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(54) **RECOMBINANT MAREK'S DISEASE VIRUSES AND USES THEREOF**

(57) The present invention relates recombinant viruses and the uses thereof. More particularly, the invention relates to novel recombinant Marek's disease viruses encoding polypeptide(s) of interest, and their use to ex-

press or deliver such polypeptides to animals, particularly poultry. The invention is particularly suited to vaccinate poultry against avian pathogens.

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DescriptionFIELD OF THE INVENTION

5 **[0001]** The present invention relates to recombinant viruses and the uses thereof. More particularly, the invention relates to novel recombinant Marek's disease viruses encoding polypeptide(s) of interest, and their use to express or deliver such polypeptides to animals, particularly poultry. The invention is particularly suited to vaccinate poultry against avian pathogens.

10 BACKGROUND OF THE INVENTION

[0002] Poultry meat and eggs are important food sources, whose consumption increases continually due to the growth of the human population and their great quality-price ratio. The recent epidemic of avian influenza focused the public opinion on poultry health as well as food safety and security. Poultry vaccine technology became a worldwide concern.

15 **[0003]** Recombinant viruses expressing pathogen proteins are commonly used as poultry vaccines against targeted pathogens. Vaccines including such viruses induce expression of foreign pathogen proteins or fragments thereof within infected cells, which can subsequently induce a specific and protective humoral immunity as well as cell-mediated immunity.

20 **[0004]** It is known that different viruses can survive in the body of an infected animal in the state of latent or persistent infection. Consequently, such viruses, in which a foreign gene derived from a pathogen has been integrated, have been developed to be used as viral-vectored vaccines increasing the duration of immunity to an immunized animal.

[0005] These viral vectors (or recombinant viruses) are based typically on avipox viruses, such as fowlpox (EP-A-0,517,292), herpes virus, such as Marek's disease virus serotypes 1, 2 and 3 (HVT) (e.g., WO-A-87/04463, 5,980,906, 5,853,733) or, alternatively, Newcastle disease virus (NDV) and avian adenoviruses.

25 **[0006]** These recombinant avian viruses display variable levels of protection. A recombinant HVT expressing IBDV VP2 has shown advantages over classical IBD vaccines (Vectormune® IBD). Other HVT vectors of interest express NDV (Vectormune® ND) or ILTV (Vectormune® LT) antigens.

30 **[0007]** One of the practical problems of HVT-based recombinant viruses is their interference when several viruses are used in combination to confer immunogenicity against distinct pathogens. Indeed, when two distinct rHVT expressing different antigens are mixed, a lower protection is caused at least against one of the disease (see e.g., Slacum G et al., 2009, The compatibility of HVT recombinants with other Marek's disease vaccines, 58th Western Poultry Disease Conference, Sacramento, CA, USA, March 23-25, p 84).

[0008] Accordingly, there is a need for new approaches to improve vaccination in animals, particularly in poultry, allowing concomitant protection against several diseases.

35 **[0009]** Multivalent HVT vectors have been developed which can express two distinct antigenic peptides (see PCT/EP2013/056839).

[0010] The present invention discloses novel recombinant viruses suitable to induce strong immune protection in animals and which may, in addition, be used in combination with other viral vaccines to procure extended immunity.

40 SUMMARY OF THE INVENTION

[0011] The present invention relates to recombinant Marek's disease viruses ("MDV") which can encode one or several polypeptide(s) of interest. The invention more specifically discloses improved MDV viruses comprising a beta-actin derived promoter and surprisingly shows that, within the context of these viruses, such constructs allow highly improved expression and induction of strong protective immunity. The invention further shows that such viruses are compatible for combined use with distinct avian herpes viruses, to induce improved immune response against distinct antigenic pathogens without substantial cross-interference.

45 **[0012]** An object of the invention therefore resides in a recombinant Marek's disease virus ("rMDV") which comprises a recombinant nucleotide sequence encoding a polypeptide of interest operably linked to a promoter derived from a chicken beta-actin promoter.

[0013] A further object of the invention is a recombinant Marek's disease virus which comprises (1) a promoter comprising a core sequence of a chicken beta-actin promoter and (2) under the control of said promoter, a recombinant nucleotide sequence encoding a polypeptide.

55 **[0014]** The recombinant nucleotide sequence may be inserted in various regions of the viral genome, preferably in a region that is non-essential for viral replication or infection, preferably into the US2 gene. As will be discussed, the rMDV of the invention may comprise one or several recombinant nucleotide sequences encoding distinct polypeptides of interest. The polypeptide of interest is more particularly an antigenic peptide, typically an antigenic peptide of an avian pathogen. The recombinant Marek's disease virus (rMDV) of the invention is preferably of serotype 1 (MDV1).

[0015] Another object of the invention resides in a composition comprising a recombinant Marek's disease virus as defined above and, optionally, a pharmaceutically or veterinary acceptable excipient or carrier and/or a suitable adjuvant.

[0016] A further object of the invention relates to a vaccine composition comprising a recombinant Marek's disease virus as defined above. Such a vaccine can be used e.g., for immunizing avians, such as poultry.

[0017] Another object of the invention resides in a recombinant Marek's disease virus or composition as defined above for use to vaccinate an avian, preferably a chicken.

[0018] Another object of the invention resides in a recombinant Marek's disease virus or composition as defined above for use to induce or stimulate an immune response in an avian, preferably a chicken.

[0019] A further object of the invention is a recombinant MDV1 as defined above, for use in combination with a further recombinant herpes virus of a distinct serotype and expressing a distinct antigen, to vaccinate an avian, preferably a chicken, by simultaneous, separate sequential or alternated administration.

[0020] In another aspect, the invention provides a method of vaccinating an animal comprising at least one administration of a composition or virus as defined above.

[0021] In a further aspect, the invention provides a method for inducing an immunogenic or protective response in an animal against one or more avian pathogens comprising at least one administration of a composition or virus as defined above.

[0022] A further object of the invention is a method for producing a recombinant Marek's disease virus comprising (i) the introduction of a recombinant nucleotide sequence encoding a polypeptide under the control of a promoter comprising a core sequence of a chicken beta-actin promoter into a nonessential region of the genome of a Marek's disease virus, (ii) optionally replicating the virus and, (iii) optionally collecting the virus.

[0023] A further object of the invention relates to a recombinant nucleic acid molecule comprising the genome of a Marek's disease virus or a fragment thereof, wherein said genome or fragment comprises a US2 gene modified by insertion of a recombinant nucleotide sequence comprising a sequence encoding a polypeptide under the control of a promoter comprising a core sequence of a chicken beta-actin promoter.

[0024] The invention also relates to a plasmid comprising a nucleic acid molecule as defined above.

[0025] Another object of the invention is a host cell, or a culture of such cells, comprising a nucleic acid molecule or a plasmid or a virus as defined above.

[0026] The invention further relates to method of immunizing an avian comprising administering to said avian an effective immunizing amount of a vaccine or composition or virus of the invention.

[0027] The invention further provides a vaccination kit for immunizing an avian which comprises an effective amount of a vaccine of the invention and a means for administering said vaccine to said avian.

[0028] The invention may be used for expressing a polypeptide in any animal, preferably for the vaccination of an avian, and it is suitable for expressing any polypeptide or peptide, specifically any immunogenic peptide of avian pathogens.

LEGEND TO THE FIGURES

[0029]

Figure 1 illustrates a schematic diagram of the Rispens genome and the location of the cloned region including the insertion site within the US2 gene.

Figure 2 shows a diagram of recombinant Rispens/IBD genome, indicating locations of Junction 1 and Junction 2 amplified in PCR reactions to confirm the genome structures of the viruses.

Figure 3 is a western blot assay detecting expression of IBDV VP2 protein by the recombinant Rispens/IBD viruses.

Figure 4 illustrates IBDV ELISA titers in SPF chickens vaccinated with recombinant Rispens/IBD using a commercial IBD ELISA kit.

Figure 5 illustrates IBDV ELISA titers in commercial white leghorn chickens vaccinated with recombinant Rispens/IBD using a commercial IBD ELISA kit

Figures 6A and 6B illustrates IBDV and NDV ELISA titers in commercial white leghorn chickens vaccinated with recombinant Rispens/IBD and recombinant HVT/ND using a commercial IBD ELISA kit and a commercial ND ELISA kit.

Figure 7 illustrates the long duration of immunity to IBDV conferred by a recombinant virus of the invention.

DETAILED DESCRIPTION OF THE INVENTION

[0030] The present invention generally relates to rMDV which comprise a recombinant nucleotide sequence encoding a product of interest placed under the transcriptional control of a chicken beta actin-derived promoter. The present invention also relates to compositions comprising such rMDV, as well as to the use thereof for vaccination of animals,

particularly poultry.

[0031] The present disclosure will be best understood by reference to the following definitions:

Definitions

[0032] The term "*virus*" designates in particular a viral particle comprising a nucleic acid molecule (e.g., a genome) encapsulated in a capsid or capsule. The term "*virus*" also designates a viral vector or an isolated viral genome.

[0033] The term "*recombinant*" designates a molecule which has been created, designed or modified using genetic technologies. In relation to a virus, the term more specifically designates a virus whose genome has been modified by insertion of at least one heterologous nucleic acid, *i.e.*, a nucleic acid (e.g., DNA) which is not found naturally in the genome of the virus, or which is found naturally in said genome but in a different form or at a different position. A recombinant virus can be manufactured by a variety of methods and, once made, can be reproduced without further use genetic technologies. In relation to a nucleic acid (e.g., a gene), the term "recombinant" indicates a construct which either does not exist naturally or which has been manipulated or cloned or rearranged so that it is e.g., flanked by sequences which do not naturally flank said sequence.

