Rural *Triatoma rubrovaria* from Southern Brazil Harbors *Trypanosoma cruzi* of Lineage IIc

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Abstract. *Triatoma infestans*, the main vector of Chagas disease, has nearly been eliminated from Brazil. Nevertheless, other triatomine species are involved in the domiciliation process, including *Triatoma rubrovaria* in Rio Grande do Sul State (RS). Previous studies showed that 1.6% of the *T. rubrovaria* specimens collected at the rural district of Quaraí, RS, were naturally infected by *Trypanosoma cruzi*. In this study, five *T. cruzi* isolates obtained from infected triatomines were characterized molecularly and biologically. Genotyping of the *T. cruzi* isolates showed that they belong to lineage IIc of *T. cruzi* (TCIIc). Biological characterization showed mototropism and myositis during acute and chronic phases of infection, respectively. Virulence and mortality rates were variable among isolates. To our knowledge, this study corresponds to the first characterization of *T. cruzi* isolates from *T. rubrovaria* and the first description of TCIIc in the sylvatic cycle of *T. cruzi* from the southern region of Brazil.

INTRODUCTION

*Trypanosoma cruzi* is the etiologic agent of Chagas disease (American trypanosomiasis), a complex zoonosis that affects 16–18 million people in Latin America. Parasite routes of infection include exposure of abraded skin to feces of infected triatomine bugs, ingestion of food contaminated with infected insects, blood transfusion, and congenital contagion. Also, transmission of *T. cruzi* can occur as domestic, peridomestic, or sylvatic cycles, depending on the ecologic behavior of the triatomine and host species involved.

Clinical presentation of Chagas disease is highly variable, involving expression as negligible, acute, indeterminate, and chronic. Pathogenesis, response to chemotherapy, geographic variation, clinical presentation, and morbidity of Chagas disease have been linked to biological, biochemical, and genetic characteristics of *T. cruzi* strains. The biological behavior of *T. cruzi* strains in laboratory animals has allowed the distinction of three different biodemes. Specific electrophoretic patterns of different enzymes (zymodemes), which may be associated with biological characteristics, differentiated *T. cruzi* strains in three major zymodemes: zymodeme I (Z1), Z2, and Z3. Analysis of the 24Ss rRNA and the mini-exon gene non-transcribed spacer sequences by polymerase chain reaction (PCR) enabled the identification of two main groups of *T. cruzi*: *T. cruzi* I (TCI) and TCII, correlated to Z1 and Z2, respectively. Parasite isolates characterized as Z3 and hybrid strains included in to rDNA group I2 or DTU1 could not be classified as TCI or TCII. More recently, genetic approaches grouped *T. cruzi* in to discrete typing units (DTUs), DTUI (TCI), and DTUII (TCII), which is subdivided in to DTUs/TCIIa to IIe. By this classification, the Z3 group is associated with TCIIa and TCIIc, and hybrid groups of *T. cruzi* are included in TCIIId and TCIIe. TCIIa and TCIIc correspond, respectively, to the Z3B and Z3A subdivisions described by Mendonça and others. TCI, TCIIa, and TCIIc predominate in sylvatic transmission cycles from the Amazon basin northward, being the main cause of Chagas disease in countries such as Venezuela and Mexico. TCIIb, TCIIId, and TCIIe groups are mainly represented by parasites related to the domestic transmission cycles in Southern Cone countries of South America including Argentina, Brazil, Bolivia, Chile, Paraguay, and Uruguay, where chagasic megasymphdromes are common by way of contrast with their virtual absence to the north of the Amazon, where TCI strains predominate. Despite many studies, differences in pathology between TCI and TCII infections remain enigmatic. Thus, genetic study of the *T. cruzi* isolates can help to clarify the intraspecific heterogeneity of the parasites, whereas studies on its biological behavior in experimental animals can shed light on the relationship of parasite genetic diversity with its pathogenicity and virulence in the host.

