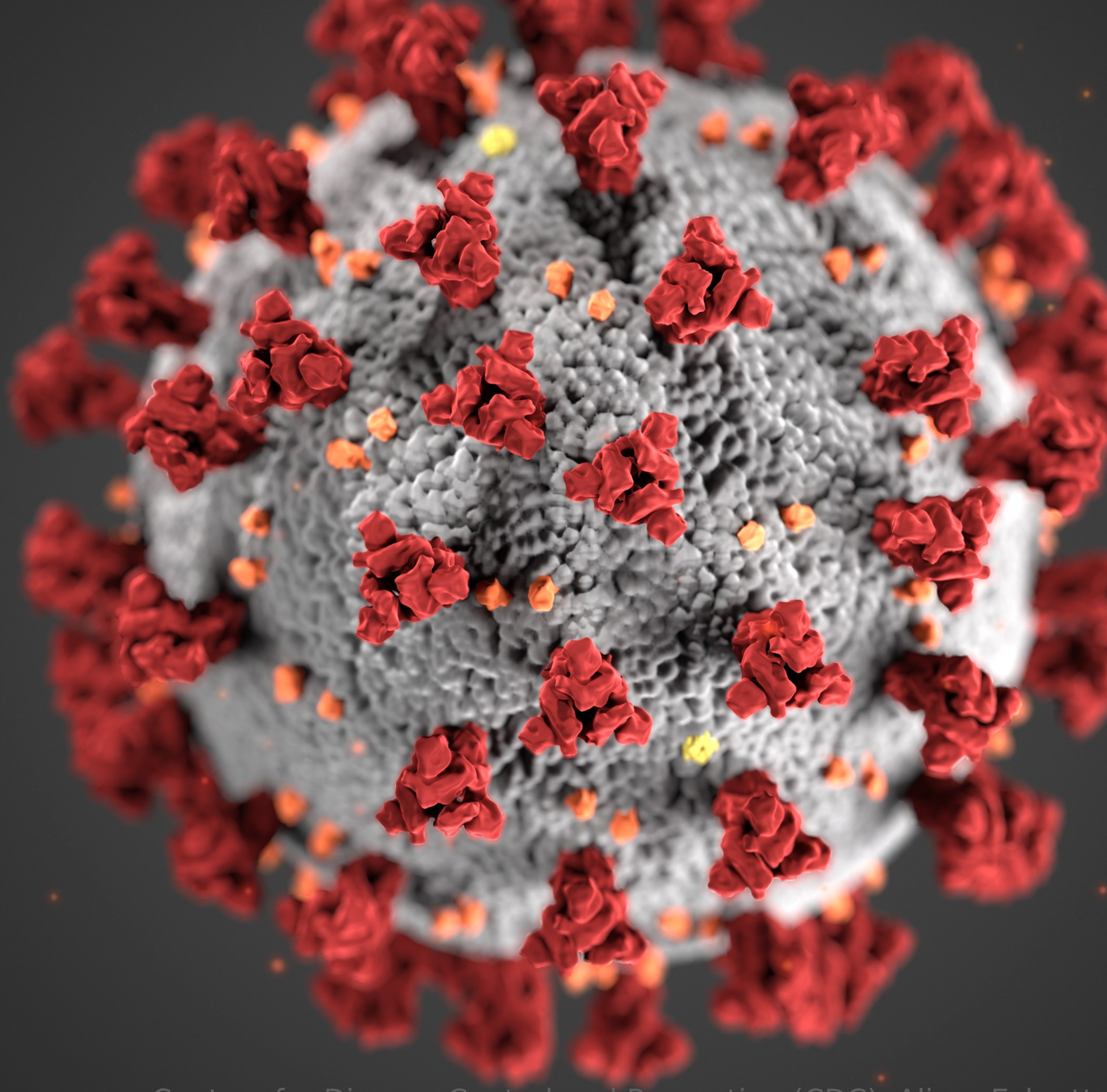
UNIVERSIDAD PERUANA
CAYETANO HEREDIA

Optimización *in silico* de señuelos basados en hACE2 con alta afinidad al dominio RBD-Spike del SARS – CoV – 2 y evaluación de su interacción *in vitro*

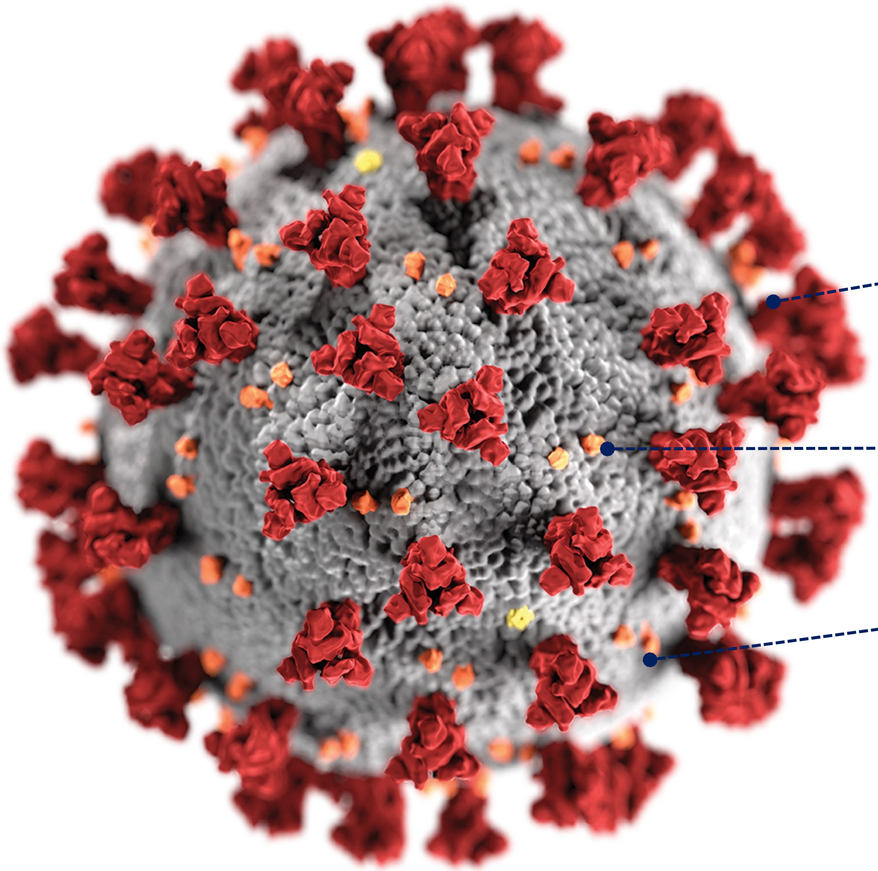
Examen de Candidatura

Lic. Mario Ricardo Ruiz Burga



Centers for Disease Control and Prevention (CDC), Alissa Eckertt y

Estructura general del SARS – CoV – 2



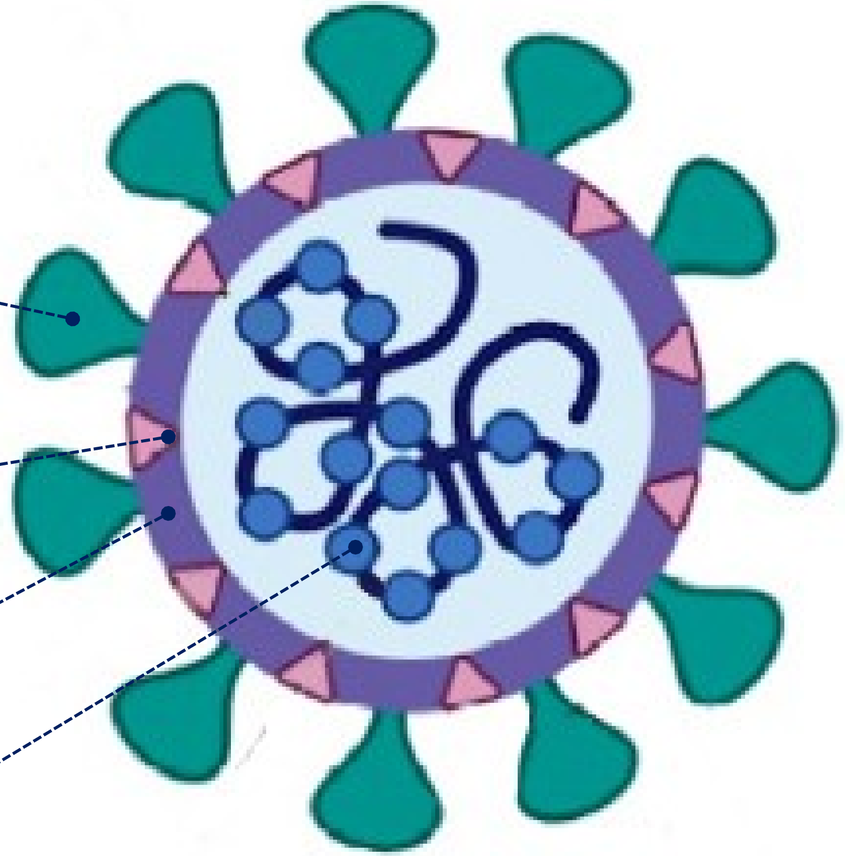
CDC, 2020

Spike (S)

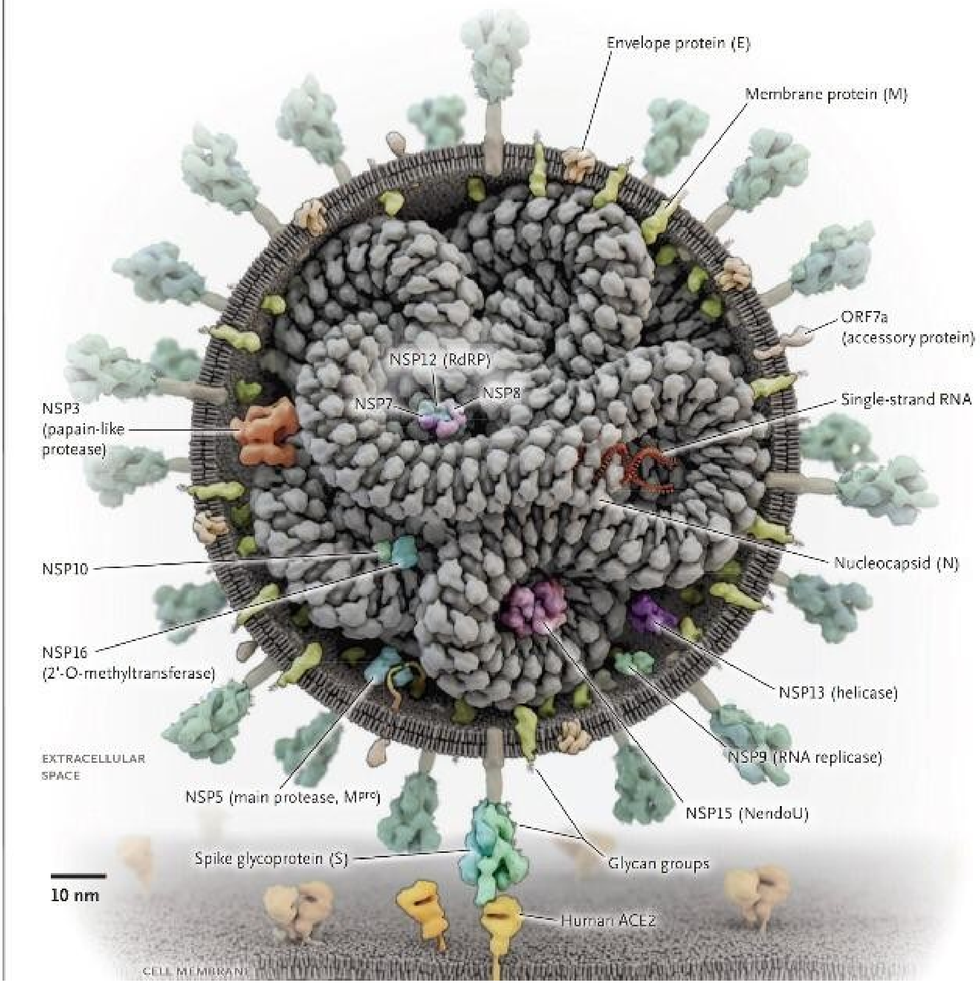
Envoltura (E)

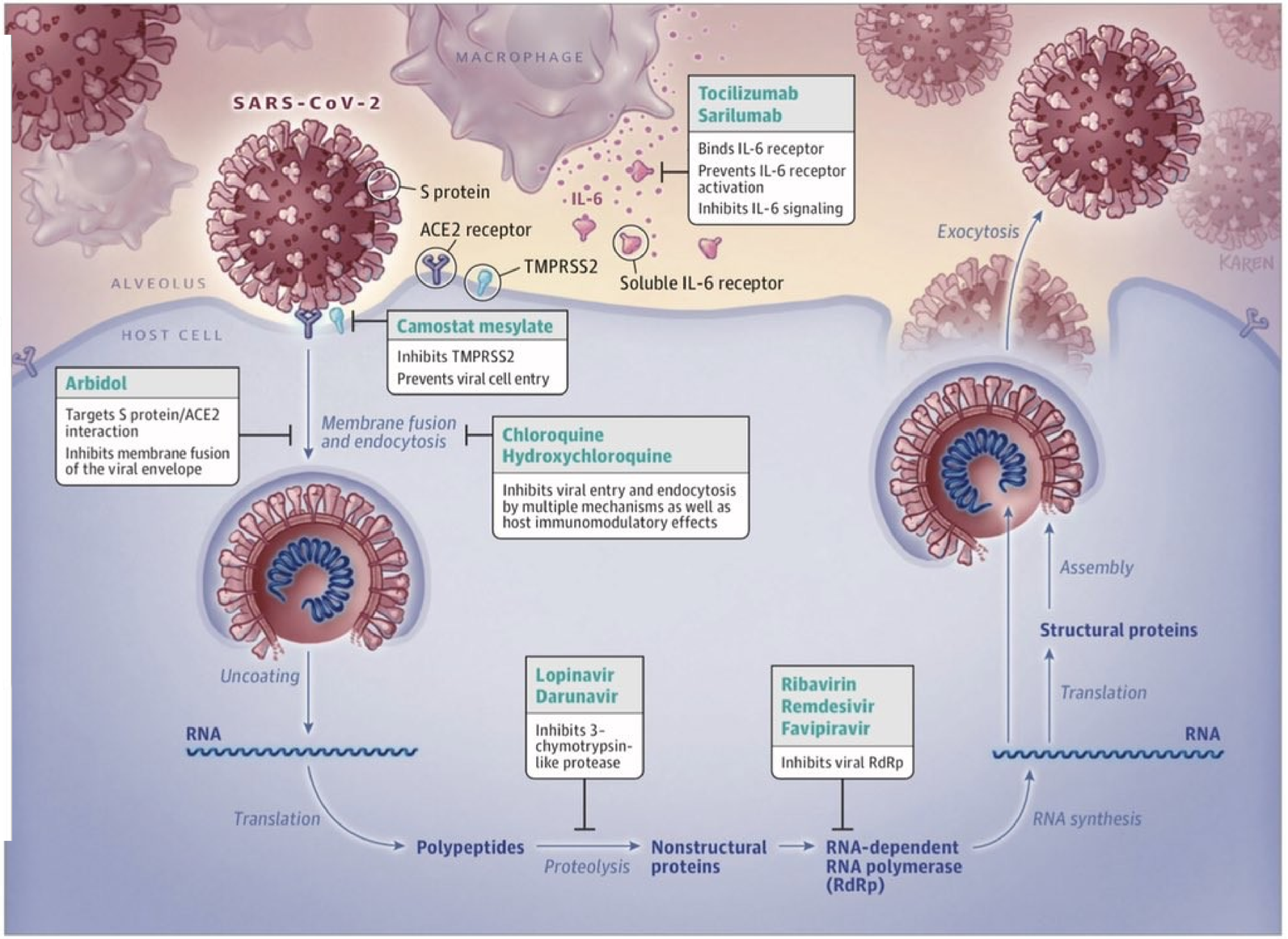
Membrana (M)

Nucleocápside (N)



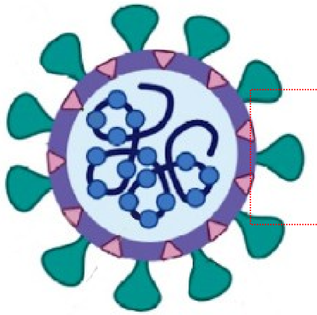
Hasöksüz et al, 2020





Schematic represents virus-induced host immune system response and viral processing within target cells. Proposed targets of select repurposed and investigational products are noted. ACE2, angiotensin-converting enzyme 2; S protein, spike protein; and TMPRSS2, type 2 transmembrane serine protease.

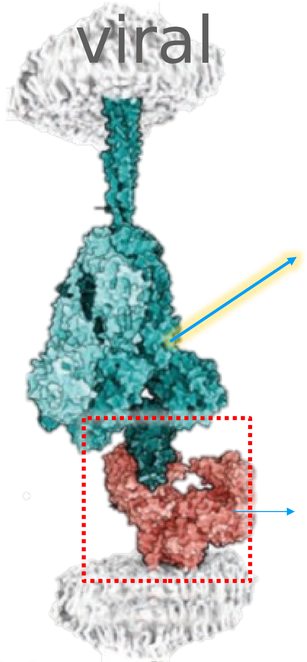
Interfaz de unión entre el SARS – CoV – 2 y la hACE2



SARS – CoV – 2

Spike S1

Envoltura viral



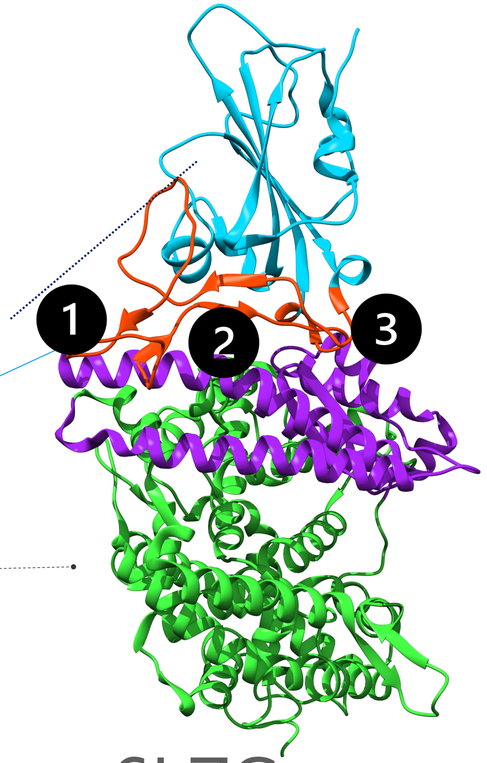
Membrana celular

Spike trimer – "down" closed

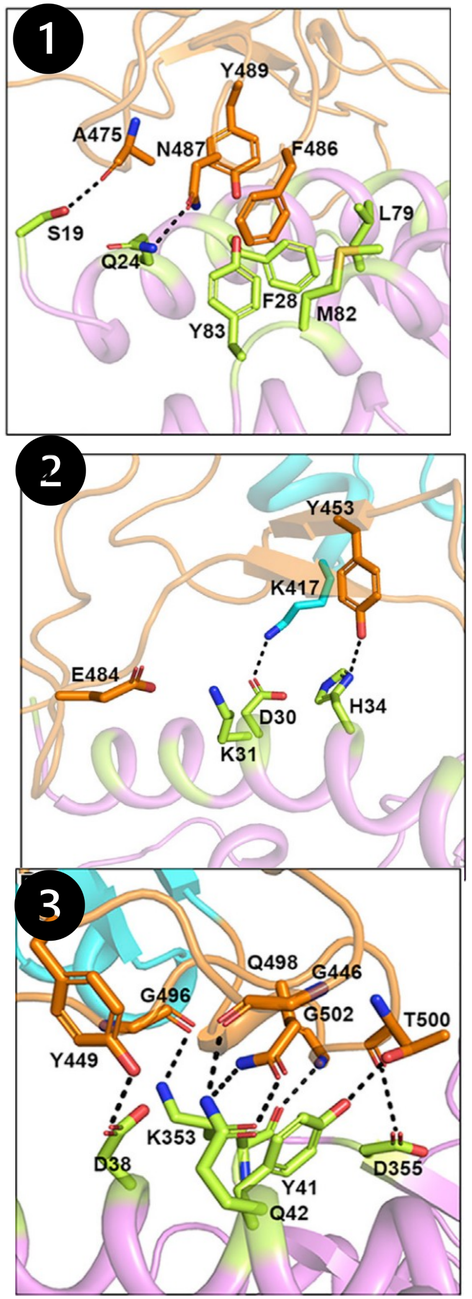
"up" RBD exposed

RBM

hACE2



6LZG

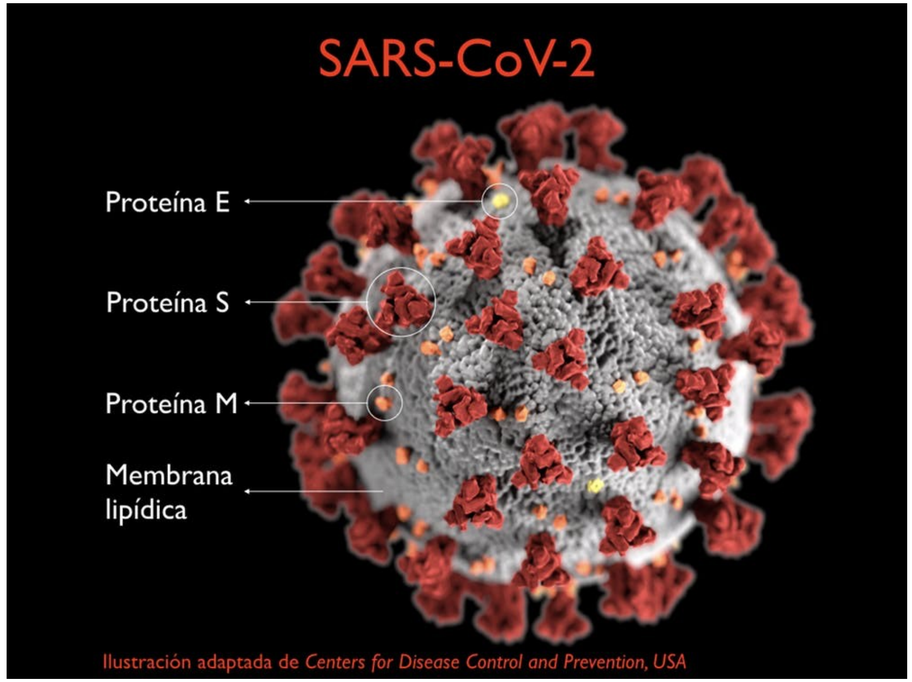


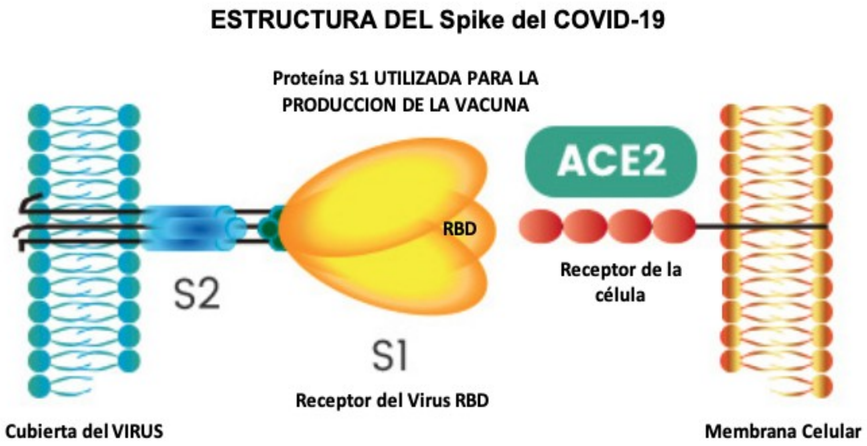
Hasöksüz et al, 2020

DeKosky, 2020

Wang et al., 2020

PROYECTO DE LA VACUNA PERUANA PARA EL COVID-19

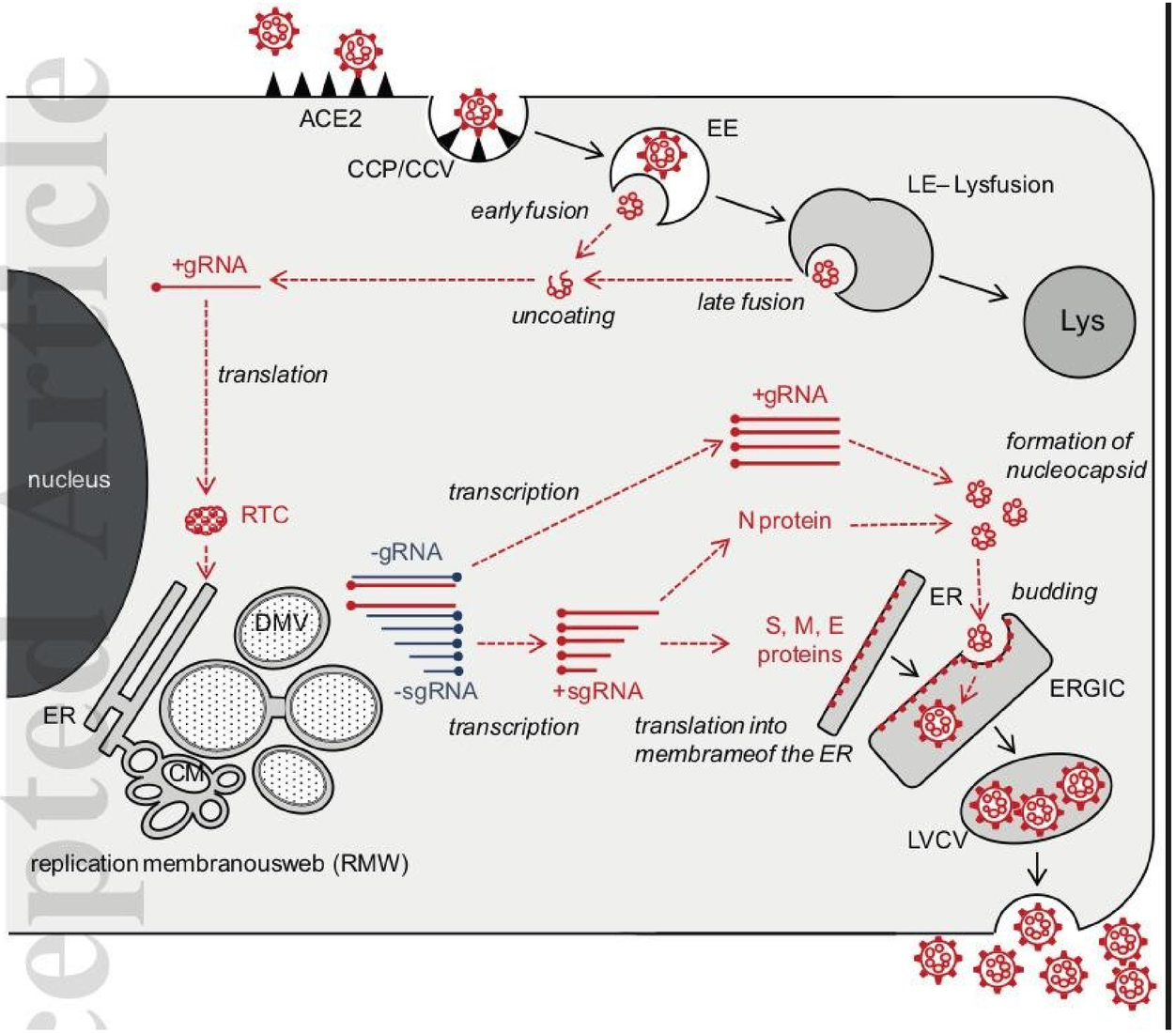


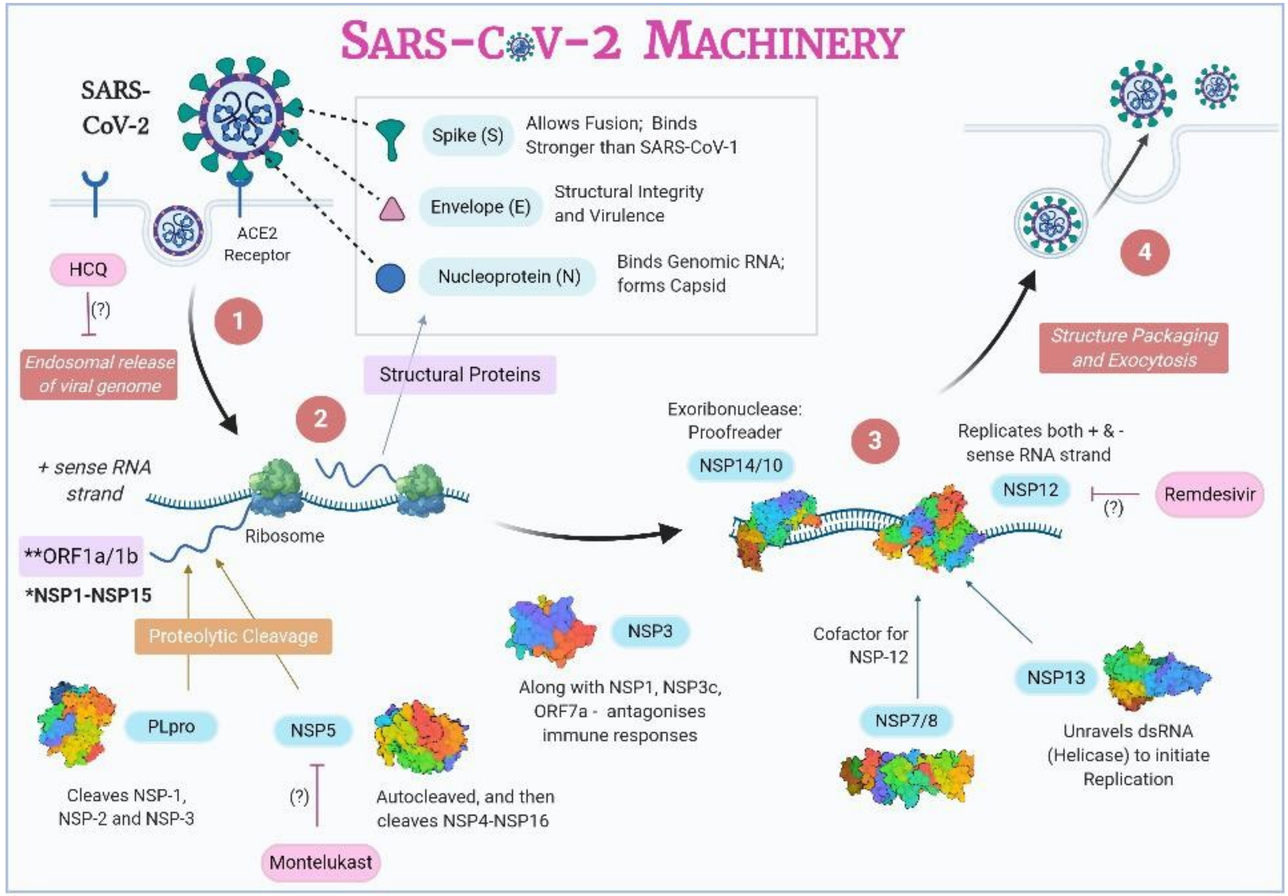


Proteinas de SARS CoV-2 evaluadas como blancos para nuestras vacunas

1. S1-S2
2. S1
3. S1 Trimerizada
4. RBD
5. RBD Dimerizada

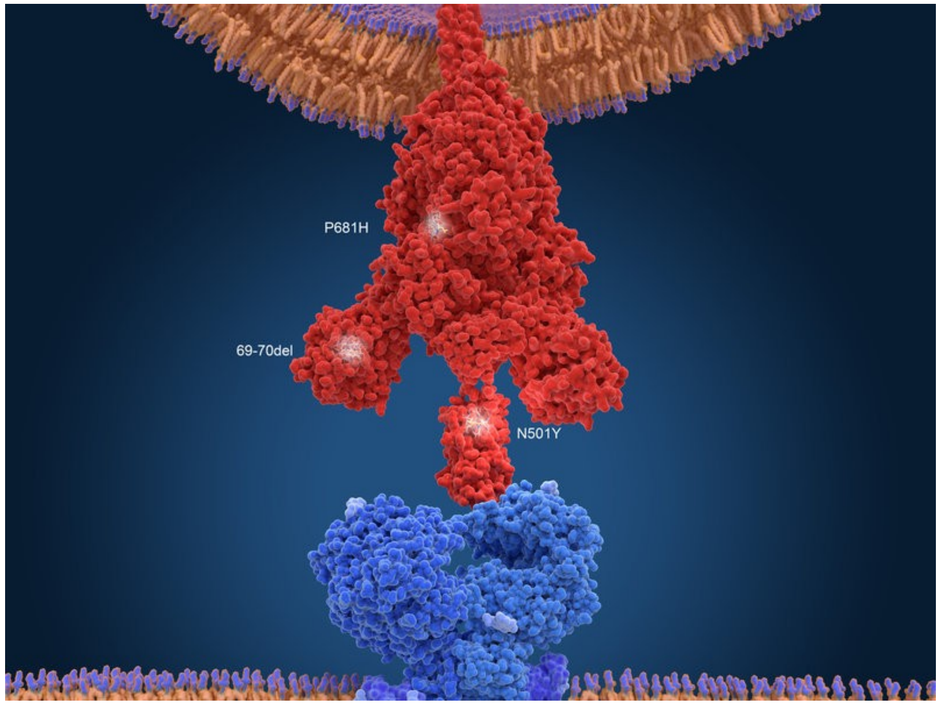
sitio crítico para atacar al virus con anticuerpos neutralizantes es la proteína Spi
roteía Spike es la “llave de entrada” para igresar a las células humanas a través
Receptor ACE2


ACE2
CCP/CCV
EE
early fusion
LE– Lysfusion
+gRNA
uncoating
late fusion
Lys
translation
nucleus
RTC
+gRNA
formation of nucleocapsid
transcription
N protein
-gRNA
DMV
ER
budding
S, M, E proteins
-sgRNA
+sgRNA
ERGIC
transcription
translation into membrameof the ER
CM
LVCV
replication membranousweb (RMW)

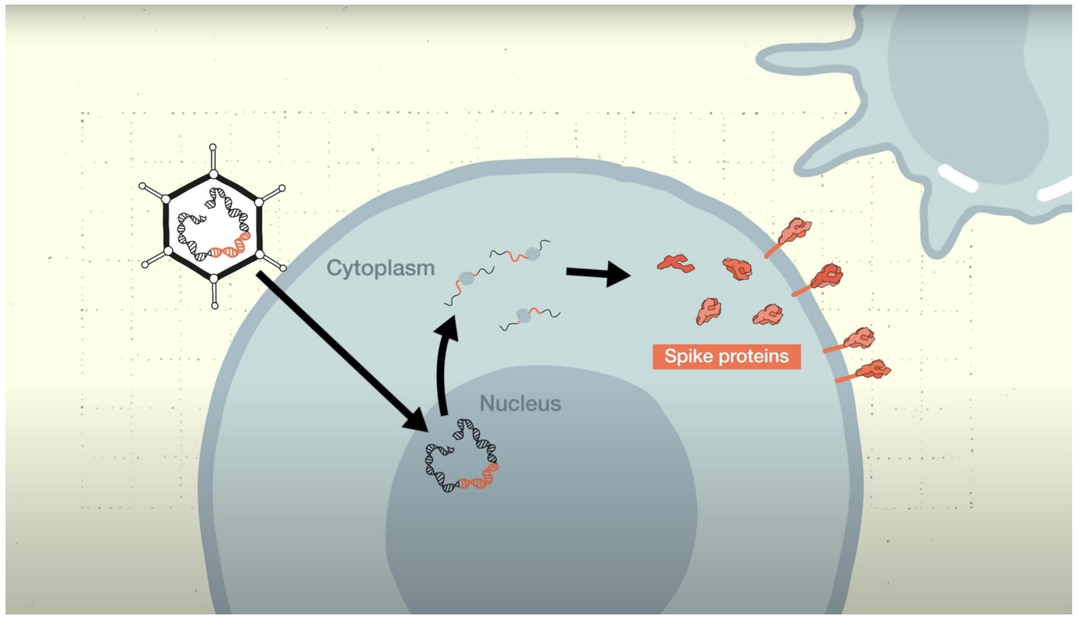

SARS-CoV-2 MACHINERY
SARS-CoV-2
Spike (S)
Allows Fusion; Binds Stronger than SARS-CoV-1
Envelope (E)
Structural Integrity and Virulence
Nucleoprotein (N)
Binds Genomic RNA; forms Capsid
ACE2 Receptor
HCQ
(?)
Endosomal release of viral genome
1
Structural Proteins
2
+ sense RNA strand
Ribosome
**ORF1a/1b
*NSP1-NSP15
Proteolytic Cleavage
PLpro
Cleaves NSP-1, NSP-2 and NSP-3
NSP5
(?)
Montelukast
Autocleaved, and then cleaves NSP4-NSP16
NSP3
Along with NSP1, NSP3c, ORF7a - antagonises immune responses
Exoribonuclease: Proofreader
NSP14/10
3
Replicates both + & - sense RNA strand
NSP12
(?)
Remdesivir
Cofactor for NSP-12
NSP7/8
NSP13
Unravels dsRNA (Helicase) to initiate Replication
4
Structure Packaging and Exocytosis

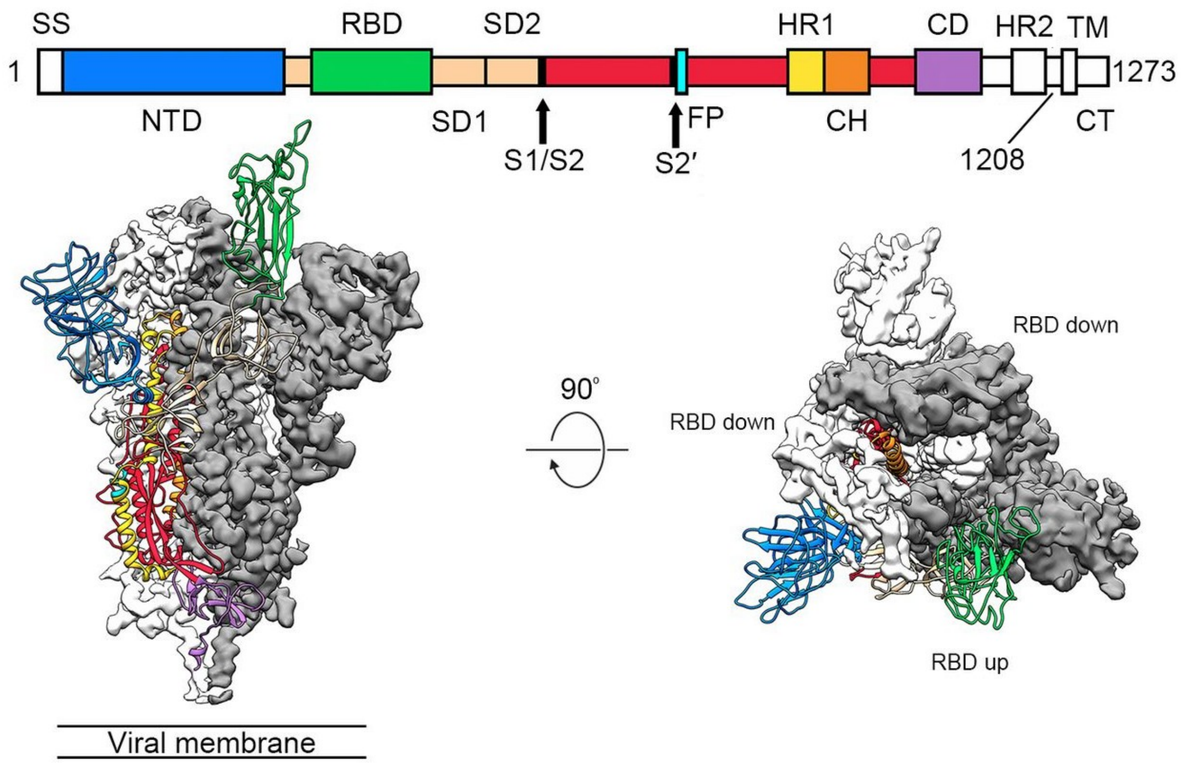
La proteína S1 de SARS-CoV-2 esta glicosilada

cuna intranasal NDV - S1 muestra sobre su superficie a la proteína S1 con el mismo patrón de glicosilación de SARS-C



Mecanismo de infección de SARS-CoV-2

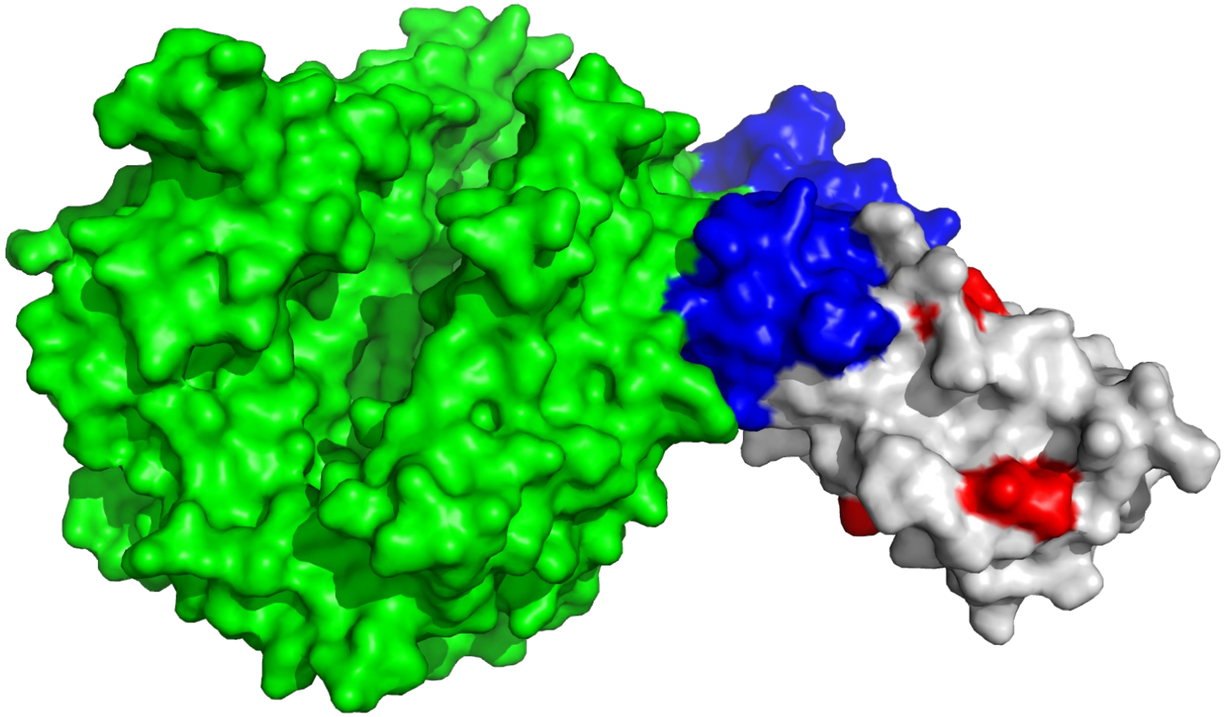


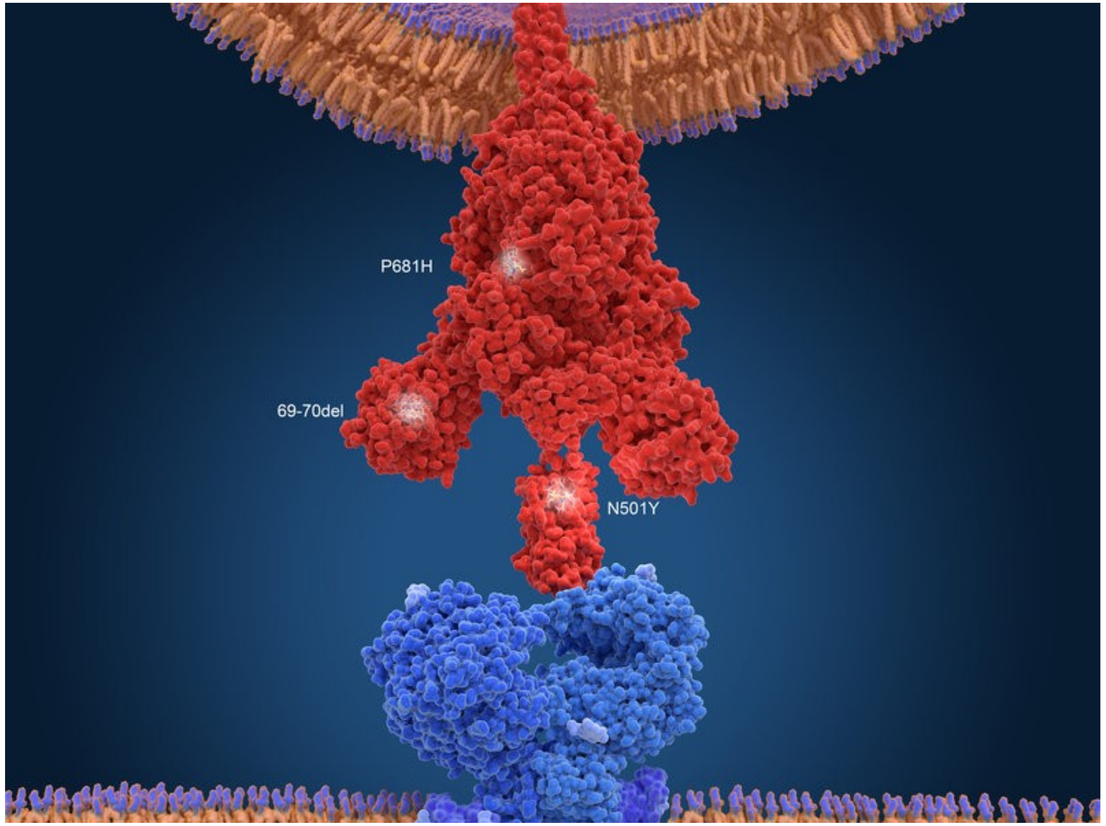


Proteinas de SARS CoV-2 evaluadas como blancos para nuestras vacunas

1. S1-S2
2. S1
3. S1 Trimerizada
4. RBD
5. RBD Dimerizada

Las vacunas que se vienen desarrollando se basan en el subdominio RBD.

