Infection, Genetics and Evolution 51 (2017) 104–107

Contents lists available at ScienceDirect

Infection, Genetics and Evolution

journal homepage: www.elsevier.com/locate/meegid

Short communication

Minicircle classes heterogeneity within the TcIII and TcIV discrete typing units of *Trypanosoma cruzi*

S. Ortiz [a], G. Osorio [b], A. Solari [a,*]

[a] Programa de Biología Celular y Molecular, ICBM, Facultad de Medicina, Universidad de Chile, Santiago, Chile
[b] Programa de Microbiología, ICBM, Facultad de Medicina, Universidad de Chile, Santiago, Chile

ARTICLE INFO

Article history:
Received 25 August 2016
Received in revised form 14 March 2017
Accepted 15 March 2017
Available online 16 March 2017

Keywords:
Trypanosoma cruzi
TcIII
TcIV
kDNA minicircles

ABSTRACT

The taxon *Trypanosoma cruzi*, causative agent of Chagas disease, is composed of several discrete typing units (DTUs) named TcI-TcVI, and Tcbat. The history of the taxon *T. cruzi* is known, even though several controversial aspects remain as the relationships between TcIII and TcIV. We analyzed cloned *T. cruzi* stocks pertaining to the seven DTUs by filter hybridization tests of PCR amplicons from minicircle variable regions and kinetoplast DNA probes. Minicircle DNA blots from the cloned stocks and filter hybridization with one TcI, one TcII, one TcV, one TcVI, three TcIII, one TcIV from North America and one TcIV kinetoplast DNA probes from South America revealed minicircle variable region cross-reaction in some *T. cruzi* DTUs probed. TcIII was heterogeneous in minicircle class composition, even though two TcIII probes revealed that a small fraction of minicircles cross-hybridized with the minicircles from the TcIII, TcV and TcVI DTUs. The minicircles of TcIV from North America cross-reacted only with TcIV from North America but not with TcIV stocks from Brazil and Bolivia. The results on minicircle cross-hybridizations are discussed in the context of RNA editing, mitochondrial function in *T. cruzi* DTUs.

1. Introduction

Kinetoplast DNA (kDNA) is a unique structurally complex mitochondrial DNA in nature with maxicircles and minicircles present in *Trypanosoma cruzi* (order Kinetoplastida). Minicircles are present in several thousand copies per network and usually nearly identical in size (0.9–2.5 kb) (Schnaufer et al., 2002). They are frequently heterogeneous in sequence and encoding gRNAs·These gRNAs are coded in the variable region of the minicircles which show absence of cross-hybridization with cloned minicircle from heterologous DTUs (Sanchez et al., 1984; Macina et al., 1985; Britto et al., 1995). TcI and TcII stocks are ancient and heterogeneous, while TcV and TcVI stocks are recent and display low genetic diversity (Lewis et al., 2011; Flores-López and Machado, 2011). The relationships between *T. cruzi* DTUs TcIII and TcIV are not sufficiently demonstrated. A three ancestor model (TcI, TcII, and TcIII) suggests two independent hybridization events between TcII and TcIII to generate TcV and TcVI. However no information on the origin of TcIV was presented in that study (de Freitas et al., 2006). Three significant clusters were found with mitochondrial genes (mtTcI, mtTcII, mtTcIII) corresponding to TcI, TcII and TcIII-TcIV-TcV-TcVI (Barnabé et al., 2016). The aim of this study is to analyze the minicircles classes present in *T. cruzi* DTUs TcIII and TcIV cloned stocks and to address the following questions: Do TcIII and TcIV cloned stocks share minicircles classes within each DTUs? Do TcIII and TcIV share minicircle classes with the other DTUs?

2. Material and methods

The panel of 21 *T. cruzi* stocks belonging to the six *T. cruzi* DTUs and Tcbat were examined by hybridization assays. We used the same DNA purified of these stocks for probes preparation and for minicircle variable region PCR assay (Table 1).

The amplification reactions were performed with oligonucleotides 121 and 122 (Wincker et al., 1994). PCR samples were transferred onto ten identical Hybond N^+ nylon membranes (Amersham, Little Chalfont, United Kingdom). The minimum amount of amplified DNA to perform filter hybridization tests is 30 ng, and under high stringency conditions any probe used should cross-hybridize and determine identity with the immobilized DNA in the membranes, unless they are heterologous DNAs. (See Fig. 1.)

