

# Dissimilar distribution of *Trypanosoma cruzi* clones in humans after chemotherapy with allopurinol and itraconazole

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**Objectives:** The aim of this work was to study the distribution of *Trypanosoma cruzi* clones after treatment failure with itraconazole or allopurinol in infected humans.

**Methods:** Blood samples from treated and untreated individuals were used to detect *T. cruzi* by PCR assays and were confirmed by hybridization tests using total kinetoplast DNA as a universal probe. Also, xenodiagnosis (XD) tests were performed with *Triatoma infestans* fed from the same group of patients. We performed Southern-blot analyses of PCR products from blood or XD samples using a panel of four genotype-specific probes: corresponding to *T. cruzi* clones TcI, TcIIb, TcIIId and TcIIe. The membranes were hybridized with radiolabelled probes and exposed in a Personal Molecular Imager.

**Results:** When comparing the presence of *T. cruzi* clones in the allopurinol-treated group with the non-treated group significant differences were only observed for XD samples. Clone TcI was present in 9/13 (69.2%) of the XD samples of the treated group, but only in 8/27 (29.6%) in the non-treated group ( $P = 0.0178$ ). When the itraconazole-treated group and the control group were compared, significant differences were found in both the blood and XD samples. In blood, the clone TcIIb was detected in 6/17 (35.5%) of the treated group and in 18/27 (66.7%) of the non-treated group ( $P = 0.0207$ ). When XD samples were analysed, the clone TcI was observed in 14/17 (82.3%) of the itraconazole-treated group but only in 8/27 (29.6%) of the control group ( $P = 0.0006$ ), which suggests resistance of this clone to itraconazole.

**Conclusions:** We detected a dissimilar distribution of *T. cruzi* clones in treated and untreated groups of patients. The presence of TcI increased in patients treated with allopurinol and itraconazole, whereas the presence of TcIIb decreased in itraconazole-treated patients. The type of *T. cruzi* clone that prevails suggests that TcI is resistant to both drugs and that TcIIb is susceptible to itraconazole.

Keywords: Chagas' disease, *T. cruzi*, protozoa

## Introduction

*Trypanosoma cruzi*, a flagellate protozoan, the agent of Chagas' disease, represents a public health problem in Central and South America.<sup>1</sup> *T. cruzi* is composed of a heterogeneous population of clones circulating in domestic and sylvatic cycles, which include humans, insect vectors and animals.<sup>2</sup> The isolation of *T. cruzi* populations from different sources has demonstrated the presence of a broad range of parasite clones with different genetic, biological, immunological, molecular and pharmacological

characteristics.<sup>3,4</sup> Several therapeutic studies have been carried out for acute and chronic cases of Chagas' disease using nifurtimox and benznidazole. They compared the efficacy and tolerance of drugs in patients, therapeutic schemes, periods of follow-up and cure evaluation criteria. These drugs have low efficacy in chronic cases; contributing factors to this may be the predominant *T. cruzi* strain in each geographic area and the current phase of the disease.<sup>5</sup> Basic studies of nifurtimox and benznidazole have illuminated the molecular basis of both the anti-*T. cruzi* activity and toxicity of these compounds.

## Distribution of *Trypanosoma cruzi* clones

Nifurtimox and benznidazole have significant activity in the acute phase, with up to 80% parasitological cure rates in treated patients.<sup>6</sup> However, their efficacy varies according to the geographical area, probably due to differences in drug susceptibility among different *T. cruzi* strains.<sup>5,6</sup> It is known that the success of treatment with nifurtimox and benznidazole is limited by the prevalence of *T. cruzi* strains resistant to these chemotherapeutics, a fact correlated with biological characteristics of the parasite. The so-called type III biotopeme is highly resistant. Strains isolated from mice previously treated with benznidazole showed increased resistance to treatment with the same drug, suggesting the selection of resistant clones to explain the persistence of infection in treated mice.<sup>7</sup> The predominance of resistant clones in *T. cruzi* strains is probably responsible for treatment failure, as seen in the endemic area of Central Brazil in patients infected with strains of the biotopeme type III, Z1 (*T. cruzi* I) as compared with those infected with biotopeme II, Z2 (*T. cruzi* II). In the past decade, benznidazole has been reported to have significant curative activity in recent chronic disease (up to a few years post-infection), with up to 60% parasitological cure rates observed in infected children of Argentina and Brazil treated with this compound.<sup>8,9</sup> Similar results were obtained in Chile with nifurtimox.<sup>10</sup> Both drugs have significant side effects, probably as a consequence of oxidative or reductive damage in the host's tissues. The most frequent collateral effects with nifurtimox and benznidazole are alterations of the digestive, cutaneous and nervous systems.<sup>11</sup> However, the low anti-parasitic activity in the chronic form of the disease is a major limitation of both compounds because ~80% of treated patients do not achieve parasitological cure. These conclusions based on the persistence of positive anti-*T. cruzi* serology and clinical evolution of these patients have now been confirmed using PCR-based methods.<sup>12</sup> The reasons for the marked difference in the anti-parasitic efficacy of nitro-heterocyclic compounds between the acute and chronic stages of the disease are not known,<sup>5</sup> but they could be related to unfavourable pharmacokinetic properties of the drugs in the chronic stages.<sup>13</sup> After the introduction of nifurtimox and benznidazole, few new compounds have been tested. The results obtained with allopurinol in experimental models and the knowledge of its mode of action led to clinical assays for the treatment of Chagas' disease. The therapeutic experience comparing allopurinol, benznidazole and nifurtimox in a prospective study with chronic cases, indicates that allopurinol was the most tolerated drug and the one with the least incidence of therapeutic abandonments.<sup>14</sup> The azole itraconazole has been shown to have anti-*T. cruzi* activity in murine models.<sup>15</sup> Parasitological evaluations by xenodiagnosis (XD) of chronic infected individuals treated with allopurinol or itraconazole revealed promising results of drug efficacy.<sup>16,17</sup>

