# Molecular characterization of Cyclophilin (TcCyP19) in *Trypanosoma cruzi* populations susceptible and resistant to benznidazole

Juciane Vaz Rêgo[a,b,e], Ana Paula Duarte[b], Daniel Barbosa Liarte[c], Francirlene de Carvalho Sousa[a], Humberto Medeiros Barreto[c], Jacqueline Búa[d], Alvaro José Romanha[b], Gandhi Rádis-Baptista[e], Silvane Maria Fonseca Murta[b*]

[a]Campus Amilcar Ferreira Sobral, Universidade Federal do Piauí-UFPI, Floriano, PI, Brazil

[b]Laboratório de Parasitologia Celular e Molecular, Centro de Pesquisa René Rachou-CPqRR/FIOCRUZ, Belo Horizonte-MG, Brazil

[c] Campus Petrônio Portela, Universidade Federal do Piauí-UFPI,Teresina-PI, Brazil

[d]Instituto Nacional de Parasitología "Dr. M. Fatala Chabén", A.N.L.I.S. Buenos Aires, Argentina

[e]Instituto de Ciências do Mar, Universidade Federal do Ceará-Labomar/UFC, Fortaleza-CE, Brazil

* Corresponding author. Tel.: +55-31-33497780, Fax: +55-31-32953115.

E-mail address: silvane@cpqrr.fiocruz.br (S..M.F. Murta), juciane@ufpi.edu.br (J.V. Rego)

*Abbreviations:* CyP-A, cyclophilin-A; TcCyp19, *T. cruzi* 19 kDa cyclophilin; BZ, Benznidazole; HGPRT, Hypoxanthine-guanine phosphoribosyltransferase; LIT, Liver-infusion tryptose; NFX, Nifurtimox; PCR, Polymerase chain reaction; qRT-PCR, real time PCR; PFGE, Pulsed-field gel electrophoresis.

**ABSTRACT**

Cyclophilin (TcCyP19), a peptidyl-prolyl *cis*/*trans* isomerase, is a key molecule with diverse biological functions that include roles in molecular chaperoning, stress response, immune modulation, and signal transduction. In this respect, TcCyP19 could serve as a potential drug target in disease-causing parasites. Previous studies employing proteomics techniques have shown that the TcCyP19 isoform was more abundant in a benznidazole (BZ)-resistant *Trypanosoma cruzi* population than in its susceptible counterpart. In this study, TcCyP19 has been characterized in BZ-susceptible and BZ-resistant *T. cruzi* populations. Phylogenetic analysis revealed a clear dichotomy between Cyphophilin A (CyPA) sequences from trypanosomatids and mammals. Sequencing analysis revealed that the amino acid sequences of TcCyP19 were identical among the *T. cruzi* samples analyzed. Southern blot analysis showed that *TcCyP19* is a single-copy gene, located in chromosomal bands varying in size from 0.68 to 2.2 Mb, depending on the strain of *T. cruzi*. Northern blot and qPCR indicated that the levels of *TcCyP19* mRNA were two-fold higher in drug-resistant *T. cruzi* populations than in their drug-susceptible counterparts. Similarly, as determined by two-dimensional gel electrophoresis immunoblot, the expression of TcCyP19 protein was increased to the same degree in BZ-resistant *T. cruzi* populations. No differences in TcCyP19 mRNA and protein expression levels were observed between the susceptible and the naturally resistant *T. cruzi* strains analyzed. Taken together, these data indicate that cyclophilin TcCyP19 expression is up-regulated at both transcriptional and translational levels in *T. cruzi* populations that were in vitro-induced and in vivo-selected for resistance to BZ.

Key words: *Trypanosoma cruzi* – Cyclophilin - benznidazole - Drug resistance

## 1. Introduction

Chagas disease, also known as American trypanosomiasis, is caused by the protozoan parasite *Trypanosoma cruzi*. It is estimated that 8 to 11 million people are infected with Chagas disease worldwide, mostly in Latin America, where it is endemic (CDC 2013). Drug treatment of chagasic patients currently involves the 5-nitrofuran nifurtimox (NFX) and the 2-nitroimidazole Benznidazole (BZ). However, both drugs cause severe side effects. NFX exerts its action via the generation of nitro anion radicals, a process known as the redox cycle (Maya et al., 2003), while BZ acts via reductive stress, which involves the covalent modification of macromolecules such as DNA, proteins, and lipids by reduced nitro intermediates (Docampo, 1990).

A previous study suggests that both BZ and NFX are prodrugs, activated by nitroreductases to produce nitrogenated radicals (Wilkinson et al., 2008). Interestingly, the deletion of copies of genes encoding two different nitroreductases, namely, old yellow enzyme (TcOYE; also named prostaglandin synthase or NADPH oxidoreductase) (Murta et al., 2006) and trypanosomal type I nitroreductase (NTR-1) (Wilkinson et al., 2008), has been associated with a phenotype of *T. cruzi*, which is resistant to NFX and BZ *in vitro*. Overexpression of enzymatic activity related to antioxidant response, like that of tryparedoxin peroxidase, ascorbate peroxidase, and iron-superoxide dismutase, has been detected in *T. cruzi* populations resistant to BZ (Nogueira et al., 2006, 2009, 2012). In addition, the hexose transporter activity was 40% lower in a BZ-resistant *T. cruzi* population than in the susceptible control (dos Santos et al., 2012). Differences in susceptibility to BZ and NFX between *T. cruzi* strains (Filardi and Brener, 1987; Murta et al., 1998; Toledo et al., 2004) and/or the genetic diversity of the host (Filardi and Brener, 1987) might explain, in part, the variations in the efficacies

of antiparasitic drugs. The mechanisms underlying drug resistance in *T. cruzi* and most parasites remain poorly understood. A more in-depth understanding of these mechanisms is essential to develop efficacious chemotherapeutic strategies for fighting Chagas disease.

