RVWFPVYGGL KPNSPSDTAQ EGKYVIYKRY NNTCPDEQDY QVRMAKSSYK
361 PGRFGGKRVQ QAILSIKVST SLGEDPVLTV PPNTVTLMGA EGRILTVGTS HFLYQRGSSY
421 FSPALLYPMT VRNKTATLHS PYTFNAFTRP GSVPCQASAR CPNSCITGVY TDPYPVVFHR
481 NHTLRGVFGT MLDNEQARLN PVSAIFDYTS RSRITRVSST STKAAYTTST CFKVVKTNKV
541 YCLSIAEISN TLFGEFRIVP LLVEILKDDR V
```

NDV HN TX/GB (P12553)(SEQ ID NO:5)

```
  1 MDRAVSQVAL ENDEREAKNT WRLIFRIAIL LLTVVTLATS VASLVYSMGA STPSDLVGIP
 61 TRISRAEEKI TSALGSNQDV VDRIYKQVAL ESPLALLNTE TTIMNAITSL SYQINGAANN
121 SGWGAPIHDP DFIGGIGKEL IVDDASDVTS FYPSAFQEHH NFIPAPTTGS GCIRIPSFDM
181 SATHYCYTHN IISSGCRDHS HSYQYLALGV LRTSATGRIF FSTLRSINLD DTQNRKSCSV
241 SATPLGCDML CSKVTETEEE DYNSAVPTLM VHGRLGFDGQ YHEKDLDVTT LFEDWVANYP
301 GVGGGSFIDS RVWFSVYGGL KPNSPSDTVQ EEKYVIYKRY NDTCPDEQDY QIRMAKSSYK
361 PGRFGGKRIQ QAILSIKVST SLGEDPVLTV PPNTVTLMGA EGRILTVGTS HFLYQRGSSY
421 FSPALLYPMT VSNKTATLHS PYTFNAFTRP GSIPCQASAR CPNSCVTGVY TDPYPLIFYR
481 NHTLRGVFGT MLDGEQARLN PASAVFDSTS RSRITRVSSS STKAAYTTST CFKVVKTNKT
541 YCLSIAEISN TLFGEFRIVP LLVEILKNDG VREARSG
```

NDV HN LaSota (AAA46659)(SEQ ID NO:7)

