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(54) Title: ANDES VIRUS VARIANTS, PHARMACEUTICAL COMPOSITIONS, AND METHODS OF TREATMENT RE-
LATING THERETO

(57) Abstract: The present disclosure relates to Andes virus (ANDV) variants comprising one or more attenuating amino acid modifi-
cations, pharmaceutical compositions and vaccines comprising Andes virus variants, and methods of treating diseases and conditions
via administration of Andes virus variants.

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ANDES VIRUS VARIANTS, PHARMACEUTICAL COMPOSITIONS, AND METHODS OF TREATMENT RELATING THERETO

Priority

[0001] This application claims priority under 35 U.S.C. § 119(e) to U.S. Provisional Application No. 63/507,569, filed June 12, 2023, the contents of which are incorporated herein by reference in their entirety.

Government Support

[0002] This invention was made with government support under Contract No. W81XWH-17-0222 awarded by the National Institute of Health. The government has certain rights in the invention.

Field

[0003] The present disclosure relates to Andes virus variants comprising one or more attenuating amino acid modifications, pharmaceutical compositions and vaccines comprising Andes virus variants, and methods of treating diseases and conditions via administration of Andes virus variants.

Background

[0004] Andes virus (ANDV) belongs to the family *Hantaviridae*, of the order *Bunyavirales* (Walker PJ et al. *Changes to virus taxonomy and the International Code of Virus Classification and Nomenclature ratified by the International Committee on Taxonomy of Viruses*. Arch Virol. 2019;164(9):2417-29). ANDV is an etiological agent of hantavirus pulmonary syndrome (HPS) or hantavirus cardiopulmonary syndrome (HCPS) that is characterized by respiratory distress, pulmonary edema, and cardiogenic shock (Jonsson CB et al. *A global perspective on hantavirus ecology, epidemiology, and disease*. Clin Microbiol Rev. 2010;23(2):412-41). Found predominantly in South America, ANDV is the only known hantavirus with documented person-to-person transmission (Martinez-Valdebenito C et al.

Person-to-person household and nosocomial transmission of Andes hantavirus, Southern Chile, 2011. Emerg Infect Dis. 2014;20(10):1629-36). Of the hantavirus animal models, Syrian hamsters challenged with ANDV develop a lethal disease recapitulating many of the features of human HPS, namely dyspnea, pulmonary edema, and fluid in the pleural cavity (Hooper JW et al. *A lethal disease model for hantavirus pulmonary syndrome.* Virology. 2001;289(1):6-14).

[0005] Hantaviruses are enveloped, negative-sense, single-stranded RNA viruses with tripartite genome. The three segments are denoted as small (S), medium (M), and large (L) encoding for the nucleoprotein (N), glycoproteins (Gn and Gc), and the RNA-dependent RNA polymerase, respectively (Elliot RM & Schmaljohn CS. Bunyaviridae. In: Knipe D, Howley P, editors. *Fields Virology*. 1. Sixth Edition ed. Philadelphia, PA: Lippincott, Williams & Wilkins; 2013. p. 1244). Hantaviruses infect microvascular endothelial cells altering the barrier properties of these cells resulting in a vascular leakage disease in the target organ, the lung in the case of ANDV infection (Gavrilovskaya I et al. *The Role of the Endothelium in HPS Pathogenesis and Potential Therapeutic Approaches.* Adv Virol. 2012;467059. PubMed PMID: 22811711; PubMed Central PMCID: PMC3395186). Efforts to map the pathogenicity of ANDV to a specific genetic segment were unsuccessful as reassortants generated with ANDV and Sin Nombre virus (SNV, an HPS-causing hantavirus found in North America), demonstrated that all three ANDV segments were necessary to induce lethality in hamsters (Brocato RL et al. *Innate immune responses elicited by Sin Nombre virus or type I IFN agonists protect hamsters from lethal Andes virus infections.* J Gen Virol. 2018. doi: 10.1099/jgv.0.001131; McElroy AK et al. *Andes virus M genome segment is not sufficient to confer the virulence associated with Andes virus in Syrian hamsters.* Virology. 2004;326(1):130-9). A reverse genetics system for hantaviruses does not currently exist.

Summary

[0006] There is currently no reverse genetics system for hantaviruses. Herein we use an approach to the development of ANDV variants through passaging in cell culture. Mutations were determined from sequenced, plaque-picked (pp) stocks and the influence of

these mutations on lethality in Syrian hamsters was observed. The attenuated ANDV variants were protective against a subsequent wild type ANDV exposure. In the absence of a reverse genetics system for hantaviruses, these results shed light on ANDV pathogenesis and highlight specific mutations required for attenuation towards a nonpathogenic phenotype.

[0007] Further, the attenuated ANDV may be utilized to form the bases of an attenuated virus hantavirus vaccine. Such a vaccine may be further attenuated by producing reassortants with Sin Nombre virus, which would not require reverse genetics. Via the discovery of a reverse genetics system, the E265K mutation may be incorporated along with other attenuating mutations.

Brief Description of the Drawings

[0008] **FIGS. 1A-E** relate to serial passaging of ANDV in Vero E6 cells leading to an accumulation of mutations and attenuation in hamsters; ANDV p2 was passaged 18 times in Vero E6 cells. **FIG. 1A** shows images of morphology changes between ANDV p2 and ANDV p20 by immunostaining. **FIG. 1B** is a table showing ANDV p2 and ANDV p20 variants were sequenced and percentages of variants with detected mutations are shown. **FIG 1C** is a graph relating to hamsters that were exposed to either 1,000 PFU of ANDV p2 or ANDV p20 by the intramuscular route; the lone hamster fatality from the ANDV p20 group is shown as a lone square at day 14 post-infection. ****, $P < 0.0001$ by log-rank test. **FIG 1D** is a graph showing RT-PCR of ANDV S-segment in hamster lungs. The lone square at 14 days and the upper three squares at 28 days for ANDV p20 indicate lung samples collected for viral sequencing. ***, $P < 0.001$ by unpaired t- test. **FIG 1E** is a table wherein, from select ANDV p20 hamster lungs, the percentage of variants with detected mutations are shown.

[0009] **FIG. 2** is a table relating to plaque-picked virus from ANDV p20 showing variable lethality; wild type ANDV p2 was used as control virus with lethal phenotype; ANDV p20 showed intermediate lethality; three plaque picks from the p20 stock, pp1, pp15, and pp17 were also tested; P20pp15 and p20pp17 were both completely attenuated whereas p20pp1 was lethal in all but one animal. N=8 hamsters for each group.