[0034] In the present description, the terms "*nucleic acid*" or "*nucleotide sequence*" are used interchangeably and refer to a nucleic acid molecule having a determined sequence, which may be deoxyribonucleotide (DNA) and/or ribonucleotide (RNA). The nucleotide sequence may be first prepared by e.g., recombinant, enzymatic and/or chemical techniques, and subsequently replicated in a host cell or an in vitro system. A nucleotide sequence preferentially comprises an open reading frame encoding a product of interest, such as a polypeptide (e.g., a peptide, protein, etc) or an RNA. The nucleotide sequence may contain additional sequences such as a transcription terminator, a signal peptide, an IRES, an intron, etc.

[0035] The term "*untranslated region*" as used herein refers to a region of nucleotides that has no ORF and do not define an amino acid sequence of protein to be expressed by translation, or a region of nucleotides in which the ORF is not involved in any of transcription, translation, or protein expression.

[0036] The term "*avian species*" is intended to encompass all kinds of avians such as birds of the class of Aves, *i.e.*, vertebrate animals which are feathered, winged, bipedal, endothermic and egg-laying. In the context of the invention, avians or avian species refer more particularly to birds with economical and/or agronomical interests, such as poultry, (such as chickens and turkeys), waterfowl poultry (such as ducks and geese) and ornamental birds (such as swans and psittacines).

[0037] The term "*vaccine*" as used herein designates an agent which may be used to cause, stimulate or amplify an immune response in an organism.

[0038] A "*product of interest*" includes, without limitation, a polypeptide (e.g., a peptide, a protein, etc.) as well as an RNA (e.g., an antisense RNA, an interfering RNA, an aptamer, etc.).

[0039] An "*immune response*" designates the development in a host of a cellular and/or antibody-mediated immune response to a composition or vaccine of interest. Usually, an "immune response" includes the production of antibodies, B cells, helper T cells, and/or cytotoxic T cells, directed specifically to an antigen or antigens included in the composition or vaccine of interest. Preferably, the immune response is protective such that resistance to new infection will be enhanced and/or the clinical severity of the disease reduced.

Marek's Disease Viruses

[0040] Marek's Disease Viruses are avian herpes viruses. Various serotypes of MDV have been reported in the art, particularly serotype 1 Marek's disease virus, such as the CVI988/Rispens strain, and serotype 2 Marek's disease virus, such as the SB1 strain. Preferred Marek's disease viruses of the invention are derived from serotypes or strains that are non-pathogenic to targeted animal (e.g., avian) species. A most preferred MDV of the invention is a recombinant MDV of serotype 1.

[0041] MDV are disclosed in publications (see for instance Kingham et al. "The genome of herpesvirus of turkeys: comparative analysis with Marek's disease viruses" - Journal of General Virology (2001) 82, 1123-1135) and generally available from collections. The sequence of the genome of MDV is also available in gene libraries (GenBank Acc. No. DQ530348 and AF291866).

[0042] The invention relates to any rMDV product or composition wherein a recombinant nucleotide sequence is operably linked to a beta actin-derived promoter. Indeed, the inventors have shown that such constructs allow highly improved expression and induction of strong protective immunity. The invention further shows that such viruses are compatible for combined use with distinct avian herpes viruses, to induce improved immune response against distinct antigenic pathogens without substantial cross-interference. As demonstrated in the examples, the inventors have surprisingly found that, in the context of a rMDV, this promoter causes higher and long lasting expression of a polypeptide, and that such expression is not altered by co-administration of a distinct avian herpes virus vector, thereby allowing

concomitant immunization against several distinct antigens.

The beta actin promoter

5 **[0043]** The sequence of chicken Beta actin promoter has been reported in the art and is available on public gene libraries. An illustrative sequence is represented in SEQ ID NO: 12.

[0044] A promoter "derived" from a chicken beta actin promoter is a promoter that comprises a sequence of a functional fragment of a chicken beta actin promoter, preferably a sequence of a transcriptionally active fragment of a chicken beta actin promoter. The promoter may further comprise additional residues or functional domains, which may be artificial and/or obtained from distinct promoter(s). A promoter derived from a chicken beta actin promoter typically comprises at least 50 consecutive nucleotides of the sequence of said chicken beta actin promoter, or a sequence having at least 90% sequence identity thereto, preferably at least 96, 97, 98 or 99% sequence identity thereto, and exhibits transcriptional activity either alone or when combined with a further domain.

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15 **[0045]** The chicken beta actin-derived promoter sequence for use in the present invention is more preferably a sequence that comprises at least a core sequence of a chicken beta actin promoter. The core sequence is typically located within the region between nucleotides - 1200 to -900 of the native promoter, even more preferably between nt -1192 and -922. A specific example of a core sequence of a chicken beta actin promoter is presented in SEQ ID NO: 5:

20 TATTTTGTGCAGCGATGGGGGCGGGGGGGGGGGGGCGCGCGCCAGGCGGGGCGGGGCGGGG
CGAGGGGCGGGGCGGGGCGAGGCGGAGAGGTGCGGCGGCAGCCAATCAGAGCGGCGCGCTCC
GAAAGTTTCCTTTTATGGCGAGGCGGCGGCGGCGGCCCTATAAAAAGCGAAGCGCGCGGC
GGGCGGGAGTCGCTGCGCGCTGCCTTCGCCCGTGCCCCGCTCCGCCCGCCTCGCGCCGCC
25 GCCCGGCTCTGACTGACCGCGT

[0046] In a particular embodiment, the promoter used in the invention contains a core sequence of a chicken beta-actin promoter which comprises (i) SEQ ID NO: 5 or (ii) a sequence having at least 90% sequence identity to SEQ ID NO: 5, preferably at least 96, 97, 98 or 99% sequence identity to SEQ ID NO: 5, and exhibiting transcriptional promoter activity.

30 **[0047]** In a particular embodiment, the invention relates to a rMDV comprising a nucleic acid operably linked to a promoter comprising SEQ ID NO: 12.

[0048] In another particular embodiment, the invention relates to a rMDV comprising a nucleic acid operably linked to a promoter comprising SEQ ID NO: 5.

35 Polypeptide of interest

[0049] The rMDV of the invention may be used to deliver and/or express any polypeptide of interest such as biologically active polypeptides or immunogenic (i.e., antigenic) polypeptides. In a preferred embodiment, the polypeptide of interest is an antigenic peptide, even more preferably a peptide or polypeptide derived from an antigen of a pathogenic organism capable of causing an infection in an animal, particularly an avian. Examples of pathogens that cause infection in avian include viruses, bacteria, fungi, protozoa, etc. The immunogenic (poly)peptide may preferably be (derived from) a surface protein, a secreted protein, or a structural protein of said pathogen, or fragments thereof. The polypeptide can be derived from any source, e.g., viral, prokaryotic, eukaryotic or synthetic.

40 **[0050]** In a preferred embodiment, the nucleic acid encodes an antigenic peptide of a bird pathogenic agent.

[0051] Specific examples of pathogenic agents include, without limitation, avian influenza virus, avian paramyxovirus type 1, also called Newcastle disease virus (NDV), avian metapneumovirus, Marek's disease virus, Gumboro disease virus, also called infectious bursal disease virus (IBDV), Infectious laryngotracheitis virus (ILVT), Infectious bronchitis virus (IBV), Escherichia coli, Salmonella species, Pasteurella multocida, Riemerella anatipestifer, Ornithobacterium rhinotracheale, Mycoplasma gallisepticum, Mycoplasma synoviae, Mycoplasmas microorganisms infecting avian species or coccidian.

[0052] Preferentially, the immunogenic polypeptide is selected from the F protein of NDV, the VP2 protein of IBDV, the gB protein of ILTV, the 40K protein of Mycoplasma galisepticum, and the surface protein hemagglutinin (HA) of the avian influenza virus, or immunogenic fragments thereof.

45 **[0053]** Within the context of the invention, the term "fragment" of a protein designates preferably a fragment comprising at least 5 consecutive amino acid residues of said protein, even more preferably from 5-100. In a preferred embodiment, such a fragment comprises at least one epitope and/or is immunogenic in vivo, i.e., can cause production of antibodies that bind the full length protein.

50 **[0054]** Specific examples of immunogenic peptides include, for instance, a peptide comprising amino acid residues

1-453 of entire VP2.

Virus construction

5 **[0055]** Gene cloning and plasmid construction are well known to one person of ordinary skill in the art and may be essentially performed by standard molecular biology techniques (Molecular Cloning: A Laboratory Manual, 4th Edition, Cold Spring Harbor Laboratory, Cold Spring Harbor, New York, USA, 2012).

10 **[0056]** As indicated above, the rMDVs of the invention comprise a recombinant sequence cloned typically into a nonessential region of the viral genome. The cloning may be accomplished by techniques known per se in the art. Typically, the recombinant viruses may be prepared by homologous recombination between the viral genome and a construct (e.g., a plasmid) comprising the nucleic acid to be inserted, flanked by nucleotides from the insertion site to allow recombination. Cloning can be made with or without deletion of endogenous sequences. In a particular embodiment, the recombinant sequence is cloned in replacement of at least part of a sequence of the genome, such as at least 50 nucleotides or more. Such deletion e.g., increases the cloning capacity of the vector.