```
MDRAVSQVAL ENDEREAKNT WRLIFRIAIL FLTVVTLAIS VASLLYSMGA STPSDLVGIP 60
TRISRAEEKI TSTLGSNQDV VDRIYKQVAL ESPLALLKTE TTIMNAITSL SYQINGAANN 120
SGWGAPIHDP DYIGGIGKEL IVDDASDVTS FYPSAFQEHL NFIPAPTTGS GCTRIPSFDM 180
SATHYCYTHN VILSGCRDHS HSYQYLALGV LRTSATGRVF FSTLRSINLD DTQNRKSCSV 240
SATPLGCDML CSKVTETEEE DYNSAVPTRM AHGRLGFDGQ YHEKDLDVTT LFGDWVANYP 300
GVGGGSFIDG RVWFSVYGGL KPNSPSDTVQ EGKYVIYKRY NDTCPDEQDY QIRMAKSSYK 360
PGRFGGKRIQ QAILSIKVST SLGEDPVLTV PPNTVTLMGA EGRILTVGTS HFLYQRGSSY 420
FSPALLYPMT VSNKTATLHS PYTFNAFTRP GSIPCQASAR CPNPCVTGVY TDPYPLIFYR 480
NHTLRGVFGT MLDGVQARLN PTSAVFDSTS RSRITRVSSS STKAAYTTST CFKVVKTNKT 540
YCLSIAEISN TLFGEFRIVP LLVEILKDDG VREARSG                          577
```

Figure 3

Figure 3A

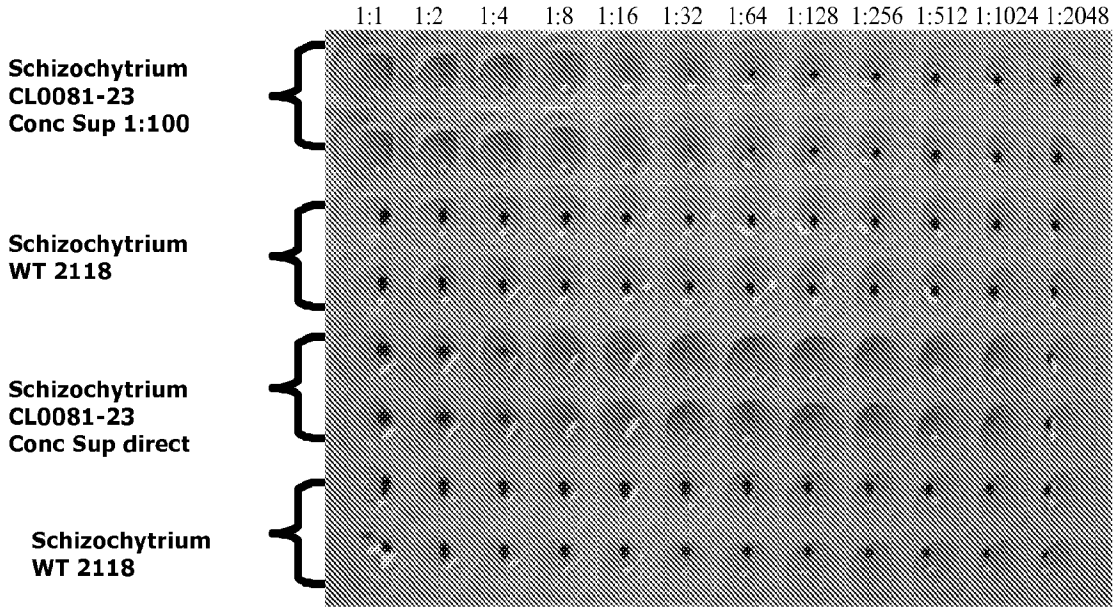


Figure 3B

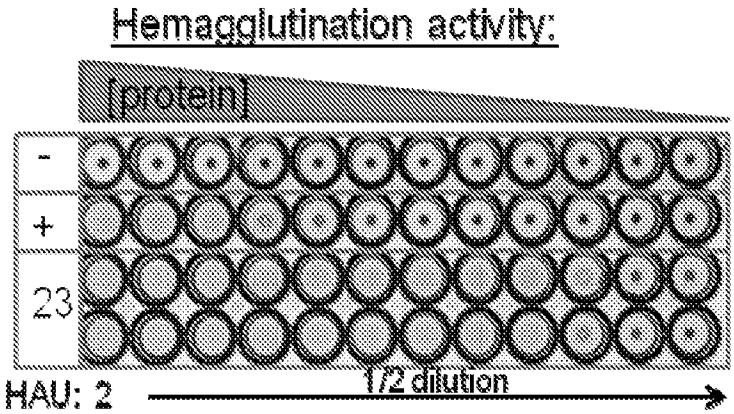


Figure 4

Secretion of HN protein by transgenic *Schizochytrium* - immunoblot analysis

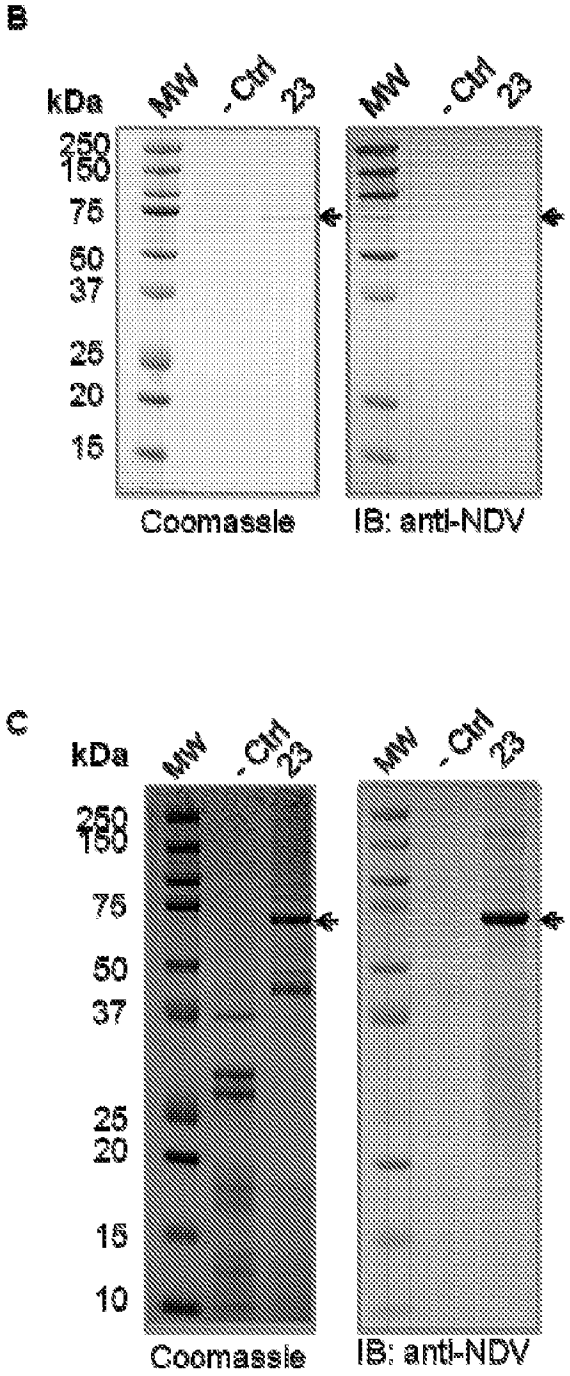
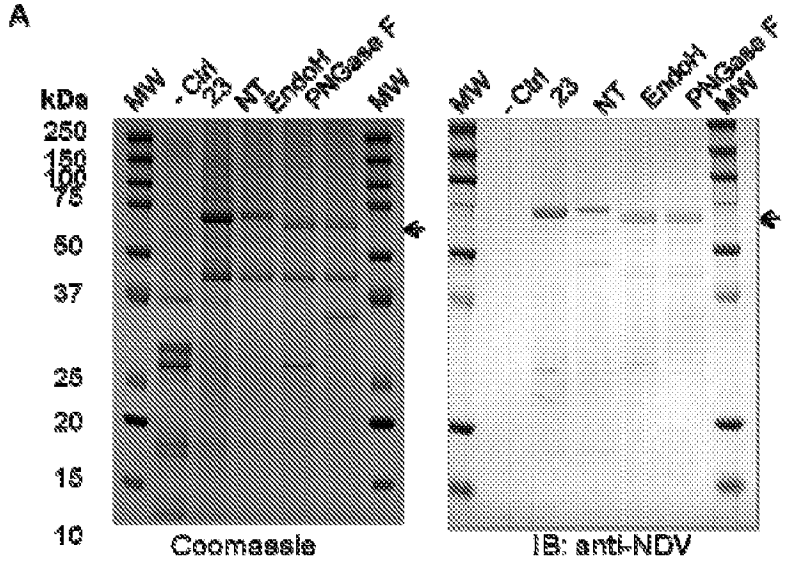


Figure 5 (1/2)


A
kDa
MW
- Ctrl
23
NT
EndoH
PNGase F
MW
250
150
100
75
50
37
25
20
15
10
Coomassie
IB: anti-NDV

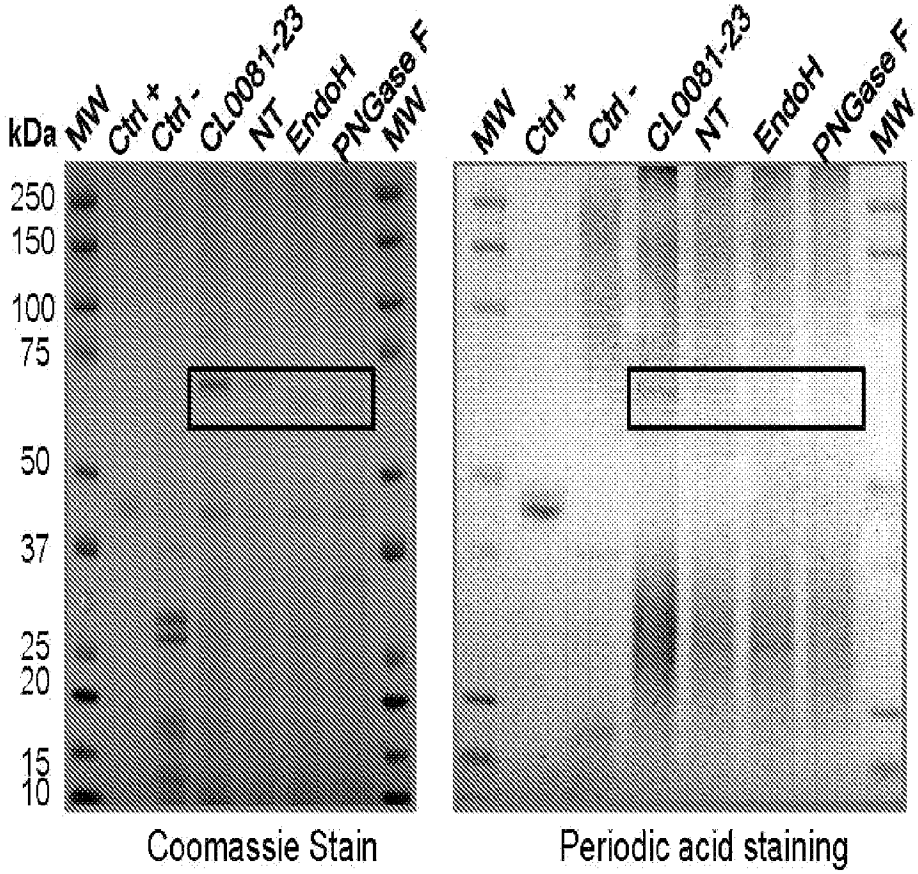
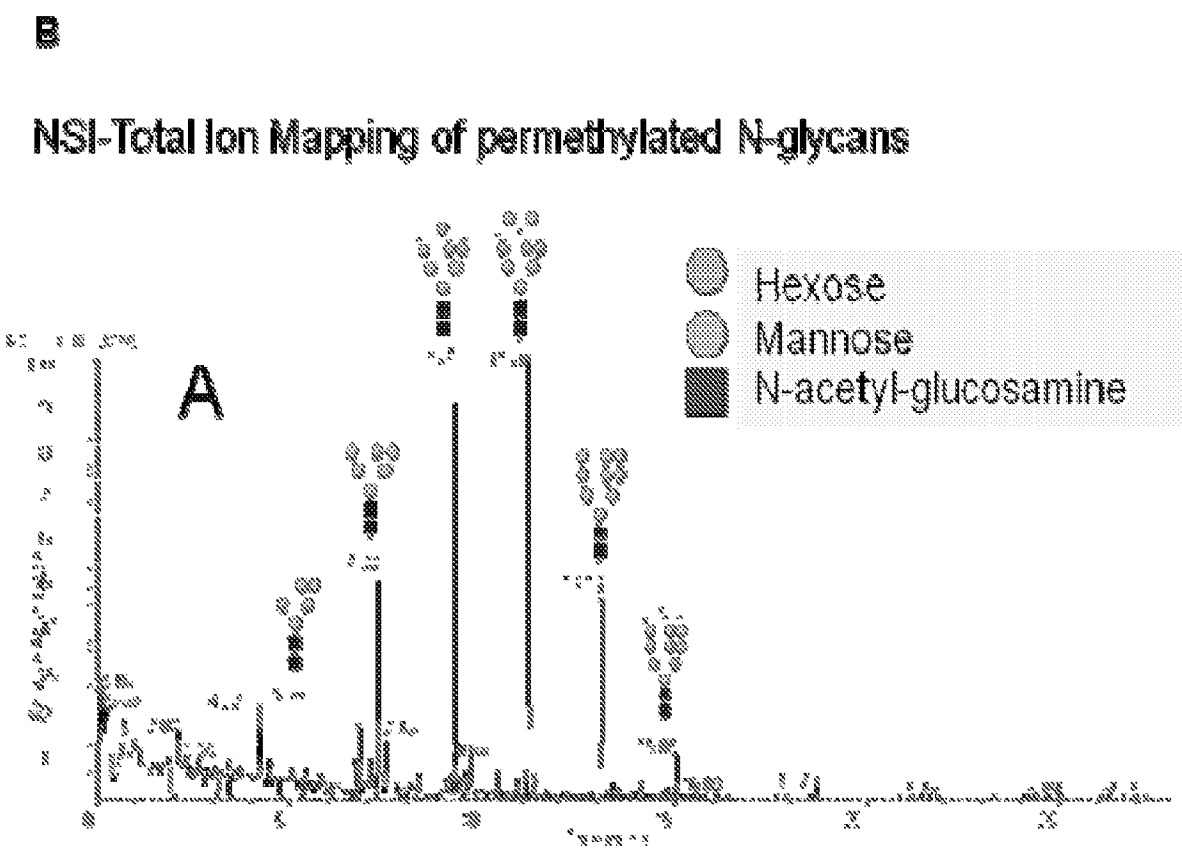

kDa
MW
Ctrl +
Ctrl -
CL0081-23
NT
EndoH
PNGase F
MW
250
150
100
75
50
37
25
20
15
10
Coomassie Stain
Periodic acid staining

Figure 5 (2/2)

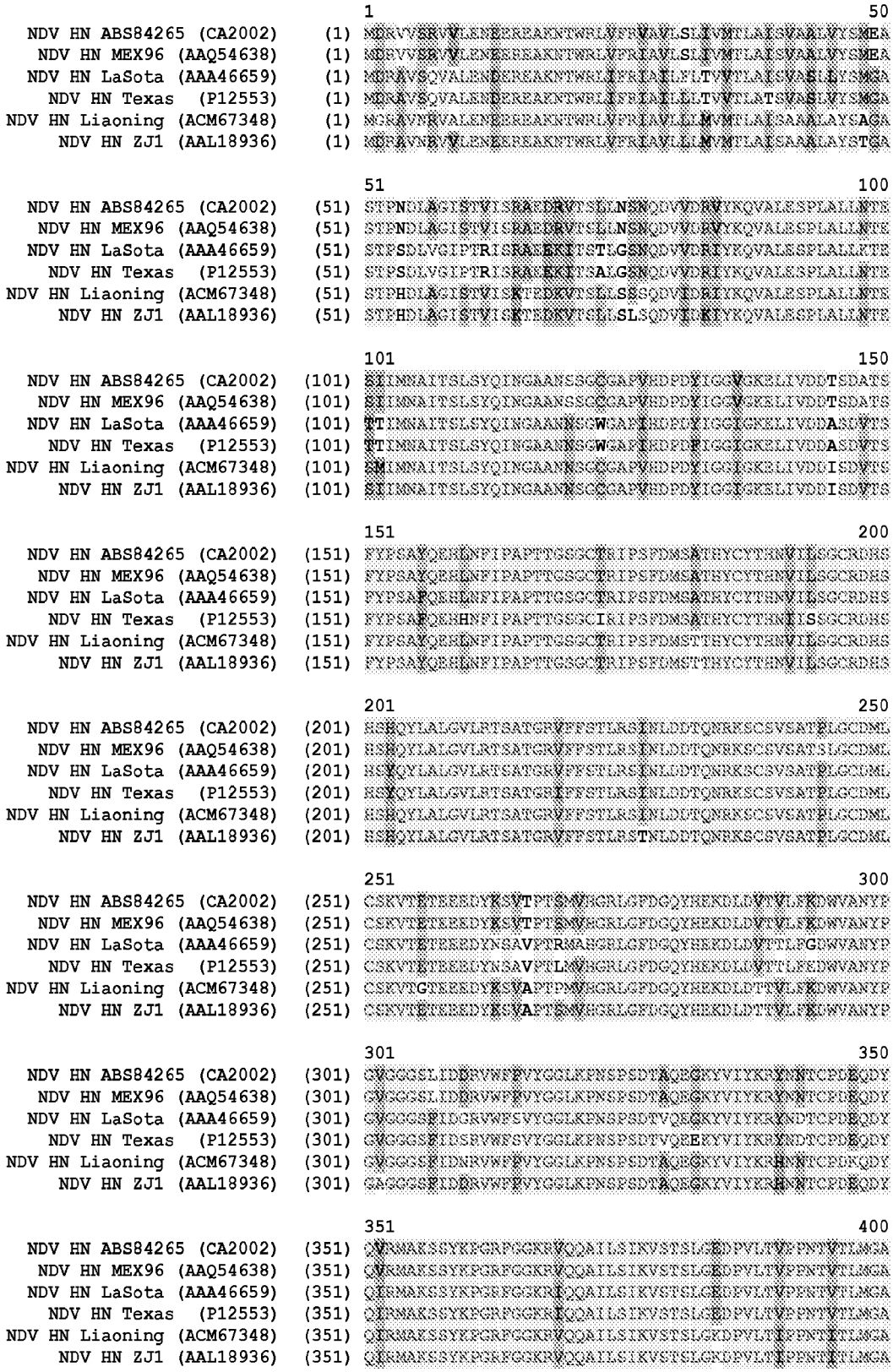
B



C

Proposed Structure	Mass [M+Na]	Charge State [z]	23
$Hex_5HexNAc_2$	1579 802	1 2	√
$Hex_6HexNAc_2$	1783 903	1 2	√
$Hex_7HexNAc_2$	1987 1005	1 2	√
$Hex_8HexNAc_2$	1106	2	√
$Hex_9HexNAc_2$	1210	2	√
$Hex_{10}HexNAc_2$	1312	2	√

Figure 6a (1/2)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NDV HN LaSota (AAA46659) (1)
NDV HN Texas (P12553) (1)
NDV HN Liaoning (ACM67348) (1)
NDV HN ZJ1 (AAL18936) (1)
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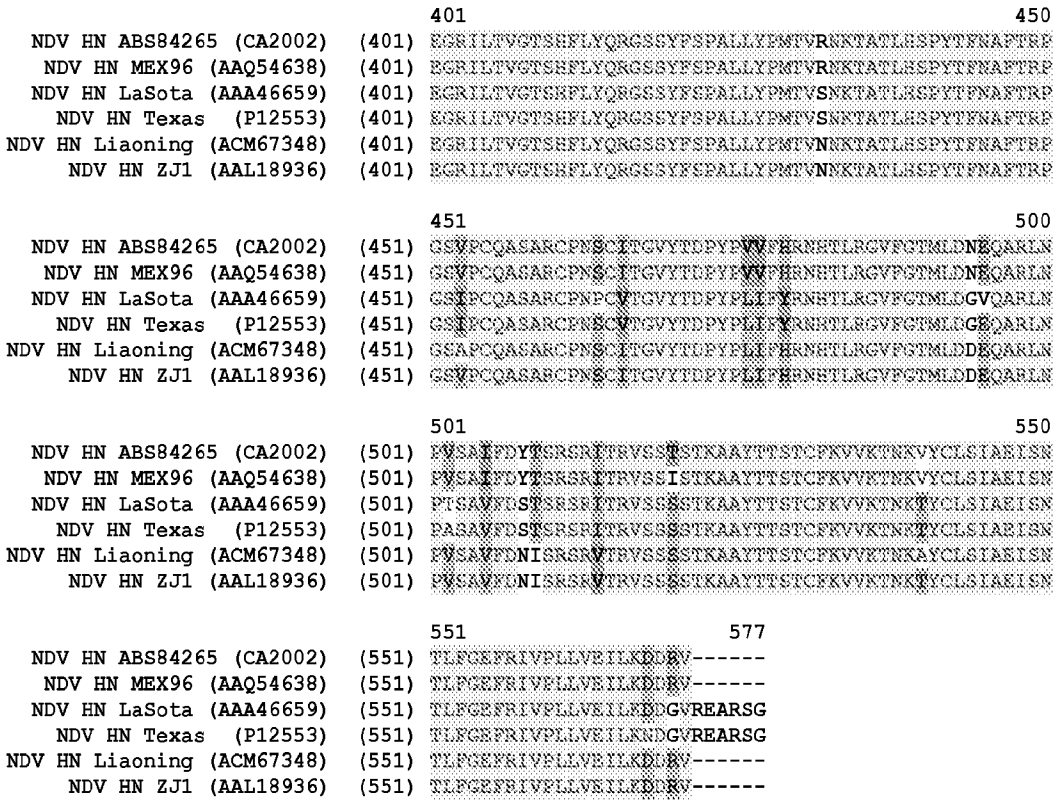
NDV HN ABS84265 (CA2002) (51)
NDV HN MEX96 (AAQ54638) (51)
NDV HN LaSota (AAA46659) (51)
