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Purification of rabies virus produced in Vero cells grown in serum free medium

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ABSTRACT

Rabies is a viral zoonosis caused by negative-stranded RNA viruses of the *Lyssavirus* genus. It can affect all mammals including humans. Dogs are the main source of human rabies deaths, contributing up to 99% of all rabies transmissions to humans. Vaccination against rabies is still the sole efficient way to fight against the disease.

Cell culture vaccines are recommended by World Health Organization (WHO) for pre and post exposure prophylaxis; among them Vero cell rabies vaccines which are used worldwide. In this work we studied the purification of inactivated rabies virus produced in Vero cells grown in animal component free conditions, using different methods. Cells were grown in VP-SFM medium in stirred bioreactor, then infected at an MOI of 0.05 with the LP2061 rabies virus strain. Collected harvests were purified by zonal centrifugation, and by chromatography supports, namely the Capto Core 700 and the monolithic CIM-QA column. Generated data were compared in terms of residual DNA level, host cell proteins (HCP) level and the overall recovery yield.

Rabies virus purification using the monolithic column resulted in the highest antigen recovery yield, equal to 94%. Capto Core 700 showed a lower yield, about 84%; whereas the purification yield by zonal centrifugation was equal to 60%. In terms of host cell residual DNA removal, zonal centrifugation was the most efficient method; the removal yield was equal to 88.5%; elimination of host cell DNA was slightly lower when using the monolithic CIM-QA (equal to 73%). Whereas Capto Core 700 showed the lowest level (49.2%). Host cell protein removal varied between 92.6% for the monolithic column and 78.6% for the zonal centrifugation. Capto Core 700 eliminated 86.5% of HCP.

1. Introduction

Rabies is a neglected viral disease; it is caused by viruses that belong to the Lyssavirus genus in the family Rhabdoviridae of the order Mononegavirales. It is a negative sense RNA enveloped virus. Virus genome encodes five proteins which are the nucleoprotein (N), phosphoprotein (P), the matrix protein (M), the glycoprotein (GP) and the RNApolymerase (L) [1,2]. Rabies virus glycoprotein is exposed at the surface of the virus as trimers; and is responsible of the synthesis of neutralizing antibodies providing protection against infection.

Rabies infection is 100% fatal if no vaccine and rabies virus immunoglobulins are administrated on time. Around 60 000 annual human deaths occurred globally; nevertheless the highest death toll is recorded in Asia and in Africa. Although dog- transmitted rabies is eradicated in North America and Western European countries, dog is still considered as the main vector and reservoir in developing countries. Children under 15 years are the main victims.

Efficacious and safe rabies vaccines for pre and post exposure treatment are available; cell culture vaccines and embryonated egg-based vaccines are recommended by World Health Organization for human use [3]. However these vaccines remain still not affordable for vulnerable populations living in endemic areas; the cost of the vaccine and the huge need worldwide are the main hurdles for an equitable and global use of human rabies vaccine.

In this respect a global strategic plan called "Zero by 2030" was launched in 2015 by WHO; the objective is to eliminate dog-mediated human rabies deaths by 2030 [4].

Vero cells are a continuous cell line established from the kidney of African Green Monkey. This cell line is now widely accepted by WHO and international regulatory agencies such as European Medicines Agency, for the manufacturing of human viral vaccines, among them rabies vaccine.

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Vero cell derived rabies vaccines are produced in China, India, Brazil, etc. to fulfill local and international needs.

In previous work we described the intensification of the upstream process to produce a rabies vaccine in Vero cells grown in serum containing medium on microcarriers and stirred bioreactor [5,6]. Cell growth and the virus replication steps were conducted using continuous culture mode. This process was further improved to eliminate animal derived products [7] to be in line with the international recommendations in terms of the use of animal derived products, for biopharmaceutical production. In our previous study, Vero cells were grown on Cytodex1 in VP-SFM medium; cells were infected by LP-2061 rabies virus strain. Multiple virus harvests were collected.

In the current study we report the purification of rabies virus harvests using continuous zonal centrifugation on sucrose gradient, and chromatography methods. Overall yields, Host cell proteins and residual host cell DNA levels were determined, and performances of the different methods were compared.

2. Material & methods

2.1. Cell line

Vero cells at passage 131, provided by the National Laboratory for Control of Biologicals (Tunis, Tunisia) and originally obtained from ATCC (CCL-81), were used in this study.

2.2. Virus strain

Louis Pasteur 2061 virus strain adapted to grow in Vero cells (LP 2061/Vero) was provided by Institut Pasteur (Paris, France).

2.3. Chemicals

All chemicals used in this study were supplied by Sigma (St. Louis, USA).

2.4. Microcarrier preparation

Cytodex1 microcarriers purchased from GE Healthcare (Uppsala, Sweden) were used in this work. Microcarriers were prepared according to the supplier instructions.

2.5. Cell culture in monolayer

Cells were cultivated in VP-SFM Medium (Invitrogen, MD, USA) at 37 °C and 5% CO_2.Cells were passaged twice per week, using Tryple Select which is a recombinant enzyme purchased from Gibco (Life Technologies, MD, USA).Cells were subcultivated at $3x10^5$ cells/mL.

2.6. Bioreactor cultures

Cultures were performed in a 7-L bioreactor (Sartorius Biobraun, Sartorius, Germany), containing 4 L as a working volume, equipped with a pitch blade impeller and a spin filter (pore size: 75 μm) fixed on the axis. During the cell proliferation step, the following conditions were maintained: pH 7.2 regulated by CO_2 sparging or addition of $NaHCO_3$ at 88 g/L, dissolved oxygen was regulated at 50% air saturation by continuous surface aeration. Temperature was maintained at 37 °C and the agitation rate at 90 rpm.

