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(54) Title: RECOMBINANT ROTAVIRUS EXPRESSION SYSTEM AND RECOMBINANT ROTAVIRUSES

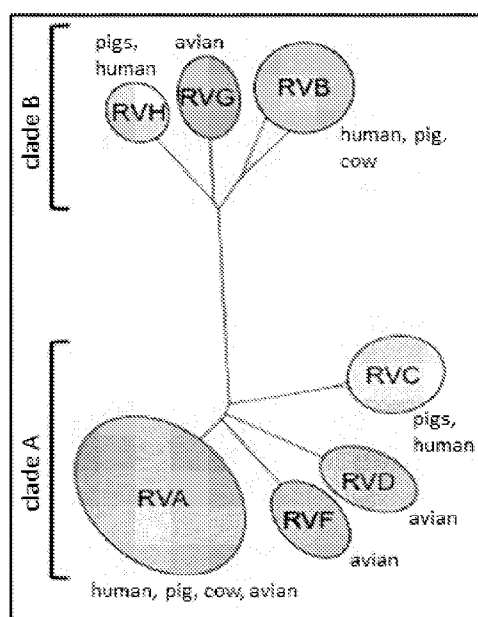


FIG. 1

(57) Abstract: Embodiments herein provide compositions, methods, uses and manufacturing procedures for rotavirus constructs and immunogenic compositions thereof. Some embodiments concern compositions that include, but are not limited to, chimeric rotaviruses of use in immunogenic compositions against rotavirus infection as well as against coronavirus infection in a subject. In certain embodiments, constructs of use herein can be generated and used where a rotavirus expression system further includes one or more nucleic acid molecules encoding one or more polypeptides of a coronavirus.

RECOMBINANT ROTAVIRUS EXPRESSION SYSTEM AND RECOMBINANT ROTAVIRUSES

PRIORITY CLAIM

[0001] This application claims the benefit of US provisional patent application number 63/012,870, filed on April 20, 2020, the provisional application is incorporated herein by reference in its entirety.

STATEMENT OF GOVERNMENT RIGHTS

[0002] This invention was made with Government support under grant AI144881 awarded by the National Institutes of Health (NIH). The Government has certain rights in this invention.

SEQUENCE LISTING

[0003] The instant application contains a Sequence Listing which is provided in the following table:

Table of Sequences

Description	Sequence	SEQ ID NO
P2A	SKFQIDKILISGDIELNPGP	1
SARS-CoV-2 S protein (MN908947.3)	MFVFLVLLPLVSSQCVNLTRTQLPPAYTNSFTRGVYYPDKVFRSSVLHST QDLFLPFFSNVTWFHAIHVSGTNGTKRFDNPVLPFNDGVYFASTEKSNIIRG WIFGTTLDSKTQSLIVNNAATNVVIKVECFQFCNDPFLGVYHKNKNSWM ESEFRVYSSANNCTFEYVSQPFLMDLEGKQGNFKNLREFVFNIDGYFKIY SKHTPINLVRDLPQGFSALEPLVDLPIGINITRFQTLLALHRSYLTTPGDSSSG WTAGAAAYYVGYLQPRTFLLKYNENGTITDAVDCALDPLSETKCTLSFT VEKGIYQTSNFRVQPTESIVRFPNITNLCPFGEVFNATRFASVYAWNKRIS NCVADYSVLVNSASFSTFKCYGVSPTKLNDLCFTNVYADSFVIRGDEVRI APGQTGKIADYNYKLPDDFTGCVIAWNSNNLDSKVGGNYNYLYRLFRKSN LKPFERDISTEIQAGSTPCNGVEGFNCYFPLQSYGFQPTNGVGYQPYRVV VLSFELLHAPATVCGPKKSTNLVKNKCVNFNFNGLTGTGVLTESNKKFLPF QQFGRDIADTTDAVRDPQTLEILDITPCSFGGVSVITPGTNTSNQVAVLYQD VNCTEVPVAIHADQLTPTWRVYSTGSNVFQTRAGCLIGAHEVNNSYEC DIP IGAGICASYQTQTN SPRRARSVASQSIIAYTMSLGAENSVAYSNN SIAIPTNF TISVTTEILPVSMTKTSVDCTMYICGDSTECSNLLQYGSFCTQLNRALTGIA VEQDKNTQEVFAQVKQIYKTPPIKDFGGFNFSQILPDPSKPSKRSFIEDLLFN KVTADAGFIKQYGDCLGDIAARDLICAQKFNGLTVLPPLL TDEMIAQYTS ALLAGTITSGWTFGAGAA LQIPFAMQMAYRFNGIGVTQNVLYENQKLIAN QFNSAIGKIQDSL SSTASALGKLQDVVNQNAQALNTLVKQLSSNF GAISSVL NDILSRDLKVEAEVQIDRLITGRLQSLQTYVTQQLIRAAEIRASANLAATKM SECVLGQSKRVDFCGKGYHLMSFPQ SAPHGVVFLHVITYVPAQEKNFTTAP AICHDGKAHFPREGVFVSNGTHWFVTQRNFYEPQIITTDNTFVSGNCDVVI	2

	GIVNNTVYDPLQPELDSFKEELDKYFKNHTSPDVLGDISGINASVVNIQKE IDRLNEVAKNLNESLIDLQELGKYEQYIKWPWYIWLGFIAGLIAIVMTIML CCMTSCCSCCLKGCCSCGSCCKFDEDDSEPVLKGVKLHYT*	
SARS-CoV-2 S1/NTD (15- 294 of SEQ ID NO: 2)	CVNLTTRTQLPPAYTNSFTRGVYYPDKVFRSSVLHSTQDLFLPFFSNVTWF HAIHVSGTNGTKRFDNPVLPFNDGVYFASTEKSNIIRGWIFGTTLDSKTQSL LIVNNATNVVIK VCEFQFCNDPFLGVYYHKNNKSWMESEFRVYSSANNCT FEYVSQPFLMDLEGKQGNFKNLREFVFKNIDGYFKIY SKHTPINLVRDLPO GFSALEPLVDLPIGINITRFQTLALHRSYLT PGDSSSGWTAGAAAYVGYL QPRTFLLKYNENGTITDAVDCALD	3
SARS-CoV-2 S1/NTD+SS (1-294 of SEQ ID NO: 2)	MFVFLVLLPLVSSQCVNLTTRTQLPPAYTNSFTRGVYYPDKVFRSSVLHST QDLFLPFFSNVTWFHAIHVSGTNGTKRFDNPVLPFNDGVYFASTEKSNIIRG WIFGTTLDSKTQSL LIVNNATNVVIK VCEFQFCNDPFLGVYYHKNNKSWM ESEFRVYSSANNCTFEYVSQPFLMDLEGKQGNFKNLREFVFKNIDGYFKIY SKHTPINLVRDLPOGFSALEPLVDLPIGINITRFQTLALHRSYLT PGDSSSG WTAGAAAYVGYLQPRTFLLKYNENGTITDAVDCALD	4
SARS-CoV-2 S1/RBD (335- 523 of SEQ ID NO: 2)	LCPFGEVFNATRFASVYAWNKRISNCVADYSVLVNSASFSTFKCYGVSP KLNDLCFTNVYADSFVIRGDEV RQIAPGQTGKIADYNYKL PDDFTGCVIAW NSNNLDSKVGGNYNLYRLFRKSNLKPFERDISTEIQAGSTPCNGVEGFN CYFPLQSYGFQPTNGVGYQPYRVVLSFELLHAPAT	5
SARS-CoV-2 S1/2xRBD (335-523 of SEQ ID NO: 2 +GGSGGS+3 35-523 of SEQ ID NO: 2)	LCPFGEVFNATRFASVYAWNKRISNCVADYSVLVNSASFSTFKCYGVSP KLNDLCFTNVYADSFVIRGDEV RQIAPGQTGKIADYNYKL PDDFTGCVIAW NSNNLDSKVGGNYNLYRLFRKSNLKPFERDISTEIQAGSTPCNGVEGFN CYFPLQSYGFQPTNGVGYQPYRVVLSFELLHAPATGGSGGSLCPFGEVFN ATRFASVYAWNKRISNCVADYSVLVNSASFSTFKCYGVSPKLNDLCFTN VYADSFVIRGDEV RQIAPGQTGKIADYNYKL PDDFTGCVIAWNSNNLDSK VGGNYNLYRLFRKSNLKPFERDISTEIQAGSTPCNGVEGFNCYFPLQSY GFQPTNGVGYQPYRVVLSFELLHAPAT	6
SARS-CoV- 2/1 S1/2xRBD (335-523 of SEQ ID NO: 2 +GGSGGS+3 22-509 of SEQ ID NO: 12)	LCPFGEVFNATRFASVYAWNKRISNCVADYSVLVNSASFSTFKCYGVSP KLNDLCFTNVYADSFVIRGDEV RQIAPGQTGKIADYNYKL PDDFTGCVIAW NSNNLDSKVGGNYNLYRLFRKSNLKPFERDISTEIQAGSTPCNGVEGFN CYFPLQSYGFQPTNGVGYQPYRVVLSFELLHAPATGGSGGSLCPFGEVFN ATKFPSVYAWERKKISNCVADYSVLVNSTFFSTFKCYGVSA TKLNDLCFSN VYADSFVVKGDDVRQIAPGQTGVIADYNYKL PDDFMGCVLAWNTRNIDA TSTGNYNYKYRYLRHGKLRPFERDISNVPFSPDGKPTPALNCYWPLNDY GFYTTTGIGYQPYRVVLSFELLNAPAT	7
SARS-CoV- 1/2 S1/2xRBD (322-509 of SEQ ID NO: 12+GGSGGS +335-523 of SEQ ID NO: 2)	LCPFGEVFNATKFPSVYAWERKKISNCVADYSVLVNSTFFSTFKCYGVSA KLNDLCFSNVYADSFVVKGDDVRQIAPGQTGVIADYNYKL PDDFMGCVL AWNTRNIDATSTGNYNYKYRYLRHGKLRPFERDISNVPFSPDGKPTPAL NCYWPLNDYGFYTTTGIGYQPYRVVLSFELLNAPATGGSGGSLCPFGEVFN NATRFASVYAWNKRISNCVADYSVLVNSASFSTFKCYGVSPKLNDLCFT NVYADSFVIRGDEV RQIAPGQTGKIADYNYKL PDDFTGCVIAWNSNNLDS KVGGNYNLYRLFRKSNLKPFERDISTEIQAGSTPCNGVEGFNCYFPLQS YGFQPTNGVGYQPYRVVLSFELLHAPAT	8
SARS-CoV-2 S1/ExRBD (311-599 of SEQ ID NO: 2)	GIYQTSNFRVQPTESIVRFPNITNLCPFGEVFNATRFASVYAWNKRISNCV ADYSVLVNSASFSTFKCYGVSPKLNDLCFTNVYADSFVIRGDEV RQIAPG QTGKIADYNYKL PDDFTGCVIAWNSNNLDSKVGGNYNLYRLFRKSNLKP FERDISTEIQAGSTPCNGVEGFNCYFPLQSYGFQPTNGVGYQPYRVVLSF ELLHAPATVCGPKKSTNLVKNKCVNFNFNGLTGTGVLTESNKKFLPFQFQ GRDIADTTDAVRDPQTLEILDITPCSFGGVSVIT	9
SARS-CoV-2 S2/core (711- 1077 of SEQ ID NO: 2)	SIAPTNFTISVTTEILPVSMTKTSVDCTMYICGDSTECNLLLQYGSFCTQLN RALTGIAVEQDKNTQEVFAQVKQIYKTPPIKDFGGFNFSQILPDPSKPSKRSF IEDLLFNKVTADAGFIKQYGDCLGDIAARDLICAQKFNGLTVPPLLTDE MIAQYTSALLAGTITSGWTFGAGAALQIPFAMQMAYRFNGIGVTQNVLYE NQKLIANQFNSAIGKIQDLSSTASALGKLQDVVNQNAQALNTLVKQLSSN FGAISSVLNDILSRDLKVEAEVQIDRLITGRQLSLQTYVTQQLIRAAEIRASA	10

	NLAATKMSECVLGQSKRVDFCGKGYHLSFQPSAPHGVVFLHVTYVPAQ EKNFTT	
SARS-CoV-2 S2w/oTM (686-1194 of SEQ ID NO: 2)	SVASQSIAYTMSLGAENSVAYSNNIAIPTNFTISVTTEILPVSMTKTSVDCT MYICGDSTECNLLLQYGSFCTQLNRALTGIAVEQDKNTQEVFAQVKQIYK TPPIKDFGGFNFSQILPDPSKPSKRSFIEDLLFNKVTADAGFIKQYGDCLGDI AARDLICAQKFNGLTVLPLLTDemiaQYTSALLAGTITSGWTFGAGAALQ IPFAMQMAYRFNGIGVTQNVLYENQKLIANQFNSAIGKIQDSLSTASALG KLQDVVNQNAQALNTLVKQLSSNFGAISSVLNDILSRDLKVEAEVQIDRLIT GRLQSLQTYVTQQLIRAAEIRASANLAATKMSECVLGQSKRVDFCGKGYH LMSFQPSAPHGVVFLHVTYVPAQEKNTTAPAICHGDKAHFPREGVVFVSN THWFTVQRNFYEPQIITDNTFVSGNCDVIGIVNNTVYDPLQPELDSFKEE LDKYFKNHTSPDVLGDISGINASVVNIQKEIDRLNEVAKNLN	11
SARS-CoV S glycoprotein (P59594)	MFIFLLFLTLTSGSDLDRCTTFDDVQAPNYTQHTSSMRGVYYPDEIFRSDTL YLTQDLFLPFYSNVTGFHTINHTFGNPVIPFKDGIYFAATEKSNVVRGWVFG STMNNKSQSVIIINNSTNVIRACNFELCDNPFFAVSKPMGTQTHTMIFDNA FNCTFEYISDAFSLDVSEKSGNFKHLREFVFNKNDGFLYVYKGYQPIDVVR DLPSGFNTLKPIFKLPLGINITNFRAILTAFSPAQDIWGTSAAYFVGYLKPT TFMLKYDENGITDAVDCSQNPLAELKCSVKSFEIDKGIYQTSNFRVPSG DVVRFPNITNLCPFGEVFNATKFPSVYAWERKKISNCVADYSVLNSTFFST FKCYGVSATKLNDLCFSNVYADSFVVKGDDVRQIAPGQTGVIADYNYKLP DDFMGCVLAWNTRNIDATSTGNYNKYRYLRHGKLRPFERDISNVPFSPD GKPCPTPALNCYWPLNDYGFYTTTGIGYQPYRVVLSFELLNAPATVCGPK LSTDLIKNQCVNFNGLTGTGVLTPSSKRFQPFQFGRDVSDFTDSVRDP KTSEILDISPCSFGGVSVITPGTNASSEVAVLYQDVNCTDVSTAIHADQLTPA WRIYSTGNVVFQTQAGCLIGAEHVDTSYECDIPIGAGICASYHTVSLRSTS QKSIVAYTMSLGADESSIAYSNNIAIPTNFSISITTEVMPVSMAKTSVDCNM YICGDSTECANLLLQYGSFCTQLNRALSGIAAEQDRNTREVFQVKQMYK TPTLKYFGGFNFSQILPDPLKPTKRSFIEDLLFNKVTADAGFMKQYGECLG DINARDLICAQKFNGLTVLPLLTDemiaAAYTAALVSGTATAGWTFGAGA ALQIPFAMQMAYRFNGIGVTQNVLYENQKLIANQFNKAIQIESLTTTST ALGKLQDVVNQNAQALNTLVKQLSSNFGAISSVLNDILSRDLKVEAEVQID RLITGRLQSLQTYVTQQLIRAAEIRASANLAATKMSECVLGQSKRVDFCGK GYHLSFQPSAPHGVVFLHVTYVPSQERNFTTAPAICHEGKAYFPREGVVF FNGTSWFITQRNFFSPQIITDNTFVSGNCDVIGIINNTVYDPLQPELDSFKE ELDKYFKNHTSPDVLGDISGINASVVNIQKEIDRLNEVAKNLNESLIDLOE LGKYEYQIKWPWYVWLGFIAGLIAIVMTILLCCMTSCCCLKGACSCGSC CKFDEDDSEPVLKGVKLHYT	12
SARS-CoV S1/NTD (16- 281 of SEQ ID NO: 12)	LDRCTTFDDVQAPNYTQHTSSMRGVYYPDEIFRSDTL YLTQDLFLPFYSNV TGFTINHTFGNPVIPFKDGIYFAATEKSNVVRGWVFGSTMNNKSQSVIIIN NSTNVIRACNFELCDNPFFAVSKPMGTQTHTMIFDNAFNCTFEYISDAFSL DVSEKSGNFKHLREFVFNKNDGFLYVYKGYQPIDVVRDLPSGFNTLKPIFK LPLGINITNFRAILTAFSPAQDIWGTSAAYFVGYLKPTTFMLKYDENGIT DAVDCSQN	13
SARS-CoV S1/NTD+SS (1-281 of SEQ ID NO: 12)	MFIFLLFLTLTSGSDLDRCTTFDDVQAPNYTQHTSSMRGVYYPDEIFRSDTL YLTQDLFLPFYSNVTGFHTINHTFGNPVIPFKDGIYFAATEKSNVVRGWVFG STMNNKSQSVIIINNSTNVIRACNFELCDNPFFAVSKPMGTQTHTMIFDNA FNCTFEYISDAFSLDVSEKSGNFKHLREFVFNKNDGFLYVYKGYQPIDVVR DLPSGFNTLKPIFKLPLGINITNFRAILTAFSPAQDIWGTSAAYFVGYLKPT TFMLKYDENGITDAVDCSQN	14
SARS-CoV S1/RBD (322- 509 of SEQ ID NO: 12)	LCPFGEVFNATKFPSVYAWERKKISNCVADYSVLNSTFFSTFKCYGVSAT KLNDLCFSNVYADSFVVKGDDVRQIAPGQTGVIADYNYKLPDDFMGCVL AWNTRNIDATSTGNYNKYRYLRHGKLRPFERDISNVPFSPDGKPCPTPAL NCYWPLNDYGFYTTTGIGYQPYRVVLSFELLNAPAT	15
SARS-CoV S1/RBD (322- 509 of SEQ ID	LCPFGEVFNATKFPSVYAWERKKISNCVADYSVLNSTFFSTFKCYGVSAT KLNDLCFSNVYADSFVVKGDDVRQIAPGQTGVIADYNYKLPDDFMGCVL AWNTRNIDATSTGNYNKYRYLRHGKLRPFERDISNVPFSPDGKPCPTPAL	16

NO: 12 +GGSGGS+3 22-509 of SEQ ID NO: 12)	NCYWPLNDYGFYTTTGIGYQPYRVVLSFELLNAPATGGSGGSLCPFGEVF NATKFPSVYAWERKKISNCVADYSVLNSTFFSTFKCYGVSATKLNLCFS NVYADSFVVKGDDVRQIAPGQTGVIADYNYKLPDDFMGCVLAWNTRNID ATSTGNYNKYRYLRHGKLRPFERDISNVFSPDGKPCPPALNCYWPLND YGFYTTTGIGYQPYRVVLSFELLNAPAT	
SARS-CoV S1/ExRBD (298-585 of SEQ ID NO: 12)	GIYQTSNFRVVPSPGDVVRFPNITNLCPFGEVFNATKFPSVYAWERKKISNCV ADYSVLNSTFFSTFKCYGVSATKLNLCFSNVYADSFVVKGDDVRQIAP GQTGVIADYNYKLPDDFMGCVLAWNTRNIDATSTGNYNKYRYLRHGKLR PFERDISNVFSPDGKPCPPALNCYWPLNDYGFYTTTGIGYQPYRVVLS FELLNAPATVCGPKLSTDILKNQCVNFNFNGLTGTGVLTPSSKRFQPFQQFG RDVSDFTDSVRDPKTSEILDISPCSFGGVSVIT	17
SARS-CoV S2/core (693- 1059 of SEQ ID NO: 12)	TIAIPTNFSISITTEVMPVSMAKTSVDCNMYICGDSTECANLLLQYGSFCTQL NRALSGIAAEQDRNTREVF AQVKQMYKTPTLKYFGGFNFSQILPDPLKPTK RSFIEDLLFNK VTLADAGFMKQYGECLGDINARDLICAQKFNGLT VLPPLL TDDMIAAYTAALVSGTATAGWTFGAGAALQIPFAMQMAYRFNGIGVTON VLYENQKQIANQFNKAISQIQESLTTTSTALGKLQDVVNQNAQALNTLVKQ LSSNFGAISSVLNDILSRDLKVEAEVQIDRLITGRLQSLQTYVTQQLIRAAEI RASANLAATKMSECVLGQSKRVDFCGKGYHLMSFPQAAPHGVVFLHVTY VPSQERNFTT	18
SARS-CoV S2w/oTM (686-1194 of SEQ ID NO: 12)	SIAYSNNTIAIPTNFSISITTEVMPVSMAKTSVDCNMYICGDSTECANLLLQY GSFCTQLNRALSGIAAEQDRNTREVF AQVKQMYKTPTLKYFGGFNFSQILP DPLKPTKRSFIEDLLFNK VTLADAGFMKQYGECLGDINARDLICAQKFNGLT TVLPPLLTDDMIAAYTAALVSGTATAGWTFGAGAALQIPFAMQMAYRFN GIGVTONVLYENQKQIANQFNKAISQIQESLTTTSTALGKLQDVVNQNAQA LNTLVKQLSSNFGAISSVLNDILSRDLKVEAEVQIDRLITGRLQSLQTYVTQ QLIRAAEIRASANLAATKMSECVLGQSKRVDFCGKGYHLMSFPQAAPHGV VFLHVTYVPSQERNFTTAPAICHEGKAYFPREGVVFVNGTSWFTQNRNFFSP QIITTDNTFVSGNCDVVGIIINNTVYDPLQPELDSFKEELDKYFKNHTSPDVD LGDISGINASVVNIQKEIDRLNEVAKNLN	19
MERS-CoV (YP_0090472 04.1)	MIHSVFLLMFLLTPTESYVDVGPDSVKSACIEVDIQQTFFDKTWPRPIDVSK ADGHIYPQGRITYSNITITYQGLFPYQGDHGDMDYVYSAGHATGTTTPQKLFVA NYSQDVKQFANGFVVRIGAAANSTGTVIISPSTSATIRKIYPAFMLGSSVGN FSDGKMGRFFNHTLVLLPDGCGTLLRAFYCILEPRSGNHCPAGNSYTSFAT YHTPATDCSDGNYNRNASLNSFKEYFNLRNCTFMYTYNITEDEILEWFGIT QTAQGVHLFSSRYVDLYGGNMFFQFATLPVYDTIKYYSIIPHSIRSISQSDRKA WAAFYVYKLQPLTFLDFSVDGYIRRAIDCGFNDLSQLHCSYESFDVESGV YSVSSFEAKPSGVSVEQAEGVECDFSPLLSGTPPQVYNFKRLVFTNCNYNL TKLLSLFSVNDFTCISQISPAAIASNCYSSLILDYFSYPLSMKSDLSVSSAGPIS QFNYKQSFNSPTCLILATVPHNLTITKPLKYSYINKCSRLLSDDRTEVPQLV NANQYSPCVSIVPSTVWEDGDYRQKLSPLEGGGWLVASGSTVAMTEQL QMFGGITVQYGTDTNSVCPKLEFANDTKIASQLGNCVEYSLYGVSGRGVF QNCTAVGVRRQRFVYDAYQNLVGYYSDDGNYCLRACVSVPVSVIYDKE TKTHATLFGSVACEHISSTMSQYSRSTRSMLKRRDSTYGPLQTPVGCVLGL VNSSLFVEDCKLPLGQSLCALPDTPSTLTPRSVRSVPGEMRLASIAFNHPIQV DQLNSSFYFKLSIPTNFSFGVTQEYIQTIIQKVTVDCKQYVCNGFQKCEQLLR EYGGQFCSKINQALHGANLRQDDSVRNLFASVKSSQSSPIIPGFGGDFNLTL EPVSISTGSRARSARSAIEDLLFDKVTIADPGYMQGYDDCMQQGPASARDLIC AQYVAGYKVLPLMDVNMEAAAYTSSLLGSIAGVGWTAGLSSFAAIPFAQSI FYRLNGVGITQQVLSENQKLIANKFNQALGAMQTGFTTTTNEAFQKVQDAV NNNAQALSKLASELSNTFGAISASIGDIIQRLDVLEQDAQIDRLINGRLTTLN AFVAQQLVRSESAALSAQLAKDKVNECVKAQSKRSGFCGQGTHIVSFVVN APNGLYFMHVGYYPSNHIEVVSAYGLCDAANPTNCIAPVNGYFIKTNNTRI VDEWSYTGSSFYAPEPITSLNTKYVAPQVTYQNISTNLPPLLGNSTGIDFQ DELDEFFKNVSTSIPNFGSLTQINTTLLDLTYEMLSLQQVVKALNESYIDLK ELGNYTYYNKWPWYIWLGFIAGLVALALCVFFILCCTGCGTNCMKGKLCN RCCDRYEEYDLEPHKVHVH	20

