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(54) Titre : VACCINS A BASE D'ADN MULTIPLES ET MULTIVALENTS IN OVO
 (54) Title: MULTIPLE AND MULTIVALENT DNA VACCINES IN OVO

(57) **Abrégé/Abstract:**

The present invention provides a multiple DNA vaccine and/or a multivalent DNA vaccine for use in acquiring embryonic immunity in fowl eggs. The multiple DNA vaccine contains two or more DNA constructs, each containing a DNA molecule encoding an avian viral protein or a fragment thereof capable of inducing a protective immune response against the avian viral disease in fowl. The multivalent DNA vaccine contains one DNA construct which contains two or more DNA molecules, each representing an avian viral gene or a fragment thereof. The multivalent DNA vaccine is capable of expressing two or more viral antigens and inducing protective immune responses against the avian viral diseases in fowl.

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(54) Title: MULTIPLE AND MULTIVALENT DNA VACCINES *IN OVO*

(57) Abstract: The present invention provides a multiple DNA vaccine and/or a multivalent DNA vaccine for use in acquiring embryonic immunity in fowl eggs. The multiple DNA vaccine contains two or more DNA constructs, each containing a DNA molecule encoding an avian viral protein or a fragment thereof capable of inducing a protective immune response against the avian viral disease in fowl. The multivalent DNA vaccine contains one DNA construct which contains two or more DNA molecules, each representing an avian viral gene or a fragment thereof. The multivalent DNA vaccine is capable of expressing two or more viral antigens and inducing protective immune responses against the avian viral diseases in fowl.



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MULTIPLE AND MULTIVALENT DNA VACCINES *IN OVO*5 FIELD OF THE INVENTION:

The present invention relates to either a multiple DNA vaccine or a multivalent DNA vaccine for use in acquiring embryonic immunity in fowl eggs and methods for preparing and using the same. The multiple DNA vaccine contains two or more DNA constructs, each containing a DNA molecule encoding an avian viral protein or a fragment thereof capable of inducing a protective immune response against an avian viral disease in fowl. The multivalent DNA vaccine contains a DNA construct which contains two or more DNA molecules. Each of the DNA molecules represents an avian viral gene or a fragment thereof. The multivalent DNA vaccine is capable of expressing two or more viral antigens and inducing protective immune responses against two or more of the avian viral diseases in fowl. Both the multiple DNA vaccine and the multivalent DNA vaccine are preferred to be injected into the amniotic fluid of the fowl egg after being fertilized for about 18 days.

BACKGROUND OF THE INVENTION:

In ovo vaccination of virus-containing vaccines was extensively described by Sharma et al. (U.S. Pat. No. 4,458,630). In particular, it teaches that live Marek's disease virus can be injected into amniotic fluid within the egg, whereafter the embryo is infected and the vaccine virus replicates to a high titer which induces the formation of protective antibodies in the treated embryo. (See Sharma (1985), Avian Diseases 29, 1155, 1167-68).

It is well-known in the worldwide poultry business that certain viral diseases, such as Marek's disease virus (MDV), infectious bursal disease virus (IBDV), Newcastle disease virus (NDV), infectious bronchitis virus (IBV), infectious laryngotracheitis virus (ILTV), avian encephalomyelitis (AEV), chick anemia virus (CAV), Fowlpox virus (FPV), avian influenza virus (AIV), reovirus, avian leukosis virus (ALV), reticuloendotheliosis virus (REV), avian adenovirus and hemorrhagic enteritis virus (HEV), may cause major outbreak and result in significant economic losses in the commercial poultry industry. Among them, MDV, IBDV, NDV and IBV, are particularly important due to their virulent nature.

Marek's Disease (MD) is a malignant, lymphoproliferative disorder disease that occurs naturally in chickens. The disease is caused by a herpesvirus: Marek's Disease Virus (MDV). MD is ubiquitous, occurring in poultry-producing countries throughout the world. Chickens raised under intensive production systems will inevitably suffer losses from MD. The symptoms of MD appear widely in the nerves, genital organs, internal organs, eyes and skin of the infected birds, causing motor trouble (due to paralysis when the nerves have been affected), functional trouble of the internal organs (due to tumors), and chronic undernourishment (if the internal organs are attacked by the virus). MD affects chickens from about 6 weeks of age, occurring most frequently between ages of 12 and 24 weeks.

At of this time, there are no methods of treating MD. The control of the disease is based primarily on management methods such as insulating growing chickens from sources of infection, the use of genetically resistant stock, and vaccination. However, management procedures are normally not cost-effective and the progress has been disappointing with respect to the selection of poultry stock with increased genetically controlled resistance. Nowadays, control of MD is almost entirely based on vaccination.

Infectious bursal disease virus (IBDV) is responsible for a highly contagious immunosuppressive disease in young chickens which causes significant losses to the poultry

industry worldwide (See Kibenge (1988), J. Gen. Virol., 69:1757-1775). Infection of susceptible chickens with virulent IBDV strains can lead to a highly contagious immunosuppressive condition known as infectious bursal disease (IBD). Damage caused to the lymphoid follicles of the bursa of Fabricius and spleen can exacerbate infections caused by other agents and reduce a chicken's ability to respond to vaccination as well (See Cosgrove (1962), Avian Dis., 6:385-3894).

IBDV is a member of the Birnaviridae family and its genome consists of two segments of double-stranded RNA (See Dobos et al (1979), J. Virol., 32:593-605). The smaller segment B (about 2800 bp) encodes VP1, the dsRNA polymerase. The larger genomic segment A (about 3000 bp) encodes a 110 kDa precursor polypeptide in a single open reading frame (ORF) that is processed into mature VP2, VP3 and VP4 (See Azad et al (1985), Virology, 143:35-44). From a small ORF partly overlapping with the polypeptide ORF, segment A can also encode VP5, a 17-kDa protein of unknown function (See Kibenge et al (1991), J. Gen. Virol. 71:569-577).