Several groups have used proteomics approaches in order to understand the mechanisms of drug resistance in protozoan parasites. In a previous study by our group, two-dimensional electrophoresis in combination with mass spectrometry was applied to detect differences in protein expression between BZ–susceptible and -resistant *T. cruzi* populations, as well as variant clones (Andrade et al., 2008). Among the proteins upregulated in BZ-resistant samples, we observed that cyclophilin TcCyP19 was more abundant in the BZ-resistant population (17LER) and clone (27R) than in their BZ-susceptible counterparts (17WTS and clone 9S).

Cyclophilin (TcCyP19) is considered an important player in several biological processes like signal transduction, protein-protein interaction, as well as protein folding and cellular stress response (Galat, 2003). This protein belongs to the peptidyl-prolyl isomerase family (PPIases), which catalyzes the *cis-trans* isomerization of prolyl-peptide bonds in biochemical pathways of cellular communication. The role of Cyclophilin (CyP) in heat shock response, in which the expression of a certain class of cyclophilins was shown to be upregulated under various stressful conditions, has been reviewed previously (Andreeva et al., 1999). The cyclophilin family comprises several isoforms, including cyclophilin TcCyP19, ranging in size from 19 to 110 kDa (Potenza et al., 2006). The most abundantly expressed cyclophilin in the epimastigote stage of *T. cruzi* is TcCyP19. This isoform is a classical cytosolic cyclophilin, with a theoretical mass of 18.782 Da (19 kDa). Búa and co-workers (2001) observed that TcCyP19 is

homologous to cyclophilin from *T. brucei* (TbCyP19), *Leishmania major* (LmCyP19), and *T. vivax* (TvCyP19). It has been reported that TcCyP19 and other cyclophilin members of *T. cruzi* (e.g., TcCyP22, TcCyP28, and TcCyP40) are also able to bind to the immunosuppressant drug cyclosporine A (CsA), which could serve to explain their role in mediating immunosuppressive responses (Potenza et al., 2006). Despite its multiple roles, the involvement of TcCyP19 in the BZ-resistant phenotype of *T. cruzi* has not yet been demonstrated.

In the present study, the phylogenetic relationship between TcCyP19 and CyPs from other organisms was established, TcCyP19 nucleotide and amino acid sequence polymorphisms were evaluated, the copy number and chromosomal location of *TcCyP19* gene was determined, and the expression of *TcCyP19* gene was analyzed at the transcriptional and translational levels. We observed a positive correlation between increased expression of TcCyP19 in *T. cruzi* populations with in vitro-induced and in vivo-selected resistance to BZ.

## 2. Materials and methods

### *2.1 Trypanosoma cruzi strains*

Eleven *T. cruzi* strains and clones were used in this study (Table I). The BZ-resistant *T. cruzi* population (17 LER) derived from the Tehuantepec cl2 susceptible wild-type strain (17 WTS) (Nirdé et al., 1995) was obtained by *in vitro* exposure to increasing concentrations of BZ (LAFEPE Pharmaceutical Laboratory of the State of Pernambuco, Vitória de Santo Antão, Brazil). Parasites of the 17 LER population are resistant to 220 µM BZ, a concentration that is 23-fold higher than the $IC_{50}$ for the 17 WTS control population. The BZ-resistant *T. cruzi* population (BZR) was derived from

the susceptible Y strain (BZS) following *in vivo* selection after 25 successive passages in mice treated with a single high dose (500 mg/kg body weight) of BZ (Murta and Romanha, 1998). We also used two clones derived from these populations: one susceptible (clone 9S) and other resistant (clone 27R). The other five *T. cruzi* strains were previously characterized according to their in vivo susceptibility to BZ and NFX (Filardi and Brener, 1987). The VL-10 strain is naturally resistant to both drugs, while CL Brener, Romano, Buriti and Berenice are susceptible (Murta et al., 1998). All eleven strains were classified as *T. cruzi* group Tc I, Tc II or Tc VI according to the nomenclature for *T. cruzi* (Zingales et al., 2009). Epimastigotes forms of all *T. cruzi* strains were grown in LIT medium, washed in PBS and the parasite pellets were used for the preparation of DNA, RNA and protein samples.

### *2.2. In silico and phylogenetic analysis of the* TcCyP19 *gene*

Similarity searches were carried out for complete TcCyP19 amino acid sequence (GenBank accession no. XM_816485) within five different *T. cruzi* genomes, using the Basic Local Alignment Search Tool (BLAST; National Center for Biotechnology Information). The genomes used for similarity search were CL Brener Esmeraldo_Like, CLBrener Non-Esmeraldo_like, SylvioX10, JRc14 and Marinkellei_B7 (available in TritrypDB - www.tritrypdb.org - version 6.0). The sequences with high identity values to *Tc*CyP19 were located within the respective genomes and the predicted proteins were identified using the ARTEMIS (version 15.0 - www.sanger.ac.uk) and BLAST. For phylogenetic analysis, the predicted protein sequences corresponding to cyclophilins were aligned using CLUSTAL-W (Larkin et al., 2007), and the phylogenetic tree was constructed using the MEGA software (Molecular Evolutionary Genetics Analysis,

version 5.2.2 – Tamura et al., 2011) with bootstrap test (1000 replicates). In addition, the amino acid sequence from *Tc*CyP19 was compared with related cyclophilin sequences from four species of *Trypanosoma* (*T. brucei*, *T. congolense*, *T. evansi* and *T. vivax*), five *Leishmania* (*L. donovani*, *L. infantum*, *L. major*, *L. mexicana* and *L. braziliensis*), and two mammals (*Mus musculus*, and *Homo sapiens*). A phylogenetic tree for this second multialignment was also constructed using the MEGA software.

