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- (71) **Applicants:** **NEW YORK BLOOD CENTER, INC.** [US/US]; 310 East 67th Street, New York, New York 10065 (US). **REGENTS OF THE UNIVERSITY OF MINNESOTA** [US/US]; 600 McNamara Alumni Center, 200 Oak Street SE, Minneapolis, Minnesota 55455-2020 (US). **OHIO STATE INNOVATION FOUNDATION** [US/US]; 1524 North High Street, Columbus, Ohio 43201 (US).
- (72) **Inventors:** **DU, Lanying**; c/o New York Blood Center, 310 East 67th Street, New York, New York 10065 (US). **JIANG, Shibo**; c/o New York Blood Center, 310 East 67th Street, New York, New York 10065 (US). **LI, Jianrong**; c/o The Ohio State University, College Veterinary Medicine, 1925 Coffey Road, Columbus, Ohio 43210 (US). **LI, Fang**; c/o University of Minnesota Medical School, 6-120 Jackson Hall, 321 Church Street S.E., Minneapolis, Minnesota 55455 (US).
- (74) **Agents:** **CULLMAN, Louis C.** et al.; K&L Gates LLP, 1 Park Plaza, 12th Floor, Irvine, California 92614 (US).
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(54) **Title:** PORCINE EPIDEMIC DIARRHEA VIRUS IMMUNOGENIC COMPOSITIONS

(57) **Abstract:** Disclosed herein are immunogenic compositions for preventing infection with porcine epidemic diarrhea virus (PEDV) wherein the immunogenic compositions comprises a PEDV peptide, and one or both of an immunopotentiator, such as an Fc fragment of immunoglobulin G, and a stabilization sequence. Also disclosed are methods of inducing immune responses against PEDV and preventing infection with PEDV in porcine subjects.



PORCINE EPIDEMIC DIARRHEA VIRUS IMMUNOGENIC COMPOSITIONS

FIELD

[0001] The present disclosure relates to the field of immunogenic compositions for the prevention and treatment of porcine epidemic diarrhea virus infection.

BACKGROUND

[0002] Porcine epidemic diarrhea virus (PEDV) is an infectious and highly contagious coronavirus widely affecting the swine industry. Newly emerging strains exhibit high mortality rates (50-90%) leading to significant economic losses. Development of safe and efficacious vaccines is essential to prevent and control the continual spread of PEDV within the swine industry.

SUMMARY

[0003] Disclosed herein are methods and immunogenic compositions for preventing and treating infection with porcine epidemic diarrhea virus (PEDV). Specifically, an immunogenic composition is provided for induction of an immune response against PEDV comprising a fusion protein comprising or consisting of a PEDV polypeptide, an immunopotentiator selected from the group consisting of the Fc fragment of immunoglobulin G (IgG), C3d, *Onchocerca volvulus* ASP-1, cholera toxin, and muramyl peptides, or a stabilization sequence, or both a immunopotentiator and a stabilization sequence.

[0004] In certain embodiments, the PEDV peptide is a PEDV spike (S) protein, a PEDV S protein S1 fragment, a PEDV S protein S2 fragment, a PEDV S protein S1 subunit N-terminal domain (S1-NTD) sequence, a PEDV S protein S1 subunit C-terminal domain (S1-CTD) sequence, a PEDV S protein fusion peptide (FP) sequence, a PEDV S protein heptad repeat (HR1 and HR2) sequence, and a PEDV S protein transmembrane (TM) sequence. In some embodiments, the PEDV peptide comprises a PEDV S protein S1-NTD sequence. In other embodiments, the PEDV peptide comprises a PEDV S protein S1-CTD sequence. In yet other embodiments, the PEDV peptide comprises both a PEDV S protein S1-NTD sequence and a PEDV S protein S1-CTD sequence.

[0005] In certain embodiments, the Fc fragment of IgG is human, mouse, rabbit, or porcine. In some embodiments, the immunopotentiator is human IgG Fc. In other embodiments, the immunopotentiator is porcine IgG Fc.

[0006] In certain embodiments, the stabilization sequence is foldon or GCN4. In other embodiments, the stabilization sequence is foldon.

[0007] In some embodiments, the polypeptide is a fusion protein. In certain embodiments, if the immunogenic composition comprises a PEDV peptide, an immunopotentiator, and a stabilization sequence, the PEDV peptide is linked to the stabilization sequence and wherein the stabilization sequence is linked to the immunopotentiator in a single polypeptide.

[0008] In some embodiments, the immunogenic composition further comprises an adjuvant.

[0009] Also disclosed herein is a method of inducing a protective immune response against a porcine epidemic diarrhea virus (PEDV) comprising administering an immunogenic composition disclosed herein to a subject in need thereof, and where the immunogenic composition induces a protective immune response against challenge with PEDV in said subject. In certain embodiments, the method further comprises administering a booster dose of the immunogenic composition to the host.

[0010] Also disclosed herein is a method of protecting pigs against infection with PEDV, comprising administering an immunogenic composition disclosed herein to a porcine subject in need thereof and thereby preventing PEDV-infection in the porcine subject. In certain embodiments, the method further comprises administering at least one booster dose of the immunogenic composition to the porcine subject.

[0011] Further disclosed herein is an immunogenic composition for use in inducing a protective immune response against PEDV in a subject in need thereof for the prevention of PEDV infection.

BRIEF DESCRIPTION OF THE DRAWINGS

[0012] FIG. 1A-M depicts the schematic structure of PEDV spike (S) protein S1 and S2 subunits (FIG. 1A) and the construction of recombinant PEDV S1-NTD-Fc (FIG. 1B), S1-CTD-Fc (FIG. 1C), S1-NTD-CTD-Fc (FIG. 1D), S1-NTD-CTD-Fd-Fc (FIG. 1E), S1-NTD-CTD-Fd (FIG. 1F), S1-NTD-CTD-His (FIG. 1G), S1-CTD-Fd-Fc (FIG. 1H), S1-CTD-Fd (FIG. 1I), S1-CTD-His (FIG. 1J), S1-NTD-Fd-Fc (FIG. 1K), S1-NTD-Fd (FIG. 1L), and S1-NTD-His (FIG. 1M) proteins. Functional domains include: signal peptide (SP), PEDV S1 subunit N-terminal domain (S1-NTD, containing amino acids 19-252 of PEDV S1), C-terminal domain (S1-CTD, containing amino acids 509-638 of PEDV S1). Both PEDV S1-NTD and S1-CTD serve as receptor-binding domain (RBD) of PEDV. FP, fusion peptide; HR1 and HR2, heptad repeats 1 and 2, respectively; TM, transmembrane domain; CP, cytoplasmic tail; Fc, human IgG Fc; Fd, foldon (Fd) trimerization motif.

[0013] FIG. 2A-B depicts SDS-PAGE (FIG. 2A) and Western blot (FIG. 2B) analysis of the expressed PEDV S1-NTD-Fc and S1-CTD-Fc proteins. The protein molecular weight marker (kDa) is indicated on the left. Protein samples with or without boiling were used for the assay. PEDV swine sera (1:2,000) were used for Western blot analysis.

[0014] FIG. 3 depicts SDS-PAGE (FIG. 3A) and Western blot (FIG. 3B) analysis of the expressed PEDV S1-NTD-CTD-Fc protein. The protein molecular weight marker (kDa) is indicated on the left. Protein samples with or without boiling were used for the assay. PEDV swine sera (1:2,000) were used for Western blot analysis.

[0015] FIG. 4 depicts IgG antibody responses induced by PEDV S1-NTD-Fc using ELISA. Mouse sera from 10 days post-3rd immunization were tested for PEDV S1-NTD-specific IgG antibody titers. The data are presented as mean $\pm$ standard deviation (SD) of five mice per group. PBS was used as the control.

[0016] FIG. 5 depicts IgG antibody responses induced by PEDV S1-CTD-Fc using ELISA. Mouse sera from 10 days post-3rd immunization were tested for PEDV S1-CTD-specific IgG antibody titers. The data are presented as mean $\pm$ SD of five mice per group. PBS was used as the control.

[0017] FIG. 6 depicts IgG antibody responses induced by PEDV S1-NTD-CTD-Fc using ELISA. Mouse sera from 10 days post-3rd immunization were tested for PEDV S1-specific (coated plates with S1-NTD-CTD-His protein) IgG antibody titers. The data are presented as mean $\pm$ SD of five mice per group. PBS was used as the control.

[0018] FIG. 7 depicts IgG antibody responses induced by PEDV S1-NTD-CTD-Fc using ELISA. Mouse sera (1:3,200 dilution) before (pre-immune) and 10 days post-1st, 2nd, and 3rd immunizations were tested for PEDV S1-specific IgG (A450) antibody responses. The data are presented as mean $\pm$ SD of five mice per group. PBS was used as the control.

[0019] FIG. 8 depicts long-term IgG antibody responses induced by PEDV S1-NTD-CTD-Fc using ELISA. Mouse sera at 0, 10, 31, 52, 83, and 121 days post-immunization were tested for PEDV S1-specific IgG antibody titers, and the data are presented as mean $\pm$ SD of five mice per group. PBS was used as the control.

[0020] FIG. 9 depicts IgG1 antibody responses induced by PEDV S1-NTD-CTD-Fc using ELISA. Mouse sera from 10 days post-3rd immunization were tested for PEDV S1-specific IgG1 antibody titers. The data are presented as mean $\pm$ SD of five mice per group. PBS was used as the control.

[0021] FIG. 10 depicts IgG2a antibody responses induced by PEDV S1-NTD-CTD-Fc using ELISA. Mouse sera from 10 days post-3rd immunization were tested for PEDV S1-

specific IgG2a antibody titers. The data are presented as mean $\pm$ SD of five mice per group. PBS was used as the control.

[0022] FIG. 11 depicts IgG3 antibody responses induced by PEDV S1-NTD-CTD-Fc using ELISA. Mouse sera from 10 days post-3rd immunization were tested for PEDV S1-specific IgG3 antibody titers. The data are presented as mean $\pm$ SD of five mice per group. PBS was used as the control.

[0023] FIG. 12 depicts IgG antibody responses induced by PEDV S1-NTD-CTD-Fd-Fc, S1-NTD-CTD-Fc, S1-NTD-CTD-Fd, and S1-NTD-CTD-His using ELISA. Mouse sera from 10 days post-3rd immunization were tested for PEDV S1-specific IgG antibody titers. The data are presented as mean $\pm$ SD of five mice per group.

[0024] FIG. 13 depicts long-term IgG antibody responses induced by PEDV S1-NTD-CTD-Fd-Fc using ELISA. Mouse sera from 1, 2, 3, 4, 5 months and 10 days post-last immunization at 6 months were tested for PEDV S1-specific IgG antibody titers. The data are presented as mean $\pm$ SD of five mice per group. PBS was used as the control.

[0025] FIG. 14 depicts long-term IgG antibody responses induced by PEDV S1-NTD-CTD-Fc using ELISA. Mouse sera from 1, 2, 3, 4, 5 months and 10 days post-last immunization at 6 months were tested for PEDV S1-specific IgG antibody titers. The data are presented as mean $\pm$ SD of five mice per group. PBS was used as the control.

[0026] FIG. 15 depicts long-term IgG antibody responses induced by PEDV S1-NTD-CTD-Fd using ELISA. Mouse sera from 1, 2, 3, 4, 5 months and 10 days post-last immunization at 6 months were tested for PEDV S1-specific IgG antibody titers. The data are presented as mean $\pm$ SD of five mice per group. PBS was used as the control.

[0027] FIG. 16 depicts long-term IgG antibody responses induced by PEDV S1-NTD-CTD-His using ELISA. Mouse sera from 1, 2, 3, 4, 5 months and 10 days post-last immunization at 6 months were tested for PEDV S1-specific IgG antibody titers. The data are presented as mean $\pm$ SD of five mice per group. PBS was used as the control.

[0028] FIG. 17 depicts neutralizing antibodies induced by PEDV S1-NTD-CTD-Fc. Mice sera from 10 days post-3rd immunization were tested for neutralization against PEDV infection, and the neutralizing antibody titers are presented as the reciprocal of the highest dilution of sera that resulted in a complete inhibition of virus-induced cytopathic effects (CPE) in at least 50% of the wells (NT₅₀). The data are presented as mean $\pm$ SD of five mice per group. PBS was used as the control.

[0029] FIG. 18 depicts antibody responses in gnotobiotic (Gn) piglets immunized with S1-NTD-CTD-Fc or S1-NTD-CTD-Fd (referred to in the figures as NTD-CTD-Fc and NTD-

CTD-Fd, respectively) and later challenged with virulent PEDV. Weekly serum samples were collected from each piglet and tested for PEDV-specific VN antibodies. The assay was repeated 3 times for each sample.

[0030] FIG. 19A-F depicts B cell responses in ileum, mesenteric lymph nodes (MLN), and spleen in Gn piglets immunized with 200 µg S1-NTD-CTD-Fc (Group A), 200 µg S1-NTD-CTD-Fd (Group B), 50 µg S1-NTD-CTD-Fd (Group D), or not-immunized (adjuvant control, Group C) and later challenged with virulent PEDV. At day 5 post challenge, mononuclear cells (MNCs) from ileum, MLN, and spleen were isolated and the frequency of IgA⁺ (FIG. 19A-C) and IgG⁺ (FIG. 19D-F) B cells in each tissue were determined by flow cytometry. * = $p < 0.05$; ** = $p < 0.01$; *** = $p < 0.005$.

[0031] FIG. 20 depicts reduced viral RNA shedding in S1-NTD-CTD-Fc (200 µg) or S1-NTD-CTD-Fd (200 µg or 50 µg) immunized Gn piglets later challenged with virulent PEDV. RNA copies in feces were quantified by real-time RT-PCR. Data were average of 3 piglets.

[0032] FIG. 21 depicts higher antibody responses in conventional piglets immunized with S1-NTD-CTD-Fc or S1-NTD-CTD-Fd and compared to an inactivated PEDV vaccine (200 µg or 500 µg), and later challenged with virulent PEDV.

[0033] FIG. 22 depicts reduction in viral RNA shedding in feces in conventional piglets immunized with S1-NTD-CTD-Fc or S1-NTD-CTD-Fd compared to an inactivated PEDV vaccine and later challenged with virulent PEDV.

DEFINITION OF TERMS

[0034] To facilitate an understanding of the following Detailed Description, Examples and appended claims it may be useful to refer to the following definitions. These definitions are non-limiting in nature and are supplied merely as a convenience to the reader.

[0035] Gene: A “gene” as used herein refers to at least a portion of a genetic construct having a promoter and/or other regulatory sequences required for, or that modify the expression of, the genetic construct.

[0036] Host: As used herein the terms “host” and “subject” refer to the recipient of the present immunogenic compositions. Exemplary hosts are mammals including, but not limited to, primates, rodents, cows, horses, dogs, cats, sheep, goats, pigs and elephants. In some embodiments the host is a pig. For the purposes of this disclosure the terms “host” and “subject” are synonymous with “vaccinee.”

[0037] Immunogen: As used herein the term “immunogen” refers to any substrate that elicits an immune response in a host. Immunogens of the present disclosure include, but are not limited to, proteins from PEDV.

[0038] Immunogenic Composition: As used herein an “immunogenic composition” refers to an expressed protein or a recombinant vector, with or without an adjuvant, which expresses and/or secretes an immunogen *in vivo* and wherein the immunogen elicits an immune response in the host. The immunogenic compositions disclosed herein may or may not be immunoprotective or therapeutic. When the immunogenic compositions may prevent, ameliorate, palliate or eliminate disease from the host then the immunogenic composition may optionally be referred to as a vaccine. However, the term immunogenic composition is not intended to be limited to vaccines.

DETAILED DESCRIPTION

[0039] Porcine epidemic diarrhea virus (PEDV) is an infectious and highly contagious coronavirus widely affecting the swine industry. The newly emerged PEDV strains contained high mortality rates (50-90%), leading to significant economic losses. Therefore, development of efficacious and safe vaccines is essential to prevent and control continual spreading of PEDV within the swine industry. The lack of currently available PEDV vaccines demonstrates an urgent need to develop vaccines against PEDV.

[0040] Like other coronaviruses, PEDV spike (S) protein plays important roles in mediating receptor binding and subsequent entering into target cells, and is thus an important vaccine target. The S protein contains S1 and S2 subunits with S1 binding target cells expressing viral receptor aminopeptidase N (APN), and the S2 being responsible for viral and target cell membrane fusion. Among various vaccine types, including killed and live attenuated virus-based, DNA-based, and subunit vaccines, protein-based subunit vaccines maintain high safety and strong stability, being able to induce sufficient efficiency in the presence of suitable adjuvants or fusion with appropriate carriers.

[0041] Disclosed herein are immunogenic compositions (PEDV subunit vaccines) comprising functional domains of the PEDV S1 subunit, such as an N-terminal domain (S1-NTD, containing amino acids 19-252 of S1 subunit), a C-terminal domain (S1-CTD, containing amino acids 509-638 of S1 subunit), a sequence containing S1-NTD-CTD (covering amino acids 19-638 of S1 subunit), and their ability to bind PEDV's sugar co-receptor N-acetylneuraminic acid (Neu5Ac) and APN receptor, respectively. Both S1-NTD and S1-CTD can serve as PEDV's receptor-binding domains (RBDs). The above residue range for PEDV S1-NTD, S1-CTD, and S1-NTD-CTD may vary, depending on the amino acids required to fold into a stable domain. Thus disclosed herein are expressed

recombinant proteins containing the two identified functional RBDs of PEDV S1 subunit, namely S1-NTD, S1-CTD, and S1-NTD-CTD fused with IgG Fc fragment and/or foldon (Fd) trimeric motif (i.e., S1-NTD-Fc, S1-NTD-Fd-Fc, S1-NTD-Fd, S1-CTD-Fc, S1-CTD-Fd-Fc, S1-CTD-Fd, S1-NTD-CTD-Fc, S1-NTD-CTD-Fd-Fc, S1-NTD-CTD-Fd, etc.), to form conformational structures and thus to increase their immunogenicity, neutralization and protection. These proteins are then used to immunize animals and to evaluate their ability to induce immune responses and neutralizing activity against PEDV infection.

