Vaccine 32 (2014) 2789–2793

Contents lists available at ScienceDirect

Vaccine

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

Development of a membrane adsorber based capture step for the purification of yellow fever virus

Tânia P. Pato[a,c], Marta Cristina O. Souza[a], Andréa N.M.R. Silva[a], Renata C. Pereira[a], Marlon V. Silva[a], Elena Caride[a], Luciane P. Gaspar[a], Marcos S. Freire[a], Leda R. Castilho[b,c,*]

[a] Oswaldo Cruz Foundation (FIOCRUZ), Bio-Manguinhos, Avenida Brasil 4365, 21045-900 Rio de Janeiro/RJ, Brazil
[b] Federal University of Rio de Janeiro, COPPE, Cell Culture Engineering Laboratory, Cx. Postal 68502, 21941-972 Rio de Janeiro/RJ, Brazil
[c] Federal University of Rio de Janeiro, IQ, Biochemistry Program, Av. Athos da Silveira Ramos 149, Bloco A, 21941-909 Rio de Janeiro/RJ, Brazil

ARTICLE INFO

Article history:
Available online 11 March 2014

ABSTRACT

Yellow fever (YF) is an endemic disease in some tropical areas of South America and Africa that presents lethality rate between 20 and 50%. There is no specific treatment and to control this disease a highly effective live-attenuated egg based vaccine is widely used for travelers and residents of areas where YF is endemic. However, recent reports of rare, sometimes fatal, adverse events post-vaccination have raised concerns. In order to increase safety records, alternative strategies should be considered, such as developing a new inactivated vaccine using a cell culture based technology, capable of meeting the demands in cases of epidemic. With this goal, the production of YF virus in Vero cells grown on microcarriers and its subsequent purification by chromatographic techniques was studied. In this work we investigate the capture step of the purification process of the YF virus. At first, virus stability was studied over a wide pH range, showing best results for the alkaline region. Considering this result and the pI of the envelope protein previously determined *in silico*, a strong anion exchanger was considered most suitable. Due to the easy scalability, simplicity to handle, absence of diffusional limitations and suitability for virus handling of membrane adsorbers, a Q membrane was evaluated. The amount of antigen adsorbed onto the membrane was investigated within the pH range for virus stability, and the best pH for virus adsorption was considered to be 8.5. Finally, studies on gradient and step elution allowed to determine the most adequate salt concentration for washing (0.15 M) and virus elution (0.30 M). Under these operating conditions, it was shown that this capture step is quite efficient, showing high product recovery ($93.2 \pm 30.3\%$) and efficient DNA clearance (0.9 ± 0.3 ng/dose).

1. Introduction

Yellow fever (YF) is a viral disease, endemic to tropical regions of Africa and South America, characterized by fever, nausea, vomiting, epigastric pain, hepatitis with jaundice, renal failure, hemorrhage, and shock. The fatality rate often is between 20 and 50% and there are no specific therapies. It is estimated that 200,000 cases occur annually in Africa, South America and Central America [1,2]. The virus is transmitted to and between humans *via* the bite of infected insects of the family *Culicidae*, of the genera *Haemagogus* (in wild areas) and *Aedes* (in urban areas). The causative agent, YF virus, has a single-stranded RNA and belongs to the *Flavivirus* genus of the *Flaviviridae* family. The virus particles are about 50 nm in size [3,4].

To control this disease a highly effective live-attenuated vaccine is available since the 1930s, manufactured in embryonated eggs, based on the virus substrains 17 DD and 17D-204. This vaccine is widely used for travelers and residents of areas in which YF is endemic. An acceptable safety profile has been well established during these decades, considering the application of more than 500 million doses. However, reports of rare, but serious adverse events post-vaccination have raised concerns [4,5]. This category of adverse event, named vaccine-associated viscerotropic disease, was first described in Brazil in 1999 and was shown to occur with a frequency of 3–4 per million doses administered and a case fatality of 60% [2].

