Vaccine 37 (2019) 3214–3220

Contents lists available at ScienceDirect

Vaccine

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

Purification of yellow fever virus produced in Vero cells for inactivated vaccine manufacture

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ARTICLE INFO

Article history:
Received 6 December 2018
Received in revised form 19 April 2019
Accepted 24 April 2019
Available online 30 April 2019

Keywords:
Yellow fever virus (YFV)
Downstream processing
Virus purification
Inactivated vaccine
Chromatographic techniques

ABSTRACT

Yellow fever (YF) is a high-lethality viral disease, endemic in tropical regions of South America and Africa, with a population of over 900 million people under risk. A highly effective attenuated vaccine, produced in embryonated eggs, has been used for about 80 years. However, egg-based production limits manufacturing capacity, and vaccine shortage led to the emergency use of a fractional dose (1/5) by the WHO in an outbreak in Africa in 2016 and by Brazilian authorities during an outbreak in 2018. In addition, rare but fatal adverse events of this vaccine have been reported since 2001. These two aspects make clear the need for the development of a new vaccine. In an effort to develop an inactivated YF vaccine, Bio-Manguinhos/FIOCRUZ started developing a new vaccine based on the production of the attenuated 17DD virus in serum-free conditions in Vero cells propagated in bioreactors, followed by chromatography-based purification and β-propiolactone inactivation.

Virus purification was studied in this work. Capture was performed using an anion-exchange membrane adsorber (Sartobind® Q), resulting in a virus recovery of 80.2 ± 4.8% and a residual DNA level of 1.3 ± 1.6 ng/dose, thus in accordance with the recommendations of the WHO (<10 ng/dose). However, the level of host cell proteins (HCP) was still high for a human vaccine, so a second chromatography step was developed based on a multimodal resin (Capto™ Core 700). This step resulted in a virus recovery of 65.7 ± 4.8% and decreased HCP levels to 345 ± 25 ppm. The overall virus recovery in these chromatography steps was 52.7%. SDS-PAGE of the purified sample showed a band with molecular mass of 56 kDa, thus consistent with the virus envelope protein (E) and corresponding to 96.7% of identified proteins. A Western blot stained with an antibody against the E protein showed a single band, confirming the identity of the sample.

1. Introduction

Yellow fever (YF) is a non-contagious viral hemorrhagic disease, transmitted by infected mosquitoes, in particular *Aedes aegypti* (urban cycle) and *Haemagogus* sp. (jungle cycle). The disease can occur in mild or severe forms, the latter being characterized by fever, nausea, vomiting, epigastric pain, hepatitis with jaundice, renal failure, hemorrhage and shock. There is no specific antiviral treatment. According to the CDC, currently there are 34 countries in Africa and 13 in South America where there is risk of transmission of yellow fever virus (YFV) [1]. The vast majority of YF cases occur in Africa, and estimates by Garske et al. [2] indicate that the yellow fever burden in Africa in 2013 was 130,000 cases with fever and jaundice or haemorrhage, including 78,000 deaths. This is comparable to the worldwide annual average number of hospitalizations and deaths caused by influenza. Non-human primates are reservoir hosts of the virus, making it impossible to eradicate the mosquito-borne jungle transmission, which primarily affects young male forestry and agricultural workers. Over the last 20 years, an increase in the number of YF cases worldwide has been observed, and possible causes could be declining population immunity to infection, deforestation, urbanization, population movements and climate change [3].

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https://doi.org/10.1016/j.vaccine.2019.04.077
0264-410X/

YF is considered an emerging or reemerging disease of high importance. The most effective means of prevention and control is vaccination, which is recommended for residents of endemic regions and travelers to risk areas [3,4]. A live-attenuated YF vaccine, based on virus produced in embryonated eggs, has been used since the 1930s in many countries, with hundreds of millions of doses administered so far, providing highly effective and durable immunity [4]. As recognized by the WHO since 2013, a single dose provides effective immunity within 30 days for 99% of vaccinated people and confers life-long protection, with no need for booster doses [3]. However, despite these excellent data, the observation of rare, but fatal adverse events raised the discussion about the risks and benefits of vaccination and about the need for the development of new vaccines [4,5].

While the adverse events have been motivating the development of new types of vaccines (inactivated, subunit or DNA), the unmet and growing worldwide demand has been reinforcing the need for developing new technology platforms, which should not be egg-based anymore and should be able to meet the growing demand for routine preventive immunization, as well as for cases of outbreaks.