While VP2 and VP3 are the major structural proteins of the virion, VP2 is the major host-protective immunogen and causes induction of neutralizing antibodies (See Becht et al. (1988), J. Gen. Virol., 69:631-640; Fahey et al. (1989), J. Gen. Virol., 70:1473-1481). VP3 is considered to be a group-specific antigen because it is recognized by monoclonal antibodies (Mabs) directed against VP3 from strains of both serotype 1 and 2 (See Becht et al (1988), J. Gen. Virol., 69:631-640). VP4 is a virus-coded protease and is involved in the processing of the precursor protein (See Jagadish et al. (1988), J. Virol., 62:1084-1087).

In the past, control of IBDV infection in young chickens has been achieved by live vaccination with avirulent strains, or principally by the transfer of maternal antibody induced by the administration of live and killed IBDV vaccines to breeder hens. Unfortunately, in recent years, virulent variant strains of IBDV have been isolated from

vaccinated flocks in the United States (See e.g., Snyder et al. (1988), *Avian Dis.*, 32:535-539; Van der Marel et al. (1990), *Dtsch. Tierarztl. Wschr.*, 97:81-83), which drastically undermine the effectiveness of using live vaccination for IBDV.

Efforts to develop a recombinant vaccine for IBDV have also been made, and the
5 genome of IBDV has been cloned (See Azad et al (1985) "Virology", 143:35-44). The VP2 gene of IBDV has been cloned and expressed in yeast (See Macreadie et al. (1990), *Vaccine*, 8:549-552), as well as in recombinant fowlpox virus (See Bayliss et al (1991), *Arch. Virol.*, 120:193-205). When chickens were immunized with the VP2 antigen expressed from yeast, antisera afforded passive protection in chickens against IBDV infection. When used in
10 active immunization studies, the fowlpox virus-vectored VP2 antigen afforded protection against mortality, but not against damage to the bursa of Fabricius.

Newcastle disease virus (NDV) is an enveloped virus containing a linear, single-strand, nonsegmented, negative sense RNA genome. Typically, virus families containing enveloped single-stranded RNA of the negative-sense genome are classified into groups
15 having non-segmented genomes (e.g., Paramyxoviridae and Rhabdoviridae) or those having segmented genomes (e.g., Orthomyxoviridae, Bunyaviridae and Arenaviridae). NDV, together with parainfluenza virus, Sendai virus, simian virus 5, and mumps virus, belongs to the Paramyxoviridae family.

The structural elements of the NDV include the virus envelope which is a lipid
20 bilayer derived from the cell plasma membrane. The glycoprotein, hemagglutinin-neuraminidase (HN) protrude from the envelope allowing the virus to contain both hemagglutinin and neuraminidase activities. The fusion glycoprotein (F), which also interacts with the viral membrane, is first produced as an inactive precursor, then cleaved post-translationally to produce two disulfide linked polypeptides. The active F protein is
25 involved in penetration of NDV into host cells by facilitating fusion of the viral envelope

with the host cell plasma membrane. The matrix protein (M), is involved with viral assembly, and interacts with both the viral membrane as well as the nucleocapsid proteins.

The main protein subunit of the NDV nucleocapsid is the nucleocapsid protein (NP) which confers helical symmetry on the capsid. In association with the nucleocapsid are the P and L proteins. The phosphoprotein (P), which is subject to phosphorylation, is thought to play a regulatory role in transcription, and may also be involved in methylation, phosphorylation and polyadenylation. The L gene, which encodes an RNA-dependent RNA polymerase, is required for viral RNA synthesis together with the P protein. The L protein, which takes up nearly half of the coding capacity of the viral genome is the largest of the viral proteins, and plays an important role in both transcription and replication.

The replication of all negative-strand RNA viruses, including NDV, is complicated by the absence of cellular machinery required to replicate RNA. Additionally, the negative-strand genome can not be translated directly into protein, but must first be transcribed into a positive-strand (mRNA) copy. Therefore, upon entry into a host cell, the virus can not synthesize the required RNA-dependent RNA polymerase. The L, P and NP proteins must enter the cell along with the genome on infection. Both the NDV negative strand genomes (vRNAs) and antigenomes (cRNAs) are encapsidated by nucleocapsid proteins; the only unencapsidated RNA species are virus mRNAs. The cytoplasm is the site of NDV viral RNA replication, just as it is the site for transcription. Assembly of the viral components appears to take place at the host cell plasma membrane and mature virus is released by budding.

In U.S. Pat. No. 5,427,791, Ahmad et al. describe the embryonal vaccination against NDV, which requires the modification of the viruses through the use of ethyl methane sulfonate (EMS). However, EMS is a mutagen so that the vaccine prepared by the use of EMS is suspected to act as a mutagen as well, which is undesirable for regular

administration of the vaccine. Nevertheless, without the modification with EMS, the NDV vaccine cannot be applied for *in ovo* vaccination as almost all of the embryos will die upon injection of the eggs with the unmodified virus.

Infectious bronchitis virus (IBV), the prototype of the family Coronaviridae, is the etiological agent of infectious bronchitis (IB). The virus has a single-stranded RNA genome, approximately 20 kb in length, of positive polarity, and is usually about 80-100 nm in size, being round with projecting 20 nm spikes. IBV is the causative agent of an acute, highly contagious disease in chickens of all ages, affecting the respiratory, reproductive and renal systems.

IBV contains three structural proteins: the spike (S) glycoprotein, the membrane glycoprotein, and the nucleocapsid protein. The spike glycoprotein is so called because it is present in the teardrop-shaped surface projections or spikes protruding from the lipid membrane of the virus. The spike protein is believed likely to be responsible for immunogenicity of the virus, partly by analogy with the spike proteins of other coronaviruses and partly by *in vitro* neutralisation experiments (See, e.g., D. Cavanagh et al. (1984), Avian Pathology, 13, 573-583). There are two spike glycoproteins, which are S1 (90,000 daltons) and S2 (84,000 daltons). The polypeptide components of the glycopolypeptides S1 and S2 have been estimated after enzymatic removal of oligosaccharides to have a combined molecular weight of approximately 125,000 daltons. It appears that the spike protein is attached to the viral membrane by the S2 polypeptide.

