A simplified roller bottle platform for the production of a new generation VLPs rabies vaccine for veterinary applications

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ABSTRACT

Rabies is a neglected disease with an estimated annual mortality of 55,000 human deaths, affecting mainly low-income countries. Over 95% of these cases result from virus transmission through the bite of infected dogs and for this reason there is a real need for a cheap and effective rabies veterinary vaccine to be used in mass vaccination campaigns. In this work, we describe the establishment of a simple platform for the production of a virus-like particles based rabies vaccine using mammalian cells and roller bottles as culture system. Adherent cells were cultured during more than 15 days and VLPs were continuously produced and secreted to the culture supernatant. Immunogenicity and protective efficacy of VLPs were tested through rabies virus neutralizing antibody test and NIH potency test. These viral particles induced high titer of long lasting neutralizing antibodies and protected mice against active virus challenge. Therefore, this development represents a promising platform for the production of a new generation and virus-free rabies vaccine candidate for veterinary applications.

1. Introduction

Although effective rabies vaccines have been available since 1885 [1], human deaths from rabies are still an important health issue worldwide [2]. As dog bites are the main cause of rabies transmission to human beings, the priority is to reduce the burden of human rabies by controlling dog rabies through diverse methodologies where stray dogs care and mass vaccination campaigns are the central and more cost-effective activities [3–5].

In the case of rabies the "One Health" approach takes central importance to achieve the WHO, the World Organization for Animal Health (OIE) and the Food and Agriculture Organization of the United Nation (FAO) goal to eliminate dog-transmitted human rabies in endemic countries by 2030 [6]. To fulfill this goal, 70% of the dogs have to be vaccinated during at least 7 consecutive years, and therefore the demand of canine rabies vaccine will be increased. A study estimates that if the production remains at the current level there will be a cumulative shortage of about 7.5 billion doses in order to meet the demand to achieve dog rabies elimination by 2030 [6]. This scenario shows the need of having more and cheaper sources of veterinary vaccines, mostly for the countries were dog rabies is endemic.

In the last decades many developments of recombinant rabies vaccine candidates have been published but, although they demonstrate the induction of humoral immune responses, some of those articles do not inform if the vaccine candidates are able to confer protection against virus challenge [1,7]. Furthermore, those recombinant antigens having an effective protective capacity in the proof of concept were not assessed in larger production scales. Thus, veterinary vaccines that are currently available in the market continue being the classical inactivated vaccines containing adjuvant, in most cases aluminium hydroxide, in its formulation.

Previously, our group developed an adherent HEK293 cell clone (adhP2E5) that continuously express immunogenic rabies virus-like particles (RV-VLPs) to the culture supernatant [8,9]. The RV-VLPs were completely characterized and we showed that they are circular enveloped viral particles containing the rabies virus glycoprotein (G) in the surface. In this work we studied the use of these stable recombinant mammalian cells for the establishment of a method for the production of RV-VLPs using roller bottles, as a simple method for the production of the recombinant rabies vaccine. This platform is fully applicable to the already available industrial facilities, avoids the manipulation of active rabies virus and the use of any adjuvant in the formulation,

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turning cheaper and safer the production of this new generation rabies vaccine for the veterinary market.

2. Materials and methods

2.1. Cells and antibodies

HEK-293 cells were cultured in Dubelcco's modified Eagle medium (DMEM, Gibco) supplemented with 10% fetal calf serum (FCS, Gibco) at $37\,°C$ with 5% CO_2.

Monoclonal antibodies and polyclonal sera, used to detect the rabies virus glycoprotein, were produced in our laboratory. Goat anti-mouse AlexaFluor 488® was purchased from Invitrogen™ (Thermo), polyclonal goat anti-mouse immunoglobulins/HRP were purchased from DAKO (Agilent) and the HRP-streptavidin complex was purchased from Sigma–Aldrich (Merk). The inactivated rabies virus vaccine used for the immunization protocol is the commercially available veterinary vaccine Rabisin® (Merial).

