*Rev. Inst. Med. Trop. Sao Paulo*
*53(5):247-253, September-October, 2011*
*doi: 10.1590/S0036-46652011000500002*

# INDUCTION OF PHAGOCYTIC ACTIVITY AND NITRIC-OXIDE PRODUCTION IN NATURAL POPULATIONS OF *Trypanosoma cruzi* I AND II FROM THE STATE OF PARANÁ, BRAZIL

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## SUMMARY

Twelve strains of *Trypanosoma cruzi* isolated from wild reservoirs, triatomines, and chronic chagasic patients in the state of Paraná, southern Brazil, and classified as *T. cruzi* I and II, were used to test the correlation between genetic and biological diversity. The Phagocytic Index (PI) and nitric-oxide (NO) production *in vitro* were used as biological parameters. The PI of the *T. cruzi* I and II strains did not differ significantly, nor did the PI of the *T. cruzi* strains isolated from humans, triatomines, or wild reservoirs. There was a statistical difference in the inhibition of NO production between *T. cruzi* I and II and between parasites isolated from humans and the strains isolated from triatomines and wild reservoirs, but there was no correlation between genetics and biology when the strains were analyzed independently of the lineages or hosts from which the strains were isolated. There were significant correlations for Randomly Amplified Polymorphic Deoxyribonucleic acid (RAPD) and biological parameters for *T. cruzi* I and II, and for humans or wild reservoirs when the lineages or hosts were considered individually.

**KEYWORDS:** *Trypanosoma cruzi;* Genetic lineages; Phagocytic index; Infectivity *in vitro*; Nitric oxide.

## INTRODUCTION

Chagas' disease, the etiologic agent of which is *Trypanosoma cruzi,* is a serious public-health problem in Latin America, where around eight million people are infected by the parasite[42]. In the United States, 50 to 100 thousand people are infected, and in Brazil, 1.9 to 3.5 million[35]. *T. cruzi* is a digenetic flagellate protozoan of the order Kinetoplastida, family Trypanosomatidae, that circulates in nature among humans, vectors, and wild and domestic reservoirs. The interaction of the parasite with natural reservoirs and triatomine bugs is known as the wild or sylvatic transmission cycle of the parasite. The colonization of non-natural habitats by triatomine vectors allowed *T. cruzi* to infect humans and domestic mammals, resulting in a domestic transmission cycle[15].

*Trypanosoma cruzi* is composed of a variety of subpopulations with different characteristics. Natural populations of the parasite display great biological, biochemical, immunological, and genetic heterogeneity[5,9,22,26,29,34,44]. This heterogeneity, together with the genetic characteristics of the host, may explain the clinical variability of Chagas' disease and the local differences in morbidity[14]. The relationship between *Trypanosoma cruzi* and humans, at least to some extent, appears to be a successful adaptation of an infectious agent that survives in the host's body, occasionally causing harm[45]. In addition to the parasite's genetic lineage, the host's genetic makeup seems to have a marked influence on the biological behavior of *T. cruzi*[8].

Two main evolutionary lineages, *T. cruzi* I and *T. cruzi* II, have been identified by different methods[4,21]. A third lineage, termed *T. cruzi* III, also exists. The *T. cruzi* III lineage is equivalent to the sublineage called TCIIc identified by BRISSE *et al.*[10], who proposed five subdivisions for DTU (discrete typing unit) II, termed IIa-e. Although the present consensus is to refer to six DTUs (TcI-VI) for these strains[52], many studies have investigated the genetic-biological relationship in terms of virulence in mice, transmissibility by triatomines, infectivity to cell cultures, and drug sensitivity *in vitro* and *in vivo,* using populations belonging to the two lineages that were formerly distinguished as *T. cruzi* I and II[23,24,30,39,46]. Populations of *T. cruzi* from northern and northwestern areas of the state of Paraná, Brazil have been determined to belong to these two major genetic lineages[51]. Strains isolated from triatomines and wild reservoirs belong to *T. cruzi* I, and isolates from humans belong to *T. cruzi* II.