15 **[0057]** For that purpose, a sequence containing the targeted region is typically first cloned into a suitable vector. According to the invention, such target region is preferably a region non-essential for virus replication or infection, such as a non-coding (or untranslated) region, or a nonessential gene. A preferred example of such a region is US2 gene. Examples of vectors include plasmids, such as pBR322, pBR325, pBR327, pBR328, pUC18, pUC19, pUC7, pUC8, or pUC9; phages such as lambda phage and M13 phage; or cosmids such as pHC79.

20 **[0058]** The target region sequence is integrated into the vector according to a conventional cloning method. The target region sequence used is preferably of sufficient length so as to allow subsequent in vivo homologous recombination with the viral genome. Preferably, the cloned target region sequence shall have at least approximately 100 nucleotides in length, typically above 300, such as between 1000 and 2000 nucleotides.

25 **[0059]** The coding and promoter sequences, for insertion into the virus, are inserted into the target region of the viral genome cloned in the vector. Insertion shall be made preferably in a manner that leaves a portion of sequence of the target region on each side of the cloned insert of a length sufficient to allow homologous recombination (e.g. of at least 50 nucleotides, preferably of at least 100 nucleotides). The coding and promoter sequences can be introduced into the cloned target region by classical restriction enzyme and ligation procedures. If appropriate, mutation(s) may be carried out at a specific site of the target region to create a new cleavage site for a restriction enzyme. Conventional mutagenesis techniques well known by a person skilled in the art may be used for that purpose, such as e.g., in vitro mutagenesis or PCR.

30 **[0060]** Vectors in which the coding and promoter sequence has been inserted into the target region obtained as above may be introduced into an MDV-infected cell or MDV genome-transfected cells using known techniques such as electroporation, calcium phosphate, lipofectin-based method, or the like. The recombinant viruses are thereby produced by recombination in said cells between the homologous regions of MDV-DNA and the vector. When the amount of the vector is in the range of 0.1 to 1000 μ g, the efficiency of generation of recombinant viruses is particularly optimized.

35 **[0061]** The resulting recombinant virus may be selected genotypically or phenotypically using known techniques of selection, e.g., by hybridization, detecting enzyme activity encoded by a gene integrated along with the recombinant nucleic acid sequences or detecting the antigenic peptide expressed by the recombinant virus immunologically. The selected recombinant virus can be cultured on a large scale in cell culture after which, recombinant virus containing peptides can be collected.

Preferred rMDVs

45 **[0062]** Preferred rMDVs of the invention comprise at least one recombinant nucleic acid inserted into the US2 gene, more preferably in replacement of at least part of the US2 gene, even more preferably between 248th and 494th nucleotides from the start codon.

[0063] Most preferred rMDVs of the present invention are based on serotype 1 MDV.

50 **[0064]** Furthermore, preferred rMDVs of the invention encode an antigenic peptide selected from the F protein of NDV, the VP2 protein of IBDV, the gB protein of ILTV, the 40K protein of Mycoplasma galisepticum, and the surface protein HA of the avian influenza virus, or fragments thereof.

[0065] According to a particular embodiment, the invention relates to a recombinant MDV1 which comprises, inserted in the US2 gene, a nucleotide sequence encoding a VP2 protein of IBDV, or a fragment thereof, under the control of a promoter comprising a core sequence of chicken beta actin promoter.

55 **[0066]** Specific examples of such rMDVs of the invention include RR013, RR030 and RR031.

[0067] In RR013, the antigen peptide sequence is located under the control of a Bac promoter of SEQ ID NO: 12, inserted in US2 gene of MDV1.

[0068] In RR030 and RR031, the antigen peptide sequence is located under the control of Coa5 promoter of SEQ ID

NO: 5, inserted in US2 gene of MDV1.

Cell cultures

5 **[0069]** The recombinant viruses of the present invention may be propagated in any competent cell cultures. After required growth of the viruses is achieved, the cells may be detached from the wells using a scraper or with trypsin and the infected cells may be separated from the supernatant by centrifugation.

10 **[0070]** Examples of competent cells include CEF, embryonated egg, chicken kidney cell, and the like. The cells or viruses may be cultured in a culture medium such as Eagle's MEM, Leibowitz-L-15/McCoy 5A (1:1 mixture) culture medium at about 37° C for 3 to 6 days. The infected cells are typically suspended in a culture medium containing 10% dimethyl sulfoxide (DMSO) and stored frozen under liquid nitrogen.

Compositions and vaccines

15 **[0071]** The invention also relates to compositions, such as vaccines, which comprise one or more recombinant MDV of the invention.

[0072] Compositions of the invention may comprise the rMDV in a pharmaceutically or veterinary acceptable vehicle or excipient. The composition may, in addition or alternatively, comprise a suitable adjuvant.

20 **[0073]** The rMDVs of the invention may be used in live form (e.g., to prepare live vaccines) or, alternatively, in inactivated, attenuated, or killed form. The production of such forms is known in the art.

25 **[0074]** The vaccine according to the present invention may further comprise a suitable solvent, such as for example an aqueous buffer or a phosphate buffer. Preferably, the vaccine also comprises additives. Additives of the present invention may be obtained from any of a number of sources including various proteins and peptides derived from animals (e.g., hormones, cytokines, co-stimulatory factors), and novel nucleic acids derived from viruses and other sources (e.g., double stranded RNA, CpG), and the like which are administered with the vaccine in an amount sufficient to enhance the immune response. In addition, any number of combinations of the aforementioned substances may provide an immunopotential effect, and therefore, can form an immunopotentiator of the present invention.

30 **[0075]** The vaccines of the present invention may further be formulated with one or more further additives to maintain isotonicity, physiological pH and stability, for example, a buffer such as physiological saline (0.85%), phosphate-buffered saline (PBS), citrate buffers, Tris(hydroxymethyl aminomethane (TRIS), Tris-buffered saline and the like, or an antibiotic, for example, neomycin or streptomycin, etc.

35 **[0076]** The route of administration can be any route including oral, ocular (e.g., by eyedrop), oculo-nasal administration using aerosol, intranasal, Cloacal in feed, in water, or by spray, in ovo, topically, or by injection (e.g., intravenous, subcutaneous, intramuscular, intraorbital, intraocular, intradermal, and/or intraperitoneal) vaccination. The skilled person will easily adapt the formulation of the vaccine composition for each type of route of administration.

[0077] Each vaccine dose may contain a suitable dose sufficient to elicit a protective immune response in avian species. Optimization of such dose is well known in the art. The amount of antigen per dose may be determined by known methods using antigen/anti-body reactions, for example by the ELISA method.

40 **[0078]** The vaccines of the invention can be administered as single doses or in repeated doses, depending on the vaccination protocol.

[0079] The vaccines of the present invention are further advantageous in that they confer to bird species up to 80% protection against the targeted avian pathogens.

45 **[0080]** The present invention further relates to the use of the vaccine as described above for immunizing avian species, such as poultry, and to method of immunizing avian species by administering an immunologically effective amount of the vaccine according to the invention. The vaccine may be advantageously administered intradermally, subcutaneously, intramuscularly, orally, *in ovo*, by mucosal administration or via oculo-nasal administration.

50 **[0081]** The present invention further relates to vaccination kits for immunizing avian species which comprises an effective amount of the multivalent vaccine as *described* above and a means for administering said components to said species. For example, such kit comprises an injection device filled with the vaccine according to the invention and instructions for intradermic, subcutaneous, intramuscular, or *in ovo* injection. Alternatively, the kit comprises a spray/aerosol or eye drop device filled with the vaccine according to the invention and instructions for oculo-nasal administration, oral or mucosal administration.

55 **[0082]** Further aspects and advantages of the invention will be disclosed in the following experimental section, which is illustrative of the claimed invention.

EXAMPLES

[0083] In the experiments, several recombinant viruses have been produced and used, which are designated as follows

(virus/insertion site/inserted genes):

RR013: Rispens/US2/Bac-VP2stc
 RR024: Rispens/US2/RSV-VP2stc
 RR025: Rispens/US2/SV40-VP2stc
 RR030: Rispens/US2/Coa5-VP2stc
 RR031: Rispens/US2/Coa5-VP2stc2
 FW023 (recombinant HVT/IBD control): HVT/UL45-46/Bac-VP2stc
 FW029 (recombinant HVT/ND control): HVT/UL45-46/Pec-F

Example 1: Construction of homology vectors

[0084] The plasmid construction was essentially performed by the standard molecular biology techniques (Molecular Cloning: A Laboratory Manual, 4th Edition, Cold Spring Harbor Laboratory, Cold Spring Harbor, New York, USA, 2012).

Construction of pRisUS2Sfi

[0085] A 2.2-kb DNA fragment of Rispens genome flanking the intended insertion site (within US2 gene) was cloned by PCR reactions, deleting a portion (0.2-kb) of the US2 gene and adding SfiI recognition site at the insertion site (Figure 1). Briefly, using DNA extracted from Rispens as a template, two PCR reactions were conducted. Primer pairs used are SEQ NO. 1 (5'- AAACGAATTCGAAGCTTGCATGCCCGCTAAGGAC -3') and SEQ NO. 2 (5'- GCCACCAGATGGAAGTGGGCAATAAGGCCGGTATGCCGTGGGCC -3'), and SEQ NO. 3 (5'- GGCCACG-GCATAACGGCCTTATTGGCCCCAGTTCCA TCTGGTGGC -3') and SEQ NO. 4 (5'- GATTACGAATTCGCCCTTTTTCATGCTGC CCCAA -3'). Another PCR reaction was conducted using a mixture of PCR products from the two previous PCR reactions as a template and SEQ NO. 1 and SEQ NO. 4 as primers. An obtained PCR fragment was cloned into pUC18 vector (GenBank Acc. No. L09136) after digestion with EcoRI and HindIII, resulting in pRisUS2Sfi.