MERS-CoV S1/NTD (26-342 of SEQ ID NO: 20)	VKSACIEVDIQQTFFDKTWPRPIDVSKADGHIYPQGRITYSNITITYQGLFPYQGDHGDYVYSAGHATGTTTPQKLFVANYSQDVKQFANGFVVRIGAAANSTGTVIISPSTSATIRKIYPAFMLGSSVGNFSDGKMGRFFNHTLVLLPDGCGTLLRAFYCILEPRSGNHCPAGNSYTSFATYHTPATDCSDGNYNRNASLNSFKEYFNLNRNCTFMYTYNITEDEILEWFGITQTAQGVHLFSSRYVDLYGGNMFQFATLPVYDTIKYYSIIPHSIRSISIQSDRKAWAAFYVYKLQPLTFLDFSVDGYIRR AIDCGFN	21
MERS-CoV S1/NTD+SS (1-342 of SEQ ID NO: 20)	MIHSVFLLMFLLTPTESYVDVGPDSVKSACIEVDIQQTFFDKTWPRPIDVSKADGHIYPQGRITYSNITITYQGLFPYQGDHGDYVYSAGHATGTTTPQKLFVANYSQDVKQFANGFVVRIGAAANSTGTVIISPSTSATIRKIYPAFMLGSSVGNFSDGKMGRFFNHTLVLLPDGCGTLLRAFYCILEPRSGNHCPAGNSYTSFATYHTPATDCSDGNYNRNASLNSFKEYFNLNRNCTFMYTYNITEDEILEWFGITQTAQGVHLFSSRYVDLYGGNMFQFATLPVYDTIKYYSIIPHSIRSISIQSDRKAWAAFYVYKLQPLTFLDFSVDGYIRRAIDCGFN	22
MERS-CoV S1/RBD (383-579 of SEQ ID NO: 20)	CDFSPLLSGTPPQVYNFKRLVFTNCNYNLTCLLSLFSVNDFTCSQISPAAIASNCYSSLILDYFSYPLSMKSDLSVSSAGPISQFNKQSFNPTCLILATVPHNLTITKPLKYSYINKCSRLSDDRTEVPQLVNANQYSPCVSIVPSTVWEDGDY YRKQLSPLEGGGWLVASGSTVAMTEQLQMFGGITVQYGT	23
MERS-CoV S1/ExRBD (359-663 of SEQ ID NO: 20)	GVYSVSSFEAKPSGVSVEQAEQVECDFSPLLSGTPPQVYNFKRLVFTNCNYNLTCLLSLFSVNDFTCSQISPAAIASNCYSSLILDYFSYPLSMKSDLSVSSAGPISQFNKQSFNPTCLILATVPHNLTITKPLKYSYINKCSRLSDDRTEVPQLVNANQYSPCVSIVPSTVWEDGDY YRKQLSPLEGGGWLVASGSTVAMTEQLQMFGGITVQYGTDTNSVCPKLEFANDTKIASQLGNCVEYSLYGVSGRGVFQNCCTAVGVRQQRFFVYDAYQNLVGYYSDDGNYCLRACVSVPSVIY	24
MERS-CoV S2/core (776-1151 of SEQ ID NO: 20)	SYFKLSIPTNFSFGVTQEYIQTITQKVTVDCKQYVCNGFQKCEQLLREYGQFCSKINQALHGANLRQDDSVRNLFASVKSSQSSPIIPGFGGDFNLTLLEPVSSITGSRARSARSAIEDLLFDKVTIADPGYMQGYDDCMQQGPASARDLCAQYVAGYKVLPLMDVNMEAAAYTSSLLGSIAGVGWTAGLSSFAAIPFAQSIFYRLNGVGITQQVLSENQKLIANKFNQALGAMQTGFTTTNEAFQKVQDAVNNAQALSKLASELSNTFGAISASIGDIIQRLDVLEQDAQIDRLINGRLTTLNAFVAQQLVRSESAALSAQLAKDKVNECVKAQSKRSGFCGQGTHIVSFVNAPNGLYFMHVGYYPSNHIEVVS	25
MERS-CoV S2w/oTM (769-1277 of SEQ ID NO: 20)	QVDQLNSSYFKLSIPTNFSFGVTQEYIQTITQKVTVDCKQYVCNGFQKCEQLLREYGQFCSKINQALHGANLRQDDSVRNLFASVKSSQSSPIIPGFGGDFNLTLLEPVSSITGSRARSARSAIEDLLFDKVTIADPGYMQGYDDCMQQGPASARDLCAQYVAGYKVLPLMDVNMEAAAYTSSLLGSIAGVGWTAGLSSFAAIPFAQSIFYRLNGVGITQQVLSENQKLIANKFNQALGAMQTGFTTTNEAFQKVQDAVNNAQALSKLASELSNTFGAISASIGDIIQRLDVLEQDAQIDRLINGRLTTLNAFVAQQLVRSESAALSAQLAKDKVNECVKAQSKRSGFCGQGTHIVSFVNAPNGLYFMHVGYYPSNHIEVVSAYGLCDAANPTNCIAPVNGYFIKTNNTRIVDEWSYTGSSFYAPEPITSLNTKYVAPQVTYQNIQNTNLPPLLGNSTGIDFQDELDEFFKNVSTSIPTNFGSLTQINTLLDLTYEMLSLQQVVKALN	26
HCoV-299E (NP_073551.1)	MFVLLVAYALLHIAGCQTTNGLNTSYSVNCNGCVGYSENVFAVESGGYIPSDFAFNWFLLTNTSSVVDGVVRSFQPLLNCLWSVSGLRFTTGFFVYFNGTGRGDCKGFSSDVLSDVIRYNLNFEEENLRRTILFKTSYGVVVFYCTNNTLVSGDAHIFPGTVLGNFYCFVNTTIGNETTSFVVGALPKTVREFVISRTGHFYINGYRYFTLGNVEAVNFVNTAETDFTVALASYADVLVNVSQTSIANIHCNSVINRLRCDQLSFDVDPDGFYSTSPIQSVLPVSVISLPVYHKHTFIVLYVDFKPQSGGKCFNCYPAGVNITLANFNETKGPLCVDTSHTTKYVAVYANVGRWSASINTGNCPSFGKVNNFVKFGSVCFSLKDIPGGCAMPVANWAYSKEYTIGSLYVSWSDGDGITGPVQPVGVSSFMNVTLTKCTKYNIDVSGVGVIRVSNDFLNGITYTSTSGNLLGFKDVTGKIYITPCNPPDQLVVYQQA VVGAMLSENFTSYGFSNVVELPKFFYASNGTYNCTDAVLTYSSFGVCADGSIIAVQPRNVSYDSVSAIVTANLSIPSNTTTSVQVEYLQITSTPIVDCSTYVCNGNVRCEVELLKQYTSACKTIEDALRNSARLESADVSEMLTFDKKAFTLANVSSFGDYNLSSVIPSLPTSGSRVAGRSAIEDILFSKLVTSGLGTVDADYKCKTKG	27

	LSIADLACAQYYNGIMVLPGVADAERMAMYTGSLIGGIALGGLTSAVSIPFSLAIQARLNYVALQTDVLQENQKILAAAFNKAMTNIVDAFTGVNDAITQTSQALQTVATALNKIQDVVNQQGNLSNHLTSQLRQNFQAISSSIQAIYDRLDTIQADQQVDRLITGRLAALNVFVSHTLTKYTEVRASRQLAQQKVNECVKSQSKRYGFCGNGTHIFSIVNAAPEGLVFLHTVLLPTQYKDVEAWSGLCVDGTNGYVLRQPNLALYKEGNYRITSRIMFEPRIPTMADFVQIENCNVTFVNISRELTIVPEYIDVNKTLQELSYKLPNYTVPDLVVEQYNQTILNLTSEISTLENKSAELNYTVQKLQTLIDNINSTLVDLKWLN RVETYIKWPWWVWLCISVVLIFFVSMMLLCCCSTGCCGFFSCFASSIRGCCSTKLPYYDVEKIHQ	
HCoV-299E S1/NTD (8-151 of SEQ ID NO: 27)	YALLHIAGCQTTNGLNTSY SVCNGCVGYSENVFAVESGGYIPSDFAFNWFL TNTSSVVDG VVRSFQPLLLNCLWSVSGLRFTTG FVYFNGTGRGDCKGFS SDVLSDVIRYNLNFEEENLRRTILFKTSYGVVVFYCTNNTLV	28
HCoV-OC43 S1/NTD+SS (1-293 of SEQ ID NO: 27)	MFLILLISLPTAF A VIGDLKCTSDNINDKDTGPPPISTDTVDVTNGLGTYYYLDRVYLNNTTLFLNGYYPTSGSTYRNMAL KGSVLLSRLWFKPPFLSDFINGIFA KVKNTKVIKDRVMYSEFPAITIGSTFVN TSYSVVVQPR TINSTQDGDNKLQGLLEVSVCQYNMCEYPQTICHPNLGNHRKEL WHLDTGVVSCLYKRNFTY DVNADYLYFHFYQEGGTFYAYFTDTGVVTKFLFN VYLGMA LSHYYVMPLTCNSKLTLEYWVTPLTSRQYLLAFNQDGIIFNAVDCMSD	29
HCoV-299E S1/RBD (192-424 of SEQ ID NO: 27)	EFVISRTGHFYINGYRYFTLGNVEAVNFNVTTAETTD FCTVALASYADVLV NVSQTSIANIY CNSVINRLRCDQLSFDVPDGFYSTSPIQSVELPVSI VSLPVYHKHTFIVLYVDFK PQSGGGKCFNCYPAGVNITLANFN ETKGPLCVDTSHTTKYVAVYANVGRWSASINTGNCPFSFGKVNNFVKFGSVCFSLKDIPGGCA MPIVANWAYS SKYYTIGSLYVSWSDGDG	30
HCoV-299E S1/ExRBD (167-506 of SEQ ID NO: 27)	YCFVNTTIGNETTSAFV GALPKTVREFVISRTGHFYINGYRYFTLGNVEAVNFNVTTAETTD FCTVALASYADVLV NVSQTSIANIY CNSVINRLRCDQLSFDVPDGFYSTSPIQSVELPVSI VSLPVYHKHTFIVLYVDFK PQSGGGKCFNCYPAGVNITLANFN ETKGPLCVDTSHTTKYVAVYANVGRWSASINTGNCPFSFGKVNNFVKFGSVCFSLKDIPGGCA MPIVANWAYS SKYYTIGSLYVSWSDGDGITGVPPQVEGVSSFMNVTL DCKCTKYNIYDVSGVG VIRVSNDTFLNGITYTSTSGNLLGFKDVT KGTIYSITPCNPPDQLVY	31
HCoV-299E S2/core (582-962 of SEQ ID NO: 27)	LSIPSNWTTSVQVEYLQITSTPIVDCSTYVCNGNVRCVELL KQYTSACKTIEDALRNSARLESADVSEMLTFDKKAFTLANVSSFGDYNLSSVIPSLPTSGSRVAGRSAIEDILFSKLVTSGLGTVDADYKKCTKGLSIADLACAQYYNGIMVLPGVADAERMAMYTGSLIGGIALGGLTSAVSIPFSLAIQARLNYVALQTDVLQENQKILAAAFNKAMTNIVDAFTGVNDAITQTSQALQTVATALNKIQDVVNQQGNLSNHLTSQLRQNFQAISSSIQAIYDRLDTIQADQQVDRLITGRLAALNVFVSHTLTKYTEVRASRQLAQQKVNECVKSQSKRYGFCGNGTHIFSIVNAAPEGLVFLHTVLLPTQYKDVEA	32
HCoV-299E S2w/oTM (582-1096 of SEQ ID NO: 27)	LSIPSNWTTSVQVEYLQITSTPIVDCSTYVCNGNVRCVELL KQYTSACKTIEDALRNSARLESADVSEMLTFDKKAFTLANVSSFGDYNLSSVIPSLPTSGSRVAGRSAIEDILFSKLVTSGLGTVDADYKKCTKGLSIADLACAQYYNGIMVLPGVADAERMAMYTGSLIGGIALGGLTSAVSIPFSLAIQARLNYVALQTDVLQENQKILAAAFNKAMTNIVDAFTGVNDAITQTSQALQTVATALNKIQDVVNQQGNLSNHLTSQLRQNFQAISSSIQAIYDRLDTIQADQQVDRLITGRLAALNVFVSHTLTKYTEVRASRQLAQQKVNECVKSQSKRYGFCGNGTHIFSIVNAAPEGLVFLHTVLLPTQYKDVEAWSGLCVDGTNGYVLRQPNLALYKEGNYRITSRIMFEPRIPTMADFVQIENCNVTFVNISRSELQTIVPEYIDVNKTLQELSYKLPNYTVPDLVVEQYNQTILNLTSEISTLENKSAELNYTVQKLQTLIDNI	33
HCoV-OC43 (YP_0095552 41.1)	MFLILLISLPTAF A VIGDLKCTSDNINDKDTGPPPISTDTVDVTNGLGTYYYLDRVYLNNTTLFLNGYYPTSGSTYRNMAL KGSVLLSRLWFKPPFLSDFINGIFA KVKNTKVIKDRVMYSEFPAITIGSTFVN TSYSVVVQPR TINSTQDGDNKLQGLLEVSVCQYNMCEYPQTICHPNLGNHRKEL WHLDTGVVSCLYKRNFTY DVNADYLYFHFYQEGGTFYAYFTDTGVVTKFLFN VYLGMA LSHYYVMPL	34

	TCNSKLTLEYWVTPLTSRQYLLAFNQDGIIFNAVDCMSDFMSEIKCKTQSIAPPTGVYELNGYTVQPIADVYRRKPNLPNCNIEAWLNDKSVPSPLNWERKTF SNCNFMSSLMFSFIQADSFTCNNIDA AKIYGMCFSSITIDKFAIPNGRKVDL QLGNLGYLQSFNYRIDTTATSCQLYYNLPAANVSVS RFPSTWNKRFGFIE DSVFKPRPAGVLTNHDVVYAQHCFKAPKNFCPCKLNGSCVSGPGKNNGI GTCPAGTNYLTCDNLCTPDITFTGT YKCPQTKSLVGIGEHCSGLAVKSDY CGGNSCTCRPQAFLGWSADSC LQGDKNIFANFILHDVNSGLTCSTDLQKA NTDIILGVCVNYDLYGILGQGIFVEVNATYYNSWQNLLYDSNGNLYGFRD YITNRTFMIRSCYSGRVSAAFHANSSEPALLFRNIKCNYVFNNSLTRQLQPI NYFDSYLGCVVNAYNSTAISVQTCDLTVGSGYCVDY SKNRRSRGAITTY RFTNFEPFTVNSVND SLEPVGGLYEIQIPSEFTIGNMVEFIQTSSPKVTIDCAA FVCGDYAACKSQLVEYGSFCDNINAILTEVNELLDTTQLQVANSLMNGVT LSTKLKDG VNFNVDDINFSPVLGCLGSECSKASSRSAIEDLLFDKVKLSDVG FVEAYNNCTGGAEIRD LICVQSYKGIKVL PPLLENQISGYTLAATSASLFPP WTAAAGVPFYLNVQYRINGLGVTMDVLSQNQKLIANAFNNALYAIQEGFD ATNSALVKIQAVVNANAEALNNLLQQLSNRFGAISASLQEILSRDALEAE AQIDRLINGRLTALNAYVSQQLSDTLVKFSAAQAMEKVNECVKSQSSRIN FCGNGNHISLVQNAPYGLYFIHFSYVPTKYVTARVSPGLCIAGDRGIAPKS GYFVN VNNTWMYTGSGYYPITENNVMSTCAVNYTKAPYVMLNTS IPNLPDFKEELDQWFKNQTSVAPDLSDYINVTFLDLQVEMNRLQEAIKVL NQSYINLKDIGTYEYYVKWPWYVWLLICLAGVAMLVLLFFICCCTGCGTS CFKKCGGCCDDYTG YQELVIKTSHDD	
HCoV-OC43 S1/NTD (21- 293 of SEQ ID NO: 34)	CTSDNINDKDTGPPPISTDTVDVTNGLGTYYVLDRVYLNNTLFLNGYYPTS GSTYRNMALKGSVLLSRLWFKPPFLSDFINGIFAKVKNTKVIKDRVMYSEF PAITIGSTFVNTSYSVVVQPR TINSTQDGDKNLQGLLEVSVQCYNMCEYPQ TICHPNLGNHRKELWHLDTGVVSCLYKRNFTYDVNADYLYFH FYQEGGTF YAYFTDTGVVTKFLFN VYLGMA LSHYYVMPLTCNSKLTLEYWVTPLTSRQ YLLAFNQDGIIFNAVDCMSD	35
HCoV-OC43 S1/NTD+SS (1-293 of SEQ ID NO: 34)	MFLILLISLPTAFAVIGDLKCTSDNINDKDTGPPPISTDTVDVTNGLGTYYVL DRVYLNNTLFLNGYYPTSGSTYRNMALKGSVLLSRLWFKPPFLSDFINGIFA KVKNTKVIKDRVMYSEFPAITIGSTFVNTSYSVVVQPR TINSTQDGDKNLQ GLLEVSVQCYNMCEYPQTICHPNLGNHRKELWHLDTGVVSCLYKRNFTY DVNADYLYFH FYQEGGTFYAYFTDTGVVTKFLFN VYLGMA LSHYYVMPL TCNSKLTLEYWVTPLTSRQYLLAFNQDGIIFNAVDCMSD	36
HCoV-OC43 S1/RBD (335- 599 of SEQ ID NO: 34)	CNIEAWLNDKSVPSPLNWERKTF SN CNFMSSLMFSFIQADSFTCNNIDA AK IYGMCFSSITIDKFAIPNGRKVDLQLGNLGYLQSFNYRIDTTATSCQLYYNL PAANVSVS RFPSTWNKRFGFIEDSVFKPRPAGVLTNHDVVYAQHCFKAP KNFCPCKLNGSCVSGPGKNNGIGT C PAGTNYLTCDNLCTPDITFTGT YK CPQTKSLVGIGEHCSGLAVKSDYCGGNSCTCRPQAFLGWSADSC LQGDKC NIFANFILHDV	37
HCoV-OC43 S1/ExRBD (310-683 of SEQ ID NO: 34)	GVYELNGYTVQPIADVYRRKPNLPNCNIEAWLNDKSVPSPLNWERKTF SN CNFMSSLMFSFIQADSFTCNNIDA AKIYGMCFSSITIDKFAIPNGRKVDLQL GNLGYLQSFNYRIDTTATSCQLYYNLPAANVSVS RFPSTWNKRFGFIEDS VFKPRPAGVLTNHDVVYAQHCFKAPKNFCPCKLNGSCVSGPGKNNGIGT CPAGTNYLTCDNLCTPDITFTGT YKCPQTKSLVGIGEHCSGLAVKSDYCG GNSCTCRPQAFLGWSADSC LQGDKNIFANFILHDVNSGLTCSTDLQKANT DIILGVCVNYDLYGILGQGIFVEVNATYYNSWQNLLYDSNGNLYGFRDYIT NRTFMIRSCYSGRVSAAF	38
HCoV-OC43 S2/core (780- 1161 of SEQ ID NO: 34)	SLEPVGGLYEIQIPSEFTIGNMVEFIQTSSPKVTIDCAAFVCGDYAACKSQLV EYGSFCDNINAILTEVNELLDTTQLQVANSLMNGVT LSTKLKDG VNFNV DINFSPVLGCLGSECSKASSRSAIEDLLFDKVKLSDVG FVEAYNNCTGGAEI RD LICVQSYKGIKVL PPLLENQISGYTLAATSASLFPPWTAAAGVPFYLNV QYRINGLGVTMDVLSQNQKLIANAFNNALYAIQEGFDATNSALVKIQAVV NANAEALNNLLQQLSNRFGAISASLQEILSRDALEAE AQIDRLINGRLTAL	39

	NAYVSQQLSDSTLVKFSAQAAMEKVNECVKSQSSRINFCGNGNHIISLVQN APYGLYFIHFSYVPTKYVTARV	
HCoV-OC43 S2w/oTM (773-1278 of SEQ ID NO: 34)	TVNSVNDLSLEPVGGLEYEIQIPSEFTIGNMVEFIQTSSPKVTIDCAAFVCGDYA ACKSQLVEYGSFCDNINAILTEVNELLDTTQLQVANSLMNGVTLSTKLKDG VNFNVDDINFSPVLGCLGSECSKASSRSAIEDLLFDKVKLSDVGFVEAYNN CTGGAEIRDLCVQSYKGKVLPLLENQISGYTLAATSASLFPWTAAG VPFYLVNQYRINGLGVTMDVLSQNQKLIANAFNNALYAIQEGFDATNSAL VKIQAVVNANAEALNNLLQQLSNRFGAISASLQEILSRLDALEAEAQIDRLI NGRLTALNAYVSQQLSDSTLVKFSAQAAMEKVNECVKSQSSRINFCGNGN HIISLVQNAPYGLYFIHFSYVPTKYVTARVSPGLCIAGDRGIAPKSGYFVNV NNTWMTGSGYYPEPITENNVVMSTCAVNYTKAPYVMLNTSIPNLPDF KEELDQWFKNQTSVAPDLSLDYINVTFDLQVEMNRLQEAIKVLN	40
HCoV-HKU1 (YP_173238.1)	MLLIIFILPTTLAVIGDFNCTNFAINDLNTTVPRISEYVVDVSYGLGTYIYILDR VYLNTTILFTGYFPKSGANFRDLSLKGTTYLSTLWYQKPFLSDFNNGIFSRV KNTKLYVNKTLYSEFSTIVIGSVFINNSYITIVVQPHNGVLEITACQYTMCEY PHTICKSKGSSRNESWHFDKSEPLCLFKKNFTYNVSTDWLYFHFYQERGTF YAYYADSGMPTTFLFSLYLGTLSSHYYVLPLTCNAISSNTDNETLQYWVTP LSKRQYLLKFDNRGVITNAVDCSSFFSEIQCKTKSLLPNTGVYDLSGFTVK PVATVHRRIPDLPCDIDKWLNNFNVPSPNWERKIFSNCNFNLSLTLRLVH TDSFSCNNFDESKIYGSFCFSIVLDKFAIPNSRRSDLQLGSSGFLQSSNYKID TTSSSCQLYYSLPAINVTINNYNPSSWNRRYGFNNFNLSHSHSVVYSRYCFSV NNTFCPCAKPSFASSCKSHKPPSASCPIGTNYRSCESTTVLDHTDWCRCSCL PDPITAYDPRSCSQKSLVGVGEHCAGFGVDEEKCGLDGSYNVSCLCSTD AFLGWSYDTCVSNRNCNIFSNFILNGINS GTTCSNDLLQPNTEVFTDVCVDY DLYGITGQGIFKEVSAVYYNSWQNLLYDSNGNIIGFKDFVTNKTYNIFPCY AGRVSAAFHQNASLALLYRNLKCSYVLNNISLTTQPYFDSYLGCVFNADN LTDYSVSSCALRMGSGFCVDYNPSSSSSRKRRSISASRYFVTFEPFNVSVFV NDSIESVGGLEYEIKIPTNFTIVGQEEFIQTNSPKVTIDCSLFVCSNYAACHDLL SEYGTFCDNINSILDEVNGLLDTTQLHVADTLMQGVTLSSNLNTNLHFDVD NINFKSLVGCLGPHCGSSSRFFEDLLFDKVKLSDVGFVEAYNNCTGGSEIR DLLCVQSFNGIKVLPPILSESQISGYTTAATVAAMFPPWSAAAGIPFSLNVQ YRINGLGVTMDVLNKNQKLIATAFNALLSIQNGFSATNSALAKIQSVVNS NAQALNSLLQQLFNKFGAISSSLQEILSRLDALEAQVQIDRLINGRLTALNA YVSQQLSDISLVKFGAALAMEKVNECVKSQSPRINFCGNGNHILSLVQNAP YGLLFMHFSYKPISEKTVLVSPGLCISGDVGIAPKQGYFIKHNDHWMFTGSS YYPEPISDKNVFMNTCSVNFTKAPLVYLNHSPKLSDFESELSHWFKNQ TSIAPNLTLNLHTINATFLDLYEMNLIQESIKSLNNSYINLKDIGTYEMYVK WPWYVWLLISFSFIIFLVLLFFICCCTGCGSACFSKCHNCCDEYGGHHDFVI KTSHDD	41
HCoV-HKU1 S1/NTD (20- 285 of SEQ ID NO: 37)	CTNFAINDLNTTVPRISEYVVDVSYGLGTYIYILDRVYLNTTILFTGYFPKSG ANFRDLSLKGTTYLSTLWYQKPFLSDFNNGIFSRVKNTKLYVNKTLYSEFS TIVIGSVFINNSYITIVVQPHNGVLEITACQYTMCEY PHTICKSKGSSRNESW HFDKSEPLCLFKKNFTYNVSTDWLYFHFYQERGTFYAYYADSGMPTTFLFSL LYLGTLSSHYYVLPLTCNAISSNTDNETLQYWVTPLSKRQYLLKFDNRGVI TNAVDCSS	42
HCoV-HKU1 S1/NTD+SS (1-285 of SEQ ID NO: 41)	MLLIIFILPTTLAVIGDFNCTNFAINDLNTTVPRISEYVVDVSYGLGTYIYILDR VYLNTTILFTGYFPKSGANFRDLSLKGTTYLSTLWYQKPFLSDFNNGIFSRV KNTKLYVNKTLYSEFSTIVIGSVFINNSYITIVVQPHNGVLEITACQYTMCEY PHTICKSKGSSRNESWHFDKSEPLCLFKKNFTYNVSTDWLYFHFYQERGTF YAYYADSGMPTTFLFSLYLGTLSSHYYVLPLTCNAISSNTDNETLQYWVTP LSKRQYLLKFDNRGVITNAVDCSS	43
HCoV-HKU1 S1/RBD (327- 599 of SEQ ID NO: 41)	CDIDKWLNNFNVPSPNWERKIFSNCNFNLSLTLRLVHTDSFSCNNFDESKI YGSFCFSIVLDKFAIPNSRRSDLQLGSSGFLQSSNYKIDTTSSSCQLYYSLPAI NVTINNYNPSSWNRRYGFNNFNLSHSHSVVYSRYCFSVNNTFCPCAKPSFAS SCKSHKPPSASCPIGTNYRSCESTTVLDHTDWCRCSCLPDPITAYDPRSCSQ	44

