Journal of Microbiological Methods 92 (2013) 220–225

Contents lists available at SciVerse ScienceDirect

Journal of Microbiological Methods

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

# Validation of a Poisson-distributed limiting dilution assay (LDA) for a rapid and accurate resolution of multiclonal infections in natural *Trypanosoma cruzi* populations

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ARTICLE INFO

*Article history:*
Received 14 September 2012
Received in revised form 5 November 2012
Accepted 6 November 2012
Available online 13 November 2012

*Keywords:*
Multiclonality
Chagas disease
*Trypanosoma cruzi*
Aneuploidy
Infrapopulation
Superinfection

ABSTRACT

*Trypanosoma cruzi* is the causative agent of American trypanosomiasis, a complex zoonotic disease that affects more than 10 million people in the Americas. Strains of this parasite possess a significant amount of genetic variability and hence can be divided into at least six discrete typing units (DTUs). The life cycle of this protist suggests that multiclonal infections may emerge due to the likelihood of contact of triatomine insects with more than 100 mammal species. To date, there have been a few studies on but no consensus regarding standardised methodologies to identify multiclonal infections caused by this parasite. Hence, the aim of this study was to develop and validate a limiting dilution assay (LDA) to identify multiclonal infections in *T. cruzi* populations by comparing the feasibility and reliability of this method with the widely applied solid phase blood agar (SPBA) methodology. We cloned reference strains belonging to three independent genotypes (TcI, TcII, and TcIV) and mixed infections (TcI + TcII) using LDA and SPBA; the comparison was conducted by calculating the feasibility and reliability of the methods employed. Additionally, we implemented LDA in strains recently isolated from *Homo sapiens*, *Rhodnius prolixus*, *Triatoma venosa*, *Panstrongylus geniculatus*, *Tamandua tetradactyla*, *Rattus rattus*, *Didelphis marsupialis* and *Dasypus novemcinctus*, with the aim of resolving multiclonal infections using molecular characterization employing SL-IR (spliced leader intergenic region of mini-exon gene), the 24Sα rDNA gene and microsatellite loci. The results reported herein demonstrate that LDA is an optimal methodology to distinguish *T. cruzi* subpopulations based on microsatellite markers by showing the absence of multiple peaks within a single locus. Conversely, SPBA showed patterns of multiple peaks within a single locus suggesting multiclonal events. The biological consequences of these results and the debate between multiclonality and aneuploidy are discussed.



## 1. Introduction

The natural life cycle of *Trypanosoma cruzi*, the etiological agent of Chagas disease, involves triatomine bugs and mammalian reservoirs. The zoonotic cycle of the parasite facilitates the occurrence of multiclonal infections within a single host because of the multifactorial interactions between the parasite, the insect vectors and/or the mammalian reservoirs (WHO, 2007; Rassi et al., 2012). Several studies have revealed the presence of different *T. cruzi* populations within the same host (Llewellyn et al., 2011; Macedo et al., 2004; Venegas et al., 2010). Additionally, the high genetic variability evidenced by at least six discrete typing units (DTUs) within the *T. cruzi* taxon facilitates the high level of diversification of the parasite (Zingales et al., 2009, 2012). Therefore, it is necessary to develop rapid and accurate strategies to obtain populations from a parasite clone with the aim of developing molecular, biological or behavioural studies depending on the area of research.

In the few last decades, many attempts have been made to resolve multiclonal infections of *T. cruzi* within hosts. The most commonly used method has been the clonal isolation of *T. cruzi* on solid medium (Goldberg and Chiari, 1980; Mondragon et al., 1999; Santos et al., 2000; Yeo et al., 2007). It has been demonstrated that clonal growth and isolation on solid agar medium by spreading epimastigotes on an agar surface with subsequent incubation showed variation in plate efficiencies that were affected by the solidification time, temperature and other factors (Gomes et al., 1991; Ng-Ying-kin and Yaphe, 1972). Using this method, fully grown colonies that reached a diameter of 1 mm were observed at around 20–35 days. In addition to the long time it takes to isolate a single colony, the effect of clumping could bias the parasite cloning procedure by merging two separate populations within the same colony. The most successful attempt at obtaining biological clones was reported by Yeo et al. (2007) who tested different culture media concentrations on strains

