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Trypanosoma cruzi infectivity assessment in "*in vitro*" culture systems by automated cell counting

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ABSTRACT

Chagas disease is an endemic, neglected tropical disease in Latin America that is caused by the protozoan parasite *Trypanosoma cruzi*. *In vitro* models constitute the first experimental approach to study the physiopathology of the disease and to assay potential new trypanocidal agents.

Here, we report and describe clearly the use of commercial software (MATLAB®) to quantify *T. cruzi* amastigotes and infected mammalian cells (BeWo) and compared this analysis with the manual one. There was no statistically significant difference between the manual and the automatic quantification of the parasite; the two methods showed a correlation analysis r^2 value of 0.9159. The most significant advantage of the automatic quantification was the efficiency of the analysis. The drawback of this automated cell counting method was that some parasites were assigned to the wrong BeWo cell, however this data did not exceed 5% when adequate experimental conditions were chosen.

We conclude that this quantification method constitutes an excellent tool for evaluating the parasite load in cells and therefore constitutes an easy and reliable ways to study parasite infectivity.

1. Introduction

Chagas disease is caused by the hemoflagellated protozoan *Trypanosoma cruzi* of the Kinetoplastidae order and Trypanosomatidae family and is a major public health concern in Latin America. Among vector-borne diseases, it is second to malaria in prevalence and mortality (Coura and Vinas, 2010).

T. cruzi has an heteroxenic life cycle. Hematophagous insects (Triatomids) serve as intermediary hosts, and mammals, including humans, serve as definitive hosts (De Souza, 2002).

Disease transmission primarily occurs via the bite of a *T. cruzi*-infected insect. Upon feeding on mammalian blood, the insect deposits feces containing infectious metacyclic trypomastigotes onto the skin. The parasites then enter the mammalian host through the skin by a mechanism that is facilitated by self-inflicted scratching and pore-forming lytic protein enzymes, such as tryalisin which can permeabilize mammalian cells, in the insect's saliva (Amino et al., 2002). Trypomastigotes are phagocytozed by antigen-presenting cells, and they then differentiate into amastigotes, the obligate cytoplasmic form. After a certain number of replications, amastigotes differentiate into trypomastigotes, escape into the circulation, and migrate toward target tissues, such as the myocardium, skeletal muscle, smooth visceral muscle, glial cells of the central nervous system, and the placenta (Kemmerling et al., 2010). Other important means of transmission are blood transfusions, organ transplants (10% of cases) (Coura and Borges-Pereira, 2012), oral infection through the ingestion of contaminated foods (Coura and Vinas, 2010; de Souza et al., 2010; Toso et al., 2011; Yoshida, 2006, 2008), and transplacental transmission in chagasic mothers (Coura and Borges-Pereira, 2012; Duaso et al., 2012; Fretes and Kemmerling, 2012; Kemmerling et al., 2010).

Although extensive public health measures to control vectors and prevent blood-borne transmission have decreased the incidence and prevalence of this disease, the currently available

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therapies do not possess high efficacies (Molina-Berríos et al., 2013).

In vitro models are the primary experimental approaches that are used to assess potential new trypanocidal agents (Triquell et al., 2009). The quantification of infected mammalian cells and of intracellular parasites (amastigotes) in *in vitro* assays is a fundamental method used to determine the effect of anti-parasitic drugs (Faundez et al., 2005). However, the manual or semi-automatic quantification of infected cells and amastigotes requires trained and experienced observers and can take many hours. Today, the development of diverse algorithms and bioimaging software allows for the automated analysis of large data sets (Carpenter et al., 2012).

Here we report the use of commercial software (MATLAB®) to quantify *T. cruzi* amastigotes in infected mammalian cells (BeWo).

2. Materials and methods

2.1. Cell culture

Green Monkey (*Cercopithecus aethiops*) renal fibroblast-like cells (VERO cells (ATCC® CCL-81)) were grown in RPMI medium supplemented with 5% fetal bovine serum (FBS) and antibiotics (penicillin–streptomycin) (Villalta and Kierszenbaum, 1982). BeWo cells (ATCC CCL-98) were grown in DMEM-F12K medium supplemented with 10% FBS, L-glutamine and antibiotics (penicillin–streptomycin) (Drewlo et al., 2008). The cells were grown at 37 °C in a humidified atmosphere with 5% CO_2. The culture medium was replaced every 24 h.

