

## QUANTIFICATION OF *TRIPANOSOMA CRUZI* IN THE HEART, LYMPH NODES AND LIVER OF EXPERIMENTALLY INFECTED MICE, USING LIMITING DILUTION ANALYSIS

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*Limiting dilution analysis was used to quantify Trypanosoma cruzi in the lymph nodes, liver and heart of Swiss and C57 Bl/10 mice. The results showed that, in Swiss and Bl/10 mice infected with T. cruzi Y strain, the number of parasites/mg of tissue increased during the course of the infection in both types of mice, although a greater number of parasites were observed in heart tissue from Swiss mice than from Bl/10. With regard to liver tissue, it was observed that the parasite load in the initial phase of infection was higher than in heart. In experiments using T. cruzi Colombian strain, the parasite load in the heart of Swiss and Bl/10 mice increased relatively slowly, although high levels of parasitization were nonetheless observable by the end of the infection. As for the liver and lymph nodes, the concentration of parasites was lower over the entire course of infection than in heart. Both strains thus maintained their characteristic tissue tropisms. The limiting dilution assay (LDA) proved to be an appropriate method for more precise quantification of T. cruzi, comparing favorably with other direct microscopic methods that only give approximate scores.*

Key words: *Trypanosoma cruzi* – limiting dilution analysis – experimental infection

*Trypanosoma cruzi*, the causative agent of Chagas' disease, is widely distributed in Central and South America. This protozoan has a complex life cycle and exists in three main morphologically distinct forms: trypomastigote (metacyclic or bloodstream), epimastigote and amastigote (Hoare, 1972). Amastigote forms are intracellular, occurring exclusively in the mammalian host.

Accurate quantification of the number of parasites present in infected tissues of mice injected with *T. cruzi* is an important parameter to be analyzed in the experimental model.

A standard method for enumerating amastigotes of *T. cruzi* in organs of infected mammalian hosts has been to carry out histological examination of tissues sections (after hematoxylin-eosin staining) under the microscope (Hanson & Roberson, 1974; Melo & Brener, 1978; Castro & Brener, 1985). How-

ever, this method is not capable of detecting small numbers of parasites in tissues, nor can it distinguish living from dead organisms. Furthermore, only a small fragment of the organ can be examined.

Recently, we described a method for estimating the number of living parasites in the heart and blood of infected mice using a limiting dilution assay (LDA). The method is similar to the one used for quantifying *Leishmania* in cutaneous lesions of infected mice (Titus et al., 1985).

In this paper, we describe the use of LDA for quantification of *T. cruzi* in the heart, lymph nodes and liver of mice during the course of acute Chagas' disease. We compare these results with those obtained by examination of histological heart sections under the microscope.

### MATERIALS AND METHODS

*Mice* – Male albino Swiss and C57 Bl/10 mice, 4-6 weeks old were obtained from the animal house at the Oswaldo Cruz Institute, Rio de Janeiro, Brazil.

*Parasites* – Two strains of *T. cruzi* were used: Y (Silva & Nussenzweig, 1953), and

This work was partially supported by FINEP and by the UNDP/World Bank/WHO Special Programme for Research and Training in Tropical Diseases.

Received 28 April 1992.

Accepted 24 June 1992.

Colombian (Federici et al., 1964). These parasite strains were maintained by serial passage through Swiss mice, with intraperitoneal injections (i.p.) of  $10^5$  bloodstream trypomastigotes from infected mice at their peak of parasitemia. The Y strain was transferred from mouse to mouse every 7 days, and the Colombian strain every 14 days.

*Limiting dilution assay for the quantification of T. cruzi in the heart, liver and lymph nodes of infected mice* – The LDA technique was performed as described by Nunes et al. (1990). Briefly, the heart, liver and lymph nodes (inguinal and para-aortic) were removed from infected mice and cut into pieces, washed twice in a tube containing PBS (pH 7.2) by agitation in a vortex mixer, dried on filter paper, and weighed. The tissues fragments were then passed through a stainless-steel mesh into a petri dish containing Liver Infusion Triptose (LIT) medium (Chiari & Camargo 1984) plus 10% bovine serum and antibiotics (penicillin, 100 U/ml; streptomycin, 100 µg/ml). After homogenization, the suspension was centrifuged at 40 g for 5 min to remove debris. The supernatant was subjected to four serial dilutions in LIT, and distributed in four groups of 24 wells in a 96-well round bottomed microtitration plates (Nunclon, Denmark). The plates were maintained for 30 days at 27 °C in a humid atmosphere containing 5% CO<sub>2</sub>. After 20 and 30 days of culture each individual well was examined under an inverted microscope and checked for parasite growth. Although complete growth of the parasites could be detected after 20 days of culture, the plates were also examined, after 30 day to confirm that negative wells had not shifted to positive.