2.2. Confocal microscopy

Cells grown on cover-slips were washed twice in PBS and fixed by incubation with 4% paraformaldehyde in PBS for 30 min at room temperature. Cells were then incubated for 30 min at room temperature with a monoclonal antibody against G glycoprotein diluted 1:1,000 in PBS, washed three times in PBS and incubated with a goat anti-mouse AlexaFluor 488® antibody diluted 1:1,000 in PBS. Thereafter, cell nuclei were counterstained with TO-PRO™-3 Iodide (Thermo) for 30 min. Cells were washed three times in PBS and the slides were mounted with glycerol for confocal microscopy analysis and examined with a Leica–TCS–SP8 confocal microscope (Leica).

2.3. RV-VLPs production

To produce RV-VLPs we used the adhP2E5 clone that was previously obtained from the original HEK-G cell line [9]. Briefly, the RV-VLPs expressing cell line 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. 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} [8].

In this work, adhP2E5 cells were cultured in 175 cm^2 T flasks (Greiner Bio-One) and harvested at exponential growth phase. 850 cm^2 roller bottles (Cellmaster™, Greiner Bio-One) were seeded with a 1.5×10^5 cells. ml^{-1} suspension in DMEM supplemented with 10% FCS, reaching a final volume of 150 ml. Then, they were cultured using a roller bottle culture system (Wheaton) at 0.2 rpm at $37\,°C$ in 5% CO_2 atmosphere. Medium exchange was performed every 48 h during the first 13 days and every 24 h during the last 5 days. Culture supernatants containing RV-VLPs were pooled, clarified by centrifugation at 200 x g for 10 min and then filtered thought a 0.6 μm and 0.2 μm filtration train.

2.4. RV-VLPs purification and characterization

The clarified supernatant was layered over a 30% sucrose cushion and centrifuged at $65,000 \times g$ for 3 h at $4\,°C$ (Beckman JA.30 rotor, Beckman Coulter). 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) and centrifuged at $100,000 \times g$ for 4 h at $4\,°C$ (Beckman JS24.15 rotor, Beckman Coulter). The banded VLPs were collected and the buffer was

exchanged using 100,000 MWCO Amicon® Ultra centrifugal units (Millipore) and RV- VLPs stabilization buffer.

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).

2.5. ELISA sandwich

The sensitization step was as follows: 96-well micro-plates (Greiner Bio-One) were coated by adding in each well 100 μl of a 1:2,000 dilution of rabbit 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) 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 cell culture supernatants were prepared (in PBS, 0.05% Tween-20, 0.2% skim milk), distributed (100 μl per well) and incubated for 1 h at $37°C$. After that, plates were incubated for 1 h with a dilution of a biotin-conjugated rabbit polyclonal antibody (diluted 1:2,000 in PBS, 0.05% Tween-20, 0.2% skim milk) and followed by 1 h incubation with HRP-streptavidin complex (Sigma–Aldrich) (diluted 1:15,000 in PBS, 0.05% Tween-20, 0.2% skim milk). Six washes were performed between each step and the reaction was revealed by adding 100 μl per well of a chromogenic substrate solution (0.5 mg/ml o-phenylenedia-mine (Sigma–Aldrich), 0.5 μl $^{-1}$ H_2O_2 30 vol., 50 mM citrate-phosphate buffer, pH 5.3) and stopped by adding 50 μl of a 0.5 M sulphuric acid solution. The optical density was measured at 492 nm in a spectrophotometer (Labsystems Multiskan®).

The World Health Organization 6th International Standard for Rabies Vaccine (NIBSC) 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 ELISA Units (EU) ml^{-1} of glycoprotein rabies virus antigen content.

3. Mice immunization and serology

3.1. Immunization plan

Female 4–5 week old BALB/c mice were intraperitoneally injected with roller produced RV-VLPs preparations without adjuvant addition (Group 1, n = 6), and boosted on day 7-post primary immunization. Positive control group was immunized with a commercially purchased inactivated rabies virus vaccine (Group 2, n = 7), and boosted on day 7-post primary immunization as well. For measurement of humoral response parameters blood samples were collected on days 17, 47, 147 and 210. Pre-immunized reference sera were collected prior injection.