Table 1. Components that can be used for design of immunopotentiator-linked PEDV vaccines

Immunogen sequences of PEDV	Stabilization molecule or method to form trimer or oligomer	Immunopotentiators or modulators
S protein	GCN4	cholera toxin
S1 sequence	Foldon	immunomodulators (such as cytokines)
S2 sequence	2,2-bipyridine-5-carboxylic acid (BPY)	bacterial LPS
S1-NTD (N-terminal domain) sequence	Disulfide bonds	Synthetic LPS mimetic RC529
S1-CTD (C-terminal domain) sequence	Facile ligation	muramyl peptides
Fusion peptide (FP) sequences	IQ	Monophosphoryl lipid A (MPL)
Heptad repeat (HRs) sequences of S protein	IZ	dsRNA complexes
Nucleocapsid (NP) sequences		CpG ODN, CTA1-DD
Membrane (M) protein sequences		IgG Fc
Envelope (E) protein sequences		C3d
		ASP-1
		TGF- β or Th2 cytokines

Table 2. Amino acid sequences of immunopotentiator-linked PEDV immunogenic compositions

PEDV spike (S) protein (amino acids 1-1386) (SEQ ID NO:1):
 MKSLTYFWLFLPVLSTLSLPQDVTRCSANTNFRRFFSKFNVQAPAVVVLGGYLP IGENQGVN
 STWYCAGQHPTASGVHGI FVSHIRGGHGFEIGISQEPFDPSGYQLYLHKATNGNTNATARLR
 ICQFPSIKTLGPTANNDVTTGRNCLFNKAI PAHMSEHSVVGITWDNDRVTVFSDKIYYFYFK
 NDWSRVATKCYNSSGCAMQYVYEPTYMYMLNVT SAGEDGI FYQPCTANCIGYAANVFATEPNG
 HIPEGFSFNNWFLLSNDSTLVHGKVVSNQPLL VNCLLAI PKIYGLGQFFSFNQITIDGVCNGA
 AVQRAPEALRFNINDTSVILAEGSIVLHTALGTNFSFVCSNSSNPHLATFAI PLGATQVPYY
 CFLKVDTYNSTVYKFLAVLPPTVREIVITKYGDVYVNGFGYLHLGLLDAVTINFTGHGTTTTD
 VSGFWTIASTNFVDALIEVQGTAIQRILYCDDPVSQ LKCSQVAFDLDDGFGFYPISSRNLLSHE

QPISFVTLP SFNDHSFVNITVSASF GGHS GANLIASDTT ING FSSFCVDTRQFTISLFYNVT
 NSYGYVSKSQDSNCPFTLQSVNDYLSFSKFCVSTSL LASACTIDLFGYPEFGSGVKFTSLYF
 QFTKGELITGTPKPLEGVTDV SFMTLDVCTKYTIYGFKGEGIIITLTNSSFLAGVYYTSDSGQ
 LLAFKNVTSGAVYSVTPCSFSEQAAYVDDDIVGVISSLSSTFNSTRELPGFFYHSNDGSNC
 TEPVLVYSNIGVCKSGSIGYVPSQSGQVKIAPT VTGNISIP TNFMSIRTEYLQLYNTPVSV
 DCATYVCNGNSRCKQLLTQYTAACKTIESALQLSARLESVEVNSMLTISDEALQLATISSFN
 GDGYNFTNVLGVSVYDPASGRVVQKRSFIEDLLFNKVVTNGLGTVDEDEYKRCSNGRSVADLV
 CAQYYSGVMVLPGVVDAEKLHMY SASLIGGMVLGGFTSAAALPFSYAVQARLNYLALQTDVL
 QRNQQLLAESFNSAIGNITSAFESVKEAISQTSKGLNTVAHALTKVQEVVNSQGAALTQLT
 VQLQHNFAQIAISSIDDIYSRLDILSADVQVDRLITGRLSALNAFVAQTLTKYTEVQASRKLAQ
 QKVNECVKSQSQR YGFCGGDGEHIFSLVQAAPQGLLFLHTVLVPSDFVDVIAIAGLCVNDEI
 ALTLREPGLVLFTH ELQNHTATEYFVSSRRMFEP RKPTVSDFVQIESCVV TYVNLTRDQLPD
 VIPDYIDVNKTLDEILASLPNRTGPSLPLDVFNATYLNLTGEIADLEQRSESLRNTTEELQS
 LIYNINNTLV DLEWLN RVETIYIKWPWWVWLIIFIVLIFVVSLLVFCCI STGCCGCCGCCAC
 FSGCCRGPR LQPYEVFEKVHVQ

PEDV S1 protein (containing PEDV S amino acids 19-740) (SEQ ID NO:2):

LPQDVTRCSANTNFRFFSKFNVQAPAVVVLGGYLP IGENQGVNSTWY CAGQHPTASGVHGI
 FVSHIRGGHGFEIGISQEPFDPSGYQLYLHKATNGNTNATARLRICQFP SIKTLGPTANNDV
 TTGRNCLFNKAI PAHMSEHSVVGITWDNDRVTVFSDKIYFYFKNDWSRVATKCYNSSGCCAM
 QYVYEPTY YMLNVT SAGEDGIFYQPCTANCIGYAANVFATEPNGHIPEGFSFNNWFLLSNDS
 TLVHGKVVS NQPLL VNCLLAIPKIYGLGQFFSFNQ TIDGVCNGAAVQRAPEALRFNINDTSV
 ILAEGSIVLHTALGTNFSFVCSNSSNPHLATFAIPLGATQVPYYCFLKVD TYNSTVYKFLAV
 LPPTVREIVITKYGDVYVNGFGYLHLGLLDAVTINFTGHGTDDDVS GFWTIASTNFVDALIE
 VQGTAIQRILYCDDPVSQ LKCSQVAFDLDDGFYPISSRNLLSHEQPISFVTLP SFNDHSFVN
 ITVSASF GGHS GANLIASDTT ING FSSFCVDTRQFTISLFYNVTNSYGYVSKSQDSNCPFTL
 QSVNDYLSFSKFCVSTSL LASACTIDLFGYPEFGSGVKFTSLYFQFTKGELITGTPKPLEGV
 TDVSFMTLDVCTKYTIYGFKGEGIIITLTNSSFLAGVYYTSDSGQLLAFKNVTSGAVYSVTPC
 SFSEQAAYVDDDIVGVISSLSSTFNSTRELPGFFYHSND

PEDV S2 protein (containing PEDV S amino acids 741-1386) (SEQ ID NO:3):

GSNCTEPVLVYSNIGVCKSGSIGYVPSQSGQVKIAPT VTGNISIP TNFMSIRTEYLQLYN
 TVSVD CATYVCNGNSRCKQLLTQYTAACKTIESALQLSARLESVEVNSMLTISDEALQLATI
 SSFNGDGYNFTNVLGVSVYDPASGRVVQKRSFIEDLLFNKVVTNGLGTVDEDEYKRCSNGRSV
 ADLVCAQYYSGVMVLPGVVDAEKLHMY SASLIGGMVLGGFTSAAALPFSYAVQARLNYLALQ
 TDVLQRNQQLLAESFNSAIGNITSAFESVKEAISQTSKGLNTVAHALTKVQEVVNSQGAALT
 QLT VQLQHNFAQIAISSIDDIYSRLDILSADVQVDRLITGRLSALNAFVAQTLTKYTEVQASR
 KLAQQKVNECVKSQSQR YGFCGGDGEHIFSLVQAAPQGLLFLHTVLVPSDFVDVIAIAGLCV
 NDEIALTLREPGLVLFTH ELQNHTATEYFVSSRRMFEP RKPTVSDFVQIESCVV TYVNLTRD
 QLPDVIPDYIDVNKTLDEILASLPNRTGPSLPLDVFNATYLNLTGEIADLEQRSESLRNTTE
 ELQSLIYNINNTLV DLEWLN RVETIYIKWPWWVWLIIFIVLIFVVSLLVFCCI STGCCGCCGC
 CCACFSGCCRGPR LQPYEVFEKVHVQ

PEDV S1-NTD-CTD protein (containing PEDV S1 amino acids 19-638) (SEQ ID NO:4):

LPQDVTRCSANTNFRFFSKFNVQAPAVVVLGGYLP IGENQGVNSTWY CAGQHPTASGVHGI
 FVSHIRGGHGFEIGISQEPFDPSGYQLYLHKATNGNTNATARLRICQFP SIKTLGPTANNDV
 TTGRNCLFNKAI PAHMSEHSVVGITWDNDRVTVFSDKIYFYFKNDWSRVATKCYNSSGCCAM
 QYVYEPTY YMLNVT SAGEDGIFYQPCTANCIGYAANVFATEPNGHIPEGFSFNNWFLLSNDS
 TLVHGKVVS NQPLL VNCLLAIPKIYGLGQFFSFNQ TIDGVCNGAAVQRAPEALRFNINDTSV
 ILAEGSIVLHTALGTNFSFVCSNSSNPHLATFAIPLGATQVPYYCFLKVD TYNSTVYKFLAV
 LPPTVREIVITKYGDVYVNGFGYLHLGLLDAVTINFTGHGTDDDVS GFWTIASTNFVDALIE

VQGTAIQRILYCDDPVSQKCSQVAFDLDDGFYPISSRNLLSHEQPISFVTLPSFNDHSFVN
ITVSASFEGHSGANLIASDTTNGFSSFCVDTRQFTISLFYNVTNSYGYVSKSQDSNCPFTL
QSVNDYLSFSKFCVSTSLASACTIDLFGYPEFGSGVKFTSLYFQFTKGELITGTPKPLEGV

PEDV S1-NTD protein (containing PEDV S1 amino acids 19-252) (SEQ ID NO:5):

LPQDVTRCSANTNFRFFFSKFNQAPAVVVLGGYLPIGENQGVNSTWYCAGQHPTASGVHGI
FVSHIRGGHGFEIGISQEPFDPSPGYQLYLHKATNGNTNATARLRICQFPSIKTLGPTANNDV
TTGRNCLFNKAI PAHMSEHSVVGITWDNDRVTVFSDKIYYFYFKNDWSRVATKCYNSSGCAM
QYVYEPTYMYMLNVT SAGEDGIFYQPCTANCIGYAANVFATEPNGHIPE

PEDV S1-CTD protein (containing PEDV S1 amino acids 509-638) (SEQ ID NO:6):

DHSFVNITVSASFEGHSGANLIASDTTNGFSSFCVDTRQFTISLFYNVTNSYGYVSKSQDS
NCPFTLQSVNDYLSFSKFCVSTSLASACTIDLFGYPEFGSGVKFTSLYFQFTKGELITGTP
KPLEGV

PEDV S1-NTD-CTD-Fc protein (containing PEDV S1 amino acids 19-638 with hFc) (SEQ ID NO:7):

LPQDVTRCSANTNFRFFFSKFNQAPAVVVLGGYLPIGENQGVNSTWYCAGQHPTASGVHGI
FVSHIRGGHGFEIGISQEPFDPSPGYQLYLHKATNGNTNATARLRICQFPSIKTLGPTANNDV
TTGRNCLFNKAI PAHMSEHSVVGITWDNDRVTVFSDKIYYFYFKNDWSRVATKCYNSSGCAM
QYVYEPTYMYMLNVT SAGEDGIFYQPCTANCIGYAANVFATEPNGHIPEGFSFNNWFLLSNDS
TLVHGKVVSNOPLLVNCLLAI PKIYGLGQFFSFNQIDGVCNGAAVQRAPEALRFNINDTSV
ILAEGSIVLHTALGTNFSFVCSNSSNPHLATFAI PLGATQVPYYCFLKVDVTYNSTVYKFLAV
LPPTVREIVITKYGDVYVNGFGYLHLGLLDAVTINFTGHGTDDDVS GFWTIASTNFVDALIE
VQGTAIQRILYCDDPVSQKCSQVAFDLDDGFYPISSRNLLSHEQPISFVTLPSFNDHSFVN
ITVSASFEGHSGANLIASDTTNGFSSFCVDTRQFTISLFYNVTNSYGYVSKSQDSNCPFTL
QSVNDYLSFSKFCVSTSLASACTIDLFGYPEFGSGVKFTSLYFQFTKGELITGTPKPLEGV
RSDKTHTCPPCPAPELLGGPSVFLFPKPKDTLMISRTPEVTCVVVDVSHEDPEVKFNWYVD
GVEVHNAKTKPREEQYNSTYRVVSVLTVLHQDWLNGKEYKCKVSNKALPAPIEKTISKAKGQ
PREPQVYITLPPSREEMTKNQVSLTCLVKGFYPSDIAVEWESNGQPENNYKTTTPVLDSDGSF
FLYSKLTVDKSRWQQGNVVFSCSVMEALHNHYTQKSLSLSPGK

PEDV S1-NTD-CTD-Fd protein (containing PEDV S1 amino acids 19-638 with foldon (Fd) sequence) (SEQ ID NO:8):

LPQDVTRCSANTNFRFFFSKFNQAPAVVVLGGYLPIGENQGVNSTWYCAGQHPTASGVHGI
FVSHIRGGHGFEIGISQEPFDPSPGYQLYLHKATNGNTNATARLRICQFPSIKTLGPTANNDV
TTGRNCLFNKAI PAHMSEHSVVGITWDNDRVTVFSDKIYYFYFKNDWSRVATKCYNSSGCAM
QYVYEPTYMYMLNVT SAGEDGIFYQPCTANCIGYAANVFATEPNGHIPEGFSFNNWFLLSNDS
TLVHGKVVSNOPLLVNCLLAI PKIYGLGQFFSFNQIDGVCNGAAVQRAPEALRFNINDTSV
ILAEGSIVLHTALGTNFSFVCSNSSNPHLATFAI PLGATQVPYYCFLKVDVTYNSTVYKFLAV
LPPTVREIVITKYGDVYVNGFGYLHLGLLDAVTINFTGHGTDDDVS GFWTIASTNFVDALIE
VQGTAIQRILYCDDPVSQKCSQVAFDLDDGFYPISSRNLLSHEQPISFVTLPSFNDHSFVN
ITVSASFEGHSGANLIASDTTNGFSSFCVDTRQFTISLFYNVTNSYGYVSKSQDSNCPFTL
QSVNDYLSFSKFCVSTSLASACTIDLFGYPEFGSGVKFTSLYFQFTKGELITGTPKPLEGV
GYIPEAPRDGQAYVRKDG EWVLLSTFL

PEDV S1-NTD-CTD-Fd-Fc protein (containing PEDV S1 amino acids 19-638 with Fd and hFc) (SEQ ID NO:9):

LPQDVTRCSANTNFRFFFSKFNQAPAVVVLGGYLPIGENQGVNSTWYCAGQHPTASGVHGI
FVSHIRGGHGFEIGISQEPFDPSPGYQLYLHKATNGNTNATARLRICQFPSIKTLGPTANNDV

TTGRNCLFNKAI PAHMSEHSVVGITWDNDRVTVFSDKIYYFYFKNDWSRVATKCYNSSGGCAM
 QYVYEPTYTYMLNVT SAGEDGIFYQPCTANCIGYAANVFATEPNGHIPEGFSFNNWFLLSNDS
 TLVHGKVVSNOPLLNVNCLLAIPKIYGLGQFFSFNQITIDGVCNGAAVQRAPEALRFNINDTSV
 ILAEGSIVLHTALGTNFSFVCSNSSNPHLATFAIPLGATQVPYYCFLKVDTYNSTVYKFLAV
 LPPTVREIVITKYGDVYVNGFGYLHLGLLDAVTINFTGHGTDDDVS GFWTIAS TNFVDALIE
 VQGTAIQRILYCDPVSQ LKCSQVAFDLDDGFYPISSRNLLSHEQPISFVTLP SFNDHSFVN
 ITVSASFVGHSGANLIASDTTNGFSSFCVDTRQFTISLFYNVTNSYGYVSKSQDSNCPFTL
 QSVNDYLSFSKFCVSTSLASACTIDLFGYPEFGSGVKFTSLYFQFTKGELITGTPKPLEGV
 GYIPEAPRDGQAYVRKDGEWVLLSTFLRSDKTHTCPPCPAPELLGGPSVFLFPPKPKDTLMI
 SRTPEVTCVVVDVSHEDPEVKFNWYVDGVEVHNAKTKPREEQYNSTYRVVSVLTVLHQDWLN
 GKEYKCKVSNKALPAPIEKTISKAKGQPREPQVYTLPPSREEMTKNQVSLTCLVKGFYPSDI
 AVEWESNGQPENNYKTTPPVLDSDGSFFLYSKLTVDKSRWQQGNV FSCSV MHEALHNHYTQK
 SLSLSPGK

PEDV S1-NTD-CTD-His protein (containing PEDV S1 amino acids 19-638 with His₆) (SEQ ID NO:10):

LPQDVTRCSANTNFRFFFSKFNQAPAVVVLGGYLP IGENQGVNSTWYCAGQHPTASGVHGI
 FVSHIRGGHGFEIGISQEPFDPSGYQLYLHKATNGNTNATARLRICQFPSIKTLGPTANNDV
 TTGRNCLFNKAI PAHMSEHSVVGITWDNDRVTVFSDKIYYFYFKNDWSRVATKCYNSSGGCAM
 QYVYEPTYTYMLNVT SAGEDGIFYQPCTANCIGYAANVFATEPNGHIPEGFSFNNWFLLSNDS
 TLVHGKVVSNOPLLNVNCLLAIPKIYGLGQFFSFNQITIDGVCNGAAVQRAPEALRFNINDTSV
 ILAEGSIVLHTALGTNFSFVCSNSSNPHLATFAIPLGATQVPYYCFLKVDTYNSTVYKFLAV
 LPPTVREIVITKYGDVYVNGFGYLHLGLLDAVTINFTGHGTDDDVS GFWTIAS TNFVDALIE
 VQGTAIQRILYCDPVSQ LKCSQVAFDLDDGFYPISSRNLLSHEQPISFVTLP SFNDHSFVN
 ITVSASFVGHSGANLIASDTTNGFSSFCVDTRQFTISLFYNVTNSYGYVSKSQDSNCPFTL
 QSVNDYLSFSKFCVSTSLASACTIDLFGYPEFGSGVKFTSLYFQFTKGELITGTPKPLEGV
 HHHHHH

PEDV S1-NTD-Fc protein (containing PEDV S1 amino acids 19-252 with hFc) (SEQ ID NO:11):

LPQDVTRCSANTNFRFFFSKFNQAPAVVVLGGYLP IGENQGVNSTWYCAGQHPTASGVHGI
 FVSHIRGGHGFEIGISQEPFDPSGYQLYLHKATNGNTNATARLRICQFPSIKTLGPTANNDV
 TTGRNCLFNKAI PAHMSEHSVVGITWDNDRVTVFSDKIYYFYFKNDWSRVATKCYNSSGGCAM
 QYVYEPTYTYMLNVT SAGEDGIFYQPCTANCIGYAANVFATEPNGHIPERSDKTHTCPPCPAP
 ELLGGPSVFLFPPKPKDTLMI SRTPEVTCVVVDVSHEDPEVKFNWYVDGVEVHNAKTKPREE
 QYNSTYRVVSVLTVLHQDWLNGKEYKCKVSNKALPAPIEKTISKAKGQPREPQVYTLPPSRE
 EMTKNQVSLTCLVKGFYPSDIAVEWESNGQPENNYKTTPPVLDSDGSFFLYSKLTVDKSRWQ
 QGNVFSCSV MHEALHNHYTQKSLSLSPGK

PEDV S1-NTD-Fd protein (containing PEDV S1 amino acids 19-252 with Fd) (SEQ ID NO:12):

LPQDVTRCSANTNFRFFFSKFNQAPAVVVLGGYLP IGENQGVNSTWYCAGQHPTASGVHGI
 FVSHIRGGHGFEIGISQEPFDPSGYQLYLHKATNGNTNATARLRICQFPSIKTLGPTANNDV
 TTGRNCLFNKAI PAHMSEHSVVGITWDNDRVTVFSDKIYYFYFKNDWSRVATKCYNSSGGCAM
 QYVYEPTYTYMLNVT SAGEDGIFYQPCTANCIGYAANVFATEPNGHIPEGYIPEAPRDGQAYV
 RKDGEWVLLSTFL