A batch culture was first started at 2-3 × 10^5 cells/mL using 3 g/L Cytodex 1 then recirculation was started after 2 days of culture as described by Trabelsi et al. [6]. For rabies virus production, pH was maintained at 7.4, pO_2 at 30% air-saturation and the temperature at 34 °C. After cell infection perfusion rate was maintained at 0.5 reactor volume/day. Samples were taken daily to determine the following parameters: cell density, cell viability, microcarriers load, virus titer, cell infection, glucose, glutamine, lactate and ammonia levels.

2.7. Virus clarification

Each single virus harvest was clarified by filtration at a flow rate of 20 mL/min through 8 μm white SCWP membrane filter (Millipore, Ireland) that has 158 cm^2 filtration area. Then the clarified harvest was inactivated with beta-propiolactone (Acros Organics, Geel, Belgium) supplied at 98% and diluted to a final dilution of 1/4000 (v/v). The beta-propiolactone was first pre diluted at 1/40 (v/v) in MilliQ sterile water then added to the clarified harvest and kept for 24 h under slow agitation at +4 °C. Thereafter, the inactivated harvest was transferred to a sterile vessel and stirred for 2 h at room temperature. Harvests were then pooled and filtered at 16 mL/min through 0.45 μm Durapore membrane filter (Millipore, Ireland); a total filtration area of 475 cm^2 was needed to insure the filtration of the pooled harvests.

2.8. Zonal centrifugation

Pooled and clarified viral harvests were purified by zonal centrifugation using the KII ultracentrifuge (Electro-Nucleonics, Inc. USA) equipped with a K-3 rotor. After sterilization with 2% (w/v) formaldehyde solution, washing with water and Phosphate Buffer Saline (PBS), 1 600 mL of a 60% sucrose solution were pumped into the rotor. The centrifuge was then accelerated to the operating speed of 35 000 rpm. The viral material was passed through the rotor at a flow rate of 5 L/h. After the completion of the product feed, the rotor was maintained at the operating speed for further 20 min for the binding step. The rotor was finally decelerated to rest and the reoriented gradient was pumped out through the bottom of the rotor at a flow rate of approximately 2 L/h. The gradient was fractionated into 50 mL aliquots.

2.9. Gradient shape

Gradient shape was determined by measuring manually the refractive index (RI) of each fraction with a refractometer from PZO (Warszawa, Poland).

2.10. Purification of rabies virus harvests using chromatography matrices

Harvests were first concentrated and buffer exchanged by Tangential flow filtration using cogent μscale System (Merck Millipore, MA, USA) at a flow rate of 27 mL/min equipped with Pellicon cassette Biomax XL (cut off 100 kDa) (Cat. N°. PXB100C50, Millipore, MA, USA), transmembrane pressure (TMP) was around 2.7 bar; the retentate was then purified by AKTÄ purifier system (GE Healthcare Life Sciences, Uppsala, Sweden) using different chromatography columns.

Monolithic chromatographic support CIMmultus QA-8 advanced composite column (BIA Separation, Ajdovščina, Slovenia) was first washed with 5 column volumes (CV) of Milli-Q water to eliminate traces of ethanol then equilibrated with 5 CV 20 mM Tris buffer, pH 7.5 (Table 1). Concentrated inactivated rabies virus, was passed through the column then eluted with 10 CV of 20 mM Tris, 1 M NaCl, pH 7.5. Collected fractions were analysed for antigen recovery.

The dynamic binding capacity of CIM-QA-8 column was determined using the AKTÄ purifier system. To obtain breakthrough

Table 1
Operating conditions for rabies virus harvests purification using chromatography methods.

Column	Equilibration buffer	Elution buffer
Capto Core 700 (Multimodal, octylamine, 1 mL)	20 mM Tris, 150 mM NaCl, pH 7.4	20 mM Tris, 1 M NaCl, pH 7.5
CIMmultus QA-8 mL Advanced Composite Column	20 mM Tris, pH 7.5	20 mM Tris, 1 M NaCl, pH 7.5

curves, increasing volumes of concentrated virus harvest was loaded into the column at a low rate of 16 mL/min. Flow through fractions were collected and rabies virus glycoprotein content was determined by ELISA, then the amount of adsorbed protein/mL of support was calculated.

Capto Core 700 Multimodal chromatography column (GE Healthcare Life Sciences, Uppsala, Sweden) was equilibrated with 10 CV (20 mM Tris, 150 mM NaCl, pH 7.4) (Table 1). Fraction of interest was collected in the flow through; the impurities were eluted using 30 CV (20 mMTris, 1 M NaCl, pH 7.5).

2.11. Analytical methods

2.11.1. Cell density and viability

Cells were counted using a hemacytometer. Cell viability was estimated via the Trypan blue exclusion method. Details are provided in Trabelsi et al. [5].

2.11.2. Rabies virus titration

Virus titer was determined according to a modified rapid fluorescence inhibition test (RFFIT) as detailed by Smith et al. [8], and expressed in Fluorescent Focus Units per ml (FFU/mL).

2.11.3. Substrates and metabolites analysis

Glucose concentration was quantified enzymatically using a glucose GOD-PAP kit (Biolabo SA, Maizy, France). Glutamine, ammonia and lactate were monitored by enzymatic assays, using specific kits from Megazyme (Wicklow, Ireland).

2.11.4. Monitoring of cell infection

Aliquots of 2 mL samples of rabies infected culture were first washed twice with PBS prior to resuspension in a minimum volume. Thereafter, 2 drops were deposited on a microscope slide and let to dry at room temperature in a laminar flow cabinet. Then, the slide was fixed with chilled 80% acetone and stained with fluorescein-labelled anti-rabies nucloeocapsid (Biorad, France).

