Short Report: European Rabbits (*Oryctolagus cuniculus*) are Naturally Infected with Different *Trypanosoma cruzi* Genotypes

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Abstract. *Trypanosoma cruzi*, the etiologic agent of Chagas disease, presents a complex life cycle, cycling between reduvid vectors and wild and domestic mammals. The European rabbit is an introduced species in America, but its role as reservoir in the wild transmission cycle of Chagas disease remains unknown. We used polymerase chain reaction, Southern blot, and hybridization tests to detect infection and characterize genotypes in rabbits from a hyperendemic area of Chagas disease in Chile. Results show 38% of infection with different genotypes. We provide evidence that rabbits are naturally infected with *T. cruzi*, which may have important epidemiologic consequences for the wild transmission cycle.

Chagas disease is a zoonosis caused by the flagellate protozoan *Trypanosoma cruzi* and transmitted by triatomine insects (Hemiptera, Reduviidae).® Native marsupials and rodents play important roles in the ecology and epidemiology of Chagas disease. In Chile, *Meprata spinolai* is the main sylvatic vector, and *T. cruzi* infection in wild mammals ranges from 5% to 50% depending on the diagnosis method used and the study area involved.® The European rabbit, *Oryctolagus cuniculus*, was introduced from Spain into central Chile in 1884.® Short generation time, low rates of predation, and increased availability of forage have led rabbits to become a serious pest; however, the impact of this species on wild transmission cycles of endemic diseases including other native host animals remains unknown.®

For several reasons, the European rabbit could be considered a suitable host for the wild transmission cycle of Chagas disease. The alimentary profile of *M. spinolai* shows that the rabbit is the most important blood source in central Chile.® Wild rabbits are nocturnal and live in groups linked to aggregated burrows nearby quarries, the preferred microhabitat described for *M. spinolai*. This vector is diurnal with a peak of activity at noon; therefore, rabbits are more vulnerable to be bitten by vectors at daytime when hiding or resting into their burrows.® Unfortunately, to our knowledge, scarce information has been published about its status of *T. cruzi* infection.® Laboratory evidence indicates that experimentally infected rabbits show patent parasitemias and some lesions similar to those of Chagasic human patients.®

The taxon *T. cruzi* is divided into two lineages, TCI and TCII, mainly infecting marsupials and placental mammals, respectively.®®®® TCII corresponds to the classic *Z_1*, and it has been proposed that TCII is subdivided into five sublineages, one corresponding to *Z_2* (TCIIb), another to *Z_3* (TCIIa), both considered ancestral, and the most recent hybrids TCIIc, TCIIId, and TCIIe.®®®® In this study, we examine the status of *T. cruzi* infection in wild European rabbits from a hyperendemic area of Chagas disease in Chile. We combine, for the first time, classic and hemi-nested polymerase chain reaction (PCR) to increase detection probabilities in cases of extremely low parasitemia. Infective *T. cruzi* genotypes are characterized by Southern blot analyses and hybridization tests using minicircle probes as described.® We provide molecular evidence to examine the importance of the European rabbit as a potential reservoir host for *T. cruzi*.

We wild-trapped European rabbits in October–December 2005 and January 2006 at Las Chinchillas National Reserve (30°30′ S, 71°06′ W), a protected area of 4,229 ha considered hyperendemic of Chagas disease in Chile.®®®® During each month, rabbits were captured during 5 consecutive nights by means of address-based sampling using 10 unbaited Tomahawk traps (Tomahawk Live Trap Company, Tomahawk, WI). Each trap was placed at the entrance of a burrow (distance among burrows ≥ 1 km). Captured animals were weighed and anesthetized with isoflurane at a dose of 13 mg/kg of body weight. Once anesthetized, 1.0–3.0 mL of blood was withdrawn by cardiac puncture using a heparinized syringe. Animals were hair-marked to avoid additional blood sampling and released in the capturing area. Blood extraction procedure was conducted following the recommendation of the Ethical Committee of the Faculty of Medicine, University of Chile.