* Corresponding author at: Programa de Biología Celular y Molecular, ICBM, Facultad de Medicina, Universidad de Chile, 8380453 Santiago, Chile.
E-mail address: asolari@med.uchile.cl (A. Solari).

http://dx.doi.org/10.1016/j.meegid.2017.03.017
1567-1348/

Table 1
Set of stocks and reference strains representing the known *T. cruzi* DTUs used in the study.

Strain	DTU	Origin	Host
Tcbat 4 TCC793	Tcbat	Brazil Sao Paulo	*Myotis levis*
Tcbat 5 TCC1122	Tcbat	Brazil Sao Paulo	*Myotis albescens*
Cuica cl1	TcI	Brazil Sao Paulo	*Philander opossum*
Sp104 cl1[a]	TcI	Chile IV Region	*Mepraia spinolai*
CBB cl3[a]	TcII	Chile Tulahuén	*Homo sapiens*
Esm cl3	TcII	Brazil São Felipe	*Homo sapiens*
X109 cl2[a]	TcIII	Paraguay Makthlawaiya	*Canis familiaris*
M6241 cl6	TcIII	Brazil Belém	*Homo sapiens*
M5631 cl5[a]	TcIII	Brazil Marajo	*Dasypus novemcinctus*
ARMA18 cl3[a]	TcIII	Paraguay Campo Loro	*Dasypus novemcinctus*
ARMA13 cl1	TcIII	Paraguay Campo Loro	*Dasypus novemcinctus*
JA2 cl2[a]	TcIII	Brazil Amazonas	*Monodelphis* sp.
CanIII cl1[a]	TcIV	Brazil Belém	*Homo sapiens*
DogTheis cl1	TcIV	USA Oklahoma	*Canis familiaris*
92122102R cl1	TcIV	USA Georgia	*Procyon lotor*
10 R26 cl1	TcIV	Bolivia Santa Cruz	*Aotus* sp.
StC10R cl1[a]	TcIV	USA Georgia	*Procyon lotor*
92.80 cl2	TcV	Bolivia Santa Cruz	*Homo sapiens*
VCF cl1[a]	TcV	Chile San Félix	*Triatoma infestans*
V195 cl1[a]	TcVI	Chile San Pedro de Atacama	*Triatoma infestans*
CH2 cl1	TcVI	Chile San Pedro de Atacama	*Triatoma infestans*

The *T. cruzi* clones used in this study are described in Lewis et al., 2011.
[a] kDNA minicircles used as probe (see Materials and methods).

Ten kDNA minicircles probes (Table 1) were obtained as described in Veas et al. (1991). Finally, the probes were labeled using the random primer method with [α-^{32}P] dATP. The electrophoresis signal and the hybridization profiles were analyzed by densitometry (NIH Image J softwate). The intensity of the radioactive signal with the corresponding identical probe was considered as 100% (positive control). The identity of any heterologous sample tested against each probe was quantified as percentage of the positive control. Signals similar to background measured in areas without DNA weren't considered (≤5%). The hybridizations were repeated twice (see Supplementary material).

3. Results and discussion

The hybridization results with the same *T. cruzi* stock are maximum (100%) (Fig.1). However when a kDNA probe from TcIII or TcIV stock hybridizes with another *T. cruzi* stock belonging to a similar DTU the hybridization signal is low or absent, indicating that each *T. cruzi* within the same DTU contains very different minicircle classes or the proportions of these are quite different. That means TcIII and TcIV stocks are heterogeneous in their minicircle classes composition. Some minicircle classes of TcIII (X109 and JA2) are found in other DTUs in very low amount suggesting they were conserved in the similar DTU TcIV, TcV and TcVI as well. Some minicicle classes of TcIV (StC 10R) are found in other similar DTUs from North America and TcVI but not in TcIV stocks from South America. Minicircle classes from TcIII and TcIV DTUs, do not hybridize with minicircles belonging to the TcI, Tcbat, and the TcII stocks used in this -study. This result also confirms that cross hybridizations are not due to remnants or the constant regions present in the kDNA probes.