3.2. Total specific antibody titer determination (indirect ELISA)

96-well micro-plates (Greiner Bio-One) 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 overnight 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 per 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 1:2,000 in PBS, 0.05% Tween-20, 0.2% skim milk). Six washes were performed between each step and the reaction was revealed by adding 100 μl per well of a chromogenic substrate solution (0.5 mg/ml o-phenylenediamine (Sigma-Aldrich), 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 of 0.5 M sulphuric acid solution and the optical density was measured at 492 nm in a spectrophotometer (Labsystems Multiskan®). OD 492 nm values exceeding the mean + 2 S.D. of negative controls (basal mice sera) were considered as positive.

3.3. Rabies virus neutralizing antibody (RVNA) determination

To analyse 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, as was previously described [8]. Briefly, lentiviral particles were produced by simultaneous cotransfection of HEK293 cells with four plasmids using the lipid reagent Lipofectamine 2000 (Invitrogen™, Thermo): the packaging construct (pMDLg/pRRE), the Rev-expressing construct (pRSV- Rev) (Addgene, Plasmid numbers #12251, #12253) [10], the transfer vector pLV-PLK-GFP [11] and 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, filtrated through 0.45 μm and frozen at −80°C prior to use. For the RVNA measurement test, serial 2-fold dilutions of sera in 50 μl of DMEM 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, 3.5×10^3 HEK293 cells in a final volume of 50 μl were added. Plates were incubated for 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) as standard. This reference preparation contains 30 IU ml^{-1} human anti-rabies immunoglobulin and is recommended for RVNA measurement.

3.4. Rabies vaccine NIH potency test

The NIH potency test (Wilbur and Aubert, 2016) was performed in order to analyse the potency of VLPs samples as follows: CF-1 mice were 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 the 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}).

4. Results

4.1. Rabies G protein expression analysis by confocal microscopy

The adhP2E5 clone [8] was thawed from the working cell bank stored in liquid nitrogen and analysed by confocal microscopy in order to confirm the correct subcellular localization of the G protein (Fig. 1). As shown in Fig. 1, G protein was specifically detected in the external membrane of the producer cells. This analysis is important due to the fact that in the RVVLPs structure the G protein is anchored in the membrane and exposed in the particle surface [8].

4.2. RV-VLPs characterization

RV-VLPs produced by adhP2E5 were purified by density gradient ultracentrifugation and characterized by transmission electron microscopy (TEM) (Fig. 2). Only one band in discontinuous iodixanol gradient was observed and enveloped round shaped particles were observed in TEM assays.

4.3. RV-VLPs production using roller bottles

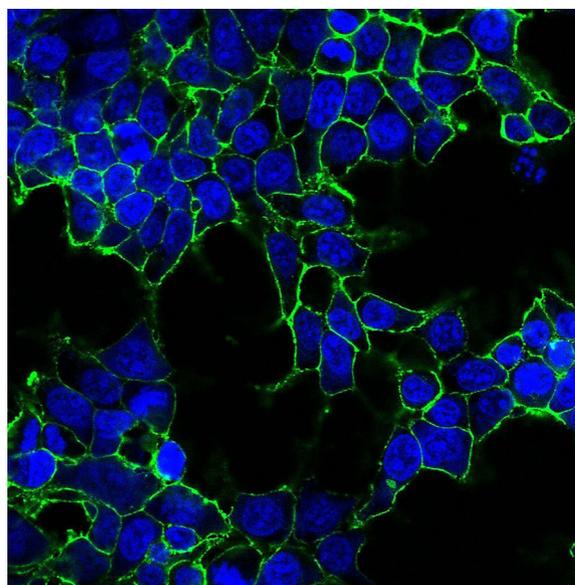To investigate the feasibility of using roller bottles as a method for

Fig. 1. Confocal microscopy of adhP2E5 clone. Cells were fixed, incubated with anti-G protein monoclonal antibody and then incubated with goat anti-mouse AlexaFluor 488® conjugated antibody. Nuclei were stained with TO-PRO™-3 Iodide. Merge image is shown.

the production of RV-VLPs we seeded 850 cm^2 roller bottles with an exponential growth adhP2E5 cell culture. Medium exchange was performed every 48 h during the first 13 days and every 24 h during the last 5 days. RV-VLPs were continuously produced and secreted to the culture supernatant (Fig. 3). We obtained a total harvest of 2.5 L per bottle that was pooled and analysed by sandwich ELISA, presenting a glycoprotein content of 19 EU ml^{-1}.