2.2. Production of the infective cellular form (trypomastigotes) of *T. cruzi*

Upon reaching confluence, VERO cells were incubated with a culture of Ypsilon strain epimastigotes (non-infective cellular form of the parasite) in the late stationary phase, which contains approximately 5% trypomastigotes (Contreras et al., 1985). The trypomastigotes were then allowed to invade VERO cells and replicate intracellularly as amastigotes. After 72 h, the amastigotes transformed back into trypomastigotes and lysed the host cells. The parasites were recovered by low-speed centrifugation (500 × *g*) to separate the trypomastigotes in the supernatant from the amastigotes in the sediment (Villalta and Kierszenbaum, 1982).

2.3. Infection of BeWo cells with *T. cruzi* trypomastigotes

BeWo cells were detached by trypsinization, sedimented, and resuspended in media containing 10% FBS. Then, 2×10^5 cells were seeded into six-well plates. The cells were allowed to adhere to the bottom of the wells for 3 h and were then challenged with the parasites at a BeWo cell:parasite ratio of 1:1. The cells were analyzed 48 h post-infection, and the parasites were identified based on nuclear size and morphology.

2.4. Microscopy analysis of BeWo cells and amastigote identification

BeWo cells were fixed in cold 90% methanol. The preparations were washed with PBS and incubated with 1 μg/ml 4′6′-diamidino-2-phenylindole (DAPI) (Molecular Probes). Then, the sections were mounted in Vectashield (ScyTek ACA) and observed on an epifluorescence microscope (Motic BA310; Hong Kong, China). Amastigote were recognized by their morphology, including nuclear size and the presence of a kinetoplast (Liempi et al., 2014).

3. Results and discussion

3.1. Quantification of in vitro infection by *T. cruzi* using MATLAB® software

T. cruzi can invade and infect any nucleated cell (De Souza, 2002; Diaz-Lujan et al., 2012). Here, we challenged the choriocarcinoma-derived cell line BeWo (ATCC CCL-98) with *T. cruzi* Ypsilon strain trypomastigotes at a parasite:cell ratio of 1:1 for 2, 4, 6, 12, and 24 h. After 48 h, the cell nuclei were stained with DAPI (Duaso et al., 2010). The parasite forms were recognized by their morphology, including nuclear size and the presence of a kinetoplast (Fig. 1). The cells were observed on an epifluorescence microscope, and the images were captured as "tiff" files (Fig. 2A). Importantly, all of the files were of the same quality (pixels) for later analysis.

The color images were converted into gray scale (Fig. 2A), equalized (Fig. 2B) (contrast and brightness adjustment), and subjected to binarization (Fig. 2C) (black and white scale). Afterwards, the small objects within the image (in this case, the parasites) were digitally eliminated (Fig. 2D), and a "watershed" algorithm was applied (Fig. 2E) to count the mammalian cell nuclei (Fig. 2F) (Gudla et al., 2008; Nandy et al., 2011).

After counting the mammalian cell nuclei, the parasites were quantified. In the equalized and binarized images, the mammalian nuclei were digitally eliminated (Fig. 2G), and the parasites were identified by the presence of smaller nuclei beneath a bright kinetoplast. The software then calculated the local variance (Fig. 2H) and counted the parasites (Fig. 2I) (Gudla et al., 2008; Nandy et al., 2011).

There was no statistically significant difference between the manual and the automatic quantification of the parasite (Fig. 3A). The two methods showed a correlation analysis r^2 value of 0.9159 (Fig. 3B). The most significant advantage of the automatic quantification was the efficiency of the analysis; it took approximately 15 min to quantify the parasites and BeWo cell nuclei of 450 files. For the manual count, each evaluator required at least 7 working days. Additionally, the automatic quantification eliminated the variability between the different individuals who performed the evaluations.

To assign the parasites to their respective mammalian cells, we defined an infected cell as containing at least three parasites localized perinuclearly. The software used the Voronoi diagram (Wakamatsu et al., 2011 Yu et al., 2010) to assign amastigotes to each nucleus and hypothetical cell (Fig. 4). The drawback of this method was that some parasites were assigned to the wrong

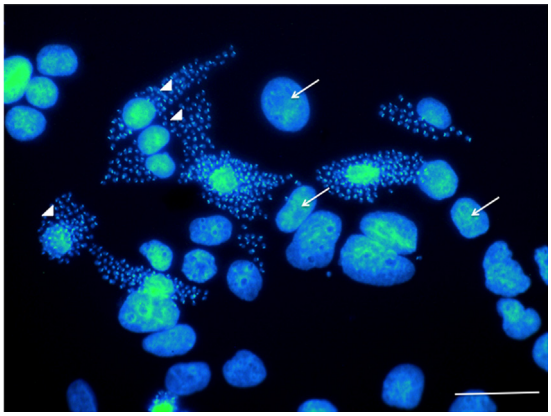

Fig. 1. Infection of BeWo cells with *T. cruzi* amastigotes. BeWo cells were challenged with *T. cruzi* Ypsilon strain trypomastigotes at a parasite:cell ratio of 1:1 for 24 h and were processed for DAPI staining after 48 h. The arrows show BeWo cell nuclei, and the arrowheads show intracellular amastigotes. Scale bar: 10 μm.

