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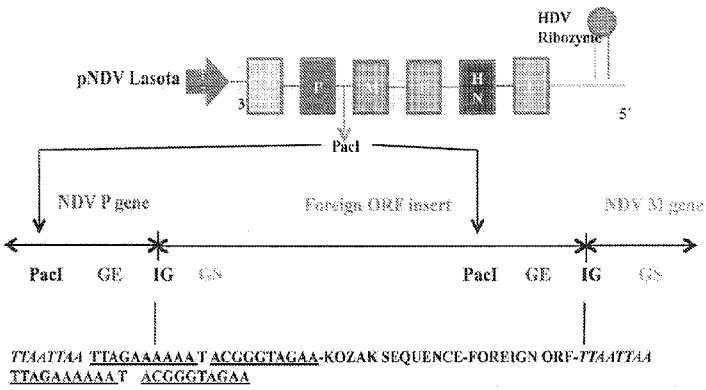
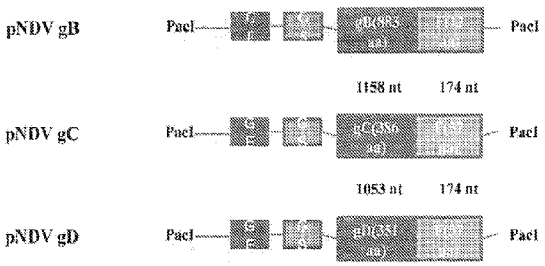
(54) Title: INFECTIOUS LARYNGOTRACHEITIS VIRUS (ILTV) VACCINE USING RECOMBINANT NEWCASTLE DISEASE VIRUS VECTOR





(57) Abstract: In this study, for the first time, protective efficacy of gD against ILTV challenge was evaluated. Immunization with recombinant Newcastle disease virus expressing ILTV gD induced a higher level of neutralizing antibodies and offered complete protection to chickens against lethal ILTV challenge. Uses of recombinant NDV as a vaccine vector are also described.

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TITLE OF THE INVENTION

Infectious Laryngotracheitis Virus (ILTV) Vaccine Using Recombinant Newcastle Disease Virus Vector

STATEMENT OF FEDERALLY SPONSORED RESEARCH AND DEVELOPEMNT

This invention was made with government support under 20116701530136 awarded by USDA. The Government has certain rights in the invention.

CROSS REFERENCE TO RELATED APPLICATIONS

This application is a Continuation-In-Part of U.S. patent application Ser. No. 13/188,392, filed July 21, 2011, still pending; which is a Continuation of 10/440,419, filed May 19, 2003; which is a Continuation-In-Part application of U.S. patent application Ser. No. 09/926,431, filed Mar. 6, 2002, now U.S. Pat. No. 7,244,558; which is National Stage entry of International Application No.: PCT/US00/06700 filed on May 5, 2000. The present patent application also claims the benefit of U.S. Provisional Patent Application Nos. 61/857,558 filed on July 23, 2013; 60/381,462 filed on May 17, 2002; 60/132,597, filed May 5, 1999; and 60/171,072, filed Dec. 16, 1999. The disclosures of each of these applications are hereby incorporated in their entirety by reference.

INTRODUCTION

The present application relates to recombinant Newcastle disease viruses useful as vaccine vectors, which when carrying one or more foreign genes, i.e. genes not found naturally in the Newcastle disease virus, are also useful as bivalent or multivalent vaccines.

BACKGROUND OF THE INVENTION

Newcastle disease is a highly contagious viral disease affecting all species of birds. The disease can vary from an asymptomatic infection to a highly fatal disease, depending on the virus strain and the host species. Newcastle disease has a worldwide distribution and is a major threat to the poultry industries of all countries. Based on the severity of the disease produced in chickens, Newcastle disease virus (NDV) strains are grouped into three main pathotypes: lentogenic (strains that do not usually cause disease in adult chickens), mesogenic (strains of intermediate virulence) and velogenic (strains that cause high mortality).

NDV is a member of the genus *Rubulavirus* in the family Paramyxoviridae. The genome of NDV is a non-segmented, single-stranded, negative-sense RNA of 15186 nucleotides (Krishnamurthy & Samal, 1998, J Gen Virol 79, 2419-2424; Phillips et al., 1998, Arch Virol 143, 1993-2002; de Leeuw and Peeters, 1999, J Gen Virol 80, 131-136). The genomic RNA contains six

genes that encode the following proteins in the order of: the nucleocapsid protein (NP), phosphoprotein (P), matrix protein (M), fusion protein (F), haemagglutinin-neuraminidase (HN) and large polymerase protein (L). Two additional proteins, V and W, of unknown function are produced by RNA editing during P gene transcription (Steward et al., 1993, J Gen Virol 74, 2539-2547).

Three proteins, i.e. NP, P and L proteins, constitute the nucleocapsid. The genomic RNA is tightly bound by the NP protein and together with the P and L proteins form the functional nucleocapsid within which resides the viral transcriptive and replicative activities. The F and HN proteins form the external envelope spikes, where the HN glycoprotein is responsible for attachment of the virus to host cell receptors and the F glycoprotein mediates fusion of the viral envelope with the host cell plasma membrane thereby enabling penetration of the viral genome into the cytoplasm of the host cell. The HN and F proteins are the main targets for the immune response. The M protein forms the inner layer of the virion.

NDV follows the general scheme of transcription and replication of other non-segmented negative-strand RNA viruses. The polymerase enters the genome at a promoter in the 3′ extragenic leader region and proceeds along the entire length by a sequential stop-start mechanism during which the polymerase remains template bound and is guided by short consensus gene start (GS) and gene end (GE) signals.

This generates a free leader RNA and six non-overlapping subgenomic mRNAs. The abundance of the various mRNAs decreases with increasing gene distance from the promoter. The genes are separated by short intergenic regions (1-47 nucleotides) which are not copied into the individual mRNAs. RNA replication occurs when the polymerase somehow switches to a read-through mode in which the transcription signals are ignored. This produces a complete encapsulated positive-sense replicative intermediate which serves as the template for progeny genomes.

Reverse-genetic techniques have been reported to recover negative-sense viruses from cloned cDNA (Conzelmann, 1996, J Gen Virol 77, 381-389). For NDV, reverse-genetic technology is currently available for avirulent strain LaSota (Römer-Oberdörfer et al., 1999, J Gen Virol 80, 2987-2995; Peeters et al., 1999, J Gen Virol 73, 5001-5009).

Infectious laryngotracheitis (ILT) is an acute respiratory disease of chickens that causes significant economic losses to poultry industry worldwide (Bagust et al., 2000, Rev Sci Tech 19, 483-492; Bagust, 1986, Avian Pathol 15, 581-595). The causative pathogen, ILTV, is a member of the genus Iltovirus in the family Herpesviridae (Bagust et al., 2000, supra; Fuchs et al., 2007, Vet Res 38, 261-279). Currently, live attenuated vaccines are used to control ILT infections. However, the live-attenuated vaccines are not satisfactory since they can revert to virulence after bird-to-bird

passage (Guy et al., 1991, Avian Dis 35, 348-355) and can induce latent infections (Hughes et al., 1991, Arch Virol 121, 213-218). Several alternative strategies have been used to develop improved ILTV vaccines (Mauricio et al., 2013, Avian Pathol 42, 195-205). One of the strategies has been the creation of ILTV deletion mutants for use as attenuated live-virus vaccines (Mauricio et al., 2013, supra). Two of the concerns of using gene deleted ILTV vaccine are the establishment of latency and the possibility that the gene-deleted vaccine virus could become virulent after recombination with different attenuated vaccine used in the same region (Sang-Won et al, 2012, Science 337, 188; Henderson et al., 1991, Am J Vet Res 52, 820-825). All studies conducted to date suggest that a virus-vectored ILTV vaccine will be most effective for prevention and control of ILT (Tong et al., 2001, Avian pathol 30, 143-148; Sun et al., 2008, Avian Dis 52, 111-117; Vagnozzi et al., 2012, Avian Pathol 41, 21-31). A vectored-vaccine will be safe and not lead to reversion to virulence or establishment of latency. However, current live virus vectored vaccines against ILT have limitations (Mauricio et al., 2013, supra; Vagnozzi et al. 2012, supra): (i) route of administration to large number of one-day old chicks, (ii) effective delivery of vaccine antigen to the mucosal surface, (iii) production cost, and (iv) incomplete protection. Therefore, there is a need to evaluate additional viral vectors to deliver ILTV antigens to chickens.

SUMMARY OF THE INVENTION

Of the eleven glycoproteins on the envelope of ILTV (Fuchs et al., 2007, supra), only glycoprotein B has been shown to be a major protective immunogen (Tong et al., 2001, supra; Sun et al., 2008, supra; York et al., 1991, Avian Pathol 20, 693-704), but the role of other glycoproteins in immunity and protection has not been evaluated. In this study, the inventors have evaluated the role of three major surface proteins (gB, gC, and gD) of ILTV in induction of neutralizing antibodies and protection in chickens using Newcastle disease virus (NDV) as a vaccine vector.

It has previously been shown that NDV expressing protective antigens of highly-pathogenic avian influenza virus and infectious bursal disease virus of chicken provided complete protection against respective challenge viruses (Nayak et al., 2009, PLoS One 4, e6509; Zhuhui et al., 2004, J Virol 78, 10054-10063). In the Examples below, three recombinant NDVs (rNDVs) which express and incorporate gB, gC, and gD of ILTV, individually, were constructed and used to immunize chickens. Results indicate that rNDV expressing ILTV gD is a safe and effective bivalent vaccine that would provide protection against both of these economically important diseases.

Reverse-genetic techniques were used in making the recombinant NDVs of the present invention from

cloned cDNA. This approach involves co-expression of the cloned cDNA of full length NDV genome and nucleocapsid proteins (the NP, P and L proteins) from transfected plasmids using the vaccinia virus/T7 RNA polymerase expression system. Within the scope of the present invention, recombinant NDV can be recovered from cDNA and the genome of NDV can be manipulated at the cDNA level. The production of infectious NDV from cloned cDNA can be used to engineer NDV carrying foreign genes. With the manipulation of the genome of NDV, one can insert foreign sequences into the NDV genome for co-expression. For example, the gene for a protective antigen of another avian pathogen or the genes for avian cytokines can be inserted into the NDV genome for co-expression.

Thus, the present invention includes multivalent genetically engineered NDV vaccines carrying genes encoding immunogens (e.g. immunogenic proteins) for pathogens of interest, such as for influenza virus, infectious bursal disease virus, rotavirus, infectious bronchitis virus, infectious laryngotracheitis virus, chicken anemia virus, Marek's disease virus, avian Leukosis virus, avian adenovirus and avian pneumovirus.

The present invention also is directed toward a genetically engineered NDV carrying avian cytokine genes. A NDV carrying at least one gene encoding an avian cytokine, e.g. an interleukin such as IL-2 and IL-4, can be used as a vaccine.

The recombinant NDV prepared by insertion of foreign genes into the NDV genome can express the foreign genes in cells infected by the recombinant NDV. As a result, the recombinant NDV can be used to express proteins of non-avian pathogens or other avian pathogens.

One of the objects of the invention is to provide a recombinant Newcastle disease virus (rNDV) comprising NP gene, P gene, M gene, F gene, HN gene and L gene. In one embodiment of the invention, the Newcastle disease virus contains a tyrosine to alanine substitution in the fusion or "F" gene at amino acid position 527. This tyrosine has been found to be conserved among different strains of NDV. The tyrosine can be substituted to any hydrophobic amino acid selected from the group: alanine, glycine, proline, methionine, leucine, etc. The inventors have found that the NDV with a 527 substitution has a higher replication magnitude than wild type NDV, results in larger plaques compared to wild type counterparts, and when a gene encoding a foreign antigen was inserted between the P and M genes, surface expression of the foreign antigen increased. Therefore, the mutation favors enhanced surface distribution of the expressed foreign protein and in turn, increases immunogenicity of the resulting vaccine. In the description that follows, it is understood that by NDV F gene is meant use of either the wild-type or the mutant form unless expressly stated.

Another object of the present invention is a recombinant antigenomic RNA or cDNA of Newcastle disease virus, comprising NP gene, P gene, M gene, F gene, HN gene and L gene in this order from a 5′ to 3′ direction, said antigenomic RNA further comprising n foreign nucleotide complexes inserted (a) before the NP gene, (b) between the P and M genes, and/or (c) between the HN and L genes, wherein n is 1, 2, 3 or 4;

each of the foreign nucleotide complexes comprising a Newcastle disease virus gene start sequence, an open reading frame of a foreign gene and a Newcastle disease virus gene end sequence in this order from the 5′ to 3′ direction, wherein the foreign gene is a gene not found naturally in the Newcastle disease virus;

wherein when n is 1, 2, 3 or 4, the foreign nucleotide complexes are the same or different; and wherein when 1, 2, 3 or 4 the foreign nucleotide complexes are inserted together or separately before the NP gene, between the P and M genes, or between the HN and L genes, the foreign nucleotide complexes are sequentially linked directly or indirectly.

Since each foreign nucleotide complex has a NDV gene start signal, i.e. GS sequence motif, upstream of the open reading frame (ORF) of the foreign gene and a NDV gene end signal, i.e. GE sequence motif, downstream of the ORF of the foreign gene, each foreign nucleotide complex forms a transcriptional unit or a gene cassette.

The recombinant antigenomic RNA or cDNA of NDV of the present invention preferably further comprises NP-P intergenic region between the NP gene and P gene, P-M intergenic region between the P gene and M gene, M-F intergenic region between the M gene and F gene, F-HN intergenic region between the F gene and HN gene, and/or HN-L intergenic region between the HN gene and L gene.

When one or more of the foreign nucleotide complexes are inserted between the P and M genes, the foreign nucleotide complexes can be inserted into the P-M intergenic region if present. Similarly, when one or more of the foreign nucleotide complexes are inserted between the HN and L genes, the foreign nucleotide complexes can be inserted into the HN-L intergenic region. Optionally, one or more of the NP-P intergenic region, P-M intergenic region, M-F intergenic region, F-HN intergenic region, and HN-L intergenic region are replaced with a single nucleotide, dinucleotide or an oligonucleotide of 3-80 nucleotides (preferably 4-60 nucleotides) in length, wherein the oligonucleotide optionally contains one or more restriction sites.

When one or more of the foreign nucleotide complexes are inserted before the NP gene, the foreign nucleotide complexes preferably are inserted into a non-coding region immediately before the ORF of the NP gene, so that the ORF of the foreign gene in each of the foreign nucleotide complexes is flanked by NDV gene start and gene end signals and

the ORF of the NP gene is preceded by a NDV gene start signal, with the GS-foreign gene ORF-GE structure preceding the GS signal for the NP ORF. Within the scope of the invention is a recombinant antigenomic RNA of NDV having one or more foreign nucleotide complexes inserted between P and M genes.