Characteristics of *T. cruzi* natural populations related to the growth kinetics of epimastigotes, metacyclogenesis, infectivity to mammal cells, and susceptibility to trypanocidal drugs can be considered natural markers of the parasites' heterogeneity as well as of the genetic characteristics of these populations[28]. Although macrophages play a crucial role in the immune response to flagellate infection, the infectivity of *T. cruzi* strains may be related to the capacity of the parasite to evade the macrophages' effector mechanisms, and nitric-oxide production is an important mechanism that restricts the replication of intracellular parasites. Together with the phagocytic index, nitric-oxide production

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is a characteristic of each isolate, and is therefore a useful parameter for comparing strains[13,17,18,25].

In this study, we determined the macrophage phagocytic index (PI) and nitric-oxide production of natural populations of *T. cruzi* isolated from wild reservoirs, triatomines, and humans, belonging to the *T. cruzi* I and II lineages from Paraná, and evaluated the existence of any correlation between genetic diversity and the PI and NO production.

## MATERIALS AND METHODS

**Parasite strains:** Table 1 shows the *T. cruzi* strains isolated in Paraná, their genetic lineages, and hosts. The trypomastigote forms used in the macrophage infections were obtained on day 8 of culture in LIT (Liver Infusion Tryptose) medium. The total counts ranged from 6.8 to 9.3 x $10^7$ parasites/mL, and the differential counts ranged from 2.4 to 7.0% trypomastigotes for the strains used. Because the strains of *T. cruzi* isolated in Paraná show a low rate of metacyclogenesis in LIT medium, with little variation among them[4], macrophages were placed in contact together with trypomastigotes and epimastigotes without purification of the trypomastigotes. For the biological characterization, the thawed strains were maintained in LIT medium until they reached the exponential growth stage.

**Table 1**
Trypanosoma cruzi strains isolated from chronic chagasic patients, wild reservoirs, and triatomines in the state of Paraná, southern Brazil.

| STRAIN | GENETIC GROUP* | HOST |
|---|---|---|
| G1 | *T. cruzi* I | *Didelphis* sp. |
| G2 | *T. cruzi* I | *Didelphis* sp. |
| G3 | *T. cruzi* I | *Didelphis* sp. |
| G249 | *T. cruzi* I | *Didelphis* sp. |
| F3 | *T. cruzi* I | *Triatoma sordida* |
| A21A | *T. cruzi* I | *Triatoma sordida* |
| N914A | *T. cruzi* I | *Triatoma sordida* |
| N120B | *T. cruzi* I | *Panstrongylus megistus* |
| 2052 | *T. cruzi* II | Human |
| 328 | *T. cruzi* II | Human |
| 379 | *T. cruzi* II | Human |
| 399 | *T. cruzi* II | Human |

According to Zalloum *et al.,* 2005.

**Cells:** Peritoneal macrophages were obtained from female BALB/c mice, 60 days old. Mice were inoculated intraperitoneally (i.p.) with 1 mL of sterile thioglycolate broth four days prior to the assay. Ketamine hydrochloride (50 mg/Kg) and xylazine (10 mg/Kg) i.p. were used to euthanize the mice. The peritoneal macrophages were harvested by washing the peritoneal cavity with cold phosphate-buffered saline (PBS).

**Macrophage infection and phagocytic index (PI):** In 24-well plates, 5 x $10^5$ macrophages were placed on sterile glass coverslips. Non-adherent cells were removed by several washes with Roswell Park Memorial Institute medium (RPMI), and the plates were maintained at 37 °C in a 5% $CO_2$ incubator. The *T. cruzi* parasites (trypomastigotes and epimastigotes) were incubated with macrophages (in order to have two trypomasigotes/macrophage) for four h in RPMI medium without serum at 37 °C. Non-adherent parasites were removed by washing the monolayers with RPMI medium. Infected macrophages were maintained in RPMI 1640 with 5% fetal calf serum at 37 ºC in a 5% $CO_2$ incubator for 20 h. The macrophages were stained with HEMA 3 Stain (Biochemical Science, Inc., USA). The phagocytic index (PI) was determined by multiplying the percentage of macrophages that had phagocytosed at least one parasite by the mean numbers of parasites per infected macrophage, as described by BUCHI & DE SOUZA[11,12]. Three hundred cells were examined. The phagocytic index (PI) was determined for the genetic lineages and hosts. The assays were carried out in quadruplicate.