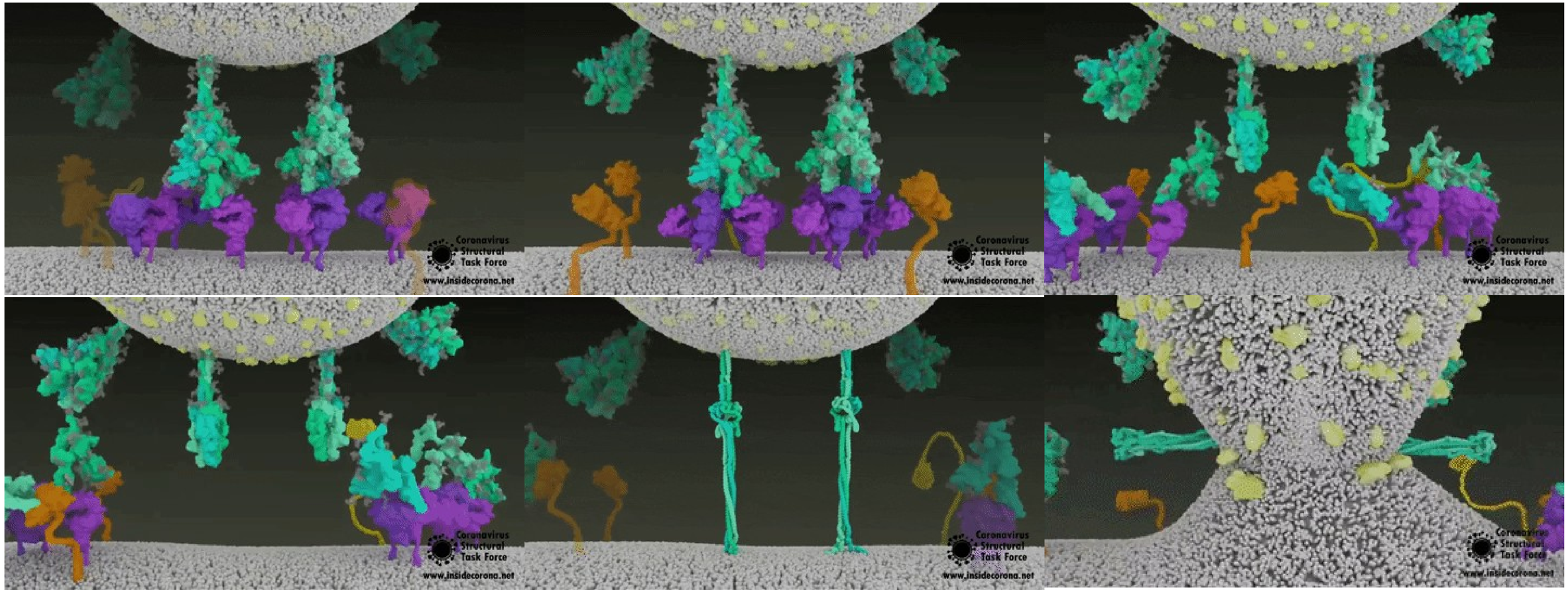


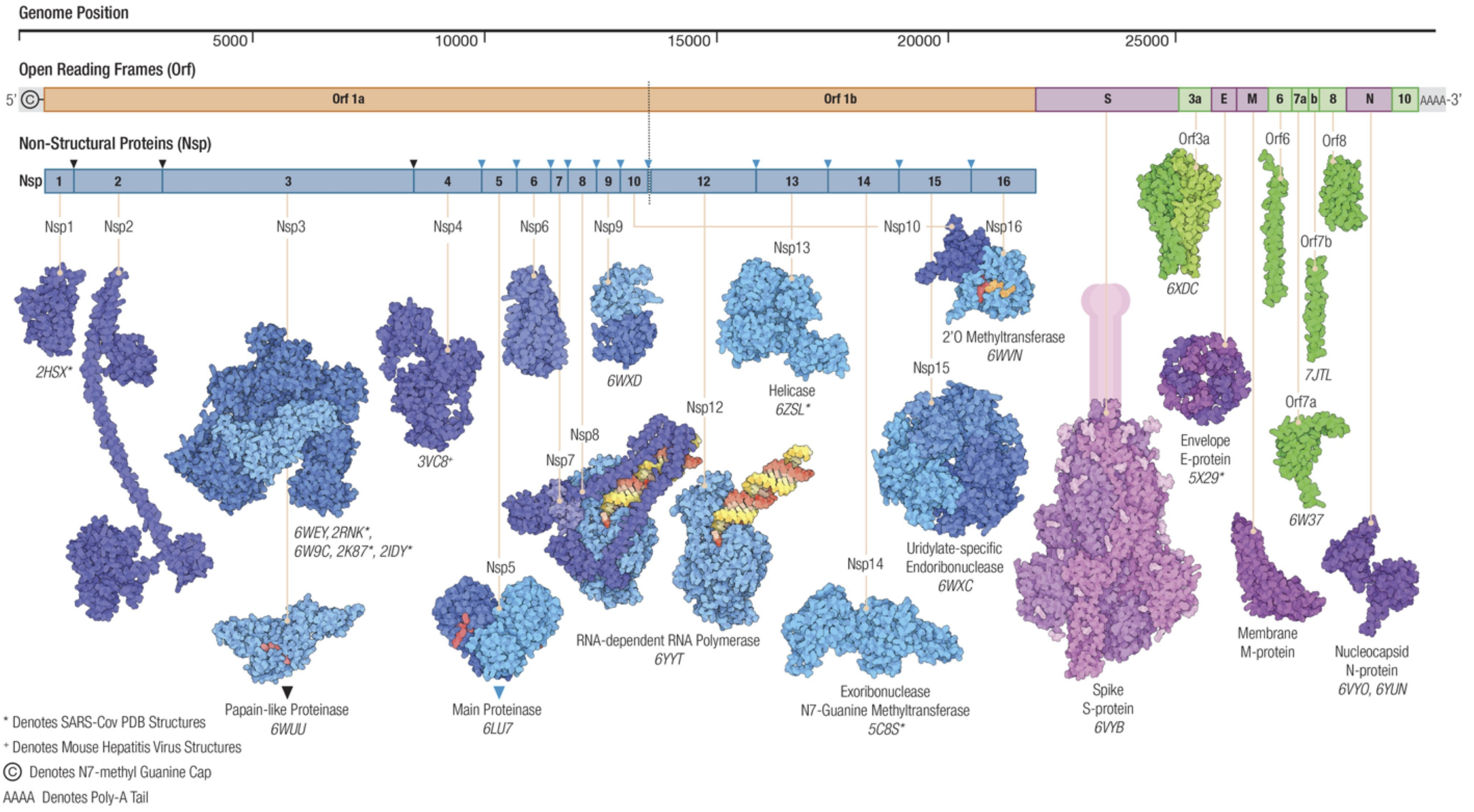


ACE2	RBD	S1	MUTATIONS

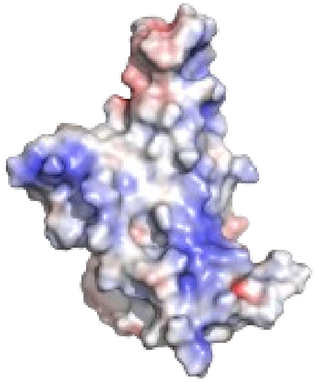
Las pruebas iniciales se basaron en RBD S1-monomérico, S1-trimérico, y S2. En todos los casos, RBD fué el antígeno que mostró las respuestas más fuertes y fué este el antígeno que se continuó evaluando

Mecanismos de entrada viral

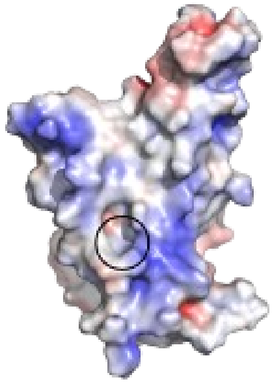



Genome Position
5000
10000
15000
20000
25000
Open Reading Frames (Orf)
5'
Orf 1a
Orf 1b
S
3a
E
M
6
7a
b
8
N
10
AAAA-3'
Non-Structural Proteins (Nsp)
Nsp
1
2
3
4
5
6
7
8
9
10
12
13
14
15
16
Nsp1
Nsp2
Nsp3
Nsp4
Nsp6
Nsp9
Nsp10
Nsp16
Nsp13
Nsp15
Nsp12
Nsp8
Nsp7
Nsp5
Nsp14
Orf3a
Orf6
Orf8
Orf7b
Orf7a
2HSX*
6WEY, 2RNK*, 6W9C, 2K87*, 2IDY*
3VC8+
6WXD
Helicase 6ZSL*
2'O Methyltransferase 6WVN
6XDC
7JTL
Envelope E-protein 5X29*
6W37
Uridylate-specific Endoribonuclease 6WXC
RNA-dependent RNA Polymerase 6YYT
Papain-like Proteinase 6WUU
Main Proteinase 6LU7
Exoribonuclease N7-Guanine Methyltransferase 5C8S*
Spike S-protein 6VYB
Membrane M-protein
Nucleocapsid N-protein 6VYO, 6YUN
* Denotes SARS-Cov PDB Structures
+ Denotes Mouse Hepatitis Virus Structures
Denotes N7-methyl Guanine Cap
AAAA Denotes Poly-A Tail

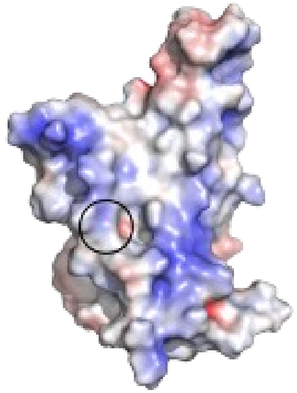
MODELLING MUTATION SPIKE-S1



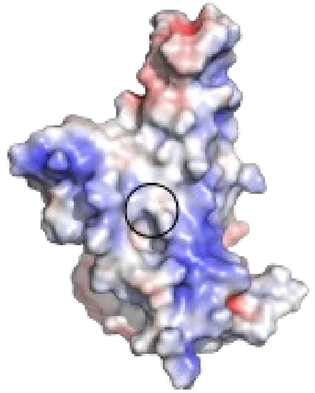
Wild Type



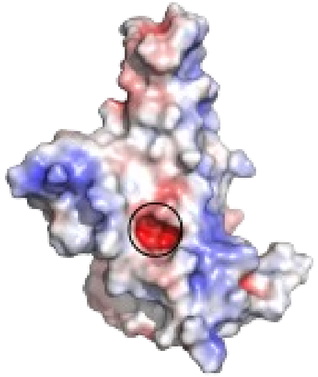
VAL_341_ILE
RMSD_SOLV: 0.040
RMSD_VACU: 0.000



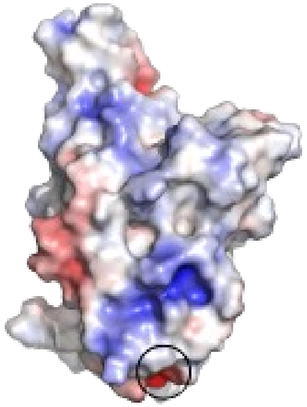
ALA_344_SER
RMSD_SOLV: 0.045
RMSD_VACU: 0.000



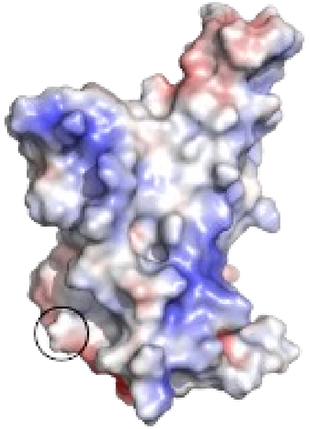
ALA_348_THR
RMSD_SOLV: 0.035
RMSD_VACU: 0.000



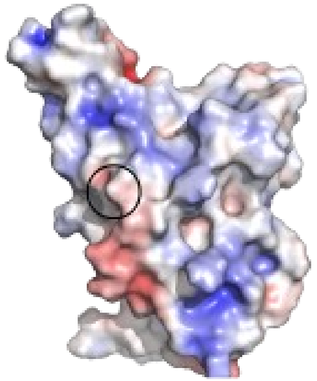
ASN_354_ASP
RMSD_SOLV: 0.058
RMSD_VACU: 0.015



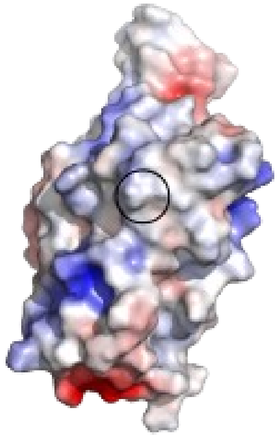
ASP_364_TYR
RMSD_SOLV: 0.064
RMSD_VACU: 0.016



VAL_367_PHE
RMSD_SOLV: 0.020
RMSD_VACU: 0.000

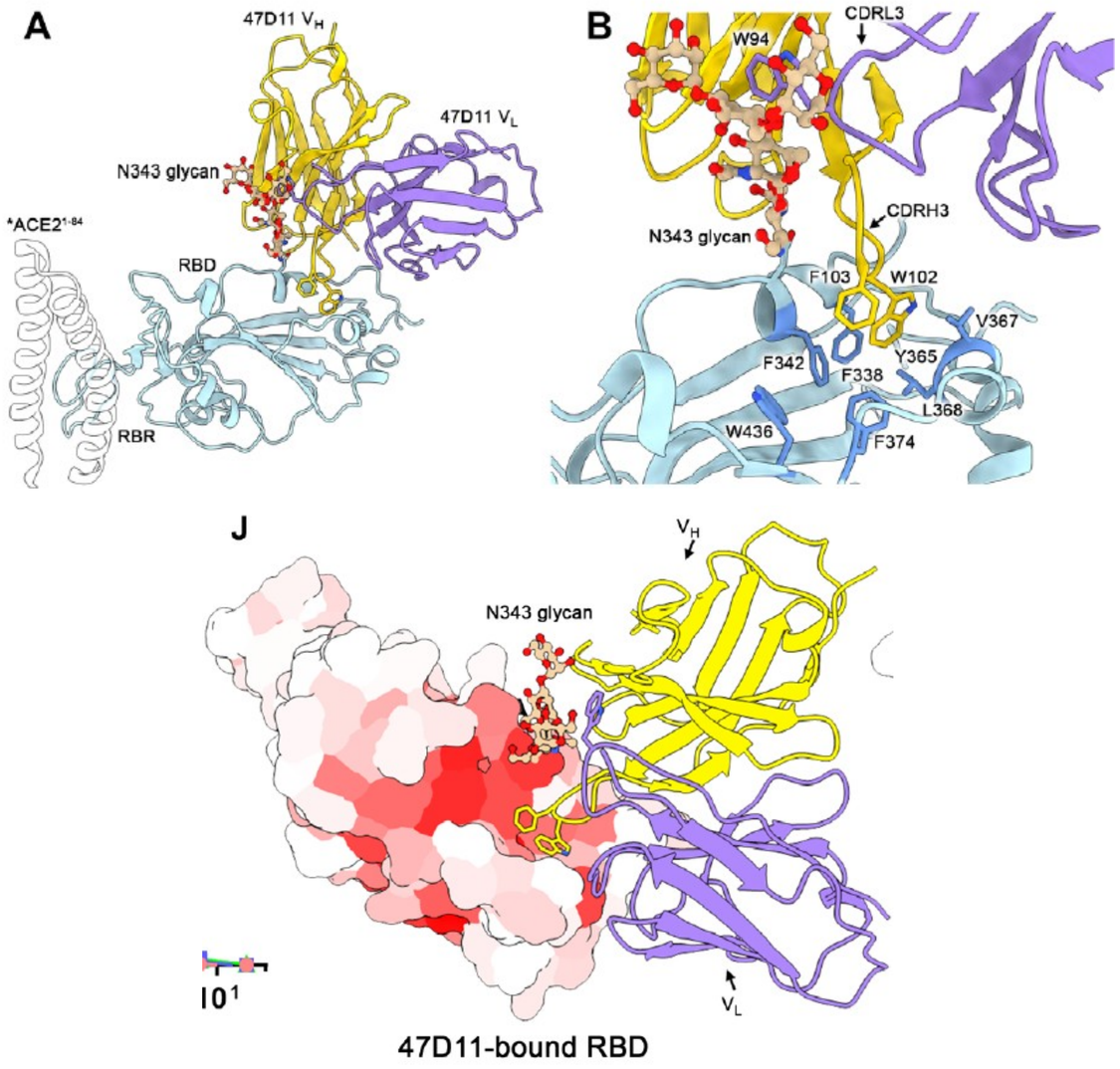


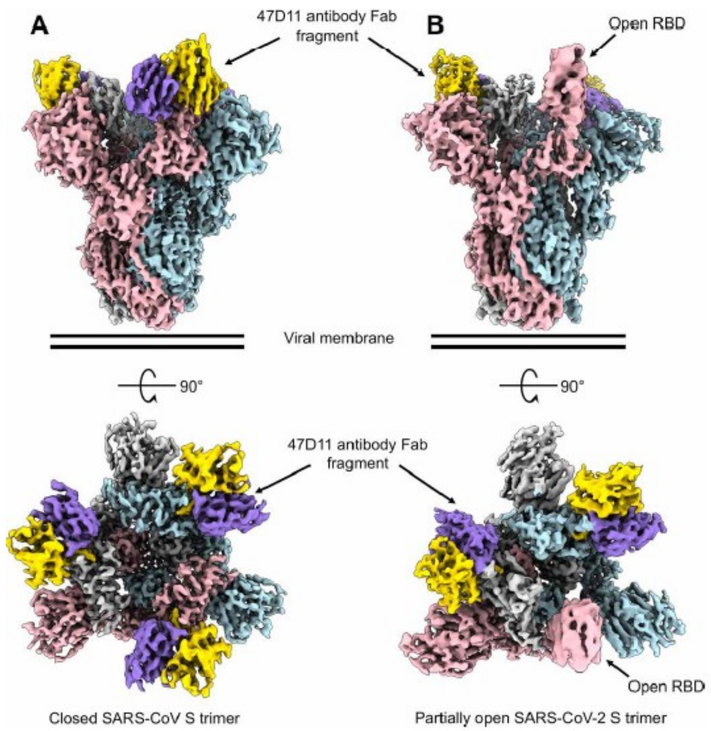
LYS_378_ARG
RMSD_SOLV: 0.033
RMSD_VACU: 0.013



ALA_435_SER
RMSD_SOLV: 0.033
RMSD_VACU: 0.005

Sitio de unión de los anticuerpos neutralizantes en la proteína Spike



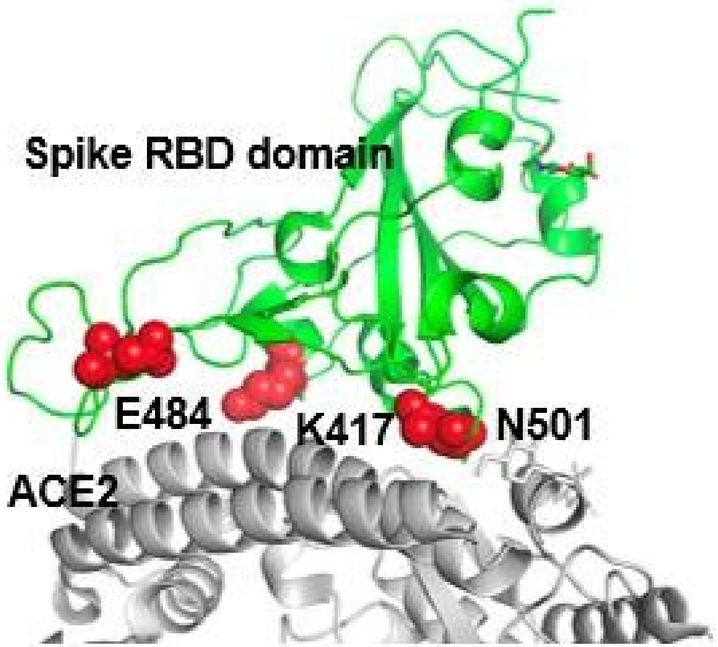


"Hydrophobic pocket" conservado

- Loop CDRL3
- W94 → N343 (SARS2)
- CDRH3
- W102/F103
- F338, F342, Y365, V367, L368, F374 y W436

Fedry *et al.*, *Sci. Adv.* 2021

Structural visualization of spike RBD-hACE2 complex



The three RBD mutations are at key residues interacting with the human ACE2 receptor and with neutralizing antibodies (NAbs)

N501Y enhances binding affinity to ACE2

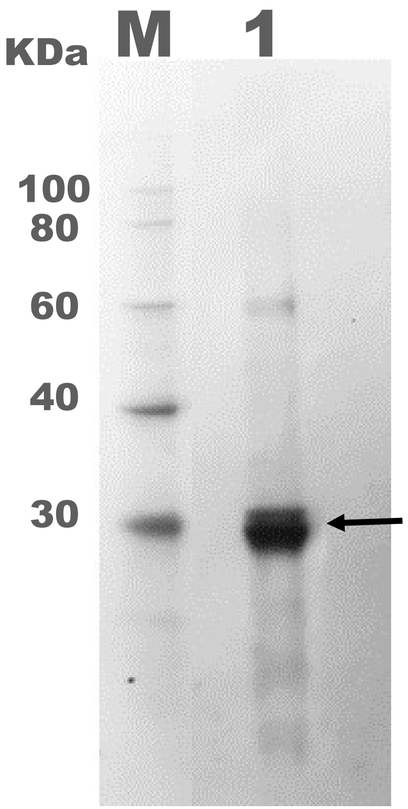
E484K enhances binding affinity to ACE2 and confers resistance to class 2 NAbs

K417N would abolish key interactions with class 1 NAbs, and likely contributes toward immune evasion at this site

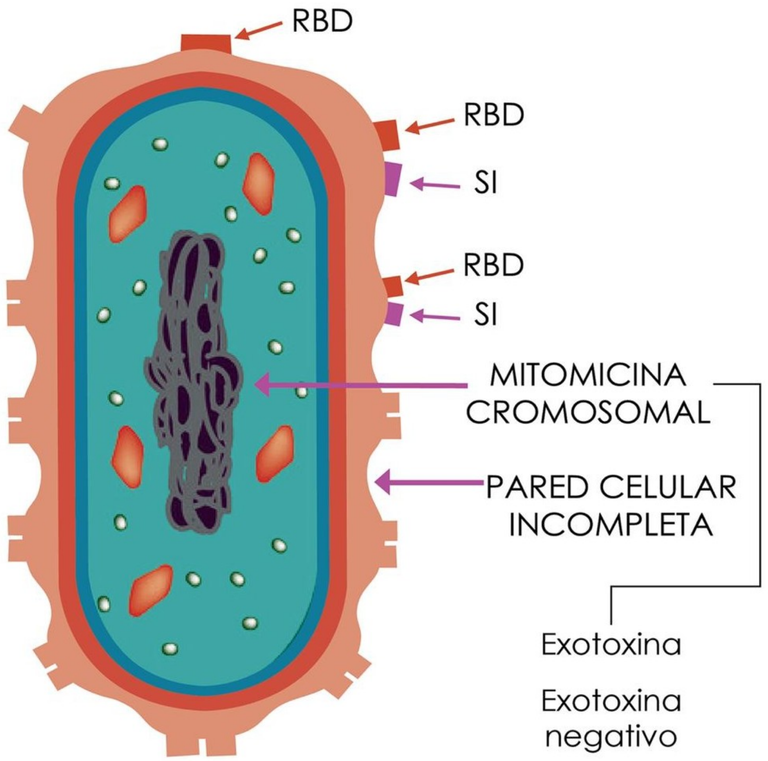
Los 3 tipos de vacuna que se vienen desarrollando

Adicionalmente esta vacuna lleva la flagelina de la Salmonella, la cual es reconocida por ser un potente inmunoestimulad

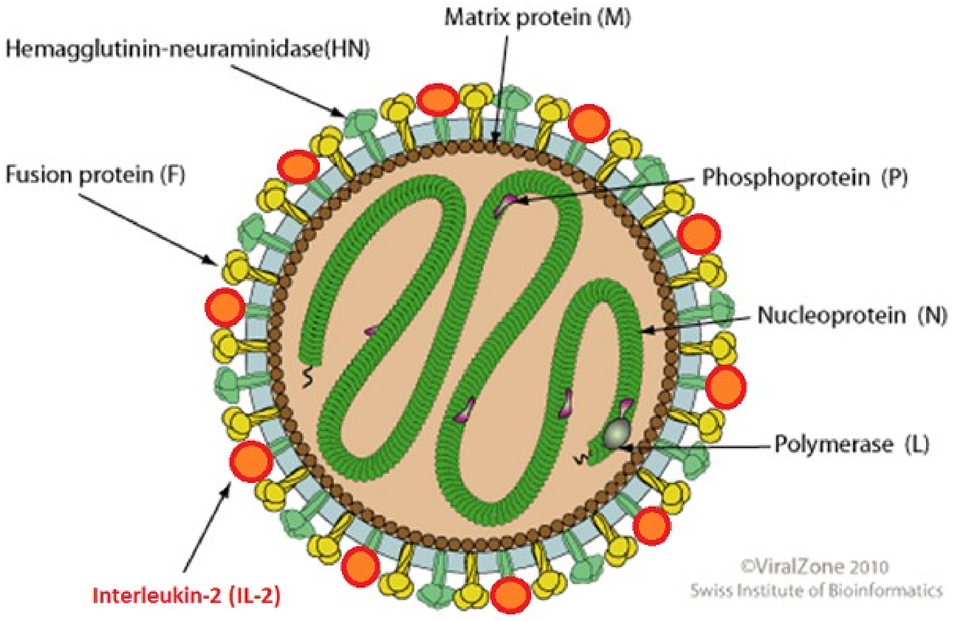
La salmonella es reconocida por inducer una respuesta Th1 y una baja respuesta Th2. Esto es importante para reducir el riesgo de exhacerbación de la respuesta inflamatoria



Proteina RBD “pura” expresada en células Sf9 – baculovirus purificada y combinada con un adyuvante



Salmonella genéticamente Modificada (no-patógena por doble Mutación) expresando RBD en la superficie (Patente FARVET)



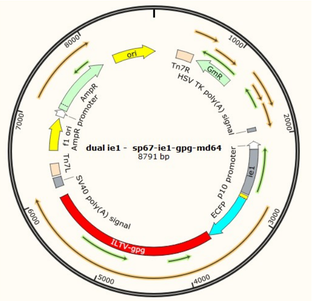
Virus de Newcastle genéticamente modificado expresando RBD en la superficie (Patente FARVET)

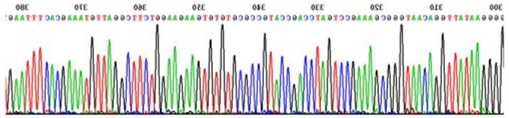
1. Tecnología ADN Recombinante:

Clonación , expresión en células de insecto

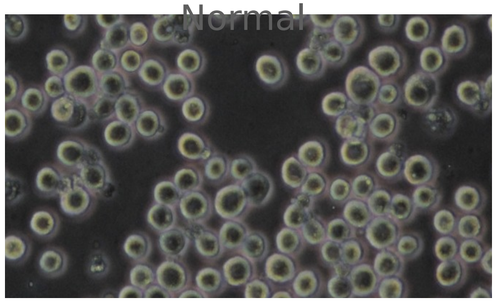
RECOMBINANT VACCINE Proteína Recombinante SPIKE COVID 19

Diseño ADN





Célula de insecto Normal



Cel. Insecto “Recombinante” Mostrando “RBD – Fluorescente ó Rojo”

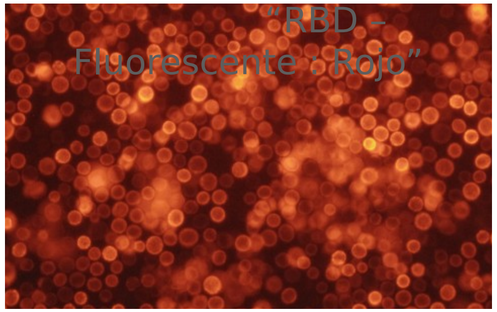
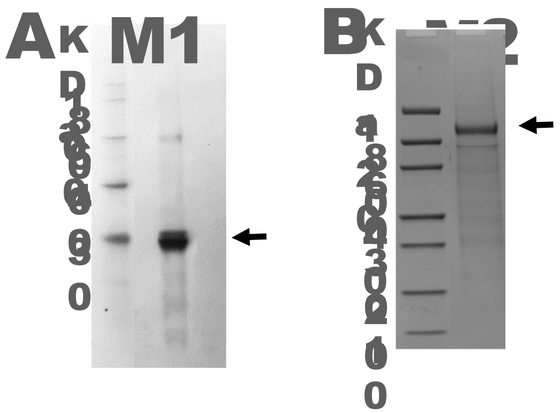


Fig. 1A Fig. 1B Fig. 1C

Figura 1. Equipos utilizados para la producción de proteínas recombinantes. (1A-1B) Biorreactores STR y controlador BIOSTAT B plus-c Twin manteniendo cultivos de células de insecto Sf9. Sartoflow**(1C)** sistema de flujo tangencial para la clarificación y concentración de sobrenadantes de cultivo.

Figura 2. Equipo automatizado para cromatografía AKTA Pure, utilizado para la purificación de proteínas recombinantes



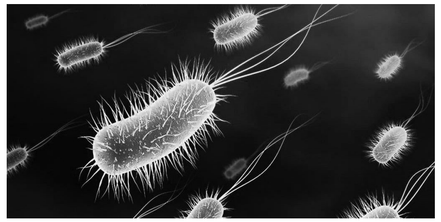
Purificacion de RBD y S1 recombinante. (A) SDS-PAGE RBD por Coomassie (B) SDS-PAGE S1 Coomassie .

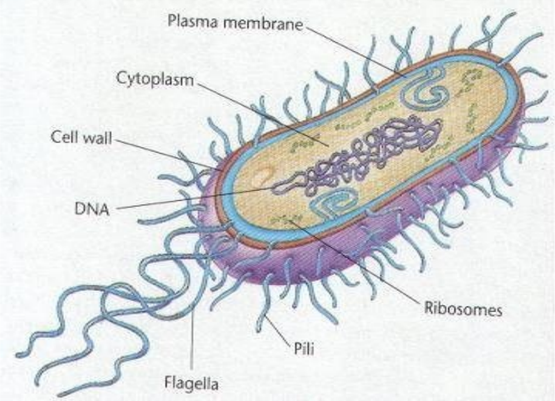
- **1.- Antígeno RBD.-** 150µg (250µl+750µl PBS)/1mL adyuvante FAR23520
- **2.- Antígeno S1.-** 150µg (250µl+750µl PBS)/1mL adyuvante FAR23520
- **3.- Antígeno S1+RBD.-** 75µg/75µg (150µl+850µl PBS)/1mL adyuvante FAR23520
- -Mezcla 50% antígeno y 50% de adyuvante (*Seppic* ISA 71 R VG – 36518T)
- - Se pasó por prensa (emulsificador) 2 veces a 40 psi

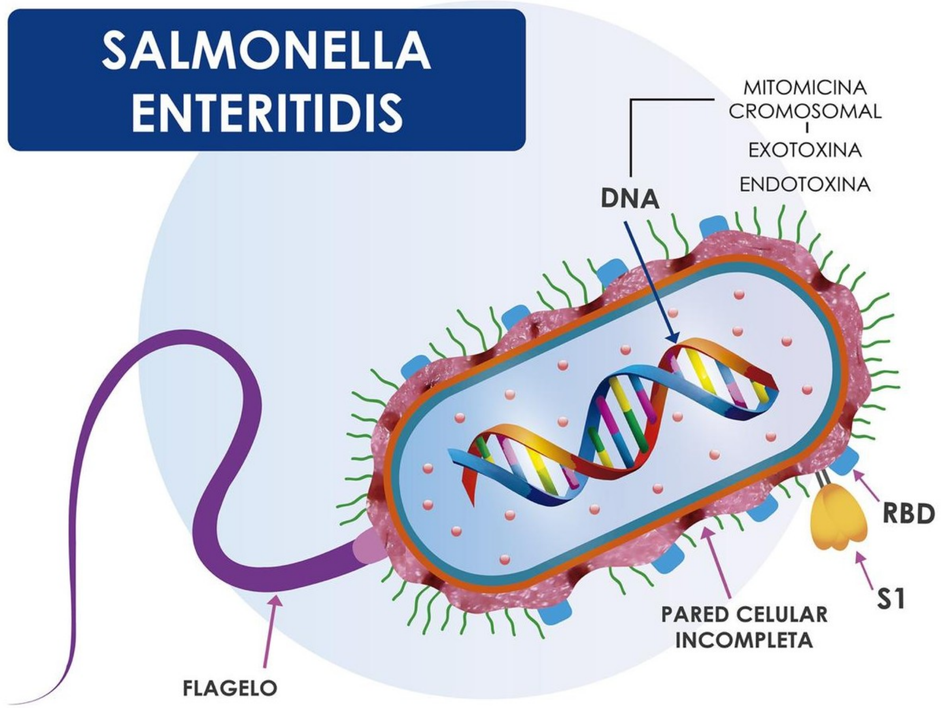
PLANTA DE BIORREACTORES DE ÚLTIMA GENERACIÓN FARVET

BIORREACTOR DE ÚLTIMA GENERACIÓN

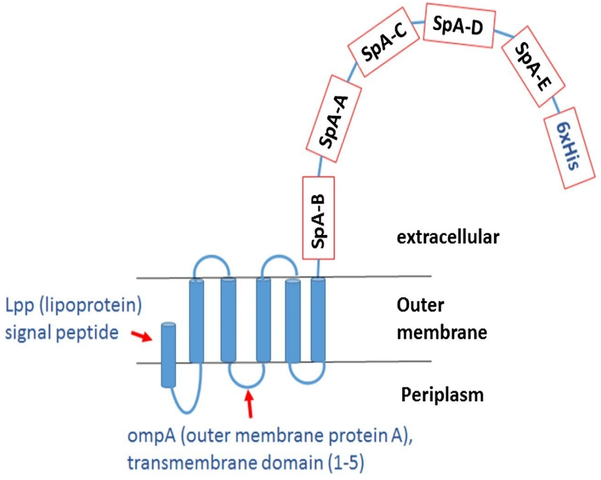
Vacuna oral vectorizada en Salmonella SPIKE COVID 19

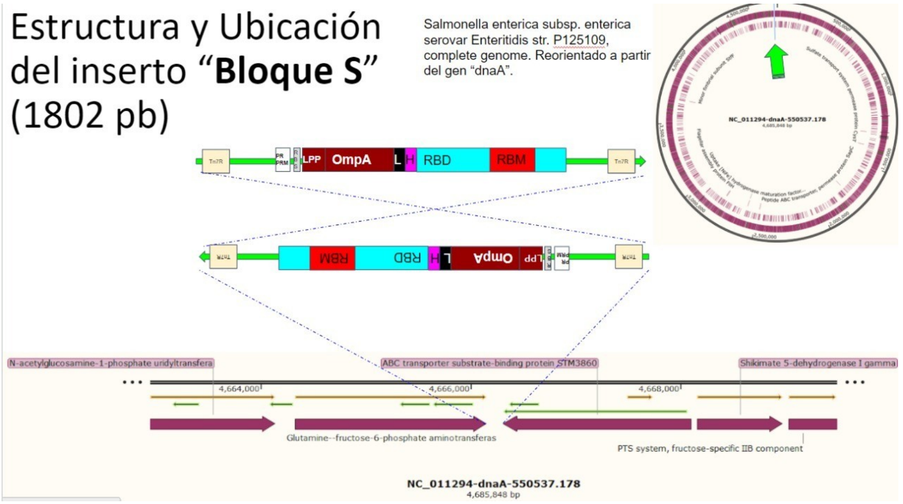






Vacfar 3925
Patente Farvet
SAC





Las 2 primeras vacunas (Proteína recombinante y Salmonella), se han evaluado

Esta evaluación se ha realizado en:
-Ratones
-Conejos
-Gallinas
-Alpacas
-Monos aotus

Los ensayos en Conejos, gallinas y alpacas, se orientaron a evaluar mayorment
la seguridad y la capacidad de elicitar anticuerpos IgG.