NDV HN Texas (P12553) (51)
NDV HN Liaoning (ACM67348) (51)
NDV HN ZJ1 (AAL18936) (51)
101 150
NDV HN ABS84265 (CA2002) (101)
NDV HN MEX96 (AAQ54638) (101)
NDV HN LaSota (AAA46659) (101)
NDV HN Texas (P12553) (101)
NDV HN Liaoning (ACM67348) (101)
NDV HN ZJ1 (AAL18936) (101)
151 200
NDV HN ABS84265 (CA2002) (151)
NDV HN MEX96 (AAQ54638) (151)
NDV HN LaSota (AAA46659) (151)
NDV HN Texas (P12553) (151)
NDV HN Liaoning (ACM67348) (151)
NDV HN ZJ1 (AAL18936) (151)
201 250
NDV HN ABS84265 (CA2002) (201)
NDV HN MEX96 (AAQ54638) (201)
NDV HN LaSota (AAA46659) (201)
NDV HN Texas (P12553) (201)
NDV HN Liaoning (ACM67348) (201)
NDV HN ZJ1 (AAL18936) (201)
251 300
NDV HN ABS84265 (CA2002) (251)
NDV HN MEX96 (AAQ54638) (251)
NDV HN LaSota (AAA46659) (251)
NDV HN Texas (P12553) (251)
NDV HN Liaoning (ACM67348) (251)
NDV HN ZJ1 (AAL18936) (251)
301 350
NDV HN ABS84265 (CA2002) (301)
NDV HN MEX96 (AAQ54638) (301)
NDV HN LaSota (AAA46659) (301)
NDV HN Texas (P12553) (301)
NDV HN Liaoning (ACM67348) (301)
NDV HN ZJ1 (AAL18936) (301)
351 400
NDV HN ABS84265 (CA2002) (351)
NDV HN MEX96 (AAQ54638) (351)
NDV HN LaSota (AAA46659) (351)
NDV HN Texas (P12553) (351)
NDV HN Liaoning (ACM67348) (351)
NDV HN ZJ1 (AAL18936) (351)

Figure 6a (2/2)



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NDV HN MEX96 (AAQ54638): SEQ ID NO:9
NDV HN LaSota (AAA46659): SEQ ID NO:7
NDV HN Texas (P12553): SEQ ID NO:5
NDV HN Liaoning (ACM67348): SEQ ID NO:17
NDV HN ZJ1 (AAL18936): SEQ ID NO:19

Sequence identity percentage:

SEQ ID NO:	9	3	7	5	17	19
9	100	99	89	88	92	92
3		100	89	88	92	92
7			100	97	88	87
5				100	87	87
17					100	97
19						100

Vector NTI 11.0 (PC) software package (Invitrogen, 1600 Faraday Ave., Carlsbad, CA).

Figure 6b (1/2)

```
                    1                                                  50
SEQ ID NO:20     (1) --------------------SAGASTPHDLAGISTVISKTEDKVTSLLSS
SEQ ID NO:15     (1) VAVLSLIVMTLAISVAALVYSMEASTPNDLAGISTVISRAEDRVTSLINS

