DEVELOPMENT OF A THERAPY BASED ON NANOANTIBODIES AGAINST THE INFLUENZA A VIRUS

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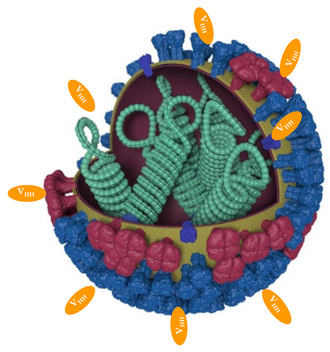
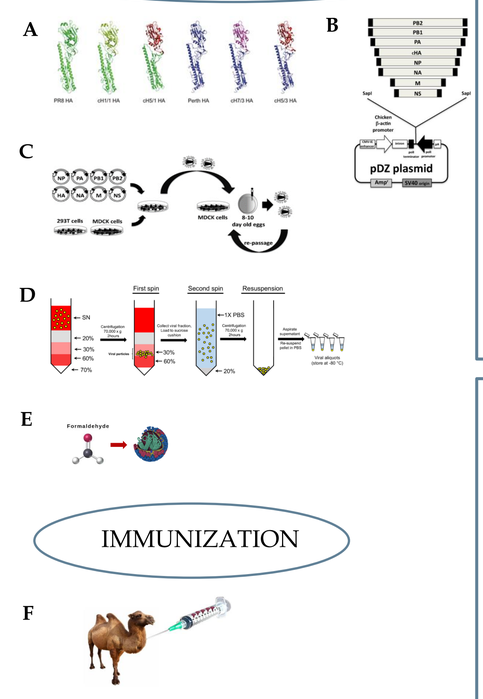
ABSTRACT

Influenza viruses are enveloped, single-stranded, negative-sense RNA viruses that belong to the family Orthomyxoviridae. Three types of Influenza viruses have been described, being types A and B the main human pathogens. They cause significant morbidity and mortality worldwide, with the elderly and children being the most susceptible patients. Influenza A viruses are divided into subtypes according to genetic and antigenic differences in the two main surface spike proteins, hemagglutinin (HA) and neuraminidase (NA). Current vaccines are an effective measure against infection, but must be reformulated yearly due to the antigenic drift of the virus. Furthermore, they do not protect against new pandemic strains. Currently, only two classes of drugs are approved for treatment: M2 ion channel blockers and NA inhibitors, but they have limited efficacy, adverse side effects, and great drug resistance. Therefore, there is a need to develop new antiviral strategies with new modes of action and reduced resistance. Nanobodies are small single domain antibody fragments that have unique properties such as the ability to recognize particular epitopes (not recognized by conventional antibodies) and their improved stability. They have been used successfully in therapeutic approaches as receptor blockers associated with pathogenic diseases or viruses, or conjugated with drug carriers. The main objective of this work was the generation of broad-spectrum neutralizing nanobodies against conserved Influenza A epitopes. Currently, the strains with the highest infection rate are H1N1 and H3N2. Therefore, a new approach of sequential vaccination has been applied in camels using synthetic viruses expressing chimeric HA molecules (cHA), which combine stem-conserved domains of group 1 (H1) or 2 (H3) with unusual domains in the head. A phage display nanobody library was subsequently generated and candidates were selected after successive rounds of biopanning. The neutralizing activity of the selected candidates against Influenza A virus is currently being analysed.



