Pre-Columbian Chagas disease in Brazil: *Trypanosoma cruzi* I in the archaeological remains of a human in Peruaçu Valley, Minas Gerais, Brazil

**Alexandre Fernandes, Alena M Iñiguez, Valdirene S Lima[1]/[2], Sheila MF Mendonça de Souza[2],
Luiz Fernando Ferreira[3], Ana Carolina P Vicente/[+], Ana M Jansen[1]**

Laboratório de Genética Molecular de Microorganismos [1]Laboratório de Biologia de Tripanosomatídeos, Instituto Oswaldo Cruz-Fiocruz, Av. Brasil 4365, 21045-900 Rio de Janeiro, RJ, Brasil [3]Laboratório de Paleoparasitologia [2]Departamento de Endemias Samuel Pessoa, Escola Nacional de Saúde Pública Sergio Arouca-Fiocruz, Rio de Janeiro, RJ, Brasil

We evaluated the presence and distribution of Trypanosoma cruzi *DNA in a mummy presenting with megacolon that was dated as approximately 560 ± 40 years old. The mummy was from the Peruaçu Valley in the state of Minas Gerais, Brazil. All samples were positive for* T. cruzi *minicircle DNA, demonstrating the presence and broad dissemination of the parasite in this body. From one sample, a mini-exon gene fragment was recovered and characterized by sequencing and was found to belong to the* T. cruzi *I genotype. This finding suggests that* T. cruzi *I infected humans during the pre-Columbian times and that, in addition to* T. cruzi *infection, Chagas disease in Brazil most likely preceded European colonization.*

Key words: Brazilian mummy - Chagas disease - ancient DNA - *Trypanosoma cruzi* I - megacolon

Trypanosoma cruzi, which infects a range of mammalian and triatomine hosts, occurs in a large extent of the American continent. American trypanosomiasis is primarily considered a wild enzootic disease. The heterogeneity of this taxon, the competency in infecting almost all tissues of its several mammalian host species and its wide distribution are most likely the outcomes of the long and independent evolutionary process of its multiple clones (Tibayrenc & Ayala 1988, Tibayrenc 2003). The heterogeneity has been a factor since the very early studies that were based on the morphology of the parasite (Chagas 1909, Brumpt 1912), where these are now being studied using biochemical and molecular tools (Tibayrenc & Ayala 1988, Tibayrenc 2003). It has not yet been possible to establish correlation between parasite heterogeneity and the resulting epidemiology, clinical features of human infection or disease (Macedo et al. 2004).

The classical hypothesis proposes that humans, probably the more recent host of *T. cruzi,* were included in its transmission cycle as a consequence of agricultural implementation and animal domestication, resulting in sedentary habits. In fact, grain storages may have attracted wild grain feeding mammals and, indirectly triatomines, establishing a transmission cycle near human shelters. Findings of mega-syndromes and histological alterations in soft tissues were shown in North American, Chilean and Peruvian mummies and, in addition, the recovery of *T. cruzi* ancient DNA (aDNA) has revealed that the human infection as well as Chagas disease preceded the colonial period in these countries (Rothhammer et al. 1985,

Fornaciari et al. 1992, Guhl et al. 1999, Ferreira et al. 2000, Reinhard et al. 2003, Aufderheide et al. 2004).

In Brazil, recent work has shown the presence of the *T. cruzi* I genotype in human remains (7000-4500 bp) from the Abrigo do Malhador archaeological site the Peruaçu Valley (PV) in the state of Minas Gerais (MG) (Lima et al. 2008), demonstrating, for the first time, the antiquity of the infection in lowland South America.

We tested the remnants of an adult male individual recovered from PV, dated 560 ± 40 years old, using the Carbon 14 method. In this individual, named Lapa do Boquete IV (LBIV), a structure that displayed the characteristics of a fecaloma was observed (Figs 1, 2). This was potentially caused by megacolon, a clinical symptom of Chagas disease. The archaeological site of Lapa do Boquete is a rock shelter used as a burial place by prehistoric people. This site is placed in a cold and dry region that favors biological material preservation (Prous & Schlobach 1997). We therefore evaluated the presence and distribution of the *T. cruzi* infection in distinct tissue samples, as well as typing the parasite by characterizing the mini-exon gene non-transcribed spacer region. Two samples of bone (rib and metacarpus) and five samples of soft tissue from different places of body were analyzed, including tissue in contact with coprolite.