**Nitric oxide (NO) determination:** For the NO production assays, the macrophages were stimulated with LPS (50 ng/µL) (Sigma L6511, Steinheim, Germany) four h before, during, and four h after the addition of the parasites. Two controls were used: uninfected macrophages and infected macrophages without LPS stimulation. The plates were maintained at 37 °C in a $CO_2$ incubator for 20 h. The assays were carried out in triplicate.

NO production was indirectly evaluated in the supernatant from the macrophage cultures, by the determination of nitrites[16]. 50 µL of cell-culture supernatant was incubated with the same volume of Griess reagent (1% sulfanilamide, 0.1% naphthyldiamine dihydrochloride, and 2.5% orthophosphoric acid), for 15 min at ambient temperature. The absorbance was determined in a spectrophotometer (Microplate Fluorescence Reader FL-600) at 530 ηm. The nitrite concentration was calculated based on a standard curve (5, 10, 30, 60 µM) of sodium nitrite ($NaNO_2$) in culture medium.

**Genetic characterization:** The genetic characterization was carried out using RAPD and SSR-PCR[51].

**Statistical analyses:** The nonparametric Mann-Whitney test and the means test were used to compare the phagocytic index in relation to the genetic lineage to which the strains belonged. The NO production was compared between the genetic lineages by analysis of variance and Tukey test. The NO production was compared between the lineages and the controls using Student´s *t*-test. The Statistica 6.0 program was used.

The nonparametric Mantel test (SAS Program - Statistical Analysis System - version 8.02) was used to evaluate the existence of correlations between genetic diversity and the phagocytic index or NO production. Mantel's Test[27] estimates the correlation (association) between two squared symmetrical distance matrices obtained from different measurements (type of data, observations) of the same group of elements[42]. Genetic distance matrices between strains, taken two by two, for RAPD and SSR-PCR data were obtained by the arithmetic complement of Jaccard's similarity coefficient. For biological distance analyses, an equal score was assigned to each biological parameter, with 1 the highest value, and the other values in proportion. To construct the biological data matrix, the values were weighted so that the results were expressed in proportion to the highest value obtained for each parameter. Next, a mean of all the weighted parameters for each strain was calculated. The biological distance between two strains was calculated

by the difference of their means. Genetic distance matrices between strains, taken two by two, for RAPD and SSR-PCR data were obtained by the arithmetic complement of Jaccard's similarity coefficient (1 - the coefficient). The significance level was 5% for all the analyses.

**Ethics Committee Evaluation:** The Ethics Committee for Animal Experiments (CEEA) of the State University of Maringá approved this study.

## RESULTS

**Phagocytic Index (PI) for macrophages infected with *T. cruzi* I and II strains isolated from different hosts:** The PIs for the strains belonging to the *T. cruzi* I lineage ranged from 1.77 to 15.02, and the PIs for the *T. cruzi* II lineage strains ranged from 2.10 to 10.46. No significant difference ($p$ = 0.1285) was observed between the mean phagocytic indexes for the genetic lineages of strains *T. cruzi* I (3.47) and II (2.32) (Fig. 1A). A statistically significant *T. cruzi* I intra-lineage variation was observed in PI ($p$ = 0.0251), but not for *T. cruzi* II ($p$ = 0.116). In the T. *cruzi I* group, 50% of the strains (G1, A21A, N120B and N914A) displayed a PI ranging from 5 to 15.0, and for the other strains the PI ranged from 1.77 to 2.89. In the *T. cruzi* II group, only one strain (399) displayed a discrepant PI.