[0010] **FIGS. 3A-J** relate to pathogenic ANDV (p2 and p20pp1) replicate to higher titers in Syrian hamsters compared to nonpathogenic ANDV (p20pp15 and p20pp17); hamsters were exposed to 1,000 PFU of the indicated ANDV variant by the intramuscular route. **FIG. 3A** is a table relating to whole blood collected from 3 hamsters/group every other day being analyzed for S-segment viral RNA by RT-PCR; the numbers above the 12 dpi indicate the number of hamsters remaining from each group. **FIG. 3B** is a graph showing 8 dpi (open symbols) and 10 dpi (closed symbols) were combined for the pathogenic ANDV (p2 and p20pp1) and nonpathogenic ANDV (p20pp15 and p20pp17). ****, $P < 0.0001$, unpaired t- test. H&E staining of FFPE lung tissue from hamsters exposed to ANDV p2 (**FIG. 3C**), ANDV p20pp1 (**FIG. 3D**), ANDV p20pp15 (**FIG. 3E**), and ANDV p20pp17 (**FIG. 3F**); asterisks indicate mild to moderate perivascular edema and hemorrhage. Arrows indicate inflammatory cells extending into adjacent alveolar lumen and expanding alveolar septa; ANDV genomic RNA (darker staining,) was detected by *in situ* hybridization (ISH) in hamsters exposed to ANDV p2 (**FIG. 3G**); ANDV p20pp1 (**FIG. 3H**), ANDV p20pp15 (**FIG. 3I**), and ANDV p20pp17 (**FIG. 3J**). Scale bars: (A, D) 50 μm , (B, C, G) 100 μm , and (H-J) 200 μm .

[0011] **FIGS. 4A-E** relate to attenuated ANDV (p20pp15 and p20pp17) protecting against lethal ANDV challenge; hamsters were exposed to 1,000 PFU of ANDV p20pp15 or p20pp17 on either Day -14, -3, or 0; and then on Day 0 they were challenged with wild type ANDV p2. Panels A and B show the antibody responses on Day 0. **FIG. 4A** is a graph showing N-ELISA reporting anti-N binding antibody. **FIG. 4B** is a graph showing, ANDV PsVNA data reporting neutralizing antibody activity. **FIG. 4C** is a graph showing Survival data. Approximately one month after challenge, sera was collected from survivors and the antibody responses were evaluated, with **FIG. 4D** being a graph showing N-ELISA. **FIG. 4E** is a graph showing ANDV and PsVNA.

Detailed Description of the Embodiments

[0012] In a first aspect of the disclosure, an Andes virus (ANDV) variant may comprise an amino acid sequence that is about 90% to about 99% identical to the sequence of the wild type ANDV. In some embodiments, the ANDV variant may comprise an amino acid sequence

that is about 50%, 60%, 70%, 80%, 90%, 95%, 96%, 97%, 98%, or 99% identical to the sequence of the wild type ANDV.

[0013] In some embodiments, the ANDV variant may comprise one or more attenuating amino acid modification(s). In some embodiments, the amino acid modification(s) may comprise a substitution at position 265. In an exemplary embodiment, the amino acid modification may comprise E265K.

[0014] In certain embodiments, a pharmaceutical composition comprising the above ANDV variants or viral particles thereof are envisioned. In some embodiments, the pharmaceutical composition may be for use in the treatment and/or prevention of an infection or disease in a subject in need thereof. In some embodiments, the infection or disease may be characterized by respiratory distress, pulmonary edema, and/or cardiogenic shock. In some embodiments, the infection or disease may be hantavirus pulmonary syndrome (HPS) or hantavirus cardiopulmonary syndrome (HCPS). In some embodiments, a vaccine comprising the above ANDV variants or viral particles, or above pharmaceutical composition, is envisioned. In some embodiments, a method of treating a disease or condition in need thereof is envisioned, wherein the method may comprise administering a therapeutically effective amount of the above pharmaceutical composition, or, alternatively, may comprise administering the above vaccine.

[0015] Through cell culture passaging, we identified Andes virus (ANDV) variants with specific mutations and evaluated their virulence in the Syrian hamster model. Our starting virus was a wild type ANDV, strain Chile virus passaged twice (ANDV p2). This ANDV p2 is highly lethal in Syrian hamsters and the disease closely resembles hantavirus pulmonary syndrome (HPS) in humans. We passaged ANDV p2 and additional 18 times in Vero E6 cells to produce a ANDV p20 stock that infected hamsters but did not cause disease. The ANDV p20 stock of virus was used to plaque-pick (pp) in an attempt to isolate virus with attenuated phenotype. One ANDV variant, p20pp1, retained the pathogenicity observed with wild type ANDV. Two ANDV variants, p20pp15 and p20pp17, were attenuated in hamsters. Reduced viremia was detected in hamsters exposed to nonpathogenic ANDV variants. Both of the nonpathogenic ANDV variants contained an E265K mutation in the ectodomain of the glycoprotein, Gn. We

conducted an experiment to determine if the attenuated ANDV were capable of protecting hamsters against wild type ANDV. We found that “vaccination” two weeks before challenge with the attenuated pp viruses elicited neutralizing antibodies and protected hamsters from lethal HPS. These attenuated viruses could also protect if given on Day -3, but not Day 0, suggesting they elicited an innate response that was capable of protecting the animals in the absence of a detectable adaptive immune response. These attenuated ANDV form the bases of a future attenuated virus hantavirus vaccine. Such a vaccine would need to be further attenuated by producing reassortants with Sin Nombre virus, which would not require reverse genetics. If a reverse genetics system is discovered, then the E265K mutation could be incorporated along with other attenuating mutations that remain to-be-discovered.

Materials and Methods

[0016] Viruses and cells. ANDV strain Chile-9717869 was propagated in Vero E6 cells (Vero C1008 ATCC CRL 1586, Manassas, VA) and twice plaque –purified resulting in a p2 stock. Cells were maintained in complete Eagle minimum essential medium with Earle’s salts (cEMEM) supplemented with 10% FBS, 10nM HEPES (pH 7.4), 200 U ml⁻¹ penicillin, 200 µg ml⁻¹ streptomycin, 1x non-essential amino acids (NEAA), 1.5 µg ml⁻¹ amphotericin B, and 50 µg ml⁻¹ gentamicin sulfate at 37°C in a 5% CO₂ incubator. All cell culture reagents were procured from Life Technologies (Gibco, Grand Island, NY).

[0017] Generation of ANDV variants. The third passage of ANDV was accomplished by initially infecting a T-25 flask of Vero E6 cells with ANDV p2 at an MOI of 0.0024. The next 17 passages were completed by taking 10 µl of the previous passage, combining with 0.5 ml cEMEM, and adsorbing onto Vero E6 cells for 1 hr (rocking every 15 min) at 37°C in a 5% CO₂ incubator. The volume was raised to 4 ml with cEMEM. Approximately 1 wk post-infection supernatant was transferred to fresh cells for infection and incubation. Plaque-picks from the ANDV p20 stock were propagated in Vero E6 cells and sequenced.