                    51                                                 100
SEQ ID NO:20    (31) SQDVIDRIYKQVALESPLALLNTESMIMNAITSLSYQINGAANNSGCGAP
SEQ ID NO:15    (51) NQDVYDRVYKQVALESPLALLNTESIIMNAITSLSYQINGAANSSGCGAP

                    101                                                150
SEQ ID NO:20    (81) VHDPDYIGGIGKELIVDDISDVTSFYPSAYQEHLNFIPAPTTGSGCTRIP
SEQ ID NO:15   (101) VHDPDYIGGVGKELIVDDTSDATSFYPSAYQEHLNFIPAPTTGSGCTRIP

                    151                                                200
SEQ ID NO:20   (131) SFDMSTTHYCYTHNVILSGCRDHSHSHQYLALGVLRTSATGRVFFSTLRS
SEQ ID NO:15   (151) SFDMSATHYCYTHNVILSGCRDHSHSHQYLALGVLRTSATGRVFFSTLRS

                    201                                                250
SEQ ID NO:20   (181) INLDDTQNRKSCSVSATPLGCDMLCSKVTGTEEEDYKSVAPTPMVHGRLG
SEQ ID NO:15   (201) INLDDTQNRKSCSVSATPLGCDMLCSKVTETEEEDYKSVTPTSMVHGRLG

                    251                                                300
SEQ ID NO:20   (231) FDGQYHEKDLDTTVLFKDWVANYPGVGGGSFIDNRVWFPVYGGLKPNSPS
SEQ ID NO:15   (251) FDGQYHEKDLDVTVLFKDWVANYPGVGGGSLIDDRVWFPVYGGLKPNSPS

                    301                                                350
SEQ ID NO:20   (281) DTAQEGKYVIYKRHNNTCPDKQDYQIRMAKSSYKPGRFGGKRVQQAILSI
SEQ ID NO:15   (301) DTAQEGKYVIYKRYNNTCPDEQDYQVRMAKSSYKPGRFGGKRVQQAILSI

                    351                                                400
SEQ ID NO:20   (331) KVSTSLGKDPVLTIPPNTITLMGAEGRILTVGTSHFLYQRGSSYFSPALL
SEQ ID NO:15   (351) KVSTSLGEDPVLTVPPNTVTLMGAEGRILTVGTSHFLYQRGSSYFSPALL

                    401                                                450
SEQ ID NO:20   (381) YPMTVNNKTATLHSPYTFNAFTRPGSAPCQASARCPNSCITGVYTDPYPL
SEQ ID NO:15   (401) YPMTVRNKTATLHSPYTFNAFTRPGSVPCQASARCPNSCITGVYTDPYPV

                    451                                                500
SEQ ID NO:20   (431) IFHRNHTLRGVFGTMLDDEQARLNPVSAVFDNISRSRVTRVSSSSTKAAY
SEQ ID NO:15   (451) VFHRNHTLRGVFGTMLDNEQARLNPVSAIFDYTSRSRITRVSSTSTKAAY

                    501                                          546
SEQ ID NO:20   (481) TTSTCFKVVKTNKAYCLSIAEISNTLFGEFRIVPLLVEILKDDRV-
SEQ ID NO:15   (501) TTSTCFKVVKTNKVYCLSIAEISNTLFGEFRIVPLLVEILKDDRV-
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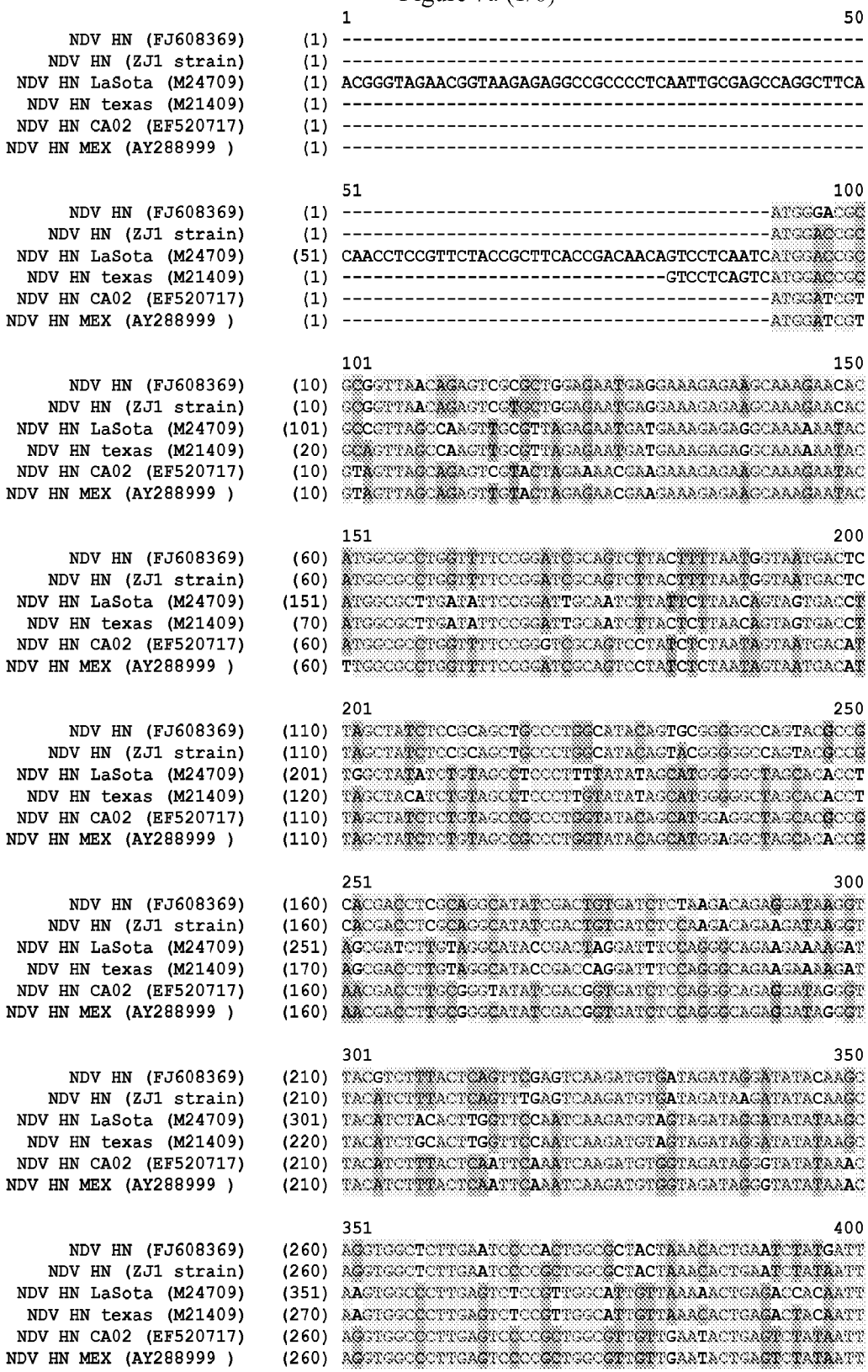
The protein identity percentage between SEQ ID NO:15 and SEQ ID NO:20 is 93%.

Figure 7a (1/6)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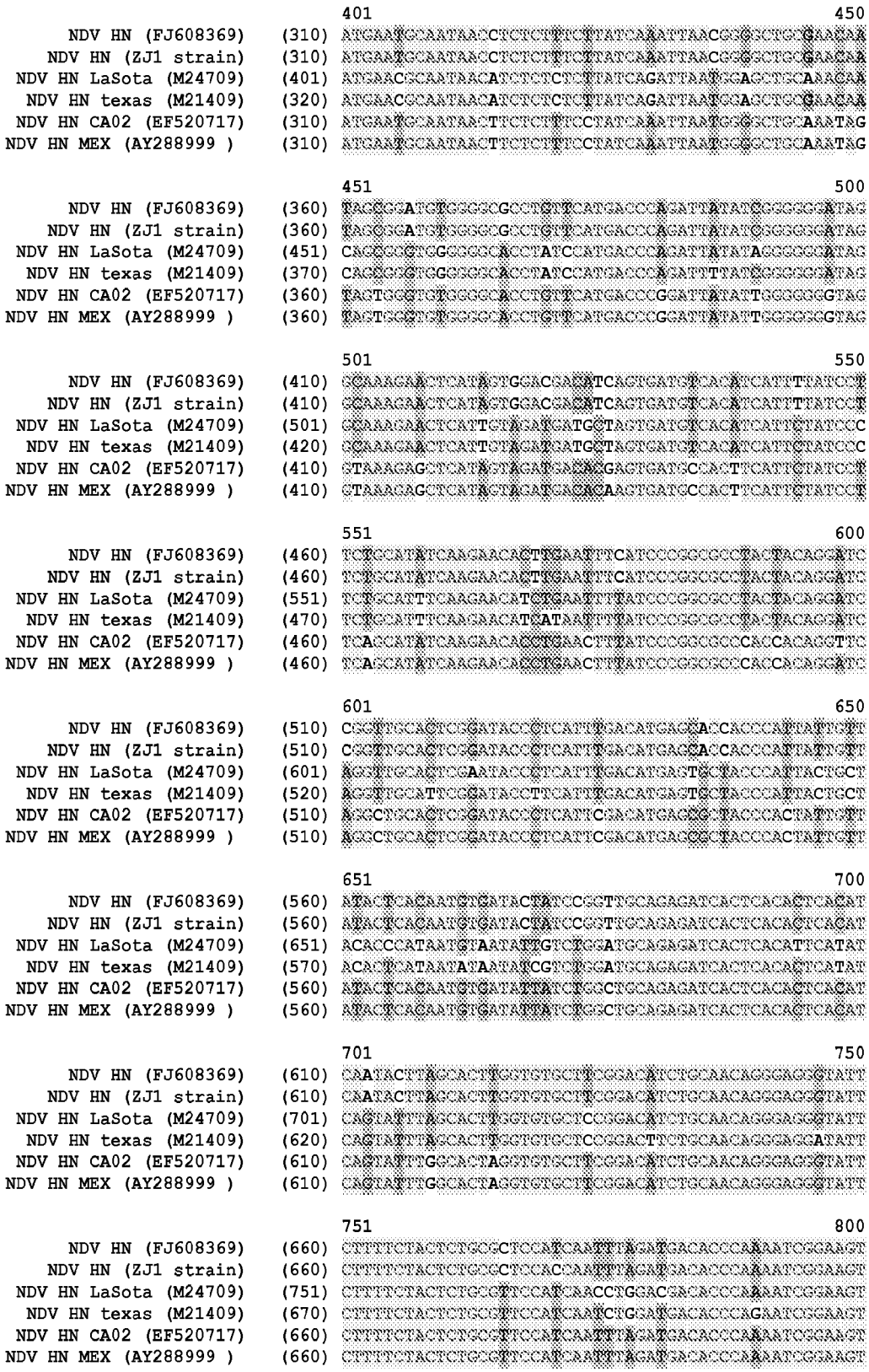
NDV HN (ZJ1 strain) (1) ----------
NDV HN LaSota (M24709) (1) ACGGGTAGAACGGTAAGAGAGGCCGCCCCTCAATTGCGAGCCAGGCTTCA
NDV HN texas (M21409) (1) ----------
NDV HN CA02 (EF520717) (1) ----------
NDV HN MEX (AY288999) (1) ----------
51 100
NDV HN (FJ608369) (1) ----------ATGGGACGC
NDV HN (ZJ1 strain) (1) ----------ATGCACCGC
NDV HN LaSota (M24709) (51) CAACCTCCGTTCTACCGCTTCACCGACAACAGTCCTCAATCATGCACCGC
NDV HN texas (M21409) (1) ----------GTCCTCAGTCATGCACCGC
NDV HN CA02 (EF520717) (1) ----------ATGCATCGT
NDV HN MEX (AY288999) (1) ----------ATGCATCGT
101 150
NDV HN (FJ608369) (10) GCGGTTAACAGAGTCGCGCTGGAGAATGAGGAAAGAGAAGCAAAGAACAC
NDV HN (ZJ1 strain) (10) GCGGTTAACAGAGTCCTGCTGGAGAATGAGGAAAGAGAAGCAAAGAACAC
NDV HN LaSota (M24709) (101) GCCGTTAGCCAAGTTGCGTTAGAGAATGATGAAAGAGAGGCAAAAAATAC
NDV HN texas (M21409) (20) GCAGTTAGCCAAGTTGCGTTAGAGAATGATGAAAGAGAGGCAAAAAATAC
NDV HN CA02 (EF520717) (10) GTAGTTAGCAGAGTCCTAGTAGAAACGAAGAAAGAGAAGCAAAGAATAC
NDV HN MEX (AY288999) (10) GTAGTTAGCAGAGTCTTAGTAGAACGAAGAAAGAGAAGCAAAGAATAC
151 200
NDV HN (FJ608369) (60) ATGGCGCTGGTTTTCCGGATCGCAGTCTTACTTTTAATGGTAATGACTC
NDV HN (ZJ1 strain) (60) ATGGCGCTGGTTTTCCGGATCGCAGTCTTACTTTTAATGGTAATGACTC
NDV HN LaSota (M24709) (151) ATGGCGCTTGATATTCCGGATTGCAATCTTATTCTTAACAGTAGTGACCT
NDV HN texas (M21409) (70) ATGGCGCTTGATATTCCGGATTGCAATCTTACTCTTAACAGTAGTGACCT
NDV HN CA02 (EF520717) (60) ATGGCGCTGGTTTTCCGGGTCGCAGTCCTATCTCTAATAGTAATGACAT
NDV HN MEX (AY288999) (60) TTGGCGCTGGTTTTCCGGATCGCAGTCCTATCTCTAATAGTAATGACAT
201 250
NDV HN (FJ608369) (110) TAGCTATCTCCGCAGCTGCCCTGGCATACAGTGCGGGGCCAGTACGCCG
NDV HN (ZJ1 strain) (110) TAGCTATCTCCGCAGCTGCCCTGGCATACAGTACGGGGCCAGTACGCCG
NDV HN LaSota (M24709) (201) TGGCTATATCTGTAGCGTCCCTTTTATATAGCATGGGGCTAGCACACCT
NDV HN texas (M21409) (120) TAGCTACATCTGTAGCGTCCCTTGTATATAGCATGGGGCTAGCACACCT
NDV HN CA02 (EF520717) (110) TAGCTATCTCTGTAGCGGCCCTGCTATACAGCATGCAGGCTAGCAGGCCG
NDV HN MEX (AY288999) (110) TAGCTATCTCTGTAGCGGCCCTGCTATACAGCATGGAGGCTAGCACACCG
251 300
NDV HN (FJ608369) (160) CACGACCTCGCAGGCATATCGACTGTGATCTCTAACACAGAGCATAAGGT
NDV HN (ZJ1 strain) (160) CACGACCTCGCAGGCATATCGACTGTGATCTCCAACACAGAACATAAGCT
NDV HN LaSota (M24709) (251) AGCGATCTTGTAGGCATACCGACTAGGATTTCCAGCGCAGAAGAAAAGAT
NDV HN texas (M21409) (170) AGCGACCTTGTAGGCATACCGACCAGGATTTCCAGCGCAGAAGAAAAGAT
NDV HN CA02 (EF520717) (160) AACGACCTTCCGGGTATATCGACGGTGATCTCCAGCGCAGAGGATAGCGT
NDV HN MEX (AY288999) (160) AACGACCTTCCGGGCATATCGACGGTGATCTCCAGCGCAGAGGATAGCGT
301 350
NDV HN (FJ608369) (210) TACGTCTTTACTCAGTTGGAGTCAAGATGTGATAGATAGGATATACAAGC
NDV HN (ZJ1 strain) (210) TACATCTTTACTCAGTTTGAGTCAAGATGTGATAGATAACATATACAAGC
NDV HN LaSota (M24709) (301) TACATCTACACTTGGTTGCAATCAAGATGTAGTAGATAGGATATATAAGC
NDV HN texas (M21409) (220) TACATCTGCACTTGGTTGCAATCAAGATGTAGTAGATAGGATATATAAGC
NDV HN CA02 (EF520717) (210) TACATCTTTACTCAATTCAAATCAAGATGTGGTAGATAGGGTATATAAAC
NDV HN MEX (AY288999) (210) TACATCTTTACTCAATTCAAATCAAGATGTGGTAGATAGGGTATATAAAC
351 400
NDV HN (FJ608369) (260) AGGTGGCTCTTGAATCGCCACTGGCGCTACTAAAGACTGAATCTATGATT
NDV HN (ZJ1 strain) (260) AGGTGGCTCTTGAATCGCCGCTGGCGCTACTAAAGACTGAATTATAATT
NDV HN LaSota (M24709) (351) AAGTGGCCCTTGAGTCTCCGTTGGCATTGTTAAAACTGAGACCACAATT
NDV HN texas (M21409) (270) AAGTGGCCCTTGAGTCTCCGTTGGCATTGTTAAACACTGAGACTACAATT
NDV HN CA02 (EF520717) (260) AGGTGGCGCTTGAGTCGCCGCTGGCGTTGTTGAATACTGAGTCTATAATT
NDV HN MEX (AY288999) (260) AGGTGGCGCTTGAGTCGCCGCTGGCGTTGTTGAATACTGAGTCTATAATT