Vectorial transmission of *T. cruzi* in Brazil has decreased substantially since 1983, because of the chemical control of *Triatoma infestans*, its major domestic vector. However, the risk for domiciliation of sylvatic triatomite species that were not targets of control actions has increased. Surveillance data obtained by the Brazilian National Health Foundation (Funasa) during the Chagas Disease Control Program have shown that *Triatoma rubrovaria* (Blanchard, 1843) (Hemiptera, Reduviidae, Triatominae) is taking over the niches of *T. infestans* and gradually invading houses in southern Brazil. *Triatoma rubrovaria*, an exclusively sylvatic species of triatomine, is found in rocky areas and peridomestic habitats in Rio Grande do Sul (RS) State of Brazil and also is widespread in Uruguay and in parts of northeastern Argentina. It is considered a generalist species, feeding on a wide variety of vertebrate and invertebrate hosts, including humans. Vectorial capability of *T. rubrovaria* to transmit *T. cruzi* has been shown experimentally and by studies on its patterns of feeding and defecation. Natural infection of *T. rubrovaria* by *T. cruzi* has been shown in Uruguay at a rate of 0.3%, and more recently, our research group showed that 1.6% of *T. rubrovaria* nymphs from a rural district of RS, Brazil, were naturally infected by *T. cruzi*.

Therefore, taking into account that the sylvatic transmission cycle of *T. cruzi* in the rural district of Quaraí, RS, Brazil, is established and considering the domiciliation risk of *T. cruzi*...
rubrovaria\textsuperscript{24,33} to know the profile of the \textit{T. cruzi} strains circulating in the sylvatic environment of the south of Brazil, we genetically and biologically characterized five \textit{T. cruzi} isolates obtained from \textit{T. rubrovaria} collected in Quaraí, RS.

\textbf{MATERIALS AND METHODS}

\textit{Trypanosoma cruzi} isolates. The five isolates of \textit{T. cruzi} used in this study were obtained from fecal samples of \textit{T. rubrovaria} collected from 23 to 25 April 2003 in rural locales of the district of Quaraí (30°22’52”S, 56°25’31”W), situated in the west bordering of the Brazilian State of Rio Grande do Sul and including Quaraí-Branquinhos I (QBI), Quaraí-Macarrão I (QMI), Quaraí-Macarrão II (QMII), Quaraí-Jarau I (QJI), and Quaraí-Jarau III (QJIII). Isolation, identification, and geographic distribution of the triatomines and precipitin tests with several vertebrate antisera on its feces have been described previously\textsuperscript{29}.

Genotyping of \textit{T. cruzi} isolates. For preparation of DNA templates, cultured epimastigotes were lysed with digestion buffer (2 mol/L NaCl, 2 mol/L Tris-HCl, pH 8.0, 0.5 mol/L EDTA, pH 8.0, 10% SDS, and 10 mg/mL Proteinase K) for 2 hours at 65°C, and DNA was extracted using the GFX DNA extraction kit (GE Healthcare UK Limited, England). Two PCR assays were used to genotype the \textit{T. cruzi} isolates: one based on the divergent domain D7 of the 24S\textsubscript{rDNA}\textsuperscript{9} and a multiplex method based on the non-transcribed spacer of the miniexon gene\textsuperscript{34}. The \textit{T. cruzi} reference strains used in the PCR assays were G, Y, and JJ for the lineages TCI, TCIIb, and TCIIa (Z3B), respectively. \textit{T. cruzi} isolates 3663 and 3869 were used as reference strains for the TCIIc lineage (Z3A)\textsuperscript{34–36}.

Sequencing of partial SSU \textit{rRNA} gene and phylogenetic inferences. A 900-bp DNA fragment containing a partial SSU \textit{rRNA} sequence (V7–V8 regions) was amplified by PCR and sequenced as previously described\textsuperscript{37} from the isolates of \textit{T. cruzi} obtained from \textit{T. rubrovaria} included in this study. Sequences of the isolates QBI and QMI were aligned among other trypanosome sequences from GenBank (accession number): \textit{T. cruzi} Peru (X53917); \textit{T. cruzi} G (AF239981); \textit{T. cruzi} Y (AF301912); \textit{T. cruzi} Colombiana (AF239980); \textit{T. cruzi} Dm28c (AF245382); \textit{T. cruzi} JJ (AY491761); and \textit{T. cruzi} CANIII (AJ009148), 3663 (AF288660), and 3869 (AF303660). Alignments were made using the general alignment in the \textit{rRNA} database (http://rrna.uia.ac.be/) as a guide and manually refined. A dendrogram was inferred using maximum parsimony analysis, whereas bootstrap analyses with 100 replicates and a similarity matrix were performed as before\textsuperscript{37}. \textit{T. cruzi marinkellei} (AJ009150) was used as outgroup for \textit{T. cruzi} isolates.