Each minicircle is replicated once/generation when released from the concatenated network, generating nicked minicircles, to avoid another replication event/generation (Kitchin et al., 1985). Available minicircle sequences show great heterogeneity in the whole *T. cruzi* taxon and within a parasite clone (Frasch et al., 1984; Macina et al., 1986; González, 1986; Degrave et al., 1988; Velazquez et al., 2008). The analysis of the variable regions from 170 minicircle sequences of four *T. cruzi* DTUs showed that only 56% exhibited significant homology and the other 44% corresponded to unique sequences (Telleria et al., 2006). These sequence studies also revealed that the most frequent mutations observed in those minicircle variable regions were mainly substitutions, transitions and also short insertions/deletions without large sequence rearrangements, suggesting they are the result of replication errors of mitochondrial DNA by DNA polymerase β, which generates a higher mutation rate in kDNA than in nuclear DNA (Schamber-Reis et al., 2012; Maldonado et al., 2015). An unequal segregation of minicircles after the number of proliferation cycles could also contribute to their heterogeneous generation. The heterogeneity of different minicircle classes in *T. cruzi* Mexican TcI stocks was observed by kDNA hybridization tests as described, or a great heterogeneity of hybridization patterns, with *T. cruzi* populations in Brazil (Britto et al., 1995; Bosseno et al., 2000). These results and other studies with highly characterized *T. cruzi* stocks from several countries of South America demonstrate that kDNA probes of *T. cruzi* DTUs TcI, TcII, Tc V and TcIV are specific in hybridization tests under high stringency conditions and hybridize only with identical minicircle sequences (Brenière et al., 1998; Arenas et al., 2012; Rumi et al., 2013; Egaña et al., 2016; Bontempi et al., 2016). The results of positive cross-hybridization in this study, using the TcIII and TcIV kDNA probes suggest that the total composition of minicircle classes varies between each TcIII and TcIV stock as reported previously in TcI and TcII stocks (Arenas et al., 2012). This observation suggests that minicircle classes heterogeneity code for the different gRNA present in a *T. cruzi* clone. Similar minicircle classes are detected between TcIII of X109 with other TcIII, and TcVI. The same occur with TcIII of JA2 with other TcIII; TcIV from North and South America, TcV and TcVI. The high similarity in minicircle classes between the JA2 clon with TcVI stocks could indicate the identity of the parental TcIII which fused with the parental TcII. This result suggests that some minor minicircle classes are conserved after the fusion of TcII with TcIII and later recombination independent events to generate TcV and TcVI. Between TcIII most of the *T. cruzi* clones tested contain very different minicircle classes to code for gRNAs. Restoration of the disrupted reading frames of mitochondrial transcript seems to be accomplished by strain specific RNA editing (Westenberger et al., 2006). The cross-hybridization signals obtained with one TcIV probe (StC10R) with two TcIV samples from North America, but not with other two TcIV samples from South America suggests that minicircle class composition is quite different between North and South American Tc IV stocks. The same differentiation was shown by phylogenetic analysis with other genetic markers (Tomasini and Diosque, 2015). This TcIV probe also revealed that some minor minicircle classes are conserved within TcIV and TcVI but not in any other DTU. The heterogeneity of minicircle classes observed within the TcIII and TcIV stocks represent different sets of gRNAs.

4. Conclusions

We report minicircle classes' heterogeneity in some *Trypanosoma cruzi* TcIII and TcIV DTUs which represent different sets of guide RNAs within those DTUs. In some TcIII stocks a fraction of minicircles classes are conserved in other TcIII, TcIV, TcV and TcVI cloned stocks but in someone is strain specific as most of the cases. Future studies should be necessary using different methodologies to validate these conclusions.

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.meegid.2017.03.017.

Acknowledgements

We are grateful to Dr. Patrick Hamilton for send us Tcbat DNA samples, and to Dr. M. A. Miles for epimastigote *T. cruzi* clones DNA samples (Epi Net project. Contract N° 223034). We are grateful to Constanza Blanco for make the densitometry measurements. This work was supported by FONDECYT-Chile 1120122 to A. Solari.