In order to increase safety records, alternative strategies have been considered, one of them aiming at developing a new inactivated vaccine, that could potentially be used in immunosuppressed

* Corresponding author at: Federal University of Rio de Janeiro, COPPE, Cell Culture Engineering Laboratory, Cx. Postal 68502, 21941-972 Rio de Janeiro/RJ, Brazil. Tel.: +55 21 2562 8336.
E-mail address: leda@peq.coppe.ufrj.br (L.R. Castilho).

http://dx.doi.org/10.1016/j.vaccine.2014.02.036
0264-410X/

individuals, persons with allergies to egg proteins, infants under 9 months of age, and even pregnant and nursing women [1].

The state vaccine producer Bio-Manguinhos/Oswaldo Cruz Foundation (Rio de Janeiro, Brazil) is the world's largest producer of attenuated yellow fever vaccine. In recent years, Bio-Manguinhos started developing an inactivated vaccine using non-egg based technology and adopting chromatographic steps to achieve high virus purification.

In recent decades significant advances in the production of virus using animal cell culture in large-scale bioreactors occurred. Comparatively less effort has been dedicated to the downstream process, which currently constitutes a major bottleneck in the production of biologicals. In order to economically manufacture high-quality viral vaccines, with high antigen concentration and low levels of impurities such as DNA and host cell protein (HCP), meeting the specifications required by regulatory agencies, the development of a simple and efficient method for purification of virus is crucial [6–8].

Widely used traditional procedures for virus purification, such as cycles of differential centrifugation, ultracentrifugation and density gradient centrifugation are almost impracticable for large-scale biomanufacturing. Therefore, in recent years chromatography has been intensively investigated as a method for efficient virus purification. However, conventional chromatography using porous resins packed in fixed beds is inappropriate for purification of large biomolecules such as virus particles, due to the small pore sizes and high shearing forces that develop inside the columns [7,9].

Replacement of resins by highly porous chromatographic supports, such as membranes and monoliths, overcomes many of the limitations associated with resins such as high back-pressure, low linear velocities and diffusional limitations. In the membrane, the convective flow significantly enhances mass transfer and large-size pores provide high accessibility even for viral particles. In addition, column packing and column testing are eliminated when using membrane absorbers or monoliths [10–12].

In the present work, the capture step for the purification process of the yellow fever virus from Vero cell culture supernatant was investigated. This is a critical step because the supernatant volume must be quickly reduced to concentrate, isolate and stabilize the antigen, transferring it to a buffer that will conserve potency/activity [6,13].

For the experiments a Q membrane adsorber was utilized. The best pH for viral stability and virus adsorption, the best salt concentration for elution and the dynamic binding capacity of the membrane were investigated.

2. Materials and methods

2.1. YF virus production

The YF virus suspension was produced in stirred bioreactors (BioFlo 110, New Brunswick Scientific, model M 1273-0054) fitted with a 3 L vessel (2.2 L working volume) at 37 °C, 70 rpm, pH 7.2 and 50% air saturation [3]. Vero cells (ECCAC) were grown on Cytodex 1 microcarriers (GE Healthcare) at a concentration of 3 g/L in a serum-free medium supplemented with glutamine to 4 mM final concentration (Gibco, Invitrogen Corp.) and Pluronic F68 to 0.01% (m/v) final concentration (Sigma Aldrich Co.). Infection was carried out on the 3rd day post-inoculation, using a multiplicity of infection (MOI) of 0.02.

At the end of the process, the culture was decanted and subsequently clarified using Sartopure PP2 Mini Caps (8.0 μm), Sartoclean CA (3.0 μm + 0.8 μm) and Sartobran P (0.45 μm + 0.2 μm) filter units (Sartorius-Stedim).

2.2. Analytical methods

Quantification of total proteins was carried out using the BCA protein assay kit (Pierce®) according to manufacturer's instructions.

DNA content was determined using Qubit™ Quantification Fluorometer (Invitrogen Corp.), according to the manufacturer's instructions.

HCP concentration was measured using a commercial Vero Cell HCP ELISA kit, according to the manufacturer's instructions.

YF virus was quantified by virus titration as described previously [14] and by an ELISA assay developed in house, which measures the virus envelope protein concentration, thus named 'specific protein' hereafter.