Minimal estimates of the number of viable *T. cruzi* per organ were calculated by the Poisson and  $\chi^2$  minimization methods (Taswell, 1981).

*Histological study of the heart* – A small fragment of heart from each animal was fixed in 10% formalin, embedded in paraffin and cut into 5 µm sections. After hematoxylin-eosin staining, the number of amastigote nests was counted and graded on a 5-point scale (Castro & Brener, 1985): (–) no parasites were detected; (+) parasites were detected after examination of more than 50 microscopic fields (40 x 10) of three sections; (++) 5, or less than 5 amastigote nests were detected per 50 microscopic fields; (+++) intermediate parasit-

ism; and (++++) parasites were detected in the majority of microscopic fields.

## RESULTS

*Quantification of T. cruzi Y strain in the heart, lymph nodes and liver of Swiss and C57 Bl/10 mice during the course of infection* – Using LDA, the number of living parasites in the heart, lymph nodes and liver of infected mice was determined at various points during the course of the infection. Table I shows that, in both Swiss and C57 Bl/10 mice, parasites were detected in heart four days after the initial infection. Parasitization of the heart reached its maximum by day 12. However the number of parasites/mg of heart tissue was much higher in Swiss than in C57 Bl/10 mice.

Parasites were detectable in the lymph nodes and liver of both Swiss and C57 Bl/10 mice four days after the initial infection (Table I). The parasite load in the lymph nodes reached its peak by day 8, with the number of *T. cruzi*/mg of tissue in both strains of mice declining thereafter. In the liver of Swiss mice, the number of parasites was observed to increase until day 8/10. The parasite load in liver was much higher than in heart until day 10.

*Quantification of T. cruzi Colombian strain in the heart, lymph nodes and liver of Swiss and C57 Bl/10 mice during the course of infection* – As with Y strain the number of parasites present in tissues of Swiss and C57 Bl/10 mice infected with the Colombian strain was estimated at several points after infection.

Table II shows that, in the early phases of infection (four days), parasites were not detected in the heart of either strain of mouse. Thereafter, the parasite load in heart of both types increased, reaching high levels.

In both strains of mouse, parasitization of the heart following infection with Colombian strain reached its maximum 26 days after infection. From that point onwards, a decrease in the number of parasites/mg of heart tissue was observed in Swiss mice (Table II). C57 Bl/10 mice did not survive after 26 days of infection.

In the liver of both Swiss and C57 Bl/10 mice, parasites were detected as early as four days after infection. The number of parasites subsequently reached its maximum at day 18, declining thereafter.



TABLE I

Quantification of *Trypanosoma cruzi* (Y strain) in the heart, liver and lymph nodes of Swiss and C57 Bl/10 mice during the course of infection as estimated by limiting dilution analysis

Day after infection	Number of <i>T. cruzi</i> per milligram of tissue					
	Heart		Liver		Lymph Nodes	
	Swiss	C57 Bl/10	Swiss	C57 Bl/10	Swiss	C57 Bl/10
2	<i>a</i>	<i>a</i>	<i>a</i>	<i>a</i>	<i>a</i>	<i>a</i>
4	5.4(±3.6)	7.19(±0.9)	56.81(±39)	132(±20)	11(±3)	5.8(±0.5)
6	65.7(±19)	104.2(±36)	1672(±890)	625(±91)	569(±420)	201(±98)
8	607.9(±360)	364(±56)	2932(±1788)	1934(±240)	2028(±1409)	411(±210)
10	3134.8(±992)	1250(±132)	6015(±3453)	1031(±85)	1651(±890)	400(±42)
12	3809.1(±753)	1333(±200)	2311(±1130)	806(±250)	292.7(±10.5)	285(±30)

Each value represents the mean (SD) of three mice.  
*a*: none detected.

TABLE II

Quantification of *Trypanosoma cruzi* (Colombian strain) in the heart, liver and lymph nodes of Swiss and C57 Bl/10 mice during the course of infection as estimated by limiting dilution analysis

Day after infection	Number of <i>T. cruzi</i> per milligram of tissue					
	Heart		Liver		Lymph Nodes	
	Swiss	C57 Bl/10	Swiss	C57 Bl/10	Swiss	C57 Bl/10
4	<i>a</i>	<i>a</i>	0.49(±0.23)	1.86(±0.94)	<i>a</i>	<i>a</i>
8	120(±28)	282(±30)	64.51(±52)	130(±56)	735(±370)	151(±70)
10	440(±360)	353(±277)	ND	ND	ND	ND
12	2904(±2205)	1678(±310)	328.95(±249)	382(±140)	3618.5(±1480)	255(±28)
16	22310(±1660)	16666(±1130)	2040(±920)	1956(±290)	1143.3(±170)	746(±80)
18	36019(±2746)	20408(±690)	7122(±5120)	3827(±417)	3982.6(±383)	2463(±141)
20	90909(±31345)	71428(±5200)	3189(±1772)	2822(±325)	5238(±2690)	4465(±218)
22	148898(±67970)	111029(±8598)	1291(±510)	545(±153)	8090(±4241)	6250(±700)
26	236036(±154150)	190722(±17548)	652.9(±490)	319(±42)	4810(±2503)	3164(±285)
36	127052(±70822)	<i>b</i>	35.3(±26)	<i>b</i>	1122(±482)	<i>b</i>

