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(54) Title: CANINE ORAL VACCINES AND METHODS OF ADMINISTRATION

(57) Abstract: This disclosure provides a method of protecting a canine against canine distemper virus, the method comprising administering a recombinant oral vaccine comprising a modified live canine adenovirus type 2 (CAV-2) vector carrying a Canine Distemper Virus (CDV) antigen to said canine, wherein the vaccine is administered orally.

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CANINE ORAL VACCINES AND METHODS OF ADMINISTRATION

FIELD OF THE INVENTION

[0001] This invention is in the field of oral vaccines, particularly canine oral vaccines.

BACKGROUND

[0002] Vaccines against major canine infectious disease have been available for three to four decades, and they have greatly reduced the incidence of these infectious diseases in dogs (Appel, MJ. 1999. Adv Vet Med. 41 :309-324). Zoetis sells several vaccines for prevention of diseases associated with various viral and bacterial diseases in dogs. The VANGUARD® line of vaccines, including core canine vaccines, is used for the vaccination of healthy dogs 6 weeks of age or older as an aid in preventing canine distemper (CD) caused by canine distemper virus (CDV), infectious canine hepatitis (ICH) caused by canine adenovirus type 1 (CAV-1), respiratory disease caused by canine adenovirus type 2 (CAV-2), canine parainfluenza (CPI) caused by canine parainfluenza (CPI) virus, and canine parvoviral enteritis caused by canine parvovirus (CPV) (Mouzin DE, et al., 2004, JAVMA, 224: 55-60).

[0003] CD is a high morbidity, high mortality viral disease occurring in unvaccinated dog populations worldwide. Approximately 50% of non-vaccinated, non-immune dogs infected with CDV develop clinical signs and approximately 90% of those dogs die (Swango LJ. 1983. Norden News 58:4-10). Infectious Canine Hepatitis (ICH), caused by CAV-1, is a universal, sometimes fatal, viral disease of dogs characterized by hepatic and generalized endothelial lesions. The respiratory disease caused by CAV-2, in severe cases, may include pneumonia and bronchopneumonia. CAV-2 vaccine has been shown to cross-protect against ICH caused by CAV-1 (Bass EP, et al., 1980, JAVMA, 177:234-242). The upper respiratory disease caused by CPI virus may be mild or subclinical, with signs becoming more severe if concurrent infection with other respiratory pathogens exists. The enteric disease caused by CPV is characterized by sudden onset of vomiting and diarrhea, often hemorrhagic, and may be accompanied by leukopenia (Appel MJ, et al., 1979, Vet Rec, 105:156-159).

[0004] Generally, puppies are vaccinated at about 6 weeks of age with so-called core vaccines, comprising antigens against CDV, CAV-2, and CPV. Major animal health companies market core canine vaccines in their companion animal vaccine franchise. However, all of these vaccines are

delivered by the parenteral route, especially subcutaneous injections. A canine vaccine that could be delivered easily would provide increased convenience of vaccine delivery to the pet, the veterinarian, and the pet owner, and allow for personnel untrained in parenteral administration techniques to deliver canine core vaccines to animals.

[0005] Oral vaccines against CDV have been disclosed previously. For example, US Publication 2010028379 discloses oral vaccines comprising modified live canine distemper. However, despite significant effort, there is no commercially available oral vaccine against CDV. Therefore, there is a need in the art for effective vaccines against CDV that can be delivered orally.

SUMMARY OF INVENTION

[0006] In one aspect, the disclosure provides a method of protecting an MDA (Maternally Derived Antibodies) -negative canine against canine distemper virus, the method comprising administering a recombinant oral vaccine comprising a modified live canine adenovirus type 2 (CAV-2) vector carrying a Canine Distemper Virus (CDV) antigen to said canine, wherein the recombinant oral vaccine is administered orally in a first dose and orally in a second dose.

[0007] In a second aspect, the disclosure provides a method of annually revaccinating a dog, the method comprising orally administering to said dog a vaccine comprising a recombinant oral vaccine comprising a modified live canine adenovirus type 2 (CAV-2) vector carrying a Canine Distemper Virus (CDV) antigen, wherein said recombinant oral vaccine is administered about a year after a previous annual revaccination or about a year after administering to said dog a first dose of an initial vaccine, wherein further said first dose of the initial vaccine is administered to said dog parenterally, and wherein said initial vaccine comprises a CDV antigen and a CAV-2 antigen. In certain embodiments of this second aspect, in said initial vaccine, the CDV antigen is

a) a modified live CDV and the CAV-2 antigen is a modified live CAV-2; or
b) encoded by a nucleic acid sequence within a modified live canarypox virus genome.

[0008] In additional or alternative embodiments of the second aspect, in said previous annual revaccination the dog is administered a vaccine wherein the CDV antigen is

a) a modified live CDV and the CAV-2 antigen is a modified live CAV-2; or

b) encoded by a nucleic acid sequence within a modified live canarypox virus genome.

[0009] In certain embodiments applicable to the first and the second aspect of the invention, in the recombinant oral vaccine, the CDV antigen is H protein, preferably comprising SEQ ID NO: 1 or a sequence that is 90% or 95% identical to SEQ ID NO: 1. In certain embodiments, in the recombinant oral vaccine SEQ ID NO: 1 is encoded by a nucleic acid sequence comprising SEQ ID NO: 2. In additional embodiments, the recombinant oral vaccine further comprises F protein of CDV, preferably comprising SEQ ID NO: 3 or a sequence that is 90% or 95% identical to SEQ ID NO: 3. In certain embodiments, in the recombinant oral vaccine SEQ ID NO: 3 is encoded by a nucleic acid sequence comprising SEQ ID NO: 3. Preferably, the nucleic acid sequence encoding the CDV antigen according to any of the embodiments of the recombinant oral vaccine as described above, is inserted into E3 region of the modified live CAV-2 vector genome.

[0010] Also described is a method according to any of the embodiments described above, wherein said recombinant oral vaccine contains at least 10^3 $TCID_{50}$ to about 10^8 $TCID_{50}$ of said modified live CAV-2 vector carrying the CDV antigen per dose, more preferably about $10^{3.5}$ $TCID_{50}$ to about $10^{4.5}$ $TCID_{50}$ of said modified live CAV-2 vector carrying the CDV antigen per dose.

[0011] Also described is a method according to any of the above-disclosed embodiments, wherein, in the recombinant oral vaccine, said modified live canine adenovirus type 2 vector carrying a CDV antigen protects against Canine Distemper Virus, Canine Adenovirus Type 1 infection, and Canine Adenovirus Type 2 infection when administered orally. Preferably, the canine vaccinated according to the methods of the first aspect of the invention has not been previously vaccinated against Canine Distemper virus and, more preferably, against Canine Adenovirus Type 2.

[0012] In certain embodiments applicable to both the first and the second aspect, the recombinant oral vaccine further comprises a modified live parvovirus, wherein said recombinant oral vaccine further protects against Canine Parvovirus infection when administered orally. Preferably, said vaccine comprises about $10^{5.0}TCID_{50}$ to about 10^{10} $TCID_{50}$ of the modified live Canine Parvovirus, more preferably, $10^{7.5}$ $TCID_{50}$ to about 10^9 $TCID_{50}$ of the modified live Canine Parvovirus.

[0013] In additional embodiments, the recombinant oral vaccine according to any embodiments of the method according to the first or second aspect of the invention further comprises modified live Canine Parainfluenza virus, wherein said recombinant oral vaccine further protects against Canine Parainfluenza virus infection when administered orally. In certain embodiments of the first aspect of the inventin, said canine has not been previously vaccinated against Canine Parainfluenza virus.

[0014] In yet additional embodiments applicable to both the first and the second aspect of the invention, the recombinant oral vaccine comprises $10^{4.5}$ $TCID_{50}$ to about 10^{8} $TCID_{50}$ of the modified live Canine Parainfluenza virus, preferably $10^{6.5}$ $TCID_{50}$ to about $10^{7.5}$ $TCID_{50}$ of the modified live Canine Parainfluenza virus. In certain embodiments appricable to the first aspect of the invention, said canine has not been previously vaccinated against Canine Parainfluenza virus.

[0015] In certain embodiments of the method disclosed herein specifically applicable to the first aspect of the invention, said canine is about 8 to about 16 weeks old at the time of the first administration of the recombinant oral vaccine. Alternatively, in the embodiments specifically applicable to the second aspect of the invention, the canine is at least 13 months old at the time of administering the recombinant oral vaccine.

[0016] In certain embodiments of the first aspect of the invention, the second dose is administered 7-35 days after the first dose, preferably about 21 days after the first dose.

[0017] In further embodiments, the methods disclosed herein further comprise the step of orally administering a third dose of the recombinant oral vaccine described herein, wherein said third dose is administered about 21 days after the second dose.

[0018] In the third and the fourth aspect, the recombinant oral vaccine is provided, for use according to the embodiments of the first and the second aspect, respectively, as described above.