Nowadays, meeting vaccine demand remains a challenge. In 2018, the Eliminate Yellow Fever Epidemics (EYE) Strategy partnership, steered by WHO, UNICEF and Gavi, was launched with the main purpose of ensuring adequate vaccine supply to all those at-risk [6].

In Africa, before 2010, most YF outbreaks were reported in 12 West African countries, and after implementation of preventive mass vaccination campaigns and routine immunization, the YF disease burden in those areas decreased dramatically. However, since 2010 there has been a shift of disease activity from West to Central and East Africa, where no preventive mass vaccination campaigns have been conducted. An urban YF outbreak began in Angola in December 2015, and spread throughout the country, Uganda and Congo along 2016, causing also cases imported to China, Kenya, and Morocco [7,8]. This African outbreak led the WHO to introduce the emergency use of a fractional (1/5) dose after depletion of the WHO YF vaccine stockpile. The outbreak was finally controlled, but hundreds of people died and the first YF cases ever to occur in Asia were reported, raising the concern about the risk of YF spreading in Asia [9].

In South America, over the last decade YF cases had been reported at moderate amount in over 10 countries. However, in December 2016 an outbreak started in Brazil, being the worst outbreak in the country in decades [10]. Mass vaccination of over 35 million people in 2017 slowed down virus transmission, but in January 2018 (restart of the rainy season) virus transmission restarted intensively, approaching highly-populated metropolitan areas, so that despite Brazil being a major YF vaccine producer the emergency use of the fractional dose had to be introduced [11]. Despite the mass vaccination campaigns, approximately 750 people died of YF in the 2016–2018 outbreak in Brazil [12].

The recent YF outbreaks in Angola and Brazil have thus given evidence that global vaccine supply remains an important challenge regarding the control of the disease. Although the worldwide annual vaccine production capacity increased from 20 to over 80 million doses between 2001 and 2018, the supply is still below the current demand [6,7,8].

In this context, Bio-Manguinhos (FIOCRUZ, Brazil), one of the largest producers of the YF live-attenuated vaccine, has been investing efforts to develop a new inactivated vaccine based on Vero cell culture, chromatographic virus purification and subsequent virus inactivation. This new vaccine could represent an alternative to the world supply limitations, increase the product safety profile and allow the immunization of patients with contraindications to the egg-derived, attenuated vaccine: immunosuppressed or thymectomized patients, children under 9 months of age, people allergic to egg protein, and lactating women [13].

This work shows results related to the development of a two-step chromatography-based purification process of the YF virus, produced in Vero cells, for the production of the new inactivated vaccine. The main goal was to establish a process that should be applicable to industrial scale production, being robust, economically feasible, easy to scale-up and efficient in the removal of critical contaminants such as host-cell protein (HCP) and DNA down to levels accepted by regulatory agencies [14]. With this main goal in mind, we built on our previous work [15], which proposed a first capture step based on ion-exchange chromatography, to further develop a polishing step and evaluate the overall performance of the two-step purification process.

Ion exchange chromatography (IEC) is a technique that can be quickly performed, offers high capacity and has the ability to concentrate the sample. Furthermore, the technique is widely used at industrial scale because of its simplicity and the low cost of buffer solutions. IEC using membrane adsorbers instead of conventional resins presents several advantages: (i) convective flow dominates, allowing the use of high flow rates at low pressure drops, and resulting in a substantial decrease in process time; (ii) the large pores provide high accessibility even to large products, such as viruses; (iii) membranes are often used as disposable units, eliminating the need for cleaning and validation procedures [16,17]. Several works have reported the successful use of membrane adsorbers [14,16,17,18] for the purification of different viral particles, and in our previous paper we have shown that an anion-exchange membrane could be successfully used for YFV capture from Vero cell culture supernatant [15].

For the polishing step, based on literature mainly focusing on influenza virus purification [19,20], multimodal chromatography based on Capto™ Core 700 resin was evaluated. It is designed for intermediate purification and polishing of viruses and other large biomolecules. The resin is based on the core bead concept. Each bead has an octylamine core and an inert highly porous outer layer. The layer excludes large molecules that are collected in the flow through, while monomeric proteins and other contaminants are not excluded by the outer layer and bind to the charged and hydrophobic ligand attached to the core [21].

Thus, in the present work we improved the capture step reported in our previous work [15], performed detailed studies to establish a polishing step and evaluated if the overall performance of the 2-step chromatography process allows meeting the regulatory requirements for clearance of critical contaminants, such as HCP, DNA and endotoxins. This work is thus an important step toward enabling the production of a purified, inactivated vaccine (PIV) from attenuated 17DD YF virus propagated in Vero cells in bioreactors under serum-free conditions.