2.11.5. SDS-PAGE analysis of viral proteins

Samples were analyzed by SDS-PAGE under reducing conditions using 10% polyacrylamide gel as described by Laemmli [9] and visualized by silver stain kit (Sigma, Merck, St Louis, USA).

2.11.6. Enzyme linked immunosorbent assay

An indirect enzyme-linked immunosorbent assay (ELISA) was performed to quantify rabies glycoprotein (GP).The presence of GP in samples was detected according to the following protocol. Briefly, 100 μL per well of either the sample or the standard (rabies virus GP) was incubated for 2 h at 37 °C. Saturation with 2% BSA (bovine serum albumin) and 0.2% Tween 20 was carried out for 2 h at room temperature, and then 100 μL of monoclonal antibody anti-glycoprotein TW1 (NIBSC, Hertfordshire, UK) diluted in 2% BSA and 0.2% Tween 20 was added to the wells and incubated for 1 hat 37 °C. Finally, 100 μL of anti-human coupled to peroxidase (Sigma Aldrich, USA) was added to the wells and incubated for 30 min at 37 °C. After the addition of tetramethylbenzidine (TMB), the reaction was stopped with 0.5 N H_2SO_4 to give a yellow colour; its intensity was measured at 450 nm then the GP concentration was calculated using a standard curve.

2.11.7. Residual host cell DNA level

The residual Vero DNA was quantified by qPCR using resDNA-SEQ quantitative VERO DNA kit (Applied Biosystems, Life Technology, Warrington, UK). Briefly, at first, the extraction of the host cell DNA was carried out according to a protocol pre-established by DNA Extraction kit (PrepSEQ Residual DNA Sample Preparation kit). To 100 μL of sample, we added 70 μL proteinase K, after brief vortexing the mixture was spin then incubated for 30 min at 56 °C. After cooling 360 μL of lysis solution was added to each tube. Then 30 μL of magnetic particles and 300 μL of binding solution were added. After homogenisation by inverting the tubes twice, the tubes were placed in a magnetic stand for 5 min, and the supernatant was discarded. Finally, after washing nucleic acids were eluted by 50 μL of the eluent buffer, collected in sterile tubes, and stored at 4 °C for a use within a week.

DNA Quantitation kit was used to quantify Vero cell DNA in the samples using the Applied Biosystems 7500 Fast instrument. A standard curve based on the material provided with the kit was also established. The results were analysed using AccuSEQ Real-Time PCR Detection software.

2.11.8. Host cell protein estimation

Host cell proteins were estimated using Vero Cell Host Cell Proteins kit (Cygnus Technologies, Southport, USA). 0.50 μL of the collected fractions containing Vero cell HCPs were mixed simultaneously with 100 μL of anti-Vero cell antibody (goat polyclonal, HRP) in microtiter strips coated with an affinity purified capture goat polyclonal anti-Vero cell antibody. The immunological reactions result in the formation of a sandwich complex of solid phase antibody-HCP-enzyme labelled. The microtiter strips were then washed to remove any unbound reactants. Thereafter the substrate, tetramethylbenzidine (TMB) was added. The amount of hydrolyzed substrate was read at 450 nm wavelength in a microtiter plate reader (Thermo Fisher Scientific, Vantaa, Finland). The concentration of Vero cell HCPs was estimated in ng/mL using a standard curve.

2.11.9. Total proteins level

The concentration of total proteins in the collected fractions was estimated using the Bradford Reagent (Sigma-Aldrich, Merck, St. Louis, USA). The method is based on the formation of a complex between 1.5 mL of the dye which is Brilliant Blue G, and 50 μL of sample. After 5 min of incubation at room temperature the absorbance was read at 595 nm. Then the amount of protein was calculated according the standard curve using a known concentration of bovine serum albumin protein.

2.11.10. Rabies vaccine potency test

The protective activity of experimental vaccines was determined according to the NIH test. Swiss mice were intraperitoneally immunized at day 0 and 7, and then intracerebrally challenged using CVS-R9 strain (obtained from the Rabies laboratory at Institut Pasteur de Tunis), as described by Wilbur et al. [10].

3. Results

3.1. Bioreactor culture of Vero cells in VP-SFM

Vero cells were grown in VP-SFM supplemented with 2 mM Glutamine, on 3 g/L Cytodex 1, in a 7-L stirred bioreactor. After a batch growth, recirculation was initiated at day 2. Data are dis-

played in Fig. 1; Vero cells exhibited an exponential cell growth; cells had continued to grow after the start of recirculation. The average specific growth rate was equal to 0.019 h^{-1}. Cell density level reached 3 × 10^6 cell/mL at day 5. Vero cells were infected at day 5 with LP-2061 rabies virus strain at an MOI of 0.05. After infection, the culture was perfused at 0.5 reactor volume/day with fresh VP-SFM medium supplemented with 2 mM glutamine. Cells had continue to grow after cell infection for as short period, then a progressive fall of cell density level was observed. At day 2 post infection, cell density level was equal to 3.88 × 10^6 cells/mL. Monitoring of cells infection by direct immunofluorescence, showed that the percentage of infected cells had increased continuously to reach 100% at day 5 post-infection. The culture was stopped when cell density had dramatically decreased. Rabies virus was secreted to the culture medium; the highest virus titer was obtained at day 10 post infection and was equal to 4.8 × 10^7 FFU/mL. Afterwards a slow decrease of the virus was noticed. Regarding substrates and metabolites profiles, a typical cell metabolism was observed. Fig. 1b showed an increase of lactate and ammonia levels as glucose and glutamine were consumed. At day 6, lactate and ammonia concentrations were equal to 9.1 mM and 2.9 mM, respectively. During cell infection, glucose consumption by the cells was decreased; similar pattern was also seen for glutamine. At the end of the culture, ammonia level was around 3.8 mM whereas glucose concentration was equal to 16 mM. Glutamine level was not limiting during the rabies virus production phase, it has reached 0.9 mM when the culture was stopped. At the end of the culture, 13 viral harvests were collected having a total volume of 26 L.