DETAILED DESCRIPTION

[0019] The following definitions are provided for a better understanding of the invention:

[0020] The term "about" as applied to a reference number refers to the reference number plus or minus 10 percent, unless the number is given as "10^N", in which case, "about 10^N" or "10^N" refers to N plus or minus 10%, inclusive, e.g., if N is 2, then about N is 1.8 to 2.2, inclusive.

[0021] The term "conservative substitution" refers to replacement of one amino acid with another, wherein both amino acids have similar properties. The following six groups each contain amino acids that are typical conservative substitutions for one another: [1] Alanine (A), Serine (S), Threonine (T); [2] Aspartic acid (D), Glutamic acid (E); [3] Asparagine (N), Glutamine (Q); [4] Arginine (R), Lysine (K), Histidine (H); [5] Isoleucine (I), Leucine (L), Methionine (M), Valine (V); and [6] Phenylalanine (F), Tyrosine (Y), Tryptophan (W), (see, e.g., US Patent Publication 20100291549).

[0022] The references to animals that "have not been vaccinated" or "have not been actively vaccinated" or the like do not encompass maternal immunity or passive antibody transfer.

[0023] The term "orally" or "oral" or the like refers to administration into the mouth of a subject, where the vaccine contacts the subject's oral mucosa.

[0024] The terms "protect", "protection", "protective immune response" and the like generally refer to the ability of the vaccines disclosed herein to reduce or eliminate the duration or the severity of at least one clinical sign of the pathogen against which the subjects are vaccinated. The clinical signs vary depending on the pathogen. A more detailed description of the protective immune response to specific viruses is provided below.

[0025] The term "subject" refers to animals to which the vaccines disclosed herein are administered. The term refers non-human animals, such as dogs, cats, horses, pigs, cattle, swine. In certain embodiments, the term also refers humans.

[0026] The term "therapeutically effective amount," in the context of this disclosure, refers to an amount of an antigen or vaccine that would induce a protective immune response in a subject receiving the antigen or vaccine.

[0027] The term "vaccine" refers to a composition containing an antigen against specific pathogen, wherein the antigen can elicit protective immune response against said pathogen. The response preferably is generated by the adaptive immune system and may include an antibody

response generated by B cells, cell-mediated immunity mediated by T-cells, or both antibody- and cell-mediated immunity.

[0028] The instant disclosure provides a recombinant oral vaccine comprising a modified live canine adenovirus type 2 (CAV-2) vector carrying an antigen from a Canine Distemper Virus. In preferred embodiments, in the recombinant oral vaccine the antigen from the Canine Distemper virus is its H-protein (Hemagglutinin) or F-protein (Fusion), as these proteins are present on the surface of the virus and are known to elicit protective immune response. In a particularly preferred embodiment, the CDV antigen in the recombinant oral vaccine is the H protein. Respective H proteins from different CDV strains are available in publicly accessible databases of genetic information. In certain embodiments, in the recombinant oral vaccine the H protein comprises an amino acid sequence that is at least 90% identical to SEQ ID NO: 1, and can be in different embodiments, at least 91% identical, or at least 92% identical, or at least 93% identical, or at least 94% identical, or at least 95% identical, or at least 96% identical, or at least 97% identical, or at least 98% identical, or at least 99% identical, or 100% identical to SEQ ID NO: 1. In the embodiments where the H protein is at least 90% identical but less than 100% identical to SEQ ID NO: 1, at least half of the differing amino acids (or at least 60%, or at least 70%, or at least 80%, or at least 90%, or at least 95%, or 100%,) are conservative substitutions.

[0029] Respective F proteins from different CDV strains are also available in publicly accessible databases of genetic information. In certain embodiments, the F protein comprises an amino acid sequence that is at least 90% identical to SEQ ID NO: 3, and can be in different embodiments, at least 91% identical, or at least 92% identical, or at least 93% identical, or at least 94% identical, or at least 95% identical, or at least 96% identical, or at least 97% identical, or at least 98% identical, or at least 99% identical, or 100% identical to SEQ ID NO: 3. In the embodiments where the H protein is at least 90% identical but less than 100% identical to SEQ ID NO: 3, at least half of the differing amino acids (or at least 60%, or at least 70%, or at least 80%, or at least 90%, or at least 95%, or 100%,) are conservative substitutions.

[0030] One way to create the modified live canine adenovirus type 2 (CAV-2) vector carrying an antigen from the Canine Distemper Virus is to recombinantly insert a nucleic acid sequence encoding the antigen from the Canine Distemper Virus according to any of the embodiments

described above into the genome of the modified live CAV-2 vector. In certain non-limiting embodiments, in the recombinant oral vaccine described herein the nucleic acid sequence encoding the antigen from the Canine Distemper Virus comprises SEQ ID NO: 2 or, in view of the redundancies in the genetic code, an equivalent of SEQ ID NO: 2. In other non-limiting embodiments of the recombinant oral vaccine described herein, the nucleic acid sequence encoding the antigen from the Canine Distemper Virus comprises SEQ ID NO: 4 or, in view of the redundancies in the genetic code, an equivalent of SEQ ID NO: 4.

[0031] The methods of recombinantly inserting foreign nucleic acids into viral vectors are well known in the art. Preferably, the nucleic acid sequence encoding the antigen from the Canine Distemper Virus is inserted into the E3 region of the genome of the modified live CAV-2 vector by any of the methods well known in the art. It is also preferred that the nucleic acid sequence encoding the antigen from the Canine Distemper Virus according to any of the embodiments described above be under operative control of a promoter. Suitable promoters should be able to direct expression of the CDV antigen in the cells of the intended host, such as for example, canines. Suitable promoters include, without limitations, SV-40 promoter, CMV promoter, RSV promoter, and other promoters known in the art.

[0032] In certain embodiments of the recombinant oral vaccine, the modified live CAV-2 vector carrying the CDV antigen according to any embodiments described herein is not enterically coated. Enteric coating generally protects viruses from the harsh environment of the stomach, but applicants have previously discovered that enteric coating of a modified live CDV inhibited the immune response to oral vaccination with the modified live CDV.

[0033] In other embodiments, in addition to the modified live CAV-2 vector carrying the antigen from a Canine Distemper Virus according to any of the embodiments, described above, the recombinant oral vaccine may comprise modified live canine parvovirus and/or modified live canine parainfluenza virus. The suitable modified live CPV and CPI viruses are known in the art. For example and without limitations, the respective CPV and CPI viruses present in VANGUARD® DAPPi vaccine (containing modified live CDV, CAV-2, CPV, and CPI) may be administered orally even though VANGUARD® DAPPi vaccine is currently approved for parenteral administration. In

certain embodiments of the recombinant oral vaccine, neither of the modified live CPV and CPI is enterically coated.

[0034] The methods of manufacturing the viruses according to any embodiments described herein are known in the art. Without limitations, the viruses may be propagated on NLDK (Norden Lab Dog Kidney) cells as per standard procedures. Briefly, canine viruses can be grown in roller bottles with or without Gentamicin in Dulbecco's Minimum Essential Media (DMEM) with 0-5% FBS. Depending on the specific virus NLDK cells are planted at an initial density of 1.2 x 10^4 to 3.5 x 10^4 cells / cm^2 and after 4-7 days of growth will be 80-100% confluent at the time of infection with virus. The cells are infected with virus at a multiplicity of infection (MOI) between 0.001 and 1.0 tissue culture infective dose 50 ($TCID_{50}$), incubated at 34.0 – 38.0 °C, rotated at 0.2 -0.5 rpm, harvested after 2-5 days, and stored at less than or equal to -40°C.

[0035] The recombinant oral vaccines described herein elicit protective immune responses against the respective pathogens if administered orally. Thus, the recombinant oral vaccines according to any embodiments described herein and containing the modified live CAV-2 vector carrying the CDV antigen elicit protective immune responses against CAV-1 infection, against CAV-2 infection, and against CDV infection. If the recombinant oral vaccine further comprises modified live CPV, then the recombinant oral vaccine also elicits a protective immune response against canine parvovirus. If the recombinant oral vaccine further comprises modified live CPI, then the recombinant oral vaccine also elicits a protective immune response against canine parainfluenza virus.

[0036] "Protective immune response" against CDV refers to reduction in duration or intensity of one or more clinical signs of CDV infection including, without limitations, CDV-caused mortality. Most preferably, the protective immune response against CDV refers to at least 95% (19 of 20) survival rate among the vaccinated dogs without showing any clinical signs of said disease in a challenge model wherein 80% (4 of 5) of the non-vaccinated dogs die of the disease, and 100% of the non-vaccinates show clinical signs after challenge.