[0018] Plaque Assay. Serial dilutions of neat virus were performed beginning at the 1:10 dilution as described (Hooper JW et al. *A lethal disease model for hantavirus pulmonary*

syndrome. Virology. 2001;289(1):6-14; Wahl-Jensen V et al. *Temporal analysis of Andes virus and Sin Nombre virus infections of Syrian hamsters*. J Virol. 2007;81(14):7449-62).

Immunostaining was performed as previously described (Hooper JW et al. *A Phase I clinical trial of Hantaan virus and Puumala virus M-segment DNA vaccines for haemorrhagic fever with renal syndrome delivered by intramuscular electroporation*. Clin Microbiol Infect. 2014;20 Suppl 5:110-7).

A fully human IgG anti-HPS glycoprotein primary antibody (Hooper JW et al. *DNA vaccine-derived human IgG produced in transchromosomal bovines protect in lethal models of hantavirus pulmonary syndrome*. Sci Transl Med. 2014;6(264):264ra162) and an anti-human horseradish peroxidase conjugated secondary antibody (Sera Care, Gaithersburg, MD).

[0019] Viral sequencing. RNA sequencing libraries were prepared using the KAPA RNA HyperPrep kit (KAPA Biosystems, Wilmington, MA, USA) following manufacturers' guidelines. Following library preparation, libraries were enriched for ANDV specific reads using the Illumina TruSeq RNA Exome enrichment reagents (using one-quarter quantities to enable singleplex enrichment) with a custom ANDV biotinylated probe set. The ANDV probe set included 201 unique probes of 120-nt in length that were designed against ANDV strain Chile-9717869 (AF291702, AF291703, and AF291704) and manufactured by Twist Biosciences (San Francisco, CA, USA). Pooled libraries were sequenced on the Illumina Miseq or NextSeq500sequencing platforms using 2 x 151 bp paired-end sequencing.

[0020] Reads were cleaned using Trimmomatic v0.38 (Bolger AM et al. *Trimmomatic: a flexible trimmer for Illumina sequence data*. Bioinformatics. 2014;30(15):2114-20) to remove Illumina adaptors and low-quality bases, followed by Prinseq-lite v0.20.4 (Schmieder R & Edwards R. *Fast identification and removal of sequence contamination from genomic and metagenomic datasets*. PloS one. 2011;6(3):e17288) to remove duplicate reads. Reference-based assemblies were done using Bowtie2 (Langmead B & Salzberg SL. *Fast gapped-read alignment with Bowtie 2*. Nat Methods. 2012;9(4):357-9) and AF291702, AF291703, and AF291704 as references for the S, M, and L segment, respectively. Duplicates were removed with Picard and a new consensus sequence was generated using a combination of Samtools

v0.1.18 (Li H et al. *The Sequence Alignment/Map format and SAMtools*. Bioinformatics. 2009;25(16):2078-9) and custom scripts (https://github.com/jtldner/Scripts/blob/master/reference-based_assembly/consensus_fasta.py). Only bases with a Phred quality score Q20 or better were used for consensus calling.

[0021] Animal manipulations. Female Syrian hamsters aged 6-8 wks (Envigo, Indianapolis, IN) were anesthetized by inhalation of vaporized isoflurane using an IMPAC 6 veterinary anesthesia machine. Once anesthetized, animals were injected by the intramuscular route (i.m., caudal thigh) with the indicated concentration of virus diluted in PBS. Blood sampling from the vena cava occurred under anesthesia. Humane endpoint conditions were established and hamsters monitored throughout the experiment. Surviving hamsters at the end of the study were deeply anesthetized with ketamine-acepromazine-xylazine (KAX) for blood collection and barbiturate overdose.

[0022] Isolation of RNA and real-time RT-PCR. Approximately 200 mg of organ tissue was homogenized in 1.0 ml of TRIzol (ThermoFisher, Waltham, MA) reagent using M tubes on the gentleMACS (Miltenyi Biotec, Auburn, CA) dissociation system on the RNA setting. RNA was extracted from TRIzol per manufacturers protocol. A NanoDrop 8000 was used to determine RNA concentration, which was then raised to 100 ng μ l⁻¹ in UltraPure distilled water. Real-time PCR was conducted on a BioRad CFX thermal cycler using an Agilent Brilliant II QRT-PCR one-step master mix. ANDV primer/probe sequences are described in (Khaiboullina SF et al. *Andes-virus-induced cytokine storm is partially suppressed by ribavirin*. Antivir Ther. 2013;18(4):575-84). Cycling conditions were 30 min at 48 °C, 10 min at 95 °C, followed by 40 cycles of 15 s at 95 °C and 1 min at 60 °C. Data acquisition occurs following the annealing step.

[0023] N-ELISA. Recombinant N protein ELISA were performed on serum samples as previously described (Hooper JW et al. *DNA vaccination with hantavirus M segment elicits neutralizing antibodies and protects against seoul virus infection*. Virology. 1999;255(2):269-78). Samples were gamma-irradiated (on dry ice) with 3 x 10⁶ rads from a 60C source.

[0024] **ANDV PsVNA.** The PsVNA using a non-replicating VSVΔG-luciferase pseudovirion system was performed as previously described (Kwilas S et al. *A hantavirus pulmonary syndrome (HPS) DNA vaccine delivered using a spring-powered jet injector elicits a potent neutralizing antibody response in rabbits and nonhuman primates*. Curr Gene Ther. 2014;14(3):200-10).

[0025] **Preparation of tissues for histology.** Tissues were fixed in 10% neutral buffered formalin, trimmed, processed, embedded in paraffin, cut at 5 to 6µm, and stained with hematoxylin and eosin (H&E).

[0026] **In situ hybridization.** To detect ANDV genomic RNA in FFPE tissues, *in situ* hybridization (ISH) was performed using the RNAscope 2.5 HD RED kit (Advanced Cell Diagnostics, Newark, CA, USA) as described previously (Liu J et al. *Molecular detection of SARS-CoV-2 in formalin fixed paraffin embedded specimens*. JCI Insight. 2020. Epub 2020/05/08. doi: 10.1172/jci.insight.139042).