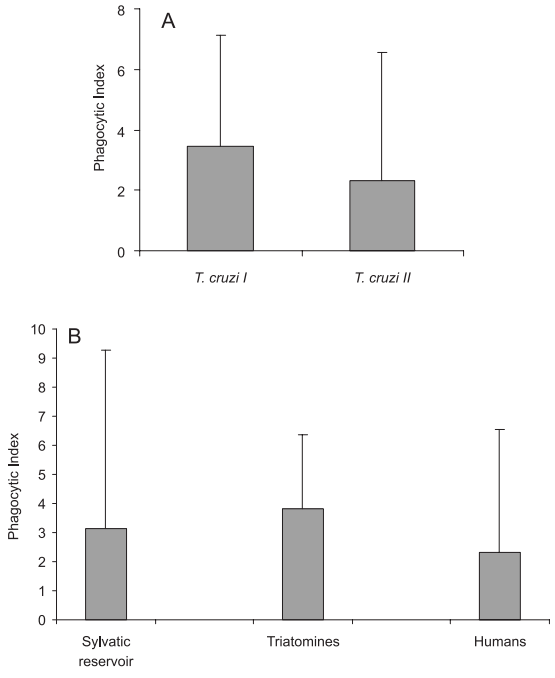


**Fig. 1 -** Mean Phagocytic Index (PI) for macrophages infected with strains of *Trypanosoma cruzi* from the state of Paraná, Brazil. (A) Strains classified as TcI and II genetic groups ($p$ = 0.1285), and (B) Phagocytic Index of strains isolated from different hosts ($p$ = 0.742949).

The PIs for the strains isolated from humans ranged from 1.84 to 10.48. Among the strains isolated from triatomines, the PIs ranged from 1.77 to 8.02, while the PIs of the *T. cruzi* strains isolated from wild reservoirs ranged from 2.44 to 15.02. There was no significant difference ($p$ = 0.7429) among the PIs of *T. cruzi* strains isolated from different hosts (Fig. 1B). The mean PIs of *T. cruzi* strains isolated from human, triatomines, and wild reservoirs were 2.33, 3.80, and 3.12 respectively. The PIs were not statistically significant for *T. cruzi* strains isolated from a specific host (humans, $p$ = 0.08; triatomines, $p$ = 0.1116; wild reservoirs, $p$ = 0.0719).

**NO production by macrophages infected with *T. cruzi* I and II strains isolated from different hosts:** The parasites from the *T. cruzi* I genetic lineage were able to inhibit NO production by the macrophages stimulated with LPS prior to ($p < 0.000$) or during the parasite infection ($p < 0.000$), but not by macrophages stimulated after the infection. The parasites from the *T. cruzi* II genetic lineage inhibited NO production in all three periods. The difference in inhibition of NO production between the lineages was significantly greater for *T. cruzi* I parasites only when the LPS stimulation was carried out prior to the infection ($p$ = 0.013) (Fig. 2A). A statistically significant intra-lineage variation in NO production was observed for *T. cruzi* I ($p$ = 0.003), but not for *T. cruzi* II ($p$ = 0.079).

Figure 2B shows the NO production by the infected macrophages, according to the different hosts from which the strains were isolated. When the LPS stimulation was carried out prior to or during the addition of the parasite, parasites isolated from humans, triatomines, and sylvatic reservoirs all significantly inhibited NO production compared to the control ($p < 0.000$).