The antigenomic RNA or cDNA can be made by inserting the one or more foreign nucleotide complexes into the noncoding region of P gene after the stop codon, but before the NDV gene end signal of the P gene. When only one foreign nucleotide complex is inserted into the noncoding region of P gene after the stop codon, the ORF of the foreign gene is preceded by a NDV gene end and NDV gene start signals, resulting in the ORF of the P gene being preceded by a NDV gene end signal, which is followed by a NDV gene start signal, the ORF of the foreign gene, and a NDV gene end signal in that order (the ORF of the following M gene is preceded by a NDV gene start signal). More foreign gene complexes can be inserted after this foreign gene complex. Similarly, the recombinant antigenomic RNA or cDNA of NDV having one or more foreign nucleotide complexes inserted between P and M genes can be made by inserting the one or more foreign nucleotide complexes into the noncoding region of M gene before the ORF of the M gene.

The present invention is also directed toward a process of preparing the recombinant antigenomic RNA of the invention, comprising the following steps:

(i) providing a cDNA comprising NP gene, P gene, M gene, F gene, HN gene and L gene in this order, said cDNA further comprising n foreign nucleotide complexes inserted (a) before the NP gene, (b) between the P and M genes, and/or (c) between the HN and L genes, wherein n is 1, 2, 3 or 4;

each of the foreign nucleotide complexes comprising a Newcastle disease virus gene start sequence, an open reading frame of a foreign gene and a Newcastle disease virus gene end sequence in this order from the 5′ to 3′ direction, wherein the foreign gene is a gene not found naturally in the Newcastle disease virus;

wherein when n is 1, 2, 3 or 4, the foreign nucleotide complexes are the same or different; and wherein when 1, 2, 3 or 4 foreign nucleotide complexes are inserted together before the NP gene, between the P and M genes, or between the HN and L genes, the foreign nucleotide complexes are sequentially linked directly or indirectly;

(ii) transcribing the antigenomic cDNA to form a mixture containing an antigenomic RNA; and thereafter

(iii) isolating the antigenomic RNA.

In some embodiments of the process of preparing the recombinant antigenomic RNA of the invention, the cDNA used in step (i), comprising NP gene, P gene, M gene, F gene, HN gene and L gene having the n foreign nucleotide complexes inserted, is prepared by (I) constructing a cDNA comprising the NP gene, P

gene, M gene, F gene, HN gene and L gene in this order; and thereafter (II) inserting the n foreign nucleotide complexes (a) before the NP gene, (b) between the P and M genes, and/or (c) between the HN and L genes. Preferably, the cDNA constructed in step (I) and/or the cDNA constructed in step (II) are in a plasmid, such as pBR322 or pGEM-7Z. In step (ii), the cDNA preferably is transcribed in cells expressing a RNA polymerase, such as T7 RNA polymerase.

The present invention is also directed toward a recombinant NDV (rNDV) comprising a recombinant antigenomic RNA carrying one or more foreign genes of the present invention. The recombinant NDV can be produced by a process comprising the following steps:

(i) providing cells capable of synthesizing T7 RNA polymerase;

(ii) cotransfecting the cells with a plasmid comprising the cDNA encoding the antigenomic RNA having one or more foreign genes inserted according to the invention, a plasmid encoding NP protein, a plasmid encoding P protein, and a plasmid encoding L protein to obtain cotransfected cells in a medium; and thereafter

(iii) isolating Newcastle disease virus from a supernatant of the medium of step (ii) to obtain the recombinant Newcastle disease virus.

The cells capable of synthesizing T7 RNA polymerase provided in step (i) can be animal cells

of an avian or mammalian species, plant cells, or cells from a cell line expressing T7 RNA polymerase.

Within the scope of the present invention are a cDNA encoding a recombinant NDV antigenomic RNA having one or more foreign genes inserted according to the invention, a cell containing the cDNA, a plasmid comprising the cDNA, a cell containing the plasmid, a cell containing the recombinant antigenomic RNA, and a recombinant NDV containing the recombinant antigenomic RNA of the invention, e.g. a recombinant NDV carrying one or more foreign genes recovered from transcription of the cDNA or the plasmid in a competent cell. The recombinant NDV containing the recombinant antigenomic RNA of the invention is preferably substantially purified. Also preferred is a substantially purified recombinant antigenomic RNA of NDV carrying one or more foreign genes prepared according to the invention.

In one embodiment, the present invention provides a cDNA encoding a recombinant antigenomic NDV RNA having one or more genes from ILTV inserted according to the invention, a cell containing the cDNA, a plasmid comprising the cDNA, a cell containing the plasmid, a cell containing the recombinant antigenomic RNA, and a recombinant NDV containing the recombinant antigenomic RNA of the invention, e.g. a recombinant NDV carrying one or more ILTV genes recovered from transcription of the cDNA or the plasmid in a competent cells.

The recombinant NDV, or rNDV, containing the one or more inserted foreign genes can be used as a

monovalent vaccine to provide immunity and protection against NDV challenge, a bivalent vaccine protective against NDV and challenge with the pathogen source of the inserted foreign nucleic acid encoding one or more immunogenic protein, or a multivalent vaccine protective against NDV and challenge with the more than one pathogen source of the inserted foreign nucleic acid encoding one or more immunogenic protein.

In another aspect, the present invention includes a bivalent vaccine to provide immunity and protection against NDV and ILTV challenge, the vaccine comprising rNDV having one or more genes from ILTV, gB, gC, gD, preferably gD.

The present invention also includes a method of vaccinating an avian animal against Newcastle disease, wherein the avian animal is in need of the vaccination, comprising administering an effective amount of the recombinant NDV optionally carrying one or more foreign genes according to the invention to the avian animal.

One of the objects of the invention is a method of treating an avian animal with an avian cytokine, wherein the avian animal is in need of the treatment, said method comprising administering an effective amount of the recombinant NDV of the invention carrying one or more foreign genes encoding one or more avian cytokines, such as avian interleukins (preferably IL-2 and/or IL-4) to the avian animal.

Another object of the invention is a method of immunizing an avian animal against an avian pathogen selected from the group consisting of influenza virus, infectious bursal disease virus, rotavirus, infectious bronchitis virus, infectious laryngotracheitis virus, chicken anemia virus, Marek's disease virus, avian Leukosis virus, avian adenovirus and avian pneumovirus, wherein the avian animal is in need of the immunization, said method comprising administering an effective amount of the recombinant NDV of the invention to the avian animal, wherein one or more the recombinant NDV carries one or more foreign genes encoding one or more immunogenic proteins of the avian pathogen against which the avian animal is immunized.

In another object, the present invention provides a method of immunizing an avian animal against ILTV, said method comprising administering an effective amount of the recombinant NDV of the present invention, wherein the NDV carries one or more ILTV genes encoding one or more immunogenic ILTV proteins. In one embodiment, the ILTV genes are gB, gC, and gD, in any combination.

Also within the scope of the invention is a method of immunizing a mammal against a non-avian pathogen, wherein the mammal is in need of the immunization, said method comprising administering an effective amount of the one or more recombinant NDV of the invention to the mammal, wherein the recombinant NDV carries one or more foreign genes encoding one or more immunogenic proteins of the

non-avian pathogen, e.g. influenza virus, SARS-causing virus, human respiratory syncytial virus, human immunodeficiency virus, hepatitis A virus, hepatitis B virus, hepatitis C virus, poliovirus, rabies virus, Hendra virus, Nipah virus, human parainfluenza 3 virus, measles virus, mumps virus, Ebola virus, Marburg virus, West Nile virus, Japanese encephalitis virus, Dengue virus, Hantavirus, Rift Valley fever virus, Lassa fever virus, herpes simplex virus and yellow fever virus, against which the mammal is immunized.

In this study, for the first time, the inventors have evaluated the protective efficacy of gD against ILTV challenge and demonstrate that ILTV gD is a major protective immunogen capable of inducing a protective immune response against ILTV in chickens. Immunization with rNDV expressing ILTV gD induced a higher level of neutralizing antibodies and offered complete protection to chickens against lethal ILTV challenge. The complete protection offered by gD can be attributed to its superior envelope incorporation and cell surface expression leading to induction of a protective immune responses.

Therefore, the ILTV gD protein can be exploited as an effective vaccine antigen for the development of safe vectored vaccines against ILT using viral and nonviral vectors. Examples of viral vectors include adenovirus, adeno-associated virus, herpesvirus, pox virus, influenza virus, retrovirus,

and other recombinant viral vectors known to a person in the art.

Therefore, in another object, the present invention provides an ILTV vaccine comprising gD. Also provided is a method for eliciting in a subject an immune response against ILTV, the method comprising administering to a subject a nucleic acid comprising a gD encoding nucleic acid. The nucleic acid comprising a gD encoding nucleic acid can be part of a vector such as a viral vector, capable of producing gD in an immunized avian or non-avian animal. In another aspect, a composition comprising gD can be administered to a subject in need thereof.

Other objects, features and advantages of the present invention will become apparent from the following detailed description. It should be understood, however, that the detailed description and the specific examples, while indicating specific embodiments of the invention, are given by way of illustration only, since various changes and modifications within the spirit and scope of the invention will become apparent to those skilled in the art from this detailed description.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1. Construction of recombinant NDVs expressing ILTV gB, gC, and gD. Schematic diagram depicting the full length antigenome of NDV strain LaSota with insertion of an added gene engineered to express the ILTV gB consisting of the complete ORF of gB fused to last 12 amino acids of NDV F protein

cytoplasmic tail, gC or gD consisting of the ectodomain of gC or gD respectively, fused to the transmembrane and cytoplasmic tail of the NDV F protein. The inserted foreign ORF was placed under the control of a set of NDV transcriptional gene end (GE) and gene start (GS) signals such that each was expressed as a separate mRNA. nt- nucleotides, aa- amino acids, IG-intergenic.

Figure 2-1 and 2-2. Western blot and flow cytometry analysis of the rNDVs expressing ILTV proteins. Fig 2-1: Expression of ILTV gB, gC, and gD in DF1 cells and their incorporation into rNDV virions. Fig 2-2: Flow cytometry analysis of the surface expression of ILTV proteins. DF1 cells were infected with rNDV gB (panel A), rNDV gC (panel B) or rNDV gD (panel C) viruses at a MOI of 5, in parallel with cells that were mock-infected or infected with the rNDV LaSota empty vector. At 24 h post-infection, the cells were probed with rabbit anti-ILTV sera, followed by incubation with Alexa Fluor 488 conjugated goat anti-rabbit IgG antibody and analyzed by Flowjo program of FACSRIA II flow cytometer. Values represent averages of the results obtained from two independent experiments.

Figure 3. Immunoelectron microscopy of purified virions of rNDV LaSota, rNDV gB, rNDV gC, and rNDV gD, analyzed using rabbit anti ILTV serum against gB (upper panel), gC (middle panel) or gD (lower panel).

Figure 4-1 and 4-2. Multicycle growth kinetics of rNDVs expressing ILTV proteins and NDV-specific

serum antibody responses in chickens at 21 days following two oculonasal immunizations with rNDVs administered either individually or in combination. Figure 4-1: Multicycle growth kinetics of rNDVs in nine-day-old SPF embryonated chicken eggs. Nine-day-old embryonated chicken eggs were inoculated with 100 PFU of each virus, and allantoic fluids from three eggs were harvested at different time points (12 h, 24 h, 36 h, 48 h, 60 h, and 72 h) after inoculation. The virus titer in allantoic fluid was determined by TCID50 assay in DF-1 cells. Values represent averages of the results obtained from two independent experiments. Figure 4-2: NDV-specific serum antibody response was determined by HI assay and all antibody titers are expressed as mean reciprocal log2 titer ± SEM (standard error of the mean). Statistical differences were calculated by one-way ANOVA with $P < 0.05$.

Figure 5A and 5B. ILTV-neutralizing antibody response post-vaccination and clinical signs score evaluation post-ILTV challenge. 5A. Chickens were immunized by the oculonasal route with rNDVs either individually or in combination. Sera were taken on days 12 (12 days following primary immunization) and 21 (7 days following booster immunization) post-vaccination and analyzed for the ability to neutralize USDA strain of ILTV in vitro. The serum-neutralizing antibody titers were expressed as mean reciprocal log2 titer (means ± SEM). Statistical differences were calculated by one-way ANOVA with $P < 0.05$. 5B. Total clinical signs were recorded daily

until 14 days post-ILTV challenge for chickens immunized with rNDVs individually (panel a) or in combination (panel b). For comparison, clinical score of birds vaccinated with Trachivax CEO and HVT-LT were also included.

DETAILED DESCRIPTION OF THE INVENTION

In some embodiments of the invention, the recombinant antigenomic RNA is from a paramyxovirus, Newcastle disease virus strain LaSota. Other NDV strains, for example, Hitchner-B1 (B1), Clone-30, Strain-F, Strain V4, Strain V4-HR, Strain-I2 and Ulster (U) can also be used.

In some embodiments of the recombinant antigenomic RNA of the present invention, n is 1, 2, 3 or 4 (preferably 2 or 3, and more preferably 2) and the foreign nucleotide complexes are different. In some embodiments of the recombinant antigenomic RNA, n is 1, 2, 3 or 4 (preferably 2 or 3, and more preferably 2) and the foreign nucleotide complexes are the same. In still some embodiments of the recombinant antigenomic RNA, n is 1 or 2.

In some of the recombinant antigenomic RNAs of the invention, the ORF of each of the foreign genes in the inserted foreign nucleotide complexes is no more than about 3000 nucleotides, no more than about 2000 nucleotides, no more than about 1500 nucleotides, no more than about 1000 nucleotides, no more than about 800 nucleotides, no more than about 500 nucleotides, or no more than about 300 nucleotides in length.

In some of the embodiments of the recombinant antigenomic RNA of the present invention, where 1, 2, 3 or 4 foreign nucleotide complexes are inserted together before the NP gene, between the P and M genes, or between the HN and L genes, the foreign nucleotide complexes are sequentially linked directly or indirectly, and the foreign nucleotide complexes have a combined length of no more than about 5000 nucleotides, no more than about 4000 nucleotides, no more than about 3000 nucleotides, no more than about 2000 nucleotides, no more than about 1000 nucleotides, or no more than about 800.