2.3. Virus stability over a wide pH range for choice of the ion exchanger

In order to choose the most suitable ion exchanger, experiments were carried out to define the pH range for best virus stability. The pH of the clarified virus suspension was adjusted with 3 M HCl or 1 M NaOH in the range of 4.5–9.0, with intervals of 0.5 pH units and then incubated for 1 h at 25 ± 5 and 5 ± 3 °C. The quantification of YF in each sample was performed by viral titer and this value was compared to the titer of the viral suspension without pH adjustment, incubated under identical conditions.

2.4. Selection the best pH for virus adsorption

The adsorption pH was selected through experiments within the stable range for the virus, where the amount of antigen adsorbed onto the membrane was quantified. With this purpose, a volume of 5 mL of virus suspension with pH previously adjusted to the range of 7.5–9.0 using 1 M NaOH was applied to a Q membrane adsorber with 75 cm^2 nominal membrane area. Sample application was performed manually, using disposable syringes. The content of YF virus in the flow-through was quantified by ELISA and viral titer. This value was compared to the titer of the original virus suspension to quantify the percentage of antigen adsorbed.

2.5. Optimization of chromatography operational conditions

Chromatography experiments were performed using Akta Purifier 10, operated with the UNICORN software (GE Healthcare). The flow rate used was 10 mL/min (122.3 cm/h), based on recommendations of the membrane manufacturer. Sanitization of the membrane adsorber was carried out with 50 mL of 1 M NaOH and equilibrium with the same volume of 0.05 M Tris, pH 8.5 (Amresco). The sample was kept in an ice bath prior to and during sample application.

The pH of the clarified virus suspension was adjusted to 8.5 with 1 M NaOH and then 200 mL were applied onto the membrane. Non-bound substances were washed out by applying 30 mL of the equilibrium buffer. In order to determine the best elution conditions, linear and step gradients were carried out by increasing NaCl concentration (0–1 M) in 0.05 M Tris buffer pH 8.5. Fractions were collected and analyzed for YF by ELISA.

Regeneration was performed with 0.05 M Tris buffer containing 1 M NaCl, pH 8.5, followed by cleaning with 1 M NaOH. After re-equilibration, filters were rinsed with 20% ethanol containing 1 M KCl prior to storage at 4 °C.

2.6. Dynamic binding capacity

The dynamic binding capacity was determined at linear velocities of 61.2, 122.3 and 244.6 cm/h, using Akta Purifier 10 and Akta Avant 150. To obtain the breakthrough curves for the Q membrane,

