UNIVERSIDAD DE INVESTIGACIÓN DE TECNOLOGÍA EXPERIMENTAL YACHAY

Escuela de Ciencias Biológicas e Ingeniería

TÍTULO: Expression of the E21b, E21d, and E21e antigenic viral protein variants of bovine viral diarrhea in the CHO cell line.

Trabajo de integración curricular presentado como requisito para la obtención del título de Licenciatura en Biología

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Urcuquí, marzo 2020

Urcuquí, 21 de mayo de 2020

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Dedicatory

I dedicate this dissertation to:

Yachay Tech University and its professors, for offering me the tools to fulfill a lifelong dream.

My parents and family, for their unwavering patience and support through the years.

My great grandmother, for always encouraging my curiosity and passion for science.

Jorge Simón

Acknowledgments

No labor of this nature can be accomplished without the help, support, and cooperation of many others. My list of acknowledgments begins with the eternal patience and guidance of my tutor, Nelson Vispo, and co-tutor Eddy Gonzales. Their efforts and trust were most effective in the development and completion of this work.

For their support and guidance in the culmination of my professional program of studies, the foundations of this dissertation, special recognition is due to my former professors: Markus Tellkamp, Francisco Álvarez, Lilian Spencer, Graciela Salum, Santiago Ballaz, Ming Ni, Fernando Gonzales, Frank Alexis, José Castillo, Sofia Carvalho, and Hugo Romero.

I also extend my appreciation to the members of the Biological Sciences Faculty at the University of Conception, especially to Jorge Toledo, Seidy Pedroso, Noralvis Fleitas, and Frank Camacho, who have given me their continued assistance and precious time to help bring this study to fruition.

Finally, I want to thank all my friends, family, and colleagues who have supported me with their kind words and have been there for me throughout this endeavor.

Jorge Simón

Resumen

El virus de la Diarrea Viral Bovina, del género *Pestivirus*, es un patógeno viral que afecta las vías respiratorias y tasas de fertilidad del ganado a nivel mundial generando substanciales pérdidas económicas para la industria ganadera. Dado el irregular desempeño de las vacunas comerciales disponibles en Chile contra la Diarrea Viral Bovina, y la fuerte inducción de respuesta inmune de la proteína de envoltura E2 de la DVB, nosotros expresamos y caracterizamos tres variantes en un sistema recombinante, de la forma truncada de E2 como base para candidatos de vacuna. Dos vectores de expresión mamífera (pCI-Neo) fueron usados para expresar las variantes de menor tamaño de la glicoproteína principal de envoltura E2, identificadas como E21b, E21d y E21e, en una línea productora estable de células de ovario de hámster chino. Estos genotipos recombinantes expresados compartían características similares electroforéticas y serológicas a las de su contraparte viral. Los vectores plasmídicos contenían una proteína fluorescente (EFGP) y un casete de expresión de resistencia a la neomicina fosfotransferasa, confiriendo resistencia a la Geneticina, para conducir la expresión del cultivo y permitir la selección de células conteniendo el plásmido. Mediante análisis por SDS-PAGE y Western Blot fueron caracterizadas las proteínas blanco expresadas y se seleccionaron las líneas celulares más productoras. Las tres variantes de E2 obtenidas fueron purificadas simultáneamente en un solo paso hasta pureza moderada a través de una cromatografía de Ni-Sepharose, obteniéndose inmunógenos listos para ser utilizadas en pruebas serológicas *in vivo*.

Palabras Clave:

Expresión de proteínas recombinantes, sistema de expresión mamífero, sistema de expresión bacterial, vacuna terapéutica.

Abstract

Bovine Viral Diarrhea Virus (BVDV), genus *Pestivirus*, is a viral pathogen that affects the respiratory tract and fertility rates in cattle worldwide, generating substantial economic losses for the livestock industry. Given the irregular performance of commercial vaccines available in Chile against bovine viral diarrhea and the strong induction of an immune response to the BVDV E2 envelope glycoprotein, we expressed and characterized three variants in a recombinant system from the truncated form of E2 as a base for a vaccine candidate. Two pCI-Neo Mammalian Expression Vectors were used to express the BVDV major envelope E2 glycoprotein small variants E21b, E21d, and E21e in a stable Chinese hamster ovary cell line. The expressed recombinant genotypes showed electrophoretic and serological characteristics like those of its viral counterpart. The plasmid vectors contained a fluorescent protein (EGFP) and a neomycin phosphotransferase resistance expression cassette, conferring Geneticin resistance, to drive the expression and allow for the selection of cells containing the plasmid. Through SDS-PAGE and Western Blot analysis, the expressed target proteins were characterized, and the most productive cell lines were selected. The three variants of E2 obtained were purified simultaneously in one step to moderate purity through a Ni-Sepharose chromatography, which yielded immunogens ready to be used in serological tests *in vivo*.

Key Words:

Recombinant protein expression, mammalian expression systems, microbial expression systems, therapeutic vaccine

Content

LIST OF FIGURES

I. INTRODUCTION

Definition

Bovine viral diarrhea virus (BVDV), from the genus *Pestivirus* of the family *Flaviviridae*[1], is a viral pathogen that affects the respiratory tract and fertility rates in cattle worldwide, generating substantial economic losses for the livestock industry. The infection usually manifests itself in cattle but can be transmitted across various species of the order Artiodactyla, which includes sheep, goats, camelids, and pigs.

The disease can present itself as one of two differentiated biotypes. Either inducing cellular death as a cytopathic biotype (cp) or failing to induce cellular death as a non-cytopathic biotype (ncp). However, cytopathogenicity does not correlate with virulence, given that the most virulent strains are of the ncp biotype[2]. Consequently, most of the cases present themselves as ncp biotype with subclinical symptoms, but the virus's effects rely on the age, time of gestation, and the immunologic state of the animal [3]. The animals that present the cp biotype exhibit clinical symptoms which vary between acute diarrhea, fever, predisposition to respiratory infections, reduced milk production, and an increased risk of abortion. In sporadic cases, the disease can also manifest itself as a highly lethal acute hemorrhagic syndrome (Mucosal disease)[4][5][6][7].

Epidemiology

Previous studies around the world show that BVD is variably widespread in most cattle-raising countries with variations most likely due to differences in cattle population structure and management practices[8][9]. BVD-endemic countries are found in both cold northern regions, such as the USA, Canada, Germany, and Switzerland, and temperate southern latitudes, such as Chile, Peru, and South Africa.

The disease causes substantial economic losses for the livestock sector, with the main impacts being decreased milk production, an immunosuppressor effect that leaves the animal susceptible to digestive and respiratory diseases[7][8], and reproductive failures leading to malformations, abortions and the birth of weak calves[12]. Infected mothers carrying the ncp strain can give birth to weakened persistently infected (PI) calves, which develop partial tolerance to the virus but behave as recurring reservoirs of the virus in treated herds[13]. In contrast, BVDV cp strains induce a higher mortality rate among infected hosts as an acute form of Mucosal disease[8][10].

The seroprevalence of the virus in endemic areas usually ranges from 30 to 60%, whereas the prevalence of PI can vary between 1 and 2%[11][12][14]. Factors such as population density, handling practices, and confinement methods all contribute to the observed differences[12][14]. In Chile, the virus was first isolated in 1985, and since then, it has been identified widely disseminated across the country's regions. Central and south-central provinces report a serologic prevalence around 60%, with an 86% prevalence in the capital region, Santiago de Chile[16]. Analysis of segments of the untranslated regions 5'UTR of the virus reveals the frequent presence of five genotypic groups within the country, BVDV-1a, BVDV-1b, BVDV-1i, BVDV-1j, and BVDV-2a[17][18][19]. Despite close genomic similarity among genotypes and subgroups, they lack common neutralizable epitopes, which explains the low or no cross-protection between the strains[20].

Economic impact

Given the high percentage of natural grassland areas in Chile, the Carnic and milk industries constitute its fourth main export product within the silvo-agricultural sector. The country is home to over 120.000 dedicated national producers who manage 1.91 million head of cattle, generating revenues worth 800 million dollars (USD) annually, with the most important international markets being the USA, China, and Europe[21].

Estimated losses in BVD-endemic countries range between 10 and 40 million dollars (USD) for every million calves born, with a reduction of up to 30% in milk production during epidemic outbreaks[22]. Countries that have introduced immunization programs were able to significantly reduce costs associated with this disease[23][24].

Morphological characteristics

BVDV, as part of the genus *Pestivirus*, is considered a small-size virus, possessing a diameter ranging from 40-60nm, with a sedimentation coefficient of 140 S and a floating density of 1.12 to 1.13 g/cm3 in agarose gradient. Mature virions contain three virus-encoded membrane proteins (Erns, E1, and E2) embedded in a lipidic envelop surrounding a single icosahedral capsid protein (C) encompassing the 12.5Kb positive-sense ssRNA genome[25][26][27][28][29] (Figure.1)[30].

