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

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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%

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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

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