The foreign gene inserted in the recombinant antigenomic RNA of the invention preferably encode a substance selected from the group consisting of chloramphenical acetyltransferase, GFP, an avian cytokine, and an immunogenic protein of influenza virus, infectious bursal disease virus, rotavirus, infectious bronchitis virus, infectious laryngotracheitis virus, chicken anemia virus, Marek's disease virus, avian leukosis virus, avian adenovirus, or avian pneumovirus. The foreign gene may encode an immunogenic protein of a non-avian pathogen, e.g. influenza virus, SARS-causing virus, human respiratory syncytial virus, human immunodeficiency virus, hepatitis A virus, hepatitis B virus, hepatitis C virus, poliovirus, rabies virus, Hendra virus, Nipah virus, human parainfluenza 3 virus, measles virus, mumps virus, Ebola virus, Marburg virus, West Nile disease virus, Japanese encephalitis virus, Dengue virus,

Hantavirus, Rift Valley fever virus, Lassa fever virus, herpes simplex virus and yellow fever virus.

When more than one foreign gene encoding the avian cytokine is inserted, the foreign genes may encode the same or different avian cytokines, such as avian interleukins, e.g. IL-2 and IL-4.

Examples of the foreign gene encoding an immunogenic protein of an avian pathogen are HA or NA gene of influenza virus, VP2 or polyprotein gene of infectious bursal disease virus, S or S1 gene of infectious bronchitis virus, glycoprotein gene of infectious laryngotracheitis virus, e.g. gB, gC, gD, the complete genome of chicken anemia virus, glycoprotein gene of Marek's disease virus, envelope gene of avian leukosis virus, avian adenovirus, and G or F gene of avian pneumovirus.

Examples of the foreign gene encoding an immunogenic protein of a non-avian pathogen are HA or NA gene of influenza virus, S or S1 gene of SARS-causing virus, G or F gene of human respiratory syncytial virus, gp60, gp120 or gp41 gene of human immunodeficiency virus, surface antigen gene of hepatitis A virus, surface antigen gene of hepatitis B virus, surface antigen of hepatitis C virus, capsid proteins gene of poliovirus, G protein gene of rabies virus, G or F protein gene of Hendra virus, G or F protein gene of Nipah virus, HN or F protein gene of human parainfluenza 3 virus, H or F protein gene of measles virus, HN or F protein gene of mumps virus, G protein gene of Ebola virus, G protein gene of Marburg virus, envelope protein gene

of West Nile disease virus, envelope protein gene of Japanese encephalitis virus, envelope protein gene of Dengue virus, glycoprotein gene of Hantavirus, glycoprotein gene of Rift Valley fever virus, G1 or G2 protein gene of Lassa fever virus, glycoprotein genes of herpes simplex virus, and glycoprotein gene of yellow fever virus.

The present invention is also directed toward an antigenomic RNA of NDV carrying one or more foreign genes inserted before the NP gene, between the P and M genes, and/or between the HN and L genes, wherein at least one of the foreign genes encodes a tumor antigen, such as pg100, MAGE1, MAGE3 and CDK4.

In the recombinant antigenomic RNA of the invention, the foreign nucleotide complexes preferably are inserted before the NP gene, and/or between the P and M genes. More preferably, at least one of the foreign nucleotide complexes is inserted before the NP gene. In some embodiments of the recombinant antigenomic RNA, at least one of the foreign nucleotide complexes is inserted before the NP gene and at least one of the foreign nucleotide complexes is inserted between the P and M genes. In some embodiments, at least one of the foreign nucleotide complexes is inserted before the NP gene and at least one of the foreign nucleotide complexes is inserted between the HN and L genes. In still some embodiments, at least one of the foreign nucleotide complexes is inserted before the NP gene, at least one of the foreign nucleotide complexes is

inserted between the P and M genes, and at least one of the foreign nucleotide complexes is inserted between the HN and L genes. In yet some embodiments, at least one of the foreign nucleotide complexes is inserted between the P and M genes. Most preferably, the foreign nucleotide complexes are inserted only before the NP gene.

NDV grows to very high titers ($<10^9$ PFU/ml) in many cell lines and eggs and elicits strong humoral and cellular immune responses in vivo. NDV naturally infects via respiratory and alimentary tract mucosal surfaces. NDV replicates in the cytoplasm of infected cells and does not undergo genetic recombination, making vaccine vectors based on the recombinant NDV carrying foreign genes stable and safe. Due to these characteristics of NDV described herein, recombinant NDVs that can express foreign genes carried in the recombinant NDVs are good vaccines, wherein the foreign genes encode immunogenic proteins of pathogens.

The recombinant NDV of the invention carrying one or more inserted foreign genes show robust expression of the foreign genes. Moreover, the recombinant NDV expressing one or more of the foreign gene can replicate in cell culture and in vivo. NDV recombinants expressing heterologous proteins could be used as multivalent vaccines.

The recombinant NDV generated from the recombinant antigenomic RNA carrying one or more foreign genes inserted according to the invention can also be used as an inactivated vaccine.

The vaccine or vaccine vector based on the recombinant NDV generated from the recombinant antigenomic RNA carrying one or more foreign genes inserted according to the invention can be administered topically, via the respiratory route, orally or via an injection. The dose of the vaccine or vaccine vector to be used can be readily determined by a person skilled in the art based on the disease, the host subject species, and the age, sex and/or health condition of the host subject involved.

This study demonstrates for the first time that ILTV gD is a major protective immunogen capable of inducing protective immune responses against ILTV infection in chickens.

As used herein, the term gD also includes analogs and truncated forms that are immunologically cross-reactive with natural gD. By gD is intended gD from other strains of ILTV, or any other newly identified strain or field isolate of ILTV.

gD can be used as a homo-oligomer, containing more than one gD monomer, e.g. gD dimers, trimers or tetramers, or any higher-order homo-oligomers of gD. The oligomers may contain one, two, or several different monomers of gD obtained from different strains of ILTV including for example USDA strain, ILTV strain 63140/C/08/BR, Strain A489, Australian CSW-1 ILTV strain, SA-2 ILTV, A-20 ILTV, Serva-ILTV, Strain V1-99, Strain Q1-96, Strain N3-04, Strain S2-04, Trachivax ILTV vaccine strain, and other strains and field isolates. Such mixed oligomers are still

homo-oligomers within the scope of this invention, and may allow more universal diagnosis, prophylaxis or treatment of ILTV.

In one embodiment, ILTV gD can be recombinantly expressed, isolated and purified using methods well known in the art. The term 'purified' as applied to proteins herein refers to a composition wherein the desired protein comprises at least 35% of the total protein component in the composition. The desired protein preferably comprises at least 40%, more preferably at least about 50%, more preferably at least about 60%, still more preferably at least about 70%, even more preferably at least about 80%, even more preferably at least about 90%, and most preferably at least about 95% of the total protein component. The composition may contain other compounds such as carbohydrates, salts, lipids, solvents, and the like, without affecting the determination of the percentage purity as used herein. An 'isolated' protein intends a composition that is at least 35% pure.

The term 'essentially purified proteins' refers to proteins purified such that they can be used for in vitro diagnostic methods and as a prophylactic compound. These proteins are substantially free from cellular proteins, vector-derived proteins or other ILTV components. The proteins of the present invention are purified to homogeneity, at least 80% pure, preferably, 90%, more preferably 95%, more preferably 97%, more preferably 98%, more preferably 99%, even more preferably 99.5%.

The term 'recombinantly expressed' used within the context of the present invention refers to the fact that the proteins of the present invention are produced by recombinant expression methods be it in prokaryotes, or lower or higher eukaryotes.

Therefore, the present invention relates to a DNA or cDNA segment which encodes ILTV gD as described above. Genome sequences from different strains of ILTV have been published and are publicly available. DNA or nucleic acid sequences to which the invention also relates include fragments of the gD gene containing protective epitopes or antigenic determinants. The sequence of nucleic acids encoding antigens may be generated in any manner, including for example, chemical synthesis or DNA replication or reverse transcription or transcription, which are based on the information provided by the sequence bases in the region(s) from which the polynucleotide is derived. In addition, combinations of regions corresponding to that of the designated sequence may be modified in ways known in the art to be consistent with an intended use. It is understood in the art that certain advantageous steps can be taken to increase the antigenicity of an encoded protein by modifying its amino acid composition. Such changes in amino acid composition can be introduced by modifying the genetic sequence encoding the protein. It is contemplated that all such modifications and variations of the ILTV gD gene are equivalents within the scope of the present invention.

The DNA encoding the desired antigen can be introduced into the cell in any suitable form including, the fragment alone, in a vector such as a linearized plasmid, a circular plasmid, a plasmid capable of replication, an episome, RNA, a viral vector, an expression vector, etc. Individual expression vectors capable of expressing the genetic material can be produced using standard recombinant techniques. Please see e.g., Maniatis et al., 1985 *Molecular Cloning: A Laboratory Manual* or *DNA Cloning*, Vol. I and II (D. N. Glover, ed., 1985) for general cloning methods.

The DNA, alone or in a vector, can be delivered by injection into the tissue of the recipient, oral or pulmonary delivery. Any of these methods can be used to deliver DNA as long as the DNA is expressed and the desired antigen is made in the cell.

The present invention more particularly relates to a composition comprising at least one of the above-specified peptides or a recombinant gD protein composition as defined above, for use as a vaccine for immunizing avian subject against ILT, comprising administering a sufficient amount of the composition possibly accompanied by pharmaceutically acceptable adjuvant(s), to produce an immune response. The vaccine composition of the present invention is expected to provide cross-protection against infection from other ILTV strains, since the immunogenic antigen gD is highly conserved between strains.

Immunogenic compositions can be prepared according to methods known in the art. The present compositions comprise an immunogenic amount of a recombinant protein or peptides as defined above, usually combined with a pharmaceutically acceptable carrier, preferably further comprising an adjuvant.

Pharmaceutically acceptable carriers include any carrier that does not itself induce the production of antibodies harmful to the individual receiving the composition. Suitable carriers are typically large, slowly metabolized macromolecules such as proteins, polysaccharides, polylactic acids, polyglycolic acids, polymeric amino acids, amino acid copolymers; and inactive virus particles. Such carriers are well known to those of ordinary skill in the art.

The immunogenic compositions typically will contain pharmaceutically acceptable vehicles, such as water, saline, glycerol, ethanol, etc. Additionally, auxiliary substances, such as wetting or emulsifying agents, pH buffering substances, preservatives, and the like, may be included in such vehicles.

Typically, the immunogenic compositions are prepared as liquid solutions or suspensions; solid forms suitable for solution in, or suspension in, liquid vehicles prior to administration may also be prepared. Immunogenic compositions used as vaccines comprise a 'sufficient amount' or 'an

immunologically effective amount' of the protein gD or a vector which will produce a sufficient amount of the gD protein in the subject. 'Immunologically effective amount', means that the administration of that amount to an individual, either in a single dose or as part of a series, is effective for treatment, as defined above. This amount varies depending upon the health and physical condition of the subject to be treated, the formulation of the vaccine, and other relevant factors. It is expected that the amount will fall in a relatively broad range that can be determined through routine trials. Usually, the amount will vary from 0.01 to 1000 ug/dose, more particularly from about 1.0 to 100 ug/dose most preferably from about 10 to 50 ug/dose.

Administration of the compounds or vaccines, disclosed herein may be carried out by any suitable means, including parenteral injection (such as intraperitoneal, subcutaneous, or intramuscular injection), in ovo injection of birds, orally, oculonasal, or by topical application to an airway surface carried out by inhalation administration, such as by creating respirable particles of a pharmaceutical formulation (including both solid particles and liquid particles) as an aerosol suspension, and then causing the subject to inhale the respirable particles. Methods and apparatus for administering respirable particles of pharmaceutical formulations are well known, and any conventional technique can be employed. Oral administration may be in the form of an ingestable liquid or solid

formulation.

The treatment may be given in a single dose schedule, or preferably a multiple dose schedule in which a primary course of treatment may be with 1–10 separate doses, followed by other doses given at subsequent time intervals required to maintain and or reinforce the response, for example, at 1–4 months for a second dose, and if needed, a subsequent dose(s) after several months. Examples of suitable treatment schedules include: (i) 0, 1 month and 6 months, (ii) 0, 7 days and 1 month, (iii) 0 and 1 month, (iv) 0 and 6 months, (v) 0 and 14 days, or other schedules sufficient to elicit the desired responses expected to reduce disease symptoms, or reduce severity of disease.

The present invention also provides kits which are useful for carrying out the present invention. The present kits comprise a first container means containing the above-described antibodies. The kit also comprises other container means containing solutions necessary or convenient for carrying out the invention. The container means can be made of glass, plastic or foil and can be a vial, bottle, pouch, tube, bag, etc. The kit may also contain written information, such as procedures for carrying out the present invention or analytical information, such as the amount of reagent contained in the first container means. The container means may be in another container means, e.g. a box or a bag, along with the written information.

All publications, including, but not limited to patents and patent applications, cited in this specification are herein incorporated by reference as if each individual publication were specifically and individually indicated to be incorporated by reference herein as though fully set forth.

The invention is further described in detail to the following experimental examples. These examples are provided for purposes of illustration only, and are not intended to be limiting unless otherwise specified. Thus, the invention should in no way be construed as being limited to the following examples, but rather, should be construed to encompass any and all variations which become evident as a result of the teaching provided therein.

The following Materials and Methods were used in the following Examples.

Materials and Methods

Cells, viruses, adjuvants and antisera production in rabbits.

Human epidermoid carcinoma, chicken embryo fibroblast, and Vero cells were grown in Dulbecco's modified Eagle medium (DMEM) containing 10% fetal bovine serum (FBS) and maintained in DMEM with 5% FBS. Chicken embryo liver cells (CELi) and chicken embryo kidney cells (CEK) were harvested from 11 -12 day-old and 18-19 day-old specific pathogen free embryonated chicken eggs, respectively, by conventional trypsin disaggregation method and were

grown in Eagle's minimal essential medium (EMEM) containing 10% FBS. The chicken-embryo-origin ILTV vaccine Trachivax was obtained from the Schering-Plough Animal Health Corp, Millsboro, DE. The Vectormune HVT-LT vaccine was obtained from the Ceva Animal Health, Lenexa, KS. The USDA challenge strain of ILTV was obtained from the National Veterinary Services Laboratory, Ames, IA, USA. The USDA ILTV challenge strain was propagated on monolayers of chicken embryo liver cells. Recombinant NDV strains were grown in 9-day-old specific-pathogen-free (SPF) embryonated chicken eggs. The modified vaccinia virus Ankara strain expressing T7 RNA polymerase was grown in primary chicken embryo fibroblast cells. The Freund's complete and the Freund's incomplete adjuvants were obtained from the Sigma-Aldrich, St-Louis, MO. The anti-ILTV antiserum was raised in rabbits against the synthetic peptides of gB, gC, and gD of ILTV. Synthetic peptides of ILTV gB, gC, and gD were obtained from GenScript USA Inc., Piscataway, NJ, USA. The sequences of the synthetic peptides used are shown in table below. Briefly, rabbits were injected with the synthetic peptides of gB, gC, and gD initially, and followed by two booster doses at 14 day interval time. Synthetic peptides were homogenized with the Freund's complete adjuvant in equal proportion for the initial dose, and for the subsequent booster doses, mixture of synthetic peptides and Freund's incomplete adjuvant in equal proportion was used. The rabbits were bled after the final booster and the serum samples were

collected. The specificity of the antiserum (designated as anti-ILTV gB, gC, and gD antisera) was determined by Western blot analysis.