	KKSLVGVGEHCAGFGVDEEKCGLDGSYNVSLCSTDAFLGWSYDTCVS NNRCNIFSNFILNGI	
HCoV-HKU1 S1/ExRBD (302-683 of SEQ ID NO: 41)	GVYDLSGFTVKPVATVHRRIPDLPCDDIDKWLNNFNVPSPNLNWERKIFSNC NFNLSLTLRLVHTDSFSCNNFDESKIYGSCFKSIVLDKFAIPNSRRSDLQLGS SGFLQSSNYKIDTTSSSCQLYYSLPAINVTINNYNPSSWNRRYGFNNFNLS HSVVYSRYCFSVNNTFCPCAKPSFASSCKSHKPPSASCPIGTNYRSCSTTV LDHTDWCRCSCLPDPITAYDPRSCSQKKSLVGVGEHCAGFGVDEEKCGLD DGSYNVSLCSTDAFLGWSYDTCVSNRCNIFSNFILNGINS GTTCSNDLLQ PNTEVFTDVCVDYDLYGITGQGIFKEVSAVYYNSWQNLLYDSNGNIIGFKD FVTNKTYNIFPCYAGRVSAAF	45
HCoV-HKU1 S2/core (782- 1162 of SEQ ID NO: 41)	SIESVGGLEYEIKIPTNFTIVGQEEFIQTNSPKVTIDCSLFVCSNYAACHDLLSE YGTFCDNINSILDEVNGLLDTTQLHVADTLMQGVTLSSNLNTNLHFDVDNI NFKSLVGCLGPHCGSSSRFFEDLLFDKVKLSDVGVFVEAYNNCTGGSEIRD LLCVQSFNGIKVLPPILESQISGYTTAATVAAMFPPWSAAAGIPFSLNVQY RINGLGVTMDVLNKNQKLIATAFNNALLSIQNGFSATNSALAKIQSVVNSN AQALNSLLQQLFNKFGAIISSSLQEILSRLDALEAQVQIDRLINGRLTALNAY VSQQLSDISLVKFGAALAMEKVNECVKSQSPRINFCGNGNHILSLVQNAPY GLLFMHFSYKPISEKTVLV	46
HCoV-HKU1 S2w/oTM (775-1281 of SEQ ID NO: 41)	NVSFVNDIESVGGLEYEIKIPTNFTIVGQEEFIQTNSPKVTIDCSLFVCSNYAA CHDLLSEYGTFCDNINSILDEVNGLLDTTQLHVADTLMQGVTLSSNLNTNL HFDVDNINFKSLVGCLGPHCGSSSRFFEDLLFDKVKLSDVGVFVEAYNNCT GGSEIRDLLCVQSFNGIKVLPPILESQISGYTTAATVAAMFPPWSAAAGIPF SLNVQYRINGLGVTMDVLNKNQKLIATAFNNALLSIQNGFSATNSALAKIQ SVVNSNAQALNSLLQQLFNKFGAIISSSLQEILSRLDALEAQVQIDRLINGRL TALNAYVSQQLSDISLVKFGAALAMEKVNECVKSQSPRINFCGNGNHILSL VQNAPYGLLFMHFSYKPISEKTVLVSPGLCISGDVGIAPKQGYFIKHNDHW MFTGSSYYYPEISDKNVVFMNTCSVNFTKAPLVYLNHSPKLSDFEELS HWFKNQTSIAPNLTLNLHTINATFLDLYYEMNLIQESIKSLN	47
HCoV-NL63 (YP_003767.1)	MKLFLILLVLPLASCFCTCNSNANLSMLQLGVPDSSSTIVTGLLPTHWFCA QSTSVYSANGFFYIDVGNHRSALHTGYDANQYYIYVTNEIGLNASVTL KICKFSRNTTFDFLSNASSSFCIVNLLFTEQLGAPLGITISGETVRLHLYNVT RTFYVPAAYKLTSLSVKCYFNYSVFSVNVNATVTNVNVTTHNGRVVNYTV CDDCNGYTDNIFSVQQDGRIPNGFPFNNWFLLTNGSTLVDGVSRLYQPLRL TCLWPVPLKSSSTGFVYFNATGSDVNCNGYQHNSVVDVMRYNLNFSANS LDNLKSGVIVFKTLQYDVLFCNSSSSGVLDTTIPFGPSSQPYCYFINSTINT THVSTFVGILPPTVREIVVARTGQFYINGFKYFDLGFIEAVNFNVTTASATDF WTVAFATFVDVLNVNVSATNIQNLLYCDSPFEKLQCEHLQFGLQDGFYSAN FLDDNVLPETYVALPIYYQHTDINFATASFGGSCYVCKPHQVNISLNGNTS VCVRTSHFSIRYIYNRVKSGSPGDSSWHIYLKSGTCPFSSKLN NFQKFTIC FSTVEVPGSCNFPLEATWHYTSYITV GALYVTWSEGNISITGVPYPVSGIREF SNLVLNNCTKYNIYDYVGTGIIRSSNQSLAGGITYVSNSGNLLGFKNVSTGN IFIVTPCNQPDQVAVYQQSIIGAMTAVNESRYGLQNLLQLPNFYVVSNGGN NCTTAVMTYSNFGICADGSLIPVRPRNSSDNGISAIITANLSIPSNNWTTSVQV EYLQITSTPIVDCATYVCNGNPRCKNLLKQYTSACKTIEDALRLSAHLETN DVSSMLTFDSNAFSLANVTSFGDYNLSSVLPQRNIRSSRIAGRSALEDLLFS KVVTSGLGTVDVDYKSC TKGLSIADLACAQYNGIMVLPGVADAERMAM YTGSLIGGMVLGGLTSA AIPFSLALQARLNYVALQTDVLQENQKILAASF NKAINNIVASFSSVNDAITQTAEAIHTVTIALNKIQDVVNQQGSALNHLTSQ LRHNFQAISNSIQAIYDRLDSIQADQQVDRLITGRLAALNAFVSQVLN KYTE VRGSRRLAQKINECVKSQSNRYGFCGNGTHIFSIVNSAPDGLLFLHTVLLP TDYKNVKAWSGICVDGIYGYVLRQPNLVLYSDNGVFRVTSRVMFQPRLPV LSDFVQIYNCNVTFVNISRVELHTVIPDYVDVNKTLQEFAQNL PKYVKPNF DLTPFNLTYNLNLSELKQLEAKTASLFQTTVELQGLIDQINSTVDL KLLNRF ENYIKWPWWVWLII SVFVLLSLLVFCCLSTGCCGCCNCLTSSMRGCCD CGSTKLPPYEFK VHVQ	48

HCoV-NL63 S1/NTD (32- 334 of SEQ ID NO: 48)	VPDNSSTIVTGLLPTHWF CANQSTSVYSANGFFYIDVGNHRSAFALHTGYY DANQYYIYVTNEIGLNASVTLKICKFSRNTTFDFLSNASSSFDCIVNLLFTEQ LGAPLGITISGETVRLHLYNVTRTFYVPAAYKLTCLSVKCYFNYSVFSVV NATVTNVNTTHNGRVVNYTV CDDCNGYTDNIFSVQQDGRIPNGFPFNNWF LLTNGSTLVDGVSRLYQPLRLTCLWPVPGLKSSTGFVYFNATGSDVNCNG YQHNSVVDVMRYNLNFSANSLDNLKSGVIVFKTLQYDVLFYCSNSSSG	49
HCoV-NL63 S1/NTD+SS (1-334 of SEQ ID NO: 48)	MKLFLILLVLPLASCFFTCNSNANLSMLQLGVPDNSSTIVTGLLPTHWF CAN QSTSVYSANGFFYIDVGNHRSAFALHTGYYDANQYYIYVTNEIGLNASVTL KICKFSRNTTFDFLSNASSSFDCIVNLLFTEQLGAPLGITISGETVRLHLYNV TRTFYVPAAYKLTCLSVKCYFNYSVFSVVNATVTNVNTTHNGRVVNYTV CDDCNGYTDNIFSVQQDGRIPNGFPFNNWFLLTNGSTLVDGVSRLYQPLRL TCLWPVPGLKSSTGFVYFNATGSDVNCNGYQHNSVVDVMRYNLNFSANS LDNLKSGVIVFKTLQYDVLFYCSNSSSG	50
HCoV-NL63 S1/RBD (375- 605 of SEQ ID NO: 48)	EIVVARTGQFYINGFKYFDLGFIEAVNFNVTTASATDFWTVAFATFVDVLV NVSATNIQNLLYCDSPFEKLQCEHLQFGLQDGFYSANFLDDNVLPETYVAL PIYYQHTDINFATASFGGSCYVCKPHQVNISLNGNTSVCVRTSHFSIRYIYN RVKSGSPGDSSWHIYKSGTCPF SSKLNNFQKFKTICFSTVEVP GSCNFPLE ATWHYTSYTIVGALYVTWSEGNS	51
HCoV-NL63 S1/ExRBD (350-687 of SEQ ID NO: 48)	YCFINSTINTTHVSTFVGLPPTVREIVVARTGQFYINGFKYFDLGFIEAVNFN VTTASATDFWTVAFATFVDVLNVVSATNIQNLLYCDSPFEKLQCEHLQFGL QDGFYSANFLDDNVLPETYVALPIYYQHTDINFATASFGGSCYVCKPHQV NISLNGNTSVCVRTSHFSIRYIYNRVKSGSPGDSSWHIYKSGTCPF SSKLNN NFQKFKTICFSTVEVP GSCNFPLEATWHYTSYTIVGALYVTWSEGNSITGVP YPVSGIREFSNLVLNNCTKYNIYDYVGTGIIRSSNQSLAGGITYVSNSGNLL GFKNVSTGNIFIVTPCNQPDQVAVY	52
HCoV-NL63 S2/core (763- 1143 of SEQ ID NO: 48)	LSIPSNWTTSVQVEYLQITSTPIVVDCATYVCNGNPRCKNLLKQYTSACKTI EDALRLSAHLETNDVSSMLTFDSNAFSLANVT SFGDYNLSSVLPQRNIRSSR IAGRSALEDLLFSKVVTSGLGTVDVDYK SCKGLSIADLACAQYYNGIMVL PGVADAERMAMYTGSLIGGMVLGGLTSAAAI PFSALQARLNYVALQTDV LQENQKILAASFNKAINNIVASFSSVNDAITQTAEAIHTVTIALNKIQDVVNQ QGSALNHLTSQLRHNFQAISNSIQAIYDRLDSIQADQQVDRLITGRLAALNA FVSQVLNKYTEVRGSRRLAQQKINECVKSQSNRYGFCGNGTHIFSIVNSAP DGLLFLHTVLLPTDYKNVKA	53
HCoV-NL63 S2w/oTM (763-1277 of SEQ ID NO: 48)	LSIPSNWTTSVQVEYLQITSTPIVVDCATYVCNGNPRCKNLLKQYTSACKTI EDALRLSAHLETNDVSSMLTFDSNAFSLANVT SFGDYNLSSVLPQRNIRSSR IAGRSALEDLLFSKVVTSGLGTVDVDYK SCKGLSIADLACAQYYNGIMVL PGVADAERMAMYTGSLIGGMVLGGLTSAAAI PFSALQARLNYVALQTDV LQENQKILAASFNKAINNIVASFSSVNDAITQTAEAIHTVTIALNKIQDVVNQ QGSALNHLTSQLRHNFQAISNSIQAIYDRLDSIQADQQVDRLITGRLAALNA FVSQVLNKYTEVRGSRRLAQQKINECVKSQSNRYGFCGNGTHIFSIVNSAP DGLLFLHTVLLPTDYKNVKAWSGICVDGIYGYVLRQPNLVLYSDNGVFRV TSRVMFQPRLPVLSDFVQIYNCNVTFVNISRVELHTVIPDYVDVNKTLQEFA QNLPKYVKPNFDLTPFNLTYNLSSSELKQLEAKTASLFQTTVELQGLIDQIN	54
Peptide Linker	<u>GGSGGS</u>	55
Vector For	<u>TGACCATTTTGATACATGTTGAACAATCAAATACAG</u>	56
Vector Rev	<u>GCTAGCCTTGTCATCGTCATCCT</u>	57
NTD For	<u>GATGACAAGGCTAGCTGTGTTAATCTTACAACCAGAACTCAATTACCCC</u>	58
NTD Rev	<u>GTATCAAAATGGTCAGTCAAGTGCACAGTCTACAGCATC</u>	59
ExRBD_For	<u>GATGACAAGGCTAGCGGAATCTATCAAACCTCTAACTTTAGAGTCCAAC CA</u>	60
ExRBD Rev	<u>GTATCAAAATGGTCATGTTATAAACTGACACCACCAAAAAGAACA</u>	61
RBD For	<u>GATGACAAGGCTAGCTTGTCGCCCTTTTGGTGAAGTTT</u>	62
RBD Rev	<u>GTATCAAAATGGTCAAGTTGCTGGTGCATGTAGAAGT</u>	63
CR_For	<u>GATGACAAGGCTAGCTCTATTGCCATACCCACAAATTTTACTATTAGTG T</u>	64

CR_Rev	<u>GTATCAAAATGGTCAAGTTGTGAAGTTCTTTTCTTGTGCAGG</u>	65
S1_For	<u>GATGACAAGGCTAGCGTGTGTTTCTTTCTTGTGTTTATTGCCACTAGTCT</u>	66
S1_Rev	<u>GTATCAAAATGGTCAACGTGCCCCCG</u>	67

BACKGROUND

[0004] Rotaviruses are one the leading causes of childhood gastroenteritis throughout the world, and have a major impact on childhood morbidity and mortality. Rotavirus vaccines have demonstrated very good safety and efficacy profiles, and their use has significantly reduced the toll of acute rotavirus gastroenteritis in both developed and developing countries.

[0005] Coronaviruses are a group of large, enveloped, positive-sense, single stranded RNA viruses. Originating in bats, zoonotic coronaviruses have been present in humans for at least 500-800 years, and are often the cause of the common cold. Of the four genera of coronaviruses (alpha, beta, gamma, and delta), characterized by different antigenic cross-reactivity and genetic makeup, only alpha- and betacoronavirus genera include strains pathogenic to humans.

[0006] Of the coronavirus species known prior to December of 2019, only six are known to cause disease in humans: HCoV-229E, HCoV-OC43, HCoV-NL63, HCoV-HKU1, severe acute respiratory syndrome coronavirus (SARS-CoV) and Middle East respiratory virus coronavirus (MERS-CoV). HCoV-229E, HCoV-OC43, HCoV-NL63, and HCoV-HKU1 are endemic locally, and have been associated mainly with mild, self-limiting disease, whereas SARS-CoV and MERS-CoV can cause severe illness. SARS-CoV and MERS-CoV are betacoronaviruses, and are among the pathogens included in the World Health Organization's list of high-priority threats.

[0007] Coronaviruses are so named for the crown-like spikes on their surface, and have two major envelope proteins. The S glycoprotein is a major antigen responsible for both receptor binding and cell fusion. The transmembrane glycoprotein (M) is involved in budding and envelope formation. A few coronavirus species have a third glycoprotein, the haemagglutinin-esterase (HE). The coronavirus genome is non-segmented, positive single-stranded RNA of about 26-32 kb, making it the longest RNA viral genome known, included from 7 to 10 open reading frames. Coronaviruses are capable of adapting quickly to new hosts through the processes of genetic recombination and mutation in vivo.

[0008] In late 2019, a new coronavirus began causing febrile respiratory illness in China. The virus, provisionally known as 2019-nCoV (2019 novel coronavirus; now known as SARS-CoV-2), was first detected in Wuhan, China. SARS-CoV-2 was sequenced and identified as a betacoronavirus belonging to the sarbecovirus, with 75-80% similarity in genetic sequence to SARS-CoV. The animal host of SARS-CoV-2 is presumed to be a bat, although an intermediate host may also have been involved. Although the initial cases were a result of zoonotic transmission, human-to-human transmission was documented soon after, in both healthcare settings and familial clusters.

[0009] Following an incubation ranging from 2-14 days, SARS-CoV-2 infection manifests as a respiratory illness termed COVID-19 (coronavirus disease 2019), with symptoms including fever, cough, and dyspnea. An early description of 41 clinical cases described patients as having serious, sometimes fatal, pneumonia, with clinical presentations very similar to those of SARS-CoV. Patients with the most severe illness developed acute respiratory distress syndrome (ARDS), requiring ICU admission and oxygen therapy.

[0010] According to the World Health Organization, as of April 9, 2020, more than 1.6 million confirmed cases of COVID-19 and 95,000 deaths had been reported worldwide, with cases being reported in 212 different countries, areas, or territories. Although the early case-fatality rate appeared to be low, the rapid spread and ease of transmission of the virus, even by asymptomatic individuals, has caused global alarm; if easily transmissible, a virus poses a significant risk at the population level. The WHO declared SARS-CoV-2 infection to be a pandemic on March 11, 2020.

[0011] As of April, 2020, 5 SARS-CoV-2 vaccines had begun Phase I clinical trials, with nearly 20 additional vaccine candidate in preclinical development. None appear to explore the possibility of a neonatal vaccine.

SUMMARY

[0012] In a first example ("Example 1), provided herein is a recombinant rotavirus comprising a gene segment including a first nucleotide sequence encoding rotavirus nonstructural protein 3 (NSP3) and a second nucleotide sequence encoding a coronavirus S protein fragment selected from: an S1 N-terminal domain; an S1 N-terminal domain with signal sequence; an S1 subunit without signal sequence; an S protein receptor binding domain; an extended S protein receptor binding domain; an S2 core domain; and an S2 subunit without a

transmembrane anchor domain, wherein the NSP3 and the coronavirus S protein fragment are encoded by a single open reading frame, separated by a self-cleavage protease domain.

[0013] In another example (“Example 2”), further to Example 1, the coronavirus is SARS-CoV-2 and: the S1 N-terminal domain is at least 95% identical to SEQ ID NO: 3; the S1 N-terminal domain with signal sequence is at least 95% identical to SEQ ID NO: 4; the S protein receptor binding domain is at least 95% identical to SEQ ID NO: 5; the extended S protein receptor binding domain is at least 95% identical to SEQ ID NO: 9; the S2 core domain is at least 95% to SEQ ID NO: 10; and the S2 subunit without a transmembrane anchor domain is at least 95% identical to SEQ ID N: 11.

[0014] In another example (“Example 3”), further to Example 1 or Example 2, further comprising a third nucleotide sequence encoding a second coronavirus S protein fragment selected from: an S1 N-terminal domain; an S1 N-terminal domain with signal sequence; an S1 subunit without signal sequence; an S protein receptor binding domain; an extended S protein receptor binding domain; an S2 core domain; and an S2 subunit without a transmembrane anchor domain, wherein the NSP3 the coronavirus S protein fragment, and the second coronavirus S protein fragment are encoded by a single open reading frame, wherein the NSP3 and the coronavirus S protein fragment are separated by a self-cleavage protease domain and the coronavirus S protein fragment and the second coronavirus fragment are separated by a molecular hinge.

[0015] In another example (“Example 4”), further to any one of Examples 1-3, the self-cleavage protease domain is a 2A cleavage element.

[0016] In another example (“Example 5”), further to any one of Examples 1-4, the self-cleavage protease domain is a tesco porcine virus 2A (P2A) element.

[0017] In another example (“Example 6”), further to any one of Examples 1-5, the self cleavage protease domain is a P2A element has a sequence having at least 80% sequence identity to SEQ ID NO: 1 (SKFQIDKILISGDIELNPGP).

[0018] In another example (“Example 7”), further to any one of Examples 1-7, the nucleotide sequence encoding the coronavirus S protein fragment is derived from a coronavirus selected from: SARS-CoV; MERS-CoV; SARS-CoV-2; HCoV-299E; HCoV-OC43; HCoV-HKU1; and HCoV-NL63.

[0019] In another example (“Example 8”), further to any one of Examples 3-7, the third nucleotide sequence encoding the second coronavirus S protein fragment is derived from a same or a different coronavirus species.

[0020] In another example (“Example 9”), further to any one of Examples 1-8, the recombinant rotavirus induces an immunological response against rotavirus and coronavirus in a subject when administered to the subject.

[0021] In another example (“Example 10”), further to any one of Examples 1-9, the recombinant rotavirus is based upon strain G1P[8].

[0022] In another example (“Example 11”), further to any one of Examples 1-10, the recombinant rotavirus is attenuated.

[0023] In another example (“Example 12”), provided herein is an immunogenic composition comprising the recombinant rotavirus of any one of Examples 1-11.

[0024] In another example (“Example 13”), further to Example 12, the immunogenic composition includes a pharmaceutically acceptable excipient.

[0025] In another example (“Example 14”), further to Example 11 or Example 12, the immunogenic composition is formulated for oral, subcutaneous, or intramuscular administration.

[0026] In another example (“Example 15”), provided herein is a method for inducing a protective immune response against rotavirus and a coronavirus in a subject, the method comprising administering an effective amount of the immunogenic composition of any one of Examples 12-14 to the subject.

[0027] In another example (“Example 16”), provided herein is a recombinant rotavirus expression system comprising: a nonstructural protein 3 (NSP3) expression vector including a nucleotide sequence encoding rotavirus (NSP3) and a nucleotide sequence encoding a coronavirus S protein fragment selected from: an S1 N-terminal domain; an S1 N-terminal domain with signal sequence; an S1 subunit without signal sequence; an S protein receptor binding domain; an extended S protein receptor binding domain; an S2 core domain; and an S2 subunit without a transmembrane anchor domain, wherein the NSP3 and the coronavirus S protein fragment are encoded by a single open reading frame, separated by a self-cleavage protease domain; a VP1 expression vector; a VP2 expression vector; a VP3 expression vector; a VP4 expression vector; a VP5 expression vector; a VP6 expression vector; a VP7 expression vector; a NSP1 expression vector; a NSP2 expression vector; a NSP4 expression vector; a

NSP5/6 expression vector; and an African Swine Fever Virus NP868R RNA capping enzyme expression vector.

[0028] In another example (“Example 17”), further to Example 16, the coronavirus is SARS-CoV-2 and: the S1 N-terminal domain is at least 95% identical to SEQ ID NO: 3; the S1 N-terminal domain with signal sequence is at least 95% identical to SEQ ID NO: 4; the S protein receptor binding domain is at least 95% identical to SEQ ID NO: 5; the extended S protein receptor binding domain is at least 95% identical to SEQ ID NO: 9; the S2 core domain is at least 95% to SEQ ID NO: 10; and the S2 subunit without a transmembrane anchor domain is at least 95% identical to SEQ ID N: 11.

[0029] In another example, (“Example 18”), further to Example 16 or Example 17, the recombinant rotavirus expression system includes a third nucleotide sequence encoding a second coronavirus S protein fragment selected from: an S1 N-terminal domain; an S1 N-terminal domain with signal sequence; an S1 subunit without signal sequence; an S protein receptor binding domain; an extended S protein receptor binding domain; an S2 core domain; and an S2 subunit without a transmembrane anchor domain, wherein the NSP3 the coronavirus S protein fragment, and the second coronavirus S protein fragment are encoded by a single open reading frame, wherein the NSP3 and the coronavirus S protein fragment are separated by a self-cleavage protease domain and the coronavirus S protein fragment and the second coronavirus fragment are separated by a molecular hinge.

[0030] In another example (“Example 19”), further to any one of Examples 16-18, each of the NSP3 expression vector, VP1 expression vector, VP2 expression vector, VP3 expression vector, VP4 expression vector, VP5 expression vector, VP6 expression vector, VP7 expression vector, NSP1 expression vector, NSP2 expression vector, NSP4 expression vector, and NSP5/6 expression vector are T7 expression vectors.

[0031] In another example (“Example 20”), further to any one of Examples 16-19, the self-cleavage protease domain is a 2A cleavage element.

[0032] In another example (“Example 21”), further to any one of Examples 16-20, the self-cleavage protease domain is a tesco porcine virus 2A (P2A) element.

[0033] In another example (“Example 22”), further to any one of Examples 16-21, the self-cleavage protease domain has a sequence having at least 80% sequence identity to SEQ ID NO: 1 (SKFQIDKILISGDIELNPGP).

[0034] In another example (“Example 23”), further to any one of Examples 16-22, the nucleotide sequence encoding the coronavirus S protein fragment is derived from a coronavirus selected from: SARS-CoV; MERS-CoV; SARS-CoV-2; HCoV-229E; HCoV-OC43; HCoV-HKU1; and HCoV-NL63.

[0035] In another example (“Example 24”), further to any one of Examples 18-23, the third nucleotide sequence encoding the second coronavirus S protein fragment is derived from a same or a different coronavirus species.

[0036] In another example (“Example 25”), further to any one of Examples 16-24, the recombinant rotavirus expression system is based upon rotavirus strain G1P[8].

[0037] In another example (“Example 26”), provided herein is a method for producing a recombinant rotavirus comprising, transfecting BHK-T7 cells with the recombinant rotavirus expression system of any one of Examples 16-25; overseeding the transfected BHK-T7 cells with MA104 cells; preparing a clarified cell lysate; and isolating recombinant rotavirus.

[0038] In another example (“Example 27”), further to Example 26, recombinant rotavirus is isolated by plaque purification.

[0039] In another example (“Example 28”), further Example 26 or Example 27, the recombinant rotavirus is attenuated.

BRIEF DESCRIPTION OF THE DRAWINGS

[0040] The following drawings form part of the present specification and are included to further demonstrate certain embodiments. Some embodiments may be better understood by reference to one or more of these drawings alone or in combination with the detailed description of specific embodiments presented.

[0041] **FIG. 1** Graphic illustration of a phylogenetic tree of rotavirus species. RVA is the major cause of rotavirus illness in humans.

[0042] **FIG. 2** Graphic illustration of a rotavirus capsid with structural proteins labeled (Left) and dsRNA genome segments showing protein products (Right).

[0043] **FIG. 3** A schematic drawing illustrating transfection of BHK-T7 cells with vectors for rotavirus (+)RNAs, a CMV vector for ASFV NP868R, and a pCAG vector for p10FAST. Briefly, three days after transfection, BHK-T7 cells are overseeded with MA104 cells in trypsin-containing media. The combined BHK-T7/MA104 cultures are harvested six days post transfection, and recombinant virus amplified by passage in MA104 cells.

[0044] **FIG. 4A** A diagram illustrating the ASFV NP868R gene that was synthesized by Genewiz and inserted in a pCMV plasmid downstream of the CMV promoter;

[0045] **FIG. 4B** A polyacrylamide gel electrophoresis (PAGE) illustrating the pCMV-NP868R plasmid and pT7 plasmids for SA11 (+)RNAs (Addgene) were used in the RG system to generate recombinant SA11 rotavirus (rSA11).

[0046] **FIG. 4C** A bar graph illustrating fold increase in the level of virus recovered in RG experiments expressing ASFV NP868R capping enzyme compared to RG experiments expressing vaccinia virus capping D1R/D12L enzymes.

[0047] **FIG. 5** A photograph of a PAGE gel illustrating electropherotypes of wildtype SA11-4F and sister strain with gene 7 rearrangement (g7re).

[0048] **FIG. 6** An illustration of protein-encoding regions of group A (RVA) rotavirus gene 7 (NSP3seg) and group C (RVC) rotavirus gene 6 (NSP3-dsRBPseg). Self-cleavage site in RVC 2A is labeled.

[0049] **FIG. 7** An illustration of a pT7/NSP3 plasmid designs incorporating various SARS-CoV-2 S protein fragments according to certain embodiments.

[0050] **FIG. 8A** A schematic depiction of the structure of the SARS-CoV-2 spike (S) gene.

[0051] **FIG. 8B** Descriptions of SARS-CoV-2 S protein fragments that can be incorporated into a recombinant rotavirus in accordance with certain embodiments. N-terminal domain (NTD); receptor binding domain (RBD); signal sequence (SS); transmembrane anchor domain (TM).

[0052] **FIG. 8C** Two 3-D renderings of the SARS-CoV-2 S protein monomer.

[0053] **FIG. 9A** A schematic representation of a rotavirus reverse genetics system according to one embodiment.

[0054] **FIG. 9B** An illustration of pT7/NSP3 plasmid design incorporating various norovirus VP1 according to certain embodiments.

[0055] **FIG. 9C** A photograph of a gel showing that the genome of rRV encoding NSP3-2A-NoV VP1 contained a 2.9-kB segment 7 RNA instead of a wildtype 1.1-kB segment 7RNA.

[0056] **FIG. 9D** A photograph of an immunoblot showing that rRV containing the 2.9-kB segment RNA produced VP1 dimers in infected cells.

