Should Trypanosoma cruzi be Called “cruzi” Complex?
A Review of the Parasite Diversity and the Potential of Selecting Population after in Vitro Culturing and Mice Infection

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**Morpho-biological diversity of** Trypanosoma cruzi **has been known since Chagas’ first works in 1909.** Several further studies confirmed the morphological differences among the parasite strains, which were isolated from different reservoirs and vectors, as well as from human beings. In the early sixties, antigenic differences were found in the parasite strains from various sources. These differences, coupled to the observation of regional variations of the disease, led to the proposal of the term cruzi complex to designate the taxon T. cruzi. Since then this protozoan has been typed in distinct biodesmes, zymodesmes and lineages which were consensually grouped into T. cruzi I, T. cruzi II and into non-grouped strains. T. cruzi genotypic characterization, initially carried out by schizodeme analysis and more recently by various other techniques, has shown a great diversity of the parasite strains. In fact, T. cruzi is formed by groups of heterogeneous sub-population, which present specific characteristics, including distinct histotropism. The interaction of the different infecting clones of the cruzi complex and the human host will determine the morbidity of the disease.

Key words: Trypanosoma cruzi - “cruzi” complex - genetic diversity

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Trypanosoma cruzi is a protozoan parasite presenting two alternating host during its life cycle: insect vectors of the Family Triatominae and vertebrates, including a wide variety of mammals. They are hemoflagellates of the Order Kinetoplastida harboring a differentiated mitochondria, the kinetoplast, corresponding to a DNA condensation located inside the single and branched organelle. (Hoare 1964).

The taxon is not composed by a homogeneous species. In the sixties, when the present molecular tools were not available, Coura et al. (1966) defended the use of the term “cruzi” complex to designate the protozoan, based on morphological variation, immunological features, distinct virulence and differences in the regional and individual pattern of Chagas disease (COURA 1966). Nowadays it is known that T. cruzi is an heterogeneous species consisting of several sub-populations of the parasite circulating among various wild and domestic vertebrates and invertebrate hosts (Morel et al. 1986, Zingales et al. 1998). Intra-specific variations among T. cruzi have been observed based on (i) the biological behavior of the strains in laboratory animals, (ii) biochemical features of the isolates, and (iii) molecular characteristics of the stocks (Bice & Zeledon 1970, Petana & Coura 1974, Miles et al. 1977, Melo & Brener 1978, Morel et al. 1980, Andrade 1985, Araujo & Chiari 1988, Carneiro et al. 1991, Macedo & Pena 1998). In order to study the role of the parasite diversity in the pathogenesis of Chagas disease, a comprehensive characterization of the populations found in nature is crucial.

As T. cruzi is composed of heterogeneous populations, the same host may be simultaneously infected by different strains. In this scenario, a major concept is the clear existence of clones that are more representative, being found in several hosts in different geographic areas of Latin America (Tybarenc & Brenière 1988, Lauria-Pires et al. 1996, 1977, Lauria-Pires & Teixeira 1996, Andrade & Campos 1998, Andrade 1999). Nowadays it is known that T. cruzi has a clonal population structure (Tibayrenc et al. 1986a, Tibayrenc & Ayala 1988, 1991). This explains the existence of independent organisms with discrete biological features explaining the finding of a great number of clones in different geographic areas (Tibayrenc & Brenière 1988).

Recent studies using molecular tools demonstrate that these major clones from endemic areas might be responsible for some clinical manifestations and chemotherapy response (Campos & Andrade 1996, Andrade & Campos 1998, Andrade 1999).

A current hypothesis suggests that the (i) heterogeneity and (ii) multicolonality of a strain determine differential tissue tropism and, consequently, variations in the clinical presentation of the disease (Andrade 1999). A correlation between the genetic diversity of T. cruzi and its pathogenicity was recently proposed as the clonal histotrophic model that is based on the two aforementioned features (Macedo et al. 1992, Oliveira et al. 1998, 1999, Macedo & Pena 1998).