Table 1. Sequences of the synthetic peptides used for the production of rabbit polyclonal anti-ILTV sera and their respective anti-ILTV antibody titers.

Peptide	Sequence	Anti-ILTV antibody titers[a] after 2nd booster
gB1 gB2 gB3	LPRGRERRQAAGRRT 432 to 445 of SEQ ID NO:2 AIGSGAPKEPQIRNR 59 to 73 of SEQ ID NO: 2 RNLFRRKPRTKEDDY 854 to 868 of SEQ ID NO: 2	Anti-ILTV gB : 6582
gC1 gC2 gC3	ELEIRGEASQPLPSK 234 to 248 of SEQ ID NO: 4 WTPPEDFEMLRPETR 255 to 269 of SEQ ID NO: 4 FSDRPLTHEESVKVE 46 to 60 of SEQ ID NO: 4	Anti-ILTV gC : 5394
gD1 gD2 gD3	LRKKNPSAPDPRPDS 246 to 260 of SEQ ID NO: 6 PEDTEHDDPNSDPDY 314 to328 of SEQ ID NO: 6 MISAAKEKEKGGPFE 75 to 89 of SEQ ID NO: 6	Anti-ILTV gD : 5987

[a] Anti ILTV titers were determined by ELISA (ProFLOCK® LT ELISA Kit, Synbiotics Corp., San Diego, CA) following the manufacturer's instructions.

Construction and generation of rNDVs containing ILTV gB, gC, and gD genes.

The ILTV gB, gC, and gD (GenBank accession number NC_06623) open reading frames were PCR amplified from the purified ILTV DNA and were subsequently cloned into the pCR 4 TOPO vector (Invitrogen). The nucleic acid sequence of gB is shown in SEQ ID NO:1, and the amino acid sequence is shown in SEQ ID NO:2. The nucleic acid sequence of gC in SEQ ID NO:3, and the amino acid sequence is shown in SEQ ID NO:4. The nucleic acid sequence of gD is shown in SEQ ID NO:5, and the amino acid sequence is shown in SEQ ID NO:6. The integrity of the gB, gC, and gD genes was confirmed by sequence analysis. To construct an insert encoding the modified gB glycoprotein, the complete ORF (excluding the stop codon) of the gB gene was fused to the last 12 amino acids of the NDV F protein cytoplasmic tail (amino acids 542-553). The gB open reading frame (ORF) was amplified by PCR using forward primer (gBF) 5´GATC*TTAATTAA*<u>*TTAGAAAAAA*</u>**T**<u>ACGGGTAGAA</u>**<u>GGCCACC</u>**atgcaatcct acatcgccgtg3´(SEQ ID NO:7)(The primer contains a PacI site (italicized), the NDV gene end transcriptional signal (italicized, underlined), the NDV gene start transcriptional signal (underlined), the T intergenic nucleotide (boldface), additional nucleotide in order to maintain the genome length as a multiple of six (italicized and bold), a six nucleotide Kozak

sequence for efficient translation (bold, underlined) and the ILTV specific sequence is in small case) and a reverse primer (gBR) 5´GATC*TTAATTAA***TCA**CATTTTTGTAGTGGCTCTCATCTGATCTAGAGTAT Tttcgtcttcgctttcttc3´ (SEQ ID NO:8) (The primer contains a PacI site (italicized), sequence specific to last 12 amino acids of the NDV F gene (underlined) and sequence specific to ILTV gB gene (small case) and a stop codon (bold face)). After amplification, the 2688 base pair product was cloned into pCR 4-Topo vector (Invitrogen) and sequenced to confirm the correct gB gene structure and the absence of any mutations. The glycoprotein gC and gD inserts were constructed by fusing the ectodomain of glycoproteins to the transmembrane domain and cytoplasmic tail (amino acids 497-553) of the NDV F protein by overlapping PCR. Briefly, the gC gene of ILTV was amplified by PCR using a forward primer (gCF) 5´ GATC*TTAATTAATTAGAAAAAA***T**ACGGGTAGAA***GGCCACC**atgcagcatcag agtactgcg 3´ (SEQ ID NO:9)(The primer and its constituents are notated similarly as described for the gBF primer) and a reverse primer (gC1) 5´-GACTGCGGGGAATCCTTGCCGCATTG-3´ (sequence represents the sequence specific to ILTV gC gene ORF at position 1133-1158 of SEQ ID NO:3). The transmembrane domain and cytoplasmic tail sequences of the NDV F gene was PCR amplified using forward primer (gC2) 5´-CAATGCGGCAAGGATTCCCCGCAGTCagcacatctgctctcattac-3´ (SEQ ID NO:10)(sequence specific to ILTV gC gene

overlap is in uppercase and NDV F gene transmembrane-specific sequence is in lower case) and a reverse primer (gCR) 5´- gatc*TTAATTAA*TCACAT TTTTGTAGTGGCTCTCATCTGATC-3´ (SEQ ID NO:11)(the PacI site is italicized and NDV F gene cytoplasmic tail-specific sequence is in uppercase). Both the fragments were ligated by overlapping PCR by using forward primer gCF and reverse primer gCR. After amplification, 1332-bp PCR product was cloned into pCR-4 Topo vector (Invitrogen) and sequenced to confirm the correct gC gene structure and absence of any mutations. To make an insert that encodes for the ILTV gD protein, the ILTV gD gene was amplified by PCR using a forward primer (gDF) 5´- GATC*TTAATTAATTAGAAAAAA***T**ACGGGTAGAA***GGCC*****GCCACC**atggaccgc catttatttttgag-3´ (SEQ ID NO:12) (The primer and its constituents are notated similarly as described for gBF primer) and a reverse primer (gD1) 5´- GGGCATGGA GACGGCATTAGAACT-3´ (SEQ ID NO:13)(sequence represents the sequence specific to ILTV gD gene ORF at position (1030-1053). The transmembrane domain and cytoplasmic tail sequences of the NDV F gene was PCR amplified using forward primer (gD2) 5´- AGTTCTAATGCCGTCTCCATG CCCagcacatctgctctcattacct-3´ (SEQ ID NO:14)(sequence specific to ILTV gD gene overlap is in uppercase and NDV F gene transmembrane-specific sequence is in lower case) and a reverse primer (gDR) 5´- gatc*TTAATTAA*TCACATTTTTGTAGTGGCTCTCATCTGATC-3´(SEQ ID NO:15)(the PacI site is italicized and NDV F gene cytoplasmic tail-specific sequence is in uppercase).

Both the fragments were ligated by overlapping PCR by using forward primer gDF and reverse primer gDR. After amplification, 1227-bp PCR product was cloned into pCR-4 Topo vector (Invitrogen) and sequenced to confirm the correct gD gene structure and the absence of any mutations.

Statistical analysis.

Statistically significant differences in data from serological analysis of different immunized chicken groups were evaluated by one-way analysis of variance (ANOVA) (for more than two groups) and t-test (between two groups) with the use of Prism 5.0 (Graph Pad Software Inc., San Diego, CA) at a significance level of $P < 0.05$. The significant differences in mean tracheal viral load post-ILTV challenge between experimental groups were determined by one-way ANOVA with Bonferroni post-test for multiple comparisons at 95% confidence intervals. The total clinical scores obtained for different groups at different days post ILTV challenge were entered into a prism 5.0 data sheet and statistically significant differences in data from clinical sign scores at different days post challenge within each group were evaluated by one-way ANOVA. Multiple pair-wise comparisons were made using Bonferroni test with 95% confidence intervals to limit the overall type-I error to 5%.

Immunofluorescence analysis of the expression of ILTV proteins.

Immunofluorescence assay was performed to evaluate the cell surface and intracellular

expression of ILTV glycoproteins. Briefly, confluent monolayers of vero cells on 4 well Lab-Tek chamber slides were infected with the recombinant viruses at a multiplicity of infection (MOI) of 0.1. At 24 h post-infection, the infected cells were either fixed with 4% paraformaldehyde for 20 min at room temperature for detection of cell surface expression, or fixed in the same manner and permeabilized with 0.2% Triton X-100 in PBS for 10 min for detection of intracellular expression. The cells were blocked for 30 min with 3% normal goat serum and incubated with 1:100 dilution of primary antibody (anti-ILTV gB, gC and gD antisera) for 1 h. The cells were then rinsed with PBS and incubated with 1:1000 dilution of Alexa Fluor 488 conjugated goat anti-rabbit immunoglobulin G antibody (Invitrogen, Carlsbad, CA) for 45 min. Subsequently, the cells were washed with PBS and analyzed with a confocal microscope. Immunofluorescence analysis of vero cells infected with rNDV LaSota (data not shown), rNDV gB, rNDV gC, and rNDV gD and processed for intracellular expression of ILTV proteins: gB, gC and gD), as well as surface expression of ILTV proteins: gB, gC and gD using rabbit anti-ILTV antisera.

Construction and generation of rNDVs containing ILTV gB, gC, and gD genes.

All the vaccine constructs in the present study were based on the recombinant avirulent NDV strain LaSota. The construction of a full-length cDNA of the antigenomic RNA of NDV strain LaSota has been

described previously (Huang et al., 2001, J Gen Virol 82, 1729-1736). In the present study, we have used a previously-described NDV derivative that had been modified to contain a unique PacI site between P and M genes. ILTV gB gene is 2652 nucleotides in length and has a guanine plus cytosine ratio of 44.53%. Detailed scanning of ILTV gB gene demonstrated sequence similarities to NDV strain LaSota gene end signals at nucleotide positions (ORF positions) 564-573 which contains poly A tail consisting of more than six repeated adenine bases flanked by "AG" nucleotides in the upstream region. These signals could be potentially read as gene ends by viral RNA polymerase leading to premature termination of transcription. Therefore, we have modified the gB gene at above said nucleotide positions by overlapping PCR methodology and without altering the amino acid sequence of the encoded protein. The wild type and modified gB with the modified nucleotides and their positions is shown in the Table 2 below. To construct an insert encoding the modified gB, the complete ORF (excluding the stop codon) of the gB gene was fused to the last 12 amino acids of the NDV F protein cytoplasmic tail (amino acids 542-553). The gC and gD inserts were constructed by fusing the ectodomain of glycoproteins to the transmembrane domain and cytoplasmic tail (amino acids 497-553) of the NDV F protein. The inserts bearing the gB, gC, and gD gene of ILTV were cloned at the unique PacI site between P and M genes of full-length NDV plasmid. The

resulting plasmids were designated as pNDV gB (SEQ ID NO: 16), pNDV gC (SEQ ID NO:17), and pNDV gD (SEQ ID NO:18), respectively, (figure 1) which were used to recover recombinant viruses designated rNDV gB (SEQ ID NO:19), rNDV gC (SEQ ID NO: 20), and rNDV gD (SEQ ID NO:21), respectively, following the procedure described (Huang et al., 2001, supra).

Table 2: Modification of gB gene

Mutagenesis at 567,570 and 573 ORF positions of ILTV gB gene
5'-AAT GAT GAA GCA GAa AAa AAa TTG CCC CTG GTT CCA TCA CTG-3' (SEQ ID NO:22)
5'-AAT GAT GAA GCA GA*g* AA*g* AA*g* TTG CCC CTG GTT CCA TCA CTG-3'(SEQ ID NO:23)
(Nucleotides at positions 567,570 and 573 in unmodified gB (top row) are shown in lower case and their modifications are shown in modified gB (bottom row) in lower case italicized. Underlined sequence in the unmodified gB (top row) indicates the sequence showing close similarities to NDV transcriptional gene end signal: TTA GAA AAA A (SEQ ID NO:24)(NDV gene end transcriptional signal).

Expression and incorporation of ILTV gB, gC, and gD by rNDVs.

The expression of ILTV gB, gC, and gD was examined by Western blot, immunofluorescence, and flow cytometry while their incorporation by rNDVs was evaluated by Western blot and immunoelectron microscopy assays as described (Nayak et al., 2009, supra; Khattar et al., 2010, Vaccine 28, 3159-70; Khattar et al., 2011, J Virol 85, 10529-41; Nayak et al., 2010, J Virol 84, 2408-20), using anti-peptide

antisera raised in rabbits against ILTV gB, gC, and gD.

DF1 cells were infected with the individual rNDV constructs and 48 h later the cells were collected and processed to prepare cell lysates. Allantoic fluid from embryonated eggs infected with the individual constructs was clarified and subjected to centrifugation on sucrose gradients to make partially purified preparations of virus particles. For purification of ILT virions, infected Chicken embryo liver cell lysates were cleared by centrifugation at 4500 × g for 15 min followed by sedimentation of ILTV by centrifugation through a cushion of 40% sucrose in phosphate buffered saline (PBS), and purified in a continuous 20–50% sucrose gradient at 25,000 rpm and 4 °C for 1 and half hour. The virions were resuspended in PBS. Total CEK cell lysates were prepared 24 h after infection with ILTV at a multiplicity (MOI) of 5 PFU per cell. These samples were analyzed by Western blot analysis using rabbit anti-ILTV gB, gC, and gD antisera (see text for details).

Flow cytometry analysis of the surface expression of ILTV proteins. DF1 cells were infected with the rNDV gB (panel A), rNDV gC (panel B) or rNDV gD (panel C) viruses at a MOI of 5, in parallel with cells that were mock-infected or infected with the rNDV LaSota empty vector. At 24 h post-infection, the cells were probed with rabbit anti-ILTV sera, followed by incubation with Alexa Fluor 488 conjugated goat anti-rabbit IgG antibody and

analyzed by Flowjo program of FACSRIA II flow cytometer. Values represent averages of the results obtained from two independent experiments.

Biological characterization of the recombinant viruses.

The multicycle growth kinetics of rNDVs expressing ILTV gB, gC, and gD were determined in SPF embryonated chicken eggs (Nayak et al., 2010 supra). The pathogenicity of recombinant viruses was determined by the mean death time (MDT) test in 9-day-old SPF embryonated chicken eggs (Nayak et al., 2010, supra).

Immunization and challenge experiments in chickens.