Figure 7a (2/6)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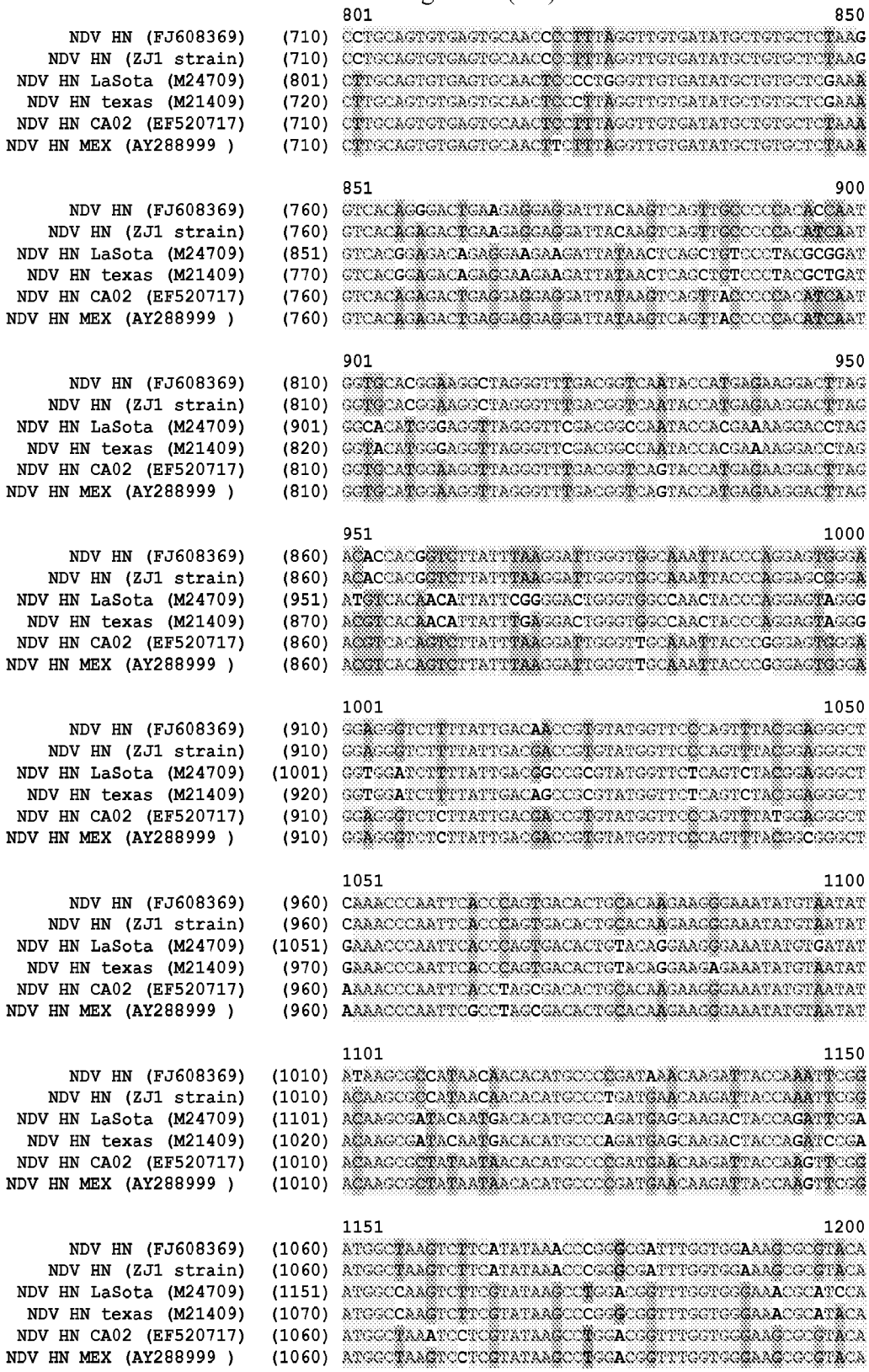
NDV HN (ZJ1 strain) (310) ATGAATGCAATAACCTCTCTTTCTTATCAAATTAACGGGCTGCGAACAA
NDV HN LaSota (M24709) (401) ATGAACGCAATAACATCTCTCTCTTATCAGATTAATGGAGCTGCAAACAA
NDV HN texas (M21409) (320) ATGAACGCAATAACATCTCTCTCTTATCAGATTAATGGAGCTGCGAACAA
NDV HN CA02 (EF520717) (310) ATGAATGCAATAACTTCTCTTTCCTATCAAATTAATGGGGCTGCAAATAG
NDV HN MEX (AY288999) (310) ATGAATGCAATAACTTCTCTTTCCTATCAAATTAATGGGGCTGCAAATAG
451 500
NDV HN (FJ608369) (360) TACCGGATGTGGGGCGCCTGTTCATGACCCAGATTATATCGGGGGGATAG
NDV HN (ZJ1 strain) (360) TAGCGGATGTGGGGCGCCTGTTCATGACCCAGATTATATCGGGGGGATAG
NDV HN LaSota (M24709) (451) CAGCCCGTGGGGGGCACCTATCCATGACCCAGATTATATACGGGGCATAG
NDV HN texas (M21409) (370) CAGCGGGTGGGGGGCACCTATCCATGACCCAGATTTTATCGGGGGGATAG
NDV HN CA02 (EF520717) (360) TAGTGGGTGTGGGGCACCTGTTCATGACCCGGATTATATTGGGGGGGTAG
NDV HN MEX (AY288999) (360) TAGTGGGTGTGGGGCACCTGTTCATGACCCGGATTATATTGGGGGGGTAG
501 550
NDV HN (FJ608369) (410) GCAAAGAACTCATAGTGGACGACATCAGTGATGTCACAACATTTTATCCT
NDV HN (ZJ1 strain) (410) GCAAAGAACTCATAGTGGACGACATCAGTGATGTCACAACATTTTATCCT
NDV HN LaSota (M24709) (501) GCAAAGAACTCATTGTAGATGATGCTAGTGATGTCACAACATTCTATCCC
NDV HN texas (M21409) (420) GCAAAGAACTCATTGTAGATCATGCTAGTGATGTCACAACATTCTATCCC
NDV HN CA02 (EF520717) (410) GTAAAGAGCTCATAGTAGATCACACGAGTGATGCCACTTCATTCTATCCT
NDV HN MEX (AY288999) (410) GTAAACAGCTCATAGTAGATCACACAAGTGATGCCACTTCATTCTATCCT
551 600
NDV HN (FJ608369) (460) TCTGCATATCAAGAACACTTGAATTTCATCCCGGCGCCTACTACAGGATC
NDV HN (ZJ1 strain) (460) TCTGCATATCAAGAACACTTGAATTTCATCCCGGCGCCTACTACAGGATC
NDV HN LaSota (M24709) (551) TCTGCATTTCAAGAACATCTGAATTTTATCCCGGCGCCTACTACAGGATC
NDV HN texas (M21409) (470) TCTGCATTTCAAGAACATCATAATTTTATCCCGGCGCCTACTACAGGATC
NDV HN CA02 (EF520717) (460) TCAGCATATCAAGAACACCTGAACTTTATCCCGGCGCCCACCACAGGTTC
NDV HN MEX (AY288999) (460) TCAGCATATCAAGAACACCTGAACTTTATCCCGGCGCCCACCACAGGATC
601 650
NDV HN (FJ608369) (510) CGGTTGCACTCCGATACCCTCATTTGACATGAGCACCACCCATTATTCTT
NDV HN (ZJ1 strain) (510) CGGTTGCACTCCGATACCCTCATTTGACATGAGCACCACCCATTATTCTT
NDV HN LaSota (M24709) (601) AGGTTGCATTCGAATACCCTCATTTGACATGAGTGCTACCCATTACTGCT
NDV HN texas (M21409) (520) AGGTTGCATTCGATACCTTCATTTGACATGAGTGCTACCCATTACTGCT
NDV HN CA02 (EF520717) (510) AGGCTGCACTCCGATACCCTCATTCGACATGAGCGCTACCCACTATTCTT
NDV HN MEX (AY288999) (510) AGGCTGCACTCCGATACCCTCATTCGACATGACCGCTACCCACTATTCTT
651 700
NDV HN (FJ608369) (560) ATACTCACAATGTGATACTATCCGGTTGCAGAGATCACTCACACTCACAT
NDV HN (ZJ1 strain) (560) ATACTCACAATGTGATACTATCCGGTTGCAGAGATCACTCACACTCACAT
NDV HN LaSota (M24709) (651) ACACCCATAATGTAATATTGTCTGGATGCAGAGATCACTCACATTCATAT
NDV HN texas (M21409) (570) ACACTCATAATATAATATCGTCTGGATGCAGAGATCACTCACACTCATAT
NDV HN CA02 (EF520717) (560) ATACTCACAATGTGATATTATCTGGCTGCAGAGATCACTCACACTCACAT
NDV HN MEX (AY288999) (560) ATACTCACAATGTGATATTATCTGGCTGCAGAGATCACTCACACTCACAT
701 750
NDV HN (FJ608369) (610) CAATACTTAGCACTTGGTGTGCTTCGGACATCTGCAACAGGGAGGGTATT
NDV HN (ZJ1 strain) (610) CAATACTTAGCACTTGGTGTGCTTCGGACATCTGCAACAGGGAGGGTATT
NDV HN LaSota (M24709) (701) CAGTATTTAGCACTTGGTGTGCTCCGGACATCTGCAACAGGGAGGGTATT
NDV HN texas (M21409) (620) CAGTATTTAGCACTTGGTGTGCTCCGGACTTCTGCAACAGGGAGGATATT
NDV HN CA02 (EF520717) (610) CAGTATTTGGCACTAGGTGTGCTTCGGACATCTGCAACAGGGAGGGTATT
NDV HN MEX (AY288999) (610) CAGTATTTGGCACTAGGTGTGCTTCGGACATCTGCAACAGGGAGGGTATT
751 800
NDV HN (FJ608369) (660) CTTTTCTACTCTGCGCTCCATCAATTTAGATGACACCCAAAATCGGAAGT
NDV HN (ZJ1 strain) (660) CTTTTCTACTCTGCGCTCCACCAATTTAGATGACACCCAAAATCGGAAGT
NDV HN LaSota (M24709) (751) CTTTTCTACTCTGCGCTTCCATCAACCTGGACGACACCCAAAATCGGAAGT
NDV HN texas (M21409) (670) CTTTTCTACTCTGCGCTTCCATCAATCTGGATGACACCCAGAATCGGAAGT
NDV HN CA02 (EF520717) (660) CTTTTCTACTCTGCGCTTCCATCAATTTAGATGACACCCAAAATCGGAAGT
NDV HN MEX (AY288999) (660) CTTTTCTACTCTGCGCTTCCATCAATTTAGATGACACCCAAAATCGGAAGT

