

VARIATION IN *TRYPANOSOMA CRUZI* CLONAL COMPOSITION DETECTED IN BLOOD PATIENTS AND XENODIAGNOSIS TRIATOMINES: IMPLICATIONS IN THE MOLECULAR EPIDEMIOLOGY OF CHILE

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Abstract. To identify *Trypanosoma cruzi* clones from chronically infected individuals, they were transferred to triatomines by the xenodiagnosis test (XD) with *Triatoma infestans*. Polymerase chain reaction (PCR) and hybridization assays were performed to detect minicircle DNA in human blood samples and triatomine feces, using probes to determine the *T. cruzi* clones present. *T. cruzi* clone 19 (TcI) resulted the most prevalent in humans, with a frequency of 0.70 compared with a frequency of 0.53 in triatomines. *T. cruzi* clone 39 (TcIIId) was the most prevalent in *T. infestans*, with a frequency of 0.65 compared with 0.33 in humans. The *T. cruzi* clone 43 (TcIIe) was not detected in blood samples; nevertheless, it was present at a rate of 0.17 in *T. infestans* feces. In conclusion, the *T. cruzi* clones are associated to each host, suggesting that selective amplification of clones occurs in human and triatomines.

INTRODUCTION

Trypanosoma cruzi, the etiologic agent of Chagas disease, is widely spread over Latin America and is transmitted by triatomine bugs. At least 10 million people are at risk of infection.¹ *T. cruzi* isolates are complex multiclonal populations that differ in their genetic, biologic, biochemical, and molecular characteristics and in their behavior in the vertebrate host. *T. cruzi* isolates differ in intrinsic characters such as antigenic composition, susceptibility to chemotherapy, isoenzyme patterns, and genomic profiles of DNA.² However, a clonal structure has been suggested for *T. cruzi* populations; a nucleotide sequence provides evidence of genetic exchange among lineages of *T. cruzi*, and *T. cruzi* hybrids can be experimentally generated.³⁻⁵ Great efforts have been made in detecting *T. cruzi* in biologic samples, and polymerase chain reaction (PCR) has shown a variable degree of efficiency. The sensitivity of the methods ranged from 96-100% compared with serologic diagnosis.^{6,7} Studies of *T. cruzi* strains to determine genetic diversity of this parasite based on a few isoenzymes described three main parasite groups or "zymodemes" (Z1, Z2, and Z3).⁸ Later, with the analysis of isoenzymatic profiles at 15 gene loci and *T. cruzi* samples isolated from a variety of vertebrate and invertebrate hosts, it was possible to identify at least 43 distinct natural isoenzyme profiles or clones.³ *T. cruzi* contains nuclear and kinetoplast DNA (nDNA and kDNA), both of which contain many repetitive sequences that are highly suitable for PCR detection. Characterization of different *T. cruzi* isolates has also been achieved by studying restriction fragment length polymorphism (RFLP) of kDNA. The parasites exhibiting the same RFLP patterns belong to a "schizodeme."⁹ Different groups of investigators have shown a close correlation between zymodemes and schizodemes, suggesting that nuclear structural genes coding for enzymes and extranuclear (kDNA) have had a parallel evolution.^{10,11} Currently, the taxon *T. cruzi* is divided into two divisions (I and II) on the basis of isoenzyme phenotypes, DNA profiles, ribosomal, and mini-exon DNA sequence polymorphisms. *T. cruzi* I corresponds to zymo-

deme Z1, and it has been proposed that *T. cruzi* II consists of five sub-lineages, one corresponding to Z2 and another to Z3.¹² The clinical evolution of *T. cruzi* infection varies widely in different patients and geographic regions. It is possible that the variability in the clinical picture of Chagas disease could be related in part to *T. cruzi* clones, harboring different pathogenicity.¹³ *T. cruzi* kDNA contains two components: minicircles and maxicircles. The minicircles have two kinds of sequence elements: a conserved region repeated four times per molecule and four variable and divergent regions intercalated among the repeats.

