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# Rabies virus-like particles expressed in HEK293 cells

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ABSTRACT

Rabies is an infectious viral disease with a mortality rate close to 100%. Currently, there is a need to generate cheaper and more immunogenic vaccines for the effective prevention of rabies, mostly in developing countries. Virus-like particles have been widely used in viral vaccine production due to their high immunogenicity and safety during the production process. Rabies virus glycoprotein is the major antigen to trigger a protective immune response and the only protein capable of generating virus neutralizing antibodies. In this study we describe the development of a recombinant stable cell line for the production of rabies virus-like particles (RV-VLPs) expressing the rabies virus glycoprotein by lentivirus-based transduction of HEK293 cells. Protein expression was analyzed by flow cytometry, fluorescence microscopy, western blot and ELISA. Particles were purified from culture supernatant and their size and morphology were studied. Furthermore, mice were immunized with RV-VLPs, formulated with adjuvant, and these particles were able to produce a specific antibody response, demonstrating that these virus-like particles present a promising rabies vaccine candidate.



## 1. Introduction

Rabies is a fatal disease responsible for approximately 60,000–70,000 annual deaths worldwide. In humans, rabies is almost invariably fatal once clinical signs occur, making this disease a severe public problem mostly in developing countries. It is transmitted to other animals and humans through close contact with saliva from infected animals [1,2]. Prevention of rabies in humans includes provision of post-exposure prophylaxis (PEP) to potentially exposed patients and pre-exposure immunization of people at frequent risk of exposure. Today, 10 millions post-exposure treatments are reported yearly among humans [3,4]. As dogs are the main vector for the transmission of rabies to humans, mass vaccination of dogs, as well as dog population management is very important for the efficacy of rabies elimination programs [1]. In developing countries, nerve-tissue vaccines are still applied for prophylaxis and in developed countries cell-based vaccines are available. However, they have to be repeatedly administered, four to five doses for PEP [1]. Although new classes of vaccines have been developed, such as protein subunit vaccines, DNA vaccines and viral vectors vaccines, most of them have not yet undergone extensive pre-clinical testing or comparison to commercially available vaccines [5]. This is why there is still a need for more immunogenic and cheaper rabies virus vaccines in order to reduce costs to prevent the disease.

Virus-like particles (VLPs) are multiprotein empty structures with characteristics and an antigenicity very similar to viruses. For enveloped viruses, VLPs are assembled expressing envelope proteins with or without the matrix proteins that form the authentic viral particle. The envelope proteins bud from plasma membrane of the cell and contain the cellular lipids and proteins present in it. VLPs have been widely used in vaccine development in the last decades as they can trigger a protective immune response with lower doses and are obtained by harmless processes [6–10].

Rabies virus is a neurotropic virus and has a small negative-stranded RNA genome of about 12 kb, which encodes five proteins: nucleoprotein (N), phosphoprotein (P), matrix (M2), glycoprotein (G) and RNA-dependent-RNA polymerase (L). Within these proteins, G is the major viral antigen, responsible for the induction of protective immunity. This protein can induce CTL and T helper cells proliferation [11,12] and is the main target for neutralizing antibodies [13]. Mebatsion et al. described that G protein possesses an autonomous exocytosis activity and that, during the replication cycle of the rabies virus, vesicles containing G protein were found in supernatant of infected cells. This G protein activity generates a "pull" effect from the outside of the membrane and plays a central role in the virus budding [14,15]. We focused in this feature to decide to produce rabies virus-like particles (RV-VLPs) expressing G

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protein in HEK-293 cells. This kind of cells has been widely used for the production of recombinant proteins and viral vectors for gene therapy and has been proposed as a novel substrate for the production of viral vaccines [16,17]. In addition, HEK-293 has been used for the production of VLPs of diverse viruses as Rift Valley fever virus, Nipah virus, Ebola virus, Dengue virus and Mumps virus [6,18–24].

In this work, we generated stable cell lines for the production of rabies virus-like particles (RV-VLPs). By lentiviral-based transduction of HEK-293 cells we developed a G protein expressing cell line (HEK-G) to produce G containing virus-like particles and evaluated the immunogenicity of these particles in mice. We found that the RV-VLPs were able to induce specific immune responses against rabies virus G protein. These results encourage the study of new VLPs as a possible rabies vaccine candidate.