*2.3. RNA and DNA preparations*

Genomic DNA and total RNA from *T. cruzi* samples were extracted as previously described (Nogueira et al., 2006). In order to prepare the molecular probes used in the Southern and Northern blot assays, a 400 bp segment corresponding to nucleotide 68 to 468 of the *TcCyP19* (GenBank accession no. XM_816485) was amplified from *T. cruzi* Y strain DNA by conventional PCR using the TcCYP19 forward primer 5' GGTGGCCAATCAGCCGGACG 3' and the TcCYP19 reverse primer 5' TCCATTGCCTTAACGACTTC 3'. For Southern blot assay, about 5 µg total DNA of different *T. cruzi* strains were digested with the restriction enzyme *Sac*I in standard reaction conditions (Invitrogen, Carlsbad, CA, USA). The Southern and northern blots were carried out using protocol previously described (Murta et al., 2006).

*2.4. DNA sequencing*

The *TcCyP19* 534 bp ORF from *T. cruzi* BZ-susceptible and -resistant populations (17WTS, 17LER, BZS, BZR, CL Brener and VL-10) was cloned into the TOPO PCR2.1 vector (Invitrogen) and amplified in *E. coli* TOP 10 F' competent cells. Minipreparations of plasmid DNA were done using the QIAprep Spin Miniprep kit

(Qiagen). Aliquots of 500 ng DNA were sequenced using the DYEnamic WET Dye Terminator Kit (GE Healthcare) in a MegaBACE 1000 DNA Analysis System (GE Healthcare), using the following primers: M13 forward 5'-GTAAAACGACGGCCAG-3', M13 reverse 5'-CAGGAAACAGCTATGAC-3' and TcCyP19 forward 5'-ATGTCGTACAAGCCGCATCA-3' and TcCyP19 reverse 5'-AGGCCTCTGGTCAACTTTAA-3'. Reaction consisted of an initial denaturation at 95°C followed by 30 cycles of 15s 95°C, 20s at 55°C and 80s at 60°C. Samples were analyzed on Mega Bace 400 sequencer (Amersham) and the data were analyzed using Phred, Phrap and Consed. Sequence variability between parasites was assessed by sequencing three colonies of each *T. cruzi* population and by sequencing each colony twice with each primer. Sequences selected for analysis were those with Phred >40. Nucleotide sequences were translated into the amino acid sequence using Transec. The nucleotide and amino acid sequences were aligned using the ClustalW 2.1 software.

*2.5. Pulsed Field Gel Electrophoresis (PFGE)*

The *T. cruzi* chromosomes were separated by PFGE in a gene navigator TM system (Amersham Pharmacia, Buckinghamshire, UK), as previously described (Murta et al. 2006). After testing different electrophoresis conditions and switch times, the best condition that provided the optimal separation for *TcCyP19* gene was: 70 s for 15 h, 90 s for 24 h, 200 s for 15 h, and 400 s for 15 h at 180 V. After electrophoresis, the gels were transferred onto Hybond nylon membranes (Amersham) as described by the manufacturer's instructions. The membranes were hybridized with the $^{32}$P-labeled *TcCyP19* gene probe.

*2.6. Quantitative real-time RT-PCR*

The protocol employed for the preparation of first strand cDNA and the procedure for real-time RT-PCR were as previously described (Nogueira et al., 2006). An ABI Prism 7000 - Sequence Detection System SDS (PE Applied Biosystems, Foster City, CA, USA) was employed in the real-time PCR amplification of first strand cDNA (5 µl) using the specific primers: 5` TGTCGTACAAGCCGCATCAC 3` (RT CYPRTF forward) and 5` CAATGCTGACGTCGAAGAAGAC 3` (RT CYPRTR 2 reverse) selected from the complete nucleotide sequence of *TcCyP19* (GenBank Accession No. XM_816485). The *T. cruzi* housekeeping gene hypoxanthine-guanine phosphoribosyltransferase (TcHGPRT) was used to normalize the amount of samples (Nogueira et al., 2006). Standard curves were prepared for each experiment using known quantities of TOPO PCR 2.1 plasmids (Invitrogen) containing the *TcCyP19* and *TcHGPRT* genes. PCR products were quantified using Sequence Detection System data analysis software and normalized to the *TcHGPRT* values for each sample.

*2.7. Two-Dimensional Gel Electrophoresis (2-DE) and Western blotting analysis*

Protein extracts were obtained by direct lysis of parasites in lysis buffer (Matrangolo et al., 2013) in a proportion of 100 µl for $3.5 \times 10^8$ epimastigote forms. After extraction, protein concentration was quantified by the Bradford method. Protein extracts (100 µg) were loaded on 7 cm non-linear IPG strips pH 3–10 (Bio-Rad) and isoelectric focused using the Protean IEF Cell (Bio-Rad), 50 µA/strip at 20$^o$C. Passive rehydration was performed for 4 h, followed by an active rehydration at 50 V for 12 h. Isoelectric focusing was increased gradually to 4,000 V and run for 16,000 V-hour. Subsequently, the second dimension electrophoretic protein separation was performed

in 12% SDS-PAGE and blotted onto nitrocellulose membranes. Blots were incubated for 16 h at 4°C with rabbit polyclonal antibody anti-cyclophilin (TcCyP19) of *T. cruzi* (1:5,000) (kindly provided by Dr. Jacqueline Búa, Universidad de Buenos Aires, Argentina). The blots were washed and then incubated for 1 h with horseradish peroxidase-conjugated anti-rabbit IgG 1:2,000 (GE Healthcare). The blots were washed, incubated with ECL Plus chemiluminescent substrate (GE Healthcare) and exposed to X-ray film. To confirm equivalent loading, SDS-PAGE containing the same samples were stained with Colloidal Coomassie Blue G-250 (Neuhoff et al., 1988). The gels stained with Coomassie Blue G250 and the imumnoblots were scanned on a GS-800 calibrated densitometer (BioRad) and submitted to comparative analysis.

