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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

with TcI and TcII metacyclic trypomastigotes. Approximately 700,000 cells/mL were infected with 4,000,000 parasites/mL of each strain and maintained in 25 $cm^2$ flasks at 37 °C with 5% $CO_2$. The first culture replacement with Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% SFB was performed on the second day of infection and thereafter every five days. The cultures were observed daily under inverted microscopy in the search for amastigotes or trypomastigotes. A total of three replicates were employed.

### 2.6. Infection in mice

A set of four Twenty-six-day ICR-CD1 mice were infected intraperitoneally with a $1 \times 10^6$ trypomastigotes of each of the strains to test the infectivity of trypomastigotes *in vivo* (two replicates per strain). The biomodels were maintained at the animal facility of the Universidad de Los Andes, Colombia, under biosecurity standards, macroambient conditions, humidity 60% ± 5, temperature 20 °C ± 2, ventilation of 10 to 15 changes per hour, housed in type T3 stainless steel cages of 48 cm of length, 26 cm of width and 16 cm of height, with a capacity of 3 animals per cage, supply of water and food *ad libitum*, following the principles for the experimentation with animals stipulated in the Bioterium and following national and international regulations. To evaluate the parasitemia, 50 μL of blood were obtained from the animal's tail, during 16, 30 and 45 days of testing, obtaining three samples per mouse and a total of 6 samples per strain (2 replicates). The samples obtained were submitted to extraction of DNA using the Roche High Pure PCR Template Preparation Kit, followed by a quantitative qPCR, following the protocol of Duffy et al., 2012.

### 2.7. Statistic analyses

The data obtained were tabulated in Excel 2015 for the performance of the metacyclogenesis curve using 2 replicates per strain. The graphs corresponding to the metacyclogenesis curve, as well as the statistical analyses (normality, ANOVA and multiple comparisons between the production of trypomastigotes during the different days of the metacyclogenesis curve), were performed in the GraphPad Prism 6 software.

## 3. Results and discussion

### 3.1. Parasites - metacyclogenesis curve

The concentration of parasites used ($1 \times 10^8$) induced the metacyclogenesis of epimastigotes from day 1 post culture (DPC) for both strains (Fig. 1), which could be related to a nutritional stress and a redox status caused by the decrease of nutrients generated by the high concentration of parasites in the medium. Nutritional stress is considered a prime factor that triggers the metacyclogenesis of the parasite, due to the activation of enzymes such as adenylate cyclase and subsequent increase of cAMP in the medium (Nogueira et al., 2015; Hamedi et al., 2015; Shaw et al., 2016). However, considering that these trypomastigotes may correspond to an earlier culture, the TTI was estimated. Both strains exhibited an increase in metacyclogenesis from 4 DPC (Fig. 1A). Despite this, when performing the ANOVA test and the analysis of multiple comparisons, statistically significant differences were only observed with TTI from 8 DPC for the MDID/BR/84/DM28 (TcI) strain (Fig. 1B), and 6 DPC for the MHOM/BR/53/Y strain (TcII) (Fig. 1C), with lower concentrations of transformed trypomastigotes for the TcI strain. These results could be related to the genetic and biological differences that have been observed between these DTUs, and are most likely associated with the fundamental role played by the parasite membrane (Abegg et al., 2017). It should be noted that the MHOM/BR/53/Y strain maintained a constant trypomastigote concentration from TTI day.

