

# Microsatellite and Mini-Exon Analysis of Mexican Human DTU I *Trypanosoma cruzi* Strains and Their Susceptibility to Nifurtimox and Benznidazole

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## Abstract

Chagas disease is caused by the protozoan parasite *Trypanosoma cruzi*, and it affects as many as 10 million people in North and South America, where it represents a major public health problem. *T. cruzi* is a parasite with high genetic diversity, and it has been grouped into 6 discrete typing units (DTUs), designated as *T. cruzi* I (TcI) to *T. cruzi* VI (TcVI). Mexican isolates from humans and from vector insects have been primarily found to be TcI, and these isolates are likely to be the strains that cause the clinical manifestations observed in Mexico. However, genetic characterization and drug susceptibility assays are limited in Mexican TcI strains. In this work, 24 Mexican *T. cruzi* strains, obtained primarily from humans, were studied with 7 locus microsatellites and mini-exon gene by PCR. Also, drug susceptibility was evaluated by growth and mobility assays. All of the human strains belonged to TcI, and they could be further grouped through microsatellite analysis into 2 subgroups (microsatellite genotypes 1 and 2), which were not related to the host clinical status or biological origin of the strain. Two strains, both from wild mammals, belonged to the TcII–TcVI groups; these strains and the CL Brener strain constituted microsatellite genotype 3. The number of alleles in each locus was lower than reported for South American strains, and a departure from the Hardy–Weinberg equilibrium was observed. The susceptibility of these strains to nifurtimox and benznidazole was heterogeneous. *T. cruzi* strains characterized as microsatellite genotypes 2 and 3 were significantly more susceptible to benznidazole than strains of microsatellite genotype 1. Only 1 Mexican strain resistant to both drugs was found in this study.

**Key Words:** Trypanosome—Chagas disease—Genetics—Nifurtimox—Benznidazole.

## Introduction

*Trypanosoma cruzi*, THE CAUSAL AGENT of Chagas disease, is present in several Latin American countries and 10 million infected inhabitants have been reported (World Health Organization 2010). In Mexico, a seroprevalence of 1.6% was reported several years ago (Velasco-Castrejón et al. 1992). However, some communities and blood banks showed seroprevalence rates higher than previously reported (Rangel-Flores et al. 2001, Galavíz-Silva et al. 2009).

The genetics of *T. cruzi* have been revised, and this species has been regrouped into 6 discrete typing units (DTUs) designated as TcI–TcVI (Zingales et al. 2009). Subgroups of TcI have also been reported, using mini-exon gene sequences and microsatellite analysis (Macedo et al. 2001, Falla et al. 2009, Llewellyn et al. 2009, Ocaña-Mayorga et al. 2010, Barnabé et al. 2011).

However, Mexican *T. cruzi* strains have not been characterized by these molecular markers. This characterization is important because differences in the biological features between Mexican and South American TcI strains, such as metacyclogenesis and growth *in vitro*, have been reported (López-Olmos et al. 1998).

The association of genetic markers with drug susceptibility in *T. cruzi* has been studied in some South American strains (Murta et al. 1998, Gomes et al. 2003). However, only a few studies have focused on the susceptibility of Mexican *T. cruzi* strains to nifurtimox or benznidazole, and the Mexican *T. cruzi* strains used in these studies have not been well characterized genetically (León-Pérez et al. 2007).

The purpose of the present work was to characterize Mexican human TcI strains through the analysis of 7 microsatellite loci and the mini-exon gene and to assay their susceptibility to nifurtimox and benznidazole *in vitro*.