La vacuna de NDV comenzó tarde en el año y se ha evaluado en
-ratones
-hamsters

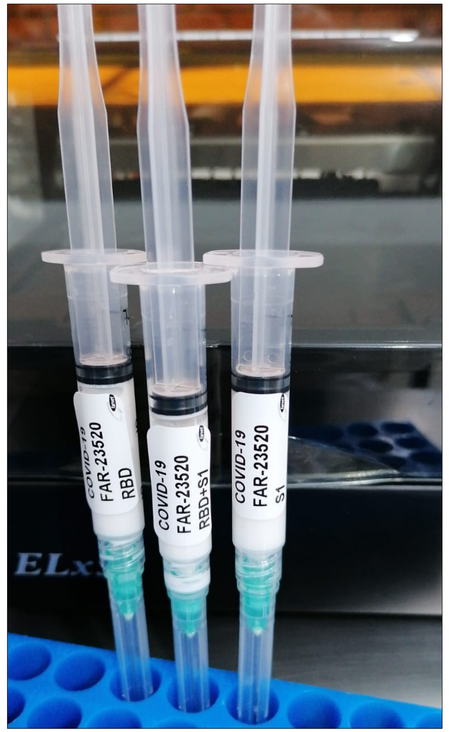
DANGER

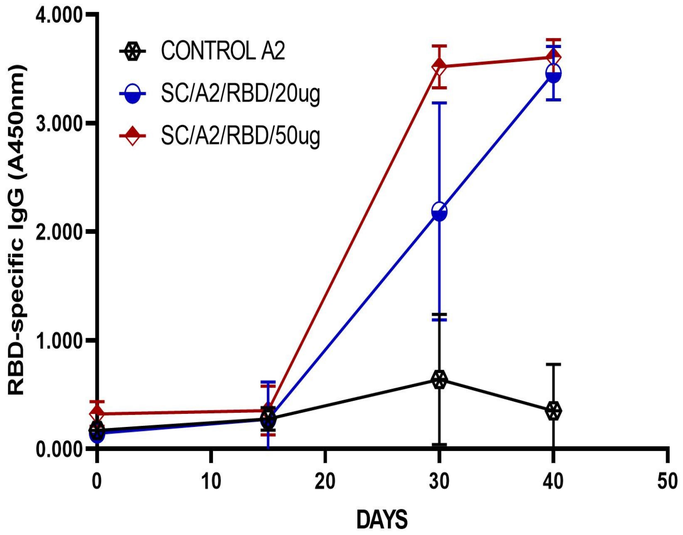
Dr. MANOLO FERNANDEZ
Vaccine

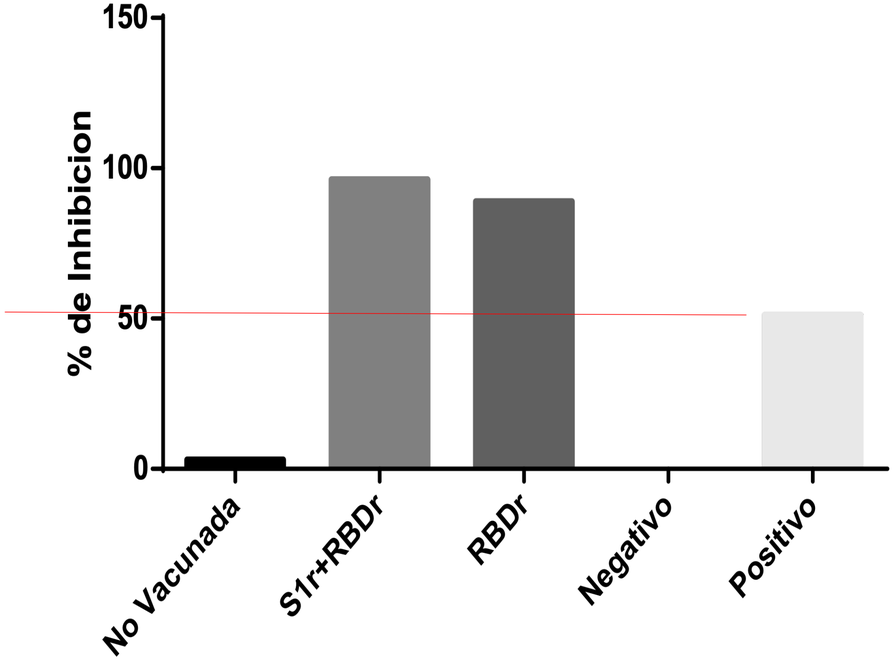
Monos AOTUS, que de Preferencia deberían ser MACACOS RHESUS

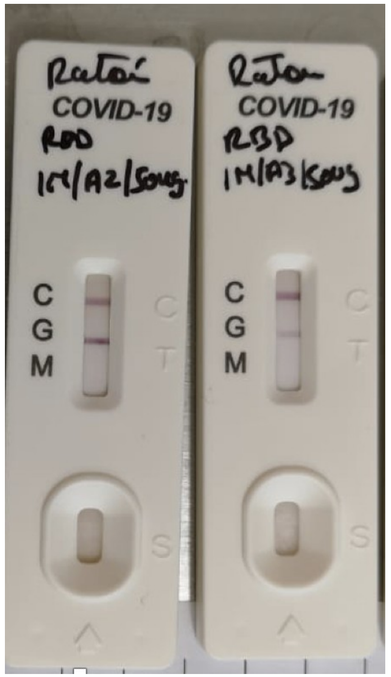
ansporte de los monos en
Jaulas/cajas especiales

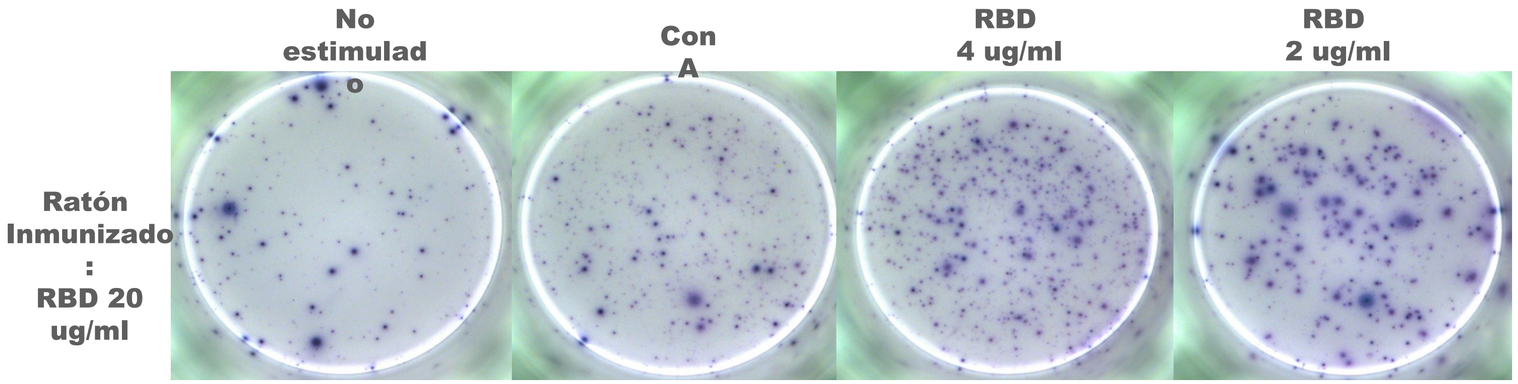
Evidencia de presencia de inmunidad humoral y celular en ratonnes inmunizados con la vacuna de Proteína RBD recombinante



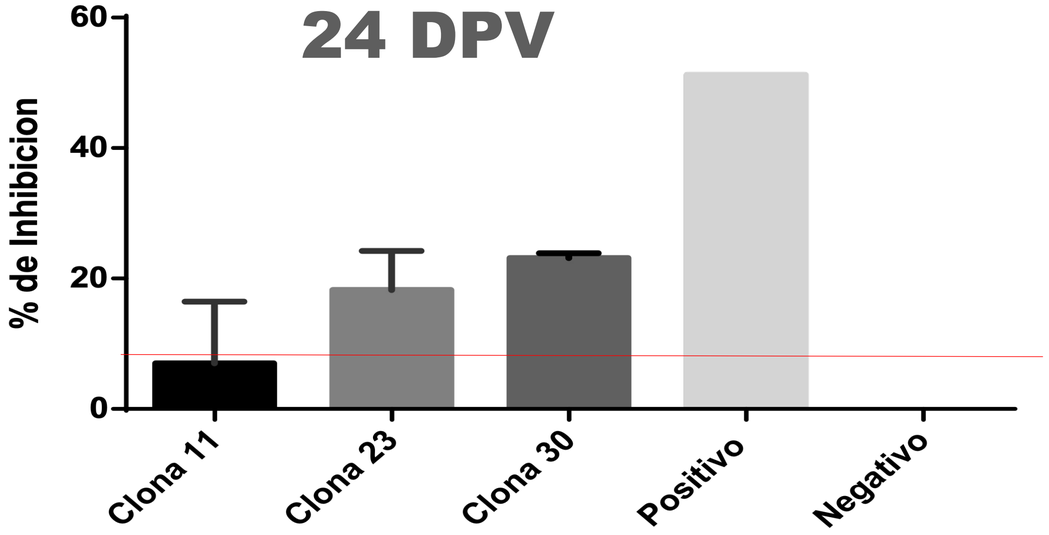


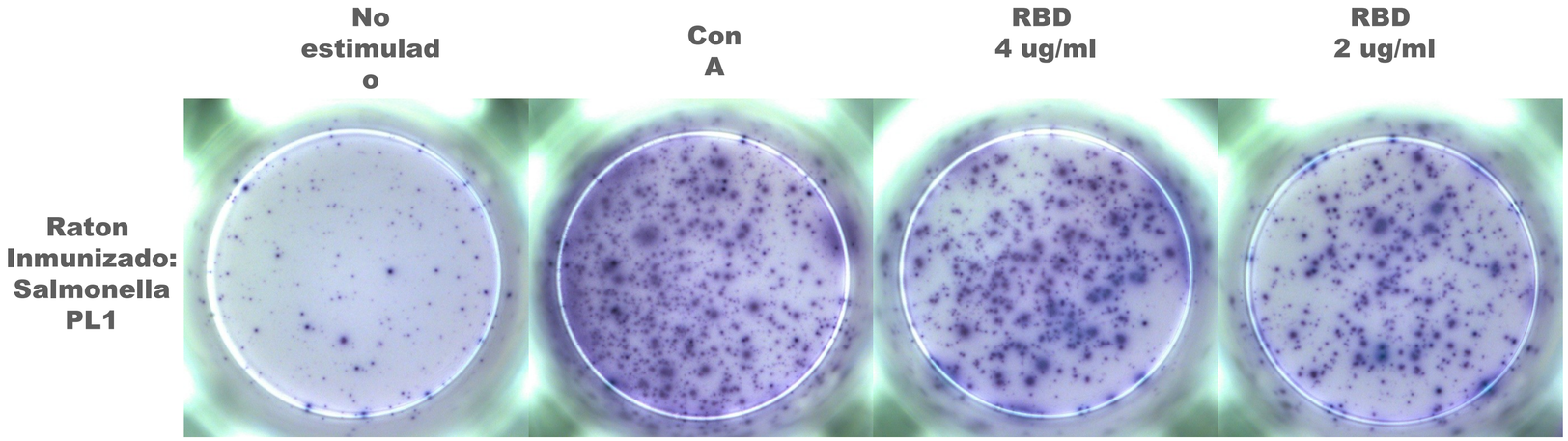




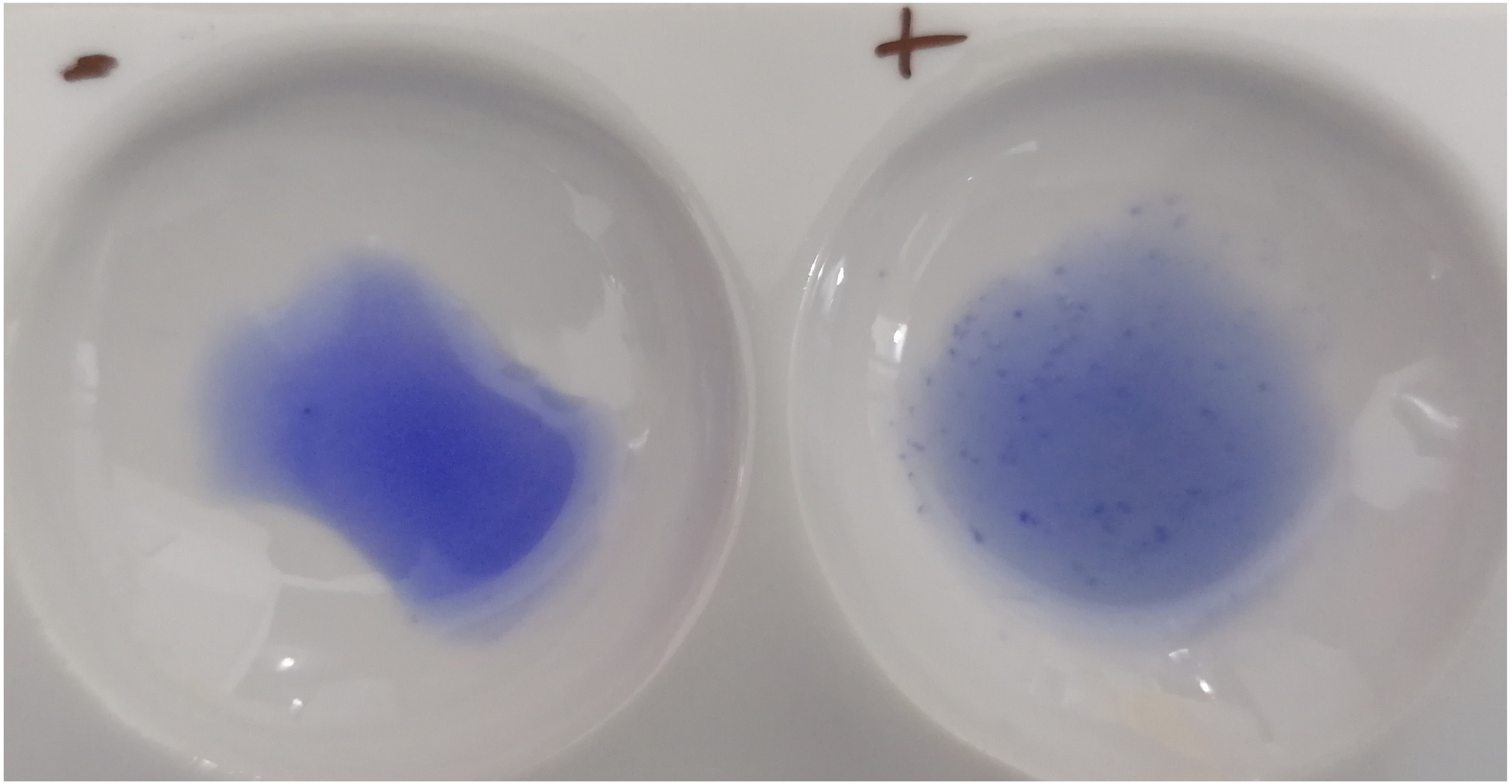


EL SUERO DE RATONES INMUNIZADOS CON SALMONELLA-RBD HA MOSTRADO CAPACIDAD DE NEUTRALIZACIÓN DEL VIRUS EN CULTIVOS CELULARES

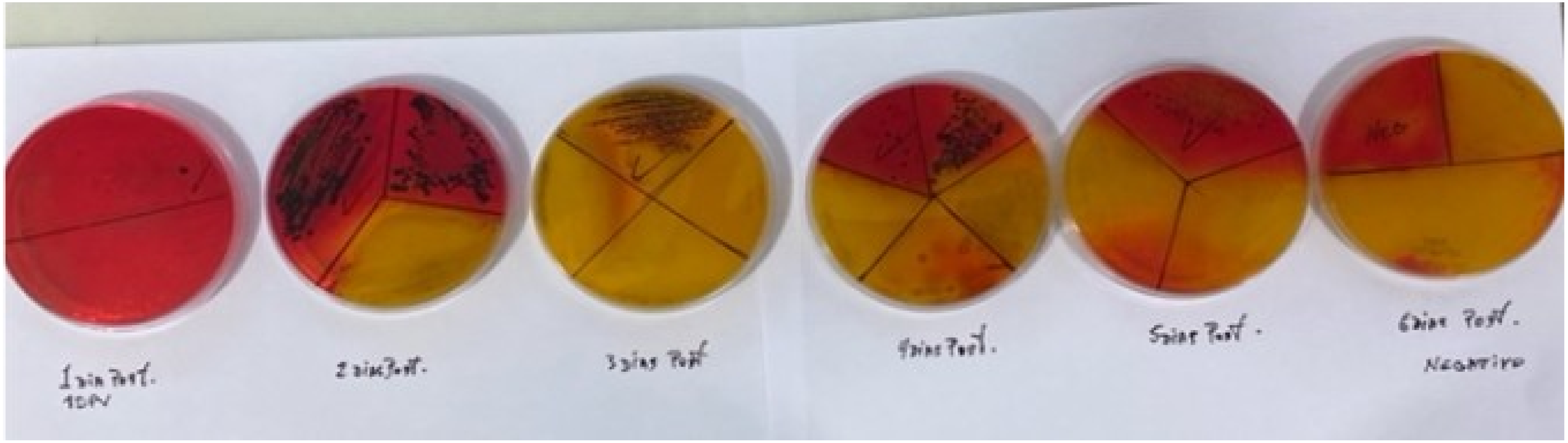




Prueba de aglutinacion rápida para detectar anticuerpos COVID 19 con Salmonella modicada en monos Aotus vacunados (21 días post-inmunización)



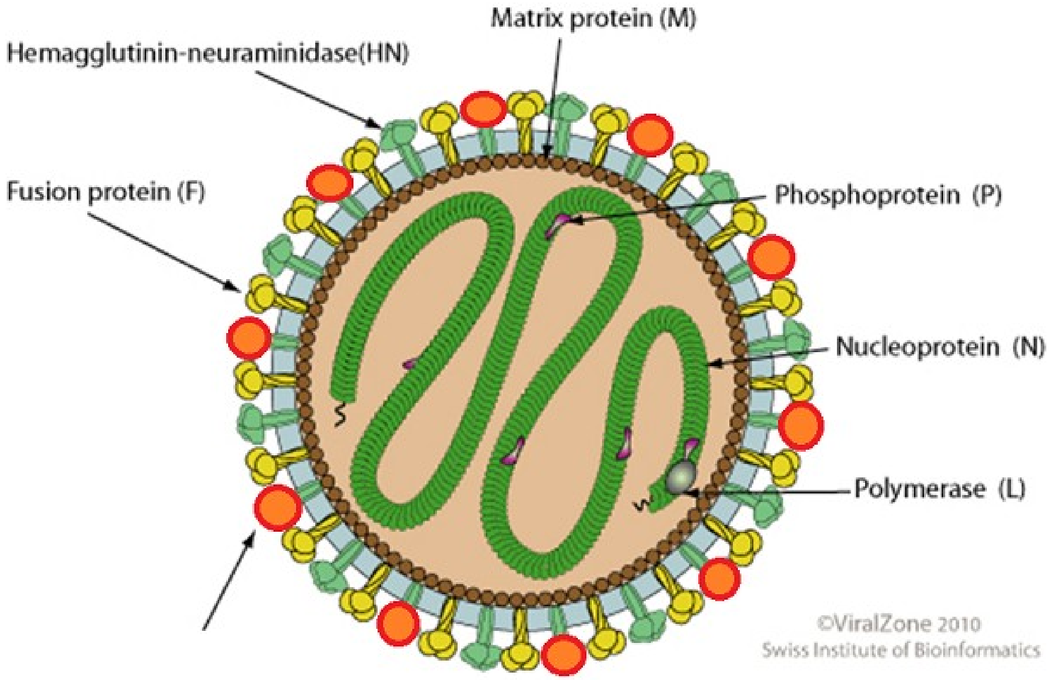
Deteccion de Salmonella RBD en heces monos inmunizados con la vacuna de salmonella: Los monos dejan de excretar la salmonella al día 6



Vacuna Peruana COVID-19 NDV-S1

Strain La Sota
Isolated in 1960
Attenuated - lentogenic - no-vurulent strain
Used in avian vaccines
Oncolytic virus:
-Has been evaluated for cancer treatment in humans
-Phase II clinical trial in cancer patients. Intravenous administration
-Other studies have used other more virulent NDV strains

SARS-Cov-2 spike gene inserted in the genome
spike protein expressed as a surface protein exposed on the virion



Spike Covid 19

Newcastle Disesase Virus -based COVID vaccine design:

Strain La Sota
Isolated in 1960
Attenuated - lentogenic - no-vurulent strain
Used in avian vaccines
Oncolytic virus:
-Has been evaluated for cancer treatment in humans
-Phase II clinical trial in cancer patients. Intravenous administration
-Other studies have used other more virulent NDV strains

SARS-Cov-2 spike gene inserted in the genome
spike protein expressed as a surface protein exposed on the virion

Review Article

Safety and Clinical Usage of Newcastle Disease Virus in Cancer Therapy

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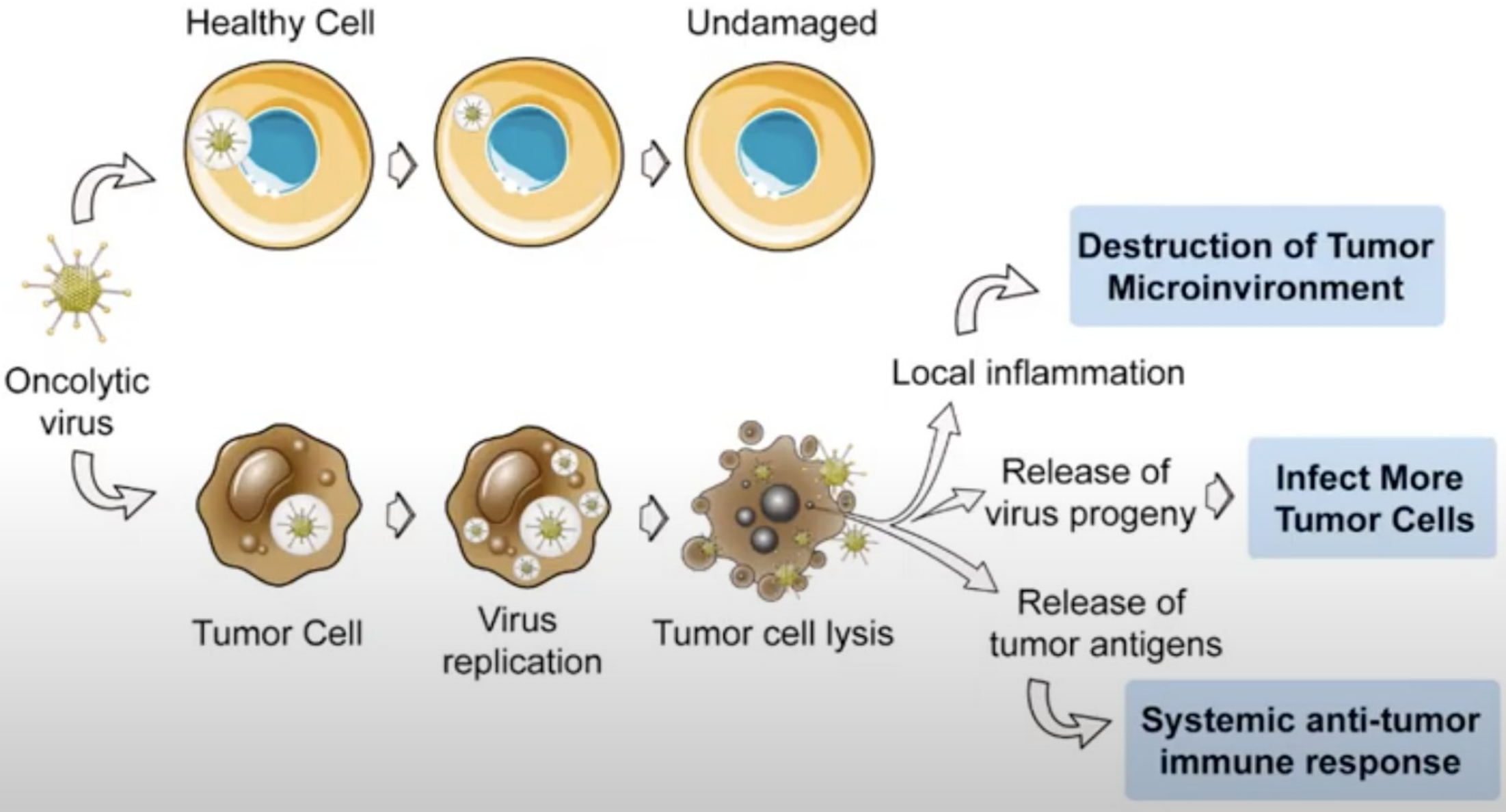
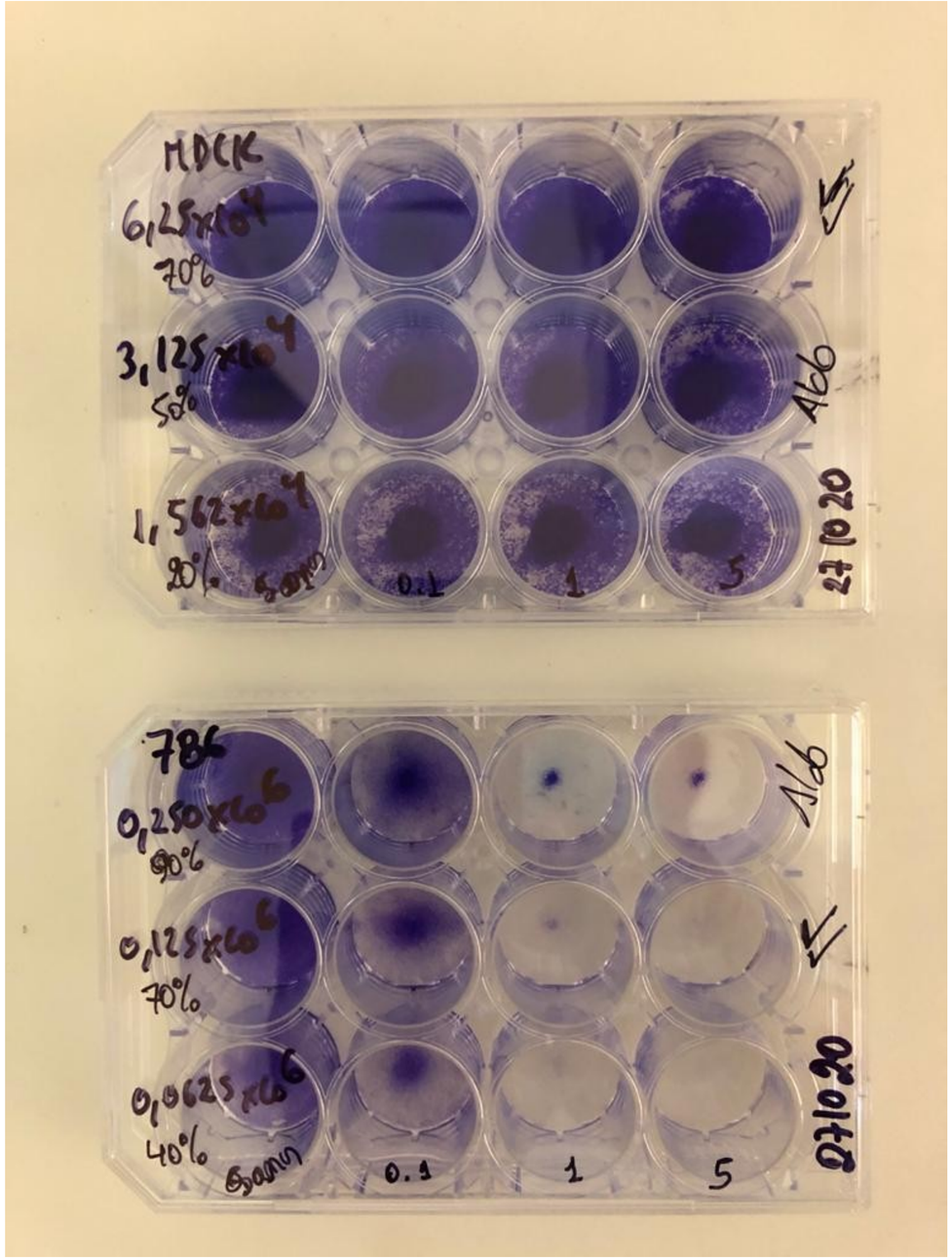
Newcastle disease virus (NDV) is an avian virus that causes deadly infection to over 250 species of birds, including domestic and wild-type, thus resulting in substantial losses to the poultry industry worldwide. Many reports have demonstrated the oncolytic effect of NDV towards human tumor cells. The interesting aspect of NDV is its ability to selectively replicate in cancer cells. Some of the studies have undergone human clinical trials, and favorable results were obtained. Therefore, NDV strains can be the potential therapeutic agent in cancer therapy. However, investigation on the therapeutic perspectives of NDV, especially human immunological effects, is still ongoing. This paper provides an overview of the current studies on the cytotoxic and anticancer effect of NDV via direct oncolysis effects or immune stimulation. Safety of NDV strains applied for cancer immunotherapy is also discussed in this paper.

ACTIVIDAD ONCOLITICA DEL VIRUS DE NEWCASTLE





	G	H	I
2			
3	OBSERVACIONES:		
4	ESPECIE		TEJIDO
5	MOUSE	FIBROBLAST	EMBRYO: NORMAL FIBRO
6	HUMAN	EPITHELIAL	KIDNEY:RENAL CELL CAR
7	RAT	STEM	DE TEJIDO ADIPOSO-EXPRI
8	MOUSE	SPINDLE	SKIN MELANOMA MOUSE
9	MOUSE	SPINDLE	SKIN MELANOMA MOUSE
10		LYMPHOBLAST	
11	BACTERIAS		
12	HUMAN	EPITHELIAL	CHORIOCARCINOMA-PLA
13	HAMSTER	FIBROBLAST	KIDNEY
14	HUMAN	FIBROBLAST	FORESKIN
15	MOUSE	MYOBLAST	MUSCULO
16	HUMAN	EPITHELIAL	COLON: ADENOCARCINOMA
17	MOUSE-C3H		GASTRIC CARCINOMA
18	HUMAN	EPITHELIAL	COLON: ADENOCARCINOMA
19	HUMAN		PROSTATA NORMAL
20	HUMAN	ENDOTHELIAL	ENDOTHELIUM: UMBILICAL
21	HUMAN	EPITHELIAL	CANCER GASTRICO
22	HAMSTER	EPITHELIAL	OVARY
23	HUMAN	EPITHELIAL	COLON: ADENOCARCINOMA

Es interesante destacar que el virus NDV es oncolítico. Por esta razón, se vienen realizando diversos estudios para evaluar el potencial efecto terapéutico de NDV en algunos tipos de cancer. Actualmente existe un estudio clínico fase II, en el cual se administra NDV vía intravenosa, a pacientes voluntarios con cancer.

Tumor Venéreo Transmisible Canino

Tumor Venéreo Transmisible Canino 07 Días Después del Tratamiento con Virus de Newcastle – Cepa FARVET

Newcastle disease virus, a host range-restricted virus, as a vaccine vector for intranasal immunization against emerging pathogens

Joshua M. DiNapoli*, Alexander Kotelkin*, Lijuan Yang*, Subbiah Elankumaran†, Brian R. Murphy*, Siba K. Samal†, Peter L. Collins*, and Alexander Bukreyev*‡

*Laboratory of Infectious Diseases, National Institute of Allergy and Infectious Diseases, National Institutes of Health, Bethesda, MD 20892; and †University of Maryland, College Park, MD 20742

Communicated by Robert M. Chanock, National Institutes of Health, Bethesda, MD, April 24, 2007 (received for review January 22, 2007)

The international outbreak of the severe acute respiratory syndrome-associated coronavirus (SARS-CoV) in 2002–2003 highlighted the need to develop pretested human vaccine vectors that can be used in a rapid response against newly emerging pathogens. We evaluated Newcastle disease virus (NDV), an avian paramyxovirus that is highly attenuated in primates, as a topical respiratory vaccine vector with SARS-CoV as a test pathogen. Complete recombinant NDV was engineered to express the SARS-CoV spike S glycoprotein, the viral neutralization and major protective antigen, from an added transcriptional unit. African green monkeys immunized through the respiratory tract with two doses of the vaccine developed a titer of SARS-CoV-neutralizing antibodies comparable with the robust secondary response observed in animals that have been immunized with a different experimental SARS-CoV vaccine and challenged with SARS-CoV. When animals immunized with NDV expressing S were challenged with a high dose of SARS-CoV, direct viral assay of lung tissues taken by necropsy at the peak of viral replication demonstrated a 236- or 1,102-fold (depending on the NDV vector construct) mean reduction in pulmonary SARS-CoV titer compared with control animals. NDV has the potential for further development as a pretested, highly attenuated, intranasal vector to be available for expedited vaccine development for humans, who generally lack preexisting immunity against NDV.

severe acute respiratory syndrome (SARS) | respiratory tract | monkey

The severe acute respiratory syndrome-associated coronavirus (SARS-CoV) emerged as a human pathogen in Southern China in 2002 and spread internationally, resulting in 8,096 infections and 774 deaths between November 1, 2002, and July 31, 2003, as well as considerable alarm and economic loss (1). There is a clear need for a safe and effective vaccine should an outbreak of a SARS-like virus reoccur in humans. In addition, the SARS experience highlighted the importance of having pretested, safe, and effective vectors on hand that can be used to accelerate initial steps of vaccine development should other new pathogenic agents emerge.

Inactivated whole-virus preparations of SARS-CoV have been shown to induce protective immunity in small-animal models (2–5) and primates (6, 7). However, large-scale growth of a highly pathogenic agent introduces the risk of laboratory infection, which indeed has occurred (8). Furthermore, incomplete inactivation can leave residual infectivity in such vaccines, posing further risk to vaccinees (9, 10). The SARS-CoV spike S glycoprotein is the viral neutralization and major protective antigen (11). Full-length or fragments of recombinant purified S have been shown to be immunogenic in small-animal models, but their safety and efficacy have not yet been evaluated in primates. Furthermore, inactivated whole-virus or protein subunit vaccines are not necessarily safe and effective: in the case of measles and respiratory syncytial virus, they induced altered immune responses, resulting in immune-mediated enhancement of disease upon subsequent exposure to the pathogen (12, 13).

Vectored vaccines offer a live-vaccine approach that does not involve the complete pathogen. A number of vectored vaccines against SARS-CoV have been described. A recombinant vesicular stomatitis virus (VSV) expressing the SARS-CoV S protein was protective in mice (14) but has not been tested in primates, and the safety of VSV in humans remains to be established. Recombinant modified vaccinia Ankara (MVA) virus expressing SARS-CoV S protein was immunogenic and protective in rodent and primate models (15–17); however, one group found that the MVA/SARS-CoV S-immunized ferrets developed hepatitis upon challenge with SARS-CoV (17). Vaccine constructs based on replication defective human adenovirus type 5 expressing a partial or full-length SARS-CoV S protein have been evaluated for immunogenicity in rats and monkeys (18, 19), but immunization depends on a high vaccine dose, and safety and protective efficacy remain to be demonstrated. We previously used an attenuated version of human parainfluenza virus type 3, a common pediatric respiratory pathogen, to express the SARS-CoV S protein and showed that a single intranasal (i.n.) and intratracheal (i.t.) inoculation was immunogenic and protective against SARS-CoV challenge in hamsters and African green monkeys (AGM) (20). This vector has a natural tropism for the respiratory tract and does not significantly spread beyond that site, properties desirable for use as a safe, restricted, topical respiratory tract vaccine. However, a concern that exists with any vector based on a common pathogen is that the adult population has significant immunity from prior exposure that will restrict infection and replication of the viral vector and reduce its immunogenicity. Indeed, comparisons of the immunogenicity of vaccinia virus-vectored and human adenovirus type 5-vectored vaccines in rodents, non-human primates, and humans demonstrated that preexisting immunity to the vector greatly reduced the immunogenicity of these vaccines (21–23).

An additional limitation of previous studies of vectored and inactivated SARS-CoV vaccines in non-human primates, the model that is most similar to humans, is that protective efficacy was measured by quantitation of challenge SARS-CoV shed in respiratory secretions (6, 7, 16, 20). It is now known that the virus

Author contributions: J.M.D., B.R.M., S.K.S., P.L.C., and A.B. designed research; J.M.D., A.K., L.Y., S.E., S.K.S., and A.B. performed research; S.K.S. contributed new reagents/analytic tools; J.M.D., B.R.M., S.K.S., P.L.C., and A.B. analyzed data; and J.M.D., B.R.M., P.L.C., and A.B. wrote the paper.

The authors declare no conflict of interest.

Abbreviations: AGM, African green monkeys; BC, Beaudette C; HAI, hemagglutination inhibition; i.n., intranasal; i.t., intratracheal; MVA, modified vaccinia Ankara; NDV, Newcastle disease virus; PBMC, peripheral blood mononuclear cell; SARS-CoV, severe acute respiratory syndrome-associated coronavirus; $TCID_{50}$, tissue culture 50% infectious dose.

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This article contains supporting information online at www.pnas.org/cgi/content/full/0703584104/DC1.

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Además del esfuerzo Peruano en Farvet, existen otros esfuerzos mundiales de buscar producir una vacuna para Covid, basada en el virus NDV. Entre ellos, los conocidos son:

-El grupo Mount Sinai/Avimex,

-El grupo Holandés en la Universidad de Wageningen en colaboración con un centro biotecnológico veterinario,

-El grupo de la Universidad de Texas. Este último ha publicado el resultado del desafío en hamsters, con los mismos resultados que los obtenidos por Farvet.

Desde hace más de 40 años, los vacunadores de pollos se han expuesto por via inhalaltoria al virus NDV, durante los procesos de inmunización, los cuales se realizan por medio de fumigación.

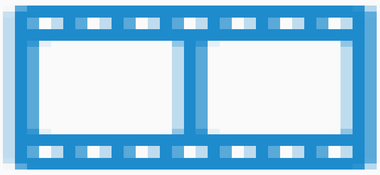
Más recientemente, los vacunadores aplican la vacuna contra el coronavirus aviar, (virus de bronquitis infecciosa IBV), la cual es vectorizada en NDV. Esto confirma que un grupo cercano a 4,000 personas en Chincha se ha expuesto y continúa haciéndolo, al virus NDV mediante inhalación del mismo. En ningún caso se han reportado efectos en la salud de estas personas.

Adicionalmente, y de manera interesante, a lo que va desde el inicio de la pandemia, los aproximadamente 900 vacunadores que aplican rutinariamente la vacuna contra el IBV vectorizada en NDV, no han reportado signos serios de un cuadro de Covid, pese a que en algunos casos, se reportaron familiares con cuadros de Covid en la casa.

MEDIANTE LA VACUNACION VIA NEBULIZACION O SPRAY, SE VACUNAN MAS DE 1,600 MILLONES DE POLLOS MENSUALMENTE, EN CENTRO Y SUDAMERICA.

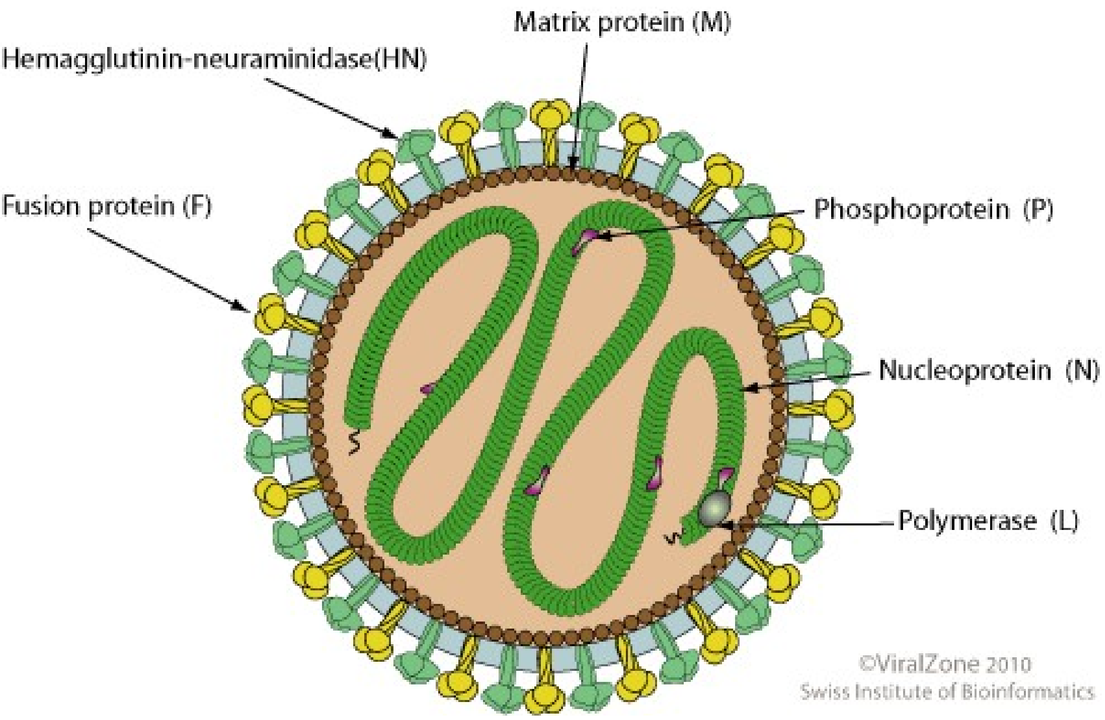
LO REALIZAN APROXIMADAMENTE 160,000 VACUNADORES EN LAS PLANTAS DE INCUBACION Y EN LAS NAVES DE CRIANZA.