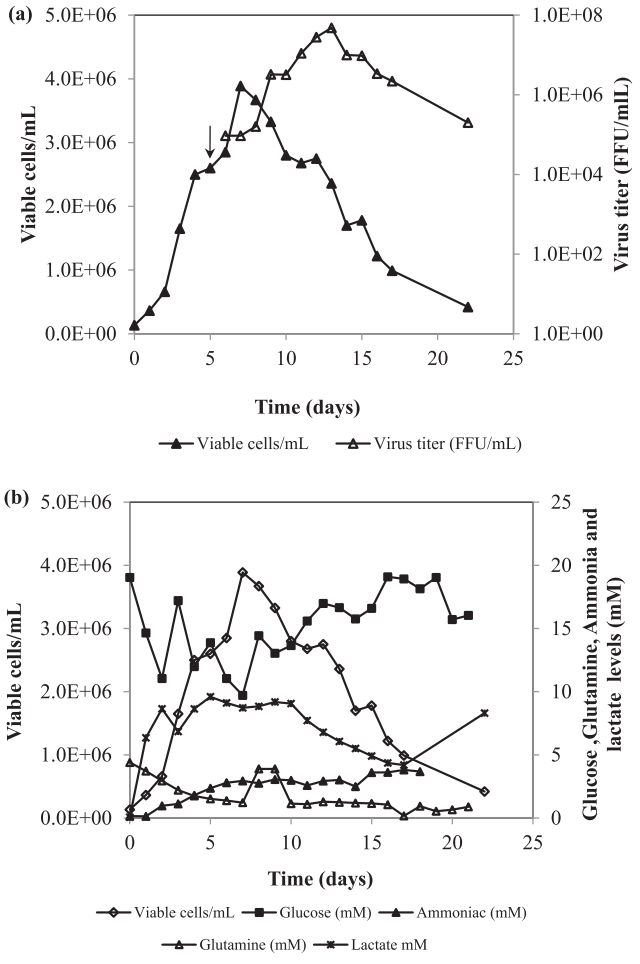

Fig. 1. Kinetics of rabies virus production in Vero cells grown in a 7-Lbioreactor on 3 g/L Cytodex1, and VP-SFM+2 mM. Recirculation culture was maintained from day 2 to day 5, then cell infection phase was conducted using perfusion mode; cells were perfused at 0.5 reactor volume/day. (↓) indicates cell infection. (a) Time course of cell density and virus titer during the culture, (b) Time course of glucose, glutamine, lactate and ammonia levels during the culture.

3.2. Zonal virus purification

Purification of clarified and inactivated rabies virus suspensions was performed on a 60% sucrose gradient using the ElectroNucleonics with K3-rotor continuous ultracentrifuge. The pooled harvests were fed to the rotor at a rate of 5 L/h. At the end of the purification, 60 fractions of 50 mL each were collected aseptically at a flow rate of 2 L/h. Collected fractions through zonal purification were then analyzed.

Data shown in Fig. 2 indicated that sucrose gradient decreased linearly from 59% to 9% (Fig. 2). Total protein levels were estimated using Bradford method, obtained results showed that total protein level increased to reach the highest value at fraction 29, which was 398 μg/mL then a continuous decrease was observed to stabilize at fraction 45 at a level of 68 μg/mL. The analysis of the same fractions by ELISA to estimate the rabies glycoprotein (GP) levels showed the same pattern than the total proteins. GP peak was located between fractions 20 and 36 at a percentage of sucrose comprised between 49% and 28%. The maximum GP level was obtained in fraction 29, and was equal to 253 μg/mL and at 37.5% sucrose.

To verify if this peak contains viral proteins, we analyzed these fractions by SDS-PAGE and performed a silver staining of the gels. The obtained results confirmed those obtained by ELISA. Fig. 3 shows the presence of several protein bands that correspond to the different rabies virus proteins. The first 200 kDa band should correspond to the polymerase (L), the second which is the most intense and having a molecular weight of 67 kDa corresponds probably to the glycoprotein (G). The other bands are probably the nucleoprotein N (57 kDa), the phosphoprotein M1 (38.5 kDa) and the matrix protein M2 (25 kDa). Note also that from fraction 23, the band having a molecular weight of 67 kDa becomes wider which suggests that the glycoprotein content is increasing. The fractions of interest (26–33) were pooled and checked for their potency according to the NIH test. They showed an activity of 61 IU/mL. Residual cellular DNA quantification by q-PCR was also carried out, and was equal to 31 pg/mL. This means a level of 1.28 pg/dose, assuming that one dose should have a potency of 2.5 IU at least. Concerning the concentrations of HCP in the fractions of interest, it was equal to 509 ng/mL which is equivalent

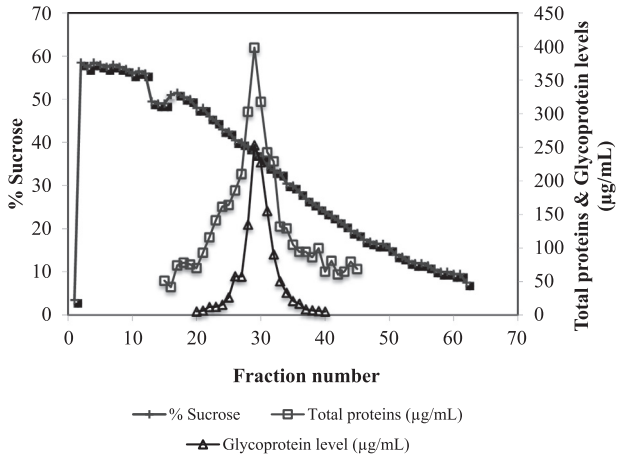

Fig. 2. Gradient profile, total proteins and glycoprotein levels of the fractions collected through zonal purification on sucrose gradient of rabies virus harvests.