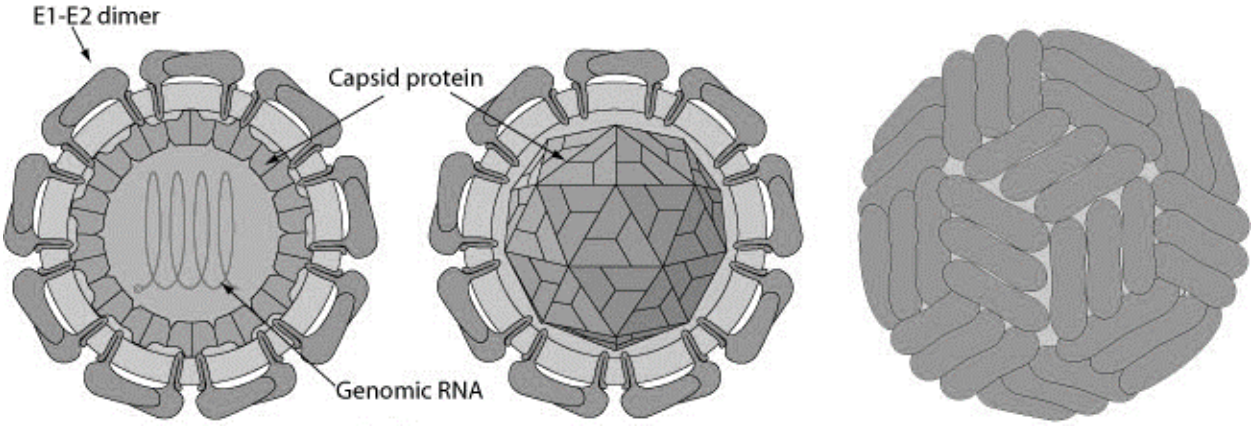


Figure.1
Schematic representation of a BVDV virion showing envelope proteins (E1, E2), Capsid protein (C), and the packaged genomic RNA.

The virus's genetic material contains approximately 4000 codons coding for a polyprotein that, after translation, becomes 12 polypeptides (Npro, C, Erns, E1, E2, P7, NS2 y NS3, NS4A, NS4B, NS5A, and NS5B). From these peptides, Npro functions as an autoprotease that splits the viral polypeptide into individual proteins, in addition to suppressing IFN-1 production to hinder the host innate immune response. C protein becomes the virion nucleocapsid, while Erns, E1, and E2 become glycoproteins integrated into the lipidic envelope. Erns possesses ribonuclease activity and provokes a virulent enhancer effect through IFN-β suppression, while E1 plays a structural role in covalently bonding with E2. E2 genomic variability is the highest among the 12 peptides, and it is involved in regulating virus entry into target cells. This protein features numerous neutralizable domains[31][32]. In contrast, the function of the P7 protein is yet to be determined. In turn, NS2 and NS3 are highly preserved proteins across strains that play a role as RNA-helicases and serine proteases with NS4A working as a serine protease cofactor. Lastly, NS4B and NS5A are components of the replicase, while NS5B functions as an RNA-dependent polymerase[31].

The virus can be inactivated by high temperatures, lipid solvents, and detergents, and it is sensitive to ethylic ether, chloroform, and trypsin. The virion resists a wide range of pH, staying stable between a pH of 5.7 to 9.3, with a pH 7.4 offering the highest stability[33][34]. It loses its infectivity after four days at 37˚C and after 45 min at 56˚C[35].

Biotypes

BVDV can be classified into two biotypes, depending on their ability to induce cell death. The cytopathic biotype is rare, generating an immuno-suppressor effect by killing lymphocytes and monocytes-macrophages in the host. It is usually associated with the highly lethal Mucosal disease, with episodes of bloody diarrhea, snout bleeding, petechial hemorrhages and ecchymosis in the mucosal membranes of the gastrointestinal tract, as well as thrombocytopenia and leukopenia[4][5][6][7]. Alternatively, the non-cytopathic biotype is predominant and does not provoke visible alterations in the infected cells but can cause reproductive dysfunction, respiratory complications and persistent diarrhea in the host [3][36][37]. Cp strains are derived from ncp strains through rare mutational events that separate ncp NS2-3 viral protein into the cp NS2 and NS3 viral proteins[31].

Genotypes and genomic variability

Analysis of 5'UTR, NS2-3, Npro, and E2 regions revealed a series of genetic markers that were used to group BVDV genotypes and subgroups according to their nucleic sequence variability [7][38][39][40]. There are fifteen genomic subgroups identified for the genotype BVDV-1, from BVDV-1a to BVDV-1o, and only two subgroups for BVDV-2, BVDV-2a, and BVDV-2b, respectively[40][41][42][43][44][45]. Among genotypes, a 60% sequential similarity was found, and an 80 to 85% similarity among subgroups within genotypes[46]. Analyses of the Npro and E2 genomic regions are usually performed to define subgroups[47], while the more conserved 5'UTR region is used to define BVDV genotypes[48]. Through the use of serological techniques, it has been possible to determine the genotypes commonly afflicting BVD-endemic countries [7][39][49][50].

E2 glycoprotein

The BVDV E2 glycoprotein is a 51 to 58kDa sized protein embedded in the external surface of the lipidic envelope[32][51]. It forms homodimers and heterodimers with glycoprotein E1 through disulfide bonds on the surface of mature virions[52][53]. E2 is the biggest of the envelope proteins and the most genomically variable one, which results in it becoming the primary inductor of neutralizing antibodies in the host after infection or vaccination[54][55][56][57][58]. At least ten immunogenic sites produce reactivity in tests with monoclonal antibodies[59]. The low homology of its nucleotide sequence in the

coding gene responsible for the induction of neutralizing antibodies gives rise to its high antigenic diversity among BVDV populations[60][61].

A recent study on crystalized BVDV E2 described the molecule as elongated and dimeric, with each monomer consisting of four antigenic domains (DA, DB, DC, and DD), linearly disposed from N-terminal to C-terminal (Figure.2) [62]. E2 possesses 17 cysteines (Cys) groups that form nine disulfide bonds, 8 of them intramolecularly, and one intermolecularly. Domains DA and DB are the most distant from the virion envelop and highly exposed on the surface, with foldings resembling those of immunoglobulins, possibly as binding sequences for cell receptors. The DC domain is rich in disulfide bonds, and it is composed of loops and antiparallel β-chains containing two glycosylation sites. The more genetically preserved DD domain has a role in the homodimerization of E2, covalently stabilizing dimers with a disulfide bond, which allows the two DD domains in each monomer to intertwine adjacently[52][62].

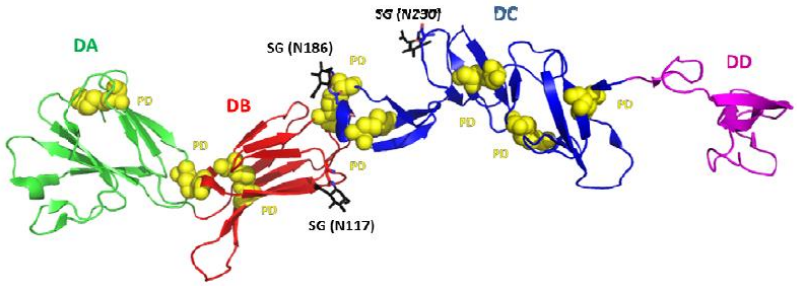


Figure.2
PyMOL graphic representation of the crystalized structure of theBVDV E2 glycoprotein. Each color represents an element, DA domain (green), DB domain (red), DC domain (blue), DD domain (magenta), disulfide bonds (yellow spheres), glycosylation sites (black).

II. PROBLEM STATEMENT

Considering productive and reproductive losses caused by BVD infections in cattle worldwide, the World Organization for Animal Health (OIE) in 2007 added BVDV to the list of reportable cattle diseases, to promote awareness and to call for prevention and control measures[63].

The main objectives of BVDV control programs are to prevent fetal infections, avoid reproductive losses, reduce the number of PI calves, and avert acute infections in mature cattle, all of which affect meat and milk production[64][65]. These goals can be achieved by preventing exposure to the virus through the identification and removal of PI calves, whose secretions and excretions act as permanent sources for viral particles. This process is slow and usually requires lab diagnosis, given the calves' partial immunity and lack of clear or sustained infection symptoms. Thus, BVDV vaccination campaigns are implemented to increase herd immunity[64].

Available market vaccines come in 2 types, modified or inactive. These are mostly monovalent and contain a reference strain BVDV-1a, in some cases bivalent, containing BVDV-1a and BVDV-2a strains. Only one vaccine has been reported to contain the BVDV-1b strain. The effective immunity conferred by the resulting neutralizing antibodies depends on the degree of similarity between neutralizable epitopes of the reference strain in the vaccine and the afflicting one. Given the antigenic differences among genotypic groups, there is almost no cross-protection granted by vaccines[39][47][66][67][68][69][70].