The immunogenicity and protective efficacy of the recombinant viruses against virulent ILTV and virulent NDV challenges were evaluated in specific pathogen free (SPF) chickens obtained from Charles River Laboratories, Wilmington, MA, USA. A total of 140 two-week-old SPF white leghorn chickens were assigned to 10 groups of 14 chickens each and received a prime-boost immunization on days 0 and 14 with the indicated virus by the indicated routes as described below (the day 0 and day 14 doses are identical). Briefly, the control group remained unvaccinated and served later as challenge controls. Group CEO and HVT-LT were vaccinated with the ILTV-CEO vaccine Trachivax and the recombinant herpes virus of turkey expressing laryngotracheitis antigens vaccine HVT-LT respectively, as per the manufacturer's recommendations. The groups gB, gC,

and gD received a virus rNDV gB, rNDV gC, and rNDV gD, respectively, by oculonasal route with a dose of 10^6 $TCID_{50}$/mL, whereas the groups gB+gC, gB+gD, gC+gD, and gB+gC+gD were immunized through the same route with a multivalent vaccine consisting of a mixture of 10^6 $TCID_{50}$/mL each of rNDV gB and rNDV gC, a mixture of 10^6 $TCID_{50}$/mL each of rNDV gB and rNDV gD, a mixture of 10^6 $TCID_{50}$/mL each of rNDV gC and rNDV gD, and a mixture of 10^6 $TCID_{50}$/mL each of rNDV gB, rNDV gC, and rNDV gD respectively. Each oculonasal immunization involved administration of allantoic fluid containing the indicated rNDVs in a total volume of 200μL (50μL in each eye and nostril). Blood was collected on days 12 and 21 and sera were separated from the blood samples for analyzing antibody response. Two weeks following booster immunization, chickens in each group were divided into two subgroups of 7 chickens each, one subgroup was transferred to enhanced BSL3 facility for virulent NDV challenge. The remaining chickens were kept in a BSL-2+ facility for virulent ILTV challenge. For virulent NDV challenge, each bird in all groups (n=7) was challenged by oculonasal route with $10^{4.5}$ EID_{50} of velogenic NDV strain Texas GB. All birds were observed daily for 2 weeks for clinical signs (death, paralysis, and torticollis) of neurotropic NDV. In order to determine the replication of challenge virus, two chickens from each group were sacrificed on 3rd day post challenge. Tissue sample (trachea, lungs, and brain) were collected, homogenized in cell culture medium

(1gm/10ml) and clarified by centrifugation. The challenge virus titers in tissue samples were determined by limiting dilution in DF-1 cells. For virulent ILTV challenge, each bird in all groups (n=7) were challenged with $6.3X10^4$ $TCID_{50}$ of a USDA ILT challenge virus in a total volume of 200μL (100μL intratracheally and 50μL in each nostril). All birds were observed daily for 14 days post challenge for clinical signs of dyspnea, conjunctivitis, depression, and mortality. A daily total clinical sign score was calculated for each group following the scoring system described by Oldoni et al. (Oldoni et al., 2009, Avian Pathol 38, 47-53). In order to determine the replication of challenge virus as well as to assess the viability of trachea, two chickens from each group were sacrificed on 4^{th} day post challenge. A part of the tracheal tissue was collected in buffered formalin for histopathology and the remaining tissue was collected in cell culture medium (1g/mL) and homogenized. The homogenate was used to determine the challenge virus titers by limiting dilutions in chicken embryo liver cells. The remaining five chickens in each group were observed daily for 14 days for disease signs and mortality following challenge. Virulent NDV Texas-GB challenge experiment was carried out in an enhanced BSL3 containment facility certified by the USDA, with the investigators wearing appropriate protective equipment. All of the animals used in this study were cared for in accordance with established

guidelines, and the experimental protocols were performed with the approval of Institutional Animal Care and Use Committee (IACUC) of the University of Maryland and under Animal Welfare Association (AWA) regulations.

Scoring of clinical signs.

Blind scoring of clinical signs was performed following the scoring system described by Oldoni et al. (supra).

Virological and serological assays.

Limiting dilution (TCID50), virus neutralization test (VNT) and hemagglutination inhibition (HI) assays were performed following standard protocols (Nayak et al., 2010, supra; Hierholzer and Killington, 1996, In Mahy and Kangro Eds. Virology Methods Manual, Academic Press, London).

Histopathology examination.

Tracheal tissues collected from the birds 4th day post ILTV challenge were processed for sections. The sections were stained with hematoxylin and eosin and the blind histological scoring was performed based on the severity of inflammation, necrosis, ulceration, and the presence of viral inclusions in the tracheal epithelium. Inflammatory, necrotic, and ulcerative lesions were scored as 0 (no lesions), + (minimal lesions), ++ (mild lesions), +++ (moderate lesions), and ++++ (severe lesions). Inclusion bodies were scored as either + (present) or – (absent). An overall histological score was given to each bird on a 0 to ++++ scale as described above.

Transfection and recovery of recombinant NDV.

Transfection was carried out as described previously (Krishnamurthy et al., 2000, Virol 278, 168-182). Briefly, HEp-2 cells (6-well plates) were infected at 1 p.f.u. per cell with modified vaccinia virus (MVA/T7) expressing T7 RNA polymerase. A mixture of three plasmids containing NDV NP, P and L gene ORFs under the control of the T7 promoter (2·5, 1·5 and 0·5 μg per well, respectively) and a fourth plasmid encoding either the NDV or NDV plus foreign genes, antigenome (5 μg) was transfected with Lipofectamine Plus (Life Technologies). Four h after transfection, cells were washed and the medium was replaced with 2 ml fresh medium (DMEM with 0% fetal calf serum and 1 μg/ml acetyl trypsin). Three days post-transfection, the supernatant was harvested for virus, clarified and used to infect fresh HEp-2 cells. Three days later, 100 μl supernatant was taken to inoculate into the allantoic cavity of 10-day-old embryonated SPF eggs. After 96 h, allantoic fluid was harvested and tested for haemagglutinating (HA) activity.

Recovery of infectious recombinant NDV from cDNA.

A recombinant vaccinia virus-based transfection system was used to recover infectious recombinant NDV from cDNA. HEp-2 cells were infected with recombinant vaccinia virus (MVA/T7) capable of synthesizing T7 RNA polymerase. Simultaneously, the cells were transfected with the recombinant NDV encoding the desired foreign antigen, along with

plasmids encoding proteins of RNP complex, namely NP (pNP), P (pP), and L (pL). In a parallel transfection, plasmid pL was excluded in the experiment to serve as a negative control. Four days after transfection, the supernatant was used in either of two different ways to recover the virus. The supernatant was either injected into the allantoic cavities of 9-day-old embryonated eggs or amplified further in HEp-2 cells and DF1 cells (chicken embryo fibroblast cell line). The allantoic fluid of the eggs injected with the transfectant gave a positive hemagglutination (HA) titer ranging from 32 to 2048. The cell culture-amplified supernatant gave NDV titers slightly in excess of 104 plaque-forming units (PFU)/ml at the end of passage 1 and slightly in excess of 108 PFU/ml at the end of passage 2. Thus, the cotransfection method of rescue resulted in efficient recovery of NDV. After passage 2, the cell culture passaged virus was plaque purified to eliminate vaccinia virus and then individual plaques were used to inject 9-day-old embryonated eggs. No plaques were visualized nor HA titer quantified in the case of negative controls, further confirming the specificity of recovery of NDV from cDNA. The recovered virus was designated, for example rNDVgB, when the foreign antigen was gB, to distinguish it from the parental wild-type NDV, or in this case, pNDV gB.

Example 1

Distinguishing features of vector Lasota having 527 mutation (Y527A) in its F gene over the wild type Lasota (WT) vector.

The Newcastle disease virus Fusion protein (F) is a major contributor to the protective immunity of the NDV vaccine and also the primary determinant of NDV virulence and pathogenicity in chickens. The cytoplasmic tail of the NDV fusion protein contains a tyrosine amino acid at position 527 (of the "F" protein, SEQ ID NO:25) which is found to be conserved among different strains of NDV. To evaluate the effect of point mutation at this conserved tyrosine residue, tyrosine was substituted to alanine, cloned into PBR322 to produce pNDVY527A (SEQ ID NO:26) and the resulting Newcastle disease virus, rNDVY527A (SEQ ID NO:27), with phenotype designated "Y527A" was compared with the wild type Lasota (WT) virus for its ability to multiply in cell culture, fusogenicity, levels of surface expression of a foreign protein, pathogenicity to chicken eggs and chicken embryos, and immunogenicity and protective efficacy in chickens against virulent NDV challenge.

Growth characteristics and fusion activity of Y527A: The multistep growth kinetics and magnitudes of replication of the Y527A and the WT viruses were determined in DF1 cells (data not shown). Both the viruses replicated exponentially until ~40 hpi, after which replication was at a plateau. The

magnitudes of replication were similar for WT and the Y527A, however, the titer of the Y527A virus was approximately 1.75 log10 higher than that of WT at 24 hpi. These results suggest that the mutagenesis in Y527A virus did not compromise its ability to multiply in cell culture but the same has been improved slightly over the WT virus. We further evaluated the fusogenicity of the Y527A virus by measuring the plaque sizes of the mutated virus on DF-1 cell monolayers and comparing them with those of the WT virus (data not shown). We observed significantly larger plaques for Y527A virus compared to their WT counterparts. These results indicated that added mutation in the Y527A virus provides for the enhanced fusogenicity compared to that of the WT virus.

Levels of surface expression of a foreign protein by recombinant viruses: In our previous study we found that the level of surface expression of a vaccine antigen is the main contributor to the immunogenicity and protective efficacy of the vaccine (Kanabagatte Basavarajappa et al., 2014, Vaccine 32, 3555-63). Therefore, to quantify and compare the levels of surface expression of a foreign antigen by the recombinant viruses, the human respiratory syncytial virus (hRSV) “F” gene was cloned into Y527A and WT viruses at a unique PmeI site present between their P and M genes. Surface expression of the foreign protein was quantified by flowcytometry using DF-1 cells and commercially available monoclonal antibodies against

hRSV "F" protein. We observed approximately 5% more surface expression of the hRSV "F" by Y527A virus compared to the surface expression of the foreign protein by the WT virus (data not shown). These results suggest that the mutation in the Y527A virus favors the enhanced surface distribution of the expressed foreign protein on the virus infected cells and presumably this would increase the immunogenicity of the vaccine.

Pathogenicity of the CT mutant viruses in embryonated chicken eggs and 1-day-old chicks. We evaluated the effect of the added mutation on viral pathogenicity using two standard pathogenicity assays, namely, the mean embryo death time (MDT) assay and the intracerebral pathogenicity index (ICPI) test. MDT values were determined in 9-day-old embryonated chicken eggs (data not shown). NDV strains are categorized into three pathotypes on the basis of their MDT values: velogenic (less than 60 h), mesogenic (60 to 90 h), and lentogenic (greater than 90 h). The MDT value of the Y527A mutant (90.60 h) was reduced by 10 h compared to that for WT (100.60 h), which is suggestive of modest increases in virulence but still they were the lentogenic viruses. The pathogenicity of the recombinant viruses was also evaluated by the ICPI test in 1-day-old chicks (data not shown). Lentogenic strains give values close to 0. The ICPI value of Y527A virus was 0.2 which is slightly higher than that for WT (0.11) which is indicative of increased pathogenicity, although the increases was modest.

Immunogenicity and protective efficacy of recombinant viruses in chickens. The immunogenicity and protective efficacy of the recombinant viruses against virulent NDV challenge was evaluated in specific pathogen free (SPF) chickens obtained from Charles River Laboratories, Wilmington, MA, USA. A total of 21 two-week-old SPF white leghorn chickens were assigned to 3 groups of 6 chickens each and received a immunization on days 0 with the indicated virus by the indicated routes as described below. The control group remained unvaccinated and served later as challenge controls. The groups Y527A and WT received a virus Y527A and WT, respectively, by oculonasal route with a dose of 10^6 TCID50/mL. Each oculonasal immunization involved administration of allantoic fluid containing the indicated recombinant viruses in a total volume of 200μL (50μL in each eye and nostril). Blood was collected on day 21 and sera were separated from the blood samples for analyzing antibody response. After 21 days, birds were transferred to enhanced BSL3 facility for virulent NDV challenge. For virulent NDV challenge, each bird in all groups (n=7) were challenged by oculonasal route with $10^{4.5}$ EID50 of velogenic NDV strain Texas GB. All birds were observed daily for 2 weeks for clinical signs (death, paralysis, and torticollis) of neurotropic NDV. In order to determine the replication of challenge virus, two chickens from each group were sacrificed on 3rd day post challenge. Tissue sample (trachea, lungs, and brain) were collected, homogenized in cell culture medium

(1gm/10ml) and clarified by centrifugation. The challenge virus titers in tissue samples were determined by limiting dilution in DF-1 cells. NDV-specific antibody responses in the sera collected on 21st day post immunization was assayed using HI test. High levels of NDV-specific serum antibodies were detected for both Y527A and WT groups (data not shown). However, the Y527A group possessed approximately 0.5 log2 higher HI titers than WT group indicating the enhanced immunogenicity of the mutated Y527A virus. Upon challenge on 22nd day post immunization with highly-virulent NDV strain Texas-GB, all of the chickens that had been immunized with recombinant viruses were completely protected from NDV challenge without any disease signs and with no evidence of challenge virus replication in the organs collected 3rd day post challenge. These results suggest that the added mutation in Y527A virus does not reduce its protective efficacy against virulent NDV challenge.

In the present study, we have investigated the effect of mutagenesis of the conserved tyrosine residue in the NDV "F" protein cytoplasmic tail by substituting alanine for tyrosine. The resulting recombinant virus was compared with wild-type Lasota virus for its ability to replicate in cell culture, fusogenicity and levels of surface expression of the foreign protein in vitro. The pathogenicities of the recombinant viruses were evaluated in vivo in embryonated chicken eggs, 1-day-old chicks and 2-week old SPF chickens. Our results indicated that

the mutated Y527A virus is superior to WT virus in all of the parameters evaluated in vitro and in vivo. Briefly, Y527A virus showed enhanced replication in cell culture, higher fusogenicity and surface expression of the foreign protein, boosted immunogenicity and protective efficacy compared to the WT virus yet maintaining the lentogenic phenotype similar to the WT virus. Therefore, the hyperfusogenic virus developed in this study may be useful in developing NDV as a better vaccine vector and as an oncolytic agent.

Example 2

Modification of the ILTV gD gene for improved incorporation in NDV envelope.

In the present study, we have generated rNDV expressing and incorporating ILTV gD protein. It has been reported that expression of foreign envelope glycoprotein by recombinant negative sense non-segmented viruses (NNSV) can result in incorporation of the foreign protein into the envelope of NNSV (DiNapoli et al., PNAS, June 2007, 1049788-9793). However, in our study, incorporation of the ILTV gD protein into envelopes of rNDV particles was not found when its intact ORF was cloned into NDV genome, but significant incorporation of gD protein into NDV particles was detected only when its ectodomain (amino acids 1-351) was fused to the NDV F protein cytoplasmic tail and transmembrane domain (amino acids 497-553), suggesting that native ILTV gD protein lack the packaging signals necessary for

their incorporation into NDV particles. These results were consistent with the previous study, which has shown that replacement of the transmembrane domain and cytoplasmic tail of the foreign envelope protein with those of a NDV envelope protein increased incorporation of the foreign glycoprotein into the NDV virion (Nayak et al., 2009, supra).