4.4. RV-VLPs immunogenicity analysis

In order to analyse the immunogenic characteristics of the RV-VLPs produced in the roller bottles, we injected mice with the VLPs present in the clarified pool harvest, without adjuvant addition or any concentration step. In a control group, we immunized the animals with a commercial rabies veterinary vaccine, based on inactivated rabies virus adsorbed on aluminium hydroxide. 10 days after the second dose mice were bled and the recovered sera analysed by indirect ELISA in order to obtain total specific antibody titers. The results showed that the RV-VLPs produced in roller bottles were able to induce a specific immune response against rabies and that the humoral immune response triggered was similar to the induced by the commercial vaccine, without significant differences between groups (Fig. 4A). Besides, the RV-VLPs induced rabies virus neutralizing antibodies (RVNA) with a titer significantly higher than the one obtained using the inactivated virus vaccine (Fig. 4B). This result demonstrate that this vaccine candidate could be a better antigen to produce RVNA, possibly due to that during the production of RV-VLPs there is no need to include inactivation processes like UV light treatment or chemical compounds like beta-propiolactone. It has been described that inactivation steps reduce the antigenicity of some viral proteins [12–15], normally because the conformational structure is affected. As neutralizing antibodies recognize external viral proteins, as is the case of the rabies virus glycoprotein which is organized as a trimer on the virion surface, if that structure is altered the induction of RVNA could be reduced. Anyway, the total specific antibodies titer shows similar results.

Further, the titer of RVNA was studied over time (Fig. 5), demonstrating that RV-VLPs produced in adherent condition using roller bottles are able to induce a long-lasting antibody response. This is particularly important for rabies because a minimum titer of 0.5 IU
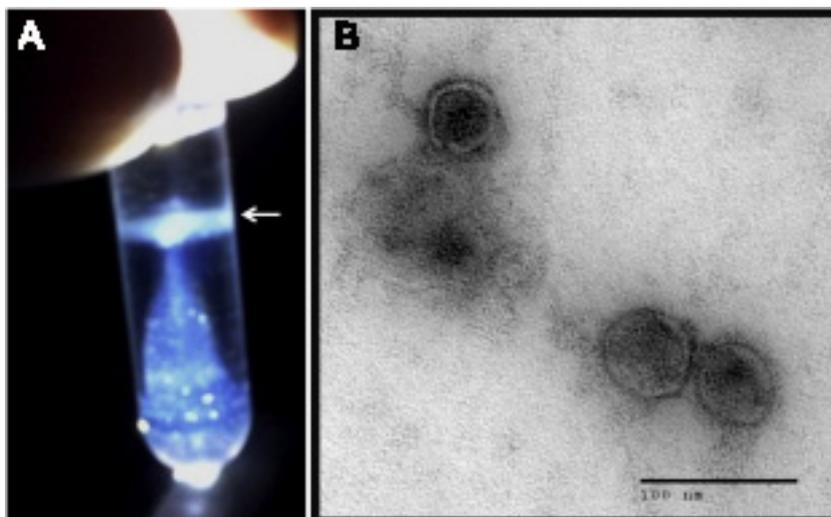
Fig. 2. RV-VLPs characterization. A. VLPs band obtained in the density gradient ultracentrifugation. B. Negative staining TEM of the purified RV-VLPs.

ml^{-1} is needed to demonstrate protection or a correct vaccination [16]. The immune response triggered with RV-VLPs maintained high titers for more than 200 days after vaccination.