### 3.2. Purification of metacyclic trypomastigotes

One of the characteristic features of epimastigote and trypomastigote stages is the difference in plasma membrane charge, as has been previously demonstrated in several studies (Abbassy et al., 1972; Gutteridge et al., 1978). We decided to test the purification of metacyclic trypomastigotes from a culture in LIT medium, which also had abundant epimastigotes (Fig. 1D–E), using the DEAE-sepharose affinity chromatography technique, where DEAE bound to the resin has a high positive charge. This compound captures the epimastigotes due to the formation of an electrostatic ionic bond between the DEAE and the parasite membrane. The trypomastigotes do not present this charge and pass through the resin and can be recovered in the eluate. The ion exchange chromatography with DEAE sepharose resin allowed the separation of 100% pure metacyclic trypomastigotes from the epimastigote stages under the conditions mentioned above. The results were obtained from visualizing three replicates of 200 parasites in one Giemsa-colored slide and daily observation for a period of 8 days of an eluate culture in LIT medium (Fig. 2C–D). Another parameter analyzed was the recovery of trypomastigotes; for this purpose, the amount of trypomastigotes before and after the separation were determined. It was observed that the technique allowed the recovery of a smaller number of trypomastigotes compared to those that were initially in the LIT media and that these differences were higher for the MHOM/BR/53/Y strain (Fig. 2A). The differential expression of some transialidases such as gp82 and gp90 among different strains has been demonstrated, which could be related to lower levels of sialic acid in the membrane that would lead to a modification in the membrane charge. Considering that the sialic acid is the main contributor to the membrane charge of the parasite and related to the infectivity capacity of *T. cruzi*, differences in the parasite yield obtained after purification were expected between strains (Franchin et al., 1997). However, we did not observe epimastigotes (stages with negative charge), in the obtained eluate which could indicate an increase in the negative charge of MHOM/BR/53/Y strain trypomastigotes and therefore an increase in the affinity for the resin, which could explain the low concentrations of trypomastigotes obtained in the eluate, confirming the high biological differences that could be associated with differences in the plasma membrane (Neira et al., 2003; Manque et al., 2000; Bayer-Santos et al., 2013).

The changes in the recovery of trypomastigotes observed when performing the technique could be due to the presence of immature stages of metacyclic trypomastigotes that still possess epimastigote characteristics like membrane charge and could be retained by the sepharose resin – DEAE (Kollien and Schaub, 2000). The Sepharose - DEAE chromatography resulted in a greater recovery of trypomastigote under these conditions (Fig. 2B) using parasites corresponding to 12 DPC. This was probably due to complete differentiation into metacyclic trypomastigotes. These results confirm the remodeling that the parasite undergoes in the plasma membrane, that is most likely associated with the function exerted by the trypomastigote stages and its relation with the infective capacity (Serrano et al., 1995; de Andrade et al., 1991; Schenkman et al., 1993; Chaves et al., 1993; Yoshida et al., 1997). Finally, we decided to evaluate some parameters and conditions that allow an efficient set-up for the technique. An optimal volume of 2 mL DEAE-sepharose was found for a 10 mL column; resin saturation was observed when $1 \times 10^9$ parasites/mL were loaded, allowing the passage of epimastigotes; moreover, the maximum recovery volume (eluate obtained) was 5 mL, since a larger volume would allow the passage of epimastigotes.

### 3.3. In vitro and in vivo evaluation

Considering that the metacyclic trypomastigotes correspond to the infective stage of the parasite, we decided to evaluate if the DEAE-sepharose chromatography could affect this characteristic. An *in vitro* test was performed to solve this concern in VERO cells and *in vivo* in ICR-