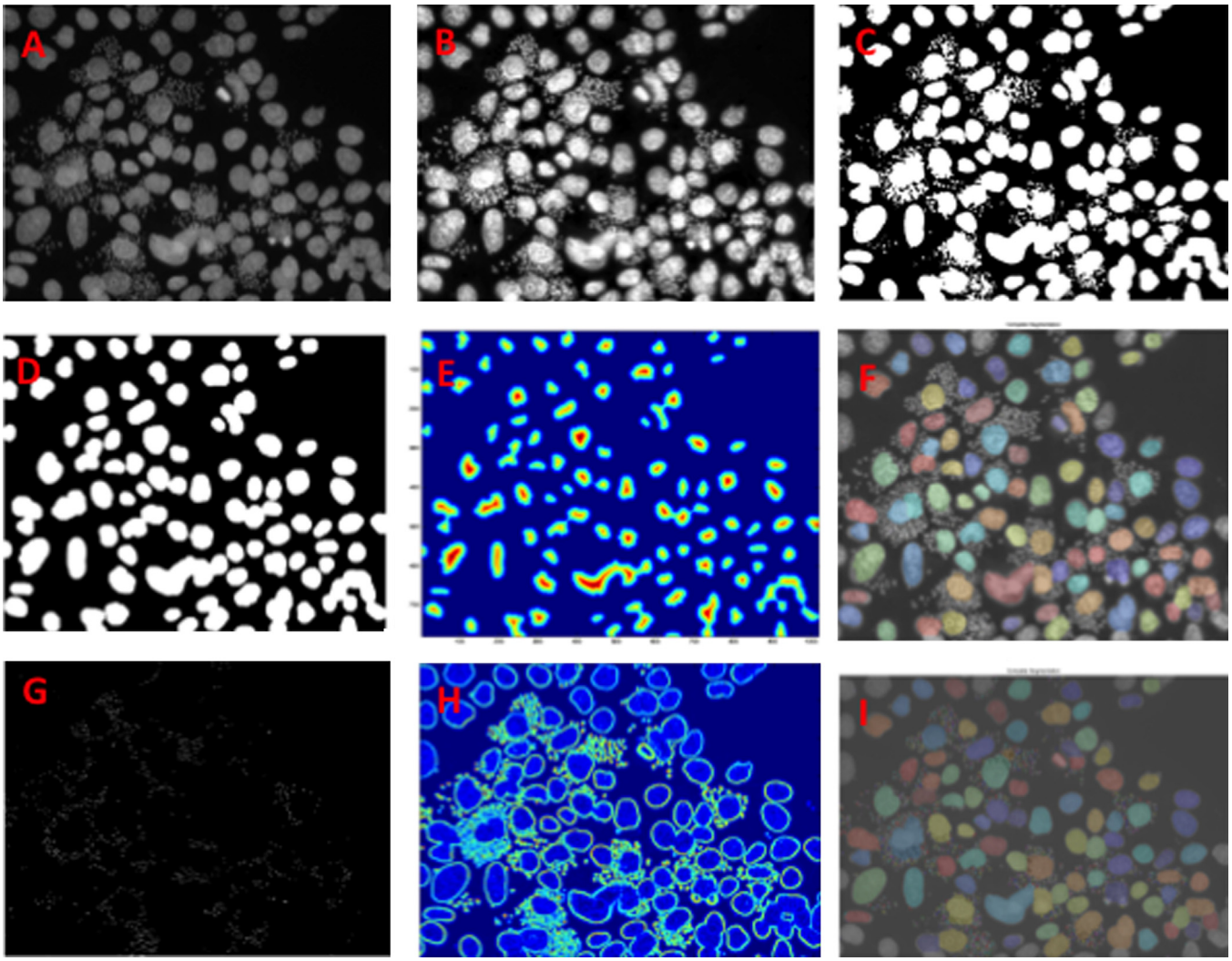

Fig. 2. Quantification of BeWo cell nuclei. Tiff files from the different experimental conditions were converted into gray scale (A), the contrast and brightness were adjusted (equalization) (B), and images were subjected to binarization (C). Then, the small objects in the image (the parasites) (D) were digitally eliminated, and a "watershed" algorithm (E) was applied to count the mammalian cell nuclei, which were randomly colored (F). Subsequently, the BeWo cell nuclei were digitally eliminated (G), and the parasites were identified by the presence of smaller nuclei beneath a bright kinetoplast. The software calculated the local variance (H) to count the parasites (I).