Because of the lack of vaccines in Chile for any strains other than BVDV-1a, BVDV-1b, and BVDV-2a, we developed this project as a means to create a novel vaccine for three variants of BVDV: BVDV-1b, BVDV-1d, and BVDV1e present in the south-central provinces[17][18][19]. For this project, we developed a protocol to express and characterize three smaller-sized immunogenic variants of a truncated form of the BVDV E2 envelop glycoprotein, given its role as the main target of the neutralizing response. We evaluated the three antigens' immunogenicity, expressed in a recombinant system of Chinese hamster ovary (CHO) cells, against the inactivated form of the virus. Through SDS-PAGE and Western Blot (WB) assays and computational predictions, we also tested the electrophoretic and serological characteristics of the variants proving them to be similar to those of its viral counterpart. We performed the transfection of the mammalian cell line using two mammalian expression vectors (pCI-Neo). The vectors contained a fluorescent protein (EGFP) and a neomycin phosphotransferase resistance expression cassette, conferring Geneticin resistance, to drive the expression and allow for the selection of cells containing the plasmid. After selection and culture expansion of the highest expression cell lines, characterized by PAGE and

Western Blot, we purified to a moderate degree the expressed immunogens simultaneously in one step through Ni-Sepharose chromatography, with the help of their polyhistidine tag. We analyzed the collected fractions by SDS-PAGE and Western Blot assays, and imaging software to assess their purity, and the resulting purified proteins were quantified by BCA and SDS-PAGE analysis.

The core guidelines for the development of this methodology were to reduce time and resources in as few steps as possible while keeping the process scalable for commercial use. Candidate immunogens for vaccines determined by this procedure were apt for later use in serological tests *in vivo*.

III. OBJECTIVES

General objective

To express in a combined manner three E2 glycoprotein variants from the envelope of the bovine viral diarrhea virus in a CHO cellular line to select in one step vaccine candidates through IMAC.

Specific objectives

To amplify and purify plasmid vectors encoding three E2 variants from the bovine viral diarrhea virus through *E. coli TOP10* cultures.

To express three E2 variants in the CHO cell line by sequential transfection of plasmids pCI-Neo-V2 and pCI-Neo-V1.

To obtain samples of immunogens of moderate to high purity using a single-step purification method by IMAC.

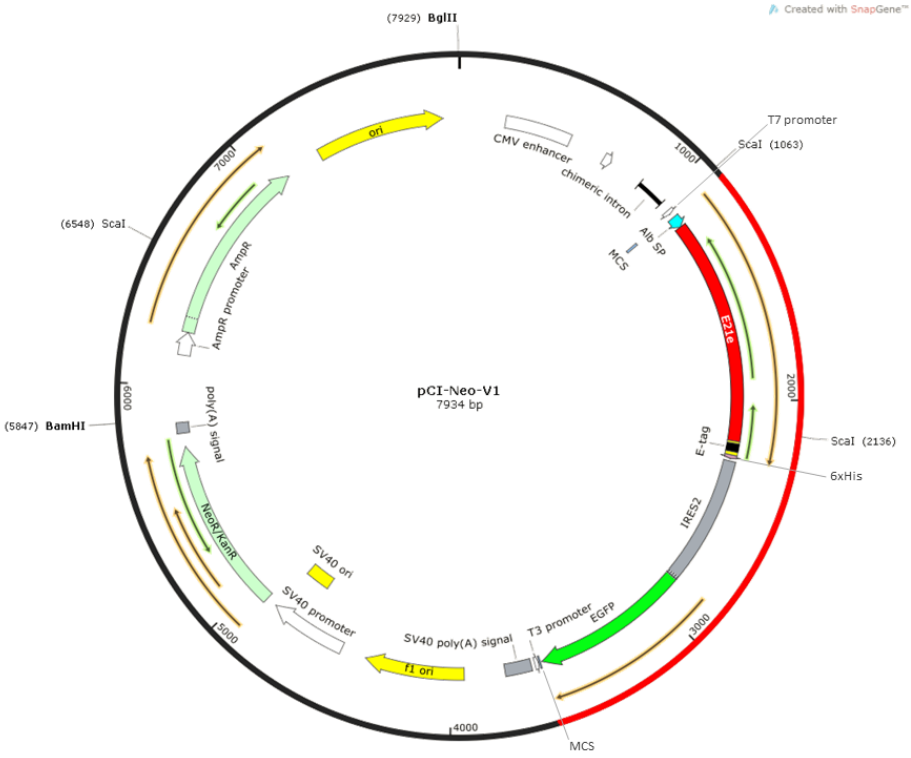
To quantify the expression levels of the viral E2 protein variants in a stable CHO producer cell line.

IV. METHODOLOGIES

Synthetic gene modification and vector construct

The pCI-Neo Mammalian Expression Vector (Promega, WI, USA) was used to express the E21b, E21d, and E21e genotypes of the surface glycoprotein E2 of the Bovine viral diarrhea virus (BVDV) in Chinese hamster ovary (CHO) cells. This vector encoded for the human cytomegalovirus (CMV) enhancer/promoter region, which promoted a strong and constitutive expression of DNA inserts in mammalian cells. It also encoded a neomycin phosphotransferase gene, a selection marker for mammalian cells by conferring them resistance to Kanamycin and Geneticin (G-418), and β-lactamase gene (AmpR) as a bacterial selection marker offering Ampicillin resistance. The vector also contained the SV40 promoter, a transient inductor for the episomal replication of the vector, located upstream of the neomycin phosphotransferase gene (NeoR). The regions downstream of CMV and flanking the cDNA inserts encoded a chimeric intron from the human β-globin gene, which increased the level of gene expression. Additionally, an SV40 Late Polyadenylation Signal in the vector added 200–250 adenosine residues to the 3'-end of the RNA transcript, enhancing RNA stability and translation.

The pCI-neo-V1 vector was designed as a bicistronic plasmid encoding two proteins, E21e (1029 bp) and a fluorescent protein (EGFP) used as a selection marker, spaced by an internal ribosome entry site (IRES). The second vector pCI-neo-V2 also was bicistronic and encoded for two E2 glycoproteins E21b (1029 bp)/E21d (1026 bp) separated by an IRES. Each vector also contained two Human Albumin (Alb) secretory signal peptides (SP) to allow the protein(s) to be secreted to the culture medium. At the end of each E2 glycoprotein, specific sequences of tags were added to facilitate the detection of expressed proteins. Variant E21b was linked to an HA-tag (YPYDVPDYA), E21d to a Strep II-tag (WSHPQFEK), and E21e to an E-tag (GAPVPYPDPLEPR). All variants also encoded a hexahistidine tail (6xHis tag) to facilitate the purification of the recombinant proteins. Both plasmids were synthesized by GenScript Company (Hong Kong) and shipped on filter paper. SnapGene 1.1.3 plasmid viewer software was used to depict the elements present in the expression vectors (Figure.3).



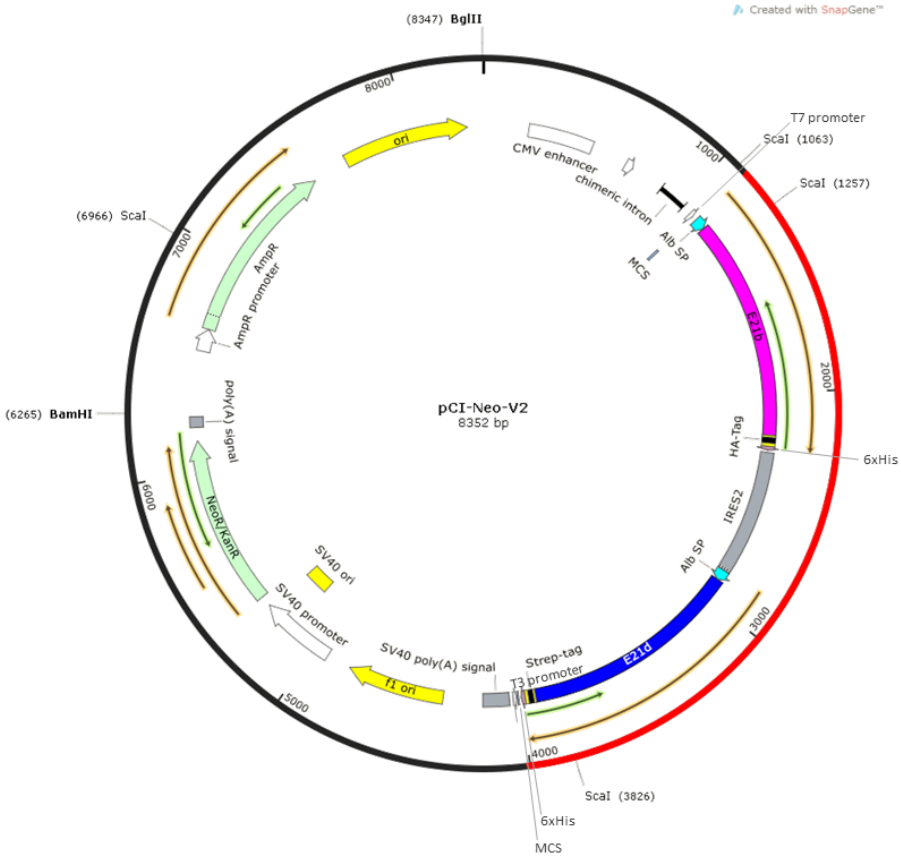


Figure.3
Map of plasmids pCI-Neo-V1 and pCI-Neo-V2. The main elements are shown. CMV: expression enhancer; chimeric intron: expression enhancer; MCS: multiple cloning region; SV40: replication inductor; Alb SP: Secretory signal peptide; Genotype sequences: E21b, E21d, E21e; Epitopes: HA-tag, Strep II-tag, E-tag; 6xHis: hexahistidine-tag; NeoR/KanR: geneticin resistance gene; AmpR: ampicillin resistance gene.