Whole genomic DNA was isolated from 0.2-mL blood samples using the EZNA kit (Omega Biotech, Doraville, GA). The DNA was concentrated by ethanol precipitation, resuspended in 50 μL of deionized water, and stored at −20°C. The amplification reaction was performed with oligonucleotides 121 and 122, which anneal to the conserved sequence blocks CSB2 and CSB3, respectively, present in the constant regions of each minicircle.®®®® The reaction mixture for the classic PCR was composed of 5 μL of the DNA sample in a volume of 50 μL with 33 cycles and conditions already described.®®®® Each experiment included a control that contained water instead of DNA and a positive control that contained purified kinetoplast (kDNA) of *T. cruzi*. The 330-bp PCR product was analyzed by electrophoresis in a 2% agarose gel and visualized by staining with ethidium bromide. To confirm infection with *T. cruzi*, Southern blot analysis and hybridization with a universal probe of kDNA was performed with 10 μL of each PCR product. The minimal amount of amplified DNA to perform hybridization tests is 30 ng, and under these conditions, any probe used should cross-react with the immobilized DNA in the membranes, unless they are heterologous DNAs. Samples subjected to electrophoresis were transferred onto Hybond N+ nylon membranes (Amersham, Little Chalfont, UK) and cross-linked with ultraviolet light to fix the DNA. The membranes were pre-hybridized for at least 2 hours at 55°C and

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hybridized with a universal probe composed of total T. cruzi kinetoplast P32-labeled DNA (1 × 10⁶ cpm/membrane). Membranes were finally washed under high stringency conditions (0.1× SSC, 0.1% SDS at 65°C). All samples were amplified by PCR and subsequently subjected to hemi-nested PCR to improve DNA amplified bands, using oligonucleotides CV1 (similar to oligonucleotide 122) and CV2, which anneal in the CSB3 and CSB1, respectively. For genotyping, different T. cruzi clones were used as DNA template to generate specific probes and determine, by hybridization tests, the lineage infecting rabbits (TCI, TCIIb, TCIIId, and TCIIe) under high stringency conditions as described.

A total of 58 rabbits were trapped (10 adults, 12 sub-adults, and 36 juveniles). Overall, 19.0% (N = 11) and 37.9% (N = 22) of the sampled rabbits were positive for T. cruzi, by classic and hemi-nested PCR, respectively. Figure 1 shows results of the same samples analyzed by classic and hemi-nested PCR. Infection rate increased significantly by using hemi-nested PCR (χ² = 5.12, df = 1, P = 0.024). The 22 positive rabbits corresponded to 4 adults, 3 sub-adults, and 15 juveniles, but no statistically significant differences were detected in the probability of infection among age classes (χ² = 1.08, df = 2, P = 0.582), suggesting that infection occurs early in their lifetime. Positive samples were subjected to genotyping with the four specific probes and showed almost all possible hybridization patterns (Table 1). Hybridization with more than one probe is indicative of mixed infection. Within the detected infections, genotypes TCIIId (68%) and TCI (41%) are the most prevalent ones, followed by TCIIe (23%) and TCIIb (14%) (single and mixed forms combined). TCIIId is significantly more abundant than the other genotypes (χ² = 12.18, df = 3, P = 0.007; single and mixed forms combined). At least one unknown genotype is circulating in the European rabbit because two blood samples did not hybridize with any of the tested probes. Single and mixed infections were observed in the same proportion (χ² = 0.38, df = 1, P = 0.55; Table 1). Genotype TCI was never found in single form, and the most common mixture was TCI + TCIIId, which was present in 27.0% of the positive samples (Table 1).