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http://dx.doi.org/10.1016/j.mimet.2012.11.002

belonging to three different genotypes (TcI, TcII and TcIV) and proposed a reliable method for obtaining *T. cruzi* clones on solid agar medium. However, this procedure could also be biased by the formation of parasite clumps that may contribute to misleading observations. Nevertheless, this method has been used in several molecular epidemiological approaches (Llewellyn et al., 2009, 2011; Messenger et al., 2012).

Limiting dilution assays (LDAs) are often used in cell culture to isolate single cells in a multiwell culture plate. The culture is diluted such that each well should theoretically contain a single cell. When an individual cell divides, the well will contain only clones of the original cell. The cloned cells can then be diluted and the process repeated, producing many clones of a single cell in a Poisson-distributed pattern (Frisan et al., 2001). LDA has been widely employed for cloning hybridoma cells and parasites such as *Plasmodium*, where parasite growth in individual microplate wells is detected by one of several methods, each of which has one or more shortcomings (Butterworth et al., 2011; Lyko et al., 2012; Trager et al., 1981). This assay has proven to be quite accurate and offers the ability to rapidly obtain viable clones from protozoan parasites. The aim of this study was to develop and validate a rapid and feasible LDA for the resolution of multiclonal infections in *T. cruzi* isolates from humans, insect vectors and/or reservoirs while comparing this method with the cloning of the solid plate agar method. Validation of this method provides the scientific community with a rapid, easy and reliable method for biological cloning of *T. cruzi* isolates.

## 2. Materials and methods

### 2.1. Parasites and limiting dilution assay (LDA)

The parasites were cultured at 25 °C in LIT-biphasic medium. To prepare 250 mL of this medium, 2 g of $Na_2HPO_4$ (Nuclear), 1 g of NaCl (Synth), 0.1 g of KCl (Dinâmica) and 44.5 mL of Milli-Q water were placed in a sterile Kitasato flask. A stainless-steel filter holder (Millipore) containing a 22 μm membrane (Millipore) was adapted to the Kitasato flask, sealed with aluminium foil and manila paper and autoclaved at 121 °C for 20 minutes. Simultaneously, 0.75 g of liver infusion (Difco) was diluted in 100 mL of heated Milli-Q water, 0.5 g of dextrose (Oxoid), and 1.25 g of Tryptose (Vetec®) and titrated to a pH of 7.4. This solution was filtered into the Kitasato flask using a vacuum. Later, 5.5 mL of 2.2% bovine haemoglobin was filtered (BBLTM). In a laminar flow hood, 30 mL of foetal bovine serum (11% final concentration) (Nutricell) and 1 mL (20 mg) of gentamicin (Sigma) were added in sterile conditions. Finally, a sterility control was performed in a steriliser at 37 °C for 48 h using BHI medium. The supplemented medium was distributed into sterile tubes with threaded caps, and parasites were maintained in an incubator at 28 °C–30 °C until the cultured parasites reached exponential phase. All stocks were synchronised using sequential passages in LIT medium every 5 days. After synchronisation, the parasites were cloned using a Poisson-distributed LDA to test the likelihood of obtaining one parasite per well after serial dilutions. The objective was to select an optimal cell dose to obtain acceptable proportions of positive wells after the serial dilutions. If the quantity of viable cells in suspension is high enough that each culture receives several cells, then all the cultures will be positive. If the concentration of cells is too low, then positive cultures would be rare. Between these extremes, the result can be quantified as a function of cell dose. We determined the grade of the dilutions according to the equation $F_0 = e^{-m}$ where $m$ is the average number of cells per millilitre and $F_0$ is the frequency of cultures receiving no cells. We tested cell doses from 25 parasites/mL to 1000 parasites/mL. We used microculture plates (Nalgene) for the LDA assay. All of the wells contained 50 μL of LIT medium, the cell dose selected was added to the first well, and 1:2 serial dilutions were performed until a total volume of 100 μL was reached. The plate was centrifuged at 4000 ×*g* for 10 minutes. Every well that should have theoretically contained one parasite according to $F_0$ calculations starting from serial dilutions of 400 parasites/mL was visualised using an inverted microscope (Nikon) to ensure the presence of one unique parasite clone (Fig. 1). The plate was incubated for 24 h at 25 °C, and a second assessment of each well was performed. In all cases, 30 microscopic fields were checked. If a well contained more than ten parasites, the well was washed with LIT medium and passed to a LIT-biphasic medium supplemented with bovine serum 5% and 200 mg/mL of gentamicin (Sigma) and 5-fluorocitosine (Sigma). The parasites were then cultured until exponential phase was reached.