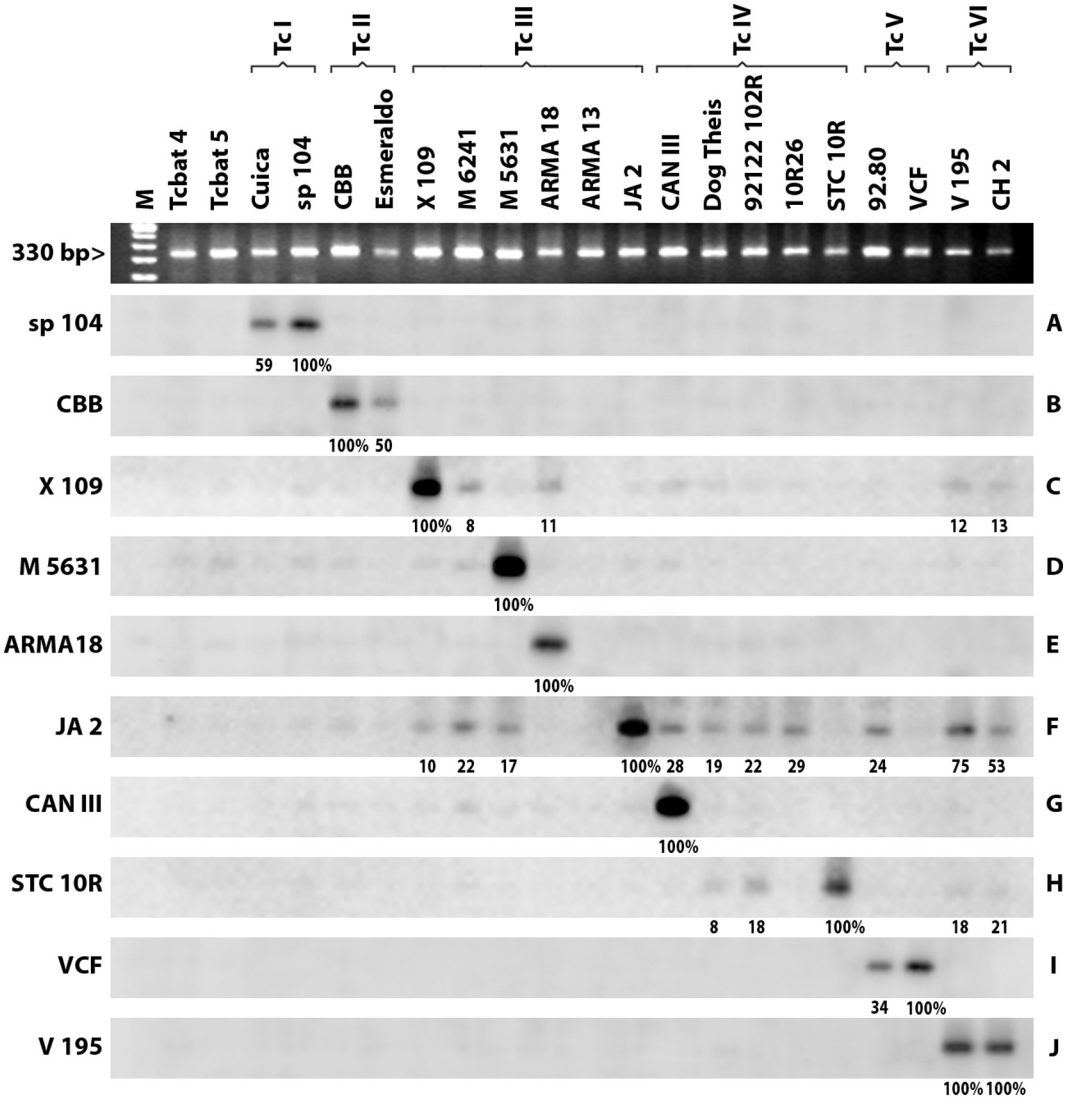

Fig. 1. Hybridization patterns of different *Trypanosoma cruzi* stocks belonging to different DTUs. Different *T. cruzi* DTUs probes (TcI-sp 104 cl 1; TcII-CBB cl 3, TcIII-X109 cl 2, M5631 cl 5, Arma 18cl 3, JA 2 cl 2; TcIV-CAN III cl 1, STC10R cl 1; TcV-VCF cl 1; TcVI-V195 cl 1). Minicircle PCR amplicons stained with ethidium bromide Lane M, 100-base pair (bp) DNA ladder. A. Hybridization with probe TcI; B. Hybridization with probe TcII; C, D, E, F. Hybridizations with probes TcIII; G, H. Hybridizations with probes TcIV; I. Hybridization with probe TcV; J. Hybridization with probe TcVI.