In order to characterize a parasite strain using the current techniques, it is necessary to isolate T. cruzi through laboratory animal inoculation, xenodiagnosis and/or in vitro
culture. These approaches can lead to the selection of a specific sub-population of the parasite that was present in the original inoculum, differing from those in the host's blood or tissue. Furthermore, due to the differential tropism of the various strains, the circulating clones and therefore available for haemoculture can be different from those that are causing the tissue lesion, supporting the clonal histotropic model of Chagas disease (Macedo & Pena 1998). Some experimental evidence strengthens this proposal (Andrade 1999) and it has been shown that clones in the cardiac tissue of some patients proved to be different from those in the esophagus. This means that different genotypes of T. cruzi present a favorite distribution in tissues of chronic chagasic patients, suggesting that the genetic variability of the parasite is one of the leading factors to the clinic form of the disease (Vago et al. 2000).

**WHY SHOULD WE CALL T. CRUZI AS T. CRUZI I AND T. CRUZI II?**

The genetic diversity of T. cruzi has been revealed by enzymatic markers (Miles et al. 1978, Miles 1983, Tibayrenc & Ayala 1988), restriction fragment length polymorphism (RFLP) of the kinetoplast DNA (Morel et al. 1980), molecular karyotypes (Henrikson et al. 1993), DNA fingerprints (Macedo et al. 1992) and by randomly amplified polymorphic DNA analysis (RAPD) (Stein et al. 1993, Tibayrenc et al. 1993). Alternatively, when markers derived from constitutive genes are used, such as the 24S α rRNA and the mini-exon genes, a different result is revealed. Based on these two targets, it has been observed that T. cruzi can be divided into two major well-defined groups that coincides with the isoenzymatic dichotomy proposed in the late seventies and corroborated in the nineties (Miles et al. 1977, 1980, Tibayrenc et al. 1993, Souto et al. 1996, Fernandes et al. 1998).

In the light of this dichotomy, T. cruzi strains are now classified into two major groups named T. cruzi I and T. cruzi II, denomination arisen from a consensus reached by specialists, who proposed the unification of the various classifications based on different markers (Anonymous 1999).

T. cruzi I is mainly observed in wild mammals and sylvan triatomines, whereas T. cruzi II is usually found in humans (Fernandes et al. 1998, Zingales et al. 1998). Fernandes et al. (1999a) revealed the presence of both groups in the sylvatic cycle of the parasite and the preferential association of T. cruzi major phylogenetic lineages with different hosts, illustrating the complexity of the parasite cycle in nature. These findings have helped to understand how the sylvatic and domestic cycles are connected and therefore, this new classification of T. cruzi has brought important contributions to the definition of the eco-epidemiology of Chagas disease.

**BIOLOGICAL DIVERSITY AND BIODEMIES**

The proper characterization of a certain T. cruzi isolate is essential to determine the putative role that the different strains may play in the pathogenesis, geographic variation, clinical presentation and morbidity of Chagas disease and also in the distinct rates of cure after specific chemotherapy (WHO 1991).

Biological behavior in laboratory animals, mostly from the Family Muridae, is one of the parameters used to characterize the different strains of T. cruzi. Although experiments conducted on T. cruzi infection in animal models do not faithfully reproduce the human infection, mouse has been widely used to study various aspects of the infection. The common use of this animal is justified by (i) the small size, (ii) large availability, (iii) easy maintenance, and (iv) simple manipulation. The mouse lineage also plays an important role and its choice depends on the type of the investigation being carried out. Different susceptibilities and survival rates are dependent of the mouse lineage and the used strain of the parasite (Andrade 2000, Araújo-Jorge 2000).

The vast majority of the biological characterizations of T. cruzi strains have been conducted on non-isogenic Swiss mice (Silva & Nussenzweig 1953, Andrade et al. 1970, 1985, Mello & Brener 1978, Andrade 1990). Swiss mouse belongs to an heterogeneous group and therefore should be considered the best representative of the characteristics of the human population from endemic areas. Nevertheless, other lineages have been used for characterization studies (Bice & Zeledón 1970, Andrade et al. 1985, Araújo & Chiari 1988, Oliveira et al. 1993). More recently, the use of isogenic lineages has been emphasized in order to obtain standard infections (Andrade 2000). However, those lineages are more used for immunopathological studies.