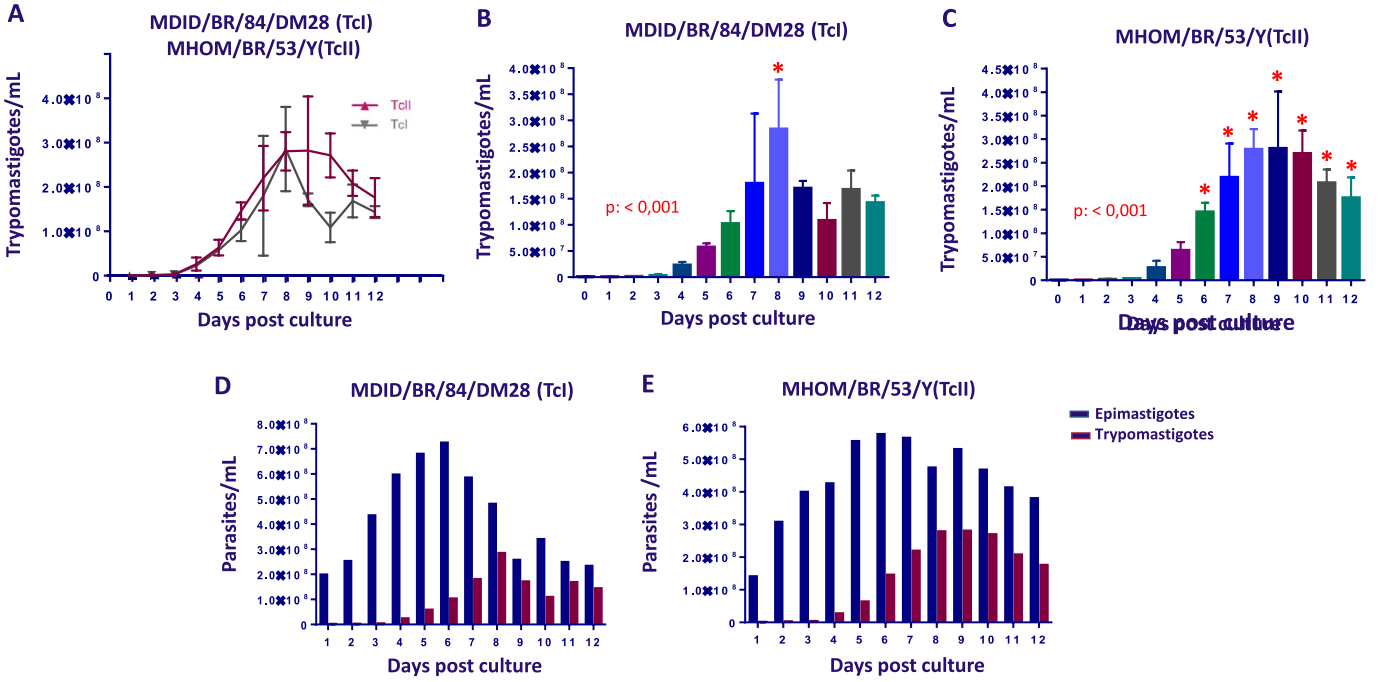


**Fig. 1.** Metacyclogenesis *in vitro*. A. Metacyclogenesis Curves TcI and TcII. B. TcI metacyclic trypomastigotes. C. TcII Metacyclic trypomastigotes. D. Comparison of epimastigotes and TcI metacyclic trypomastigotes. E. Comparison of epimastigotes and TcI metacyclic trypomastgiotes. The results were statistically significant when $p < 0.05$. The results were obtained using 3 replicates.