## 3. Results

### *3.1. In silico and phylogenetic analysis of the* TcCyP19 *gene*

Similarity searches were carried out between the *TcCyP19* sequence (GenBank accession XM_816485) and a local copy of the *T. cruzi* database of five different *T. cruzi* genomes. Altogether, 79 sequences similar to that of *TcCyP19* were found in the five *T. cruzi* genomes (13 to 19 sequences were identified from each genome). The predicted protein sequence of the gene sequences identified was analyzed and compared to the NCBI non-redundant database, and 61 were found to share the highest similarity with cyclophilins. Of these, 24 proteins (including *TcCyP19*) had molecular weights ranging from 19 to 23 kDa, which is similar to the molecular weight of *TcCyP19*. A phylogenetic tree was constructed using these 24 cyclophilin sequences (ranging from 18.7 to 23.7 kDa). A cyclophilin having a molecular weight of 35 kDa was used as the outgroup (Fig. 1S - supplementary data). The results showed that these cyclophilins

were grouped into two divergent branches. One of the branches included cyclophilins having molecular weights of 18.7 kDa (TcCyP19), 21.4–23.7 kDa, and 21.0–21.1 kDa, while the other encompassed cyclophilins having molecular weights of 21.6–21.7 and 19.7–19.8 kDa.

In order to compare the similarity of the TcCyP19 amino acid sequence with cyclophilin-A (CyPA) sequences of different organisms, a neighbor-joining phylogenetic tree was constructed (Fig. 1). This phylogenetic tree shows a clear dichotomous divergence between trypanosomatids and mammalian CyPA sequences. The amino acid sequence of *TcCyP19* is closely related to sequences from other species of *Trypanosoma* and *Leishmania*, sharing identities ranging between 84% and 90% and 78% and 81%, respectively.

### *3.2. Sequencing data*

DNA sequencing of the *TcCyP19* gene from *T. cruzi* populations that were susceptible (CL Brener, 17WTS, and BZS), naturally resistant (VL-10), and with in vitro-induced (17 LER) and in vivo-selected (BZS) resistance to BZ was performed in order to investigate whether point mutations could be associated with the BZ-resistance phenotype. Multiple-sequence alignment of the *TcCyP19* gene nucleotide sequences revealed three nucleotide mutations (positions 150, 261 and 390) that do not lead to amino acid substitutions (Fig. 2S - supplementary data). No association was found between nucleotide mutations and BZ-resistant phenotype. These three nucleotide mutations are strain-specific.

### *3.3. Copy number of the* TcCyP19 *gene*

Southern blot assays were carried out using samples of *T. cruzi* genomic DNA that had been digested with the endonuclease *Sac*I; which has one restriction site within the reference *TcCyP19* sequence (GenBank accession no. XM_816485). Hybridization of the blots of *Sac*I-digested DNA with a *TcCyP19*-specific probe revealed two fragments of 7.8 and 1.5 Kb in all *T. cruzi* samples analyzed (Fig. 3S - supplementary data).

The copy number ratio of the *TcCyP19* gene per genome for the pair 17WTS/17LER and BZS/BZR was also determined by real-time PCR. Considering that *T. cruzi* contains 0.33 pg of DNA (Moser et al., 1989) and that *TcHGPRT* is a single-copy gene (Allen and Ullman, 1994), *TcCyP19* copy numbers were estimated using 200, 100, 50, and 25 ng of genomic DNA. It was found that the copy number ratio of the *TcCyP19* gene was the same for 17WTS/17LER and BZS/BZR populations (data not shown), indicating that the copy number of the *TcCyP19* gene is not different in the genome of BZ-resistant *T. cruzi* populations.

### *3.4. Chromosomal location of the* TcCyP19 *gene*

Chromosomes from *T. cruzi* strains were separated by pulsed-field gel electrophoresis (PFGE) (Fig. 2A). Chromosome hybridization with the *TcCyP19*-specific probe showed that this gene is present in chromosomal bands that range from 680 to 2200 kb (Fig. 2B). A correlation between the chromosomal location of the *TcCyP19* gene and *T. cruzi* group was observed for the *T. cruzi* strains analyzed in this study (Table I). However, no correlation between chromosomal location of the *TcCyP19* gene and the drug-resistant phenotype was established.

*3.5. Levels of* TcCyP19 *mRNA expression*

Levels of *TcCyP19* mRNA in the parasite populations were first investigated by northern blot analysis. A 1.4-kb transcript was detected in northern blots of total RNA derived from BZ-susceptible and -resistant *T. cruzi* strains, following hybridization with a $^{32}$P-labelled *TcCyP19*-specific probe (Fig. 3A). Quantitative controls using a ribosomal RNA probe are shown in Fig. 3B. Comparative densitometric analysis revealed that *TcCyP19* mRNA levels were at least 2-fold higher in BZ-resistant 17LER and BZR populations than in their susceptible counterparts 17WTS and BZS. We did not observed any comparative difference in the levels of *TcCyP19* mRNA among other *T. cruzi* strains and clones analyzed.