4.5. NIH potency test for rabies vaccine

Finally, the VLPs harvest produced in roller bottles, without the addition of adjuvant or any concentration step, was analysed by the NIH potency test the gold-standard assay to measure the potency of rabies vaccine. We obtained a potency of 1 IU/ml, demonstrating that this recombinant vaccine candidate is not only able to induce a potent antibody response but also to induce the protection against a challenge with rabies virus.

5. Discussion

In the present work we evaluated the possibility of using an adherent mammalian cell line stably expressing highly immunogenic RV-VLPs in a roller bottles, in order to show that this technology could be applied in the rabies veterinary vaccine production industry.

Rabies vaccine has a central role in the prevention and treatment of the disease. Both pre and post-exposure prophylaxis in humans are based on the administration of the vaccine. On the other hand, vaccination of pets and stray animals through mass vaccination campaigns is the chosen strategy of the international health authorities to eliminate dog-transmitted human rabies [6]. The most affected countries by human rabies are placed in Asia and Africa, where more than 97% of the cases are related with dog bites, whereas in some countries of Latin America and the Caribbean dog rabies is still being endemic.

In some countries nerve tissue vaccines remain in production although its replacement is highly recommended by the WHO [17]. Moreover, in the production of cell culture-based vaccines two differential stages have to be performed. The first one involves the cell growing phase and no production is achieved. Once the desired cell concentration is obtained the culture is infected and the productive phase starts. Thus, only half of the overall process is indeed productive, so the obtained doses have to be related with the entire amount of medium that was used during all the batch culture, including the cell expansion stage. Conversely, in the case of the production of RV-VLPs using our the stable HEK293 clone [9], the entire culture time is productive so all the harvest contains VLPs, even in the first days after the culture seed (Fig. 3). This feature gives to this platform an important advantage over the classical virus production due to the fact that the cell expansion phase is not needed and there is no culture medium
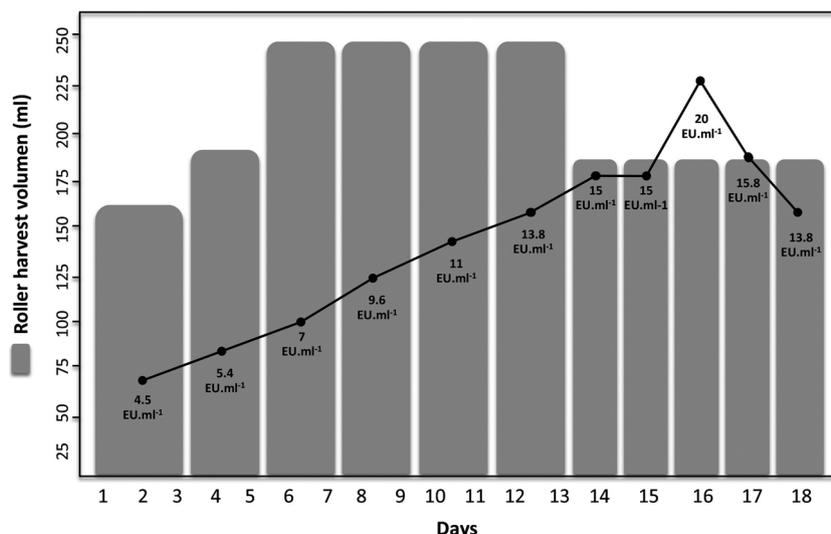