Construction of the homology vectors

[0086] Utilizing the plasmid pRisUS2Sfi, various homology vectors containing a promoter and IBDV VP2 gene from standard challenge strain (VP2-STC) were constructed. These homology vectors were used to construct recombinant Rispens/IBD (RR013, RR024, RR025, RR030, and RR031). First, pRisUS2Sfi was cleaved with SfiI and dephosphorylated with Alkaline Phosphatase Shewanella sp. S1B1 Recombinant (PAP) (Funakoshi #DE110). The Bac promoter-VP2-STC cassette was obtained by BglII digestion of p45/46bacVP2-STC#11 (U.S. Pat. No. 6,764,684) and inserted into the SfiI-digested pRisUS2Sfi, resulting in pRisUS2bacVP2stc. This plasmid, pRisUS2bacVP2stc, was used to construct a recombinant Rispens/IBD, RR013. Homology plasmids containing a partial core sequence (SEQ No. 5) of Bac promoter (Coa5 promoter) were also constructed. The Coa5 promoter was obtained from the plasmid pGICOA (U.S. Pat. No. 6,866,852) by BglII and XbaI digestion, and ligated with an XbaI-EcoRI fragment (6.3-kb) and an EcoRI-BglII fragment (0.1-kb) of p45/46bacVP2-STC#11, resulting in p45/46COA5VP2-STC#11. The Coa5 promoter-VP2-STC cassette was then cut out from p45/46COA5VP2-STC#11 by BglII digestion and ligated with the SfiI-digested pRisUS2Sfi, resulting in pRisUS2Coa5VP2stc. This plasmid, pRisUS2Coa5VP2stc was used to construct RR030. Another homology vector with the Coa5 promoter was constructed having a different sequence between the Coa5 promoter and the VP2-STC gene. After PCR using pRisUS2Coa5VP2stc as a template and SEQ No. 6 (5'-GTGGGGACCCGAGGATTTTG -3') and SEQ No. 7 (5'- GCGTCTAGA GGATCGATCCACCGGTCGCCACCATGACAAACCTGCAAGATCA -3') as primers, an obtained DNA fragment was digested with XbaI and Sall, and then inserted into an XbaI-Sall fragment (5.2-kb) of pRisUS2Coa5VP2stc. The resultant plasmid, pRisUS2Coa5VP2stc2, was used to make RR031. A RSV promoter-VP2-STC cassette and a SV40 promoter-VP2-STC cassette were obtained by digestion of p44-45d46RsvVP2 and p44-45d46SV40VP2 (EP patent 12305390), respectively, with BglII. These fragments were ligated with the SfiI-digested pRisUS2Sfi, resulting in pRisUS2RSVVP2stc and pRisUS2SV40VP2stc. These plasmids were used to construct RR024 and RR025, respectively.

Example 2: Construction of recombinant Rispens

[0087] Construction of recombinant Rispens was conducted by homologous recombination either in cultured cells or *E. coli*. For homologous recombination in cultured cells, viral DNA of wild type Rispens virus was prepared as described by Morgan et al. (Avian Diseases, 34:345-351, 1990). Approximately 2 µg of the Rispens DNA and 1 µg of one of the homology vector were transfected into approximately 10⁷ CEF cells by electroporation using Nucleofector II (Lonza, Basel, Switzerland). The transfected cells were added to Leibovitz's L-15 (Life Technologies Corp., Cat. #41300-39),

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McCoy's 5A Medium (Life Technologies Corp., Cat. #21500-061) (1:1) and 4% calf serum [LM (+) medium], planted in 96-well tissue culture plates, and then incubated at 37°C in 4-5% CO₂ for 5-7 days until Rispens plaques became visible. The cells were then detached from the plates by trypsinization, transferred equally to two 96-well plates with CEF, and incubated for 4 to 6 days until plaques were observed. Screening was conducted by the black plaque assay, staining only plaques expressing IBDV VP2 protein. Briefly, one of the two plates was fixed with methanol:acetone mixture (1:2) and incubated with anti-IBDV VP2 monoclonal antibody R63 (ATCC #: HB-9490). Next, incubated with biotinylated anti-mouse IgG antibody (Vector Laboratories, Cat# BA-9200) and then with VECTASTAIN ABC-AP kit (Vector Laboratories, Cat# AK-5000), plaques expressing VP2 protein were stained by addition of NBT/BCIP solution (Roche Applied Science, Cat# 1681451). Wells containing stained recombinant plaques were identified and cells from the corresponding wells on the other 96-well plate were trypsinized. The cells were then diluted in fresh secondary CEF cells and transferred to 96-well plates to complete the first round of purification. The purification procedure was repeated until all plaques were stained positively in the black plaque assay. For homologous recombination in *E. coli*, DH10B *E. coli* strain carrying Rispens genome as bacterial artificial chromosomes (BAC) and plasmid pKD46 (GenBank Acc. No. AY048746) was transfected with 1 µg of one of the homology vector after induction of recombination enzymes by addition of 10 mM L-arabinose. Transfection was conducted by electroporation using Gene Pulser Xcell (Bio-Rad Laboratories) at 1.75kV, 25µF, and 200 ohm. After transfection, the *E. coli* was planted onto Luria-Bertani (LB) agar plates, and incubated overnight at 37°C. *E. coli* clones carrying an appropriate insert containing the VP2 gene were identified by PCR using a primer pair amplifying a region between VP2 gene and the insertion site region of Rispens genome. The primers are SEQ NO. 6 and SEQ NO. 8 (5'- TGA ACTACACAAA ATTGATACTGA -3'). Rispens BAC DNA was extracted from clones that contained the insert and transfected into CEF using Nucleofector II (Lonza). The transfected CEF was planted in 96-well plates and incubated at 37°C in 4-5% CO₂ for 5-7 days until Rispens plaques became visible. Plaques expressing VP2 protein were purified as described above.

Example 3: Verification of genome structure

[0088] Genome structures of the recombinant Rispens/IBD were verified by two PCR reactions amplifying junction regions (Junction 1 and Junction 2) at each end of the inserted genes. Figure 2 shows where Junction 1 and Junction 2 are located in the recombinant virus genome. The primer pairs used in the PCR reactions are SEQ NO. 9 (5'-GAG-GCGGACGTAAATGGAGA -3') and SEQ NO. 8 for Junction 1, and SEQ NO. 10 (5'-GCCAGGGAATCCAGGGAAAAA-GAC -3') and SEQ NO. 11 (5'-TATGAGCGGCAGTTATCGTGT -3') for Junction 2. Expected sizes of PCR products were observed with all of the recombinant Rispens, confirming that these recombinant Rispens have the expected genome structures.

Example 4: Expression of an inserted antigen by recombinant Rispens

[0089] Expression of the VP2 protein by the recombinant Rispens/IBD was confirmed by the black plaque assay and the Western blot assay. Procedures for the black plaque assay are described in Experiment 2. The western blot was conducted using CEF cells infected with the recombinant viruses and anti-IBDV VP2 monoclonal antibody R63. Briefly, CEF cells in 6-well plates were infected with one of the recombinant viruses or the parent Rispens strain at a multiplicity of infection of approximately 0.1. Three days post inoculation, cells were harvested with trypsin and centrifuged at 913 x g for 5 minutes. The pellet was washed with PBS and resuspended with 100 µl of PBS. After adding the same volume of 2 x SDS sample buffer (130 mM Tris-Cl (pH6.8), 6% SDS, 20% Glycerol, 10% 2-Mercaptoethanol and 0.01% Bromo Phenol Blue), cell suspension was boiled for 5 minutes. The samples were separated by SDS-PAGE using 12% polyacrylamide gel and transferred to a PVDF membrane (Immobilon-P, Millipore). The membrane was dried completely and then incubated with the R63 monoclonal antibody. After the R63 antibody was washed off, biotinylated anti-mouse IgG antibody (Vector Laboratories, Cat# BA-9200) and then with VECTASTAIN ABC-AP kit (Vector Laboratories, Cat# AK-5000). Protein bound with the R63 monoclonal antibody was visualized by addition of NBT/BCIP solution (Roche Applied Science, Cat# 1681451). As shown in Figure 3, protein bands of 40 kilodaltons (kDa), which was the expected size of the VP2 protein, was observed only in the lanes with the recombinant virus infected cells. Recombinant Rispens with an entire or a partial Bac promoter, i.e. RR013, RR030, and RR031, expressed more VP2 protein than RR024, which has a RSV promoter.

Example 5: Efficacy of recombinant Rispens in chickens - The beta actin promoter provides highly superior protection

[0090] Efficacy of the recombinant Rispens viruses expressing the IBDV VP2 gene was evaluated against virulent IBDV challenge. Three recombinant Rispens/IBD viruses (RR013, RR024 and RR025) were used in which expression of the antigen is driven by different promoters. Specific pathogen free (SPF) white leghorn chickens at one day of age

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were divided into six groups and chicks in Groups 3 through 5 were vaccinated subcutaneously with approximately 3000 plaque forming units (pfu)/0.2 ml of one of the recombinant Rispens (Group 3, RR013: Group 4, RR024: Group 5, RR025). Chicks in Group 6 were vaccinated with FW023 (rHVT/IBD-STC#11: U.S. patent 6,764,684), which was a recombinant HVT/VP2 control. Chicks in Group 1 (non-immunized, non-challenged negative control) and chicks in Group 2 (non-immunized, challenged positive control) were left unvaccinated. The chickens were bled each week between 2 and 7 weeks of age for evaluation of humoral immunity against IBDV. Anti-IBDV antibodies were quantitated with a commercial IBDV ELISA kit (Idexx Laboratories, FlockChek IBD). At 7 weeks of age, all chickens except Group 1 were challenged with 10^3 mean embryo infectious dose (EID₅₀) of virulent IBDV standard challenge (STC) strain via oral route. Chickens were observed daily for clinical signs associated with IBD, such as depression and death. Seven days post challenge, chickens were necropsied and observed for grossly observable bursal lesions such as edema, discoloration, atrophy, hemorrhage, and yellow or gelatinous exudates. Weights of body and bursa were also measured at necropsy for calculation of B/B index, which is the ratio between the weight of the bursa and the body weight of challenged birds divided by the same ratio of non-challenged birds.

