Acta Tropica 108 (2008) 26–34

Contents lists available at ScienceDirect

Acta Tropica

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

Biological characterization of *Trypanosoma cruzi* stocks from domestic and sylvatic vectors in Sierra Nevada of Santa Marta, Colombia

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ARTICLE INFO

Article history:
Received 11 December 2007
Received in revised form 12 August 2008
Accepted 21 August 2008
Available online 30 August 2008

Keywords:
Chagas disease
Colombia
Trypanosoma cruzi
Histopathology
Parasitic load
qPCR

ABSTRACT

Sierra Nevada of Santa Marta is one of the most endemic regions of Chagas disease in Colombia. In this study, we compared the biological behavior and genetic features of *Trypanosoma cruzi* stocks that were isolated from domestic and sylvatic insects in this area. *Rhodnius prolixus* (from domestic environments) and *Triatoma dimidiata* (from sylvatic, peridomestic and domestic environments) are the most important vectors in this region. Genetic characterization showed that all stocks corresponded to *T. cruzi* I, but LSSP-PCR analyses indicated that some genotypes were present in both environments. Biological characterization *in vitro* showed a low growth rate in sylvatic *T. cruzi* stocks and in some domestic *T. cruzi* stocks, possibly indicating the presence of stocks with similar behavior in both transmission cycles. In parallel, *in vivo* behavioral analysis also indicated that *T. cruzi* stocks are variable and this species did not show a correlation between the environments where they were isolated. In addition, all stocks demonstrated a low mortality rate and histopathological lesions in heart, skeletal muscle and colon tissue. Moreover, our data indicated that experimentally infected chagasic mice displayed a relation between their myocardial inflammation intensity, parasitism tissue and parasite load using the qPCR.

In conclusion, our results indicate that the *T. cruzi* stocks present in SNSM have similar biological behavior and do not show a correlation with the different transmission cycles. This could be explained by the complex transmission dynamics of *T. cruzi* in Sierra Nevada of Santa Marta, where hosts, vectors (e.g., *T. dimidiata*) and reservoirs circulate in both environments due to the close contact between the two transmission cycles, favoring environment overlapping. This knowledge is an important key to understanding the epidemiology and pathology of Chagas disease in this Colombian region. Furthermore, our findings could be of significant use in the design of control strategies restricted to a specified endemic region.

1. Introduction

Trypanosoma cruzi, the etiologic agent of Chagas disease, has infected 17 million people in South America (Moncayo, 2003). Approximately 30% of infected people develop chronic disease characterized by a variety of pathological features, including cardiomyopathy, megacolon and megaesophagus (Kirchhoff, 1996). This diversity of symptoms has been attributed to a group of complex interactions between host, parasites and environmental and social factors (Andrade and Magalhães, 1996; Macedo et al., 2002); however, the correlation between these variables has remained inconclusive.

Analysis of both the D7 domain of the 24Sα ribosomal RNA genes as well as the intergenic regions of spliced leader RNA (SL-RNA) genes allowed identification of two major phylogenetic groups among the *T. cruzi* isolates, designated as TcI and TcII (Souto et al., 1996; Fernández et al., 1998). *T. cruzi* lineages have a particular distribution throughout the American continent, with some of them predominant in certain geographical areas and transmission cycles. In Colombia, *T. cruzi* I is the predominant lineage associated with both sylvatic and domestic cycles (Márquez et al., 1998; Cuervo et al., 2002; Montilla et al., 2002; Triana et al., 2006; Salazar et al., 2006; Herrera et al., 2007). However, *T. cruzi* II was recently detected in blood of chronic chagasic patients (Zafra et al., 2008).

Sierra Nevada of Santa Marta (SNSM) is an isolated mountain mass, which rises abruptly from the tropical lowlands in the North coast of Colombia. SNSM is considered one of the most endemic regions for Chagas disease in Colombia (Dib et al., 2000; Dib and Ariza, 2000). This endemicity is favored by the presence of infected vectors such as *Rhodnius prolixus*, which is restricted to domestic environments, and *Triatoma dimidiata* from sylvatic, peridomestic and domestic environments (Agudelo et al., 2005). Analysis with different molecular markers indicated that *T. cruzi* stocks from *R.*

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doi:10.1016/j.actatropica.2008.08.006

Table 1
Biological and geographic origins of the *T. cruzi* populations studied

Environment	Stock	Biological origin	Geographic origin
Sylvatic	Mg8	*T. dimidiata*	Tinajas, Magdalena, SNSM
	Mg9	*T. dimidiata*	Tinajas, Magdalena, SNSM
	SN11	*T. dimidiata*	Gumake, Guajira, SNSM
Domestic	SN3	*R. prolixus*	Manzanal, Guajira, SNSM
	SN5	*R. prolixus*	Nimaiyshi, Magdalena, SNSM
	SN8	*R. prolixus*	Kasakumake, Guajira, SNSM
	SN9	*R. prolixus*	Umandita, Guajira, SNSM
	SN10	*R. prolixus*	Umandita, Guajira, SNSM

prolixus and *T. dimidiata* in SNSM were classified as *T. cruzi* I. However, RAPD and paraflagellar protein gene sequences were able to differentiate stocks from both environments (Dib et al., 2005).

Since differences have been observed in the biological behavior and genetic background of *T. cruzi* stocks isolated from different hosts and vectors (Devera et al., 2002; Toledo et al., 2002; Cummings and Tarleton, 2003; Martins et al., 2006), and in order to understand the pathogenesis of the Chagas disease in this region, the aim of this study was to evaluate the biological behavior of *T. cruzi* stocks isolated from insect vectors of sylvatic and domestic environments in the SNSM. We provide the biological characterization of *T. cruzi* stocks from two different vectors circulating in different ecological niches and describe the association between tissue parasitism (TP) with parasite load in cardiac tissue and inflammatory processes. Our results showed that some genotypes were present in both environments and that the *T. cruzi* stocks present in SNSM had similar biological behavior. In addition, no relationship between the different transmission cycles was found.

