Coexistence of *Trypanosoma cruzi* Genotypes in Wild and Periodomestic Mammals in Chile

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Abstract. Epidemiologic evidence suggests a preferential association of *Trypanosoma cruzi* genotypes TCI and TCII with marsupials and placental mammals, respectively. We identify *T. cruzi* genotypes from 117 infected mammals. Minicircle DNA amplified by polymerase chain reaction and hybridization with a panel of four specific probes showed frequencies for the *T. cruzi* genotypes TCI, TCIIb, TCIIId, and TCIIe of 38%, 41%, 26%, and 9%, respectively, in wild mammals. In peridomestic mammals, frequencies for the same clones were 29%, 33%, 43%, and 14%, respectively. As a whole, mixed infections are found in more than 31% of the cases, which indicates the coexistence of multiclonal strains circulating in nature, and the absence of specific associations between *T. cruzi* genotypes and reservoir hosts, including marsupials. The direct characterization of parasite genotypes emphasizes the importance of obtaining unbiased epidemiologic information from parasite-endemic areas. Results are discussed in the context of competition or facilitation of *T. cruzi* genotypes within hosts.

INTRODUCTION

Specificity between host and parasites is the ability of a particular parasite to infect a host more efficiently than another host and, conversely, the ability of a host to resist infection by one parasite more efficiently than another parasite. Theoretical models suggest that competition within hosts would generate selection for pathogens with greater virulence.

In the Americas, there are more than 150 wild mammal species infected with the protozoan parasite *Trypanosoma cruzi*, the causative agent of Chagas disease in humans. Some of these reservoirs play an important role in the maintenance and interaction of domestic, peridomestic, and wild cycles of American trypanosomiasis. These reservoirs include marsupials, edentates, and rodents. The proportion of infected reservoirs is variable according to the local epidemiologic situation, and its magnitude depends on the density and rates of infected triatomine vectors in each ecologic niche.

*Trypanosoma cruzi* lineages diverged between 37 and 88 million years ago. Genotype TCI is indigenous to South America, and genotype TCII was introduced more recently after the connection of the Americas in the Pliocene epoch (5 million years ago) by North American placental mammals. In Chile, several species of wild, synanthropic, and peridomestic mammals are infected with *T. cruzi*.

South American mammals descended from successive groups of newly arrived animals. Estimates suggest that 38–100 million years ago South America fauna was composed of marsupials, edentates, and ungulates. Approximately 38 million years ago during the Oligocene epoch, two main groups, the caviomorph rodents and primitive primates, were introduced from North America. Similar to other wild hosts, native marsupials and cricetid rodents do not show severe pathology when infected with *T. cruzi*. However, in more ancestral mammals, experimental infections should not be considered benign. This observation suggests that those mammals of greater antiquity in the Americas have had a longer association with *T. cruzi*, which enables some degree of host-parasite coevolution that results in attenuated pathogenicity.

*Trypanosoma cruzi* genotypes are complex multiclonal populations that propagate asexually and differ in their genetic and biologic characteristics and behavior in vertebrate hosts. Currently, the taxon *T. cruzi* is divided into two lineages (genotypes): TCI and TCII. Genotype TCI corresponds to the classic zymodeme Z1 (multilocus genotypes [clonets] 1–25). Genotype TCII includes five sub-genotypes: TCIIa (clonets 27–29), TCIIb (clonets 30–34), TCIIc (clonets 35–37), TCIIId (clonets 38–39), and TCIIe (clonets 40–43). Genotype TCIIb corresponds to Z2 and genotypes TCIIa and TCIIc correspond to Z3. It has been proposed that the fusion between genotypes TCI and TCIIb generated the heterozygous hybrids TCIIa and TCIIc, and fusion between genotypes TCIIb and TCIIc generated the hybrids TCIIId and TCIIe.

In this study, we report *T. cruzi* genotypes circulating in four wild and one peridomestic mammal hosts from central Chile. We identified the *T. cruzi* genotypes directly by Southern blot analysis of minicircle polymerase chain reaction (PCR)–amplified DNA with a panel of specific probes for each parasite lineage. We also examined specific associations between *T. cruzi* genotypes in wild or peridomestic mammals, mixed infections, and aggregation or segregation among *T. cruzi* genotypes.