[0037] "Protective immune response" against CAV-1 refers to reduction in duration or intensity of one or more clinical signs of CAV-1 infection including, without limitations, CDV-caused mortality. Most preferably, the protective immune response against CAV-1 refers to at least 95%

(19 of 20) survival rate of the vaccinated dogs survive without showing any clinical signs of canine hepatitis in a challenge model wherein 80% of the non-vaccinated dogs do show clinical signs.

[0038] "Protective immune response" against CAV-2 refers to reduction in duration or intensity of one or more clinical signs of CAV-2 infection including, without limitations, CDV-caused mortality. Most preferably, the protective immune response against CAV-2 refers to a significant difference in clinical signs between vaccinated and non-vaccinated dogs, in a challenge model wherein at least 60% of the non-vaccinated dogs show clinical signs of the disease.

[0039] "Protective immune response" against CPV refers to reduction in duration or intensity of one or more clinical signs of CPV infection including, without limitations, CDV-caused mortality. In the most preferred embodiment, the protective immune response against CPV refers to at least 95% (19 of 20) survival rate of the vaccinated dog during the observation period without showing more than one of the of following criteria of infection: temperature of ≥103.4, lymphopenia of ≥50 percent of pre-challenge normal, mucus or blood in the feces, and hemagglutination levels ≥1:64 in a 1:% dilution of feces, in a challenge model wherein more than 80% of non-vaccinated dogs exhibit at least three of the above signs.

[0040] The protective immune response against CPI refers to a significant reduction in virus isolation (shedding) in the vaccinates as compared to non-vaccinated animals.

[0041] As noted above, the recombinant oral vaccines may elicit the protective immune response after two oral administrations according to the first aspect of the invention or maintain (or elicit) the protective immune response after an annual revaccination according to the second aspect of the invention. In other words, in the first aspect of the invention, the parenteral administration is not needed to elicit the protective immune response against the target pathogen, as described above. When the recombinant oral vaccine administered orally in multiple doses, according to the fist aspect of the invention, the second oral dose of the recombinant oral vaccine follows the first oral dose by 7 to about 35 days, or by 14 to 35 days or by 7 to 28 days, or by 14 to 28 days or by about 21 days. Optionally, in certain embodiments, the second oral dose of the recombinant oral vaccine may be followed by a third oral dose, wherein the third oral dose by 7 to about 35 days, or by 14 to 35 days or by 7 to 28 days, or by 14 to 28 days or by about 21 days. It is preferred that the animals that receive the recombinant oral

vaccine (whether in two or three oral doses) according to the first aspect of the invention have not been actively vaccinated against CDV prior to receiving the first oral dose of CDV via the recombinant oral vaccine described herein.

[0042] In yet other embodiments of the first aspect of the invention, regardless of whether two or three oral doses of the recombinant oral vaccines described herein are administered, the methods further entail annual revaccinations of the recombinant oral vaccines accomplished via an oral administration of any of the vaccines disclosed herein, about one year after the first administration or a preceding annual oral administration of the vaccine, as would be understood by one of ordinary skill in the art.

[0043] In the second aspect, the recombinant oral vaccines described herein may be used for revaccinations, e.g., annual revaccinations that occur about one year from administering first and second dose of a different vaccine and/or about one year from administering a previous annual revaccination, either with the vaccine described herein or the different vaccine. The term "different vaccine" is interchangeable with the term "initial vaccine" and refers to vaccines containing the same antigen(s) as the vaccine according to the invention (in this case, at least a CDV antigen and a CAV-2 antigen) but not in the form of a recombinant CAV-2 vector comprising the CDV antigen, e.g., CDV H gene.

[0044] Different vaccines suitable for use as the initial vaccines are known in the art and include, without limitations VANGUARD® line (e.g., VANGUARD® DAP containing modified live CAV-2 virus, modified live CDV virus, and modified live CPV virus), or vaccines of RECOMBITEC ® line (e.g., RECOMBITEC ® C3 or RECOMBITEC ® C4) or NOBIVAC® line (e.g., NOBIVAC® CANINE 1-DAPPv). In this aspect, the puppy does not need to be MDA-negative and can be vaccinated with the initial vaccine as early as 4 weeks of age, as instructed by the manufacturer of the respective initial vaccines. One year after the administration of the first dose of said different vaccine, or one year after a previous annual revaccination with said different vaccine, the dog undergoes the annual revaccination comprising orally administering to said dog the recombinant oral vaccine according to any embodiment described above.

[0045] The modified live viruses in the recombinant oral vaccines may be present in the following amounts per dose (whether the first dose, the second dose, the optional third dose according to the first aspect, or the annual revaccination dose according to the first or the second aspect):

modified live CAV-2 carrying the CDV antigen according to any embodiment of the invention: from $10^{2.5}$ $TCID_{50}$ to about 10^{8} $TCID_{50}$, more preferably, from 10^{3} $TCID_{50}$ to about 10^{8} $TCID_{50}$, more preferably, from 10^{3} $TCID_{50}$ to about 10^{6} $TCID_{50}$, from 10^{3} $TCID_{50}$ to about $10^{5.5}$ $TCID_{50}$, from 10^{3} $TCID_{50}$ to about 10^{5} $TCID_{50}$, from 10^{3} $TCID_{50}$ to about $10^{4.5}$ $TCID_{50}$, from about $10^{3.5}$ $TCID_{50}$ to about $10^{5.5}$ $TCID_{50}$, from $10^{3.5}$ $TCID_{50}$ to about 10^{5} $TCID_{50}$, from $10^{3.5}$ $TCID_{50}$ to about $10^{4.5}$ $TCID_{50}$, from $10^{3.5}$ $TCID_{50}$ to about 10^{4} $TCID_{50}$, from about $10^{3.7}$ $TCID_{50}$ to about $10^{5.5}$ $TCID_{50}$, from $10^{3.7}$ $TCID_{50}$ to about 10^{5} $TCID_{50}$, from $10^{3.7}$ $TCID_{50}$ to about $10^{4.5}$ $TCID_{50}$, from $10^{3.7}$ $TCID_{50}$ to about 10^{4} $TCID_{50}$, or from $10^{3.7}$ $TCID_{50}$ to about 10^{4} $TCID_{50}$;

modified live CPV, if present: $10^{5.5}$ $TCID_{50}$ to about 10^{10} $TCID_{50}$, more preferably $10^{6.5}$ $TCID_{50}$ to about 10^{9} $TCID_{50}$, or $10^{6.5}$ $TCID_{50}$ to about 10^{8} $TCID_{50}$, or $10^{7.5}$ $TCID_{50}$ to about 10^{10} $TCID_{50}$, or $10^{7.5}$ $TCID_{50}$ to about 10^{9} $TCID_{50}$;

modified live CPI, if present: $10^{4.5}$ $TCID_{50}$ to about 10^{8} $TCID_{50}$, more preferably $10^{5.5}$ $TCID_{50}$ to about 10^{8} $TCID_{50}$, or $10^{6.5}$ $TCID_{50}$ to about 10^{8} $TCID_{50}$, or $10^{7.5}$ $TCID_{50}$ to about 10^{8} $TCID_{50}$, or $10^{4.5}$ $TCID_{50}$ to about 10^{7} $TCID_{50}10^{5.5}$ $TCID_{50}$ to about 10^{6} $TCID_{50}$, more preferably $10^{6.5}$ $TCID_{50}$ to about 10^{8} $TCID_{50}$, or $10^{6.5}$ $TCID_{50}$ to about 10^{7} $TCID_{50}$.

[0046] In addition to the antigens, the recombinant oral vaccines described herein may contain other components, including without limitations pharmaceutically acceptable excipients, including carriers, solvents, and diluents, isotonic agents, buffering agents, stabilizers, preservatives, immunomodulatory agents (e.g., interleukins, interferons, and other cytokines), vaso-constrictive agents, antibacterial agents, antifungal agents, and the like. Typical carriers, solvents, and diluents include water, saline, dextrose, ethanol, glycerol, and the like. Representative isotonic agents include sodium chloride, dextrose, mannitol, sorbitol, lactose, and the like. Useful stabilizers include gelatin, albumin, and the like.

[0047] As used herein, "a pharmaceutically acceptable carrier" includes any and all solvents, dispersion media, coatings, adjuvants, stabilizing agents, diluents, preservatives, antibacterial and antifungal agents, isotonic agents, adsorption delaying agents, and the like. The carrier(s)

must be "acceptable" in the sense of being compatible with the components of the invention and not deleterious to the subject to be immunized. Typically, the carriers will be sterile and pyrogen-free, and selected based on the mode of administration to be used. It is well known by those skilled in the art that the preferred formulations for the pharmaceutically accepted carrier which comprise the vaccines are those pharmaceutical carriers approved in the applicable regulations promulgated by the United States (US) Department of Agriculture, or equivalent government agency in a non-US country. Therefore, the pharmaceutically accepted carrier for commercial-production of the vaccines is a carrier that is already approved or will be approved by the appropriate government agency in the US or foreign country.