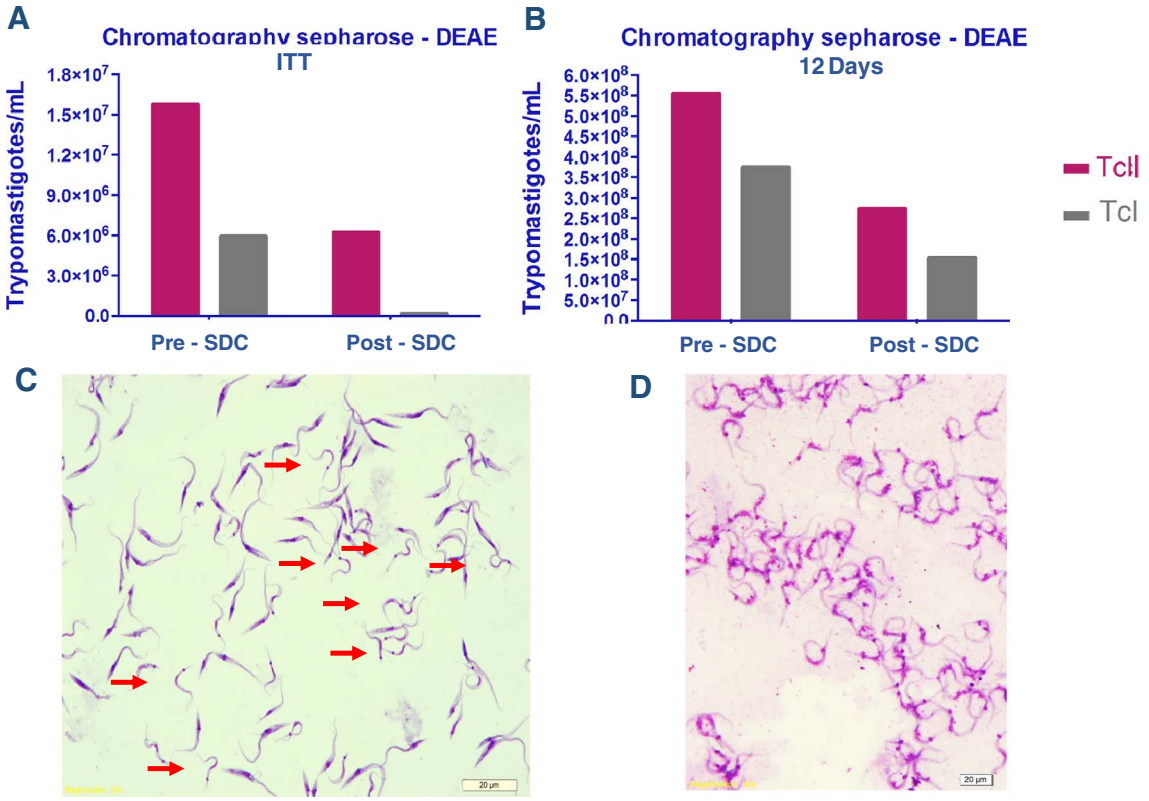


**Fig. 2.** Sepharose Chromatography - DEAE. A. Chromatography day time of trypomastigotes increment (TTI). B. Chromatography day 12, C. Metacyclic trypomastigotes and epimastigotes in Liver Infusion Tryptose (LIT) medium. B. Trypomastigotes obtained by chromatography in sepharose-DEAE.

CD1 mice. The *in vitro* analysis showed the ability of trypomastigotes of the TcI (MDID/BR/84/DM28) and TcII (MHOM/BR/00/Y) strains to develop, where amastigote nests and free trypomastigotes were observed at 11 days of infection (Fig. 3A–B). When the *in vivo* infectivity evaluation of both strains was carried out, both mice were positive by qPCR; one of the mice infected with the MHOM/BR/00/Y strain showed a decrease in motor activity, piloerection and finally death. The samples were positive for *T. cruzi* from day 16 until the end of the test