[0057] FIG. 9E A photograph of a gel showing a segment 7 RNA encoding NSP1 and the astrovirus VP90 protein. The rRV contained a 3.6-kB segment 7 RNA, revealing the remarkable flexibility of the rotavirus genome and its capacity to accommodate extra RNA.

[0058] FIG. 10A A schematic illustration of the domains of the SARS-CoV-2 S protein expressed by rSA11. S protein trimers are cleaved at the S1/S2 junction by furin proconvertase and at the S2' site by the TMPRSS2 serine protease. The S1 fragment contains a signal sequence (SS), N-terminal domain (NTD), receptor binding domain (RBD), receptor binding motif (RBM), coiled-coil (CC), and two heptad repeats (HR1, HR2). The S2 fragment contains a trimeric core region, transmembrane anchor (TM), and fusion domain.

[0059] FIG. 10B A schematic illustration of portions of the S protein expressed by recombinant rotaviruses are indicated.

[0060] FIG. 10C Ribbon representations of the closed conformation of the trimeric S protein (PDB 6VXX) showing locations of the RBD (magenta), extended RBD (ExRBD, cyan), NTD (blue), core (CR, gold) domains and the S1 cleavage product (green).

[0061] FIG. 11 Schematic illustrations of plasmids with modified segment 7 (NSP3) cDNAs used to generate rSA11 viruses expressing regions of the SARS-CoV-2 S protein. Illustration indicates nucleotide positions of the coding sequences for NSP3, porcine teschovirus 2A element, 3xFLAG (FL), and the complete S1 or portions of the S1 (NTD, ExRBD, and RBD) and S2 (CR) proteins. The red arrow notes the position of the 2A translational stop-restart site, and the asterisk notes the end of the ORF. Sizes (aa) of encoded NSP3 and S products are in parenthesis. T7 (T7 RNA polymerase promoter sequence), Rz (Hepatitis D virus ribozyme), UTR (untranslated region).

[0062] FIG. 12A A photograph of a gel showing the properties of rSA11/NSP3-CoV2/S viruses expressing regions of the SARS-CoV-2 S protein. Double-stranded RNA of rSA11/NSP3-fS1 serially passaged twice (P1 and P2) in MA104 cells.

[0063] FIG. 12B A photograph of a gel showing the properties of rSA11/NSP3-CoV2/S viruses expressing regions of the SARS-CoV-2 S protein. dsRNA was recovered from MA104 cells infected with plaque-purified rSA11 isolates, resolved by gel electrophoresis, and detected by ethidium-bromide staining. RNA segments of rSA11/wt are labeled 1 to 11. Sizes (kbp) of segment 7 RNAs (black arrows) of rSA11 isolates are indicated.

[0064] **FIG. 12C** A photograph of plaque assays performed using MA104 cells and detected by crystal-violet staining.

[0065] **FIG. 12D** A graph showing the titers reached by rSA11 isolates were determined by plaque assay. Bars indicate standard deviations calculated from three separate determinations.

[0066] **FIG. 13A** A photograph of a gel showing the expression of SARS-CoV-2 S products by rSA11 viruses. Whole cell lysates (WCL) were prepared from cells infected with rSA11 viruses and examined by immunoblot assay using FLAG antibody to detect S products (NTD, ExRBD, RBD, CR, S1, and 2A read-through products) and antibodies specific for rotavirus NSP3 and VP6 and proliferating cell nuclear antigen (PCNA). Red asterisks identify 2A read-through products and blue asterisks identify 2A cleavage products

[0067] **FIG. 13B** A photograph of a gel showing the expression of SARS-CoV-2 S products by rSA11 viruses. Lysates prepared from MA104 cells infected with rSA11wt, rSA11/NSP3-fRBD and rSA11/NSP3-fExRBD were examined by immunoblot assay using antibodies specific for RBD (ProSci 9087), rotavirus VP6, and PCNA.

[0068] **FIG. 13C** A photograph of a gel of lysates prepared from MA104 cells infected with rSA11/wt, rSA11/NSP3-fRBD and rSA11/NSP3-fExRBD viruses were examined by immunoprecipitation assay using a SARS-CoV-2 S1 specific monoclonal antibody (GeneTex CR3022). Lysates were also analyzed with a NSP2-specific polyclonal antibody. Antigen-antibody complexes were recovered using IgA/G beads, resolved by gel electrophoresis, blotted onto nitrocellulose membranes, and probed with FLAG (fRBD and fExRBD) and NSP2 antibody. Molecular weight markers are indicated (kDa). Red arrows indicate fRBD and fExRBD. fRBD comigrates near the Ig light chain (Ig/L). Ig heavy chain, Ig/H).

[0069] **FIG. 14** A photograph of a gel showing the production of RBD and ExRBD by rSA11 viruses during infection. MA104 cells were mock infected or infected with rSA11/wt, rSA11/NSP3-fRBD, or rSA11/NSP3-fExRBD (MOI of 5). Lysates were prepared from the cells at 0, 4, 8, or 12 h p.i. and analyzed by immunoblot assay using antibodies specific for FLAG, NSP3, VP6, and PCNA. Red asterisks identify 2A read-through products. Positions of molecular weight markers are indicated (kDa).

[0070] **FIG. 15A** A gel showing the impact of genome size on rotavirus particle density. MA104 cells were infected with rSA11/wt, rSA11/NSP3-fExRBD, or rSA11/NSP3-fS1 viruses at an MOI of 5. At 12 h p.i., the cells were recovered, lysed by treatment with non-ionic

detergent, and treated with EDTA to convert rotavirus virions into DLPs. DLPs were banded by centrifugation in CsCl gradients and densities (g/cm^3) were determined using a refractometer.

[0071] FIG. 15B A gel of MA104 cells were infected with rSA11/wt, rSA11/NSP3-fExRBD, or rSA11/NSP3-fS1 viruses at an MOI of 5. At 12 h p.i., the cells were recovered, lysed by treatment with non-ionic detergent, and treated with EDTA to convert rotavirus virions into DLPs. DLPs were banded by centrifugation in CsCl gradients and densities (g/cm^3) were determined using a refractometer.

[0072] FIG. 15C A gel of lysates from rSA11/wt and rSA11/NSP3-fS1 infected cells were combined and their DLP components banded by centrifugation in a CsCl gradient.

[0073] FIG. 15D A photograph of the electrophoretic profile of the dsRNA genomes of DLPs recovered from CsCl gradients. RNAs derive from DLPs in panel A RNA segments of rSA11/wt are labeled 1 to 11. Positions of segment 7 RNAs are indicated with red arrows,

[0074] FIG. 15E A photograph of the electrophoretic profile of the dsRNA genomes of DLPs recovered from CsCl gradients. RNAs derive from DLPs in panel B and C. RNA segments of rSA11/wt are labeled 1 to 11. Positions of segment 7 RNAs are indicated with red arrows,

[0075] FIG. 16A Photographs of gels showing the genetic stability of rSA11 strains expressing SARS-CoV-2 S domains. rSA11 strains were serially passaged 5 to 6 times (P1 to P5 or P6) in MA104 cells. Genomic RNAs were recovered from infected cell lysates and analyzed by gel electrophoresis. Positions of viral genome segments are labeled. Position of modified segment 7 (NSP3) dsRNAs introduced into rSA11 strains are denoted with black arrows. Genetic instability of the modified segment 7 (NSP3) dsRNA of rSA11/NSP3-fS1 yielded R1-R4 RNAs during serial passage

[0076] FIG. 16B Photographs of gels showing genomic RNAs prepared from large (L1-L4) and small (S1-S4) plaque isolates of P6 rSA11/NSP3-fS1. Segment 7 RNAs are identified as R1-R3, as in (A).

[0077] FIG. 16C Schematics showing the organization of R1-R3 sequences determined by sequencing of segment 7 RNAs of L1, S1, and S3 plaque isolates. Sequence deletions are indicated with dashed lines. Regions of the S1 ORF that are no longer encoded by the R1-R3 segment 7 RNAs are indicated by slashed green-white boxes.

[0078] FIG. 17 A summary of the properties of recombinant tRV/NSP-3-2A-CoV2 strains.

[0079] **FIG. 18** A summary of the primers used to produce pT7/NSP3-2A-CoV2 plasmids.

DEFINITIONS

[0080] As used herein, the singular form “a”, “an”, and “the” includes plural references unless indicated otherwise.

[0081] The term "treating" as used herein, unless explicitly stated or implied otherwise, includes administering to a human or an animal patient at least one dose of a pharmaceutical formulation. “Treating” also refers to lessening the likelihood and/or severity of at least one disease as well as limiting the length/duration of an illness, or the severity of an illness, or inducing a protective immune response. Treating a patient may or may not result in a cure of the disease or condition. The term “treating” refers to partially or completely alleviating, ameliorating, delaying onset of, improving, inhibiting initiation of or progression of, relieving, and/or reducing incidence of one or more symptoms or causes of a particular disease, disorder or condition. The term also refers to inhibiting infection in a patient. Treatment may be administered to a subject who does not exhibit signs of a disease, disorder, and/or condition, and/or to a subject who exhibits only early signs of a disease, disorder, and/or condition, including an infection, for the purpose of decreasing the risk of developing pathology associated with the disease, disorder, and/or condition.

[0082] As used herein, unless explicitly stated otherwise or clearly implied otherwise, the terms “therapeutically effective dose,” “therapeutically effective amounts,” “effective dose” and the like, refer to any amount of a recombinant rotavirus that has a net positive effect on the health and wellbeing of a human or other animal. Therapeutic effects may include an improvement in longevity, quality of life and the like, and may also include a reduced susceptibility to developing a disease, disorder, and/or condition, or slow or prevent deteriorating health or wellbeing. The effects of treatment may be realized after a single dose and/or treatment or they may be realized after a series of doses and/or treatments. A "therapeutically effective amount" or “effective amount” in general means any amount that, when administered to a subject or animal for treating a disease or eliciting an immune response in the subject or animal, is sufficient to affect the desired degree of treatment for the disease, disorder, and/or condition, or to elicit a protective immune response, at a reasonable benefit/risk ratio applicable to medical treatment. The specific therapeutically effective dose for any particular subject can depend upon

a variety of factors, including the particular disease, disorder, and/or condition being treated; virulence of the specific recombinant rotavirus employed; the specific pharmaceutical composition employed; the age, body weight, general health, sex and diet of the patient; the time of administration, and route of administration; the duration of the treatment; and like factors well-known in the medical arts.

[0083] Pharmaceutical formulations described herein may be suitable for oral, parenteral (including subcutaneous, intradermal, intramuscular and intravenous) and/or rectal administration. The formulations may be presented in unit dosage form and may be prepared by any of the methods well known in the art of pharmacy. All methods include the step of bringing into association the active ingredient (i.e., a recombinant rotavirus of the present disclosure) with a carrier. In general, the formulations are prepared by uniformly and intimately bringing into association the active ingredient with a liquid carrier or, a finely divided solid carrier or both, and then, if necessary, forming the associated mixture into the desired dosage form.

[0084] Pharmaceutical formulations of the present disclosure suitable for oral administration can be presented as discrete units, such as a capsule, cachet, tablet, or lozenge, each containing a predetermined amount of the active ingredient; as a powder or granules; as a solution or a suspension in an aqueous liquid or non-aqueous liquid such as a syrup, elixir or a draught, or as an oil-in-water liquid emulsion or a water-in-oil liquid emulsion. The formulation can also be a bolus, electuary, or paste.

[0085] Pharmaceutical formulations of the present disclosure suitable for parenteral administration can include aqueous and non-aqueous sterile injectable solutions, and may also include an adjuvant, an antioxidant; a buffer; a bacteriostat; a solution which renders the composition isotonic with the blood of the recipient; and aqueous and non-aqueous sterile suspensions which can contain, for example, a suspending agent and a thickening agent. The formulations can be presented in a single unit-dose or multi-dose containers, and can be stored in a lyophilized condition requiring the addition of a sterile liquid carrier prior to use.

[0086] The term “pharmaceutically acceptable carrier or excipient”, unless stated or implied otherwise, can be used herein to describe any ingredient other than the active component(s) that can be included in a formulation (e.g., carriers and adjuvants). The choice of carrier and/or adjuvant will to a large extent depend on factors such as the particular mode of

administration, the effect of the carrier on solubility and stability, and the nature of the dosage form.

[0087] Unless explicitly stated otherwise or clearly implied otherwise, the term “immunogenic composition” can be used to refer to a composition (e.g., a pharmaceutical formulation) that induces an immune response in a subject when introduced to the subject; for example, a vaccine.

DETAILED DESCRIPTION

[0088] Embodiments herein provide compositions, methods, uses and manufacturing procedures for recombinant rotaviruses and immunogenic compositions thereof. Some embodiments concern compositions that include, but are not limited to, chimeric rotaviruses of use in immunogenic compositions against rotavirus infection and coronavirus infection in a subject. In certain embodiments, recombinant rotaviruses are generated using a rotavirus reverse genetics system that includes an expression vector encoding rotavirus NSP3 and one or more coronavirus spike (S) protein fragments.

[0089] FIG. 1 depicts a phylogenetic tree of rotavirus species. RVA is the major cause of rotavirus illness in humans. Referring now to FIG. 2, rotaviruses have a non-enveloped icosahedral capsid that encloses a genome consisting of 11 segments of double-stranded (ds)RNA. The G and P genotypes of rotavirus isolates are defined by the outer capsid proteins VP7 (glycoprotein) and VP4 (protease-sensitive attachment protein), respectively. The rotavirus genome segments are mostly monocistronic, containing a single open-reading-frame (ORF) that encodes a structural protein (VP1-VP4, VP6-VP7) or nonstructural protein (NSP1-NSP6). Two of the nonstructural proteins, the interferon antagonist NSP1 and translation regulator NSP3, are not essential for virus replication in cell culture; however, these proteins can affect progeny yields. During rotavirus genome replication, viral (+)RNAs not only direct protein synthesis but also act as templates for dsRNA synthesis. Segment 5 encodes the interferon antagonist NSP1, a non-essential viral protein. Others have used RG systems to generate recombinant rotaviruses with modified segment 5 RNAs that express truncated NSP1 and GFP and UnaG reporter proteins. With this system, recombinant SA11 viruses have been made in which the ORF of segNSP1 has been partially deleted and replaced with a foreign reporter protein (eGFP or mCherry). However, no recombinant rotaviruses have been reported that express a foreign protein, without sacrificing the coding potential of one of the viral ORFs.

[0090] In a first aspect, provide herein is a recombinant rotavirus expression system rotavirus. In certain embodiments, the recombinant rotavirus expression system comprises a rotavirus nonstructural protein 3 (NSP3) expression vector including a nucleotide sequence encoding rotavirus (NSP3) and a nucleotide sequence encoding a coronavirus S protein fragment. In such embodiments, NSP3 and the coronavirus S protein fragment are encoded by a single open reading frame, separated by a self-cleavage protease domain. The recombinant rotavirus expression system further include a VP1 expression vector; a VP2 expression vector; a VP3 expression vector; a VP4 expression vector ;a VP5 expression vector; a VP6 expression vector; a VP7 expression vector; a NSP1 expression vector; a NSP2 expression vector; a NSP4 expression vector;a NSP5/6 expression vector; and an African Swine Fever Virus (ASFV) NP868R RNA capping enzyme expression vector. Unlike previous recombinant rotavirus expression systems that rely on modification of segment 5, modification of segment 7, as is done in the present system, does not result in interruption or deletion of any portion of the segment's ORF. The recombinant rotavirus expression system allows for up to 2.5 kB of foreign (i.e., non-rotavirus) sequence to be added to the rotavirus genome.

[0091] The expression vectors used to express VP1-VP7, NSP1-NSP5/6, and ASFV NP868R RNA capping enzyme can be any appropriate expression vector capable of expression in a selected cell line. In some embodiments, BHK-T7 cells are transfected with the recombinant rotavirus expression system, and thus the expression vectors used to express VP1-VP7, NSP1-NSP5/6 are T7 expression vectors. In some embodiments, the expression vector used to express ASFV NP868R RNA capping enzyme is a CMV expression vector.

[0092] The self-cleavage protease domain is provided between the NSP3 polypeptide and the coronavirus S protein fragment, so that NSP3 and the coronavirus S protein fragment are automatically separated during translation. That is, the self-cleavage protease domain separates NSP3 from the non-rotavirus polypeptide.

[0093] In certain embodiments, the self-cleavage protease domain is a 2A cleavage element. Many viruses use 2A 'self-cleavage' elements to generate more than one protein from a single ORF. 2A elements are roughly 20 amino acids in length and end with a conserved Pro-Gly-Pro motif. During translation of the 2A element, the ribosome fails to form a peptide bond between the Gly-Pro residues, thus disconnecting the protein product synthesized upstream of the residues from any protein product synthesized downstream of the residues. The presence of a

2A element causes the upstream protein to end with a few extra 2A-derived residues and the downstream polypeptide to start with a Pro residue. In particular embodiments, the self-cleavage protease is a tesco porcine virus 2A (P2A) element. The P2A element has the sequence SKFQIDKILISGDIENPGP (SEQ ID NO: 1). In some embodiments the 2A element has a sequence having at least 80%, at least 85%, at least 90%, at least 95%, at least 98%, at least 99%, or 100% sequence identity to SEQ ID NO: 1.

[0094] In some embodiments, the nucleotide sequence encoding the coronavirus S protein fragment is derived from a coronavirus such as, but not limited to, SARS-CoV; MERS-CoV; SARS-CoV-2; HCoV-229E; HCoV-OC43; HCoV-HKU1; and HCoV-NL63.

[0095] HCoV-229E, HCoV-OC43, HCoV-NL63, and HCoV-HKU1 are endemic locally, and have been associated mainly with mild, self-limiting disease (e.g., the common cold). SARS-CoV causes severe acute respiratory syndrome (SARS), with cases being first reported in Asia in February 2003. The virus spread to more than two dozen countries, with about 8,000 people worldwide developing SARS. The 2003 SARS epidemic was contained, with not case of SARS being reported since 2004. MERS-CoV similarly causes severe acute respiratory illness (Middle East Respiratory Syndrome, or MERS). First identified in Saudi Arabia in 2012, MERS-CoV does not pass easily from person to person; person-to-person transmission primarily occurred in healthcare settings. In late 2019, a new coronavirus began causing febrile respiratory illness in China. The virus, provisionally known as 2019-nCoV (2019 novel coronavirus; now known as SARS-CoV-2), was first detected in Wuhan, China. SARS-CoV-2 was sequenced and identified as a betacoronavirus belonging to the sarbecovirus, with 75-80% similarity in genetic sequence to SARS-CoV.

[0096] As with other coronaviruses, SARS-CoV-2 is an enveloped virus characterized by the presence of large spikes formed by trimers of the viral spike (S) protein. In addition to mediating virus entry, the S protein is a major inducer of host immune responses. The coronavirus S protein includes a large ectodomain, a single-pass transmembrane anchor domain, and a short intracellular tail. The ectodomain includes a receptor binding subunit S1 and a membrane-fusion subunit S2. The S protein has been demonstrated to be cleaved at an S1/S2 cleavage site by host cell proteases during infection. During virus entry, viral attachment results from S1 binding to a receptor on the host cell, while S2 fuses the host and viral membranes,

allowing viral genome entry into the host cell. Receptor binding and membrane fusion are early and essential to the coronavirus infection cycle.

[0097] The SARS-CoV S1 subunit includes a conserved Receptor Binding Domain (RBD), which recognizes the angiotensin-converting enzyme 2 (ACE2). A 14 aa domain of the SARS-CoV S1 subunit has been demonstrated to complex with ACE2. Eight of these residues are strictly conserved in SARS-CoV-2, implicating ACE2 as the receptor for SARS-CoV-2. This receptor binding motif (RBM) directs specific binding to ACE2. The S1 subunit also includes a signal sequence (SS). The S2 domain is responsible for S protein homo-trimerization and includes core and transmembrane anchor domains, which function as fusion domains, promoting virus entry via fusion of the viral envelope with the host cell membrane.

[0098] In certain embodiments, the coronavirus S protein fragment is one of: an S1 subunit N-terminal domain; an S1 subunit N-terminal domain with the signal sequence; an S1 receptor binding domain; an extended form of the receptor binding domain; an S1 subunit lacking the signal sequence; an S2 core domain; or an S2 subunit lacking the transmembrane anchor domain.

[0099] In particular embodiments, the corona S protein fragment is a SARS-CoV-2 S protein fragment. FIG. 8A depicts the SARS-CoV-2 spike gene. As illustrated, the S1 and S2 subunits are produced by furin cleavage. The S1 fragment includes a signal sequence (SS), N-terminal domain (NTD), receptor binding domain (RBD), and receptor binding motif (RBM). The S2 subunit includes a trimeric core region and a transmembrane (TM) anchor domain. FIG. 8B provides those domains of the SARS-CoV-2 S protein S1 and S2 subunits that can be expressed by the recombinant rotavirus expression system described herein. FIG. 8B provides size and location information, where the positional information is provided with reference to the full-length amino acid sequence for the SARS-CoV-2 S protein (SEQ ID NO: 2). FIG. 8C provides 3-dimensional illustrations of an SARS-CoV-2 S protein monomer, identifying locations of the RBD, extended RBD, NTD, and core domains.

[00100] In those embodiments where the S protein fragment is a SARS-CoV-2 S protein fragment: the full length S protein shares at least 95% amino acid sequence identity with SEQ ID NO: 2; the S1 N-terminal domain has an amino acid sequence that shares at least 95% amino acid sequence identity with amino acids 15-294 of SEQ ID NO: 2 (SEQ ID NO: 3); the S1 N-terminal domain with signal sequence has an amino acid sequence that shares at least 95% amino

acid sequence identity with amino acids 1-294 of SEQ ID NO: 2 (SEQ ID NO: 4); the S1 subunit without signal sequence has an amino acid sequence that shares at least 95% amino acid sequence identity with amino acids 15-685 of SEQ ID NO: 2; the S protein receptor binding domain has an amino acid sequence that shares at least 95% amino acid sequence identity with amino acids 335-523 of SEQ ID NO: 2 (SEQ ID NO: 5); an extended S protein receptor binding domain has an amino acid sequence that shares at least 95% amino acid sequence identity with amino acids 311-599 of SEQ ID NO: 2 (SEQ ID NO: 9); the S2 core domain has an amino acid sequence that shares at least 95% amino acid sequence identity with amino acids 711-1077 of SEQ ID NO: 2 (SEQ ID NO: 10); and the S2 subunit without a transmembrane anchor domain has an amino acid sequence that shares at least 95% amino acid sequence identity with amino acids 686-1194 of SEQ ID NO: 2 (SEQ ID NO: 11). Illustrations of the NSP3-P2A-coronavirus S protein fragment are provided in FIG. 7.

[00101] In other embodiments, the S protein fragment is derived from a coronavirus other than SARS-CoV-2, such as, for example, SARS-CoV; MERS-CoV; HCoV-229E; HCoV-OC43; HCoV-HKU1; and HCoV-NL63.

[00102] In those embodiments where the S protein fragment is a SARS-CoV S protein fragment: the full length S protein shares at least 95% amino acid sequence identity with SEQ ID NO: 12; the S1 N-terminal domain has an amino acid sequence that shares at least 95% amino acid sequence identity with amino acids 16-281 of SEQ ID NO: 12 (SEQ ID NO: 13); the S1 N-terminal domain with signal sequence has an amino acid sequence that shares at least 95% amino acid sequence identity with amino acids 1-281 of SEQ ID NO: 12 (SEQ ID NO: 14); the S1 subunit without signal sequence has an amino acid sequence that shares at least 95% amino acid sequence identity with amino acids 16-685 of SEQ ID NO: 12; the S protein receptor binding domain has an amino acid sequence that shares at least 95% amino acid sequence identity with amino acids 322-509 of SEQ ID NO: 12 (SEQ ID NO: 15); an extended S protein receptor binding domain has an amino acid sequence that shares at least 95% amino acid sequence identity with amino acids 298-585 of SEQ ID NO: 12 (SEQ ID NO: 17); the S2 core domain has an amino acid sequence that shares at least 95% amino acid sequence identity with amino acids 693-1059 of SEQ ID NO: 12 (SEQ ID NO: 18); and the S2 subunit without a transmembrane anchor domain has an amino acid sequence that shares at least 95% amino acid sequence identity with amino acids 686-1194 of SEQ ID NO: 12 (SEQ ID NO: 19).

[00103] In those embodiments where the S protein fragment is a MERS-CoV S protein fragment: the full length S protein shares at least 95% amino acid sequence identity with SEQ ID NO: 20; the S1 N-terminal domain has an amino acid sequence that shares at least 95% amino acid sequence identity with amino acids 26-342 of SEQ ID NO: 20 (SEQ ID NO: 21); the S1 N-terminal domain with signal sequence has an amino acid sequence that shares at least 95% amino acid sequence identity with amino acids 1-342 of SEQ ID NO: 20 (SEQ ID NO: 22); the S1 subunit without signal sequence has an amino acid sequence that shares at least 95% amino acid sequence identity with amino acids 26-768 of SEQ ID NO: 20; the S protein receptor binding domain has an amino acid sequence that shares at least 95% amino acid sequence identity with amino acids 383-579 of SEQ ID NO: 20 (SEQ ID NO: 23); an extended S protein receptor binding domain has an amino acid sequence that shares at least 95% amino acid sequence identity with amino acids 359-663 of SEQ ID NO: 20 (SEQ ID NO: 24); the S2 core domain has an amino acid sequence that shares at least 95% amino acid sequence identity with amino acids 776-1151 of SEQ ID NO: 20 (SEQ ID NO: 25); and the S2 subunit without a transmembrane anchor domain has an amino acid sequence that shares at least 95% amino acid sequence identity with amino acids 769-1277 of SEQ ID NO: 20 (SEQ ID NO: 26).

[00104] In those embodiments where the S protein fragment is an HCoV-299E S protein fragment: the full length S protein shares at least 95% amino acid sequence identity with SEQ ID NO: 27; the S1 N-terminal domain has an amino acid sequence that shares at least 95% amino acid sequence identity with amino acids 8-151 of SEQ ID NO: 27 (SEQ ID NO: 28); the S1 N-terminal domain with signal sequence has an amino acid sequence that shares at least 95% amino acid sequence identity with amino acids 1-293 of SEQ ID NO: 27 (SEQ ID NO: 29); the S1 subunit without signal sequence has an amino acid sequence that shares at least 95% amino acid sequence identity with amino acids 8-581 of SEQ ID NO: 27; the S protein receptor binding domain has an amino acid sequence that shares at least 95% amino acid sequence identity with amino acids 192-424 of SEQ ID NO: 27 (SEQ ID NO: 30); an extended S protein receptor binding domain has an amino acid sequence that shares at least 95% amino acid sequence identity with amino acids 167-5065 of SEQ ID NO: 27 (SEQ ID NO: 31); the S2 core domain has an amino acid sequence that shares at least 95% amino acid sequence identity with amino acids 582-962 of SEQ ID NO: 27 (SEQ ID NO: 32); and the S2 subunit without a

transmembrane anchor domain has an amino acid sequence that shares at least 95% amino acid sequence identity with amino acids 582-1096 of SEQ ID NO: 27 (SEQ ID NO: 33).