LA PRACTICA MASIVA DE VACUNACION SE REALIZA HACE MAS DE 50 AÑOS

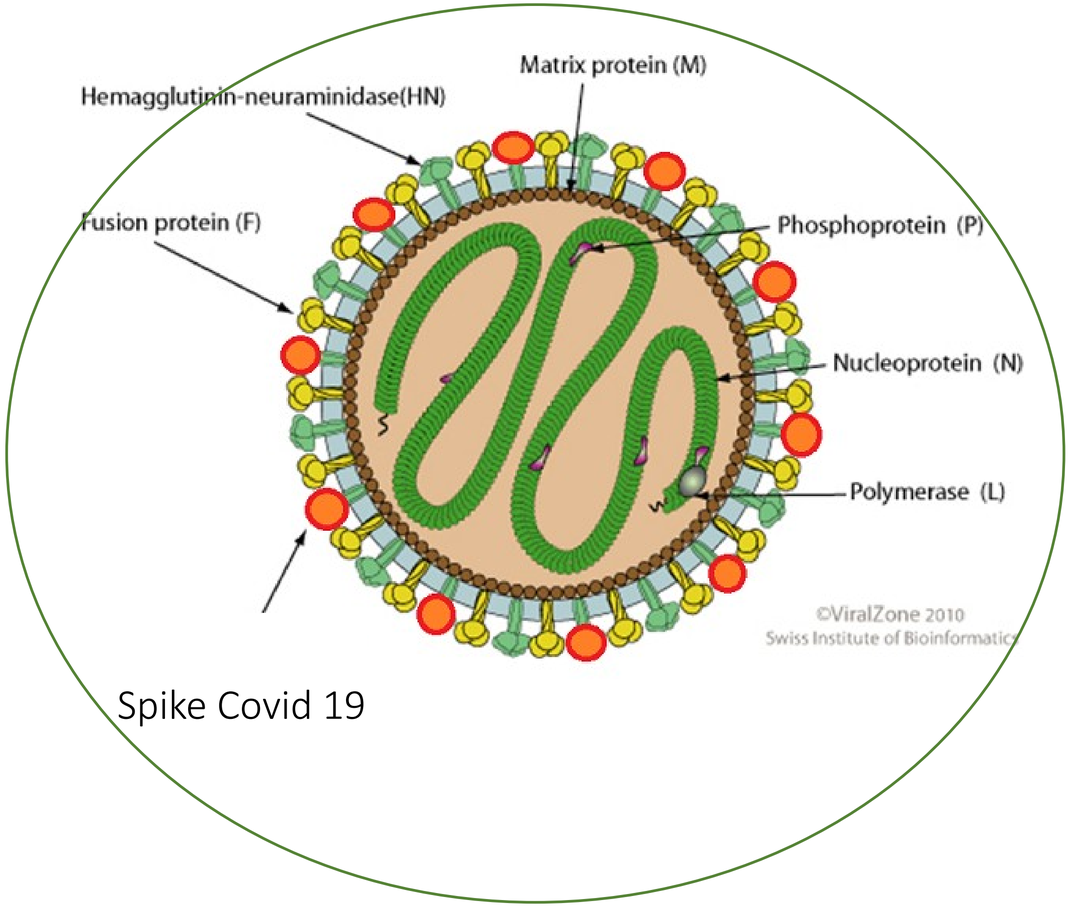


VACUNACION POR SPRAY CON EL VIRUS DE NEWCASTLE

NDV Cuya Superficie Contiene Spike SARS COV 2

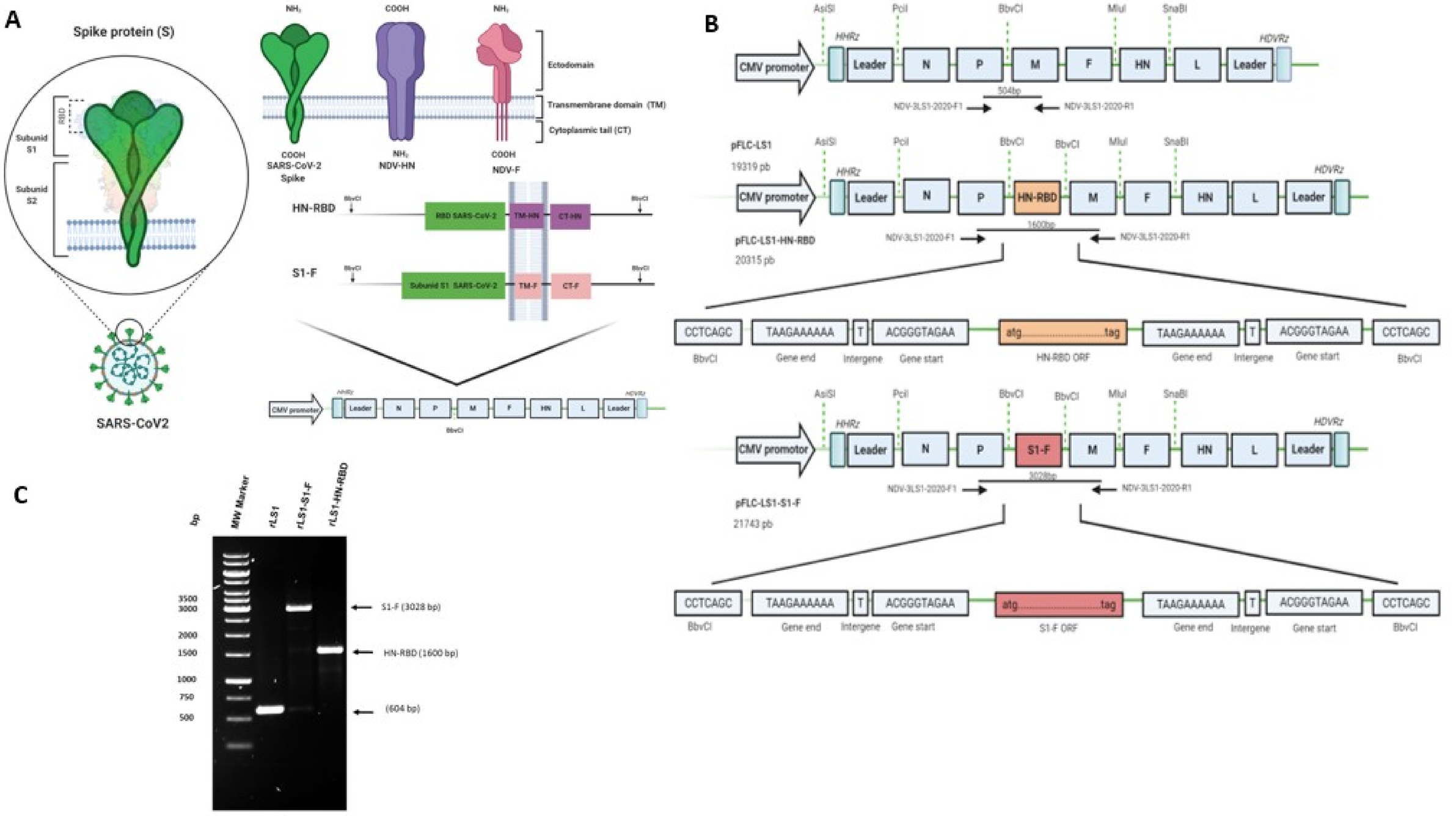


NDV Wild Type

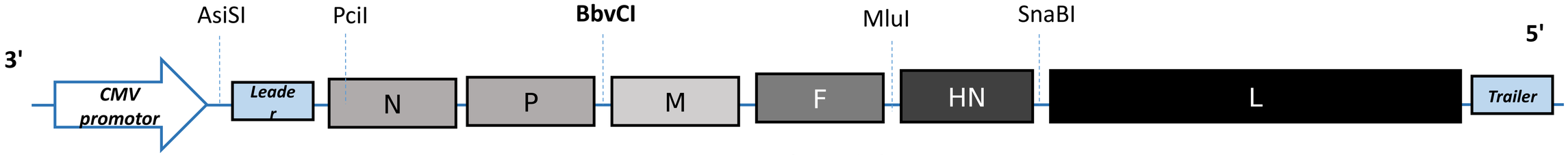


NDV Spike Covid 19

Patente FARVET


A
Spike protein (S)
RBD
Subunid S1
Subunid S2
SARS-CoV2
NH2
COOH
NH2
Ectodomain
Transmembrane domain (TM)
Cytoplasmic tail (CT)
COOH
SARS-CoV-2 Spike
NH2
NDV-HN
COOH
NDV-F
HN-RBD
BbvCI
RBD SARS-CoV-2
TM-HN
CT-HN
S1-F
Subunid S1 SARS-CoV-2
TM-F
CT-F
CMV promoter
HHRz
Leader
N
P
M
F
HN
L
HDVRz
B
AsiSI
PciI
BbvCI
MluI
SnaBI
504bp
NDV-3LS1-2020-F1
NDV-3LS1-2020-R1
pFLC-LS1
19319 pb
1600bp
pFLC-LS1-HN-RBD
20315 pb
CCTCAGC
TAAGAAAAAA
T
ACGGGTAGAA
atg.....tag
HN-RBD ORF
Gene end
Intergene
Gene start
S1-F
3028bp
pFLC-LS1-S1-F
21743 pb
S1-F ORF
C
bp
MW Marker
rLS1
rLS1-S1-F
rLS1-HN-RBD
3500
3000
2000
1500
1000
750
500
S1-F (3028 bp)
HN-RBD (1600 bp)
(604 bp)

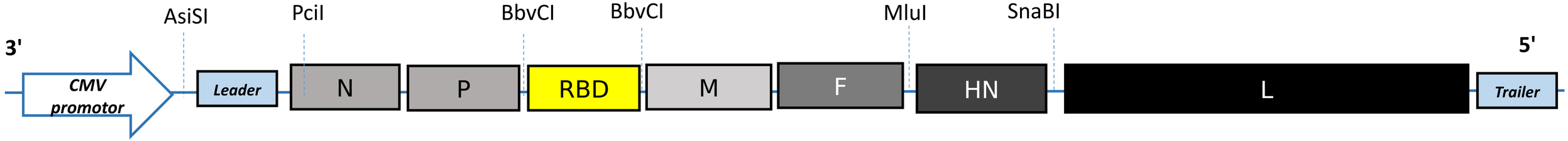
CONSTRUCCIÓN *IN SÍLICO* DEL rNDV-RBD/SARS-CoV-2



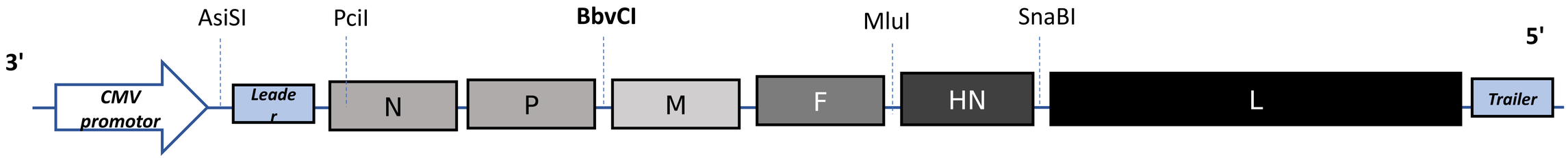
Genoma rNDV (15186 bp)

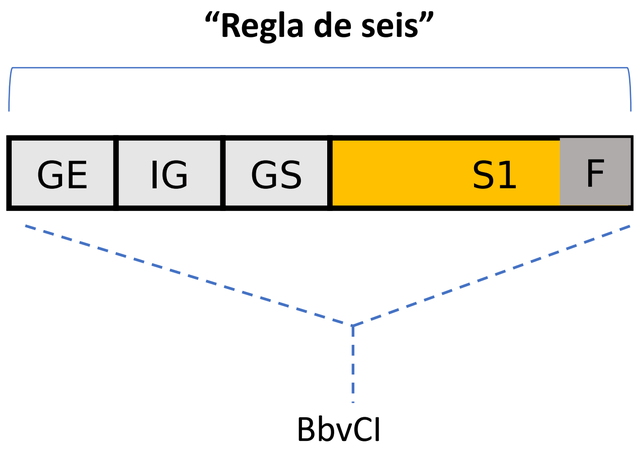
Genoma rNDV-S1/F-SARS-CoV-2 (17610 bp)

CONSTRUCCIÓN *IN SÍLICO* DEL rNDV-S1/SARS-CoV-2

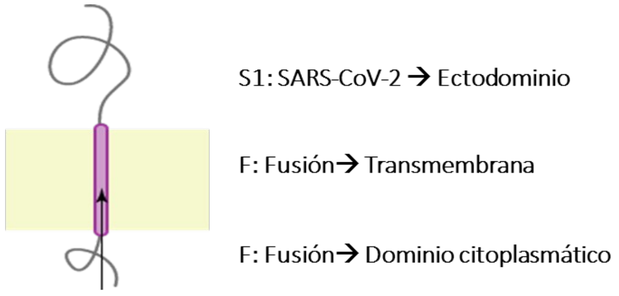


Genoma rNDV (15186 bp)

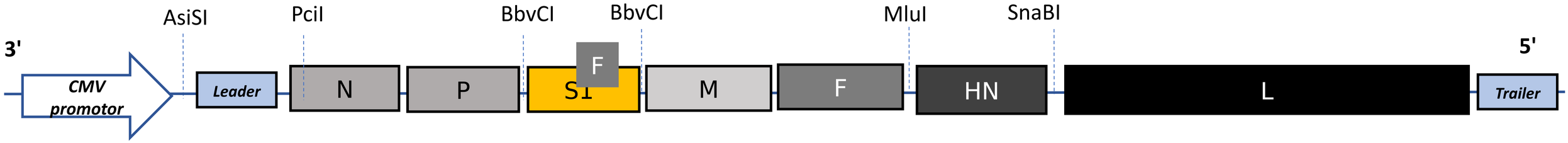




GE: GEN END
IG: INTERGENIC REGION
GS: GEN START
S1: GEN FORÁNEO DEL SARS-CoV-2

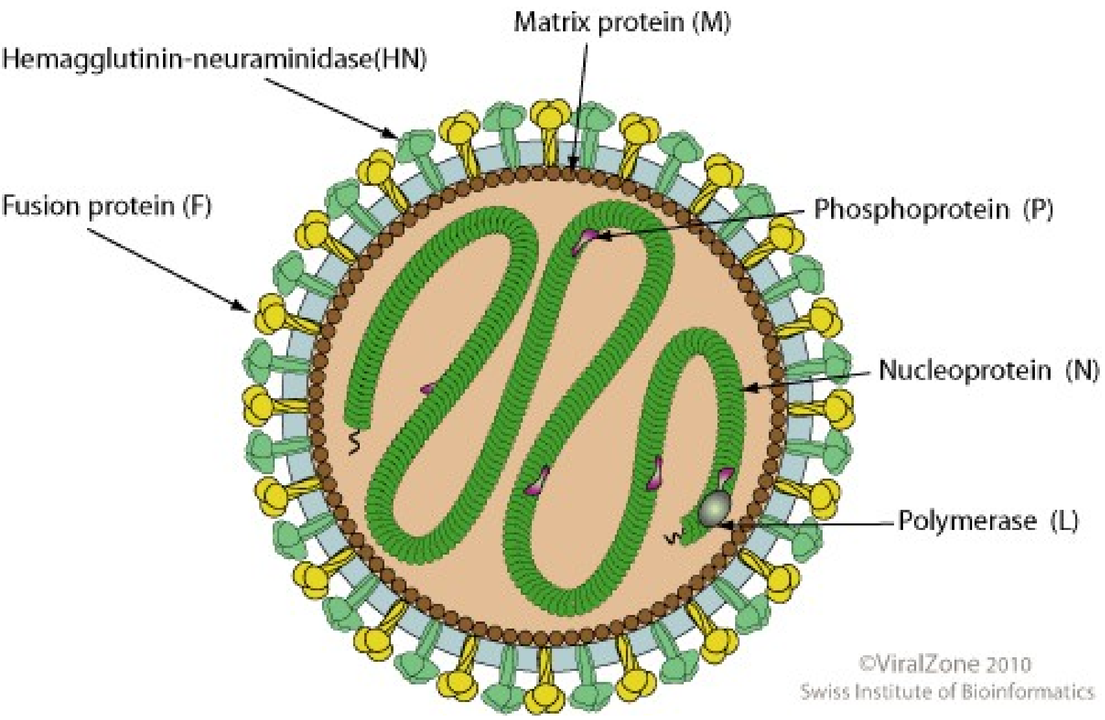


Incorporación la proteína S1 en el virión

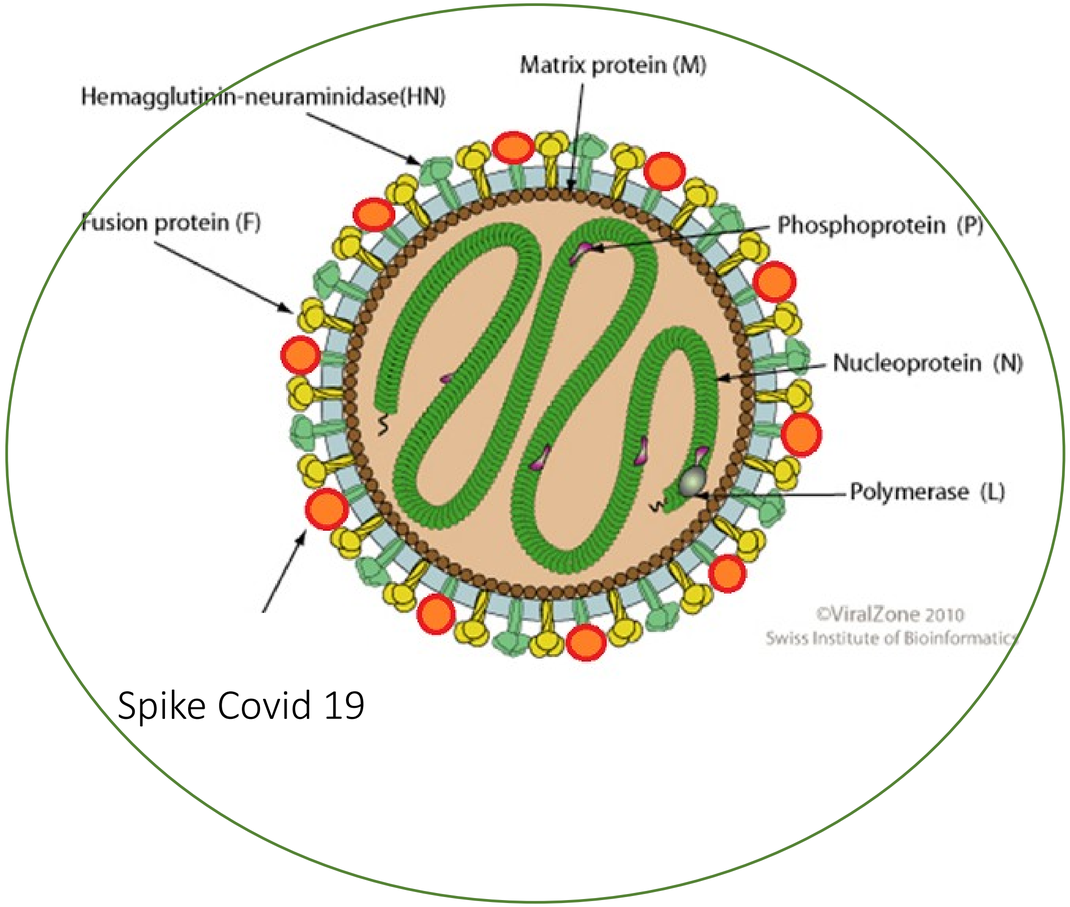


Genoma rNDV-S1/F-SARS-CoV-2 (17610 bp)

NDV Cuya Superficie Contiene Spike SARS COV 2



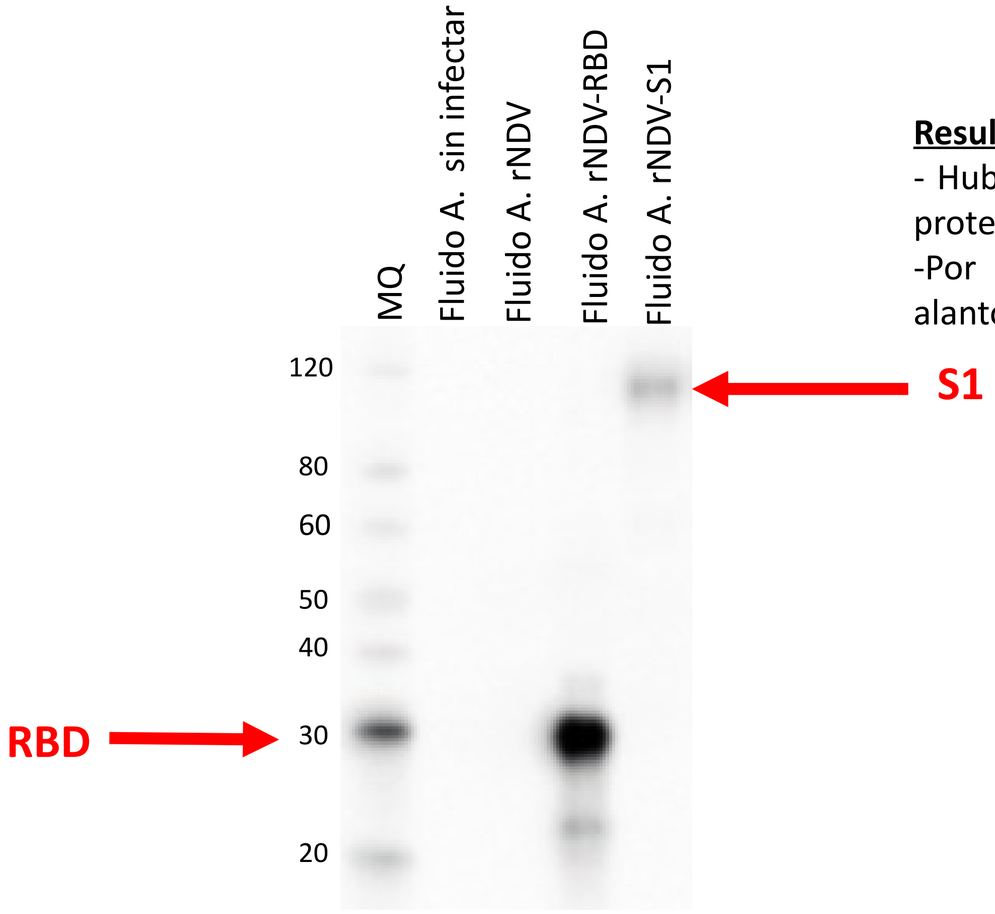
NDV Wild Type



NDV Spike Covid 19

Patente FARVET

INCORPORACIÓN DE LA PROTEINA RBD Y S1 EN EL VIRIÓN

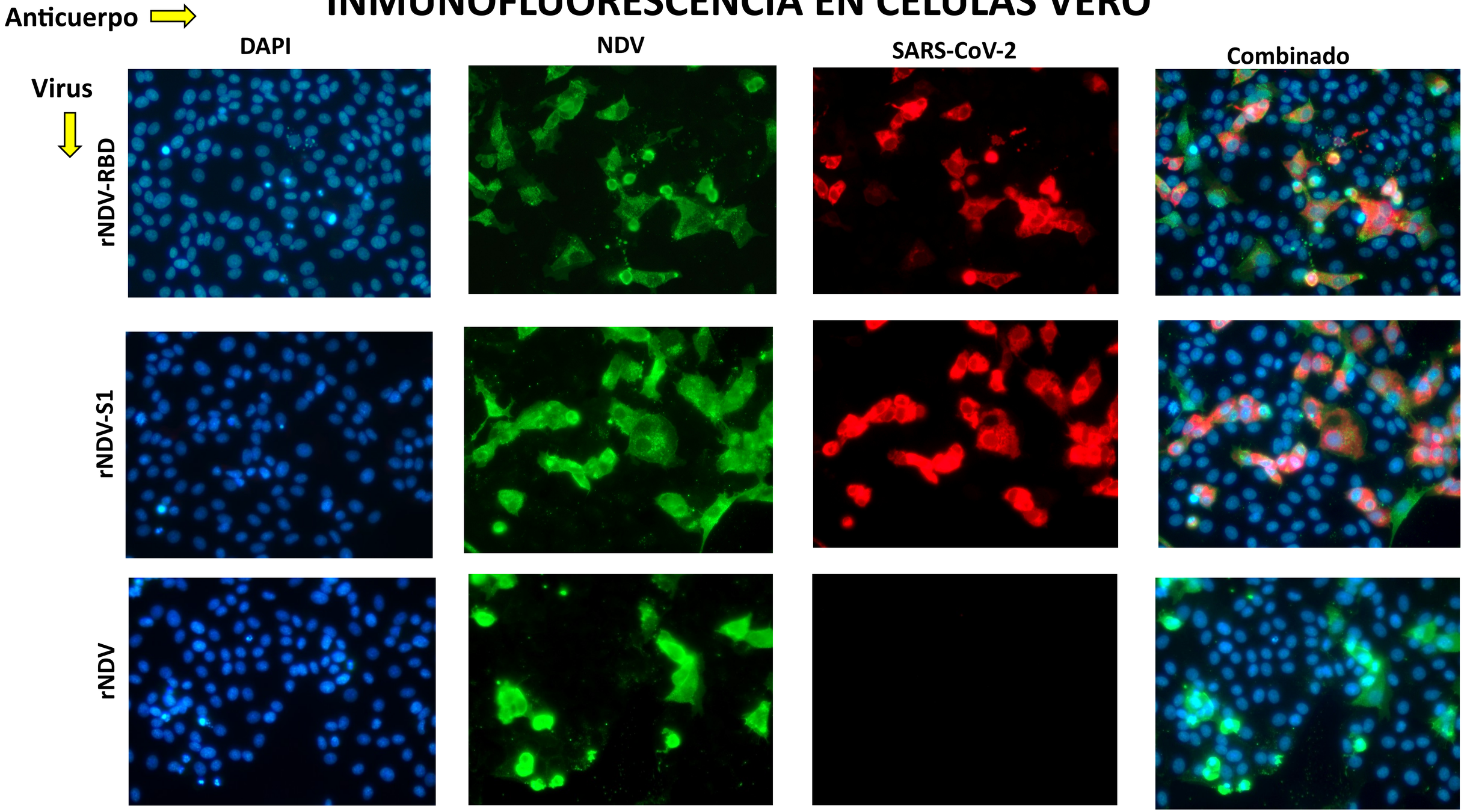


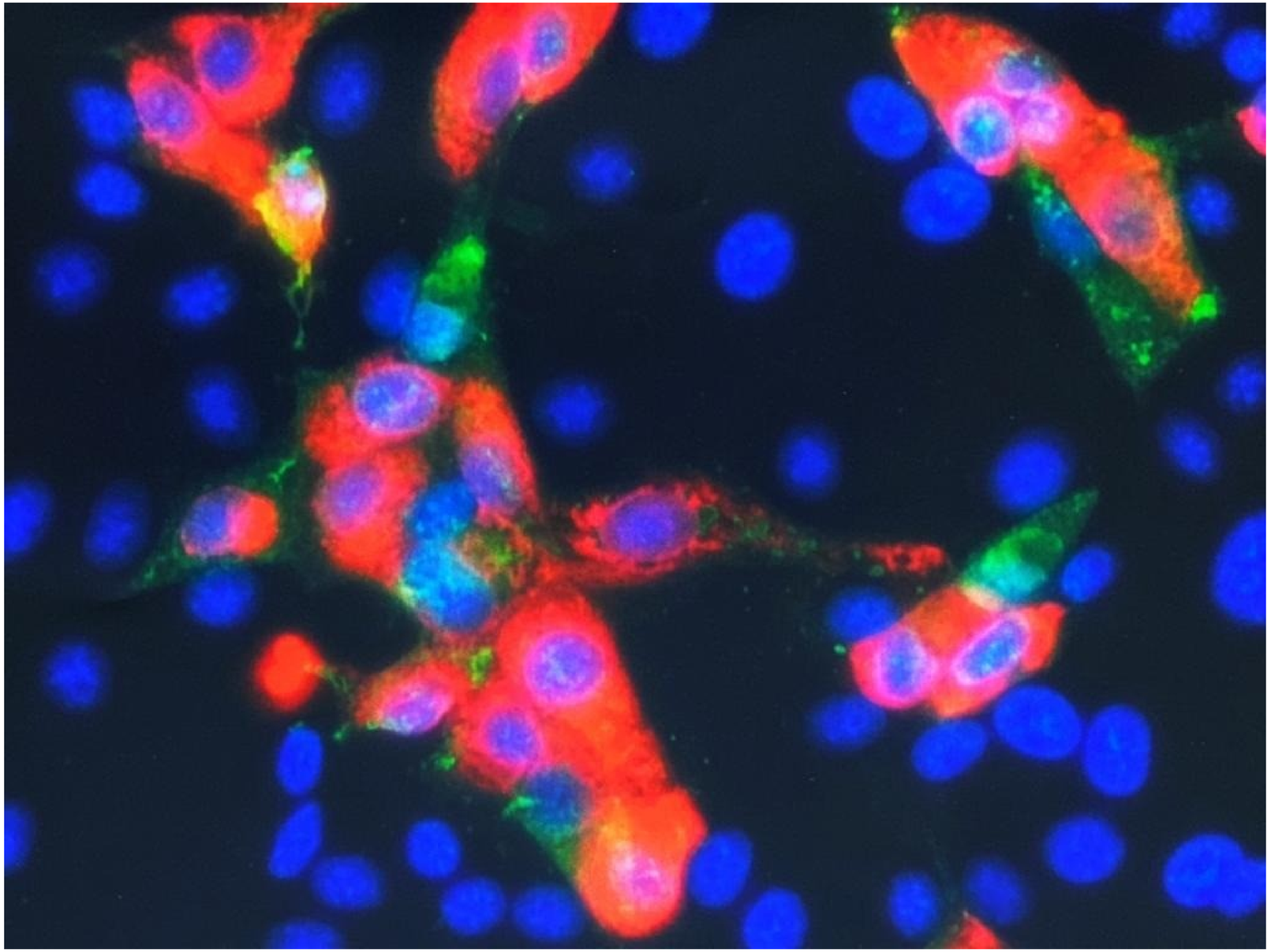
Resultados:

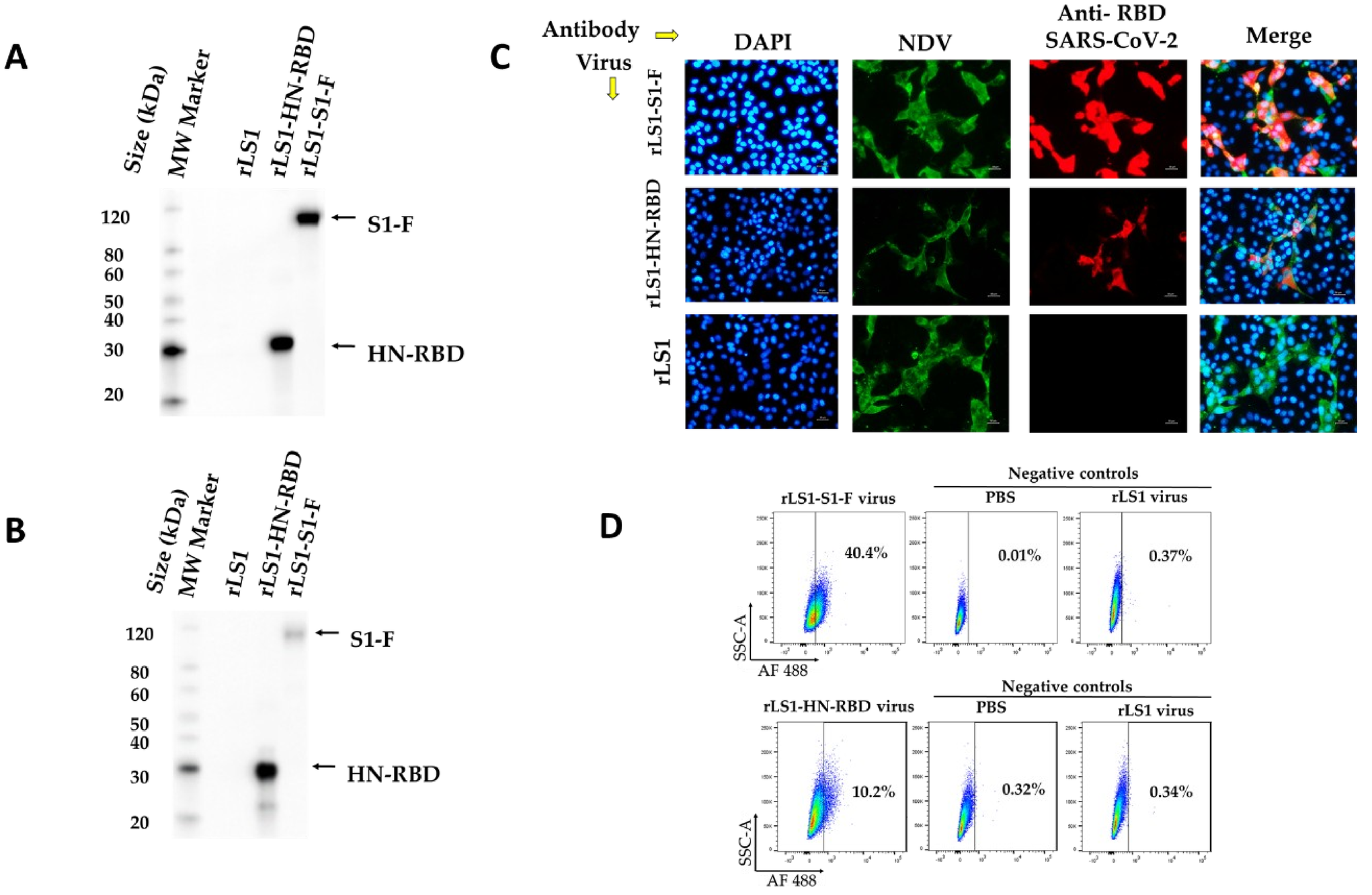
- Hubo reactividad con ambas bandas de aprox. 30 y 100 Kda que corresponden a la proteína RBD y S1 del rNDV-RBD/SARS-CoV-2 y rNDV-S1/SARS-CoV-2 respectivamente.

-Por lo contrario, no hubo reactividad en las muestras correspondientes a fluido alantoideo sin infectar y al fluido infectado con rNDV parental.