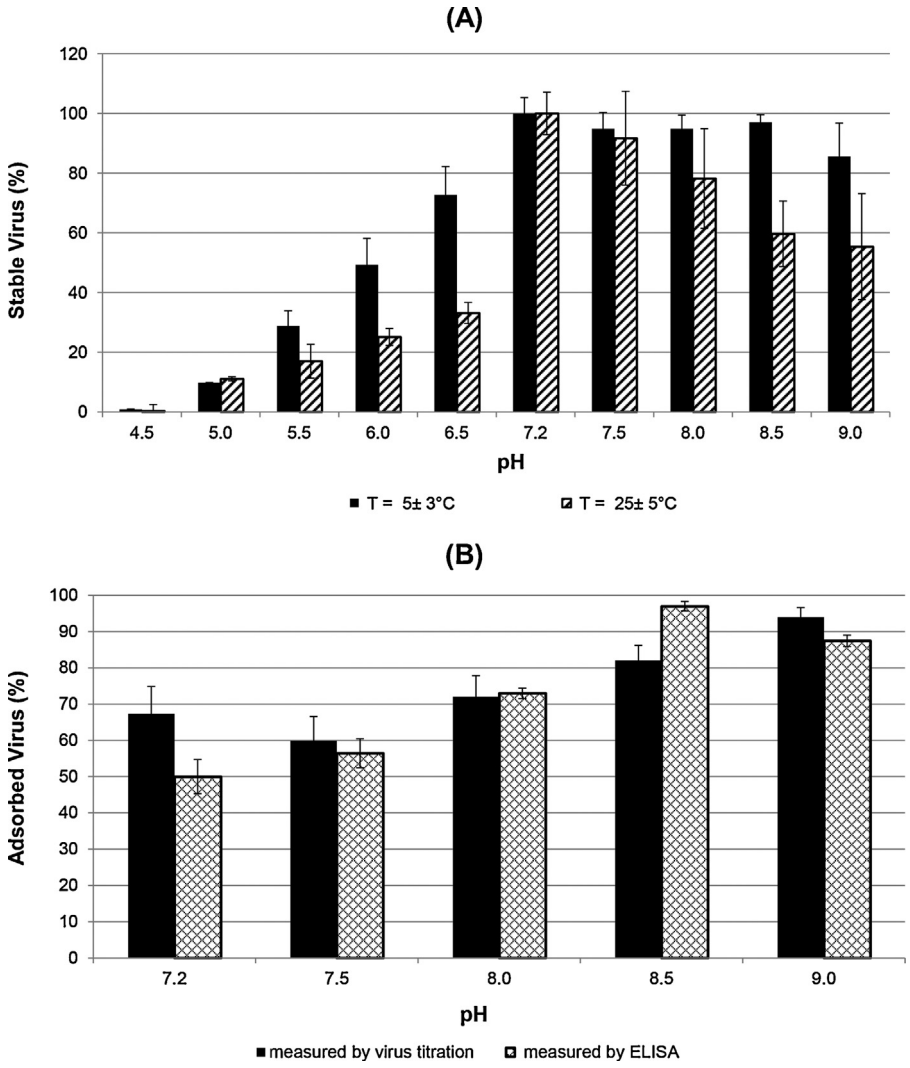

Fig. 1. (A) Virus stability (based on virus titer) in a wide pH range (4.5–9.0) after 1 h of incubation at 5 ± 3 °C and 25 ± 5 °C. (B) Virus adsorption onto the membrane adsober in the pH range of virus stability. YF quantification was performed by virus titer and ELISA. Data shown represent the average of three independent experiments and their standard deviation.

the viral suspension was applied until the maximum working pressure of the membrane (0.6 MPa). Flow-through fractions were collected and analyzed by ELISA to determine the amount of specific protein adsorbed per membrane area.

3. Results and discussion

3.1. Virus stability over a wide pH range

Although the final aim of the work is to develop an inactivated vaccine from the attenuated YF strain, it was considered that monitoring virus stability by titration prior to the inactivation step and choosing operational conditions favoring virus stability would decrease the chances of affecting virus structure and consequently its immunogenicity.

Fig. 1A shows the percentage of stable virus, based on virus titer, in a pH range of 4.5–9.0 after 1 h of incubation at 5 ± 3 °C and 25 ± 5 °C. The virus was unstable at pH values below to 6.5, but in the neutral and alkaline region it was rather stable at the lower temperature (5 ± 3 °C), with a decrease in titer being observed only at pH 9.0. However, at 25 °C a decrease in titer was observed already at pH 8.0. These results confirm literature information stating that flaviviruses are optimally stable at pH 8.4–8.8 and sensitive to acid pH [15].

The pI of the YF envelope protein had been previously determined *in silico* using ExPASy (Expert Protein Analysis System) to be approximately 6.38. Based on these results, the group exchanger Q (quaternary ammonium) was chosen for the capture step, because within the virus stability range the pH could be set at least 1 pH unit above the virus pI, keeping the virus negatively charged to interact with the anion exchanger.

3.2. Selection of pH for virus adsorption onto the Q membrane

Virus adsorption onto the Q membrane was evaluated in the pH range of stability (7.5–9.0) by virus titer and ELISA (Fig. 1B). In this alkaline region the percentage of adsorbed virus increased with increasing pH. However, due to virus stability, the pH of 8.5 was chosen for the adsorption step.