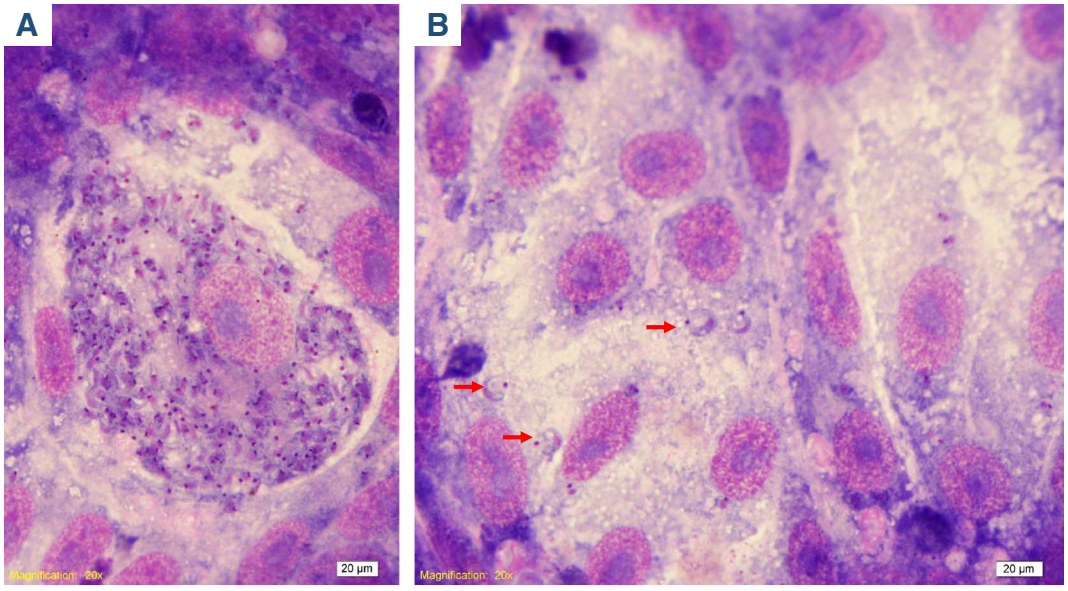


**Fig. 3.** *In vitro* and *in vivo* evaluation. A. Amastigotes of *T. cruzi* in Vero cells. B. Peripheral blood trypomastigotes in Vero cells.

(16, 30, 45 days). These results are related to the age of the mice and the development of a potent immune system. One of the major disadvantages presented by the chromatography techniques previously described with cellulose-DEAE and the sephadex, was the decrease in the infective capacity of the obtained trypomastigotes (Abbassy et al., 1972; Chao and Dusanic, 1984). In the present study our results have shown that the trypomastigotes obtained by DEAE-sepharose maintain their infectivity both *in vivo* and *in vitro*.

## 4. Conclusions

The methodology herein described allowed the complete purification of metacyclic trypomastigotes cultured in LIT medium which contained epimastigote forms prior to purification. The technique allowed a greater recovery of fully differentiated metacyclic trypomastigotes. The infectivity of trypomastigotes was not affected either *in vitro* or *in vivo*. This technique is a fast and efficient alternative for purifying metacyclic trypomastigote stages with a high purity and also without affecting their infectivity, which represents a technique of great utility in the study of specific characteristics of this parasite stage and also for the comprehension of the *T. cruzi* life cycle and studies related to host-pathogen interactions.

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## References

Abbassy, S.N., Seed, T.M., Kreier, J.P., 1972. Isolation of the trypomastigote form of *Trypanosoma cruzi* from a mixture of the trypomastigotes and epimastigote forms of the parasite by use of a DEAE-cellulose column. J. Parasitol. 58, 631–632.

Abegg, C.P., Abreu, A.P., Silva, J.L., Araújo, S.M., Gomes, M.L., Ferreira, É.C., Toledo, M.J., 2017. Polymorphisms of blood forms and in vitro metacyclogenesis of *Trypanosoma cruzi* I, II, and IV. Exp. Parasitol. 176, 8–15 (doi: 10.1016/j.exppara.2017.02.013).

de Andrade, A.F., Esteves, M.J., Angluster, J., Gonzales-Perdomo, M., Goldenberg, S., 1991. Changes in cell-surface carbohydrates of *Trypanosoma cruzi* during metacyclogenesis under chemically defined conditions. J. Gen. Microbiol. 137 (12), 2845–2849 (PubMed PMID: 1791438).

Avila, A.R., Dallagiovanna, B., Yamada-Ogatta, S.F., Monteiro-Góes, V., Fragoso, S.P., Krieger, M.A., Goldenberg, S., 2003. Stage-specific gene expression during *Trypanosoma cruzi* metacyclogenesis. Genet. Mol. Res. 31 (2(1)), 159–168 (Review. PubMed PMID: 12917812).

Bayer-Santos, E., Cunha-e-Silva, N.L., Yoshida, N., Franco da Silveira, J., 2013. Expression and cellular trafficking of GP82 and GP90 glycoproteins during *Trypanosoma cruzi* metacyclogenesis. Parasit. Vectors 6, 127. http://dx.doi.org/10.1186/1756-3305-6-127.