## 2. Materials and methods

### 2.1. Cells, antibodies and vaccines

HEK-293 cells were cultured in Dubelcco modified Eagle medium (DMEM, Gibco, USA) supplemented with 10% fetal calf serum (FCS, Gibco, USA) at 37 °C with 5% $CO_2$. For the development of stable cell lines, selection of recombinant cells was achieved supplementing media with puromycin (Sigma–Aldrich, USA). For the evaluation of the immune response, the Vero-G cell line was cultured in Minimum Essential Medium (MEM, Gibco, USA) supplemented with 10% FCS.

Monoclonal antibodies and polyclonal sera, used to detect the rabies virus glycoprotein expression in selected recombinant HEK-293 cell lines, were produced in our laboratory. Goat anti-mouse AlexaFluor 488® was purchased from Invitrogen™ (USA) and polyclonal goat anti-rabbit immunoglobulins/HRP were purchased from DAKO (Denmark).

The inactivated rabies virus vaccines used for the immunization protocol were commercially available: human rabies vaccine (VERORAB, Sanofi Pasteur, France) and the veterinary vaccine (Bagovac Rabia, BIOGÉNESIS-BAGÓ, Argentina).

### 2.2. Plasmid construction and lentivirus production

To produce lentiviral vectors for the expression of the rabies virus protein, G sequence was cloned in the self-inactivating (SIN) lentiviral vector construct (pLV-PLK) [25]. Briefly, the G protein sequence was amplified from a cloning vector (pGlycoG) by PCR using the following primers: G-fw: 5′-TGGGTACCGCTAGCGCCACCATGCC-3′; G-rev: 5′-ATGAGCGGCCGCTCATCACAGTCCG-3′, containing the restriction enzymes sites *Nhe*I and *Not*I, respectively. The fragment was cloned in pLV-PLK generating the pLV-G transfer vector.

Lentiviral particles were produced by simultaneous co-transfection of HEK-293 cells with four plasmids using the lipid reagent Lipofectamine 2000 (Invitrogen™, USA). These plasmids were the packaging construct (pMDLg/pRRE), the VSV-G expressing construct (pMDG), the Rev-expressing construct (pRSV-Rev) [26,27], and the transfer vector pLV-G. 48 h after transfection the supernatant was harvested, clarified by low speed centrifugation and frozen at −80 °C prior to use. The titer was calculated with a HIV-1 p24 ELISA kit (QuickTiter™ Lentivirus Titer Kit, Cell Biolabs Inc., USA) following manufacturer recommendations.

### 2.3. Generation of stable cell lines

HEK-293 cells were seeded at a concentration of $3 \times 10^4$ cells/ml in 6-well plates and incubated for 24 h. The supernatant was removed and the transduction was performed adding 1 ml of lentivirus stock. 96 h post-transduction the cells were incubated with puromycin as the selection agent to obtain stable recombinant cell lines. A multi-step gradual selection protocol was employed based on previous descriptions [25]. Briefly, the cells were incubated from 1 to 250 μg/ml of puromycin, the selection agent was gradually changed every 7 days on the same plates. This cell line was called HEK-G. Finally, a master cell bank and a working cell bank were constructed and conserved in liquid nitrogen.

### 2.4. Analysis of G protein expression by HEK-G cell line

To determine the expression of RV glycoprotein, HEK-G cell line was harvested, resuspended in DMEM basal media and incubated for 30 min with a G protein specific monoclonal antibody (diluted 1:1000 in PBS, 0.1% BSA). Cells were washed (PBS, 0.1% BSA), followed by a 30 min incubation with an AlexaFluor 488® conjugated goat anti-mouse antibody (diluted 1:000 in PBS, 0.1% BSA). After that, cells were analyzed by flow cytometry in a GUAVA EasyCyte cytometer (Millipore, France).

To confirm the expression of the G protein, HEK-G was seeded over polylysine treated glass slides and cultured for 48 h. After that, the cells antigen slides were fixed in 4% paraformaldehyde for 30 min, followed by 1 h incubation with a G protein specific monoclonal antibody (diluted 1:100 in PBS, 0.1% BSA). Cells were washed three times and incubated for 1 h with an AlexaFluor 488® conjugated goat anti-mouse antibody (diluted 1:500 in PBS, 0.1% BSA). After three washes, cells were dyed with a DAPI solution (4′,6-diamidino-2-phenylindole) in a final concentration of 1 μg/ml and analyzed by fluorescence microscopy (Olympus BX-51TRF, Olympus Optical Co. filters Ltd., Tokyo, Japan) and images were acquired with a high resolution camera (QImaging® Go-3, QImaging, Surrey, BC, Canada).