[0027] Briefly, forty ZZ ISH probes targeting ANDV genomic RNA fragment were designed and synthesized by Advanced Cell Diagnostics. Tissue sections were deparaffinized with xylene, underwent a series of ethanol washes and peroxidase blocking, and were then heated in kit-provided antigen retrieval buffer and digested by kit-provided proteinase. Sections were exposed to ISH target probe pairs and incubated at 40°C in a hybridization oven for 2 h. After rinsing, ISH signal was amplified using kit-provided Pre-amplifier and Amplifier conjugated to alkaline phosphatase and incubated with a Fast Red substrate solution for 10 min at room temperature. Sections were then stained with hematoxylin, air-dried, and cover slipped.

[0028] **Statistical analyses.** Graphs were generated using GraphPad Prism 8. Survival curves were compared using Kaplan-Meier survival analysis with log-rank tests. *P* values were adjusted by step-down Bonferroni correction to account for multiple comparisons. Viral RNA levels were compared using unpaired t tests.

Results

Serial passaging of ANDV in cell culture

[0029] To test if serial passaging can induce mutations, and the effect of these mutations on lethality of hamsters, we started with ANDV strain Chile that had been passaged twice, ANDV p2, and performed a total of 20 cell culture passages in Vero E6 cells. After 20 passages (ANDV p20), we observed that plaques show distinct morphological differences compared to ANDV p2, with most ANDV p20 plaques being larger, rounder and darker (**FIG. 1A**). To elucidate the mutations, even numbered ANDV passages were sequenced. Five mutations were observed between ANDV p2 and ANDV p20 across the three RNA strands (**FIG. 1B**). No amino acid changes were observed with a single mutation, Ant1441G, occurring in the 3'NCR. A single amino acid change, E265K was observed on the p20 M-segment, and two amino acid changes, K127E and E1445K, were observed on the p20 L-segment. Three of the mutations, the M segment E265K and the L segment K127E and E1445K, mutation change the amino acid charge: the M segment E265K and L segment E1445K mutations changes from the negatively charged glutamic acid to lysine, while the L segment K125E mutation makes the reverse change.

ANDV p20 is attenuated in hamsters

[0030] An initial experiment to test the lethality of ANDV p20 in hamsters showed attenuating mutations resulted in a non-lethal phenotype in 7/8 hamsters challenged with 1,000 PFU (**FIG. 1C**). Lungs collected from ANDV p20 hamsters had significantly less viral RNA present when compared to ANDV p2 infected hamsters (**FIG. 1D**). Sequenced ANDV strains from select lungs indicating that the ANDV p20 hamster that died had a large percentage (approximately 98%) of a K125T mutation that is located in the catalytic region of the endonuclease (**FIG. 1E**).

ANDV p20 plaque picked variants

[0031] Given the variation of ANDV p20, twice plaque-picked (pp) ANDV p20 variants were sequenced. Selected plaque-picks, based on completion of sequence data and variability of

mutations, were analyzed in the hamster model to determine the mutation effect on lethality (Table 1).

Table 1

	S Segment	M Segment	L segment					
	Ant1441G	E265K	K125T	K127E	E1445K	Q2122R	T2151A	Survival
ANDV p20	63.76%	16.68%	0.55%	41.78%	100%	24.16%	15.3%	88%
ANDV p20pp1	A	E	K	K	K	Q	T	13%
ANDV p20pp17	A	K	K	K	K	Q	T	100%
ANDV p20pp15	A	K	T	K	K	Q	T	100%

[0032] Groups of 8 hamsters each were challenged with 1,000 PFU of the indicated ANDV p20 plaque-pick variant by the intramuscular route (**FIG. 2** and **Table 1**). These data show that, while the E1445K mutation is heavily selected for in all ANDV p20 plaque-pick variants, this mutation alone is not attenuating in the ANDV/hamster model (ANDV p20pp1, 1/8 hamsters surviving). The M-segment E265K is associated with 100% lethality.

[0033] The kinetics of two nonpathogenic ANDV variants (ANDV p20pp17 and p20pp15), and pathogenic ANDV variants (ANDV p2 and ANDV p20pp1) were analyzed (**FIGS. 3A-3J**). At 8 and 10 days post infection (dpi), viremia is approximately 3 logs higher in pathogenic compared to nonpathogenic ANDV ($p < 0.0001$, t-test, **FIG. 3B**). Lung tissue was collected during peak viremia indicating mild to moderate perivascular edema and hemorrhage admixed with lymphocytes, heterophils, and macrophages extending into adjacent alveolar lumen and expanding alveolar septa from hamsters exposed to pathogenic ANDV variants and minimal edema and reduced numbers of infiltrating macrophages and heterophils from hamsters exposed to nonpathogenic ANDV variants (**FIGS. 3C-3F**). Using *in situ* hybridization (ISH) to detect genomic RNA, there is a striking difference between

pathogenic and nonpathogenic ANDV variants in the lungs of exposed hamsters (**FIGS. 3G-3J**) indicating nonpathogenic ANDV variants have reduced viremia and dissemination to the target organ.

Protective efficacy of pre-exposure to ANDV variants

[0034] The possible vaccine potential of attenuated ANDV variants was evaluated in a dual challenge experiment. Hamsters were exposed to nonpathogenic ANDV variants p20pp15 and p20pp17 on either minus (-) 14 dpi (to evaluate the protective adaptive immune response) or -3 dpi (to evaluate the protective innate immune response) and subsequently exposed to a lethal dose of ANDV p2. Serum collected just prior to ANDV p2 challenge on 0 dpi indicate that all animals exposed to ANDV variants p20pp15 and p20pp17 on -14 dpi had detectable antibodies to nucleocapsid (**FIG. 4A**) and neutralizing antibody (**FIG. 4B**). Hamsters were monitored for survival following ANDV p2 (**FIG. 4C**). All hamsters exposed only to ANDV p2 met euthanasia criteria on 10 and 11 dpi. Two hamsters from the ANDV p20pp17 -3 dpi/ANDV p2 0 dpi group met euthanasia criteria on 10 dpi (75% survival). All hamsters from remaining groups survived to the end of the study. Serum collected at the end of the study indicate all surviving animals had developed a robust antibody response to one or the combination of ANDV variants (**FIGS. 4D and 4E**).