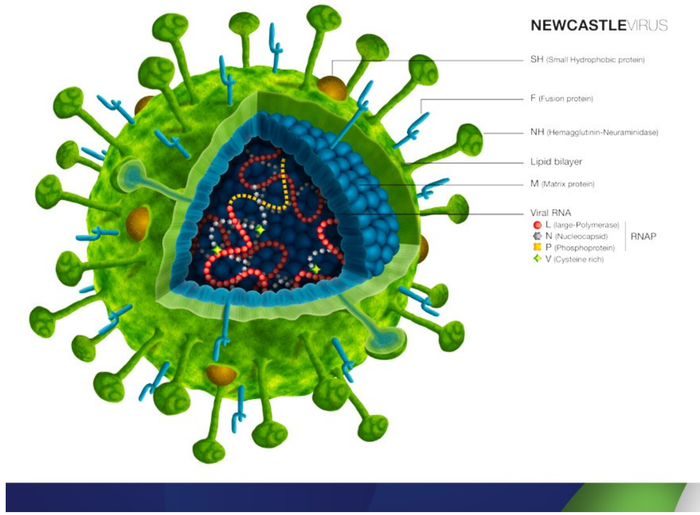
INMUNOFLUORESCENCIA EN CÉLULAS VERO

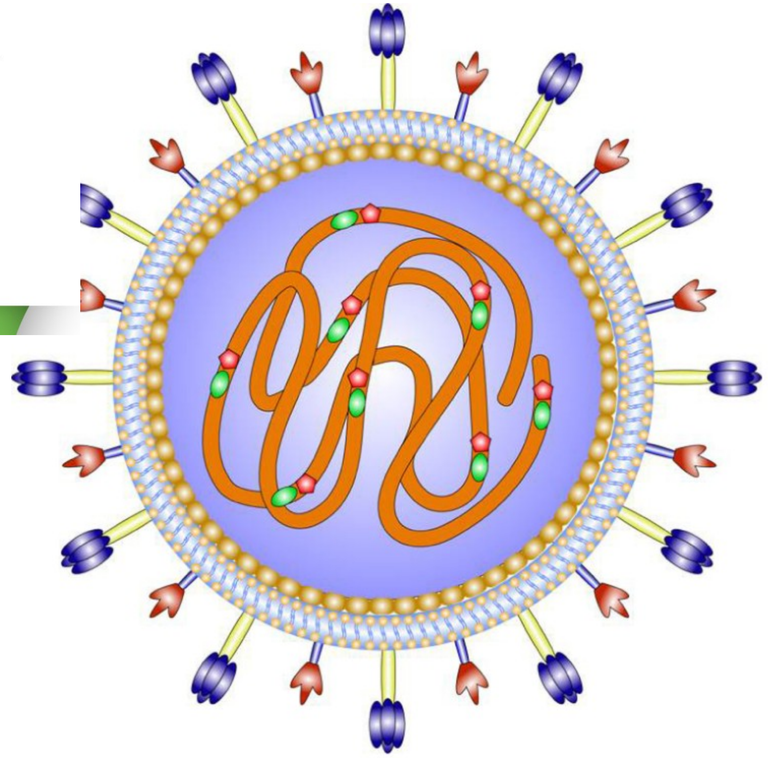





A
Size (kDa)
MW Marker
rLS1
rLS1-HN-RBD
rLS1-S1-F
120
80
60
50
40
30
20
S1-F
HN-RBD
B
Size (kDa)
MW Marker
rLS1
rLS1-HN-RBD
rLS1-S1-F
120
80
60
50
40
30
20
S1-F
HN-RBD
C
Antibody
Virus
DAPI
NDV
Anti- RBD SARS-CoV-2
Merge
rLS1-S1-F
rLS1-HN-RBD
rLS1
D
Negative controls
rLS1-S1-F virus
PBS
rLS1 virus
40.4%
0.01%
0.37%
SSC-A
AF 488
Negative controls
rLS1-HN-RBD virus
PBS
rLS1 virus
10.2%
0.32%
0.34%
SSC-A
AF 488

Virus de Newcastle (Wild-type)



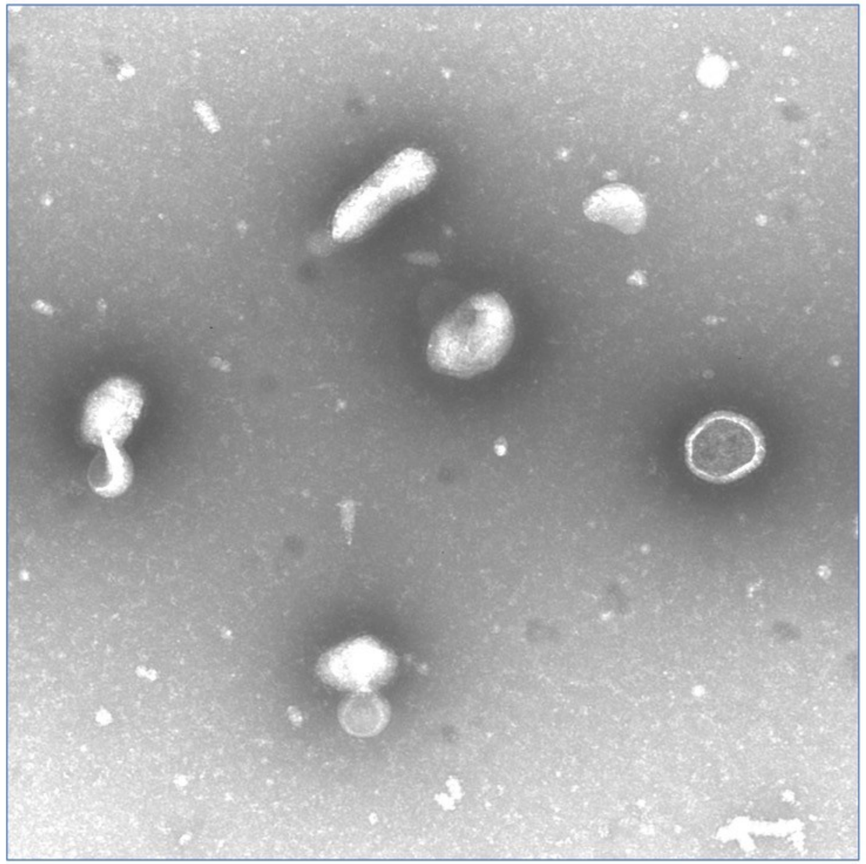


M (Matrix protein)
HN (Hemagglutinin neuraminidase)
F (Fusion protein)

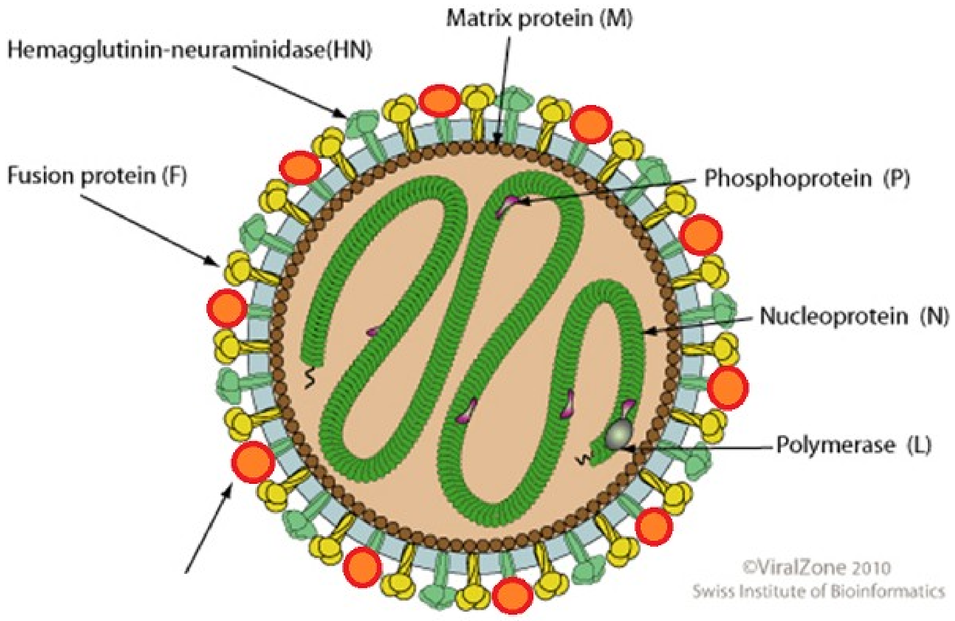
N (Nucleocapsid)
P (Phosphoprotein)
L (Large polymerase)

Microscopía electrónica

Sample 001// Rabbit anti-S 1:10 + PAG15



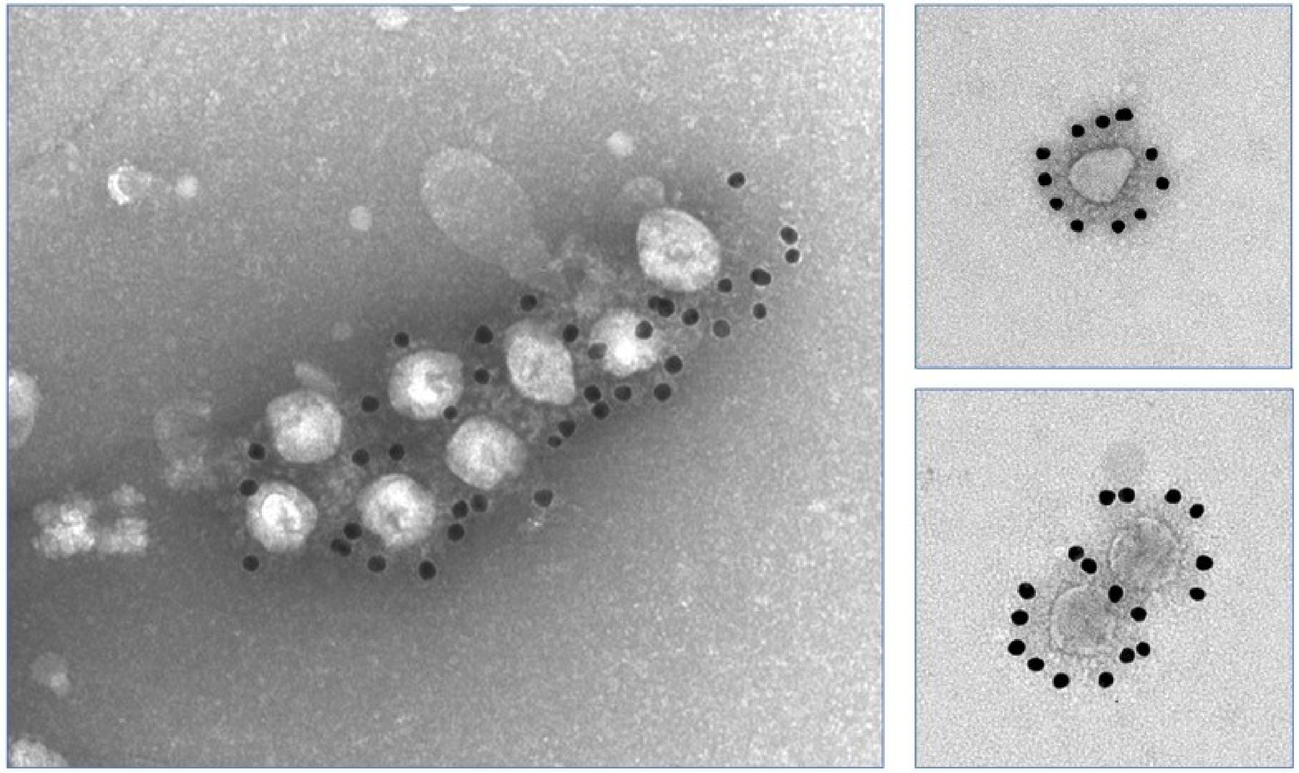
Inmuno-micorscopía electrónica de la vacuna nasal NDV-S1



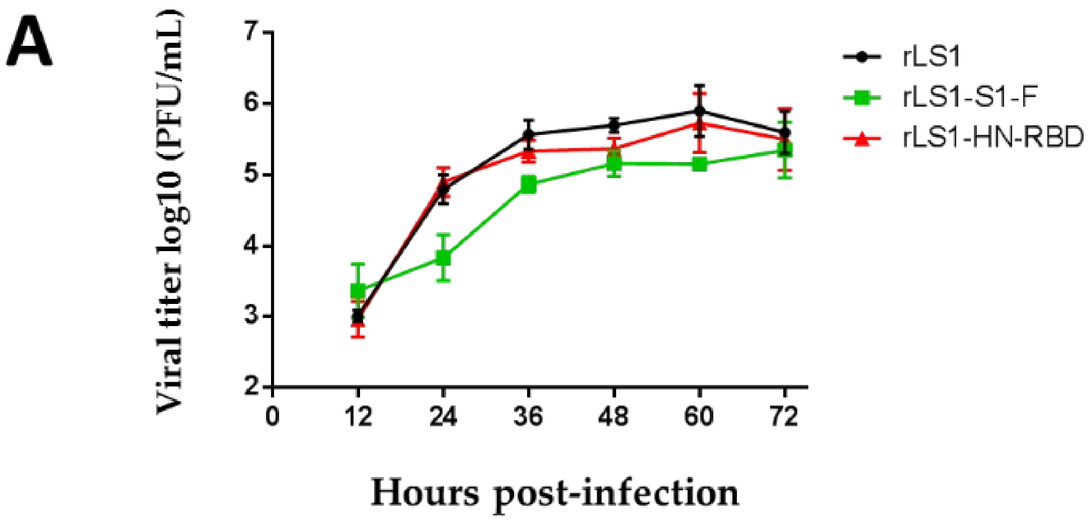
Spike Covid 19

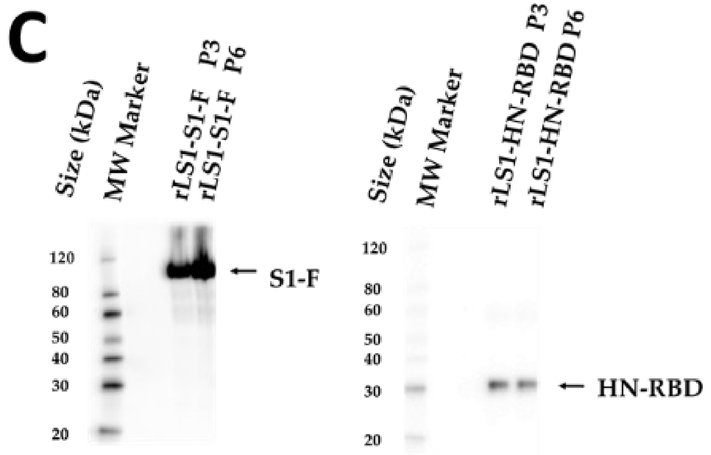
NDV Spike Covid 19

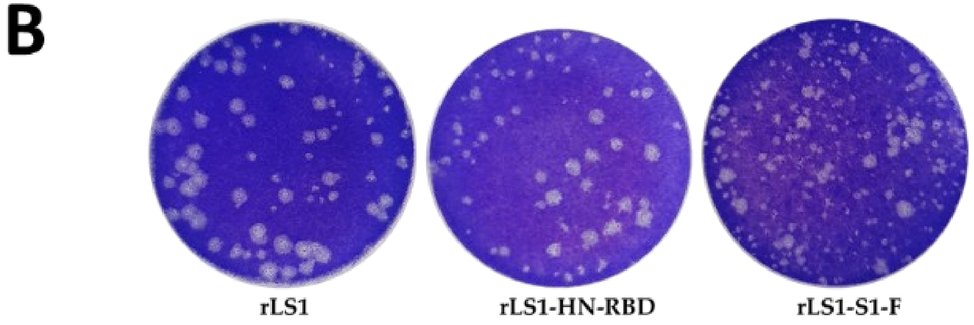
SARS-CoV-2// Rabbit anti-S 1:100 + PAG15

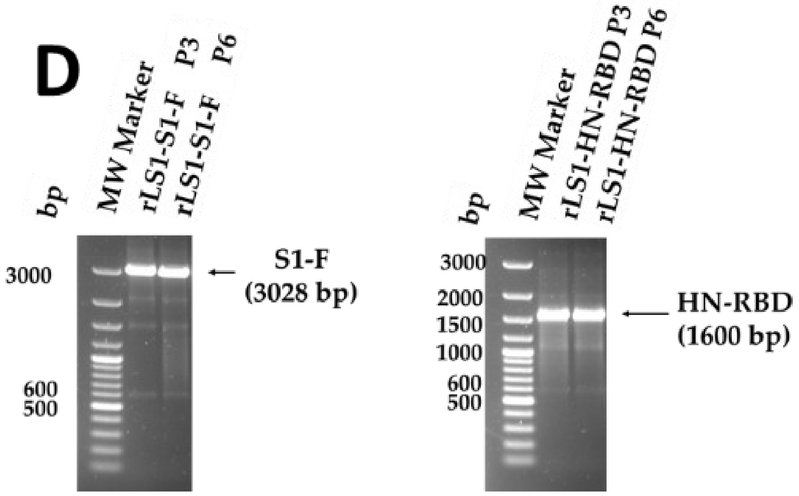


Propiedades de replicación del virus y estabilidad genética

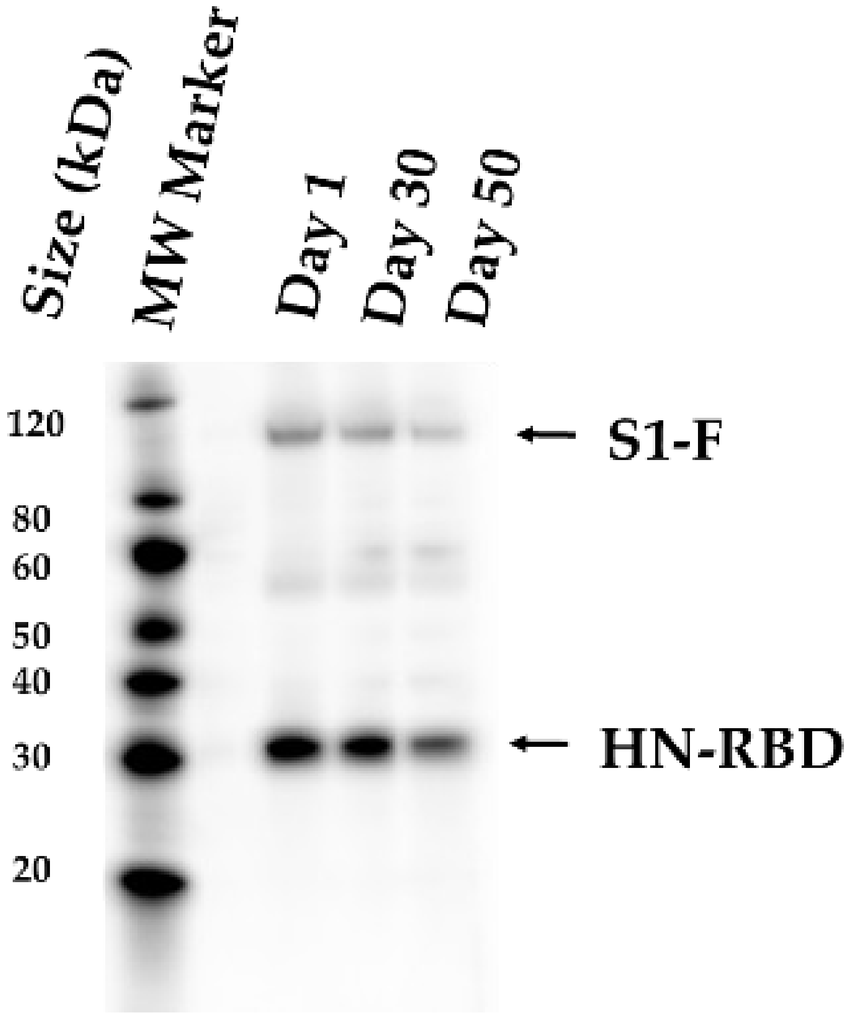




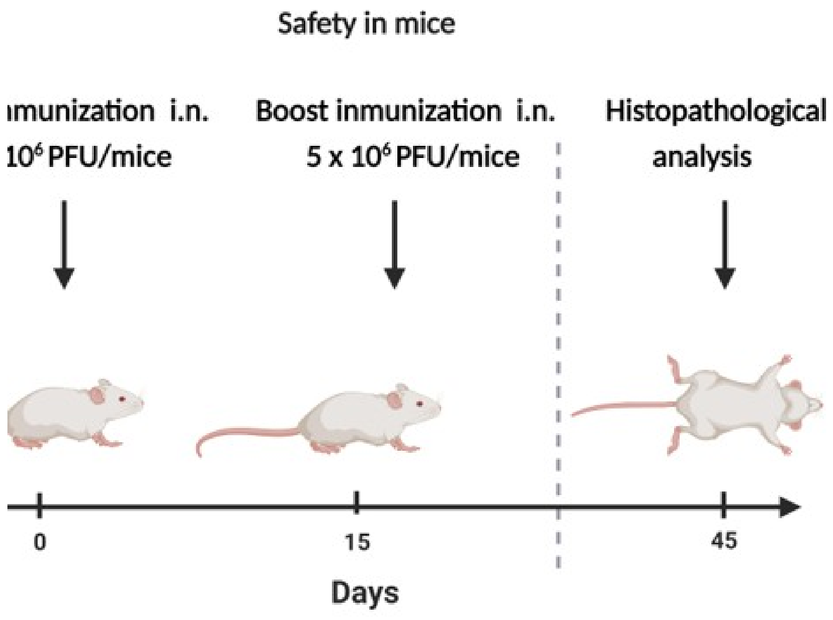


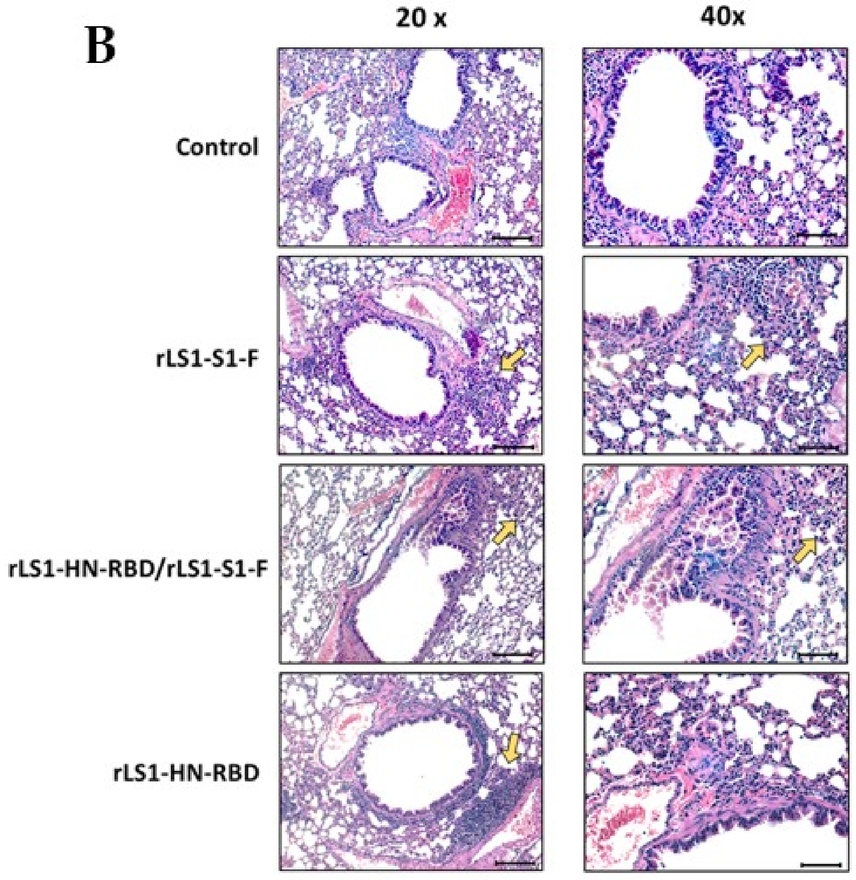


Estabilidad de la vacuna liofilizada



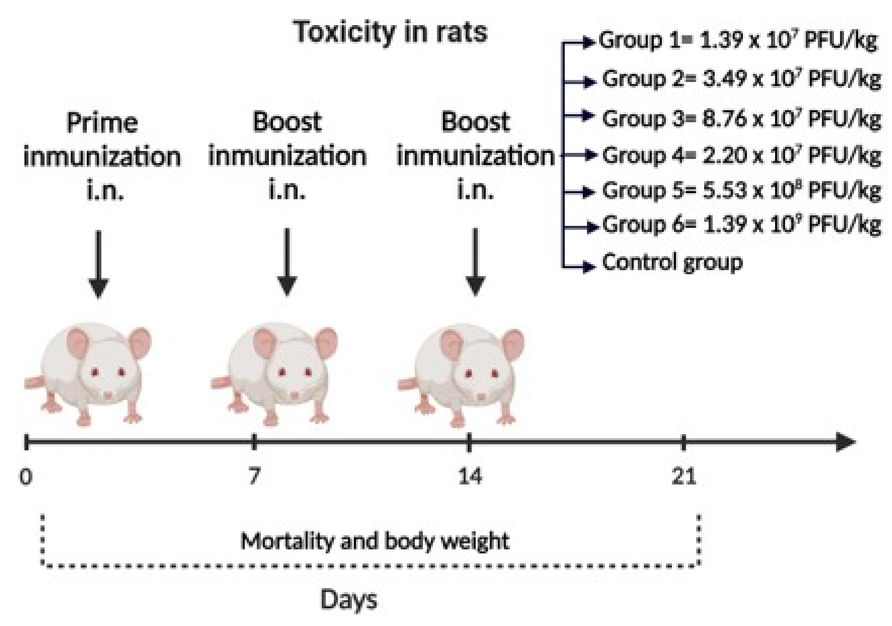
Pruebas de seguridad (ratones)



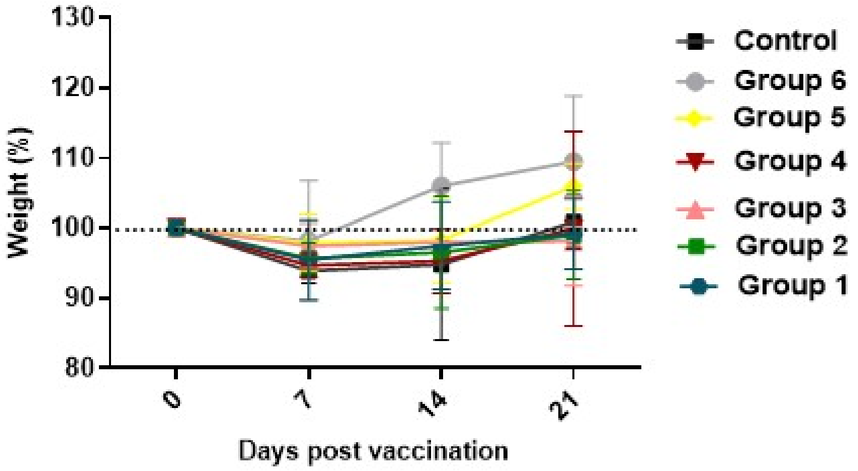


Pruebas de toxicidad (ratas)

A

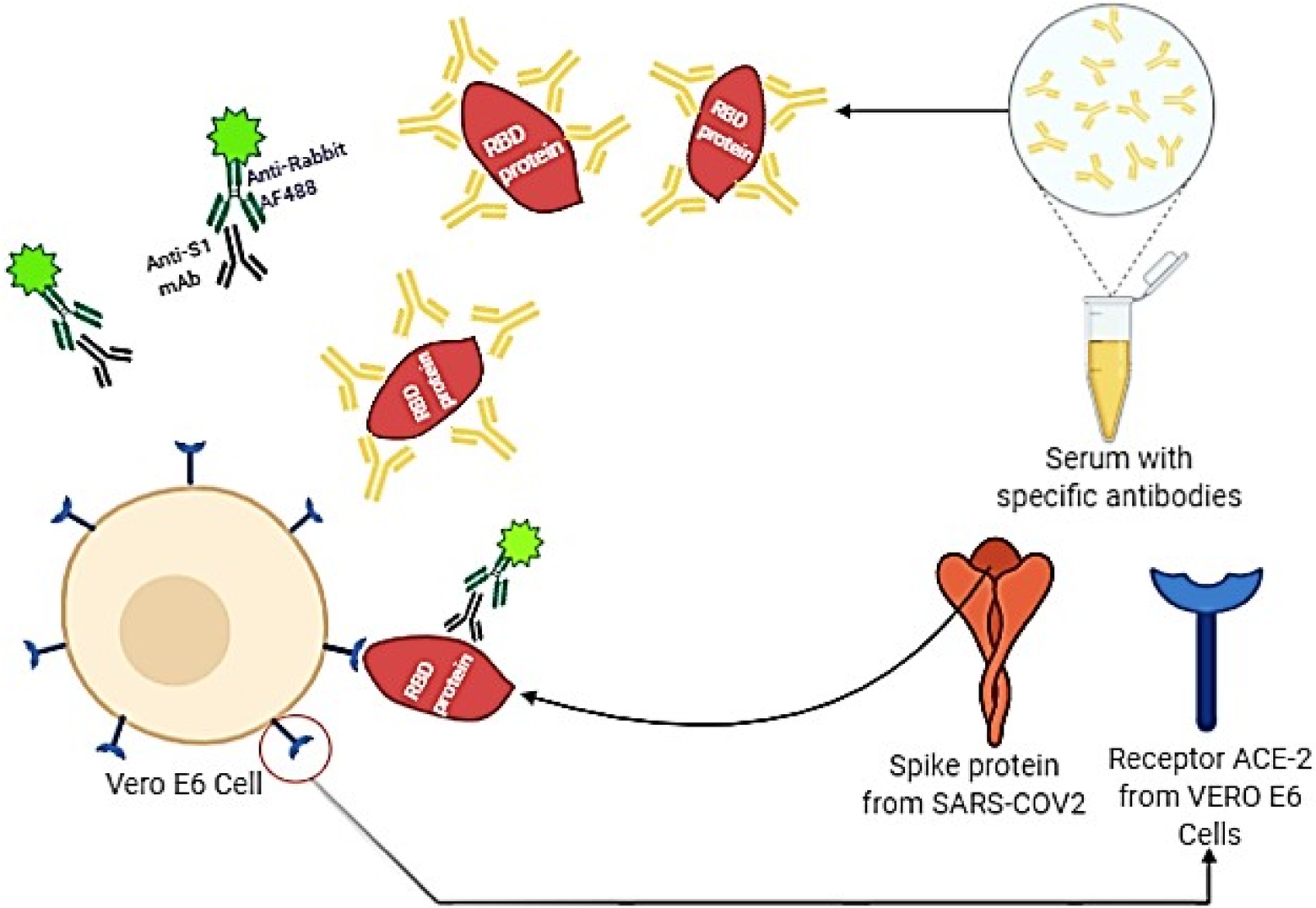


B



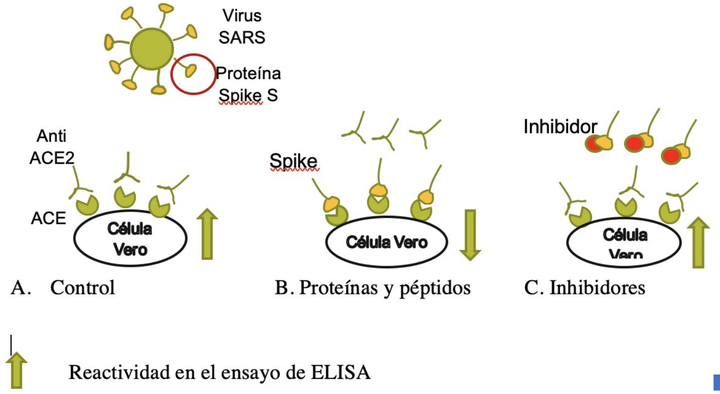
Ensayo de Bloqueo

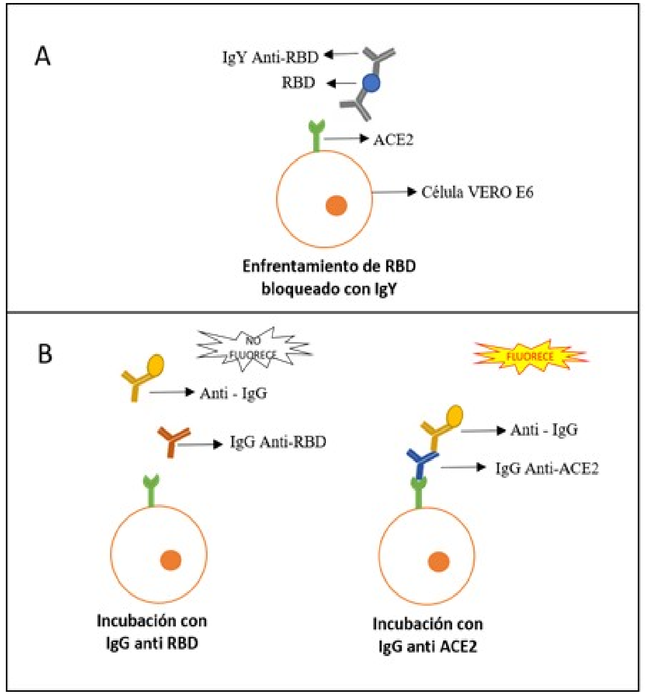
Anticuerpos del suero bloquean la unión de RBDr a VERO E6



MEDICIÓN DE LA CAPACIDAD DE NEUTRALIZACIÓN DE LA PROTEÍNA VIRAL

-Citometría de flujo
-Kit comercial de análisis de neutralización
-Prueba in-house libre de virus





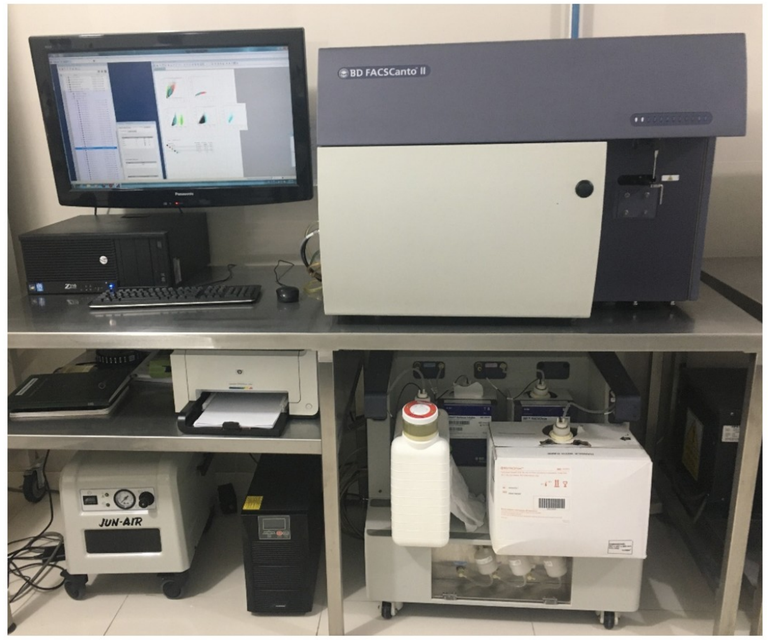
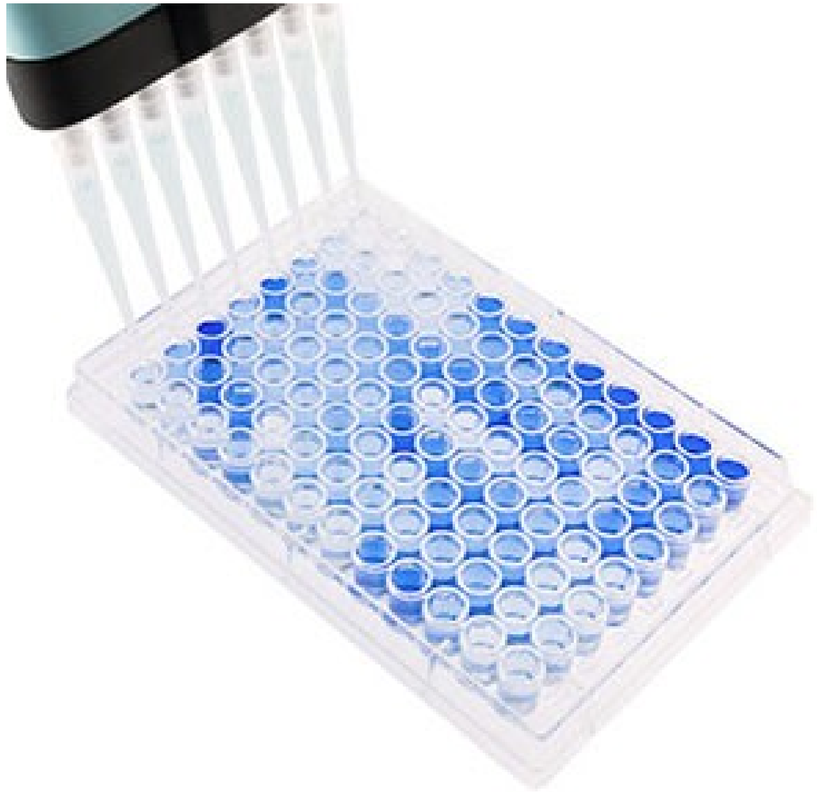
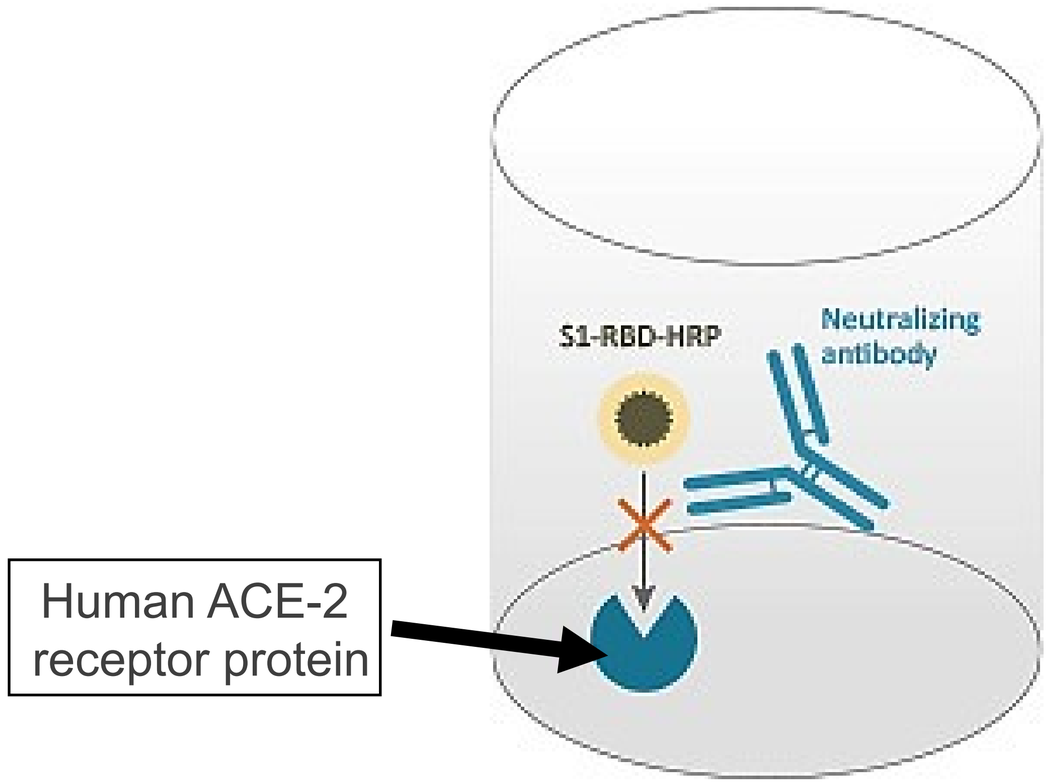


Figura 3. Equipo de citometría de flujo en FARVET para identificación de patrones celulares.

SARS-COV-2 Neutralizing Antibody ELISA Kit





Species Independent

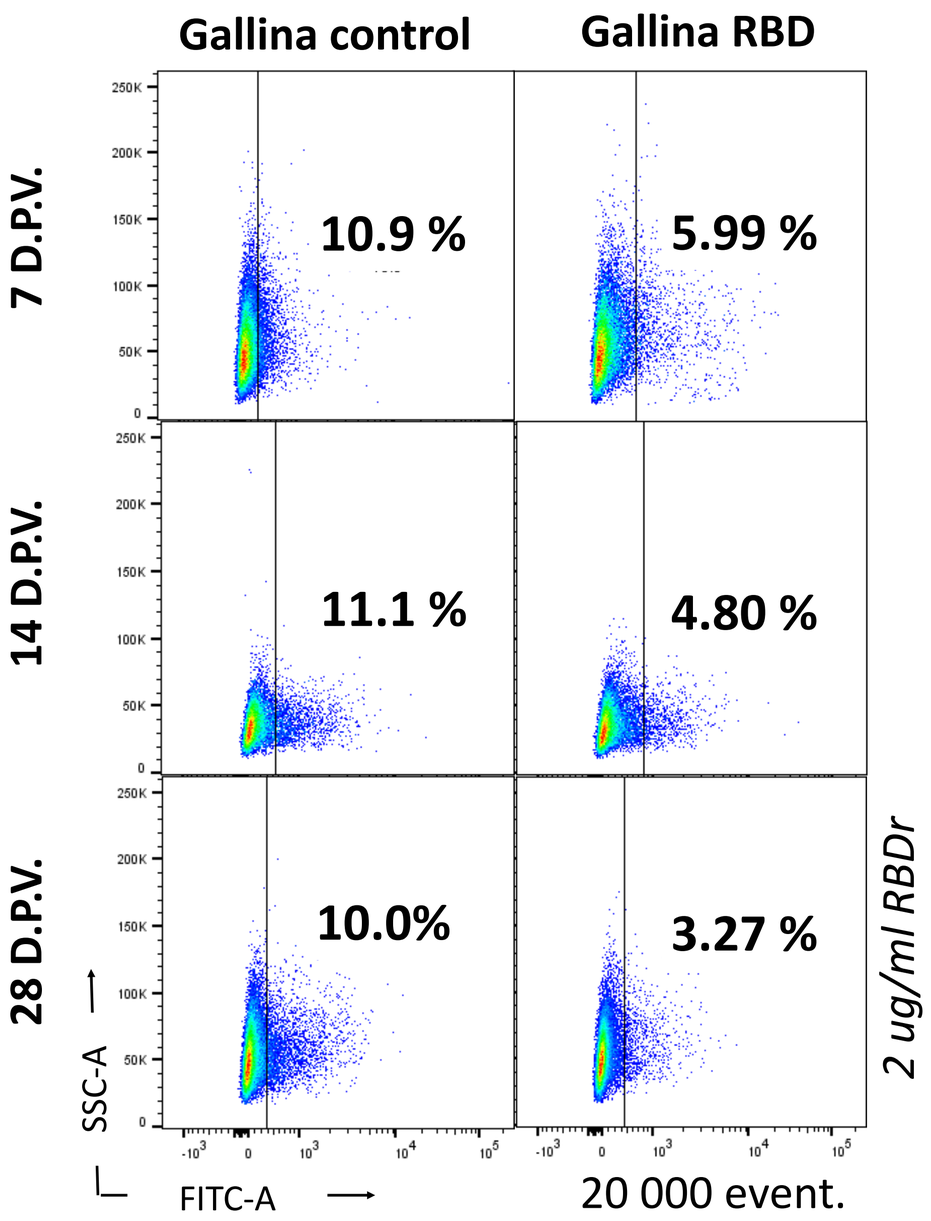
Isotype Independent

High Specificity

High Sensitivity

Safe & Scalable

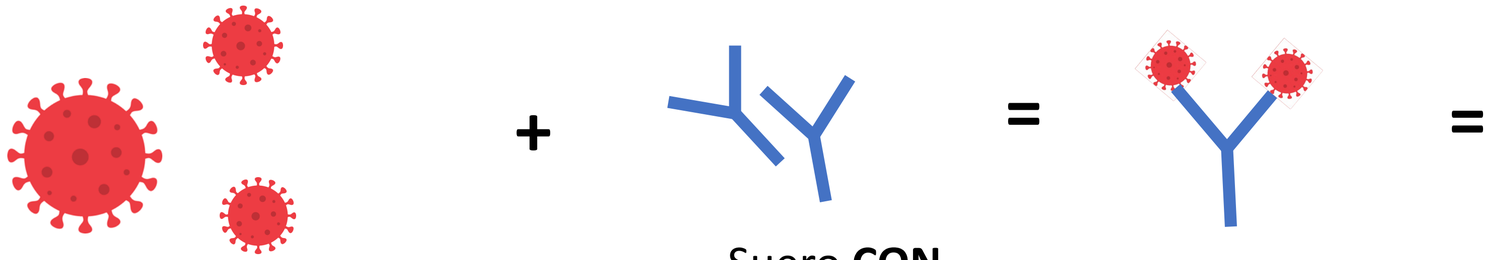
Ensayo de Bloqueo de sueros de animales vacunadas con RBDr



Días post vacunación (D.P.V.)	7		14		28	
Tratamiento	Control	RBDr	Control	RBDr	Control	RBDr
% Unión	10.9	5.99	11.1	4.8	10	3.27
% Bloqueo	0	45.1	0	56.8	0	67.3

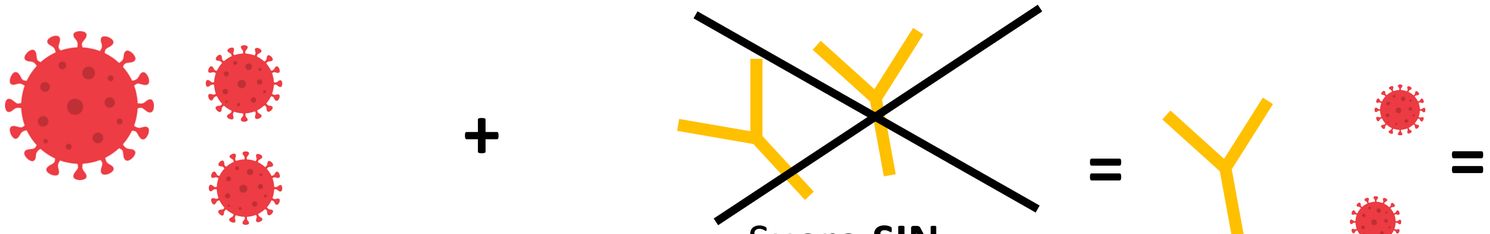
NEUTRALIZACIÓN POR REDUCCIÓN DEL NÚMERO DE PLACAS (PRNT)

Fundamento

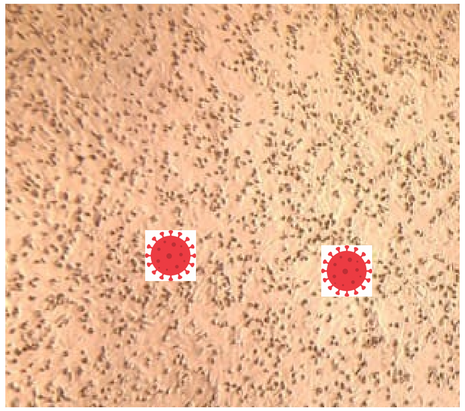


Suero **CON**
anticuerpos específicos

Célula normal



Suero **SIN**
anticuerpos específicos

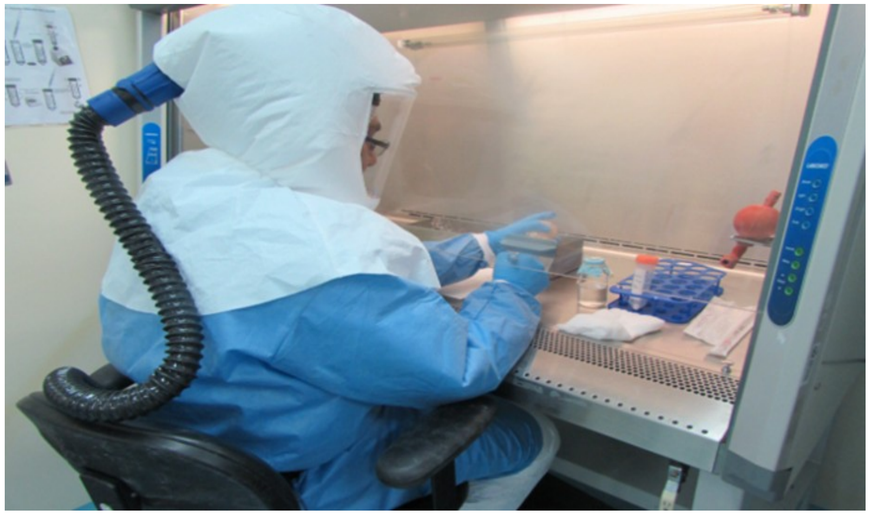


ECP

METODOLOGÍA: Obtención del virus (National Institute of health + FARVET)

Aislamiento virus SARS-CoV-2 → **Confirmación del aislamiento** → **Producción de antígeno viral** → **Titulacion viral (UFP/mL)**

Semilla viral



PCR
IFI

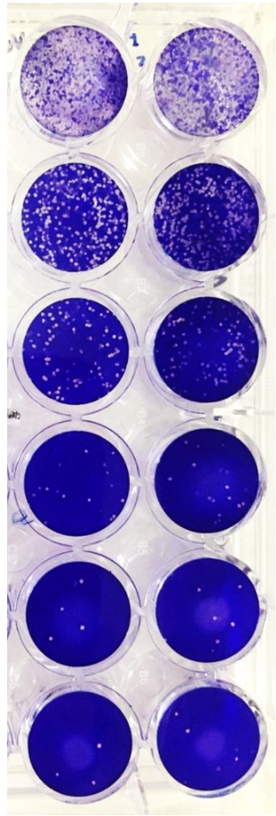
Stock viral

Cuantos virus hay?