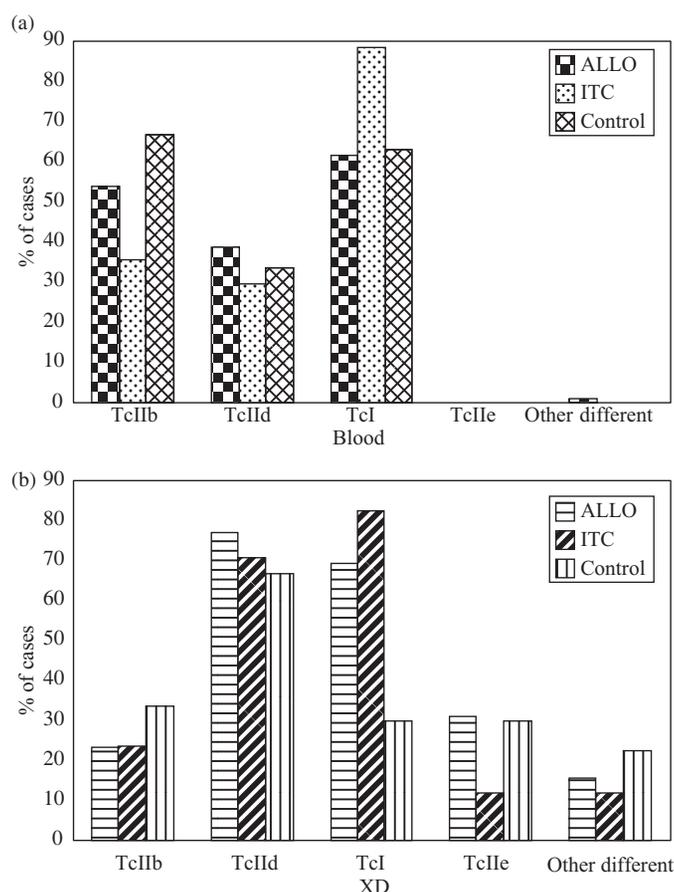
## Materials and methods

The distribution of *T. cruzi* clones after treatment with itraconazole and allopurinol in humans 12 years after completion of therapy was studied. Infected subjects (untreated) studied here inhabit an endemic area free of vectorial transmission. They were treated with allopurinol ( $n = 13$ ; 8.5 mg/kg/day for 60 days; 300 mg tablets; Silesia Laboratories, Chile) or with itraconazole ( $n = 17$ ; 6 mg/kg/day divided into two doses per day over a 120 day period; 100 mg capsules; Janssen Laboratories, Beerse, Belgium). Supervision for compliance was performed by direct observed therapy at the rural

outpatient clinics. Each patient was serologically and clinically evaluated every year. Twenty-seven infected individuals without previous treatment against *T. cruzi* were considered as a non-treated group (control group). The study was approved by the Ethics Committee of the Faculty of Medicine of the University of Chile, and informed consent was obtained from each individual. Flow cytometry of anti-live trypomastigote antibodies (FC-ALTA) was performed to detect active infection. XD tests were also performed. This test was carried out using two boxes with seven uninfected third-instar nymphs of *Triatoma infestans* each, fed with peripheral blood of the infected individual.<sup>18</sup> Microscopic examination of insect faeces was performed 30, 60 and 90 days after feeding and a pool was obtained to perform PCR assays, directed to *T. cruzi* minicircle DNA whether they resulted positive or negative. Blood samples from infected individuals were used to detect *T. cruzi* by PCR assays performed in triplicate and were confirmed by hybridization tests using total kinetoplast DNA as a universal probe, as described by Solari *et al.*<sup>10</sup> All PCR-amplified DNA from blood or XD samples was further analysed by Southern blot with a panel of four genotype-specific probes. These were from *T. cruzi* clones, sp104cl1, CBBcl3, NRcl3 and v195cl1, corresponding to clones, 19 (TcI), 32 (TcIIb), 39 (TcIIc) and 43 (TcIIe), respectively, and prepared as described by Torres *et al.*<sup>19</sup> The probes were radiolabelled with <sup>32</sup>P and membranes were exposed and analysed in a Personal Molecular Imager-FX (Bio-Rad, USA).  $\chi^2$  was used as a statistical method to analyse the results.