[0048] The recombinant oral vaccine compositions optionally may include vaccine-compatible pharmaceutically acceptable (i.e., sterile and non-toxic) liquid, semisolid, or solid diluents that serve as pharmaceutical vehicles, excipients, or media. Diluents can include water, saline, dextrose, ethanol, glycerol, and the like. Isotonic agents can include sodium chloride, dextrose, mannitol, sorbitol, and lactose, among others. Stabilizers include albumin, among others.

[0049] The recombinant oral vaccines according to any of the embodiments of the first or the second aspect may be non-adjuvanted. In other embodiments, the recombinant oral vaccines may further comprise an adjuvant. Suitable adjuvants include, without limitations, salts of N-(2-Deoxy-2-L-leucylamino-b-D-glucopyranosyl)-N-octadecyldodecanoylamide, e.g., acetate salt, alone or in combination with other adjuvanting compounds such lecithin and DDA (dimethyl dioctadodecyl ammonium bromide), or PAM3CSK4.

[0050] In yet other embodiments, the recombinant oral vaccines disclosed herein may further contain mucoadhesives. Suitable mucoadhesives include, without limitations natural or synthetic hydrophilic substances which have organic functional groups (carboxyl, hydroxyl, and amino groups) or hydrogen bonds. Some known mucoadhesive polymers are carbomers, cellulose derivatives, alginates, lectins, and tiolated polymers. Specific mucoadhesives include CARBOPOPL® (a lightly cross-linked polyacrylic acid (PAA)), chitosan.

[0051] The recombinant oral vaccines may be administered in the doses having volume of between 0.25 and about 5 ml, e.g., between bout 0.5 ml and about 3 ml, or about 0.5 and 2 ml, or about 1 ml.

[0052] Preferably, in the first aspect, the vaccines are given to dogs that are MDA-negative and are about eight weeks of age or older (e.g., about 10 weeks, about 12 weeks, about 14 weeks, etc). Thus, the first dose would be given to a dog at about four weeks of age (or about six weeks, about eight weeks, about 10 weeks, about 12 weeks, about 14 weeks, etc.), followed by subsequent doses according to the timetable given herein. If the recombinant oral vaccine is administered in the annual revaccination dose, then at the time of the annual revaccination the dog can be as young as about 14 months old (if the puppy had to wait until until it is MDA negative, and only then was orally vaccinated according to the methods of the first aspect of the invention).

[0053] In contrast, if the puppy was vaccinated with the initial vaccine which is different from the recombinant oral vaccine described herein and if the recombinant oral vaccine is administered in the annual revaccination dose, then at the time of the annual revaccination the dog can be as young as 13 months old.

[0054] All publications cited in the specification, both patent publications and non-patent publications, are indicative of the level of skill of those skilled in the art to which this invention pertains. All these publications are herein fully incorporated by reference, to the same extent as if each individual publication were specifically and individually indicated as being incorporated by reference.

[0055] The invention will now be described with reference to the following non-limiting examples.

EXAMPLES

Example 1. Preparation of CAV-2 vector carrying CDV H gene

[0056] The recombinant Canine Adenovirus type 2 expressing the hemagglutinin (H) protein of canine distemper virus (rCAV-2::CDV-H) was generation by an initial transfection of the virulent Manhattan strain of CAV-2 with a plasmid containing a mCherry expression cassette and flanking sequences of gene E3 of the CAV-2 genome (rCAV-mC) cultured in Norden Lab dog kidney (NLDK) cells. A subsequent transfection of rCAV-mC with a plasmid containing a CDV-H expression cassette and the same flanking sequences of the E3 gene was performed to replace mCherry with the H gene from the Onderstepoort CDV strain.

[0057] The expression plasmid pSV40 that was used to construct pCAV-SV40-H was acquired by Zoetis VMRD, Kalamazoo, MI from GenScript.

[0058] The expression plasmid pCAV-SV40-H that was used to replace mCherry and generate CAV-SV40-H was created by cloning the SV40 promoter from pSV40 into NotI-AscI sites of pCAV-CMV-H by digestion and ligation.

[0059] Trypsin was confirmed negative for the following specific extraneous agents in accordance with 9CFR extraneous testing:

Fluorescent antibody (FA) on MDBK cells: bovine adenovirus I, bovine adenovirus III, bovine adenovirus V, bovine parvovirus, bovine viral diarrhea virus I, bovine viral diarrhea virus II, rabies, infectious bovine rhinotracheitis, parainfluenza-3;

FA on Vero cells: bovine respiratory syncytial virus, blue tongue virus, bovine reovirus, bovine rotavirus;

FA on ST cells: porcine adenovirus, porcine hemagglutinating encephalomyelitis, porcine parvovirus, swine influenza virus H1N1 and N3N2, transmissible gastroenteritis;

FA on MA104 cells: porcine reproductive respiratory syndrome, porcine rotavirus;

Absence of extraneous agents was confirmed by CPE and HA on MDBK, Vero, MA 104, and ST cells;

Negative for porcine circovirus strain I &II;

[0060] Pre-irradiated serum was confirmed negative for the following specific extraneous agents in accordance with 9CFR extraneous testing:

FA on MDBK cells: bovine adenovirus I, bovine adenovirus III, bovine adenovirus V, bovine parvovirus, bovine viral diarrhea virus I, bovine viral diarrhea virus II, rabies;

FA on Vero cells: bovine respiratory syncytial virus, blue tongue virus, bovine reovirus, bovine rotavirus;

FA on CRFK cells: bovine reovirus;

[0061] Absence of extraneous agents was confirmed by CPE and HA on MDBK cells, Vero cells, CRFK cells.

[0062] To generate CAV-mC, transfer plasmid pCAV-CMV-mC was linearized by PacI restriction enzyme digestion. The linearized plasmid was transfected into NLDK cells with Lipofectamine 3000 (Life Technologies) and infected with parental CAV-2 virus in 6-well plate. Four days post transfection/infection, the transfected/infected cells were harvested and subject to 4 plaque purifications for red fluorescent plaques by agarose overlay in 6-well plates. One purified fluorescent plaque was further tested by PCR to confirm there was no wild-type CAV virus contamination. This plaque was scaled up in a T-75 flask. The cells and supernatant of the T-75 flask culture was harvested, aliquoted and stored at -80o C freezer. This recombinant virus was designated as CAV-mC.

[0063] To generate pCAV-SV40-H, the SV40 promoter from pSV40 was cloned into the NotI-AscI sites of pCAV-CMV-H by digestion and ligation. Transfer plasmid pCAV-SV40-H was linearized by PacI restriction enzyme digestion. The linearized plasmid was transfected into NLDK cells with Lipofectamine 3000 and infected with CAV-mC virus in a 6-well plate. Four days post transfection/infection, the transfected/infected cells were harvested and subject to 4 plaque purifications for non-fluorescent plaques by agarose overlay in 6-well plates. Seven purified non-fluorescent plaques were further PCR confirmed to contain SV40-H insert.

[0064] Three purified plaques were further passed/expanded, the whole cultures were harvested and stored at -80 C freezer. These 3 plaques were designated as CAV-SV40-H clone 1, clone 2, and clone 3. The passage 3 of the 3 clones were further subjected to sequencing confirmation of the inserts. PCR amplified insert region (SV40-H) of each clone was confirmed to have the correct sequences. IFA staining of the recombinant viruses infected cell culture with available dog anti-CDV sera did not give definitive confirmation of CDV-H expression due to high background.

[0065] One CAV-SV40-H clone was selected as a candidate to advance forward to pre-master seed virus bank. This clone was further expanded through two passages (p4 and p5) by infection of NLDK cells cultured in DMEM medium containing 2 mM L-glutamine, and 10 µg/mL gentamicin to scale up for pre-MSV. Passage 5 material was then inoculated onto NLDK cells cultured in DMEM medium containing L glutamine and 0,05% gentamicin. The pre-MSV was designated

"CAV-SV40-H #1 p6 Lot 221202-011." P6 indicates 6 passages for scaling up (after clonal purification). The pre-MSV was frozen by storage at -80°C.

[0066] The CAV-SV40-H pre-MSV candidate Lot 221202-011 was tested for purity, potency, and identity. Purity was tested using the Modified Sterility/Purity test method and Mycoplasma using the Sigma Aldrich Lookout Mycoplasma qPCR Detection kit, ref: MP0400A-1KT. Potency and identity were tested by IFA method on CAV-SV40H infected NLDK cells.

[0067] Briefly, potency and identity was determined by IFA method on CAV-SV40-H infected NLDK cells. CAV-SV40-H pre-MSV Lot 221202-011 was titrated on NLDK to determine potency. Wells containing CPE were tested for CAV-2 using goat polyclonal antibody specific for CAV-2 as the primary antibody and detected by a bovine anti-goat–IgG Alex Fluor 488 probe (green fluorescence). The CDV-H identity was determined using canine polyclonal antibody as the primary antibody and detected by a rabbit anti-dog labeled with Cy3 probe (red fluorescence). The dual label assay allows for CAV-SV40-H infected cultures to be assessed for CAV-2 and CDV-H expression simultaneously and demonstrate that each well was fluorescent for both.