The manipulation and decontamination of samples were performed as recommended for aDNA work (Hofreiter et al. 2001). The aDNA extraction was done according to the Geneclean® Kit for Ancient DNA protocol (Bio101). Two regions were targeted for the detection of *T. cruzi* DNA: the minicircle conserved region of kDNA for diagnostics and the mini-exon non-transcribed spacer region for genotyping. The primers for the kDNA were S67 (5'-TGGTTTTGGGAGGGG(G/C)(G/C)(G/T)TCAA(A/C)TTT) and S34 (5'TATATTACACCAAC-CCCAATCGAACC) (Sturm et al. 1989), generating

the 121 bp fragment from the conserved minicircle regions and (5'-CCCCCCTCCCAGGCCACACTG), TC1 (5'-GTGTCCGCCACCTCCTTCGGGCC) and TC2 (5'-CCTGCAGGCACACGTGTGTGTG) were the primers used for the mini-exon, amplifying 350 and 300 bp fragments for *T. cruzi* I and *T. cruzi* II genotypes, respectively (Souto et al. 1996). As a control for aDNA quality and to determine the mtDNA haplogroup of LBIV, the HVS-I region (185 bp) was amplified using primers L16209 (5'-CCATGCTTACAAGCAAGT) and H16356 (5'-GTCATCCATGGGGACGAGAA) (Handt et al. 1996). The mtDNA haplogroup of laboratory staff was also determined in order to verify possible DNA contamination. Each PCR reaction for human and *T. cruzi* was performed in a final volume of 50 µL (2.5 mM MgCl$_2$, 0.4 mM dNTPs, 200 ng of each primer, 2.5 U of Taq DNA polymerase (Platinum® - Invitrogen) and 5 µL of aDNA. The PCR cycles were 94ºC, 55ºC and 72ºC, for 20 s, each encompassing 40 amplification cycles. aDNA PCR products were visualized on a 8% polyacrylamide gel. Mini-exon amplicons of the *T. cruzi* I (DM28 strain) and *T. cruzi* II (Y strain) genotypes were used as molecular type markers in a 3% agarose gel electrophoresis.

Human and *T. cruzi* amplicons were cloned into the pGEM-T Easy Vector System (Promega). The clones obtained were sequenced on both strands using an ABI PRISM Big Dye Terminator Cycle Sequencing Ready Reaction Kit (Applied Biosystems-Perkin Elmer) in ABI PRISM 3730 Automated DNA Sequencer (Applied Biosystems Perkin Elmer). PCR products were also directly sequenced as described above. The CHROMAS v1.45 (School of Health Science, Griffith University, Queensland, Australia) and BioEdit v5.0.9 (Department of Microbiology, North Carolina State University) programs were used for the sequence analysis.

An aliquot of 10 µL of total aDNA extraction of each LBIV sample was denatured and fixed onto nylon membranes (Hybond-XL Amersham Pharmacia Biotech) according to protocol. The membrane was hybridized with an α-^{32}P-dCTP probe using a RadPrime DNA Labeling System kit (Life Technologies) following manufacturer's specifications. The probe consisted of a *T. cruzi* DNA pool including kinetoplast total DNA and mini-exon amplicons of the *T. cruzi* I (DM28 strain) and *T. cruzi* II (Y strain) genotypes. Hybridization was carried out in SSC 6X, SDS 0.5%, Denhardt 5X and 50% formamide at 50ºC overnight. The membrane was washed at 65ºC in SSC 0.1X and SDS 0.1% and exposed to X-ray film (Kodak® Biomax MS Film) with an intensifying screen.

We successfully recovered human mtDNA HVS-I region from all seven samples and the sequence analysis based on Brazilian HVS-I database (Alves-Silva et al. 2000) of four clones revealed the presence of a unique haplotype, exhibiting the haplogroup A signatures. The sequence was deposited in GenBank (accession number EU567083). Segments from the minicircle conserved regions were amplified in all seven samples, demonstrating infection and parasite dissemination in the ancient human body. One minicircle sequence was deposited in GenBank (accession number EU605980). We recovered the 350 bp mini-exon target corresponding to the *T. cruzi* I
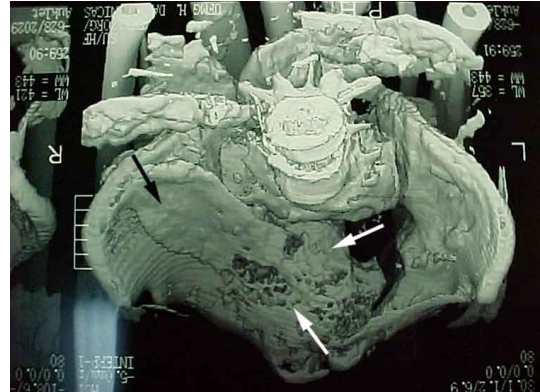

Fig. 1: tomographic image from LBIV pelvis showing in situ a big coprolite (arrows) indicative of megacolon.