Figure 7a (3/6)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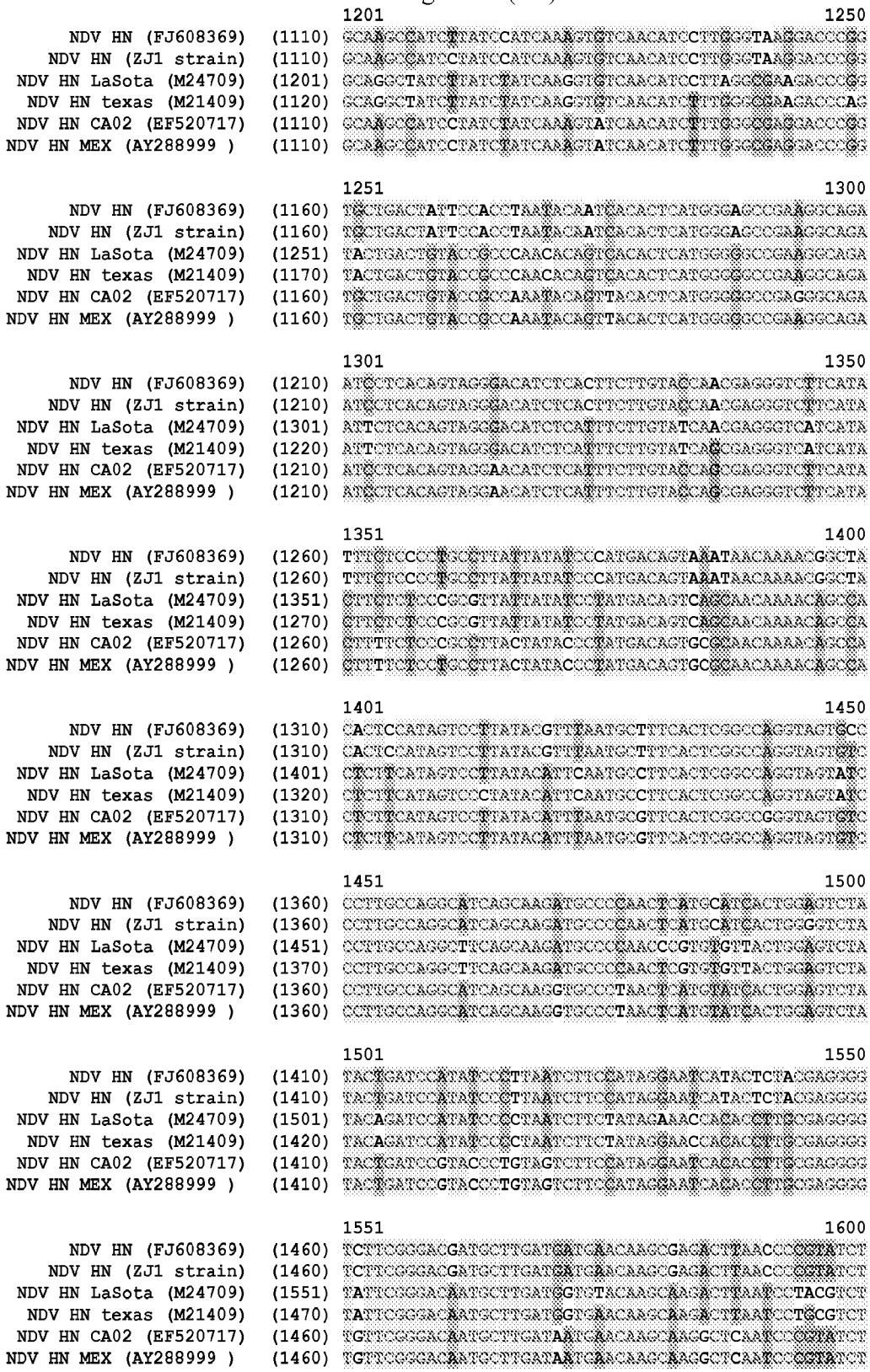
NDV HN (ZJ1 strain) (710) CCTGCAGTGTGAGTGCAACCCCTTTAGGTTGTGATATGCTGTGCTCTAAG
NDV HN LaSota (M24709) (801) CTTGCAGTGTGAGTGCAACTCCCCTGGTTGTGATATGCTGTGCTCGAAA
NDV HN texas (M21409) (720) CTTGCAGTGTGAGTGCAACTCCCTTAGTTGTGATATGCTGTGCTCGAAA
NDV HN CA02 (EF520717) (710) CTTGCAGTGTGAGTGCAACTCCTTTAGGTTGTGATATGCTGTGCTCTAAA
NDV HN MEX (AY288999) (710) CTTGCAGTGTGAGTGCAACTTCTTTAGGTTGTGATATGCTGTGCTCTAAA
851 900
NDV HN (FJ608369) (760) GTCACAGGGACTGAAGAGCAGGATTACAAGTCAGTTGCCCCCACACCAAT
NDV HN (ZJ1 strain) (760) GTCACACAGACTGAAGAGCAGGATTACAAGTCAGTTGCCCCCACATCAAT
NDV HN LaSota (M24709) (851) GTCACGGAGACAGAGCAAGAAGATTATAACTCAGCTGTCCCTACGCGGAT
NDV HN texas (M21409) (770) GTCACGCAGCACAGAGCAAGAAGATTATAACTCAGCTGTCCCTACGCTGAT
NDV HN CA02 (EF520717) (760) GTCACACAGACTGAGGAGCAGGATTATAAGTCAGTTACCCCCACATCAAT
NDV HN MEX (AY288999) (760) GTCACACAGACTGAGGAGCAGGATTATAAGTCAGTTACCCCCACATCAAT
901 950
NDV HN (FJ608369) (810) GGTGCACGCAAGGCTAGGGTTTGACGGTCAATACCATGAGAAGGACTTAG
NDV HN (ZJ1 strain) (810) GGTGCACGCAAGGCTAGGGTTTGACGGTCAATACCATGAGAAGGACTTAG
NDV HN LaSota (M24709) (901) GGCACATCGGAGGTTAGGGTTCGACGGCCAATACCACGAAAGGACCTAG
NDV HN texas (M21409) (820) GGTACATCGGAGGTTAGGGTTCGACGGCCAATACCACGAAAGGACCTAG
NDV HN CA02 (EF520717) (810) GGTGCATCGAAGGTTAGGGTTTGACGGTCAGTACCATGAGAAGGACTTAG
NDV HN MEX (AY288999) (810) GGTGCATCGAAGGTTAGGGTTTGACGGTCAGTACCATGAGAAGGACTTAG
951 1000
NDV HN (FJ608369) (860) ACACCACGGTCTTATTTAAGGATTGGGTTGCAAATTACCCAGGAGTCGGA
NDV HN (ZJ1 strain) (860) ACACCACGGTCTTATTTAAGGATTGGGTTGCAAATTACCCAGGAGCCGGA
NDV HN LaSota (M24709) (951) ATGTCACAACATTATTCGGGGACTGGGTGGCCAACTACCCAGGAGTAGGG
NDV HN texas (M21409) (870) ACGTCACAACATTATTTGAGGACTGGGTGGCCAACTACCCAGGAGTAGGG
NDV HN CA02 (EF520717) (860) ACGTCACAGTCTTATTTAAGGATTGGGTTGCAAATTACCCGGGAGTCGGA
NDV HN MEX (AY288999) (860) ACGTCACAGTCTTATTTAAGGATTGGGTTGCAAATTACCCGGGAGTGGGA
1001 1050
NDV HN (FJ608369) (910) GGAGGGTCTCTTTATTGACAACCGTGTATGGTTCCCAGTTTACGCAGGGCT
NDV HN (ZJ1 strain) (910) GGAGGGTCTCTTTATTGACGACCGTGTATGGTTCCCAGTTTACGCAGGGCT
NDV HN LaSota (M24709) (1001) GGTGGATCTCTTTATTGACGGCCGCGTATGGTTCTCAGTCTACGCAGGGCT
NDV HN texas (M21409) (920) GGTGGATCTCTTTATTGACAGCCGCGTATGGTTCTCAGTCTACGCAGGGCT
NDV HN CA02 (EF520717) (910) GGAGGGTCTCTTATTGACGACCGTGTATGGTTCCCAGTTTATGCAGGGCT
NDV HN MEX (AY288999) (910) GGAGGGTCTCTTATTGACGACCGTGTATGGTTCCCAGTTTACGGCGGGCT
1051 1100
NDV HN (FJ608369) (960) CAAACCCAATTCACCCAGTGACACTCCACAAGAACGGAAATATGTAATAT
NDV HN (ZJ1 strain) (960) CAAACCCAATTCACCCAGTGACACTCCACAAGAACGGAAATATGTAATAT
NDV HN LaSota (M24709) (1051) GAAACCCAATTCACCCAGTGACACTGTACAGGAACGAAATATGTGATAT
NDV HN texas (M21409) (970) GAAACCCAATTCACCCAGTGACACTGTACAGGAAGAGAAATATGTAATAT
NDV HN CA02 (EF520717) (960) AAAACCCAATTCACCTAGCGACACTCCACAAGAACGCAAATATGTAATAT
NDV HN MEX (AY288999) (960) AAAACCCAATTCGCCTAGCGACACTCCACAAGAACGCAAATATGTAATAT
1101 1150
NDV HN (FJ608369) (1010) ATAAGCGCCATAACAACACATGCCCCGATAAACAAGATTACCAAATTCGG
NDV HN (ZJ1 strain) (1010) ACAAGCGCCATAACAACACATGCCCTGATGAACAAGATTACCAAATTCCG
NDV HN LaSota (M24709) (1101) ACAAGCGATACAATGACACATGCCCAGATGAGCAAGACTACCAGATTCGA
NDV HN texas (M21409) (1020) ACAAGCGATACAATGACACATGCCCAGATGAGCAAGACTACCAGATCCGA
NDV HN CA02 (EF520717) (1010) ACAAGCGCTATAATAACACATGCCCCGATGAACAAGATTACCAAGTTCGG
NDV HN MEX (AY288999) (1010) ACAAGCGCTATAATAACACATGCCCCGATGAACAAGATTACCAAGTTCGG
1151 1200
NDV HN (FJ608369) (1060) ATGGCTAAGTCTTCATATAAACCCGCGCGATTTGGTGGAAAGCGCGTACA
NDV HN (ZJ1 strain) (1060) ATGGCTAAGTCTTCATATAAACCCGCGCGATTTGGTGGAAAGCGCGTACA
NDV HN LaSota (M24709) (1151) ATGGCCAAGTCTTCGTATAAGCCTGCACGTTTGGTGGCAAACGCATACA
NDV HN texas (M21409) (1070) ATGGCCAAGTCTTCGTATAAGCCCGCGCGTTTGGTGGCAAACGCATACA
NDV HN CA02 (EF520717) (1060) ATGGCTAAATCCTCGTATAAGCCTGCACGTTTGGTGGCAAGCGCGTACA
NDV HN MEX (AY288999) (1060) ATGGCTAAGTCCTCGTATAAGCCTGCACGTTTGGTGGCAAGCGCGTACA

Figure 7a (4/6)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NDV HN (FJ608369) (1110)
NDV HN (ZJ1 strain) (1110)
NDV HN LaSota (M24709) (1201)
NDV HN texas (M21409) (1120)
NDV HN CA02 (EF520717) (1110)
NDV HN MEX (AY288999) (1110)
1251 1300
NDV HN (FJ608369) (1160)
NDV HN (ZJ1 strain) (1160)
NDV HN LaSota (M24709) (1251)
NDV HN texas (M21409) (1170)
NDV HN CA02 (EF520717) (1160)
NDV HN MEX (AY288999) (1160)
1301 1350
NDV HN (FJ608369) (1210)
NDV HN (ZJ1 strain) (1210)
NDV HN LaSota (M24709) (1301)
NDV HN texas (M21409) (1220)
NDV HN CA02 (EF520717) (1210)
NDV HN MEX (AY288999) (1210)
1351 1400
NDV HN (FJ608369) (1260)
NDV HN (ZJ1 strain) (1260)
NDV HN LaSota (M24709) (1351)
NDV HN texas (M21409) (1270)
NDV HN CA02 (EF520717) (1260)
NDV HN MEX (AY288999) (1260)
1401 1450
NDV HN (FJ608369) (1310)
NDV HN (ZJ1 strain) (1310)
NDV HN LaSota (M24709) (1401)
NDV HN texas (M21409) (1320)
NDV HN CA02 (EF520717) (1310)
NDV HN MEX (AY288999) (1310)
1451 1500
NDV HN (FJ608369) (1360)
NDV HN (ZJ1 strain) (1360)
NDV HN LaSota (M24709) (1451)
NDV HN texas (M21409) (1370)
NDV HN CA02 (EF520717) (1360)
NDV HN MEX (AY288999) (1360)
1501 1550
NDV HN (FJ608369) (1410)
NDV HN (ZJ1 strain) (1410)
NDV HN LaSota (M24709) (1501)
NDV HN texas (M21409) (1420)
NDV HN CA02 (EF520717) (1410)
NDV HN MEX (AY288999) (1410)
1551 1600
NDV HN (FJ608369) (1460)
NDV HN (ZJ1 strain) (1460)
NDV HN LaSota (M24709) (1551)
NDV HN texas (M21409) (1470)
NDV HN CA02 (EF520717) (1460)
NDV HN MEX (AY288999) (1460)