[00105] In those embodiments where the S protein fragment is an HCoV-OC43 S protein fragment: the full length S protein shares at least 95% amino acid sequence identity with SEQ ID NO: 34; the S1 N-terminal domain has an amino acid sequence that shares at least 95% amino acid sequence identity with amino acids 21-293 of SEQ ID NO: 34 (SEQ ID NO: 35); the S1 N-terminal domain with signal sequence has an amino acid sequence that shares at least 95% amino acid sequence identity with amino acids 1-293 of SEQ ID NO: 34 (SEQ ID NO: 36); the S1 subunit without signal sequence has an amino acid sequence that shares at least 95% amino acid sequence identity with amino acids 21-772 of SEQ ID NO: 34; the S protein receptor binding domain has an amino acid sequence that shares at least 95% amino acid sequence identity with amino acids 335-599 of SEQ ID NO: 34 (SEQ ID NO: 37); an extended S protein receptor binding domain has an amino acid sequence that shares at least 95% amino acid sequence identity with amino acids 310-683 of SEQ ID NO: 34 (SEQ ID NO: 38); the S2 core domain has an amino acid sequence that shares at least 95% amino acid sequence identity with amino acids 780-1161 of SEQ ID NO: 34 (SEQ ID NO: 39); and the S2 subunit without a transmembrane anchor domain has an amino acid sequence that shares at least 95% amino acid sequence identity with amino acids 773-1278 of SEQ ID NO: 34 (SEQ ID NO: 40).

[00106] In those embodiments where the S protein fragment is an HCoV-HKU1 S protein fragment: the full length S protein shares at least 95% amino acid sequence identity with SEQ ID NO: 41; the S1 N-terminal domain has an amino acid sequence that shares at least 95% amino acid sequence identity with amino acids 20-285 of SEQ ID NO: 41 (SEQ ID NO: 42); the S1 N-terminal domain with signal sequence has an amino acid sequence that shares at least 95% amino acid sequence identity with amino acids 1-285 of SEQ ID NO: 41 (SEQ ID NO: 43); the S1 subunit without signal sequence has an amino acid sequence that shares at least 95% amino acid sequence identity with amino acids 20-774 of SEQ ID NO: 41; the S protein receptor binding domain has an amino acid sequence that shares at least 95% amino acid sequence identity with amino acids 327-599 of SEQ ID NO: 41 (SEQ ID NO: 44); an extended S protein receptor binding domain has an amino acid sequence that shares at least 95% amino acid sequence identity with amino acids 302-683 of SEQ ID NO: 41 (SEQ ID NO: 45); the S2 core domain has an amino acid sequence that shares at least 95% amino acid sequence identity with amino acids

782-1162 of SEQ ID NO: 41 (SEQ ID NO: 46); and the S2 subunit without a transmembrane anchor domain has an amino acid sequence that shares at least 95% amino acid sequence identity with amino acids 775-1281 of SEQ ID NO: 41 (SEQ ID NO: 47).

[00107] In those embodiments where the S protein fragment is an HCoV-NL63 S protein fragment: the full length S protein shares at least 95% amino acid sequence identity with SEQ ID NO: 48; the S1 N-terminal domain has an amino acid sequence that shares at least 95% amino acid sequence identity with amino acids 32-334 of SEQ ID NO: 48 (SEQ ID NO: 49); the S1 N-terminal domain with signal sequence has an amino acid sequence that shares at least 95% amino acid sequence identity with amino acids 1-334 of SEQ ID NO: 48 (SEQ ID NO: 50); the S1 subunit without signal sequence has an amino acid sequence that shares at least 95% amino acid sequence identity with amino acids 32-762 of SEQ ID NO: 48; the S protein receptor binding domain has an amino acid sequence that shares at least 95% amino acid sequence identity with amino acids 375-605 of SEQ ID NO: 48 (SEQ ID NO: 51); an extended S protein receptor binding domain has an amino acid sequence that shares at least 95% amino acid sequence identity with amino acids 350-687 of SEQ ID NO: 48 (SEQ ID NO: 52); the S2 core domain has an amino acid sequence that shares at least 95% amino acid sequence identity with amino acids 763-1143 of SEQ ID NO: 48 (SEQ ID NO: 53); and the S2 subunit without a transmembrane anchor domain has an amino acid sequence that shares at least 95% amino acid sequence identity with amino acids 763-1277 of SEQ ID NO: 48 (SEQ ID NO: 54).

[00108] In some embodiments, a single open reading frame encodes rotavirus NSP3 and two coronavirus S protein fragments. The two coronavirus S protein fragments can be the same S protein fragment, two different S protein fragments from the same coronavirus species, orthologous S protein fragments from two different coronavirus species, or non-orthologous S protein fragments from two different coronavirus species. In certain embodiments, the two coronavirus S protein fragments are in tandem, separated by a molecular hinge. Suitable molecular hinges are known in the art, and include, for example, GGSGGS (SEQ ID NO: 55).

[00109] For example, a single rotavirus ORF can encode rotavirus NSP3 and two copies of the SARS-CoV-2 receptor binding domain (RBD) (i.e., two copies of SEQ ID NO: 5). When separated by the linker GGSGGS (SEQ ID NO: 55), the 2x SARS-CoV-2 S1 RBD peptide has the amino acid sequence of SEQ ID NO: 6. Further examples include, but are not limited to, SARS-CoV-2/SARS-CoV S1 RBDs (SEQ ID NO: 7), SARS-CoV/SARS-CoV-2 S1 RBDs (SEQ

ID NO: 8), and 2x SARS-CoV RBD (SEQ ID NO: 16). Other combinations that include non-RBD domains, as well as S protein fragments from other coronavirus species are also contemplated.

[00110] In certain embodiments, the 2A element is fused to a fluorescent reporter protein to allow for visualization of construct expression.

[00111] In particular embodiments, the coronavirus S protein fragment(s) is capable of inducing an immunological response against the coronavirus from which it originated. In such embodiments, a resulting recombinant rotavirus expresses both the rotavirus polypeptides and the coronavirus S protein fragment, allowing the recombinant rotavirus to be used as a dual vaccine, eliciting protective immune responses to both the rotavirus and the coronavirus. In embodiments where S protein fragments from two different coronavirus species are encoded by a single NSP3 ORF, recombinant rotaviruses express the rotavirus NSP3 and the S protein fragment of each coronavirus species, allowing the recombinant rotavirus to be used as a trivalent vaccine, eliciting protective immune responses to both the rotavirus and both coronavirus species.

[00112] In some embodiments, the recombinant rotavirus expression system is based upon rotavirus strain G1P[8]. That is, one or more of the rotavirus gene segments (i.e., VP1-VP7 segments and NSP1-NSP5/6 segments) originate from strain G1P[8]. In some embodiments, the recombinant rotavirus expression system includes gene segments from two or more viral rotavirus strains, resulting in a reassortant virus. For example, in some embodiments, at least one of the gene segments is a human G1P[8] strain gene segment, and at least one of the gene segments is a simian SA11 strain gene segment. In other embodiments, all rotavirus gene segments of the recombinant rotavirus expressions system are G1P[8] strain gene segments.

[00113] In some embodiments, the recombinant rotavirus expression system and expressed recombinant rotaviruses can be tailored for use in, for example, humans, livestock, or poultry. Using a particular rotavirus strain as the basis for the expression system or recombinant virus, or two or more rotavirus strains to produce a reassortant virus, a recombinant rotavirus expression system and the resulting recombinant rotaviruses can be tailored for use in a desired species.

[00114] In another aspect, provided herein are recombinant rotaviruses obtainable from the recombinant rotavirus expression systems described herein. In some embodiments,

recombinant rotaviruses obtainable from the recombinant rotavirus expression systems described herein express all rotavirus proteins in their entirety in addition to the coronavirus S protein fragment(s). As provided above, in some embodiments the coronavirus S protein fragment can induce an immune response to one or more coronavirus species in addition to rotavirus. In this regard, the recombinant rotavirus can confer dual immunity to two different viruses simultaneously, or trivalent immunity to three different viruses simultaneously.

[00115] As the recombinant rotavirus is obtained from the described recombinant rotavirus expression systems, it will necessarily include a nucleotide sequence encoding rotavirus nonstructural protein 3 (NSP3) and a nucleotide sequence encoding a coronavirus S protein fragment (or two coronavirus S protein fragments in tandem), wherein the NSP3 and the coronavirus S protein fragment(s) are encoded by a single open reading frame, separated by a self-cleavage protease domain. The details of these elements are provided above in relation to the recombinant rotavirus expression systems.

[00116] In some embodiments, the recombinant rotavirus is attenuated. Attenuated rotavirus strains are known, such as attenuated G1P[8]. Thus, in some embodiments, recombinant rotavirus expression systems and the resulting recombinant rotavirus can be based on an attenuated strain.

[00117] In another aspect, provided herein are immunogenic compositions comprising a recombinant rotavirus described herein. Immunogenic compositions may further comprise, for example a pharmaceutically acceptable excipient. The immunogenic compositions can be formulated for administration to the subject for delivery orally, subcutaneously, intramuscularly, intradermally, intranasally, topically, transdermally, parenterally, gastrointestinally, transbronchially, transalveolarly, or mucosally. In some embodiments, immunogenic compositions are formulated for oral, subcutaneous, or intramuscular administration.

[00118] In another aspect, provided herein are methods for inducing a protective immune response against rotavirus and a second virus in a subject. In some embodiments, such a method includes administering an effective amount an immunogenic composition described above to the subject.

[00119] In another aspect, provided herein are methods for producing a recombinant rotavirus. In certain embodiments, such methods include transfecting BHK-T7 cells with the recombinant rotavirus expression system described herein; overseeding the transfected BHK-T7

cells with MA104 cells; preparing a clarified cell lysate; and isolating recombinant rotavirus. In some embodiments, plasmid mixtures used for transfecting BHK-T7 cells include 1x levels of plasmid vectors encoding VP1-VP7, NSP1, NSP3, and NSP4, and about 3x levels or higher of plasmid vectors encoding NSP2 and NSP5. Transfected BHK-T7 cells are overseeded with MA104 cells to promote amplification of the recombinant rotavirus. Consistent with these embodiments, the method may further include the step of freeze-thawing the BHK-T7/MA104 cell cultures. For example, following amplification of the recombinant rotavirus in BHK-T7/MA104 cells, the cells may be freeze-thawed three times. In some embodiments, cells are centrifuged at low speed to remove large debris. In certain embodiments, recombinant viruses in cell lysates are amplified by passage in MA104 cells, plaque isolated, and amplified again in MA104 cells.

[00120] In some embodiments, the recombinant rotavirus is isolated by plaque purification. In certain embodiments, the produced recombinant rotavirus is attenuated.

[00121] In some embodiments reverse genetics is used to generate recombinant rotaviruses that express, as separate products, portions of the SARS-CoV-2 S protein, including its immunodominant RBD. These embodiments may include COVID-19 combination vaccines which induce immunological protective responses against both rotavirus and SARS-CoV-2. In some embodiments this combination vaccine is used in infants and young children. Some of these embodiments allow the widespread distribution and administration of COVID-19-targeted vaccines by piggy backing onto current rotavirus immunization programs used in the USA and many other countries, both developed and developing.

[0100] Some embodiments include a 18.6-kbp rotavirus dsRNA incorporating as much as 2.2-kbp of foreign sequence, sufficient to encode the SARS-CoV-2 S1 protein. The coding capacity provided by 1.0-1.5-kbp of extra sequence is sufficient to produce recombinant rotaviruses that encode the SARS-CoV-2 NTD, RBD, or S2 core along with trafficking signals that can promote engagement of S products with antigen-presenting cells and naive B-lymphocytes.

EXAMPLES

[00122] Kanai *et al.*, *Entirely plasmid-based reverse genetics system for rotaviruses*, PROC NATL ACAD SCI USA 2017; 114(9):2349-2354, developed a plasmid-based reverse genetics (RG)

system that allows genetic modification of any of the 11 dsRNA genome segments of simian rotavirus SA11. The RG system includes eleven SA11 T7 transcription vectors and three CMV support vectors - two expressing vaccinia virus D1R and D12L RNA capping enzymes and one expressing reovirus p10FAST fusion protein.

[00123] To enhance the recovery of recombinant virus by the RG system, the capping-enzyme gene of African swine fever virus (ASFV) NP868R, a protein with triphosphatase, guanylyltransferase, and methyltransferase activities was synthesized. Then, the two support vectors expressing vaccinia virus D1R/D12L were replaced with a single vector expressing the African Swine Fever Virus (ASFV) NP868R RNA capping enzyme. Accordingly, the modified RG system contains eleven (pT7) T7-promoter plasmids expressing SA11 (+)RNAs, a CMV-promoter plasmid expressing the ASFV NP868R capping enzyme, and a pCAG vector expressing p10FAST fusion protein. Referring now to FIG. 3, in this modified rotavirus RG system, baby hamster kidney cells expressing T7 polymerase (BHK-T7) are transfected with T7 vectors for rotavirus (+)RNAs, a CMV vector for ASFV NP868R, and a pCAG vector for p10FAST. Three days after transfection, BHK-T7 cells are overseeded with MA104 cells in trypsin-containing media. The combined BHK-T7/MA104 cultures are harvested six days post transfection, and recombinant virus amplified by passage in MA104 cells.

[00124] Referring now to FIG. 4, the experiments provided that replacing the D1R/D12L support vectors with an NP868R-expression vector enhanced recovery of recombinant virus from the rotavirus RG system. About 10-fold more recombinant virus were recovered in RG experiments expressing ASFV NP868R capping enzyme than vaccinia virus capping D1R/D12L enzymes. (FIG. 4C). The modified RG system uses a single support plasmid (NP868R) in place of the originally described two support plasmids (vaccinia virus D1R & D12L) to generate capping activity. The ASFV NP868R capping enzyme promotes greater recovery of recombinant virus than the vaccinia virus D1R and D12L capping enzymes.

[00125] The collective size of the 11 rotavirus genome segments is about 18.5 kB. However, a number of naturally occurring rotavirus variants have been recovered with genomes that are significantly larger, in some cases approaching 20 kB. The increased size typically results from the introduction of sequence duplication within a genome segment, yielding viral variants that display unusual genome profiles upon gel electrophoresis (FIG. 5). The most commonly characterized rotavirus variants have sequence duplications in the segNSP1 and

segNSP3 dsRNAs. The longest segNSP1 duplication described so far is about 1.2 kB. The longest segNSP3 duplication is about 0.9 kB, yielding an atypical genome segment with a size of about 2.0 kB instead of its wild type about 1.1 kB size. Notably, these rotavirus variants are genetically stable and, in general, grow about as well as wildtype viruses. It is also possible to use RG systems to create genetically stable recombinant rotaviruses that maintain sequence duplications. Accordingly, the ability of rotaviruses to carry extra genetic information of greater than 1kB suggests that it is possible to re-engineer the virus to produce a foreign protein that is more than 300 amino acids in size.

[00126] Many viruses use 2A 'self-cleavage' elements to generate more than one protein from a single ORF. 2A elements are roughly 20 amino acids in length and end with a conserved Pro-Gly-Pro motif. During translation of the 2A element, the ribosome fails to form a peptide bond between the Gly-Pro residues, thus disconnecting the protein product synthesized upstream of the residues from any protein product synthesized downstream of the residues. The presence of a 2A element causes the upstream protein to end with a few extra 2A-derived residues and the downstream polypeptide to start with a Pro residue. While group A rotaviruses, like those that formulate rotavirus vaccines, do not use 2A elements to produce viral proteins, group C rotaviruses do. In particular, the segNSP3 dsRNA of group C viruses contains an ORF that encodes an NSP3 protein that is functionally and structurally similar to RVA NSP3, but due to a downstream 2A element, also produces a second protein (dsRBP) - a double-strand RNA binding protein that inhibits protein kinase R (PKR) activation (see FIG. 6). Based on the properties of the group C segNSP3 dsRNA, one can create group A rotaviruses with an analogous segNSP3 dsRNA (*i.e.*, a rotavirus that expresses a functional NSP3 protein and, through downstream placement of a 2A element, a second foreign protein). There is precedence for using 2A elements to drive the expression of extra proteins for other members of the *Reoviridae*. For example, recombinant mammalian reoviruses have been generated with 2A elements that direct expression of the HIV gag protein and the eel epi fluorescence green protein (UnaG). More recently, recombinant rotaviruses have been described that contain re-engineered segNSP1 dsRNA, wherein the NSP1 ORF has been largely replaced with a 2A element fused to a fluorescent reporter protein. In the present disclosure, 2A self-cleavage elements were used to produce recombinant SA11 rotaviruses that express all 12 viral proteins plus an additional separate foreign protein.

[00101] Modified reverse genetics system. The plasmid-based rotavirus RG system developed by Kanai *et al.* relies on co-transfection of BHK-T7 cells with three types of plasmids: (1) eleven T7 transcription (pT7) vectors that direct the expression of the 11 rotavirus (+)RNAs; (2) two CMV-pol II plasmids that direct the expression of the vaccinia virus D1R and D12L RNA capping enzymes; and (3) a CMV-pol II plasmid that directs the expression of the avian reovirus p10FAST fusion protein. The system was inefficient, leading the inventors to make changes that improved the recovery of recombinant virus by at least 10-fold (FIG. 4). With the modified system, recombinant SA11 viruses with mutations in several genome segments (*e.g.*, NSP1, NSP2, NSP3, and/or VP3) have been generated. The major changes that were made to the RG system are replacing the two plasmids for the vaccinia virus capping enzyme complex with a single plasmid encoding the African swine fever virus (ASFV) NP868R capping enzyme and discontinuing use of the p10FAST plasmid. These results provide that a vector expressing p10FAST is no longer necessary for the modified reverse genetics system disclosed herein.

[00127] This modified reverse genetics system and its ability to express a foreign protein are described by Philip *et al.*, J Virol 2019 Nov 26;93(24). pii: e01616-19; Philip *et al.*, Microbiol Resour Announc 2019 Jul 3;8(27). pii: e00523-19; and Philip *et al.*, J Vis Exp 2020, e61039 (in press), each of which is expressly incorporated by reference in its entirety. Recombinant rotaviruses were found to be well-growing, genetically stable, and to efficiently express a foreign protein. Generated recombinant rotaviruses include those that express all or a portion of the capsid proteins of human norovirus (NoV; CP1, P, or P2), hepatitis E virus (HEV; VP1); and human astrovirus (HAstrV; VP34, VP70, or VP90). Recombinant rotaviruses expressing various fluorescent reporter proteins have also successfully been generated, and are contemplated herein.

[00128] FIG. 9 highlights the inventors' ability to recover recombinant rotaviruses (rRV) expressing foreign antigens. FIG. 9A illustrates the reverse genetics that was used to replace the rotavirus segment 7 (NSP3) RNA with an analogous segment encoding both NSP3 and a foreign antigen (NoV VP1) as separate proteins. FIG. 9B illustrates the plasmid design for expression of NSP3 and NoV VP1 from segment 7 as separate proteins, driven by an intervening 2A self-cleaving element. The genome of rRV encoding NSP3-2A-NoV VP1 contained a 2.9-kB segment 7 RNA instead of a wildtype 1.1-kB segment 7 RNA (see FIG. 9C). rRV containing the 2.9-kB segment RNA produced VP1 dimers in infected cells (see FIG. 9D). A similar approach

was used to generate rRV with a segment 7 RNA encoding NSP1 and the astrovirus VP90 protein. The rRV contained a 3.6-kB segment 7 RNA, revealing the remarkable flexibility of the rotavirus genome and its capacity to accommodate extra RNA (see FIG. 9E).

[00129] To generate recombinant rotaviruses expressing foreign proteins, a T7 transcription vector-based reverse genetics system was used to replace the NSP3 ORF of the rotavirus segment 7 dsRNA with an ORF encoding NSP3 fused to a foreign protein. Inserted at the fusion junction was a porcine teschovirus 2A-like translation stop-restart (self-cleaving) element designed to direct the separate expression of NSP3 and the foreign protein. In some cases, 3xFLAG and 6xHis tags were placed at the ends of the foreign ORF sequence to facilitate detection of foreign protein. Through engineering of segment 7, the inventors determined that as much as 2.5 kB of foreign sequence could be added to the rotavirus genome and that recombinant rotaviruses could be generated that expressed large-sized foreign protein including the 56-kDa NoV VP1 protein, and the 70- and 90-kDa HAstrV VP70 and VP90 proteins, respectively.

[0102] Method for generating recombinant viruses. A modification of the plasmid-based reverse genetics system reported by Kanai *et al* (2017) was used to generate rRVs. Day 0: BHK-T7 cells were seeded into 12-well plates (about 2×10^5 cells/well) in G418-free Glasgow/FBS+ medium. Day 1: Plasmid mixture was prepared that contained 0.8 µg each of the 11 SA11 rotavirus pT7 plasmids and 0.8 µg of pCMV-NP868R. The plasmid combination was added to 100 µl of pre-warmed (37°C) Opti-MEM (Gibco, 31985-070) and mixed by gently pipetting up and down. Afterwards, 25 µl of TransIT-LTI transfection reagent (Mirus, MIR2305) was added, and the transfection mixture gently vortexed and incubated at room temperature for 20 min. During the incubation period, BHK-T7 cells in 12-well plates were washed once with Glasgow/FBS- complete medium and the cells overlaid with 1 ml of SMEM complete medium [MEM Eagle Joklik (Lonza 04-719Q), 10% tryptone-peptide broth, 2% NEAA, 1% penicillin-streptomycin, 1% glutamine). The transfection mixture was added drop-by-drop to the SMEM complete medium in the 12-well plates and the plates returned to a 37°C, 5% CO₂ incubator. Day 3: 2×10^5 MA104 cells in 0.25 ml of M199/FBS-free complete medium were added to plate wells, along with trypsin to a final concentration of 0.5 µg/ml. Day 6: Cells in plates were freeze-thawed 3-times and the lysates were placed in 1.5 ml microfuge tubes. After centrifugation at

500 x g for 10 min (4°C), 300 µl of the clarified lysates were transferred onto MA104 monolayers in 6-well plates containing 2 ml of M199/FBS-free complete medium and 0.5 µg/ml trypsin. The plates were incubated in a 37°C, 5% CO₂ incubator for 7 days or until complete cytopathic effects (CPE) were observed. Typically, complete CPE occurred at 4-6 days for wells containing rRV. Recombinant viruses are plaque purified and analyzed by sequencing.

[0103] Modified segment 7 (NSP3) expression vectors containing SARS-CoV-2 S sequences. To examine the possibility of using rotavirus as an expression platform for regions of the SARS-CoV-2 S protein, we replaced the NSP3 ORF in the pT7/NSP3SA11 transcription vector with a cassette comprised of the NSP3 ORF, a porcine teschovirus 2A element, and a coding sequence of the S protein (FIG. 11). The cassette included a flexible GAG hinge between the coding sequence for NSP3 and the 2A element and a 3x FLAG (f) tag between the coding sequences for the 2A element and the S region. This approach was used to generate a set of vectors (collectively referred to as pT7/NSP3-CoV2/S vectors) that contained coding sequences for SARS-CoV-2 S1 (pT7/NSP3-2A-fS1), NTD (pT7/NSP3-2A-fNTD), RBD (pT7/NSP3-2A-fRBD), an extended form of the RBD (ExRBD) (pT7/NSP3-2A-fExRBD), and the S2 core region (CR) including its fusion domains (pT7/NSP3-2A-fCR) (FIG. 10). The S sequences were inserted into the pT7/NSP3SA11 vector at the same site as used before in the production of recombinant SA11 (rSA11) rotaviruses expressing FPs.

[0104] Recovery of rSA11 rotaviruses with segment 7 dsRNA containing S sequences. To generate rSA11 viruses, BHK-T7 monolayers were transfected with a complete set of pT7/SA11 expression vectors, except pT7/NSP3SA11 was replaced with a pT7/NSP3-CoV2/S vector, and a CMV expression plasmid (pCMV-NP868R) encoding the capping enzyme of African swine fever virus. In transfection mixtures, plasmids encoding rotavirus NSP2 (pT7/NSP2SA11) and NSP5 (pT7/NSP5SA11) were included at levels three-fold greater than the other pT7/SA11 vectors. BHK-T7 cells were overseeded with MA104 cells two days following transfection. The BHK-T7/MA104 cell mixture was freeze-thawed three days later, and the rSA11 viruses were recovered by plaque isolation and amplified by 1 or 2 cycles of growth in MA104 cells prior to characterization. Properties of the rSA11 viruses are summarized in FIG. 18.

[0105] Based on gel electrophoresis, rSA11 viruses generated with pT7/NSP3-S vectors (collectively referred to as rSA11/NSP3-CoV2/S viruses) contained segment 7 dsRNAs that

were much larger than that of wildtype rSA11 (rSA11/wt) virus (FIG. 12). Sequence analysis confirmed that the segment 7 dsRNAs of the rSA11/NSP3-CoV2/S viruses matched the segment 7 sequences present in the pT7/NSP3-CoV2/S vectors (data not shown). The re-engineered segment 7 dsRNA of virus isolate rSA11/NSP3-fS1 had a length of 3.3 kbp, accounting for its electrophoretic migration near the largest rotavirus genome segment (segment 1), which is likewise 3.3 kbp in length (FIG. 18, FIG. 12A). The segment 7 dsRNA of rSA11/NSP3-fS1 contains a 2.2-kbp foreign sequence insertion, the longest foreign sequence that has been introduced into the segment 7 dsRNA, or for that matter, any rotavirus genome segment. The previously longest 7 dsRNA engineered into rSA11 was the 2.4-kbp segment 7 dsRNA of rSA11/NSP3-fmRuby-P2A-fUnaG, which contained a cassette that encoded three proteins (NSP3, UnaG, mRuby). The total genome size of rSA11/NSP3-fS1 is 20.8 kbp, 12% greater than that of rSA11/wt. This is the largest genome known to exist within a rotavirus isolate and demonstrates the capacity of rotavirus to replicate and package large amounts of foreign sequence.