10^{-1}

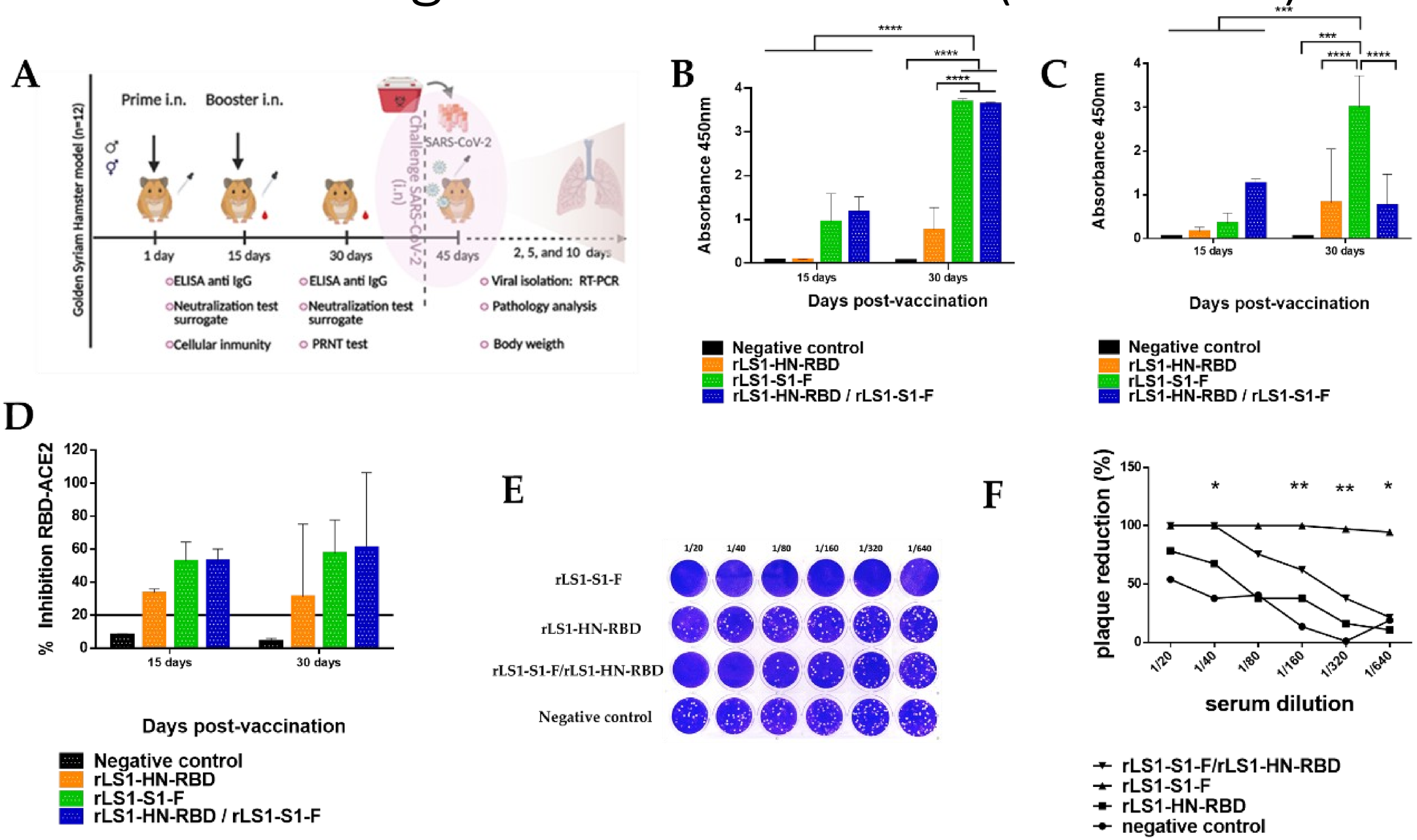
10^{-2}

10^{-3}



6×10^{5} UFP/mL

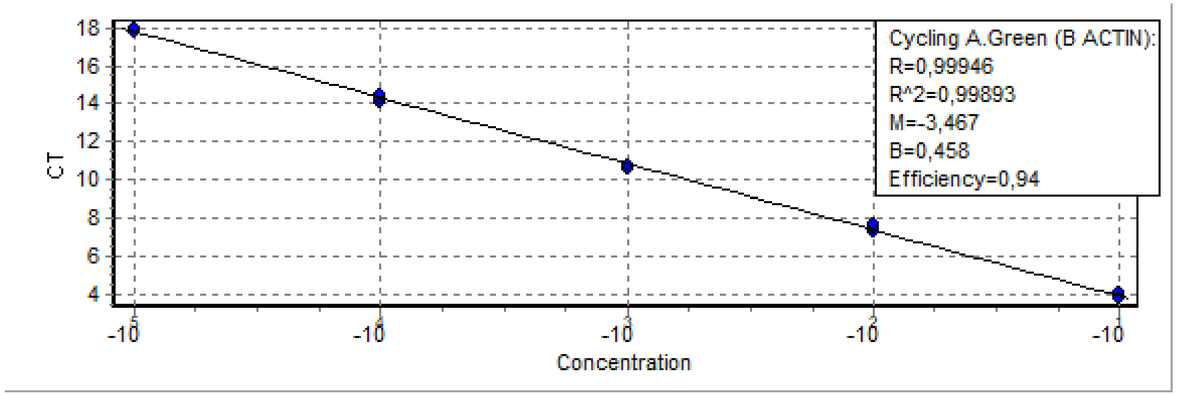
Inmunogenicidad- humoral (hamsters)



Medición de respuesta inmune celular por medio de la cuantificación de expresión de citoquinas mediante qPCR

Eficiencia por qPCR del reference gene (house keeping)

B actina



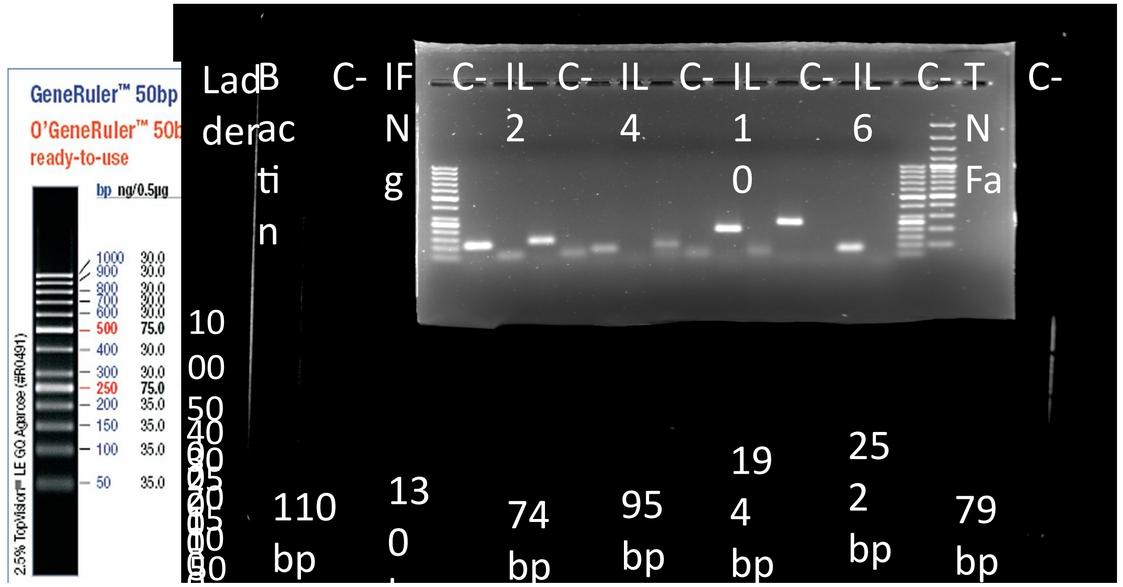
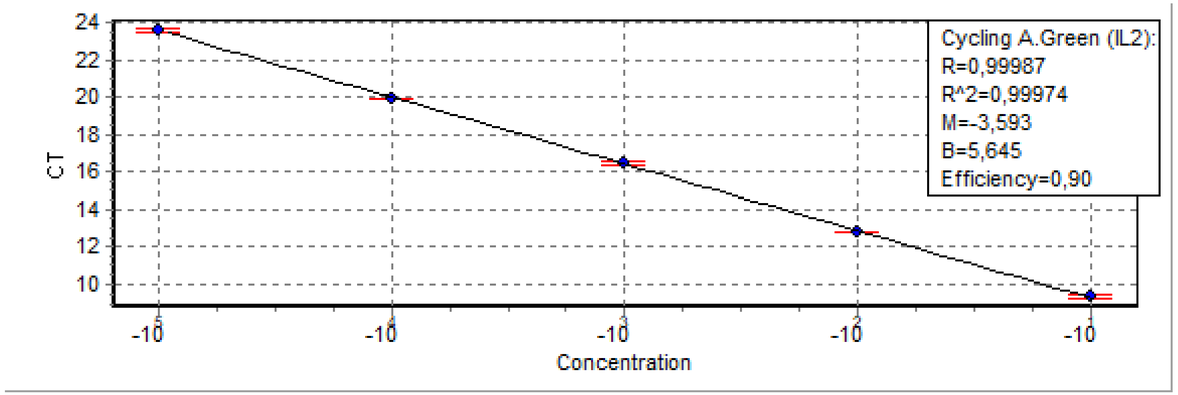


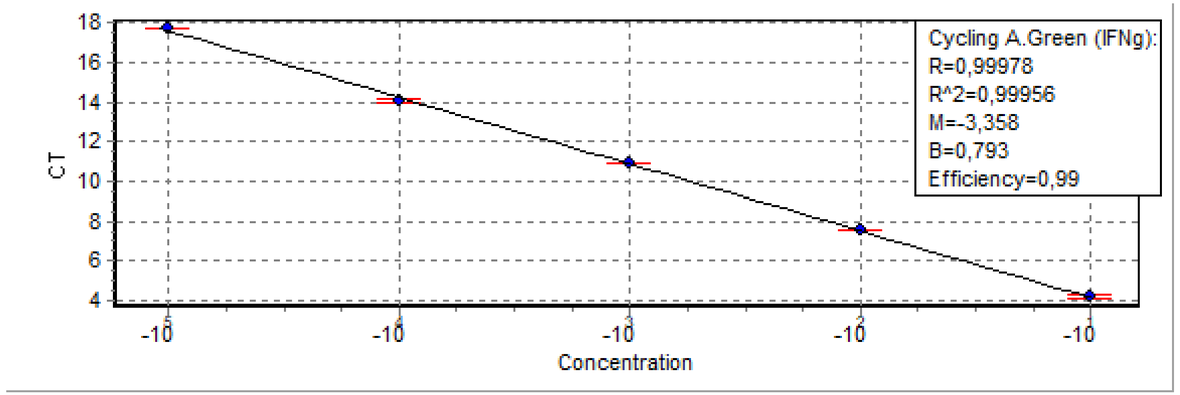
Fig. Presencia de bandas del tamaño indicado para el house keeping y las citocinas

Eficiencia por qPCR de las citocinas

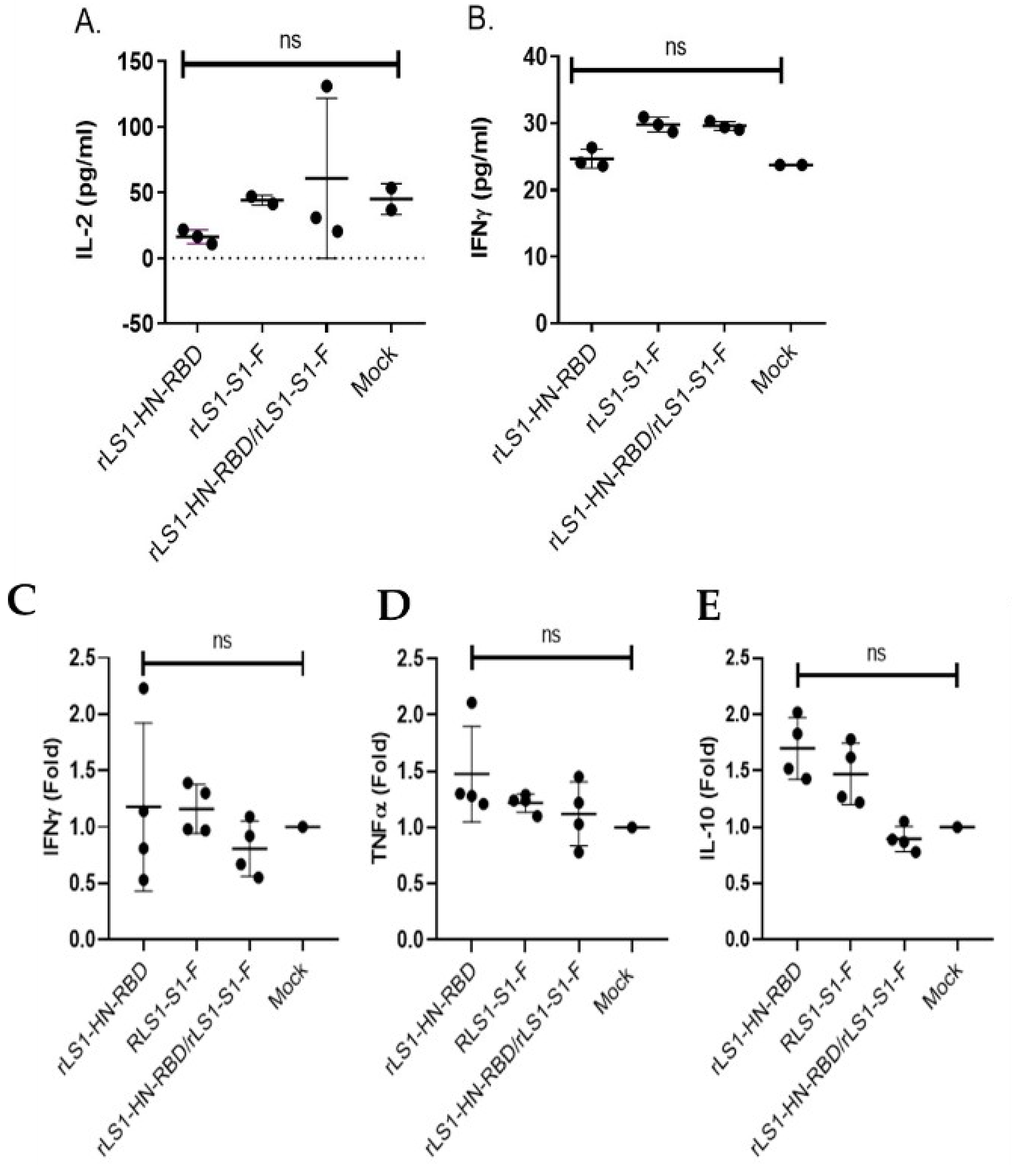
IL2



IFNg



Inmunogenicidad- celular (hamsters)



EFICACIA DE LA VACUNA: Ensayo de desafío (Hamster dorado Sirio)

- Ensayo del “DESAFÍO” en modelo animal validado: Hamster dorado Sirio
- Tuvimos que construir un contenedor de seguridad BSL3
- Inmunizar a los animals
- Desafiar a los animales con el virus SARS-CoV-2 (colaboración con el INS)
- Monitorear la variación de la carga viral así como la viabilidad viral a nivel del sistema respiratorio (q-RT-PCR)
- Monitorear el daño a nivel celular del tejido pulmonar de los animales (necropsia)
- Monitorear variaciones en el peso
- Monitorear el grado de movilidad de los animales

frontiers in Immunology

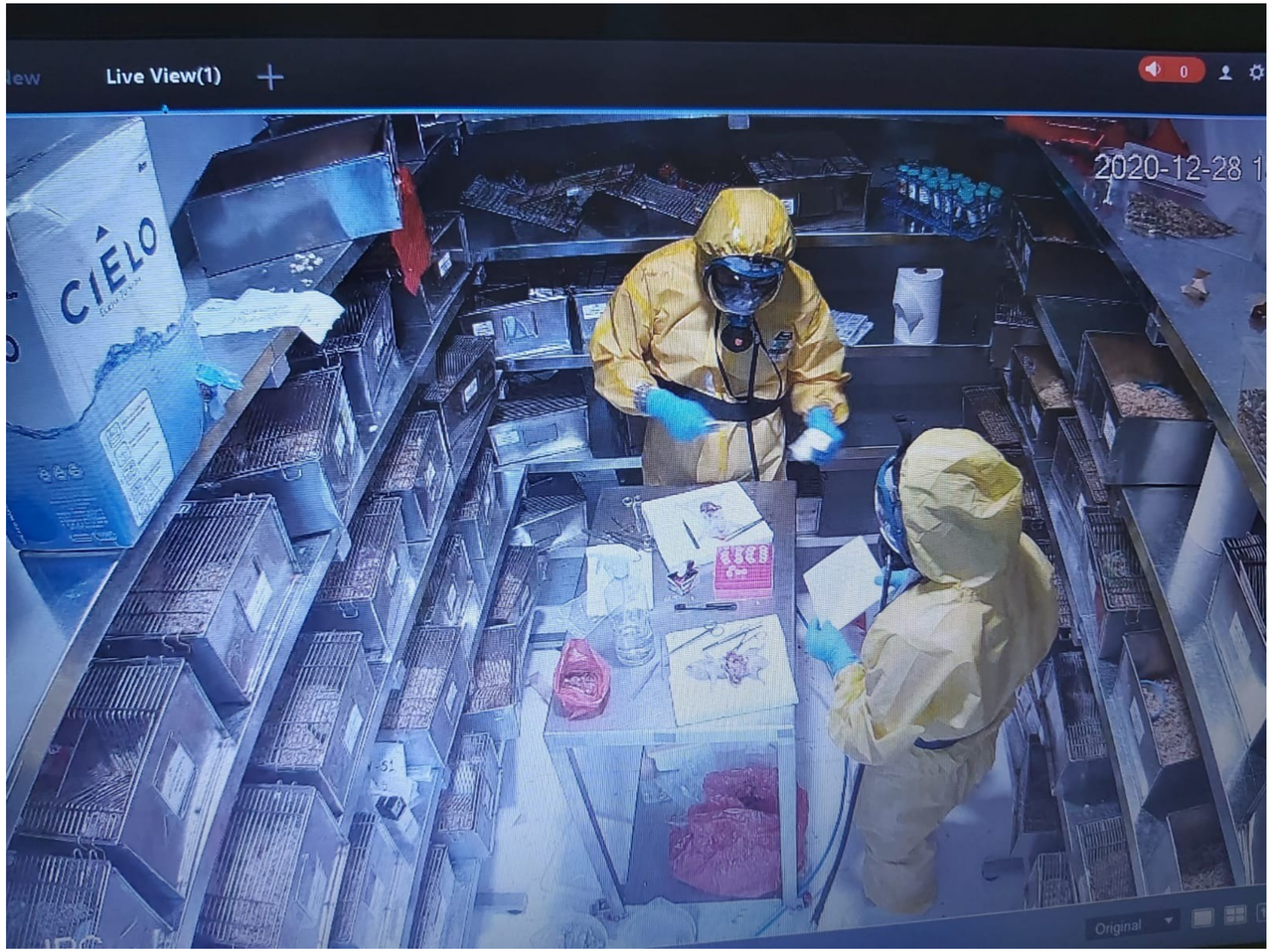
Syrian Hamster as an Animal Model for the Study on Infectious Diseases

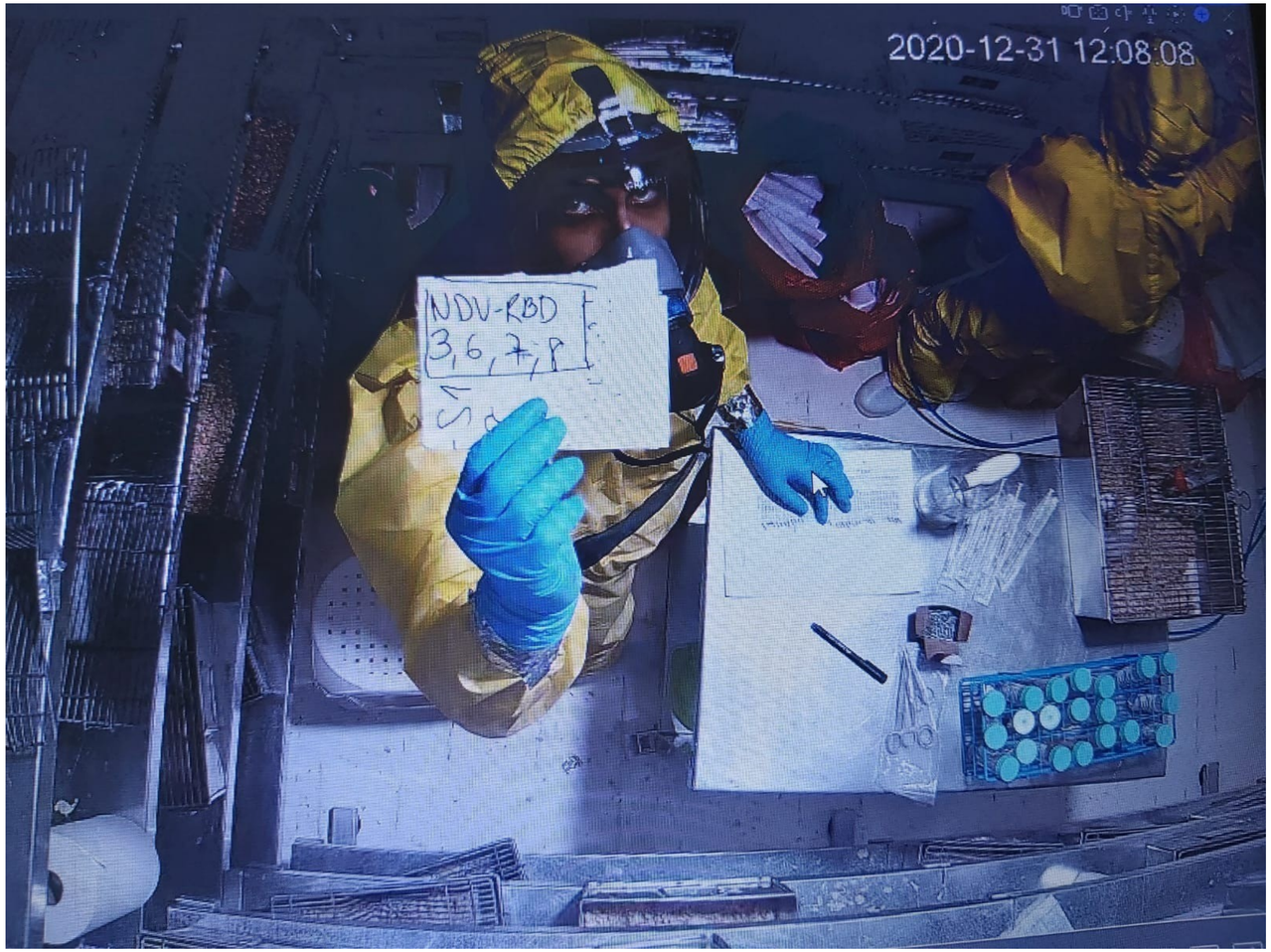
Jinxin Miao, Louisa S. Chard, [...], and Yaohe Wang

BSL3

ALCOHOL

Challenge: 10^5 PFU in 20uL nasal delivered

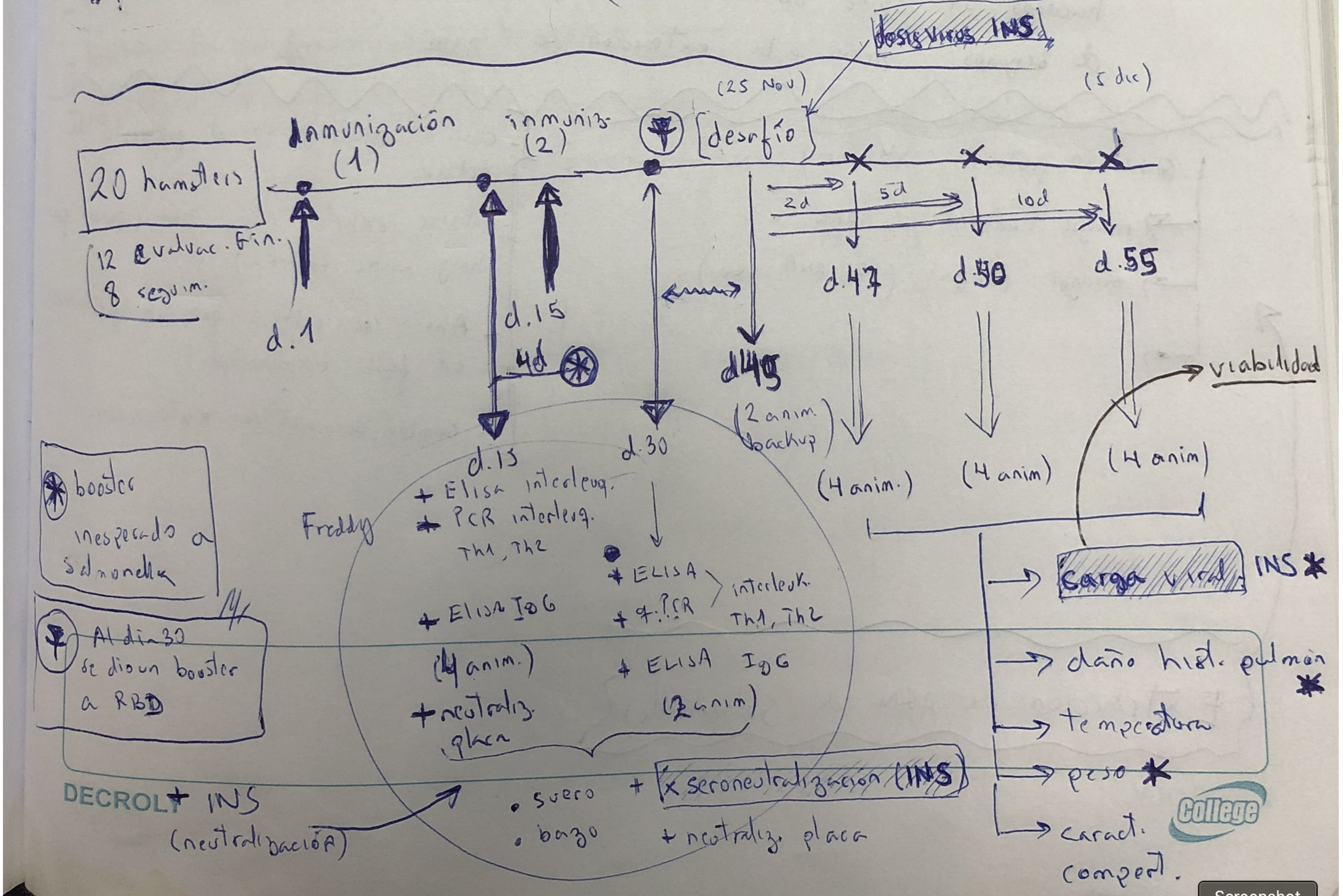




Grupos de vacunación para la prueba de desafío de hamsters

- Vacunados con NDV nasal
  - NDV-RBD (12 hamsters)
  - NDV-S1 (12 hamsters)
  - NDV- RBD+S1 (12 hamsters)

- Grupo Placebo (12 hamsters)


dosis virus INS
(25 Nov)
(5 dic)
inmunización (1)
inmuniz. (2)
desafío
20 hamsters
(12 evaluac. fin.
8 seguim.)
d.1
d.15
4d
d.15
d.30
d.45
(2 anim. backup)
2d
5d
10d
d.47
d.50
d.55
(4 anim.)
(4 anim)
(4 anim)
viabilidad
carga viral INS
daño hist. pulmón
temperatura
peso
caract. compert.
boster inesperado a salmonella
Al día 30 se dio un booster a RBD
Freddy
+ Elisa interleuq.
+ PCR interleuq. Th1, Th2
+ Elisa IgG
(4 anim.)
+ neutraliz. placa
+ ELISA
+ PCR
interleuk. Th1, Th2
+ ELISA IgG
(2 anim)
DECROLY INS
(neutralización)
• suero
• bazo
+ seroneutralización (INS)
+ neutraliz. placa

Resultados del ensayo pre-clínico de la vacuna nasal de NDV

Características de la vacuna nasal NDV-S1

Tipo de vacuna: Vectorizada en el virus de Newcastle (NDV). Cepa lentogénica La Sota.

Antígeno viral: Proteína S1 del SARS-CoV-2, expuesta en la membrana del NDV

Producción de la vacuna: Cultivo en huevos SPF fertilizados (embrión de pollos).

Purificación: Filtración tangencial a partir del fluido alantoideo

Presentación 1: Virus NDV-S1 vacunal liofilizado en una cantidad total de 10^6 PFU por dosis

Presentación 2: Virus NDV-S1 vacunal en solución líquida, en un volumen de 0.5mL en una cantidad total de 10^6 PFU por dosis.

Preparación de la dosis: La vacuna NDV-S1 liofilizada, se solubiliza con 0.5 mL de agua destilada

Administración: Vía intranasal (0.25 mL en cada fosa nasal)

Número de dosis: 2 (segunda dosis aplicada 15 días después de la primera dosis)

Capacidad de actualización: La vacuna NDV-S1 tiene la capacidad de ser actualizada con un nuevo antígeno S1 de interés, en 30-45 días, para empezar a ser producida.

Evaluaciones pre-clínicas realizadas:

A la fecha se han completado las pruebas correspondientes a los ensayos pre-clínicos, en ratones, ratas y hamsters. Específicamente se han realizado pruebas de:

-Seguridad: Análisis histopatológico de órganos en ratones vacunados con 2 dosis

-Toxicidad aguda: Evaluación de mortalidad y signos clínicos en ratas inmunizadas con dosis 100 veces mayor de lo esperado a utilizar.

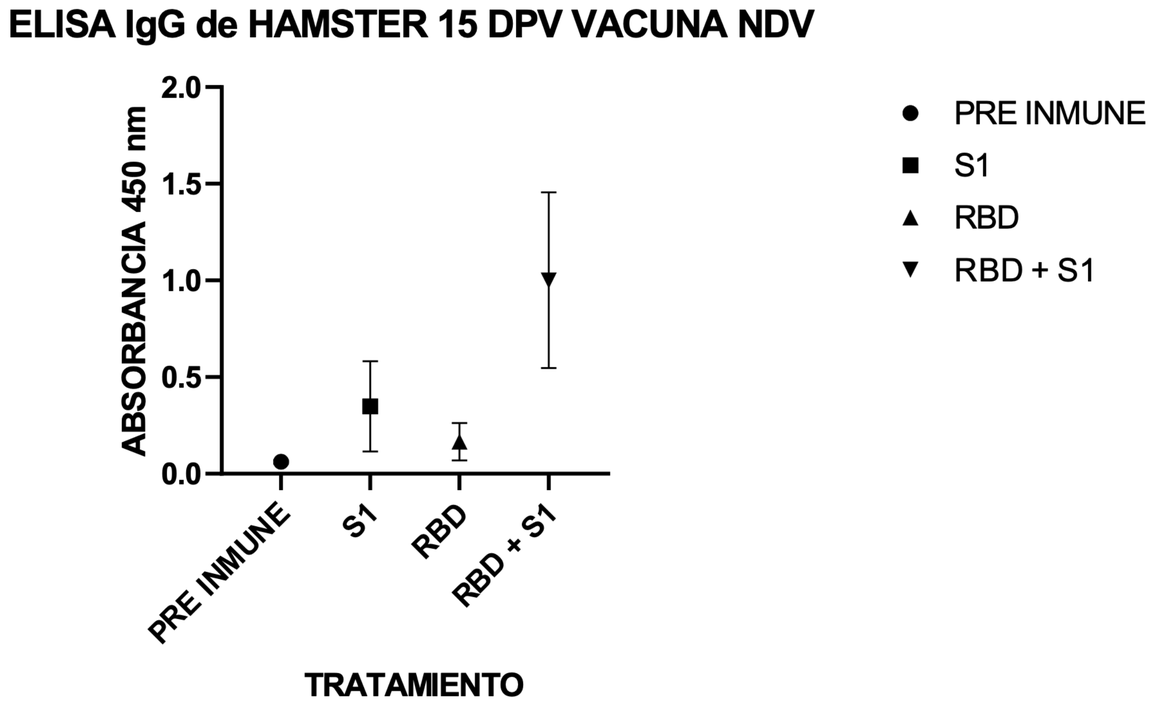
-Tolerancia: Evaluación de mortalidad y signos clínicos en ratas que reciben 3 dosis de la vacuna hasta con concentraciones 100 veces mayor de lo esperado a utilizar.

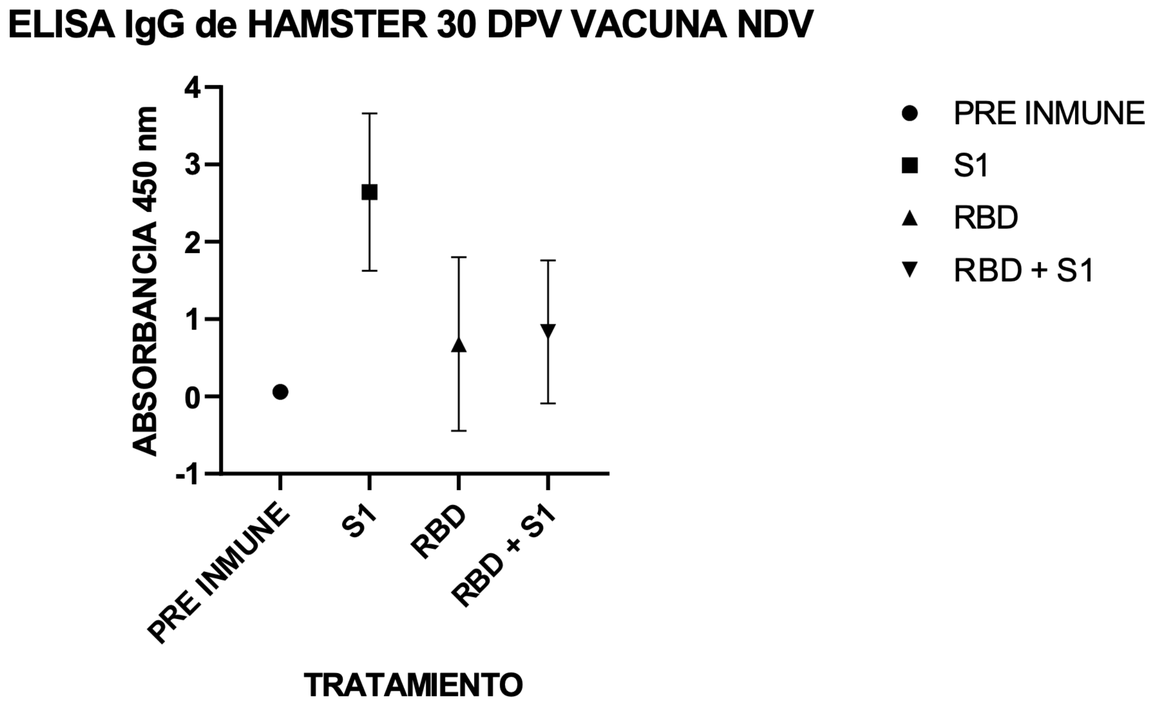
-Inmunogenicidad: En hamsters
* Anticuerpos IgG detectados por ELISA
* Neutralización de proteína RBD recombinante en un ensayo en placa
* Neutralización del virus SARS-CoV-2 en placa
* Inmunidad celular evalúa proliferación de linfocitos T CD4 por interferón gamma otras citoquinas

-Estabilidad:
*NDV liofilizado es estable a 8C por al menos 2 meses, con una disminución de las unidades formadoras de placas (PFU) menor al 5%. NDV en solución líquida es estable a 8C por 15 días, con una disminución de las unidades formadoras de placas (PFU) menor al 5%.
*La expresión del gen S1 / RBD es estable por varios pasajes del virus

-Eficacia: Hamsters desafiados con SARS-CoV-2 en ambiente BSL3. Se evalúa:
(1) Carga viral en pulmón
(2) Viabilidad viral en pulmón
(3) Daño histológico a nivel de pulmón y traquea
(4) Variación del peso corporal
(5) Grado de movilidad de los animales.