Example 3

Generation of rNDVs expressing gB, gC, and gD genes of ILTV

In order to obtain a NDV recombinant which will express and incorporate gB in its envelope, several rNDVs containing chimeric gB were generated (data not shown). However, it was found that when the complete ORF of gB fused to the last 12 amino acids of NDV F protein cytoplasmic tail in a recombinant rNDV gB (figure 1), the gB was incorporated into the envelope of NDV. The expression and incorporation of ILTV gC and gD were achieved when their ectodomain was fused to the cytoplasmic tail and transmembrane domain of NDV F protein creating rNDV gC and rNDV gD (described above, figure 1), respectively. The genetic stability of the ILTV genes was confirmed by passaging the recombinant viruses in embryonated chicken eggs. Our results showed that the integrity of the added genes and the expression of the foreign proteins were preserved even after 10 egg passages.

Example 4

Expression and incorporation of ILTV glycoproteins by recombinant viruses

The expression and incorporation of ILTV glycoproteins by recombinant viruses were analyzed by western blot using rabbit anti-ILTV peptide sera. All the three proteins of ILTV that were expressed and incorporated by rNDVs reacted in western blot with the anti-ILTV gB, gC and gD antisera (figure 2-1). Western blot analysis detected two bands in purified virus preparations and lysates of cells infected with ILTV and rNDV gB viruses (figure 2-1A): these represented (i) the uncleaved monomeric precursor form of gB with an apparent molecular weight of >100 kDa and (ii) C-terminal cleavage product of gB with an apparent molecular weight of 58kDa (Poulsen and Keeler, 1997, J Gen Virol 78, 2945-2951). Western blot analysis detected 60 kDa band of gC and 42 kDa band of gD (figure 2-1B and C) in ILTV infected cell lysate and ILTV purified virus. However, a band slightly higher than ILTV control was detected in lysates of cell infected with rNDV gC and rNDV gD and purified virus preparations of rNDV gC (figure 2-1B and C), which was likely due to the fused cytoplasmic tail and transmembrane domains of NDV F protein. Approximately 50kDa band of gD was detected in the lysate of cells infected with rNDV gD virus. Further, the increase in molecular weight to ~ 65 to 70 kDa of the chimeric gD observed in rNDV gD purified virions (figure 2-1C) was presumably due to

an artificial aggregation of gD with itself or other proteins occurring during virion preparation. As expected, the ILTV gB, gC, and gD were not detected in lysate of cells infected with rNDV LaSota virus. Immunofluorescence studies showed expression of gB, gC and gD in the cytoplasm and surface of vero cells confirming the internal and surface expression of the foreign proteins. Flowcytometry results indicated a higher level of cell surface expression of gD than cell surface expression of gB and gC (figure 2-2). The magnitude of surface expression is in the order: gD>gC>gB with 11-fold and 1.5-fold more surface expression of gD and gC, respectively, compared to the surface expression of gB on infected DF-1 cells. In parallel with the flowcytometry results, the results of immunoelectron microscopy indicated the enhanced incorporation of gD into the envelopes of recombinant viruses compared to the incorporation of gB and gC into the NDV particles (figure 3).

Example 5

Biological characterization of rNDVs expressing ILTV proteins

The results of multicycle growth kinetics of rNDVs (figure 4-1) indicated the similar growth patterns for rNDV LaSota, rNDV gD, and rNDV gB viruses. At 72 hour post-inoculation, the maximum titers for rNDV LaSota and rNDV gD viruses were similar, but the rNDV gB virus achieved the final titer which was approximately one half log lower

compared to rNDV LaSota virus. The rNDV gC grew more slowly and attained the final titer which was approximately two logs lower than that of rNDV LaSota virus. The pathogenicities of the rNDVs were evaluated by MDT test in 9-day old embryonated SPF chicken eggs. The MDTs for the recombinant viruses were 110 h (rNDV LaSota), 125 h (rNDV gB), 124 h (rNDV gC), and 122 h (rNDV gD) which indicated that the rNDVs expressing ILTV proteins were lentogenic viruses (an NDV strain is considered lentogenic or avirulent, if the MDT value is>90 h (Alexander, DJ, 1989, Newcastle disease, p. 114-120. In, HG Purchase et al., Eds. A Laboratory Manual for the Isolation and Identification of Avian Pathogens, 3rd Ed. American Association for Avian Pathologists, Inc. Kennett Square. PA) and the addition of ILTV genes further decreased the virulence of the NDV vector.

Example 6

Immunogenicity and protective efficacies of rNDVs against virulent NDV challenge

NDV-specific antibody responses in the sera collected on 21st day post immunization was assayed using HI test. High levels of NDV-specific serum antibodies with no statistically significant differences ($P < 0.05$) were detected for all of the immunized groups except control, CEO, and HVT-LT vaccinated birds (figure 4-2). Upon challenge on 42nd day post immunization with highly-virulent NDV strain Texas-GB, all of the chickens that had been immunized with rNDVs either individually or in

combination were completely protected from NDV challenge without any disease signs and with no evidence of challenge virus replication in the organs collected 3rd day post challenge. In contrast, all of the chickens in the unvaccinated control group, CEO, and HVT-LT vaccinated groups died within 3 days after challenge. Therefore, these results suggested that expression of the ILTV glycoproteins does not interfere with protective immunity of NDV LaSota vaccine.

Example 7

ILTV specific serum neutralizing antibody (NAb) responses following immunization with rNDVs

The ability of sera taken after primary and secondary immunization to neutralize the virulent USDA ILTV strain was evaluated by virus neutralization test (VNT) (figure 5A). The salient findings are that vector-expressed ILTV gB and gC do not or barely induce detectable virus neutralizing antibodies, whereas gD alone induces higher titers than any combination or an attenuated whole virus ILTV vaccine. Furthermore, it is remarkable that the gD-expressing HVT-based vaccine does not induce detectable neutralizing antibodies. In addition, negligible and inconsistent presence of NAbs were found for CEO, gB, gB+gD, gC+gD and gB+gC+gD vaccinated groups with some birds in each group completely lacking the NAb response, while all birds vaccinated with rNDV gD vaccine showed the presence of neutralizing antibody activity in various titers

differed from bird to bird. These results suggested that gD expressed by rNDV gD induce a very good neutralizing antibody response in chickens.

Example 8

Protective efficacy of rNDVs against virulent ILTV replication in trachea

To determine the protective efficacy of rNDVs vaccines against virulent ILTV challenge, the challenge virus titers in the tracheal tissue collected from birds on 4^{th} day post-challenge were titrated by limiting dilution and are shown in table 1. Our results suggested that rNDV gD and rNDV gB + rNDV gD vaccines were very efficient in preventing challenge virus replication in trachea. The kinetics of challenge virus replication in the trachea was monitored by taking tracheal swabs for the recovery of challenge virus from the remaining 5 birds in each group on 5^{th} and 7^{th} days post-challenge. Surprisingly, none of the tracheal swabs were positive for ILTV, indicating peak challenge virus titers occurs in trachea up to 4th day post-challenge.

Example 9

Histopathology examination

Histopathological scoring of tracheal tissue collected on 4^{th} day post challenge with ILTV is shown in table 2. Our results suggested that gD expressed by rNDV is very efficient in preventing challenge virus replication in trachea which is

reflected by the absence of histopathology as well as viral inclusions in the tracheal epithelium.

Table 3. Histopathological characterization of tracheal tissue samples from chickens after challenge with USDA strain of ILTV.

Group	Animal ID	Overall score	Necrosis	Ulceration	Inflammation	Viral Inclusions
Control group	Bird 1	++++	++++	++++	+++	+
	Bird 2	++++	++++	++++	+++	+
CEO	Bird 1	+	0	0	+	0
	Bird 2	+	0	0	+	0
HVT-LT	Bird 1	++	0	0	++	0
	Bird 2	0	0	0	0	0
gB	Bird 1	+++	+	+++	++	+
	Bird 2	+++	++	+++	+++	+
gC	Bird 1	++	+	+	++	+few[a]
	Bird 2	++	0	0	++	0
gD	Bird 1	++	0	0	++	0
	Bird 2	0	0	0	0	0
gB+gC	Bird 1	++	0	0	+++	+few
	Bird 2	+	0	0	+	0
gB+gD	Bird 1	+++	0	0	+++	0
	Bird 2	+++	0	0	+++	+few
gC+gD	Bird 1	+++	0	0	+++	+few
	Bird 2	++	0	0	++	0
gB+gC+gD	Bird 1	++	0	0	+++	+few
	Bird 2	+++	++	++	+++	+

Histology scoring: 0, no lesions; +, minimal; ++, mild;+++, moderate; ++++, severe; +, viral intra-nuclear inclusions present.

[a] Tracheas with few inclusions usually had only focal lesions with the inclusions

Example 10

Clinical signs score evaluation

Clinical sign scores were recorded for all of the experimental groups from day 1 to 14 post-challenge and are summarized in figure 5B. Briefly, all of the chickens in the control and gB groups showed severe clinical signs until 9th and 6th dpi, respectively. Birds in the gC group showed mild disease signs on 3rd and 4th dpi with signs recorded until 6th dpi. The birds immunized with rNDV gD and multivalent vaccines consisting of combinations of rNDVs did not show disease signs until 14th dpi with the exception of gB + gC + gD group showed severe disease signs between 3rd to 5th dpi. Total clinical scores of gB or gC group were statistically significantly ($P<0.05$) differed while those for gD and multivalent vaccinated groups did not differ significantly from total clinical scores obtained for CEO and HVT-LT groups. The birds immunized with CEO and HVT-LT vaccines showed optimum protection with few birds in HVT-LT group displayed respiratory dyspnea and depression until 5th dpi. The detailed summary of the mortalities in each experimental group following ILTV challenge is given in table 4. These results suggested that rNDV gD and multivalent vaccines consisting of combinations of two rNDVs offered optimal protection without apparent clinical signs and mortality.

Group	Mean tracheal viral load at 4 day post challenge for the indicated groups	No. of survivors on day 14 post-challenge with USDA ILTV/ total no. of birds
Control	6.0[a] (±0.50)[A]	2/5
Trachivax	0.0[B]	5/5
HVT-LT	0.0[B]	5/5
gB	5.5 (±0.50)[A]	4/5
gC	2.2 (±2.2)[A]	4/5
gD	0.0[B]	5/5
gB+gC	2.3 (±2.3)[A]	5/5
gB+gD	0.0[B]	5/5
gC+gD	1.8 (±1.8)[A]	5/5
gB+gC+gD	1.0 (±1.0)[A]	5/5

Table 4. Tracheal viral load in chickens on 4th day post challenge with USDA strain of ILTV and survival of chickens after ILTV challenge.

Discussion

ILT is a highly contagious and economically important disease of poultry world-wide. Currently available vaccine strategies against ILT are not ideal and the knowledge about the protective antigens of ILTV is limited. Therefore, we have

used recombinant NDV to evaluate the role of three major ILTV envelope glycoproteins gB, gC, and gD in immunity and protection. These three envelope proteins of ILTV were chosen because they were found to be the major protective antigens in other herpesviruses (Fischer et al., 1003, Vaccine 21, 1732-1741; Hong et al., 2002, Vaccine 20, 1205-1214; Lukacs et al., 1985, J Virol 53, 166-173; Hampl et al., 1984, J Virol 52, 583-590; Zuckermann et al., 1990, J Virol 64, 802-812; Ober et al., 1998, J Virol 72, 4866-4873; Babiuk et al., 1987, Virology 159, 57-66; Chase et al., 1989, J Gen Virol 70, 1561-1569; van Drunen Little-van den Hurk et al., 1990, Vaccine 8, 358-368; Gao et al., 1994, Vaccine 12, 145-152, Zhu and Letchworth, 1996, Vaccine 14, 61-69).

Glycoprotein B (gB) has previously been shown to be an important target for cellular and humoral immune responses capable of conferring protective immunity against ILTV infection (Tong et al, 2001, supra; Sun et al., 2008, supra; York and Fahey, 1991, Avian Pathol 20, 693-704). Likewise, gC in other herpesviruses has been shown to be a target for cellular and humoral immune responses capable of inducing neutralizing antibodies and T-cell immune responses (Fischer et al., 2003, Vaccine 21, 1732-1741; Hong et al., 2002, Vaccine 20, 1205-1214; Lukas et al., 1985, J Virol 53, 166-173; Hampl et al., 1984, J Virol 52, 583-590). However, in this study, immunization with rNDV gB or rNDV gC vaccine did not induce the immune response sufficient to

offer complete protection against ILTV challenge. We presume that the incomplete protection offered by these vaccines was due to inefficient envelope incorporation and cell surface expression of gB or gC which might have led to inadequate immune activation leading to partial protection, since there has been a previous report correlating the levels of foreign protein expression and the extent of protection offered by the recombinant viral vaccine (Roberts et al., 2004, J Virol 78, 3196-3199). Further, previous studies with recombinant fowl pox virus (rFPV) vector expressing gB gene of ILTV provided variable protection against morbidity but 100% protection against mortality after virulent ILTV challenge in chickens (Tong et al., 2001, supra; Chen et al., 2011, FEMS Immunol Med Microbiol 63, 289-295). In addition, in another study with rFPV co-expressing NDV fusion (F) and hemagglutinin proteins (HN) and ILTV gB induce detectable ELISA antibody titers against NDV and ILTV, but failed to elicit significant HI titers against NDV (Sun et al., 2008, supra). The immunization of chickens with rFPV based bivalent vaccine against ND and ILT offered 70% protection from death against NDV challenge and 100% protection against ILTV induced mortality, but 70% protection against ILTV induced clinical signs. In our study, immunization of chickens with rNDV based ILTV gB vaccine induces higher levels of HI antibody titers against NDV and detectable levels of NAb titers against ILTV. Following challenge, the vaccine offered complete

protection against NDV challenge and 80% protection against ILTV induced mortality, but failed to protect chickens against ILTV associated respiratory signs. The discrepancy in results between these studies could be due to the differences in the vector systems used to express the ILTV gB. The poor protective efficacy of rNDV gB vaccine against ILTV challenge was attributed to its poor immunogenicity which is represented by the presence of lower neutralizing antibody titers in chickens immunized with rNDV gB vaccine.

In this study, for the first time, we have evaluated the protective efficacy of gD against ILTV challenge. Immunization with rNDV expressing ILTV gD induced a higher level of neutralizing antibodies and offered complete protection to chickens against lethal ILTV challenge. The complete protection offered by gD can be attributed to its superior envelope incorporation and cell surface expression leading to induction of protective immune responses.