Each value represents the mean of three mice.  
*a*: none detected.  
*b*: all mice died.  
 ND: not done.

Parasites could be detected in the lymph nodes of both strains of mouse 8 days after the initial infection (Table II), with the parasite load subsequently reaching its peak at day 22.

It should be noted, however, that in Swiss mice the number of parasites/mg of tissue increased at day 12, declined at day 16, and subsequently increased again to reach its maximum at day 22. In C57 Bl/10 mice, on the other hand, lymph node parasitization increased progressively during the course of the infection until day 22.

*Quantification of T. cruzi in the heart of Swiss and C57 Bl/10 mice during the course of*

*infection, using LDA. Comparison of these results with those obtained by examination of histological sections under the microscope –* When examined under the microscope, heart sections from Swiss and C57 Bl/10 mice infected with Y strain failed to show parasitization four and six days, respectively, after infection (Table III). Using LDA, however, it was possible to quantify parasites in heart tissue obtained from the same animals after four days of infection.

Although the number of parasites detectable in the heart of both strain of mouse increased substantially during the course of the infection, as revealed by LDA, only very few

TABLE III

Quantification of *Trypanosoma cruzi* (Y strain) as estimated by limiting dilution analysis (LDA) and scores in crosses by microscopical examination of histological sections

Swiss			C57 Bl/10		
Day after Infection	Number of <i>T. cruzi</i> /mg of tissue (LDA)	Scores by microscopical examination	Number of <i>T. cruzi</i> /mg of tissue (LDA)	Scores by microscopical examination	
2	<i>a</i>	—	<i>a</i>	—	
4	7.2	—	8.2	—	
6	91	+	100	—	
8	454	+++	385	+	
10	3310	+++	1304	+++	
12	4000	+++	1413	++	

Data obtained from a representative experiment.  
*a*: none detected.

TABLE IV

Quantification of *Trypanosoma cruzi* (Colombian strain) as estimated by limiting dilution analysis (LDA) and scores in crosses by microscopical examination of histological sections

Swiss			C57 Bl/10		
Day after Infection	Number of <i>T. cruzi</i> /mg of tissue (LDA)	Scores by microscopical examination	Number of <i>T. cruzi</i> /mg of tissue (LDA)	Scores by microscopical examination	
4	<i>a</i>	—	<i>a</i>	—	
8	100	—	264	—	
10	801	—	370	+	
12	3706	+	1461	+	
16	24989	++	15848	++	
18	54400	+++	20000	+++	
20	76911	++++	67699	++++	
22	111288	++++	104978	++++	
26	345004	++++	178315	+++	
36	178678	+++	<i>b</i>	<i>b</i>	

Data obtained from a representative experiment.  
*a*: none detected.  
*b*: all mice died.

amastigote nests (+) could be detected at day 8 by examination of heart sections under the microscope. Furthermore, in Swiss mice, an identical score (+++) was registered on days 8, 10 under the microscope, whereas quite different numbers were established using LDA. Conversely, similar numbers of parasites were detected at day 10 and 12 in C57 Bl/10 using LDA, whereas the same days gave different scores when heart sections were examined under the microscope.

In experiments using Colombian strain, the LDA method was able to detect parasites in

heart homogenate in the early phase of infection (eight days). When heart sections were examined under the microscope, however, parasites could only be detected at days 10 and 12 after the infection in Swiss and C57 Bl/10 mice, respectively.

#### DISCUSSION

In a previous paper (Nunes et al., 1990), we described how the LDA method could be used for quantification of *T. cruzi* in the heart tissue of experimentally infected mice. The purpose of the present investigation has been

to extend those preliminary experiments, in order to use LDA to quantify *T. cruzi* not only in the heart, but also in the lymph nodes and liver of both Swiss and C57 Bl/10 mice during the course of the infection. In addition, we set out to compare the data obtained using LDA with equivalent data acquired by examination of histological sections of heart tissue under the microscope.