PEDV S1-NTD-Fd-Fc protein (containing PEDV S1 amino acids 19-252 with Fd and hFc) (SEQ ID NO:13):

LPQDVTRCSANTNFRFFFSKFNQAPAVVVLGGYLP IGENQGVNSTWYCAGQHPTASGVHGI
 FVSHIRGGHGFEIGISQEPFDPSGYQLYLHKATNGNTNATARLRICQFPSIKTLGPTANNDV

TTGRNCLFNKAIPAHMSEHSVVGITWDNDRVTVFSDKIYYFYFKNDWSRVATKCYNSGGCAM
 QYVYEPTYMLNVTSAAGEDGIFYQPCTANCIGYAANVFATEPNGHIPEAPRDGQAYV
 RKDGEWVLLSTFLRSDKTHTCPPCPAPELLGGPSVFLFPPKPKDTLMISRTPEVTCVVVDVS
 HEDPEVKFNWYVDGVEVHNAKTKPREEQYNSTYRVVSVLTVLHQDWLNGKEYKCKVSNKALP
 APIEKTISKAKGQPREPQVYTLPPSREEMTKNQVSLTCLVKGFYPSDIAVEWESNGQPENNY
 KTTTPVLDSDGSFFLYSKLTVDKSRWQQGNVFCFSVMHEALHNHYTQKSLSLSPGK

PEDV S1-NTD-His protein (containing PEDV S1 amino acids 19-252 with His₆) (SEQ ID NO:14):

LPQDVTRCSANTNFRFFFSKFNQAPAVVVLGGYLPIGENQGVNSTWYCAGQHPTASGVHGI
 FVSHIRGGHGFEGISQEPFDPSGYQLYLHKATNGNTNATARLRICQFPSIKTLGPTANNDV
 TTGRNCLFNKAIPAHMSEHSVVGITWDNDRVTVFSDKIYYFYFKNDWSRVATKCYNSGGCAM
 QYVYEPTYMLNVTSAAGEDGIFYQPCTANCIGYAANVFATEPNGHIPEHHHHHH

PEDV S1-CTD-Fc protein (containing PEDV S1 amino acids 509-638 with hFc) (SEQ ID NO:15):

DHSFVNITVSASFSGGHSGANLIASDTTNGFSSFCVDTRQFTISLFYNVTNSYGYVSKSQDS
 NCPFTLQSVNDYLSFSKFCVSTSLASACTIDLFGYPEFGSGVKFTSLYFQFTKGELITGTP
 KPLEGVRSDKTHTCPPCPAPELLGGPSVFLFPPKPKDTLMISRTPEVTCVVVDVSHEDPEVK
 FNWYVDGVEVHNAKTKPREEQYNSTYRVVSVLTVLHQDWLNGKEYKCKVSNKALPAPIEKT
 ISKAKGQPREPQVYTLPPSREEMTKNQVSLTCLVKGFYPSDIAVEWESNGQPENNYKTTTPVL
 DSDGSFFLYSKLTVDKSRWQQGNVFCFSVMHEALHNHYTQKSLSLSPGK

PEDV S1-CTD-Fd protein (containing PEDV S1 amino acids 509-638 with Fd) (SEQ ID NO:16):

DHSFVNITVSASFSGGHSGANLIASDTTNGFSSFCVDTRQFTISLFYNVTNSYGYVSKSQDS
 NCPFTLQSVNDYLSFSKFCVSTSLASACTIDLFGYPEFGSGVKFTSLYFQFTKGELITGTP
 KPLEGVGYIPEAPRDGQAYVRKDGEWVLLSTFL

PEDV S1-CTD-Fd-Fc protein (containing PEDV S1 amino acids 509-638 with Fd and hFc) (SEQ ID NO:17):

DHSFVNITVSASFSGGHSGANLIASDTTNGFSSFCVDTRQFTISLFYNVTNSYGYVSKSQDS
 NCPFTLQSVNDYLSFSKFCVSTSLASACTIDLFGYPEFGSGVKFTSLYFQFTKGELITGTP
 KPLEGVGYIPEAPRDGQAYVRKDGEWVLLSTFLRSDKTHTCPPCPAPELLGGPSVFLFPPK
 KDTLMISRTPEVTCVVVDVSHEDPEVKFNWYVDGVEVHNAKTKPREEQYNSTYRVVSVLTVL
 HQDWLNGKEYKCKVSNKALPAPIEKTISKAKGQPREPQVYTLPPSREEMTKNQVSLTCLVK
 GFYPSDIAVEWESNGQPENNYKTTTPVLDSDGSFFLYSKLTVDKSRWQQGNVFCFSVMHEAL
 HNHYTQKSLSLSPGK

PEDV S1-CTD-His protein (containing PEDV S1 amino acids 509-638 with His₆) (SEQ ID NO:18):

DHSFVNITVSASFSGGHSGANLIASDTTNGFSSFCVDTRQFTISLFYNVTNSYGYVSKSQDS
 NCPFTLQSVNDYLSFSKFCVSTSLASACTIDLFGYPEFGSGVKFTSLYFQFTKGELITGTP
 KPLEGVHHHHHH

[0042] In some embodiments, the stabilization sequence comprises a sequence that stabilizes the PEDV sequence in a trimer or oligomer configuration. As used herein, the terms stabilization sequence, trimeric motif and trimerization sequence are interchangeable

and equivalent. Suitable stabilization sequences include, but are not limited to, foldon, a 27 amino acid region of the C-terminal domain of T4 fibrin (GYIPEAPRDGQAYVRKDG EWLLSTFL, SEQ ID NO:19 or GSGYIPEAPRDGQAYVRKDG EWLLSTFL, SEQ ID NO:20), GCN4 (MKQIEDKIEEILSKIYHIENEIARIKKLIGEV; SEQ ID NO. 21), IQ (RMKQIEDKIEEIESKQKKIENEIARIKK; SEQ ID NO. 22), or IZ (IKKEIEAIKKEQ EAIKKKIEAIEK; SEQ ID NO. 23). Other suitable stabilization methods include, but are not limited to, 2,2-bipyridine-5-carboxylic acid (BPY), disulfide bonds, and facile ligation.

[0043] In other embodiments, the immunopotentiator comprises a sequence to enhance the immunogenicity of the immunogenic composition. Suitable immunopotentiators include, but are not limited to, the Fc fragment of human IgG, C3d (a complement fragment that promotes antibody formation binding to antigens enhancing their uptake by dendritic cells and B cells), ASP-1 (*Onchocerca volvulus* homologue of the activation associated secreted gene family) (see US 20060039921, which is incorporated by reference herein for all it discloses regarding ASP-1 adjuvants), cholera toxin, muramyl peptides and cytokines.

[0044] In some embodiments, the immunopotentiator is an immunoglobulin Fc fragment. The immunoglobulin molecule consists of two light (L) chains and two heavy (H) chains held together by disulfide bonds such that the chains form a Y shape. The base of the Y (carboxyl terminus of the heavy chain) plays a role in modulating immune cell activity. This region is called the Fc (fragment, crystallizable) region, and is composed of two heavy chains that contribute two or three constant domains depending on the class of the antibody. By binding to specific proteins, the Fc region ensures that each antibody generates an appropriate immune response for a given antigen. The Fc region also binds to various cell receptors, such as Fc receptors, and other immune molecules, such as complement proteins. By doing this, the Fc region mediates different physiological effects including opsonization, cell lysis, and degranulation of mast cells, basophils and eosinophils. Exemplary immunoglobulin Fc fragments include, but are not limited to, human IgG Fc (SEQ ID NO:24), mouse IgG Fc (SEQ ID NO:25), rabbit IgG Fc (SEQ ID NO:26), and porcine IgG Fc (SEQ ID NO:27). Also within the scope of the present disclosure is a fragment of any one of SEQ ID NOs:24-27 which retains the immunomodulatory activity of the intact IgG Fc sequence of SEQ ID NOs:24-27.

[0045] Disclosed herein are immunogenic compositions comprising functional domains of the PEDV S1 subunit, namely the NTD and the CTD, fused with the IgG Fc fragment and/or foldon (Fd) trimeric motif to form conformational structures to thus increase the composition's immunogenicity, neutralization, and protection characteristics. These proteins

are then used to immunize animals and to evaluate their ability to induce immune responses and neutralizing activity against PEDV infection.

[0046] In some embodiments, pFUSE-hIgG1-Fc (human Fc, hFc), pFUSE-mIgG2a-Fc2 (murine Fc, mFc), or pFUSE-rIgG2-Fc2 (rabbit Fc, rFc) vectors are used for construction of the disclosed fusion proteins. In other embodiments, the fusion proteins can be expressed from other mammalian cell expression vectors, including, but not limited to, pcDNA3.1, pcDNA6-His, pJW4303, PEE13.1, PEE14.1, pCMV-NEO-BAM, pSV2, and pCMV1, 2, 3, 4, 5, 6. In other embodiments, the fusion proteins can be expressed from insect cell expression vectors including, but not limited to, pAcGP67, pFastBac Dual, and pMT/V5-His-TOPO. In yet other embodiments, the fusion proteins can be expressed from *E. coli* expression vectors including, but not limited to, pET, pET-SUMO, and pGEX vectors with GST.

[0047] The following expression systems are suitable for use in expressing the disclosed fusion proteins: mammalian cell expression systems such as, but not limited to, the pcDNA and GS Gene expression systems; insect cell expression systems such as, but not limited to, Bac-to-Bac, baculovirus and DES expression systems; and *E. coli* expression systems including, but not limited to, pET, pSUMO and GST expression systems.

[0048] Advantages of proteins expressed in mammalian cell expression systems include the follows. The mammalian cell expression system is a relatively mature eukaryotic system for expression of recombinant proteins. It is more likely to achieve correctly folded soluble proteins with proper glycosylation, making the expressed protein maintain its native conformation and keep sufficient bioactivity. This system can either transiently or stably express recombinant antigens, and promote signal synthesis. Recombinant proteins expressed in this way may keep good antigenicity and immunogenicity. However, both insect and bacterial expression systems provide inexpensive and efficient expression of proteins which may be appropriate under certain conditions.

[0049] The purification systems are dependent on whether a tag is linked or fused with the PEDV protein sequence. When the fusion proteins are fused with IgG Fc vectors, Protein A or Protein G affinity chromatography is used for the purification. If the fusion proteins are fused with GST proteins, the GST columns will be used for the purification. If the fusion proteins link with 6xHis tag at the N- or C-terminal, the expressed proteins are be purified using His tag columns. If no tag is linked with recombinant proteins, the expressed proteins could be purified using fast protein liquid chromatography (FPLC), High performance liquid chromatography (HPLC) or other appropriate chromatography.

[0050] In certain embodiments, the immunogenic compositions further comprise or are administered with an adjuvant. Adjuvants suitable for use in animals and/or humans include, but are not limited to, Freund's complete or incomplete adjuvants, SIGMA ADJUVANT SYSTEM[®] (SAS; Sigma Aldrich, St. Louis, MO), Ribi adjuvants, MF59[®] (an oil-in-water emulsion adjuvant; Novartis AG, Basel, Switzerland), MONTANIDE[®] ISA 51 or 720 (a mineral oil-based or metabolizable oil-based adjuvant; Seppic SA, Puteaux, FR), aluminum hydroxide, -phosphate or -oxide, HAVLOGEN[®] (an acrylic acid polymer-based adjuvant, Intervet Inc., Millsboro, DE), monophosphoryl lipid A (MPLA, a derivative of lipid A), glucopyranosyl lipid adjuvant (GLA)-AF (aqueous formulation) or SE (stable emulsion), polyacrylic acids, oil-in-water or water-in-oil emulsion based on, for example a mineral oil, such as BAYOL[™] or MARCOL[™] (Esso Imperial Oil Limited, Canada), or a vegetable oil such as vitamin E acetate, saponins, and *Onchocerca volvulus* activation-associated protein-1 (ASP-1) (see US 7,700,120, which is incorporated by reference herein for all it discloses regarding ASP-1 adjuvants). However, components with adjuvant activity are widely known and, generally, any adjuvant may be utilized that does not adversely interfere with the efficacy or safety of the vaccine and/or immunogenic composition.

[0051] Vaccine and immunogenic compositions according to the various embodiments disclosed herein can be prepared and/or marketed in the form of a liquid, frozen suspension or in a lyophilized form. Typically, vaccines and/or immunogenic compositions prepared according to the present disclosure contain a pharmaceutically acceptable carrier or diluent customarily used for such compositions. Carriers include, but are not limited to, stabilizers, preservatives and buffers. Suitable stabilizers are, for example SPGA, TWEEN[®] compositions (such as are available from A.G. Scientific, Inc., San Diego, CA), carbohydrates (such as sorbitol, mannitol, starch, sucrose, dextran, glutamate or glucose), proteins (such as dried milk serum, albumin or casein) or degradation products thereof. Non-limiting examples of suitable buffers include alkali metal phosphates. Suitable preservatives include, but are not limited to, thimerosal, merthiolate and gentamicin. Diluents include, but are not limited to, water, aqueous buffer (such as buffered saline), alcohols and polyols (such as glycerol).

[0052] Also disclosed herein are methods for inducing an immune response to a PEDV using the disclosed immunogenic compositions (PEDV subunit vaccines). Generally, the vaccine or immunogenic composition may be administered subcutaneously, intradermally, intranasally, mucosally, or intramuscularly in an effective amount to prevent infection from the PEDV of interest and/or treat an infection from the PEDV. An effective amount is defined as an amount of immunizing immunogenic composition that will induce immunity in the

immunized animals, against challenge by a virulent virus. Immunity is defined herein as the induction of a significant higher level of protection in a population of the animal after immunization compared to a non-immunized group.

[0053] Further disclosed herein is the administration several doses of the immunogenic compositions in a prime-boost strategy. After an initial immunization, a plurality of booster doses of immunogenic composition can be provided to the subject. In certain embodiments, at least one booster immunization is administered 1 week, 2 weeks, 3 weeks, 4 weeks, 6 weeks, 2 months, 3 months, 4 months, or more after an initial immunization. The number of boosters can be 1, 2, 3, 4, or more booster immunizations.

[0054] Further, in various formulations of the vaccines and/or immunogenic compositions, suitable excipients, stabilizers, and the like may be added.

EXAMPLES

Example 1

Construction, expression and immunogenicity of recombinant PEDV fusion proteins

[0055] **Construction, expression and purification of recombinant proteins.** The construction, expression and purification of recombinant PEDV proteins fused with Fd and/or Fc of human IgG were performed as follows. Briefly, genes encoding amino acids 19-252, 509-638, and 19-638, respectively, of PEDV S protein S1 subunit were amplified by PCR using synthesized codon-optimized PEDV S sequences (GenBank: AII20254) as the template, and inserted into the pFUSE-hlgG1-Fc2 expression vector (hereinafter named Fc) to produce PEDV S1-NTD-Fc, S1-CTD-Fc, and S1-NTD-CTD-Fc. PEDV S1-NTD-Fd-Fc, S1-CTD-Fd-Fc, or S1-NTD-CTD-Fd-Fc was constructed by adding Fd at the C-terminus of S1-NTD, S1-CTD, or S1-NTD-CTD and then inserted into the Fc vector. PEDV S1-NTD-Fd, S1-CTD-Fd, S1-NTD-CTD-Fd, S1-NTD-His, S1-CTD-His, and S1-NTD-CTD-His were constructed by adding Fd to the C-terminus of S1-NTD, S1-CTD, or S1-NTD-CTD, and then inserted into pJW4303 expression vector (for S1-NTD-Fd, S1-CTD-Fd, or S1-NTD-CTD-Fd), or directly inserting S1-NTD, S1-CTD, or S1-NTD-CTD to this vector (for S1-NTD-His, S1-CTD-His, or S1-NTD-CTD-His), all of which contain a C-terminal His₆ tag from the vector. The sequence-confirmed recombinant plasmids were transfected into 293T cells seeded 24 h before transfection, followed by replacing culture medium with serum-free DMEM 8-10 h later, and collecting supernatant containing expressed proteins 72 h post-transfection. The recombinant proteins with Fc tag (i.e., S1-NTD-Fc, S1-CTD-Fc, S1-NTD-CTD-Fc, S1-NTD-Fd-Fc, S1-CTD-Fd-Fc, and S1-NTD-CTD-Fd-Fc) were purified by Protein A affinity chromatography, and the proteins with His₆ tag (i.e., S1-NTD-Fd, S1-CTD-Fd, S1-NTD-CTD-

Fd, S1-NTD-His, S1-CTD-His, and S1-NTD-CTD-His) were purified by NI-NTA SUPERFLOW[®] (Qiagen, Hilden, DE).

[0056] SDS-PAGE and Western blot. The purified proteins were analyzed by SDS-PAGE and Western blot. Briefly, the proteins were separated by 10% Tris-glycine gels (either native or boiled for 10 min), which were then stained with Coomassie Blue or transferred to nitrocellulose membranes for Western blot analysis. After blocking with 5% non-fat milk in PBST (phosphate buffered saline with TWEEN[®]) overnight at 4°C, the blots were incubated for 1 h at room temperature with anti-PEDV swine sera (1:2,000). After three washes, the blots were then incubated with horseradish peroxidase (HRP)-conjugated goat anti-pig IgG (1:3,000) for 1 h at room temperature. Signals were visualized with ECL (enhanced chemiluminescence) Western blot substrate reagents and HYPERFILM[®] (Amersham, Little Chalfont, UK).

[0057] Mouse immunization and sample collection. Mice were subcutaneously (s.c.) prime-immunized with recombinant proteins (10 µg/mouse) plus MF59[®] adjuvant and boosted twice with the same immunogen and adjuvant at 3-week intervals. Sera were collected before immunization and 10 days post-each immunization to detect PEDV S1-specific IgG antibodies and neutralizing antibodies.

[0058] ELISA. Collected mouse sera were tested for PEDV S1-specific antibody responses by ELISA. Briefly, 96-well ELISA plates were respectively precoated with recombinant proteins overnight at 4°C and blocked with 2% non-fat milk for 2 h at 37°C. Serially diluted mouse sera were added to the plates and incubated at 37°C for 1 h, followed by four washes. Bound antibodies were incubated with HRP-conjugated goat anti-mouse IgG (1:3,000), IgG1 (1:2,000), IgG2a (1:3,000), and IgG3 (1:2,000) for 1 h at 37°C. The reaction was visualized by substrate 3,3',5,5'-tetramethylbenzidine (TMB) and stopped with 1N H₂SO₄. The absorbance at 450 nm (A450) was measured by ELISA plate reader.