Discussion

[0035] ANDV separates itself from other hantaviruses in its documented person-to-person transmission (Martinez-Valdebenito C et al. *Person-to-person household and nosocomial transmission of andes hantavirus, Southern Chile, 2011*. Emerg Infect Dis. 2014;20(10):1629-36), super-spreader events (Martinez VP et al. *"Super-Spreaders" and Person-to-Person Transmission of Andes Virus in Argentina*. N Engl J Med. 2020;383(23):2230-41), and uniform lethality of HPS disease in hamsters (Hooper JW et al. *A lethal disease model for hantavirus pulmonary syndrome*. Virology. 2001;289(1):6-14). In the absence of a hantavirus reverse genetics system, serial passaging of ANDV in cell culture was used to generate mutations in

the viral genome. This is an approach that has shown that minimal changes in the viral genome can have profound effects on maintenance in the viral reservoir (Lundkvist A et al. *Cell culture adaptation of Puumala hantavirus changes the infectivity for its natural reservoir,*

Clethrionomys glareolus, and leads to accumulation of mutants with altered genomic RNA S segment. J Virol. 1997;71(12):9515-23; Nemirov K et al. *Adaptation of Puumala hantavirus to cell culture is associated with point mutations in the coding region of the L segment and in the noncoding regions of the S segment.* J Virol. 2003;77(16):8793-800) and pathogenicity in animal models (Safronetz D et al. *Pathophysiology of hantavirus pulmonary syndrome in rhesus macaques.* Proc Natl Acad Sci U S A. 2014;111(19):7114-9). ANDV p2 which is typically uniformly lethal was passaged through 18 additional cell culture passages, resulted in a virus stock that was highly attenuated with significant reduced lethality in Syrian hamsters. Using twice-plaque picked ANDV variants, specific mutations were evaluated for pathogenicity in the hamster model. The E265K mutation, located in the ectodomain of Gn, appears to be central to determining pathogenicity by our analysis. This amino acid is located in a site known to be the target of antibodies targeting a related hantavirus (Li S et al. *A Molecular-Level Account of the Antigenic Hantaviral Surface.* Cell Rep. 2016;16(1):278), in addition to ANDV (Haese N et al. *Antiviral Biologic Produced in DNA Vaccine/Goose Platform Protects Hamsters Against Hantavirus Pulmonary Syndrome When Administered Post-exposure.* PLoS Negl Trop Dis. 2015;9(6):e0003803).

[0036] Recently, Warner, *et al.*, published a comparative analysis of ANDV Chile 9717869 isolated from the natural reservoir and CHI-7913 isolated from a lethal human case of HPS (Warner BM et al. *Differential pathogenesis between Andes virus strains CHI-7913 and Chile-9717869 in Syrian Hamsters.* J Virol. 2021. Epub 2021/02/26. doi:10.1128/JVI.00108-21). In this study, hamsters exposed to CHI-7913 had no lethality and reduced histopathology and viral RNA detected in the serum and organs when compared to hamsters exposed to ANDV Chile 9717869. Twenty-three amino acid changes were detected between these two ANDV strains that did not include the E265K mutation we have identified or mutations in the endonuclease region of L. These results, combined with our own, highlight the need for a hantavirus reverse

genetics system to tease apart the impact of specific viral genome mutations on virus fitness and host immune responses to infection.

[0037] There are currently no FDA-approved vaccines or treatments for hantaviruses, including ANDV. Several vaccines targeting ANDV have been demonstrated efficacious in animal models (i.e. virus-vectored recombinant vaccines) (Brown KS et al. *Vesicular stomatitis virus-based vaccine protects hamsters against lethal challenge with Andes virus*. J Virol. 2011;85(23):12781-91; Saffronetz D et al. *Adenovirus vectors expressing hantavirus proteins protect hamsters against lethal challenge with andes virus*. J Virol. 2009;83(14):7285-95) and an ANDV DNA vaccine is currently in a clinical trial (Brocato RL & Hooper JW. *Progress on the Prevention and Treatment of Hantavirus Disease*. Viruses. 2019;11(7)). Reassortant viruses have also been considered a putative vaccine approach for hantaviruses. These viruses, generated by genetic reassortment of pathogenic and nonpathogenic hantaviruses *in vitro*, generate humoral immunity in a similar manner to wild-type viruses (Brocato RL et al. *Innate immune responses elicited by Sin Nombre virus or type I IFN agonists protect hamsters from lethal Andes virus infections*. J Gen Virol. 2018. doi: 10.1099/jgv.0.001131; McElroy AK et al. *Andes virus M genome segment is not sufficient to confer the virulence associated with Andes virus in Syrian hamsters*. Virology. 2004;326(1):130-9; Handke W et al. *Generation and characterization of genetic reassortants between Puumala and Prospect Hill hantavirus in vitro*. J Gen Virol. 2010;91(Pt 9):2351-9). To add to the vaccine possibilities, the use of the nonpathogenic ANDV variants as live attenuated vaccines were evaluated in hamsters. ANDV p20pp15 and p20pp17 elicited a robust neutralizing antibody response, detected 2 weeks following exposure and protected hamsters from lethal disease. Moreover, exposure to ANDV p20pp15 only 3 days prior to a lethal ANDV p2 challenge was completely protective. Recently, monoclonal antibodies to ANDV have been described (Duehr J et al. *Neutralizing Monoclonal Antibodies against the Gn and the Gc of the Andes Virus Glycoprotein Spike Complex Protect from Virus Challenge in a Preclinical Hamster Model*. mBio. 2020;11(2):e00028-20; Garrido JL et al. *Two recombinant human monoclonal antibodies that protect against lethal Andes hantavirus infection in vivo*. Sci Transl Med. 2018;10(468)). These identified antibodies do

not map to the region of Gn containing the E265K mutation. However, as new ANDV monoclonal antibodies are discovered, these variants could be used to identify escape potential.

[0038] In conclusion, these attenuated ANDV form the bases of a future attenuated virus hantavirus vaccine. Such a vaccine would need to be further attenuated by producing reassortants with Sin Nombre virus, which would not require reverse genetics. If a reverse genetics system is discovered, then the E265K mutation could be incorporated along with other attenuating mutations that remain to-be-discovered.

[0039] All identified publications mentioned herein are hereby incorporated by reference to the same extent as if each such publication was specifically and individually indicated to be incorporated by reference in its entirety. While the disclosure has been described in connection with exemplary embodiments, it will be understood that it is capable of further modifications and this application covers any variations, uses, or adaptations following, in general, the principles of the disclosure and including such departures as come within known or customary practice within the art to which the disclosure pertains.

What Is Claimed Is:

1. An Andes virus (ANDV) variant comprising an amino acid sequence that is about 90% to about 99% identical to the sequence of wild type ANDV.
2. The ANDV variant of claim 2, comprising one or more attenuating amino acid modifications.
3. The ANDV variant of claim 2, wherein said one or more attenuating amino acid modifications comprise a substitution at position 265.
4. The ANDV variant of claim 2, wherein said one or more attenuating amino acid modifications comprise E265K.
5. A pharmaceutical composition comprising the ANDV variant of any one of claims 1-4 or viral particles thereof.
6. The pharmaceutical composition of claim 5 for use in one or more of treatment or prevention of an infection or disease in a subject in need thereof.
7. The pharmaceutical composition of claim 6, wherein said infection or disease is characterized by respiratory distress, pulmonary edema, and/or cardiogenic shock.
8. The pharmaceutical composition of claim 7, wherein said infection or disease is selected from the group consisting of hantavirus pulmonary syndrome (HPS) or hantavirus cardiopulmonary syndrome (HCPS).
9. A vaccine comprising the ANDV variant of any one of claims 1-4 or viral particles thereof, or the pharmaceutical composition of any one of claim 5-8.
10. A method of treating a disease or condition in a subject in need thereof, comprising administering a therapeutically effective amount of the pharmaceutical composition of any one of claims 5-8.
11. A method of preventing a disease or condition in a subject in need thereof, comprising administering the vaccine of claim 9.