Our results are consistent with the results of previous studies reported in other herpesviruses in which glycoprotein D provided higher level of protection against the respective challenge viruses (Khattar et al., 2010, supra; Bennett et al., 1999, J Med Virol 57, 47-56; Zakhartchouk et al., 1999, J Gen Virol 80, 1263-9; Heineman et al., 2004, Vaccine 30, 2558-65). However, it is important to mention that the commercial vectored vaccine HVT-LT also express ILTV gD together with ILTV gI in one virus recombinant (Vagnozzi et al., 2012, supra), but only

immunization with rNDV gD induce detectable neutralizing antibodies, and confers better protection than HVT-LT. This observed discrepancy can be explained by the fact that the herpes virus of turkey is a strong inducer of cell-mediated immunity (Fabienne et al., 2010, Vaccine 28, 823-833), but NDV elicits strong humoral and cellular immune responses (DiNapoli et al., 2007, J Virol 81, 11560-11568) which is reflected by the presence of higher levels of neutralizing antibodies in chickens immunized with rNDV gD vaccine. Further, our results indicated that, neutralizing antibodies against gD were highly effective in blocking ILTV attachment and entry, as was shown by the absence of histopathology and viral inclusions in the tracheal epithelium post-ILTV challenge. In addition, the intranasal immunization of rNDV gD vaccine might have induced robust mucosal immunity at the respiratory tract, the portal of entry for ILTV, and hence preventing ILTV colonization and replication in the tracheal tissue.

In the present study, the apparent inability of the multivalent vaccines where gD is a part of the combination to completely prevent the challenge virus replication in trachea could be due to interference or competition for growth among the rNDVs expressing gB, gC or gD. These results were consistent with the results of previous study in which rNDV expressing HPAIV M2 protein interfered with the replication of rNDV expressing HA or NA proteins when administered to chickens as a

multivalent vaccine (Nayak et al., 2010, supra). In our study, failure to achieve similar levels of envelope incorporation of gB, gC and gD into NDV particles render us unable to clearly evaluate the role of each of these proteins in immunity and protection against virulent ILTV challenge. It is notable that immunization with rNDV gB + rNDV gC or HVT-LT vaccine did not induce a neutralizing antibody response but protected 100% of chickens against virulent ILTV challenge. This is consistent with the fact that humoral antibody response cannot stand alone to provide complete protection against ILT infection (York and Fahey, 1990, Arch Virol 115, 289-297; Fahey and York, 1990, J Gen Virol 2401-2405; Fahey et al., 1983, Avian Pathol 12, 505-514; Honda et al., 1994, J Vet Med Sci 56, 1051-1055). Therefore, to thoroughly assess the immunogenicity and protective efficacy of rNDV vectored ILTV vaccines, the mucosal and cell-mediated immunity would need to be evaluated.

In summary, for the first time we have evaluated the potential of recombinant NDV as a vaccine vector for ILTV. Our study showed that rNDV gD elicited immune response specific to NDV and ILTV and provided complete protection against highly virulent NDV and ILTV challenges. These results demonstrated that ILTV gD is a major protective antigen capable of inducing neutralizing antibodies. The immune response induced by rNDV gC or rNDV gB or multivalent rNDV combinations was not adequate enough to confer complete protection against

virulent ILTV challenge. Further, the NDV-vectored vaccine expressing gD alone was superior to a combination vaccine consisting of rNDVs expressing gB, gC, and gD. Therefore, the rNDV-based ILTV gD vaccine generated in this study for the protection of both NDV and ILTV will be highly beneficial to the poultry industry worldwide and could be the promising vaccine candidate to replace the existing ILTV vaccines.

What is claimed is:

1. A cDNA comprising

a recombinant Newcastle disease virus (NDV) polynucleotide encoding NDV proteins NP, P, M F, HN and L wherein the F protein contains a tyrosine to alanine substitution at position 527 of SEQ ID NO:25, and wherein the NDV proteins are positioned between a T7 promoter and a hepatitis delta virus ribozyme sequence.

2. A vector comprising the cDNA of claim 1.

3. The vector of claim 2 wherein said vector is pNDVY527A identified in SEQ ID NO:26.

4. A cell comprising the vector of claim 3.

5. A method for producing recombinant Newcastle disease virus comprising:

(i) providing cells capable of synthesizing T7 RNA polymerase;

(ii) cotransfecting the cells with a plasmid comprising the cDNA of claim 1, and a mixture of plasmids encoding NP, P, and L proteins; and

(iii) isolating recombinant Newcastle disease virus from medium of cotransfected cells.

6. The method of claim 5, wherein said plasmid in (ii) is pNDVY527A identified in SEQ ID NO:26.

7. A recombinant Newcastle disease virus (rNDV) produced by the method of claim 6, wherein said rNDV is rNDVY527A identified in SEQ ID NO:27.

8. A cDNA comprising

(i) a recombinant Newcastle disease virus (NDV) polynucleotide encoding NDV proteins NP, P, M F, HN and L wherein the F protein contains a tyrosine to alanine substitution at position 527 of SEQ ID NO:25, and wherein the NDV proteins are positioned between a T7 promoter and a hepatitis delta virus ribozyme sequence;

and

(ii) one or more polynucleotide encoding an antigen of interest, wherein said one or more polynucleotide is inserted before the NP gene, between the P and M genes, and/or between the HN and L genes.

9. The cDNA of claim 8 wherein said one or more polynucleotide encoding an antigen is inserted between the P and M genes.

10. The cDNA of claim 9 wherein the antigen of interest is from a virus selected from the group consisting of influenza virus, infectious bursal disease virus, rotavirus, infectious bronchitis virus, infectious laryngotracheitis virus, chicken anemia virus, Marek's disease virus, avian leukosis virus, avian adenovirus and avian pneumovirus

11. The cDNA of claim 10 wherein said antigen is a surface glycoprotein from Infectious Laryngotracheitis virus (ILTV).

12. The cDNA of claim 11 wherein said antigen is one or more ILTV antigen gB, gC, and/or gD, in any combination.

13. A vector comprising the cDNA of claim 12.

14. The vector of claim 13 wherein the vector is a plasmid.

15. The vector of claim 14 wherein said vector is pNDVgB identified in SEQ ID NO:16, pNDVgC identified in SEQ ID NO:17, or pNDVgD identified in SEQ ID NO:18.

16. A cell comprising one or more vector of claim 15.

17. A method for producing recombinant Newcastle disease (rNDV) virus comprising:

(i) providing cells capable of synthesizing T7 RNA polymerase;

(ii) cotransfecting the cells with one or more plasmid of claim 15, and a mixture of plasmids encoding NP, P and L proteins; and

(iii) isolating rNDV from medium of cotransfected cells.

18. A rNDV virus produced by:

(i) providing cells capable of synthesizing T7 RNA polymerase;

(ii) cotransfecting the cells with one or more plasmid of claim 13, and plasmids encoding NP, P and L proteins; and

(iii) isolating rNDV from medium of cotransfected cells.

19. A rNDV virus produced by:

(i) providing cells capable of synthesizing T7 RNA polymerase;

(ii) cotransfecting the cells with one or more plasmid of claim 15, and plasmids encoding NP, P and L proteins; and

(iii) isolating rNDV from medium of cotransfected cells.

20. The rNDV of claim 18, wherein said rNDV is any of rNDVgB identified in SEQ ID NO:19, rNDVgC identified in SEQ ID NO:20, and rNDVgD identified in SEQ ID NO:21.

21. A bivalent vaccine protective against challenge with Newcastle disease virus and ILTV, said vaccine comprising one or more rNDV of claim 18.

22. A bivalent vaccine protective against challenge with Newcastle disease virus and ILTV, said vaccine comprising one or more rNDV of claim 19.

23. The bivalent vaccine of claim 22 wherein said rNDV is rNDVgD.

24. The bivalent vaccine of claim 21 wherein said rNDV is rNDVgB and rNDVgC.

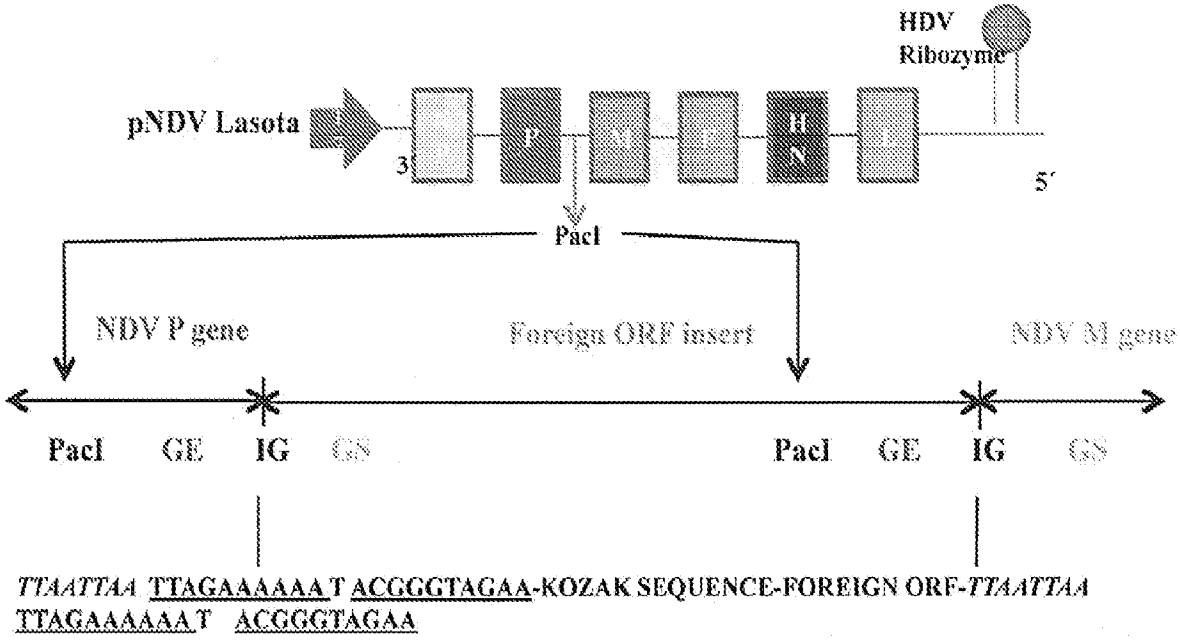
25. A method for stimulating a protective immune response in an avian animal against Newcastle disease and ILT comprising administering to said avian animal an immunologically sufficient amount of rNDV according to claim 19.

26. An immunogenic composition comprising, in a physiologically acceptable vehicle, recombinant NDV according to claim 18.

27. An immunogenic composition comprising, in a physiologically acceptable vehicle, recombinant NDV according to claim 19.

28. An immunogenic composition protective against ILTV challenge in an avian animal comprising ILTV gD in a vector.

29. The composition of claim 28 wherein said vector is a recombinant viral vector chosen from the group consisting of: NDV, adenovirus, adeno-associated virus, herpesvirus, pox virus, influenza virus, and retrovirus.


HDV
Ribozyme
pNDV Lasota
3'
5'
PacI
NDV P gene
Foreign ORF insert
NDV M gene
PacI GE IG GS
PacI GE IG GS
TTAATTAA TTAGAAAAAA T ACGGGTAGAA-KOZAK SEQUENCE-FOREIGN ORF-TTAATTAA
TTAGAAAAAA T ACGGGTAGAA

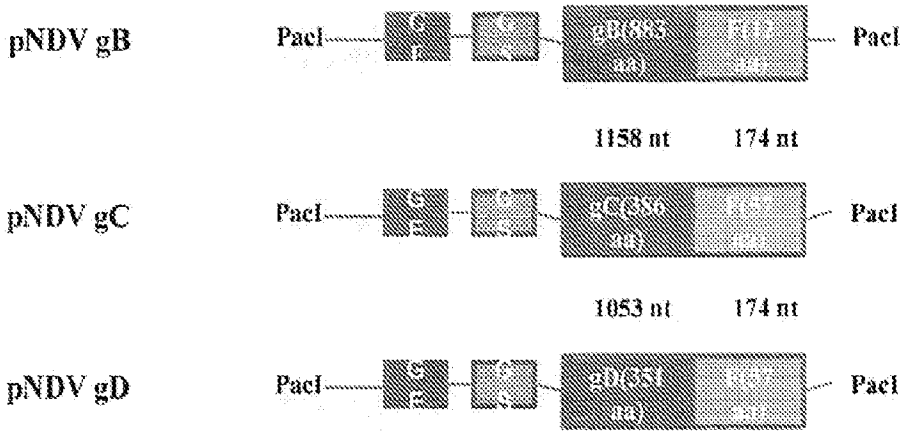

pNDV gB
PacI
PacI
1158 nt
174 nt
pNDV gC
PacI
PacI
1053 nt
174 nt
pNDV gD
PacI
PacI

Fig 2-1

A
~100 kDa
~ 58 kDa
ILTV-CEK lysate
rNDV gB lysate
rNDV-Lasota lysate
ILTV purified virus
rNDV gB purified virus
rNDV-Lasota purified virus
B
60 kDa
ILTV-CEK lysate
rNDV gC lysate
rNDV-Lasota lysate
ILTV purified virus
rNDV gC purified virus
rNDV-Lasota purified virus
C
~65 kDa
~50 kDa
42 kDa
ILTV-CEK lysate
rNDV gD lysate
rNDV-Lasota lysate
ILTV purified virus
rNDV gD purified virus
rNDV-Lasota purified virus

Fig 2-2

A
Medium fluorescent intensity (MFI)
1000
800
600
400
200
0
Mock
rNDV Lasota
rNDV gB
B
Medium fluorescent intensity (MFI)
1500
1000
500
0
Mock
rNDV Lasota
rNDV gC
C
Medium fluorescent intensity (MFI)
10000
8000
6000
4000
2000
0
Mock
rNDV Lasota
rNDV gD