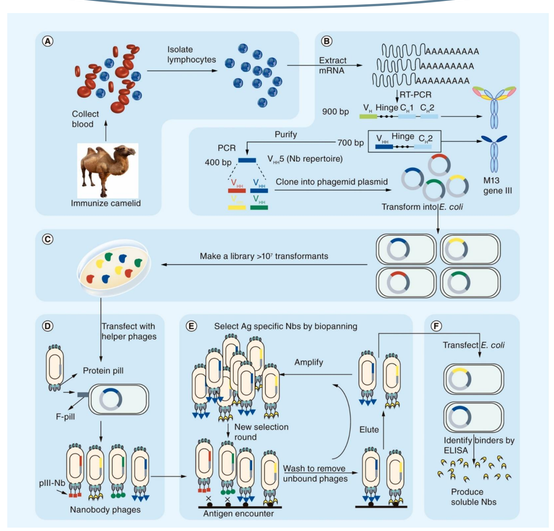
MATERIALS AND METHODS

INACTIVE RECOMBINANT VIRUSES



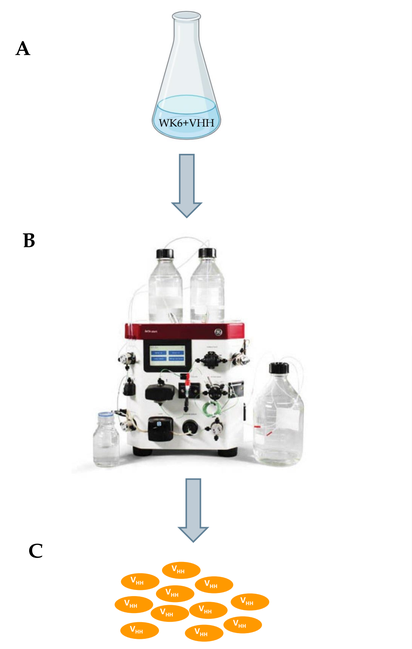
(A) cHAs selection. **(B)** Cloning of cHAs into the ambiense plasmid pDZ. **(C)** Generation of chimeric viruses with the transfection of pDZ plasmids in a coculture of MDCK-Siat 1 and Hek-293T for their subsequent amplification in embryonated eggs. **(D)** Concentration of amplified viruses in allantoic fluid using sucrose gradient. **(E)** Inactivation of viruses with formaldehyde. **(F)** Serial immunization of camel with inactive synthetic viruses

NANOBODY LIBRARY



(A) After camelid immunization with the antigen of interest, anti-coagulated peripheral blood is collected to isolate lymphocytes. **(B)** The first-strand cDNA is prepared from RNA and used as a template to amplify the Nb repertoire. Two consecutive PCR reactions are performed. In the first, two fragments are amplified: one fragment of ~900 bp, corresponding to the VHs-hinge-CH1-CH2 region of the H-chain of conventional antibodies, and a second fragment of ~700 bp, encompassing the VHHs-hinge-CH2 domains of heavy-chain antibodies. After electrophoresis separation, the 700 bp PCR fragment is purified and used as a template in a second PCR to amplify the specific VHHs sequences of ~400 bp (Nb repertoire). Then, the amplified Nb repertoire is cloned into a phagemid plasmid. This vector contains M13 gene III, which encodes the pIII protein, a helper phage surface protein located at one end of M13 tubular structure particle. **(C)** The generated plasmid collection is transformed into an *Escherichia coli* amber suppressor strain. **(D)** Transformants cells harboring the Nb library are infected with M13K07 helper phages to produce virus particles with the cloned Nb at their tips due to the fusion to pIII protein. **(E)** Isolated phage particles are used to perform serial rounds of panning to select Ag-specific Nb-phages. Eluted phage particles from each round are added to exponential growing cells to amplify and to enrich the sample with Ag-specific Nbs. **(F)** Part of this infected cells are plated and used to identify high affinity binders Nbs.

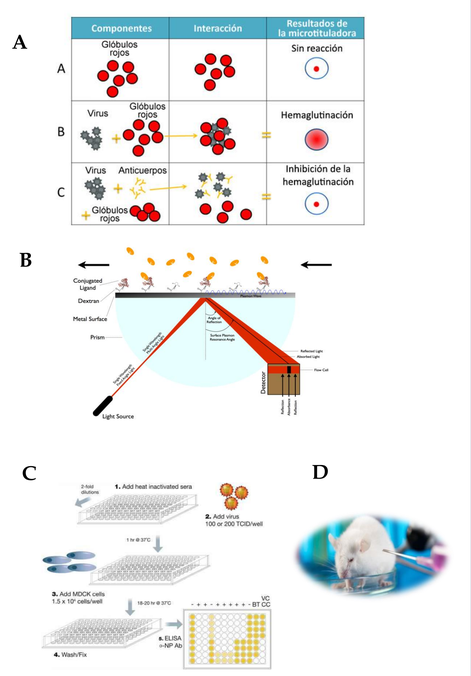
VHH EXPRESSION AND PURIFICATION



(A) Individual colonies are growth, and after protein expression induction, the periplasmic extract containing the Nb. **(B)** Positive Nb in phage ELISA is purified by Fast Protein Liquid Chromatography (FPLC) with Ni+ Column. **(C)** Soluble positive Nb (VHH).