[0059] Neutralization assay. Neutralizing antibody titers of mouse sera against PEDV infections were detected as follows. Briefly, serial 2-fold dilutions of mouse sera were incubated with 100 50% tissue culture infective dose (TCID₅₀) of PEDV VBS2 strain for 1 h at 37°C prior to addition to the monolayer of Vero CCL-81 cells in five replicates. Virus supernatant was removed and replaced with fresh medium. Cytopathic effect (CPE) in each well was observed daily and recorded on day 5 post-infection. The neutralizing antibody titers were expressed as the highest dilution of mouse sera that completely prevented virus-induced CPE in at least 50% of the wells (NT₅₀).

[0060] Recombinant proteins containing NTD and CTD of the PEDV S1 subunit were highly expressed and purified that induced potent antibody responses with neutralizing activity in immunized animals.

[0061] The recombinant PEDV proteins were constructed as shown in Figure 1B-M. The purified PEDV S proteins were characterized and their reactivity determined using PEDV-specific antibodies. Recombinant proteins containing amino acids 19-252, 509-638, and 19-638 of PEDV S1, respectively, were fused with human IgG Fc (i.e., PEDV S1-NTD-Fc, S1-CTD-Fc, and S1-NTD-CTD-Fc) (FIG. 1B-D), and expressed at a high level in the culture supernatant of transfected 293T cells, and purified to high purity (FIG. 2A, 3A). The molecular weight of these proteins was shown as twice in the native (non-reducing, dimer) versus the boiled (reducing, monomer) samples (FIG. 2A, 3A), and reacted strongly with hyperimmune sera of gnotobiotic swine immunized with inactivated PEDV (FIG. 2B, 3B), suggesting that the expressed Fc-fused PEDV S proteins were capable of forming a stable, dimeric conformational structure and showing high specificity to PEDV.

[0062] Analysis of the IgG antibody endpoint titers revealed that high titers of IgG antibodies were induced by the S1-NTD-Fc, S1-CTD-Fc, and S1-NTD-CTD-Fc immunogenic compositions, respectively, against S1-NTD, S1-CTD, and S1-NTD-CTD proteins (FIG. 4-6), suggesting their specificity for the S1 of PEDV. Results also showed that the IgG antibody induced by S1-NTD-CTD-Fc rapidly reached a high level after the 2nd immunization, and then maintained this similar level after the 3rd immunization (FIG. 7). As expected, only background levels of IgG antibody response were induced in the control mice injected with PBS (FIG. 4-7).

[0063] To investigate the kinetics of antibody responses elicited by S1-NTD-CTD-Fc, the mice were observed for up to about 121 days post-immunization and their serum antibody titers periodically determined. Results depicted in FIG. 8 demonstrated that PEDV S1-specific IgG reached at the highest titers after the 2nd immunization. In spite of a slight decrease afterwards, IgG antibodies were still maintained at high levels during the detection period of four months, indicating that PEDV S1-NTD-CTD-Fc has the capability to induce highly potent, long-term specific IgG antibody responses in the immunized mice.

[0064] To elucidate the IgG subtypes induced by S1-NTD-CTD-Fc, IgG1, IgG2a and IgG3 antibodies were detected in sera 10 days post-3rd immunization. As expected, high titers of IgG1 (indicative of a Th2 response) (FIG. 9), IgG2a (indicative of a Th1 response) (FIG. 10), and IgG3 (FIG. 11) antibodies were induced specifically targeting PEDV S1 protein. However, only background level of IgG subtypes was induced in the control immunized (PBS injected)mice (FIG. 9-11). The above data confirm the ability of PEDV S1-

NTD-CTD-Fc in the induction of high levels of specific antibody subtypes in the immunized mice.

[0065] IgG antibody responses induced by S1-NTD-CTD-Fd-Fc, S1-NTD-CTD-Fc, S1-NTD-CTD-Fd, and S1-NTD-CTD-His were compared using mouse sera at 10 days post-3rd immunization (FIG. 12). The data showed that S1-NTD-CTD-Fd-Fc and S1-NTD-CTD-His induced the highest and the lowest antibody responses, respectively, while S1-NTD-CTD fused with Fc (S1-NTD-CTD-Fc) or Fd (S1-NTD-CTD-Fd) were able to induce higher titers of antibodies than S1-NTD-CTD without fusion tags (i.e., S1-NTD-CTD-His).

[0066] Further evaluation of the immunogenicity of S1-NTD-CTD-Fd-Fc, S1-NTD-CTD-Fc, S1-NTD-CTD-Fd, and S1-NTD-CTD-His in inducing long-term immune responses revealed that all of these proteins can induce high IgG antibody responses in mice and maintain the response for at least 6 months (FIG. 13-16). Particularly, S1-NTD-CTD fused with Fd and Fc (S1-NTD-CTD-Fd-Fc) induced the highest titers of antibody responses (FIG. 13), followed by S1-NTD-CTD-Fd (FIG. 15) and S1-NTD-CTD-Fc (FIG. 14).

[0067] Neutralizing antibodies induced by S1-NTD-CTD-Fc were evaluated in mouse sera collected at 10 days post-3rd immunization and tested using a Vero CCL-81 cell-based PEDV neutralization assay. Results depicted in FIG. 17 demonstrated that high titers of neutralizing antibodies were elicited in the immunized mice against PEDV infection, while the PBS control group did not induce any detectable neutralizing antibody. These data confirm the ability of S1-NTD-CTD-Fc to induce neutralizing antibody responses.

[0068] In summary, recombinant PEDV-immunoenhancer fusion proteins formed stable, conformational structures, and induced highly potent immune responses in immunized mice. Particularly, strong neutralizing antibodies were elicited by S1-NTD-CTD-Fc that neutralized PEDV infection, indicating the high potential of this vaccine candidate to effectively protect animals from PEDV infection. These data suggest the possibility for further development of these recombinant PEDV S1 proteins as effective and safe subunit vaccines against PEDV. It provides a novel and useful means for future development of vaccines and antiviral agents against PEDV and other coronaviruses with similar spike glycoprotein structures.

Example 2

PEDV Virus Stocks

[0069] **Isolation and propagation of PEDV strain VBS10.** Intestinal contents were collected from piglets with severe diarrhea on two farms in Ohio, USA. PEDV RNA was detected in the samples by reverse transcription polymerase chain reaction (RT-PCR) and the strain was designated PEDV VBS10. Intestine contents from infected piglets were

homogenized in cell culture medium (DMEM, Dulbecco's Modified Eagle Medium). The suspension was centrifuged at 5,000xg for 10 min at 4°C. The supernatant was collected and filtered through a 0.45 µm pore size filter. The supernatant was further filtered through a 0.22 µm pore-size filter and used as an inoculum for virus isolation. This inoculum was designated as passage 0 (P0).

[0070] To propagate the virus, T25 flasks of Vero cells were inoculated with 0.5 ml of filtered supernatant. Two hours after infection, the inoculum was removed and washed three times with cell culture medium (MEM, Minimum Essential Media). The infected Vero cells were maintained in 3 ml of MEM supplemented with 10% fetal bovine serum (FBS), 2 mM L-glutamine, 0.1 mg/ml gentamicin, 20 unit/ml penicillin, 20 µg/ml streptomycin, and 0.25 µg/ml amphotericin. Twelve hours post-infection, the medium was replaced with 3 ml of MEM supplemented with tryptose phosphate broth (TPB, 0.3%), yeast extract (0.02%), and trypsin 250 (10 µg/ml). When an extensive cytopathic effect (CPE) was observed, the cells were subjected to freeze-thaw three times. The cell culture mixtures were centrifuged at 5,000xg for 10 min at 4°C. The supernatant was collected and designated as P1. The P1 supernatants were used to inoculate new Vero cells for a second passage (P2). After 3 passages, typical CPEs such as cell enlargement, cell-cell fusion, syncytium formation, and death were observed. The presence of PEDV at each passage was confirmed by RT-PCR.

[0071] Enumeration of infectious PEDV by plaque assay. PEDV plaque assays were performed in Vero CCL81 cells. Briefly, cells were seeded into six-well plates at a density of 2×10^6 cells per well. After 24 h of incubation, cell monolayers were infected with 400 µl of a 10-fold dilution series of PEDV, and the plates were incubated for 1 h at 37°C, with agitation every 10 min. The cells were overlaid with 2.5 ml of DMEM containing 0.25% agarose, 0.018% TPB, 0.02% yeast extract, 5 µg/ml trypsin 1:250, 10 UI/ml penicillin-streptomycin, 0.05 mg/ml gentamicin, and 0.05 mg/ml kanamycin. After 48 h incubation, the plates were fixed in 10% formaldehyde, and the plaques were visualized by staining with 0.05% (wt/vol) crystal violet.

[0072] Preparation of aluminum formulated PEDV inactivated antigens. For formalin inactivation, purified PEDV stocks were incubated with 0.05% formalin (ThermoFisher, Waltham, MA) at 37°C for 18 h. The formalin was then neutralized by 0.05 M sodium metabisulfite for 24 h at 4°C. Loss of viral infectivity was confirmed by titration of inactivated virus preparations in cell culture. For vaccine preparation, 1.2 mg of formalin inactivated PEDV was formulated with 3 ml of Alhydrogel Aluminum Hydroxide Gel Adjuvant (InvivoGen, San Diego, CA) in 1 ml of PBS. This vaccine is hereinafter referred to as

inactivated PEDV vaccine. The products were stored at 4°C and inoculated to pigs on the same day.

Example 3

Efficacy of PEDV subunit vaccine in gnotobiotic piglets

Materials and Methods

[0073] All animal protocols were approved by the Institutional Laboratory Animal Care and Use Committee of The Ohio State University (IACUC-OSU). Gnotobiotic (Gn) piglets were delivered into a sterile environment via Cesarean section from a specific-pathogen-free gravid sow and housed in germ-free isolation units. Piglets were fed a milk-replacement diet (PERMALAT®, Permalatt Products, Inc., Bremen, IN) and maintained as described elsewhere. A total of 15 one-week-old Gn piglets were randomly divided into 5 groups of 3 piglets/group. Fecal swabs and blood for serum were collected before immunization and before PEDV challenge in addition to the other times discussed herein. Piglets of groups 1-3 were immunized with 200 µg S1-NTD-CTD-Fd, 200 µg S1-NTD-CTD-Fc, and 50 µg S1-NTD-CTD-Fd, respectively. Piglets in group 4 were immunized intramuscularly with 0.5 ml of MF59® adjuvant alone. Piglets in group 5 were immunized intramuscularly with 0.2 ml of MF59® adjuvant alone and served as unchallenged controls. At day 14 post-immunization, piglets in groups 1-3 were boosted with 200 µg S1-NTD-CTD-Fd, 200 µg S1-NTD-CTD-Fc, and 50 µg S1-NTD-CTD-Fd, respectively (the same immunogenic composition as used in the initial immunization). At day 17 post-booster immunization, piglets in groups 1-4 were challenged orally with 5.0 ml of DMEM containing 10² PFU of PEDV. After challenge, the piglets were observed and evaluated daily for weight and body temperature changes, and clinical signs of PEDV infection. Daily rectal mucosal/fecal swabs were collected from each piglet and diarrhea/fecal consistency score was assigned to each using a subjective scale wherein 0 = normal, 1 = creamy, 2 = pasty and 3 = watery. Pigs with fecal consistency scores of 2 or 3 were scored as diarrhea-positive. Piglets were terminated at PID 5. The day of challenge with infectious agent is PID 0, and thus PID is five days after infection.

[0074] Prior to termination, a blood sample for serum was collected from heavily sedated piglets and each piglet received EUTHASOL® (Virbac Corporation, Fort Worth, TX) solution intravenously to effect. Intestinal contents from the duodenum, proximal jejunum, ileum, transverse colon, and descending colon were collected from each pig. For this, approximately 2 cm of intestine segment was clipped at both ends, 0.5 ml of OPTI-MEM® (ThermoFisher) was injected by a sterile syringe into the intestinal lumen, the lumen was gently massaged and then all the liquid was withdrawn with the same syringe. Samples were homogenized, and infectious virus particles were detected by plaque assay or real-time RT-

PCR. Adjacent intestinal tissue segments (duodenum, jejunum, ileum, and colon), lung, kidney, liver, and spleen were collected from each pig for viral detection and histologic examination.

[0075] Additionally, mononuclear cells (MNC) from the ileum, mesenteric lymph nodes (MLN), and spleen were isolated. Briefly, ileum, spleen, and MLN tissues were cut into tiny pieces and only ileum tissues were treated with Type II collagenase after treating with EDTA and dithiothreitol. Cell suspensions were obtained after passing the digested tissues of ileum, spleen, and MLN through stainless steel 80 μ m mesh screen (CELLECTOR[®], Bellco Glass, Inc., Vineland, NJ). The harvested MNCs were subjected to density gradient centrifugation with 43% and 70% PERCOLL[®] (GE Healthcare Biosciences AB, Uppsala, Sweden), and the cells in the interface were collected and filtered through 40 μ m cell strainer (BD Falcon, MA) and re-suspended in enriched-RPMI (E-RPMI, RPMI containing 10% FBS, 200 μ M HEPES, 1 mM sodium pyruvate, 25 μ M 2-ME, 1x non-essential amino acid, and 1x antibiotic and antifungal). The viability of cells was confirmed by trypan blue dye exclusion, and the cells counted.

[0076] MNCs isolated from ileum, MLN, and spleen were plated in 24-well cell culture plate (25×10^6 cells/well) in 2 ml E-RPMI in the presence of semi-purified PEDV viral antigen (25 μ g/ml), and cells treated with medium alone or lipopolysaccharide (25 μ g/ml) were included as controls. Cells were cultured for 6 days at 39°C with 5% CO₂, and 0.5 ml of E-RPMI was added to each well on every second day. Supernatants were collected to measure PEDV-specific IgA and IgG antibody by ELISA. Cells were harvested, washed using PBS, re-suspended in E-RPMI, counted, and used for elucidating the frequency of IgA+ and IgG+ B cells by flow cytometry.

[0077] The frequencies of IgA+ and IgG+ B cells from 100,000 acquired events of immunostained MNCs were determined by flow cytometry. Briefly, MNCs were stimulated with PEDV whole virus-derived antigen as described above and immunostained with mouse anti-pig IgA mAb (Clone K60 1F1, AbD Serotec, Raleigh, NC) followed by goat anti-mouse IgG1 conjugated to APC/CY7 and rabbit anti-pig IgG conjugated to Texas Red. Subsequently, cells were fixed, permeabilized, and then intracellular stained using FITC conjugated rat anti-mouse CD79 β antibody (Clone AT107-2, AbD Serotec), which was shown to cross-react with pig B cells. Cells were acquired using BD ARIA[™] II flow cytometer (Bio-Rad Laboratories, Hercules, CA) and analyzed using the FLOWJO[®] software (FlowJo, LLC, Ashland, OR).

Results

[0078] As shown in FIG. 18, Gn piglets immunized with 200 µg S1-NTD-CTD-Fd, 200 µg S1-NTD-CTD-Fc, or 50 µg S1-NTD-CTD-Fd developed PEDV-specific antibody responses. There was no significant difference in antibody titer at days 7, 14, and 21 post-immunization. However, the antibody titer in the 200 µg S1-NTD-CTD-Fc group was significantly higher than the 200 µg S1-NTD-CTD-Fd and 50 µg S1-NTD-CTD-Fd groups at day 28 post-immunization ($P < 0.05$). No antibody was detected in adjuvant control group.

[0079] All three immunization groups induce IgA and IgG responses in ileum, MLN, and spleen. The IgA response in ileum in the 200 µg S1-NTD-CTD-Fc and 200 µg of S1-NTD-CTD-Fd groups was significantly higher than in the 50 µg S1-NTD-CTD-Fd and adjuvant control groups (FIG. 19A). All three immunization groups induce significantly higher IgA in MLN than DMEM control (FIG. 19B). The IgA response in spleen in the 200 µg S1-NTD-CTD-Fc group was significantly higher than in the 200 µg S1-NTD-CTD-Fd and 50 µg S1-NTD-CTD-Fd groups (FIG. 19C). Similar results were observed for IgG responses in ileum, MLN, and spleen (FIG. 19D-F). This result demonstrated that the PEDV subunit vaccines are capable of inducing IgA and IgG responses in piglets and the ability of these PEDV subunit vaccines to induce IgA and IgG can be ranked as 200 µg S1-NTD-CTD-Fc > 200 µg S1-NTD-CTD-Fd > 50 µg S1-NTD-CTD-Fd.

[0080] At day 28 post-immunization, piglets were challenged with PEDV VBS10. After challenge, clinical signs were monitored. At PID 5, all piglets were euthanized, and intestinal tissues were collected for histology and immunohistochemistry. These data were summarized in Table 3. All three piglets in the non-immunized, challenged group developed severe watery diarrhea (score of 3.0) at PID 1 (Table 1), and watery diarrhea persisted for 5 days (termination day). Piglets immunized with 50 µg S1-NTD-CTD-Fd had creamy to watery diarrhea (score of 2.0) at PID 2. Piglets in the 200 µg S1-NTD-CTD-Fd and 200 µg S1-NTD-CTD-Fc groups had soft feces at PID 2, and pasty to creamy diarrhea (score of 1.5) at PIDs 3-5. The non-immunized and unchallenged control piglets did not develop any clinical signs of PEDV infection.

[0081] Gross pathological changes (score 3) were observed in non-immunized challenged piglets. The perineum, ventral abdomen, and hind legs were coated with yellow adherent diarrhetic feces. Upon opening the abdomen, dilated gas and fluid-filled small intestine with thin translucent walls were observed. The cecum, spiral colon and terminal colon were dilated and filled with liquid yellow intestinal content. Ascites, hydrothorax and thymic atrophy were detected in the piglets. Mild gross pathological changes (score 1) were

observed in the 200 µg S1-NTD-CTD-Fd and 200 µg S1-NTD-CTD-Fc groups. Moderate gross pathological changes (score 2) were observed in the 50 µg S1-NTD-CTD-Fd group.

[0082] Histologic lesions in the small intestine were observed in the non-immunized challenged group. Severe villous atrophy was found in the duodenum, jejunum and ileum. The villous changes were associated with extensive intestinal epithelial degeneration and necrosis. Moderate histologic lesions (score 2.0) were observed in the 50 µg S1-NTD-CTD-Fd group whereas only mild (score 1.0 to 1.5) lesions were found in the jejunum and ileum in the 200 µg S1-NTD-CTD-Fd and 200 µg S1-NTD-CTD-Fc groups. No lesions were found in the non-immunized unchallenged group.

[0083] The presence of viral antigen in the small intestine was also evaluated by immunohistochemistry (IHC) (Table 3). A high level of PEDV antigens (score 3.0) were detected in the duodenum, jejunum and ileal enterocytes in the non-immunized challenged group. A moderate level (score 2.0) of antigen was detected in small intestinal tissues in the 50 µg S1-NTD-CTD-Fd group. There was minimal antigen (score 1.0 to 1.5) present in the duodenum and jejunum tissues in the 200 µg S1-NTD-CTD-Fd and 200 µg S1-NTD-CTD-Fc groups. No antigen was detected in the non-immunized unchallenged group.