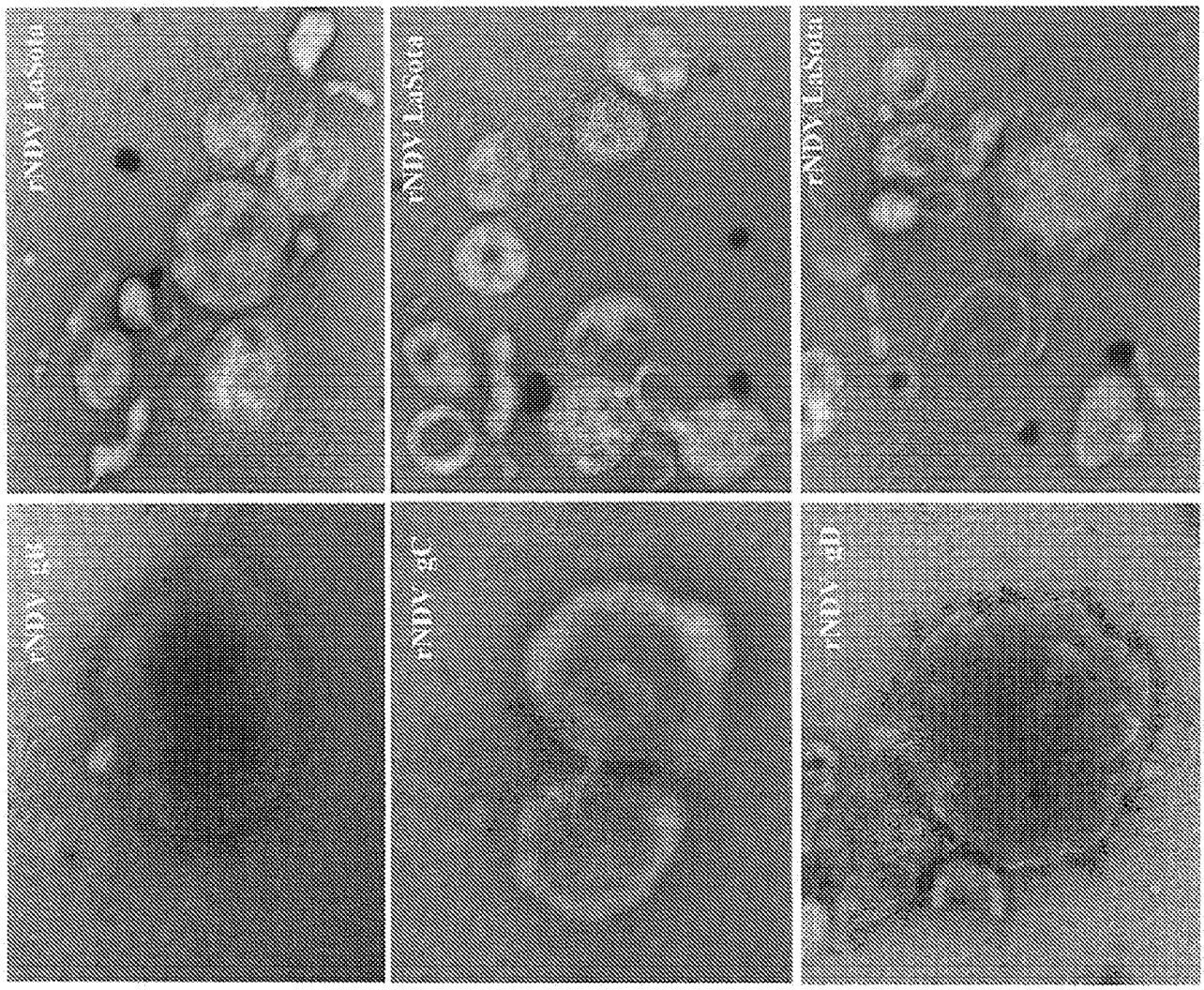

rNDV-GFP
rNDV-LaSota
rNDV-GFP
rNDV-LaSota
rNDV-GFP
rNDV-LaSota

Figure 3

Virus growth in embryonated eggs

Figure 4-1

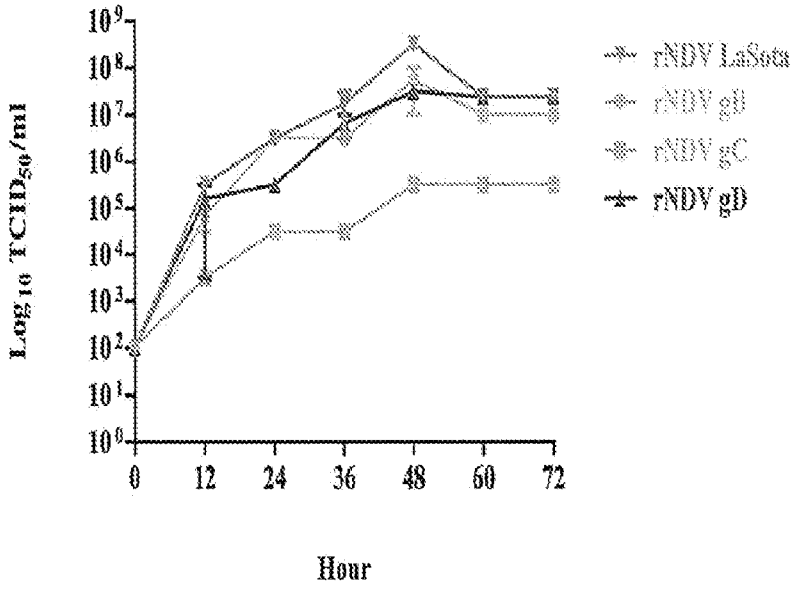


Figure 4-2

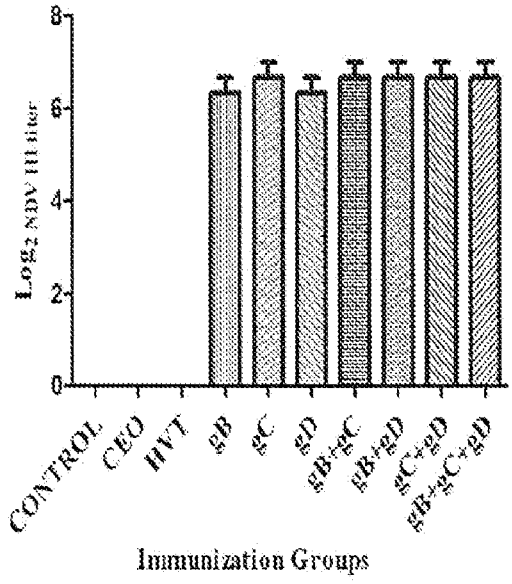


Fig 5-A

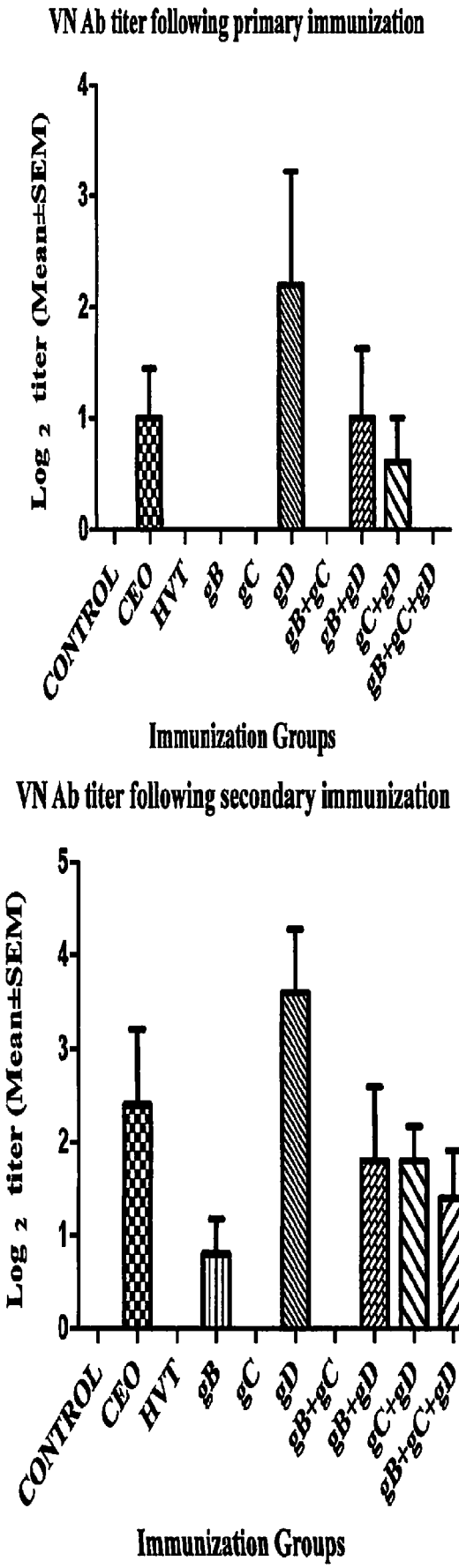
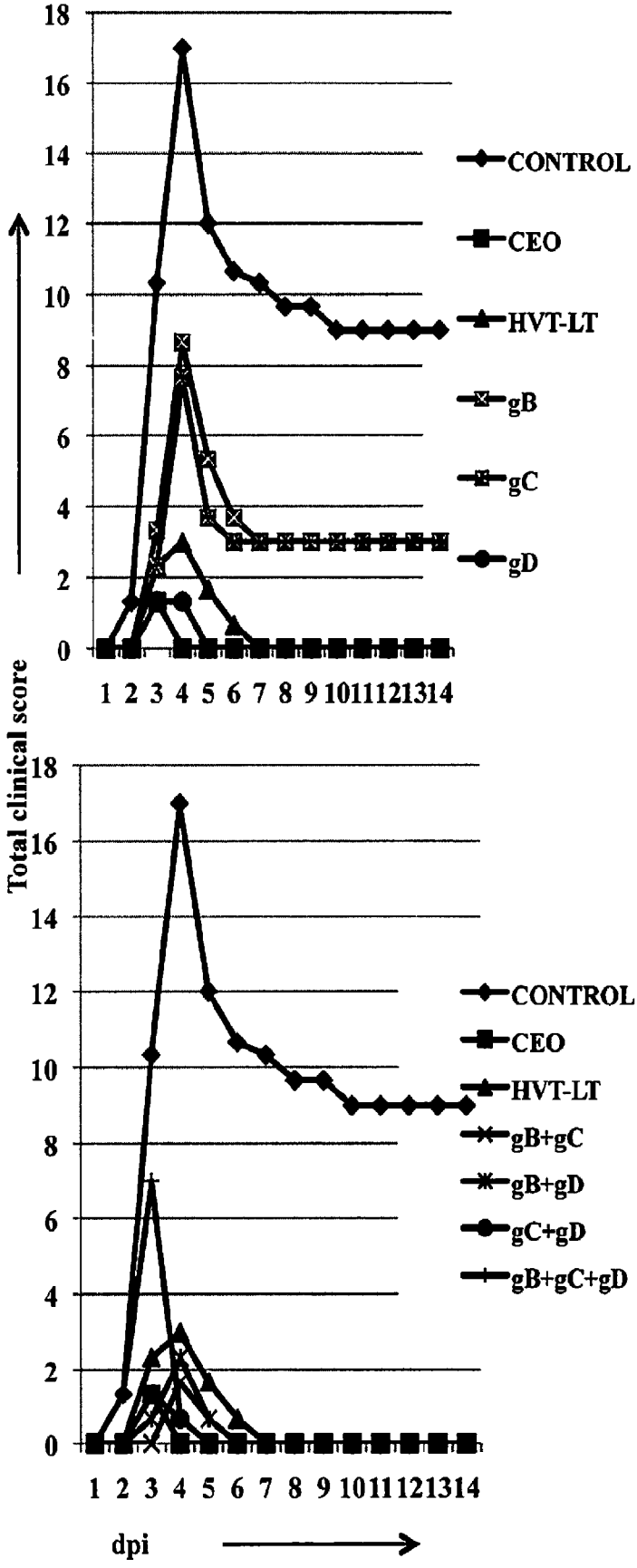

VN Ab titer following primary immunization
Log 2 titer (Mean±SEM)
CONTROL
CEO
HVT
gB
gC
gD
gB+gC
gB+gD
gC+gD
gB+gC+gD
Immunization Groups
VN Ab titer following secondary immunization
Log 2 titer (Mean±SEM)
CONTROL
CEO
HVT
gB
gC
gD
gB+gC
gB+gD
gC+gD
gB+gC+gD
Immunization Groups

Fig 5-B


Total clinical score
CONTROL
CEO
HVT-LT
gB
gC
gD
1 2 3 4 5 6 7 8 9 10 11 12 13 14
CONTROL
CEO
HVT-LT
gB+gC
gB+gD
gC+gD
gB+gC+gD
1 2 3 4 5 6 7 8 9 10 11 12 13 14
dpi

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US2014/047395

A. CLASSIFICATION OF SUBJECT MATTER
IPC(8) - A61K 39/12 (2014.01)
CPC - A61K 39/245 (2014.11)
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC(8) - A61K 39/00, 39/12, 39/295; C12N 7/00, 7/01 (2014.01)
USPC - 424/184.1, 199.1; 435/235.1; 536/23.1, 23.4

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched
CPC - A61K 39/00, 39/12, 39/245; C12N 15/85, 15/86 (2014.11) (keyword delimited)

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

Orbit, Google Patents, PubMed
Search terms used: immunogenic composition protect against iltv challenge avian gd viral vector recombinant

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	US 2013/0101619 A1 (COOK et al) 25 April 2013 (25.04.2013) entire document	28, 29
A	US 2007/0178115 A1 (TANG et al) 02 August 2007 (02.08.2007) entire document	28, 29
A	US 6,045,803 A (AUDONNET et al) 04 April 2000 (04.04.2000) entire document	28, 29
A	US 6,913,751 B2 (COCHRAN et al) 05 July 2005 (05.07.2005) entire document	28, 29
A	US 2013/0129780 A1 (GARCIA et al) 23 May 2013 (23.05.2013) entire document	28, 29

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

* Special categories of cited documents:
"A" document defining the general state of the art which is not considered to be of particular relevance
"E" earlier application or patent but published on or after the international filing date
"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
"O" document referring to an oral disclosure, use, exhibition or other means
"P" document published prior to the international filing date but later than the priority date claimed
"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
21 November 2014	11 DEC 2014
Name and mailing address of the ISA/US Mail Stop PCT, Attn: ISA/US, Commissioner for Patents P.O. Box 1450, Alexandria, Virginia 22313-1450 Facsimile No. 571-273-3201	Authorized officer: Blaine R. Copenheaver PCT Helpdesk: 571-272-4300 PCT OSP: 571-272-7774

Form PCT/ISA/210 (second sheet) (July 2009)

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US2014/047395

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

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

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

2. ☒ Claims Nos.: 1-27
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

Claims 1-27 are deemed unsearchable due to applicant's failure to provide a valid electronic or paper sequence listing.

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

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

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

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

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

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

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

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest and, where applicable, the payment of a protest fee.

☐ The additional search fees were accompanied by the applicant's protest but the applicable protest fee was not paid within the time limit specified in the invitation.

☐ No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US2014/047395

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

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

   a. (means)

   ☐ on paper

   ☐ in electronic form

   b. (time)

   ☐ in the international application as filed

   ☐ together with the international application in electronic form

   ☐ subsequently to this Authority for the purposes of search

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

3. Additional comments:

ISA/225 mailed on 04 August 2014. No approved electronic sequence listing was submitted in response to the ISA/225. The electronic sequence listing filed on 29 September 2014 contained errors and could not be entered into ISA/US's search system/tool.