In the assays where macrophages were stimulated with LPS four h after the parasite infection, the parasites isolated from wild reservoirs did

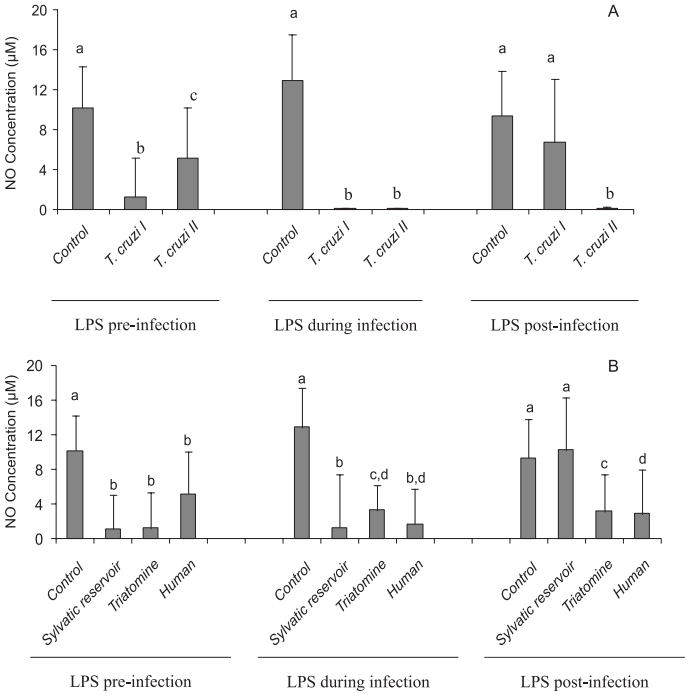


**Fig. 2 -** Nitric oxide (NO) production by macrophages from BALB/c mice infected with *Trypanosoma cruzi* strains from the state of Paraná, Brazil, and stimulated with LPS four h before, during, and after the parasite infection. Control group refers to NO production by non-stimulated macrophages. (A) Parasites from genetic lineages TcI and II and (B) Strains isolated from wild reservoirs, triatomines, and humans. For each experimental period (before, during, and after), bars marked with different letters are statistically different ($p < 0.05$). LPS: Lipopolysaccharide.

not inhibit NO production compared to the control ($p = 0.9463$), whereas the parasites isolated from humans and triatomines significantly inhibited NO production ($p = 0.00015$ for humans; $p = 0.0017$ for triatomines). The inhibition of NO production was significantly lower for parasites isolated from humans ($p < 0.0002$) than for those isolated from triatomines.

**Correlation between genetics and biology:** No correlation between genetics and biology was observed, when all *T. cruzi* lineages and hosts were analyzed together by the Mantel test, using the genetic distance and matrices from four biological parameters (phagocytic index, mean number of parasites per infected macrophage, percentage of infected macrophages, and nitric oxide production) (r = 0.023 and p = 0.4506).

When the biological parameters and the genetic distance were tested for *T. cruzi* I or *T. cruzi* II by RAPD or SSP-PCR, a significant correlation was found only for RAPD when the lineages were considered individually (*T. cruzi* I: r = 0.430, $p = 0.0142$; *T. cruzi* II: r = 0.80834, $p = 0.0379$). When biological parameters and the genetic distance were tested by type of host and RAPD or SSP-PCR, a significant correlation was found only for RAPD and humans (r = 0.80834, $p = 0.0379$) or wild reservoirs (r = 0.843, $p = 0.0411$).