*TcCyP19* mRNA levels were complementarily determined by quantitative real-time RT-PCR to confirm the northern blot results. The amount of *TcCyP19* cDNA in the samples of *T. cruzi* was normalized by the single-copy housekeeping gene *TcHGPRT*, used as an internal reference. The results, shown in Fig 3C, indicate that the levels of transcription of the *TcCyP19* gene were 2-fold higher in the 17LER and BZR populations compared to that in the 17WTS and BZS populations. No differences in the levels of transcription of the *TcCyP19* gene were detected between the other *T. cruzi* BZ-susceptible and -resistant sample pairs, i.e., CL Brener *versus* VL-10.

*3.6. Levels of TcCyP19 protein expression*

2-DE western blotting analysis showed that the anti-TcCyP19 polyclonal antibody recognized two spots with the expected size of 19 kDa and an isoelectric point (p*I*) of 7.0 to 8.5 in the pair 17LER/17WTS and three spots in the BZR/BZS pair (Fig. 4A), which coincides with that for TcCyP19. These spots, with p*I* values ranging from 7.0 to

8.5, correspond to the different isoforms of TcCyP19, probably owing to post-translational modifications of the protein (Fig. 4). These findings confirm the protein identity and are suggestive of post-translational modifications of TcCyP19. According to the relevant literature, cyclophilin-A of *L. infantum* has one site of acetylation, justifying the occurrence of isoforms with more acidic p*I* (Rosenzweig et al., 2008). In fact, the polyclonal antibody anti-TcCyP19 might recognize TcCyP19 and other cyclophilin isoforms of similar molecular weight but different isoelectric points.

Comparative analysis between Coomassie Blue-stained protein profiles of BZS/BZR and 17WTS/17LER *T. cruzi* populations showed that the protein load was similar between the samples tested (Fig. 4A and 4C). We selected the same region of both the Coomassie Blue-stained gels of 17WTS/17LER and BZS/BZR to perform the densitometric analysis. In this analysis, the protein expression of spots recognized by anti-TcCyP19 antibody was compared between the 17WTS/17LER and BZS/BZR pairs and normalized using the Coomassie Blue-stained protein profile. Densitometric analysis of the spots showed that the levels of protein expression of the two spots were higher in the 17LER BZ-resistant population than in its susceptible counterpart, i.e., 17 WTS. In the BZS/BZR populations, we identified three spots as cyclophilin TcCyP19. One of them had higher expression levels in the BZS population, while the other two were more highly expressed in the BZ-resistant population BZR than in the susceptible population 17WTS (Fig. 4).

In addition, we comparatively assessed the TcCyP19 protein expression of a BZ-susceptible *T. cruzi* strain (CL Brener) and that of a *T. cruzi* strain (VL-10) naturally resistant to BZ, using western blot one-dimensional gel electrophoresis (1D). No

differences were observed in the expression levels of TcCyP19 protein between both the *T. cruzi* strains analyzed (data not shown).

## 4. Discussion

Cyclophilin-A is a member of the peptidyl-prolyl *cis*/*trans* isomerase class of enzymes and it is a key molecule in diverse biological processes, including molecular chaperoning, protein folding, and protein trafficking (Bell et al., 2006). In the present study, the gene encoding *TcCyP19* was characterized from populations and strains of *T. cruzi* that are susceptible or resistant to benznidazole (BZ). The ORF of *TcCyP19* (TcCLB.506925.300) is 534 bp in length and encodes a protein of 177 amino acids, with a predicted mass of 19 kDa. Phylogenetic analyses of the amino acid sequences of CyPs revealed a clear dichotomy between trypanosomatid and mammalian cyclophilin sequences. CyP19 proteins from *Trypanosoma* spp. and *Leishmania* spp. are the closest related groups in this family. In addition, sequence analysis revealed that the amino acid sequences of the TcCyP19 protein are identical among the *T. cruzi* samples analyzed.

Southern blot analysis showed that the *TcCyP19* gene is present in the parasite genome as a single copy in BZ-resistant and -susceptible *T. cruzi* populations. These data are concordant with the results of a study by Potenza et al. (2006), who reported the presence of a single copy for each of the *TcCyP* genes analyzed in the *T. cruzi* genome (i.e., *TcCyP19, TcCyP20, TcCyp22, TcCyP25, TcCyP28, TcCyP34,* and *TcCyP40*). In the present study, the *TcCyP19* gene was located in chromosome bands varying in size from 0.68 to 2.2 Mb, depending on the strain of *T. cruzi*. In partial agreement, Búa and co-workers (2001) observed that the *TcCyP19* gene is located in two chromosomal bands of sizes 2.0 and 2.2 Mb in the CL Brener *T. cruzi* clone, whereas other

cyclophilin isoforms are found dispersed in the *T. cruzi* genome. These differences could be due to *T. cruzi* chromosomal rearrangements or cyclophilin-like sequences that could be recognized by the *TcCyP19* probe.

Northern blot and qPCR indicated that the levels of *TcCyP19* mRNA were two-fold higher in *T. cruzi* populations with in vitro-induced and in-vivo selected resistance to BZ compared to that in drug-susceptible counterparts. In accordance with these results, analytical 2DE immunoblot showed an increase in TcCyP19 protein expression level in the BZ-resistant *T. cruzi* populations. However, no differences in *TcCyP19* mRNA and protein expression levels were observed between the susceptible and naturally resistant *T. cruzi* strains analyzed, indicating different drug resistance mechanisms in these samples. Many studies evaluating drug resistance mechanisms in parasites were based on models produced by artificial induction of resistance. In contrast, there is very little information available on the biochemical mechanisms underlying drug resistance in field isolates. The data presented here show that TcCyP19 protein expression level is increased in *T. cruzi* populations that were in vitro-induced and in-vivo selected for resistance to BZ, a situation that is different from that observed in the naturally resistant population. In agreement with our results, Villareal et al. (2005) observed that the mechanisms associated with natural resistance to drugs differ from those associated with induced resistance. The mechanism of drug resistance, such as that to BZ, is often complex and multifactorial, including molecules associated with the host immune system, which may interfere with the susceptibility of the parasite to the drug (Murta et al., 1999). Studies to determine whether overexpression of TcCyP19 in the susceptible population will confer the BZ-resistant phenotype to these parasites

are required to confirm our hypothesis that TcCyP19 may be involved in *T. cruzi* resistance to BZ.