[0091] Table 1 summarizes the results.

Table 1. Protection of recombinant Rispens against virulent IBDV challenge in SPF chickens (Efficacy trial 1)

Group number	Group	# chickens	B/B Index	# dead after challenge	# with bursal lesions/ # total	% protection
1	NINC	13	1.00	0	0/13	Not applicable
2	NICC	13	0.47	0	13/13	0%
3	RR013	15	0.95	0	2/15	87%
4	RR024	15	0.77	0	7/15	53%
5	RR025	15	0.59	0	10/15	33%
6	FW023	13	0.93	0	1/13	92%

NINC = non-immunized, non-challenged negative controls
 NICC = non-immunized, challenged positive controls
 RR013: Rispens/US2/Bac-VP2stc
 RR024: Rispens/US2/RSV-VP2stc
 RR025: Rispens/US2/SV40-VP2stc
 FW023 (recombinant HVT/IBD control): HVT/UL45-46/Bac-VP2stc

[0092] All chickens in Group 2 (challenged positive control) developed gross bursal lesions typical of IBD, while all chickens in Group 1 (non-challenged negative control) remained free from such lesions. Protection provided by RR013 (Group 3) was 87% (13/15) and it was equivalent to protection from FW023 recombinant HVT/IBD control (Group 6). B/B Index of group 3 was 0.95, suggesting no significant atrophy in bursa. Surprisingly, RR024 (Group 4) and RR025 (Group 5) gave only partial protections (53% and 33%). All vaccinated groups gave higher IBD ELISA titers starting from 3 weeks of age (Figure 4). Titers in RR013 vaccinated chickens were consistently higher than those in RR024 or RR025 vaccinated chickens, which is consistent with protection results after challenge.

[0093] In conclusion, RR013 with a Bac promoter, provided substantially superior humoral and protective immunity as compared to RR024 and RR025, both of which contain non-Bac promoters.

Example 6: Efficacy of recombinant Rispens in chickens - Activity of different beta actin-derived promoters

[0094] The efficacy of RR013, RR030, and RR031, all of which contain an entire or a partial Bac promoter, was investigated in commercial layer (white leghorn) chickens with maternal antibodies. Chickens at one day of age were divided into six groups and chicks in Groups 3 through 5 were vaccinated subcutaneously with approximately 3000 plaque forming units (pfu)/0.2 ml of one of the recombinant Rispens (Group 3, RR013: Group 4, RR030: Group 5, RR031). Chicks in Group 6 were vaccinated with FW023, which was a recombinant HVT/VP2 control. Chicks in Group 1 (non-immunized, non-challenged negative control) and chicks in Group 2 (non-immunized, challenged positive control) were left unvaccinated. The chickens were bled each week between 1 and 7 weeks of age and tested for presence of anti-IBDV antibodies with a commercial IBDV ELISA kit (IDEXX IBD Ab Test: IDEXX Laboratories). Challenge was conducted two times at 5 weeks of age and 7 weeks of age. A half of the chickens in each group were challenged at 5 weeks of age and the other half were challenged at 7 weeks of age. For challenge, 10^3 EID₅₀ of virulent IBDV STC strain was administered via oral route. Observation and evaluation after challenge were conducted in the same manner as in

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Example 5.

[0095] Tables 2 and 3 summarize the results.

Table 2. Protection of recombinant Rispens against virulent IBDV challenge at 5 weeks of age in commercial layer chickens (Efficacy trial 2)

Group number	Group	# chickens	B/B Index	# dead after challenge	# with bursal lesions/# total	% protection
1	NINC	20	1.00	0	0/20	Not applicable
2	NICC	22	1.08	3	22/22	0%
3	RR013	20	1.09	0	2/20	90%
4	RR030	22	1.08	0	1/22	95%
5	RR031	22	1.21	0	1/22	95%
6	FW023	21	1.24	1	4/21	81%

NINC = non-immunized, non-challenged negative controls
 NICC = non-immunized, challenged positive controls
 RR013: Rispens/US2/Bac-VP2stc
 RR030: Rispens/US2/Coa5-VP2stc
 RR031: Rispens/US2/Coa5-VP2stc2
 FW023 (recombinant HVT/IBD control): HVT/UL45-46/Bac-VP2stc

Table 3. Protection of recombinant Rispens against virulent IBDV challenge at 7 weeks of age in commercial layer chickens (Efficacy trial 2)

Group number	Group	# chickens	B/B Index	# dead after challenge	# with bursal lesions/# total	% protection
1	NINC	19	1.00	0	0/20	Not applicable
2	NICC	19	0.55	5	19/19	0%
3	RR013	19	0.96	0	3/19	84%
4	RR030	22	0.96	1	2/22	91%
5	RR031	20	0.91	0	4/20	80%
6	FW023	21	0.97	1	4/21	81%

[0096] After challenge at 5 weeks of age, all groups vaccinated with recombinant Rispens containing a BAC promoter gave excellent protection (90% for RR013, 95% for RR030, and 95% for RR031), while all of chickens in non-immunized challenged positive control (Group 2) developed gross bursal lesions typical of IBD (Table 2). FW023 recombinant HVT/IBD control gave a protection at 81%, which was lower than recombinant Rispens groups. All chickens in Group 1 (non-challenged negative control) remained free from such lesions. High B/B Index in non-immunized challenged positive control (Group 2) while exhibiting typical IBD gross lesions in bursa may indicate involvement of remnant maternally derived antibodies, leading to the delay in development of bursal lesions. After challenge at 7 weeks of age, all groups vaccinated with recombinant Rispens again containing a BAC promoter gave excellent protection (84% for RR013, 91% for RR030, and 80% for RR031), while all of chickens in non-immunized challenged positive control (Group 2) developed gross IBD bursal lesions (Table 3). B/B Indices of the vaccinated groups were above 0.91, suggesting no significant atrophy in bursa. IBDV ELISA results show that after decay of maternally derived antibodies from 1 week to 4 weeks, all vaccinated groups gave higher IBD ELISA titers (Figure 5). Titers in RR030 and RR031 vaccinated chickens were higher than those in FW023 vaccinated chickens between 5 and 7 weeks of age.

[0097] In summary, RR013, RR030, and RR031, all of which contain an entire or a partial Bac promoter combined with the VP2 gene, gave excellent protection in commercial white leghorn layer chickens with maternal antibodies at 5 and 7 weeks of age after vaccination at one day of age.

Example 7: Stability of recombinant Rispens of the invention

[0098] In this example, the stability of RR030 and RR031 was evaluated in cell culture (*in vitro*) and in chickens (*in vivo*). For *in vitro* stability, viruses were passed in chicken embryo fibroblasts (CEF) 20 times. For *in vivo* stability, viruses were inoculated to one day of age commercial layer (white leghorn) chickens and then re-isolated from the vaccinated chickens at 5 and 7 weeks of age by inoculating peripheral blood lymphocytes of the chickens onto CEF. Viruses after *in vitro* passages and viruses isolated from vaccinated chickens were tested by PCR and the black plaque assay using R63 anti-IBDV VP2 monoclonal antibody.

[0099] PCR assay detected expected bands with all of tested samples, suggesting that there was no detectable deletion or change in the virus genome. After the black plaque assay using R63 anti-IBDV VP2 monoclonal, all plaques were stained black with all tested samples.

[0100] Therefore, RR030 and RR031 are genetically and phenotypically stable *in vitro* and *in vivo*.

Example 8: Duration of immunity of Rispens/IBD

[0101] In order to investigate duration of immunity to IBDV conferred by Rispens/IBD, chickens vaccinated with RR030 were monitored for IBDV ELISA titers through 41 weeks of age. More particularly, one day of age commercial layer (white leghorn) chickens with maternal antibodies were vaccinated subcutaneously with approximately 3000 pfu/0.2 ml of the recombinant Rispens/IBD (RR030). The chickens were bled weekly between 1 and 7 weeks of age and biweekly thereafter. Sera were tested for presence of anti-IBDV antibodies with a commercial IBDV ELISA kit (IDEXX IBD Ab Test: IDEXX Laboratories). As shown in Figure 7, after maternal antibodies against IBDV waned through 3 weeks of age, ELISA titers started to increase. Titers continued to increase up to S/P values of more than 2.5 through 25 weeks of age, and high level of IBDV ELISA titers were maintained through 41 weeks of age. This result demonstrates that Rispens/IBD provides exceptionally long duration of immunity against IBDV.