The aim of this study was to determine the different *T. cruzi* clones circulating in the blood of chronically infected individuals and those detected in *Triatoma infestans* after xenodiagnosis (XD). This evaluation is important to determine the distribution of *T. cruzi* clones in the two hosts (vertebrate and invertebrate) of the Chagas disease transmission cycle. These results support the selection of specific clones by the different hosts implicated in Chagas disease transmission cycle.

MATERIALS AND METHODS

Patients and blood samples. We tested 57 chronically infected individuals (average, 40 years old) of the IV and V regions of Chile who were positive by conventional serology enzyme-linked immunosorbent assay (ELISA) and indirect immunofluorescence (IFI). Also, flow cytometry was performed for detection of active infection.¹⁴ Two milliliters of blood was mixed with a similar volume (6 mol/L) of the buffer Guanidine HCl and 0.2 mol/L EDTA for PCR assays. XD was performed simultaneously. The samples were incubated at 98°C for 15 minutes, and DNA was extracted by phenol extraction and frozen at -20°C until used.

Informed consent. Informed consent was obtained from all human adult participants. The project was approved by the Ethical Committee of the Medicine Faculty, University of Chile.

XD and triatomine samples. This test was carried out using two cylindrical wooden boxes with seven uninfected third-instar nymphs of *T. infestans* each, as described previously.¹⁵ Microscopic examination of insect feces was performed 30, 60, and 90 days after feeding, and a pool of these samples was

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collected to be used in PCR. The pool of triatomine feces for the PCR test consisted of all the samples obtained at 30, 60, and 90 days from the triatomines fed with peripheral blood of the infected individuals, no matter whether the XD was positive or negative at these time. The material was placed in Diamond medium and incubated at 98°C for 15 minutes and centrifuged at 4,000 rpm for 3 minutes, and the supernatant was passed through a P6 column to remove low-molecular-weight DNA polymerase inhibitors. Finally, the wash through was collected and frozen at -20°C until used.

DNA extraction and PCR amplification. PCR assays were performed in triplicate from blood and triatomine feces, according to conditions described.⁷ Essentially, 800 µL of the blood mixture was phenol extracted. The final aqueous phase was mixed with 1/10 volume of 3 mol/L ammonium acetate and 2 volumes of cold absolute ethanol and left overnight at -20°C to precipitate the DNA. The pellet obtained was centrifuged (12,000 rpm for 15 minutes at 4°C) and washed in 70% ethanol. The pellet was dried to 4°C and resuspended in 50 µL of sterile distilled water. PCR amplification of DNA was performed three times. The reaction mixture was composed of 5 µL of the DNA sample in a volume of 50 µL and conditions already described using primers 121 (5'-AAATAATGTACGGG (T/G) GAGATGCATGA-3'), and 122 (5'-GGTTCGATTGGGGTTGGTGTAAATATA-3'). PCR assays are performed in a DNA clean chamber to avoid contamination. Every 22 samples, a single amplification of a DNA preparation from a confirmed infected individual was included as positive control, and a tube from a confirmed non-infected individual was included as a negative control of PCR. The same was done for triatomine samples. A similar protocol was used as a PCR internal control for amplification of the human β-globin gene sequences. Finally a fraction of the reaction mixture was electrophoresed on 2% agarose gels, denatured, transferred to a nylon membrane, and cross-linked by UV irradiation.

Hybridization with universal and specific radioactive probes. Hybridization was performed using total kDNA as a universal probe¹⁶ and exposed in a Personal Molecular Imager-FX (Bio-Rad Laboratories, Hercules, CA). The hybridization assays were performed at least three times with the universal probe and specific probes.