Plasmid replication in bacterial hosts

Plasmids were recovered from filter papers by cutting a quarter of the paper and vortexing in buffer TE (10 mM Tris-Cl, pH 8.0, 1 mM EDTA) for 10 min. Ten microliters of this suspension were mixed in 40µl of chemically competent *E. coli TOP10*, incubated on ice for 10 min, and subjected to a thermic shock for 90 sec at 42˚C and 1 min on ice. Transformed bacteria were grown in liquid LB medium for 1 h at 37˚C and 140 revolutions per minute (rpm) in a shaker. A 30 µl sample was seeded on a 100 mm plate of LB-Agar supplemented with Ampicillin 100 µg/ml and incubated for 18 h at 37˚C. Single-picked colonies were inoculated and grown in 5 ml of LB supplemented with Ampicillin 100 µg/ml for 18 h at 37˚C and 140 rpm in a shaker.

Plasmid purification

Plasmid purification was performed by the alkaline lysis method. Bacteria were collected by centrifugation after 3 min at 8000 rpm. The supernatant was decanted and resuspended by vortex in 300 µl of resuspension buffer (50 mM Tris-HCl, pH 7.5, 10 mM EDTA, 100 µg/ml RNase A). Then, 300 µl of lysis buffer (0.2 M NaOH, 1% SDS) was added, mixed thoroughly by tube inversion ten times, and incubated for 5 min at room temperature. Later, 300 µl of neutralization buffer (1.32 M Potassium acetate, pH 4.8) was added and mixed in by tube inversion. The tube was centrifuged for 15 min at 12.000 rpm, and the supernatant transferred to a new microfuge tube. The previous step was repeated, and the plasmid DNA was precipitated by adding 0.7 volumes of Isopropanol, followed by centrifugation for 5 min at 12.000 rpm. The flow-through liquid was discarded, and the DNA pellet was washed in 1 ml of 70% ethanol and centrifuged for 5 min at 12.000 rpm. Plasmids were resuspended in 50 µl of distilled water and quantified by absorbance at 260 nm. All centrifugations were performed at room temperature.

The purified plasmids were analyzed by enzymatic digestion with *Sca I* and Agarose gel electrophoresis. The plasmid maxipreparation was performed with the QIAGEN Plasmid Maxi Kit (QIAGEN, Hilden, Germany) and the resulting purified plasmids were digested with the restriction enzymes BamH I and Bgl II. The obtained fragments were separated by Agarose gel electrophoresis and the band with the genes of interest was extracted from the agarose by a GeneJET Gel Extraction Kit (Thermofisher, MA, USA).

Characterization of the plasmid

Enzymatic digestion with *Sca I* was performed to corroborate plasmid identity. The digestion was performed in a total volume of 20 µl with NEBuffer™ 3.1 (100 mM NaCl, 10 mM MgCl2, 50 mM Tris-HCl, 100 µg/ml BSA, pH 7.9), with 500 ng of each plasmid and 10 U of Sca I (*Sca I* 1 µL at 10 U/µL) for 1 h at 37°C. The DNA fragments were then separated by a 0.8% Agarose gel electrophoresis and visualized under UV light. A 1kb ladder molecular weight marker (New England Biolabs, MA, USA) was used to determine the relative electrophoretic mobility of DNA fragments, and the electrophoresis profile was compared to a simulation in the SnapGene 1.1.3 plasmid viewer software.

Plasmid extraction

The plasmid maxipreparation purification from a 100 mL culture grown at 37˚C and 300 rpm for 12 h was achieved by using a QIAGEN Plasmid Maxi Kit (QIAGEN, Hilden, Germany). The bacterial cells were harvested by centrifugation at 10.000 rpm for 15 mins at 4˚C, the supernatant discarded, and the pellets resuspended in 10 ml of buffer P1 (50 mM Tris-HCl, pH 8.0, 10 mM EDTA, 100 ug/mL RNase A). Then, 10 ml of buffer P2 (200 mM NaOH, 1% SDS) were added, mixed thoroughly by inversion six times, and incubated at room temperature for 5 min. Later, 10 ml of chilled buffer P3 (3.0 M Potassium acetate, pH 5.5) were added and mixed thoroughly by inversion six times, followed by incubation on ice for 20 min. The mixture was centrifuged at 15.000 rpm for 30 min at 4˚C, and the supernatant containing the plasmid DNA was quickly transferred to a clean 50 ml tube and centrifuged again at 15.000 rpm for 15 min at 4˚C. The supernatant was quickly collected and added to a gravity-flow anion exchange column QIAGEN Genomic-tip 100/G, previously equilibrated with 10 ml of QBT buffer (750 mM NaCl, 50 mM MOPS, pH 7, 15% Isopropanol, 0.15% Triton X-100). The column was washed twice with 30 ml of QC buffer (1.0 M NaCl, 50 mM MOPS, pH 7, 15% Isopropanol), and the DNA eluted with 15 ml of QF buffer (1.25 M NaCl, 50 mM Tris-Cl, pH 8.5, 15% Isopropanol) in a 50 ml tube. The DNA was precipitated by adding 0.7 volumes of room-temperature Isopropanol, mixed by inversion, and centrifuged at 12.000 rpm for 30 min at 4˚C, after which the supernatant was carefully decanted. The DNA pellets were then washed with 5 ml of room-temperature 70% ethanol and centrifuged at 12.000 rpm for 10 min; the supernatant was discarded. The pellets were left to air-dry for 5 min and dissolved in 1 ml of TE buffer (10 mM Tris-Cl, pH 8.0, 1 mM EDTA).

Plasmid digestion

For the linearization of the 10 µg of circular vector DNA, enzymatic digestions were performed with the restriction enzymes *BamHI* and *Bgl II* (*BamHI* 1 µL at 20 U/µL and *Bgl II* 2 µL at 10 U/µL) in a total volume of 50 µl with NEBuffer™ 3.1 (100 mM NaCl, 50 mM Tris-HCl, 10 mM MgCl2, 100 µg/ml BSA, pH 7.9. The DNA fragments were then separated by 0.8% Agarose gel electrophoresis. Finally, a block of agarose containing the mammalian expression cassette was excised from the agarose gel.

Plasmid extraction for transfection

The extraction of the DNA fragment from the agarose gel block was carried out with the GeneJET Gel Extraction Kit (Thermofisher, MA, USA). The gel section containing the mammalian expression cassette was weighed and transferred to a 1.5 ml tube along with Volume Buffer from the kit in a 1:1 relation. The sample was incubated for 10 min at 60˚C in a thermoshaker, inverted every 3 min, and vortexed briefly, obtaining a yellowish appearance at this point. From the kit, a purification column was assembled inside a collection tube, 800 µl of the sample was added to the column and centrifuged for 1 min at 13.000 rpm. The filtrated product in the collection tube was discarded, and the column returned to the collection tube. Seven hundred microliters of washing buffer from the kit were diluted 1:6 in 70% ethanol, added to the column, and centrifuged under the same parameters as before. The filtrate in the tube was discarded, and the column returned to the collection tube. The column, now empty, was centrifuged a third time as before. The column was transferred to a microcentrifugation 1.5 ml tube from the kit, 50 µl of Elution buffer were carefully added to its center and centrifuged for 1 min at 13.000 rpm. The column was discarded, the purified DNA fragment was quantified by absorbance at 260 nm by spectrophotometry and stored at -20˚C.

Mammalian vector transfection

The CHO cells were thawed and grown in RPMI medium supplemented with 10% of fetal bovine serum (FBS), 1% of L-Glutamine and 1% of Penicillin-Streptomycin (P-S) and incubated at 37˚C in a CO_2 incubator (5% CO_2, pH 7.2). All CHO cell adherent cultures were grown until confluence under the same conditions unless indicated differently. The initial sample had gone through 11 doubling cycles. Samples from the culture after the transfection were removed every 15 days and kept in storage at -80˚C.

Transfection was carried out with a Lipofectamine 2000 Transfection Reagent kit (Invitrogen, MA, USA) on two separate occasions. Initially, the pCI-Neo-V2 vector was inserted in a wild type CHO cell line to

express E21b and E21d protein variants, creating the CHO-V2 cell lines. After expansion and characterization, the most expressive CHO-V2 cell line was transfected with the pCI-neo-V1 vector, generating the CHO-V2-V1 cell lines.