References

Arenas, M., Campos, R., Coronado, X., Ortiz, S., Solari, A., 2012. *Trypanosoma cruzi* genotypes of insect vectors and patients with Chagas of Chile studied by means of cytochrome b gene sequencing, minicircle hybridization, and nuclear gene polymorphisms. Vector Borne Zoonotic Dis. 12, 196–205.

Barnabé, C., Mobarec, H.I., Jurado, M.R., Cortez, J.A., Brenière, S.F., 2016. Reconsideration of the seven discrete typing units within the species *Trypanosoma cruzi*, a new proposal of three reliable mitochondrial clades. Infect. Genet. Evol. 39, 176–186.

Bontempi, I.A., Bizai, M.L., Ortiz, S., Manattini, S., Fabbro, D., Solari, A., Diez, C., 2016. Simple methodology to directly genotype *Trypanosoma cruzi* discrete typing units in single and mixed infections from human blood samples. Infect. Genet. Evol. 43, 123–129.

Bosseno, M.F., Espinoza, B., Sánchez, B., Brenière, S.F., 2000. Mexican *Trypanosoma cruzi* stocks: analysis of minicircle kDNA homologies by cross-hybridization. Mem. Inst. Oswaldo Cruz 95, 473–476.

Brenière, S.F., Bosseno, M.F., Telleria, J., Bastrenta, B., Yacsik, N., Noireau, F., Alcazar, J.L., Barnabé, C., Wincker, P., Tibayrenc, M., 1998. Different behavior of two *Trypanosoma cruzi* major clones: transmission and circulation in young Bolivian patients. Exp. Parasitol. 89, 285–295.

Britto, C., Cardoso, M.A., Ravel, C., Santoro, A., Pereira, J.B., Coura, J.R., Morel, C.M., Wincker, P., 1995. *Trypanosoma cruzi*: parasite detection and strain discrimination in chronic chagasic patients from northeastern Brazil using PCR amplification of kinetoplast DNA and nonradioactive hybridization. Exp. Parasitol. 81, 462–471.

Degrave, W., Fragoso, S.P., Britto, C., van Heuverswyn, H., Kidane, G.Z., Cardoso, M.A., Mueller, R.U., Simpson, L., Morel, C.M., 1988. Peculiar sequence organization of kinetoplast DNA minicircles from *Trypanosoma cruzi*. Mol. Biochem. Parasitol. 27, 63–70.

Egaña, C., Pinto, R., Vergara, F., Ortiz, S., Campos, R., Solari, A., 2016. Fluctuations in *Trypanosoma cruzi* discrete typing unit composition in two naturally infected triatomines: *Mepraia gajardoi* and *M. spinolai* after laboratory feeding. Acta Trop. 160, 9–14.

Flores-López, C.A., Machado, C.A., 2011. Analyses of 32 loci clarify phylogenetic relationships among *Trypanosoma cruzi* lineages and support a single hybridization prior to human contact. PLoS Negl. Trop. Dis. 5, e1272.

Frasch, A.C., Sánchez, D.O., Stoppani, A.O., 1984. Homogeneous and heterogeneous minicircle subpopulations in *Trypanosoma cruzi* kinetoplast DNA. Biochim. Biophys. Acta 782, 26–33.

de Freitas, J.M., Augusto-Pinto, L., Pimenta, J.R., Bastos-Rodrigues, L., Gonçalves, V.F., Teixeira, S.M., Chiari, E., Junqueira, A.C., Fernandes, O., Macedo, A.M., Machado, C.R., Pena, S.D., 2006. Ancestral genomes, sex, and the population structure of *Trypanosoma cruzi*. PLoS Pathog. 2, e24.

González, A., 1986. Nucleotide sequence of a *Trypanosoma cruzi* minicircle. Nucleic Acids Res. 14, 9217.

Kitchin, P.A., Klein, V.A., Englund, P.T., 1985. Intermediates in the replication of kinetoplast DNA minicircles. J. Biol. Chem. 260, 3844–3851.

Lewis, M.D., Llewellyn, M.S., Yeo, M., Acosta, N., Gaunt, M.W., Miles, M.A., 2011. Recent, independent and anthropogenic origins of *Trypanosoma cruzi* hybrids. PLoS Negl. Trop. Dis. 5, e1363.