Biological characterization of \textit{T. cruzi}. Biological characterization of the five \textit{T. cruzi} isolates QBI, QMI, QMII, QJI, and QJIII was performed on Swiss mice using infectivity, parasitemia, and mortality parameters. The maintenance and care of animals complied with the National Institutes of Health guidelines for the human use of laboratory animals. To carry out the parasitemic curve of each \textit{T. cruzi} isolate, groups of five old Swiss mice were intraperitoneally inoculated with 1,800 trypomastigotes obtained from the blood of a previous infected mouse. Parasitemia was evaluated three times a week by microscopic examination of peripheral blood after the third day after infection for 68 days\textsuperscript{38} and expressed as logarithms of the media of parasites in peripheral blood of five mice for each \textit{T. cruzi} isolate. Considering the parasitemic peak, parasitemia was classified as low (1,000 trypomastigotes/μL of total blood); intermediate (varied from 1,000 to 10,000 trypomastigotes/μL of total blood); and high (> 10,000 trypomastigotes/μL of total blood).

\textbf{Histopathologic characterization of \textit{T. cruzi}.} For tissue tropism and histopathologic studies, for each \textit{T. cruzi} isolate, groups of 21 Swiss mice, 20 days old, were intraperitoneally inoculated with 0.1 mL of whole infected blood presenting at concentrations of 34,220, 2,000, 2,000, 3,820, and 92,000 trypomastigotes of QBI, QJI, QJIII, QMI, and QMII, respectively. Heart, skeletal muscles, esophagus, liver, spleen, and colon of groups of three mice\textsuperscript{7} infected with each \textit{T. cruzi} isolate were collected at acute (7, 10, 14, 21, and 30 days) and chronic (150 and 180 days) phases of infection after CO\textsubscript{2} euthanasia. Organs were fixed in 10% formalin, included in paraffin blocks, serially sectioned (4 μm thick), and stained with hematoxylin and eosin (H&E). Three histologic sections of each organ per mouse were analyzed for inflammation process, which was quantitatively classified as discrete (+), moderate (+++), and intense (++++) by the presence of focal, multifocal, and diffuse inflammatory infiltrate, respectively.\textsuperscript{40} For the parasite load, the following criteria was adopted: discrete (+) when the parasites were rare; moderate (+++) when they were detected in some foci; and intense (++++) when they were seen diffusively.\textsuperscript{40} Classification of the isolates in biodemes was performed according to Andrade.\textsuperscript{41}
Comparison of V7–V8 rRNA sequences from T. cruzi isolates obtained from T. rubrovaria showed 100% of sequence similarity. Sequences from these isolates shared 93.6%, 95.4%, and 96.8% of sequence similarity, respectively, with sequences from the reference strains of T. cruzi TCI (G), TCIIb (Y), and TCIIa (JJ). Highest similarity (~99%) of these new isolates was shared with the reference strains of TCIIc (isolates 3669 and 3663 (TCIIc); isolates of T. cruzi obtained from Triatoma rubrovaria: QB1 and QMI.

Biological behavior of T. cruzi isolates. Virulence of T. cruzi isolates was studied through determination of the parasitemic curve, mortality rate, and pre-patent period. Pre-patent period was short for all strains, occurring at Day 5 after infection for the isolates QJI, QJII, QMII, and QBI and at Day 7 after infection for the QMI isolate. Parasitemia was highly variable (Figure 3), being considered low for isolate QBI, intermediate for isolates QJI, QJIII, and QMI, and high for isolate QMII. Parasitic peak was reached in 21 days for isolates QMI and QMII and after 31 days for stocks QBI, QJI, and QJIII. Thus, the most virulent T. cruzi isolate was QMII, followed by QMI. During the studied period, one mouse infected with isolate QMI and four mice infected with QMII isolate died in the parasitic peak.