2. Materials and methods

2.1. YF virus production

Virus production was performed in Vero cells cultured on microcarriers in bioreactors under serum-free conditions as described by Mattos et al. [22], who suggested improvements to the original protocol proposed by Souza et al. [23].

The working virus seed used to infect Vero cells in the bioreactor had a titer of 9.25×10^7 pfu/mL and was produced in monolayer Vero cells from virus seed stock of sub-strain 17-DD, passage 287 belonging to Laboratory of Virological Technology (Bio-Manguinhos/FIOCRUZ).

Virus suspension harvested from the bioreactor was decanted and clarified using a combination of 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, Germany).

2.2. Virus purification

The capture step of YF virus from Vero cell culture supernatant was carried out as described in our previous study [15,24] with only one modification: the introduction of sorbitol (Neosorb®, Roquette, France) as a stabilizer in the feed and in the equilibration and elution buffers.

A polishing step was investigated using Capto™ Core 700 resin (GE Healthcare), prepacked in a HiScreen™ column (4.7 mL packed volume, 0.77 cm diameter and 10 cm bed height), and coupled to an Äkta Purifier 10 System, operated with Unicorn software (GE Healthcare, Sweden). Some operating variables were tested (Table 1) to obtain the best results for recovery and HCP removal. In two out of the 4 conditions tested, sterile pyrogen-free human serum albumin (HSA) was added to the eluate of the capture step from a pharmaceutical HSA stock solution (Blaubimax® 20% m/v from Blau, Brazil).

Column regeneration was performed with 10 mL of 1 M NaOH + 30% (v/v) isopropanol (40 cm/h), and equilibration was carried out with 25 mL of 50 mM Tris + 0.3 M NaCl + 8% sorbitol, pH 8.5 (500 cm/h). Protein concentration in the Äkta system was monitored by absorbance at 280 nm.

2.3. Analytical methods

DNA content was determined using Qubit™ Quantification Fluorometer (Invitrogen, USA), following the methodology described in the manufacturer's instructions.

Because the attenuated virus was used to infect to Vero cells, virus inactivation could be performed as the last step and all the process could be monitored by YF titration. Virus titer was quantified by plaque titration on Vero cell monolayers as described previously [25].

An enzyme-linked immunosorbent assay (ELISA), developed in house by Laboratory of Immunological Technology (Bio-Manguinhos/Fiocruz), was used to measure envelope (E) protein content. For this assay, 96-well plates were coated with anti-E monoclonal antibody. After sample incubation, peroxidase labeled anti-E antibody and then tetramethyl benzidine (TMB) were added.

Host cell protein (HCP) concentration was measured using a commercial Vero Cell HCP ELISA kit (Cygnus Technologies, USA), according to the manufacturer's instructions.

Endotoxin content was determined using the Kinetic Chromogenic LAL – *Limulus Amebocyte Lysate* assay (Endochrome-K™, Charles River, USA), according to the manufacturer's instructions.

The purity profile of the sample after each purification step was verified by SDS-PAGE, performed using 4–12% Novex® NuPAGE® gels and the XCell SureLock System® (Thermo Fisher Scientific, USA). Silver staining was carried out according to the literature [26]. After scanning, the gel was analyzed using the ImageMaster® software (GE Healthcare, Sweden) for band quantification by densitometry.

To perform Western blot, non-stained gels were electrotransferred to nitrocellulose membrane (Trans-Blot Transfer Medium) using a Trans-Blot Semi-Dry Transfer Cell System (BIO-RAD, USA). E protein detection was carried out using a polyclonal rabbit serum provided by Chagas [27] and an alkaline phosphatase-conjugated anti-rabbit IgG (Sigma-Aldrich, A3812).

Table 1
Recovery (%) and HCP level (ppm) in purified product obtained under four different conditions evaluated for the polishing step, using the resin Capto™ Core 700.

#	Operating conditions	Recovery (%)	HCP (ppm)
1	1.6 mL/min (200 cm/h)	25.6	131
2	3.9 mL/min (500 cm/h)	67.0	454
3	1.6 mL/min (200 cm/h), 1% (m/v) HSA*	78.1	7017
4	1.16 mL/min (150 cm/h), 0.5% (m/v) HSA*	65.7	363

* Final concentration of HSA in the capture step eluate, which was fed to the polishing step.