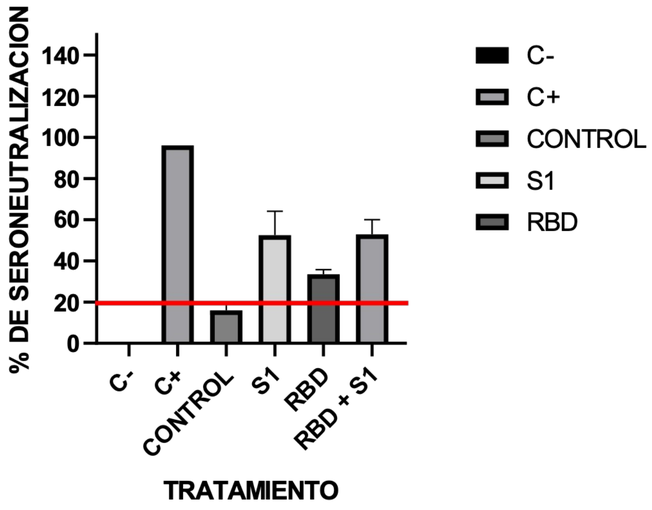
RESULTADOS DE SEGUIMIENTO SEROLOGICOS PREVIO AL DESAFÍO
DE HAMSTERS CON LA VACUNA NDV
ELISA IgG anti-RBD



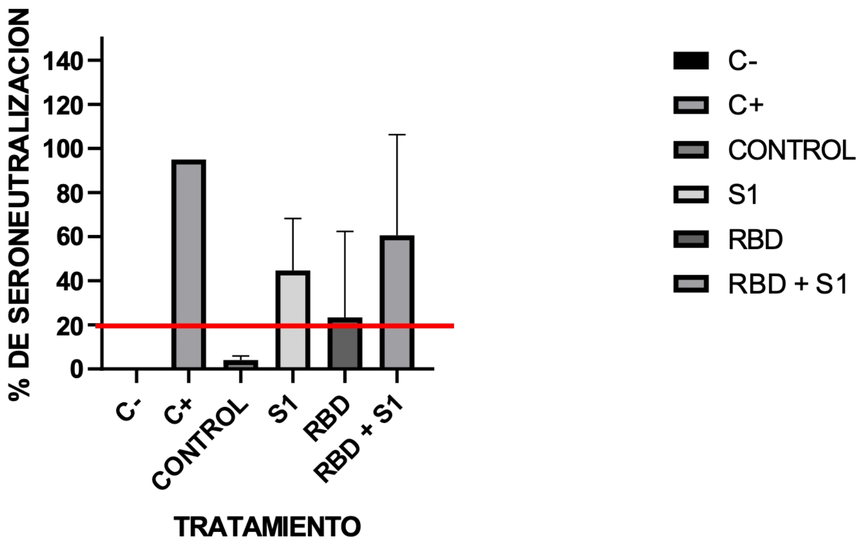


SEGUIMIENTO SEROLOGICO PREVIO AL DESAFÍO DE HAMSTERS CON LA VACUNA NDV
Seroneutralización de RBD en placa

PORCENTAJE DE SERONEUTRALIZACION HAMSTERS 15 DPV VACUNA NDV

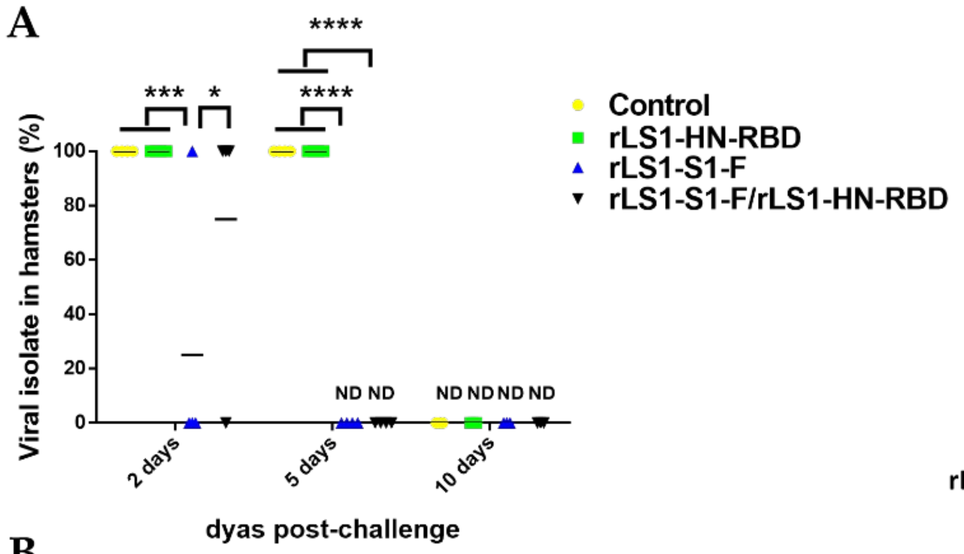


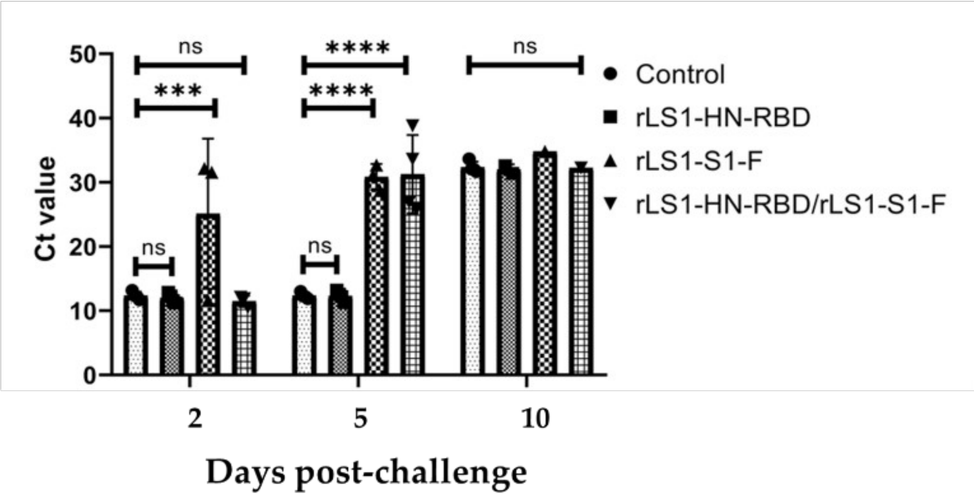
PORCENTAJE DE SERONEUTRALIZACION HAMSTERS 30 DPV VACUNA NDV

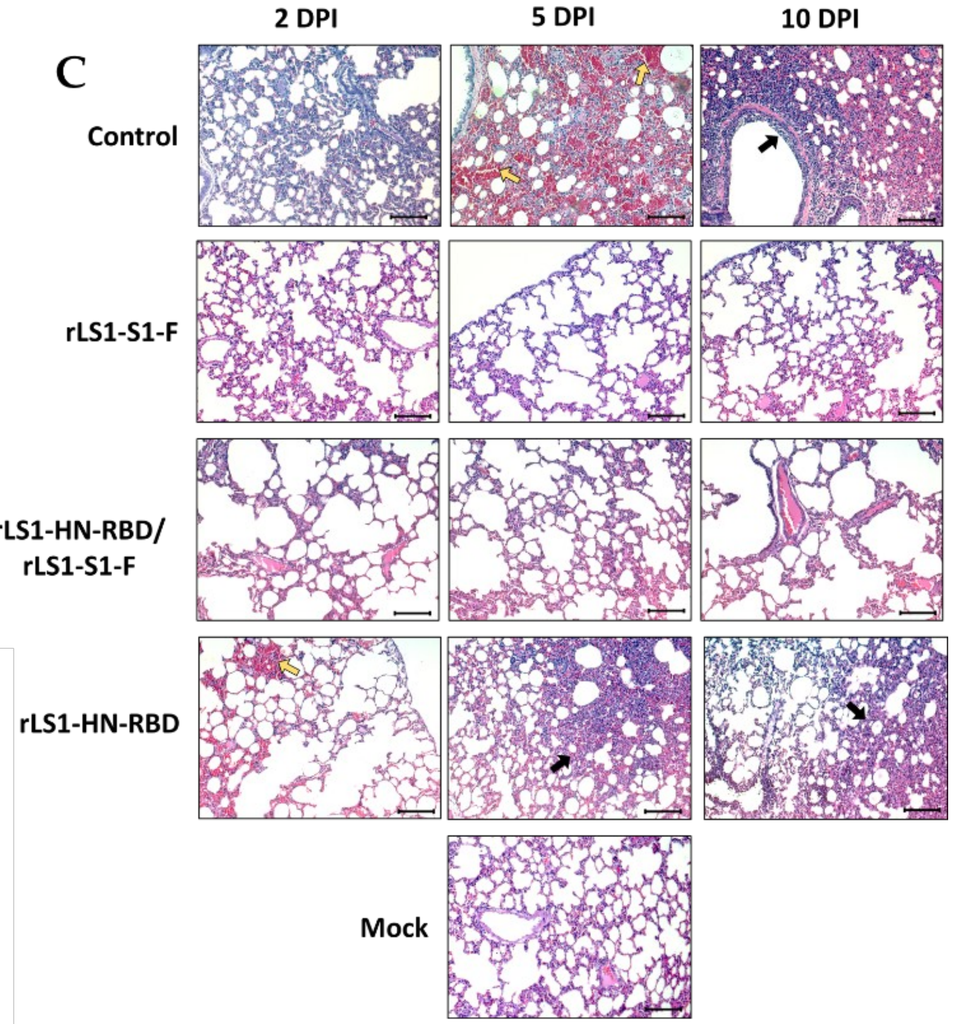


El análisis histopatológico de los órganos (pulmón y traquea) de los animales desafiados, mostraron inflamación y necrosis en los animales placebo, y no hubo evidencia de daño significativo en los animales vacunados

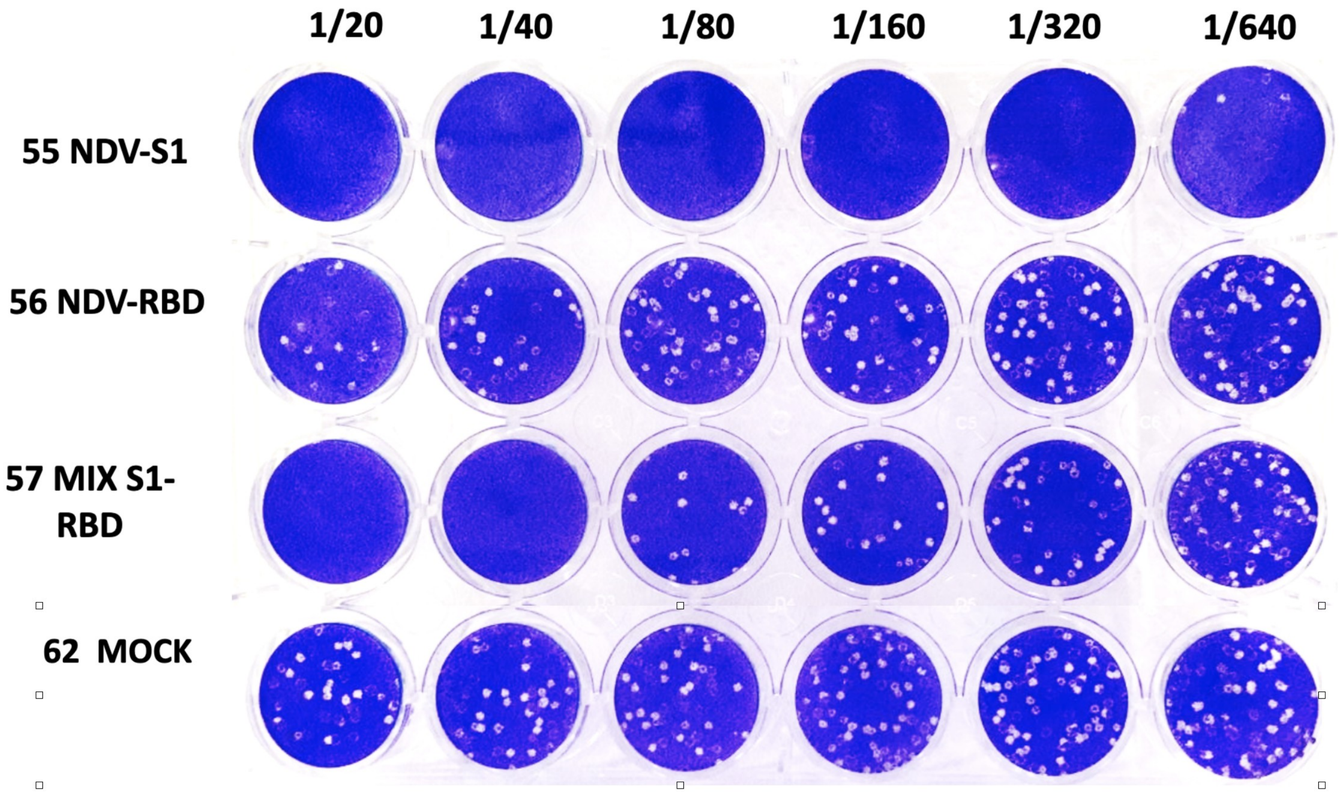
Eficacia de la vacuna:
-Análisis histopatológico de pulmón
-Viabilidad viral (cultivo)
-Carga viral (RT-PCR)





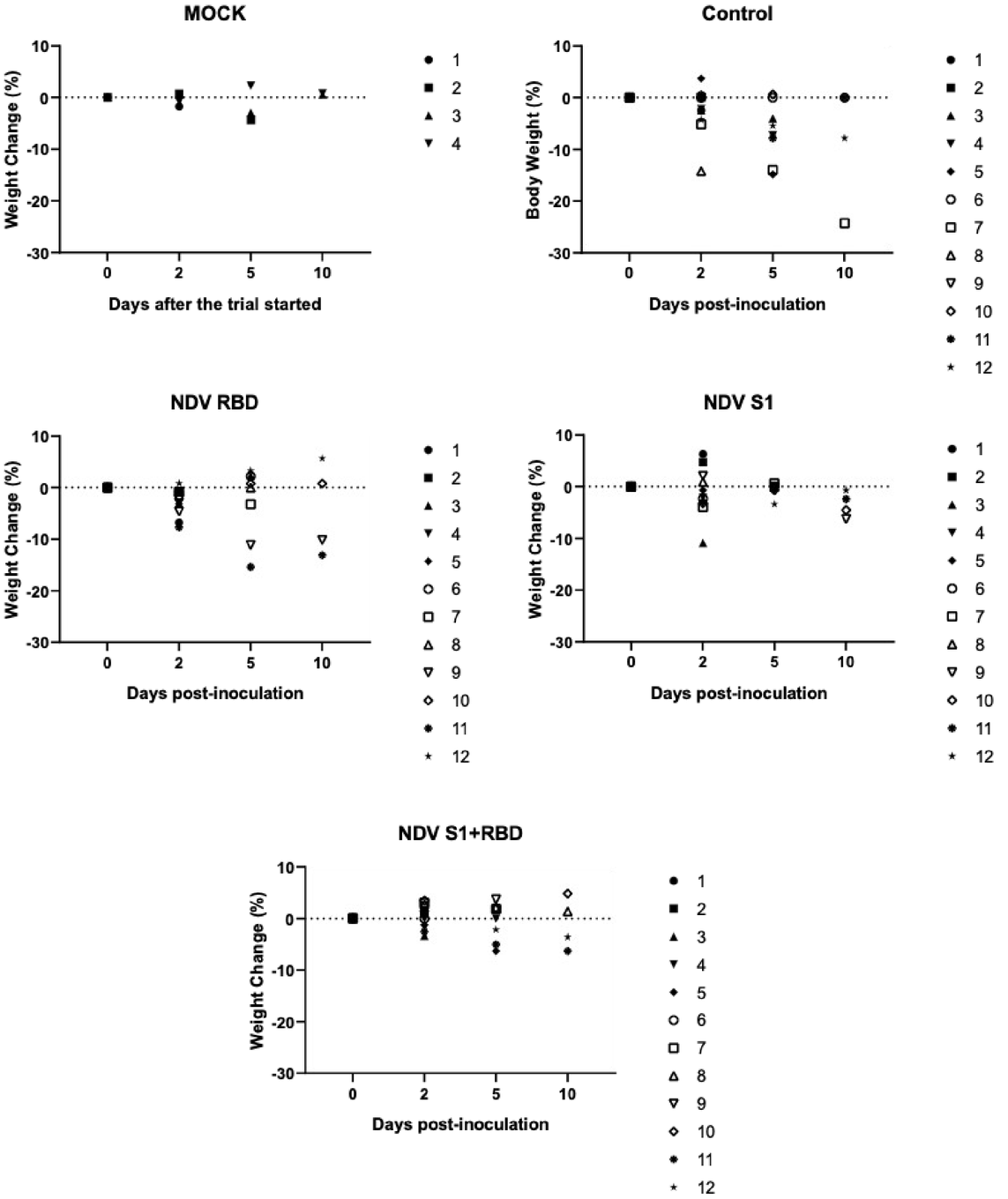


Eficacia de la vacuna:
-Neutralización del virus SARS-CoV-2 en placa en cultivo celular PRNT,

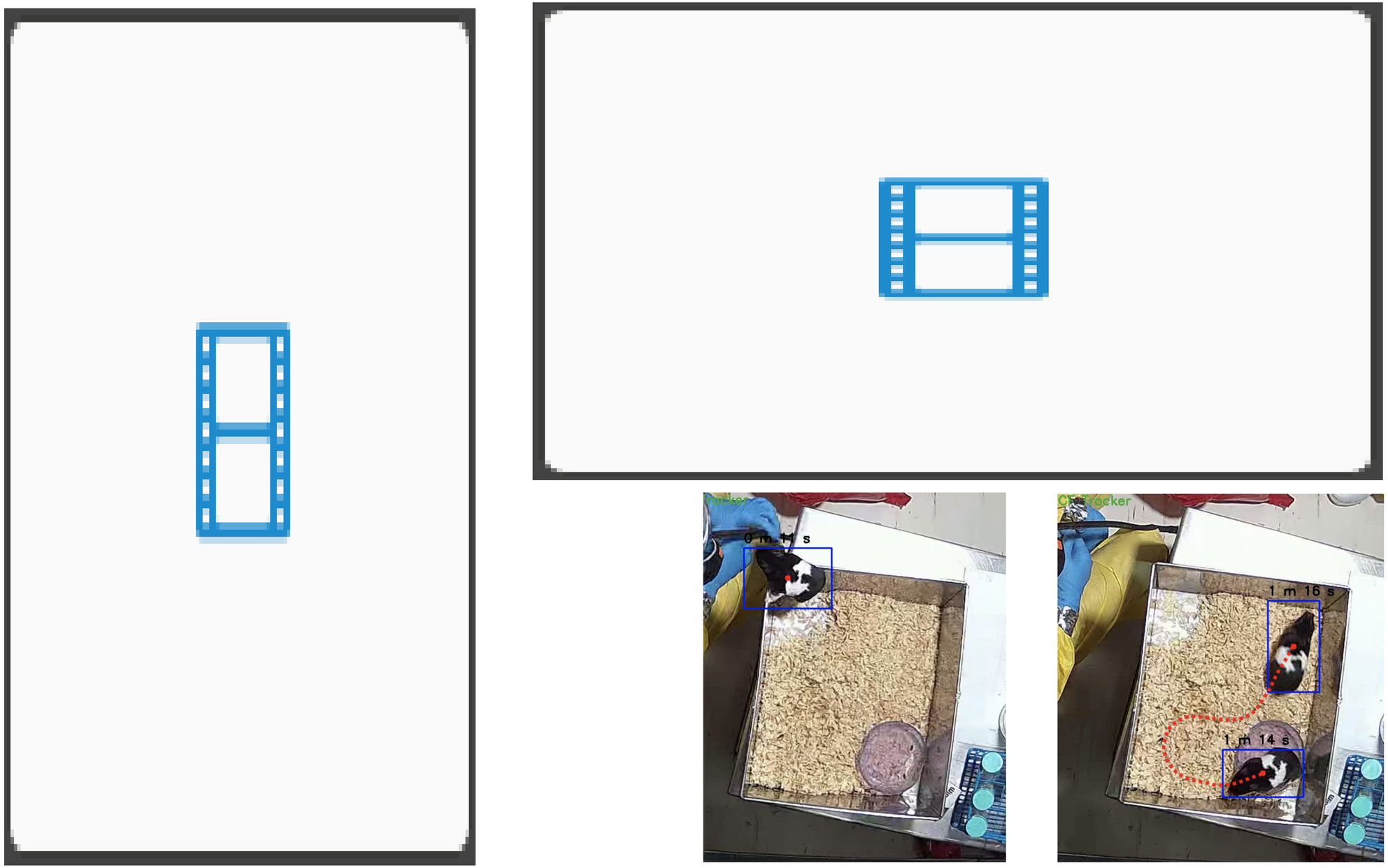


MUESTRAS PROCESADAS							
CODIGO DE MUESTRA	PORCENTAJE DE NEUTRALIZADION						TITULO A 100%
	1/20	1/40	1/80	1/160	1/320	1/640	
N-55	100.0	100.0	100.0	100.0	97.3	94.6	1/160
N-56	78.4	67.6	37.8	37.8	16.2	10.8	<1/20
N-57	100.0	100.0	75.7	62.2	37.8	21.6	1/40
N-58	51.4	48.6	29.7	8.1	10.8	0.0	<1/20
N-59	100.0	100.0	78.4	45.9	27.0	35.1	1/40
N-60	81.1	86.5	78.4	64.9	29.7	29.7	<1/20
N-61	100.0	94.6	62.2	67.6	10.8	-5.4	1/20
N-62	54.1	37.8	40.5	13.5	-5.4	18.9	<1/20

Eficacia de la vacuna:
-Variación de peso corporal

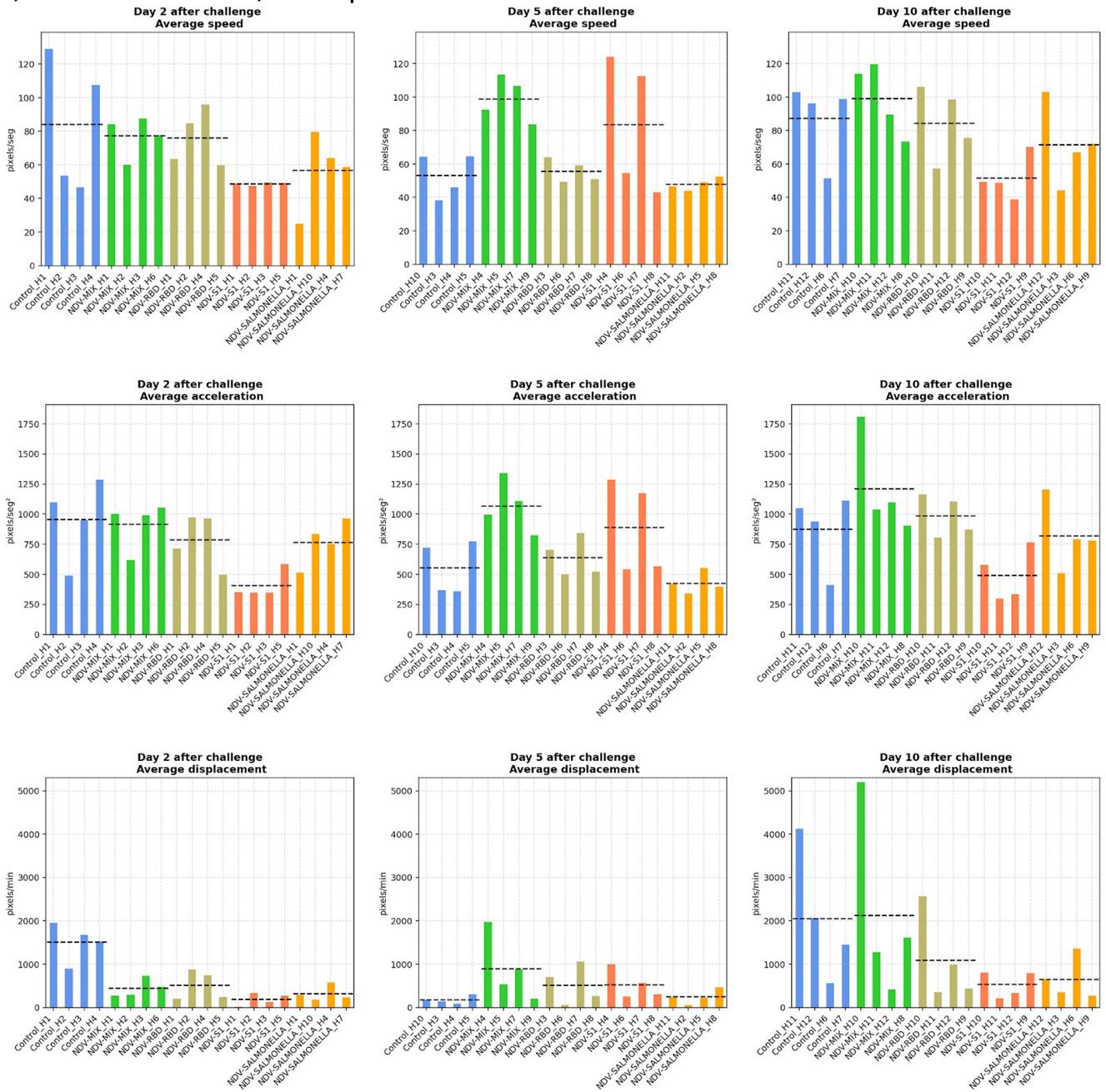


Higher mobility in vaccinated animales compared to placebo after challenge



(a) (b)

Eficacia de la vacuna:
-Movilidad (velocidad, aceleración, desplazamiento



Advantages of the nasal-NdV vaccines

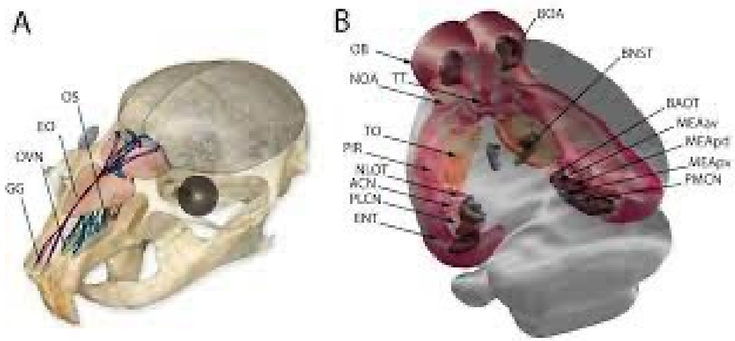
-La vacuna ha demostrado ser segura en ratones
-Capacidad de levantar respuesta humoral (IgM, IgG)
-Capacidad de levanter respuesta celular (linfocitos T CD4 – interferon gama)
-Capacidad de neutralizar la proteína viral in-vitro
-Capacidad de sero-neutralización del virus SARS-CoV-2 (colaboración INS)
-Facilidad y velocidad de producción
-Es de administración por vía nasal (no va a requerir de un ejército de técnicos para inmunizar). Podrían participar promotores comunitarios.
-Importante estabilidad (liofilizada: más de un año a 8C)
-Bajo costo de producción
-Facilidad para cambiar el antígeno en un función de las mutaciones mas prevalentes

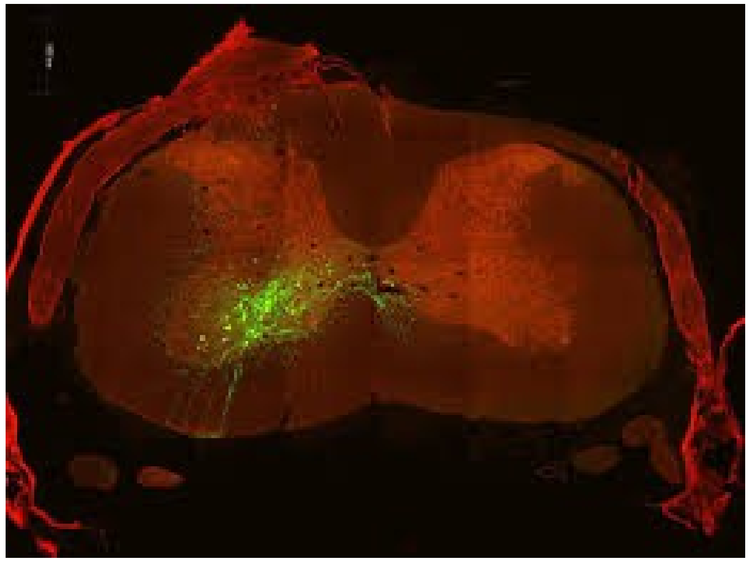
Ensayos clínicos Fase I, II y III

- Phase I: (N=50) Phase II: (N=200) Phase III: (5,000)
- Limitations:
  - Production of doses -> GMP Laboratory
    - Tercerizing in GMP laboratory
  - Clinical phases in Peru (Funded by Peruvian government and private sector)
    - Fase I:(N=50)
    - Fase II:(N=200)
    - Fase III:(N=5,000)

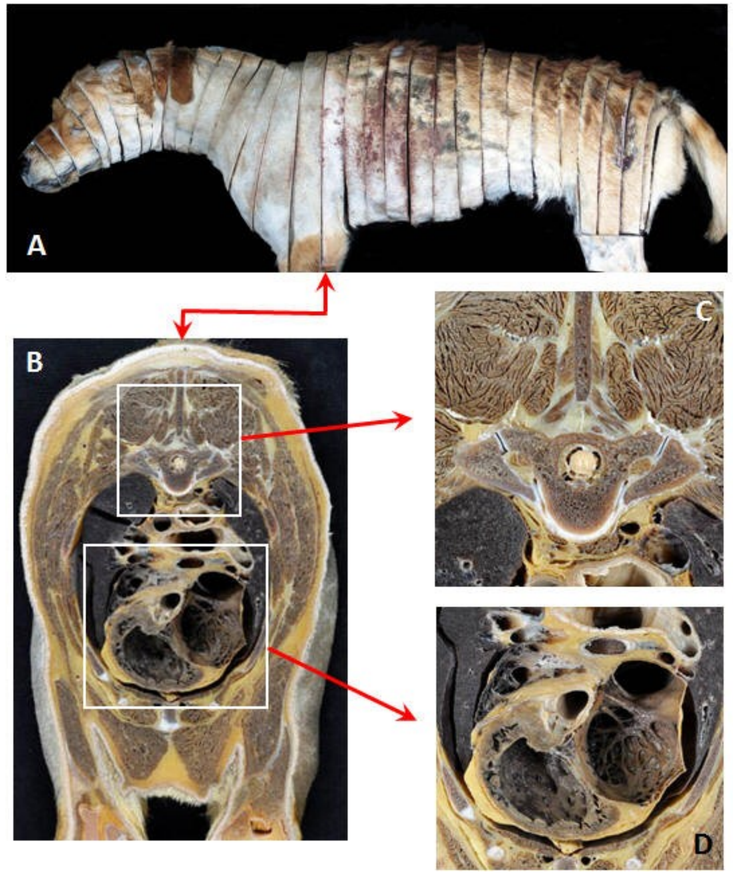
Próximos estudios:

Seguimiento del patrón del desplazamiento del virus NDV luego de la inmunización nasal, evaluado por fluorescencia





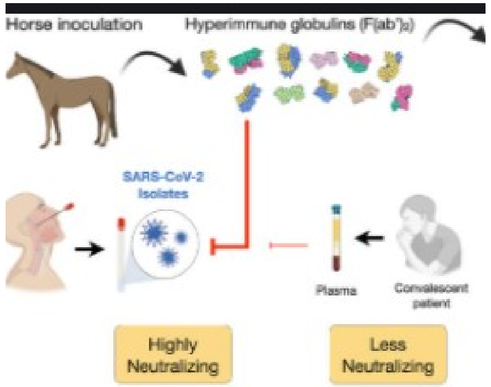
Corte transversal de médula espinal inyectada con vectores virales que expresan la proteína fluorescente verde (GFP) y procesada con inmunofluorescencia



Anticuerpos IgY de gallina purificados del huevo como tratamiento para casos severos de COVID-19

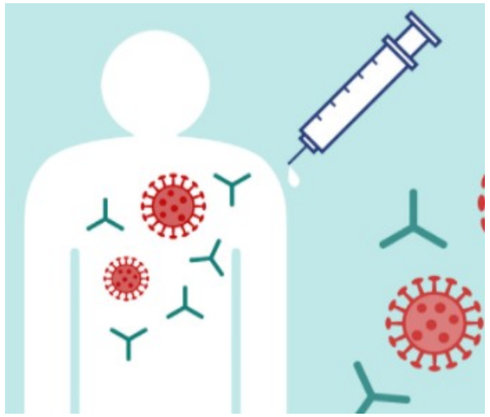
Neutralización de la interacción entre el RBD de la proteína Spike la hACE2

Inmunoterapia



- Plasma convaleciente
- Anticuerpos recombinantes
  - Humanos
  - Equinos
  - Camélidos sudamericanos
  - IgY de huevo de gallina

Vacunación

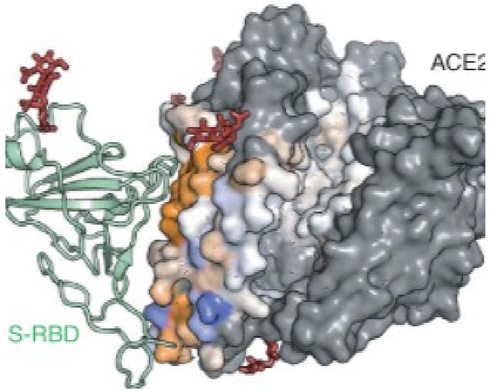


Inducen anticuerpos

- Virus Inactivado
- Vacunas recombinantes
- Vacunas de mRNA

Chen et al., 2020, H. Zhou et al., 2020

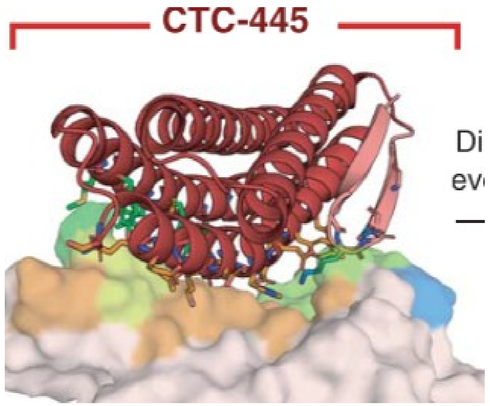
Ingeniería de proteínas



hACE2 soluble,
Proteína completa

Li et al., 2020

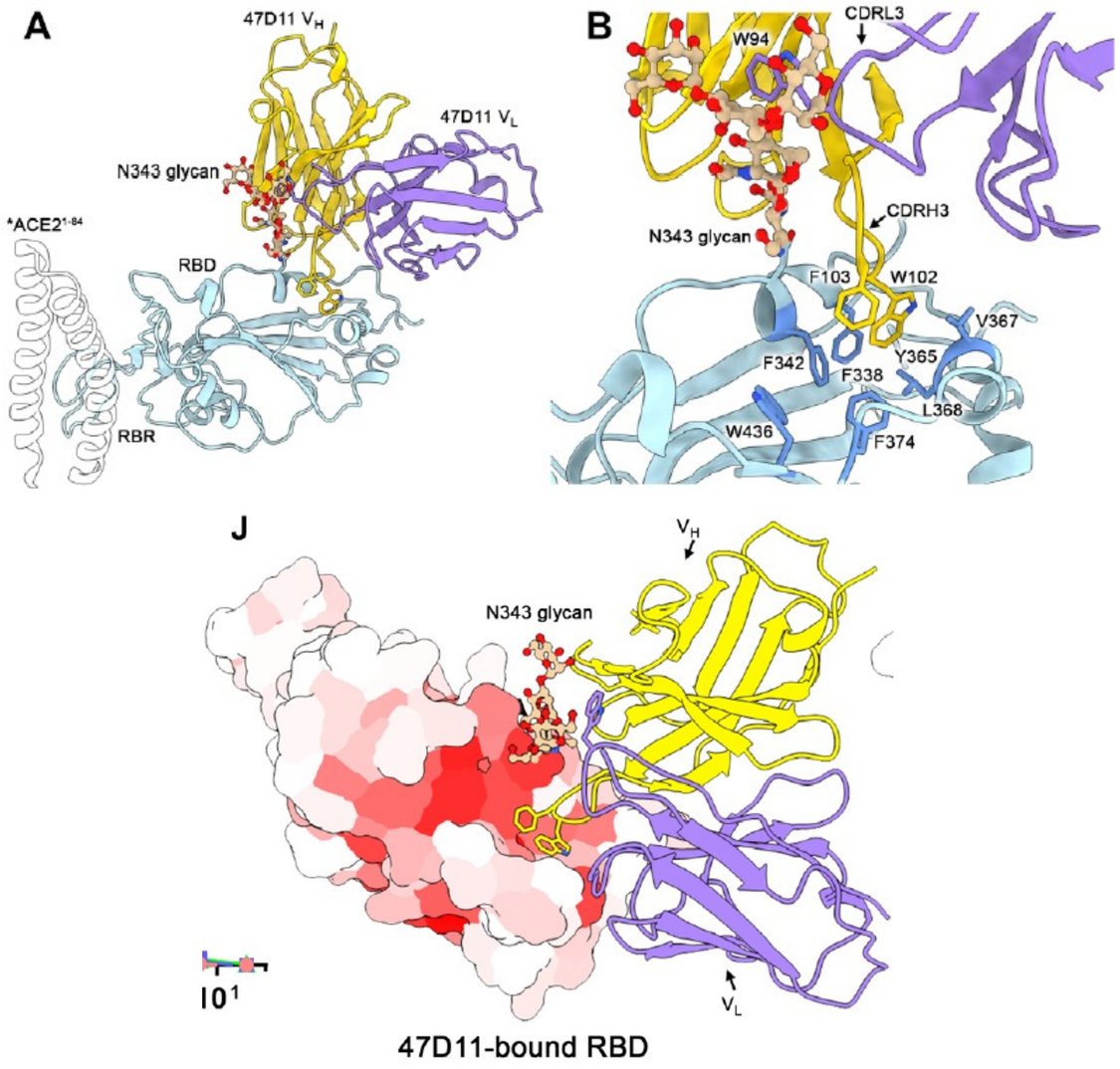
Diseño de proteínas de novo

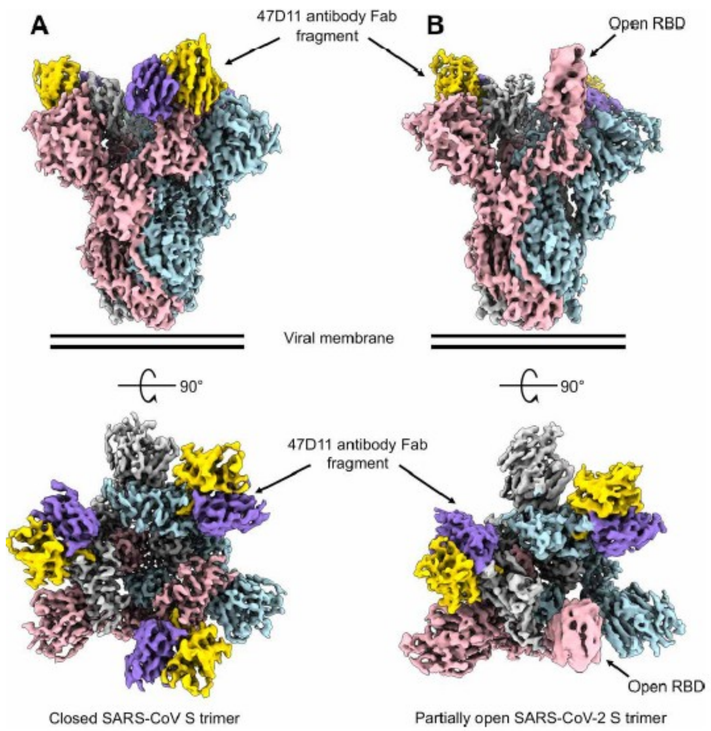


Diseño de proteínas de novo
Y de microproteínas con alta afinidad.