Tissue tropism and histopathologic lesions during the acute and chronic phases of infection of all five T. cruzi isolates are summarized in Table 1. Fifteen mice infected with each T. cruzi isolate were examined during acute phase, and all isolates presented a discrete to intense inflammatory process in liver lobular portion (60%; 45/75), in liver portal system (30.7%; 23/75), in skeletal muscle (37.3%; 28/75), and in heart (33.3%; 25/75). Amastigote nests were observed in hearts of mice infected with QJIII (4.7%), QMI (9.5%), and QMII (27.8%); in skeletal muscle of mice infected with QMI (33.3%) and QMII (38.9%); and in livers of mice infected with QMII (5.5%; Figure 4A–C). Inflammatory process and amastigote nests were not observed in the colon, esophagus, and spleen of infected mice collected in the acute phase.

During the chronic phase of infection, a total of 27 mice were analyzed (3 mice infected with isolate QMII died before 150 days of infection). Mice infected with each T. cruzi isolate showed a discrete to intense inflammatory process in the liver lobular portion (74.1%; 20/27), in the liver portal system (11.1%; 3/27), in skeletal muscle (44.4%; 12/27), and in the...
Table 1: Degree of histopathologic lesions and parasitism in mice infected with T. cruzi isolates obtained from T. rubrovaria

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*Parasitism was observed only in the acute phase of infection; QJI, QJIII, QMI, QMII, and QBI were classified as biodeme III and QMII as biodeme II as described by Andrade.

DISCUSSION

Molecular characterization of the T. cruzi isolates by PCR analysis of the 24S rRNA D7 domain and of the non-transcribed spacer of the mini-exon genes showed that all the isolates belonged to the TCIIc (Z3A) group. The lineages IIA and IIC of T. cruzi are predominantly sylvatic and present a wide distribution in the Amazon region, being also reported in the Brazilian state of Bahia, Venezuela, Colombia, and in the north up to the United States. As far as we know, this is the first description of IIC T. cruzi lineage in the southern region of Brazil. There are indications that these T. cruzi lineages evolved in a terrestrial habitat in burrows and in rocky locations with the triatomine tribe Triatoma, which include bugs from genera Panstrongylus and Triatoma, in association with edentates and/or ground dwelling marsupials. Thus, the ecologic behavior of T. rubrovaria is in accordance with its association with the TCIIc group.

Very little data on characterization of T. cruzi isolates obtained from the sylvatic T. cruzi transmission cycle in the countries of the southern region of South America are available. Evidence for the occurrence of TCIIc in this region has been shown in one specimen of domestic T. infestans and more recently in armadillos and in a short-tailed opossum but not in T. infestans from the Paraguayan Chaco region. Also, in Argentina, TCIIc was found in one skunk specimen, in a few T. infestans specimens, and in dogs from a sylvatic environment. Thus, the finding of TCIIc in T. rubrovaria indicates that this and other triatomines species with terrestrial ecologic behavior can participate in the sylvatic T. cruzi cycle involving the TCIIc group in the Southern Cone countries of America.

There is evidence that TCIIa (Z3B) and TCIIc (Z3A) have emerged from hybridization events between lineages TCIIb and TCI.49 Subdivision of the Z3 stocks in the clusters Z3A and Z3B could be performed because of the genetic outline of the ribosomal cistron. Use of other genetic parameters such as the recent study on chromosome size polymorphism showed intragroup T. cruzi Z3 variability such as the clustering of TCIIc 3663 with hybrid stocks of T. cruzi. To verify the relatedness of the TCIIc isolates from T. rubrovaria with representatives of TC groups I and II based on the ribosomal small subunit, a sequence of a 900-bp fragment was compared among the T. cruzi strains described in Figure 2. Using this strategy, we observed that all isolates from the Brazilian South showed high similarity to TCIIc isolates 3663 and 3869 from the Amazon Basin, suggesting a common origin. Geographic distance of these isolates in Brazil could be caused by a lack of information about T. cruzi sylvatic isolates from different animal and bug groups of others Brazilian localities. Thus, to understand the epidemiologic characteristics of the TCIIc
group, more studies on T. cruzi isolates from various species of vertebrates and bugs with different ecologic behaviors must be performed, including specimens from the southern region of Brazil.