[0084] After PEDV challenge, daily feces were collected, and the presence of viral RNA in feces was quantified by real-time RT-PCR. As shown in FIG. 20, viral RNA was not detectable in all three immunization groups at day 1. At day 2, there was no significant difference in RNA titer in the 200 µg S1-NTD-CTD-Fc, 200 µg S1-NTD-CTD-Fd, and challenge control groups. The 50 µg S1-NTD-CTD-Fd group had significantly lower RNA titer compared to other three groups ($P < 0.05$). At days 3 and 4, RNA titer in the 200 µg S1-NTD-CTD-Fc and 200 µg S1-NTD-CTD-Fd groups was significantly lower than 50 µg S1-NTD-CTD-Fd and challenge control groups. These data demonstrated piglets immunized with PEDV subunit vaccines had reduced viral RNA shedding in feces.

Table 3: Protection efficacy of PEDV subunit vaccine in gnotobiotic piglets

Groups ^A	Onset of clinical signs (days)	Diarrhea score ^B	Pigs with clinical signs ^C	Gross lesion ^D	Histologic lesions ^D			IHC ^E		
					D	J	I	D	J	I
200 µg S1-NTD-CTD-Fc	3	1.5	2/3	1.0	1.0	1.0	0.5	1.0	1.0	0
200 µg S1-NTD-CTD-Fd	3	1.5	2/3	1.0	1.0	1.0	1.0	1.0	1.0	0
50 µg S1-NTD-CTD-Fd	2	2.0	3/3	2.0	2.0	2.0	2.0	2.0	1.0	1.0
Challenge control	1	3.0	3/3	3.0	3.0	3.0	3.0	3.0	3.0	3.0
Adjuvant control	None	0	0/3	0	0	0	0	0	0	0

^A One-week-old gnotobiotic piglets were immunized with the indicated PEDV subunit vaccine candidates.

^B Diarrhea was scored for each piglet. 0 = normal, 1 = pasty, 2 = creamy and 3 = watery. Pigs with fecal consistency scores of 2 or 3 were scored as diarrhea-positive.

^C Clinical signs include diarrhea, vomiting, and progressive dehydration.

^D Gross and histologic lesion were scored for each piglet/tissue. 0 = no change; 1 = mild; 2 = moderate; 3 = severe. D = duodenum; J = jejunum; I = ileum

^E PdCV antigen expression was scored for each tissue. 0 = no positive cells; 1 = less than 20% of crypt epithelial cells were positive; 2 = 20-50% of cells were positive; 3 = more than 50% of cells were positive.

[0085] Collectively, these data demonstrated that (i) piglets in non-immunized challenged control group developed severe clinical signs and pathological lesion of PEDV infection; (ii) piglets immunized with 200 µg S1-NTD-CTD-Fd or 200 µg S1-NTD-CTD-Fc had significant reduction in clinical signs, pathological lesions, and antigen expression in small intestine; and (iii) the protection efficacy of the PEDV subunit vaccine is dose-dependent, and the efficacy of S1-NTD-CTD-Fd at dose of 200 µg was significantly higher than at a dose of 50 µg. These data also demonstrated that PEDV subunit vaccines induced protective immunity in Gn piglets.

Example 4

Efficacy of PEDV subunit vaccine in conventional piglets

Materials and Methods

[0086] One-week-old healthy conventional piglets were purchased from Hartley Farm, Circleville, OH. These piglets were seronegative for major porcine diseases including PdCV, PEDV, TEGV, PCV, and PRRSV. Thirty one-week-old conventional piglets were randomly divided into six groups (5 piglets/group) and were housed in six separate rooms. Piglets in group 1 were immunized intramuscularly with 0.5 ml MF59[®] adjuvant alone and served as unchallenged controls. Piglets in group 2 were immunized with 0.5 ml MF59[®] adjuvant alone. Piglets in group 3 were immunized with 200 µg S1-NTD-CTD-Fd. Piglets in group 4 were immunized with 200 µg S1-NTD-CTD-Fc. Piglets in group 5 were immunized with an inactivated PEDV vaccine containing 500 µg PEDV antigen. Fourteen days post-immunization, piglets in groups 3-5 were boosted with the respective vaccine at same dose. Fourteen days post-booster immunization, piglets in groups 2-5 were challenged with 5 ml of DMEM containing 10² PFU of the PEDV VBS10 (piglets in group 2 were challenged 28 days after the initial control immunization). After challenge, the piglets were observed and evaluated daily for weight and body temperature changes, and clinical signs of PEDV infection. Diarrhea score was monitored and feces were collected daily. Piglets were terminated at PID 5. At the termination, intestinal tissues were collected for histology and IHC.

[0087] Fecal samples and intestinal contents were processed as described above, with collected tissue samples ground in liquid nitrogen, and dispersed through 20 gauge needle/syringe for at least 20 times before RNA extraction for quantification of viral genomic RNA by RT-qPCR. The serum samples were used directly for RNA extraction. The total RNA was extracted using an RNeasy[®] Mini Kit (Qiagen, Valencia, CA) as directed by the manufacturer. Two-step reverse transcription (RT) was conducted using primer targeting on the N gene of PEDV using SUPERScript III[®] transcriptase kit (Life Technologies, Carlsbad, CA) following the manufacturer's protocol. The RT products were then used to perform real-time PCR using primers and probes specifically targeting the N gene of PEDV (Applied Biosystems, Foster City, CA) on a STEPONE[®] Real Time PCR system (Applied Biosystems). A standard plasmid for PEDV was constructed by inserting the sequence of entire N gene into pGEM[®] T-easy vector (Promega, Madison, WI). The plasmid of known concentration was serial diluted ten-fold to generate a standard curve for real-time PCR. The amplification cycles were: 2 min at 50°C, 10 min at 95°C, 40 cycles of 15 s at 95°C, 1 min at 60°C. The threshold for detection of fluorescence above background was set within the

exponential phase of the amplification curve. For each assay, 10-fold dilutions of standard plasmid were generated, and negative controls samples and ddH₂O were included in each assay.

[0088] In addition to intestine segments from each pig, portions of lung, kidney, spleen, and liver were also collected at necropsy for histologic examination. Tissues were fixed in 10% (v/v) phosphate-buffered formalin for 24-36 h, dehydrated in a graded ethanol series, embedded in paraffin, cut in 5 µm sections, and collected on glass slides. The sections were de-paraffinized, rehydrated and then stained with hematoxylin and eosin. Slides were examined by conventional light microscopy by a pathologist blinded to the experimental groups.

[0089] Five-micron sections of paraffin-embedded tissues were placed onto positively charged slides. After deparaffinization, sections were incubated with target retrieval solution (Dako, Carpinteria, CA) for antigen retrieval. After blocking, a primary anti-PEDV serum antibody from PEDV-infected convalescent sows was incubated for 30 min, 22°C followed by incubation with a biotinylated horse anti-pig IgG secondary antibody (Vector Laboratories, Burlingame, CA). Slides were further incubated with ABC Elite complex to probe biotin (Vector Laboratories) and then developed using a 3,3'-diaminobenzidine (DAB) chromogen Kit (Dako); hematoxylin was used as a counterstain. Tissue sections from PEDV-infected and uninfected samples were used as positive and negative controls respectively.

[0090] Serum samples were collected from each piglet and tested for PEDV-specific VN antibodies. Briefly, two-fold dilutions of the serum samples were incubated with 100 PFU/well of PEDV at 37°C for 1 h. The mixtures were then transferred to confluent Vero CCL-81 cells in 6-well plates for plaque assay. After fixation and staining, the plaques were counted and 50% plaque reduction titers were calculated as the PEDV-specific VN antibody titers. The assay was repeated 3 times for each sample.

Results

[0091] The efficacy of PEDV subunit vaccines and inactivated PEDV vaccine was compared in conventional piglets. As shown in FIG. 21, conventional piglets immunized with 200 µg S1-NTD-CTD-Fd or 200 µg S1-NTD-CTD-Fc demonstrated similar antibody titers to PEDV ($P>0.05$). However, antibody titer in both PEDV subunit vaccine groups were significantly higher compared to inactivated PEDV vaccine at days 21 and 28 post-immunization. In addition, piglets immunized with 500 µg of inactivated PEDV vaccine had significantly higher antibody titer compared to those immunized with 200 µg of inactivated PEDV vaccine ($P<0.05$). No antibody was detected in non-immunized control group.

[0092] At day 28 post immunization, piglets were challenged with PEDV VBS10. After challenge, clinical signs were monitored. At PID 5, all piglets were euthanized, and intestinal tissues were collected for histology and immunohistochemistry. These data were summarized in Table 4. All five piglets in non-immunized challenged group developed severe watery diarrhea (score of 3.0) at PID 1 (Table 4), and watery diarrhea persisted for 5 days (termination day) and at termination had severe gross lesions (score 3). The small intestine had severe histologic lesion (score 3.0) and high levels of PEDV antigen expression (score 3.0). Overall, piglets immunized with 200 µg S1-NTD-CTD-Fd, 200 µg S1-NTD-CTD-Fc, or 500 µg inactivated PEDV vaccine had significantly reduced clinical signs, gross and histologic lesions, and antigen expression in small intestine; these three groups had similar levels of protection efficacy. Piglets immunized with 200 µg of inactivated PEDV vaccine had moderate (score 2) level of clinical signs, gross and histologic lesions, and antigen expression. A high dose (500 µg) of inactivated PEDV vaccine had higher protection efficacy compared to low dose (200 µg) of inactivated PEDV vaccine. Piglets in normal control group (no immunization or challenge) had no clinical signs, pathological lesions, or PEDV antigen. Collectively, these data demonstrated that PEDV subunit vaccine induced protective immunity in conventional piglets.

Table 4: Protection efficacy of PEDV subunit vaccine in conventional piglets

Groups ^A	Onset of clinical sign (days)	Diarrhea score	Pigs with clinical signs	Gross lesion	Histologic lesions			IHC		
					D	J	I	D	J	I
200 µg NTD-CTD-Fc	3	1.5	3/5	1.0	1.0	1.0	0.5	1.0	1.0	0
200 µg NTD-CTD-Fd	3	1.5	4/5	1.0	1.5	1.0	1.0	1.0	1.0	0
200 µg inactivated	2	2.0	5/5	2.0	2.0	2.0	2.0	2.0	2.0	1.0
500 µg inactivated	3	1.5	4/5	1.5	1.5	1.0	0.5	1.0	1.5	0.5
Challenge control	1	3.0	5/5	3.0	3.0	3.0	3.0	3.0	3.0	3.0
Adjuvant control	None	0	0/5	0	0	0	0	0	0	0

^A One-week-old conventional piglets were immunized with the indicated PEDV vaccine candidate. At day 14 after primary immunization, piglets were boosted with the same PEDV subunit vaccine. At day 28 post immunization, piglets were challenged with PEDV VBS10. After challenge, clinical signs were monitored. At PID 5, all piglets were euthanized, and intestinal tissues were collected for histology and immunohistochemistry.

D = duodenum; J = jejunum; I = ileum

[0093] After challenge, daily feces were collected, and the presence of viral RNA in feces was quantified by real-time RT-PCR. As shown in FIG. 22, piglets in the non-immunized challenge group had high levels (approximately 7.0 log genomic RNA copies/g feces) of PEDV RNA from PIDs 1-5. Piglets immunized with 500 µg or 200 µg of inactivated PEDV vaccine had 4-5 log RNA at PID 1-2. Interestingly, no viral RNA was detected in the 200 µg S1-NTD-CTD-Fc or 200 µg S1-NTD-CTD-Fd groups at PIDs 1-2. Overall, piglets immunized with 200 µg S1-NTD-CTD-Fc or 200 µg S1-NTD-CTD-Fd had significantly lower viral RNA shedding compared to piglets immunized with 500 µg or 200 µg of the inactivated PEDV vaccine ($P < 0.05$). These data demonstrated that the PEDV subunit vaccines had significantly higher efficacy in reducing viral RNA shedding compared to an inactivated PEDV vaccine.

[0094] Unless otherwise indicated, all numbers expressing quantities of ingredients, properties such as molecular weight, reaction conditions, and so forth used in the specification and claims are to be understood as being modified in all instances by the term "about." Accordingly, unless indicated to the contrary, the numerical parameters set forth in the specification and attached claims are approximations that may vary depending upon the desired properties sought to be obtained by the present invention. At the very least, and not as an attempt to limit the application of the doctrine of equivalents to the scope of the claims, each numerical parameter should at least be construed in light of the number of reported significant digits and by applying ordinary rounding techniques. Notwithstanding that the numerical ranges and parameters setting forth the broad scope of the invention are approximations, the numerical values set forth in the specific examples are reported as precisely as possible. Any numerical value, however, inherently contains certain errors necessarily resulting from the standard deviation found in their respective testing measurements.

[0095] The terms "a," "an," "the" and similar referents used in the context of describing the invention (especially in the context of the following claims) are to be construed to cover both the singular and the plural, unless otherwise indicated herein or clearly contradicted by context. Recitation of ranges of values herein is merely intended to serve as a shorthand method of referring individually to each separate value falling within the range. Unless otherwise indicated herein, each individual value is incorporated into the specification as if it were individually recited herein. All methods described herein can be performed in any suitable order unless otherwise indicated herein or otherwise clearly contradicted by context. The use of any and all examples, or exemplary language (e.g., "such as") provided herein is intended merely to better illuminate the invention and does not pose a limitation on the

scope of the invention otherwise claimed. No language in the specification should be construed as indicating any non-claimed element essential to the practice of the invention.

[0096] Groupings of alternative elements or embodiments of the invention disclosed herein are not to be construed as limitations. Each group member may be referred to and claimed individually or in any combination with other members of the group or other elements found herein. It is anticipated that one or more members of a group may be included in, or deleted from, a group for reasons of convenience and/or patentability. When any such inclusion or deletion occurs, the specification is deemed to contain the group as modified thus fulfilling the written description of all Markush groups used in the appended claims.

[0097] Certain embodiments of this invention are described herein, including the best mode known to the inventors for carrying out the invention. Of course, variations on these described embodiments will become apparent to those of ordinary skill in the art upon reading the foregoing description. The inventor expects skilled artisans to employ such variations as appropriate, and the inventors intend for the invention to be practiced otherwise than specifically described herein. Accordingly, this invention includes all modifications and equivalents of the subject matter recited in the claims appended hereto as permitted by applicable law. Moreover, any combination of the above-described elements in all possible variations thereof is encompassed by the invention unless otherwise indicated herein or otherwise clearly contradicted by context.

[0098] Specific embodiments disclosed herein may be further limited in the claims using consisting of or consisting essentially of language. When used in the claims, whether as filed or added per amendment, the transition term "consisting of" excludes any element, step, or ingredient not specified in the claims. The transition term "consisting essentially of" limits the scope of a claim to the specified materials or steps and those that do not materially affect the basic and novel characteristic(s). Embodiments of the invention so claimed are inherently or expressly described and enabled herein.

[0099] Furthermore, numerous references have been made to patents and printed publications throughout this specification. Each of the above-cited references and printed publications are individually incorporated herein by reference in their entirety.

[00100] In closing, it is to be understood that the embodiments of the invention disclosed herein are illustrative of the principles of the present invention. Other modifications that may be employed are within the scope of the invention. Thus, by way of example, but not of limitation, alternative configurations of the present invention may be utilized in accordance

with the teachings herein. Accordingly, the present invention is not limited to that precisely as shown and described.

What is claimed is:

1. An immunogenic composition for induction of an immune response against porcine epidemic diarrhea virus (PEDV) comprising a polypeptide comprising:
a PEDV peptide; and
an immunopotentiator selected from the group consisting of a Fc fragment of immunoglobulin G, C3d, *Onchocerca volvulus* ASP-1, cholera toxin and muramyl peptides,
or
a stabilization sequence, or
both an immunopotentiator and a stabilization sequence.
2. The immunogenic composition of claim 1, wherein the PEDV peptide is a PEDV spike (S) protein, a PEDV S protein S1 fragment, a PEDV S protein S2 fragment, a PEDV S protein S1 subunit N-terminal domain (S1-NTD) sequence, a PEDV S protein S1 subunit C-terminal domain (S1-CTD) sequence, a PEDV S protein fusion peptide (FP) sequence, a PEDV S protein heptad repeat (HR1 and HR2) sequence, or a PEDV S protein transmembrane (TM) sequence.
3. The immunogenic composition of claim 1, wherein the PEDV peptide comprises a PEDV S protein S1-NTD sequence.
4. The immunogenic composition of claim 1, wherein the PEDV peptide comprises a PEDV S protein S1-CTD sequence.
5. The immunogenic composition of claim 1, wherein the PEDV peptide comprises both a PEDV S protein S1-NTD sequence and a PEDV S protein S1-CTD sequence.
6. The immunogenic composition of claim 1, wherein the Fc fragment of immunoglobulin G is human, mouse, rabbit, or porcine.
7. The immunogenic composition of claim 6, wherein the immunopotentiator is human IgG Fc.
8. The immunogenic composition of claim 6, wherein the immunopotentiator is porcine IgG Fc.
9. The immunogenic composition of claim 1, wherein the stabilization sequence is foldon or GCN4.
10. The immunogenic composition of claim 9, wherein the stabilization sequence is foldon.

11. The immunogenic composition of claim 1, wherein the polypeptide is a fusion protein.
12. The immunogenic composition of claim 1, wherein if the immunogenic composition comprises a PEDV peptide, an immunopotentiator, and a stabilization sequence, the PEDV peptide is linked to the stabilization sequence and the stabilization sequence is linked to the immunopotentiator in a single polypeptide.
13. The immunogenic composition of claim 1, further comprising an adjuvant.
14. A method of inducing a protective immune response against a porcine epidemic diarrhea virus (PEDV) comprising:
administering the immunogenic composition of claim 1 to a subject in need thereof; and
wherein the immunogenic composition induces a protective immune response against challenge with PEDV in said subject.
15. The method according to claim 14, wherein the immunogenic composition further comprises an adjuvant.
16. The method according to claim 14, wherein the method further comprises administering a booster dose of the immunogenic composition to the host.
17. A method of protecting pigs against infection with PEDV, comprising:
administering the immunogenic composition of claim 1 to a porcine subject in need thereof and thereby preventing PEDV-infection in the porcine host.
18. The method according to claim 17, wherein the immunogenic composition further comprises an adjuvant.
19. The method according to claim 17, wherein the method further comprises administering at least one booster dose of the immunogenic composition to the porcine subject.
20. The immunogenic composition of any one of claims 1-13 for use in inducing a protective immune response against PEDV in a subject in need thereof for the prevention of PEDV infection.