The day before the first transfection, two wells in a 24-Well microplate were seeded, each with $1x10^5$ non-transfected cells as counted by Neubauer haemocytometry, using 500 µl of growth medium RPMI without antibiotics. One of the wells acted as a control group containing cells to be treated with lipofectamine 2000, to test for cytotoxicity. The other well contained the cells to be transfected with the pCI-Neo-V2 vector. All cells were incubated at 37˚C in a CO_2 incubator, and the medium was refreshed 1 h before the transfection. A medium consisting of 2 µl of lipofectamine diluted in 50 µl of Opti-MEM3 I Medium was left incubating 5 min at room temperature. The medium was then mixed with 0.8 µg of purified plasmid DNA diluted in 50 µl of Opti-MEM I Reduced Serum. The oligomer-lipofectamine complex, total volume of 100 µl, was left incubating 20 min at room temperature and later applied to one well gently mixing it by rocking back and forth. Cells grown in the complex were transfected with the pCI-Neo-V2 vector. Another medium consisting of 2 µl of lipofectamine diluted in 50 µl of Opti-MEM3 I Medium was left incubating 5 min at room temperature, and then added to the other well (control well). The 24-well microplate was incubated at 37˚C in a CO_2 incubator for 6 h, after which its medium was changed to RPMI plus FBS 10%, then left incubating for 24 h. The control well and the well with transfected cells were transferred into a P6-well plate, dilution 1:6, with growth medium supplemented with the selection antibiotic (RPMI, 10% FBS, 120 µg/ml Geneticin antibiotic (G-418), 1% L-Glutamine and 1% Penicillin-Streptomycin (P-S)).

For the second transfection, three wells were seeded in a 24-well microplate with $1x10^5$ cells from the most expressive CHO-V2 cell lines, under the same growth conditions as the first transfection. One well was designed as a negative control group containing cells to be treated with lipofectamine 2000, to test for cytotoxicity. A second well, holding cells to be transfected with a plasmid coding for EGFP (pEGFP), was designed as a positive control group to assess the IRES-linked expression of EGFP from the pCI-Neo-V1 vector. A third well contained the cells to be transfected with the pCI-Neo-V1 vector. The complexes were prepared following the first transfection formulation, applying 50 µl of lipofectamine-Opti-MEM3 I Medium to the negative control well, a 100 µl pEGFP oligomer-lipofectamine complex to the positive control well, and a 100 µl pCI-Neo-V1 oligomer-lipofectamine complex to the well to be transfected with the pCI-Neo-V1 vector. The rest of the procedure for incubation, growth, and medium change was performed as previously.

Culture selection and cloning

The transfected cells were kept at 37˚C in a CO_2 incubator in which they were progressively being expanded and finally seeded into P6-well plates. Resistance to Geneticin antibiotic was used as a selection marker to select stable cultures integrated with the transfected gene from both vectors, pCI-Neo-V2, and pCI-Neo-V1. Fifteen days after the transfection with the pCI-Neo-V2 vector, a cellular suspension of 0.01 cells/ml with growth medium plus antibiotic was prepared. Each well of a 96-well microplate was seeded with 100 µl of the suspension, in an attempt to seed one cell/well. Sixteen colonies expressing Geneticin resistance were obtained and expanded, replacing the medium every three days. Their supernatant was characterized by SDS-PAGE and Western Blot assays. The most expressive colony of E2 variants received the insertion of the pCI-Neo-V1 vector. The resulting CHO-V2-V1 cell lines were monitored by GFP fluorescence and sorted by fluorescence-activated cell sorting (FACS) to clone wells containing one fluorescent cell in a 96-well plate. Eighteen fluorescent colonies developed on the plate, expressing a fluorescence intensity of 1000 times higher than the cells used as a negative expression control of GFP. These colonies were grown over 21 days, exchanging medium every three days. Using a phase-contrast and fluorescence microscope in an OLYMPUS IX81 microscope, the three most EGFP-expressing colonies were expanded to confluence into 6-well plates. The supernatant was collected and characterized by SDS-PAGE and Western Blot analytical techniques to assess the E2 variants' expression levels. Cells were kept at -80˚C as a backup.

Supernatant collection and protein precipitation

Cell supernatants were precipitated to be analyzed by SDS-PAGE and Western Blot analysis. Once the cultures achieved confluence, the growth medium was changed to 10% RPMI, 120 µg/ml G-418, 1% L-Glutamine, and 1% Penicillin-Streptomycin (P-S), without FBS. During the medium change, washes with Phosphate-buffered saline buffer (PBS) (137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 1.8 mM KH_2PO_4, pH 7.4) were performed thoroughly and repeated twice before adding the new RPMI medium. After 24 h, the extracellular medium was collected and centrifuged for 10 min at 5000 rpm to eliminate floating cells and debris. The supernatants were stored at -20˚C until used.

The protein precipitation from the culture supernatant was done following a TCA protein precipitation protocol. The starting material for all samples was 1 ml of cell culture supernatant, to which 100 µl of 1.5 mg/ml Sodium deoxycholate were added. The mixture was vortexed for 1 min and left to rest at room temperature for 10 min. Next, 110 µl of TCA 76% were added, and the mixture was vortexed for 1 min.

Then, it was centrifuged at 13.000 rpm for 15 min, and the supernatant was discarded. The precipitate was washed with cold acetone and vortexed for 1 min. Subsequently, the mixture was centrifuged at 10.000 rpm for 5 min, and the supernatant was discarded. The resulting pellet was dissolved in 25 µl of Loading Buffer 2x (0.125 M Tris-HCl, 4% SDS, 20% v/v Glycerol, 0.2 M DTT, 0.02% Bromophenol Blue, pH 6.8).

SDS-PAGE and Western Blot assays

SDS-PAGE analysis was performed in all cases under reducing conditions with a 10% polyacrylamide gel. The loading buffer and tank buffer used were provided with a Mini-PROTEAN Tetra Cell system (Bio-Rad Laboratories, CA, USA). The molecular weight marker AccuRuler RGB Plus Prestained Protein Ladder (MaestroGen, Taiwan) was used for assessing relative electrophoretic mobility of proteins. The protein bands were visualized using Coomassie blue staining. For Western blot analysis, the proteins separated on the SDS-polyacrylamide gels were transferred to a Bio-Rad's 0.45 µm pore–size nitrocellulose membrane at 25 V, 0.3 A for 30 min with a Trans-Blot Turbo Transfer System module. The exposed protein binding sites on the membranes were blocked by incubation at room temperature with 25 ml of skim milk–PBS (5% milk) for 1 h in a shaker at moderate speed. After four washes, the membranes were incubated for 12 h at 4˚C in 10 ml of skim milk-PBS (2% milk) with one of three primary antibodies, mAb Anti-HA-tag 1/1250 (Thermofisher, MA, USA), mAb Anti-Strep-tag II 1/1000 (Novus Biologicals, CO, USA) or pAb Anti-E-tag 1/2000 (Novus Biologicals, CO, USA). The membrane was washed four times and incubated for 1 h in darkness at room temperature with 10 ml of skim milk-PBS (2% milk) with one of three high-affinity secondary antibodies, Donkey-anti-Mouse Alexa Fluor 790 1/30000 (Jackson ImmunoResearch, PA, USA) for HA-tag, Donkey-anti-Rabbit Alexa Fluor 790 1/20000 (Jackson ImmunoResearch, PA, USA) for Strep II-tag, and Rabbit-anti-Goat F ITC 1/20000 (Dako Products, CA, USA) for E-tag. After four washes, membranes were scanned in the Odyssey CLx Near-Infrared Fluorescence Imaging System (LI-COR Biosciences, NE, USA). All washes were performed with TBS/Tween/SDS buffer (50 mM Tris, 150 mM NaCl, 0.1% Tween 20, 0.01% SDS).

Protein purification and analysis

The glycoproteins E2 variants were purified through Ni Sepharose affinity resin chromatography by way of their polyhistidine tag (6xHis), initially by a small volume gravity-flow column and later by an FPLC system (ÄKTAprime plus system). The gravity-flow column was designed as a proof of concept to determine the matrix ability to separate histidine-tagged proteins from the rest of the medium and to

assess the optimal concentrations of the Imidazole elutions and wash. These elutions were later characterized by Dot-Blot. In contrast, the elutions from the ÄKTAprime plus LC system were analyzed using SDS-PAGE and Western Blot assays, and imaging software. The final purified protein fraction was dialyzed, concentrated, and quantified using a Pierce BCA Protein Assay Kit (Thermofisher, MA, USA).

The 6% Ni-Sepharose matrix used in both purifications was composed of 34 µm beads of highly cross-linked agarose, to which a chelating group has been coupled (Ni^{2+}). This method of purification is a type of immobilized metal-affinity chromatography (IMAC), and it was selected given its extensive use to purify recombinant proteins and antigenic peptides with high selective interaction, allowing purification of histidine-tagged proteins up to 95% homogeneity in just one step.

A mobile phase was prepared to consist of 2 ml of culture supernatant expressing the E2 protein, diluted 1:1 with buffer B (5 mM Imidazole, 50 mM Tris, 300 mM NaCl, pH 7.5 by HCL). The collected supernatant was free of FBS and cell debris. The 4 ml mixture was loaded onto the 12 ml column containing a packed matrix of 3 ml of 6% nickel-sepharose resin. Once loaded, the mobile phase was collected as an unbound fraction. Two washes were then performed with 10 ml Equilibrium buffer B. Later, a stepped gradient of Imidazole. 6 ml, was applied and collected (25 mM, 50 mM, 150 mM, 200 mM, and 400 mM Imidazole).