IBV has been wide-spread in countries where an intensive poultry industry has been developed. Young chickens up to 4 weeks of age are most susceptible to IBV, infection leading to high rates of morbidity and to mortality resulting from secondary bacterial infection. Infection also results in a drop in egg production, or failure to lay at full potential,

together with an increase in the number of down-graded eggs with thin, misshapen, rough and soft-shells produced, which can have a serious economic effect.

Administering live vaccines to a developing chick in the egg (*in-ovo*) has proven to be a fast (40,000 eggs per hour), effective (100% of the eggs receive the vaccine), and labor saving (\$100,000 per year per hatchery) method to vaccinate baby chicks against certain diseases before they hatch.

The first *in-ovo* vaccination machine for use on chicken hatching eggs was developed by Embrex, Inc., of Raleigh, N.C. in the late 1980s. (See U.S. Pat. Nos. 5,056,464 and 5,699,751). This *in-ovo* machine is currently used in about 80% of the U.S. broiler hatcheries, primarily for administering MD vaccines. The popularity of this machine, which has proven to be safe and effective in vaccination of chicks against MD, is also being used increasingly to administer IBD vaccines and ND vaccines.

In the invention to be presented in the following sections, a DNA-mediated immunization (collectively "DNA vaccines") will be introduced. There are two kinds of DNA vaccines, *i.e.*, a multiple DNA vaccine and a multivalent DNA vaccine. The multiple DNA vaccine of the present invention contains a combination of two or more DNA construct, each containing a single DNA molecule which is a viral gene or a fragment thereof. The multivalent DNA vaccine of the present invention contains two or more viral genes or fragments thereof linking together in one DNA construct. The viral genes or fragments used in preparation of either the multiple DNA vaccine or the multivalent DNA vaccine are those that encode viral peptides which are antigenic to and can induce both the humoral and the cellular immune system in a host. The DNA vaccines are preferably applied to the egg by needles. The injection of the DNA vaccines *in ovo* leads to surprisingly strong immune responses which include not only antibody induction and T-cell

activation with cytokine secretion, but also the production of cytotoxic T lymphocytes (CTL).

SUMMARY OF THE INVENTION:

5 The present invention provides a multiple DNA vaccine for *in ovo* injection. The multiple DNA vaccine contains two or more DNA constructs, each DNA construct expressing an antigenic protein of an avian virus causing avian viral disease in fowl. The antigenic protein of the avian virus is capable of inducing a protective immune response against an avian viral disease. The multiple DNA vaccine is preferred to inject into the egg, particularly the amniotic fluid of the egg, of the fowl. The egg is preferred to be fertilized for about 18 days. The preferred fowl includes chicken, turkey, duck, and goose.

10 The DNA construct contains a DNA molecule and a vector. The vector can be a plasmid or a viral carrier. The preferred vector is a plasmid. Examples of the plasmid include, but are not limited to, pcDNA3, pVAX1, pSectag, pTracer, pDisplay, pUC system plasmid (such as pUC7, pUC8, pUC18), and pGEM system plasmid. Alternatively, any plasmid which contains a promoter such as CMV promoter, SV40 promoter, RSV promoter, and β -actin promoter, can also be used for preparing the DNA construct. The most favorable plasmid is pcDNA3. The preferred viral carrier is one selected from the group consisting of a baculovirus, a herpes virus, and a pox virus.

20 Examples of the avian virus include, but are not limited to Marek's disease virus (MDV), infectious vursal disease virus (IBDV), Newcastle disease virus (NDV), infectious bronchitis virus (IBV), infectious laryngotracheitis virus (ILTV), avian encephalomyelitis (AEV), avian leukosis virus (ALV), fowlpox virus (FPV), avian paramyxovirus (APV), duck hepatitis virus (DHV), and hemorrhagic enteritis virus (HEV).

The DNA molecules that are particularly suitable for inducing a protective immune response against the avian viral diseases as shown above include, but are not limited to, the entire of gB gene of Marek's Disease virus (MDV) having the DNA sequence of SEQ ID NO:1 or a fragment thereof; the entire VP2 gene of infectious bursal disease virus (IBDV) having the DNA sequence of SEQ ID NO:2 or a fragment thereof; the entire HN gene of Newcastle disease virus (NDV) having the DNA sequence (which is from bases 6321 to 8319) of SEQ ID NO:3 or a fragment thereof (i.e., SEQ ID NO:3 is the entire genome of the NDV); the entire S1 gene of infectious bronchitis virus (IBV) having the DNA sequence of SEQ ID NO:4 or a fragment thereof; the entire glycoprotein G gene of infectious laryngotracheitis virus (ILTV) having the DNA sequence of SEQ ID NO:5 or a fragment thereof; the entire VP1, VP0, or VP3 gene of avian encephalomyelitis virus (AEV) or a fragment thereof (the VP1 gene has the DNA sequence of SEQ ID NO:6; the VP0 gene has the DNA sequence of SEQ ID NO:7; and the VP3 gene has the DNA sequence of SEQ ID NO:8); the entire paraglycoprotein G gene of avian parainfluenza virus (APV) having the DNA sequence of SEQ ID NO:9 or a fragment thereof; the entire type A penton base gene of hemorrhagic enteritis virus (HEV) having the DNA sequence of SEQ ID NO:10 or a fragment thereof; and the entire envelope antigen gene of fowlpox virus (FPV) having the DNA sequence of SEQ ID NO:11 or a fragment thereof.

One preferred example of the DNA vaccine contains two DNA constructs, each containing a DNA molecule capable of expressing a gene or a fragment thereof which is from Marek's disease virus (MDV), infectious bursal disease virus (IBDV), Newcastle disease virus (NDV), or infectious bronchitis virus (IBV).

Another preferred example of the multiple DNA vaccine contains three or more DNA constructs, each containing a DNA molecule capable of expressing a gene or a

fragment thereof which is from Marek's disease virus (MDV), infectious vursal disease virus (IBDV), Newcastle disease virus (NDV), or infectious bronchitis virus (IBV).