### 2.2. Comparison of LDA and solid phase plate cloning

We selected the reference strains TcI (Dm28c isolated from *Didelphis marsupialis* in Venezuela), TcII (Y isolated from *Triatoma infestans* in Brazil) and TcIV (CANIII isolated from *Homo sapiens* in Brazil). These reference strains were maintained in LIT-biphasic medium and provided by Universidad del Tolima, Colombia, South-America. Growth curves were performed for each DTU in LIT medium, and the duplication time for each genotype was calculated as previously reported (Mejía-Jaramillo et al., 2009). The number of parasites was determined using a Neubauer chamber every 24 h for 30 days, and the parasites were cloned during the logarithmic phase using both LDA and solid phase plate cloning. A comparison between the LDA and the solid phase plate cloning methods was conducted according to Yeo et al. (2007) to validate the LDA. To determine whether this method is able to resolve multiclonal infections, $10^3$ TcI (Dm28c) cells were mixed with $10^3$ TcII (Y) cells. The time that each clone took to reach exponential phase was determined. After obtaining ten viable biological clones from each genotype and the mixed culture (TcI + TcII) by the two different methods, the clones were grown to exponential phase, and DNA was isolated from 200 μL aliquots of each culture using a Qiamp kit (Qiagen, Switzerland). The two methods were compared in terms of reliability (obtaining a clonal population from a single cell) and feasibility (the number of viable clones isolated in a period of time).

### 2.3. Triatomines, humans and reservoirs cloning of isolates

Two different approaches were used to test the applicability of this assay. i) Three recent multiclonal isolates (no more than 2 passages) from humans in the acute phase of Chagas disease (LER, EH and SMA), triatomines (NC2—*Rhodnius prolixus*, TV—*Triatoma venosa* and PGPA2—*Panstrongylus geniculatus*) and reservoirs (SLDm1—*D. marsupialis*, NR1—*Rattus rattus* and SLDN1—*Dasypus novemcinctus*) were subjected to LDA to obtain 10 biological clones from each isolate. These isolates were maintained in LIT-biphasic medium in the laboratory. ii) The second group consisted of triatomine faeces from ten *R. prolixus* captured in *Attalea* palms and blood from *Tamandua tetradactyla* and *D. marsupialis* positive for *T. cruzi* infection. The number of parasites in the faeces and blood was determined using a Neubauer chamber and set to a concentration of 100 parasites/mL according to the cell dose formula ($F_0 = e^{-m}$). All of the wells contained 10 μL (200 mg/mL) of gentamicin and 5-fluorocytosine at 200 mg/mL to avoid contamination. The plates were centrifuged, and the wells were checked for the presence of single parasites using an inverted microscope. The plates were incubated for 48 h, and when at least 10 parasites per well were observed, the wells were washed twice and transferred to LIT-biphasic medium; in this case, 20 clones were obtained from each host. When cultures for both approaches reached the logarithmic phase, 200 μL aliquots were harvested for DNA extraction and molecular analyses to test the feasibility and reliability.