Brenière, S.F., Waleckx, E., Barnabé, C., 2016. Over six thousand *Trypanosoma cruzi* strains classified into Discrete Typing Units (DTUs): attempt at an inventory. PLoS Negl. Trop. Dis. 10 (8), e0004792 (doi:10.1371/journal.pntd.0004792).

Camargo, E.P., 1964. Growth and differentiation in *Trypanosoma cruzi*. I. Origin of metacyclic trypano-somes in liquid media. Rev. Inst. Med. Trop. 6, 93–100.

Camargo, R.E., Uzcanga, G.L., Bubis, J., 2004. Isolation of two antigens from *Trypanosoma evansi* that are partially responsible for its cross-reactivity with *Trypanosoma vivax*. Vet. Parasitol. 13 (123(1–2)), 67–81 (PubMed PMID: 15265572).

Castanys, S., Osuana, A., Camarro, F., Ruiz-Perez, L.M., 1984. Purification of metacyclic forms of *Trypanosoma cruzi* by Percoll discontinuous gradient centrifugation. Z. Parasitenkd. 70, 443–449.

Cestari, I., Ramirez, M.I., 2010. Inefficient complement system clearance of *Trypanosoma cruzi* metacyclic trypomastigotes enables resistant strains to invade eukaryotic cells. Gruner AC, ed. PLoS ONE 5 (3), e9721 (doi:10.1371/journal.pone).

Chao, D., Dusanic, D.G., 1984. Comparative studies of the isolation of metacyclic trypomastigotes of *Trypanosoma cruzi* by DEAE ion exchange chromatography. Zhonghua Min. Guo Wei Sheng Wu Ji Mian Yi Xue Za Zhi 17 (3), 146–152 (PubMed PMID: 6391853).

Chaves, L.B., Briones, M.R., Schenkman, S., 1993. Trans-sialidase from *Trypanosoma cruzi* epimastigotes is expressed at the stationary phase and is different from the enzyme expressed in trypomastigotes. Mol. Biochem. Parasitol. 61 (1), 97–106 (PubMed PMID: 8259137).

De Lima, A.R., Medina, R., Uzcanga, G.L., Noris Suárez, K., Contreras, V.T., Navarro, M.C., Arteaga, R., Bubis, J., 2006. Tight binding between a pool of the heterodimeric alpha/beta tubulin and a protein kinase CK2 in *Trypanosoma cruzi* epimastigotes. Parasitology 132 (Pt 4), 511–523.

Duffy, T., Cura, C.I., Ramirez, J.C., Abate, T., Cayo, N.M., Parrado, R., et al., Schijman, A.G., 2012. Analytical performance of a multiplex real-time PCR assay using TaqMan probes for quantification of *Trypanosoma cruzi* satellite ADN in blood samples. PLoS Negl. Trop. Dis. 7 (1), e2000 (10.1371/journal.pntd.0002000).

Franchin, G., Pereira-Chioccola, V.L., Schenkman, S., Rodrigues, M.M., 1997. Passive transfer of a monoclonal antibody specific for a sialic acid-dependent epitope on the surface of *Trypanosoma cruzi* trypomastigotes reduces infection in mice. Infect. Immun. 65 (7), 2548–2554.

Garcia, E.S., Genta, F.A., de Azambuja, P., Schaub, G.A., 2010. Interactions between intestinal compounds of triatomines and *Trypanosoma cruzi*. Trends Parasitol. 26 (10), 499–505 (doi: 10.1016/j.pt.2010.07.003. Review. PubMed PMID: 20801082).

Gutteridge, W.E., Cover, B., Gaborak, M., 1978. Isolation of blood and intracellular forms of *Trypanosoma cruzi* from rats and other rodents and preliminary studies of their metabolism. Parasitology 76, 159–176.