## DISCUSSION

Although the PIs were not statistically different for *T. cruzi* I and II, the values varied significantly among strains of *T. cruzi* I. Also the values observed for NO production varied significantly among strains of *T. cruzi* I. This finding may be explained by the source of the strains of *T. cruzi* I, from wild reservoirs and triatomines. The biological characteristics of strains can be influenced by the parasite-host relationship. BÉRTOLI *et al.*[8] showed that the infectivity of the strains analyzed in their study was related to the host from which the strains were isolated, but was not related to the genetic lineage. This difference in behavior may also be a consequence of the isolated behavior of G1 (wild reservoir) and A21A, N914A and N120B (bugs) strains, which displayed significantly higher PI values than the others in the *T.cruzi* I group. In the case of strain A21A, it was shown that it is a mixture (Tc I+Tc II), in contrast to samples N914A and N120B, which are pure Tc I[36]. In another article from our group (in publication[2]), using the rRNA gene analysis (24Sα) and mitochondrial DNA (subunit 2 of cytochrome oxidase gene - COII) we found that the G1 strain also belongs to the *T. cruzi* I group. Even though the *T. cruzi* samples used in this study were randomly selected, they were all obtained from a very limited area in the state of Paraná. Also, no information on whether or which of these strains are composed by more than one clone (aside from strain A21A) is yet available. Thus, further studies to characterize the presence of polyclonal strains as well as the use of other *T. cruzi* isolates, obtained from other regions and from different hosts will help to elucidate the true significance of this variability. The PI of *T. cruzi* strains was lower in the strain isolated from humans, than from the wild reservoirs or triatomines, although not significantly. Because the PI for strains isolated from triatomines was higher than the others, we can infer that the host influences the PI. As reported elsewhere, biological characteristics can be determined by factors related to the host from which the strain has been isolated[37,41]. One report[41] clearly showed biological differences between three strains classified as *T. cruzi* I, with respect to parasitemia, tissue tropism, pathogenicity, and mortality. Other researchers have also discussed the influence that the host can have on the parasite, both with its metabolic versatility and with the wide range of environmental conditions where the host population lives[50].

In natural populations, the *T. cruzi* I strains showed a mean PI higher than that shown by the *T. cruzi* II strains. Other studies have demonstrated that clones of the *T. cruzi* II lineage show a lower percentage of infectivity to Vero cells or to BALB/c mice[39,47]. Although these results are in line with our data, infectivity is a much more complex phenomenon. In other studies with *T. cruzi* I and II strains, metacyclic trypomastigotes from *T. cruzi* II were more infective than were those from *T. cruzi* I[33]. Conversely, FERNANDES *et al.*[20], using extracellular amastigotes of the same strains, demonstrated that *T. cruzi* I displayed much higher infectivity than *T. cruzi* II. Metacyclic trypomastigotes and amastigotes from these lineages depend on different signaling mechanisms to invade various types of host cells[19,20,33].

Our data indicated a difference in NO production by natural populations of *T. cruzi* belonging to the genetic lineages *T. cruzi* I and *T. cruzi* II, or by strains isolated from different hosts. *T. cruzi* I parasites and strains isolated from wild reservoirs or triatomines showed the greatest suppression of NO production. These results are in agreement with reports by other authors[36,38] that *T. cruzi* has developed strategies to evade NO-mediated anti-microbial activity by suppressing its production. The ability of *T. cruzi* I parasites to escape from NO is in agreement with other biological characteristics of this genetic group, which include its greater infectivity, rate of intracellular replication, and virulence[23,39,47].

When LPS is added together with the parasite, the macrophages are so strongly stimulated that NO production is greatly suppressed. The suppression of production of a reagent against an exaggerated stimulus is not a rare event in the physiology of the immune system[1]. Experimentally, the NO levels were undetectable for two different genetic lineages of parasites. If we consider parasites isolated from different hosts, we find that the inhibition of NO production is higher for parasites isolated from wild reservoirs and humans than from triatomines, although parasites isolated from humans inhibited NO production similarly to the parasites isolated from triatomines. BÉRTOLI *et al.*[8] demonstrated a significantly higher infectivity to mice for strains isolated from wild reservoirs than those from humans and triatomines, illustrating the influence of the host on the virulence of *T. cruzi* strains. In the present study, the influence of the host on NO production was not as distinct; however, it was stronger than the influence of genetic lineage, because the strains isolated from wild reservoirs and triatomines, from the same genetic lineage (*T. cruzi* I), suppressed NO production at different rates.

When the infected macrophages are stimulated with LPS, the NO concentration can be understood as a result of the intrinsic capacity of each genetic lineage or strain. This stimulus is not strong and does not induce the down-regulation of NO production, as observed in macrophages stimulated with LPS prior to the infection. Under these conditions, the parasites from *T. cruzi* I that were isolated from wild reservoirs seemed to be a more efficient inducer of NO production than the *T. cruzi* II parasites. This could explain the higher infectivity, parasitemia, pathogenicity, and mortality in mice of *T. cruzi* I parasites[41], as NO acts as an inflammatory mediator in the *T. cruzi* infection.