[0106] The segment 7 dsRNAs of virus isolates, rSA11/NSP3-fNTD, -fRBD, -fExRBD, and -fCR, were determined to have lengths of 2.1, 1.8, 2.1, and 2.3 kbp, respectively (FIG. 18), and as expected from their sizes, migrated on RNA gels between rotavirus genome segments 3 (2.6 kbp) and 5 (1.6 kbp) (FIG. 12B). The segment 7 dsRNAs of the rSA11/NSP3-fNTD, -fRBD, -fExRBD, and -fCR isolates contained foreign sequence insertions of 1.0, 0.7, 1.0, and 1.2 kbp, respectively, significantly smaller than the 2.1-kbp foreign sequence insertion of rSA11/NSP3-fS1. The smaller sizes of the foreign-sequence inserts contained in the segment 7 RNAs of rSA11/NSP3-fNTD, -fRBD, -fExRBD, and -fCR may provide the additional genetic space necessary for re-engineering the S-protein products of these viruses to include routing and localization tags capable of enhancing antigen recognition and processing by immune cells. Particularly valuable may be the inclusion of tags that promote interaction of the S-protein products with antibody heavy-chain (Fc) receptors (e.g., FcRn), enable aggregation or multivalent presentation of the products, or increase the efficiency of synthesis or secretion of the products.

[0107] Consistent with previous studies examining the phenotypes of rSA11 isolates expressing FPs (18-19), the sizes of plaques formed by rSA11/NSP3-CoV2/S viruses were smaller than plaques formed by rSA11/wt (FIG. 12C). Similarly, rSA11 viruses containing S-

protein coding sequences grew to maximum titers that were up to 0.5-1 log lower than rSA11/wt (FIG. 12D). The reason for the smaller plaques and lower titers of the rSA11/NSP3-CoV2/S viruses is unknown, but may reflect the longer elongation time likely required for the viral RNA polymerase to transcribe their segment 7 dsRNAs during viral replication. Alternatively, it may reflect the longer time required to translate segment 7 (+)RNAs that contain S-protein coding sequences.

[0108] Expression of S coding sequences by rSA11 rotaviruses. To determine whether the rSA11/NSP3-CoV2/S viruses expressed products from their S sequences, lysates prepared from MA104 cells infected with these viruses were examined by immunoblot assay using FLAG- and RBD-specific antibodies (FIGs. 4A, B). Immunoblots probed with FLAG antibody showed that rSA11/NSP3-fNTD, -fExRBD, -fRBD, and -fCR viruses generated S products and that their sizes were as predicted for an active 2A element in the segment 7 ORF: fNTD (34.8 kDa), fExRBD (35.2 kDa), fRBD (24.3 kDa), and fCR (42.9 kDa) (FIG. 18). Immunoblot assays indicated that the rSA11/NSP3-fExRBD yielded higher levels of S product than any of the other rSA11/NSP3-CoV2/S viruses. The basis for the higher levels of the fExRBD product is unclear, but does not correlate with increased levels of expression of other viral products, such as NSP3 and VP6. Nonetheless, the high levels of ExRBD expression by rSA11/NSP3-fExRBD suggests that such viruses may be best suited in pursuing the development of combined rotavirus/COVID vaccines.

[0109] FLAG antibody did not detect the expected 79.6-kDa fS1 product in cells infected with rSA11/NSP3-fS1 (FIG. 13A). The explanation for this is unknown, may relate to modifications made to the S protein during its synthesis. Notably, the S1 coding sequence in the segment 7 ORF includes an N-terminal signal sequence which, in SARS-CoV-2 infected cells, is cleaved from the S1 protein during synthesis on the endoplasmic reticulum (ER). Cleavage of the signal sequence may have removed the upstream 3x FLAG tag from a S1 product, preventing its detection by the FLAG antibody. Alternatively, because the membrane anchor domain is located at the C-terminus of the SARS-CoV-2 S protein, it is possible that the S1 product was secreted from rSA11/NSP3-fS1-infected cells and thus lost. It is also possible that glycosylation and/or degradation of the 79.6 kDa-S1 product by ER-associated proteases may have prevented the protein's detection. Finally, because rotavirus usurps and possibly remodels the ER in support of

glycoprotein (NSP4 and VP7) synthesis and virus morphogenesis may perturb ER-interaction with the S signal sequence in such a way to prevent S1 synthesis.

[0110] Interestingly, all the rSA11/NSP3-CoV2/S viruses, including rSA11/NSP3-fS1, generated 2A read-through products that were detectable using FLAG antibody (FIG. 13A). Thus, the 2A stop-start element in the rSA11/NSP3-2A-CoV2/S viruses was not fully active, which is consistent with previous reports analyzing the functionality of 2A elements within cells. However, with the exception of the rSA11/NSP3-fS1, all the viruses generated more 2A-cleaved S product than read-through product. Mutation of residues in and around the 2A element, including the inclusion of flexible linker sequences, may decrease the relative frequency of read through.

[0111] Lysates from MA104 cells infected with rSA11/wt, rSA11/NSP3-fRBD, and rSA11/NSP3-fExRBD were also probed with RBD-specific polyclonal antibody prepared against a peptide mapping to the C-terminal end of the RBD domain (ProSci 9087). The RBD antibody recognized the fExRBD product of the rSA11/NSP3-fExRBD virus, but not the fRBD product of rSA11/NSP3-fRBD (FIG. 13B), presumably because the latter product lacked the peptide sequence used in generating the ProSci RBD antibody. To gain insight into whether the fRBD and fExRBD products folded into native structures mimicking those present in the SARS-CoV-2 S protein, lysates prepared from MA104 cells infected with rSA11/NSP3-fRBD and rSA11/NSP3-fExRBD were probed by pulldown assay using an anti-RBD conformation-dependent neutralizing monoclonal antibody (GeneTex CR3022). As shown in FIG 13C, the CR3022 immunoprecipitate included fExRBD, indicating that this product included a neutralizing epitope found in authentic SARS-CoV-2 S protein. Thus, at least some of the RBD product of rSA11/NSP3-fExRBD has likely folded in a conformation capable of inducing a protective antibody response. Unlike the successful pulldown of ExRBD with CR3022 antibody, it was not clear if the antibody likewise immunoprecipitated the fRBD product of rSA11/NSP3-fRBD. This uncertainty stems from the light chain of the CR3022 antibody obscuring the electrophoretically closely-migrating fRBD product in immunoblot assays (FIG. 13C).

[0112] Expression of the ExRBD and RBD products by rSA11s during rotavirus infection. To gain insight into fExRBD and fRBD expression during virus replication, MA104 cells were infected with rSA11/wt, rSA11/NSP3-fExRBD or rSA11/NSP3-fRBD and then harvested at intervals between 0 and 12 hr p.i. Analysis of the infected cell lysates by

immunoblot assay showed that fExRBD and fRBD were readily detectable by 4 h p.i., paralleling the expression of rotavirus proteins NSP3 and VP6 (FIG. 14). Increased levels of fExRBD and fRBD were present at 8 and 12 h p.i., without obvious accumulation of FLAG-tagged products of smaller sizes. Thus, the fExRBD and fRBD products appear to be relatively stable.

[0113] Density of rSA11 virus particles containing S sequences. The introduction of S sequences into the rSA11/NSP3-CoV2/S viruses increased the size of their viral genomes by 1.0 to 2.5 kbp beyond that of SA11/wt. Assuming the rSA11/NSP3-CoV2/S viruses are packaged efficiently and contain a complete constellation of 11 genome segments, the increased content of dsRNA within the core of rSA11/NSP3-CoV2/S particles should cause their densities to be greater than that of SA11/wt particles. To explore this possibility, rSA11/wt (18.6-kbp genome), rSA11/NSP3-fExRBD (19.5 kbp) and rSA11/NSP3-fS1 (20.8 kbp) were amplified in MA104 cells. The infected-cell lysates were then treated with EDTA to convert rotavirus virions (triple-layered particles) into double-layered particles (DLPs). The particles were centrifuged to equilibrium on CsCl gradients and the density of the DLP bands determined by refractometry (FIG. 15). The analysis indicated that the density of rSA11/NSP3-fExRBD DLPs (1.386 g/cm^3) was greater than SA11/wt DLPs (1.381 g/cm^3) (panel A) and similarly, the density of rSA11/NSP3-fS1 DLPs (1.387 g/cm^3) was greater than SA11/wt DLPs (1.38 g/cm^3) (panel B). Analysis of the banded DLPs by gel electrophoresis confirmed that they contained the expected constellation of eleven genome segments. To confirm that the density of rSA11/NSP3-fS1 DLPs was different than rSA11/wt DLPs, infected-cell lysates containing each of these viruses were pooled, treated with EDTA, and the viral DLPs in the combined sample banded by centrifugation on a CsCl gradient (FIG. 15E). Analysis of the gradient revealed the presence of two bands of particles, indicating that rSA11/NSP3-fSA11-fS1 and rSA11/wt DLPs were of different densities. Gel electrophoresis of the combined DLP bands showed, as expected, that both rSA11/NSP3-fSA11-fS1 and rSA11/wt were present. Taken together, these results demonstrate that rSA11/NSP3-CoV-2/S virions contain complete genome constellations despite the fact that their genome sizes are significantly greater than that of wildtype SA11 virus. Indeed, the 20.8-kbp rSA11/NSP3-fS1 genome is 12% greater in size than the 18.6-kbp rSA11/wt genome (FIG. 18). Thus, the rotavirus core has space to accommodate large amounts of additional foreign sequence. How the dsRNA within the core is re-distributed to accommodate large amounts of

additional sequence is not known, but clearly the core remains a transcriptionally-active nanomachine despite the additional sequence. Whether other genome segments can be engineered similarly to segment 7 of rSA11/NSP3-fS1 to include 2 kb of additional sequence remains to be determined. The maximum packaging capacity of the core also remains to be determined.

[0114] Genetic stability of rSA11 rotaviruses containing S sequences. The genetic stability of the rSA11/NSP3-CoV2/S viruses were assessed by serial passage, with a fresh monolayer of MA104 cells infected with 1:1000 dilutions of cell lysates at each round. Electrophoretic analysis of the dsRNAs recovered from cells infected with rSA11/NSP3-fNTD, -fRBD, -ExRBD, or -ExCR showed no changes in the sizes of any of the 11 genome segments over 5 rounds of passage (P1-P5), including segment 7, indicating that these viruses were genetically stable (FIG. 16A). In contrast, serial passage of rSA11/NSP3-S1 showed evidence of instability (FIG. 16A). By the third round of passage, novel genome segments were appearing that were smaller than the 3.3-kbp segment 7 RNA. With continued passage, four novel segments (R1 to R4) became prominent and the 3.3-kbp segment 7 RNA was no longer detectable, suggesting that the high-passage virus pools (P3-P6) were populated by variants containing segment 7 RNAs derived from the 3.3-kb segment 7 RNA through internal sequence deletion. To evaluate this possibility, 8 variants were recovered from the P6 virus pool by plaque isolation, 4 with a large (L) plaque phenotype and 4 with a small (S) plaque phenotype. Electrophoretic analysis of the genomes of the variants showed that none contained the 3.3-kbp segment 7 RNA (FIG. 16B). Instead, 6 variants (L1, L2, L3, L4, S2, and S4) contained the R3 segment, and the other two variants contained either the R1 (S1) or R2 (R2) segment. No variants were recovered that contained the novel R4 segment.

[0115] Sequencing showed that the R1, R2, and R3 segments were in fact derivatives of the 3.3-kbp segment 7 RNA (FIG. 16C). The R1, R2, and R3 RNAs all retained the complete 5'- and 3'-UTRs and NSP3 ORF of segment 7, but contained sequence deletions of 1.0 (R1), 1.5 (R2), or 1.8 (R3) kbp of S1 coding sequence. The fact that 6 of the 8 variants isolated by plaque assay contained the R3 segment suggests that variants with this RNA may have a growth advantage over variants with the R1, R2, or R4 RNAs. Although genetic instability gave rise to rSA11/NSP3-fS1 variants lacking portions of the S1 ORF, none were identified that lacked portions of the NSP3 ORF. This suggests that NSP3 may be essential for virus replication, which

would explain the failure of previous efforts by us to recover viable rSA11s encoding truncated forms of NSP3 through insertion of stop codons in the NSP3 ORF (data not shown). To gain a better understanding of the diversity of deletions introduced into the segment 7 (NSP3-fS1) RNA during serial passage, the total population of viral RNAs in high passaged virus pools are being examined by direct RNA sequencing.

[0116] Cell culture. Embryonic monkey kidney cells (MA104) were grown in medium 199 (M199) containing 5% fetal bovine serum (FBS) and 1% penicillin-streptomycin. Baby hamster kidney cells expressing T7 RNA polymerase (BHK-T7) were provided by Dr. Ulla Buchholz, Laboratory of Infectious Diseases, NIAID, NIH (Bethesda, Maryland, USA), and were propagated in Glasgow minimum essential media (GMEM) containing 5% heat-inactivated fetal bovine serum (FBS), 10% tryptone-peptide broth, 1% penicillin-streptomycin, 2% non-essential amino acids, and 1% glutamine (41). BHK-T7 cells were grown in medium supplemented with 2% Geneticin (Invitrogen) with every other passage.

[0117] Plasmid construction. Recombinant SA11 rotaviruses were prepared using plasmids (pT7/VP1SA11, pT7/VP2SA11, pT7/VP3SA11, pT7/VP4SA11, pT7/VP6SA11, pT7/VP7SA11, pT7/NSP1SA11, pT7/NSP2SA11, pT7/NSP3SA11, pT7/NSP4SA11, and pT7/NSP5SA11) obtained from the Addgene plasmid repository [https://www.addgene.org/Takeshi_Kobayashi/] and pCMV-NP868R (Y. Kanai, *et al.*, Proc. Natl Acad Sci USA 114:2349-2354). The plasmid pT7/NSP3-P2A-fUnaG was produced, as described elsewhere, by fusing a DNA fragment containing the ORF for P2A-3xFL-UnaG to the 3'-end of the NSP3 ORF in pT7/NSP3SA11. A plasmid (pTWIST/COVID19spike) containing a full-length cDNA of the SARS-CoV-2 S gene (GenBank MN908947.3) was purchased from Twist Bioscience. The plasmids pT7/NSP3-2A-fNTD, pT7/NSP3-2A-fExRBD, pT7/NSP3-2A-fRBD, pT7/NSP3-2A-fCR, and pT7/NSP3-2A-S1 were made by replacing the UnaG ORF in pT7/NSP3-2A-fUnaG with ORFs for the NTD, ExRBD, RBD, CR, and S1 regions, respectively, of the SARS-CoV-2 S protein, by In-Fusion cloning. DNA fragments containing NTD, ExRBD, RBD, CR, and S1 coding sequences were amplified from pTWIST/COVID19spike using the primer pairs NTD_For and NTD_Rev, ExRBD_For and ExRBD_Rev, RBD_For and RBD_Rev, CR_For and CR_Rev, and S1_For and S1_Rev, respectively (FIG 17). Transfection quality plasmids were prepared commercially

(www.plasmid.com) or using Qiagen plasmid purification kits. Primers were provided by and sequences determined by EuroFins Scientific.

[0118] Recombinant viruses. The reverse genetics protocol used to generate recombinant rotaviruses was described in detail previously. To summarize, BHK-T7 cells were transfected with SA11 pT7 plasmids and pCMV-NP868R using Mirus TransIT-LT1 transfection reagent. Two days later, the transfected cells were overseeded with MA104 cells and the growth medium (serum-free) adjusted to a final concentration of 0.5 µg/ml porcine pancreatic trypsin (Type IX, Sigma Aldrich). Three days later, the BHK-T7/MA104 cell mixture was freeze-thawed 3-times and the lysates clarified by low-speed centrifugation. Recombinant virus in clarified lysates were amplified by one or two rounds of passage in MA104 cells maintained in serum-free medium containing 0.5 µg/ml trypsin. Individual virus isolates were obtained by plaque purification and typically amplified 1 or 2 rounds in MA104 cells prior to analysis. Viral dsRNAs were recovered from infected-cell lysates by Trizol extraction, resolved by electrophoresis on Novex 8% polyacrylamide gels (Invitrogen) in Tris-glycine buffer, and detected by staining with ethidium bromide. Viral dsRNAs in gels were visualized using a BioRad ChemiDoc MP Imaging System. The genetic stability of plaque isolated rSA11s was assessed by serial passage as described previously.

[0119] Immunoblot analysis. MA104 cells were mock infected or infected with 5 PFU of recombinant virus per cell and harvested at 8 h p.i. Cells were washed with cold phosphate-buffered saline (PBS), pelleted by low-speed centrifugation, and lysed by resuspending in lysis buffer [300 mM NaCl, 100 mM Tris-HCl, pH 7.4, 2% Triton X-100, and 1x EDTA-free protease inhibitor cocktail (Roche cOmplete)]. For immunoblot assays, lysates were resolved by electrophoresis on Novex linear 8-16% polyacrylamide gels and transferred to nitrocellulose membranes. After blocking with phosphate-buffered saline containing 5% non-fat dry milk, blots were probed with guinea pig polyclonal NSP3 (Lot 55068, 1:2000) or VP6 (Lot 53963, 1:2000) antisera, mouse monoclonal FLAG M2 (Sigma F1804, 1:2000), rabbit monoclonal PCNA [13110S, Cell Signaling Technology (CST), 1:1000] antibody or rabbit anti-RBD (ProSci 9087; 1:200) antibody. Primary antibodies were detected using 1:10,000 dilutions of horseradish peroxidase (HRP)-conjugated secondary antibodies: horse anti-mouse IgG (CST), anti-guinea pig IgG (KPL), or goat anti-rabbit IgG (CST). Signals were developed using Clarity Western ECL Substrate (Bio-Rad) and detected using a Bio-Rad ChemiDoc imaging system.

[0120] Immunoprecipitation assay. Mock-infected and infected cell lysates were prepared as above. Lysates were mixed with a SARS-CoV-2 S1 specific monoclonal antibody (GeneTex CR3022, 1:150 dilution) or an NSP2 monoclonal antibody (#171, 1:200). After incubation at 4°C with gentle rocking for 18 h, antigen-antibody complexes were recovered using Pierce magnetic IgA/IgG beads (ThermoFisher Scientific), resolved by gel electrophoresis, and blotted onto nitrocellulose membranes. Blots were probed with FLAG antibody (1:2000) to detect fRBD and fExRBD and NSP2 antibody (1:2000).

[0121] CsCl gradient centrifugation. MA104-cell monolayers in 10-cm cell culture plates were infected with rSA11 viruses at an MOI of 5 and harvested at 12 h p.i. Cells were lysed by adjusting media to 0.5% Triton X100 (Sigma) and incubation on ice for 5 min. Lysates were then clarified by centrifugation at 500 x g at 4°C for 6 min. The clarified lysates were adjusted to 10 mM EDTA and incubated for 1 h at 37°C to cause the conversion of rotavirus TLPs to DLPs (36). CsCl was added to samples to a density of 1.367 g/cm³ and samples were centrifuged at 110,000 x g with a Beckman SW55Ti rotor at 8°C for 22 h. Fractions containing viral bands were recovered using a micropipettor and fraction densities were determined using a refractometer.

[0122] Genetic stability of rSA11 viruses. Viruses were serially passaged on MA104-cell monolayers using 1:1000 dilutions of infected cell lysates prepared in serum-free M199 medium and 0.5 µg/ml trypsin. When cytopathic effects reached completion (4-5 days), cells were freeze-thawed twice in their medium, and lysates were clarified by low-speed centrifugation. To recover dsRNA, clarified lysates (600 µl) were extracted with Trizol (ThermoFisher Scientific). The RNA samples were resolved by electrophoresis on 8% polyacrylamide gels and the bands of dsRNA detected by ethidium-bromide staining.

[0123] GenBank accession numbers. Segment 7 sequences in rSA11 viruses have been deposited in Genbank: wt (LC178572), NSP3-P2A-fNTD (MW059024), NSP3-P2A-fRBD (MT655947), NSP3-P2A-ExRBD (MT655946), NSP3-P2A-fCR (MW059025), NSP3-P2A-S1 (MW059026), NSP3-P2A-S1/R1 (MW353715), NSP3-P2A-S1/R2 (MW353716), and NSP3-P2A-S1/R3 (MW353717). See also FIG. 18.

[0124] We have shown that reverse genetics can be used to generate recombinant rotaviruses that express, as separate products, portions of the SARS-CoV-2 S protein, including its immunodominant RBD. These results indicate that it may be possible to develop rotaviruses

as vaccine expression vectors, providing a path for generating oral live-attenuated rotavirus-COVID-19 combination vaccines able to induce immunological protective responses against both rotavirus and SARS-CoV-2. Such combination vaccines would be designed for use in infants and young children and would allow the widespread distribution and administration of COVID-19-targeted vaccines by piggy backing onto current rotavirus immunization programs used in the USA and many other countries, both developed and developing. In addition, our findings raise the possibility that through the use of rotavirus as vaccine expression platforms, rotavirus-based combination vaccines could be made against other enteric viruses including norovirus, astrovirus, and hepatitis E virus.

[0125] We have determined that the 18.6-kbp rotavirus dsRNA can accommodate as much as 2.2-kbp of foreign sequence, which is sufficient to encode the SARS-CoV-2 S1 protein. However, in our hands, rSA11s encoding S1 were not genetically stable and failed to express the appropriate S1 product, for reasons that are uncertain but under further investigation. Rotaviruses carrying large amounts of foreign sequence are characteristically genetically unstable (this study and data not shown), but those with foreign sequences of <1.0-1.5-kbp are stable over 5-10 rounds of serial passage at low MOI and, thus, can be developed into vaccine candidates. The coding capacity provided by 1.0-1.5-kbp of extra sequence is sufficient to produce recombinant rotaviruses that encode the SARS-CoV-2 NTD, RBD, or S2 core along with trafficking signals that can promote engagement of S products with antigen-presenting cells and naive B-lymphocytes. Current work is underway to gain insight how successful rotaviruses expressing SARS-CoV-2 products are in inducing neutralizing antibodies in immunized animals.

[0126] While the disclosed subject matter is amenable to various modifications and alternative forms, specific embodiments are described herein in detail. The intention, however, is not to limit the disclosure to the particular embodiments described. The disclosure is intended to cover all modifications, equivalents, and alternatives falling within the scope of the disclosure as defined by the appended claims.

[0127] Similarly, although illustrative methods may be described herein, the description of the methods should not be interpreted as implying any requirement of, or particular order among or between, the various steps disclosed herein. However, certain embodiments may require certain steps and/or certain orders between certain steps, as may be explicitly described herein and/or as may be understood from the nature of the steps themselves (e.g., the

performance of some steps may depend on the outcome of a previous step). Additionally, a “set,” “subset,” or “group” of items (e.g., inputs, algorithms, data values, etc.) may include one or more items, and, similarly, a subset or subgroup of items may include one or more items. A “plurality” means more than one.

[0128] As the terms are used herein with respect to ranges, “about” and “approximately” may be used, interchangeably, to refer to a measurement that includes the stated measurement and that also includes any measurements that are reasonably close to the stated measurement, but that may differ by a reasonably small amount such as will be understood, and readily ascertained, by individuals having ordinary skill in the relevant arts to be attributable to measurement error, differences in measurement and/or manufacturing equipment calibration, human error in reading and/or setting measurements, adjustments made to optimize performance and/or structural parameters in view of differences in measurements associated with other components, particular implementation scenarios, imprecise adjustment and/or manipulation of objects by a person or machine, and/or the like.

CLAIMS

What is claimed is:

1. A recombinant rotavirus comprising a gene segment including a first nucleotide sequence encoding rotavirus nonstructural protein 3 (NSP3) and a second nucleotide sequence encoding a coronavirus S protein fragment selected from:
 - an S1 N-terminal domain;
 - an S1 N-terminal domain with signal sequence;
 - an S1 subunit without signal sequence;
 - an S protein receptor binding domain;
 - an extended S protein receptor binding domain;
 - an S2 core domain; and
 - an S2 subunit without a transmembrane anchor domain,wherein the NSP3 and the coronavirus S protein fragment are encoded by a single open reading frame, separated by a self-cleavage protease domain.
2. The recombinant rotavirus of claim 1, wherein the coronavirus is SARS-CoV-2 and:
 - the S1 N-terminal domain is at least 95% identical to SEQ ID NO: 3;
 - the S1 N-terminal domain with signal sequence is at least 95% identical to SEQ ID NO: 4;
 - the S protein receptor binding domain is at least 95% identical to SEQ ID NO: 5;
 - the extended S protein receptor binding domain is at least 95% identical to SEQ ID NO: 9;
 - the S2 core domain is at least 95% to SEQ ID NO: 10; and
 - the S2 subunit without a transmembrane anchor domain is at least 95% identical to SEQ ID N: 11.
3. The recombinant rotavirus of claim 1, further comprising a third nucleotide sequence encoding a second coronavirus S protein fragment selected from:
 - an S1 N-terminal domain;
 - an S1 N-terminal domain with signal sequence;
 - an S1 subunit without signal sequence;

- an S protein receptor binding domain;
- an extended S protein receptor binding domain;
- an S2 core domain; and
- an S2 subunit without a transmembrane anchor domain,

wherein the NSP3 the coronavirus S protein fragment, and the second coronavirus S protein fragment are encoded by a single open reading frame, wherein the NSP3 and the coronavirus S protein fragment are separated by a self-cleavage protease domain and the coronavirus S protein fragment and the second coronavirus fragment are separated by a molecular hinge.

4. The recombinant rotavirus of claim 1, wherein the self-cleavage protease domain is a 2A cleavage element.
5. The recombinant rotavirus of claim 1, wherein the self-cleavage protease domain is a tesco porcine virus 2A (P2A) element.
6. The recombinant rotavirus of claim 5, wherein the P2A element has a sequence having at least 80% sequence identity to SEQ ID NO: 1 (SKFQIDKILISGDIELNPGP).
7. The recombinant rotavirus of claim 1, wherein the nucleotide sequence encoding the coronavirus S protein fragment is derived from a coronavirus selected from: SARS-CoV; MERS-CoV; SARS-CoV-2; HCoV-299E; HCoV-OC43; HCoV-HKU1; and HCoV-NL63.
8. The recombinant rotavirus of claim 3, wherein the third nucleotide sequence encoding the second coronavirus S protein fragment is derived from a same or a different coronavirus species.
9. The recombinant rotavirus of claim 1, wherein the recombinant rotavirus induces an immunological response against rotavirus and coronavirus in a subject when administered to the subject.