The biological behavior of T. cruzi strains is based on presented virulence and pathogenicity; thus, it is essential to define these concepts. Virulence is the capacity of the parasite to multiply inside a laboratory host. Pathogenesis, on the other hand, refers to a more intrinsic and constant characteristic of the parasite and is related to the ability of producing tissue lesions and mortality (Andrade 1985).

Many studies of biological characterization have demonstrated that T. cruzi strains originating from humans, reservoirs and vectors from distinct geographic regions show different behavior in laboratory animals (parasitaemia, tissue tropism and mortality rates) (Badinez 1945, Brand et al. 1949, Talaiáferro & Pizzi 1955, Deane et al. 1963, Andrade & Magalhães 1997). This phenomenon is influenced by environmental and immunological factors, virulence, pathogenicity and possible selection of strains and clones after interacting with vectors and vertebrate hosts. The combination of several of these factors might explain the variability in the biological behavior of the parasite (Andrade et al. 1970, Bice & Zeledón 1970, Magalhães et al. 1996).

Vianna (1911) showed that T. cruzi could be found in distinct tissues of the vertebrate host, although the parasite prefers certain cellular groups, according to Baldínez's theories in 1945. Since then, other authors demonstrated that the strain tropism is an important biological characteristic to be considered in laboratory animals (Andrade et al. 1970, Bice & Zeledón 1970, Andrade 1974, Hanson & Roberson 1974, Melo & Brener 1978).

Some strains prefer muscle tissue (skeletal or cardiac) and are called miotropic, others are called reticulotropic strains because they prefer the phagocytic mononuclear

The first attempt to group *T. cruzi* strains was based on morpho-biological criteria. Chagas (1909) observed the presence of circulating parasites of wide and slim forms. Brener and Chiari (1963) and Brener (1965) confirmed this finding demonstrating that these forms from distinct stocks, circulate in mice with different percentages. The slim forms are predominant in strains of extreme virulence and tropism to macrophages (Brener 1965, Andrade 1974). The wide forms are more common in mitropic strains and low virulence stocks. In addition, the slim forms are more susceptible to the host immune system (Brener & Chiari 1963, Brener 1969). Brener (1977) suggested the classification of the stocks in two polar types based on morphology and on tissue tropism, describing an aggressive pole represented by the Y strains and a benign pole exemplified by the CL strain. It is important to emphasize that these are “elements for characterization” and are not, by themselves, sufficient to classify the *T. cruzi* strains.

The main problem regarding the biological characterization is the comparison of the strains that can be extremely difficult if the analysis is made stock by stock, due to their great variety. Taking into account this diversity and using biological parameters of the mouse-parasite interaction, such as parasitaemia, morphology, tissue tropism and histopathologic lesions, three groups of *T. cruzi* strains were proposed and named biomes (Andrade 1974).

Biodeme I, which prototypes are the Y and the Peruvian strains, consists of a fast in vitro growth, high parasitaemia and mice mortality, which occurs between the 7th and 12th day post-infection. They present mostly slim trypomastigote forms and present tropism to macrophages in the initial phase of the infection. The São Felipe strain is the prototype of biodeme II that presents a slow multiplication with peaks of irregular parasitaemia between the 12th and 20th day post-infection, period of the highest mortality. In the initial phase of the infection, wide forms with a low percentage of slim forms predominate, as well as mitotropic, damaging the heart muscle. Biodeme III, which prototype is the Colombian strain, multiplies slowly with peaks of late parasitaemia between the 20th and 30th day after the inoculum and low mortality rate – around the 50th day of infection. Wide forms are common, damaging specially the skeletal muscle. Furthermore, these biological types correspond to specific patterns of zymodemes (Andrade et al. 1983, Andrade & Magalhães 1997).