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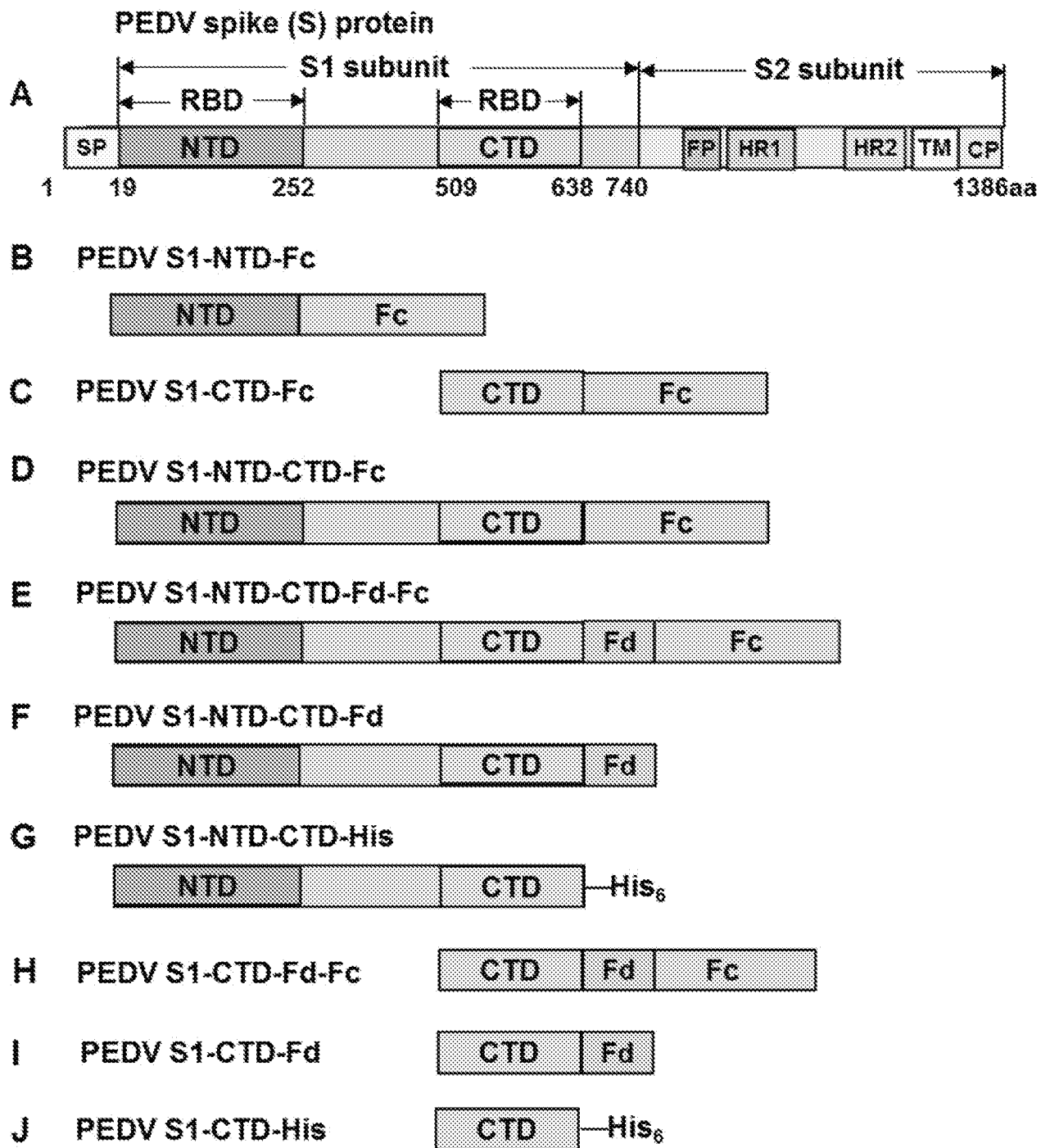


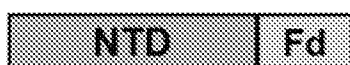
FIG. 1A-J

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K PEDV S1-NTD-Fd-Fc



L PEDV S1-NTD-Fd



M PEDV S1-NTD-His

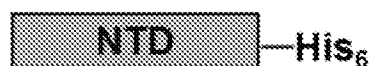


FIG. 1K-M

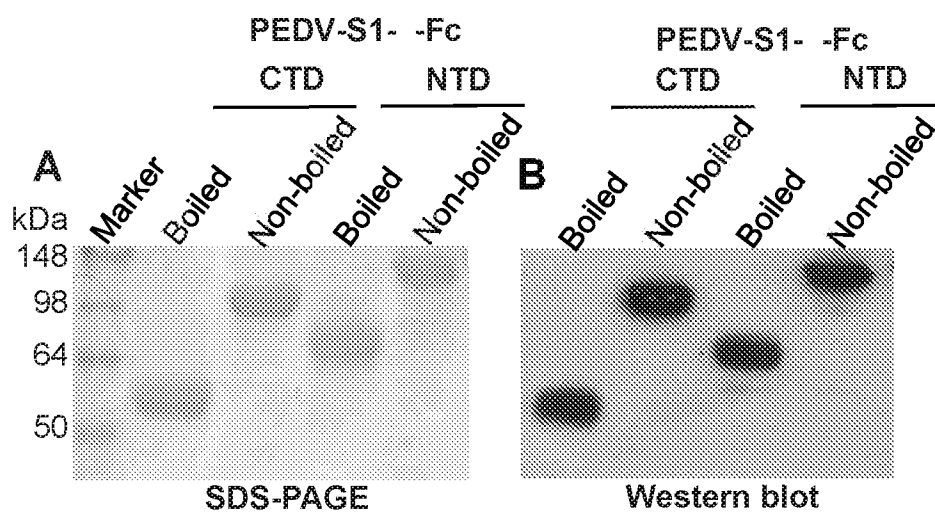


FIG. 2

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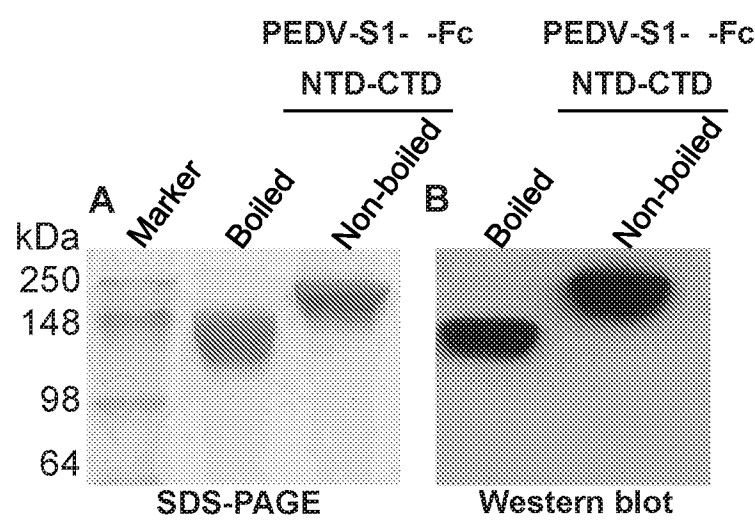


FIG. 3

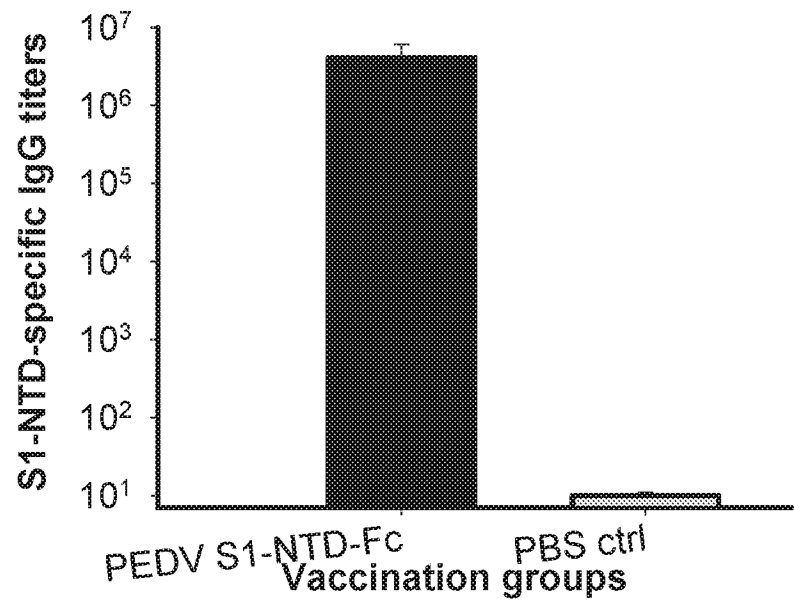


FIG. 4

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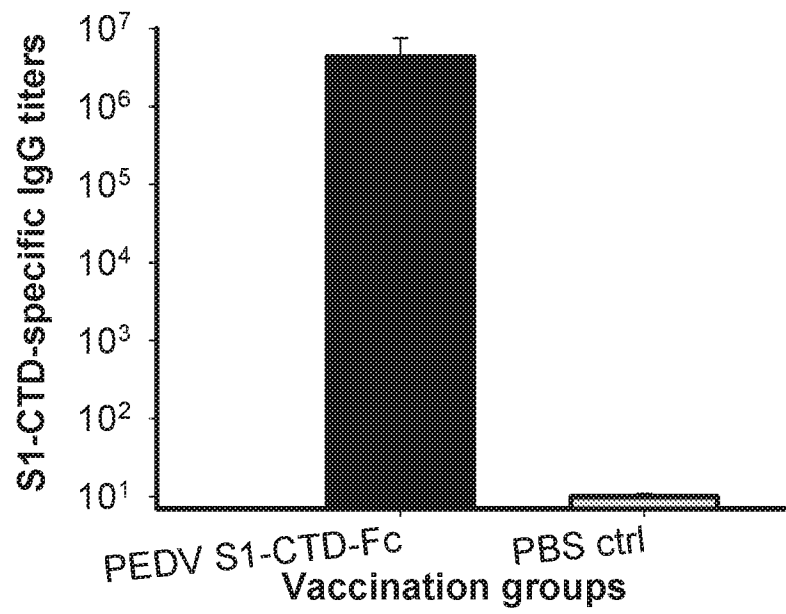


FIG. 5

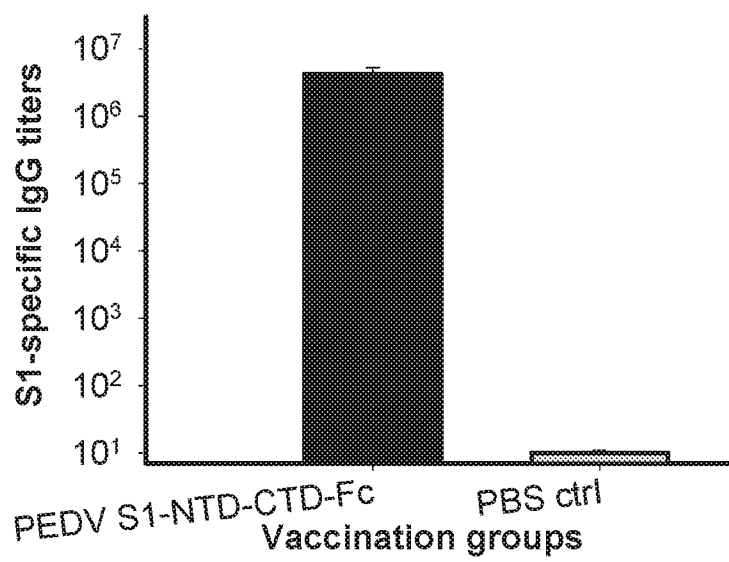


FIG. 6

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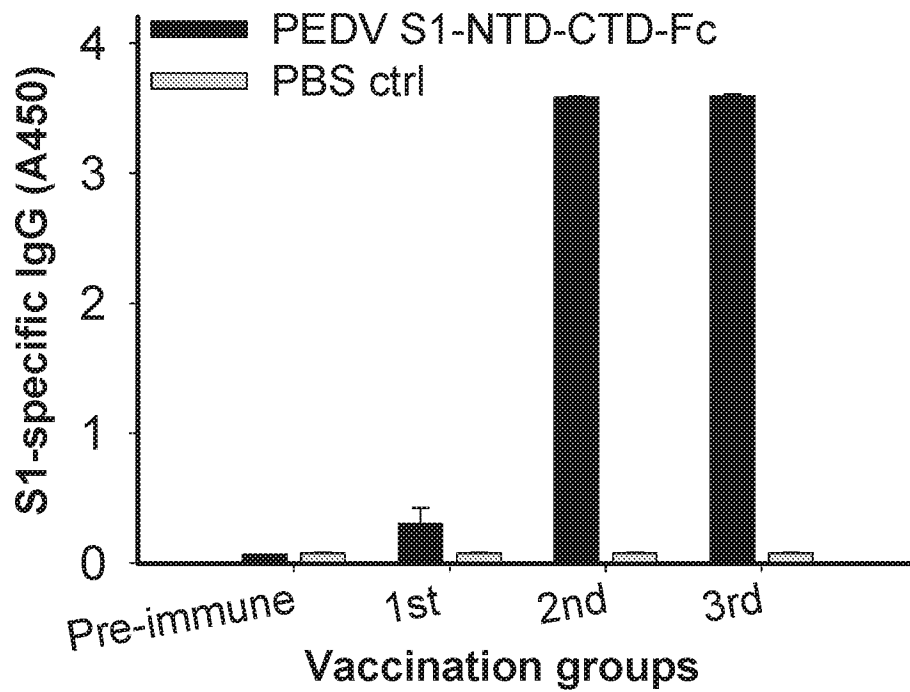


FIG. 7

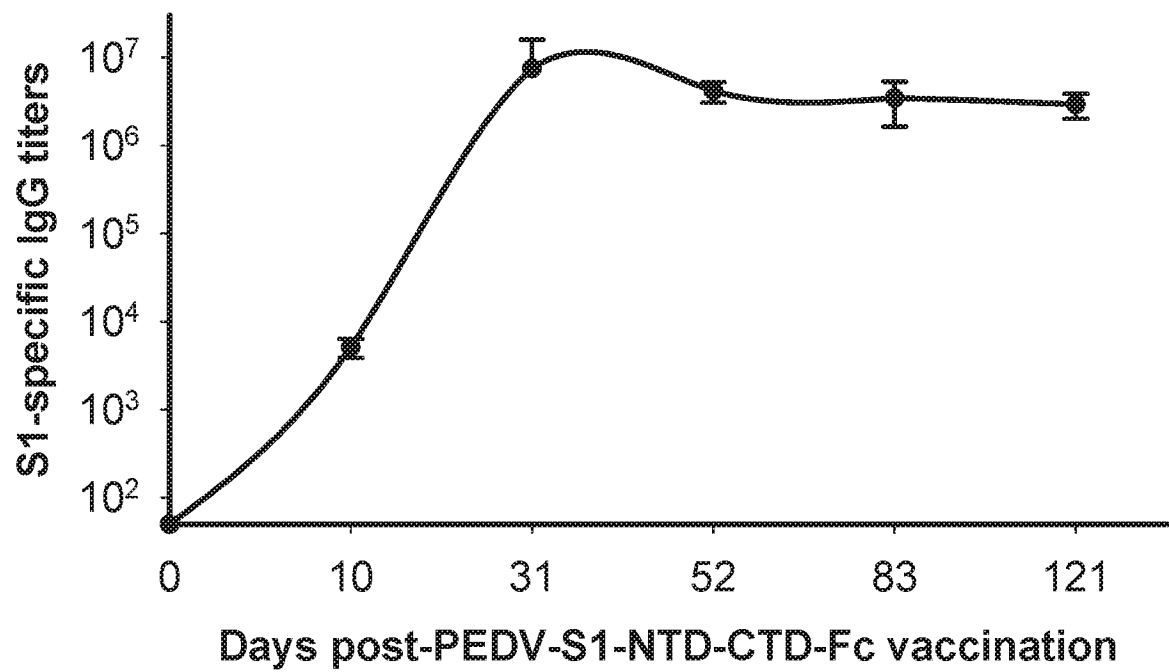


FIG. 8

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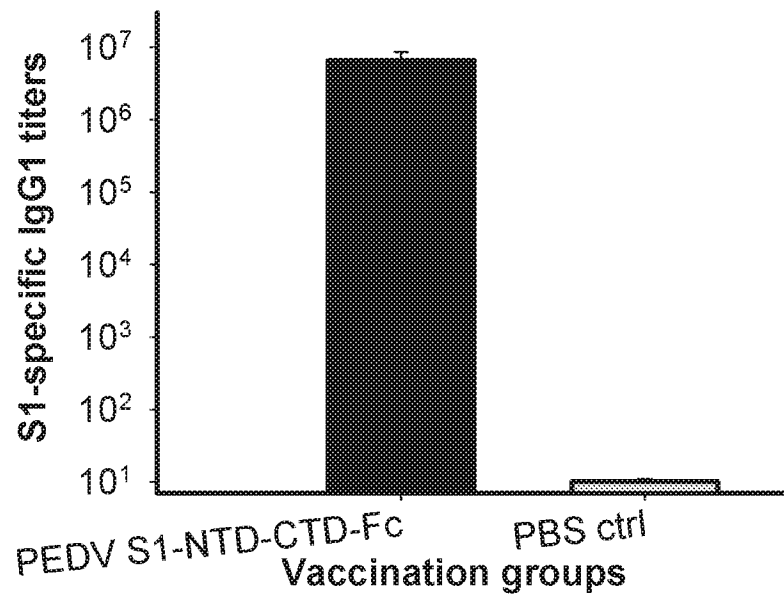


FIG. 9

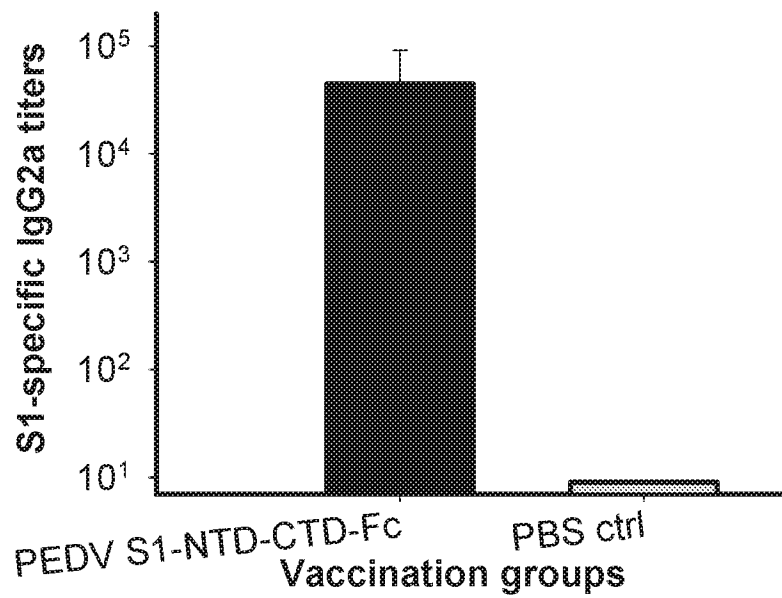


FIG. 10

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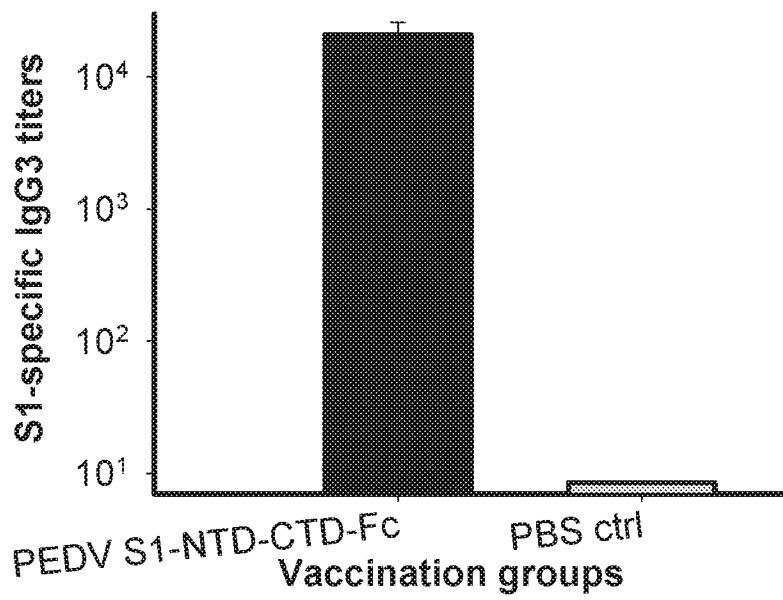