10. The recombinant rotavirus of claim 1, wherein the recombinant rotavirus is based upon strain G1P[8].
11. The recombinant rotavirus of claim 1, wherein the recombinant rotavirus is attenuated.
12. An immunogenic composition comprising the recombinant rotavirus of any one of claims 1-11.
13. The immunogenic composition of claim 12, further comprising a pharmaceutically acceptable excipient.
14. The immunogenic composition of claim 13, wherein the immunogenic composition is formulated for oral, subcutaneous, or intramuscular administration.
15. A method for inducing a protective immune response against rotavirus and a coronavirus in a subject, the method comprising administering an effective amount of the immunogenic composition of claim 12 to the subject.
16. The method of claim 15, wherein the immunogenic composition comprises a pharmaceutically acceptable excipient and is formulated for oral, subcutaneous, or intramuscular administration.
17. A recombinant rotavirus expression system comprising:
 - a nonstructural protein 3 (NSP3) expression vector including a nucleotide sequence encoding rotavirus (NSP3) and a nucleotide sequence encoding a coronavirus S protein fragment selected from:
 - an S1 N-terminal domain;
 - an S1 N-terminal domain with signal sequence;
 - an S1 subunit without signal sequence;
 - an S protein receptor binding domain;
 - an extended S protein receptor binding domain;

an S2 core domain; and
an S2 subunit without a transmembrane anchor domain,
wherein the NSP3 and the coronavirus S protein fragment are encoded by a single
open reading frame, separated by a self-cleavage protease domain;

a VP1 expression vector;
a VP2 expression vector;
a VP3 expression vector;
a VP4 expression vector;
a VP5 expression vector;
a VP6 expression vector;
a VP7 expression vector;
a NSP1 expression vector;
a NSP2 expression vector;
a NSP4 expression vector;
a NSP5/6 expression vector; and
an African Swine Fever Virus NP868R RNA capping enzyme expression vector.

18. The recombinant rotavirus expression system of claim 17, wherein the coronavirus is SARS-CoV-2 and:

the S1 N-terminal domain is at least 95% identical to SEQ ID NO: 3;
the S1 N-terminal domain with signal sequence is at least 95% identical to SEQ ID NO: 4;
the S protein receptor binding domain is at least 95% identical to SEQ ID NO: 5;
the extended S protein receptor binding domain is at least 95% identical to SEQ ID NO: 9;
the S2 core domain is at least 95% to SEQ ID NO: 10; and
the S2 subunit without a transmembrane anchor domain is at least 95% identical to SEQ ID N: 11.

19. The recombinant rotavirus expression system of claim 17, further comprising a third nucleotide sequence encoding a second coronavirus S protein fragment selected from:

- an S1 N-terminal domain;
- an S1 N-terminal domain with signal sequence;
- an S1 subunit without signal sequence;
- an S protein receptor binding domain;
- an extended S protein receptor binding domain;
- an S2 core domain; and
- an S2 subunit without a transmembrane anchor domain,

wherein the NSP3 the coronavirus S protein fragment, and the second coronavirus S protein fragment are encoded by a single open reading frame, wherein the NSP3 and the coronavirus S protein fragment are separated by a self-cleavage protease domain and the coronavirus S protein fragment and the second coronavirus fragment are separated by a molecular hinge.

20. The recombinant rotavirus expression system of claim 17, wherein each of the NSP3 expression vector, VP1 expression vector, VP2 expression vector, VP3 expression vector, VP4 expression vector, VP5 expression vector, VP6 expression vector, VP7 expression vector, NSP1 expression vector, NSP2 expression vector, NSP4 expression vector, and NSP5/6 expression vector are T7 expression vectors.
21. The recombinant rotavirus expression system of claim 17, wherein the self-cleavage protease domain is a 2A cleavage element.
22. The recombinant rotavirus expression system of claim 17, wherein the self-cleavage protease domain is a tesco porcine virus 2A (P2A) element.
23. The recombinant rotavirus expression system of claim 21, wherein the 2A element has a sequence having at least 80% sequence identity to SEQ ID NO: 1 (SKFQIDKILISGDIELNPGP).
24. The recombinant rotavirus expression system of claim 17, wherein the nucleotide sequence encoding the coronavirus S protein fragment is derived from a coronavirus selected from:

SARS-CoV; MERS-CoV; SARS-CoV-2; HCoV-229E; HCoV-OC43; HCoV-HKU1; and HCoV-NL63.

25. The recombinant rotavirus expression system of claim 17, wherein the third nucleotide sequence encoding the second coronavirus S protein fragment is derived from a same or a different coronavirus species.
26. The recombinant rotavirus expression system of claim 23, wherein the recombinant rotavirus expression system is based upon rotavirus strain G1P[8].
27. A method for producing a recombinant rotavirus, the method comprising:
 - transfecting BHK-T7 cells with the recombinant rotavirus expression system of any one of claims 17-26;
 - overseeding the transfected BHK-T7 cells with MA104 cells;
 - preparing a clarified cell lysate; and
 - isolating recombinant rotavirus.
28. The method of claim 27, wherein recombinant rotavirus is isolated by plaque purification.
29. The method of claim 27, wherein the recombinant rotavirus is attenuated.

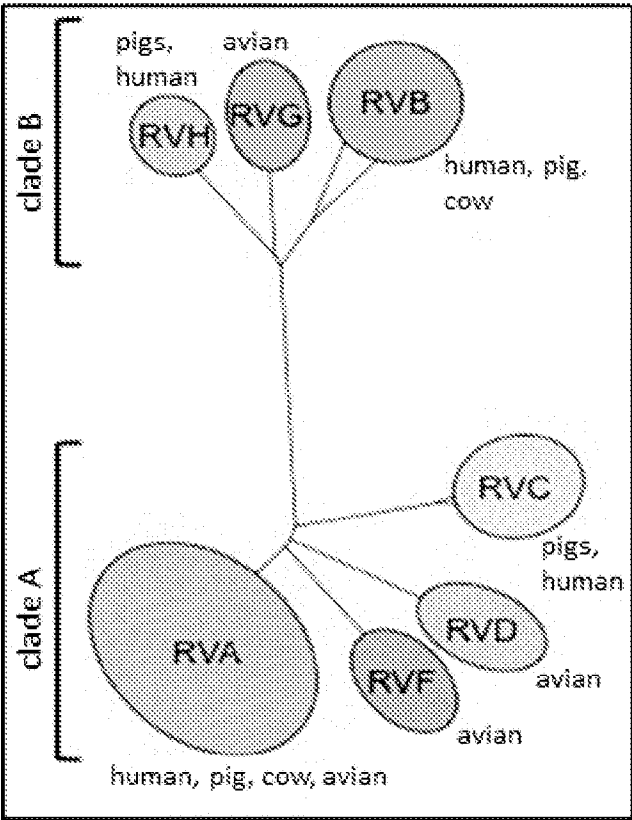


FIG. 1

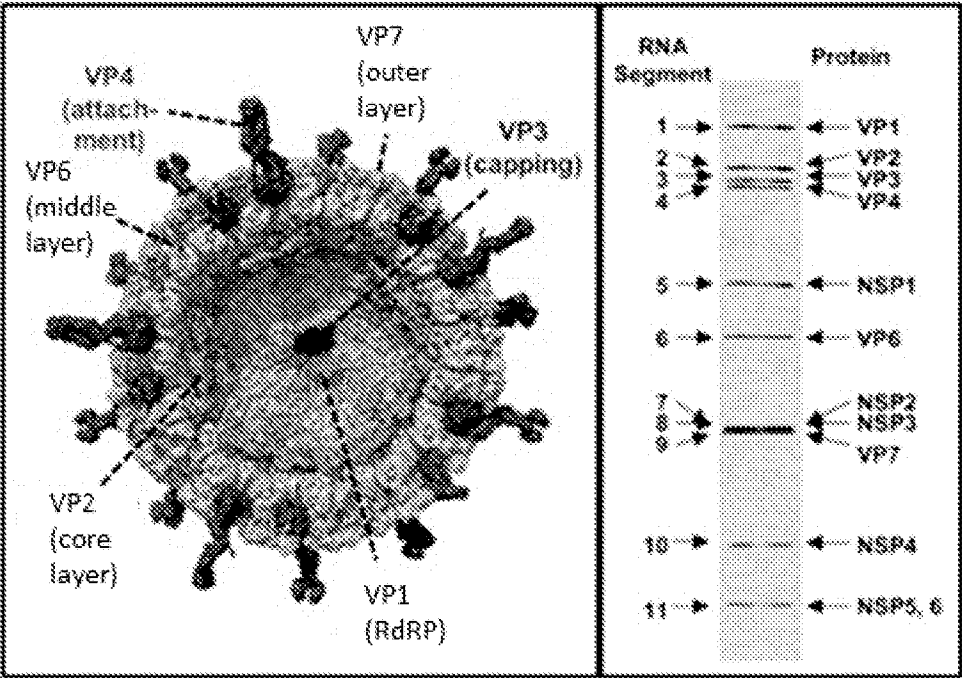


FIG. 2

2/17

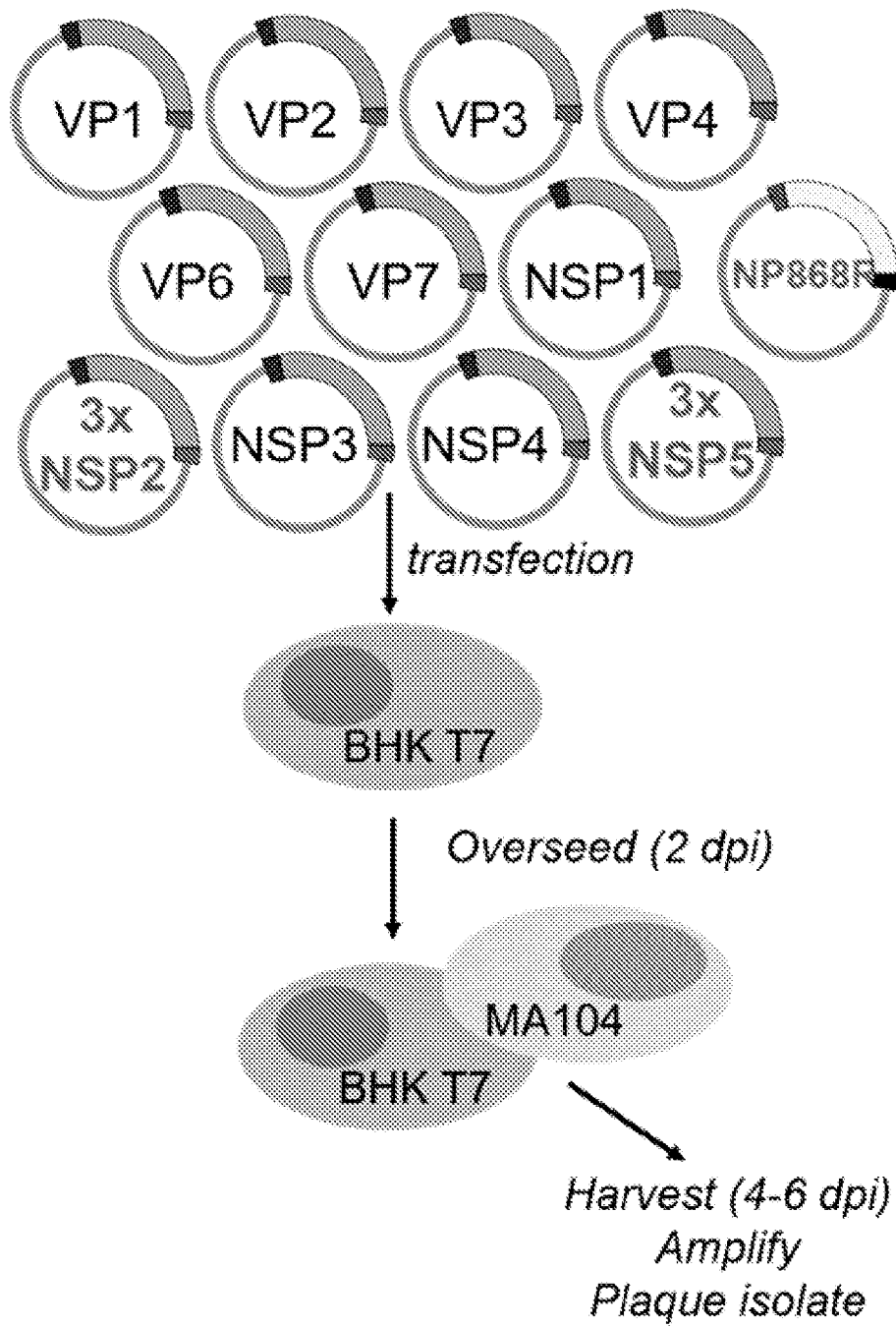


FIG. 3

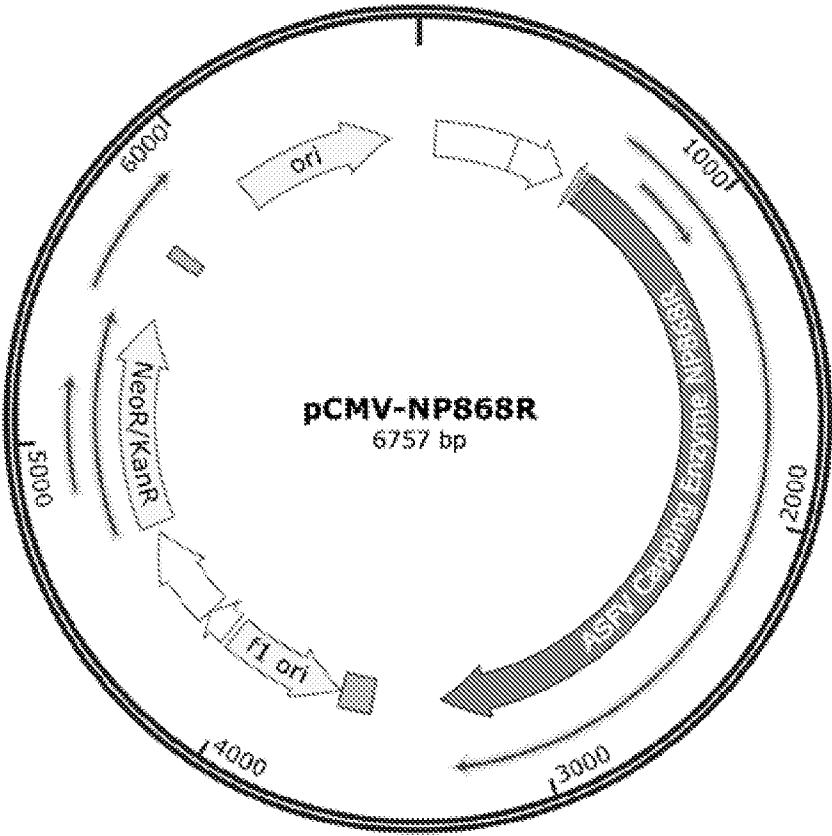


FIG. 4A

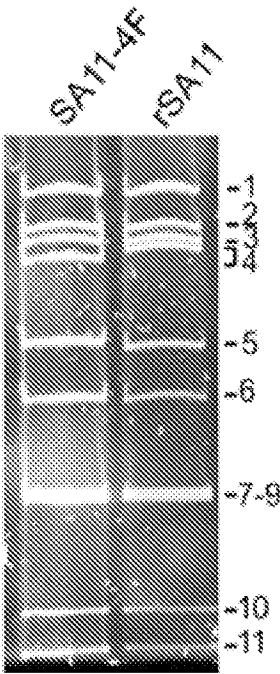


FIG. 4B

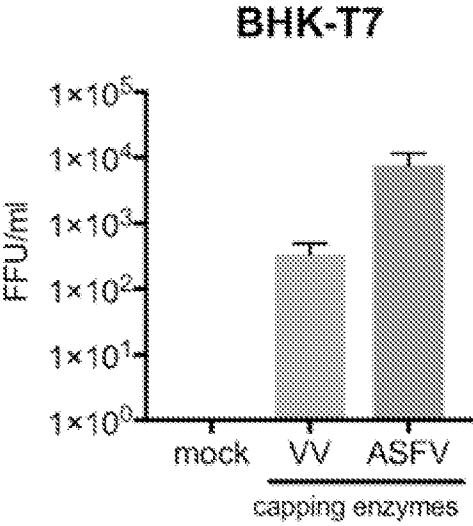


FIG. 4C

4/17

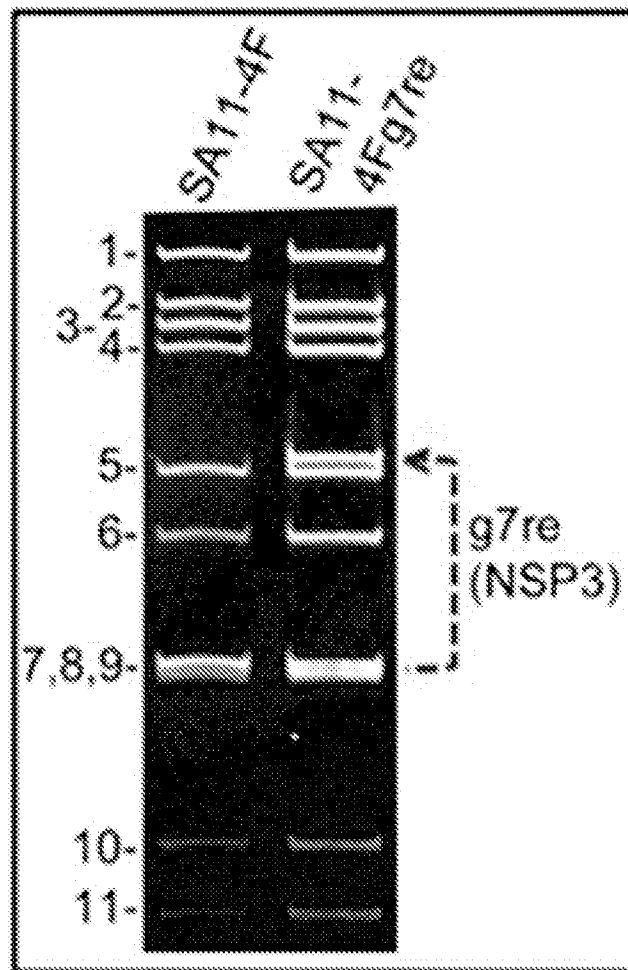


FIG. 5

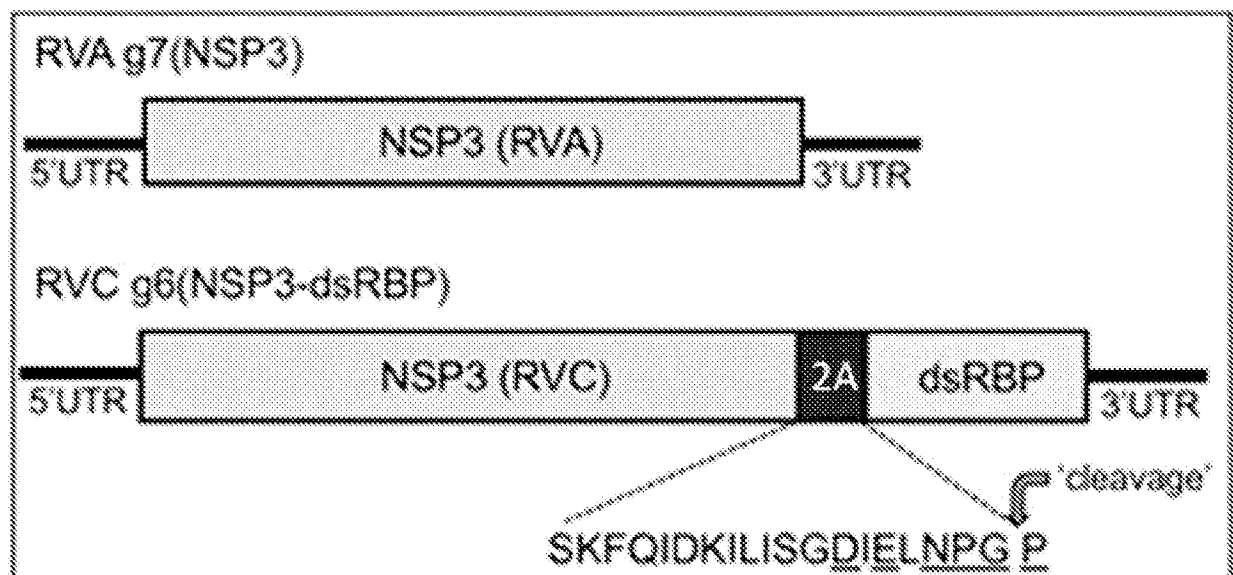


FIG. 6

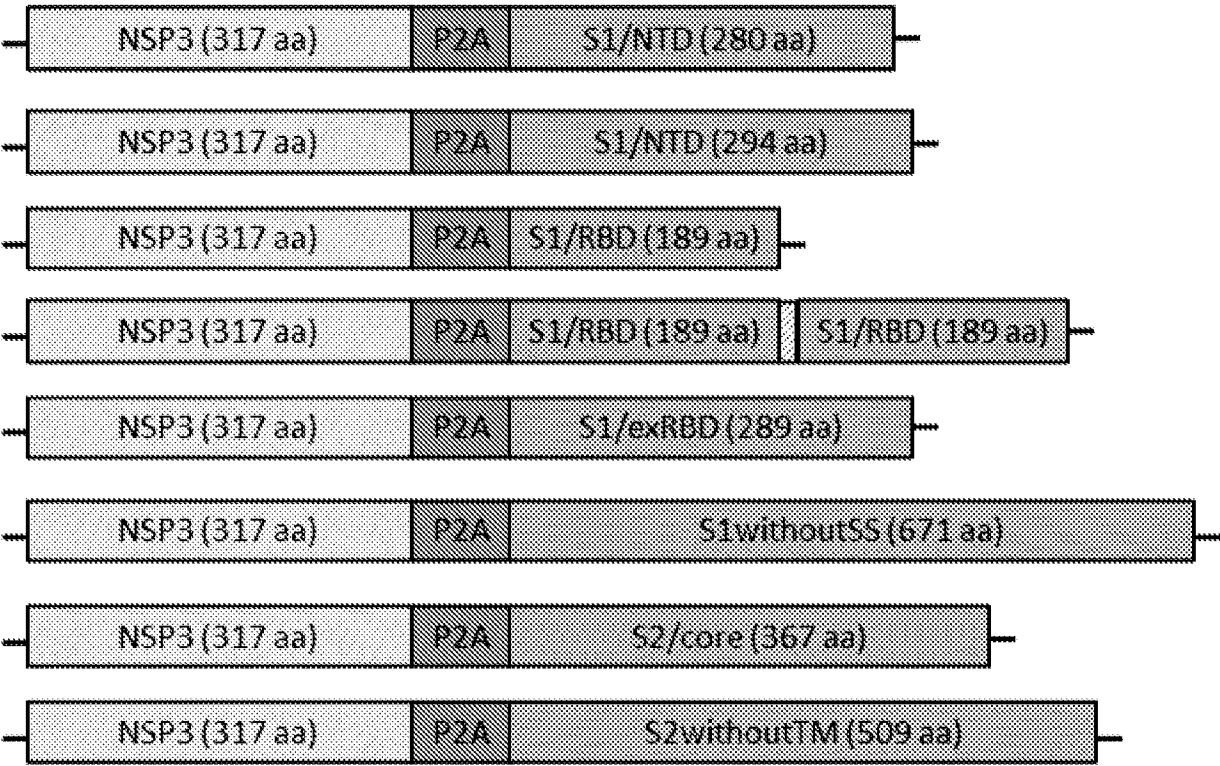


FIG. 7

6/17

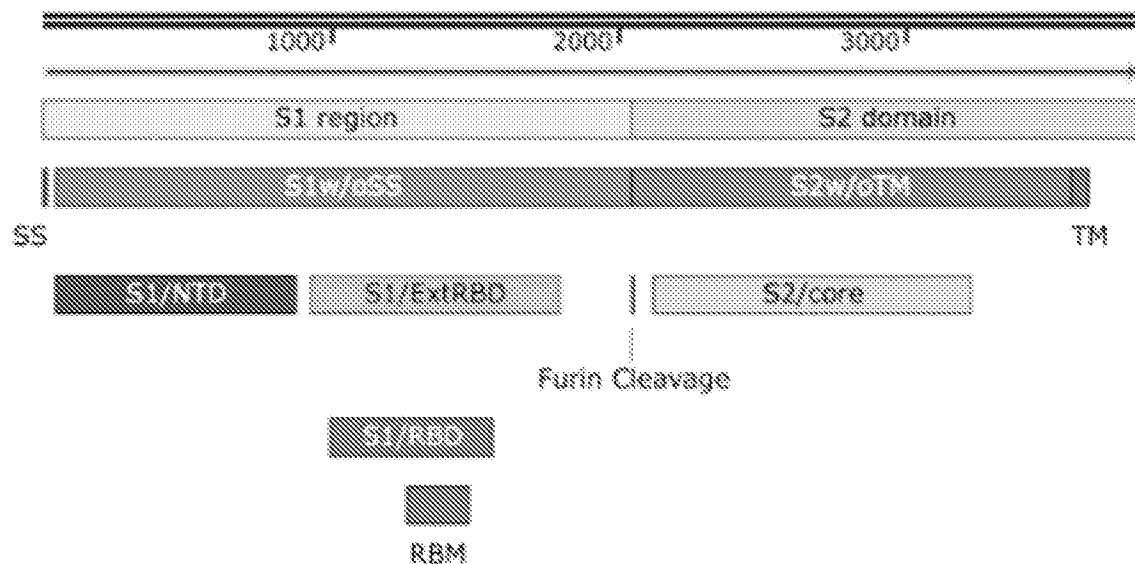


FIG. 8A

Recombinant rotavirus*	pT7 transcription plasmid**	COVID-19 protein***	Length (aa)	MW (kDa)	Position (aa)	Length (nt)	Position (nt)
rRV-NSP3-2A-CoV19/RBD	NSP3-2A-CoV19/RBD	S1/RBD	169	21.3	335-523	567	1003-1566
rRV-NSP3-2A-CoV19/ExRBD	NSP3-2A-CoV19/ExRBD	S1/ExRBD	289	32.2	311-599	867	931-1797
rRV-NSP3-2A-CoV19/2xRBD	NSP3-2A-CoV19/2xRBD	S1/2xRBD	364	42.9	335-523	567	1003-1566
rRV-NSP3-2A-CoV19/NTD	NSP3-2A-CoV19/NTD	S1/NTD	289	31.6	15-294	840	43-882
rRV-NSP3-2A-CoV19/NTD+SS	NSP3-2A-CoV19/NTD+SS	S1/NTD+SS	294	33.4	1-294	882	1-882
rRV-NSP3-2A-CoV19/core	NSP3-2A-CoV19/S2/core	S2/core	367	39.9	711-1077	1101	2131-3231
rRV-NSP3-2A-CoV19/S2w/TM	NSP3-2A-CoV19/S2w/oTM	S2w/oTM	509	55.6	586-1194	1527	2986-3592

*Can be any strain of rotavirus, including SA11 or Wa or reassortant viruses. (RBD, receptor binding domain; ExRBD, extended receptor binding domain; 2xRBD, protein containing two copies of RBD separated by molecular hinge (GGG-GGG) or any other hinge; NTD, N-terminal domain; SS, membrane signal sequence; TM, transmembrane anchor domain).