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## Materials And Methods

### Parasite culture

Epimastigotes of 20 Mexican *T. cruzi* strains from humans with different clinical conditions (acute cases, chronic asymptomatic cases, and chronic chagasic cardiomyopathy) were obtained from different geographical areas of Mexico. Additionally, 2 samples from vectors (*Triatoma barberi* and *Triatoma picturata*) and 2 from wild mammals (*Philander opossum* and *Didelphis virginiana*) were also studied (Table 1). Epimastigotes were cultured in liver infusion and tryptose medium (LIT medium), supplemented with 10% fetal bovine serum (FBS) and 25  $\mu$ /mL of hemin. Cultures of parasites were maintained at 28°C according to López-Olmos et al. (1998). Two South American reference strains were included in this study—Silvio (TcI) and CL Brener (TcVI).

### DNA isolation

A total of 20 mL of parasite culture with 40–60  $\times 10^6$  parasites/mL was used to isolate DNA. This isolation was performed following extraction with phenol-chloroform from cell pellets, as previously reported (Macedo et al. 1992). The DNA obtained was maintained at –20°C until use.

### Mini-exon gene PCR

The intergenic region of the *T. cruzi* mini-exon gene was amplified using 3 oligonucleotides, as previously described (Souto et al. 1996). The amplification products were resolved for 30 min in 1.5% agarose gels at 115 volts and stained with ethidium bromide for 15 min. The gels were photographed

with a Gel Logic 200 transilluminator (Kodak, USA). The size of amplicons was determined by comparison with a DNA ladder of 100 bp (Invitrogen, USA).

### Microsatellite assay

Seven pairs of previously described microsatellite primers were used: SCLE11, MCL05, MCLG10, MCLF10, MCLE01, MCLE08, and SCLE10 (Oliveira et al. 1998). PCR was performed using a reaction mixture in a final volume of 50  $\mu$ L containing 20 pmol of each primer, 0.2 mM of each deoxyribonucleotide triphosphate (dNTP; Invitrogen, USA), 1  $\times$  PCR buffer, 4 mM MgCl<sub>2</sub> (Invitrogen, USA), 200 ng of DNA template, and 0.5 U of Platinum *Taq* DNA polymerase (Invitrogen, USA). The cycling conditions were standardized as 94°C for 10 min, 95°C for 1 min, 59°C for 2 min, and 72°C for 2 min. This cycle was followed by 30 cycles of a denaturation step at 95°C for 1 min; an annealing step at 59°C for 1 min (for SCLE11, MCLG10, MCLF10, SCLE10, and MCLE01), 57°C for 1 min (for MCL05), or 61°C for 1 min (for MCLE08) and an extension step at 72°C for 1 min. The amplification products were resolved with 6% non-denaturing acrylamide gels stained with ethidium bromide. Analysis of amplicons was performed as described above.

### Genetic and phylogenetic analysis

The presence, absence, number, and size of microsatellite PCR products in different strains were observed for the construction of a microsatellite binary data matrix. The phylogenetic reconstruction was performed with the MrBayes 3.1.2. program, using the Bayes theorem and simulation model of

