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# Purification of *Trypanosoma cruzi* metacyclic trypomastigotes by ion exchange chromatography in sepharose-DEAE, a novel methodology for host-pathogen interaction studies

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ABSTRACT

Metacyclic trypomastigotes are essential for the understanding of the biology of *Trypanosoma cruzi*, the agent of Chagas disease. However, obtaining these biological stages in axenic medium is difficult. Techniques based on charge and density of the parasite during different stages have been implemented, without showing a high efficiency in the purification of metacyclic trypomastigotes. So far, there is no protocol implemented where sepharose-DEAE is used as a resin. Therefore, herein we tested its ability to purify metacyclic trypomastigotes in Liver Infusion Triptose (LIT) medium cultures. A simple, easy-to-execute and effective protocol based on ion exchange chromatography on Sepharose-DEAE resin for the purification of *T. cruzi* trypomastigotes is described. *T. cruzi* strains from the Discrete Typing Units (DTUs) I and II were used. The strains were harvested in LIT medium at a concentration of $1 \times 10^7$ epimastigotes/mL. We calculated the time of trypomastigotes increment (TTI). Based on the data obtained, Ion exchange chromatography was performed with DEAE-sepharose resin. To verify the purity and viability of the trypomastigotes, a culture was carried out in LIT medium with subsequent verification with giemsa staining. To evaluate if the technique affected the infectivity of trypomastigotes, *in vitro* assays were performed in Vero cells and *in vivo* in ICR-CD1 mice. The technique allowed the purification of metacyclic trypomastigotes of other stages of *T. cruzi* in a percentage of 100%, a greater recovery was observed in cultures of 12 days. There were differences regarding the recovery of metacyclic trypomastigotes for both DTUs, being DTU TcI the one that recovered a greater amount of these forms. The technique did not affect parasite infectivity *in vitro* or/and *in vivo*.

## 1. Introduction

The kinetoplastid parasite *Trypanosoma cruzi* is the etiologic agent of Chagas disease, a tropical pathology that affects around 8 million people around the world (http://www.who.int/mediacentre/factsheets/fs340/en/). This parasite exhibits remarkable genetic variability and is subdivided into at least 6 discrete typing units (DTUs): *T. cruzi* I–VI and a recent described genotype associated to bats (TcBat) (Zingales et al., 2009; Brenière et al., 2016). These DTUs are associated with different clinical manifestations, epidemiological cycles of transmission and geographical regions (Hernández et al., 2016).

*T. cruzi* has a complex life cycle that occurs among humans, mammalian reservoirs and triatomine insects of the subfamily Reduviidae, displaying several morphological stages with distinct antigenic characteristics (Tyler and Engman, 2001). One of the most important processes during its life cycle is metacyclogenesis, which is defined as the transformation of replicative epimastigotes into infective metacyclic trypomastigotes (Kollien and Schaub, 2000). They develop in the rectum of the triatomine, and are implicated in the transmission of the parasite to the vertebrate host (Avila et al., 2003; Garcia et al., 2010).

*T. cruzi* infective metacyclic trypomastigotes have been fundamental for the understanding of the biology of the parasite, as well as for the

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infection of cells and insects. Some methods are available to recover pure metacyclic trypomastigotes from axenic media such as Liver Infusion Tryptose (LIT), Triatomine Artificial Urine (TAU) and M16. Pure (100%) metacyclic trypomastigotes cannot be obtained using such media and as a result, cultures containing epimastigote stages are obtained (Camargo, 1964; Abegg et al., 2017). Therefore, in the eve of Next Generation Sequencing era and the new technologies that have arisen to conduct host-pathogen interaction studies, it is pivotal to have an easy, fast and reliable tool to purify total metacyclic trypomastigotes in the sample (Camargo, 1964; Castanys et al., 1984). Among them, density separation with percoll (Castanys et al., 1984; Rimoldi et al., 1986), and separation techniques based on the differential plasma membrane charge between epimastigotes and trypomastigotes using ion exchange chromatography with resins such as cellulose-DEAE, dE − 52 and sephadex, have been developed for the purification of metacyclic trypomastigotes (Abbassy et al., 1972; Gutteridge et al., 1978; Chao and Dusanic, 1984). These methods include the purification of blood and intracellular forms of *T. cruzi* (Gutteridge et al., 1978; Schmatz and Murray, 1981; de Sousa and 33, 1983).

The extensive use of chromatography has permitted purifying metacyclic trypomastigotes for the association of specific molecules expressed on this parasite stage membrane that are either involved in cell invasion (mainly transialidases as the gp82 and gp90), insect epithelial cell invasion and adhesion to gastric mucin (Neira et al., 2003; Manque et al., 2000; Bayer-Santos et al., 2013), effective drug evaluation for the different *T. cruzi* stages (Orrego et al., 2014; Villamizar et al., 2017) and molecular characterization of membrane protein families from metacyclic trypomastigotes involved in invasion and resistance (Martins et al., 2015; Cestari and Ramirez, 2010). This methodology has also been employed to purify parasite proteins such as alkaline kinase, coimmunoprecipitation between CK2 proteins and tubulin, as well as extracting proteins expressed in tissues infected with the parasite (Morris et al., 1990; Santana et al., 1992; de Lima et al., 2006). Moreover, it has been also used to obtain *Trypanosoma evansi* antigens (Camargo et al., 2004), *Fasciola hepatica* hemoproteins and some bacterial antigens from *Escherichia coli* and virulence factors from *Helicobacter pylori* (McGonigle and Dalton, 1995; Sigdel et al., 2004; Shih et al., 2013; Hong et al., 2017). However, to date, no protocol has been reported where sepharose-DEAE is used as a resin for *T. cruzi*. Based on the above-mentioned data, and considering the necessity of purifying metacyclic trypomastigotes. The aim of this work was to describe a simple, easy and effective protocol based on ion exchange chromatography in sepharose-DEAE resin for the purification of *T. cruzi* metacyclic trypomastigotes.