**BIOCHEMICAL CHARACTERIZATION**

**Enzyme electrophoresis**

Enzyme electrophoresis uses raw and soluble extracts of an organism to evaluate the particular activity of a protein after electrophoresis and incubation with an appropriate substrate, revealing the product of the activity through a color reaction. Under controlled conditions, the differences in the isoenzymatic mobility imply in genetic differences (Miles 1985, Miles & Cibulskis 1986). The results are analyzed through simple visual inspection or through mathematical procedures, based on rates of similarity or genetic distances (Ready & Miles 1980, Miles 1985, Miles & Cibulskis 1986).

Enzyme electrophoresis profiles (alanin aminotransferase, ALAT, and aspartate aminotransferase, ASAT) were introduced for the study of trypanosomes simultaneously by Kilgour and Godfrey (1973) and Bagster and Parr (1973), who managed to classify the African trypanosomes into species and sub-species.

For New World trypanosomes, Toyé (1974) was the first to use isoenzymes, observing differences between *T. cruzi* samples. Miles et al. (1977, 1978, 1980, 1981a, b) performed several studies on isoenzyme variability among *T. cruzi* stocks from Northeast, Brazil and subsequently, from different regions of this country, employing six enzymes: ALAT, ASAT, glucosephosphate isomerase (GPI), glucose 6 phosphate dehydrogenase (G6PDH), malic enzyme (ME) e phosphoglucomutase (PGM), determining the existence of two different groups of strains named zymodemes 1 (Z1) and 2 (Z2).

Zymodeme 1 grouped marsupials and wild triatomine stocks and Z2 clustered domestic isolates derived from humans and domiciliated mammals. Additional studies showed a third zymodeme (Z3), also associated with sylvan environments. The diversity in Z2 is especially clear, although this heterogeneity is found in all the three zymodemes (Miles et al. 1977, 1980). This clustering was further confirmed by numerical taxonomy (Miles et al. 1978, Barrett et al. 1980, Ready & Miles 1980).

Romanha (1982) and Carneiro et al. (1990) extended the multiloci enzyme electrophoresis (MLEE) analysis (eight enzymes – ALAT, ASAT, ME, PGM, G6PD, GPI, 6-phosphate dehydrogenase – 6PGD, and malate dehydrogenase – MDH), classifying the isolates into four different isoenzymatic groups (ZA, ZB, ZC e ZD), identifying a zymodeme harboring the wild strains (ZB) and another one characteristic of the domestic transmission cycle of the parasite named ZA that corresponded to the aforementioned Z2 (Miles et al. 1980).

Tibayrenc and Ayala (1988) analyzed the enzymatic profile of 645 samples of *T. cruzi* using 15 loci. The samples varied in their geographic origin and had been isolated from a great number of triatomine bugs and vertebrate hosts. A high genetic variability was detected and 43 distinct zymodemes, alternatively named clonets, were identified. Based on the findings of the same clonet in distant geographic areas, the authors proposed a clonal population structure for *T. cruzi*.


The stability of the T. cruzi isoenzymes has also been the target of investigation by several groups and non-coherent results have been described. Although the stability of the enzymatic profile, especially when cloned populations are studied, has been observed (Dvorak et al. 1980, Miles & Cibulkis 1986, Gomes et al. 1991), variations have also been evidenced. Hence, it can be concluded that the isoenzymatic profile is not always stable (Romanha et al. 1979, Romanha 1982, Goldberg & Pereira 1983, Tanuri & Almeida 1984, Bogliolo & Godfrey 1987, Carneiro et al. 1990).

Indeed, even when clones are used, the results can be discordant. Campos and Andrade (1996) studied clones and sub-clones of the 21SF strains showing stable profiles. On the other hand, Gomes et al. (1991) observed isoenzymatic homogeneity in some clones and heterogeneity in others. Goldberg and Pereira (1983) and Montamat et al. (1997) also observed isoenzymatic variations in cloned populations. Alves et al. (1993) emphasized the possibility of reversible changes in enzymatic profiles in T. cruzi strains kept in vitro culture for a long period.