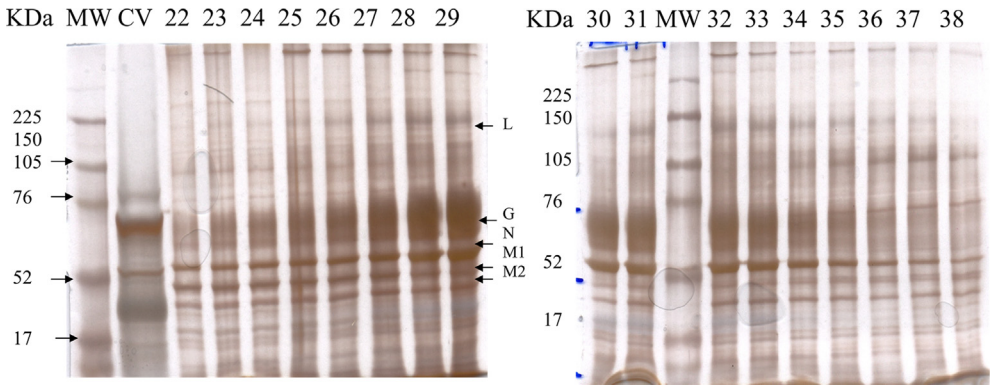

Fig. 3. SDS-PAGE analysis of the fractions collected through zonal purification. MW molecular weight marker. Lines 22-38 stand for zonal purified fractions. CV: commercial vaccine (Rabipur), G: Rabies virus glycoprotein.

to 20.9 ng/dose. The overall recovery yield of the antigen (GP level) obtained by zonal purification was around 60%.

3.3. Rabies virus purification by chromatography resins

We investigated the use of alternative methods to zonal centrifugation, to check if we could increase the overall purification yield of the inactivated rabies virus harvests produced in Vero cells. For this purpose, we used the AKTA purifier system and different chromatography columns which are Capto Core 700 column and the CIMmultus QA-8, an anion exchange advanced composite column.

300 mL of clarified and inactivated viral harvests were concentrated 6 fold by tangential flow ultrafiltration on Pellicon cassette Biomax XL with a cut-off 100 kDa, then purified on Capto Core 700 column as described in Materials & Methods. After purification, the flow through and 20 fractions of 1 mL were analyzed by SDS-PAGE and ELISA to determine glycoprotein content.

The results are indicated in Fig. 4, absorbance at 280 nm showed a major peak at fraction F3 related to the impurities coming from the loaded sample. The collected fractions were also analyzed by SDS-PAGE; intense bands were seen in F3 fraction; this correlates with absorbance at 280 nm level (Fig. 4a). Fig. 4b also indicates that viral proteins were mainly collected in the flow through fraction.

Glycoprotein quantification by ELISA indicates that the flow through fraction exhibited the highest glycoprotein level, and was equal to 13.3 μg/mL. The purification yield of the glycoprotein antigen was equal to 84%. Residual Vero cell DNA was equal to 7.25 pg/mL. In addition HCP level in the Flow through fraction was equal to 1784.4 ng/mL.

Similar approach was applied with the CIMmultus QA-8 column; this anion exchange column was chosen as a capture step based on the pI of the rabies virus glycoprotein which is around neutrality [11]. Dynamic binding capacity experiments were conducted; increasing volumes (from 10 mL to 190 mL) of concentrated and clarified viral harvest were loaded; flow through fractions were collected and their GP content was determined by ELISA. GP level in the flow through factions increased with the loaded volume of viral harvest. The column shows at least a capacity of 550 μg/mL of support.

300 mL of clarified, inactivated and 4.5-fold concentrated viral harvests were loaded on the CIMQA column at flow rate 16 mL/min. The column was first equilibrated with equilibration buffer (20 mM Tris, pH 7.5). The adsorbed virus was eluted with 20 mM Tris, 1 M NaCl, pH 7.5. 14 fractions of 8 mL each were collected during purification, then analyzed by SDS-PAGE, and glycoprotein level was estimated by ELISA.

Fig. 5a showed that monitoring of the absorbance at 280 nm of the fractions collected showed two peaks, the first one was significantly higher than the other peak. The analysis of these fractions by SDS-PAGE and silver staining allowed to visualize the specific bands of the rabies virus proteins from fraction F5–F8 (Fig. 5b). The highest peak of absorbance corresponds to the contaminant proteins (F3 and F4 fractions). The analysis of these samples by ELISA to determine GP contents follows the same profile than the SDS-PAGE analysis (Fig. 5c). The Highest glycoprotein level was equal to 308 μg/mL, and was seen for fraction 8.

Purification recovery yield was equal to 94% when considering all quantified fractions (from F5 to F8). Residual cellular DNA and HCP levels were determined in F8; they were respectively equal to 3.35 pg/mL and 3372.5 ng/mL in fraction 8.

4. Discussion

To achieve the goal of Zero human dog-mediated rabies deaths by 2030 as planned by WHO and its partners, it is of tremendous importance to encourage rabies vaccine manufacturing in low and middle-income countries, where the disease is prevailing. In addition, it is known that one of the major challenges to control and prevent rabies in endemic countries is the shortage of high quality vaccines [12]. In this context, and to set up a national human rabies vaccine manufacturing capability, we were focused during the last decade on process intensification of human rabies vaccine in Vero cells [5,6]. We demonstrated in previous studies that the use of continuous culture mode can allow a significant improvement of the number of vaccine doses produced per batch [6].