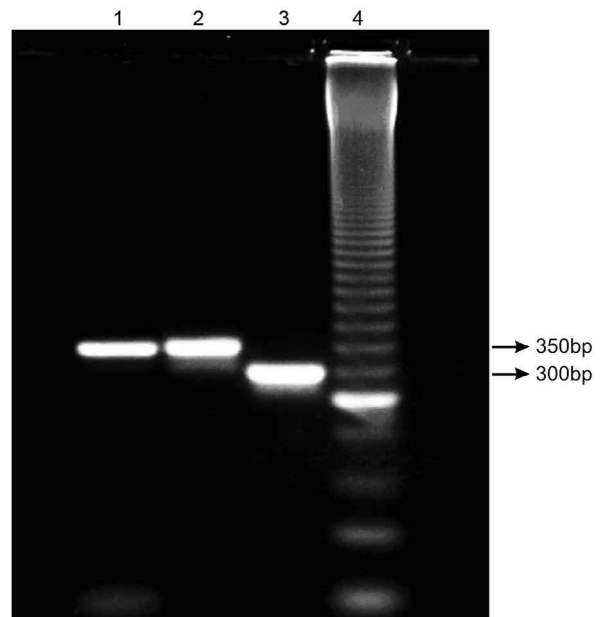Fig. 2: coprolite in situ (arrow) view inside the intact pelvis behind the articulate sacrum.

Fig. 3: PCR amplification of the mini-exon gene non-transcribed spacer region visualized on 3% agarose gel electrophoresis; Lane 1: aDNA from LBIV soft tissue sample; Lane 2: *T. cruzi* I genotype; Lane 3: *T. cruzi* II genotype; Lane 4: 50 bp DNA ladder (Invitrogen); Lane 5: PCR negative control.

genotype from a soft tissue sample that was in contact with coprolite (Fig. 3). The sequencing of this amplicon confirmed its identity (accession number EU570218). The LBIV mini-exon sequence showed 99% similarity with Cutia strain belonging to the *T. cruzi* I genotype. The quality and relative amount of aDNA recovery from the LBIV samples were assessed by hybridization assay. This confirmed the presence of *T. cruzi* aDNA in two LBIV samples, soft tissue and bone, validating the molecular data.

T. cruzi I is recognized as the agent of Chagas disease from the Amazon basin northwards (Aguilar et al. 2007). We report the presence of *T. cruzi* I in ancient human remains found in the central part of Brazil, outside the Amazonian region and from the Brazilian pre-Columbian period. This individual belongs to mtDNA haplogroup A, one of the founder human haplogroups in the Americas (Horai et al. 1993, Fagundes et al. 2008). The body displayed megacolon syndrome, a typical outcome of the chronic phase of Chagas disease, showing the occurrence of Chagas disease in the Brazilian prehistoric period. Previous work in the same archaeological area of the PV also revealed the presence of infection by the *T. cruzi* I genotype in a skeleton dating back to 7000-4500 bp (Lima et al. 2008). The identification of *T. cruzi* infection in ancient human remains from South American countries has been largely demonstrated by aDNA analysis. No megavisceral lesions, however, have yet to be associated with the recovery of *T. cruzi* sequences.

Interestingly, we have found *T. cruzi* infection by genotype I in an individual with the presence of megacolon, a syndrome that is not associated to this sort of manifestation (Macedo et al. 2004). Moreover, our study is the first to link the pre-Columbian *T. cruzi* with Chagas disease in Brazil. The presence of *T. cruzi* in all tested tissues of this mummy shows that this individual displayed a relevant and broadly dispersed parasitic load.

The possibility of detecting *T. cruzi* aDNA from both soft and hard tissues, in addition to the success in identifying the main genotypes of *T. cruzi* in ancient mammalian tissues, offers an exceptional tool to unravel several questions concerning the natural history of *T. cruzi* that still remain unanswered.

REFERENCES

Aguilar HM, Abad-Franch F, Dias JC, Junqueira AC, Coura JR 2007. Chagas disease in the Amazon Basin. *Mem Inst Oswaldo Cruz 102* (Suppl. I): 47-56.

Alves-Silva J, da Silva Santos M, Guimarães PE, Ferreira AC, Bandelt HJ, Pena SD, Prado VF 2000. The ancestry of Brazilian mtDNA lineages. *Am J Hum Genet 67*: 444-461.