FIG. 1A

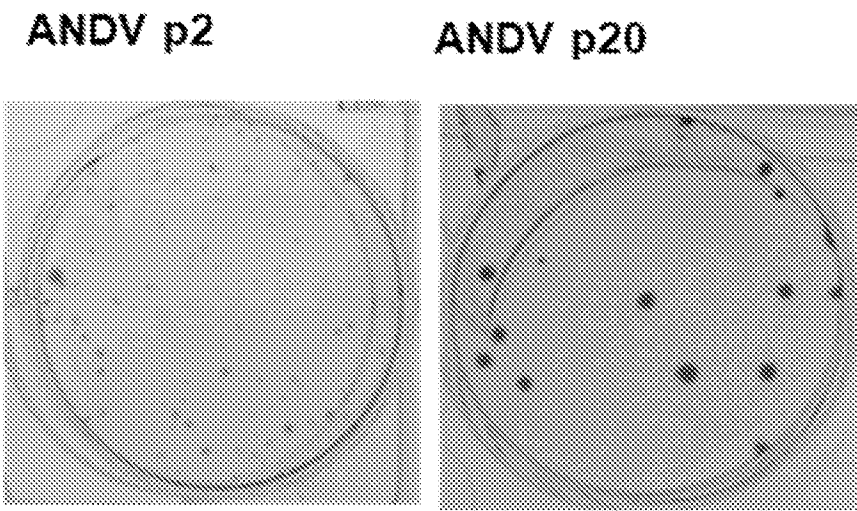


FIG. 1B

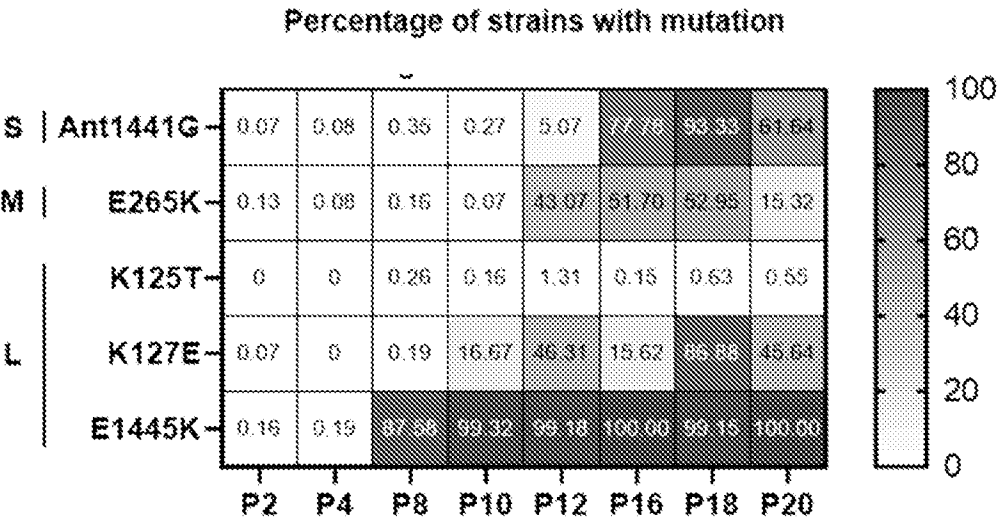


FIG. 1C

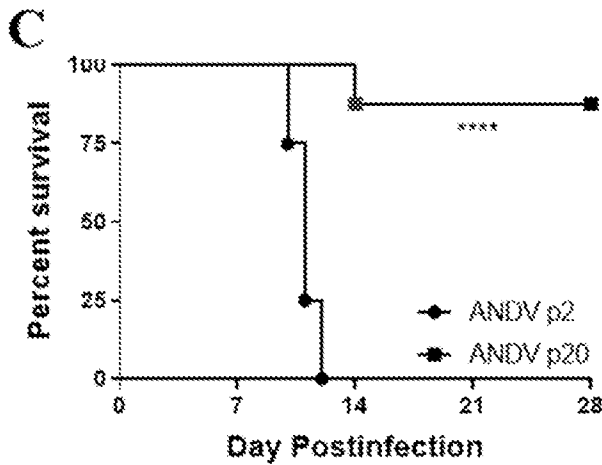


FIG. 1D

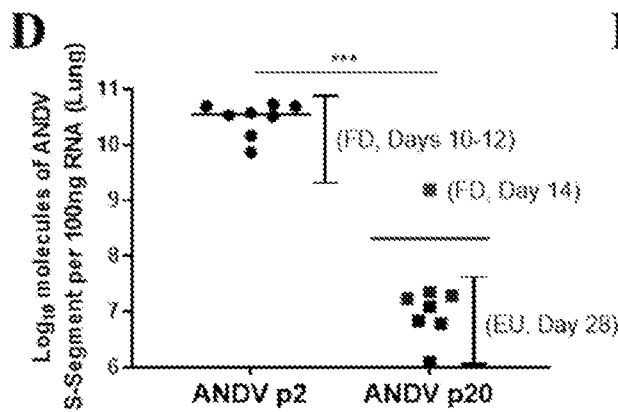


FIG. 1E

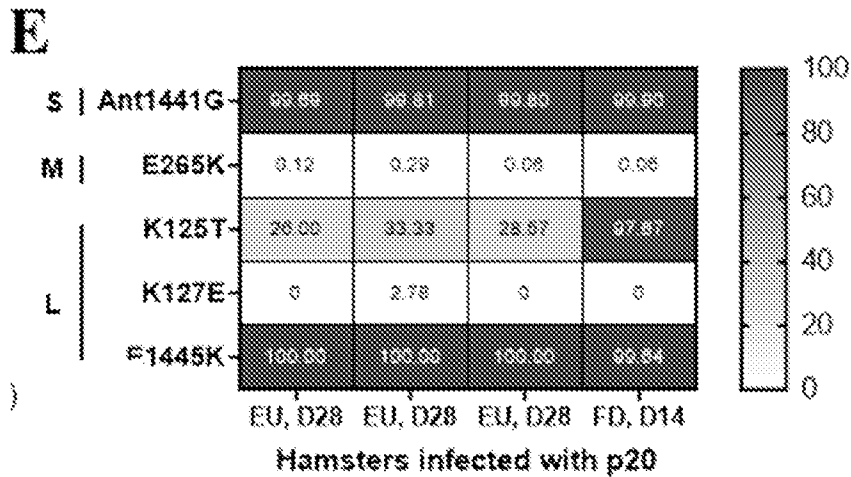


FIG. 2

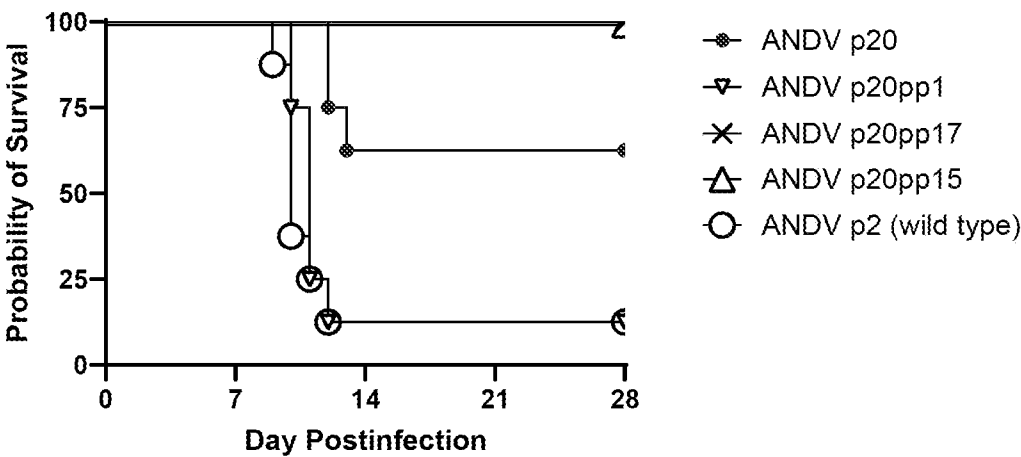


FIG. 3A

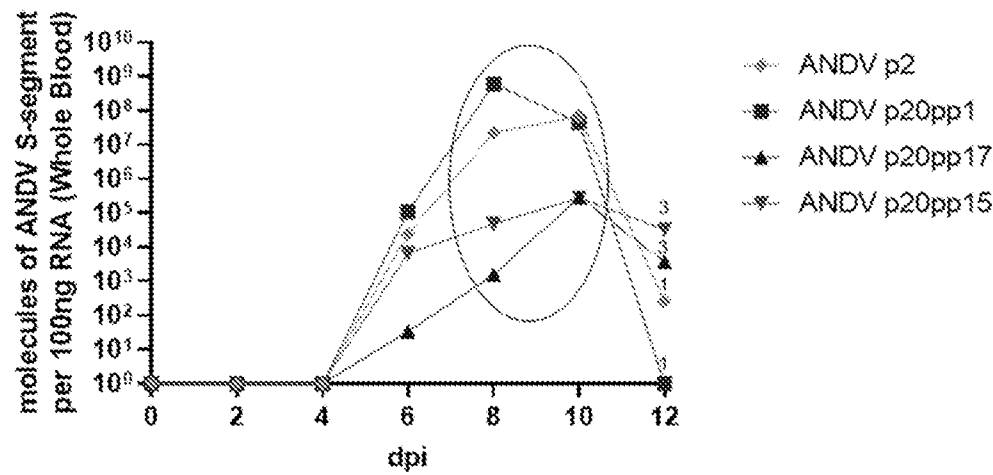


FIG. 3B

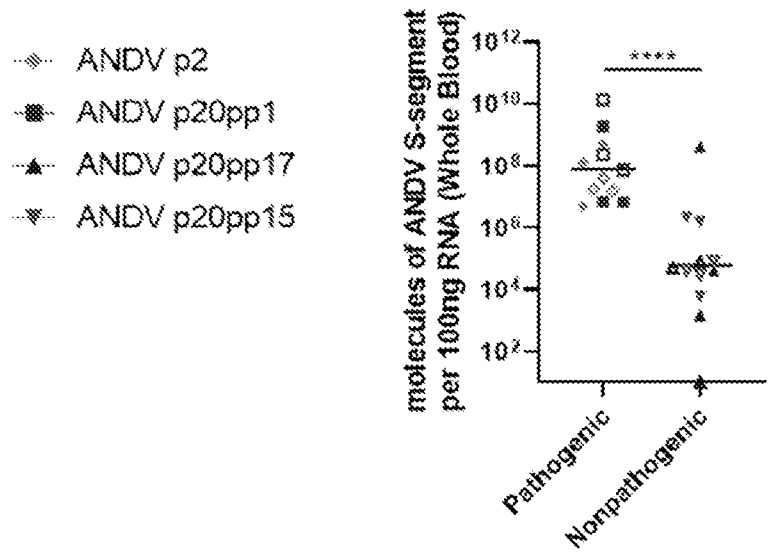


FIG. 3C