Schizodeme analysis

Molecular biology contributed with several technical approaches, which allowed the identification and classification of strains and clones, without the need of previous isolation and culturing of the parasites (Sturm et al. 1989, Ávila et al. 1991, Veas et al. 1991, Brenière et al. 1992, Vago et al. 1996, Andrade et al. 1999, Bosseno et al. 2000). However, the use of cultivated populations of parasites for DNA extraction is a constrain still faced in most of the molecular techniques, leading to an eventual selection of clones present in the original mix.

One of the techniques that does require previously cultivated parasites is the RFLP of the mitochondrial genome (kinetoplast DNA – kDNA) or schizodeme analysis. The RFLP of the kDNA was first introduced by Mattei et al. (1977) for the intrinsic classification of trypanosomes. Later, Morel et al. (1980) employed this method for genotyping T. cruzi strains and proposed the term schizodeme to refer to groups of parasites presenting the same RFLP of the kDNA. This technique is based on the electrophoretic separation of restriction fragments of the kDNA. Since then, it has been widely used (Deane et al. 1984a, Carreño et al. 1987, Gomes et al. 1991, Mimori et al. 1992, Solari et al. 1992, Alves et al. 1994, Gonzalez et al. 1995, Jaramillo et al. 1999).

Although considered as stable markers, the high heterogeneity of some strains can lead to the observation of different profiles when these strains are kept in laboratory animals or in vitro culture, as these conditions could favor the selection of sub-populations of the parasite (Morel et al. 1980, 1986, Deane et al. 1984a, b, Gonçalves et al. 1984, Carneiro et al. 1990, Gomes et al. 1991, Alves et al. 1994).

Mini-exon gene

Concerning the nuclear DNA, there are several available molecular tools to characterize T. cruzi strains. The mini-exon gene (ME) is present in the nuclear genome of the Kinetoplastida in nearly 200 copies arranged in tandemly-repeated sequences, consisting of three distinct regions: exon, intron and intergenic region. The exon is a 39 bp sequence, highly conserved among the components of the Order, being added post-transcriptionally to all nuclear messenger RNAs (mRNAs). The intron is moderately conserved among the species of the same genus or sub-genus and the intergenic region or non-transcribed spacer (NTS-ME) is particularly dissimilar among the species. In relation to T. cruzi in particular, a hypervariable spot of the NTS-ME can be amplified through the polymerase chain reaction (PCR), making it possible to classify different isolates into the two main taxonomic groups – T. cruzi I and T. cruzi II (Fernandes 1996, Souto et al. 1996, Fernandes et al. 1998). A more practical approach was recently propose to type T. cruzi strains using a multiplex PCR based on the NTS-ME, allowing to determine if a certain isolate is T. cruzi I, T. cruzi II, T. cruzi Z3 or T. rangeli (Fernandes et al. 2001).

RAPD analysis

Differences among the strains, their genetic diversity or variability, are easily revealed through the use of RAPD analysis, which proved to be an useful tool for trypanosomatids and other protozoan parasites (Dias Neto et al. 1993, Steindel et al. 1993, 1994, Tibayrenc et al. 1993, Gomes et al. 1995, Fernandes et al. 1997, Urdaneta et al. 2001). Since its introduction in 1990, this technique has been used in taxonomy and typing studies of microorganisms. It is a PCR-mediated technique that uses short primers of random sequences, which can amplify anonymous fragments of the target DNA, generating a multiple band patterns that can be strain-specific. The advantages of this technique are (i) the requirement of minimum amounts of target DNA, (ii) the acceptance of an unlimited number of primers, (iii) no requirement of previous knowledge of the sequences to be amplified (Welsh & McClelland 1990, Williams et al. 1990, Steindel et al. 1993).