The presence of the three E2 variants in each purification step was analyzed by Dot Blot to determine the optimal wash and elution concentrations of Imidazole. For the Dot Blot analysis, a Bio-Dot Microfiltration Apparatus (Bio-Rad Laboratories, CA, USA) was used. The analyzed samples were collected from the cell medium supernatant of the CHO Wild Type (CHO WT), the unbound fraction, and the five elutions at different Imidazole concentrations. A nitrocellulose membrane was mounted on the apparatus, washed thoroughly with 20% methanol, and left to dry. Next, with the vacuum pump turned on, 1 ml from each sample supernatant was slowly applied to the membrane. Each sample was applied in triplicate, one for each peptide tag to be detected (HA-tag, Strep II-tag, and E-tag). After 5 min, the pump was turned off, and the membrane was removed and cut into three strips, one for each tag to be detected. Afterward, the strips were blocked by incubation at room temperature with 50 ml of skim milk–PBS (5% milk) for 1 h in a shaker at moderate speed. Then, the strips were incubated, washed, and exposed to primary and secondary antibodies just as described in the Western Blot assays. Finally, the strips were scanned in the Odyssey CLx Near-Infrared Fluorescence Imaging System (LI-COR Biosciences, NE, USA).

For a higher volume chromatography, a GE AKTA Prime Plus system (GE Healthcare, IL, USA) with a 40 ml HiTrap XK26 pre-packed column of 6% Ni-sepharose was used. The column was first equilibrated with 200

ml of buffer B. Next, an extract of 200 ml of culture supernatant, from the highest expression cell line and free of FBS and cell debris, was prepared 1:1 as a mobile phase with 200 ml of buffer B. The cell supernatant was previously vortexed and filtrated through a 0.45 µm membrane to eliminate cellular debris. The mixture was left recirculating during all night. Then, a 120 ml wash was performed with 25 mM Imidazole, followed by two elutions of 120 ml with 200 mM and 400 mM Imidazole. All fractions were analyzed by SDS-PAGE and Western Blot assays, and the imaging software Fiji ImageJ 152p[72] and CurveExpert Professional 2.6.5[73]. Next, the 200 mM Imidazole fraction was dialyzed for 24 h in PBS solution, and then concentrated to half its volume in a water-polyethylene glycol 6000 mixture.

Protein quantification

The concentrated fraction was quantified by BCA assay using a Pierce BCA Protein Assay Kit. A standard curve of absorbance was prepared versus micrograms of protein to determine its amount by interpolation from the curve. In a 96-well, seven microplate wells were prepared with a range of diluted albumin standards (BSA) thoroughly mixed with working reagent (WR), while an eighth microwell contained the unknown sample mixed with WR. The initial volume of the BSA standards and the unknown sample was 25 µl, and 200 µl for the WR, ratio 1:8. The BSA final concentration spanned from 125 µg/ml to 2 mg/ml. The plates were incubated at 37˚C for 30 min and then cooled to measure absorbance at 562 nm on a plate reader. The results were plotted as a standard curve in the GraphPad Prism 8.0.1.244 graphing software[74], and the protein concentration of the unknown sample was interpolated from the curve.

V. RESULTS, INTERPRETATION, AND DISCUSSION

Verifying the plasmid identity

After the transformation, samples subjected to digestion with *Sca I* revealed a digestion profile that coincided with the expected pattern (Figure.4), confirming the identity of the mammalian expression vectors pCI-Neo-V1 and pCI-Neo-V2.

Figure.4
Identification of vectors pCI-Neo-V1 and pCI-Neo-V2 digested with the restriction enzyme Sca I by Gel electrophoresis.
Lane MW: Theoretical molecular weight marker 10 kb; Lane 1: Expected pCINeo-V1 digested bands of 1073, 2449 and 4412 bp; Lane 2: Expected pCINeo-V2 digested bands of 194, 2449, 2569, and 3140 bp; Lane 3: 1 kb DNA Ladder molecular weight marker; Lane 4: Experimental digested pCI-Neo-V1 bands; Lane 5: Experimental digested pCI-Neo-V2 bands.

Vector preparation for transfection

Afterward, the plasmid DNA was purified to high purity, and the resulting plasmid DNA was subjected to digestion with *BamHI* and *Bgl II* (Figure.5). This digestion allowed to remove the *Ori* and *AmpR* regions in the vector, previously used in the bacterial host, as recommended by FDA directives on the production of biotherapeutic proteins. This digestion also allowed to linearize the plasmid DNA for more efficient integration into the mammalian host by means of the non-homologous end-joining repair pathway.

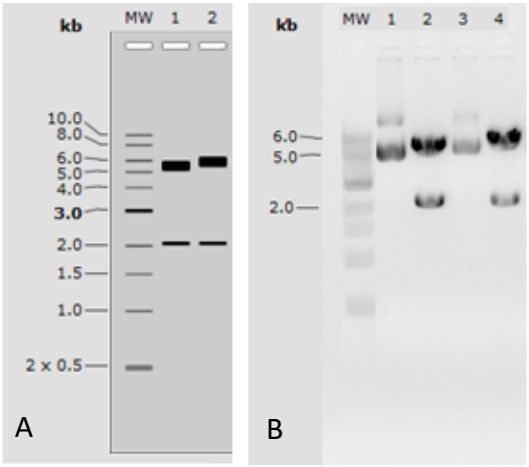


Figure.5
Identification of vectors pCI-Neo-V1 and pCI-Neo-V2 digested with restriction enzyme BamHI and Bgl II by Gel electrophoresis.
Image A: Expected bands pattern. Lane MW: Theoretical molecular weight marker 10 kb; Lane 1: pCINeo-V1 digested bands of 2082 and 5852 bp; Lane 2: pCINeo-V2 digested bands of 2082 and 6270 bp.
Image B: Experimental bands pattern. Lane MW: 1 kb DNA Ladder molecular weight marker; Lane 1: Undigested pCI-Neo-V1 band; Lane 2: Digested pCI-Neo-V1 bands; Lane 3: Undigested pCI-Neo-V2 band; Lane 4: Digested pCI-Neo-V2 bands.

Transfection and selection of stable expressing cultures

Following the *BamHI* and *Bgl II* digestion, the plasmid DNA bands were purified and used to transfect a cell line of Chinese hamster ovary cells (CHO). As a host, CHO cells were used due to their availability, previously confirmed competence in-situ for recombinant protein expression, and for its frequent use as an FDA approved cell line for high expression and production of bio-therapeutic proteins.

An initial transfection in the mammalian host with the purified vector pCI-Neo-V2 resulted in 16 colonies in a 96-well plate expressing resistance to Geneticin as a selection marker. Once expanded, the culture supernatants of these colonies were characterized by SDS-PAGE and Western Blot analysis to assess the expression of the viral glycoprotein variants E21b and E21d. A cell line showing moderate expression of E21b and E21d was selected as the most fitting cell line for transfection with the E21e variant (Figure.6).

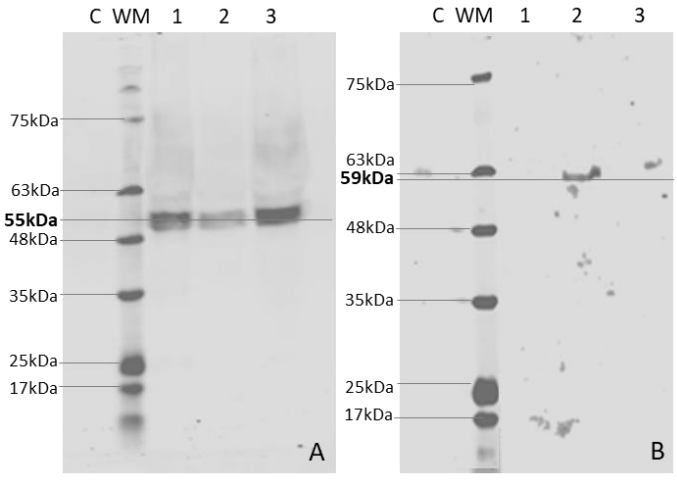


Figure.6
Detection of HA-tag and Strep II-tag in three CHO-V2 cell culture supernatants by SDS-PAGE and WB analytical techniques.
Lane C: Control group CHO wild type sample; Lane WM: AccuRuler RGB Plus Prestained Protein Ladder weight molecular marker 25-75kDa; Image A: Band profile of three transfected cultures screened for HA-tag; Lane 1: CHO-V2 culture with high expression of HA-tag; Lane 2: CHO-V2 culture with medium expression of HA-tag; Lane 3: CHO-V2 culture with the highest expression of HA-tag.
Image B: Band profile of three transfected cultures screened for Strep II-tag; Lane 1: CHO-V2 culture with very low expression or Strep II-tag; Lane 2: CHO-V2 culture with medium expression or Strep II-tag; Lane 3: CHO-V2 culture with low expression of Strep II-tag.

The Western Blot results showed intense defined bands of HA-tag at their expected size, corresponding to strong E21b protein expression. Likewise, the Strep II-tag protein was detected at the expected size, but the bands were weak in most samples, implying a low expression of the E21d genotype. A likely reason for this behavior was the innately reduced expression of the E21d gene due to its loci in the vector after the internal ribosome entry site (IRES), which usually reduces the translation efficiency of the protein that follows it.

