Molecular Epidemiology of Chagas Disease in the Wild Transmission Cycle: The Evaluation in the Sylvatic Vector *Mepraia spinolai* from an Endemic Area of Chile

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**Abstract.** The sylvatic transmission cycle of Chagas disease in Chile is composed of wild mammals and insects of the genus *Mepraia*. We determined infection rates and *Trypanosoma cruzi* genotypes in *Mepraia spinolai*. We collected 227 insects from two ecologically contrasting areas to assess *T. cruzi* infection. Polymerase chain reaction (PCR)-amplified minicircle DNAs were characterized by Southern blot and hybridization tests with genotype-specific probes. Infection in insects from the more fertile area was almost 2-fold higher than in the poorer area. The genotype TCI was the most prevalent and other genotypes such as TCIIf, TCIId, and TCIJe were found at lower rates. The areas differed in their genotypic distribution but not in their genotype diversity. We suggest that the difference in abundance and richness of mammals between the areas may be producing the detected infection levels in vectors. Our results are compared with those reported for mammals from the same area.

**INTRODUCTION**

Chagas disease vectors are hematophagous reduviid (Hemiptera: Heteroptera) insects belonging to the subfamily Triatominae and capable of transmitting *Trypanosoma cruzi*. Two Triatominae genera occur in Chile: *Triatoma* and *Mepraia*. *Triatoma infestans* has been the main domestic vector of *T. cruzi*, now controlled through widespread interventions of the Southern Cone Initiative. Meanwhile, *Mepraia spinolai* is almost entirely sylvatic, and only occasionally reported at homes or in peridomestic habitats. It is the only species of Triatomininae with wing polymorphism, and an aggressive behavior to feed on any potential host. This species presents a longer generation time compared with *T. infestans* and different feeding and defecation behavior. *Mepraia spinolai* is often found in rocky sylvatic ecotopes associated with small rodents and rabbits and also in peridomestic ecotopes, such as henhouses and goat corrals. *Mepraia spinolai* is endemic to Chile and distributed between the 22° and 33°S. Unlike other triatomines, this species is usually a diurnal bug, but it is also active at night.

Even though the human blood index for *M. spinolai* indicates that this species is not an important vector of *T. cruzi*, the insect reaches high population densities in stone walls or dry rivers near human dwellings, suggesting an increasing risk of Chagas disease transmission in these localities. A previous study reported by Botto-Mahan and others in the same geographic area showed 46.2% of *T. cruzi* infection in *M. spinolai* using molecular evidence, which is much higher than that reported for the same area using microscopic observation. In addition, their results indicate that younger nymphs are as infected as the older ones, therefore, equally important in parasite transmission.

*Trypanosoma cruzi* possesses a heterogeneous structure and is composed of different genotypes, which circulate in domestic and wild cycles, involving human, mammal reservoirs and vectors. Different genetic variants have been detected in several hosts and geographic regions. Phenotypic and genotypic analyses have allowed the division of *T. cruzi* into *T. cruzi* I (TCI) and *T. cruzi* II (TCII). The latter is subdivided into five subgroups, IIA–IIE. *Trypanosoma cruzi* kineoplast DNA (kDNA) contains two components: minicircles and maxicircles. The minicircles are abundant (10,000–20,000/cell) and have two kinds of sequence elements: a conserved region repeated four times per molecule and four variable and divergent regions intercalated among the conserved repeats. Oligonucleotides containing conserved minicircle DNA sequences have been used for the detection of the parasite by polymerase chain reaction (PCR) assays in blood and/or vector samples. Moreover, probes from the highly variable regions of minicircles are directed toward groups of *T. cruzi* populations allowing characterization and studying host-parasite relationships by hybridization tests.