FIG. 11

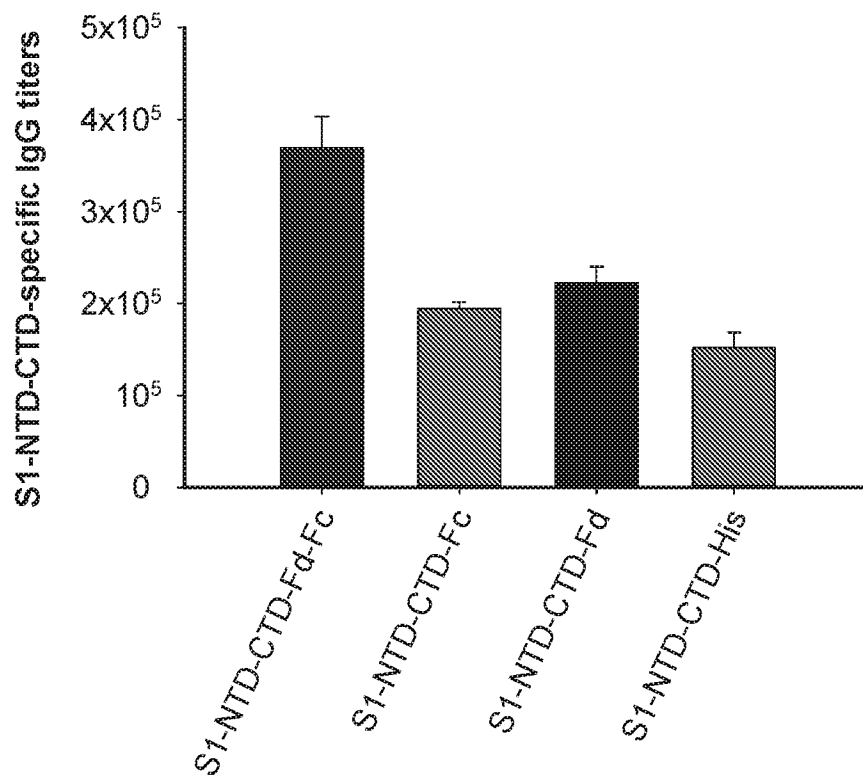


FIG. 12

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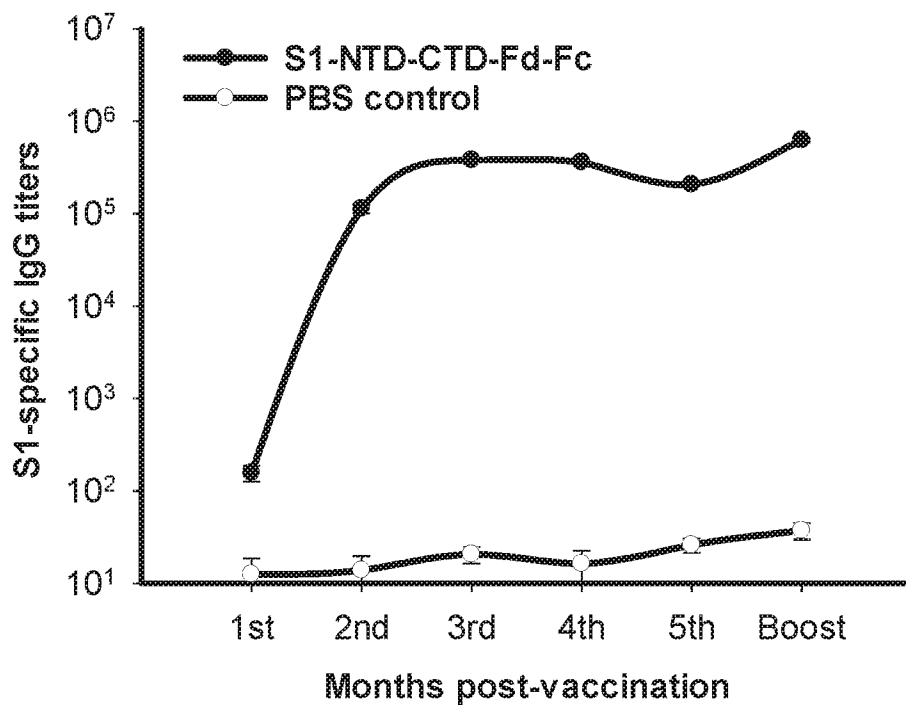


FIG. 13

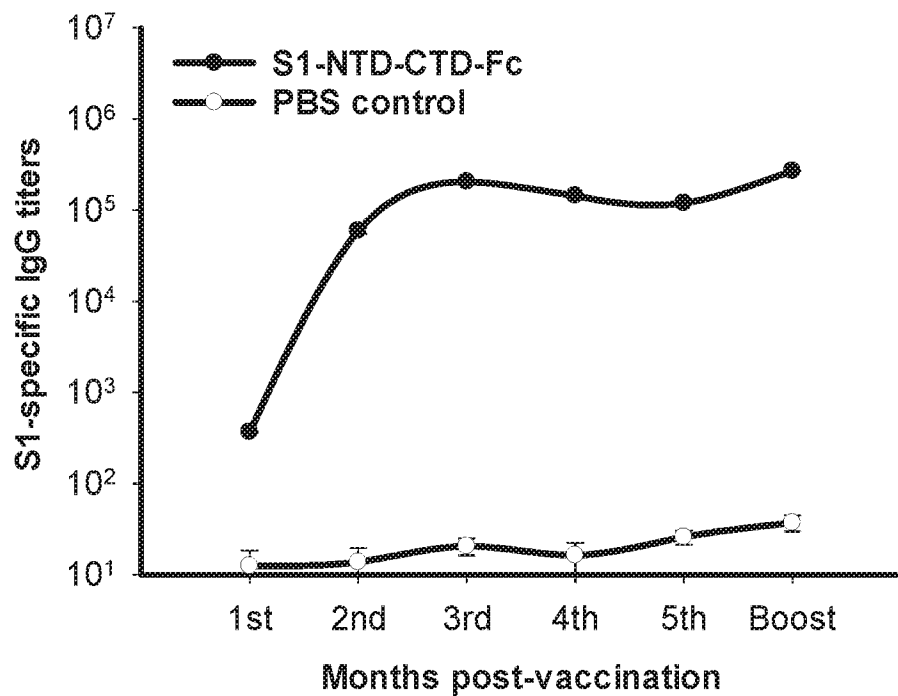


FIG. 14

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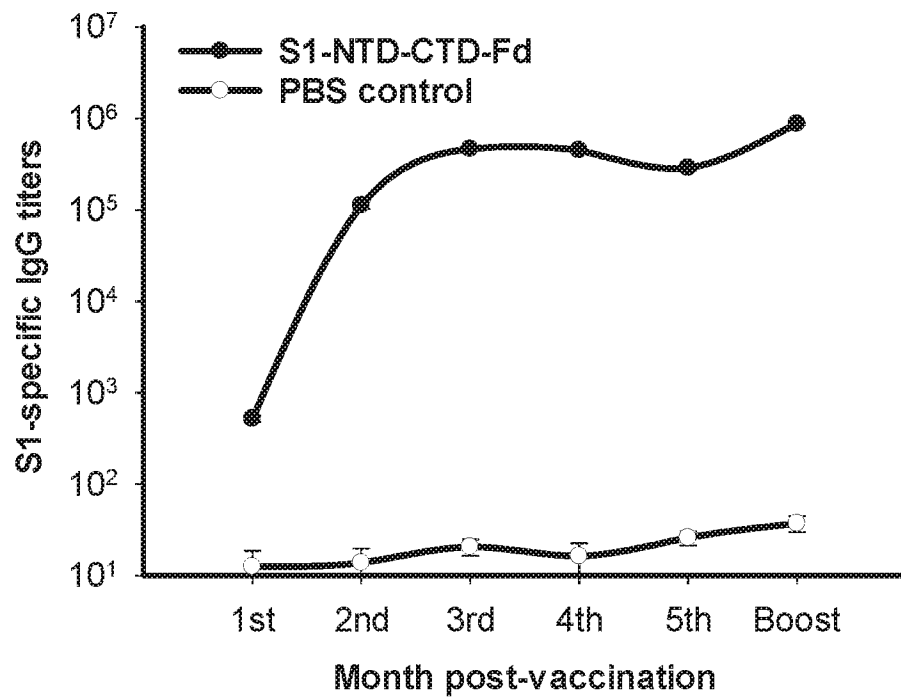


FIG. 15

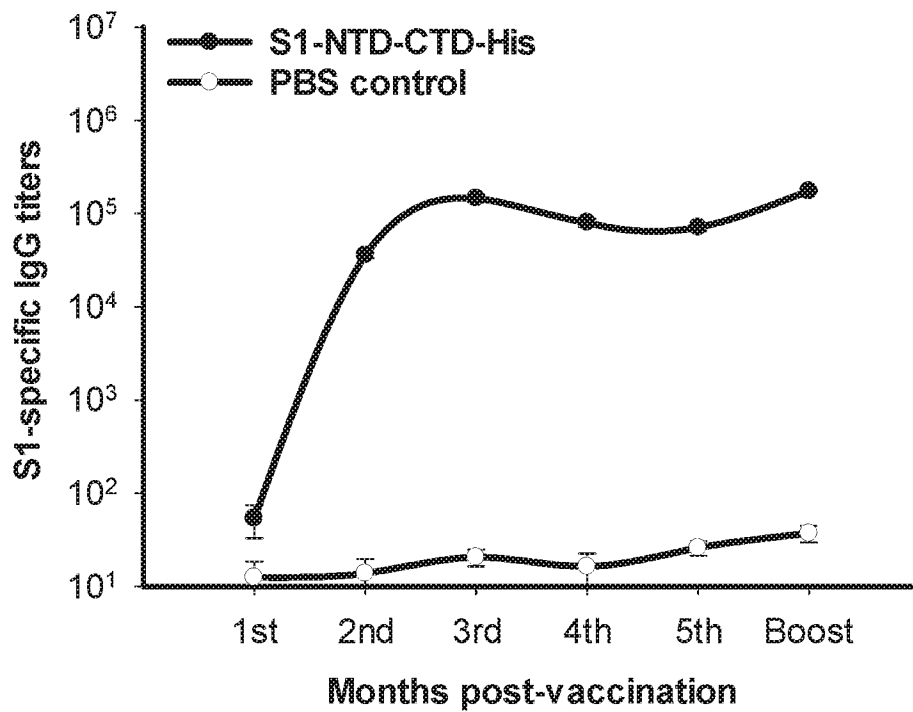


FIG. 16

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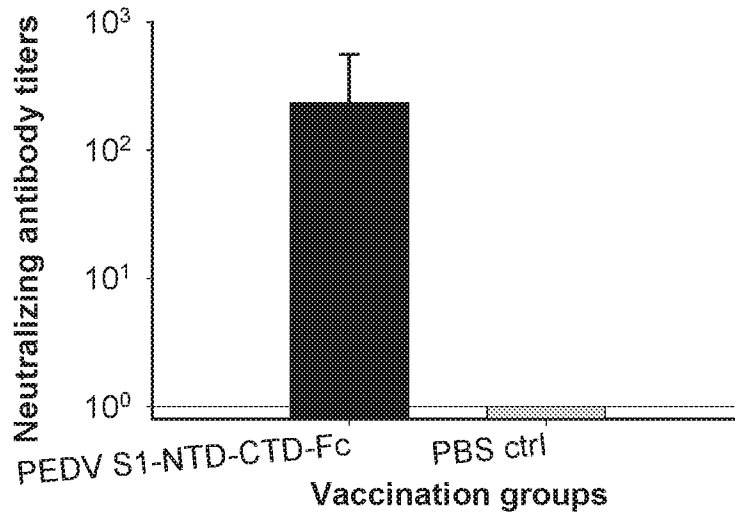


FIG. 17

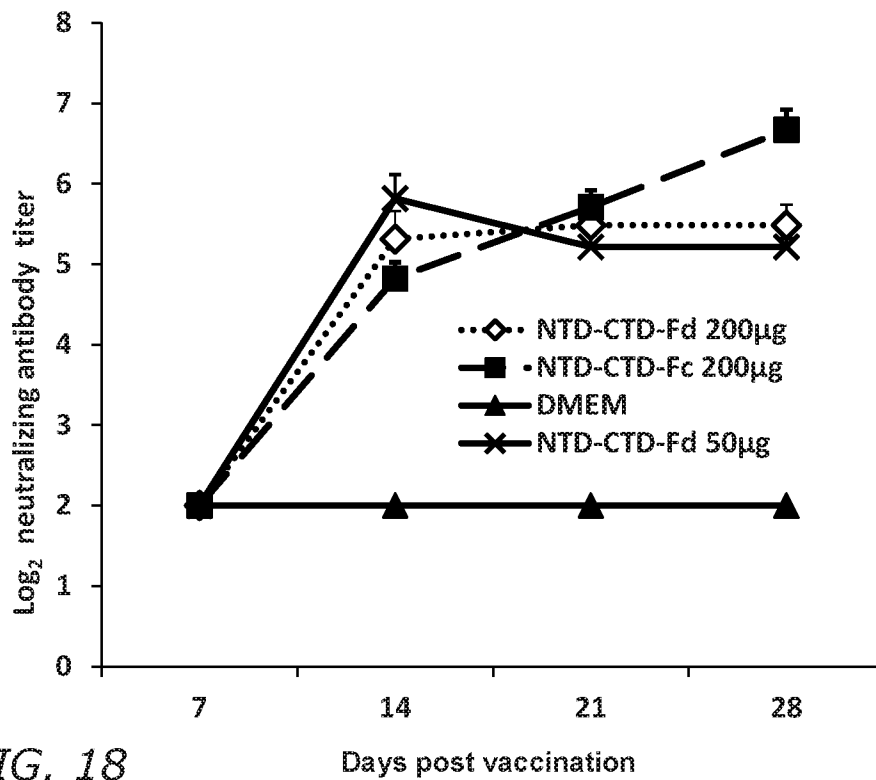


FIG. 18

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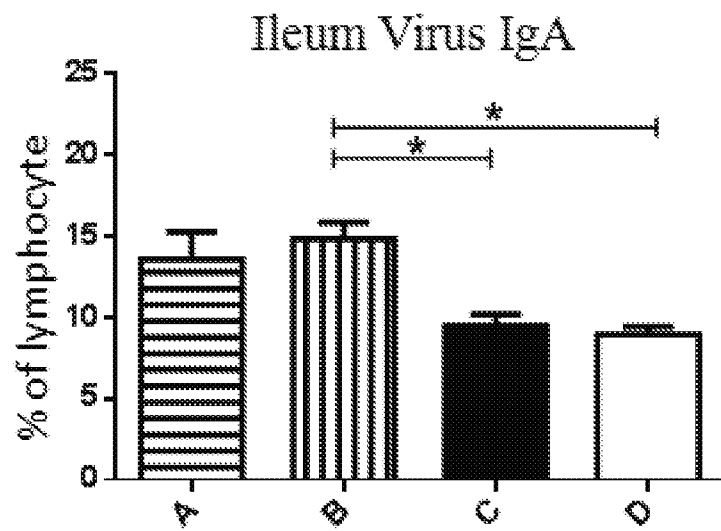


FIG. 19A

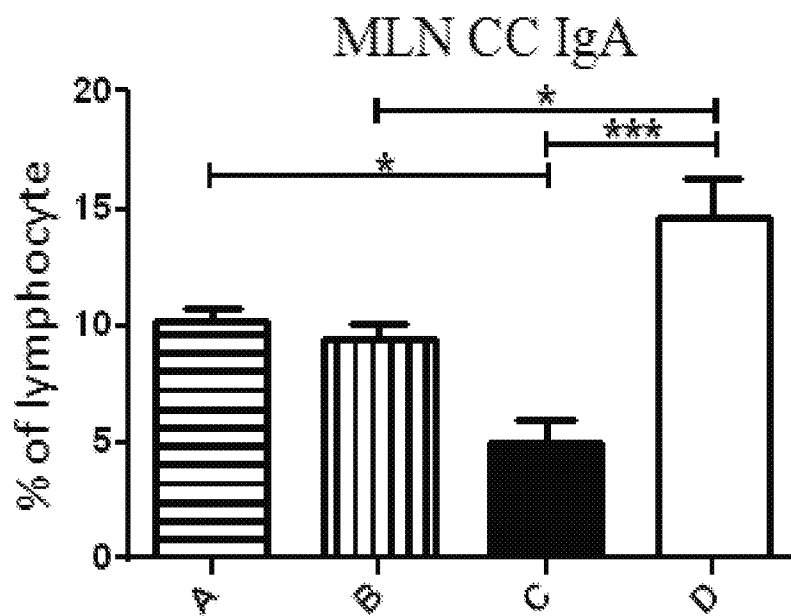


FIG. 19B

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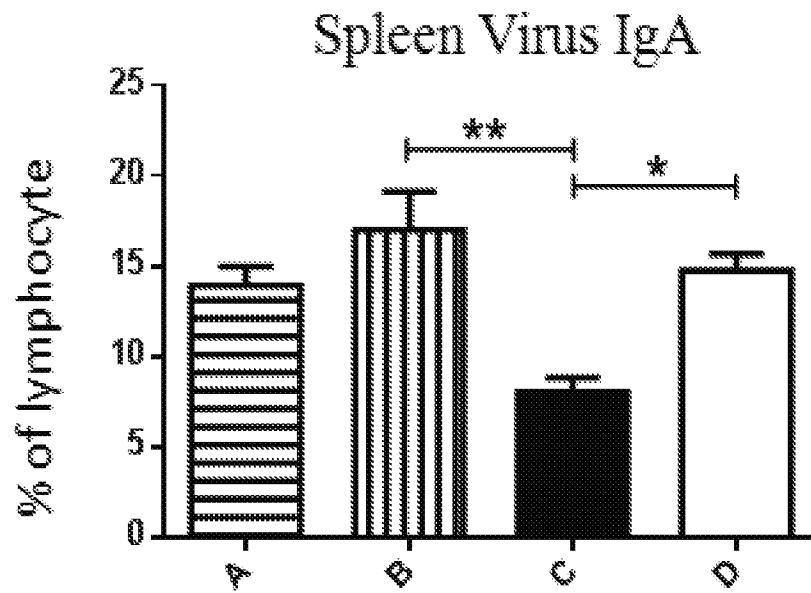