To enhance the quality of biopharmaceuticals including vaccines, it is nowadays highly encouraged to avoid the use of animal derived products such as fetal calf serum, trypsin, gelatin, etc. from vaccine production and formulation [13,14]. In this line, we had studied in the current study Vero cells growth on Cytodex1 microcarriers in 7-L bioreactor in the animal component free medium VP-SFM. Recirculation culture mode during the cell proliferation step allowed the achievement of $3x10^6$ cells/mL, at day 5 after the start of the culture. This performance is lower than the level that we had previously reached when the culture was performed in a 2-L bioreactor [7]. One explanation can be the design of the bioreactor, specifically the device of agitation which was different from the 2L bioreactor. Nevertheless, our data are higher than those reported by Mattos et al. [15] who reached 1.5×10^6 cells/mL, using 1.5 g/L Cytodex 1 and once microcarriers colonization was optimized. Thomassen and Bakker [13] obtained a cell density concentration of 5×10^6 cells/mL during Vero cells culture

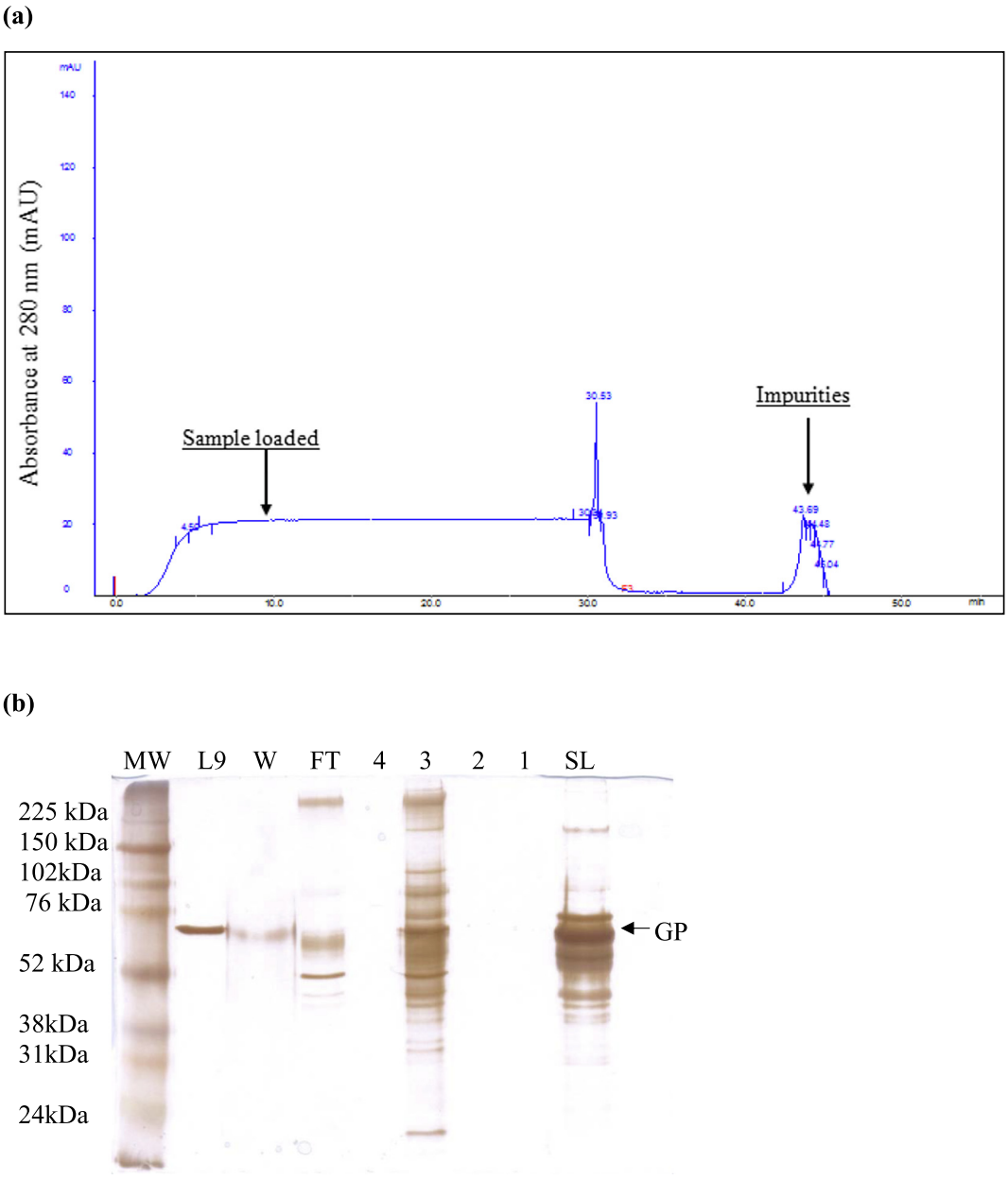

Fig. 4. (a) Viral harvests purified on Capto Core 700 column by AKTA purifier System. (b) Characterization of purified rabies virus. Protein samples were separated with polyacrylamide gel electrophoresis and visualized by silver staining. (MW) Molecular weight, (W): wash, (SL): Sample loaded, (FT): Flow through, (L9): positive control of rabies virus glycoprotein (GP), 1 to 4: fraction number.

in VP-SFM in a bench top bioreactor using 3 g/L Cytodex 1; however they used a high cell concentration at the inoculation (0.6×10^6 cells/mL).