Aufderheide AC, Streitz W, Madden M, Streitz J, Buikstra J, Guhl F, Arriaza B, Renier C, Wittmers LE Jr, Fornaciari G, Allison M 2004. A 9,000-year record of Chagas' disease. *Proc Natl Acad Sci USA 101*: 2034-2039.

Brumpt E 1912. Le *Trypanosoma cruzi* évolue chez *Conorhinus megistus, Cimex lectularius* et *Ornithodorus moubata*. Cycle evolutif de ce parasite. *Bull Soc Pathol Exot Filiales 5*: 360-367.

Chagas C 1909. Nova tripanozomiaze humana. Estudos sobre a morfologia e o ciclo evolutivo do *Schizotrypanum cruzi*, n. gen., n. sp. Ajente etiolójico de nova entidade mórbida do homem. *Mem Inst Oswaldo Cruz 1*: 159-118.

Fagundes NJ, Kanitz R, Eckert R, Valls AC, Bogo MR, Salzano FM, Smith DG, Silva WA Jr, Zago MA, Ribeiro-dos-Santos AK, Santos SE, Petzl-Erler ML, Bonatto SL 2008. Mitocondrial population genomics supports a single pre-Clovis origin with coastal route for the people of the Americas. *Am J Genet 82*: 583-592.

Ferreira LF, Britto C, Cardoso MA, Fernandes O, Reinhard K, Araújo A 2000. Paleoparasitology of Chagas disease revaled by infected tissues from Chilean mummies. *Acta Trop 75*: 79-84.

Fornaciari G, Castagna M, Viacava P, Togneti A, Bevilaqua G, Segura EL 1992. Chagas' disease in Peruvian Inca mummy. *Lancet 339*: 128-129.

Guhl F, Jaramillo C, Vallejo GA, Yockteng R, Cardenas-Arroyo F, Fornaciari G, Arriaza B, Aufderheide AC 1999. Isolation of *Trypanosoma cruzi* DNA in 4,000-year-old mummified human tissue from northern Chile. *Am J Phys Anthropol 108*: 401-407.

Handt O, Krings M, Ward RH, Pääbo S 1996. The retrieval of ancient human DNA sequences. *Am J Hum Genet 59*: 368-376.

Horai S, Kondo R, Nakagawa-Hattori Y, Hayashi S, Sonoda S, Tajima K 1993. Peopling of the Americas, founded by four major lineages of mitochondrial DNA. *Mol Biol Evol 10*: 23-47.

Hofreiter M, Serre D, Poinar HN, Kuch M, Pääbo S 2001. Ancient DNA. *Nat Rev Genet 2*: 353-359.

Lima V, Iñiguez A, Otsuki K, Ferreira LF, Araújo A, Vicente A, Jansen A 2008. Chagas disease by *Trypanosoma cruzi* lineage I in hunter-gatherer ancient population in Brazil. *Emerg Infect Dis 14*: 1001-1002.

Macedo AM, Machado CR, Oliveira RP, Pena SD 2004. *Trypanosoma cruzi*: genetic structure of populations and relevance of genetic variability to the pathogenesis of Chagas disease. *Mem Inst Oswaldo Cruz 99*: 1-12.

Prous A, Schlobach MC 1997. Sepultamentos Pré-históricos do Vale do Peruaçu. *Rev Mus Arq Etnol São Paulo 7*: 3-21.

Reinhard A, Fink TM, Skiles J 2003. A case of megacolon in Rio Grande valley as a possible case of Chagas disease. *Mem Inst Oswaldo Cruz 98* (Suppl. I): 165-172.

Rothhammer F, Allison M, Nunez L, Standen V, Arriaza B 1985. Chagas' disease in pre-Columbian South America. *Am J Phys Anthropol 68*: 495-498.

Souto RP, Fernandes O, Macedo AM, Campbell DA, Zingales B, Souto RP 1996. DNA markers define two major phylogenetic lineages of *Trypanosoma cruzi*. *Mol Biochem Parasitol 83*: 141-152.

Sturm NR, Degrave W, Morel C, Simpson L 1989. Sensitive detection and schizodeme classification of *Trypanosoma cruzi* cells by amplification of kinetoplast minicircle DNA sequences: use in diagnosis of Chagas' disease. *Mol Biochem Parasitol 33*: 205-214.

Tibayrenc M 2003. Genetic subdivisions within *Trypanosoma cruzi* (Discrete Typing Units) and their relevance for molecular epidemiology and experimental evolution. *Kinetoplastid Biol Dis 2*: 12.

Tibayrenc M, Ayala FJ 1988. Isozyme variability in *Trypanosoma cruzi*, the agent of Chagas disease: Genetic, taxonomical, and epidemiological significance. *Evolution 42*: 277-292.