MATERIALS AND METHODS

Collecting area. Wild and peridomestic mammals were obtained from Las Chinchillas National Reserve (Aucó, Region IV, 30°30′S, 71°06′W). This reserve is in a hyperendemic zone of Chagas disease in Chile. The collecting site corresponds to a zone with caprine, equine, and bovine cattle and free-ranging introduced rabbits (*Oryctolagus cuniculus*) and wild mammals. The collection area has native rodents (*Phyllostis darwini*, *Octodon degus*, *Abrothrix olivaceus*, *Oligoryzomys longicaudatus*, and *Abrocoma benetti*) and a native marsupial (*Thylamys elegans*). The mean ± SE number of mammals per hectare in the summer is 7.5 ± 1.1. The most prevalent
mammalian species are *P. darwini*, *A. olivaceus*, *O. degus*, and *T. elegans* (ratio = 7:6:1:5:1, respectively). Blood collection and DNA isolation. A total of 158 wild mammals were captured using traps (H. B. Sherman Trap Company, Tallahassee, FL) in diurnal and nocturnal periods during the summer of 1999 and 2000 (*P. darwini*, n = 55; *O. degus*, n = 46; *A. olivaceus*, n = 44; and *T. elegans*, n = 13). Each animal was weighed and anesthetized with isoflurane at a dose of 13 mg/kg of body weight. Once anesthetized, 0.5–1.0 mL of blood was withdrawn by cardiac puncture using heparinized tuberculin syringes. Animals were hair-marked to avoid additional blood sampling and released in the capture area. Blood collection from peridomestic mammals (*C. hircus*, n = 42) was performed by jugular vein puncture from a previously cleaned skin area. Blood extraction procedures were conducted following the recommendation of the Ethical Committee of the Faculty of Medicine, University of Chile (Santiago, Chile).

Whole genomic DNA was isolated from blood samples by using the EZNA kit (Omega Biotech, Inc., Doraville, GA). The DNA was concentrated by ethanol precipitation, resuspended in 50 μL of deionized sterile water, and stored at −20°C.

Polymerase chain reaction assay. The amplification reactions were performed in triplicate with oligonucleotides 121 and 122, which anneal to the four constant regions in minicircles of *T. cruzi*. The DNA samples for PCR were boiled for 10 minutes and 5 μL of supernatant was used as DNA template. Each experiment included a negative control that contained water instead of DNA and a positive control that contained purified DNA of *T. cruzi*. The 330-basepair PCR product was analyzed by electrophoresis in a 2% agarose gel and visualized by staining with ethidium bromide.

Southern blot analysis. To confirm infection with *T. cruzi*, Southern blot analysis was performed with 10 μL of each PCR product. Samples were subjected to electrophoresis, transferred onto Hybond N+ nylon membranes (Amersham, Little Chalfont, United Kingdom), and cross-linked with ultraviolet light to fix the DNA. The membranes were prehybridized for at least 2 hours at 55°C and hybridized with total *T. cruzi* kinetoplast P32-labeled DNA (1 × 106 cpm/membrane). After hybridization, each membrane was washed three times for 30 minutes with 2× SSC (0.3 M NaCl, 0.03 M sodium citrate), 0.1% sodium dodecyl sulfate at 55°C, and analyzed with the Molecular Imager FX (Bio-Rad Laboratories, Hercules, CA). For genotyping, different *T. cruzi* clones were used as probes to determine the parasite lineage infecting each animal (TCI, clonet 20 sp.104 cl1; TCIIB, clonet 33 CBB cl3; TCIId, clonet 39; NR cl13; and TCIIE, clonet 43 v195 cl1). Construction of specific probes was performed as described. These probes (approximately 250 basepairs) do not contain the constant region that cross-hybridizes with minicircles of other *T. cruzi* genotypes. This method has been validated by hybridization with probes constructed by PCR amplification of *T. cruzi* DNA of other specific genotypes. Hybridizations with genotype-specific *T. cruzi* probes were repeated twice, and genotyping was conducted with samples that showed a DNA fragment.

Statistical analyses. All proportion comparisons were performed using chi-square analyses. To examine aggregation/segregation among *T. cruzi* genotypes, we constructed a presence or absence matrix of *T. cruzi* genotypes for each mammalian species analyzed and calculated a metric of co-occurrence for each matrix, the C score, by using the EcoSim version 7.72 software. This metric corresponds to the average number of checkerboard units that are found for each pair of *T. cruzi* genotype species and enables testing for non-random patterns of species co-occurrence. When *T. cruzi* genotypes were aggregated, we expected the C score to be smaller than expected by chance (observed [O] < expected [E]). When *T. cruzi* genotypes were segregated, we expected the opposite, i.e., O > E. We calculated the observed indices for each presence or absence matrix and compared them with simulated indices calculated for 5,000 randomly assembled null matrices. These matrices measured the tail probability that each observed index is higher or lower than that expected by chance. Simulated matrices were assembled by Monte Carlo procedures using the algorithm fixed-fixed (FF). In the FF algorithm, the observed row and column totals are maintained in the simulation. The marsupial *T. elegans* was not included in any statistical analysis because of the small number of infected animals (n = 6).