TABLE 1. ORIGIN AND GENOTYPE OF MEXICAN *Trypanosoma cruzi* STRAINS

Strain nomenclature	Geographical origin	Biological origin	Clinical status <sup>a</sup>	<i>T. cruzi</i> group <sup>b</sup>	Microsatellite genotype
TBAR/MX/0000/Querétaro	Querétaro	<i>T. barberi</i>		TcI	1
TPIR/MX/0000/Guaymas	Sonora	<i>T. picturata</i>		TcI	1
MHOM/MX/1994/INC1	Oaxaca	Human	CCC	TcI	1
MHOM/MX/1994/INC5	Guanajuato	Human	CCC	TcI	1
MHOM/MX/2000/INC6	Oaxaca	Human	CCC	TcI	1
MHOM/MX/2001/INC7	Veracruz	Human	CCC	TcI	1
MHOM/MX/2001/INC8	Veracruz	Human	CCC	TcI	1
MHOM/MX/2001/INC9	Guerrero	Human	CCC	TcI	1
MHOM/MX/0000/H4	Yucatán	Human	Asymptomatic	TcI	1
MHOM/MX/2000/INC10	Guanajuato	Human	Asymptomatic	TcI	1
MHOM/MX/0000/EA	Morelos	Human	Asymptomatic	TcI	1
MHOM/MX/2001/Mor1	Morelos	Human	Asymptomatic	TcI	1
MHOM/MX/2001/Mor4	Morelos	Human	Asymptomatic	TcI	1
MHOM/MX/2001/Mor5	Morelos	Human	Asymptomatic	TcI	1
MHOM/MX/2001/Mor6	Morelos	Human	Asymptomatic	TcI	1
MHOM/MX/2001/Mor8	Morelos	Human	Asymptomatic	TcI	1
MHOM/MX/2001/Mor9	Morelos	Human	Asymptomatic	TcI	1
MHOM/MX/1994/Ninoa	Oaxaca	Human	Asymptomatic	TcI	1
MHOM/MX/2001/Mor3	Morelos	Human	Asymptomatic	TcI	2
MHOM/MX/1998/JJO	Jalisco	Human	Acute case	TcI	2
MHOM/MX/1998/JRA	Jalisco	Human	Acute case	TcI	1
MHOM/MX/0000/H1	Yucatán	Human	Acute case	TcI	1
MPHI/MX/1991/Ver3	Veracruz	<i>P. opossum</i>		c	3
MDID/MX/1991/Ver6	Veracruz	<i>D. virginiana</i>		c	3

<sup>a</sup>CCC indicates chronic chagasic cardiopathy cases; asymptomatic indicates chronic chagasic asymptomatic or indeterminate cases.

<sup>b</sup>According to nomenclature by Zingales et al. (2009).

<sup>c</sup>Similarity to CL Brener strain by mini-exon gene analysis and microsatellite products.

Markov Chain Monte Carlo (MCMC) to calculate the posterior probabilities of the trees. Analyses were performed for a data set of 1 million generations, with sampling trees every 1000 generations. Trees with a probability with a lower score than those at stationary phase (burn in) were discarded from the analysis. Generated trees reaching the stationary phase were collected and used to build a majority rule consensus tree as reported previously (Huelsenbeck et al. 2001). The Arlequin program (version 2000) was used for calculating the Hardy–Weinberg data, as described previously (Schneider et al. 2000).

*Drug susceptibility assays*

Epimastigotes were obtained after 5 days of cultivation (log phase of growth), and  $1 \times 10^6$  parasites/mL were seeded in 24-well culture plates (Costar, USA) in LIT medium supplemented as mentioned above. Benznidazole (*N*-benzyl-2-nitro-1-imidazolacetamide; Roche, Switzerland) and nifurtimox (tetrahydro-3-methyl-4-[(5-nitro-furfurylidene)amine]-2-methyl-tetrahydro-1,4-thiazine-4,4-dioxide; Bayer, Germany) were dissolved in dimethylsulfoxide (DMSO; Sigma, USA), and serial dilutions were made ranging from 0.038 to 384.6  $\mu$ M in the cultures. Controls with DMSO alone (1% v/v) were included in all of the experiments. The plated parasites were incubated at 28°C for 24 and 48 h. Three independent experiments were performed for each evaluation.

*IC<sub>50</sub> determination*

The number and motility of parasites were determined by counting in a Neubauer chamber, and the data were used to construct dose–response curves. The drug concentration inhibiting 50% of parasite growth (IC<sub>50</sub>G) or motility (IC<sub>50</sub>M) was determined using a formula previously reported by Villarreal et al. (2004).

*Statistical analysis*

The statistical significance between IC<sub>50</sub> values was determined with a paired 2-tailed *t*-test ( $p < 0.05$ ), as reported previously (Mezencev et al. 2009).

**Results**

*Mini-exon PCR*

The characteristic 350-bp product was obtained in 22 (91.7%) of the 24 strains, indicating that they belonged to DTU TcI as Silvio strain (Table 1). Only 2 strains (Ver3 and Ver6) presented a 300-bp product, indicating that they do not belong to TcI. By mini-exon gene PCR, it is not possible to discriminate among DTUs TcII through TcVI.