Example 2: Generation of CAV-2 virus expressing CDV F-protein.

[0068] The materials and methods in this example are similar to those disclosed in Example 1. The nucleic acid sequence of CDV F protein (SEQ ID NO: 4). pCAV-CMV2-F that contains CDV F gene under a truncated CMV promoter was synthesized by GenScript. Transfer plasmid pCAV-CMV2-F was linearized by PacI restriction enzyme digestion. The linearized plasmid was transfected into NLDK cells with Lipofectamine 3000 (Life Technologies) and infected with CAV-mC virus in 6-well plate. Four days post transfection/infection, the transfected/infected cells were harvested and subject to 4 plaque purifications for non-fluorescent plaques by agarose overlay in 6-well plates. Six non-fluorescent plaques from the 4^{th} plaque purification were picked. PCR of N and C-terminus of CMV2-F insert showed all were positive. Three of the plaques were passed in T-25 flask for 3 times and stored at -80° C freezer. The 3 recombinant viruses were designated as CAV-CMV2-F clone 1, clone 2, clone 3. DNAs of the passage 3 viral cultures were extracted, and PCR was performed to confirm the stability.

Example 3. Canine Adenovirus Type 2 carrying CDV antigens confers protective response to CDV challenge.

[0069] The objective of the study was to assess the efficacy of two modified live vaccines when administered orally in dogs against a Canine Distemper Virus challenge administered intravenously (IV) at a dilution of 1:50 approximately three weeks post-third vaccination. The vaccines utilized a recombinant canine adenovirus-2 (rCAV-2) expressing the canine distemper virus (CDV) H or F protein.

[0070] Thirty (30) beagles, approximately 7-10 weeks of age on Day 0, were randomly assigned to three treatment groups, 10 dogs per treatment group, using a randomized complete block-design, with block based on dam first and then date of birth. Animals in treatment group T01 served as controls receiving saline. Animals in treatment group T02 received rCAV-2::CDV H. Animals in treatment group T03 received rCAV-2::CDVH & rCAV-2::CDVF. Vaccinations were administered orally in the buccal pouch on Days 0, 21, and 42. All animals were challenged with CDV Snyder Hill Strain 1:50 dilution intravenously on Day 63.

[0071] At the time of the first vaccination, the age of the puppies varied from 7 weeks, 4 days to 8 weeks, 6 days. Dogs were healthy and seronegative (SN Titer <2) to CDV.

[0072] Feed was consistent with the standard practices of the testing facility. Diet was dry food suitable for the age and nutritional requirements of the animals, moistened if necessary, and provided *ad libitum* at least once daily through the course of the study. Canned food or non-medicated nutritional supplements were also be given as needed. Lot numbers of dry and canned food and supplements were documented. Water was available *ad libitum* at all times. Dogs were given water bowls in addition to the automatic watering system during the first few weeks upon arrival to the vaccination phase facility.

[0073] Anti-parasitic and/or antibiotic treatments were administered at the supplier site according to their procedures. Animals received additional pretreatments as determined by ARS Veterinarians as needed upon arrival for up to three (3) days.

[0074] Serology samples (approximately 6 mL) were collected in SST tubes from all animals on Days 0, 21, and 42 (prior to each vaccination) Day 63 (prior to challenge), and 84. Nasal swabs were collected using polyester swabs and sterile collection tubes containing approximately 3.0

mL of Viral Transport Media (VTM supplemented with antibiotics) prior to Day 0. Fecal swabs were collected (one swab per 3.0 mL VTM tube) prior to Day 0. Clinical Observations were recorded on Day -1, twice on Day 0 (prior to vaccination and 3-6 hours post-vaccination), once daily on Days 1-7 and 20, twice on Day 21 (prior to vaccination and 3-6 hours post-vaccination), once daily on Days 22-28 and 41, twice daily on Day 42 (prior to vaccination and 3-6 hours post-vaccination), and once daily on Days 43-49 during the vaccination phase. Clinical Observations were recorded on Day 62, twice on Days 63-68, three times on Days 69-75, twice on Days 76-83 and one on Day 84 during the challenge phase. Tympanic temperatures were recorded once on Day -1, twice on Day 0 (prior to vaccination and 3-6 hours post-vaccination), once daily on Days 1-7 and 20, twice on Day 21 (prior to vaccination and 3-6 hours post-vaccination), once daily on Days 22-28 and 41, twice daily on Day 42 (prior to vaccination and 3-6 hours post-vaccination), and once daily on Days 43-49 during the vaccination phase. Tympanic temperatures were recorded once on Day 62, twice on Day 63 (prior to challenge and approximately 3-6 hours post-challenge), and at least once daily Days 64 to 84 during the challenge phase.

Post-challenge, the veterinarians considered the humane endpoints of the study.

Potency assays

[0075] A monoclonal antibody against the CDV H protein was not available at the time the study was performed. Zoetis study B6563 was performed to generate this antibody using the beacon. Therefore, no results were available for the CDV H protein utilized in T02 and T03. The amount of the CAV-2 carrying the H protein of CDV was quantifying by determining the amount of CAV-2. The amounts of the antigens per dose were as follows: in T02: total CAV-2 was $10^{3.7}$ $TCID_{50}$, in T03: total CAV-2 was $10^{3.7}$ $TCID_{50}$, CAV-2 carrying F-protein was $10^{3.42}$ $TCID_{50}$. The challenge material was administered in the amount of $10^{3.3}$ $TCID_{50}$ per dose.

Results

[0076] The outcome criteria were based on 9 CFR 113.306: Immunogenicity of Canine Distemper Vaccine. All animals in the study must have been seronegative to CDV on Day 0 (prior to vaccination). The control animals must have remained seronegative until challenge.

[0077] Disease/mortality was the primary variable. Nine of 10 vaccinates must have survived the observation period without showing disease. The efficacy of the vaccine was declared when a vaccinated group (T02, T03 and/or T04) was significantly different than the control group (T01) at alpha = 0.10 (two-sided).

[0078] The outcome criteria for this study were met in T02 when fever was not included in post-challenge clinical signs of disease. In 9 CFR section 113.306 "clinical disease" is defined by 1) Fever. 2) Clinical signs canine distemper virus. 3) Any neurologic sign or moribund behavior. 4) death. All of the animals had a fever observed during at least one time point post-challenge. Fevers were also recorded post-vaccination in all treatment groups including the control animals who received saline.

[0079] A difference in the severity of fever between the control animals and the vaccinated animals was observed. Duration of fevers between the groups of animals could not be assessed, as 80% of the control animals had died by 8 days post-challenge (Study Day 72). In addition to numerically higher fevers than the vaccinated groups, the control animals also exhibited the clinical signs of agitation, anorexia, dehydration, depression, diarrhea, ocular discharge, muscle tremors, pain, bleeding, photophobia, vomiting, and death (euthanasia by reaching humane end-points). Therefore, the definition of disease was modified to not include fever in any of the treatment groups.

[0080] All vaccinated groups (T02-T04) showed a significant difference from the control group (T01) in the frequency of disease when fever was excluded from the other clinical signs in the definition of disease. All of the animals in the T01 control group had disease while none of the animals in T02 had disease (P = <0.0001), and two of the animals in T03 had disease (P = 0.0003). The data are summarized in Table 1.

Table 1. Frequency Distributions of Clinical Observations of Disease With and Without Fever

	Disease (fever included)		Disease (fever not included)				Total No. of Animals
	Yes		No		Yes		
Treatment	No. of Animals	%	No. of Animals	%	No. of Animals	%	
T01	10	100.00	0	0.00	10	100.00	10
T02	10	100.00	10	100.00	0	0.00	10

Treatment	Disease (fever included)		Disease (fever not included)				Total No. of Animals
	Yes		No		Yes		
	No. of Animals	%	No. of Animals	%	No. of Animals	%	
T03	10	100.00	8	80.00	2	20.00	10

[0081] Without fever, the stratified prevented fraction of disease was 100% for the rCAV-2::CDV-H vaccinates, and 80% for the rCAV-2::CDV-H / rCAV-2::CDV-F vaccinates. All of the 90% lower bounds were above zero for all vaccinated groups (Table 2).

Table 2. Treatment Comparisons and CMH Estimates of Stratified Prevented Fraction with Confidence Limits of Disease without Fever

Contrast	P-value	Significant at 0.05 level	Stratified Prevented Fraction	90% Lower bound	90% Upper bound
T01 vs T02	<.0001	Yes	1.00		
T01 vs T03	0.0003	Yes	0.800	0.309	0.942

Mortality

[0082] No dogs (0%) in treatment groups T02 (rCAV-2 CDV H) or T03 (rCAV-2 CDV H & rCAV-2 CDV F) were removed from study and/or died post-challenge (Table 3). Eight dogs (80%) in the control group T01 were removed from study post-challenge (Table 3).