Example 9: Efficacy of recombinant Rispens in chickens - No interference with distinct HVT

[0102] In this example, the compatibility between recombinant Rispens/IBD (RR030) containing a BAC promoter and recombinant HVT/ND (FW029) (rHVT/NDV: U.S. patent 6,866,852) was investigated. One day of age commercial layer (white leghorn) chickens with maternal antibodies were divided into six groups and vaccinated. Group 1 was vaccinated subcutaneously with approximately 3000 pfu/0.2 ml of the recombinant Rispens/IBD (RR030) alone. Group 2 received a mixture of RR030 and recombinant HVT/ND (FW029), both at 3000 pfu/0.2 ml. A group of chicks (Group 3) was vaccinated only with FW029. Chicks in Group 4 were vaccinated with FW023, which was a recombinant HVT/VP2 control. Chicks in Group 5 (non-immunized, challenged positive control) and in Group 6 (non-immunized, non-challenged negative control) were left unvaccinated. The chickens were bled each week between 2 and 7 weeks of age and tested for presence of anti-IBDV antibodies with a commercial IBDV ELISA kit (IDEXX IBD Ab Test: IDEXX Laboratories) and for presence of anti-NDV antibodies with a commercial NDV ELISA kit (IDEXX NDV Ab Test: IDEXX Laboratories). Challenge with either virulent IBDV or virulent NDV was conducted at 7 weeks of age. All of the chickens in Group 1 (RR030 alone) and Group 4 (FW023 alone), and a half of the chickens in Group 2 (RR030 + FW029) and Group 5 (non-immunized, challenged positive control) received IBDV challenge with 10³ EID₅₀ of virulent IBDV STC strain via oral route, while all of the chickens in Group 3 (FW029 alone) and the other half of the chickens in in Group 2 (RR030 + FW029) and Group 5 (non-immunized, challenged positive control) received NDV challenge with 10³ EID₅₀ of very virulent (neurotropic velogenic) NDV Texas GB strain via intramuscular injection into the femoral region. Observation and evaluation after IBDV challenge were conducted in the same manner as in Example 5 and 6. For NDV challenge, the chickens were observed daily for 14 days for clinical signs typical of neurotropic velogenic ND such as depression, neurological symptoms, and deaths.

[0103] The results are summarized in Table 4.

Table 4. Compatibility between recombinant Rispens/IBD and recombinant HVT/NDV in commercial layer chickens (Efficacy trial 3)

Group number	Group	# chickens	IBD challenge		ND challenge	
			# with bursal lesions/# total	% protection	# with ND clinical signs/# total	% protection
1	RR030	17	2/17	88%	N/A	N/A

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(continued)

Group number	Group	# chickens	IBD challenge		ND challenge	
			# with bursal lesions/# total	% protection	# with ND clinical signs/# total	% protection
2	RR030 + FW029	33	1/16	94%	2/17	88%
3	FW029	17	N/A	N/A	2/16	88%
4	FW023	16	1/16	94%	N/A	N/A
5	NICC	31	15/15	0%	16/16	0%
6	NINC	15	N/A	N/A	N/A	N/A
RR030: Rispens/US2/Coa5-VP2stc FW029 (recombinant HVT/ND control): HVT/UL45-46/Pec-F FW023 (recombinant HVT/IBD control): HVT/UL45-46/Bac-VP2stc NICC = non-immunized, challenged positive controls NINC = non-immunized, non-challenged negative controls						

[0104] RR030 combined with FW029 (Group 2) provided excellent protection against both IBD and ND challenges. After IBDV challenge, RR030 combined with FW029 (Group2) provided 94% (15/16) protection, which was equivalent to protection by RR030 alone (Group 1) at 88% (15/17) protection. In Group 4 vaccinated with FW023, 94% of the chickens were protected. All chickens in non-immunized, challenged positive control (Group 5) developed gross bursal lesions. Following NDV challenge, 88% (15/17) protection was observed with Group 2 (RR030 combined with FW029), while FW029 (14/16) alone provided 88% protection. All chickens in the challenged positive control (Group 5) showed clinical signs of ND. ELISA results also demonstrated that humoral immunity developed by RR030 or FW029 did not decrease when these two viruses were combined (Figures 6A and 6B).

[0105] In conclusion, this example demonstrates a lack of interference between recombinant Rispens/IBD containing a Bac construct and recombinant HVT/ND.

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15 ggaaggaaat gggcggggag ggccttcgtg cgtcgccgcg ccgccgtccc cttctccatc 1380
tccagcctcg gggctgtccg cagggggacg gctgccttcg ggggggacgg ggcagggcgg 1440
20 ggttcggctt ctggcgtgtg accggcgggg tttatatctt cccttctctg ttcctccgca 1500
gcccc 1506

25 Claims

1. A recombinant Marek's disease virus of serotype 1 (MDV1) which comprises (1) a promoter consisting of a chicken beta-actin derived promoter and (2) under the control of said promoter, a recombinant nucleotide sequence encoding a polypeptide.
30
2. The recombinant MDV1 of claim 1, wherein the promoter consists of the core sequence of a chicken beta-actin promoter.
3. The recombinant MDV1 of anyone of claims 1 to 2, wherein the core sequence of a chicken beta-actin promoter comprises SEQ ID NO: 5 or a sequence having at least 90% sequence identity to SEQ ID NO: 5 and exhibiting transcriptional promoter activity.
35
4. The recombinant MDV1 of anyone of the preceding claims, wherein said promoter and said recombinant nucleotide sequence are inserted into a non-essential region of the virus, preferably into the US2 gene of said virus.
40
5. The recombinant MDV1 of anyone of the preceding claims, wherein the recombinant nucleotide sequence encodes an antigenic peptide.
6. The recombinant MDV1 of claim 5 wherein the antigenic peptide is an antigenic peptide from an avian pathogen.
45
7. The recombinant MDV1 of claim 6, wherein the antigenic peptide is chosen among an antigenic peptide of avian paramyxovirus type 1, preferably the F protein of Newcastle disease virus (NDV) or a fragment thereof, an antigenic peptide of Gumboro disease virus, preferably the VP2 protein of the Infectious bursal disease virus (IBDV) or a fragment thereof, an antigenic peptide of the infectious laryngotracheitis virus (ILT), preferably the gB protein or a fragment thereof, an antigenic peptide of Mycoplasma galisepticum, preferably the 40K protein or a fragment thereof, and an antigenic peptide of the avian influenza virus, preferentially a surface protein hemagglutinin (HA) or a fragment thereof.
50
8. The recombinant MDV1 of claim 7, wherein the antigenic peptide is a VP2 protein of IBDV or an immunogenic fragment thereof.
55
9. The recombinant MDV1 of anyone of the preceding claims, wherein the virus comprises a further recombinant nucleotide sequence encoding a distinct polypeptide.

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- 5
10. The recombinant MDV1 of anyone of the preceding claims, wherein said MDV1 comprises, inserted into a non-essential region, a recombinant nucleotide sequence encoding an antigenic peptide under the control of a promoter comprising SEQ ID NO: 5 or a sequence having at least 90% sequence identity to SEQ ID NO: 5 and exhibiting transcriptional promoter activity.
11. A composition comprising a recombinant Marek's disease virus of anyone of claims 1 to 10 and, optionally, a pharmaceutically or veterinary acceptable excipient or carrier and/or a suitable adjuvant.
- 10
12. A recombinant MDV1 of anyone of claims 1 to 10, or a composition of claim 11, for use to vaccinate an avian, preferably a chicken.
13. A recombinant MDV1 of anyone of claims 1 to 10 expressing a first antigen, for use in combination with a distinct recombinant avian virus expressing a second distinct antigen, to vaccinate an avian, preferably a chicken, by simultaneous, separate sequential or alternated administration.
- 15
14. The recombinant MDV1 for use of claim 13, wherein the MDV1 expresses a VP2 antigen, and the distinct avian virus is an HVT expressing a distinct antigen.
15. A method for producing a recombinant MDV1 of anyone of claims 1 to 10 comprising (i) the introduction of a recombinant nucleotide sequence encoding a polypeptide under the control of a promoter comprising a core sequence of a chicken beta-actin promoter into a nonessential region of the genome of a MDV1 virus, (ii) optionally replicating the virus and, (iii) optionally collecting the virus.
- 20
16. A recombinant nucleic acid molecule comprising the genome of a MDV1 or a fragment thereof, wherein said genome or fragment comprises a US2 gene modified by insertion of a recombinant nucleotide sequence comprising a sequence encoding a polypeptide under the control of a promoter comprising a core sequence of a chicken beta-actin promoter.
- 25
17. A plasmid comprising a nucleic acid molecule of claim 16.
- 30
18. A host cell comprising a nucleic acid molecule of claim 16 or a plasmid of claim 17.
- 35
- 40
- 45
- 50
- 55