### 2.5. ELISA assay for VLPs detection

The sensitization step was as follows: 96-well micro-plate (Greiner Bio-One, Germany) was coated by adding in each well 100 μl of an appropriate dilution of horse polyclonal sera against rabies virus in carbonate buffer pH 9.6 and incubating for 1 h at 37 °C and over night at 4 °C. After six washes (PBS, 0.05% Tween-20) the wells were blocked with 200 μl of 1% BSA in PBS for 1 h at 37 °C and washed again. Serial 2-fold dilutions of the cell culture supernatants were prepared (in PBS, 0.05% Tween-20, 0.1% BSA), distributed (100 μl/well) and incubated for 1 h at 37 °C. After that, the plate was incubated for 1 h with a dilution of a rabbit polyclonal antibody (diluted 1:4000 in PBS, 0.05% Tween-20, 0.1% BSA) and followed by 1 h incubation of a HRP conjugated goat anti-rabbit immunoglobulins (diluted 1:2000 in PBS, 0.05% Tween-20, 0.1% BSA). Six washes were performed between each step and the reaction was revealed adding 100 μl/well of a chromogenic substrate solution (0.5 mg/ml o-phenylenediamine (Sigma–Aldrich, USA), 0.5 μl/ml $H_2O_2$ 30 vol., 50 mM citrate-phosphate buffer, pH 5.3). The reaction was stopped by adding 50 μl of a 0.5 M sulphuric acid solution, and the optical density was measured at 492 nm in a spectrophotometer (Labsystems Multiskan®).

### 2.6. VLPs purification

HEK-G cell line was cultured in T-175 tissue culture flasks (Greiner Bio-One, Germany) with DMEM media supplemented with 1% FCS, and the supernatants were harvested once per 2 days. The harvest was clarified at 1000 × *g* to remove cells and then centrifuged at 10,000 × *g* to remove any cellular remaining debris. After a 0.45 μm filtering, the clarified supernatant was layered over a 10 ml 30% sucrose cushion in 25 mm × 104 mm Ultra Centrifuge tubes (Beckman, USA) and centrifuged at 65,000 × *g* for 3 h (Avanti J-30I, Beckman, USA). The supernatant and sucrose cushion was

poured off and the pellet resuspended in 40 ml PBS, pH 7.4. The preparation was centrifuged again in the same conditions and the final pellet resuspended in 300 μl PBS, pH 7.4.

### 2.7. Western blot

Concentrated preparations of VLPs were mixed with 4× sample buffer (2% SDS, 10% glycerol, 50 mM Tris–HCl pH 6.8, 10% 2-mercaptoethanol, 0.05% bromophenol blue), boiled for 3 min, and separated by 12% SDS-PAGE. Proteins were transferred onto PVDF membranes (Bio-Rad Laboratories Inc., USA). Membrane was blocked (TBS, 5% skim milk) and proteins were detected with an anti-rabies rabbit polyclonal serum (diluted 1:2000 in TBS, 0.5% skim milk). HRP conjugated goat anti-rabbit immunoglobulin was used as the secondary antibody and the reaction was visualized using a chemiluminescence reagent (Pierce® ECL Western Blotting Substrate, Thermo Scientific, USA).

### 2.8. Electron microscopy

RV-VLPs were analyzed by negative staining electron microscopy. Sucrose cushion purified particles were adsorbed to a formvar-coated 300-mesh copper grid for 2 min. Excess was removed with filter paper and grids were then negatively stained with 2% uranyl acetate for 2 min. Samples were examined using a transmission electron microscope (TEM) Jeol JSM-100 CX II (Jeol, Japan) and digital images were acquired with a CCD 785 ES1000W camera (Gatan, USA).

### 2.9. Mice immunization

Female 4–5 week old BALB/c mice were intramuscularly injected with purified VLPs preparations and boosted on day 12-post primary immunization. Freund' incomplete adjuvant was used (Sigma–Aldrich, USA) and two different amounts of VLPs were employed: 3 μg (group A, $n = 5$) and 0.3 μg (group B, $n = 5$). Positive control groups were immunized with commercially purchased inactivated rabies virus vaccines: VERORAB human vaccine (C group, $n = 4$) and BagovacRabia veterinary vaccine (D group, $n = 4$). For measurement of humoral response parameters blood samples were collected on day 19. Pre-immunized reference sera were collected prior injection.

### 2.10. Evaluation of humoral immune response

Rabies virus G protein specific antibodies were determined in mice sera by ELISA and flow cytometry using a method developed in our laboratory.

#### 2.10.1. ELISA

96-Well micro-plates (Greiner Bio-One, Germany) were coated by adding in each well 100 μl of an appropriate dilution of RV-VLPs preparation in carbonate buffer pH 9.6 and incubating for 1 h at 37 °C and over night at 4 °C. After six washes (PBS, 0.05% Tween-20), wells were blocked with 200 μl 2% skim milk in PBS for 1 h at 37 °C and washed again. Serial 2-fold dilutions of sera samples were prepared (in PBS, 0.05% Tween-20, 0.2% skim milk), distributed (100 μl/well) and incubated for 1 h at 37 °C. After that, plates were incubated for 1 h with HRP conjugated rabbit anti-mouse immunoglobulins (diluted 1:2000 in PBS, 0.05% Tween-20, 0.2% skim milk). Six washes were performed between each step and the reaction was revealed adding 100 μl/well of a chromogenic substrate solution (0.5 mg/ml o-phenylenediamine (Sigma), 0.5 μl/ml $H_2O_2$ 30 vol., 50 mM citrate-phosphate buffer, pH 5.3). The reaction was stopped by adding 50 μl of 0.5 M sulphuric acid solution, and the optical density was measured at 492 nm in a spectrophotometer (Labsystems Multiskan®). An OD 492 nm value exceeding the mean + 2 S.D. of negative controls (basal mice sera) was considered as positive.

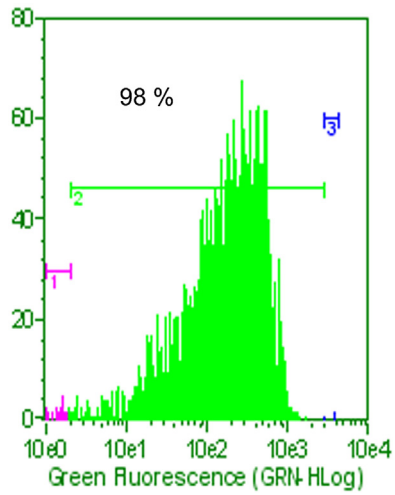


**Fig. 1.** Evaluation of the expression of G protein by recombinant cell lines using flow cytometry. Cells were incubated with a monoclonal antibody against G protein and AlexaFluor 488® conjugated goat anti-mouse was used as secondary antibody. Cells were analyzed with a GUAVA EasyCyte cytometer and the data were acquired with GUAVA ExpressPlus software. Marker 1: negative cells. Marker 2: cells that express the G protein.

#### 2.10.2. Flow cytometry

In this method, the antigen is the G protein anchored in the plasma membrane of a recombinant Vero-G cell line. Briefly, $1 \times 10^5$ total cells were incubated with 4-fold dilutions of mice immune sera for 30 min at RT and then washed twice with MEM media. After that, suspension was incubated with AlexaFluor 488® conjugated goat anti-mouse antibody (diluted 1:1000 in MEM) for 30 min at RT, washed twice and analyzed by flow cytometry (GUAVA EasyCyte, Millipore, France). Data was acquired with GUAVA ExpressPlus software.

## 3. Results

### 3.1. Production of rabies virus-like particles (RV-VLPs)

This work describes the generation of a stable cell line for the production of RV-VLPs. A G protein expressing cell line (HEK-G) was developed to produce G particles.

HEK-293 cells were transduced with lentiviral vectors and recombinant cells were selected with puromycin. By flow cytometry, HEK-G was analyzed to verify the expression of the G protein, obtaining 98% of positive cells (Fig. 1). Membrane localization of the glycoprotein was confirmed by fluorescence microscopy (Fig. 2).

Once the cellular expression of the RV proteins was confirmed, supernatant of the stable cell line was analyzed by Sandwich ELISA. RV-VLPs were found to be secreted to the supernatant as shown in Fig. 3. Supernatant of HEK-G was concentrated by ultracentrifugation through sucrose cushion reaching a 75-fold concentrated sample. Antigenicity and morphology of the purified RV-VLPs were analyzed by western blot and negative staining electron microscopy (TEM). RV-VLPs were able to be recognized by antibodies present in hyper-immune rabbit serum and the G protein band was visible at the expected size (62–65 kDa) (Fig. 4). TEM showed round particles with diameters between 50 and 60 nm in diameter (Fig. 5).

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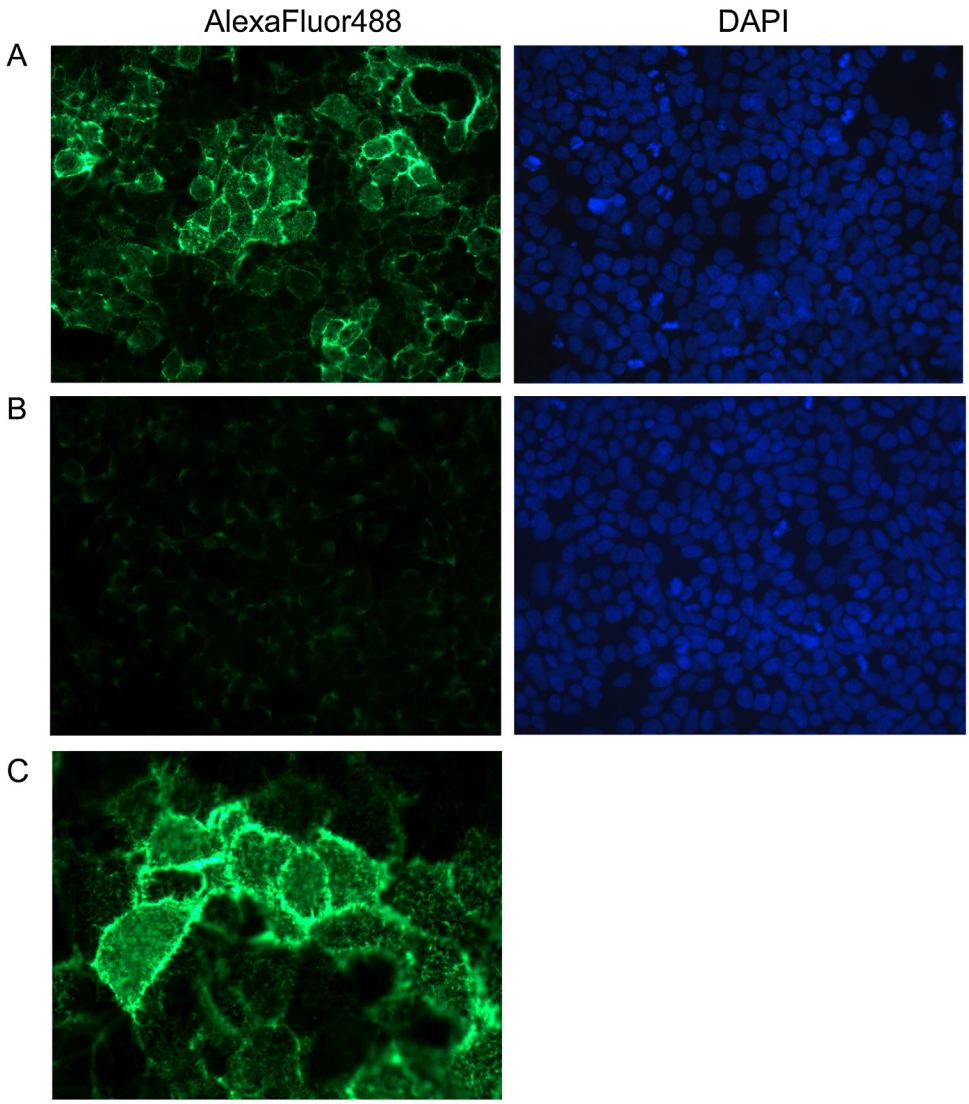


**Fig. 2.** Fluorescence microscopy to detect G protein. (A) HEK-G cell line was fixed, incubated with an anti-G protein antibody and then incubated with a goat anti-mouse AlexaFluor 488® conjugated antibody. Nuclei were stained with DAPI. (B) Non-transduced HEK293 cells were used as negative control. (C) Magnification of a specific field where the membrane localization of the glycoprotein was confirmed.

Although there have been no reports of Replication Competent Lentivirus (RCL) occurrences to date [28,29], it is recommended to perform an assay to evaluate its potential generation. A variety of methods have been developed including: a product enhanced reverse transcriptase (PERT) assay that involves the vector's reverse transcriptase, a PCR-based assay that detects Psi-Gag sequences from a recombination event between vector and packaging constructs, PCR-based assays to detect the VSV-G Env used for pseudotyping and an ELISA-based p24 Gag antigen assay. In our case, we analyzed HEK-G cell line using QuickTiter™ Lentivirus Titer Kit (Cell Biolabs Inc., USA). The result of the assay was negative as expected (data not shown).

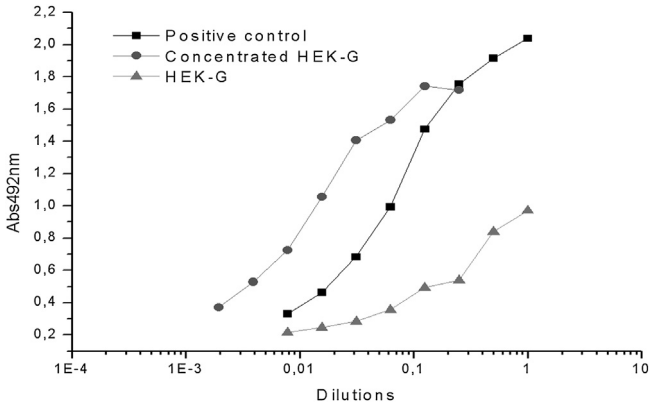


**Fig. 3.** RV-VLPs were detected in supernatants of HEK-G cell line by Sandwich ELISA specific for rabies virus. Sucrose cushion concentrated supernatants were analyzed in the same way. Inactivated Rabies virus was used as a positive control.

### 3.2. Evaluation of RV-VLPs immunogenicity

To determine whether RV-VLPs could induce specific immune responses to rabies virus major antigen, we inoculated mice with VLPs preparation using Freund' Incomplete adjuvant. BALB/c mice were injected twice by intramuscular route at two antigen doses: 0.3 and 3 μg. Mice were injected with commercial vaccines (VERORAB human vaccine and BagovacRabia veterinary vaccine) in order to compare the elicited immune responses. Blood samples were analyzed by flow cytometry using a method developed in our laboratory. Sera samples of the immunized animals were able to recognize the G protein present in the membrane of the Vero-G cell line (Fig. 6). On the other hand, high titers of specific antibodies

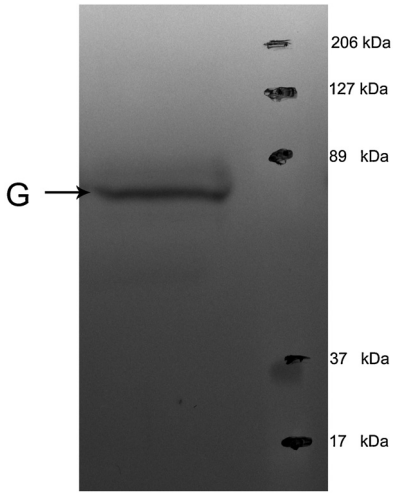


**Fig. 4.** Western blot analysis of RV-VLPs. Using an anti-Rabies serum G protein band was detected in the expected size (62–65 kDa).

were detected by indirect ELISA. In addition, we observed that the magnitude of the G protein specific antibodies induced by RV-VLPs was comparable to those induced by commercial vaccines and that the antibody levels induced with 0.3 μg of RV-VLPs were comparable with those obtained with 3 μg, showing that these particles could be injected at low doses. The obtained results indicate that RV-VLPs are immunogenic and capable of inducing specific antibody responses against rabies virus.

## 4. Discussion

Currently, inactivated vaccines are mostly used for prevention of rabies throughout the world. Cell-based vaccines are used in developed countries, but nerve tissue-based vaccines are still used in developing countries, where rabies is a real problem. Sometimes chemical inactivation changes the structure of viral antigens, decreasing the immunogenicity of the vaccine and adding challenges to immunization [1,5]. On the other hand, recombinant subunit vaccines, based on vaccination with a single viral protein or peptides, have offered an effective alternative to live virus vaccines, but usually more doses of higher concentrations of antigen are needed to have the same protective effect due to their weak immunogenicity. This leads to a need for a large dose or, alternatively, the addition of adjuvant [30]. VLPs are commonly more