3.3. Optimization of chromatography operational conditions

In order to first determine the range of NaCl concentration where the virus elutes, chromatography runs were carried out both adopting linear gradient elution and step elution. The chromatographic profile under linear gradient elution is shown in Fig. 2A. The percentage of virus recovery in each fraction, quantified by ELISA, is also shown. The product of interest was eluted at a salt

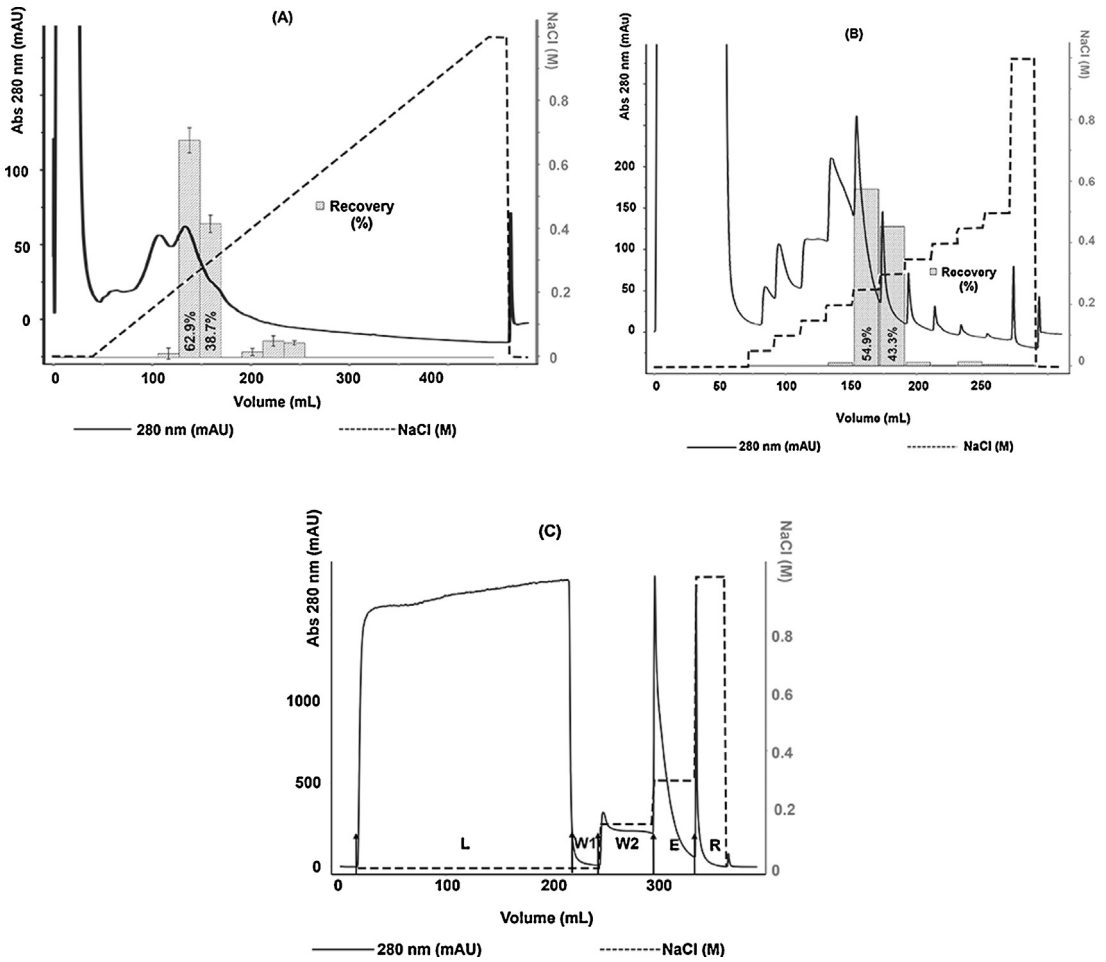

Fig. 2. (A) Linear gradient elution of YF virus showing percentage recovery in each fraction (by ELISA). Elution was carried out by linearly increasing [NaCl] in 0.05 M Tris buffer (pH 8.5). (B) Step elution of YF virus showing percentage recovery in each fraction (by ELISA). Elution was carried out by stepwise increasing [NaCl] in 0.05 M Tris buffer (pH 8.5). (C) Chromatogram of final capture step. Arrows indicate the steps of sample loading (L), washing with no NaCl (W1), washing with 0.15 M NaCl (W2), elution (E) and regeneration (R).

concentration around 0.25 M to 0.30 M. This result was confirmed in the step gradient, shown in Fig. 2B. Furthermore, it was found that impurities are eluted at lower salt concentrations, where the presence of the product of interest was not detected. Therefore we concluded that washing with 0.15 M NaCl would be adequate for process performance. Based on these results, final process configuration was established to include a washing step at 0.15 M NaCl and elution at 0.3 M NaCl.