As mentioned above, macrophages were placed in contact together with trypomastigotes and epimastigotes without purification of the

trypomastigotes. The trypomastigote-macrophage ratio was the same for all strains (2 trypomastigotes/macrophage). As far as we could determine, this procedure did not affect the phagocytic index or NO production. It is well established[40] that whereas trypomastigotes escape from the phagosome and divide in the cytoplasm, epimastigotes do not escape and will be killed. Therefore, the NO evaluated in this experiment was produced by activated macrophages and is known to be cytotoxic to *T. cruzi*[31,32]. Internalization of *T. cruzi* trypomastigotes by macrophages triggers the assembly of the NADPH oxidase complex, which does not interfere with sustained NO production (~24 h)[3].

There was no correlation between the genetic and biological parameters when *T. cruzi* lineages and hosts were considered together, although a significant correlation with RAPD was observed when data from both lineages or from human or wild reservoirs were tested separately. This result can be understood by considering that although some parameters may be directly related to each genetic marker, this relationship becomes diluted in the whole and the result is then not significant. Each particular parameter can be defined by multiple genes, and the molecular technique or the parameter evaluated may affect the result. The finding of a non-significant association between phylogenetic lineages and susceptibility to benznidazol suggests that different genes may be involved with this biological characteristic[48]. Similarly, another study related many different genes to metacyclogenesis[7]. A lack of correlation between genetics and biology has been suggested for *T. cruzi* strains in Mexico, because the biological characteristics may be determined by different factors from those identified in the genotypic characterization[41]. Another aspect possibly related to the lack of correlation between genetics and biology in our study is the existence of the third *T. cruzi* lineage[21,52], which was not considered here since it was only reported recently. Additional factors may influence the relationship between these strains. The host may influence the parasite, affecting its metabolic versatility over the wide range of environmental conditions where the hosts live[50]. Also, another study found that half of the triatomines analyzed in Paraná were co-infected by parasites from both *T. cruzi* I and II lineages[43].

Our data showed that biological parameters, including the Phagocytic Index, did not vary between the *T. cruzi* I and II genetic lineages, but did vary among *T. cruzi* strains isolated from a specific host. Nitric oxide production was inhibited by parasites of both lineages, and was related to the host from which the parasites were isolated, both depending on the stimulation status of the macrophage. The analysis also showed a correlation between biological parameters and the genetic distance of the *T. cruzi* strains.

## RESUMO

### Indução da atividade fagocitária e produção de óxido nítrico numa população natural de *Trypanosoma cruzi* I e II do Estado do Paraná, Brasil

Doze cepas de *Trypanosoma cruzi* isoladas de reservatórios silvestres, triatomíneos e de pacientes chagásicos crônicos do Estado do Paraná, Brasil, classificadas como Tc I e II foram usadas para avaliar a correlação entre genética e diversidade biológica. Índice fagocítico (IF) e produção de óxido nítrico (ON) *in vitro* foram os parâmetros biológicos utilizados. O IF de cepas *T. cruzi* I e II não diferiram significativamente assim como o IF de cepas isoladas de humanos, triatomíneos ou de reservatórios silvestres. Há diferença estatística na inibição da produção de ON entre *T. cruzi* I e II e entre parasitos isolados de humanos e de cepas isoladas de triatomíneos e reservatórios silvestres, mas não foi observada correlação entre genética e biologia quando as cepas foram analisadas independentemente da linhagem ou hospedeiros das quais elas foram isoladas. Observou-se correlação significativa para amplificação aleatória do DNA polimórfico e parâmetros biológicos de Tc I ou II e para os seres humanos ou reservatório silvestre quando linhagens ou hospedeiros são consideradas separadamente.

## ACKNOWLEDGEMENTS

This study was supported by grants from the *Coordenação de Aperfeiçoamento de Pessoal de Nível Superior* (CAPES-PROAP) of Brazil. Leila Zalloum holds a scholarship from CAPES.