3. Results and discussion

3.1. Capture step

In virus purification processes, it is important to maintain the stability of the virus particle throughout the process in order not to affect immunogenicity. After our previous work [15], virus quantifications carried out over time in samples eluted from the anion-exchange capture step and stored at −70 °C revealed loss of viral stability and suggested the need for a stabilizer.

Most common stabilizers used in vaccine formulations include carbohydrates (such as sucrose, lactose, maltose, and trehalose) or sugar alcohols (such as sorbitol and mannitol) [28]. Since sorbitol is successfully used in the live-attenuated YF vaccine commercialized by Bio-Manguinhos/FIOCRUZ, it was selected as a stabilizer for the inactivated vaccine candidate.

Due to the inclusion of sorbitol in the feed and buffers of the anion-exchange step, as well as to changes in the starting material (use of a Vero cell line from ECACC instead of the ATCC cell line used in [15]), the performance of the capture step after these adaptations was evaluated, resulting in a recovery of 80.2 ± 4.8% and a residual DNA level of 1.3 ± 1.6 ng/dose (supposing a human dose containing 8 μg of E protein). This DNA level is in accordance with the recommendations of the WHO (<10 ng/dose) [29]. The human dose size was estimated as 4-fold the mouse dose size used in a proof-of-concept study using the inactivated vaccine developed herein, where Pereira et al. [30] showed that a regimen of 3 doses (2 μg each) was able to elicit neutralizing antibodies and confer 100% protection against lethal challenge in immunized mice.

The residual level of HCP after the anion-exchange capture step was 85,970 ± 37,833 ppm, which is non-satisfactory for a vaccine candidate for human use. Although there are no clear specifications for maximum HCP levels established by regulatory agencies, and despite maximum acceptable HCP levels vary for different products depending on the number of doses and the amount of antigen administered in each dose, acceptable HCP levels are usually in the range of dozens to a few hundreds of ppm (e.g. ng of HCP/mg of active ingredient) [31]. Thus, HCP levels obtained in this purification step were too high and, for this reason, the second chromatography step was designed with the main aim of reducing HCP and polishing the sample.

3.2. Polishing step

The first operating conditions investigated for the multimodal chromatography step using a HiScreenTM Capto™ Core 700 column were the amount of sample injected and the linear velocity applied. When 1 column volume (CV) of eluate of the capture step was injected at two linear velocities (200 cm/h and 500 cm/h), recovery of E protein antigen was below 10% (data not shown). However, when 5 CVs were injected, recovery of E protein antigen was 25.6% at 200 cm/h and 67.0% at 500 cm/h (Table 1).

At the linear velocity of 200 cm/h (which corresponds to a flow rate of 1.6 mL/min), reduction of host cell protein was significant, decreasing from above 80,000 ppm in the capture eluate down to 131 ppm, which is in the range applicable for vaccines of human

use. At 500 cm/h (3.9 mL/min), HCP was reduced to 454 ppm. A comparison of these two conditions showed that a decrease in flow rate, leading to an increase in residence time, had two different consequences: longer residence times can increase HCP reduction, but can cause a decrease in virus recovery, possibly due to part of the virus being able to penetrate the outer shell, which has a cut-off in the same order of size as the virus particles.

As an additional observation, it was verified that samples purified by these protocols and kept at −70 °C experienced a decrease in virus titer, measured as plaque forming units/mL. This could be due to aggregation of viral particles in the purified samples. Although the final aim was to develop an inactivated vaccine, we considered it important to keep the virus stable throughout the purification process, because this would mean conserving the structure of the virus particles and thus eliciting the generation of the correct antibodies when used as a vaccine.

Thus, based on the literature, we decided to investigate the use of human serum albumin (HSA) as a stabilizer that could provide protection against interfacial tensions and inhibit aggregation [32]. HSA was first added at a concentration of 1% (m/v) to the eluate of the capture step, which was fed to the polishing step. Aiming to prioritize HCP removal, the linear velocity was set at the lower value tested previously (200 cm/h or 1.6 mL/min).

As shown in Table 1, recovery in the polishing step increased (78.1%) under this third condition (1% HSA and 200 cm/h), when compared to the condition without the use of HSA. However, HCP levels were very high (7017 ppm), possibly because the high load of albumin injected into the column (50 mg HSA/mL resin) could be saturating the ligands of the resin core and preventing HCP to bind to the core, thus leading to high HCP concentration in the flow-through.