*Microsatellite analysis*

Eighteen strains from humans and 2 strains isolated from vectors were named microsatellite genotype 1 (Table 1). Two strains isolated from humans (JJO and Mor3) were different from microsatellite genotype 1 in the MCL05 locus, and these strains were named microsatellite genotype 2. Furthermore, the Ver3 and Ver6 strains were labeled microsatellite genotype 3 by their different amplified profile (Fig. 1). No more than 3 alleles were found in each locus, ranging between 90 and 450 bp (Table 2). Microsatellite data from the population genetic analysis showed departures from the Hardy–Weinberg equilibrium ( $p < 0.001$ ). Differences were found between the observed and expected heterozygosity in 5 out of 7 microsatellite loci (Table 2). The statistical tests for microsatellite loci showed that these loci were strongly associated with homozygosity within the strains ( $p < 0.001$ ). A phylogenetic tree was built using the microsatellite data, which demonstrated a clear separation of 2 groups with high posterior probability values (Fig. 2).

*Effect of benznidazole and nifurtimox on parasite growth and mobility*

Nine Mexican strains of *T. cruzi* were selected to evaluate their susceptibility to nifurtimox and benznidazole, based on the clinical status of infected patients and the geographic origin of the host. Six strains from microsatellite genotype 1, 2 from microsatellite genotype 2, and 1 from microsatellite genotype 3 were chosen.

The Mexican strains of *T. cruzi* had different susceptibilities to these drugs. The greatest effect of the drugs on motility and

FIG. 1. PCR products obtained with the primers indicated (left) of the different Mexican strains of *T. cruzi* (indicated above). The presence or absence and the product sizes were used to determine genotypes. A representative experiment is shown.