In this study, we examined the status of T. cruzi infection in the European rabbit introduced in central Chile more than a century ago. Molecular evidence obtained from classic PCR method indicates that 19% of sampled rabbits from a hyperendemic area of Chagas disease are infected with the protozoan T. cruzi. However, results with hemi-nested PCR show that 38% of sampled rabbits are T. cruzi positive. The fact that classic PCR is underestimating T. cruzi infection level could be because of extremely low parasitemias in naturally infected rabbits. It is worth to point out that hemi-nested PCR can detect parasites at very low parasitemia under 0.25 equivalents of a parasite genome per milliliter, which is the detection sensitivity of a classic PCR test directed to amplify minicircles. Despite the detection method used here, the percentage of infection in rabbits is much lower than the 61% reported for native mammals and 50% for peridomestic goats (Capra hircus, an introduced species to be raised as livestock) from the same hyperendemic area. At least two non-exclusive hypotheses could be stated to explain this finding. First, the lower infection percentage detected for rabbits could simply be the result of higher mortality rates during the acute phase of the infection, which translates in less infected rabbits when sampling endemic areas. Second, rabbits could actively repel kissing bug attacks or have stronger immune systems to destroy T. cruzi compared with other mammals. Behavioral assays and experimental T. cruzi infections of native, domestic, and introduced mammals are needed to test the hypotheses suggested.

In contrast to other studies, we did not detect evidence of exclusive infection by some T. cruzi genotypes for rabbits, considering only those identified genotypes. The genotype TCIIId (single and mixed) was the most prevalent one, infecting 68% of positive rabbits. This observation is interesting because, in native mammal hosts from the same area, the ancestral genotypes TCI and TCIIb are the predominant ones but are not exclusive. This result is consistent with the idea that TCIIId, TCIIe, or hybrids are circulating at very low parasitemias in mammal hosts and can only be detected by hemi-nested PCR. This information is relevant because, even though TCI is a pathogenic genotype, TCIIId is thought to be more pathogenic for humans and is able to elicit preferential antibodies compared with other genotypes. Similarly, TCIIId in single and mixed forms is the most prevalent genotype in peridomestic goats from the same area. Probably, the immune systems of these introduced mammals, unfamiliar with T. cruzi, are responding in similar ways to the spectrum of genotypes circulating in the wild transmission cycle of Chagas disease in north-central Chile.

The significance of a host species as reservoir of a vector-borne trypanosomiasis mainly depends on its prevalence of infection, the host capacity to infect triatomines, and the rate of host-vector contact. We suggest that the European rabbit is part of the wild transmission cycle of Chagas disease in Chile.

### Table 1

<table>
<thead>
<tr>
<th>Genotypes</th>
<th>Number of cases</th>
<th>Percentage of all detected infections</th>
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</thead>
<tbody>
<tr>
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<td></td>
</tr>
<tr>
<td>TCI</td>
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</tr>
<tr>
<td>TCIIb</td>
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<td>9</td>
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<tr>
<td>TCIIId</td>
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<td>27.3</td>
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<tr>
<td>TCIIe</td>
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<td>13.6</td>
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<tr>
<td>Mixtures</td>
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<td></td>
</tr>
<tr>
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<td>27.3</td>
</tr>
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<td>9.1</td>
</tr>
<tr>
<td>Unknown</td>
<td>2</td>
<td>9.1</td>
</tr>
</tbody>
</table>

Figure 1. A. T. cruzi DNA amplified by classic PCR and stained with ethidium bromide. B. Hybridization profile of A with universal probe. C. T. cruzi DNA amplified by hemi-nested PCR and stained with ethidium bromide. Rabbit samples (1 and 5–9) represent those with very low parasitemia detected only by hemi-nested PCR. The 330- and 290-bp products represent positive assays for A/B and C, respectively.
because it 1) is the most represented blood source inside the digestive tract of M. spinolai, 2) has a partially overlapping home range with M. spinolai, and, as shown in this study, 3) is highly infected with T. cruzi.\textsuperscript{6,7}

In Chile, rabbits are hunted and consumed by local people. Orally transmitted Chagas disease is a rare form of T. cruzi transmission by ingestion of contaminated food, generally associated with massive parasitic transmission.\textsuperscript{20} We cannot discard the possibility of T. cruzi transmission to humans by handling raw meat and/or consumption of undercooked meat containing infective stages. This implies that the European rabbit may potentially have a role in the domestic transmission cycle of Chagas disease in Chile.

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REFERENCES