Since the manufacturer's datasheet indicates a dynamic binding capacity at 200 cm/h of 13 mg ovalbumin/mL resin [21], a new experiment was designed, aiming at a compromise between virus recovery and HCP removal.

Under this fourth condition, a lower HSA concentration tested (0.5% m/v) resulted in the injection of 25 mg of HSA/mL resin, still higher than the dynamic capacity for ovalbumin of the multimodal resin. Since the addition of HSA had contributed to an increase in recovery, under this condition linear velocity was decreased to 150 cm/h (1.16 mL/min) in an attempt to enhance HCP clearance.

As shown in Table 1, under condition 4 there was a decrease down to 65.7% in recovery compared to condition 3. However, HCP levels were considerably reduced (to 345 ppm), reaching levels close to condition 2. It is important to note that after storage at −70 °C, the sample purified under condition 4 remained stable, maintaining constant the viral titer and E protein content.

Replicate experiments under this condition showed that it was reproducible, with an average recovery of 65.7 ± 4.8% and HCP levels of 345 ± 25 ppm. Fig. 1 shows a typical chromatogram of this condition, whereby most viral particles are recovered in the highlighted area of the plot.

Virus recovery and residual levels of HCP under conditions 2 and 4 are similar. The significant difference between them is the use of HSA as a stabilizer of viral particles, possibly preventing the aggregation of particles and providing protection against interfacial tensions. However, condition 2 uses a higher linear speed, being advantageous for use in industrial processes. Thus, future steps of this work should evaluate if the addition of HSA immediately after obtaining the purified material under condition 2 would also help maintaining viral stability. Furthermore, given the tendency of eliminating raw materials of animal or human origin in the manufacture of vaccines or biotherapeutic products for human use, the feasibility of using recombinant albumin to preserve the stability of the yellow fever virus should also be evaluated.

3.3. Analysis of identity, purity and morphology of purified viral particles

Fig. 2A shows a SDS-PAGE gel with samples from the whole process. Whereas the first capture step (lane 5) is primarily a concentration step, a significant decrease in protein complexity after the polishing step (lane 6) was observed. In this lane, a high intensity band with a molecular mass of about 56 kDa, compatible with the E protein mass, was detected. This band corresponded to 96.7% of identified proteins (Fig. 2B), demonstrating the high level of purity of the sample. This analysis of purity would be impaired by the presence of HSA, so these data refer to the purification under condition 2.

Fig. 2C shows a transmission electron microscopy image at 44,000-fold magnification of the purified sample, under condition

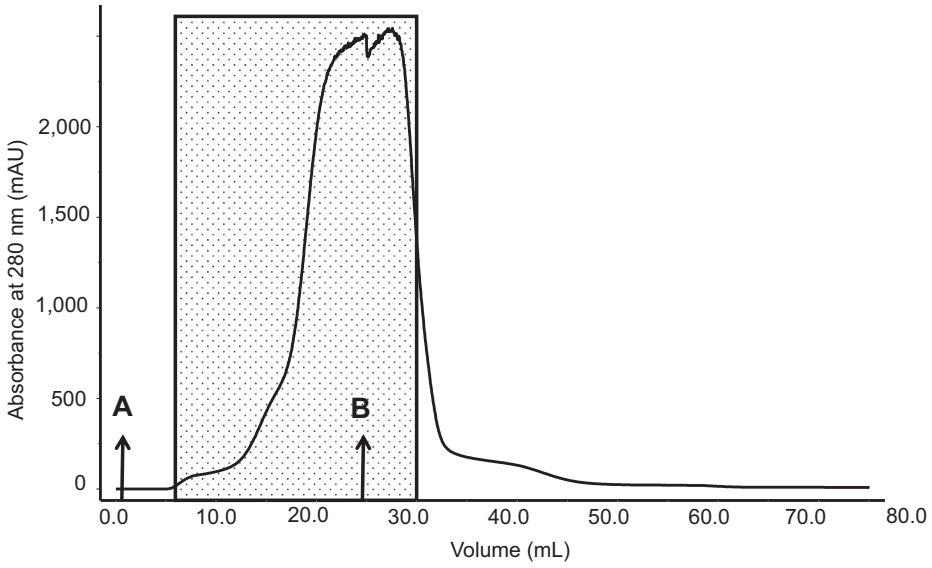

Fig. 1. Chromatographic profile of the polishing step under condition 4: HiScreen™ Capto™ Core 700 column, flow rate 1.16 mL/min (150 cm/h), sample injection volume of 25 mL, HSA 0.5% m/v. The arrows indicate: (A) sample application; (B) washing with 50 mM Tris + 0.3 M NaCl + sorbitol, pH 8.5. The area highlighted is the volume range where most viral particles were recovered, as measured by E-protein ELISA.