The present invention also provides a method for vaccinating fowl egg and a method for preparing the multiple DNA vaccine. The method for vaccinating fowl egg includes
5 injecting into the fowl egg the multiple DNA vaccine as shown above. The method for preparing the multiple DNA vaccine includes ligating a DNA molecule to a plasmid or virus carrier to form a DNA construct; and then mixing two or more said DNA constructs to form the multiple DNA vaccine. The insertion of the DNA molecule into the vector can be achieved by conventional method, *i.e.*, by ligation the DNA molecule with an enzyme such
10 as T4 DNA ligase when both the genes and the desired vector have been cut with the same restriction enzyme(s) as complementary DNA termini are thereby produced. For pcDNA3, the preferred restriction enzymes are BamH1 and EcoR1.

In another embodiment, there is provided a multivalent DNA vaccine for *in ovo* injection. The multivalent DNA vaccine comprises a DNA construct containing two or
15 more DNA molecules linked together with a vector. Each of the DNA molecules expresses an antigenic protein of an avian virus, which is capable of inducing a protective immune response against that avian viral disease in fowl. The multivalent DNA vaccine is preferred to be injected into a fowl egg. Each of the DNA molecules of the multivalent DNA vaccine is a gene or a fragment thereof from Marek's disease virus (MDV), infectious vursal disease
20 virus (IBDV), Newcastle disease virus (NDV), infectious bronchitis virus (IBV), infectious laryngotracheitis virus (ILTV), avian encephalomyelitis (AEV), avian leukosis virus (ALV), fowlpox virus (FPV), avian parainfluenza virus (APV), duck hepatitis virus (DHV), and hemorrhagic enteritis virus (HEV).

DETAILED DESCRIPTION OF THE INVENTION:

Traditional avian vaccines comprise chemically inactivated virus vaccines or modified live-virus vaccines. Inactivated vaccines require additional immunizations which are not only expensive to produce but also laborious to administer. Further, some infectious virus particles may survive the inactivation process and may cause disease after administration to the animal.

In general, attenuated live virus vaccines are preferred over inactivated vaccines because they evoke an immune response often based on both humoral and cellular reactions. Such vaccines are normally based on serial passage of virulent strains in tissue culture. However, the attenuation process induces mutations of the viral genome, resulting in a population of virus particles heterogeneous with regard to virulence and immunizing properties. In addition, it is well known that the traditional attenuated live virus vaccines can revert to virulence resulting in disease outbreaks in inoculated animals and the possible spread of the pathogen to other animals.

Thus, it is advantageous for the industry to employ vaccines based on recombinant DNA technology. The resulting DNA vaccines only contain and express the necessary and relevant immunogenic material that is capable of eliciting a protective immune response against the pathogens and would not display above mentioned disadvantages of the live or inactivated vaccines.

For the purpose of preparing multiple DNA vaccines or multivalent recombinant DNA vaccines, the DNA sequence of the gene (also used interchangeably as "DNA molecule") need not contain the full length of DNA encoding the polypeptides. In most cases, a fragment of the gene which encodes an epitope region should be sufficient enough for immunization. The DNA sequence of an epitope region can be found by sequencing the corresponding part of other viral strains and comparing them. The major antigenic

determinants are likely to be those showing the greatest heterology. Also, these regions are likely to lie accessibly in the conformational structure of the proteins. One or more such fragments of genes encoding the antigenic determinants can be prepared by chemical synthesis or by recombinant DNA technology. These fragments of genes, if desired, can be
5 linked together or linked to other DNA molecules.

Also, the viral genes need not be in DNA. In fact, some of the frequently found avian viral diseases are caused by double- or single-stranded RNA viruses. For example, Marek's disease virus is a double-stranded RNA virus, while infectious bursal disease virus (IBDV), Newcastle disease virus (NDV) and infectious bronchitis virus (IB) are single-
10 stranded RNA viruses. The RNA viral sequences, however, can be reverse-transcribed into DNA using RT-Polymerase chain reaction (RT-PCR) technology and then incorporated into a vector by the conventional recombinant DNA technology.

In addition, because of the degeneracy of the genetic code it is possible to have numerous RNA and DNA sequences that encode a specified amino acid sequence. Thus, all
15 RNA and DNA sequences which result in the expression of a polypeptide having the antibody binding characteristics are encompassed by this invention.

To construct a recombinant DNA vaccine, either univalent or multivalent, the DNA sequence of the viral gene can be ligated to other DNA molecules with which it is not associated or linked in nature. Optionally, the DNA sequence of a viral gene can be ligated
20 to another DNA molecule, *i.e.*, a vector, which contains portions of its DNA encoding fusion protein sequences such as β -galactosidase, resulting in a so-called recombinant nucleic acid molecule or DNA construct, which can be used for transformation of a suitable host. Such vector is preferably derived from, *e.g.*, plasmids, or nucleic acid sequences present in bacteriophages, cosmids or viruses.

Specific vectors which can be used to clone nucleic acid sequences according to the invention are known in the art and include either a plasmid or a virus carrier. Examples of the plasmid include, but are not limited to, pBR322, pcDNA3, pVAX1, pSectag, pTracer, pDisplay, pUC system plasmids (e.g., pUC7, pUC8, pUC18), pGEM system plasmids, Bluescript plasmids or any other plasmids where CMV promoter, SV40 promoter, RSV promoter, or β -actin promoter is included. The preferred plasmid is pcDNA3. Examples of the virus carrier include, but are not limited to, bacteriophages (e.g., λ and the M13-derived phages), SV40, adenovirus, polyoma, baculoviruses, herpes viruses (HVT) or pox viruses (e.g., fowl pox virus).

10 The methods to be used for the construction of a recombinant nucleic acid molecule are known to those of ordinary skill in the art. For example, the insertion of the nucleic acid sequence into a cloning vector can easily be achieved by ligation with an enzyme such as T4 DNA ligase when both the genes and the desired cloning vehicle have been cut with the same restriction enzyme(s) so that complementary DNA termini are thereby produced.

15 Alternatively, it may be necessary to modify the restriction sites so as to produce blunt ends either by digesting the single-stranded DNA or by filling in the recessive termini with an appropriate DNA polymerase. Subsequently, blunt end ligation with an enzyme such as T4 DNA ligase may be carried out. If desired, any restriction site may be produced by ligating linkers onto the DNA termini. Such linkers may comprise specific
20 oligonucleotide sequences that encode restriction site sequences. The restriction enzyme cleaved vector and nucleic acid sequence may also be modified by homopolymeric tailing.