## Results

Results with this panel of specific probes revealed complex hybridization patterns for blood and insect samples, indicative of *T. cruzi* infection with more than one clone in some patients. No cure was assessed by FC-ALTA or by PCR with blood and XD samples. Percentages of positive samples detected by PCR of blood samples [89.5% (51/57)] and PCR of XD samples [91.2% (52/57)] increased to 100% and 96.5% (55/57), respectively, with the hybridization test. We predicted that the lower or higher percentage of a particular *T. cruzi* clone should be indicative of parasite susceptibility or resistance to chemotherapy (although a quantification of parasitaemia levels was not performed in the present study). When comparing the presence of *T. cruzi* clones in the allopurinol-treated group with the control group significant differences were only observed for XD samples (Figure 1). Clone TcI was present in 9/13 (69.2%) of the XD samples of the treated group, but only in 8/27 (29.6%) in the control group ( $P = 0.0178$ ), which suggests resistance of this clone to allopurinol. When the itraconazole-treated group and the control group were compared, significant differences were found in both the blood and XD samples. In blood, the clone TcIIb was detected in 6/17 (35.5%) of the treated group and in 18/27 (66.7%) of the control group ( $P = 0.0207$ ), which suggests susceptibility of this clone to itraconazole. On the other hand, when XD samples were analysed, the clone TcI was observed in 14/17 (82.3%) of the itraconazole-treated group but only in 8/27 (29.6%) of the control group ( $P = 0.0006$ ), which suggests resistance of this clone to itraconazole. Therefore, in the itraconazole-treated group, a lower percentage of clone TcIIb was detected as compared with the control group in blood, and a higher percentage of clone TcI was detected in the treated group than in the control group, suggesting susceptibility and resistance to the drug, respectively. No significant differences were found for the *T. cruzi* clones TcIIc and



**Figure 1.** Percentage of cases infected with different *T. cruzi* clones detected in blood (a) and triatomine samples (b) of infected individuals untreated and treated with allopurinol (ALLO) and itraconazole (ITC).

TcIIe in the treated (allopurinol and itraconazole) and non-treated groups.

## Discussion

The frequencies of each *T. cruzi* clone found in the humans and *T. infestans* were different. Therefore, they have different adaptations in vertebrate and invertebrate hosts.<sup>20</sup> It is a frequent observation that, in chronic chagasic patients, a *T. cruzi* clone may be selected during the long-term interaction and differential tissue tropism that may interfere with *T. cruzi* subpopulation distribution. These 'filters' might select those populations or clones that are more apt in the new environment.<sup>19</sup> In this work *T. cruzi* clones TcIIId and TcIIe, and TcI and TcIIb are preferentially adapted to *T. infestans* and humans, respectively. Therefore, in situations with parasitological cure failure, as shown here, the best adapted *T. cruzi* clone to a host will be associated with the resistance to chemotherapy. In contrast, the lower adaptation of *T. cruzi* clones to a host will be associated with drug susceptibility. In summary, our study allows a more refined method to approach parasite drug resistance or susceptibility by use of two samples from each patient (blood and XD). Investigations into *T. cruzi* phylogenetic diversity and chemotherapy efficacy association, with nifurtimox and benznidazole, in mice infected with

different *T. cruzi* clones, revealed that TcIIb was highly susceptible and TcI highly resistant to these drugs.<sup>3</sup> More recently, other authors confirmed this association between phylogenetic diversity and chemotherapeutic response in the murine experimental model. They observed that TcI is resistant to benznidazole and itraconazole during the acute and chronic phases, while TcIIb is susceptible to itraconazole.<sup>21</sup> These results agree with those described here for the first time in humans, suggesting that susceptibility or resistance to a drug depends on the *T. cruzi* genotype. This result indicates that the appropriate drug or drugs to treat patients should depend upon the infective *T. cruzi* clone, or mixture of *T. cruzi* clones, present in a particular host. Unfortunately, the *T. cruzi* I clone is prevalent in many endemic areas of South America and is resistant to the currently used anti-chagasic drugs, thus new drugs effective against different *T. cruzi* clones circulating in nature are needed.

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## Transparency declarations

None to declare.

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