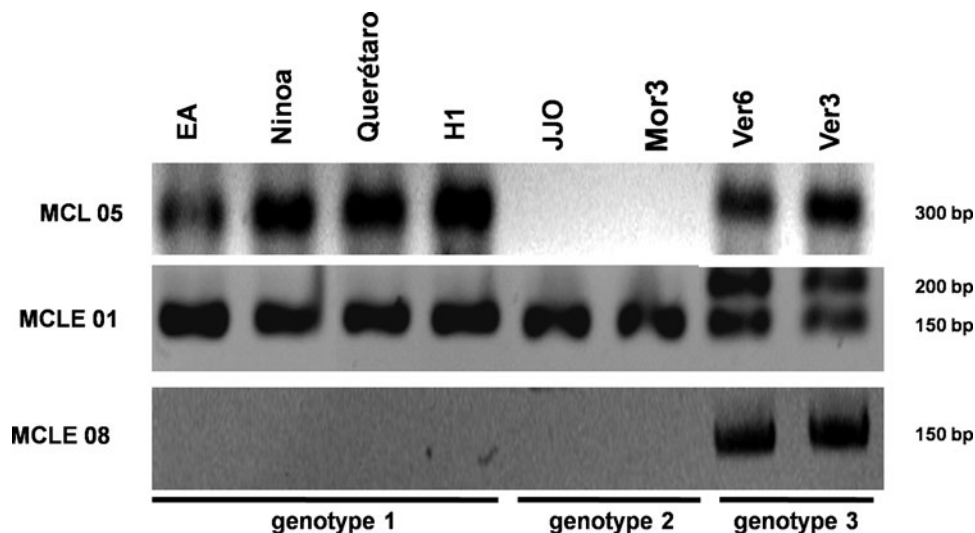


TABLE 2. MICROSATELLITES OF MEXICAN DTU TcI STRAINS

Marker	Repeat sequence <sup>a</sup>	Size allele (bp) <sup>b</sup>	Number of alleles found	Heterozygosity observed/expected
MCLG 10	(CA) <sub>8</sub>	90* 300* 200**	3	0.88/0.61
SCLE 11	(AC) <sub>9</sub>	140* 160**	2	0.00/0.24
SCLE 10	(GT) <sub>2</sub> (TG) <sub>10</sub>	250* 450**	2	0.00/0.24
MCLF 10	(CA) <sub>2</sub> A(CA) <sub>14</sub>	150* 200**	2	0.00/0.24
MCLE 08	(CA) <sub>2</sub> AA(CA) <sub>12</sub>	150**	1	ML
MCLE 01	(CA) <sub>9</sub>	150* 200**	2	0.11/0.14
MCL 05	(TC) <sub>9</sub> (GT) <sub>4</sub>	300***	1	ML

<sup>a</sup>As reported by Oliveira et al. (1998)

<sup>b</sup>Products observed in microsatellite genotype 1 and 2 (\*), genotype 3 (\*\*), and genotype 1 and 3 (\*\*\*). DTU, Discrete typing units; ML, monomorphic locus.

growth was reached after treatment for 48 h. Benznidazole treatment showed that Mexican strains belonging to microsatellite genotypes 2 and 3 (JJO, Mor 3, and Ver6) had IC<sub>50</sub>G values that were significantly lower than those of other strains (Table 3). Silvio and H1 were the only strains with an IC<sub>50</sub>G value significantly higher than the other strains. When mobility was analyzed, some strains required concentrations higher than 384.6 μM to reach their IC<sub>50</sub>M.

In the presence of nifurtimox, low IC<sub>50</sub>G values were obtained for the Ninoa, Querétaro, Ver6, and CL Brener strains (*p* < 0.05). When the IC<sub>50</sub>G values of benznidazole versus nifurtimox were compared, JJO, Mor 3, Ver6, and EA were more susceptible to benznidazole. In contrast, the Ninoa strain was more susceptible to nifurtimox. The comparison of IC<sub>50</sub>M values showed that parasite motility was more affected in JJO, Mor 3, and Ver6 by benznidazole, whereas INC9 and INC10

