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Purification by membrane technology of an intracellular *Ehrlichia ruminantium* candidate vaccine against heartwater

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Abstract

This work describes the development of a downstream process based upon membrane technology for the purification of *Ehrlichia ruminantium* (ER) elementary bodies, which can be used as an inactivated vaccine against heartwater for wild and domestic ruminants.

Currently, ER purification is performed by a time consuming multistep centrifugation leading to a high level of host endothelial cell protein contamination. Herein, a simple and scaleable process based on depth filtration for clarification, and tangential flow filtration for concentration to effectively recover ER from infected endothelial cell microcarrier cultures is described. Specifically, depth filtration using 20 and 3 μm pore size membranes was applied to remove microcarriers from the bulk culture while tangential flow filtration was used to simultaneously remove additional cell debris and concentrating the ER to an appropriate level of volume reduction. The effects of transmembrane pressure and tangential filtration mode on ER purification were evaluated; three purification processes were compared to the commonly used centrifugation technique. Results showed that an ER recovery yield of 58% and volume reduction of 87% was achievable in less than 1 h of processing time when using membrane-based processes.

This process enables a rapid purification of ER elementary bodies with a minimum of unit operations, reducing the overall cost of the vaccine production; similar approaches may be applied for the purification of other obligate intracellular bacteria with emerging impact on human and animal health.

Keywords: *Ehrlichia ruminantium*; Downstream processing; Depth filtration; Tangential flow filtration; Heartwater vaccine

1. Introduction

Ehrlichia ruminantium (ER), formerly *Cowdria ruminantium*, an obligate intracellular bacterium of vascular endothelial cells, is the causative agent of heartwater, an acute and frequently fatal tick-borne disease of domestic and wild ruminants, ranking as one of the most important vector borne afflictions of livestock in sub-Saharan Africa and the Caribbean islands [1–3]. With increased trade movement of animals, heartwater may also represent a significant threat to the domestic livestock industry in the more developed northern region [1]. At the moment, a vaccine based on the inactivated elementary bodies of the bacterium produced in endothelial cell cultures is the best candidate for protection against heartwater [4–6].

Recently, a cost-effective scaleable process for mass production of ER has been developed to overcome many of the disadvantages of the current process, in particular, limited production scale and use of serum containing medium [7]. As large quantities of ER can now be produced using stirred tank bioreactors and microcarrier technology, it is relevant to develop an efficient purification process for large-scale application.

Different strategies have been used to recover ER from cell suspension, namely sucrose or Percoll density gradient centrifugation [8,9], immunoadsorbent and cellular affinity chromatography [10,11], differential lysis method [12] and magnetic cell separation [13]. Presently, a multistep centrifugation strategy is being used [6]; although it is possible to achieve high amount of purified ER for vaccination purposes, it has drawbacks as it leads to detrimental effects on the

Abbreviations: ER, *Ehrlichia ruminantium*; DV, diafiltration volume; TMP, transmembrane pressure; VCF, volume concentration factor

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organisms, reducing the final bacterial yields, and high level of host cell protein contamination, while being time consuming, and not amenable to scale-up. Additionally, this methodology has been mostly limited to laboratory scale utilization with bacterial suspension obtained from static culture.

To overcome these issues and develop a robust and scaleable downstream process allowing for high yields of purified intact ER, it is necessary to envisage both the efficient separation of microcarriers from bulk culture as well as the ER from the cellular debris. Herein, a fast and simple method for ER purification using membrane technology is proposed; indeed, since cost reduction is critical for veterinary vaccine production, expensive chromatographic processes are avoided. Furthermore, disposable membrane technology deals away with cleaning and sterilizing issues [14].

Depth and tangential flow filtration are frequently used in the biotechnology industry [15–17]. Depth filtration can be used for bulk clarification: particulates too large to pass through the pores of the membrane will accumulate in the depth of the filtration media, while smaller molecules pass through. In tangential flow filtration, as the fluid is pumped tangentially along the surface of the membrane, the retained components do not build up at the surface of the membrane as they are swept along by the tangential flow.

The downstream process developed herein reflects the advantages of these two filtration techniques. The process was designed as two steps: (i) clarification by depth filtration for an efficient removal of microcarriers, cells and some cell debris and (ii) crossflow filtration (using cassettes or hollow fibers) to obtain highly concentrated stocks of intact ER for vaccination purposes. As mentioned above, owing to the extremely labile nature of ER and the difficulty in maintaining the integrity and viability of the isolated organisms [2,18] special attention was paid to optimize the recovery yield of the intact bacteria. For this procedures with respect to time, transmembrane pressure (TMP), volume concentration factor (VCF) and diafiltration volume (DV) were evaluated along the different steps of the purification.