FIG. 3D

FIG. 3E

FIG. 3F

Pathogenic

Nonpathogenic

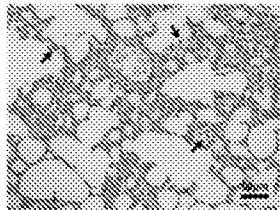
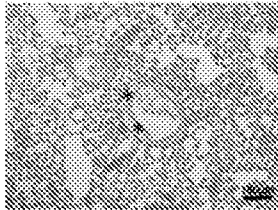
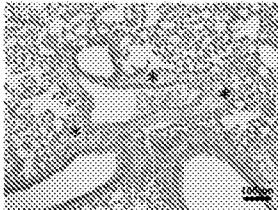
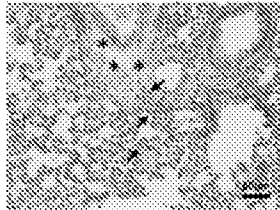


FIG. 3G

FIG. 3H

FIG. 3I

FIG. 3J

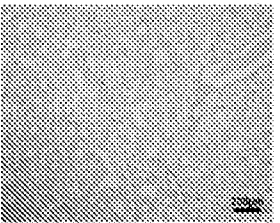
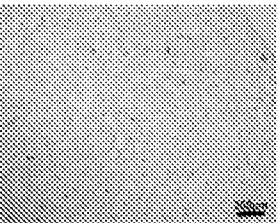
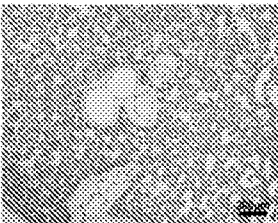
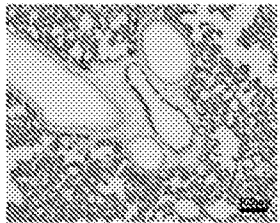