Specific probes. The probes used in this study were from *T. cruzi* clones: CBBcl3, NRcl3, sp104cl1, and v195cl1. They correspond to clones 32 (TcIib), 39 (TcIId), 19 (TcI), and 43 (TcIie), respectively, and were prepared as described.¹⁷ Clone sp104 was first isolated from *Mepraia spinolai* (silvatic cycle). Meantime the CBB and NR clones were isolated from chronically infected individuals, and clone v195 from *T. infestans* (domestic cycle). Probes were hybridized against a panel containing minicircle amplicons of *T. cruzi* clones as control.

RESULTS

Samples of blood and triatomines fed from all infected individuals were evaluated by PCR and hybridization tests with the universal probe and then with a panel of four specific probes. Positives were considered those cases that PCR and hybridization with the universal probe showed the 330 bp corresponding to minicircle DNA. Controls with non-infected humans and triatomines used were performed in parallel and

resulted negative (data not shown). Figure 1A shows a representative experiment with PCR results from blood and *T. infestans* of a selected group of studied infected individuals. Figure 1B shows the Southern analysis with the universal probe that confirmed PCR results and increased the detection sensitivity obtained by the PCR assay. Figure 1, C-F, also shows the hybridization patterns obtained after Southern analysis with the four specific probes. Probes revealed all possible patterns of hybridization; there were samples positive with zero, one, two, three, and four probes. The hybridization results for the 57 chronically infected individuals are summarized in Table 1. Subsequently, we analyzed 1) infection rates of blood samples and triatomine bugs infected with each *T. cruzi* clone and 2) infection rates of mixed infections in each host. Most of the individuals were infected with *T. cruzi* clones 19 (TcI) or 32 (TcIib) ($P = 0.0001$) or a mixture of both clones in their blood. However, when this analysis was performed with triatomines fed from the same infected individuals, the most prevalent *T. cruzi* clones detected were 39 (TcIId) and 19 (TcI). Generally, frequencies of *T. cruzi* clones found in peripheral blood and triatomine feces were different. For example: in blood, the most frequent clone was 19 (TcI) (0.70); however, in triatomines, the most frequent was 39 (TcIId) (0.65). Percentages of infected individuals in which a single *T. cruzi* clone was detected in blood were 26.3%, 17.5%, and 8.8% for the *T. cruzi* clones 19 (TcI), 32 (TcIib), and 39 (TcIId), respectively. These values contrast with those found in triatomines, which were 7.0%, 0%, and 19.3%, respectively. Statistical analysis indicated significant differences in detection of clones 32 (TcIib) and 39 (TcIId) among hosts. Interestingly, clone 43 (TcIie) was not detected in blood; nevertheless, this clone was detected at a frequency of 0.17 in *T. infestans* ($P = 0.0001$). Table 1 also shows that in 45.5% and 54.2% of the cases, mixed infections were detected in blood and triatomine samples, respectively. Analyzing peripheral blood, 1.7% of the samples did not hybridize with any specific probe tested; this increased to 17.5% in triatomine samples. These remaining samples were probably infected by *T. cruzi* clones different from 19 (TcI), 32 (TcIib), 39 (TcIId), or 43 (TcIie). The results of the analysis of representative cases are shown in Table 2. In case 19, a single clone, clone 19 (TcI), was detected in blood, whereas in feces, the following clones were detected: 32 (TcIib), 39 (TcIId), and 43 (TcIie). In case 99, clone 32 (TcIib) was detected in blood and clone 43 (TcIie) in triatomines. Finally, in case 48, several *T. cruzi* clones were detected in blood, but none were found in *T. infestans*, and the converse was seen in case 46 (Table 2). Similar cases were found in other samples shown in Table 2. These results show the importance of analyzing *T. cruzi* genotypes in both systems in parallel. It is important to notice that we were using four well-selected probes that have a high percentage of detection (98% in blood samples and 82% in triatomines) of the infecting *T. cruzi* clones circulating in chronically infected individuals. Identical profiles of *T. cruzi* with a single clone in both samples were only detected in three infected individuals (5% of cases; data not shown), suggesting that most infected individuals were infected with mixed *T. cruzi* clones.