Hamedi, A., Botelho, L., Britto, C., Fragoso, S.P., Umaki, A.C., Goldenberg, S., Bottu, G., Salmon, D., 2015. In vitro metacyclogenesis of *Trypanosoma cruzi* induced by starvation correlates with a transient adenylyl cyclase stimulation as well as with a constitutive upregulation of adenylyl cyclase expression. Mol. Biochem. Parasitol. 200 (1–2), 9–18 (doi: 10.1016/j.molbiopara.2015.04.002. Epub Apr 23. PubMed PMID: 25912925).

Hernández, C., Cucunubá, Z., Flórez, C., Olivera, M., Valencia, C., et al., Ramírez, J.D., 2016. Molecular diagnosis of chagas disease in Colombia: parasitic loads and discrete typing units in patients from acute and chronic phases. PLoS Negl. Trop. Dis. 10 (9),

e0004997 (doi:10.1371/journal.pntd.0004997).
Hong, Z.-W., Yang, Y.-C., Pan, T., Tzeng, H.-F., Fu, H.-W., 2017. Differential effects of DEAE negative mode chromatography and gel-filtration chromatography on the charge status of Helicobacter pylori neutrophil-activating protein. Ho PL, ed. PLoS ONE 12 (3), e0173632 (doi:10.1371/journal.pone.0173632).
Kollien, A.H., Schaub, G.A., 2000. The development of *Trypanosoma cruzi* in triatominae. Parasitol. Today 16 (9), 381–387.
Manque, P.M., Eichinger, D., Juliano, M.A., Juliano, L., Araya, J.E., Yoshida, N., 2000. Characterization of the cell adhesion site of *Trypanosoma cruzi* metacyclic stage surface glycoprotein gp82. Mansfield JM, ed. Infect. Immun. 68 (2), 478–484.
Martins, N.O., de Souza, R.T., Cordero, E.M., Cortez, D., Cortez, C., da Silveira, J.F., et al., 2015. Molecular characterization of a novel family of *Trypanosoma cruzi* surface membrane proteins (TcSMP) involved in mammalian host cell invasion. PLoS Negl. Trop. Dis. 9 (11), e0004216 (doi:10.1371/journal.pntd.0004216).
McGonigle, S., Dalton, J.P., 1995. Isolation of Fasciola hepatica haemoglobin. Parasitology 111 (Pt 2), 209–215 (PubMed PMID: 7675536).
Morris, S.A., Wittner, M., Weiss, L., Hatcher, V.B., Tanowitz, H.B., Bilezikian, J.P., Gordon, P.B., 1990. Extracellular matrix derived from *Trypanosoma cruzi* infected endothelial cells directs phenotypic expression. J. Cell. Physiol. 45 (2), 340–346 (PubMed PMID: 1700983).
Neira, I., Silva, F,.A., Cortez, M., Yoshida, N., 2003. Involvement of *Trypanosoma cruzi* metacyclic trypomastigote surface molecule gp82 in adhesion to gastric mucin and invasion of epithelial cells. Infect. Immun. 71 (1), 557–561 (doi:10.1128/IAI.71.1.557-561.2003).
Nogueira, N.P., Saraiva, F.M.S., Sultano, P.E., Cunha, P.R.B.B., Laranja, G.A.T., Paes, M.C., et al., 2015. Proliferation and differentiation of *Trypanosoma cruzi* inside its vector have a new trigger: redox status. PLoS ONE 10 (2), e0116712 (doi:10.1371/journal.pone.0116712).
Orrego, P.R., Olivares, H., Cordero, E.M., Bressan, A., Cortez, M., Araya, J.E., et al., 2014. A cytoplasmic new catalytic subunit of calcineurin in *Trypanosoma cruzi* and its molecular and functional characterization. PLoS Negl. Trop. Dis. 8 (1), e2676 (doi:10.1371/journal.pntd.0002676).