With respect to rabies virus production phase, the use of perfusion allowed the harvest of 26 L of virus suspension; the average of viral titer was equal to 1.4×10^7 FFU/mL. The maximal virus titer was 5×10^7 FFU/mL; which is slightly lower than the data described in Rourou et al. [7]. This difference is probably due to the lower cell density concentration reached in the current work.

Viral harvests which were pooled, after clarification and inactivation, were purified by zonal centrifugation in a sucrose density gradient. The fractions of interest, 11 in total showing the highest of GP content as determined by ELISA, were pooled and checked for their antigenic activity according to the NIH potency test. They showed an activity of 61 UI/mL. The yield obtained was around 60%, this performance was similar to the data reported by Kumar et al. [16]. Nevertheless in our study the pooled fractions collected after zonal centrifugation showed a residual cell DNA level that meets the required standard of 100 pg/dose whereas in Kumar's study a second zonal centrifugation run was performed to meet the requirements of DNA content and residual serum protein. It is worth to specify that in our case, since the process is conducted under serum free conditions, the vaccine cannot be contaminated by serum-derived proteins.

Despite these acceptable performances, zonal gradient ultracentrifugation shows several limitations such as limited scalability and productivity; it is also a cumbersome method [17]. Therefore to improve the overall recovery yield and identify an alternative to the zonal gradient ultracentrifugation method, we had already assessed in a previous work several chromatography matrices (Sephacryl S200, Sephacryl S300, Sepharose 4FF, ...) for rabies virus harvests purification. Nevertheless, the yield obtained was

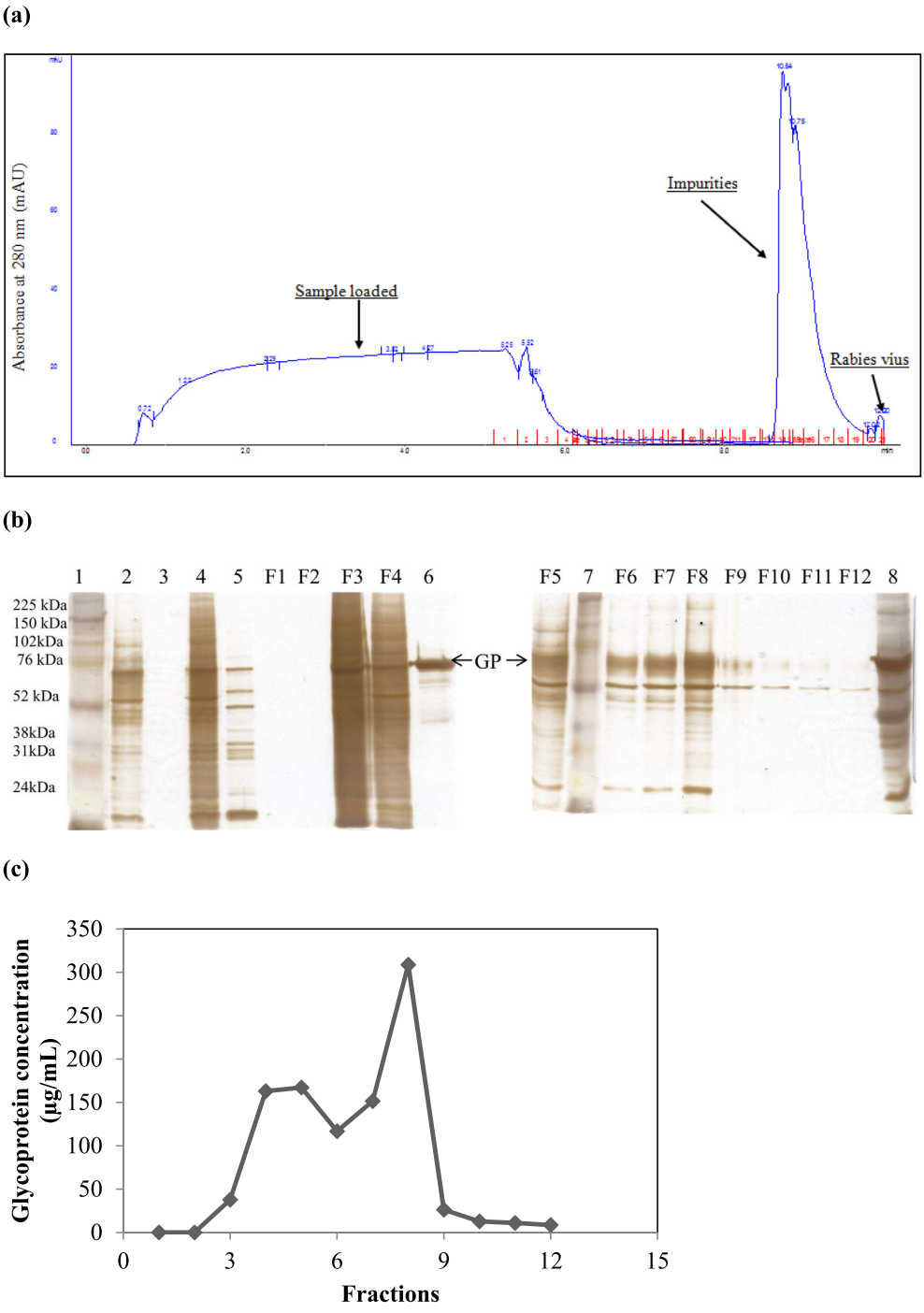

Fig. 5. (a) Viral harvests purification using CIMQA column by AKTA purifier System. (b) Characterization of purified rabies virus. Protein samples were separated with polyacrylamide gel electrophoresis and visualized by silver staining. : (1, 7) Molecular weight marker, (2) sample before TFF, (3) wash, (4) Sample loaded, (5) Flow through, (6) positive control of rabies virus glycoprotein (GP), (8) commercial vaccine (Rabipur), from F1 to F12: fraction number. (c) Rabies glycoprotein level of the fractions collected through CIMQA column purification.

not high, and was around 40% in the best case [18]. Recently, the replacement of chromatography resins by membrane adsorbers and monoliths for virus purification was reported in numerous studies. These latter methods show numerous advantages over resin chromatography; they are more efficient and robust; in addition they exhibit higher productivity and are easier to operate [19]. Hence to enhance the yield, we had tested in the current work the new chromatography medium Capto Core 700 which was designed for virus purification and other large biomolecules, and the monolithic chromatographic column (CIM-QA-8).