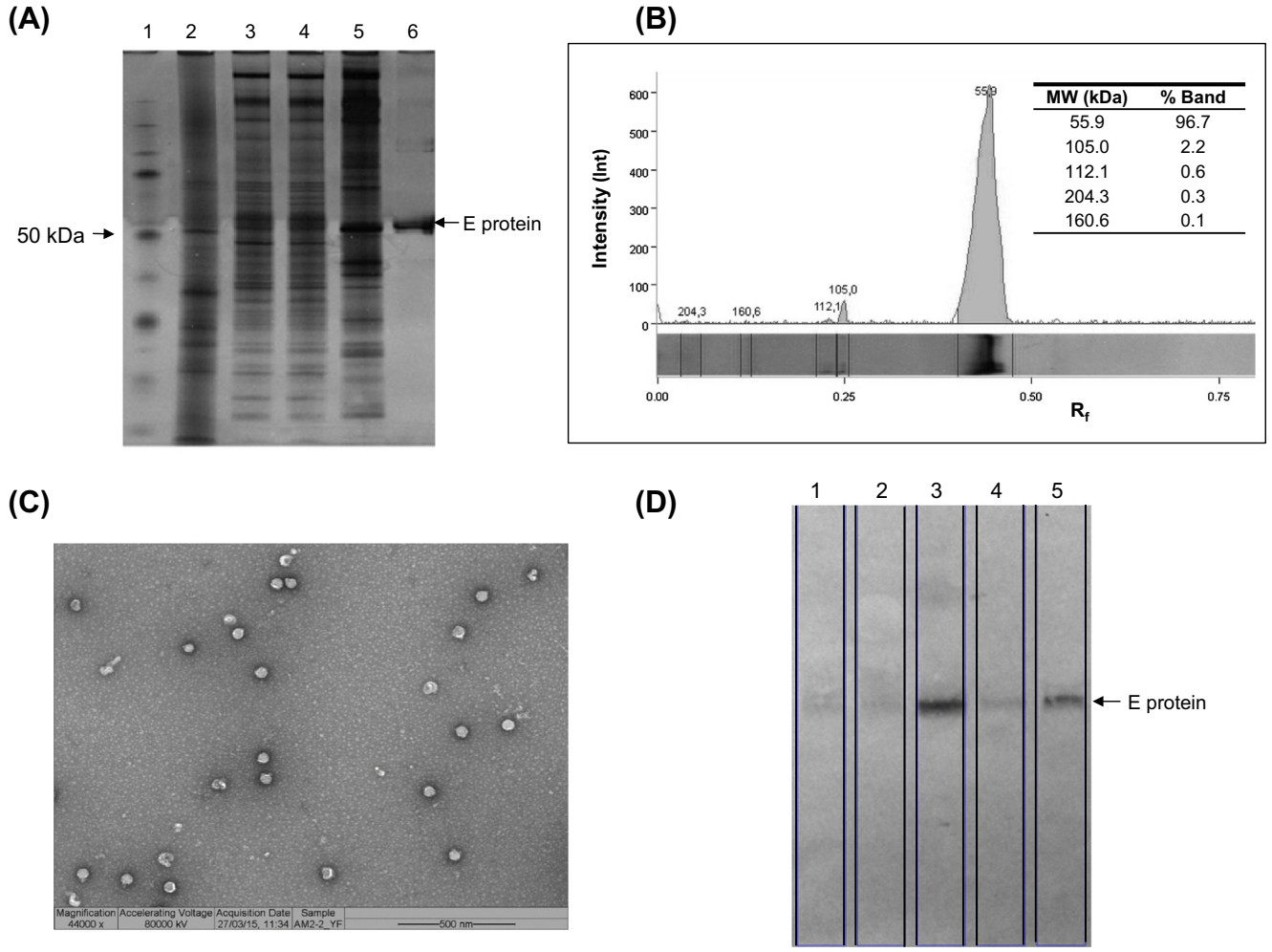

Fig. 2. Purity, identity and morphology of the purified virus. (A) SDS-PAGE, silver staining, 15 μL sample in each lane. Lane 1: Molecular mass marker; lane 2: Vero-HCP commercial standard; lane 3: Clarified viral suspension; lane 4: Clarified viral suspension after adjusting to pH 8.5; lane 5: Eluate of 1st purification step; lane 6: Purified product (after 2nd purification step, under condition 2). (B) ImageMaster® software (GE Healthcare) analysis of YFV purified under condition 2 of polishing step. (C) Transmission electron microscopy (44,000× magnification) of YFV purified under condition 2 of polishing step. (D) Western blot with anti-E antibody. Lane 1: Clarified viral suspension; lane 2: Clarified viral suspension after adjusting to pH 8.5; lane 3: Eluate of 1st purification step; lane 4: Purified product (after 2nd purification step, under condition 2); lane 5: Purified product (after 2nd purification step, under condition 4). Conditions as in Table 1 (condition 2: no HSA, 500 cm/h; condition 4: 0.5% m/v HSA, 150 cm/h).

Table 2
Recovery and residual levels of HCP, DNA and endotoxin for the 2-step purification process developed in the present work.

Step	Recovery (%)	HCP (ppm)	HCP (%)	DNA (ng/dose)*	Endotoxin (EU/dose)*
Clarified Viral Suspension**	–	1,233,802 ± 501,694	123.38 ± 50.17	89.12 ± 26.68	–
Capture (Sartobind®Q MA75)	80.2 ± 4.8	85,970 ± 37,833	8.60 ± 3.78	1.30 ± 1.55	–
Polishing (Capto™ Core 700)	65.7 ± 4.8	345 ± 25	0.04 ± 0.01	1.17 ± 0.35	0.29
Overall yield of both chromatography steps was 52.7%					

* A dose containing 8 μg of viral E protein (quantified by ELISA) was considered.
** Clarified Viral Suspension after addition of sorbitol and pH adjusted to 8.5.

2. The image shows the expected morphology and size for yellow fever virus, thus confirming that the integrity of the virus particles was maintained throughout the downstream processing steps, as intended.

Fig. 2D shows a Western blot using anti-E antibody for detection. E protein band was detected in all lanes, confirming the identity of the sample in each processing step, and for purification both under conditions 2 and 4. Equal volumes of sample were applied, so that band intensity varied according to the concentration of E protein in the sample, which was higher after the anion-exchange step.

3.4. Consolidated purification process

Table 2 presents virus recovery, residual levels of HCP and DNA in the clarified Vero cell culture supernatant and after the capture (Sartobind® Q) and polishing steps (Capto™ Core 700), as well as the overall yield of the 2-step process developed in this study.

Overall yield of both chromatography steps was 52.7%, thus in agreement with literature data about purification of different viral particles. Weigel et al. [19] reported an overall recovery of 68% for the purification of influenza virus, whereas for adenoviral vector an overall yield of 52% was reported [18]. For virus-like particles