Rimoldi, M.T., Katzin, V.J., González Cappa, S.M., de Bracco, M.M., 1986. Isolation by Percoll gradient centrifugation and radiolabeling of bloodstream forms of *Trypanosoma cruzi*. Rev. Argent. Microbiol. 18 (1), 41–44 (PubMed PMID: 3120241).
Santana, J.M., Grellier, P., Rodier, M.H., Schrevel, J., Teixeira, A., 1992. Purification and characterization of a new 120 kDa alkaline proteinase of *Trypanosoma cruzi*. Biochem. Biophys. Res. Commun. 30 (187(3)), 1466–1473 (PubMed PMID: 1417823).
Schenkman, R.P., Vandekerckhove, F., Schenkman, S., 1993. Mammalian cell sialic acid enhances invasion by *Trypanosoma cruzi*. Infect. Immun. 61 (3), 898–902.
Schmatz, D.M., Murray, P.K., 1981. *Trypanosoma cruzi*: selective isolation of pure trypomastigotes from cultured muscle cells. J. Parasitol. 67 (4), 517–521 (PubMed PMID: 7021789).
Serrano, A.A., Schenkman, S., Yoshida, N., Mehlert, A., Richardson, J.M., Ferguson, M.A., 1995. The lipid structure of the glycosylphosphatidylinositol-anchored mucin-like sialic acid acceptors of *Trypanosoma cruzi* changes during parasite differentiation from epimastigotes to infective metacyclic trypomastigote forms. J. Biol. Chem. 10 (270(45)), 27244–27253 (PubMed PMID: 7592983).
Shaw, A.K., Kalem, M.C., Zimmer, S., 2016. Mitochondrial gene expression is responsive to starvation stress and developmental transition in *Trypanosoma cruzi*. mSphere 1 (2), e00051–16 (doi:10.1128/mSphere.00051-16).
Shih, K.-S., Lin, C.-C., Hung, H.-F., Yang, Y.C., Wang, C.A., Jeng, K.C., Fu, H.W., 2013. One-step chromatographic purification of *Helicobacter pylori* neutrophil-activating protein expressed in *Bacillus subtilis*. PLoS ONE 8 (4), e60786 (doi:10.1371/journal.pone.0060786).
Sigdel, T.K., Cilliers, R., Gursahaney, P.R., Crowder, M.W., 2004. Fractionation of soluble proteins in *Escherichia coli* using DEAE-, SP-, and phenyl sepharose chromatographies. J. Biomol. Tech. 15 (3), 199–207.
de Sousa, M.A., 1983. A simple method to purify biologically and antigenically preserved bloodstream trypomastigotes of *Trypanosoma cruzi* using DEAE-cellulose columns. Mem. Inst. Oswaldo Cruz 78 (3), 317–333 (PubMed PMID: 6361445).
Tyler, K.M., Engman, D.M., 2001a. The life cycle of *Trypanosoma cruzi* revisited. Int. J. Parasitol. 31 (5-6), 472–481 (Review. PubMed PMID: 11334932).
Villamizar, L.H., Cardoso, M., de Andrade, J., Teixeira, M.L., Soares, M.J., 2017. Linalool, a Piper aduncum essential oil component, has selective activity against *Trypanosoma cruzi* trypomastigote forms at 4 °C. Mem. Inst. Oswaldo Cruz 112 (2), 131–139 (doi:10.1590/0074-02760160361).
Yoshida, N., Dorta, M.L., Ferreira, A.T., Oshiro, M.E., Mortara, R.A., Acosta-Serrano, A., Favoreto Júnior, S., 1997. Removal of sialic acid from mucin-like surface molecules of *Trypanosoma cruzi* metacyclic trypomastigotes enhances parasite-host cell interaction. Mol. Biochem. Parasitol. 84 (1), 57–67 (PubMed PMID: 9041521).
Zingales, B., Andrade, S.G., Briones, M.R.S., Campbell, D.A., Chiari, E., Fernandes, O., et al., 2009. A new consensus for *Trypanosoma cruzi* intraspecific nomenclature: second revision meeting recommends TcI to TcVI. Mem. Inst. Oswaldo Cruz 104, 1051–1054.