Macina, R.A., Sanchez, D.O., Affranchino, J.L., Engel, J.C., Frasch, A.C.C., 1985. Polymorphisms within minicircle sequence classes in the kinetoplast DNA of *Trypanosoma cruzi* clones. Mol. Biochem. Parasitol. 16, 61–74.

Macina, R.A., Sanchez, D.O., Gluschankof, D.A., Burrone, O.R., Frasch, A.C.C., 1986. Sequence diversity in the kinetoplast DNA minicircles of *Trypanosoma cruzi*. Mol. Biochem. Parasitol. 21, 25–32.

Maldonado, E., Rojas, D.A., Moreira-Ramos, S., Urbina, F., Miralles, V.J., Solari, A., Venegas, J., 2015. Expression, purification, and biochemical characterization of recombinant DNA polymerase beta of the *Trypanosoma cruzi* TcI lineage: requirement of additional factors and detection of phosphorylation of the native form. Parasitol. Res. 114, 1313–1326.

Rumi, M.M., Pérez Brandán, C., Gil, J.F., D'Amato, A.M., Ragone, P.G., Lauthier, J.J., Tomasini, N., Cimino, R.O., Orellana, V., Lacunza, C.D., Nasser, J.R., Basombrío, M.A., Diosque, P., 2013. Benznidazole treatment in chronic children infected with *Trypanosoma cruzi*: serological and molecular follow-up of patients and identification of discrete typing units. Acta Trop. 128, 130–136.

Sanchez, D.O., Frasch, A.C.C., Carrasco, A.E., Gonzalez-Cappa, E.M., de Isola, E., Stoppani, A.O., 1984. Rapid evolution of kinetoplast DNA minicircle subpopulations in *Trypanosoma cruzi*. Mol. Biochem. Parasitol. 11, 169–178.

Schamber-Reis, B.L., Nardelli, S., Régis-Silva, C.G., Campos, P.C., Cerqueira, P.G., Lima, S.A., Franco, G.R., Macedo, A.M., Pena, S.D., Cazaux, C., Hoffmann, J.S., Motta, M.C., Schenkman, S., Teixeira, S.M., Machado, C.R., 2012. DNA polymerase beta from *Trypanosoma cruzi* is involved in kinetoplast DNA replication and repair of oxidative lesions. Mol. Biochem. Parasitol. 183, 122–131.

Schnaufer, A., Domingo, G.J., Stuart, K., 2002. Natural and induced dyskinetoplastic trypanosomatids: how to live without mitochondrial DNA. Int. J. Parasitol. 32, 1071–1084.

Telleria, J., Lafay, B., Virreira, M., Barnabé, C., Tibayrenc, M., Svoboda, M., 2006. *Trypanosoma cruzi*: sequence analysis of the variable region of kinetoplast minicircles. Exp. Parasitol. 114, 279–288.

Tomasini, N., Diosque, P., 2015. Evolution of *Trypanosoma cruzi*: clarifying hybridisations, mitochondrial introgressions and phylogenetic relationships between major lineages. Mem. Inst. Oswaldo Cruz 110, 403–413.

Veas, F., Brenière, S.F., Cuny, G., Brengues, C., Solari, A., Tibayrenc, M., 1991. General procedure to construct highly specific kDNA probes for clones of *Trypanosoma cruzi* for sensitive detection by polymerase chain reaction. Cell. Mol. Biol. 37, 73–84.

Velazquez, M., Diez, C.N., Mora, C., Diosque, P., Marcipar, I.S., 2008. *Trypanosoma cruzi*: an analysis of the minicircle hypervariable regions diversity and its influence on strain typing. Exp. Parasitol. 120, 235–241.

Westenberger, S.J., Cerqueira, G.C., El-Sayed, N.M., Zingales, B., Campbell, D.A., Sturm, N.R., 2006. *Trypanosome cruzi* mitochondrial maxicircles display species and strain-specific variation and a conserved element in the non-coding region. BMC Genomics 7, 60–78.

Wincker, P., Britto, C., Pereira, J.B., Cardoso, M.A., Oelemann, W., Morel, C.M., 1994. Use of a simplified polymerase chain reaction procedure to detect *Trypanosoma cruzi* in blood samples from chronic chagasic patients in a rural endemic area. Am.J.Trop. Med. Hyg. 51, 771–777.