**RESULTS**

We analyzed 158 and 42 blood samples from wild and peridomestic infected mammals, respectively, by PCR in triplicate. Overall, 61% of the wild and 50% of the peridomestic mammals were positive for *T. cruzi*. The prevalence of *T. cruzi* infection in wild mammal species ranged from 46% to 71% (*O. olivaceus* = 71%, *O. degus* = 61%, *P. darwini* = 56%, and *T. elegans* = 46%). Samples were considered for genotyping when at least two, intensely stained, 330-basepair PCR-positive bands were observed (Figure 1A).

Genotyping of *T. cruzi* in wild and peridomestic mammals. We found singly or mixed *T. cruzi* genotypes in the analyzed species. Figure 1A shows a representative experiment with PCR results from a selected group of infected mammals. Figure 1B–E shows Southern blot analysis with the four specific probes. Probes showed all possible hybridization patterns: samples that hybridized with zero, one, two, or three probes. Hybridization with more than one probe indicates a mixed infection. Hybridization results with the controls validated this method because each probe cross-reacted only with the homologous sample.

Genotype distributions are shown in Table 1. At least four genotypes were circulating in wild species, but in different proportions. For example, genotypes TCI and TCIIB are more prevalent in *P. darwini* and *O. degus* than in *A. olivaceus*. Genotype TCIId is more prevalent in *P. darwini* and *A. olivaceus* than in *O. degus*. Statistical analyses showed significant differences in the distribution of genotypes in *P. darwini* ($\chi^2 = 12.83$, degrees of freedom [df] = 4, $P = 0.012$) and *O. degus* ($\chi^2 = 9.62$, df = 4, $P = 0.047$), which indicated that some genotypes are more prevalent than others. The most ancestral genotypes (TCI and TCIIB) were present in the highest proportions in these two mammalian species. The distribution of genotypes in *A. olivaceus* did not show statistically significant differences ($\chi^2 = 5.51$, df = 4, $P = 0.239$). Results from the most ancestral species (*T. elegans*) with different probes show a high proportion of TCI (n = 5). However, the other three genotypes (TCIIB, TCIId, and TCIIE) showed lower prevalences. Four animals had pure genotypes.
(TCI, n = 3 and TCIIb, n = 1), and two marsupials had mixed genotypes (TCI + TCIIb and TCI + TCIIe).

Only the four described T. cruzi genotypes in single or mixed forms circulated in periodomestic mammals. The most frequent genotypes were TCIIb, TCIId, and TCI; TCIIe circulated at a low rate (Table 1). However, the distribution of T. cruzi genotypes in goats did not show statistically significant differences ($\chi^2 = 3.52, df = 3, P = 0.318$). The most common genotypes in wild mammals were the most ancestral genotypes (TCI and TCIIb). However, the most common

**Table 1**

<table>
<thead>
<tr>
<th>Hosts</th>
<th>TCI</th>
<th>TCIIb</th>
<th>TCIIId</th>
<th>TCIIe</th>
<th>Unknown</th>
<th>No.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild mammals</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Phyllotis darwini</em></td>
<td>0.42</td>
<td>0.45</td>
<td>0.23</td>
<td>0.26</td>
<td>0.06</td>
<td>31</td>
</tr>
<tr>
<td><em>Abrothrix olivaceus</em></td>
<td>0.29</td>
<td>0.36</td>
<td>0.23</td>
<td>0.23</td>
<td>0.10</td>
<td>31</td>
</tr>
<tr>
<td><em>Octodon degus</em></td>
<td>0.43</td>
<td>0.43</td>
<td>0.32</td>
<td>0.13</td>
<td>0.14</td>
<td>28</td>
</tr>
<tr>
<td><em>Thylamis elegans</em></td>
<td>0.83</td>
<td>0.17</td>
<td>0.17</td>
<td>0.17</td>
<td>0</td>
<td>6</td>
</tr>
<tr>
<td>Mean</td>
<td>0.41</td>
<td>0.40</td>
<td>0.25</td>
<td>0.21</td>
<td>0.09</td>
<td>–</td>
</tr>
<tr>
<td>Peridomestic mammals</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Capra hircus</em></td>
<td>0.29</td>
<td>0.33</td>
<td>0.43</td>
<td>0.14</td>
<td>–</td>
<td>21</td>
</tr>
</tbody>
</table>

* Frequencies include total genotypes in single and mixed forms. Values in parenthesis indicate percentages of hosts singly infected by identified T. cruzi genotypes.
genotype in peridomestic mammals was the most recent hybrid (TCIIId). However, this observation did not show a statistically significant difference. Thus, there is no evidence that some T. cruzi genotypes are associated with either wild or peridomestic reservoirs.