The present invention provides two kinds of DNA vaccines. The first kind is a multiple DNA vaccine, which includes two or more of univalent DNA vaccines, each containing a DNA sequence encoding at least one polypeptide affording protection against
25 one viral disease such as Marek's disease virus (MDV), infectious bursal disease virus

(IBDV), Newcastle disease virus (NDV), infectious bronchitis virus (IBV), infectious laryngotracheitis virus (ILTV), avian encephalomyelitis (AEV), Fowlpox virus (FPV), avian influenza virus (AIV), avian leukosis virus (ALV), duck hepatitis virus B genome, and hemorrhagic enteritis virus (HEV), inserted into a commercially available plasmid.

5 The second kind is a multivalent recombinant DNA vaccine, which contains two or more genes or gene fragments from different viruses. These genes or gene fragments are carried by a useful vector, which can be either a plasmid or a virus carrier. The multivalent recombinant DNA vaccine encodes two or more antigenic polypeptides which afford protection against at least two viral diseases including, but not limited to, MD, IBD, ND or
10 IB. The viral genes or gene fragments are operatively attached to the vector in reading frame so that they can be expressed in a host. The different structural DNA sequences carried by the vector may be separated by termination and start sequences so that the proteins can be expressed separately or they may be part of a single reading frame and therefore be produced as a fusion protein by methods known in the art.

15 The preferred DNA sequences include, but are not limited to, the entire of gB gene of Merk's Disease virus (MDV) having the DNA sequence of SEQ ID NO:1 or a fragment thereof; the entire VP2 gene of infectious bursal disease virus (IBDV) having the DNA sequence of SEQ ID NO:2 or a fragment thereof; the entire HN gene of Newcastle disease virus (NDV) having the DNA sequence of SEQ ID NO:3 or a fragment thereof; the entire S1
20 gene of infectious bronchitis virus (IBV) having the DNA sequence of SEQ ID NO:4 or a fragment thereof.

The DNA sequence encoding the gB polypeptide of MDV has the nucleic acid sequence as SEQ ID NO:1. The DNA sequence contains 3650 bp of linear DNA.

The DNA sequence encoding the VP2 polypeptide of IBDV has the nucleic acid sequence as SEQ ID NO:2. The DNA sequence contains 3004 bp of linear DNA molecule which is reversely transcribed from IBDV's RNA template.

The DNA sequence of the entire genome of NDV contains 15186 bps of DNA, wherein (1) base No. 56 to 1792 encodes NP polypeptide, which is nucleocapsid protein; (2) base No. 1804-3244 encodes P polypeptide, which is a phosphoprotein; (3) base No. 3256-4487 encodes M polypeptide, which is a matrix protein; (4) base No. 4498-6279 encodes F polypeptide, which is a fusion protein; (5) base 6321-8319 encodes HN polypeptide, which is a hemagglutinin-neuraminidase; (6) base No. 8370-15073 encodes L polypeptide, which is a large polymerase protein. The NDV genome has the DNA sequence as SEQ ID NO:3.

The DNA sequence of the S1 polypeptide contains 1611 bp of linear DNA sequence as shown in SEQ ID NO:4, which is reversely transcribed from IBV's RNA templates.

The following experimental designs are illustrative, but not limiting the scope of the present invention. Reasonable variations, such as those occur to reasonable artisan, can be made herein without departing from the scope of the present invention.

I. Materials and Methods

(A) Virus and vaccines

Avian infectious bronchitis virus (IBV), infectious bursal disease (IBD) and Newcastle disease (ND) vaccines were purchased from Intervet Inc.

20 (B) Viral RNA isolation and RT-PCR

Two hundred microliter recovered attenuated vaccines (Intervet Inc.) were resolved in iced cold GTC buffer (4 M guanidium isothiocyanate, 25mM sodium citrate, pH 7.0, 0.5% Sarkosyl, 0.1 M - mercaptoethanol) and sodium acetate (pH 4). An equal volume of phenol-chloroform (1:1) was added and placed on ice for 15 minutes after vortexing. The aqueous phase was collected after centrifuge and the RNA was precipitated with an equal

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volume of isopropanol. RNA was pelleted by centrifugation at 12000 rpm for 20 min at 4°C and then suspended in diethylpyrocarbonate (DEPC) treated deionized distill water and stored at -70°C.

(C) Oligonucleotides

5 Oligonucleotide primers for RT PCR amplification were purchased from Promega, and were designed according to the genome of the Avian infectious bronchitis virus (Beaudette CK strain), Newcastle disease virus (Lasota strain) and Infectious bursa
disease virus respectively. The sequences of the primers used for PCR were :

10 IBS IF' 5' CGGGATCCGCCGCCGCCATGTTGGTAACACCTCTT 3'; (SEQ ID
NO:12)

IBS IR' 5' CGGAATTCTTAACGTCTAAAACGACGTGT 3'; (SEQ ID NO:13)

NDF F' 5' CGGGATCCGCCGCCGCCATGGGCTCCAGACCTTCTACC 3'; (SEQ
ID NO:14)

15 NDF R' 5' CCGCTCGAGTTACATTTTTGTAGTGGCTCTCATT 3'; (SEQ ID NO:15)

NDHN F' 5' CGGGATCCGCCGCCGCCATGGACCGCGCCGTTAGGCAAG 3';
(SEQ ID NO:16)

NDHN R' 5' GCTCTAGATTACTCAACTAGCCAGACCTG 3'; (SEQ ID NO:17)

20 IBDVP2F' 5' CGGGATCCGCCGCCGCCATGACAAACCTGCAAGAT 3'; (SEQ ID
NO:18)

IBDVP2R' 5' CGGAATTCTTACCTTATGGCCCGGATTAT 3'. (SEQ ID NO:19)

(D) Reverse transcription polymerase reaction (RT PCR)

Reverse transcription of IBV, NDV and IBDV RNA were carried out at 42°C for 30
min in 2.5 x *Taq* buffer (200 mM NaCl, 15 mM Tris-HCl, pH7.4, 15 mM MgCl₂, 15 mM β
25 mercaptoethanol, and 0.25 mM each of dATP, dCTP, dGTP, and dTTP). In addition to the