In the RS, Fernandes and others\textsuperscript{51} studied 35 T. cruzi strains on the basis of biological behavior in mice and isoenzyme electrophoresis patterns. The authors found that T. cruzi isolates obtained from chagasic patients (domiciliary transmission cycle) were characterized as Z2 and ZB (TCIIb) and strains isolated from the sylvatic vector P. megistus were Z1 (TCI).

In this work, we showed the presence of T. cruzi IIC. Therefore, study of T. cruzi parasite–host interaction from domestic and sylvatic cycles in RS State can shed light on the evolution, population structure, and epidemiology of Chagas disease.

Biological characteristics of T. cruzi isolates assessed through experimental infection in laboratory animals represent an important tool for understanding different aspects of the epidemiology of Chagas disease.\textsuperscript{7} According to Andrade,\textsuperscript{5} pathogenicity and virulence patterns of T. cruzi enable its classification in biodeme I, II, or III. Furthermore, a specialist committee recommendation\textsuperscript{52} established that T. cruzi isolates typed as biodeme III are equivalent to T. cruzi I and isolates typed as biodeme II are equivalent to T. cruzi II. However, in the Andrade’s biodeme proposal, there is no prototype of T. cruzi IIC. Therefore, to verify the occurrence of specific biological characteristics of the five TCIIc isolates, infectivity, histopathology, tissue tropism, and mortality rates were evaluated in experimental animals according to Andrade’s classic studies. T. cruzi isolates QJI, QJIII, QMI, and QBI showed low parasitemia and mortality rates characteris-
tic of bionome III, whereas isolate QMII induced more intense inflammatory infiltration and a higher mortality rate characteristic of bionome II. These data indicate that in Quaraí, RS, the circulating T. cruzi parasites present heterogeneous biological behavior that may underline the genetic intravariability of the T. cruzi Z3 group.\textsuperscript{18}

Comparison of tissue tropism of T. cruzi strains with specific kinetoplast DNA profiles in different inbred mice (BALB/c, DBA2, and C57BL/6) and the outbred Swiss mice showed that differential biological behavior of T. cruzi isolates are attributed to both parasite and host genetic background.\textsuperscript{53,54} leading to experimental evidence of the “clonal-histotropic” model of Chagas disease.\textsuperscript{55} Thus, T. cruzi natural isolates correspond to a population of parasites composed of distinct clones, which are selected according to the genetic potential of each vertebrate host.\textsuperscript{56,57} In the chronic phase of infection, the TCIIc isolates analyzed in this work showed tissue tropism in heart, skeletal muscles, liver, and colon, suggesting the presence of different parasite clones. To verify this premise, further analysis of cloned strains from each isolate of T. cruzi obtained from T. rubrovaria in Quaraí should be done to study intraspecific variations at the biological, immunologic, biochemical, and genetic levels.

Triatoma rubrovaria presents pre-adaptive characteristics to the domiciliary ecotope because adult bugs have been found in the peridomestic and intradomestic habitats.\textsuperscript{24} Recently, the potential adaptation of this bug to new environments was suggested by its genomic plasticity shown by the high intraspecific genetic variability detected in populations of T. rubrovaria from Brazil, Uruguay, and Argentina through molecular markers.\textsuperscript{58} In our previous study, precipitation assays performed on the collected bugs showed one specimen of T. rubrovaria positive to human antiserum in Bragançó, a settlement closer to the urban perimeter of Quaraí.\textsuperscript{29} This study on the genetic and biological characterization of T. cruzi isolates obtained from T. rubrovaria showed its pathogenicity and virulence in experimental animals. Together these data show a need for permanent entomologic surveillance in the south region of Brazil to prevent re-introduction of the urban Chagas disease. Furthermore, recently, in Santa Catarina, a state in southern Brazil, there was an outbreak of acute Chagas disease with three fatal human victims caused by the ingestion of sugar cane juice containing T. bimaculata, an exclusive sylvatic triatomine species, contaminated with TCI and TCHI isolates.\textsuperscript{59} Thus, in addition to the domiciliation possibility of T. rubrovaria, entomologic surveillance may also avert episodes of acute Chagas disease infection by oral transmission through ingestion of food contaminated with infected bugs.

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