## 2. Materials and methods

### 2.1. Parasites - metacyclogenesis curves

A total of $1 \times 10^8$ epimastigotes/mL from the MDID/BR/84/DM28 (TcI), and MHOM/BR/53/Y (TcII) strains were cultured in LIT medium supplemented with 5% inactivated fetal calf serum, 5% $CO_2$ at 26 °C. The concentration of parasites was determined daily by Neubauer chamber and the discrimination between stages (epimastigotes and trypomastigotes) was evaluated in Giemsa stained slides. This allowed the differentiation of these stages based on the location of the kinetoplast, nucleus and modifications in the flagellum. Epimastigotes present a compact nucleus in the middle of the cytoplasm, a kinetoplast located in the anterior part of the parasite, and just after this is observed the flagellum. On the other hand, metacyclic trypomastigotes show an elongated nucleus, kinetoplast located in the posterior part of the parasite and finally a flagellum that surrounds the cytoplasm of the parasite from the posterior to the anterior section. With the data obtained during approximately 12 days, curves of metacyclogenesis were performed and the day where a significant increase of trypomastigote forms was recorded with respect to day 0. This was defined as time of trypomastigotes increment (TTI). A total of 3 replicates (3 cultures) for each of the strains were used to avoid bias in the evaluation of metacyclogenesis.

### 2.2. Purification of metacyclic trypomastigotes

The ion exchange chromatography technique on sepharose membrane-DEAE was standardized to obtain a medium of pure metacyclic trypomastigotes (free of epimastigotes), as follows:

The stationary phase corresponded to the DEAE sepharose resin and the mobile one to PBS plus 4.5% glucose (PBG). A pH = 8.0 is required for the compound coupled to the DEAE-sepharose to maintain a negative charge. Subsequently, 2 mL of DEAE-sepharose were added to a column that allowed the flow of PBG (the resin was equilibrated to pH 8 by adding 30 mL of PBG). The pH of the column was verified before further development of the technique. A total of three replicates of epimastigote-trypomastigote cultures from each of the strains (MDID/BR/84/DM28 (TcI), and MHOM/BR/53/Y (TcII)) that were on the TTI day were used from 10 mL cultures in LIT medium, and were centrifuged at 2500 rpm. The supernatant was discarded and the pellet obtained was resuspended in PBG, and further centrifugation was performed to remove residues from the LIT medium that could interfere with the chromatography. The supernatant was discarded again and the pellet was retained. Finally, the parasites were suspended in 5 mL of PBG, and transferred to the equilibrated DEAE column. 5 mL of eluate were collected and then centrifuged, the supernatant was discarded with a Pasteur pipette to prevent loss of trypomastigotes of the pellet preserving 1 mL of the content. In order to confirm the results, a total of 3 replicates were performed, the replicates made were analyzed by the methodologies Sections 2.3 and 2.4 described in this article. The procedure described above was performed for each of the three replicates of both strains.

### 2.3. Giemsa staining and quantification of trypomastigotes

The parasites obtained were quantified in a Neubauer chamber and Giemsa staining was performed to evaluate the efficiency in the recovery of trypomastigotes (discriminating between the parasite stages present). For the quantification of trypomastigotes obtained, a 1:10 dilution was made with 10 μL of the eluate obtained and 90 μL of PBG. An aliquot of 10 μL of the dilution was dispensed in the Neubauer chamber. Reading was performed by counting the parasites present in the 4 quadrants of the chamber, followed by the calculation of the average of total parasites on the number of quadrants, and multiplying this result by 10,000 and the dilution used, which in this case corresponds to 10. The evaluation and differentiation between *T. cruzi* stages was carried out using the previously described methodology by observing the kinetoplast, nucleus and flagellum. The concentration of trypomastigotes before and after the purification was calculated with the data obtained.

### 2.4. Cultivation in LIT medium

One of the characteristics that *T. cruzi* exhibits is the replication ability only present in the amastigote and epimastigote stages (Tyler and Engman, 2001). Therefore, a culture of the final eluate in LIT medium supplemented with 5% fetal bovine serum was performed for 8 days to evaluate the purity of the trypomastigotes collected in the eluate. If there were epimastigotes in the eluate, this could represent evidence of concentration increase and subsequently presence of pure epimastigotes in the LIT culture.

### 2.5. Cell culture

Infections in VERO semiconfluent cells (Vero (ATCC® CCL-81™) were performed to verify *in vitro* susceptibility of Vero cells to infection