FIG. 19C

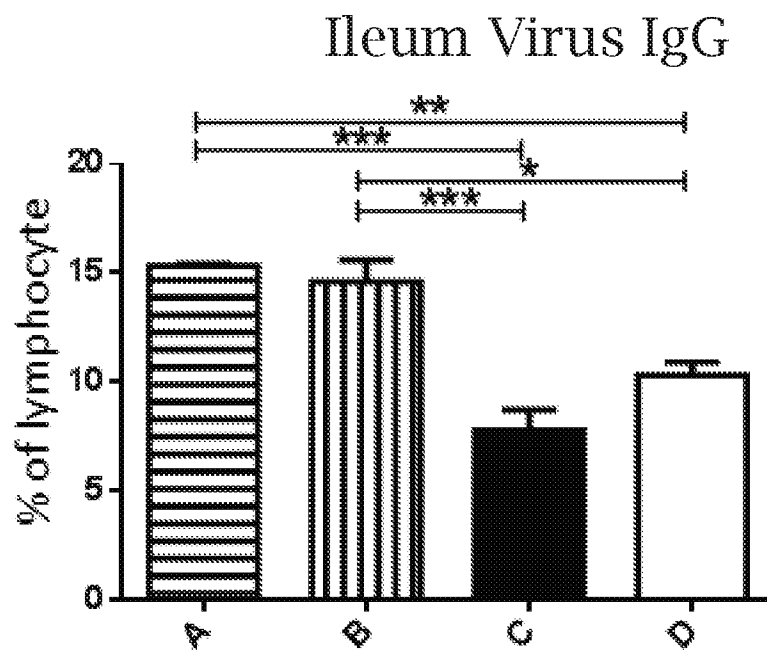


FIG. 19D

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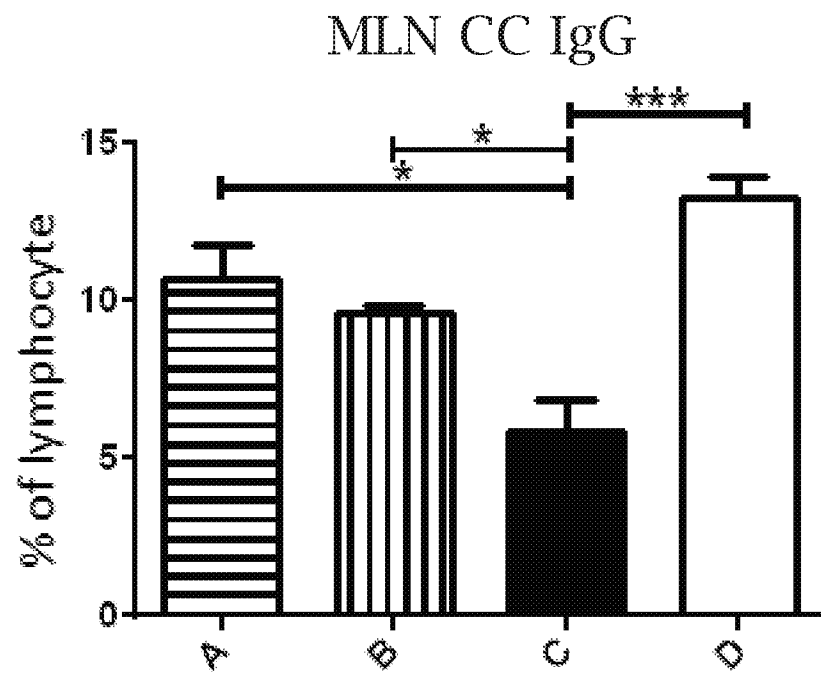


FIG. 19E

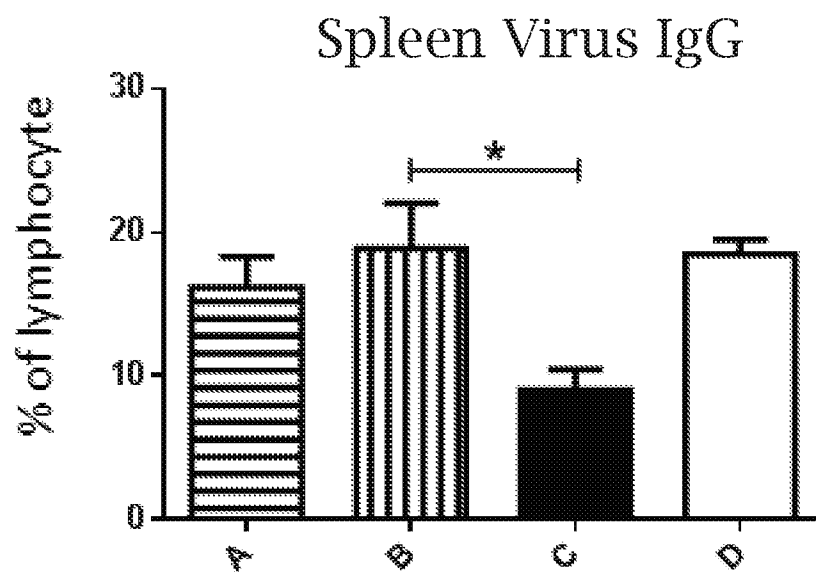


FIG. 19F

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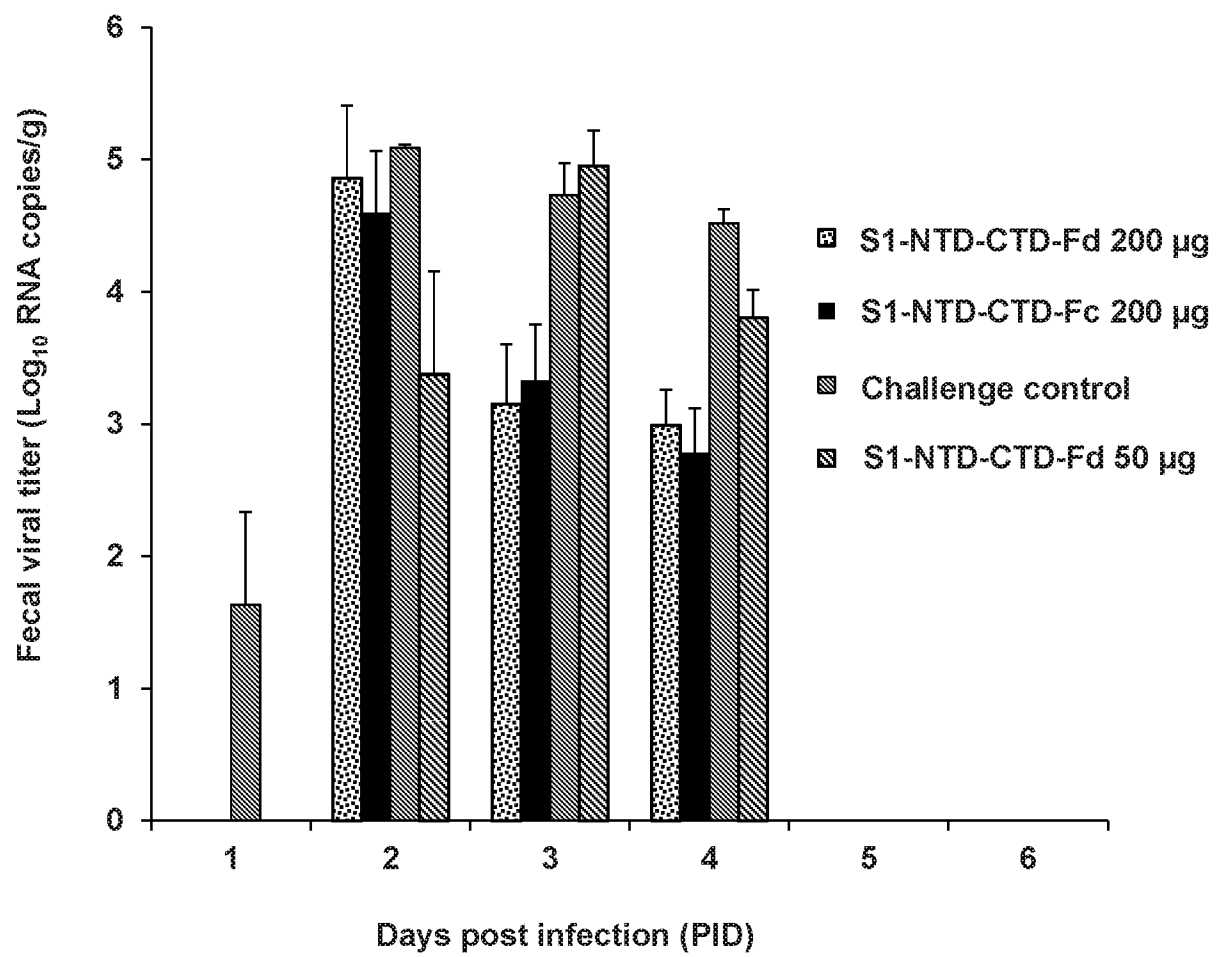


FIG. 20

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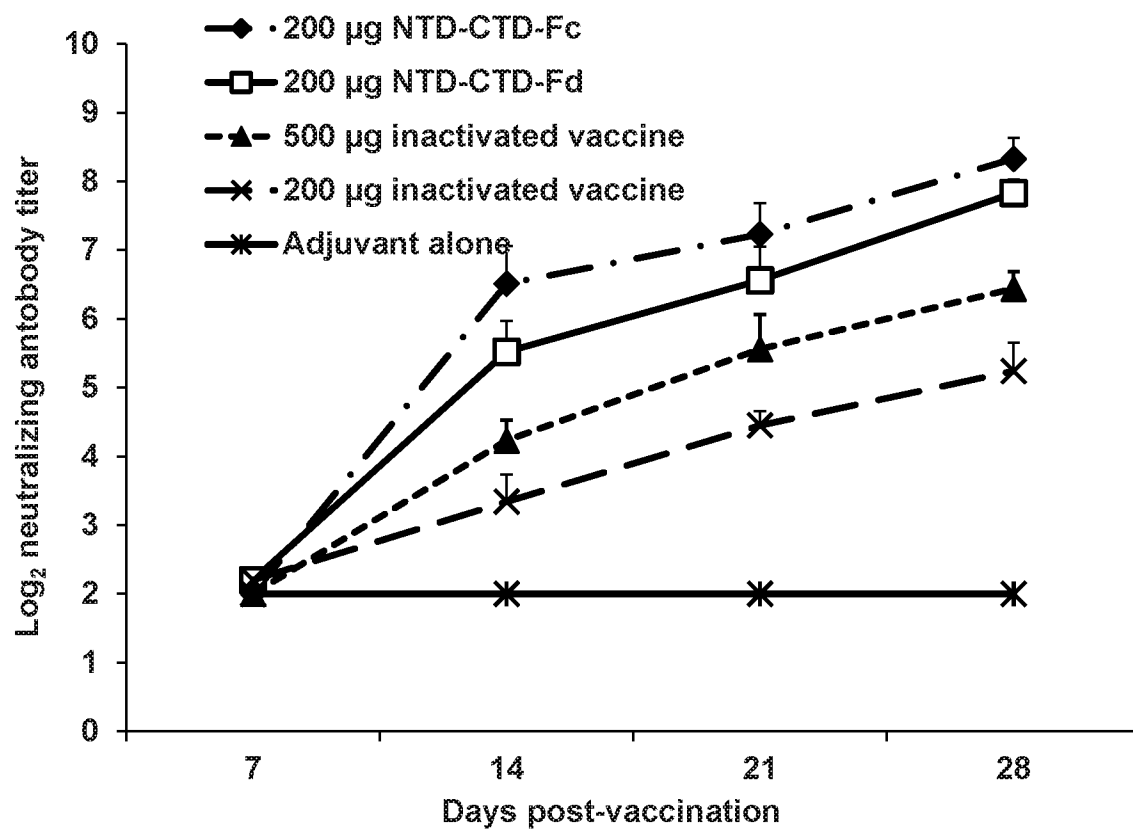


FIG. 21

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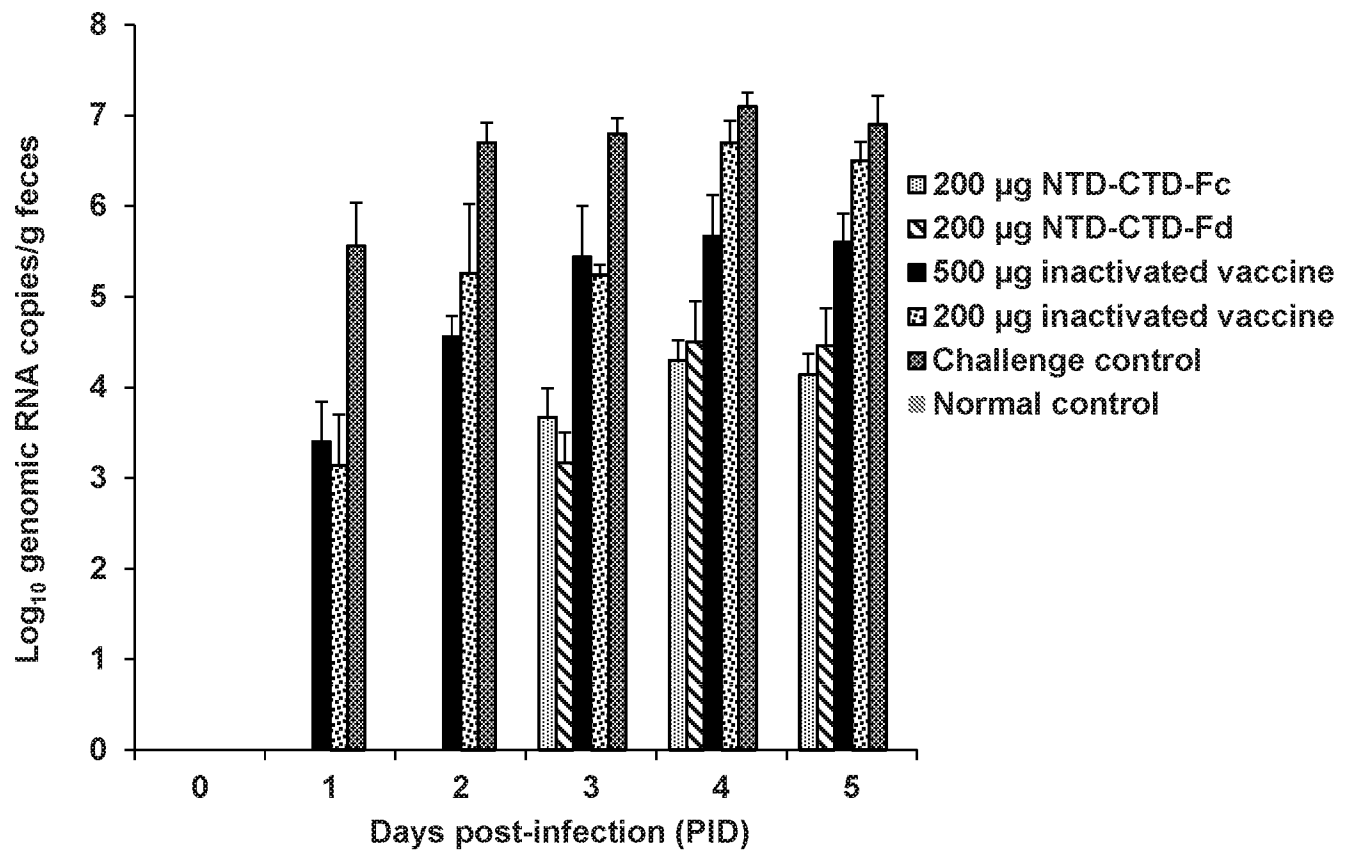


FIG. 22

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US 16/17600

A. CLASSIFICATION OF SUBJECT MATTER

IPC(8) - A61K 39/215, C07K 14/165 (2016.01)

CPC - A61K 39/215, C12N 7/00, C07K 14/165, A61K 39/00, C12N 2770/20034

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

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC(8) - A61K39/215, C07K14/165 (2016.01)

CPC - A61K39/215, C12N7/00, C07K14/165, A61K39/00, C12N2770/20034

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

USPC - 424/184.1, 424/221

Keyword search; search terms below

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

PatBase, Google scholar, Google Patents, porcine epidemic diarrhea virus, PEDV, vaccination, immunization, immunogenic composition, vaccine, spike protein, S1, S1-CTD, S1-NTD, C terminal domain, N terminal domain, Fc, GCN4, Foldon, stabilization sequence, muramyl peptide, C3d, Onchocerca volvulusASP-1, cholera toxin, coronavirus, SARS, subunit vacc

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X ----- Y	CN 104262488 A (PULAI KE BIOLOGICAL ENGINEERING CO LTD.) 07 January 2015 (07.01.2015) abstract, para [0005], [0006], [0007], [0008], [0009], [0011], [0014], [0015], [0024], [0032], [0102], [0103], [0104], [0105], [0106]	1-2, 4, 6-8, 11, 13-19, 20/(1-2,4,6-8,11,13) ----- 3, 5, 9, 10, 12, 20/(3,5,9,10,12)
Y	LEE et al. The N-terminal Region of the Porcine Epidemic Diarrhea Virus Spike Protein is Important for the Receptor Binding. Korean J Microbiol Biotechnol, 31 December 2011 (31.12.2011) Volume 39 No. 2 pp 140-145. abstract, page 143 col 2 para 1 to page 144 col 1 para 1.	3, 5, 20/(3,5)
Y	WO 2014/134439 A1 (NEW YORK BLOOD CENTER, INC.) 04 September 2014 (04.09.2014) para [0007], [0011], [0012], [0044]	9, 10, 12, 20/(9,10,12)

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

* Special categories of cited documents:

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

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

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

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

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

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

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

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

"&" document member of the same patent family

Date of the actual completion of the international search

04 April 2016 (04.04.2016)

Date of mailing of the international search report

02 MAY 2016

Name and mailing address of the ISA/US

Mail Stop PCT, Attn: ISA/US, Commissioner for Patents
P.O. Box 1450, Alexandria, Virginia 22313-1450

Facsimile No. 571-273-8300

Authorized officer:

Lee W. Young

PCT Helpdesk: 571-272-4300

PCT OSP: 571-272-7774

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US 16/17600

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. ☒ forming part of the international application as filed:

☒ in the form of an Annex C/ST.25 text file.

☐ on paper or in the form of an image file.

b. ☐ furnished together with the international application under PCT Rule 13*ter*.1(a) for the purposes of international search only in the form of an Annex C/ST.25 text file.

c. ☐ furnished subsequent to the international filing date for the purposes of international search only:

☐ in the form of an Annex C/ST.25 text file (Rule 13*ter*.1(a)).

☐ on paper or in the form of an image file (Rule 13*ter*.1(b) and Administrative Instructions, Section 713).

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

3. Additional comments: