Vaccine 33 (2015) 4238–4246

Contents lists available at ScienceDirect

Vaccine

journal homepage: www.elsevier.com/locate/vaccine

Immunogenic virus-like particles continuously expressed in mammalian cells as a veterinary rabies vaccine candidate

Diego Fontana, Ricardo Kratje, Marina Etcheverrigaray, Claudio Prieto*

Laboratorio de Cultivos Celulares, Facultad de Bioquímica y Ciencias Biológicas, Universidad Nacional del Litoral, Ciudad Universitaria, Paraje "El Pozo"-C.C. 242, S3000ZAA Santa Fe, Argentina

ARTICLE INFO

Article history:
Available online 11 April 2015

Keywords:
Rabies
Vaccine
Virus-like particles
HEK293 cells
Serum free media

ABSTRACT

Rabies is one of the most lethal infectious diseases in the world, with a mortality approaching 100%. There are between 60,000 and 70,000 reported annual deaths, but this is probably an underestimation. Despite the fact that there are vaccines available for rabies, there is a real need of developing more efficacious and cheaper vaccines. This is particularly true for veterinary vaccines because dogs are still the main vector for rabies transmission to human beings. In a previous work, we described the development and characterization of rabies virus-like particles (RV-VLPs) expressed in HEK293 cells. We showed that RV-VLPs are able to induce a specific antibodies response. In this work, we show that VLPs are able to protect mice against virus challenge. Furthermore, we developed a VLPs expressing HEK-293 clone (sP2E5) that grows in serum free medium (SFM) reaching high cell densities. sP2E5 was cultured in perfusion mode in a 5 L bioreactor for 20 days, and the RV-VLPs produced were capable of triggering a protective immune response without the need of concentration or adjuvant addition. Further, these VLPs are able to induce the production of rabies virus neutralizing antibodies. These results demonstrate that RV-VLPs are a promising rabies vaccine candidate.

1. Introduction

Virus-like particles (VLPs) are empty and self-assembled structures that mimic conformation of native virus. In the last decades, VLPs have been widely used for vaccine development and some products are commercially available [1,2]. Based on their particulate nature and repetitive antigen presentation, VLPs have several benefits over soluble antigens that have shown to fail in some vaccine approaches. On the other hand, their safety and immunogenic properties give advantages against attenuated or inactivated virus-based vaccines, because reversion is not possible and the deleterious effects of inactivation agents are not present [3–5]. Furthermore, VLPs production does not introduce high levels of viral containment, if compared to the production of active virus. Therefore, it offers an extra benefit in the structural cost of the process (costly biosafety level 3 or higher production facilities are not required) and reduces the final vaccine cost.

In the last years, lots of advances have been made for developing new subunits vaccines in the veterinary field [6–8]. Virus-like particles are one of the most important approaches due to their intrinsic immunogenic properties that can trigger both arms of the immune system, as well as VLPs are accurate candidates for generating vaccines with DIVA characteristics (Differentiating Infected from Vaccinated Animals). DIVA vaccines allow mass vaccination of animals without comprising the identification of infected individuals, ensuring the safe trade of products from vaccinated animals [9,10]. The porcine circovirus type 2 (PCV2) VLP-based vaccine is licensed and commercially available (Porcilis PCV®, manufactured by Intervet International, The Netherlands) and several other animal vaccine candidates are being developed.

Rabies is one of the most fatal existing viral diseases, with a mortality of about 100% when it is not treated. There are between 60,000 and 70,000 reported deaths per year, but this is probably an underestimation due to the lack of health surveillance in some developing countries where this disease is endemic [11]. Although it may be the oldest known infectious disease, rabies is still neglected after more than 100 years of vaccination [12,13]. Prevention of rabies depends on pre-exposure prophylaxis of people under frequent exposure, and post-exposure vaccination for individuals in contact with the virus. On the other hand, mass vaccination of dogs is one of the most cost-effective ways to reduce human infections, but normally this intervention is not achieved or prioritized by governments, mostly in developing countries. With a vaccination coverage level of 70%

* Corresponding author. Tel.: +54 342 456 4397; fax: +54 342 456 4397.
E-mail addresses: cprieto@fbcb.unl.edu.ar, claudiocesarprieto@gmail.com (C. Prieto).

http://dx.doi.org/10.1016/j.vaccine.2015.03.088
0264-410X/

canine rabies could be eliminated. In view of the economic and public health demands, it is important to have a source of cheap, safe and efficacious vaccines against rabies because dogs are still the main source of human and livestock exposure [14,15].

Rabies virus is a negative-stranded RNA genome that consists of 11,932 nucleotides and encodes five proteins [16,17]. Glycoprotein G is the major antigen, the target of neutralizing antibodies and inducer of CTL and T helper proliferation [18,19]. This protein forms homotrimers and is anchored to the membrane envelope of the virion [20,21]. Different kinds of vaccine candidates have been developed for rabies [22,23], most of them based on recombinant expression of the glycoprotein G in different expression systems [24-28], as well as DNA or viral vectors carrying one or more copies of the sequence of this immunogenic protein [29–34]. Nevertheless, none of these attempts have yet undergone pre-clinical trials or extensive comparison with commercially available vaccines.

In our previous work [35] we described the development of novel rabies virus-like particles (RV-VLPs) expressing only the external glycoprotein in HEK293 cells. We showed the budding of RV-VLPs from the plasma membrane of the cell to the supernatant and we confirmed the immunogenicity of the purified particles in vaccinated animals. In the present work, we described the development and characterization of a VLPs producing clone growing in a serum-free medium (SFM) and the immunization studies achieved with the RV-VLPs produced in those conditions. We confirmed that RV-VLPs are able to protect mice against rabies virus challenge and can be injected without adjuvant. Besides, we showed that RV-VLPs are able to induce rabies virus neutralizing antibodies in mice. Finally, we demonstrate that RV-VLPs are able to be produced in a 5 L bioreactor working in continuous mode in SFM with high productivity. In summary, these results indicate that RV-VLPs are a promising veterinary rabies vaccine candidate.

2. Materials and methods

2.1. Adherent and suspension cultures

Adherent HEK293 cells producing RV-VLPs were cultured in T-flasks at 37 °C with 5% CO_2 in Dubelcco's modified Eagle medium (DMEM, Gibco, USA) supplemented with 10% fetal calf serum (FCS, Gibco, USA). The cell line was cloned by dilution limit method and clones analyzed by flow cytometry.

The selected adhP2E5 clone was adapted to suspension growth using a serum-free media (EXCELL™ 293, SAFC Bioscience, USA) performing a direct adaptation protocol recommended by the manufacturer. Suspension HEK293 cells (sP2E5) were cultured in erlenmeyer flasks (Corning, USA) at 37 °C with 5% CO_2, agitated at 160 rpm using a shaking incubator cabinet (CERTOMAT® CT plus, Sartorius, Germany).

2.2. Bioreactor cultures

Cultures were performed in a stirred tank bioreactor (Bioengineering Inc, Switzerland) with a 4.5 l working volume equipped with a spin filter for perfusion. During the culture, pH was set at 7.2, pO_2 at 20% air-saturation, temperature at 37 °C and agitation rate at 100 rpm. Media exchange and perfusion rate were regulated according to the growth profile. Samples were collected daily in order to determine cell density, cell viability, ammonium, lactate and glucose levels.

2.3. Cell counting and metabolite analysis

Cells were stained with trypan blue and counted using a Neubauer haemocytometer. Glucose and lactate concentrations were measured using the Reflectoquant® system (Merck, EE.UU). In this system test strips (with specific enzymes for glucose and lactate) are incubated with the test sample and the developed color is determined reflectometrically. Measuring ranges are 1–100 mg l^{-1} and 3–60 mg l^{-1} for glucose and lactic acid, respectively. Ammonium concentration was determined using a commercial kit (SB, Argentina) based on the Berthelot reaction [36].

2.4. Antibodies

Monoclonal antibody (AB5, purified from ascitis, titer $1{:}2 \times 10^5$) and rabbit polyclonal anti-serum (titer $1{:}4 \times 10^6$) were produced in our laboratory using the commercial rabies vaccine as immunogen. Monoclonal antibody recognizes rabies glycoprotein anchored in the membrane of infected and/or transduced cells [35]. Goat anti-mouse AlexaFluor 488® was purchased from Invitrogen™ (USA) and polyclonal goat anti-rabbit immunoglobulins/HRP were purchased from DAKO (Denmark).

2.5. ELISA assay for VLPs detection

The sensitization step was as follows: 96-well micro-plates (Greiner Bio-One, Germany) were coated with 100 μl of an appropriate dilution of the polyclonal anti-serum in carbonate buffer pH 9.6 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 1% BSA in PBS for 1 h at 37 °C and washed again. Serial 2-fold dilutions of the samples were prepared (in PBS, 0.05% Tween-20, 0.1% BSA), distributed (100 μl $well^{-1}$) and incubated for 1 h at 37 °C. After that, plate were incubated for 1 h with biotin conjugated rabbit polyclonal antibodies (diluted 1:2000 in PBS, 0.05% Tween-20, 0.1% BSA), followed by 1 h incubation with HRP-conjugated streptavidin (diluted 1:10,000 in PBS, 0.05% Tween-20, 0.1% BSA; Amdex, GE Healthcare). Six washes were performed between each step and the reaction was revealed by adding 100 μl/well of 0.5 mg ml^{-1} o-phenylenediamine (Sigma-Aldrich, USA), 0.5 μl ml^{-1} H_2O_2 30 vol., 50 mM citrate-phosphate buffer, pH 5.3). The reaction was stopped by adding 50 μl 0.5 M sulphuric acid solution, and the optical density was determined at 492 nm (Labsystems Multiskan®).

The World Health Organization 6th International Standard for Rabies Vaccine (NIBSC, United Kingdom) was used as standard. For in vitro assays (such as ELISA or single radial immunodiffusion test) the ampoule contains 3.3 IU Pitman Moore rabies virus glycoprotein antigen. VLPs were quantified against this standard and the concentration expressed as IU ml^{-1} of glycoprotein rabies virus antigen content.

2.6. Flow cytometry

Cells were harvested, resuspended in basal medium and incubated for 30 min with G protein specific monoclonal antibody (diluted 1:1000 in PBS, 0.1% BSA). Cells were washed (PBS, 0.1% BSA), and incubated for 30 min with AlexaFluor 488® conjugated goat anti-mouse antibody (diluted 1:1000 in PBS, 0.1% BSA).Cells were analyzed by flow cytometry in a GUAVA EasyCyte cytometer (Millipore, France).

2.7. Fluorescence microscopy

Cells were seeded over glass slides and cultured for 48 h. Cells 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), washed three times and incubated for 1 h with AlexaFluor 488® conjugated goat anti-mouse antibody (diluted 1:500 in PBS, 0.1% BSA). After three washes, cells were dyed with DAPI solution (4′,6-diamidino-2-phenylindole) 1 μg ml^{-1} and analyzed by fluorescence microscopy (Eclipse Ti, Nikon, Japan). Images

were acquired with a high resolution camera (Digital sight DS-U3, Nikon, Japan).

2.8. VLPs purification

Harvest were centrifuged at 1000 × g for 10 min to remove cells and then at 10,000 × g for 10 min to remove any remaining cellular debris. After 0.45 μm filtration, the clarified supernatant was layered over a 30% sucrose cushion and centrifuged at 25,000 rpm for 3 h at 4 °C (Beckman SW28 rotor, Beckman Coulter, USA). The supernatant and sucrose cushion were poured off, the pellet was resuspended in RV-VLPs stabilization buffer (50 mM Tris–HCl, 0.15 M NaCl, 1.0 mM EDTA, pH 7.4), loaded over a 20–30–40–50% iodixanol density gradient (OptiPrep™, Axis-Shield, Scotland) and centrifuged at 35,000 rpm for 18 h at 4 °C (Beckman SW50.1 rotor, Beckman Coulter, USA). The banded VLPs were collected and analyzed to determine particle density by 340 nm absorbance measurement, as described by the density gradient medium manufacturer. Finally, buffer exchange was performed using 100,000 MWCO Amicon® Ultra centrifugal units (Millipore, USA) and RV-VLPs stabilization buffer.

2.9. 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 10% SDS–PAGE. Proteins were transferred onto PVDF membranes (Bio-Rad Laboratories Inc., USA). Membranes were blocked with TBS 5% skim milk (50 mM Tris–HCl, pH 7.5, 150 mM NaCl) and proteins were detected with anti-rabies rabbit polyclonal serum (diluted 1:2000 in TBS, 0.5% skim milk). HRP conjugated goat anti-rabbit immunoglobulin was used as secondary antibody and the reaction was visualized using a chemiluminescence reagent (Pierce® ECL Western Blotting Substrate, Thermo Scientific, USA).

2.10. Electron microscopy

RV-VLPs were analyzed by negative staining electron microscopy. 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).

2.11. Immunogold electron microscopy

Purified particles were adsorbed to a formvar-coated 300-mesh copper grid for 2 min. Excess was removed with filter paper, washed twice with 2% BSA in TBS and samples were blocked with 2% gelatine in TBS for 30 min at RT. Monoclonal antibody anti-glycoprotein was diluted 1:100 in BSA/TBS and adsorbed onto the grid for 1 h at room temperature. Following three washes with BSA/TBS, secondary gold-conjugated antibody (Colloidal Gold-AffiniPure goat anti-mouse IgG, Jackson ImmunoResearch, USA) diluted 1:20 in BSA/TBS was added and incubated for 1 h at RT. Grids were washed and 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).

2.12. Dynamic light scattering (DLS)

Purified VLPs were filtered through 0.45 μm and analyzed on a Nano ZS particle-size analyzer (Malvern Zetasizer, Malvern Instruments Ltd, UK). Ten consecutive measurements were taken on a single sample. The specific equipment software (Nanov510) was used to convert the intensity-based measurement to a size distribution based on the number of particles. The average hydrodynamic diameter of the particles was calculated as the mean size of particle population ± standard deviation (SD) of 10 independent experiments.

2.13. Rabies vaccine potency test

NIH potency test [37] was performed with two goals: on the one hand, to analyze the efficacy of RV-VLPs as a vaccine candidate, due to the fact that immunized animals are challenged with active virus and the percentage of survivors are recorded. On the other hand, as there is a dose–response curve together with a standard vaccine, this assay is used to analyze the potency of the measured VLPs sample. Thus, NIH potency test for rabies vaccine was performed as follows: CF-1 mice are injected intraperitoneally at days 0 and 7 with 0.5 ml of experimental or international reference vaccine dilutions (1:5; 1:25, 1:125, 1:625 in PBS). At day 14, mice were intracerebrally challenged with 0.03 ml of a dilution of virus standard strain (CVS strain) containing 25 LD_{50} and observed for 14 days. Mortality was recorded and vaccine potency expressed in International Units per ml (IU ml^{-1}).

VLPs were also prepared in the stabilizer recommended for veterinary rabies vaccine [38] with aluminum hydroxide gel as an adjuvant (Sigma-Aldrich, USA) to compare results.

2.14. Rabies virus neutralizing antibodies measurement

To analyze the presence of RVNA in sera of vaccinated animals, an in vitro assay using recombinant lentivirus pseudotyped with the rabies glycoprotein and carrying the Green Fluorescent Protein sequence (GFP) was performed. Some examples of pseudotypes being used for virus neutralizing antibodies measurement were previously reported [39].

Lentiviral particles were produced by simultaneous co-transfection of HEK293 cells with four plasmids using the lipid reagent Lipofectamine 2000 (InvitrogenTM, USA): the packaging construct (pMDLg/pRRE), the Rev-expressing construct (pRSV-Rev), the transfer vector pLV-PLK-GFP [40–42] and, alternatively, the Vesicular Stomatitis Virus glycoprotein (pMDG construct) or the Rabies Virus glycoprotein (PV strain, using the pZC-GlycoG construct developed in our laboratory). 48 h after transfection the supernatant was harvested, clarified by low speed centrifugation and frozen at −80 °C prior to use. We prepared two lentivirus stocks, one with the RV glycoprotein for the specific neutralization assays and the other with the VSV glycoprotein to be used as a control.

For the RVNA measurement test, serial 2-fold dilutions of sera in 50 μl of DMEM basal medium, using 96-well micro-plates, were made. After that, 50 μl of lentivirus supernatant, with a titer of 1×10^6 TU ml^{-1}, was added and incubated for 1 h at 37 °C. Finally, 5×10^3 HEK293 cells in a final volume of 50 μl were added. The plaque was incubated 72 h at 37 °C and the percentage of fluorescent cells measured by flow cytometry.

The concentration of RVNA was calculated using the World Health Organization 2nd International Standard for anti-rabies immunoglobulin (NIBSC, United Kingdom) as a standard. This reference preparation contains 30 IU ml^{-1} of rabies antibody and is recommended to be used for RVNA measurement.

3. Results

3.1. Obtaining producer clones and particle analysis

The RV-VLPs expressing cell line (HEK-G; [35]) was cloned by dilution limit method. Obtained clones were analyzed by flow cytometry and adhP2E5 was chosen as the producer one, because of the 6-fold increase in the glycoprotein expression level when compared with the cell line (Fig. 1). The fluorescence intensity measured by flow cytometry (X-mean) correlates with the amount of RV-VLPs in the supernatant (data not shown). However, the specific RV-VLPs productivity was calculated by sandwich ELISA, obtaining a value of rabies glycoprotein content of 1.1×10^{-6} IU cel^{-1} d^{-1}, between 5 and 6 times higher than the productivity of the recombinant cell line with a value of 0.2×10^{-6} IU cel^{-1} d^{-1}. The cellular expression of rabies glycoprotein was analyzed by fluorescent microscopy, to show the homogeneous expression over the plasmatic membrane (Fig. 2). Finally, master and working cell banks were constructed and conserved in liquid nitrogen.

RV-VLPs produced by adhP2E5 were purified by density gradient ultracentrifugation and characterized by dynamic light scattering (DLS), transmission electron microscopy (TEM) and immunogold labeled TEM (Fig. 3). Enveloped and round shaped particles were seen in TEM assays with a hydrodynamic diameter of 64 ± 24 nm in average measured by DLS. This value is within the range of the VLPs previously described in the bibliography (between 20 and 150 nm; [4]) and is smaller than the rabies virus (180×70 nm, bullet shaped virus, [16]). The immunoreactivity of the VLP surface glycoprotein was verified by staining purified unfixed VLPs. The use of unfixed particles assures that only the surface-exposed antigens are reactive. Immunogold labeling technique using rabies glycoprotein specific monoclonal antibody and 6 nm gold labeled secondary antibody was performed. Particles were viewed by TEM after negative staining. Gold particles bound to RV-VLP's surface were seen.

3.2. Potency test of RV-VLPs produced in adherent cultures

To investigate the protection effect of RV-VLPs, potency was analyzed performing the NIH rabies vaccine potency test recommended by WHO [37]. RV-VLPs prepared from the crude clarified supernatant of adhP2E5 having a glycoprotein content of 1.45 IU ml^{-1} and concentrated RV-VLPs with a concentration of 11.7 IU^{-1} ml were analyzed. The results showed a vaccine potency of 0.8 IU^{-1} ml for the supernatant and 1.1 IU^{-1} ml for ultracentrifugated RV-VLPs. These results indicate that concentrated RV-VLPs are able to trigger a protective immune response in mice and meets WHO requirements with regard to veterinary vaccine potency. On the other hand, although the antigen content of the supernatant was tenfold lower, the potency obtained for this sample was considerably high, although it did not reach the minimum potency required (1 IU ml^{-1}; [38]). This could be due to the better integrity of the RV-VLPs present in fresh supernatant compared with the concentrated VLPs after two rounds of ultracentrifugation. As is well known, size, spatial structure and particulate nature of VLPs are key features for the development of the immune response [4,5,43].

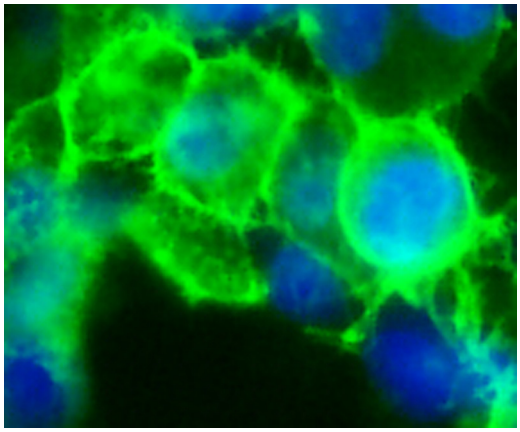

Fig. 2. Fluorescence microscopy of clone P2E5. Cells were fixed, incubated with anti-G protein antibody and then incubated with goat anti-mouse AlexaFluor 488® conjugated antibody. Nuclei were stained with DAPI. Merge image is shown.

3.3. Serum free medium adaptation

AdhP2E5 cells were adapted to grow in SFM conditions following the media manufacturer's recommendations. Briefly, cells were subcultured in DMEM 5% FCS for 3 passages and then directly subcultured in EXCELL™ 293 medium. After a lag phase, growth rates returned to normal values and viability above 95%, without the formation of aggregates or clumps (Fig. 4). adhP2E5 adapted to grow in SFM was renamed as sP2E5. Master and working cell banks were constructed and tested for bacteria, fungi and mycoplasma (data not shown). In batch conditions sP2E5 reaches cell densities of 8×10^6 cells ml^{-1} after a 7 days culture (Fig. 5). Cell growth and metabolism kinetics parameters were calculated (Table 1). The homogeneous expression of the rabies glycoprotein in the

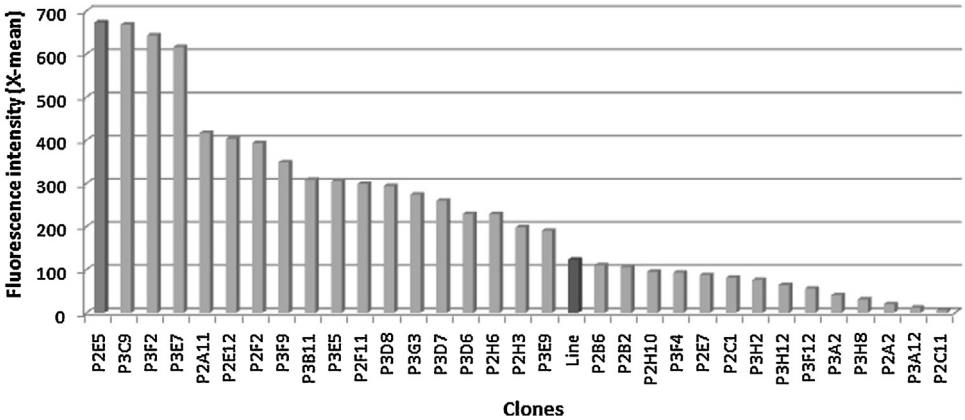

Fig. 1. Clone analysis and selection by flow cytometry. Cells were incubated with a monoclonal antibody against rabies glycoprotein and then with a goat anti-mouse AlexaFluor 488® conjugate. A GUAVA EasyCyte cytometer was used and data were acquired with the GUAVA ExpressPlus software. Clones were compared with the stable cell line and sP2E5 was chosen by its high expression level of rabies glycoprotein.

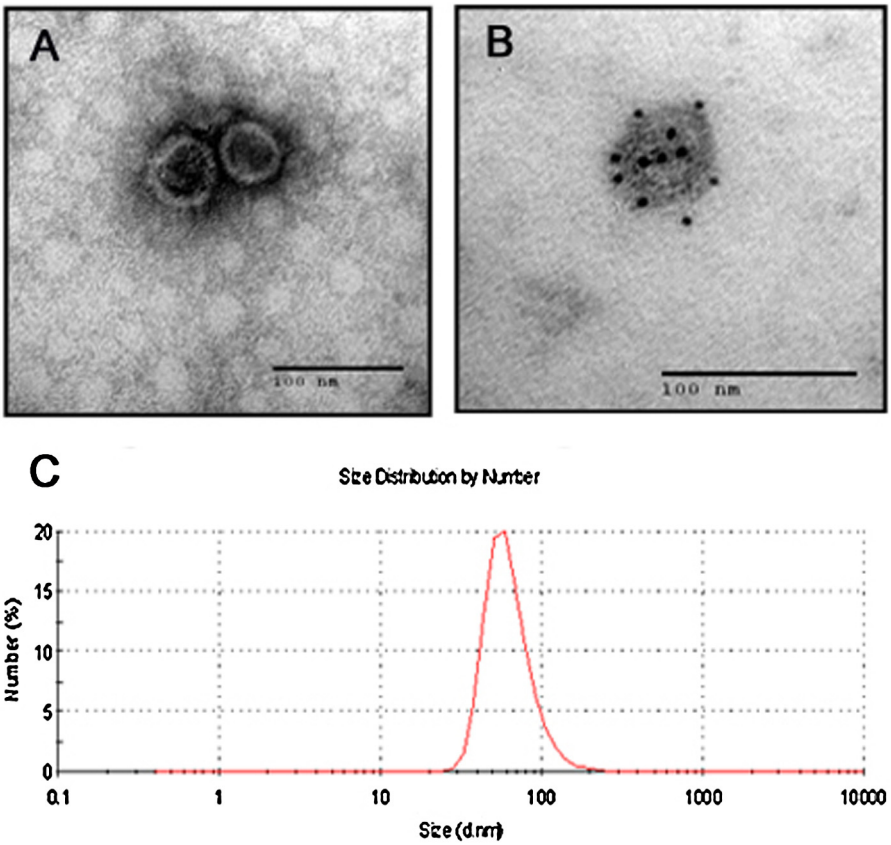

Fig. 3. Virus-like particles characterization. (A) RV-VLPs purified by density gradient ultracentrifugation were analyzed by negative staining MET. (B) Antigen presence in purified RV-VLPs was analyzed by gold-labeled MET. A secondary antibody conjugated to 6 nm gold particles was used. (C) Dynamic light scattering of RV-VLPs in solution. The average hydrodynamic diameter of the particles was calculated as the mean size of particle's population ± standard deviation (SD) of ten independent experiments.

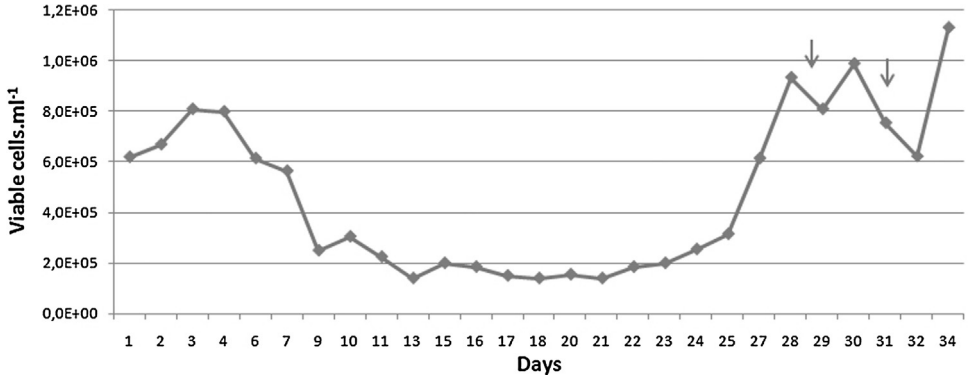

Fig. 4. Serum free medium adaptation of clone sP2E5. After three consecutive passages in DMEM 5% FBS, adhP2E5 was directly subcultured in EXCELLTM 293 media in an Erlenmeyer flask (day 0). Arrows show fresh medium addition.

membrane was analyzed by flow cytometry and RV-VLPs expression confirmed by Western blot (Fig. 6).

3.4. Potency test of RV-VLPs produced in batch conditions with SFM

RV-VLPs produced in shaker flasks in SFM were purified by ultracentrifugation and prepared either in RV-VLPs stabilization buffer without adjuvant or in the stabilizer recommended for veterinary rabies vaccine [38] with aluminum hydroxide gel as an adjuvant. NIH potency test for rabies vaccine was performed separately for both samples to analyze the effect of adjuvant addition to the VLPs preparation. VLPs without adjuvant showed an activity of 1.3 IU ml^{-1} and ultracentrifugation purified RV-VLPs with the addition of adjuvant showed a specific protection activity of 1.5 IU ml^{-1}. This result confirms that the VLPs produced in SFM are highly immunogenic and trigger an immune response able to protect mice against rabies virus challenge. On the other hand, the addition of aluminum as adjuvant did not significantly increase the potency of the VLPs. Thus, RV-VLPs could be injected without the need of adjuvant.

3.5. Rabies virus neutralizing antibodies detection

To analyze the induction of rabies virus neutralizing antibodies (RVNA) during immunization of mice with RV-VLPs, we performed

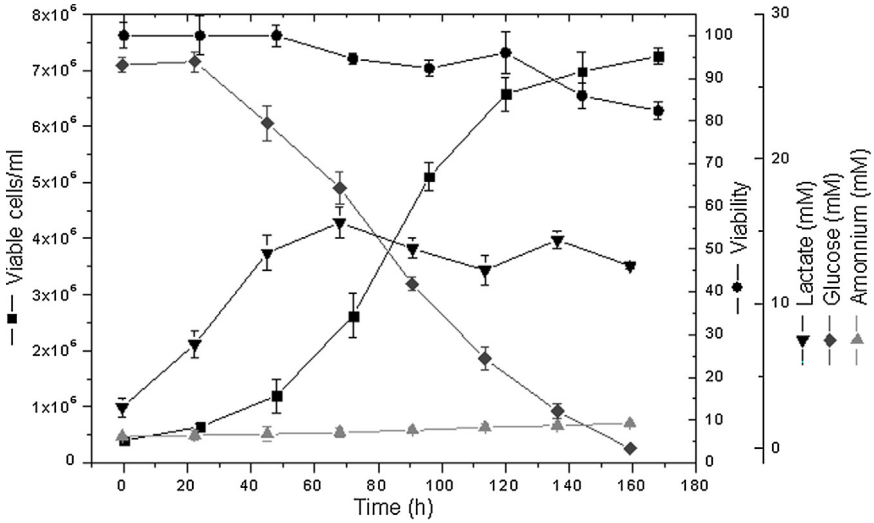

Fig. 5. Clone sP2E5 growth curve. Cells were seeded at 5 × 105 cells ml^{-1} in an Erlenmeyer flask with 30 ml SFM. Metabolite and growth parameters were analyzed for batch cultures.

Table 1
Cell growth and metabolism kinetics parameters for clone sP2E5 in batch conditions.

μ_{max}	0.028 h^{-1}
$q_{lactate}$	0.3 pmol cel^{-1} h^{-1}
$q_{glucose}$	0.09 pmol cel^{-1} h^{-1}
$q_{ammonium}$	0.001 pmol cel^{-1} h^{-1}

an in vitro assay using a recombinant lentivirus pseudotyped with the rabies glycoprotein. When RVNA interact with the rabies glycoprotein present in the lentivirus surface, the recombinant vectors that carry the GFP sequence are not able to transduce HEK293 cells added in the well and no fluorescent cells are detected in the flow cytometry assay. In this case, we analyze sera of mice injected with RV-VLPs expressed in SFM and injected without adjuvant. We performed the same immunization protocol that is described for the NIH potency test, but measuring RVNA instead of protection at day 14. The results showed that antibodies present in the samples were able to block the lentivirus transduction. The controls showed that this assay is specific as no transduction inhibition was observed when a non-reactive serum was analyzed or when a reactive serum was mixed with VSV glycoprotein pseudotyped lentivirus (Fig. 7).

On the other hand, as an International Standard for anti-rabies immunoglobulin was used as a reference, a titer of RVNA expressed in International Units per milliliter was calculated. All the serum presented a titer much higher than 0.5 IU ml^{-1} (Table 2), the value that WHO considers an indication of adequate vaccination, with an average value of 15.2 IU ml^{-1}. Although this method of RVNA measurement should be validated against the standard methods (MNT and RFFIT, [44]), in this case it is useful to confirm that RV-VLPs trigger a specific humoral immune response and induce the production of RVNA.

3.6. RV-VLPs production in perfusion mode in 5 L bioreactor

sP2E5 was cultured in SFM using a 5 L bioreactor in perfusion mode for 20 days, reaching cell densities higher than

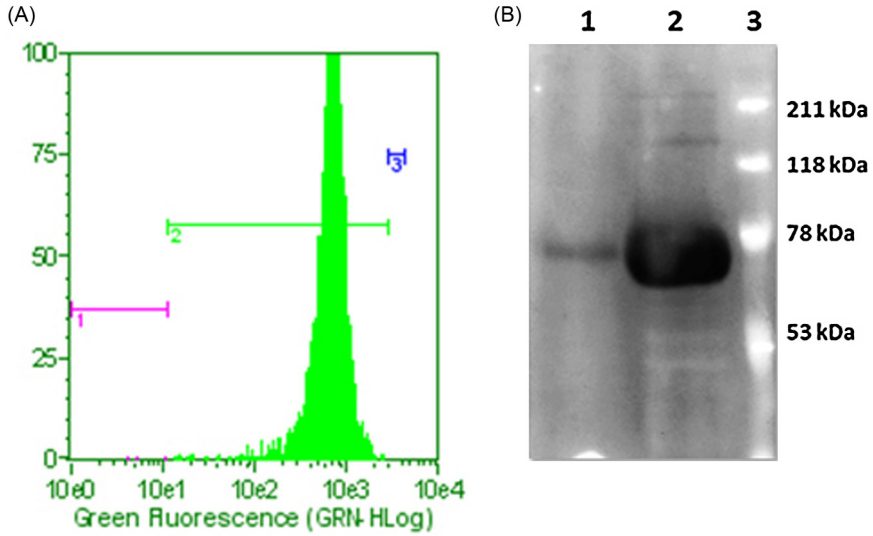

Fig. 6. (A) Flow cytometry of sP2E5, showing the homogeneous expression of the rabies glycoprotein. Cells were incubated with a monoclonal antibody against G protein and AlexaFluor 488® conjugated goat anti-mouse was used as secondary antibody. Cells were analyzed with a GUAVA EasyCyte cytometer and the data were acquired with GUAVA ExpressPlus software. (B) Western blot analysis of RV-VLPs expressed by sP2E5. The glycoprotein band corresponds to the expected size (62–65 kDa). Lane 1: supernatant of sP2E5. Lane 2: RV-VLPs expressed by sP2E5 purified by density gradient ultracentrifugation. Lane 3: molecular weight markers.

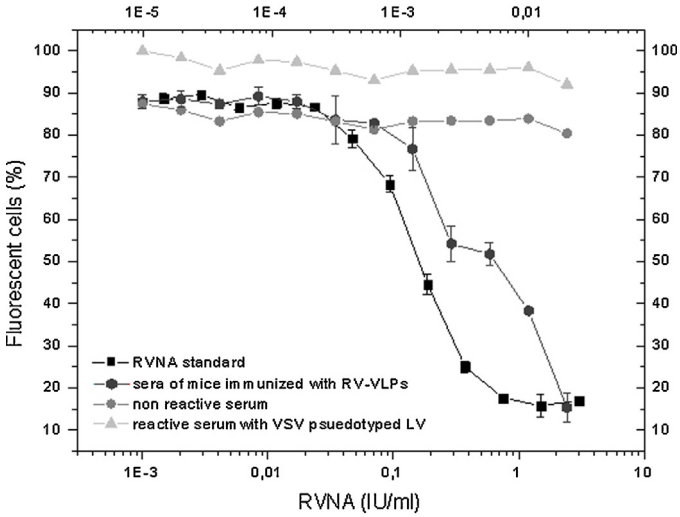

Fig. 7. Rabies virus neutralizing antibodies analysis. Serial dilutions of sera of vaccinated animals were incubated with recombinant lentivirus pseudotyped with the rabies glycoprotein and carrying the green fluorescent protein sequence. Percentage of fluorescent cells (transduced cells) was measured by flow cytometry. An international reference for rabies immunoglobulin was used as a standard.

Table 2
Rabies virus neutralizing antibodies titer for each mouse immunized with RV-VLPs.

Mouse	RVNA (IU ml^{-1})
1	10.5
2	16.6
3	21.0
4	12.7

1.6×10^7 cells ml^{-1}. Perfusion rate varied between 0.2 and 0.8 reactor volumes per day as it was necessary (Fig. 8). The rabies virus glycoprotein content in the harvest was 9.1 IU ml^{-1} measured by ELISA, compared with the 6th International Standard for Rabies Vaccine. The potency of the RV-VLPs produced in the bioreactor was analyzed. Clarified crude supernatant was used to perform NIH potency test for rabies vaccine, obtaining a value of 1.3 IU ml^{-1}. These results indicate that RV-VLPs can be produced in SFM, in continuous mode culture.

4. Discussion

Rabies is a fatal disease that can be completely prevented using vaccines, but is still generating thousands of human deaths worldwide [45]. Nowadays, cell culture-based vaccines are available, but nerve-tissue vaccines are still used in developing countries. Although the effectiveness of these vaccines is proved, they have to be repetitively administrated (between 3 and 8 doses in pre-exposure and post-exposure regimens currently recommended by the World Health Organization and the Advisory Committees in Immunization Procedures [46]). Another drawback of the existing vaccines against rabies is the high cost per dose, which is the main reason why rabies is considered a disease of poverty.

Some approaches to develop a recombinant rabies vaccine have been made, but up to now none of them have been licensed or extensively compared with commercially available vaccines [22,23]. VLPs are an excellent candidate for the development of a recombinant rabies vaccine due to the lack of the reversion risk or deleterious effect of the inactivation processes present in the classic virus vaccines protocols. The high immunogenicity described for VLPs, compared with the recombinant subunit vaccines, and the ability to activate both arms of the immune system [3–5,43,47] are features that encourage the development of a VLP-based rabies vaccine.

In a previous work [35], we developed and characterized rabies-virus like particles obtained by expressing the rabies virus glycoprotein in HEK293 cells. We showed that RV-VLPs are capable of triggering a specific humoral immune response with high antibody titers in mice. We also observed that the magnitude of the glycoprotein specific antibodies induced by RV-VLPs was comparable to that induced by commercial vaccines.

In the present work, we showed that RV-VLPs expressed in HEK293 with animal-protein free media were able to trigger a protective immune response showing high immunogenicity when injected without the addition of adjuvant. Moreover, RV-VLPs injected without adjuvant were able to induce the production of neutralizing antibodies. It is known that RVNA is not the only parameter of the immune response to rabies, but is the most widely measured to determine protection resulting from rabies vaccination [48]. It has been described that RVNA alone can result in viral clearance from the central nervous system (CNS) of experimentally infected mice [49] and most rabies-specific antibodies are directed to epitopes on the rabies virus glycoprotein, the only protein exposed in the virus surface.

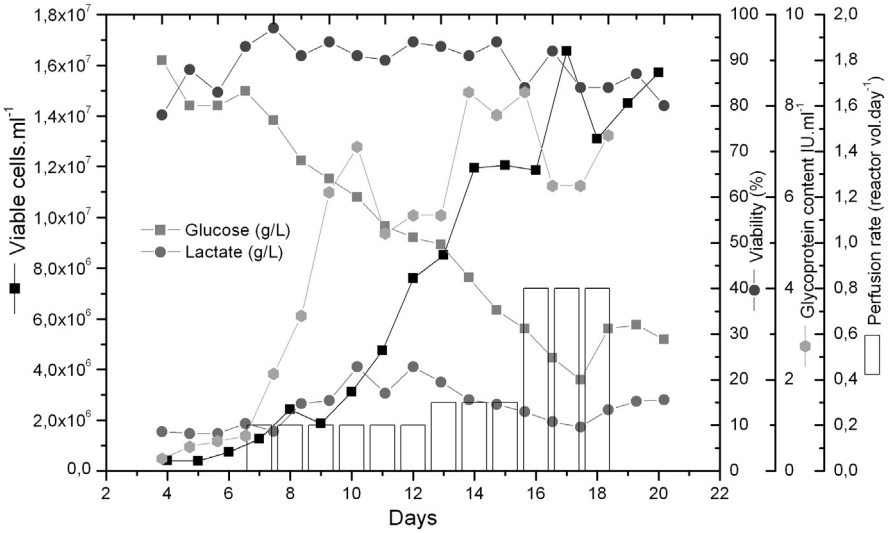

Fig. 8. Culture of sP2E5 in 5 L bioreactor in perfusion mode. Profiles of cell density, cell viability, glucose, lactate, RV-VLPs concentrations and perfusion rate are shown.

In recent years, VLPs have gained importance in the development of veterinary vaccines. A number of VLPs of different kinds of viruses have been under study, some of them are enveloped VLPs expressed in mammalian cell lines [8,10]. Rabies veterinary vaccines are based on inactivated virus produced in animal cells, as BHK-21 cell line, using FCS for media supplementation. As the active virus has to be manipulated, the production laboratory required costly biosafety level 3 (BSL3). Instead, the RV-VLPs described in this work can be produced under standard laboratory conditions, a positive feature that reduces costs of production. On the other hand, we showed the development of a RV-VLPs expressing clone that growths in SFM (sP2E5). Replacement of FCS in media supplementation as well as the use of substances of animal origin during production processes is highly encouraged by regulatory authorities, due to the high risk of contamination with viruses, prions, mycoplasma, etc. Besides, high quality FCS is particularly expensive, making its replacement a central issue, contributing to the reduction of the total cost of the process.

Further, we demonstrated that sP2E5 clone was able to continuously express RV-VLPs when cultured in a 5 L bioreactor in perfusion mode during an extended period of time. This is a key feature of the production process as VLPs are being expressed constantly, and separated cell growth and infection phases during culture are not required.

Thus, these immunogenic RV-VLPs, produced at high cell density continuous culture, in standard laboratory facilities and without the use of FCS represent a novel platform for the production of a biosecure and economic recombinant rabies vaccine.

Conflict of interest statement

The authors have declared no conflict of interest.

Acknowledgment

Part of this work was done at the Palomares-Ramírez group in the Instituto de Biotecnología, Universidad Nacional Autónoma de México, with the technical assistance of Ana Ruth Pastor. We thank the staff of Unidad de Microscopía Electrónica of the Instituto Nacional de Salud Pública de México for the technical support in the electron microscopy experiments and we thank Lic. Susana Russo of Departamento de Rabia y Pequeños Animales, Coordinación de Virología (DILAB-SENASA), for the help with the NIH potency test.

References

[1] Roldão A, Mellado MCM, Castilho LR, Carrondo MJT, Alves PM. Virus-like particles in vaccine development. Expert Rev Vaccines 2010;9(10):1149–76.
[2] Rodríguez-Limas WA, Sekar K, Tyo K. Virus-like particles: the future of microbial factories and cell-free systems as platforms for vaccine development. Curr Opin Biotechnol 2013;24:1089–93.
[3] Noad R, Roy P. Virus-like particles as immunogens. Trends Microbiol 2003;11(9):438–44.
[4] Grgacic E, Anderson DA. Virus-like particles: passport for immune recognition. Method 2006;40:60–5.
[5] Ludwig C, Wagner R. Virus-like particles—universal molecular toolboxes. Curr Opin Biotechnol 2007;18:537–45.
[6] Meeusen E, Walker J, Peters A, Pastoret P, Jungersen G. Curr Stat of Vet Vaccines. Clin Microbiol Rev 2007;20(3):489–510.
[7] Brun A, Bárcena J, Blanco E, Borrego B, Dory D, Escribano JM, et al. Current strategies for subunit and genetic viral veterinary vaccine development. Virus Res 2011;157:1–12.
[8] Crisci E, Bárcena J, Montoya M. Virus-like particles: the new frontier of vaccines for animal viral infections. Vet Immunol Immunopathol 2012;148:211–25.
[9] Liu F, Ge S, Li L, Wu X, Liu Z, Wang Z. Virus-like particles: potential veterinary vaccine immunogens. Res Vet Vaccine 2011;93(2):553–9.
[10] Liu F, Wu X, Li L, Ge S, Liu Z, Wang Z. Virus-like particles: promising platforms with characteristics of DIVA for veterinary vaccine design. Comp Immunol Microbiol Infect Dis 2013;36(4):343–52.
[11] World Health Organization. Rabies vaccines WHO position paper. Wkly Epidemiol Rec 2007;49:425–36.
[12] Dodet B, Durrheim DN, Rees H. Rabies: underused vaccines, unnecessary deaths. Vaccine 2014;32:2017–9.
[13] Bourhy H, Dautry-Varsat A, Hotez PJ, Salomon J. Rabies, still neglected after 125 years of vaccination. PLoS Negl Trop Dis 4(11):e839.
[14] Meslin FX, Briggs DJ. Eliminating canine rabies, the principal source of human infection: what will it take? Antiviral Res 2013;98:291–6.
[15] Shwiff S, Anderson A, Hampson K. Potential economic benefits of eliminating canine rabies. Antiviral Res 2013;98(2):352–6.
[16] Sokol F, Stancek D, Koprowski H. Structural proteins of rabies virus. J Virol 1971;7(5):241–9.
[17] Jackson AC. Rabies. In: Aminoff M, Boller F, Swaab D, editors. Handb Clin Neurol, 123. Amsterdam: Elsevier; 2014. p. 601–18.
[18] Cho S, Narahara H, Mifune K, Kawai A, Murine. T cell clones directed to rabies virus: isolation and some of their properties. J Gen Virol 1987;68: 115–23.
[19] Celis E, Ou D, Dietzschold B, Koprowski H. Recognition of rabies and rabies-related viruses by T cells derived from human vaccine recipients. J Virol 1988;62(9):3128–34.
[20] Gaudin Y, Ruigrok RW, Tuffereau C, Knossow M, Flamand A. Rabies virus glycoprotein is a trimer. Virology 1992;187(2):627–32.
[21] Okumura A, Harty RN. Rabies virus assembly and budding. Adv Virus Res 2011;79:23–32.
[22] Ertl HC. Novel vaccines to human rabies. PLoS Negl Trop Dis 2009;3(9):e515.
[23] Hicks DJ, Fooks AR, Johnson N. Developments in rabies vaccines. Clin Exp Immunol 2012;169:199–204.
[24] Patiño SF, Astray R, Pereira CA, Suazo TCA, Zucatelli R, Mendoça RZ, Jorge SAC. Transient expression of rabies virus glycoprotein (RVGP) in *Drosophila melanogaster* Schneider 2 (S2) cells. J Biotechnol 2014 (In press).
[25] Burger SR, Remaley AT, Danley JM, Moore J, Muschel RJ, Wunner WH, et al. Stable expression of rabies virus glycoprotein in Chinese hamster ovary cells. J Gen Virol 1991;72:359–67.
[26] Tomar N, Chandra R, Kumar R, Tiwari AK, Kumar A. Expression of rabies virus glycoprotein gene into eukaryotic system and determination of potential T-cell epitopes. Indian J Exp Biol 2011;49:594–9.
[27] Tiwari S, Roy S, Tuli R, Mishra DK, Singh A, Singh PK. High level expression of a functionally active cholera toxin B: rabies glycoprotein fusion protein in tobacco seeds. Plant Cell Rep 2009;28:1827–36.
[28] Koraka P, Bosck B, Cox M, Chubet R, van Amerongen G, Bengtsson LK, et al. A recombinant rabies vaccine expressing the trimeric form of the glycoprotein confers enhanced immunogenicity and protection in outbred mice. Vaccine 2014 (In press).
[29] Shen CF, Lanthier S, Jacob D, Montes J, Beath A, Beresford A, et al. Process optimization and scale-up for production of rabies vaccine live adenovirus vector (AdRG1.3). Vaccine 2012;30(2):300–6.
[30] Tan Y, Liang H, Chen A, Guo X. Co-expression of double or triple copies of the rabies virus glycoprotein gene using a 'self-cleaving' 2A peptide-based replication-defective human adenovirus serotype 5 vector. Biologicals 2010;38:586–93.
[31] Rim Benmaamara R, Astray RM, Wagner R, Pereira CA. High-level expression of rabies virus glycoprotein with the RNA-based Semliki Forest Virus expression vector. J Biotechnol 2009;139:283–90.
[32] Weyera J, Rupprecht CE, Nel LH. Poxvirus-vectored vaccines for rabies—a review. Vaccine 2009;27:7198–201.
[33] Saxena S, Sonwane AA, Dahiya SS, Patel CL, Saini M, Rai A, et al. Induction of immune responses and protection in mice against rabies using a self-replicating RNA vaccine encoding rabies virus glycoprotein. Vet Microbiol 2009;136(1–2):36–44.
[34] Kaur M, Rai A, Bhatnagar R. Rabies DNA vaccine: no impact of MHC class I and class II targeting sequences on immune response and protection against lethal challenge. Vaccine 2009;27:2128–37.
[35] Fontana D, Kratje R, Etcheverrigaray M, Prieto C. Rabies virus-like particles expressed in HEK293 cells. Vaccine 2014;32:2799–804.
[36] Berthelot MPE. Violet d'aniline. Repert Chim Appl 1859;1:284.
[37] Wilbur LA, Aubert MFA. The NIH test for potency. In: Meslin FX, Kaplan MM, Koprowsky H, editors. Laboratory techniques in rabies. Geneva, Switzerland: WHO; 1996. p. 360–8.
[38] Reculard P. Cell culture vaccines for veterinary use. In: Meslin FX, Kaplan MM, Koprowsky H, editors. Laboratory techniques in rabies. Geneva, Switzerland: WHO; 1996. p. 314–23.
[39] Wright E, Temperton NJ, Marston D, McElhinney LM, Fooks AR, Weiss RA. Investigating antibody neutralization of lyssaviruses using lentiviral pseudotypes: a cross-species comparison. J Gen Virol 2008;89:2204–13.
[40] Prieto C, Fontana D, Etcheverrigaray M, Kratje R. A strategy to obtain recombinant cell lines with high expression levels. Lentiviral vector-mediated transgenesis. BMC Proc 2011;5(8):7.
[41] Dull T, Zufferey R, Kelly M, Mandel RJ, Nguyen M, Trono D, et al. A third-generation lentivirus vector with a conditional packaging system. J Virol 1998;72:8463–71.
[42] Naldini L, Blomer U, Gallay P, Ory D, Mulligan R, Gage FH, et al. In vivo gene delivery and stable transduction of nondividing cells by a lentiviral vector. Science 1996;272:263–7.

[43] Raghunandan R. Virus-like particles: innate immune stimulators. Expert Rev Vaccines 2011;10(4):409–11.
[44] Moore SM, Hanlon CA. Rabies-specific antibodies: measuring surrogates of protection against a fatal disease. PLoS Negl Trop Dis 2010;4(3):e595.
[45] World Health Organization. WHO expert consultation on rabies. Second report. World Health Organ Tech Rep Ser; 2013. p. 982.
[46] Briggs D. The role of vaccination in rabies prevention. Curr Opin Virol 2012;2:309–14.
[47] Braun M, Jandus C, Maurer P, Hammann-Haenni A, Schwarz K, Bachmann MF, et al. Virus-like particles induce robust T helper immune responses. Eur J Immunol 2012;42(2):330–4.
[48] Wunderli PS, Shaddock JH, Schmid DS, Miller TJ, Baer GM. The protective role of humoral neutralizing antibody in the NIH potency test for rabies vaccines. Vaccine 1991;9(9):638–42.
[49] Dietzschold B. Antibody-mediated clearance of viruses from the mammalian central nervous system. Trends Microbiol 1993;1:63–6.