Fig. 3. Production of RV-VLPs in a roller bottle platform. Cells were seed and incubated during 18 days. Supernatant was harvest and the medium exchanged every 48 h during the first 13 days and every 24 h during the last 5 days. Each harvest was analysed by ELISA sandwich and the RV-VLPs concentration was measured.
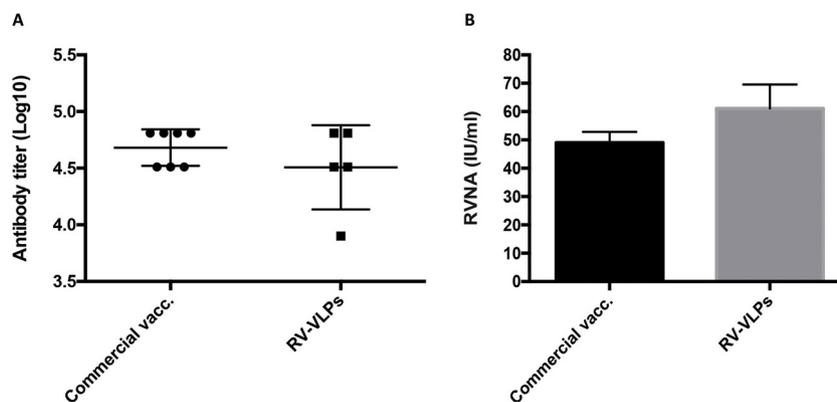
Fig. 4. Humoral immune response triggered by RV-VLPs in mice. 10 days after second dose sera of animals was analysed. A. Total specific antibody titer was measure by indirect ELISA. B. Rabies virus neutralizing antibodies were quantified by pseudotyped lentivirus assays in vitro. Results are expressed as mean ± SEM.
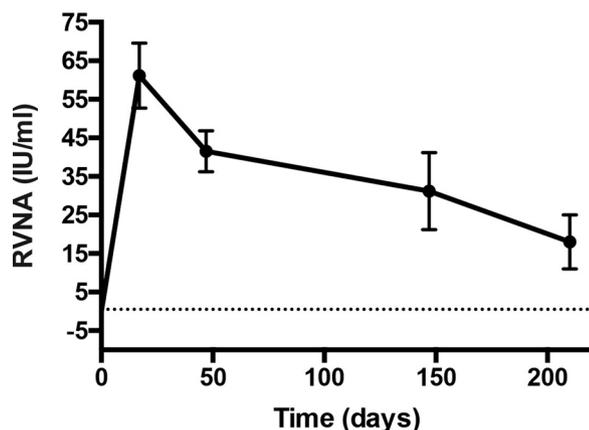

Fig. 5. Rabies virus neutralizing antibodies over time. Sera from vaccinated mice (n = 6) were obtained at days 17, 47, 147 and 210, and analysed by pseudotyped lentivirus assay. Dotted line indicate a titer of 0,5 IU ml^{-1}.

consumption without production.

Besides, we propose the use of the roller bottle method as it is one of the best-established static systems for the production of vaccines. Due to the simplicity and robustness of this system, some vaccines manufacturing processes still rely on this culture system [18], even for rabies. The possibility of changing the culture platform based on inactivated viral vaccines produced in BHK-21 by the use of this RV-VLPs production platform could be a simple solution for companies that are already producing the veterinary vaccine in roller bottles and are looking forward to reduce costs and moreover, as there is no need of manipulating the rabies virus, making the process safer and avoiding laborious work to maintain and validate viral seeds stocks. On the other hand, there is no need of an inactivation step and therefore no risk of incomplete inactivation or any residual viral activity. Besides, as our results show, RV-VLPs injected without adjuvant addition induce an excellent and long-lasting antibody response with potency values similar to the commercial veterinary vaccine that is formulated with aluminium hydroxide. The possibility is another way to reduce costs and make the process simpler. It is known that manipulation of roller bottles carries relatively high sterility risk, when performing medium exchange and harvest but, on the other hand, in comparison to bioreactors, static systems require a lower operator skill level and lower investment costs, making their implementation at large scale still an affordable and competitive option for manufacturers with reduced facility complexity [19,18].

6. Conclusions

In conclusion, we have demonstrated that RV-VLPs can be stably produced in roller bottles with an interesting overall productivity due to the fact that during all the culture process the VLPs are being produced and secreted to the supernatant. We have shown that these RV-VLPs are able to induce a specific antibody response when injected without adjuvant with a, slightly higher RVNA titer in comparison with the induced by the commercial rabies vaccine. Further, this RVNA response was maintained over time with titers above the limit of 0.5 IU ml^{-1}, which indicates an adequate vaccination [16]. Finally, the NIH potency test showed the required value for veterinary rabies vaccines, demonstrating that the RV-VLPs produced in this cell platform are able to confer protection against rabies virus challenge.

Conflict of interest

The authors declare that there is no conflict of interest regarding the publication of this article.

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