Figure 1

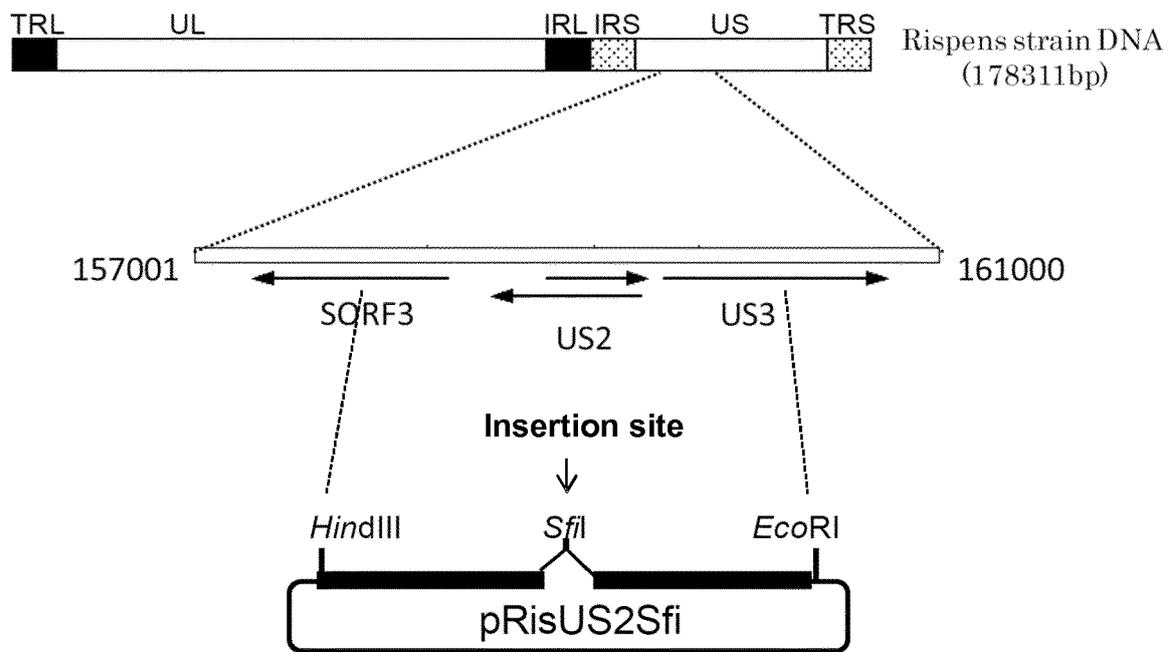


Figure 2

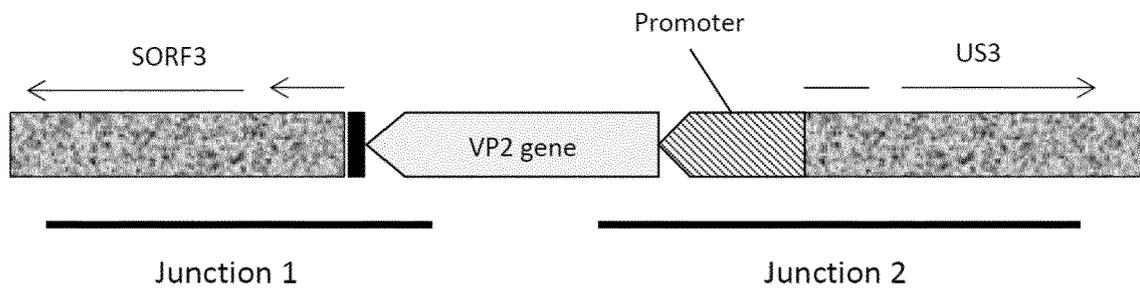
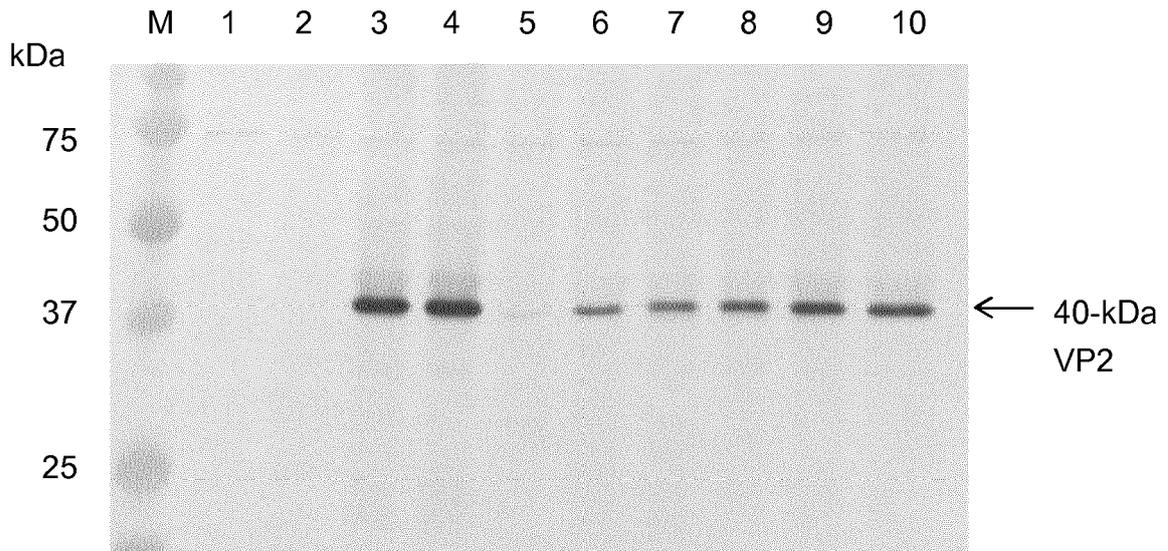


Figure 3



M: Precision Plus Protein All Blue Standards (Bio-Rad, Cat# 161-0373)