The supernatant was screened using SDS-PAGE and Western Blot techniques, which identified the most expressive cell line of the E21b and E21d variants (Figure.6; Lane 2). Next, a sample from this culture was transfected with the vector pCI-Neo-V1 and sorted by FACS, resulting in 18 colonies from a 96-well plate expressing Geneticin resistance and the EGFP protein. The three most fluorescent colonies were selected

to be expanded using phase-contrast and fluorescence microscopy (Figure.7). Each cell in the selected colonies expressed at least a fluorescence intensity 1000 times higher than the cells used as a negative expression control of GFP in the transfection, which indicated a strong expression of the BVDV E21e variant.

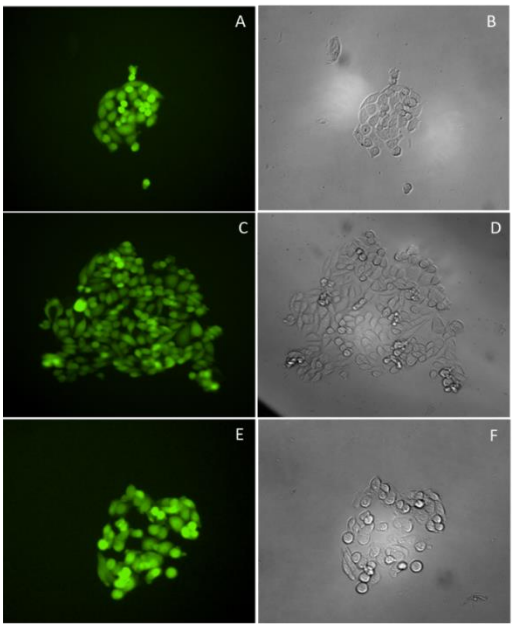


Figure.7
EGFP expression of three CHO-V2-V1 colonies seen through a fluorescence microscope OLYMPUS IX81, 10X.
Image A, C and E: Fluorescence microscopy capture of the three most expressive EGFP colonies.
Image B, D and F: Phase-contrast microscopy capture of the three most expressive EGFP colonies.

Once these cell lines became confluent in 6-well plates, their supernatants were screened for the expression of the three E2 glycoprotein genotypes (Figure.8).

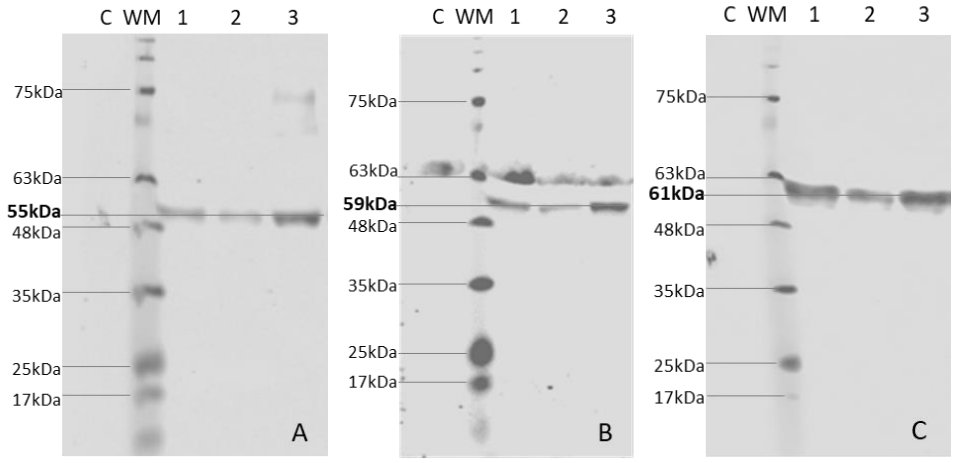


Figure.8
Detection of HA-tag, Strep II-tag, and E-tag in three CHO-V2-V1 cell culture supernatants by SDS-PAGE and WB analytical techniques. Lane C: Control group CHO wild type; Lane WM: AccuRuler RGB Plus Prestained Protein Ladder weight molecular marker 25-75kDa. Image A: Band profile of three CHO-V2-V1 cell cultures screened for HA-tag; Lane 1 and Lane 2: Cultures with low expression of HA-tag; Lane 3: Culture with the highest expression of HA-tag. Image B: Band profile of three CHO-V2-V1 cell cultures screened for Strep II-tag; Lane 1 and Lane 2: Cultures with moderate and low expression of Strep II-tag, respectively; Lane 3: Culture with the highest expression of Strep II-tag. Image C: Band profile of three CHO-V2-V1 cell cultures screened for E-tag; Lane 1 and Lane 2: Cultures with moderate expression of E-tag; Lane 3: Cultures with the highest expression of E-tag.

The SDS-PAGE and Western Blot analysis identified the CHO-V2-V1 cell line with the highest expression of the three peptide tags (Figure.9; Lane 3), showing strongly defined bands at their expected sizes. This inferred a cell line with high expression of the E21b, E21d, and E21e glycoprotein variants of the BVDV. In addition, the Anti-Strep-tag II antibody recognized bands in the control group at 63kDa due to unspecific interactions with culture supernatant components, likely other secreted cell proteins. Another possible cause of this behavior was the higher concentration of Anti-Strep-tag II mAb used in the Western Blot, compared to the other antibodies.

In order to identify the protein variants, each protein size was first defined through the NCBI GenBank database. Glycoproteins E21b, E21d, and E21e theoretical weights considering their potential glycosylation sites were 55kDa, 59kDa, and 61kDa, respectively. Each variant carried a polyhistidine tag (6xHis) for purification purposes and a specific synthetic peptide tag to detect and differentiate them by immunofluorescence, based on antibody-tag affinity. SDS-PAGE profile bands corresponding to the protein variant E21b were shown at 55kDa, and it possessed an HA-tag detected using a primary mAb Anti-HA-tag. The bands for the variant E21d were displayed at 59kDa, and it presented a Strep II-tag that detected using a primary mAb Anti-Strep-tag II. Finally, the bands for the genotype E21e were shown at 61kDa, and it had an E-tag detected using a primary pAb Anti-E-tag.

Purification of the protein variants

The selected high expression clone was expanded, and 2 ml of its supernatant was collected to be purified through the flow-gravity column. The results showed that elutions with 200 mM Imidazole presented the highest concentration of HA-tag and E-tag, thus implying the same for their corresponding E21b and E21e glycoproteins. Meanwhile, the Strep II-tag had a stronger concentration at 150 mM and 250 mM Imidazole, suggesting the same for the presence of the genotype E21d (Figure.9).

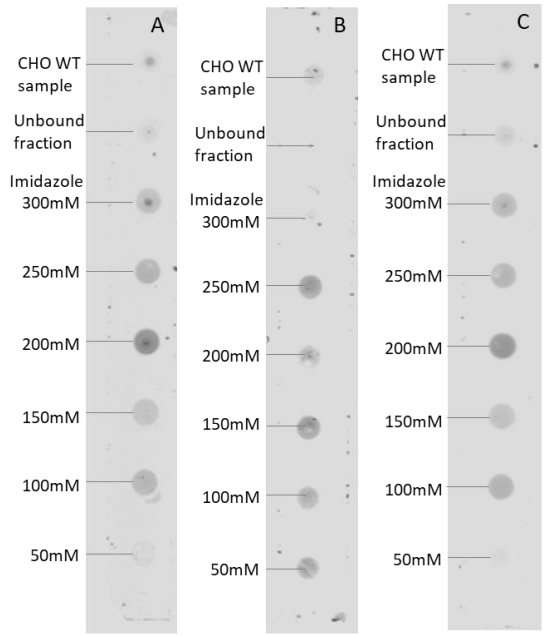


Figure.9
Detection of HA-tag, Strep II-tag, and E-tag at different concentrations of Imidazole elutions by Dot Blot.
CHO WT sample and Unbound fraction showed no significant presence of the peptide tags; Strip A: Highest concentration of HA-tag detected at 200 mM Imidazole; Strip B: Highest concentrations of Strep II-tag detected at 150 and 250 mM Imidazole; Strip C: Highest concentration of E-tag detected at 200 mM Imidazole.

Once proved the feasibility of the purification method, the high expression culture was grown into six 150 mm Petri dishes. From these cultures, 200 ml of cell supernatant were collected to be purified through the GE AKTA Prime Plus system (GE Healthcare, IL, USA). The first wash with 25 mM Imidazole generated the first peak containing proteins weakly bound to the matrix. A second peak was generated by elution using 200 mM Imidazole, also showing traces of proteins, whereas a final wash with Imidazole 400 mM presented almost no traces (Figure.10). The resulting product of the purification was collected in fractions of 5 ml each.