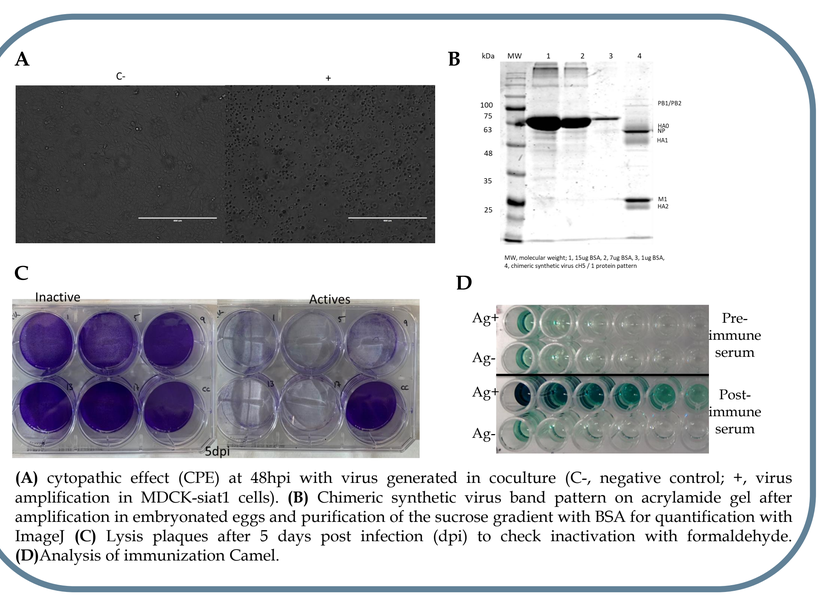
FUTURE PROSPECTS

NEUTRALIZING ACTIVITY

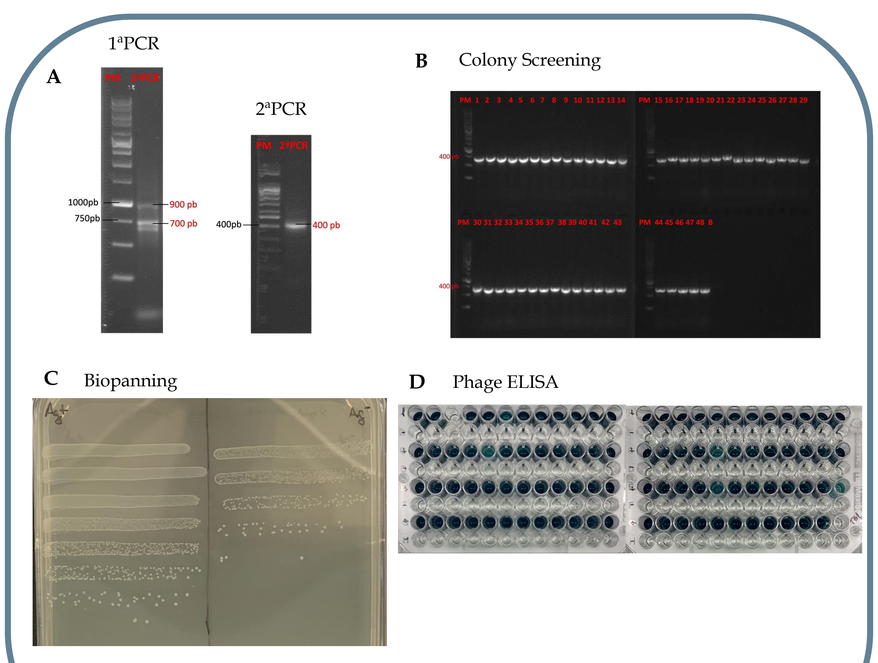


(A) Hemagglutination **(B)** Affinity by Surface Plasma Resonance **(C)** Microneutralization assays *in vitro* **(D)** Prophylaxis and microneutralization assays *in vivo* .

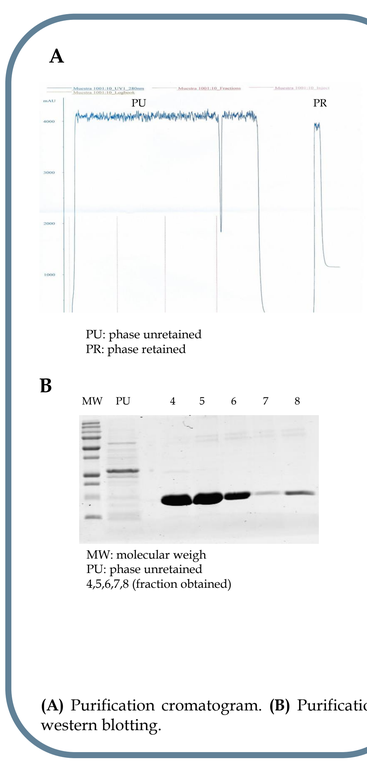
RESULTS



(A) cytopathic effect (CPE) at 48hpi with virus generated in coculture (C-, negative control; +, virus amplification in MDCK-siat1 cells). **(B)** Chimeric synthetic virus band pattern on acrylamide gel after amplification in embryonated eggs and purification of the sucrose gradient with BSA for quantification with ImageJ **(C)** Lysis plaques after 5 days post infection (dpi) to check inactivation with formaldehyde. **(D)**Analysis of immunization Camel.



(A) PCRs to amplify the Nbs repertory from camel cDNA. lymphocites. **(B)** Colony screening of library. **(C)** Bio-paninng to select positives candidates. **(D)** Phage ELISA to identify positives candidates against influenza virus.



PU: phase unretained
PR: phase retained

MW: molecular weigh
PU: phase unretained
4,5,6,7,8 (fraction obtained)

(A) Purification cromatogram. **(B)** Purification western blotting.

CONCLUSIONS

- Different nanobodies were positively identified against *Influenza* A. The nanobodies were expressed and purified.
- Subsequent *in vitro* and *in vivo* studies will be performed to analyze the neutralizing activity.

ACKNOWLEDGMENTS

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