Taq buffer, the reaction mixture (40 μ l) also contained viral RNA, 2.4 U of avian myeloblastosis virus (AMV) reverse transcriptase (Promega), 16 U of RNasin (Promega), and 0.01 nmol reverse primer (*IBDVP2R*, *NDF F*, *NDHNF* or *IBSIR*). The final volume of the reaction mixture was 40 μ l. After reverse transcription, the following reagents were added to the reverse transcription mixture: 0.02 nmol of each nucleotide triphosphate (dATP, dCTP, dGTP, dTTP), 0.01 nmol of forward primer (*IBDVP2F*, *NDF R*, *NDHN R* or *IBSIF*) and 1.5 U of *Taq* DNA polymerase (Stratagene). Water was then added to a final volume of 100 μ l. The reaction was carried out for 32 cycles in a Thermal Cycler (Perkin Elmer-Cetus). Each PCR cycle consisted of 1 min of denaturation at 94°C, 1 min of annealing at 57°C, and 2 min of DNA chain elongation at 72°C .

(E) Preparation of DNA constructs

The plasmids pCMV-VP2, pCMV-S1, pCMV-NDF and pCMV NDHN were constructed with the VP2, S1, NDF and NDHN genes from IBD vaccine, IBV vaccine and NDV vaccine respectively, placed downstream of the commercial plasmid pcDNA3. (Invitrogen, U.S.A.). All of the genes were inserted into the pcDNA3 vector using restriction enzymes *BainHI*, *EcoRI*, *XbaI* and *XhoI* (underlined characters in the sequence of the primers). Sequences of the all genes in the pcDNA3 vector were verified by sequencing in both directions.

20 (F) Preparation of DNA and DNA delivery

The quantity of plasmid DNA that had been purified by affinity chromatography (Qiagen, Inc.) was determined by spectrophotometric measurements at 260 and 280 nm. The DNA in aliquots to 100 p.g was suspended in 100 μ .l of PBS (0.14M NaCl, 10mM sodium phosphate, pH 7.4). For DNA delivery, 1cc syringe with a 20 gauge 1 and 1/2 inch needle were used.

25 For the *in-ovo* groups, the embryos (18-day-old fertilized and developing eggs from the

setting trays) were injected with 0.1 milliliters of DNA vaccine (100 µg) into the large end of each egg through the air cell with a needle. The eggs were then transferred into the hatchery where they remained until they hatched at about 21 days of age. For the IM (Intramuscular), all of the vaccines (1/5 dose of live vaccines) were injected into the
5 chicken's thoracic muscle at 10 days post hatchery.

II. Experimental design

Specific Pathogen Free (SPF) fertilized eggs (n=60) were randomized into 12 groups. All groups (five eggs each group), all eggs were given 100 µl in volume each. 100 µg pCMV-NDF+ 100 µg pCMV-NDHN mixture was injected in each egg of group A, 100
10 µg pCMV-S1 was injected in each egg of group B, 100 µg pCMV-VP2 was injected in each egg of group C, 100 µg pCMV-NDF + 100 µg pCMV-NDHN+100 µg pCMV-S1(ND+IB) was injected in each egg of group D, 100 µg pCMV-NDF+ 100 µg pCMV-NDHN+100 µg pCMV-VP2 (ND+IBD) was injected in each egg of group E, 100µg pCMV-VP2+100 µg pCMV-VP2 mixture (IB+IBD) was injected in each egg of group F, 100 µg pCMV-NDF+
15 100 µg pCMV-NDHN+100 µg pCMV-S1+100 µg pCMV-VP2 mixture (ND+IB+IBD) was injected in each egg of group G, one dose of commercialized in-ovo IBD vaccine (Embrex, Inc) was injected in each egg of group H as positive control, 100 ul PBS was injected in each egg of group I, J, K and L. All chickens in this experiment were given 100 µl in
20 volume (1/5 dose of live vaccines), injected into the chicken's thoracic muscle each at 10 days post hatchery. Chickens in group A and I were injected with NDV vaccine, group B and J were injected with IBV vaccine, group C and K were injected with IBDV vaccine, group D were injected with the mixture of NDV+IB vaccines, group E were injected with the mixture of NDV+IBD vaccines, group F were injected with the mixture of IB+IBD vaccines and group G and L were injected with the mixture of NDV, IB and IBD vaccines.

III. Serology detection

All of the serum samples were collected at 10 days (injected with low dose live vaccines at the same time), 17 days, 24 days and 31 days post hatchery . The antibody titers were detected by ELISA using IB, IBD and NDV antibody test kits which purchased from IDEXX Laboratories, Inc. All of the samples were detected duplicated. Dilute test samples five hundred fold (1:500) with sample diluents prior to being assayed. The test procedure was applied according to the kit's manual. For the assay to be valid, measure and record absorbance values at 650 nm, A (650). The relative level of antibody in the unknown was determined by calculating the sample to positive (S/P) ratio. Endpoint titers were calculated using the formula: $\text{Log}_{10}\text{Titer} = 1.09(\text{Log}_{10}\text{S/P}) + 3.36$

Results

As shown in Table 1, the results demonstrated that, for the detection of anti-IBD antibodies, the IBDV recombinant antigens VP2 could be expressed and played the role of primary stimulation. The titers increased rapidly after a low dose vaccine booster. The titers of group C, E, F and G at 17 days post hatchery (i.e. 7 days post IM injection) were significantly higher than those of group K and L. Most importantly, the expression of IBDV antigen was not interfered by other monovalent DNA vaccines (NDV and IBV). The same results were also applied to IB and NDV DNA vaccines. The titers of group B, D, F and G were higher than those of group J and L at 17 days post hatchery (Table 2) and the titers of group A, D, E and G were higher than those of group I and L at 17 days post hatchery (Table 3). The only unpredicted result was the anti-NDV titer could not be highly induced by the triple valent DNA vaccine (Table 3, group G), but anti-IBD and anti-IB did (Tables 1 and 2, group G).