Regarding T. cruzi, the RAPD analysis is judged as a useful tool to obtain highly variable DNA markers, establishing genetic relations among the isolates (Dias Neto et al. 1993, Steindel et al. 1993, Tibayrenc et al. 1993, Souto et al. 1996, Fernandes et al. 1997, Bastrenta & Brenière 1998, Brisse et al. 1998, 2000, Murta et al. 1998). Although complex band profiles can be evidenced individually, when these patterns are numerically analyzed, it is observed that T. cruzi, as a taxon, can be divided into two main groups (Tibayrenc 1995, Souto et al. 1996, Murta et al. 1998, Brisse et al. 2000). These two groups correspond to the major phylogenetic lineages of the parasite coinciding to T. cruzi I and II (Steindel et al. 1993, Tibayrenc et al. 1993, 1994, 2000).
Internal region typing (RFLP-ITS rDNA) The ribosomal RNA (rRNA) genes (rDNA) are highly conserved and bear potential for phylogenetic analysis of trypanosomatids. In these protozoa, the rDNA is found as repeated sequences. The coding regions of the large and the small sub-units are separated by internal transcribed spacers (ITSs) flanking the 5.8S rRNA gene. The ITSs present sequences of great variability and as they are flanked by highly conserved segments, it is possible to design PCR primers that anneal in these latter regions (Copolillo et al. 1995). Cupolillo et al. (1995) standardized, using Leishmania as a model, the RFLP of the ITS rDNA, which was called Intergenic Region Typing (IRT). The amplified products correspond to RFLP of the ITS rDNA, which was called Intergenic Region Typing (IRT). The amplified products correspond to the 5.8S rRNA gene plus the two flanking ITSs. Variation in the sequences of the ITSs rDNA proved that the methodology was able to distinguish among Leishmania species (Cupolillo et al. 1995). This method was adapted and applied to the molecular characterization of T. cruzi strains (Fernandes et al. 1999b). Preliminary results showed that the RFLP-ITS rDNA is powerful to distinguish intra-specific variability in T. cruzi. It also allowed geographical clustering of the isolates belonging to T. cruzi II (Santos et al. pers. commun.) and confirmed the division of the taxon in two main groups.

Mendonça et al. (2002) recently analyzed the genetic diversity among Z3 stocks from the Brazilian Amazon using the RFLP-ITS rDNA approach, showing that such isolates could be divided into two sub-groups with distinct phylogenetic origins (Kawashita et al. 2001).

Microsatellites The microsatellites are a class of a tandem-array repetitive DNA – in general around 1 to 6 bp – that are dispersed in the eukaryotic genome. They can be classified according to the number of bases and thus are called mononucleotides, dinucleotides, trinucleotides, tetranucleotides, pentanucleotides and hexanucleotides (Weber 1990). Their number of copies of the repeated unit in a specific loci is extremely polymorphic and variation in the number of the repeats occurs also between the alleles.

The mutation rate of the microsatellites varies from $10^{-6}$ to $10^{-2}$ (Harr et al. 2000). This rate changes according to the number of the repeats in the array (Bachtrog et al. 2000, Xu et al. 2000).

Due to this polymorphism, microsatellites are considered election markers with great applications in the biomedical areas such as ecology, population genetics and phylogenetic reconstruction (Oliveira et al. 1998, Hawley & Mori 1999, Bachtrog et al. 2000, Harr et al. 2000).

The main strategy employed in the analysis of microsatellites polymorphism is the PCR amplification of the loci, using a pair of specific primers that flank the segment containing the repeats, with further analysis of the size of the generated fragments (Hawley & Mori 1999).

For T. cruzi, microsatellite analysis has been used for population genetic studies, phylogenetic reconstruction, and to define the clonality of a certain strain (Oliveira et al. 1998, Macedo et al. 2001). The methodology was originally developed by Oliveira et al. (1998) as a PCR-based technique that amplifies polymorphic loci of microsatellite, with cytosine-adenine (CA) repeats of the T. cruzi genome. A genomic library of the CL Brener strain, enriched 15 times to CA repeats was used and 17 microsatellites (CA)$_n$ were selected where $n > 6$, out of which 8 were polymorphic.