Current information suggests that the agriculture has played multiple roles in the evolution of animal pathogens into human pathogens. Those roles included both generation of the large human populations necessary for the evolution and persistence of human crowd diseases, and generation of large populations of domestic animals, with which farmers came into much closer and more frequent contact than hunter/gatherers have had with wild animals. Moreover, these domestic animals served as efficient conduits for pathogen transfers from wild animals to humans. Wildlife represents a source of zoonotic diseases; therefore, as human settlements encroach on more rural areas with high numbers of infected vectors and reservoirs, the contact between humans and infected vectors will be expected to increase. In addition, poor human housing conditions provide potential access to the insect vectors.

In this study, we examined whether *T. cruzi* infection and genotype distribution in *M. spinolai* depend on biotic features of the habitat (i.e., vegetation cover and native mammal richness) and anthropogenic influence (i.e., human activity and cattle traffic) in a protected area of north-central Chile. Results from this study are compared with *T. cruzi* infection and genotype distribution for sylvatic mammals.

**MATERIAL AND METHODS**

Population under study. *Mepraia spinolai* insects were captured from two areas inside Las Chinchillas National Reserve, Coquimbo Region, Chile (31°30’S, 71°06’W). Individuals were manually collected from December 2001 to March 2002 by
two trained people in 3 consecutive days for 2 hours within the time of maximum insect activity (11:00 to 14:00 hours). Captured insects were classified by nymphal stage and individually kept to avoid cross-contamination. In the laboratory, all insects were maintained under optimal growing conditions (27°C; 75% relative humidity [RH]) until samples of intestinal content were obtained.

**Areas of study.** The two collecting areas (Population 1 and Population 2, hereafter) are approximately 8 km apart; vegetation is characterized by thorny scrub composed mainly of spiny dicots, bromeliads, and cacti, depending on sun exposure. Population 1 is located in a hill nearby a seasonal sandy stream with caipirine, equine, and bovine cattle, free-ranging rabbits (Oryctolagus cuniculus), wild rodents (Phyllotis darwini, Octodon degus, Abrothrix sp., Oligoryzomis longicaudatus, and Abrocoma bennetti), and a native marsupial (Thylamys elegans). This area presents abundant vegetation cover, moderate human activity, and regular traffic of domestic animals. On the other hand, Population 2 corresponds to an abandoned stony quarry with extremely low vegetation cover and native mammal abundance/richness (only the rodent Abrothrix sp.), null human activity, and traffic of domestic animals.

**Triatomine samples.** The intestinal content of each insect was removed through abdominal extrusion and mixed with 200 μL of phosphate buffered saline (PBS) buffer, boiled for 10 minutes, centrifuged at 10,000g, and individually frozen at −20°C for PCR assay. A sample of 1 to 5 μL of this crude pre-boiled extract was used as DNA template in 50 μL of final volume. Intestinal contents were free of fresh blood; therefore, no DNA extraction was required. Samples failing to amplify were used on a positive control with kDNA to ensure that the lack of amplicons was not caused by PCR inhibitors.

**PCR reaction.** The PCR was performed as previously reported using primers 121 (5′-AAA TAA TGT TGG TGG GTG TGG TGT-3′) and 122 (5′-GGG TTC GAT GAG ATG CAT GA-3′) to amplify the variable region of minicircle DNA.\(^{24}\) Each experiment included positive and negative controls. Samples were tested in triplicate, and a reaction was considered positive when at least two of the three assays showed positive results. False negatives were evaluated by incubation of the DNA sample on a positive control. The PCR products were analyzed by electrophoresis on 2% agarose gels and visualized by staining with ethidium bromide. A 330-basepair product indicated a positive result.

**Southern analysis.** These analyses were performed using 10 μL of each PCR assay. The PCR products were electrophoresed, transferred onto Hybond N+ nylon membranes (Amersham, Little Chalfont, UK), and cross-linked by UV light for DNA fixation. After transferring PCR products, membranes were pre-hybridized for at least 2 hours at 55°C, and finally hybridized overnight with total kDNA labeled by random priming with \(^{32}P\) (1 × 10⁶ cpm/membrane) as probe for diagnosis confirmation. After hybridization, membranes were washed three times for 30 minutes each with 2 × SSC, 0.1% sodium dodecyl sulfate (SDS) at 55°C, and later exposed in the Molecular Imager FX (Bio-Rad Laboratories, Hercules, CA).