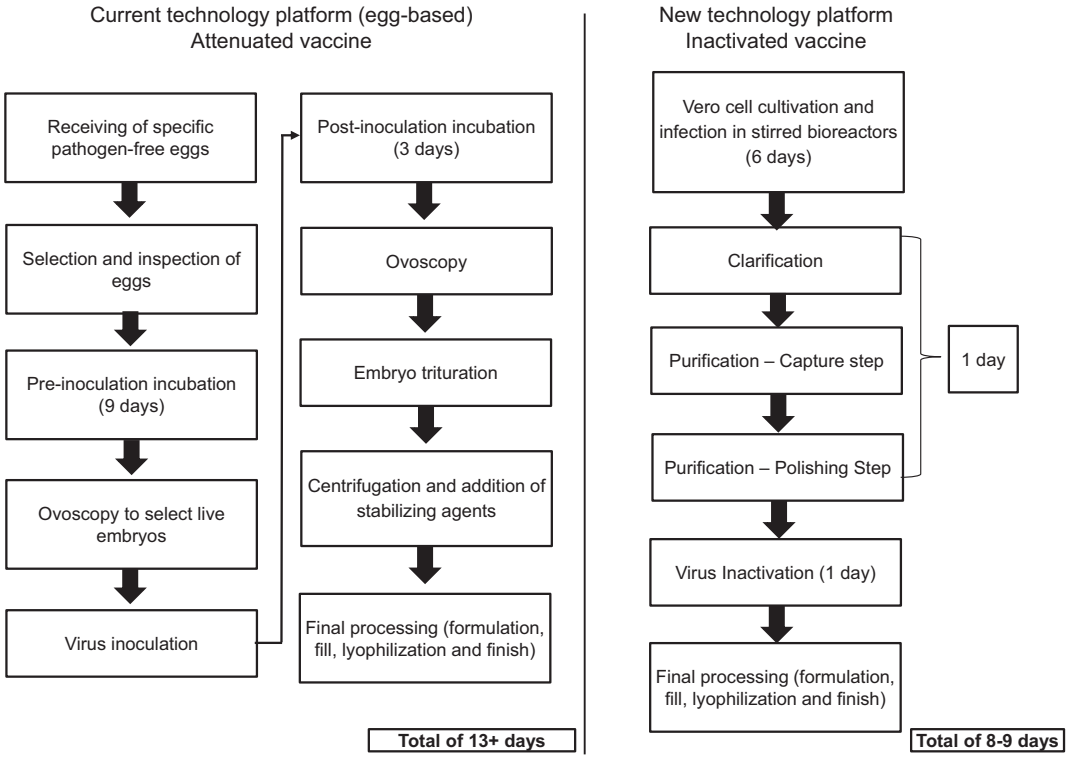

Fig. 3. Comparison of the current egg-based production process for the yellow fever live-attenuated vaccine and the new process proposed herein for a new inactivated vaccine.

(VLPs) of rotavirus, Vicente et al. [17] found a recovery of 46%, whereas membrane-base purification of recombinant baculoviruses resulted in an overall yield of 40% [33].

DNA levels obtained meet the requirements of the WHO [29], but it would be interesting to determine the size of DNA fragments, since some agencies establish that they should be smaller than 200 base pairs [34].

There is no clear specification for residual levels of HCP by regulatory authorities. Thus, the values achieved in the present work would have to be submitted for case-by-case evaluation by regulators. Important aspects to be considered to evaluate theses limits are: (i) maximum administered dose (μg antigen/body weight); (ii) frequency and length of administration; (iii) data from nonclinical and clinical studies [31].

4. Comparison to the current egg-based, attenuated vaccine technology and outlook

The design of purification processes for vaccine manufacturing must keep in mind different requirements [35]: (i) easy scale-up and ability to process large volumes; (ii) preservation of antigen structure; (iii) final product meeting specifications of regulatory agencies; (iv) economic feasibility.

Based on the data presented herein, the process designed in the present work is likely to meet these requirements, considering the scalability of the anion-exchange membrane adsorber step and the multimodal chromatography step, as well as the integrity of the final purified yellow fever virus particles. Residual DNA levels were below the limits established by the WHO, and HCP levels were in a range that is common for approved human vaccines.

When compared in mice immunogenicity and challenge studies to the current egg-based live-attenuated YF vaccine, a regimen of 3 doses of 2 μg of the inactivated vaccine produced by the technology proposed herein and adsorbed to aluminum hydroxide (alum) was equally able to confer 100% protection to mice challenged intracerebrally with YFV [30].

In terms of the manufacturing technology, the process proposed herein, based on cultivation and infection of Vero cells in stirred-tank bioreactors, followed by clarification, chromatography purification steps and beta-propiolactone inactivation, uses robust unit operations and results in a process that is shorter and more simple than the current egg-based process used for the live-attenuated vaccine (Fig. 3).

The yield of the current egg-based production platform is of about 200–400 doses per specific pathogen-free (SPF) egg. According to the data obtained in this work (E protein content in the clarified supernatant of 62.8 μg/mL), considering a 40% overall purification yield to account also for clarification and any other losses, and estimating a dose of 8 μg, one liter of the clarified supernatant would yield approximately 3140 doses. Thus a 320-L bioreactor batch would result in about 1 million doses. To produce the same amount of egg-based vaccine doses, 2500–5000 SPF eggs are required. We know that this is a preliminary comparison of technologies, since the exact human dose and vaccination schedule to be adopted for the new inactivated vaccine are not known yet and will possibly involve 3 doses, as compared to a single dose for the current live-attenuated vaccine.

The advantages related to the scalability of the technology proposed herein, avoiding future shortages of yellow fever vaccine in the world as experienced in the last few years, and the advantages related to the elimination of the rare, but fatal adverse effects related to the live-attenuated vaccine, indicate how promising the proposed production platform can be for the introduction of a new yellow fever vaccine, which could play a very important role in the future, contributing to protect the world population from YFV. As stated by Paules and Fauci [9], public health awareness and preparedness regarding this reemerging threat are critical at this moment, since YF is a "historically devastating disease" and

YFV is "the most severe arbovirus ever to circulate in the Americas".

Conflict of interest

The authors declared that there is no conflict of interest.

Acknowledgements

The authors wish to thank the Brazilian research funding agencies Faperj, CNPq and Capes for financial support.

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