2. Materials and methods

2.1. Parasites

Three *T. cruzi* stocks isolated from sylvatic *T. dimidiata* and five from domestic *R. prolixus* were used. The origin of each stock is described in Table 1. All stocks were maintained at 28 °C in liver infusion tryptose (LIT) medium, supplemented with 10% fetal bovine serum (FBS) (Camargo, 1964).

2.2. Genetic characterization

2.2.1. Amplification of the intergenic region of the miniexon genes

DNA from epimastigote forms was obtained using the salting-out method (Miller et al., 1988). Amplification was done in 0.2-mL microcentrifuge tubes containing 25 μL of reaction mixture. Primers for amplification of the intergenic region of *T. cruzi* miniexon genes were 5′-GTGTCCGCCACCTCCTTCGGGCC-3′ (TC1, group II-specific), 5′-CCTGCAGGCACACGTGTGTGTG-3′ (TC2, group I-specific) and 5′-CCCCCCTCCCAGGCCACACTG-3′ (TC, common to groups I and II) (Souto et al., 1996). These primers allow amplification of a 350-bp fragment for *T. cruzi* I (TC I) and a 300-bp fragment for *T. cruzi* II (TC II). The reaction mixture contained 1 μL of DNA template, 50 mM KCl, 10 mM Tris–HCl (pH 8.0), 0.1% Triton X-100, 1.5 mM of $MgCl_2$, enzyme buffer 10×, 200 μM of dNTPs, 10 pmol of each primer and 2.5 U of Taq polymerase (Fermentas, Burlington, Ontario, Canada). Amplification was performed with an initial denaturation step at 94 °C for 1 min, followed by 27 cycles at 94 °C for 30 s, 55 °C for 30 s, and 72 °C for 30 s, with a final extension at 72 °C for 10 min (Souto et al., 1996).

Ten microliters of each product was analyzed by electrophoresis on 1.5% agarose gels in 1× TBE (89 mM Tris borate, 2 mM EDTA [pH 8.3]); the products were detected by ethidium bromide staining.

2.2.2. Genetic characterization by LSSP-PCR

Ten microliters of each PCR product was run on a 1.5% low-melting-point agarose gel and stained with ethidium bromide. Bands corresponding to 350 bp were cut from the gel, diluted to 1:10 in double distilled water and heated to 65 °C for 20 min. One microliter of the dilution was used as a template for the LSSP-PCR reaction (Vago et al., 1996). LSSP-PCR was performed in a 25-μL reaction volume at 50 mM KCl, 1.5 mM $MgCl_2$, 120 pmol of the TC primer, 200 μM of each dNTP and 4 U of Taq polymerase (Fermentas).

Amplification was carried out with 3 min of initial denaturing at 94 °C, followed by 35 cycles at 94 °C for 45 s, 30 °C for 45 s, 72 °C for 45 s and a final cycle at 72 °C for 10 min (Pena et al., 1994). Fifteen microliters of the amplification product from each of the stocks was analyzed by electrophoresis on 3% agarose gels stained with ethidium bromide and visualized under UV light. Each parasite stock was analyzed by LSSP-PCR in duplicate.

2.3. Biological characterization

2.3.1. In vitro growth curve

A total of 3×10^5 epimastigote forms of each *T. cruzi* stock were grown in 2.5 mL of LIT medium with 10% FBS at 28 °C. The relative growth of the population was established by the relationship between the number of parasites at an indicated time (Nt) and the number of parasites at the beginning of the experiment (Nt0). The number of parasites was determined every 24 h in a hemocytometer chamber for 32 days. Each count was made in triplicate. Average and standard deviation values were calculated (Zaidenberg et al., 2000).

2.3.2. Mice infection

Trypomastigotes were obtained by infecting Vero cells with parasites obtained from late stationary culture in LIT medium. The infected Vero cell monolayers were cultured in RPMI-1640 media supplemented with 2.5% FBS. Trypomastigotes diluted in a 0.85% NaCl physiological solution were used to infect male *Mus musculus* balb/c mice, 4–6 weeks of age, and these mice were used as a blood trypomastigote form source to infect experimental group mice (Tânia et al., 2000).

Groups of five balb/c mice, 4–6 weeks old, were inoculated intraperitoneally with 5×10^4 blood trypomastigotes of each *T. cruzi* stock. The experimental sample size was estimated statistically by a recursive equation used in a block experimental design, a method that is accepted in this type of study (Festing, 2002). One group of five noninfected mice was used as control. Parasitemia was evaluated every 3 days following the Brener modified method (Brener, 1962; Arias and Ferro, 1988). Biological parameters were evaluated with some modifications as described by Toledo et al. (2002) and Martins et al. (2006). Briefly, the prepatent period (PPP in days) was considered as the first day with positive fresh blood examination; the patent period (PP in days) as the period with positive fresh blood examination; the maximum parasitemia (MP) as the maximum number of trypomastigotes detected in 1 mL of blood; the day of maximum parasitemia (DMP in days); and the mortality rate expressed in cumulative percentage of deaths recorded during the course of infection (%MORT). These parameters were determined for the group of mice that was kept for continued follow-up of infection and served as reference to sacrifice the other experimental groups.

Mice were maintained under controlled laboratory conditions and all experimental procedures were done according to the local ethics committee's requirements.