1: CEF control

2: Wild type Rispens

3: RR013 Clone 1

4: RR013 Clone 2

5: RR024

6: RR030 Clone 1

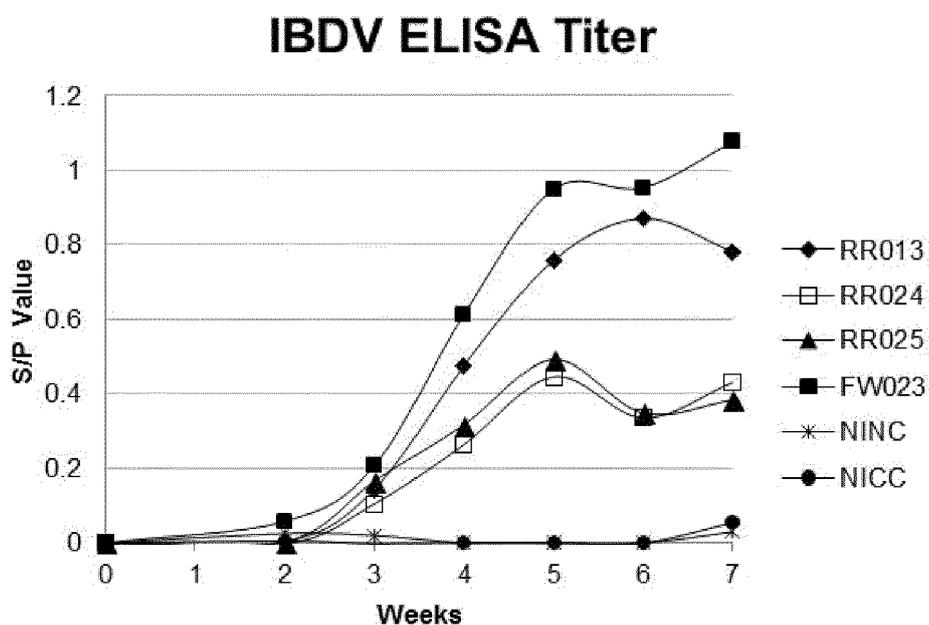
7: RR030 Clone 2

8: RR030 Clone 3

9: RR031 Clone 1

10: RR031 Clone 2

Figure 4



IDEXX IBD Ab Test: IDEXX Laboratories

RR013: Rispens/US2/Bac-VP2stc

RR024: Rispens/US2/RSV-VP2stc

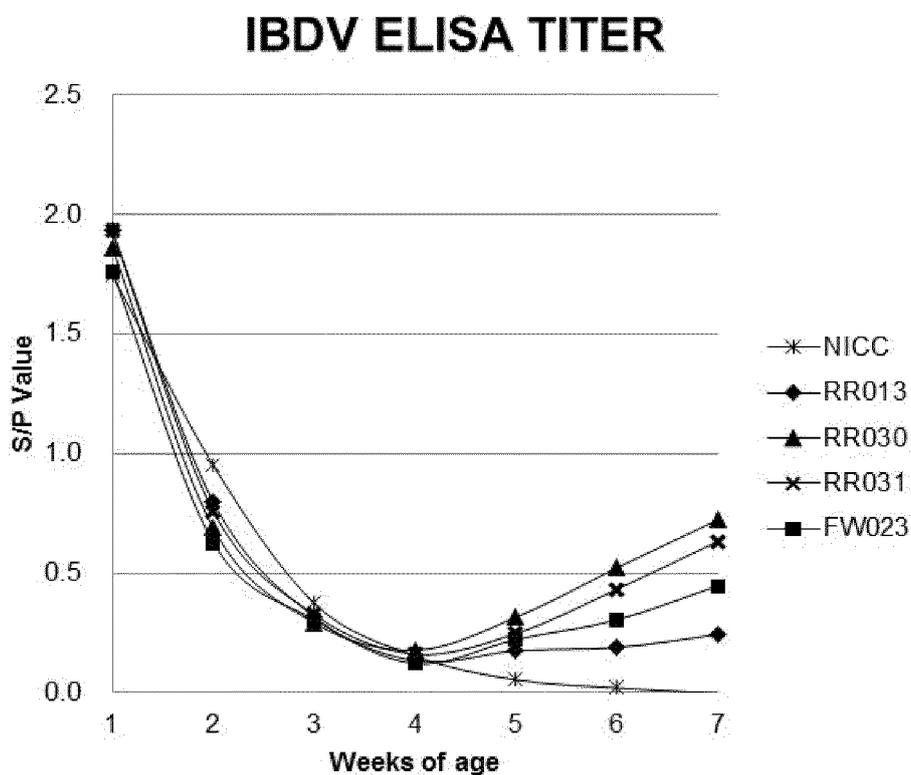
RR025: Rispens/US2/SV40-VP2stc

FW023 (recombinant HVT/IBD control): HVT/UL45-46/Bac-VP2stc

NINC = non-immunized, non-challenged negative controls

NICC = non-immunized, challenged positive controls

Figure 5



IDEXX IBD Ab Test: IDEXX Laboratories

NICC = non-immunized, challenged positive controls

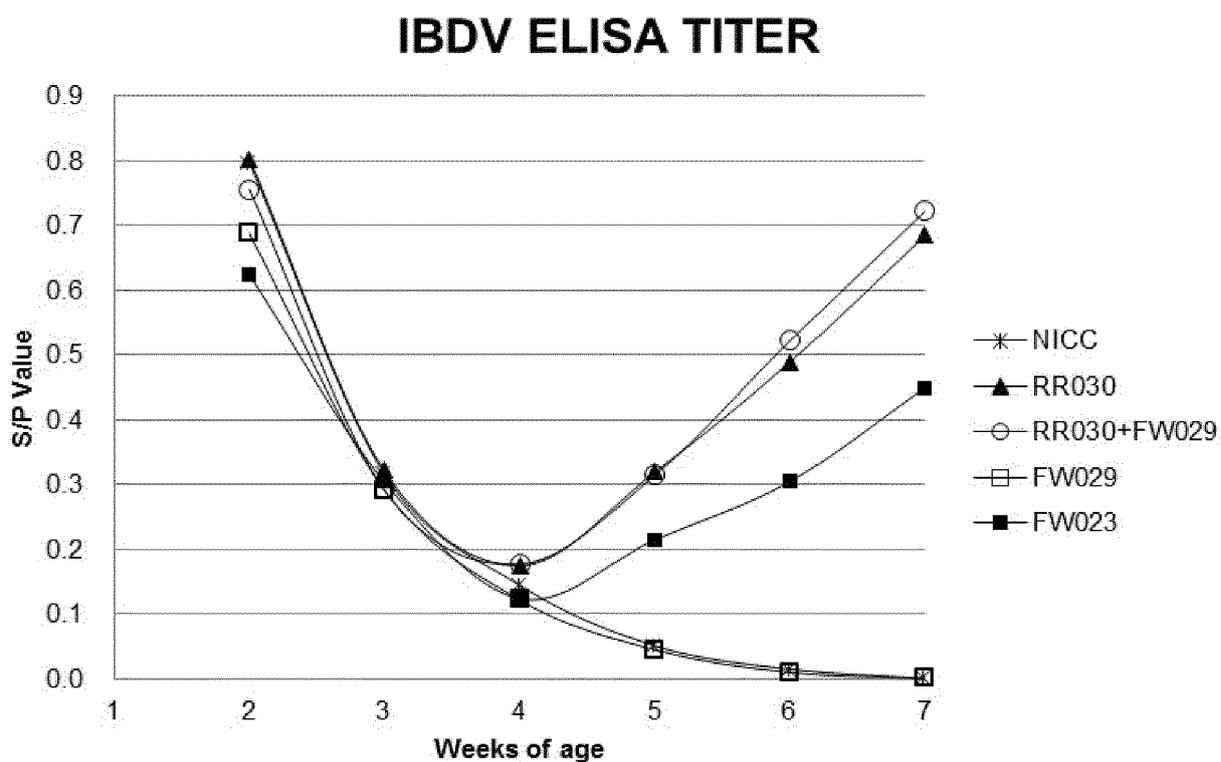
RR013: Rispens/US2/Bac-VP2stc

RR030: Rispens/US2/Coa5-VP2stc

RR031: Rispens/US2/Coa5-VP2stc2

FW023 (recombinant HVT/IBD control): HVT/UL45-46/Bac-VP2stc

Figure 6A



IDEXX IBD Ab Test: IDEXX Laboratories

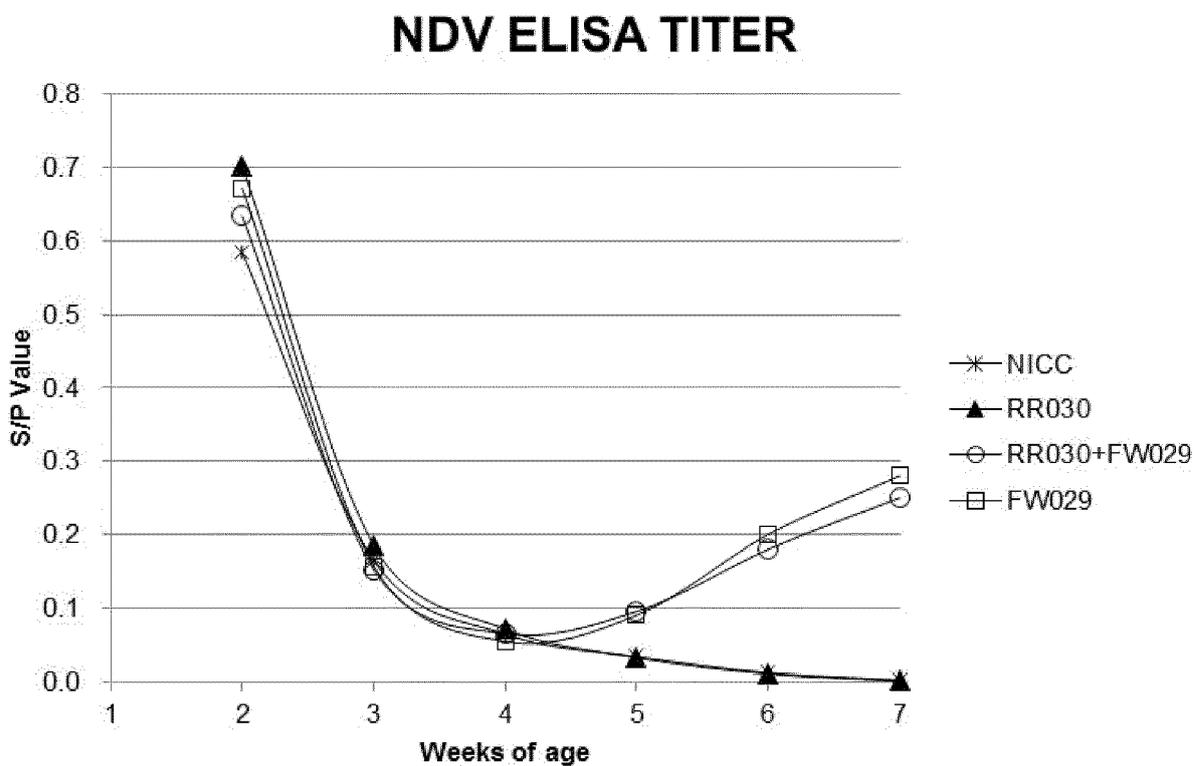
NICC = non-immunized, challenged positive controls

RR030: Rispens/US2/Coa5-VP2stc

FW029 (recombinant HVT/ND control): HVT/UL45-46/Pec-F

FW023 (recombinant HVT/IBD control): HVT/UL45-46/Bac-VP2stc

Figure 6B



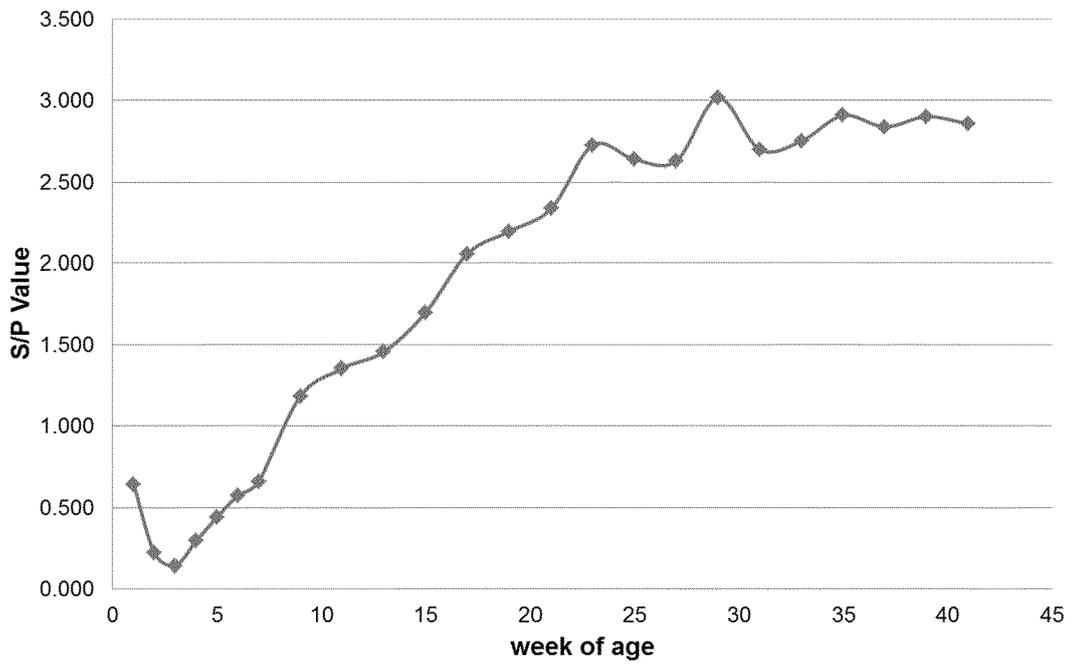
IDEXX NDV Ab Test: IDEXX Laboratories

NICC = non-immunized, challenged positive controls

RR030: Rispens/US2/Coa5-VP2stc

FW029 (recombinant HVT/ND control): HVT/UL45-46/Pec-F

Figure 7





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X : particularly relevant if taken alone Y : particularly relevant if combined with another document of the same category A : technological background O : non-written disclosure P : intermediate document			

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