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Received: 22 September 2010
Accepted: 8 September 2011

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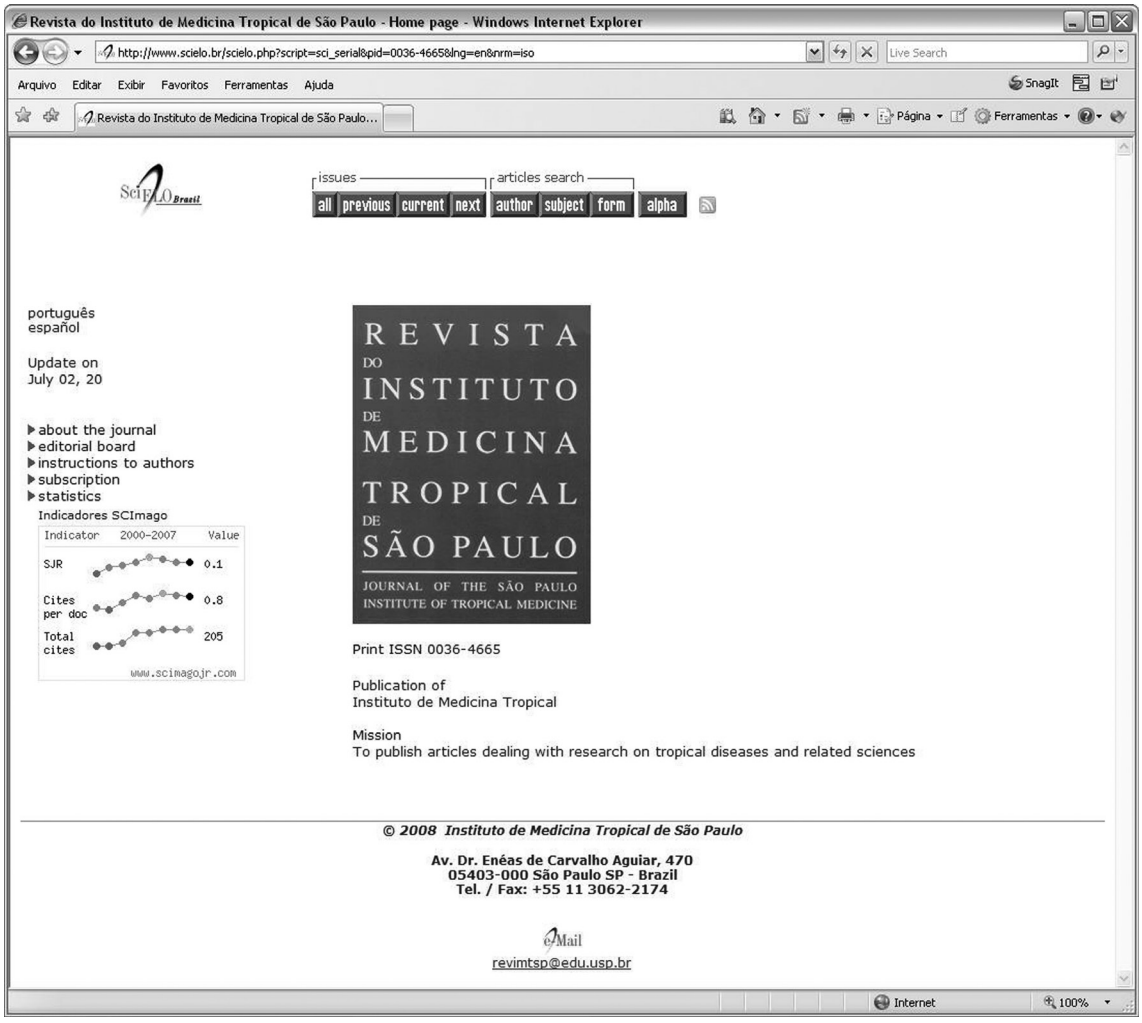


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