**Can be any type of plasmid containing a T7 RNA polymerase promoter that directs transcription of rotavirus cDNAs, including modified segment 7 cDNAs that express NSP2-2A fused to CoV19 spike (S) protein domains.

***Examples of domains of the COVID-19 (CoV19, SARS-CoV2) spike (S) protein to be expressed by strains of recombinant rotavirus generated by reverse genetics.

FIG. 8B

7/17

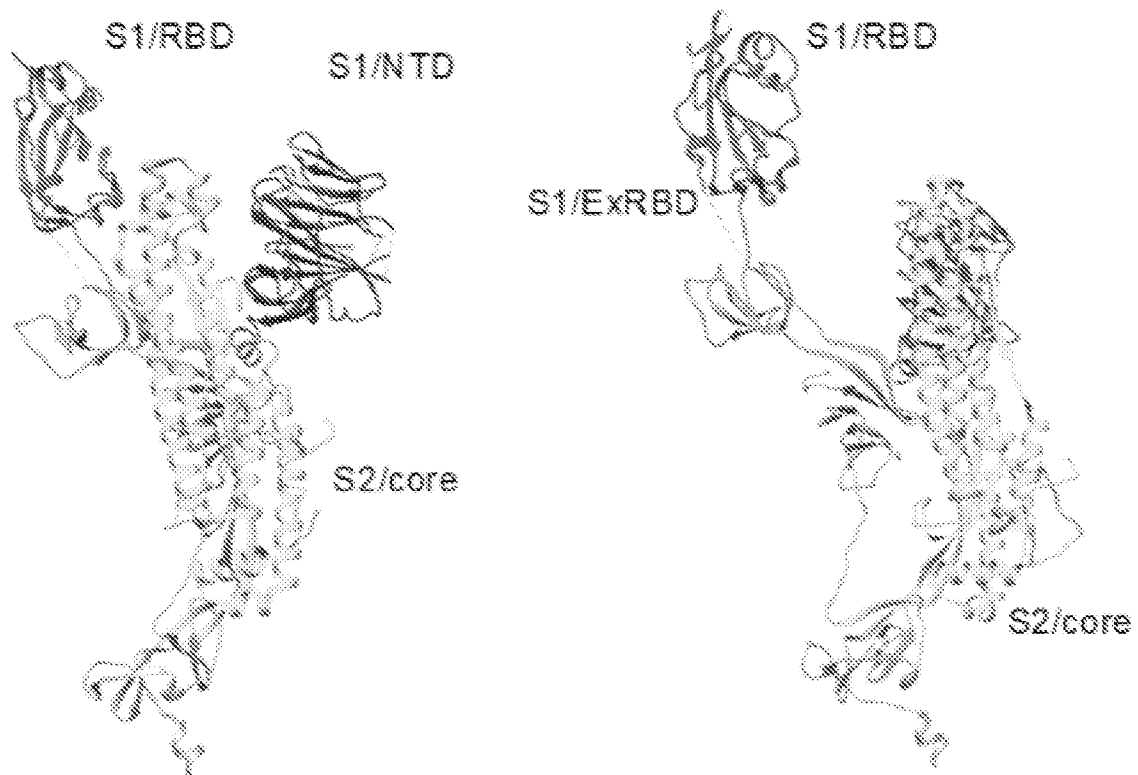


FIG. 8C

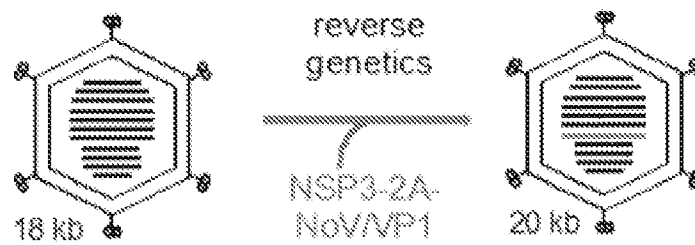


FIG. 9A

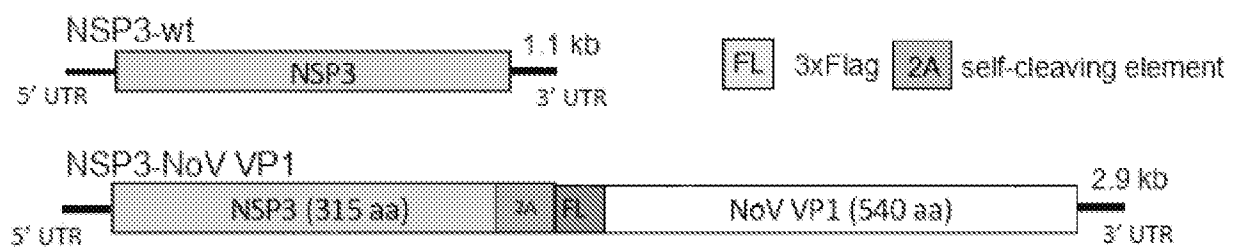


FIG. 9B

8/17

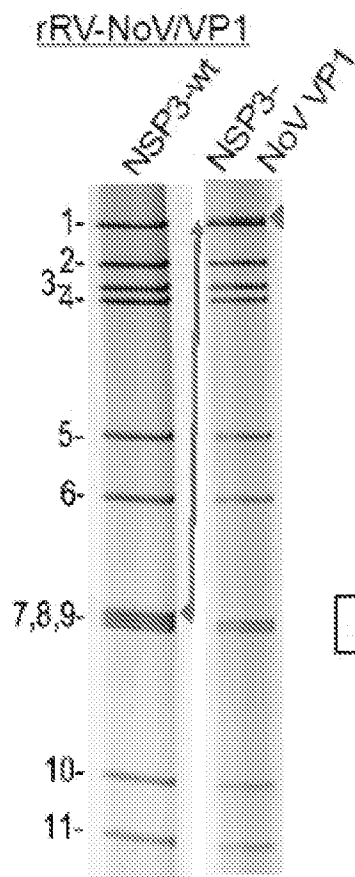


FIG. 9C

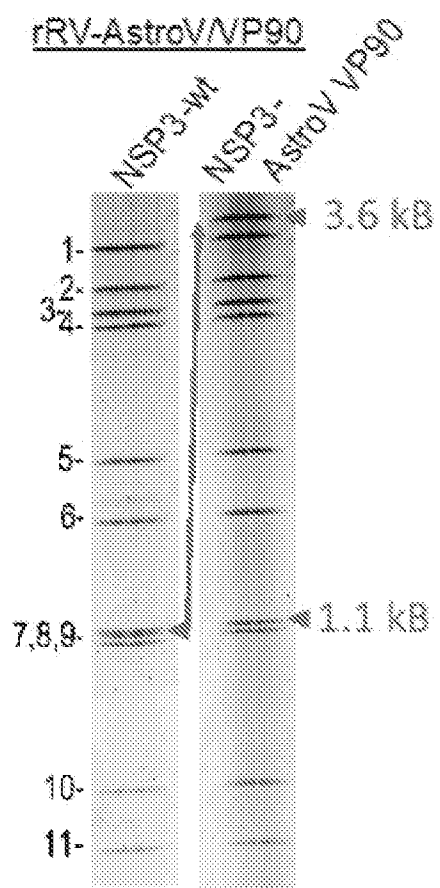


FIG. 9E

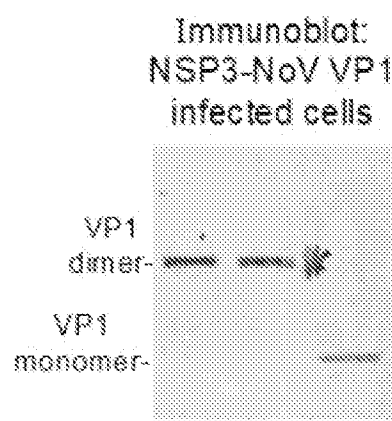


FIG. 9D

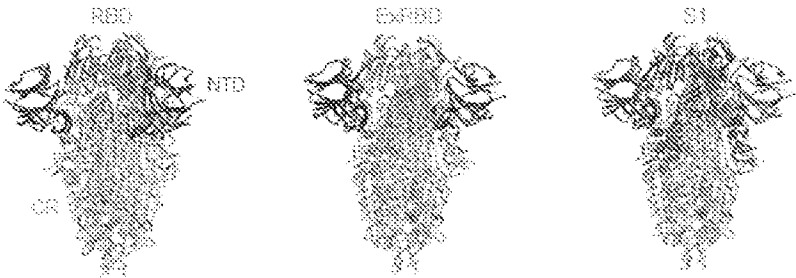
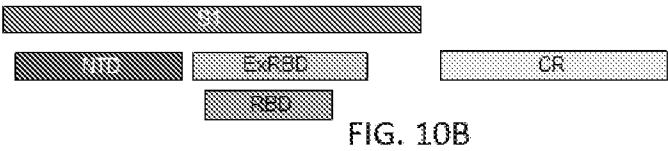
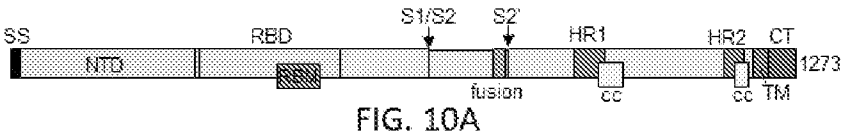


FIG. 10C

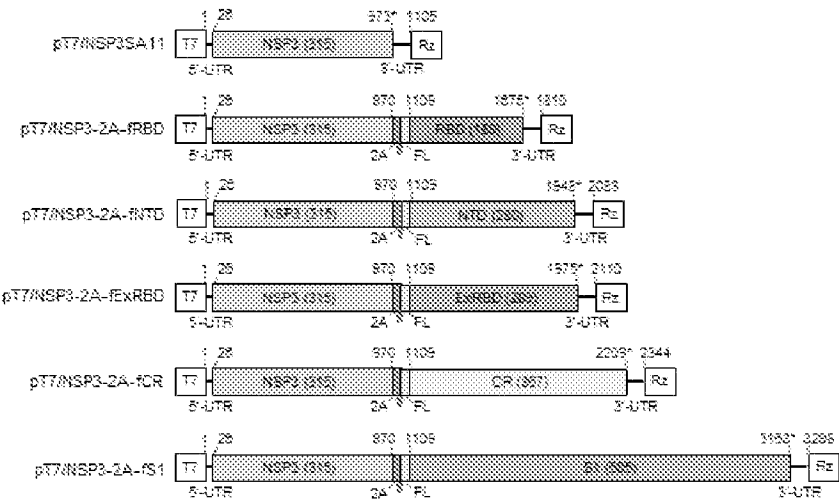
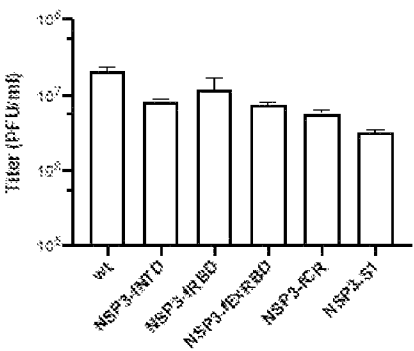
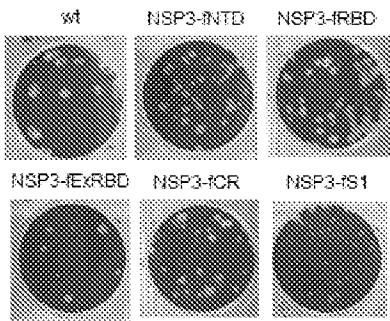
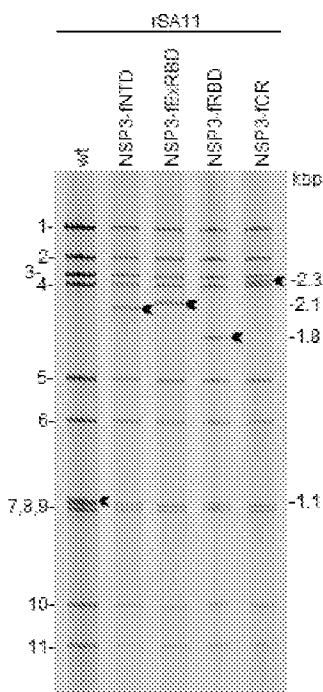
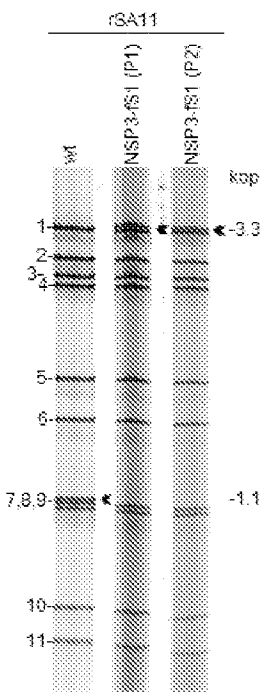


FIG. 11



12/17

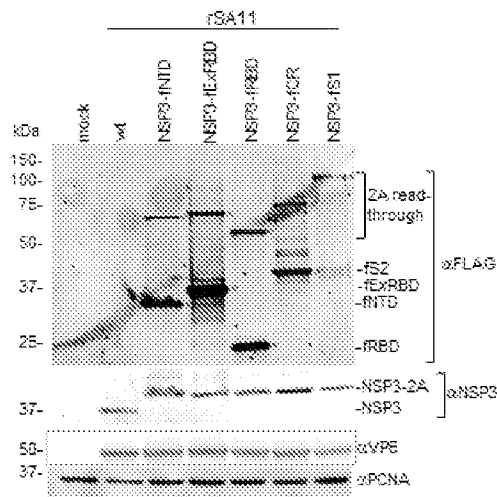


FIG. 13A

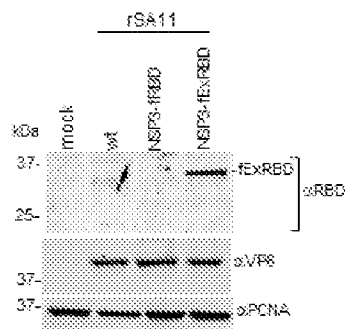


FIG. 13B

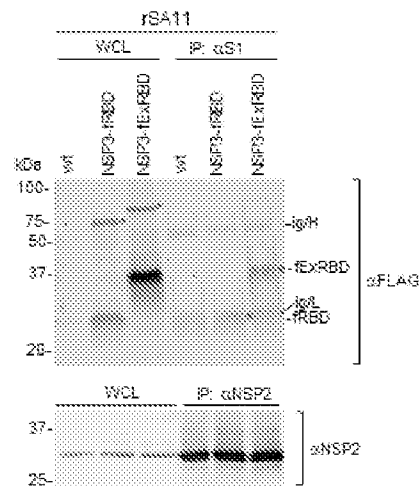


FIG. 13C

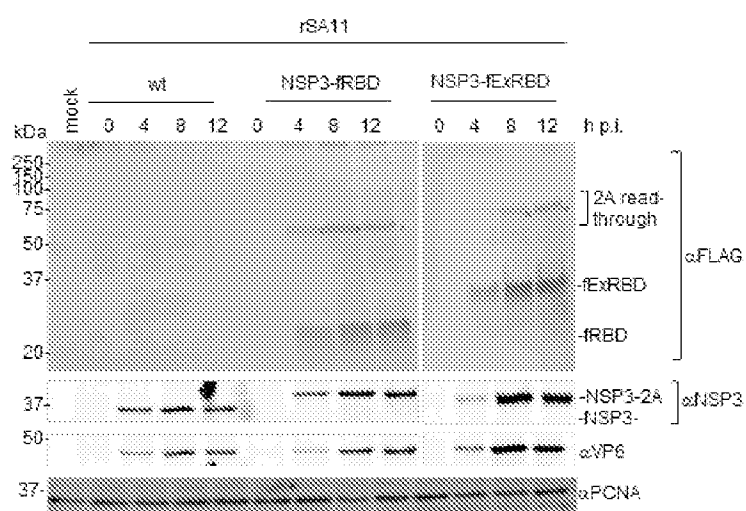
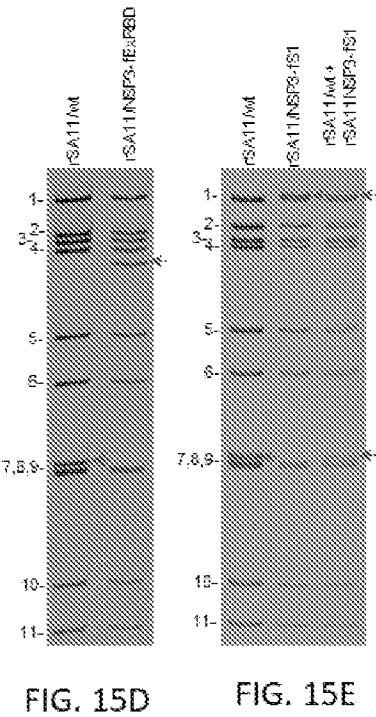
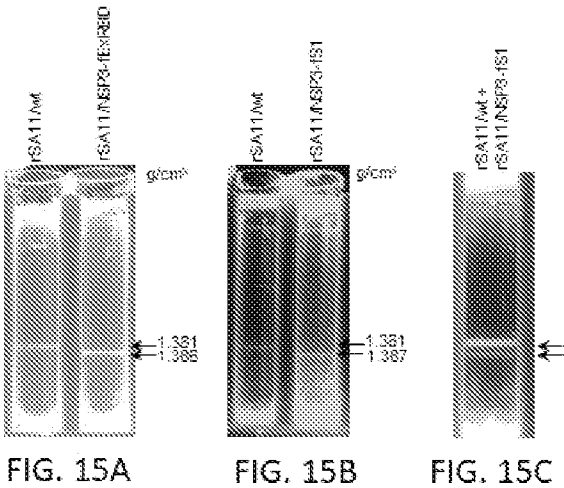


FIG. 14



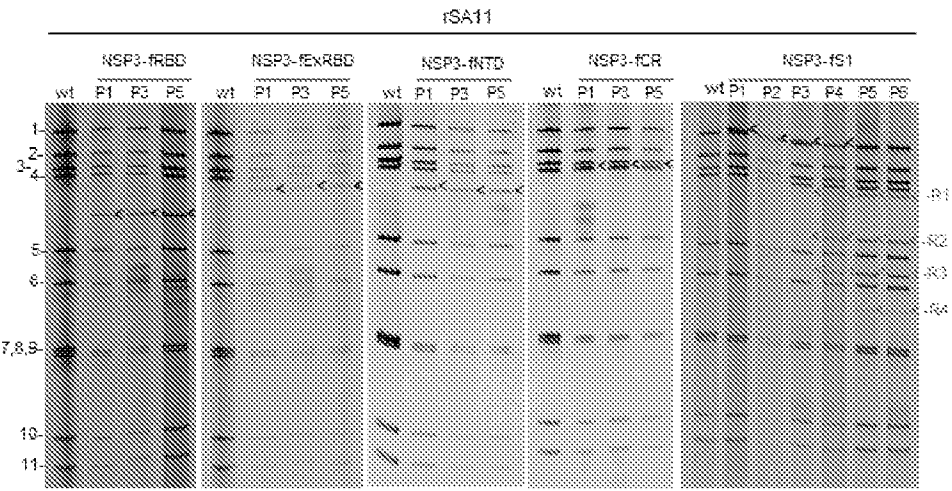


FIG. 16A

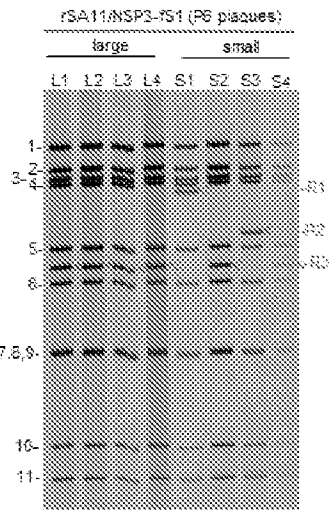


FIG. 16B

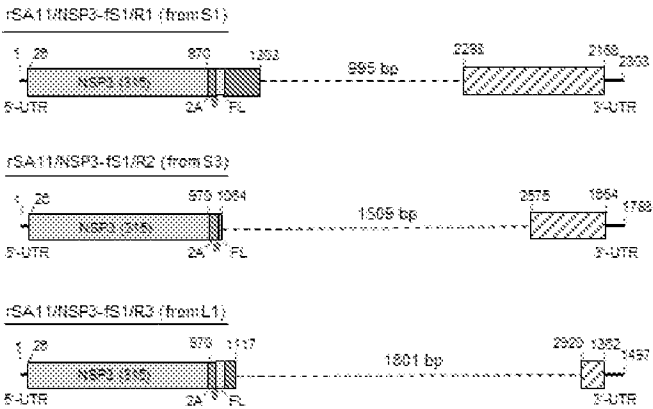


FIG. 16C

16/17

Properties of recombinant rRV/NSP3-2A-CoV2 strains.								
Virus strain			Genome segment 7					
			RNA (bp)	Protein product				NCBI accession #
Abbreviated name	Formal name*	Genome size/ increase over wt (bp)		uncleaved (aa)	2A cleaved (aa)	uncleaved (kDa)	2A cleaved (kDa)	
rSA11/wt	RVA/Simian-lab/USA/SA11wt/2019/G3P[2]	18,559/0	1105	315	nd	36.4	nd	LC178572
rSA11/NSP3-INTD	RVA/Simian-lab/USA/SA11(NSP3-P2A-CoV2:INTD)/2020/G3P[2]	19,537/976	2083	641	336 + 305	73.2	38.5 + 34.8	MW059024
rSA11/NSP3-fRBD	RVA/Simian-lab/USA/SA11(NSP3-P2A-CoV2:fRBD)/2020/G3P[2]	19,264/705	1810	550	336 + 214	62.7	38.5 + 24.3	MT655947
rSA11/NSP3-fExRBD	RVA/Simian-lab/USA/SA11(NSP3-P2A-CoV2:fExRBD)/2020/G3P[2]	19,564/1005	2110	650	336 + 314	74.7	38.5 + 35.2	MT655946
rSA11/NSP3-fCR	RVA/Simian-lab/USA/SA11(NSP3-P2A-CoV2:fCR)/2020/G3P[2]	19,798/1239	2344	728	336 + 392	81.4	38.5 + 42.9	MW059025
rSA11/NSP3-fS1	RVA/Simian-lab/USA/SA11(NSP3-P2A-CoV2:fS1)/2020/G3P[2]	20,752/2193	3298	1046	336 + 710	118.1	38.5 + 79.6	MW059026
rSA11/NSP3-fS1/R1	RVA/Simian-lab/USA/SA11(NSP3-P2A-CoV2:fS1/R1)/2020/G3P[2]	19,757/1198	2303	431	336 + 95	49.6	38.5 + 11.1	MW353715
rSA11/NSP3-fS1/R2	RVA/Simian-lab/USA/SA11(NSP3-P2A-CoV2:fS1/R2)/2020/G3P[2]	19,333/683	1788	367	336 + 31	42.1	38.5 + 3.7	MW353716
rSA11/NSP3-fS1/R3	RVA/Simian-lab/USA/SA11(NSP3-P2A-CoV2:fS1/R3)/2020/G3P[2]	18,951/392	1497	410	336 + 74	47.2	38.5 + 8.8	MW353717

* Formal strain names were assigned according to Matthijnssens et al (44). nd: not determined, no 2A cleavage site present; wt: wild type

FIG. 17

17/17

Primers used to produce pT7/NSP3-2A-CoV2 plasmids.	
Primer	Sequence
Vector_For	TGACCATTTTGATACATGTTGAACAATCAAATACAG
Vector_Rev	GCTAGCCTTGTCATCGTCATCCT
NTD_For	GATGACAAGGCTAGCTGTGTTAATCTTACAACCAGAACTCAATTACCCC
NTD_Rev	GTATCAAAATGGTCAGTCAAGTGCACAGTCTACAGCATC
ExRBD_For	GATGACAAGGCTAGCGGAATCTATCAAACCTCTAACTTTAGAGTCCAACCA
ExRBD_Rev	GTATCAAAATGGTCATGTTATAACACTGACACCACCAAAAAGAACA
RBD_For	GATGACAAGGCTAGCTTGTGCCCTTTTGGTGAAGTTT
RBD_Rev	GTATCAAAATGGTCAAGTTGCTGGTGCATGTAGAAGT
CR_For	GATGACAAGGCTAGCTCTATTGCCATACCCACAAATTTTACTATTAGTGT
CR_Rev	GTATCAAAATGGTCAAGTTGTGAAGTTCTTTTCTTGTGCAGG
S1_For	GATGACAAGGCTAGCGTGTGTTGTTTTCTTGTTTTATTGCCACTAGTCT
S1_Rev	GTATCAAAATGGTCAACGTGCCCGCCG

FIG. 18

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US2021/028195

A. CLASSIFICATION OF SUBJECT MATTER

IPC(8) - A61K 39/215; A61P 31/14; C07K 14/14 (2021.01)

CPC - A61K 39/215; C07K 14/14; C12N 2720/12341; C12N 2720/12343; C12N 2770/20034 (2021.05)

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)

see Search History document

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

see Search History document

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

see Search History document

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	WO 2020/014654 A1 (THE TRUSTEES OF INDIANA UNIVERSITY et al) 16 January 2020 (16.01.2020) entire document	1-29
Y	US 2006/0257852 A1 (RAPPUOLI et al) 16 November 2006 (16.11.2006) entire document	1-29
Y	AHMED et al., Preliminary Identification of Potential Vaccine Targets for the COVID-19 Coronavirus (SARS-CoV-2) Based on SARS-CoV Immunological Studies, Viruses, 25 February 2020 (25.02.2020), Vol. 12, No. 3: 254, Pgs. 1-15. entire document	2, 18
E	PHILIP et al., Rotavirus as an Expression Platform of Domains of the SARS-CoV-2 Spike Protein, Vaccines, 03 May 2021 (03.05.2021), Vol. 9(5), 449, Pgs. 1-19. entire document	1-29
P, X	US 2020/0407402 A1 (THE SCRIPPS RESEARCH INSTITUTE) 31 December 2020 (31.12.2020) entire document	1-29

☐ Further documents are listed in the continuation of Box C.☐ See patent family annex.

* Special categories of cited documents:

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

"D" document cited by the applicant in the international application

"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

29 July 2021

Date of mailing of the international search report

SEP 08 2021

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INTERNATIONAL SEARCH REPORT

International application No.

PCT/US2021/028195

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 13ter.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 13ter.1(a)).

☐ on paper or in the form of an image file (Rule 13ter.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:

SEQ ID NOs: 1, 3-5, and 9-11 were searched.