25

Table 1. Serum Samples Detected by IDEXX IBD Antibody Test Kit (Ab Titers Correspond to the Average Titers \pm SD)

Immunization and Sample Collection Schedule (days)				
Animal Group	10 Days PH*	17 Days PH	24 Days PH	31 Days PH
C(IBD)	\square^{**}	4535 \pm 1267	16623 \pm 3105	21254 \pm 3852
E(IBD+ND)	\square	1685 \pm 655	17339 \pm 2185	19041 \pm 2967
F(IBD+IB)	\square	8252 \pm 2205	10057 \pm 1295	17561 \pm 2006
G(IBD+IB+ND)	\square	9111 \pm 1701	13127 \pm 1763	16694 \pm 2134
H(IBD positive)	6553 \pm 851	13025 \pm 2131	18015 \pm 1592	18853 \pm 2614
K(PBS/IBD)	\square	\square	1853 \pm 302	17002 \pm 2965
L(PBS/IBD+IB+ND)	\square	\square	6923 \pm 1168	18063 \pm 2531

*P H: post hatchery

5 ** \square : average titers less than 396 (be considered negative by IDEXX kit)

Table 2. Serum Samples Detected by IDEXX IB Antibody test kit (Ab Titers Correspond to the Average Titers \pm SD)

10

Immunization and Sample Collection Schedule (days)				
Animal Group	10 Days PH*	17 Days PH	24 Days PH	31 Days PH
B(IB)	\square^{**}	441 \pm 117	2426 \pm 264	3214 \pm 877
D(IB+ND)	\square	586 \pm 182	805 \pm 221	1988 \pm 501
F(IB+IBD)	\square	509 \pm 89	685 \pm 186	1192 \pm 237
G(IBD+IB+ND)	\square	499 \pm 81	688 \pm 78	2551 \pm 531
J(PBS/IB)	\square	\square	485 \pm 76	1662 \pm 441
L(PBS/IBD+IB+ND)	\square	\square	819 \pm 202	1332 \pm 488

*P H: post hatchery

** \square : average titers less than 396 (be considered negative by IDEXX kit)

15

Table 3. Serum Samples Detected by IDEXX ND Antibody Test Kit (Ab Titers Correspond to the Average Titers \pm SD).

5

Immunization and Sample Collection Schedule (days)				
Animal Group	10 Days PH*	17 Days PH	24 Days PH	31 Days PH
A(ND)	<input type="checkbox"/>	466 \pm 101	2394 \pm 456	8103 \pm 2198
D(ND+IB)	<input type="checkbox"/>	706 \pm 140	1778 \pm 378	6811 \pm 2206
E(ND+IBD)	<input type="checkbox"/>	517 \pm 104	3021 \pm 411	5991 \pm 1695
G(IBD+IB+ND)	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	783 \pm 201
I(PBS/ND)	<input type="checkbox"/>	<input type="checkbox"/>	1853 \pm 324	3912 \pm 304
L(PBS/IBD+IB+ND)	<input type="checkbox"/>	<input type="checkbox"/>	4027 \pm 662	5807 \pm 1996

*P H: post hatchery

**: average titers less than 396 (be considered negative by IDEXX kit)

SEQUENCE LISTING

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 50 <213> infectious laryngotracheitis virus (ILTV)

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 <210> 6
 <211> 810
 <212> DNA
 <213> avian encephalomyelitis virus (AEV)
 30
 <400> 6
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 55 gcggtgccag tggtcagctc attcactgac acatctagca aaacagtgat gaatacatat 780
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 <212> DNA
 <213> avian encephalomyelitis virus (AEV)

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 20 gggggcctca tagcagctct cgtaccttat gaccaggttg acattgaatc aattgttgcc 480
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 25 gtgccatata tatacagtcg aggttgttac aaccttagga actcagcata ctccatttgg 600
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 30 gcacag 726

35 <210> 8
 <211> 735
 <212> DNA
 <213> avian encephalomyelitis virus (AEV)

40 <400> 8
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 45 tcaagtaccg ggggggaatt ggtgcatcat ttttcacagt ggacgtccat tccgtgcctt 180
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 <211> 1500
 <212> DNA
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 <211> 1440
 <212> DNA
 <213> hemorrhagic enteritis virus (HEV)

10 <400> 10
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 <211> 2995

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<212> DNA

<213> fowlpox virus (FPV)

<400> 11

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60

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CLAIMS

1. A multiple DNA vaccine for *in ovo* injection comprising: two or more DNA constructs, each DNA construct expressing an antigenic protein of an avian virus causing avian viral disease in fowl; wherein said antigenic protein of said avian virus is capable of inducing a protective immune response against said avian viral disease in said fowl; and said multiple DNA vaccine is for injection into egg of said fowl, wherein said avian virus is one selected from the group consisting of Marek's disease virus (MDV), infectious bursal disease virus (IBDV), Newcastle disease virus (NDV), infectious bronchitis virus (IBV), infectious laryngotracheitis virus (ILT), avian encephalomyelitis virus (AEV), avian leukosis virus (ALV), fowlpox virus (FPV), avian parainfluenza virus (APV), duck hepatitis virus (DHV), and hemorrhagic enteritis virus (HEV).
2. The multiple DNA vaccine according to claim 1, wherein each said DNA construct comprises a DNA molecule encoding said antigenic protein of said avian virus and a vector.
3. The multiple DNA vaccine according to claim 2, wherein said vector is a plasmid.
4. The multiple DNA vaccine according to claim 3, wherein said plasmid is one selected from the group consisting of pcDNA3™, pVAXI™, pSectag™, pTracer™, pDisplay™, pUC™ system plasmid, and pGEM[®] system plasmid.
5. The multiple DNA vaccine according to claim 3, wherein said plasmid comprises a promoter which is selected from the group consisting of a CMV promoter, SV40 promoter, RSV promoter, and β -actin promoter.
6. The multiple DNA vaccine according to claim 2, wherein said DNA molecule is an entire gB gene of Marek's Disease virus (MDV) comprising the DNA sequence of SEQ ID NO:1 or a fragment thereof.
7. The multiple DNA vaccine according to claim 2, wherein said DNA molecule is an entire VP2 gene of infectious bursal disease virus (IBDV) comprising the DNA sequence of SEQ ID NO:2 or a fragment thereof.