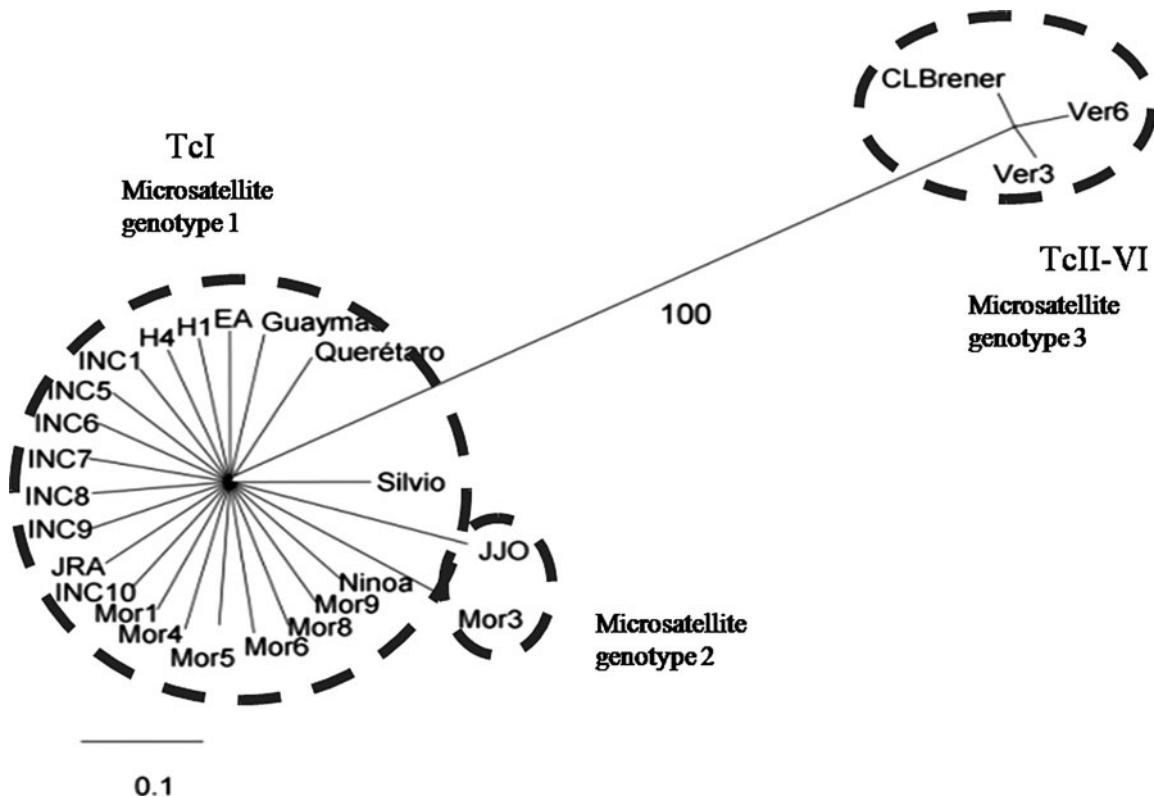


FIG. 2. Phylogenetic tree of Mexican *T. cruzi* strains. The microsatellites data matrix was analyzed with the MrBayes program. Values of the nodes indicate the posterior probability by Markov Chain Monte Carlo (MCMC) analysis.

TABLE 3. DRUG INHIBITORY CONCENTRATION AT 48 HOURS

Strain	MG <sup>a</sup>	Benznidazole ( $\mu$ M)		Nifurtimox ( $\mu$ M)	
		IC <sub>50</sub> G	IC <sub>50</sub> M	IC <sub>50</sub> G	IC <sub>50</sub> M
Silvio	1	63.3 ± 22.8 <sup>b</sup>	NF	73.4 ± 23 <sup>b</sup>	NF
MHOM/MX/0000/EA	1	0.7 ± 0.02 <sup>b,c</sup>	5.7 ± 1.1	7 ± 0.8	6.5 ± 0.15
MHOM/MX/0000/H1	1	61.99 ± 14.17 <sup>b</sup>	NF	59.2 ± 11 <sup>b</sup>	NF
MHOM/MX/2001/INC9	1	7.1 ± 3.9	NF	4.5 ± 1.19	10.2 ± 1.7 <sup>c</sup>
MHOM/MX/2000/INC10	1	12.27 ± 4.2	NF	5 ± 0.7	9 ± 2.7 <sup>c</sup>
MHOM/MX/1994/Ninoa	1	8.21 ± 1.8	NF	0.21 ± 0.18 <sup>b,c</sup>	NF
TBAR/MX/0000/Querétaro	1	4.29 ± 2.9	17.7 ± 11.6	0.38 ± 0.3 <sup>b</sup>	0.9 ± 0.3 <sup>b</sup>
MHOM/MX/1998/JJO	2	0.38 ± 0.3 <sup>b,c</sup>	0.85 ± 0.3 <sup>c</sup>	7.7 ± 3.5	NF
MHOM/MX/2001/Mor3	2	0.8 ± 0.5 <sup>b,c</sup>	3.7 ± 2.8 <sup>c</sup>	7.24 ± 1.8	NF
MDID/MX/1991/Ver6	3	0.04 ± 0.01 <sup>b,c</sup>	0.07 ± 0.09 <sup>b,c</sup>	0.68 ± 0.5 <sup>b</sup>	6 ± 0.8
CL Brener	3	5.54 ± 0.44	69.92 ± 3.4 <sup>b,c</sup>	0.51 ± 0.2 <sup>b,c</sup>	6.38 ± 0.01

<sup>a</sup>Microsatellite genotype according to Table 1.

<sup>b</sup>Statistical differences ( $p < 0.05$ ) respect other strains with the same drug.