Figure 7a (5/6)

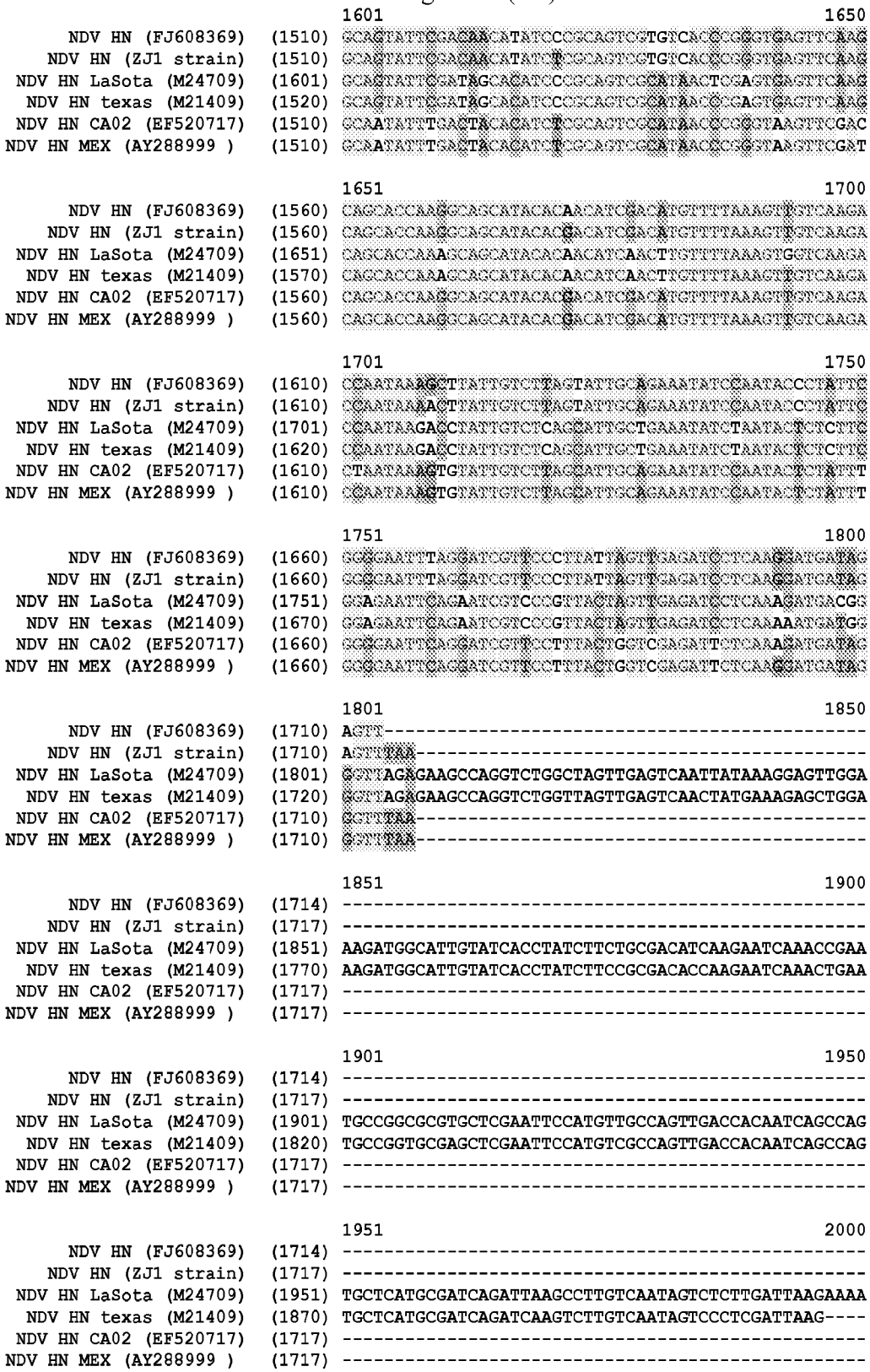
1601 1650
NDV HN (FJ608369) (1510)
NDV HN (ZJ1 strain) (1510)
NDV HN LaSota (M24709) (1601)
NDV HN texas (M21409) (1520)
NDV HN CA02 (EF520717) (1510)
NDV HN MEX (AY288999) (1510)
1651 1700
NDV HN (FJ608369) (1560)
NDV HN (ZJ1 strain) (1560)
NDV HN LaSota (M24709) (1651)
NDV HN texas (M21409) (1570)
NDV HN CA02 (EF520717) (1560)
NDV HN MEX (AY288999) (1560)
1701 1750
NDV HN (FJ608369) (1610)
NDV HN (ZJ1 strain) (1610)
NDV HN LaSota (M24709) (1701)
NDV HN texas (M21409) (1620)
NDV HN CA02 (EF520717) (1610)
NDV HN MEX (AY288999) (1610)
1751 1800
NDV HN (FJ608369) (1660)
NDV HN (ZJ1 strain) (1660)
NDV HN LaSota (M24709) (1751)
NDV HN texas (M21409) (1670)
NDV HN CA02 (EF520717) (1660)
NDV HN MEX (AY288999) (1660)
1801 1850
NDV HN (FJ608369) (1710)
NDV HN (ZJ1 strain) (1710)
NDV HN LaSota (M24709) (1801)
NDV HN texas (M21409) (1720)
NDV HN CA02 (EF520717) (1710)
NDV HN MEX (AY288999) (1710)
1851 1900
NDV HN (FJ608369) (1714)
NDV HN (ZJ1 strain) (1717)
NDV HN LaSota (M24709) (1851)
NDV HN texas (M21409) (1770)
NDV HN CA02 (EF520717) (1717)
NDV HN MEX (AY288999) (1717)
1901 1950
NDV HN (FJ608369) (1714)
NDV HN (ZJ1 strain) (1717)
NDV HN LaSota (M24709) (1901)
NDV HN texas (M21409) (1820)
NDV HN CA02 (EF520717) (1717)
NDV HN MEX (AY288999) (1717)
1951 2000
NDV HN (FJ608369) (1714)
NDV HN (ZJ1 strain) (1717)
NDV HN LaSota (M24709) (1951)
NDV HN texas (M21409) (1870)
NDV HN CA02 (EF520717) (1717)
NDV HN MEX (AY288999) (1717)

Figure 7a (6/6)

```
                                2001
      NDV HN (FJ608369)   (1714) --
    NDV HN (ZJ1 strain)   (1717) --
 NDV HN LaSota (M24709)   (2001) AA
  NDV HN texas (M21409)   (1916) --
 NDV HN CA02 (EF520717)   (1717) --
NDV HN MEX (AY288999 )    (1717) --
```

NDV HN CA02 (EF520717): SEQ ID NO:2
NDV HN MEX (AY288999): SEQ ID NO:8
NDV HN LaSota (M24709): SEQ ID NO:6
NDV HN texas (M21409): SEQ ID NO:4
NDV HN (FJ608369): SEQ ID NO:16
NDV HN (ZJ1 strain): SEQ ID NO:18

Sequence identity percentage:

SEQ ID NO:	2	4	6	8	16	18
2	100	83.9%	83.5%	98.9%	87.2%	87.9%
4		100	83.8%	84.2%	82.8%	82.9%
6			100	96.3%	82.1%	82.2%
8				100	87.5%	88.2%
16					100	98.4%
18						100

Vector NTI 11.0 (PC) software package (Invitrogen, 1600 Faraday Ave., Carlsbad, CA).

Figure 7b (1/4)

Alignment of SEQ ID NO:1 and SEQ ID NO:2

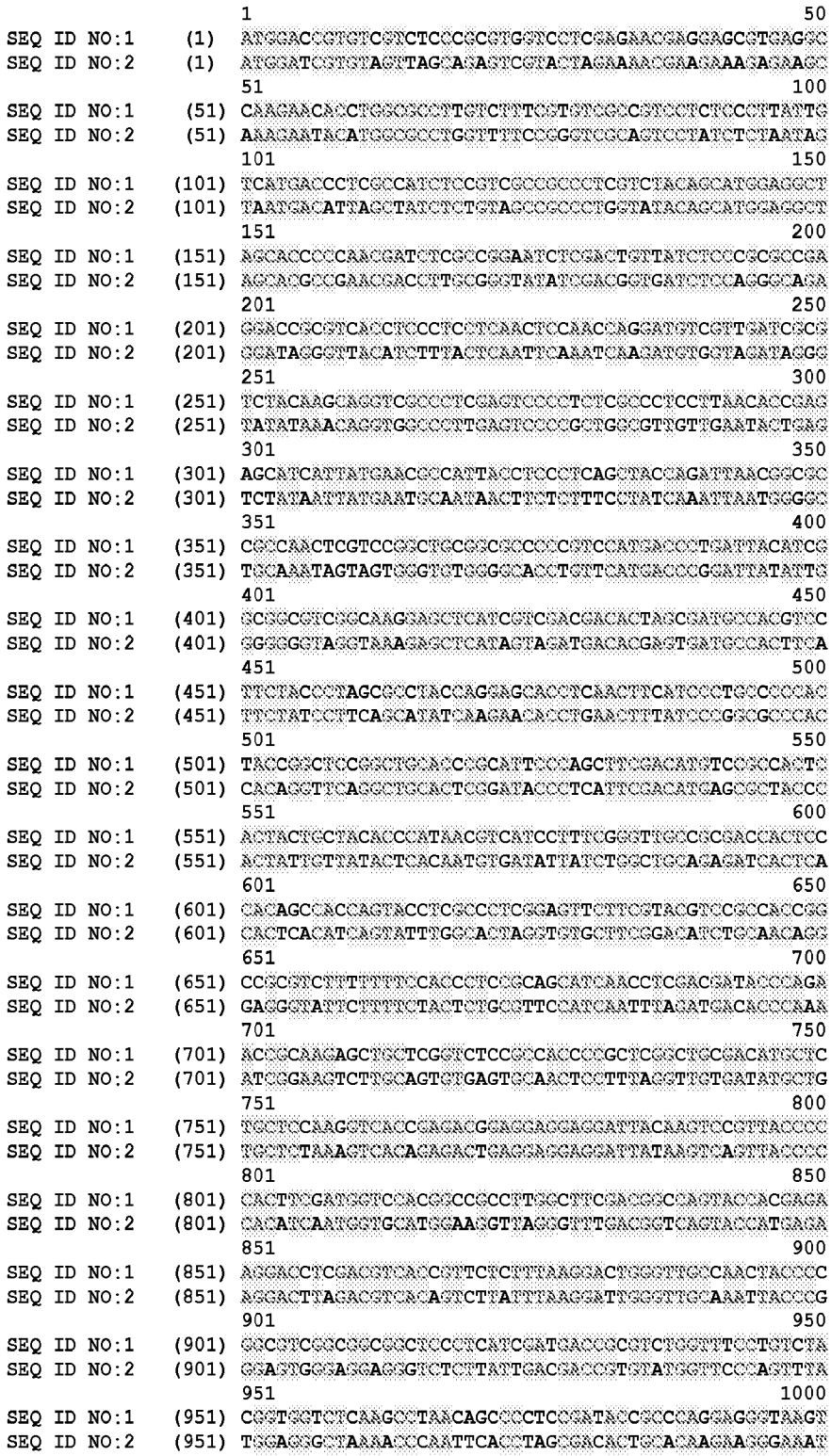

Figure 7b (2/4)

```
                    1001                                                1050
SEQ ID NO:1  (1001) ACGTGATCTACAAGCGCTACAACAACACCTGCCCTGACGAGCAGGATTAC
SEQ ID NO:2  (1001) ATGTAATATACAAGCGCTATAATAACACATGCCCCGATGAACAAGATTAC
                    1051                                                1100
SEQ ID NO:1  (1051) CAGGTCCGCATGGCCAAGTCCTCGTACAAGCCCGGTCGTTTCGGCGGCAA
SEQ ID NO:2  (1051) CAAGTTCGGATGGCTAAATCCTCGTATAAGCCTGGACGGTTTGGTGGAA
                    1101                                                1150
SEQ ID NO:1  (1101) GCGCGTCCAGCAGGCCATTCTCTCGATCAAGGTCTCGACCAGCCTCGGAG
SEQ ID NO:2  (1101) GCGCGTACAGCAAGCCATCCTATCTATCAAAGTATCAACATCTTTGGGCG
                    1151                                                1200
SEQ ID NO:1  (1151) AGGACCCGTGCTCACCGTCCCCCTAACACCGTCACCTTATGGCGCC
SEQ ID NO:2  (1151) AGGACCCGGTGCTGACTGTACCGCCAAATACAGTTACACTCATGGGCCC
                    1201                                                1250
SEQ ID NO:1  (1201) GAGGGCCGCATCCTCACCGTCGGTACCTCCCACTTCCTCTACCAGCGCGG
SEQ ID NO:2  (1201) GAGGGCAGAATCCTCACAGTAGGAACATCTCATTTCTTGTACCAGCGAGG
                    1251                                                1300
SEQ ID NO:1  (1251) CTCGAGCTACTTTTCCCCTGCCCTTCTTTACCCCATGACTGTTCGCAACA
SEQ ID NO:2  (1251) GTCTTCATACTTTTCTCCCGCCTTACTATACCCTATGACAGTGCGCAACA
                    1301                                                1350
SEQ ID NO:1  (1301) AGACTGCTACCCTCCACAGCCCCTACACCTTTAACGCCTTCACGCGCCCC
SEQ ID NO:2  (1301) AACAGCCACTCTTCATAGTCCTTATACATTTAATGCGTTCACTCGGCCG
                    1351                                                1400
SEQ ID NO:1  (1351) GCAAGCGTCCCCTGCCAGGCGAGCGCCCGCTGCCCTAACTCCTGCATTAC
SEQ ID NO:2  (1351) GGTAGTGTCCCTTGCCAGGCATCAGCAAGGTGCCCTAACTCATGTATCAC
                    1401                                                1450
SEQ ID NO:1  (1401) CGGCGTCTACACCGACCCTTACCCTGTCGTCTTTCACCGCAACCATACCC
SEQ ID NO:2  (1401) TGGAGTCTATACTGATCCGTACCCTGTAGTCTTCCATAGGAATCACACCT
                    1451                                                1500
SEQ ID NO:1  (1451) TTCGCGGCGTCTTCGGTACTATGCTTGATAACGAGCAGGCCCGCGTCAAC
SEQ ID NO:2  (1451) TGCGAGGGTGTTCGCGACAATGCTTGATAATGAACAAGCAAGGCTCAAT
                    1501                                                1550
SEQ ID NO:1  (1501) CCCGTCTCCGCCATTTTCGACTACACTTCCCGCTCCCGTATCACCCGCGT
SEQ ID NO:2  (1501) CCCGTATCTGCAATATTTGACTACACATCTCGCAGTCGCATAACCCGGGT
                    1551                                                1600
SEQ ID NO:1  (1551) CTCCTCCACCTCCACCAAGGCCGCCTACACCACCTCCACCTGCTTTAAGG
SEQ ID NO:2  (1551) AAGTTCGACCAGCACCAAGGCAGCATACACGACATCGACATGTTTTAAAG
                    1601                                                1650
SEQ ID NO:1  (1601) TTGTCAAGACTAACAAGGTCTACTGCCTCTCCATCGCCGAGATTAGCAAC
SEQ ID NO:2  (1601) TTGTCAAGACTAATAAAGTGTATTGTCTTAGCATTGCAGAATATCCAAT
                    1651                                                1700
SEQ ID NO:1  (1651) ACCCTCTTCGGAGAGTTCCGCATTGTCCCCCTGCTCGTCGAGATCCTCAA
SEQ ID NO:2  (1651) ACTCTATTTGGGGAATTCAGGATCGTTCCTTTACTGGTCGAGATTCTCAA
                    1701        1716
SEQ ID NO:1  (1701) GGACGATCGCGTTTAA
SEQ ID NO:2  (1701) AGATGATAGGGTTTAA
```