Interestingly, in the presence of BZ or other stress conditions, an increase in the expression of several proteins of distinct biological function is observed. Consequently, TcCyP19 might contribute to counteract the chemical stress stimulus by increasing, for example, the activity of protein folding, which is necessary for efficient expression of functional polypeptides. The correlation between drug resistance and increased chaperonin activity has been observed in *L. donovani* (Kumar et al., 2010) and *T. cruzi* (Andrade et al., 2008). Interestingly, CyPA also has a protective role in murine cells, functioning as an antioxidant against oxidative stress (Doyle et al., 1999; Hong et al., 2004). Additionally, in humans, CyPA binds peroxiredoxins, thereby increasing the peroxidase activity by their ability to transfer electrons (Lee et al., 2001). In analogy to this role, our data are indicative that the increased expression of TcCyP19 could in turn favor the expression and activity of enzymes associated with antioxidant defense (Nogueira et al., 2006, 2009, 2012). This might serve in detoxifying the parasite and might confer BZ-resistance — a hypothesis that warrants further investigation.

Cyclophilin-A is also implicated in biological processes such as tumor resilience and progression. For instance, microarray analysis has shown that CyPA can upregulate the expression of cytokines and genes related to drug resistance (Chen et al., 2008). Furthermore, it was observed that elevated CyPA expression contributes to the drug-resistance phenotype in cancer cells (Yang et al., 2011). Another study revealed that the overexpression of CyPA could promote cancer cell proliferation, cell migration/invasion, apoptosis inhibition, and drug-resistance phenotype in various cancer cell types (Obchoei et al., 2009). These data suggest that CyPA is a good target

for cancer chemotherapy. Interestingly, some studies suggest that TcCyPs are promising targets for the treatment of Chagas disease (Búa et al., 2008; Carraro et al., 2007). Cyclophilins bind cyclosporin A (CsA), an immunosuppressive antimicrobial drug, and non-immunosuppressive CsA analogues possess higher activity against *T. cruzi* (Búa et al., 2008). In conclusion, given that our results show that TcCyP19 is upregulated in BZ-resistant *T. cruzi* populations and that several lines of evidence implicate CyPs as a promising chemotherapeutic target against cancer and parasitic diseases, our work constitutes a starting point for further investigation into the role of TcCyPs in the mechanism of drug resistance and as a target for Chagas disease chemotherapy.

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# Legends to Figures - Rego et al.

**Fig. 1:** Neighbour-joining phylogenetic tree of CyPs sequences of *Trypanosoma cruzi* and other organisms with a high similarity degree. The numbers shown are bootstrap values. Bootstrap is a method that provides assessments of confidence for each clade of an observed tree, based on the proportion of bootstrap trees showing that same clade (Efron et al., 1996).

**Fig. 2:** Chromosomal location of the *TcCyP19* gene in BZ-resistant and susceptible *T. cruzi* strains. (A) Chromosomal bands from the *T. cruzi* strains were separated by PFGE and stained with ethidium bromide. (B) Southern blots of the chromosomes were hybridised with a $^{32}$P-labeled *TcCyP19*-specific probe. Whole chromosomes from *Saccharomyces cerevisae* were used as molecular weight markers

**Fig. 3:** Levels of *TcCyP19* mRNA in BZ-resistant and susceptible *T. cruzi* strains. (A) Northern blot profile of total RNA extracts from the *T. cruzi* strains obtained using a $^{32}$P-labeled *TcCyP19* -specific probe. (B) The quantitative control was used agarose gel containing the RNA, stained with ethidium bromide. (C) Number of cDNA molecules (copy number ratio) of *TcCyP19* (x $10^6$). Values were normalized to those obtained for the *TcHGPRT* and are presented as the means (± S.D.M.) of triplicate real-time RT-PCR analyses from three independent experiments.

**Fig. 4:** 2-DE gels of proteins and Western blot analysis of TcCyP19 expression in the benznidazole-susceptible and -resistant *T. cruzi* populations. Proteins (100 µg) were loaded on 7 cm, non-linear IPG strips of pH 3–10, submitted to isoelectric focusing and separated on 12% SDS-PAGE. The gels were stained with Colloidal Coomassie Blue G250 (A and C) or blotted onto nitrocellulose membranes (B and D). The blots were probed with a rabbit polyclonal antibody anti-cyclophilin-A of *T. cruzi* (1:5,000) and developed using ECL.