DISCUSSION

The biologic behavior of *T. cruzi* natural clones is linked to their phylogenetic diversity. Those *T. cruzi* clones that are

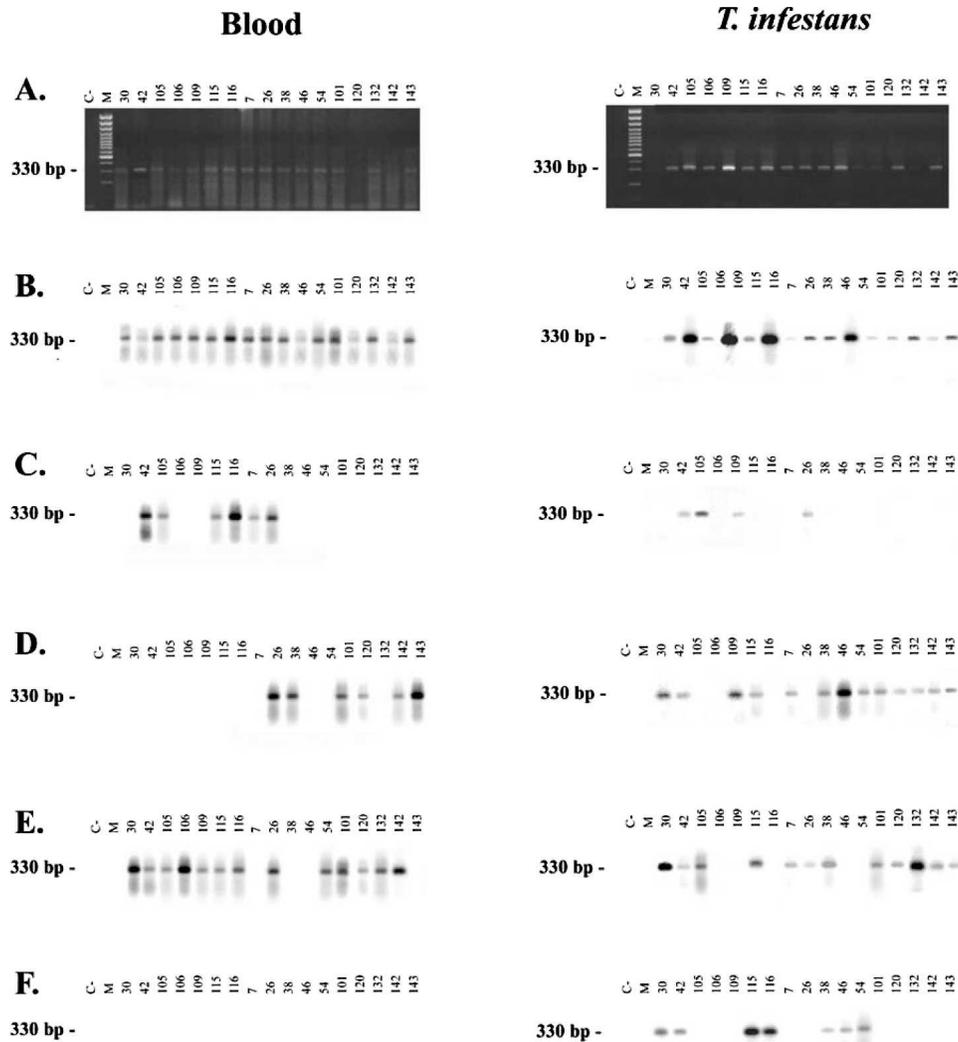


FIGURE 1. Hybridization patterns of blood and *T. infestans* used in xenodiagnosis. (A) Ethidium bromide staining from blood and *T. infestans*. (B) Hybridization with the universal probe. (C–F) Patterns of hybridization with probes TcIIb, TcIIId, TcI, and TcIIe, respectively.