FIG. 4A

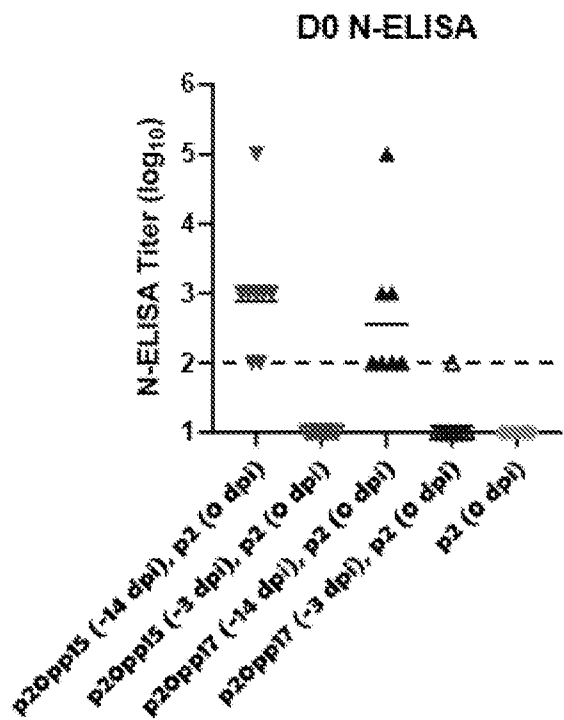


FIG. 4B

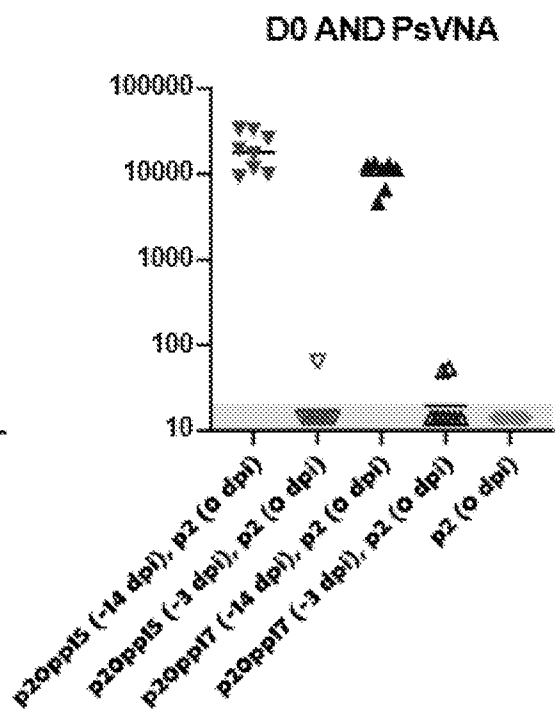
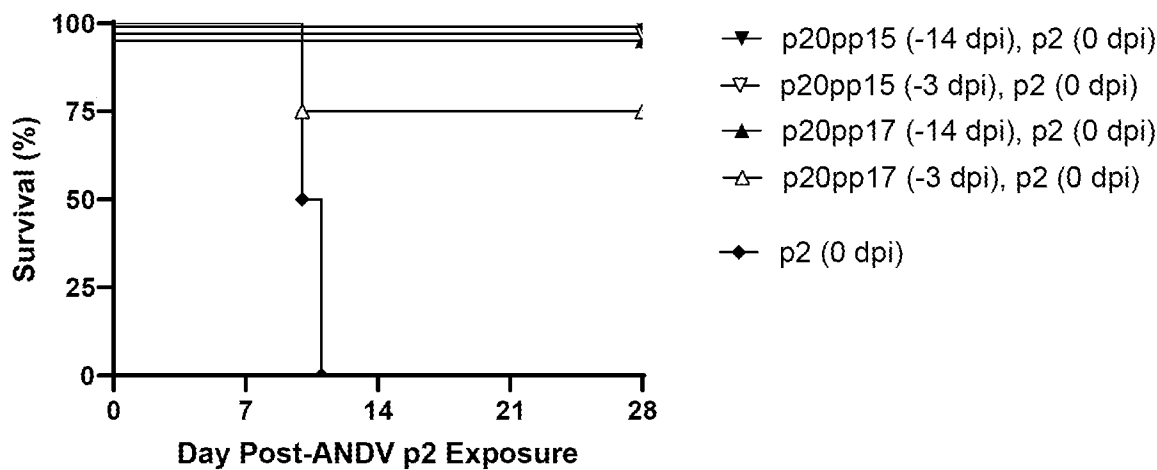


FIG. 4C



INTERNATIONAL SEARCH REPORT

International application No.

PCT/US2024/033437

Box No. II Observations where certain claims were found unsearchable (Continuation of item 2 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:
2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:
3. ☒ Claims Nos.: **9-11**
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US2024/033437

A. CLASSIFICATION OF SUBJECT MATTER IPC: A61K 39/12 (2024.01); A61P 31/14 (2024.01); C12N 7/04 (2024.01) CPC: A61K 39/12 ; A61P 31/14 ; C12N 7/00 ; A61K 2039/5254 ; C12N 2760/12121 ; C12N 2760/12134 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 2022/073144 A1 (PONTIFICIA UNIVERSIDAD CATOLICA DE CHILE) 14 April 2022 (14.04.2022) entire document	5-8
X Y	BELLOMO et al., Andes Virus Genome Mutations That Are Likely Associated with Animal Model Attenuation and Human Person-to-Person Transmission, mSphere, Vol. 8, No. 3, 25 April 2023, Pgs. 1-14, [retrieved 05 August 2024]. Retrieved from the Internet <URL: https://doi.org/10.1128/msphere.00018-23>. entire document	1, 2 5-8
A	WARNER et al., Differential Pathogenesis between Andes Virus Strains CHI-7913 and Chile-9717869 in Syrian Hamsters, Journal of Virology, Vol. 95, No. 10, 26 April 2021, Pgs. 1-15, [retrieved 05 August 2024]. Retrieved from the Internet <URL: https://doi.org/10.1128/jvi.00108-21>. entire document	1-8
☐ 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 08 August 2024 (08.08.2024)		Date of mailing of the international search report 20 August 2024 (20.08.2024)
Name and mailing address of the ISA/US Mail Stop PCT, Attn: ISA/US Commissioner for Patents P.O. Box 1450, Alexandria, VA 22313-1450 Facsimile No. 571-273-8300		Authorized officer MATOS TAINA Telephone No. 571-272-4300