**Figure 1**

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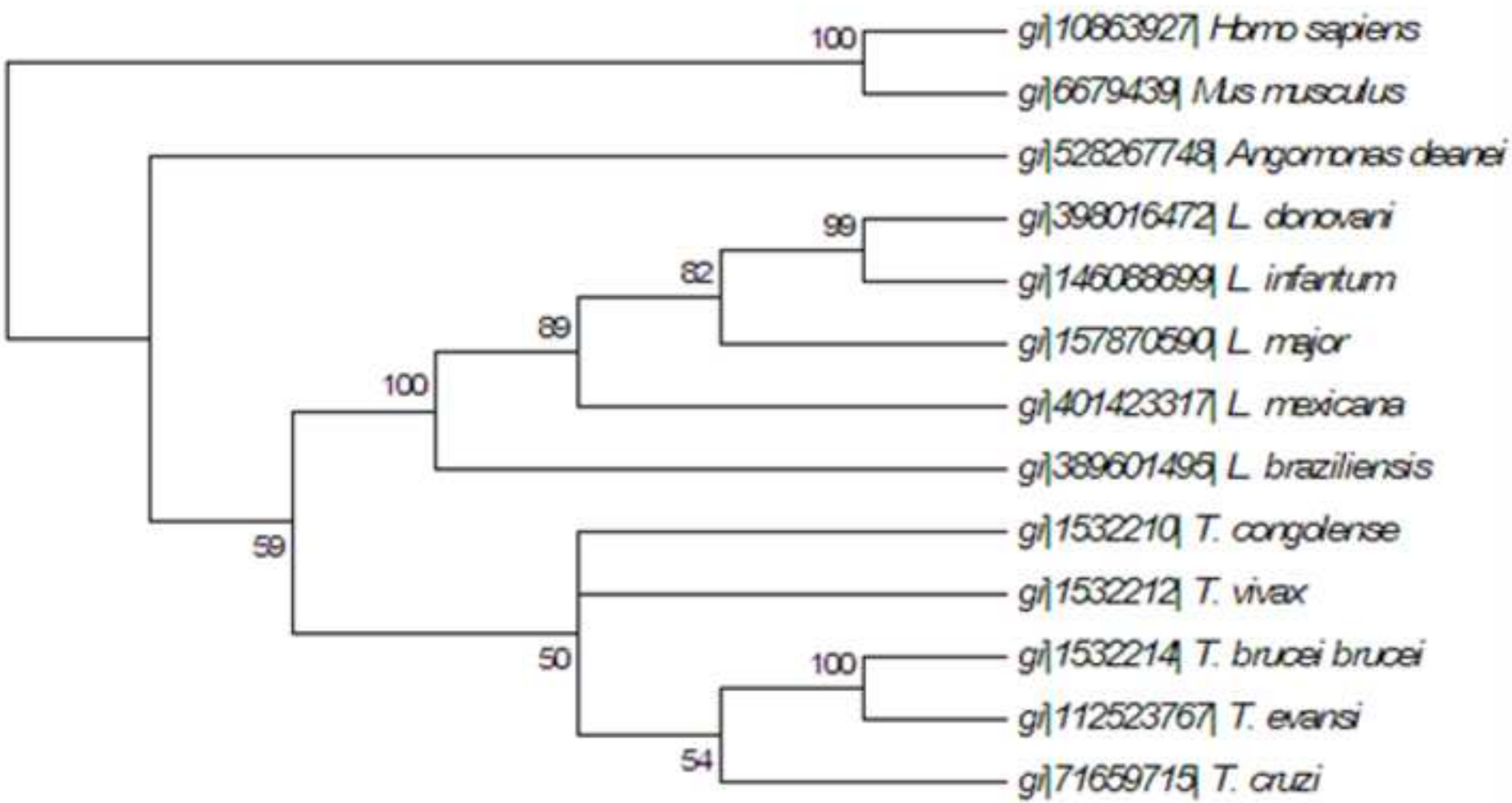

**Figure 2**

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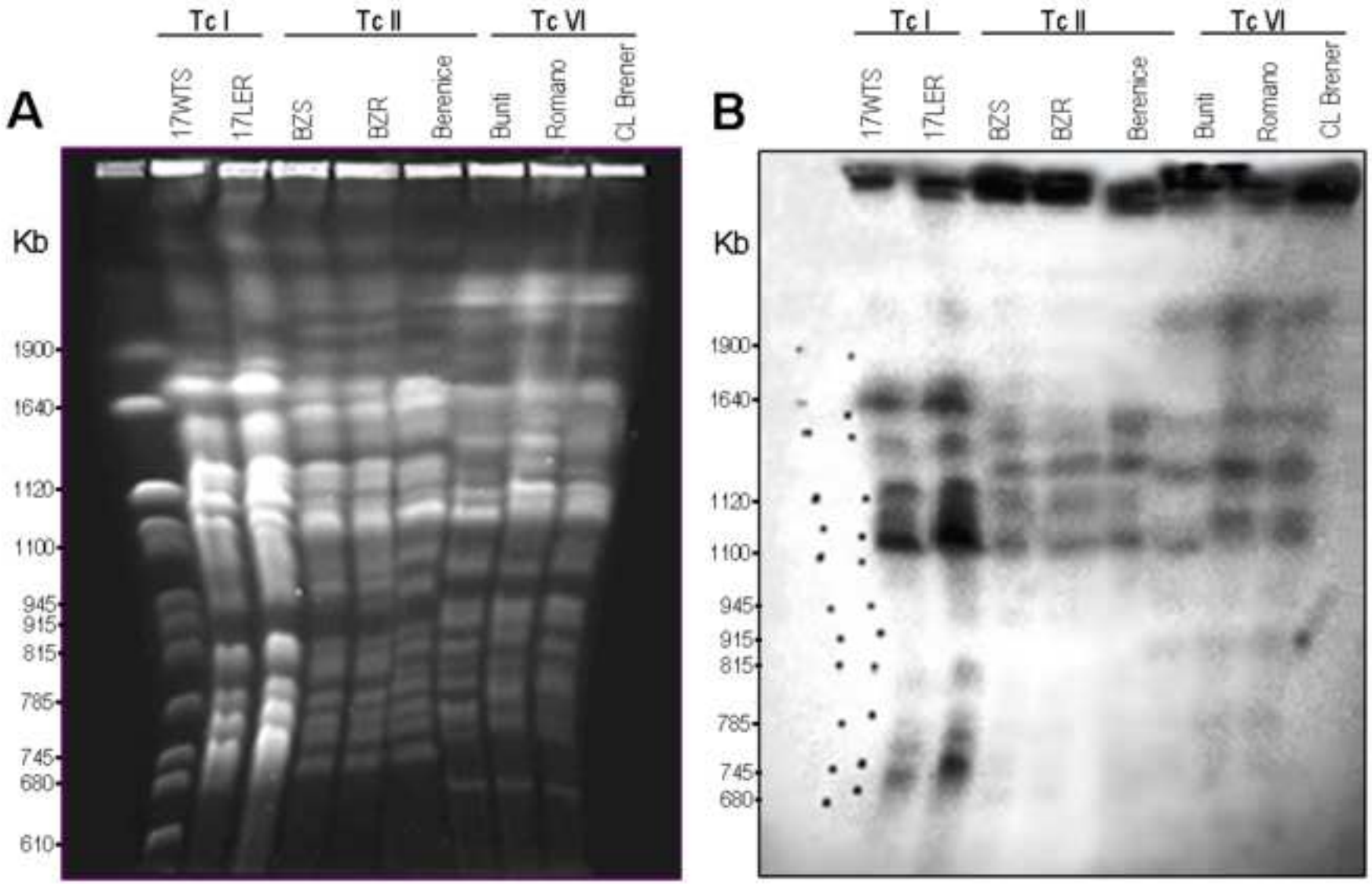