2. Materials and methods

2.1. Cell line and bacterial strain

Bovine aortic endothelial (BAE) cells and *E. ruminantium* Gardel (ERG) were kindly provided by Dr. D. Martinez (CIRAD/EMVT, France) and routinely cultured as described elsewhere [18]. Briefly, whenever the monolayer reached 100% confluence the BAE cells were either trypsinized and subcultured with a splitting ratio of 1/2 or infected with ER. In static culture conditions, the ER Gardel (ERG) strain was routinely propagated in BAE cells using a serum-containing culture medium and a multiplicity of infection (MOI) of 400 ER/cell [7,18].

2.2. E. ruminantium production

Production of ER Gardel was performed in 2 l bioreactors (B. Braun, Germany), as previously described by our group [7]. Briefly, after BAE cell inoculation at 0.25×10^6 cells/ml on a non-porous microcarrier, Cytodex 3 (6 g/l) (GE Healthcare, USA), the microcarrier culture was maintained for 96 h at 37 °C, pH 7.2 and 30% of dissolved oxygen (bubble-free system), using a 3D-blade segment impeller. After 96 h post-inoculation, the confluent beads were allowed to settle down, the supernatant was completely removed from the vessel and, after one medium exchange to serum-free culture conditions, the cells growing in microcarriers were inoculated with the bacterial suspension (66 ERG per BAE cell); then cells plus bacteria were incubated for 2 h at 37 °C and 5% CO_2 (at 30 rpm) to allow the bacteria to adhere to the host cells. Afterwards, serum-free culture medium was added to a final volume of 2 l. No additional media exchanges were performed. In these studies, the ERG used was from passage 30 to 70. The ER Gardel culture bulk was harvested at 113 hpi (hours post-infection), identified as the best time of harvesting to obtain the maximum amount of intact ER Gardel elementary bodies, the extracellular infective stage of the bacterium [18].

2.3. Downstream processing

2.3.1. Multistep centrifugation process

The multistep centrifugation methodology was performed as described elsewhere [6] with some minor modifications. Briefly, and as mentioned above, the ER Gardel suspension (constituted by free ER, cell debris and Cytodex 3 microcarriers) was collected at 113 hpi [18] and passed through a syringe and 26 Gauge 3/8 inch needle to increase ER yield and to avoid the formation of ER aggregates. After serial washing steps with phosphate-buffered saline (PBS, pH 7.4) to remove the maximum amount of Cytodex 3, the supernatant was cleared of cellular debris by centrifuging at $1800 \times g$ for 15 min at 4 °C. The resulting supernatant was then subjected to high-speed centrifugation at 4 °C ($20{,}000 \times g$, 30 min) in order to pellet ER. The resulting pellet was re-suspended in PBS for further analysis by real time PCR (to assess recovery yields of intact ER) and total protein determination.

2.3.2. Membrane based process

Prior to use, all membranes were flushed with deionized water. All the experiments were performed with NaCl (150 mM) since previous results have shown its ability to sustain ER integrity (and corresponding antigenic properties), during long-term storage (up to 3 months) at −20 °C [19]. All processes described below were performed in a sterile environment (inside a laminar flow hood) at room temperature (approximately 20 °C).

- *Clarification step*: Bulk from a 2 l bioreactor was harvested; then, using a peristaltic pump (Watson-Marlow, USA) at a flowtrough of 400 ml/min the bulk liquid was passed through one or two polypropylene depth filters (Sartorius, Germany) with 0.2 m^2 filtration area, with 20 and/or 3 μm pore size, respectively. Filter performance was evaluated by comparing the number of intact ER in the initial bacterial suspension with the harvested filtered ER. Harvests were then purified through tangential flow filtration in a laminar flow hood at room temperature.

- *Tangential flow filtration*: Tangential flow filtration was conducted using a flat sheet Sartocon Slice 200 cassette (Sartorius, Germany) with a Hydrosart membrane and a nominal filtration surface area of 0.02 m^2. A peristaltic pump was used to pump the feed solution during filtration, the retentate was returned to the input reservoir while the permeate was removed to a separate vessel. To determine the optimal operation parameters initial feed volumes of 500 and 600 ml were used. Afterwards, 2 l were processed in order to obtain the vaccine.

Different transmembrane pressures (TMP) were tested (5×10^4, 8×10^4, 13×10^4, 15×10^4 and 17×10^4 Pa) for three different crossflow rates (30, 40 and 54 ml/min). Samples of the retentate and permeate were collected and analyzed by real time PCR for monitoring intact ER titers over time. Afterwards, in order to improve the ER recovery yields and exchange the media, diafiltration was tested. This is a specialized type of ultrafiltration process in which the retentate is diluted with buffer and re-ultrafiltered, to reduce the concentration of soluble permeate components and further increase the concentration of retained components; increasing diafiltration volume (1, 2, 2.5, 5 and 7.5) were tested and final samples were analyzed for intact ER and total protein content.

Another strategy was also evaluated using hollow fiber cartridges coupled to a QuixStand Benchtop System (GE Healthcare, USA). After clarification with a depth filter of 20 μm, a microfiltration step was performed with a hollow fiber