**Trypanosoma cruzi genotyping.** For T. cruzi genotyping, four different T. cruzi clones isolated in Chile (sp 104c11, CBBcl3, NRcl3, and v195c11) corresponding to TCI, TCIIb, TCIIId, and TCIIe, respectively, were used to generate specific probes. Construction of minicircle probes was performed as previously described, and radio-labeled as described for total kDNA.\(^{23,24}\)

**Statistical analyses.** Infection level comparison between populations was performed using \(\chi^2\) analysis. Genotype distribution within each population was compared by the same statistical test. A replicated G-test of goodness of fit was used to compare genotype distributions between populations.\(^{25}\) A genotype diversity index (H: Shannon-Wiener function) was calculated for each population.\(^{26}\)

**RESULTS**

A total of 227 nymphs of M. spinolai were captured at the Reserve (both populations combined). We captured 113 in Population 1 (number of individuals per stage: 28 I, 27 II, 23 III, 22 IV, 13 V) and 114 in Population 2 (number of individuals per stage: 16 I, 27 II, 24 III, 24 IV, 23 V). Adult individuals are not represented because of extremely low abundance during the collecting periods.

Results from PCR assays indicated that 76.1% (N = 86) and 40.4% (N = 46) of the nymphs captured in Population 1 and Population 2, respectively, were infected with T. cruzi. The infection level in Population 1 was significantly higher than in Population 2 (\(\chi^2 = 29.81, \text{degrees of freedom} \ [df] = 1, P < 0.0001\)), probably showing an association between ecologic features and infection rate in M. spinolai.

Positive samples were subjected to genotyping with four specific probes. Figure 1A shows a representative experiment with PCR results from a selected group of infected M. spinolai. Figure 1B–D show Southern blot analyses with the four specific probes and mixed infections with up to two genotypes. Probes showed all possible hybridization patterns: samples that hybridized with zero (unknown genotype), one, two, three, and four probes (Table 1). Hybridization with more than one probe is indicative of mixed infection. Within the positive individuals for Population 1, the genotype TCI was the most abundant (59.8%), followed by TCIIb (20.9%), TCIIId (16.3%), and TCIIe (10.5%) (single and mixed genotypes combined). The same tendency was detected for Population 2 (TCI = 52.1%, TCIIb = 10.9%, TCIIId = 8.7%, TCIIe = 8.7%). In both populations, at least one unknown genotype is circulating because...
TABLE 1

Distribution of Trypanosoma cruzi genotypes infecting Mepraia spinolai nymphs from two study areas (Population 1 and Population 2) located at Las Chinchillas National Reserve, Chile

<table>
<thead>
<tr>
<th>T. cruzi genotypes</th>
<th>Population 1</th>
<th>Population 2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Single</td>
<td></td>
<td></td>
</tr>
<tr>
<td>TCI</td>
<td>35 (40.1%)</td>
<td>18 (39.1%)</td>
</tr>
<tr>
<td>TCIb</td>
<td>3 (3.5%)</td>
<td>1 (2.2%)</td>
</tr>
<tr>
<td>TCIId</td>
<td>8 (9.3%)</td>
<td>0</td>
</tr>
<tr>
<td>TCIle</td>
<td>3 (3.5%)</td>
<td>6 (12.5%)</td>
</tr>
<tr>
<td>Mixtures</td>
<td></td>
<td></td>
</tr>
<tr>
<td>TCI + TCIb</td>
<td>10 (11.6%)</td>
<td>2 (4.3%)</td>
</tr>
<tr>
<td>TCI + TCIId</td>
<td>1 (1.2%)</td>
<td>2 (4.3%)</td>
</tr>
<tr>
<td>TCI + TCIle</td>
<td>2 (2.3%)</td>
<td>0</td>
</tr>
<tr>
<td>TCI + TCIb + TCIId</td>
<td>2 (2.5%)</td>
<td>1 (2.2%)</td>
</tr>
<tr>
<td>TCIb + TCIle</td>
<td>1 (1.2%)</td>
<td>0</td>
</tr>
<tr>
<td>TCIId + TCIle</td>
<td>1 (1.2%)</td>
<td>0</td>
</tr>
<tr>
<td>TCI + TCIb + TCIId + TCIle</td>
<td>2 (2.3%)</td>
<td>1 (2.2%)</td>
</tr>
<tr>
<td>Unknown†</td>
<td>18 (20.9%)</td>
<td>18 (39.1%)</td>
</tr>
</tbody>
</table>