**Single versus mixed genotypes.** In wild mammals, single and mixed genotypes were observed in similar proportions (59% and 41%, respectively). However, peridomestic animals had more T. cruzi genotypes in a single form than mixed with other lineages (81% and 19%, respectively). Statistical analysis showed marginally significant differences between proportions of wild and peridomestic mammals infected with one or more T. cruzi genotypes ($\chi^2 = 3.56, df = 1, P = 0.059$).

**Segregation among T. cruzi genotypes.** The distribution of frequencies observed in wild mammals suggests aggregation and segregation among different genotypes (Table 2). Single genotypes (TCI, TCIIb, TCIIId, and TCIIe) were found in 16%, 21%, 15%, and 9%, respectively, of the 90 animals tested. The most common mixed genotype was TCI + TCIIb, which was present in 14% of samples analyzed (Table 2). Because TCIIId was commonly observed as a single genotype in wild animals, it is likely that this genotype would be found in mixtures with other T. cruzi genotypes. However, mixed genotypes with TCIIId were rarely observed (frequencies near zero). A similar distribution of T. cruzi genotypes was observed in C. hircus. Genotypes TCI, TCIIb, and TCIIId were observed as single genotypes in 24%, 24%, and 29% of the samples tested, but mixed genotypes were not detected.

To determine if these frequencies indicated aggregation or segregation among T. cruzi genotypes, null model analyses were performed. A summary of the null model analyses using the FF algorithm is shown in Table 3. The C score did not differ significantly from metrics calculated for simulated matrices for P. darwini, A. olivaceus, and C. hircus. This finding indicated that T. cruzi genotypes appear to be randomly assembled. However, the C score did not detect randomness in the occurrence of T. cruzi genotypes in O. degus, which suggested that T. cruzi genotypes in this wild rodent are segregated.

**Table 2** Percentage of single and mixed Trypanosoma cruzi genotypes in wild and peridomestic mammals*

<table>
<thead>
<tr>
<th>Genotypes</th>
<th>Phyllotis darwini (n = 31)</th>
<th>Abrotrix olivaceus (n = 31)</th>
<th>Octodon degus (n = 28)</th>
<th>Capra hircus (n = 21)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Single</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>DTU I</td>
<td>0.13</td>
<td>0.13</td>
<td>0.11</td>
<td>0.24</td>
</tr>
<tr>
<td>DTU IId</td>
<td>0.19</td>
<td>0.26</td>
<td>0.18</td>
<td>0.29</td>
</tr>
<tr>
<td>DTU IIe</td>
<td>0.13</td>
<td>0.16</td>
<td>0.18</td>
<td>0.05</td>
</tr>
<tr>
<td>Mixtures</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>DTUs I + IId</td>
<td>0.19</td>
<td>0.06</td>
<td>0.18</td>
<td>–</td>
</tr>
<tr>
<td>DTUs I + IIe</td>
<td>–</td>
<td>0.03</td>
<td>0.07</td>
<td>–</td>
</tr>
<tr>
<td>DTUs IId + IId</td>
<td>0.03</td>
<td>0.06</td>
<td>–</td>
<td>0.05</td>
</tr>
<tr>
<td>DTUs IIb + IIe</td>
<td>0.03</td>
<td>0.03</td>
<td>–</td>
<td>0.10</td>
</tr>
<tr>
<td>DTUs IId + IIe</td>
<td>0.03</td>
<td>–</td>
<td>0.07</td>
<td>–</td>
</tr>
<tr>
<td>DTUs I + IIb + IIe</td>
<td>0.03</td>
<td>–</td>
<td>–</td>
<td>0.05</td>
</tr>
<tr>
<td>DTUs I + IId + IIe</td>
<td>0.03</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Others</td>
<td>0.06</td>
<td>0.10</td>
<td>0.14</td>
<td>–</td>
</tr>
</tbody>
</table>

*Thylamys elegans was not included because of a small sample size.