Cao et al., 2020, Linsky et al., 2

Sitio de unión de los anticuerpos neutralizantes en la proteína Spike



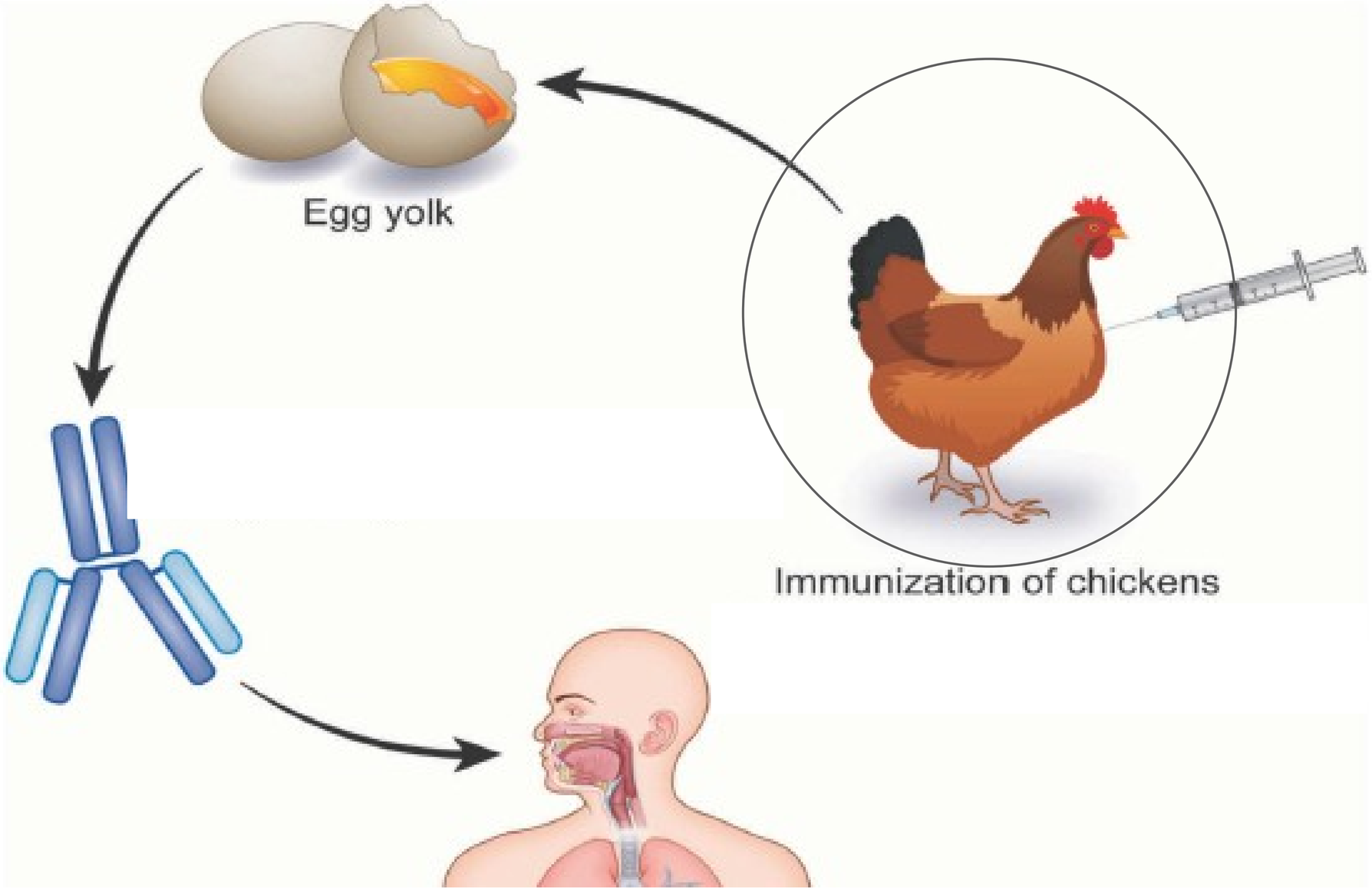


"Hydrophobic pocket" conservado

- Loop CDRL3
- W94 → N343 (SARS2)
- CDRH3
- W102/F103
- F338, F342, Y365, V367, L368, F374 y W436

Fedry *et al.*, *Sci. Adv.* 2021

Anticuerpos IgY para la inmunoprofilaxis y terapia de infecciones respiratorias



Abbas, A., El-Kafrawy, S., Sohrab, S., & Azhar, E. (2018). IgY antibodies for the immunoprophylaxis and therapy of respiratory infections. *Human Vaccines & Immunotherapeutics*, *15*(1), 264-275. doi: 10.1080/21645515.2018.1514224

Flujo de Trabajo

Inoculación de RBD del lisado celular en gallinas

Verificación de presencia de IgY anti-RBD en suero de gallinas

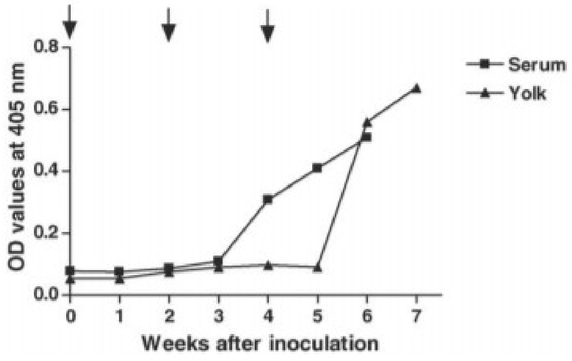


Fig. 1. Patterns of SARS coronavirus antibodies from sera and yolks of immunized SPF chickens. The arrows indicate the immunization times.

Fu Chao-Yang et al., 2005

Colecta de los huevos de las gallinas inoculadas

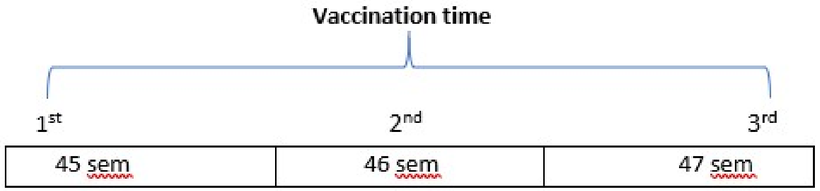
Purificación de IgY anti-RBD a partir de las yemas de huevos

Verificación de presencia de IgY anti-RBD en las yemas de huevo de gallina

Administración de IgY anti-RBD a animales

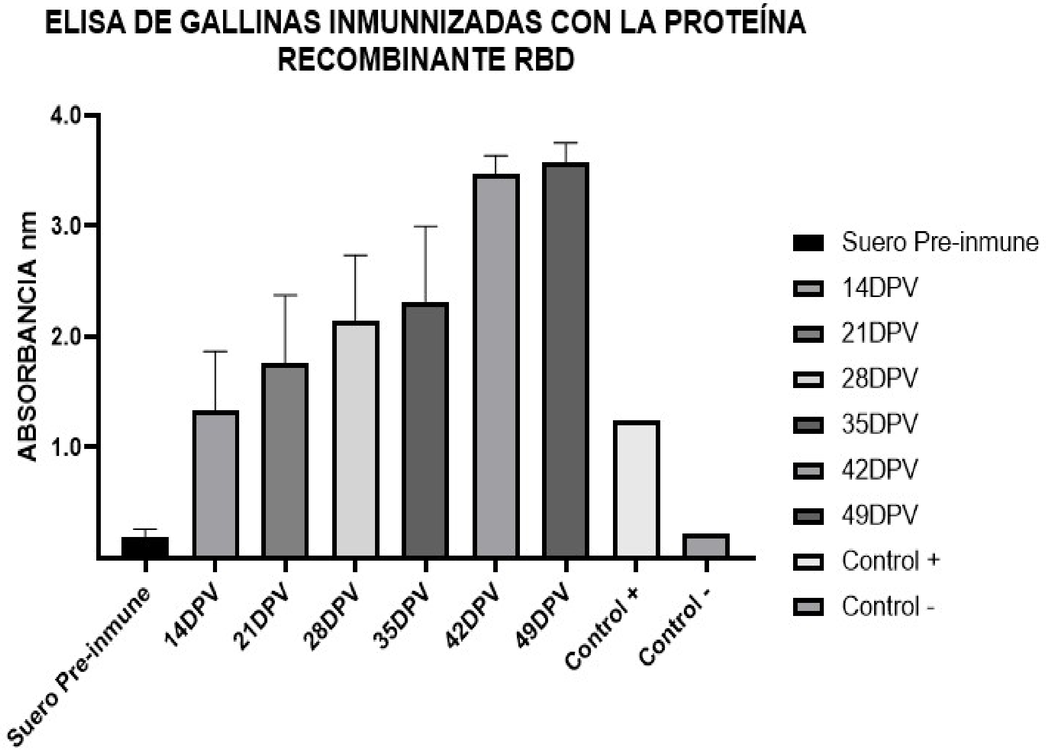
1. Inoculación de RBD del lisado celular en gallinas

- **Vía de inoculación:** Intramuscular y subcutánea
- **Adyuvante:** ISA 71R VG
- **Dosis:** 50% de aceite con 50% antígeno en un volumen final de 2 mL
- **Suspensión-prensa:** 04 veces 40psi/1 vez 80psi
- **Modelo animal:** Gallinas

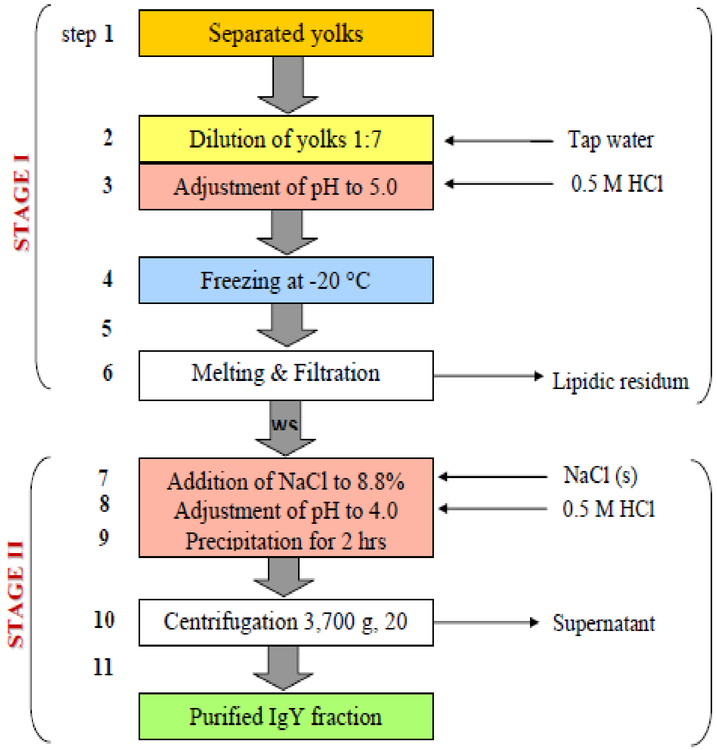


Collect sera at 0 and 10 days post-each vaccination

Vaccination	Date	Dose
1st.	09/05/2020	2mL
2nd.	16/05/2020	2mL
3rd.	23/05/2020	2mL

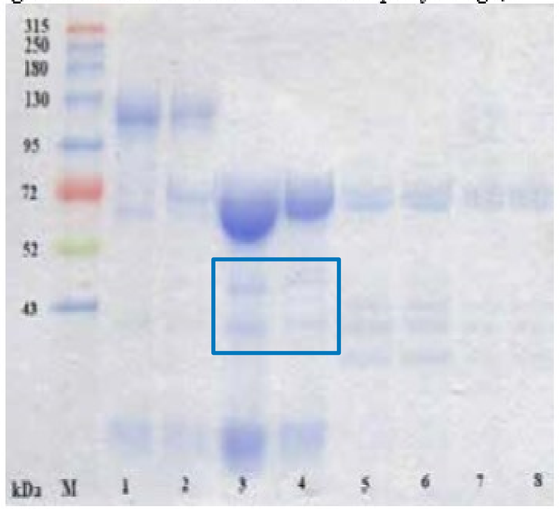


2. Obtención y purificación de la proteína IgY mediante “salting-out”

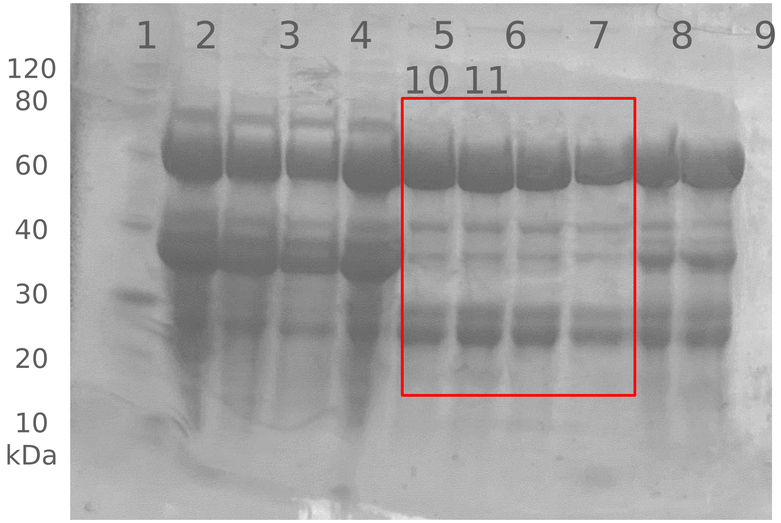


Hodek et al., 2013

Figure 1.*The SDS-PAGE profile of IgY (M: marker, 1 and 2: standard IgY non-reduced,3 and 4: standard IgY reduced, 5 and 6: WSF, 7 and 8: purified IgY).*



Wibawan et al., 2018



Line 1: Ladder; lines 2, 3, 4, 5: WSF of inmunized yolks (RBD antigen) using HCL, HCL + slow freezing method, lemon and vinegar, respectively; lines 6, 7, 8, 9: purified IgY using HCL, HCL + slow freezing method, lemon and vinegar, respectively; Lines 10, 11: Purified IgY using the gallus inmunotech inc. IgY EggsPress Purification Kit, respectively.

3. Obtención y purificación de la proteína IgY mediante “PEG”

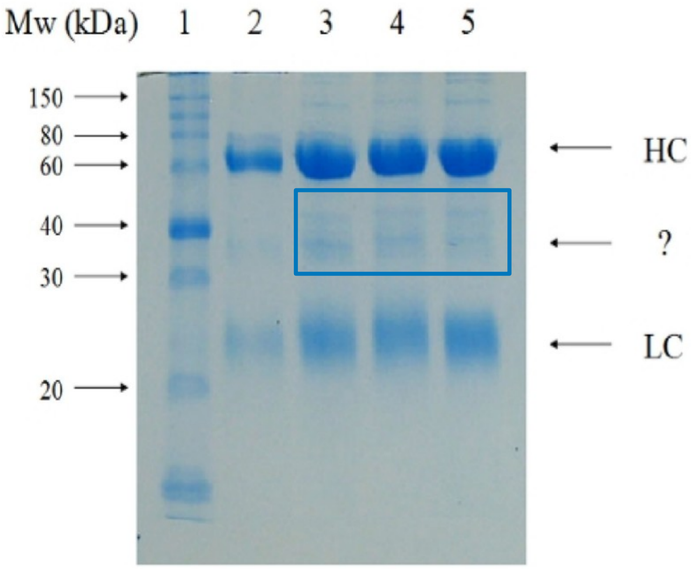
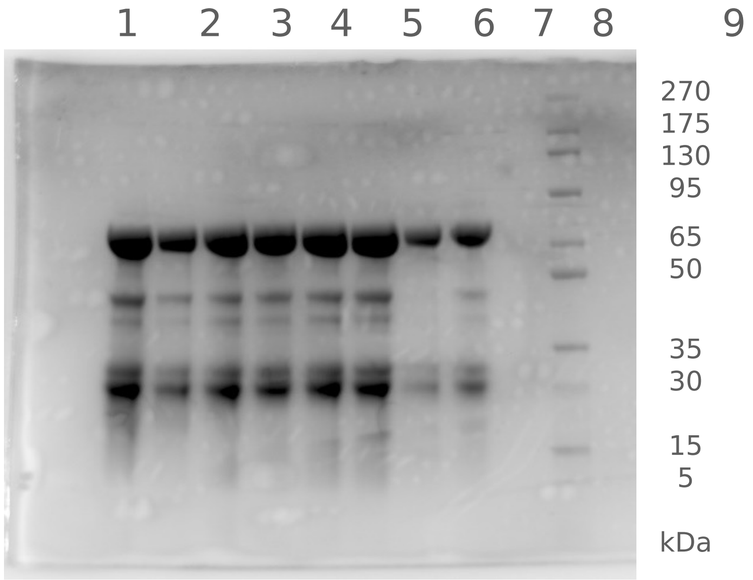


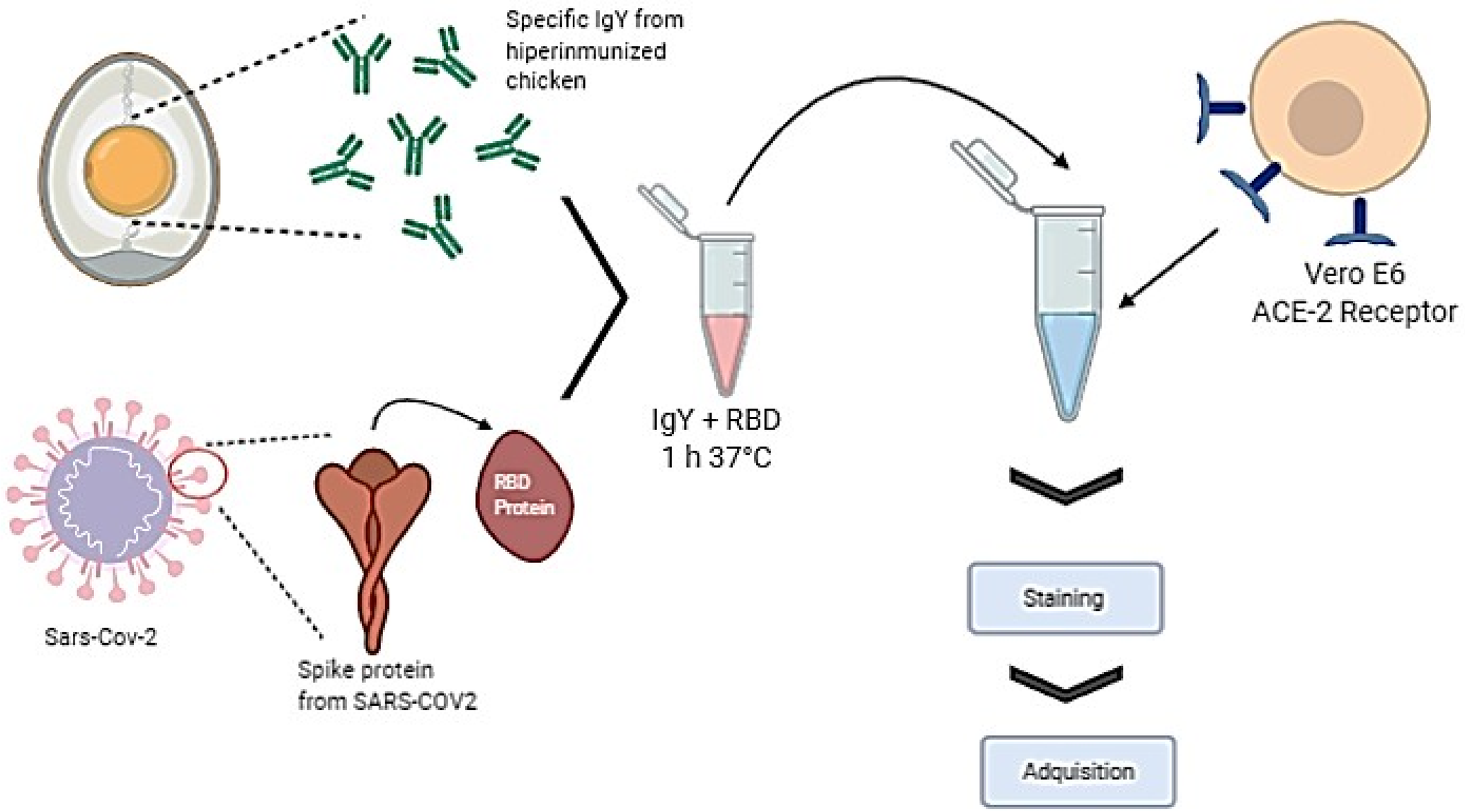
Figure 4. Polyacrylamide-Gelelectrophoresis of individual IgY preparations from different eggs under reducing conditions
Lane 1 - Molecular weight marker, Lane 2 - IgY-standard, Lane 3-5 - different IgY samples prepared as described in the protocol. These are final preparations according to step 10 of the protocol. HC - heavy chains, LC - light chaines, ? - minor impurities corresponding to molecular weight around 35 kDa (probably the C-terminal fragment of vitellogenin II precursor, [Klimentzou *et al.*, 2006]).

Pauly et al., 2011

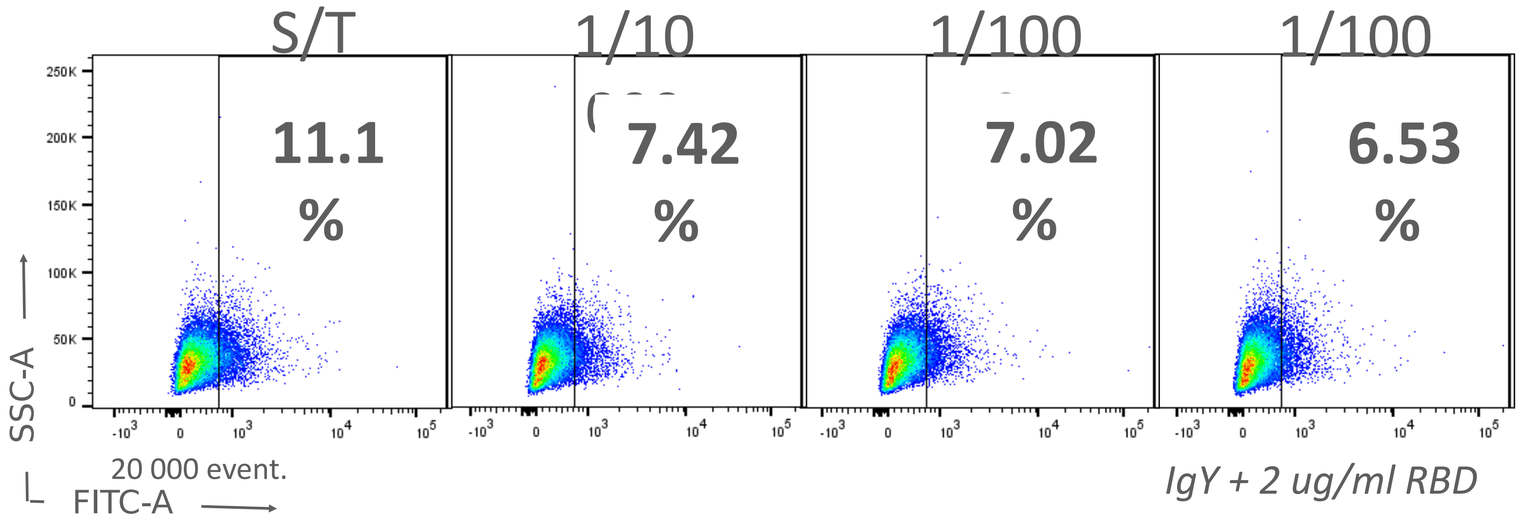


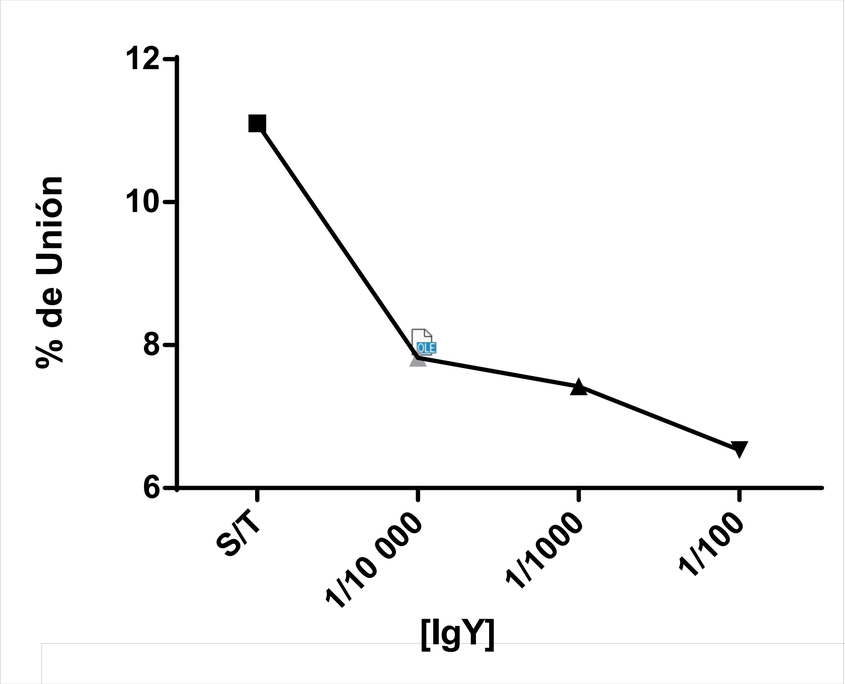
Line 1-6: IgY purified by PEG method (10 ug), Line 7-8:
IgY purified by PEG method (5 ug), Line 9: Ladder

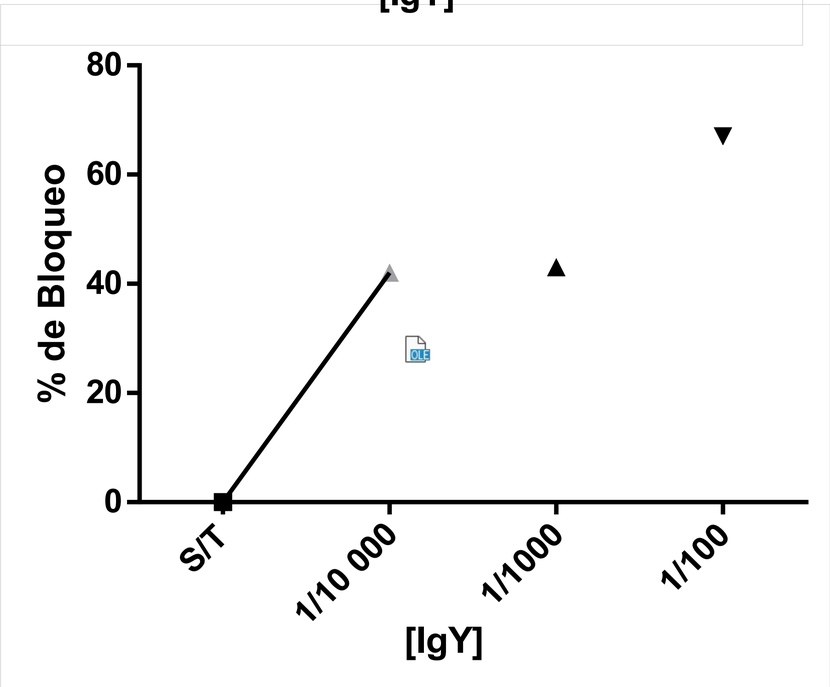
Ensayo de bloqueo con yemas de huevos de gallinas Hiper-inmunizadas



Ensayo de bloqueo con las diluciones de IgY en suero de Ratón





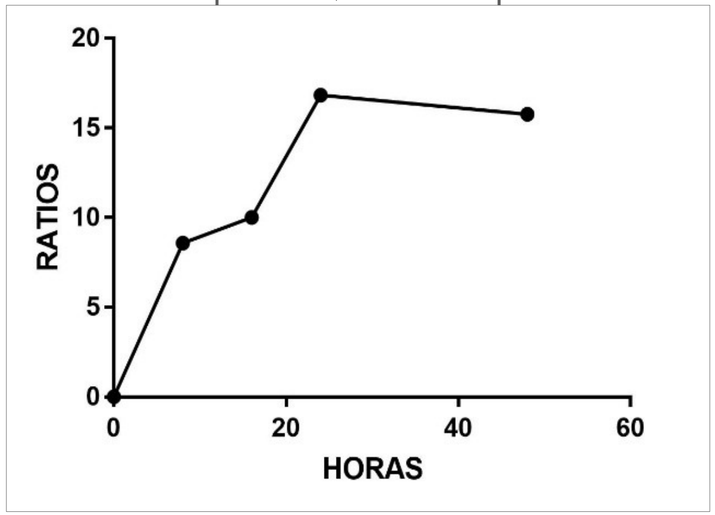


	11.1	7.42	7.02	6.53
	0	**42**	**43**	**67**

4. Administración de IgY-anti RBD en animales

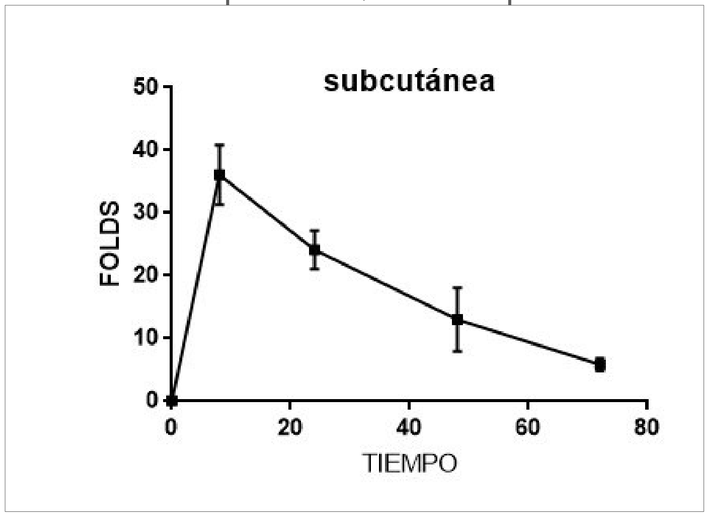
Administración subcutánea

CONEJOS



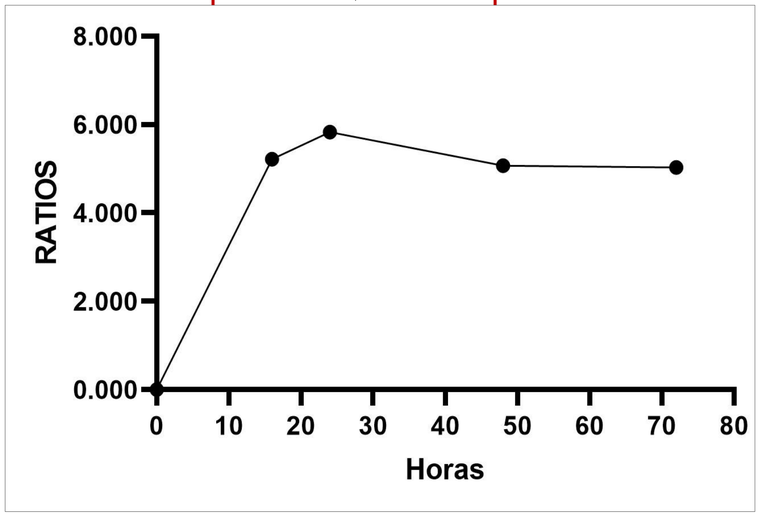
Yemas de huevos: 21 DPV aprox.
Peso de conejos: 3

RATONES



Yemas de huevos: 35 DPV aprox.
Peso de ratones: 25 –

CERDOS

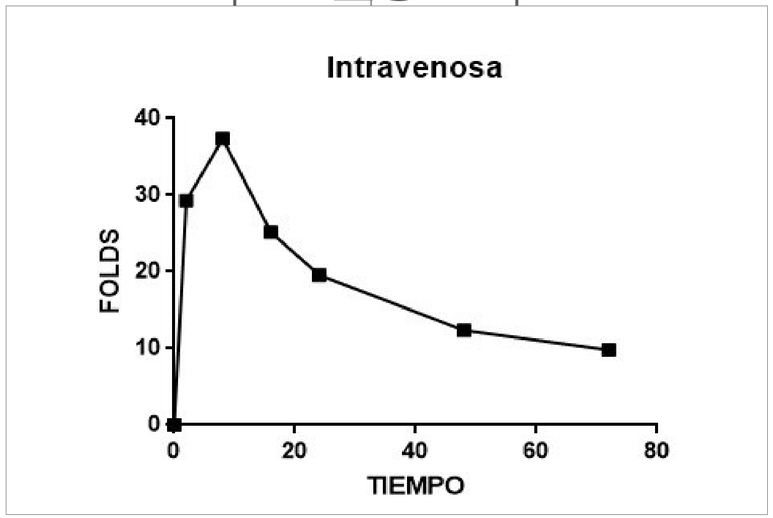


Yemas de huevos: 50 DPV aprox.
Peso de cerdos: 20

5. Administración de IgY-anti RBD en animales

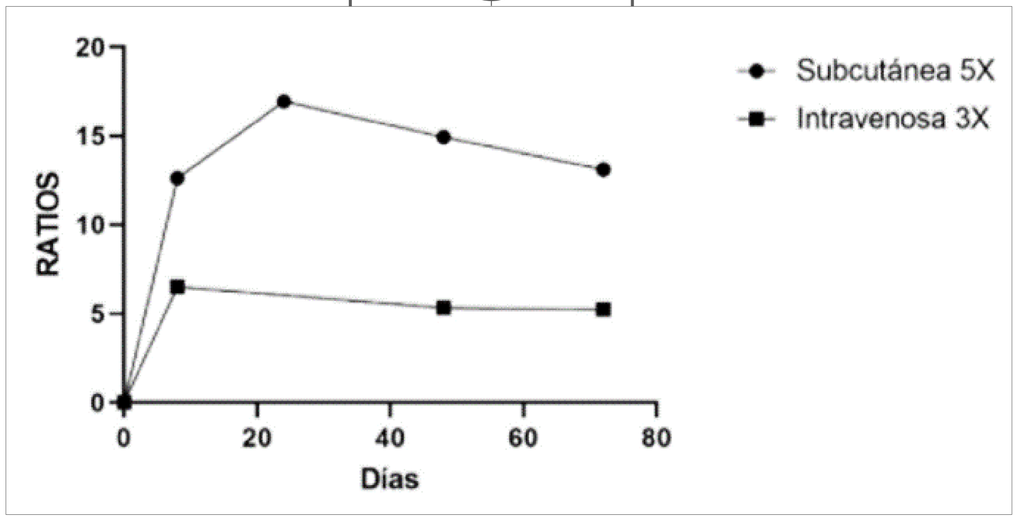
Administración intravenosa

RATONES



Yemas de huevos: 35 DPV aprox.
Peso de ratones: 25 –

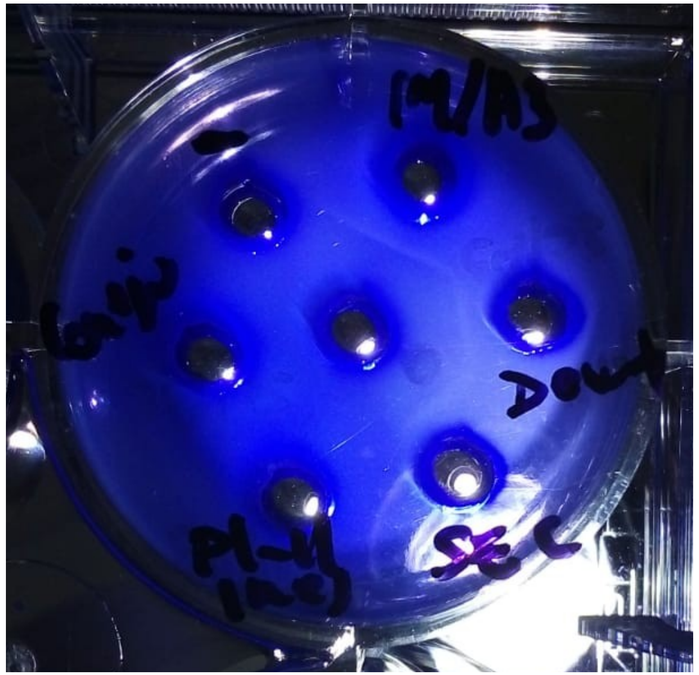
CERDOS



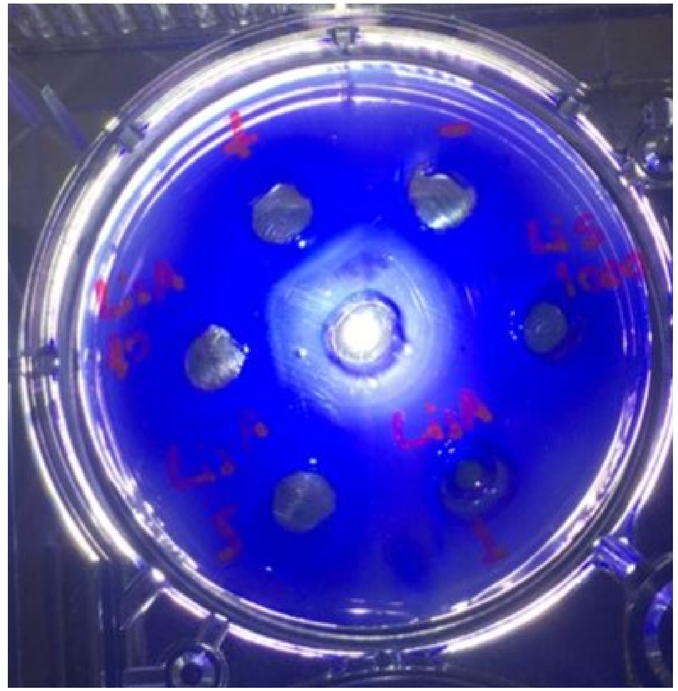
Yemas de huevos: 60 DPV aprox.
Peso de cerdos: 20 kilos

6. Inmunodifusión doble de Ouchterlony

Establecimiento de controles positivos y negativos

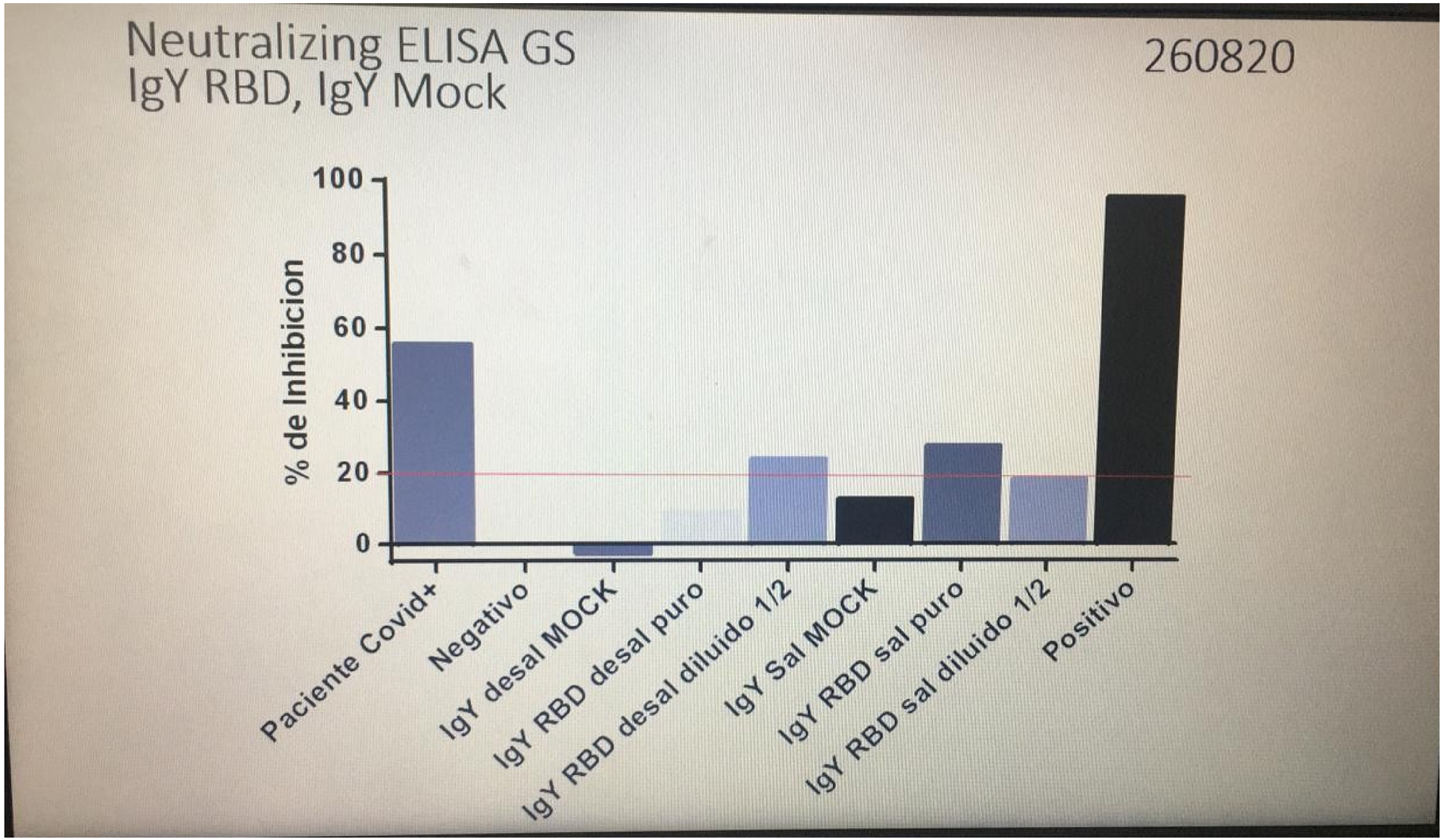


Positivo para IgY desalado y purificado por SEC en Sephadex G-75 (10 semanas PV)

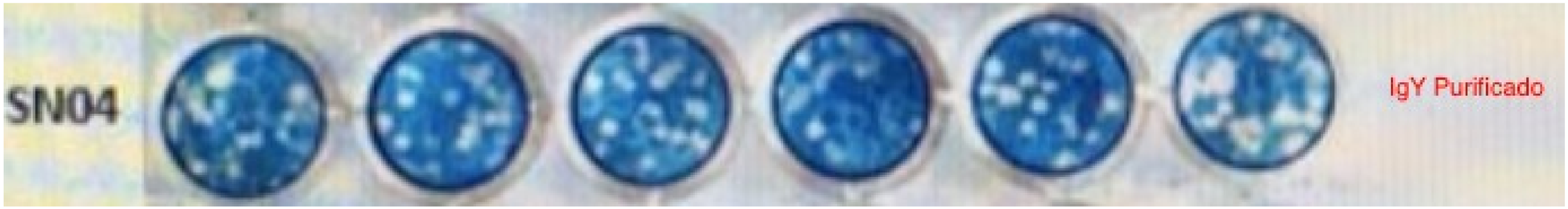


Positivo para IgY sin desalar 10 mg/mL (10 semanas PV)

7. Neutralización – huevos de 10 semanas



7. Neutralización



Yema de huevo de 74DPV aproximadamente

Neutralización de suero de cerdo con SARS-CoV 2. Se administró 75 mg/kg de IgY anti-RBD de yemas de huevo de 50DPV aprox., en total 1.5 g de IgY anti-RBD en un volumen de 15 mL en la zona subcutánea de un cerdo de 20-25 Kg.

Neutralización de suero de ratón con SARS-CoV 2. Se administró 125 mg/kg de IgY anti-RBD de yemas de huevo 35DPV aprox., en total 3.75 mg de IgY anti-RBD en un