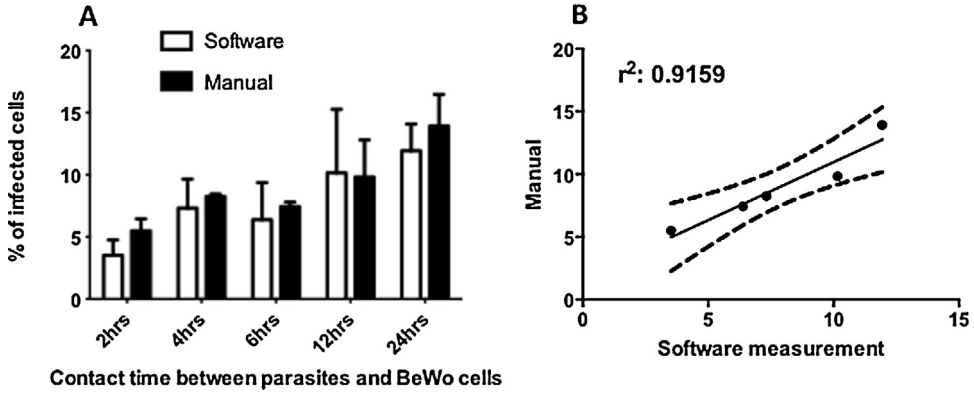

Fig. 3. Quantification of infection of BeWo cells with *T. cruzi*. BeWo cells were challenged with *T. cruzi* Ypsilon strain trypomastigotes at a parasite:cell ratio of 1:1 for 2, 4, 6, 12, and 24 h. After 48 h, the cells were processed for DAPI nuclear staining. In A, the percentages of infected BeWo cells after different incubation times as determined automatically with MATLAB software (white bars) or manually (black bars) are shown. There was no statistically significant difference between the two quantification methods for any condition. The data were analyzed by Student's *t* test. In B, the correlation analysis of both methods is shown.

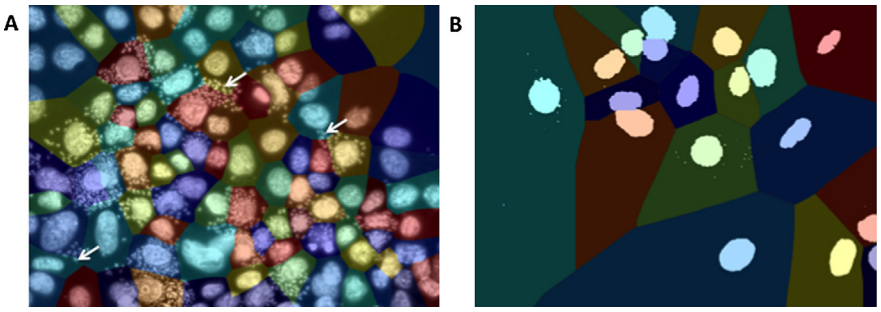

Fig. 4. Assignment of the parasites to their respective BeWo cell. Images of the tiff files, which show the quantification of BeWo cell nuclei (See Fig. 2I), were subjected to Voronoi diagram analysis. A: Image corresponding to a highly confluent culture. B: Image corresponding to a semi-confluent culture. Note that a significant number of parasites were assigned to the wrong BeWo cell (arrows) in A, but not in B.

BeWo cell; between 2.55% and 4.6% of amastigotes were assigned to the wrong cell. The lowest percentage of wrong assigned parasite corresponds to conditions in which a low concentration of BeWo cells were seeded and challenged with the corresponding parasite:cell ratio (Fig. 4B). Conditions in which the BeWo cells were confluent and challenged with a high parasite concentration, the percentage of wrong assigned parasite were above 10% (Fig. 4A). Therefore, this analysis needs adequate experimental conditions, in which the concentration of mammalian cells and parasites must be carefully chosen. Only, experiments done with mammalian cells in semi-confluence can be considered reliable (Fig. 4B). The use of immunofluorescence methods to determine cell limits should correct the false assignation of the parasites. However, this methodology would increase the duration and cost of the analysis. Currently, we are working on other experimental approaches, such as the combination of images with fluorescent nuclei and the respective phase contrast images, to improve the analysis.

We conclude that this quantification method constitutes an excellent tool for evaluating the parasite load in cells and therefore constitutes an easy and reliable ways to study parasite infectivity.

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