**Table 3** Summary of null model analyses of the co-occurrence of Trypanosoma cruzi strains in wild and peridomestic mammal hosts using the FF null model algorithm*

<table>
<thead>
<tr>
<th>Hosts</th>
<th>Observed C-score</th>
<th>Mean C-score simulated (SD)</th>
<th>$PO &lt; PE$</th>
<th>$PO &gt; PE$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild mammals</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Phyllotis darwini</td>
<td>60.5</td>
<td>59.8 (1.2)</td>
<td>0.783</td>
<td>0.267</td>
</tr>
<tr>
<td>Abrotrix olivaceus</td>
<td>55.3</td>
<td>55.6 (0.5)</td>
<td>0.509</td>
<td>0.740</td>
</tr>
<tr>
<td>Octodon degus</td>
<td>41.8</td>
<td>39.9 (0.7)</td>
<td>0.975</td>
<td>0.033</td>
</tr>
<tr>
<td>Peridomestic mammals</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Capra hircus</td>
<td>29.7</td>
<td>29.4 (0.4)</td>
<td>0.705</td>
<td>0.385</td>
</tr>
</tbody>
</table>

* $PO < PE =$ probability of observed value of the index was significantly less than the expected value by chance ($p < 0.05$); $PO > PE =$ probability of the observed value of the index was significantly greater than the expected value by chance ($p < 0.05$). Number in bold shows statistical significance.

**DISCUSSION**

In this study, hybridization results for mammals in an area of Chile hyperendemic for T. cruzi showed at least four PCR-amplified genotype products that do not cross-hybridize. Another interesting finding was T. cruzi minicircles in a large proportion of animals that hybridized with amplified products from different parasite clones without sharing extensive homology. This result is interpreted as mixed infections rather than T. cruzi clones with hybrid minicircle contents generated by sexual recombination. This alternative is reasonable because many natural T. cruzi genotypes are hybrid lines.25,35,36 In a recent study, the hybrid groups TCIIId/TCIIe showed a TCIIId-type array. This study suggested that hybrid groups originated in the Chile-Bolivia region, and thereafter adapted to different reservoirs in South America.37 This observation correlates with the presence of TCIIId in a high percentage of wild mammals.

Marsupials are the oldest mammalian reservoirs for T. cruzi. However, unlike opossums (Didelphis sp.) from Paraguay and Argentina Chaco, which are infected mainly with genotype TCI,38,39 the marsupial T. elegans in Chile showed a high rate (83%) of infection with parasites of this genotype. Other genotypes were also present but in lower proportions. Unfortunately, statistical analyses were not conclusive because an insufficient number of animals were analyzed. This association between marsupials and genotype TCI has also been observed in the central valleys of Bolivia.40 In North America, ribosome II (TCI) is found exclusively in D. marsupialis41 and ribosome I (TCII) is found in placental mammals.42 In Paraguay, which has a large diversity of T. cruzi, a revision of all published T. cruzi lineages showed that 98% of 399 Didelphis species contained genotype TCI and 91% of 35 armadillos studied contained genotype TCII.38 Conversely, 96% of 460 TCI isolates were obtained from arboreal mammals, and 63% of 82 TCIi isolates were obtained from terrestrial mammals, which suggested that an association between arboreal mammals and genotype TCI and terrestrial mammals and genotype TCII.38 In the Atlantic rainforest of Brazil, analysis of T. cruzi isolates from the two most ancient and important reservoir hosts (D. albiventris and the caviomorph rodent Trichomys aperoides) showed that 40% of isolates from D. albiventris were genotype TCI. This finding is contrary to results of a previous study in which a closely related species (D. marsupialis) contained the TCI genotype, and T. aperoides contained genotypes TCI and TCII.43 In
The coexistence of mixed parasite infections in mammals in wild and peridomestic cycles of Chagas disease may depend on several factors. Trypanosoma cruzi genotypes circulating in the blood can be affected by the vertebrate host immune system, intrinsic properties of each T. cruzi clone, such as a histotrophic preference for different tissues, or clinical phase of the disease. However, our results show the existence of different T. cruzi genotypes within several hosts. Infected mammalian species sampled in this study may be survivors, i.e., those animals able to cope with infection. Studies with larger animal populations from different parasite-endemic areas are needed before conclusions can be made regarding facilitation/competition among different T. cruzi genotypes and host-parasite associations in this system. Molecular epidemiology of chronically infected human patients from the same geographic area showed that the most common genotypes in blood are the ancestral TCI and TCIIb genotypes. These results are similar to those found in wild mammals, which suggested that these patterns can be detected in different hosts, and that the domestic and wild transmission cycles for T. cruzi are similar, as previously reported.

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