Table 3. Frequency Distribution of Mortality by Treatment Group

Treatment	Mortality				Total Number of Observations
	No		Yes		
	Number	%	Number	%	
T01	2	20.0	8	80.0	10
T02	10	100.0	0	0.0	10
T03	10	100.0	0	0.0	10

Clinical Observations

Vaccination Phase

[0083] Several dogs in all three treatments groups were noted with fever (≥39.5°C) following first, second, and third vaccination; however, dogs in treatment group T02 were noted with fever

on Day -1 and all four treatment groups on Day 0 prior to first vaccination; T01, T03, and T04 on Day 20 and T02-T04 on Day 21 prior to second vaccination; and T02-T03 on Day 41 and all four treatment groups on Day 42 prior to vaccination. There were no additional clinical signs (agitation, anorexia, ataxia, cough, dehydration, depression, diarrhea, moribund, mucopurulent nasal discharge, muscle tremors, pain, petechiae, photophobia, respiratory distress, seizure, swim, vomiting, or other clinical signs) observed in any treatment group following first vaccination.

Challenge Phase

[0084] All animals (100%) in all treatment groups showed an elevated temperature or fever (≥39.5°C) at some observation period post-challenge, however, fevers were also seen frequently in the majority of animals prior to challenge.

[0085] The frequency distributions of ever having a clinical sign post-challenge are listed in Table 4. The T02 group, receiving the CAV-2::CDV-H construct orally, never showed clinical signs of distemper post-challenge.

Table 4. Frequency Distributions of Clinical Signs of Disease Post-Challenge by Treatment Group

Clinical Sign	Treatment	Present Post Challenge?				Total No. of Animals
		No		Yes		
		No. of Animals	%	No. of Animals	%	
Agitation	T01	9	90.00	1	10.00	10
	T02	10	100.00	0	0.00	10
	T03	10	100.00	0	0.00	10
Anorexia	T01	2	20.00	8	80.00	10
	T02	10	100.00	0	0.00	10
	T03	10	100.00	0	0.00	10
Cough	T01	10	100.00	0	0.00	10
	T02	10	100.00	0	0.00	10
	T03	10	100.00	0	0.00	10

Clinical Sign	Treatment	Present Post Challenge?				Total No. of Animals
		No		Yes		
		No. of Animals	%	No. of Animals	%	
Dehydration	T01	1	10.00	9	90.00	10
	T02	10	100.00	0	0.00	10
	T03	10	100.00	0	0.00	10
Depression	T01	0	0.00	10	100.00	10
	T02	10	100.00	0	0.00	10
	T03	10	100.00	0	0.00	10
Diarrhea	T01	8	80.00	2	20.00	10
	T02	10	100.00	0	0.00	10
	T03	10	100.00	0	0.00	10
Fever	T01	0	0.00	10	100.00	10
	T02	0	0.00	10	100.00	10
	T03	0	0.00	10	100.00	10
Mucopurulent Ocular Discharge	T01	9	90.00	1	10.00	10
	T02	10	100.00	0	0.00	10
	T03	10	100.00	0	0.00	10
Muscle Tremors	T01	9	90.00	1	10.00	10
	T02	10	100.00	0	0.00	10
	T03	10	100.00	0	0.00	10
Other*	T01	4	40.00	6	60.00	10
	T02	10	100.00	0	0.00	10
	T03	8	80.00	2	20.00	10
Pain	T01	6	60.00	4	40.00	10
	T02	10	100.00	0	0.00	10
	T03	10	100.00	0	0.00	10
Petechiae	T01	4	40.00	6	60.00	10
	T02	10	100.00	0	0.00	10
	T03	10	100.00	0	0.00	10

Clinical Sign	Treatment	Present Post Challenge?				Total No. of Animals
		No		Yes		
		No. of Animals	%	No. of Animals	%	
Photophobia	T01	7	70.00	3	30.00	10
	T02	10	100.00	0	0.00	10
	T03	10	100.00	0	0.00	10
Vomiting	T01	6	60.00	4	40.00	10
	T02	10	100.00	0	0.00	10
	T03	10	100.00	0	0.00	10

*No animals (0%) in any treatment group were observed with the following clinical signs post-challenge: ataxia, moribund, mucopurulent nasal discharge, respiratory distress, seizure, swim. Other clinical signs included head tremors, hunched posture, decreased body condition, reluctance to stand, stiff gait, vocalizing, and blood in stool.

Serology

[0086] As expected, all animals in T02 (rCAV-2::CDV-H) and T03 (rCAV-2::CDV-H / rCAV-2::CDV-F) seroconverted to CDV (Table 5), CAV-1 (Table 6), and CAV-2 (Table 7) pre-challenge as measured by SN antibody titers. The level of the CAV-1 and CAV-2 titers are such that protection from disease is expected.

Table 5. Geometric Means Summary of CDV Titers by Treatment Group

Treatment	Day of Study	Number of Animals	Geometric Mean	Minimum	Maximum
T01	-1	10	1.0	1.0	1.0
	20	10	1.0	1.0	1.0
	41	10	1.0	1.0	1.0
	62	10	1.0	1.0	1.0
	71	1	1.0	1.0	1.0
	72	7	1.0	1.0	1.0
	84	2	304.4	256.0	362.0
T02	-1	10	1.0	1.0	1.0

Treatment	Day of Study	Number of Animals	Geometric Mean	Minimum	Maximum
	20	10	4.2	1.0	23.0
	41	10	29.9	11.0	64.0
	62	10	59.7	23.0	181.0
	84	10	1782.8	512.0	2896.0
T03	**-1**	10	1.0	1.0	1.0
	20	10	1.9	1.0	11.0
	41	10	38.1	16.0	91.0
	62	10	84.3	11.0	256.0
	84	10	1722.1	1024.0	2896.0

Table 6. Geometric Means Summary of CAV-1 Titers by Treatment Group

Treatment	Day of Study	Number of Animals	Geometric Mean	Minimum	Maximum
T01	**-1**	10	1.0	1.0	1.0
	20	10	1.0	1.0	1.0
	41	10	1.0	1.0	1.0
	62	10	5.5	5.5	5.5
	71	1	5.5	5.5	5.5
	72	6	5.5	5.5	5.5
	84	2	5.5	5.5	5.5
T02	**-1**	10	1.0	1.0	1.0
	20	10	208.2	64.0	512.0
	41	10	831.7	362.0	2048.0
	62	10	776.0	256.0	2048.0
	84	10	891.4	362.0	2048.0
T03	**-1**	10	1.0	1.0	1.0
	20	10	111.7	23.0	256.0
	41	10	461.4	181.0	1024.0
	62	10	675.5	362.0	1448.0
	84	10	512.0	181.0	1024.0

Table 7. Geometric Means Summary of CAV-2 Titers by Treatment Group

Treatment	Day of Study	Number of Animals	Geometric Mean	Minimum	Maximum
T01	-1	10	1.0	1.0	1.0
	20	10	1.0	1.0	1.0
	41	10	1.0	1.0	1.0
	62	10	1.0	1.0	1.0
	71	1	1.0	1.0	1.0
	72	7	1.0	1.0	1.0
	84	2	1.0	1.0	1.0
T02	-1	10	1.0	1.0	1.0
	20	10	54.1	1.0	512.0
	41	10	446.7	23.0	1448.0
	62	10	187.2	11.0	1448.0
	84	10	1217.7	512.0	2896.0
T03	-1	10	1.0	1.0	1.0
	20	10	87.8	3.0	362.0
	41	10	461.4	64.0	1024.0
	62	10	362.0	32.0	1448.0
	84	10	724.0	362.0	1448.0

[0087] All dogs (100%) were negative to CDV, CAV-1, and CAV-2 on Day -1. T01 control animals remained negative to all fractions until post-challenge, at which time the two remaining animals seroconverted to CDV at Day 84. All dogs (100%) in T02 and T03 converted to positive SN titers to CDV, CAV-1, and CAV-2 during the vaccination phase of the study (Table 8).

Table 8. Frequency Distributions of Overall Ever Positive Serum Neutralization (SN) Titer Values (>32) During Vaccination Phase by Treatment Group

	CDV SN Ever Positive				CAV-1 SN Ever Positive				CAV-2 SN Ever Positive				Total No. of Obs.
	No		Yes		No		Yes		No		Yes		
Trt	No.	%	No.	%	No.	%	No.	%	No.	%	No.	%	
T01	10	100.0	0	0.0	10	100.0	0	0.0	10	100.0	0	0.0	10
T02	0	0.0	10	100.0	0	0.0	10	100.0	0	0.0	10	100.0	10

Trt	CDV SN Ever Positive				CAV-1 SN Ever Positive				CAV-2 SN Ever Positive				Total No. of Obs.
	No		Yes		No		Yes		No		Yes		
	No.	%	No.	%	No.	%	No.	%	No.	%	No.	%	
T03	0	0.0	10	100.0	0	0.0	10	100.0	0	0.0	10	100.0	10

Conclusions

[0088] The study was valid. All animals were healthy and seronegative to canine distemper virus (<2) prior to Day 0. Eighty (80) percent of the control animals met mortality endpoints and were humanely euthanized. The remaining 20% of animals had multiple observation periods of clinical signs of disease typical of canine distemper virus. These criterial fulfill the challenge outcomes according to 9 CFR 113.306.