A typical chromatogram for this final process is shown in Fig. 2C. Average yield of the product was 93.2 ± 30.3% (by ELISA), which is in a similar range to that achieved in other studies also utilize ion exchangers for the purification of viral particles [7,12].

The DNA content in the eluted fraction was 0.9 ± 0.3 ng/dose, considering a dose established according to preliminary immunogenicity studies. This value is lower than required by regulatory agencies (10 ng/dose) [16].

Regarding host-cell protein impurities, although in literature a level of 100 ppm (HCP per specific protein) is usually mentioned as maximum acceptable HCP concentration [17,18], the limit yet to be defined by regulatory agencies will probably vary for different

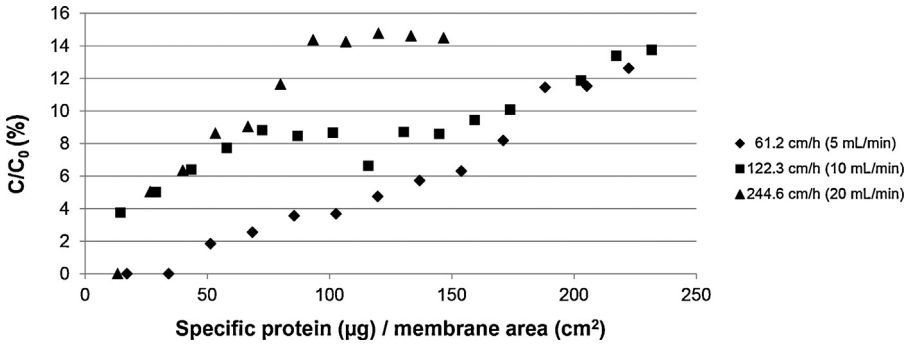

Fig. 3. Breakthrough curves obtained by applying clarified yellow fever virus suspension at three different linear velocities to Q membrane adsorber.

vaccines and different biopharmaceuticals, because both the number of doses can vary significantly and the amount of specific protein in a final dose can be considerably different. In our study, the specific protein we quantified is the envelope protein of the YF virus and the relation of the concentration of this protein to the concentration of the final active ingredient is not known yet. Anyway, the level of host cell protein we obtained was considerably high ($317{,}622 \pm 97{,}969$ ppm, or ng HCP/mg specific protein), indicating the need for targeting HCP in a subsequent step.

3.4. Dynamic binding capacity

Fig. 3 shows breakthrough curves determined for the Q membrane adsorber at different linear velocities. A small decrease in binding capacity can be observed with increasing linear velocity. Since this membrane has been used for a capture step, where speed is an important feature, a linear velocity 122.3 cm/h was chosen for which at 10% breakthrough a dynamic capacity approximately 170 μg of specific protein per membrane area (cm^2) was determined.

4. Conclusions

In this work, starting from the theoretical pI of the YF envelope protein and after determining virus stability as a function of pH, a Q anion-exchange membrane was chosen for the capture step of YF virus from Vero cell supernatant. Subsequently, adsorption tests enabled determination of the best pH for adsorption (8.5) and chromatography runs allowed determining the best type of elution (stepwise increase in NaCl concentration), as well as the best NaCl concentration for washing (0.15 M) and elution (0.3 M).

The data obtained in chromatographic runs under these conditions demonstrate that the Q membrane adsorber is a promising option for YF virus capture from cell culture supernatant in the large-scale process. High viral recovery was achieved (93%) and eluted fractions already showed acceptable levels of DNA (0.9 ng/dose). Furthermore, the membrane showed good dynamic capacity even for viral particles. Levels of the HCP remained above the limits, but could be targeted in a subsequent polishing stage.

Conflicts of interest

No conflicts of interest are declared.

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