*All values in parentheses indicate percentages of hosts infected by identified T. cruzi genotypes.
†This group could include single or mixed genotypes.

DISCUSSION

In this study, we examined T. cruzi infection levels in the endemic vector M. spinolai from two areas (Population 1 and Population 2) located inside a Reserve, which differ in their ecologic features (i.e., biotic and abiotic aspects). We found significantly more infected insects in the bug population located in an area with more vegetation cover, higher abundances and richness of mammal hosts, and more anthropogenic influence in terms of cattle and human traffic (Population 1) than in the less fertile population (Population 2). This heterogeneous or patchy distribution of the infection in vector populations had been previously described in other endemic areas of Chile. Unfortunately, the ecologic characteristics were not described to raise conclusions about areas of higher endemcity.27 We propose that the features of the ecologic environment would determine the higher or lower insect infection rate, and this would ultimately depend on vegetation abundance as a resource for mammal hosts.

In both populations, the genotype TCI was significantly more abundant, followed by TCIb, TCIId, and TCIle at much lower rates. However, at least one still unidentified genotype is circulating in the vector M. spinolai. Unknown genotypes can be genetically slightly different from those studied here, because they do not cross-hybridize with any of the probes used. The same situation has been reported in other endemic areas, where genetically related T. cruzi clones exhibit an absence of cross-hybridization between minicircles DNAs.28 Probably, these unidentified T. cruzi genotypes are TCIb, TCIId, or genetically distant TCI, TCIIb, TCIId, and TCIle to the T. cruzi clones used for probes construction.29 Our results indicate that M. spinolai individuals from the two studied populations present similar genotype diversity and are infected by similar parasite genotypes (not considering the unidentified ones), but differ in their genotype distribution. Notwithstanding, characterization of the still unidentified genotype(s) is crucial to understand the whole scenario.

We found that the genotype TCI was predominant in this study and distributed singly and combined among the wild vectors, a finding that is consistent with the observation that the genotype TCI along with TCIIb are the most pure and ancestral ones.29 Our results show that TCI is the most abundant genotype in the vector M. spinolai, and, therefore, with a higher chance to be transmitted to native mammals and probably to cattle and humans when in contact. Molecular epidemiology studies of Chagas disease of the sylvatic M. spinolai are useful not only to detect pathogens, but also their heterogeneity and dynamics of propagation.

Molecular evidence reported for wild mammals belonging to the wild transmission cycle of Chagas disease in central Chile shows high levels of infection (i.e., 61%) and variable frequencies of genotypes TCI, TCIIb, TCIId, and TCIle, being the first two of the most represented ones.23,30 In peridomestic mammals, frequencies for the same genotypes varied slightly with a preference for TCIIId.23,31 In our study, we detected an infection level for insect vectors similar to those described in wild mammals, but unlike native mammals; M. spinolai individuals present much lower frequencies of the ancestral genotype TCIIb. Interestingly, TCI was prevalent in the marsupial Thylamis elegans, same as described in other endemic areas.32 Interactions between T. cruzi and the insect vectors continue to be an interesting subject to be intensely explored. Competition within-host generating selection of parasites is a regularly described phenomenon.33 Furthermore, the intrahost competition among parasites of different genotypes has been described as a main factor shaping parasite ecology and evolution.34 No studies on the evolution of mixed infections are available, although it is to be expected that the development of the parasites will be profoundly influenced inside the blood-sucking insect.

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