Sequence identity percentage between SEQ ID NOs:1 and 2: 72.4%

Figure 7b (3/4)

Alignment of SEQ ID NO:16 and SEQ ID NO:22

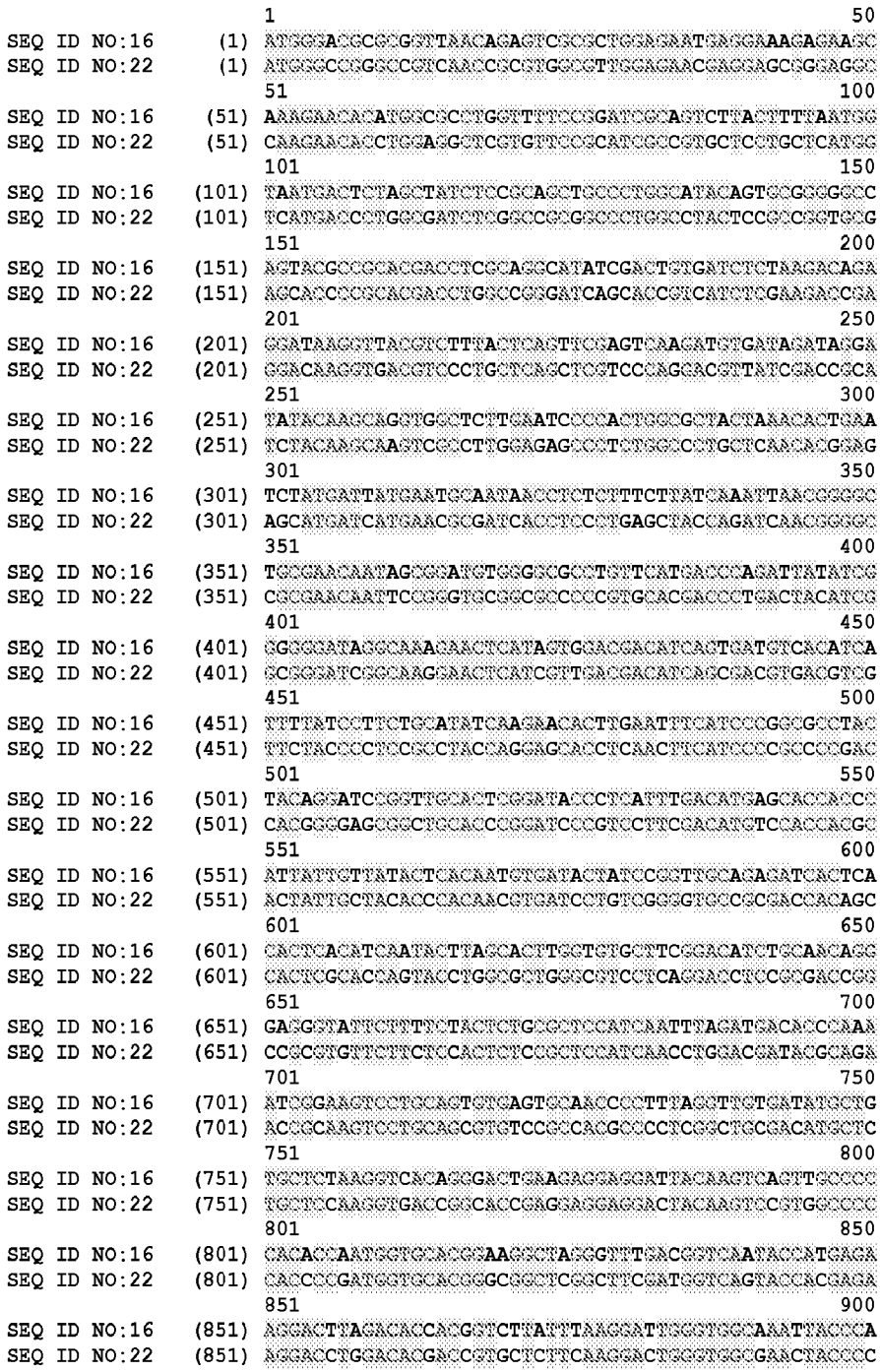

Figure 7b (4/4)

```
                    901                                                 950
SEQ ID NO:16   (901) GGAGTGGGAGGAGGGTCTTTTATTGACAACCGTGTATGGTTCCCATTTTA
SEQ ID NO:22   (901) GGTGTGGGGGGCGGTAGCTTCATCGACAACAGAGTCTGGTTCCCCGTGTA
                    951                                                1000
SEQ ID NO:16   (951) CGGAGGGCTCAAACCCAATTCACCCAGTGACACTGCACAAGAAGGGAAAT
SEQ ID NO:22   (951) CGGGGGCCTGAAGCCCAACTCCCCCTCCGACACGGCCCAGGAGGGGAAGT
                   1001                                                1050
SEQ ID NO:16  (1001) ATGTAATATATAAGCGCCATAACAACACATGCCCCGATAAACAAGATTAC
SEQ ID NO:22  (1001) ACGTCATCTACAAGCGGCACAACAACACCTGCCCGGACAAGCAGGACTAT
                   1051                                                1100
SEQ ID NO:16  (1051) CAAATTCGGATGGCTAAGTCTTCATATAAACCCGGCGATTTGGTGGAAA
SEQ ID NO:22  (1051) CAGATCCGGATGGCCAAAAGCTCCTACAAGCCCGCCGCTTCGGGGCAA
                   1101                                                1150
SEQ ID NO:16  (1101) GCGCGTACAGCAAGCCATCTTATCCATCAAAGTGTCAACATCCTTGGGTA
SEQ ID NO:22  (1101) GAGAGTCCAGCAGGCGATCCTCTCCATCAAGGTGAGCACGAGCCTCGCCA
                   1151                                                1200
SEQ ID NO:16  (1151) AGGACCCGGTGCTGACTATTCCACCTAATACAATCACACTCATGGGAGCC
SEQ ID NO:22  (1151) AGGACCCGGTCCTGACCATCCCCCCGAACACCATCACCCTCATGGGCGCC
                   1201                                                1250
SEQ ID NO:16  (1201) GAAGGCAGAATCCTCACACTAGGGACATCTCACTTCTTGTACCAACGAGG
SEQ ID NO:22  (1201) GAGGGGAGAATCCTCACTGTCGGCACCTCCCACTTCCTGTACCAGCGGGG
                   1251                                                1300
SEQ ID NO:16  (1251) GTCTTCATATTTCTCCCGTGCCTTATTATATCCCATGACAGTAAATAACA
SEQ ID NO:22  (1251) CAGCTCGTACTTCAGCCCGGCGCTCCTGTACCCGATGACCGTCAACAACA
                   1301                                                1350
SEQ ID NO:16  (1301) AAACGCCTACACTCCATAGTCCTTATACGTTTAATGCTTTCACTCGGCCA
SEQ ID NO:22  (1301) AGACCGCCACGCTGCACTCGCCCTACACCTTCAACGCCTTCACCCGCCCC
                   1351                                                1400
SEQ ID NO:16  (1351) GGTAGTGCCCCTTGCCAGGCATCAGCAAGATGCCCCAACTCATGCATCAC
SEQ ID NO:22  (1351) GGCAGCGCCCCGTGCCAGGCCTCCGCCCGCTGCCCGAACTCGTGCATCAC
                   1401                                                1450
SEQ ID NO:16  (1401) TGGAGTCTATACTGATCCATATCCCTTAATCTTCCATAGGAATCATACTC
SEQ ID NO:22  (1401) CGGGGTCTACACCGACCCTTACCCGCTGATCTTCCACCGCAACCACACGC
                   1451                                                1500
SEQ ID NO:16  (1451) TACGAGGGGTCTTCCGGACGATGCTTGATGATGAACAAGCGACACTTAAC
SEQ ID NO:22  (1451) TCAGGGGGGTGTTCGGGACCATGCTCGACGACGAGCAGCCTCGCCTGAAC
                   1501                                                1550
SEQ ID NO:16  (1501) CCCGTATCTGCAGTATTCGACAACATATCCCGCAGTCGTGTCACCCGGGT
SEQ ID NO:22  (1501) CCCGTCAGCGCCGTCTTCGACAACATCTCCCGCAGCCGCGTCACGACAGT
                   1551                                                1600
SEQ ID NO:16  (1551) GAGTTCAAGCAGCACCAAGGCAGCATACACAACATCGACATGTTTAAAG
SEQ ID NO:22  (1551) CTCCTCGTCCTCGACGAAGGCCGCGTACACCACGTCCACCTGCTTCAAGC
                   1601                                                1650
SEQ ID NO:16  (1601) TTGTCAAGACCAATAAAGCTTATTGTCTTAGTATTGCAGAATATCCAAT
SEQ ID NO:22  (1601) TGCTTAAGACCAACAAAGCCTACTGCCTCTCCATCGCTGAGATCTCCAAC
                   1651                                                1700
SEQ ID NO:16  (1651) ACCCTATTCGGGGAATTTAGGATCGTTCCCTTATTAGTTGAGATCCTCAA
SEQ ID NO:22  (1651) ACCCTCTTCGGCGAGTTCCGGATCGTGCCCCTCTTGGTGGAGATCCTGAA
                  1701     1713
SEQ ID NO:16  (1701) GGATGATAGATTT
SEQ ID NO:22  (1701) GGACGACCGCGTG
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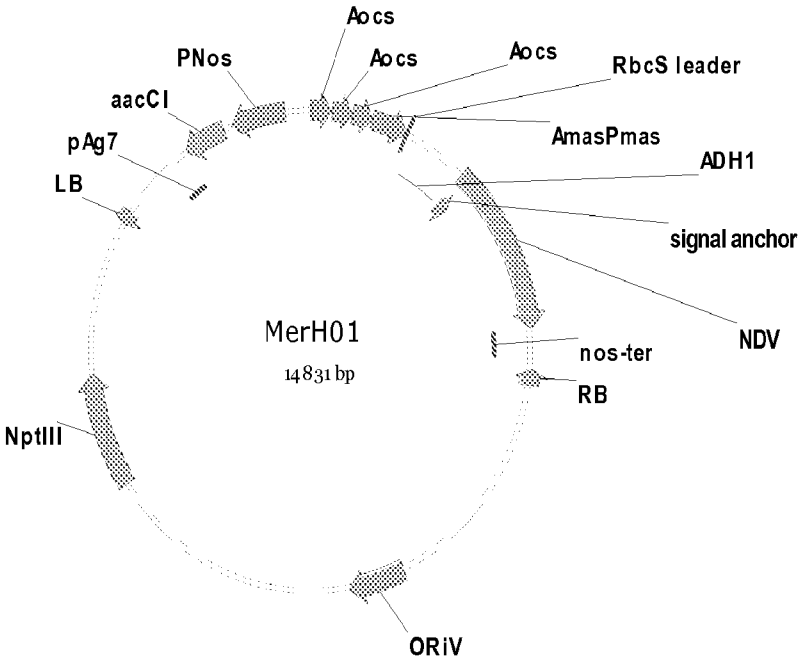
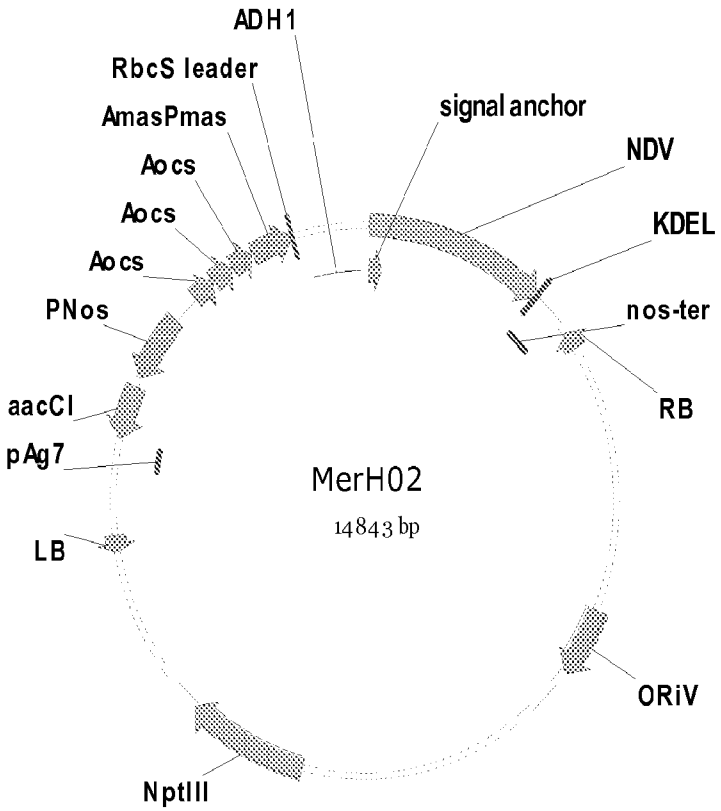
The identity percentage between SEQ ID NO:16 and SEQ ID NO:22 is 73%.

Figure 7c (1/2)


Aocs
Aocs
Aocs
PNos
RbcS leader
aacCI
AmasPmas
pAg7
ADH1
LB
signal anchor
MerH01
14831 bp
NDV
nos-ter
RB
NptIII
ORiV


ADH1
RbcS leader
signal anchor
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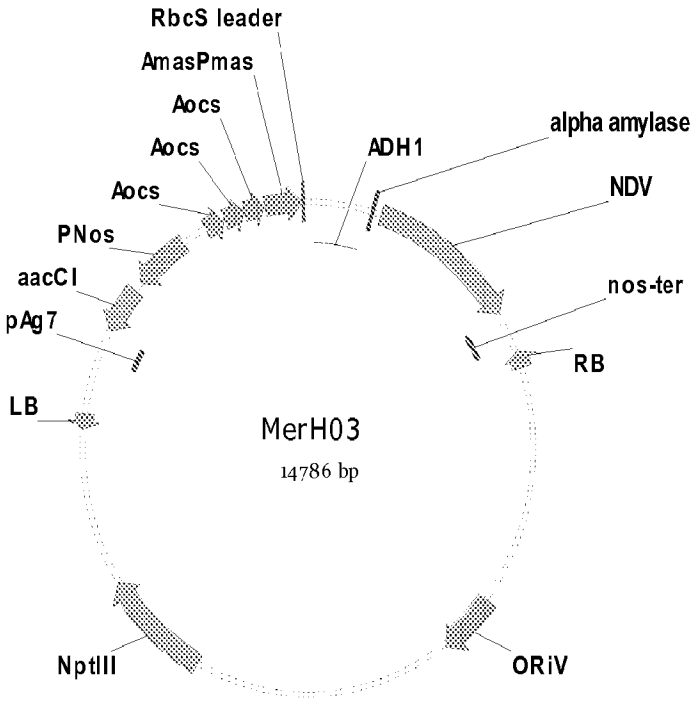
NDV
Aocs
Aocs
KDEL
Aocs
PNos
nos-ter
RB
aacCI
pAg7
MerH02
14843 bp
LB
ORiV
NptIII

Figure 7c (2/2)


RbcS leader
AmasPmas
Aocs
Aocs
Aocs
PNos
aacCI
pAg7
LB
ADH1
alpha amylase
NDV
nos-ter
RB
MerH03
14786 bp
NptIII
ORiV

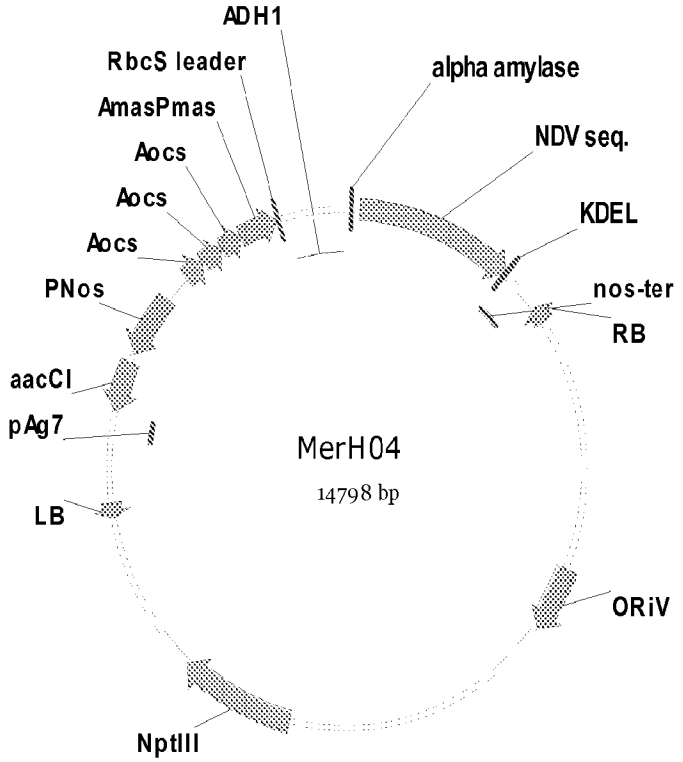
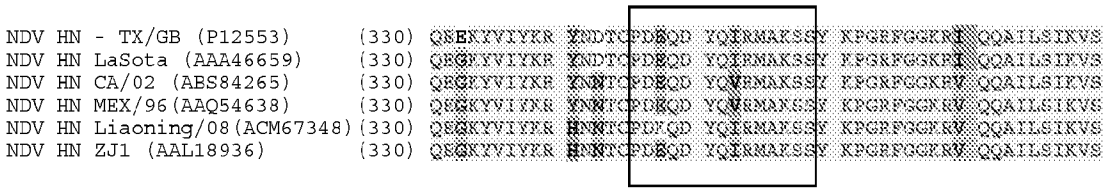

ADH1
RbcS leader
AmasPmas
Aocs
Aocs
Aocs
PNos
aacCI
pAg7
LB
alpha amylase
NDV seq.
KDEL
nos-ter
RB
MerH04
14798 bp
ORiV
NptIII

Figure 8

HN Linear Epitope Region (boxed region)



The alignment shows the amino acids between position 330 and position 379 of below protein sequences:

NDV HN - TX/GB (P12553): SEQ ID NO:5
NDV HN LaSota (AAA46659): SEQ ID NO:7
NDV HN CA/02(ABS84265): SEQ ID NO:3
NDV HN MEX/96 (AAQ54638): SEQ ID NO:9
NDV HN Liaoning/08(ACM67348): SEQ ID NO:17
NDV HN ZJ1 (AAL18936) SEQ ID NO:19

NDV HN Linear Epitope Region 1 (SEQ ID NO:10): PDEQDYQIRMAKSS

NDV HN Linear Epitope Region 2 (SEQ ID NO:11): PDEQDYQVRMAKSS

NDV HN Linear Epitope Region 3 (SEQ ID NO: 28): PDKQDYQIRMAKSS

Figure 9

Location and Presence of Glycosylation Sites in NDV Strains

Glycosylation Site*	Sequence	Glycosylation Site Presence by Strain		
		LaSota (SEQ ID NO:7)	CA/02 (SEQ ID NO:3)	TX/GB (SEQ ID NO:5)
119	NSS/NNS	X**	X	X
341	NNT/NDT	X	X	X
433	NKT	X	X	X
481	NHT	X	X	X
508				
538	NKT	X	-***	X

*There are six potential sites for glycosylation. The glycosylation site is indicated at the amino acid position of the protein sequence.
** : presence of the glycosylation site
*** : absence of the glycosylation site.

Figure 10

Graphical Feature Map of the NDV HN Protein (SEQ ID NO:3)

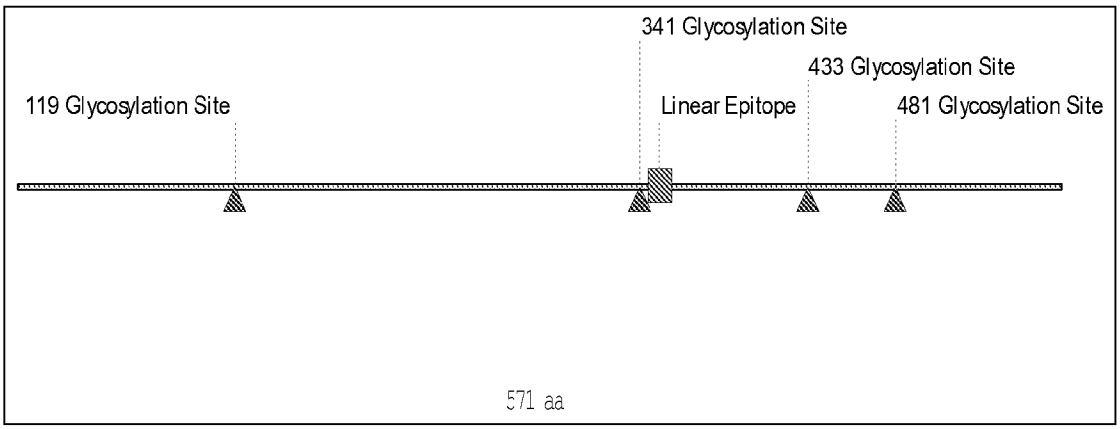


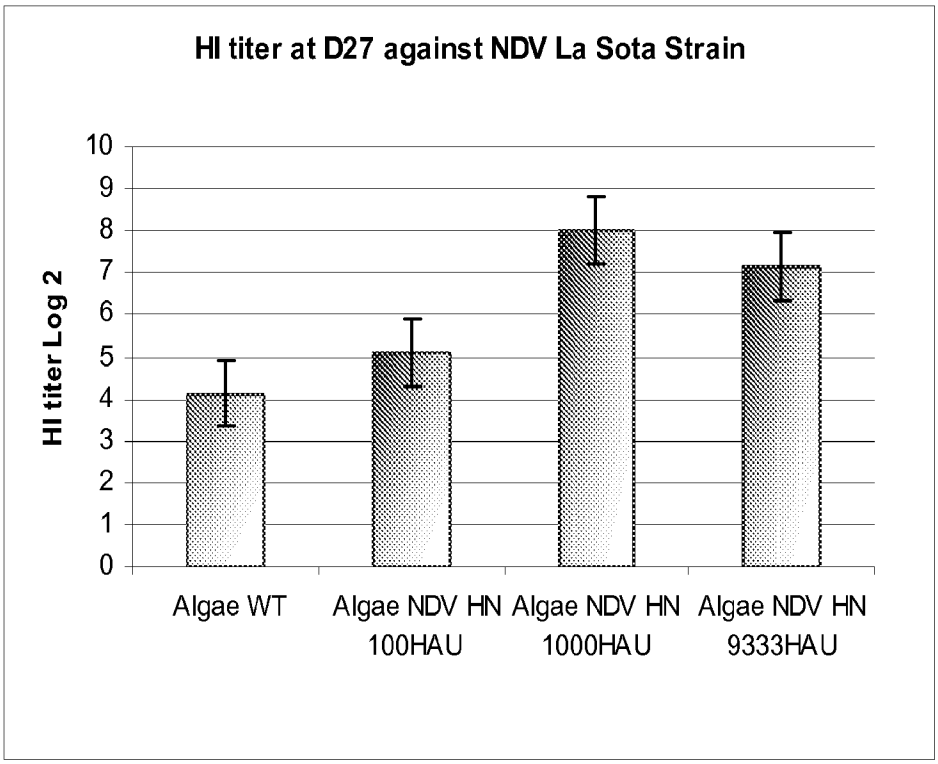
Figure 11

Peptide sequence analysis of expressed NDV HN (SEQ ID NO:3) in algae

Species: Newcastle disease virus
Name: Hemagglutinin-neuraminidase
Identified by 32 peptides covering 68% of the protein sequence.

```
1   MDRVVSRVVL ENEEREAKNT WRLVFRVAVL SLIVMTLAIS VAALVYSMEA STPNDLAGIS TVISRAEDRV TSLLNSNQDV
81  VDRVYKQVAL ESPLALLNTE SIIMNAITSL SYQINGAANS SGCGAPVHDP DYIGGVGKEL IVDDTSDATS FYPSAYQEHL
161 NFIPAPTTGS GCTRIPSFDM SATHYCYTHN VILSGCRDHS HSHQYLALGV LRTSATGRVF FSTLRSINLD DTQNRKSCSV
241 SATPLGCDML CSKVTETEEE DYKSVTPTSM VHGRLGFDGQ YHEKDLDVTV LFKDWVANYP GVGGGSLIDD KVWFPVYGGL
321 KPNSPSDTAQ EGKYVIYKRY NNTCPDEQDY QVRMAKSSYK PGRFGGKRVQ QAILSIKVST SLGEDPVLTV PPNTVTLMGA
401 EGRILTVGTS HFLYQRGSSY FSPALLYPMT VRNKTATLHS PYTFNAFTRP GSVPCQASAR CPNSCITGVY TDPYPVVFHR
481 NHTLRGVFGT MLDNEQARLN PVSAIFDYTS RSRITRVSST STKAAYTTST CFKVVKTNKV YCLSIAEISN TLFGEFRIVP
561 LLVEILKDDR V
```

Figure 12



Group	Mortality	Protection (%)
Algae WT	12/12	0
Algae NDV HN 100 HAU	8/12	33
Algae NDV HN 1000 HAU	0/12	100
Algae NDV HN 9333HAU	0/12	100