phylogenetically distant tend to show very distinct properties for various biologic parameters such as *in vitro* sensitivity to antichagasic drugs and infectivity in cell cultures among others.¹⁸ These results confirm to a large extent previous data of the distribution of *T. cruzi* clones reported in Bolivia. Indeed, these authors provided evidence that the distribution of the two major *T. cruzi* clones in humans was significantly different from that observed in *T. infestans* of the same area.¹⁹ In this study, we analyzed for the first time the distribution of different *T. cruzi* clones in human subjects and *T. infestans* fed with peripheral blood of the same human subjects (XD). As a whole, the frequencies of each *T. cruzi* clone found in the two hosts were different. Therefore, they have different adaptations in vertebrate and invertebrate hosts. Minicircle DNA was detected in blood and triatomine samples by PCR; however, when this assay was complemented with a further hybridization test and a universal probe, detection in blood samples increased from 87.8% to 100% of the cases and in triatomines from 86.6% to 96.4% of the cases. This can be explained by the higher sensitivity of the radioactive signal than ethidium bromide staining. The clone 19 (TcI) was the

most frequent in humans and clone 39 (TcIIId) in triatomines. Interestingly, 39 (TcIIId) and 43 (TcIIe) clones are genetically very related and more adapted to the invertebrate host than human hosts. These results contrast with those obtained from blood of 0- to 10-year-old chagasic patients of Chile. Frequencies obtained with the same method for similar *T. cruzi* clones differ in prevalence. The difference can be explained by temporal fluctuation of *T. cruzi* clones during the natural course of Chagas disease and/or by different geographical distribution of these clones in Chile.⁷ Others different *T. cruzi* clones are probably circulating. They can be genetically slightly different from those studied here because they do not cross-hybridize with the probes used, same as that reported in Mexico where genetically related *T. cruzi* clones exhibit an absence of cross-hybridization between minicircles kDNA.²⁰ Most probably, these *T. cruzi* clones are TcIIa or TcIIc, which have been described in other endemic areas.²¹ These unknown *T. cruzi* clones may be present mixed with the others or alone, as determined here in 1 blood sample and in 10 triatomine samples. Moreover, these other *T. cruzi* clones seem to be even more adapted to the invertebrate host be-

TABLE 1

Detection of *T. cruzi* clones TcI, TcIIb, TcIIId, and TcIIe in blood and *T. infestans*

Clones	Blood	<i>T. infestans</i>
Only TcIIb	10 (17.5%)	0
Only TcIIId	5 (8.8%)	11 (19.3%)
Only TcI	15 (26.3%)	4 (7.0%)
Only TcIIe	0	1 (1.7%)
TcIIb + TcIIId	1 (1.7%)	2 (3.5%)
TcIIb + TcI	12 (21.0%)	2 (3.5%)
TcIIb + TcIIe	0	1 (1.7%)
TcIIId + TcI	5 (8.8%)	15 (26.3%)
TcIIId + TcIIe	0	0
TcI + TcIIe	0	1 (1.7%)
TcIIb + TcIIId + TcI	8 (14.0%)	3 (5.3%)
TcIIb + TcIIId + TcIIe	0	2 (3.5%)
TcIIb + TcI + TcIIe	0	1 (1.7%)
TcIIb + TcIIId + TcI + TcIIe	0	4 (7.0%)
Others	1 (1.7%)	10 (17.5%)
Total	57	57
Frequency of TcIIb	0.54	0.26
Frequency of TcIIId	0.33	0.65
Frequency of TcI	0.70	0.53
Frequency of TcIIe	0	0.17
Two or more clones	45.5%	54.2%

cause they are more abundant than in the vertebrate host. However, with the panel of probes used, we are confident that the most important *T. cruzi* clones circulating in humans were detected. The genotyping studies of infecting *T. cruzi* clones in chronically infected individuals show that the systems are complementary, because different *T. cruzi* clones are usually found in blood and triatomines. Using both samples, we think that is possible to obtain a complete profile of *T. cruzi* clones circulating in a single infected individual because each one shows a differential behavior to a particular host. This represents the real molecular epidemiology picture of Chagas disease in an endemic area of Chile, because this methodology directly detects *T. cruzi* clones in a host, in contrast to previous studies that used parasite cultures, bypassing the final interpretation of epidemiologic data.^{8,22} The selection of specific *T. cruzi* clones in humans has been explained by probable differences in infectivity and/or parasitemia control by the immune system.^{19,23} 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* sub-population distribution.²⁴ There is clear evidence that supports the selective role of the vertebrate host.²⁵ These “filters” might select