The methodology was further incrementated with the use of fluorescent primers to the PCR and analysis of the amplified fragments in automatic sequencer determining the size of the alleles (Oliveira et al. 1998). This approach clearly showed that T. cruzi strains are diploid and multiclonal (Oliveira et al. 1998, 1999).

The strains that display one or two peaks (one or two alleles, corresponding to diploid) are considered monoclinal. The appearance of more than two peaks in different loci, is indicative that more than one population is present (polyclonality). Oliveira et al. (1998, 1999) observed that the majority of the strains isolated from chronic chagasic patients are clonal while those from non-human hosts are multiclonal.

Selection of T. cruzi populations: the role of vertebrate hosts and culture media

T. cruzi isolates are composed of heterogeneous populations of the parasite and in vitro culturing and animal inoculation can select certain sub-populations of this mixture (Deane et al 1984a, b, c, Gonçalves et al. 1984, Lamel et al. 1985, Miles & Cibulkis 1986, Morel et al. 1986, Aguiar 2002).

These “filters” might select those populations or clones that are more apt to the new environment. In the case of in vitro culturing, this selection can be achieved by changing the composition of the nutrients in the media. In vertebrate hosts the immune system may have an important role in this phenomenon (Lana et al. 1996, Oliveira et al. 1998).

Animal infection

The long-term maintenance of T. cruzi in laboratory animals leads to alterations in the biological features of the original strain. Regarding virulence for instance, the results are odd: reports of declination (Menèzes, 1970a, b, Brener et al. 1974, Schlemper Jr. 1982, Araújo 2000) and intensification (Schlemper Jr. 1982, Carneiro et al. 1991) are described.

Veloşo et al. (1996) reported a decline in the T. cruzi virulence after long-term maintenance in dogs. Lana and Chiari (1986) described similar results in mice and dogs, comparing the original Berenice strain and another stock isolated from the same patient 16 years afterwards.

There are clear evidences that support the selective role of the vertebrate host. Reports of changes in the isoenzymes profiles and in the RFLP of the kDNA after animal inoculation of an isolate are common (Deane et al. 1984a,

Morel et al. (1986), after maintaining *T. cruzi* in C3H mice for 18 months, observed that 5 out of 13 strains changed their isoenzymes and RFLP of the kDNA profile, confirming the selection of sub-populations of the parasite. After infecting mice with two different *T. cruzi* strains, Deane et al. (1984a, b, c) could re-isolate only one of them. They also confirmed the potential of the vertebrate host, in this case marsupial, in selecting sub-populations of the parasite when evidenced changes in the schizodeme analysis. More recently, after maintaining the Munantá strain in Balb/c mice, Santana et al. (1998) detected that from 9 of the studied enzymes, only one presented variation.

**In vitro culturing**

The long-term in vitro culturing of *T. cruzi* leads to the decrease in the virulence and infectivity of the strain to laboratory animals and this phenomenon has been explained by the selection of sub-populations that are better adapted to the in vitro culture media (Goble 1951, Pizzi & Prager 1952b, Cavalheiro & Collares 1965, Lambbrecht 1965, Bice & Zeledón 1970, Chiari et al. 1973, Chiari 1974a, b, Postan et al. 1983, Deane et al. 1984a, Magalhães et al. 1985, Alves et al. 1993, Contreras et al. 1994).

Pizzi and Parger (1952a) inoculated sequentially, C3H mice with *T. cruzi* culture forms, every 10 days for 6 years, and observed the decline of infectivity corroborated by the absence of parasitaemia after the last infections. Cavalheiro and Collares (1965) demonstrated that the Y strain presents its biological characteristics changed after in vitro culturing. Menezes (1970a, b) and Chiari (1974a, b) observed a decline in the infectivity and virulence of a *T. cruzi* strain after long-term maintenance in culture media.

Some populations develop better than others do in vitro systems, which results in modifications in the original genetic profile (Engel et al. 1982). Clones with a short duplication time might be selected by culture when compared to those that present long duplication time (Mangi 1995).