[0089] The stratified prevented fractions for disease without fever in the T02 (rCAV-2::CDV-H) vaccinated group was 100% ($P < 0.0001$) and 80% ($P = 0.0003$) for T03 (rCAV-2::CDV-H and rCAV-2::CDV-F). All of the 90% lower confidence bounds were above 0 for all vaccinated groups.

[0090] Because elevated temperatures were observed in all treatment groups both pre- and post-challenge, analysis was performed both with and without fever in the disease definition. Without fever, 100% of the control animals were diseased post-challenge, none of the T02 animals (rCAV-2::CDV-H) experienced disease post-challenge and two (20%) of the T03 animals (rCAV-2::CDV-H & rCAV-2 CDV F) were diseased post-challenge.

[0091] The control animals remained seronegative to CDV prior to challenge; post-challenge, the two remaining animals seroconverted to CDV by Day 84, showing exposure to the challenge virus. All vaccinated groups (T02, T03) showed an anamnestic response to challenge by geometric mean titers.

[0092] As expected, the animals in T02 and T03 also seroconverted to CAV-1 and CAV-2 due to the rCAV-2 vaccine vector.

[0093] Based on results of this study, rCAV-2::CDV-H construct (T02) appeared to be more effective than rCAV-2::CDV-H & rCAV-2 CDV F (T03) as none of the animals in this group ever presented with disease when fever was excluded, and this vaccine was completely protective

against the intravenous CDV Snyder Hill challenge. The combination of the two rCAV-2 constructs (T03) did not perform any better than the monovalent construct (T02).

CLAIMS

1. A method of protecting an MDA-negative canine against canine distemper virus, the method comprising administering a recombinant oral vaccine modified live canine adenovirus type 2 (CAV-2) vector carrying a Canine Distemper Virus (CDV) antigen to said canine, wherein the recombinant oral vaccine is administered orally in a first dose and orally in a second dose.

2. A method of annually revaccinating a dog, the method comprising orally administering to said dog a vaccine comprising a recombinant oral vaccine comprising a modified live canine adenovirus type 2 (CAV-2) vector carrying a Canine Distemper Virus (CDV) antigen, wherein said recombinant oral vaccine is administered about a year after a previous annual revaccination or about a year after administering to said dog a first dose of an initial vaccine, wherein further said first dose of the initial vaccine is administered to said dog parenterally, and wherein said initial vaccine comprises a CDV antigen and a CAV-2 antigen.

3. The method of claim 2, wherein in said initial vaccine, the CDV antigen is

c) a modified live CDV and the CAV-2 antigen is a modified live CAV-2; or

d) encoded by a nucleic acid sequence within a modified live canarypox virus genome.

4. The method according to claim 2 or claim 3, wherein in said previous annual revaccination the dog is administered a vaccine wherein the CDV antigen is

c) a modified live CDV and the CAV-2 antigen is a modified live CAV-2; or

d) encoded by a nucleic acid sequence within a modified live canarypox virus genome.

5. The method of any one of claims 1-4, wherein in said recombinant oral vaccine the CDV antigen is H protein.

6. The method of any one of claims 1-5, wherein in said recombinant oral vaccine the CDV antigen comprises SEQ ID NO: 1 or a sequence that is 90% or 95% identical to SEQ ID NO: 1.

7. The method of claim 6, wherein in said recombinant oral vaccine said CDV antigen is a conservatively substituted variant of SEQ ID NO: 1.

8. The method of any one of claims 1-7, wherein in said recombinant oral vaccine the genome of the modified live CAV-2 vector comprises a nucleic acid sequence encoding the CDV antigen.

9. The method of claim 8, wherein in said recombinant oral vaccine the nucleic acid sequence encoding the CDV antigen is under operative control of SV-40 promoter.

10. The method of any one of claims 8 or 9 wherein in said recombinant oral vaccine the nucleic acid sequence comprises SEQ ID NO: 2.

11. The method of any one of claims 8-10, wherein in said recombinant oral vaccine the nucleic acid sequence encoding the CDV antigen is inserted into E3 region of the modified live CAV-2 vector genome.

12. The method of any one of claims 1-11, wherein said recombinant oral vaccine contains at least 10^3 $TCID_{50}$ to about 10^8 $TCID_{50}$ of said modified live CAV-2 vector carrying the CDV antigen per dose.

13. The method of claim 12, wherein said recombinant oral vaccine contains about $10^{3.5}$ $TCID_{50}$ to about $10^{4.5}$ $TCID_{50}$ of said modified live CAV-2 vector carrying the CDV antigen per dose.

14. The method of any one of claims 1 and 5-13, wherein said canine has not been previously vaccinated against Canine Distemper virus.

15. The method according to any one of claims 1-14, wherein said modified live canine adenovirus type 2 vector carrying a CDV antigen protects against Canine Distemper Virus, Canine Adenovirus Type 1 infection, and Canine Adenovirus Type 2 infection when administered orally.

16. The method according to claim 15 wherein said canine has not been previously vaccinated against Canine Adenovirus Type 2.

17. The method according to any one of claims 1-16, wherein the recombinant oral vaccine further comprises a modified live parvovirus and wherein said recombinant oral vaccine further protects against Canine Parvovirus infection when administered orally.

18. The method according to claim 17, wherein said recombinant oral vaccine comprises about $10^{5.5}$ $TCID_{50}$ to about 10^{10} $TCID_{50}$ of the modified live Canine Parvovirus.

19. The method according to claim 17, wherein said recombinant oral vaccine comprises $10^{7.5}$ $TCID_{50}$ to about 10^{9} $TCID_{50}$ of the modified live Canine Parvovirus.

20. The method according to any one of claims 17-19 wherein said canine has not been previously vaccinated against Canine Parvovirus.

21. The method according to any one of claims 1-20, wherein the recombinant oral vaccine further comprises modified live Canine Parainfluenza virus, and wherein said recombinant oral vaccine further protects against Canine Parainfluenza virus infection when administered orally.

22. The method according to claim 19, wherein said recombinant oral vaccine comprises $10^{4.5}$ $TCID_{50}$ to about 10^{8} $TCID_{50}$ of the modified live Canine Parainfluenza virus.

23. The method according to claim 19, wherein said recombinant oral vaccine comprises $10^{6.5}$ $TCID_{50}$ to about $10^{7.5}$ $TCID_{50}$ of the modified live Canine Parainfluenza virus.

24. The method according to any one of claims 21-23 wherein said canine has not been previously vaccinated against Canine Parainfluenza virus.

25. The method according to any one of claims 1, 5-24, wherein at the time of administration of the recombinant oral vaccine said canine is about 8 to about 16 weeks old, or the method according to any one of claims 2-13, 15, 17-19, or 21-23 wherein said canine is at least 13 months old at the time of administration of the recombinant oral vaccine.

26. The method according to any one of claims 1, 5-25, wherein the second dose of said recombinant oral vaccine is administered 7-35 days after the first dose of said recombinant oral vaccine.

27. The method according to any one of claims 1, 5-26, wherein the second dose of said recombinant oral vaccine is administered about 21 days after the first dose of said recombinant oral vaccine.

28. The method according to any one of claims 1, 5-27, comprising the step of orally administering a third dose of the recombinant oral vaccine, wherein said third dose is administered about 21 days after the second dose of the recombinant oral vaccine.

29. The method according to any one of claims 1-28 wherein in recombinant oral vaccine the modified live CAV-2 vector is not enterically coated, and wherein the modified live canine Parvovirus if present in said recombinant oral vaccine, is not enterically coated and wherein the modified live canine parainfluenza virus, if present, is not enterically coated.

30. A recombinant oral vaccine comprising a modified live canine adenovirus type 2 (CAV-2) vector carrying a Canine Distemper Virus (CDV) antigen for use in a method of protecting an MDA-

negative canine against CDV infection, to said canine, **characterized in that** the recombinant oral vaccine is administered orally in a first dose, and orally in a second dose.

31. A recombinant oral vaccine comprising a modified live canine adenovirus type 2 (CAV-2) vector carrying a Canine Distemper Virus (CDV) antigen for use in a method of of annually revaccinating a dog, the method comprising orally administering to said dog the recombinant oral vaccine about a year after a previous annual revaccination or about a year after administering to said dog a first dose of an initial vaccine, wherein further said first dose of the initial vaccine is administered to said dog parenterally, and wherein said initial vaccine comprises a CDV antigen and a CAV-2 antigen.