8. The multiple DNA vaccine according to claim 2, wherein said DNA molecule is an entire HN gene of Newcastle disease virus (NDV) comprising the DNA sequence of bases 6321 to 8319 in SEQ ID NO:3 or a fragment thereof.
9. The multiple DNA vaccine according to claim 2, wherein said DNA molecule is an entire S 1 gene of infectious bronchitis virus (IBV) comprising the DNA sequence of SEQ ID NO:4 or a fragment thereof.
10. The multiple DNA vaccine according to claim 2, wherein said DNA molecule is an entire glycoprotein G gene of infectious laryngotracheitis virus (ILTV) comprising the DNA sequence of SEQ ID NO:5 or a fragment thereof.
11. The multiple DNA vaccine according to claim 2, wherein said DNA molecule is an entire VP1, VP0, or VP3 gene of avian encephalomyelitis virus (AEV) or a fragment thereof; wherein said VP1 gene comprises the DNA sequence of SEQ ID NO:6; said VP0 gene comprises the DNA sequence of SEQ ID NO:7; and said VP3 gene comprises the DNA sequence of SEQ ID NO:8.
12. The multiple DNA vaccine according to claim 2, wherein said DNA molecule is an entire paraglycoprotein G gene of avian parainfluenza virus (APV) comprising the DNA sequence of SEQ ID NO:9 or a fragment thereof.
13. The multiple DNA vaccine according to claim 2, wherein said DNA molecule is an entire type A penton base gene of hemorrhagic enteritis virus (HEV) comprising the DNA sequence of SEQ ID NO: 10 or a fragment thereof.
14. The multiple DNA vaccine according to claim 2, wherein said DNA molecule is an entire envelope antigen gene of fowlpox virus (FPV) comprising the DNA sequence of SEQ ID NO: 11 or a fragment thereof.
15. The multiple DNA vaccine according to claim 1, wherein said multiple DNA vaccine is for injection into an amniotic fluid of said egg.
16. The multiple DNA vaccine according to claim 15, wherein said egg is for fertilization for about 18 days.
17. The multiple DNA vaccine according to claim 1, wherein said DNA vaccine comprises two DNA constructs, each containing a DNA molecule capable of expressing a gene or a fragment thereof which encodes the antigenic protein of an avian virus, which is, for each DNA construct, independently selected from

the group consisting of Marek's disease virus (MDV), infectious vursal disease virus (IBDV), Newcastle disease virus (NDV), infectious bronchitis virus (IBV), infectious laryngotracheitis virus (ILTV), avian encephalomyelitis virus (AEV), avian leukosis virus (ALV), fowlpox virus (FPV), avian parainfluenza virus (APV), duck hepatitis virus (DHV), and hemorrhagic enteritis virus (HEV).

18. The multiple DNA vaccine according to claim 1, wherein said DNA vaccine comprises three or more DNA constructs, each containing a DNA molecule capable of expressing a gene or a fragment thereof which encodes the antigenic protein of an avian virus, which is, for each DNA construct, independently selected from the group consisting of Marek's disease virus (MDV), infectious vursal disease virus (IBDV), Newcastle disease virus (NDV), infectious bronchitis virus (IBV), infectious laryngotracheitis virus (ILTV), avian encephalomyelitis virus (AEV), avian leukosis virus (ALV), fowlpox virus (FPV), avian parainfluenza virus (APV), duck hepatitis virus (DHV), and hemorrhagic enteritis virus (HEV).

19. The multiple DNA vaccine according to claim 1, wherein said fowl is one selected from the group consisting of chicken, turkey, duck, and goose.

20. A use of said multiple DNA vaccine according to claim 1 for vaccinating fowl egg by injection into said fowl egg .

21. A method for preparing said multiple DNA vaccine according to claim 1 comprising ligating a DNA molecule to a plasmid to form a DNA construct; mixing two or more said DNA constructs to form said multiple DNA vaccine; wherein said DNA molecule comprises a gene or a fragment thereof which encodes the antigenic protein of an avian virus, which is selected from the group consisting of Marek's disease virus (MDV), infectious vursal disease virus (IBDV), Newcastle disease virus (NDV), infectious bronchitis virus (IBV), infectious laryngotracheitis virus (ILTV), avian encephalomyelitis virus (AEV), avian leukosis virus (ALV), fowlpox virus (FPV), avian parainfluenza virus (APV), duck hepatitis virus (DHV), and hemorrhagic enteritis virus (HEV).

22. A multivalent DNA vaccine for *in ovo* injection comprising: a DNA construct containing two or more DNA molecules, wherein each of said DNA molecules express an antigenic protein of an avian virus causing avian viral disease in fowl; wherein said antigenic protein of said avian virus is capable of

inducing a protective immune response against said avian viral disease in said fowl; and said multivalent DNA vaccine is for injection into egg of said fowl, which encodes the antigenic protein of an avian virus, which is, for each DNA construct, independently selected from the group consisting of Marek's disease virus (MDV), infectious vursal disease virus (IBDV), Newcastle disease virus (NDV), infectious bronchitis virus (IBV), infectious laryngotracheitis virus (ILTIV), avian encephalomyelitis virus (AEV), avian leukosis virus (ALV), fowlpox virus (FPV), avian parainfluenza virus (APV) duck hepatitis virus (DHV), and hemorrhagic enteritis virus (HEV).

23 . The multivalent DNA vaccine according to claim 22, wherein said DNA construct further comprises a plasmid .

24 . The multivalent DNA vaccine according to claim 23, wherein said plasmid is one selected from the group consisting of pcDNA3™, pVAX1™, pSectag™, pTracer™, pDisplay™, pUC™ system plasmid, and pGEM[®] system plasmid.

25 . The multiple DNA vaccine according to claim 23, wherein said plasmid comprises a promoter which is selected from the group consisting of a CMV promoter, SV40 promoter, RSV promoter, and,β-actin promoter.

26 . A use of said multivalent DNA vaccine according to claim 22 for vaccinating fowl egg by injection into said fowl egg.