Contreras et al. (1994) maintained two *T. cruzi* strains for three years, with successive passages in cultures, detecting after this period that the metacyclogenesis had reduced, declining the virulence.

Lima et al. (1995) studied three clones of a stock isolated from a naturally infected marsupial, which were maintained during four years in LIT medium with monthly passages, and compared their growth kinetics and their duplication time. The studied parameters were considered non-stable and should not be used as biological markers, emphasizing that in vitro culturing does select *T. cruzi* sub-populations.

Rodriguez et al. (1998) examined seven *T. cruzi* strains isolated from different hosts in Colombia. One of them presented four distinct enzyme electrophoresis profiles out of 13, after being maintained during 35 in vitro passages.

Long-term in vitro culturing is determinant of phenotypic alterations – decrease in infectivity, virulence, and changes in enzyme electrophoresis profiles, and also of genotypic modifications, such as changes in the RFLP of the kDNA (Romanha et al. 1979, Romanha 1982, Goldberg & Pereira 1983, Tanuri & Almeida 1984, Morel et al. 1986, Bogliolo & Godfrey 1987, Carneiro et al. 1990).

Alves et al. (1993) observed different isoenzymatic profiles in distinct clones of the *T. cruzi* Y strain that were submitted to long-term in vitro culturing. Later on, it was verified that these changes were followed by alterations in the RFLP of the kDNA. This phenomenon is called transkinetoplastid (Alves et al. 1994) and consists of fast changes in the kDNA minicircles population leading to different restriction profiles. Transkinetoplastid phenomenon was primarily observed in cloned cells of *Leishmania amazonensis* submitted to drug selection and is judged to be reversible. Probably components of the culture medium interfere with the transcription of certain genes, regulating essential activities of the kDNA minicircles, which transcripts are necessary to the proper mitosis (Lee et al. 1992).

**Human infection**

*T. cruzi* strains isolated form, different triatomine bugs, and vertebrate hosts have shown to be multiclonal populations (Dvorak 1984, Murfin et al. 1985, Morel et al. 1986, Oliveira et al. 1998, 1999). In a chronic chagasic patient, these populations may be selected during the long human-*T. cruzi* interaction and differential tissue tropism may interfere with the distribution of the sub-populations (Vigo et al. 2000). This justify the common finding of homogeneous population being isolated from chronic chagasic patients (Gomes et al. 1998, Oliveira et al. 1999) when compared to stocks isolated during acute phases and from components of the sylvatic cycle (triatomine and mammals) (Morel et al. 1980, Fernandes et al. 1997, Oliveira et al. 1998, Solari et al. 1998).

Berenice, the first human case of Chagas disease, can exemplify a clear selective role being played by the human host. Two *T. cruzi* populations were isolated from this patient: the first one when she was 55 years old and the second when she was 71 years old. The zymodemes and the schizodemes profiles were different between them (Lana et al. 1996) and could be attributed to a re-infection with a new parasite strain or by the presence of an heterogeneous population in the patient since the very first isolate. The long-term interaction of Berenice and *T. cruzi* promoted the selection of a different *T. cruzi* population that was evidenced by the differences found in the second stock.

No correlation between the genetic variability of the parasite with the clinical outcome of the disease has been established. Probably the understanding if a patient will be asymptomatic or present the cardiac or digestive form of the disease will only be possible with the complete characterization of the parasite and of the complex human-parasite relationship that is evidenced in Chagas disease.

**“cruzi” complex**

The different biological and molecular approaches that have been used to study *T. cruzi* have shown the diversity of the distinct isolates of the parasite. The recent...
proposal of defining two groups to designate the major phylogenetic lineages of the protozoan, based on several molecular targets, have received support from the scientific community. However, there are still points that need to be addressed, such as the phylogenetic origin of Z3 and the significance of genetic hybrids.

The concept of “cruzi” complex, proposed by Coura et al. (1966) should be revitalized not as a taxonomic nomenclature but as a clear form of depicting the diversity found in the taxon T. cruzi.

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