<sup>c</sup>Statistical differences ( $p < 0.05$ ) comparing the same strain and parameter with different drugs. IC, Inhibitory concentration; NF, IC<sub>50</sub>M higher than 384.6  $\mu$ M.

were more susceptible to nifurtimox. Finally, the Silvio and H1 strains were considered resistant to both drugs *in vitro* because more than 50  $\mu$ M was needed to affect their growth and motility.

**Discussion**

Six DTUs of *T. cruzi* have been identified and designated as TcI–TcVI (Zingales et al. 2009). In Mexico, most *T. cruzi* strains have been genetically determined to belong to TcI (Bosseno et al. 2002, León-Pérez et al. 2007, Gómez-Hernández et al. 2011) and TcII–TcVI strains have been reported only in a recent serological study, in wild mammals and the feces of vectors (López-Olmos et al. 1998, Bosseno et al. 2009, Risso et al. 2011). In this work, we confirmed that the Mexican *T. cruzi* strains isolated from humans belonged to TcI, as reported in Guatemala, Colombia, and Venezuela (Añez et al. 2004, Ruíz-Sánchez et al. 2005, Falla et al. 2009). In the present work, 2 other strains from wild mammals belonging to TcII–TcVI groups were also identified, but their association with any specific DTU remains to be determined.

Several strains of TcI from Latin American countries have been analyzed with microsatellite analysis, and at least 2 subgroups have been established (Macedo et al. 2001, Llewellyn et al. 2009). In the present work, the analyzed TcI Mexican strains were grouped into 2 microsatellite genotypes. Most strains were grouped as microsatellite genotype 1, and only 2 strains were subgrouped as microsatellite genotype 2. These strains were not geographically related. Only 2 strains (Ver3 and Ver6) were microsatellite genotype 3. However, a more careful characterization is needed to determinate to which genetic group (TcII–TcVI) they belong.

The effect of nifurtimox and benznidazole on parasite motility and growth was evaluated. Only the Mexican strain H1 was resistant to both drugs, according to the criteria established in previous studies, because it had IC<sub>50</sub>G and IC<sub>50</sub>M values higher than 50  $\mu$ M (Nirdé et al. 1995, Villarreal et al. 2005). The IC<sub>50</sub>M and IC<sub>50</sub>G values for the other Mexican strains were heterogeneous, and they were comparable with values reported for South American strains, such as Tulahuén

2 (TcIV), Dm28c (TcI), Silvio (TcI), Esmeraldo (TcII), and Y (TcII) (Cinque et al. 1998, Vieites et al. 2008, Wilkinson et al. 2008, Luna et al. 2009).

We found a possible correlation between genetic characteristics (microsatellite genotype 2) and susceptibility to the drug benznidazole, described previously only in South American strains (Murta et al. 1998, Gomes et al. 2003, Toledo et al. 2003).

Previous studies reported the effect of both drugs either on parasite motility or growth. It has been shown that loss of motility *in vitro* can be correlated with the death of the parasite (Jacobs et al. 2003). However, this parameter can only give an incomplete picture of the trypanocidal effect, because it has also been reported that some compounds affect only the motility and the growth and viability of the parasite are not affected (Arantes et al. 2011). It has also been demonstrated that some compounds with anti-*T. cruzi* activity can affect growth but not motility, allowing the parasite to survive (Zaverucha et al. 2003).

In this work, the effect of nifurtimox and benznidazole on both parameters was evaluated to determine if differences existed between them. We show the importance of evaluating both motility and growth to determine the real trypanocidal effect of the drugs because growth can be affected more than motility in some strains. It would be important to assess the effect of any drug on both parameters, particularly those recently proposed for the control of the parasite.

In the future, it will be important to study the role of genes such as type I nitroreductase (NTR) and OYE in benznidazole susceptibility in Mexican strains and to study possible markers of this biological response (Murta et al. 2006, Mejía-Jaramillo et al. 2011). In addition, it will be interesting to analyze whether microsatellite genotypes 1 and 2 described in the present study can be confirmed by a different set of genetic markers.

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### Author Disclosure Statement

No competing financial interests exist.

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