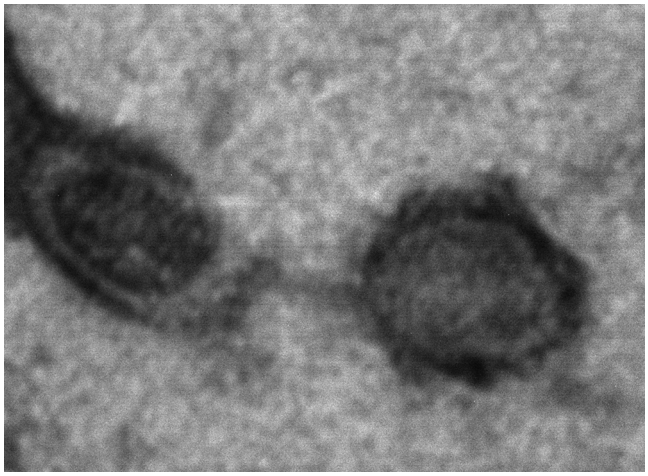

**Fig. 5.** Transmission Electron Microscopy of sucrose cushion purified RV-VLPs.

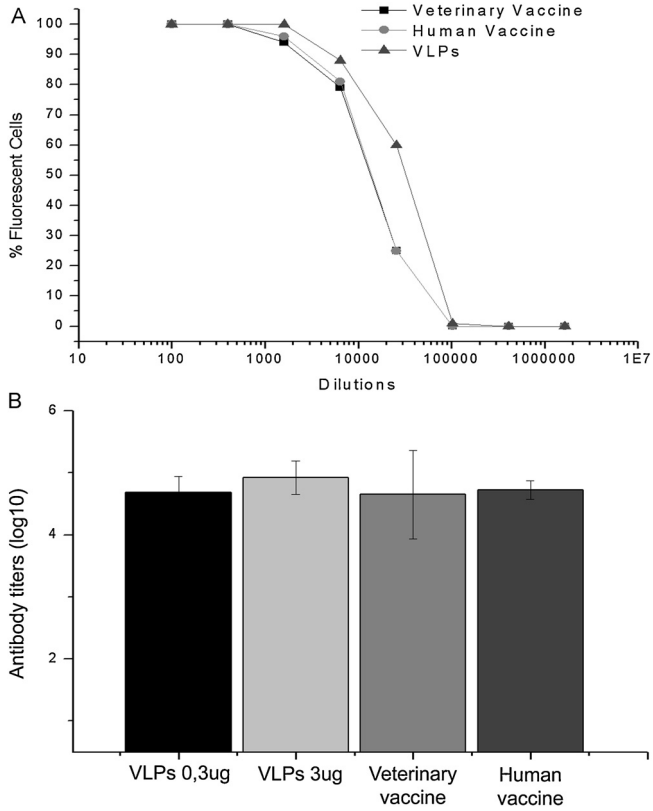


**Fig. 6.** Humoral immune responses in Balb/c mice elicited by RV-VLPs immunization. (A) Serial 4-fold dilutions of pooled sera were incubated with a suspension of Vero-G cell line that expresses the G protein in the membrane and were analyzed by flow cytometry. (B) Sera samples were analyzed by ELISA using VLPs preparation as sensitizing antigen. Immunizations with commercial vaccines were made as positive controls.

immunogenic than subunit or recombinant protein immunogens, and are able to stimulate both T- and B-cell immunity. Since VLPs provide the spatial structure for display of conformational epitopes, they are most likely to mimic the native virus structure, enhancing the production of neutralizing antibodies. This may be especially true for surface proteins of enveloped viruses, where enveloped VLPs allow the vaccine proteins to be presented in their natural state rather than as the soluble ectodomains alone. VLPs may therefore offer a safe and effective approach for the induction of antibodies to surface proteins, as rabies G protein, where soluble forms of the proteins have failed to be effective [9]. This is a principal concern because the major immunological correlate of protection produced by vaccination is the production of neutralizing antibodies [31]. The possibility to develop a rabies virus vaccine based on a virus-like particles technology seems to be very promising, mostly because there is no need to work with the active virus, in which case proper equipment and facilities with the appropriate biosafety levels are a must. The results obtained in this work encourage the study of this novel RV-VLPs as a possible vaccine candidate.

*Conflict of interest:* The authors have declared no conflict of interest.

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