32. The recombinant oral vaccine according to claim 31, wherein in said initial vaccine, the CDV antigen is

a) a modified live CDV and the CAV-2 antigen is a modified live CAV-2; or
b) encoded by a nucleic acid sequence within a modified live canarypox virus genome.

33. The recombinant oral vaccine according to claim 31 or 32, wherein in said previous annual revaccination the dog is administered a vaccine wherein the CDV antigen is

a) a modified live CDV and the CAV-2 antigen is a modified live CAV-2; or
b) encoded by a nucleic acid sequence within a modified live canarypox virus genome.

34. The recombinant oral vaccine for use according to any one of claims 30-33, wherein the CDV antigen is H protein.

35. The recombinant oral vaccine for use according to any one of claims 30-34, wherein in said recombinant oral vaccine the CDV antigen comprises SEQ ID NO: 1 or a sequence that is 90% or 95% identical to SEQ ID NO: 1.

36. The recombinant oral vaccine for use according to claim 35, wherein in said recombinant said CDV antigen is a conservatively substituted variant of SEQ ID NO: 1.

37. The recombinant oral vaccine for use according to any one of claims 30-36, wherein in said recombinant oral vaccine the genome of the modified live CAV-2 vector comprises a nucleic acid sequence encoding the CDV antigen.

38. The recombinant oral vaccine for use according to claim 37, wherein in said recombinant oral vaccine the nucleic acid sequence encoding the CDV antigen is under operative control of SV-40 promoter.

39. The recombinant oral vaccine for use according to any one of claims 37 or 38, wherein in said recombinant oral vaccine the nucleic acid sequence encoding the CDV antigen comprises SEQ ID NO: 2.

40. The recombinant oral vaccine for use according to any one of claims 37-39, wherein in said recombinant oral vaccine the nucleic acid sequence encoding the CDV antigen is inserted into E3 region of the modified live CAV-2 vector.

41. The recombinant oral vaccine for use according to any one of claims 30-40, wherein said recombinant oral vaccine contains at least 10^3 $TCID_{50}$ to about 10^8 $TCID_{50}$ of said modified live CAV-2 vector carrying the CDV antigen per dose.

42. The recombinant oral vaccine for use according to claim 41, wherein said recombinant oral vaccine contains about $10^{3.5}$ $TCID_{50}$ to about $10^{4.5}$ $TCID_{50}$ of said modified live CAV-2 vector carrying the CDV antigen per dose.

43. The recombinant oral vaccine for use according to any one of claims 30, 34-42, wherein said canine has not been previously vaccinated against Canine Distemper virus.

44. The recombinant oral vaccine for use according to any one of claims 30-43, wherein said modified live canine adenovirus type 2 vector carrying a CDV antigen protects against Canine Distemper Virus, Canine Adenovirus Type 1 infection, and Canine Adenovirus Type 2 infection when administered orally.

45. The recombinant oral vaccine for use according to claim 44 wherein said canine has not been previously vaccinated against Canine Adenovirus Type 2.

46. The recombinant oral vaccine for use according to any one of claims 30-45, wherein the recombinant oral vaccine further comprises a modified live parvovirus and wherein said vaccine further protects against Canine Parvovirus infection when administered orally.

47. The recombinant oral vaccine for use according to claim 46, wherein said recombinant oral vaccine comprises about $10^{6.5}$ $TCID_{50}$ to about 10^{10} $TCID_{50}$ of the modified live Canine Parvovirus.

48. The recombinant oral vaccine for use according to claim 46, wherein said recombinant oral vaccine comprises about $10^{5.5}$ $TCID_{50}$ to about 10^{9} $TCID_{50}$ of the modified live Canine Parvovirus.

49. The recombinant oral vaccine for use according to any one of claims 46-48 wherein said canine has not been previously vaccinated against Canine Parvovirus.

50. The recombinant oral vaccine for use according to any one of claims 30-49, wherein the recombinant oral vaccine further comprises modified live Canine Parainfluenza virus, and wherein said recombinant oral vaccine further protects against Canine Parainfluenza virus infection when administered orally.

51. The recombinant oral vaccine for use according to claim 50, wherein said recombinant oral vaccine comprises about $10^{4.5}$ $TCID_{50}$ to about 10^{8} $TCID_{50}$ of the modified live Canine Parainfluenza virus.

52. The recombinant oral vaccine for use according to claim 50, wherein said vaccine comprises $10^{6.5}$ $TCID_{50}$ to about $10^{7.5}$ $TCID_{50}$ of the modified live Canine Parainfluenza virus.

53. The recombinant oral vaccine for use according to any one of claims 50-52 wherein said canine has not been previously vaccinated against Canine Parainfluenza virus.

54. The recombinant oral vaccine for use according to any one of claims 30, 34-53, wherein said canine is about 8 to about 16 weeks old at the time of administration of said recombinant oral vaccine, or the recombinant oral vaccine for use according to any one of claims 31-42, 44, 46-48, 50-52, wherein said canine is at least 13 months old at the time of administration of said recombinant oral vaccine.

55. The recombinant oral vaccine for use according to any one of claims 30, 34-54, wherein the second dose is administered 7-35 days after the first dose.

56. The recombinant oral vaccine for use according to any one of claims 30, 34-55, wherein the second dose is administered about 21 days after the first dose.

57. The recombinant oral vaccine for use according to any one of claims 30, 34-56, wherein the method is further characterized by oral administration of a third dose of the vaccine, wherein said third dose is administered about 21 days after the second dose.

58. The recombinant oral vaccine for use according to any one of claims 30-57 wherein in said recombinant oral vaccine the modified live CAV-2 vector is not enterically coated, and wherein the modified live canine Parvovirus if present in said recombinant oral vaccine, is not enterically

coated and wherein the modified live canine parainfluenza virus, if present in said recombinant oral vaccine, is not enterically coated.

INTERNATIONAL SEARCH REPORT

International application No
PCT/US2024/052376

A. CLASSIFICATION OF SUBJECT MATTER
INV. A61K39/12 C12N15/86
ADD.

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
A61K C07K C12N

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

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

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	WO 98/00166 A1 (RHONE MERIEUX INC [FR]; FISCHER LAURENT [US]) 8 January 1998 (1998-01-08) examples 16-18 examples 24-27 claims 13, 34 page 56, lines 36-37 page 57, lines 32-34 ----- -/--	1-58

[x] Further documents are listed in the continuation of Box C.

[x] See patent family annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier application or patent but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance;; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance;; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
20 January 2025	17/02/2025

Name and mailing address of the ISA/	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Fax: (+31-70) 340-3016	Irion, Andrea

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INTERNATIONAL SEARCH REPORT

International application No
PCT/US2024/052376

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
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Y	FISCHER L ET AL: "Vaccination of puppies born to immune dams with a canine adenovirus-based vaccine protects against a canine distemper virus challenge", VACCINE, ELSEVIER, AMSTERDAM, NL, vol. 20, no. 29-30, 4 October 2002 (2002-10-04), pages 3485-3497, XP004381813, ISSN: 0264-410X, DOI: 10.1016/S0264-410X(02)00344-4 abstract	1-58
Y	CLIQUET FLORENCE ET AL: "Oral vaccination of dogs: a well-studied and undervalued tool for achieving human and dog rabies elimination", VETERINARY RESEARCH, vol. 49, no. 1, 13 July 2018 (2018-07-13), XP093240488, London, UK ISSN: 1297-9716, DOI: 10.1186/s13567-018-0554-6 Retrieved from the Internet: URL:http://link.springer.com/content/pdf/10.1186/s13567-018-0554-6.pdf> page 2, line 2, paragraph r	1-58
Y	ZHANG ET AL: "Oral vaccination of dogs (Canis familiaris) with baits containing the recombinant rabies-canine adenovirus type-2 vaccine confers long-lasting immunity against rabies", VACCINE, ELSEVIER, AMSTERDAM, NL, vol. 26, no. 3, 3 December 2007 (2007-12-03), pages 345-350, XP022419013, ISSN: 0264-410X, DOI: 10.1016/J.VACCINE.2007.11.029 the whole document	1-58

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INTERNATIONAL SEARCH REPORT

International application No.

PCT/US2024/052376

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

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

   a. [X] forming part of the international application as filed.

   b. [ ] furnished subsequent to the international filing date for the purposes of international search (Rule 13*ter*.1(a)).

      [ ] accompanied by a statement to the effect that the sequence listing does not go beyond the disclosure in the international application as filed.

2. [ ] With regard to any nucleotide and/or amino acid sequence disclosed in the international application, this report has been established to the extent that a meaningful search could be carried out without a WIPO Standard ST.26 compliant sequence listing.

3. Additional comments:

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No
PCT/US2024/052376

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