Figure 3

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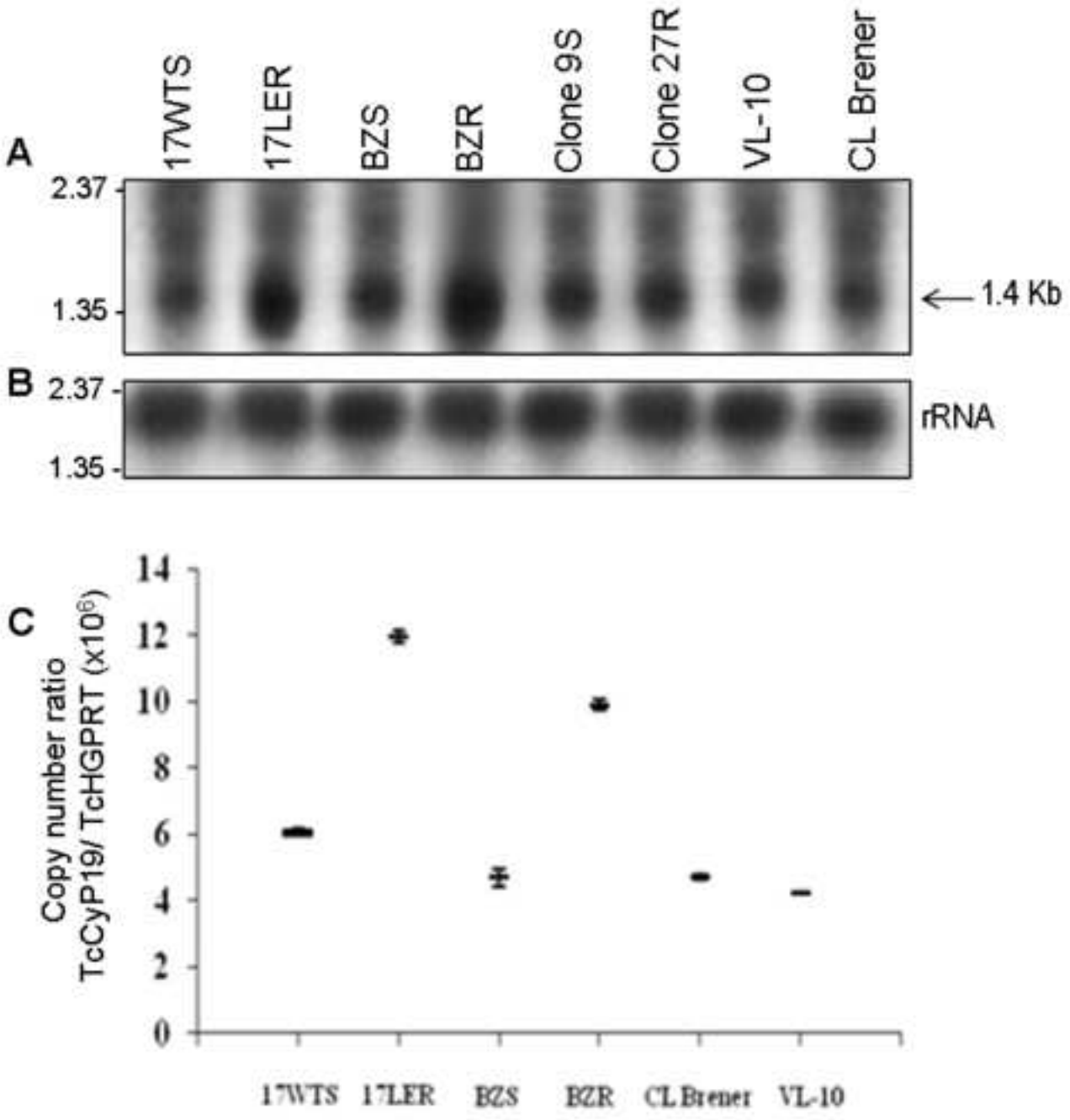

Figure 4

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