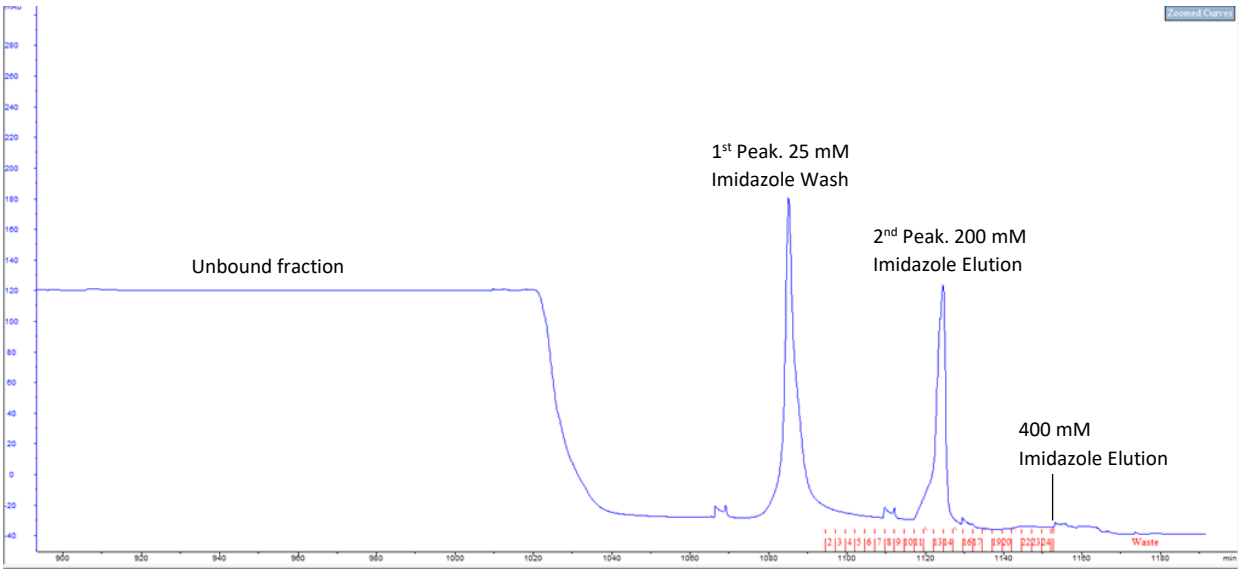


Figure.10
Chromatogram profile of the purification of three E2-envelop protein variants (BVDV) from a high-expression transfected CHO cell line. Chromatographic column: HiTrap XK26 pre-packed column of 6% Ni-sepharose matrix.

Chromatogram, fraction analysis, and quantification

A curve fitting analysis of the chromatogram delimited by peak area measurements, using Fiji ImageJ 152p and CurveExpert Professional 2.6.5 software[72][73], determined that the second peak containing the eluted proteins represented 23.58% of the detectable constituents in the application sample. The goodness-of-fit of the parameterized function to the data had a coefficient of determination (r^2) of 0.9899. The chromatography resolution was 3.59, with the peak's height, sharpness, and separation denoting a high degree of selectivity and efficiency from the Ni-Sepharose matrix to isolate the epitope tags, and therefore the target proteins from the cellular medium.

Samples from the fractions collected in the first and second peak were characterized by SDS-PAGE electrophoretic method, showing a wide size range of proteins present. Nevertheless, the fraction corresponding to the 200 mM Imidazole elution mostly showed protein bands corresponding to the expected size of the E2 variants (Figure.11 A). Likewise, the Western Blot immunodetection revealed dense bands in the 200 mM elution corresponding to the HA-tag, Strep II-tag, and E-tag, suggesting a strong presence of the E2 glycoprotein variants in such fraction (Figure.11 B). In contrast, given the lack of peaks at the 400 mM Imidazole elution, it implied an irrelevant amount of protein present. Thus, the 400 mM Imidazole fraction was excluded from the SDS-PAGE and Western Blot assays, and software analysis.

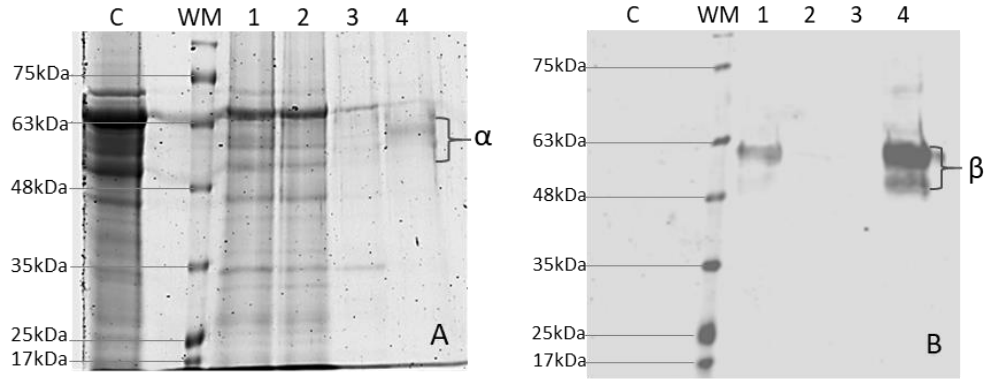


Figure.11
SDS-PAGE and Western Blot analysis of the collected fractions obtained from the purification of E2 proteins (BVDV). Lane C: Control group CHO wild type; Column WM: AccuRuler RGB Plus Prestained Protein Ladder weight molecular marker 25-75kDa; Lane 1: CHO-V2-V1 initial sample; Column 2: Unbound fraction; Column 3: Washed fraction, 25 mM Imidazole; Column 4: Eluted fraction, 200 mM Imidazole. Bracket α: Bands corresponding to the E2 variants with expected weights of 55kDa, 59kDa and 61kDa. Bracket β: Immunodetection of the three peptide tags (HA-tag, Strep II-tag and E-tag) associated to the E2 genotypes.

Further analysis of the SDS-PAGE profile using densitometry by Fiji ImageJ 152p and CurveExpert Professional 2.6.5 imaging software, revealed an approximate 72% purity index for the three combined E2 glycoproteins in the 200 mM Imidazole elution.

Next, the 200 mM Imidazole fraction was dialyzed in PBS solution and concentrated in a water-polyethylene glycol 6000 mixture to half its initial volume. A 25 µl sample of this concentrated fraction was quantified by the BCA assay, and the results were analyzed by the GraphPad Prism 8.0.1.244 graphing software. An interpolation of the optical density (OD) of the sample with the BSA standard curve of absorbance reported 106 µg of total proteins. Thus, after considering the reported purity index and quantification values, we estimated that the IMAC purification method yielded in one step 4.24 µg/µl of our combined immunogens of interest.

VI. CONCLUSIONS AND RECOMMENDATIONS

In Chile, BVDV is recognized as a leading cause of respiratory tract issues, fertility problems, and reduced production of milk and meat in cattle. The recurring nature of the disease, along with the lack of available vaccines to prevent it represents substantial economic losses for the livestock industry. Thus, this research project developed a methodology to generate vaccine candidates against three common variants of the disease in Chile.

In the present paper, we reported the induction and expression in a mammalian cell line of three protein variants derived from the E2 protein of BVDV. We characterized their expression and purified them by IMAC for later use as potential immunogens and diagnostic agents. From the results discussed in the previous chapter, these following conclusions can be drawn.

Using a bacterial host strain of *E. coli TOP10*, the plasmid vectors pCI-Neo-V1 and pCI-Neo-V2 were effectively replicated and purified to be used for transfections. Through these vectors, three highly immunogenic variants of the E2 protein were induced and expressed simultaneously in a transfected Chinese ovary hamster cell line. The three recombinant protein genotypes E21b, E21d, and E21e of the E2 membrane glycoprotein from the BVDV were simultaneously purified in a single step using a Ni-Sepharose chromatography. Through a rapid production method, it was possible to strongly express, filter to moderate purity, and quantify the three combined immunogens. These antigens possess a biological and medical interest as active ingredients for the development of therapeutic vaccine candidates against non-preventable strains of BVDV in the south-central provinces of Chile. To that end, the employed methodology was designed to be scalable, and with the least number of steps possible. Given its intended use in farm animals, the obtained purified vaccine candidates were apt for testing in the subsequent *in vivo* stage. The results obtained suggest that this methodology constitutes a promising approach to design and produce future recombinant vaccines against BVDV.

After completion of the experiment, certain factors were deemed eligible for improvement. Alternative immunoreactive methods such as ELISA are advised to be employed to select and characterize the expression of producer cell lines, particularly in cases where SDS-PAGE and Western Blot assays reported the low presence of the target proteins. Another condition to improve was the selection of 200 mM Imidazole for the protein's elution in the FPLC. At such value, the E21b and E21e variants showed to elute in the highest concentrations. However, the E21d genotype revealed a higher presence in elutions at 150 mM and 250 mM Imidazole, suggesting the use of either concentration for elution in an analogous

chromatography to increase the obtained amount of E21d protein. Due to time constraints, the E2 variants were only purified through an immobilized metal affinity chromatography, but if a higher protein purity was needed, one or more purification techniques could be performed in conjunction with the IMAC. For this end, a size exclusion chromatography or a hydrophobic interaction chromatography was considered to ensure a protein purity closer to homogeneity.

Once the E2 variants were expressed, characterized, and purified, the *in vivo* test phase of the experiment commenced. For this stage, it is expected a diagnosis of the immune responses in sheep inoculated with the vaccine. The responses are being compared to those of sheep manifesting clinical symptoms of the ncp and cp biotypes. The detection of specific antibodies in the ovine serum is being mediated by enzyme-linked immune (ELISA) assays. Preliminary results, during the time of writing of this thesis project, show immune responses in inoculated sheep with high similarity to those suffering the illness, hinting the viability of the immunogens as vaccine candidates.

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