Capto Core 700 medium has a dual-functionality, size separation and binding chromatography. Small contaminant molecules enter into the beads where they are captured, whereas large molecules that have a molecular mass higher that 700 kDa are excluded and collected in the flowthrough [20]. . In the current study, the highest yield obtained was 84.5%, which was 2-fold higher than the previous yield obtained when using size exclusion chromatography columns [18]. This yield was also 1.5 fold higher than that obtained by zonal centrifugation. In addition, residual level DNA level in the fraction of interest was under the threshold of

100 pg/mL [14,21]. Likewise, Kumar et al. [22] showed that rabies virus purification yield was enhanced by 50% in comparison with zonal chromatography when the antigen was purified in loosely packed DEAE-sepharose CL-6B column. Lagoutte et al. [23] also reported the use of Capto Core 700 for the purification of a VLP influenza vaccine produced in *Escherichia coli*; they found a recovery yield of 89% although further purification steps were needed to get rid of contaminant proteins derived from the expression host.

Affinity and ion exchange chromatography methods were used in several studies to purify rabies virus produced in Vero cells cultivated in serum-supplemented medium. For instance, Kulkarni et al. [17] mentioned that the cellufine sulfate affinity matrix showed higher recovery yield that sucrose gradient centrifugation, although no details in terms of antigen recovery yield and impurity removal were provided. Similarly Yu et al. [24] reported the purification rabies virus in sepharose gel chromatography without providing any indication about the chromatography step; antigen recovery yield was around 80%. In another study, Li et al. [25] stated that rabies virus produced in Vero cells grown in M199 medium supplemented with animal serum, was purified by gel filtration chromatography using sepharose 4FF matrix, after 0.45 μm clarification and concentration by tangential flow filtration. However the performance in term of antigen recovery was not described in this study. Rabies virus harvests purification using monolithic chromatographic column was also investigated in this study. Monolithic columns represent a new generation of chromatographic matrices with efficient mass transfer and better hydrodynamic properties. They are distinguished by large flow-through channels, which allow fast and efficient separation of large molecules such as plasmid DNA and viruses. In this work, we used CIM-QA-8 column for rabies virus purification. The recovery yield was close to 94%. It was the highest yield obtained compared to the other methods tested. Residual DNA level detected in the fractions of interest complies with 100 pg/dose limit [14,26–28].

Monolith chromatography columns were used for the purification of different viruses [29]. In this line, Venkatachalam et al. [30] described the use of a weak anion-exchange monolithic column to purify enterovirus 71. They reported a recovery yield of 55%; no data were indicated about residual DNA and host cell protein removal. Moreover, Banjac et al. [31] studied influenza A and B virus purification by ion exchange monolith chromatography; they showed that CIM QA resulted in a higher recovery yield than CIM DEAE; the recovery yield varied between 70.8 ± 32.3% and 87 ± 30%. Total protein removal was between 93.3 ± 0.4% and 98.6 ± 0.2%, DNA removal was depending on the use of benzonase.

Compared to the other methods assessed in the current study, rabies virus purification using CIM QA column appears to be the best method in terms of antigen recovery yield and host cell residual DNA level (Table 2). Besides, this method allows virus harvest concentration as the sucrose gradient centrifugation method did. By contrast with Capto Core 700 no concentration was seen since this method is working in the flow through mode. Monolith purification was also the most efficient method for the removal of residual cellular DNA (Table 2). Regarding host-cell protein impurities, all methods fail to reach the acceptable HCP level by regulatory agencies which is 100 ppm (1–100 ng per mg of specific drug product) [20], a polishing step should be implemented to reach this target. CIM-QA monolith purification will be selected to set up a downstream process of rabies virus produced in Vero cells grown in serum free medium. The scalability of the method will be studied, and the implementation of the polishing step as well. The potency of the vaccine purified according to the new process will be also determined by the NIH assay.

Table 2
Comparison of rabies virus purification using different methods.

	Antigen recovery yield (%)[a]	Residual DNA level (pg/mL)[a]	HCP level (ng/mL)[a]
Sucrose gradient ultracentrifugation	60 ± 9	26.6 ± 5	509 ± 45
Capto Core 700 purification	87 ± 3	7.25 ± 1	1784.4 ± 100
CIM-QIA purification	96 ± 2	3.35 ± 1[b]	3372.5 ± 150[b]

[a] Two batches were considered for these calculations.
[b] Only the fraction showing the highest antigen level was considered for the estimation.

5. Conclusion

In this work, we demonstrated that monolithic support CIM-QA column can be used for rabies virus harvests purification obtained during Vero cells culture in serum free medium and in stirred bioreactor. This method allows the achievement of the highest recovery yield, residual cell DNA complies with the international requirements; this method was also the most efficient for the removal of HCP; nevertheless a polishing step to comply with the HCP threshold should be achieved subsequently.

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Declaration of Competing Interest

No conflict of interest is declared by the authors.

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