TABLE 2

Different profiles of *T. cruzi* clones in human and triatomine samples

Patient no.	<i>T. cruzi</i> clones in human blood	<i>T. cruzi</i> clones in <i>T. infestans</i>	Total number of identified clones
10	TcI	TcI	1
19	TcI	TcIIb, TcIIId, TcIIe	4
26	TcIIb, TcIIId, TcI	TcIIb, TcIIId, TcI, TcIIe	4
36	TcIIb, TcI	–	2
38	TcIIId	TcIIId, TcI, TcIIe	3
46	–	TcIIId, TcI, TcIIe	3
48	TcIIb, TcIIId, TcI	–	3
94	TcI	TcIIb, TcIIe	3
99	TcIIb	TcIIe	2

Representative cases of all studied.

those populations or clones that are more apt in the new environment. Also development and metacyclogenesis (i.e., the passage of epimastigotes to trypomastigotes) is different in *T. cruzi* clones, which depends on the genetic nature of both parasite and vector.^{26,27} It has been shown that the infection of *T. cruzi* is possibly regulated by biochemical factors induced in different parts of the insect, for example, peptide hemolytic with lytic activity, which destroys several *T. cruzi* clones with different kinetics of lysis or lectins with different specificities in stomach, intestine, and hemolymph in *R. prolixus*, which represents a regulatory mechanism of interactions between the parasite and the vector.²⁸ In an effort to show that *T. cruzi* clones have different adaptations in the diverse hosts, groups of investigators analyzing biclonal experimental infections of *T. cruzi* in *T. infestans* observed that almost one half of the biclonal infections were not detectable after the conclusion of the *T. cruzi* cycle, with important differences in the detection of these double infections according to the *T. cruzi* clones used. The elimination of a clonal genotype seems to be a frequent but not constant pattern in the biclonal infections of *T. cruzi*. This selection could be independent of the involved *T. cruzi* clone; nevertheless, it is possible that *T. infestans* would determine the persistence of certain clones and not of others. As a whole, the finding described here of one *T. cruzi* clone in one host and a different clone in the other allows us to conclude that almost all circulating parasites are differentially adapted to each host and represent mixtures of *T. cruzi* clones in ratios not possible to determinate by the standard PCR used here that is not quantitative. The expected number of chronically infected individuals presenting mixed infections in the studied areas should be very important in epidemiologic studies because mixed infections can be acquired in two ways: 1) infection by a vector presenting mixed infections and 2) re-infection with different *T. cruzi* clonal genotypes. Mixed infections reported here were in 45.5% of human and 54.2% of triatomine samples, which are the same figures reported in young Chilean patients or Bolivian patients and triatomines,^{7,19,29} suggesting that humans are early infected with *T. cruzi* mixture. It has been proposed that the biologic relevance of these mixtures may be great, for example, the phenomena of clonal cooperation and clonal hitch-hiking.³⁰ Moreover, a genotype that does not proliferate in invertebrate hosts has the possibility of proliferating in the vertebrate after transmission, allowing the constant in the nature of a large diversity of *T. cruzi* clones.

Received April 29, 2005. Accepted for publication January 13, 2006.

Acknowledgments: The authors thank Carezza Botto-Mahan and M. Leonor Bustamante for valuable comments on this manuscript.

Financial support: This work was supported by Grants DI-Sal 03/06-2, Fondecyt 1040731, and Fondecyt 1040762. Additional support was obtained from the following grants Postgraduate Department fellowship PG/83/2003, University of Chile.

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