The *T. cruzi* strains used in this study (Y and Colombian) were chosen because they display different behavior in mice. They had been previously characterized according to their morphobiological parameters in mice and according to their isoenzyme patterns (Andrade, 1974, 1985; Romanha et al., 1979; Andrade et al., 1983). Andrade (1974) classified these strains as belonging to Type I and III, respectively.

Our present study shows that, using LDA, it is possible to quantify living parasites in tissues, even in the early stages of an infection.

The results obtained using Y strain confirmed the characteristic tissue tropism of this strain. In the heart of Swiss mice, the number of parasites/mg of tissue increased during the course of the infection, reaching its maximum when the number of bloodstream trypomastigotes had already declined. These data concur with results obtained by Melo & Brener (1978) showing low parasitism of the heart during the course of infection with the Y strain. These authors observed no correlation between levels of parasitemia and numbers of intracellular parasites in heart revealed by examination under the microscope.

The liver of Swiss mice infected with Y strain displayed a much higher number of parasites than the heart and lymph nodes on day 10, when analyzed using LDA. Levels of parasitism in liver and in blood were observed to show parallel profiles (data not shown).

Based on their observations of parasitism in spleen, liver, lymph nodes and bone marrow, Andrade (1974), Brener (1976) and Melo & Brener (1978) highlighted the macrophagotropism displayed by Y strain. In later stages of infection, when mortality rates were high, these same authors observed a preferential parasitization of skeletal and cardiac muscles.

In the case of Colombian strain, the highest levels of parasitization detected by LDA occurred in heart. Heart parasitization increased until the 26th day of infection, at which point parasitemia levels were very high. A close correlation between the numbers of trypomastigotes in blood and the parasite load in heart was observed.

These results suggest that Colombian strain display a predominately myocardial tropism, when compared to Y strain. Andrade & Andrade (1968) reported that Colombian strain induces more intense parasitism of the heart than the Y strain.

In spite of the classical myotropism displayed by Colombian strain, we nonetheless observed higher levels of parasitization in the lymph nodes and liver of Swiss mice during the acute phase of the infection with this strain than with Y strain. This may be due in part to the slow multiplication rate and late parasitemic peak observed to occur with Colombian strain. Hudson & Britten (1985) suggested that different *T. cruzi* strains may display different tropisms, even though amastigotes can in fact grow in any tissue.

Several studies have used different inbred strains of mice to investigate host humoral and cellular immune responses and parasite behavior. In the case of *T. cruzi*, the resistance of different strains of mice to infection by the parasite has been studied by several groups (Pizzi & Prager, 1952; Trischmann et al., 1978; Corsini et al., 1980; Trischmann & Bloom, 1982). Andrade (1985) studied six inbred strains of mice infected with three different strains of *T. cruzi* (classified into Types I, II and III), and concluded that the three parasite strains maintained their characteristics regardless of the mouse strain.

In the present study, two strains of *T. cruzi* were found to maintain their basic behavior in C57 Bl/10 mice with regard to tissue tropism, survival time and parasitemia curves (data not shown).

The patterns of tissue parasitization induced by Y and Colombian strain in C57 Bl/10 were more uniform than in Swiss mice.

The results obtained using this LDA method, suggest that both Swiss and C57 Bl/10 mice have similar susceptibilities to Colombian strain.



In both strains of mouse, Y strain showed an initial preference for parasitization of the liver rather than other tissues, up until the 10th day of infection.

It has been shown experimentally, that different strains of *T. cruzi* belonging to distinct types induce characteristic patterns of infection in different vertebrate species. Andrade & Andrade (1968) demonstrated that a strain isolated from a patient with acute Chagas' disease caused the same clinical and pathological picture when inoculated into dogs, mice and rats. These results suggest that patterns of infection depend on the intrinsic characteristics of particular parasite types, even if the severity of the disease in each instance may depend on the host.

This limiting dilution assay was able to detect living parasites much earlier than the technique based on counting of amastigote nests in histological sections under the microscope.

Melo & Brener (1978) reported that parasites could be detected under the microscope in heart tissue sections of mice infected with the Y strain only at the 7th day of infection. Using the same method, we obtained similar results whereas with LDA we were able to detect parasites in the heart of mice at the 4th day. Furthermore, samples giving identical scores with crosses gave a variety of results when the number of parasites present was estimated using LDA. The major limitations of the first technique are that the number of amastigote nests in the histological sections does not accurately reflect the number of parasites present, since only a small fragment from one organ is examined, and that the method does not distinguish living from dead organisms. LDA allows quantification of *T. cruzi* in the entire heart, which is important, since heart parasitization is not commonly distributed equally throughout the organ.

LDA is an efficient method for quantification of parasites in tissues and should be of value in other types of investigations, for example when assessing *in vivo* responses to chemotherapy or when studying the effects arising from manipulation of the host immune system, especially when kinetic studies are involved.

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