Genetic Diversity and Genetic Exchange in *Trypanosoma cruzi*: Dual Drug-resistant "Progeny" from Episomal Transformants

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Extensive characterisation of Trypanosoma cruzi *by isoenzyme phenotypes has separated the species into three principal zymodeme groups, Z1, Z2 and Z3, and into many individual zymodemes. There is marked diversity within Z2. A strong correlation has been demonstrated between the strain clusters determined by isoenzymes and those obtained using random amplified polymorphic DNA (RAPD) profiles. Polymorphisms in ribosomal RNA genes, in mini-exon genes, and microsatellite fingerprinting indicate the presence of at least two principal* T. cruzi *genetic lineages. Lineage 1 appears to correspond with Z2 and lineage 2 with Z1. Z1 (lineage 2) is associated with* Didelphis*. Z2 (lineage 1) may be associated with a primate host. Departures from Hardy-Weinberg equilibrium and linkage disequilibrium indicate that propagation of* T. cruzi *is predominantly clonal. Nevertheless, two studies show putative homozygotes and heterozygotes circulating sympatrically: the allozyme frequencies for phosphoglucomutase, and hybrid RAPD profiles suggest that genetic exchange may be a current phenomenon in some* T. cruzi *transmission cycles. We were able to isolate dual drug-resistant* T. cruzi *biological clones following copassage of putative parents carrying single episomal drug-resistant markers. A multiplex PCR confirmed that dual drug-resistant clones carried both episomal plasmids. Preliminary karyotype analysis suggests that recombination may not be confined to the extranuclear genome.*

Key words: *Trypanosoma cruzi* - genetic diversity - genetic exchange - transfection

Trypanosoma cruzi occurs widely in many mammal and triatomine bug species (Hemiptera: Reduviidae) in the New World, although there are few human infections in the United States or the Amazon basin. It has been estimated that there are around 16 to 20 million people infected and 100 million exposed to infection. There are five main domestic triatomine vector species: *Triatoma infestans* (southern cone countries and Peru), *Rhodnius prolixus* (northern South America and Central America), *Panstrongylus megistus* (eastern and central Brazil), *Triatoma brasiliensis* (northeastern Brazil) and *Triatoma dimidiata* (Central America).

The diverse clinical outcome of human *T. cruzi* infection and biological differences between *T. cruzi* strains led to the hypothesis that *T. cruzi* was a heterogeneous species. *T. cruzi* strains were reported to differ antigenically, in virulence and histotropism in experimental animals, in infectivity to triatomine bug species and in susceptibility to drugs. The diversity of *T. cruzi* was first conclusively confirmed by phenotypic characterization of isolates using isoenzyme electrophoresis. There were shown to be at least three main *T. cruzi* strain groups or principal zymodemes (Z1, Z2, Z3) and many distinct strains within these three major groups. The diversity within Z2 was particularly marked. *T. cruzi* Z2 was originally described from central and eastern Brazil in domestic transmission cycles where heart disease and megasyndromes were reported to be common. *T. cruzi* Z1 was predominantly sylvatic where Z2 occured but was found in both sylvatic and domestic transmission cycles north of the Amazon basin. *T. cruzi* Z3 has so far been primarily associated with *Panstrongylus geniculatus* and burrowing animals, such as the armadillo, and has rarely been isolated from humans. *T. cruzi* Z1 appeared to be associated with opossums, especially the genus *Didelphis*. Host associations for Z2 were less obvious. It was suggested that the original mammalian host for Z2 could be guinea pigs in the sylvatic cycle in Bolivia and that Z2 may have spread with *T. infestans*

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to the six southern cone countries of South America (Argentina, Bolivia, Brazil, Chile, Paraguay, Uruguay) and to southern Peru. *T. cruzi* transmission cycles were described as either (a) non-overlapping or discontinuous, where there are separate domestic and sylvatic transmission cycles in a single locality (e.g. Bahia, Brazil), (b) as overlapping or continuous, where the same vector and similar *T. cruzi* strains occur in adjacent domestic and sylvatic cycles (e.g. parts of Venezuela) or (c) enzootic, where there is abundant sylvatic transmission, but domestic transmission has not yet become established (e.g. the Amazon basin and the USA) (Miles 1998).

Isoenzyme characters could be used in numerical taxonomy to estimate the similarities or genetic distances between *T. cruzi* strains. A series of strains was selected, many of which are biological clones, so that experimental work could represent naturally occurring epidemiologies (Table).

TABLE

Trypanosoma cruzi World Health Organization reference strains

M/HOM/PE/00/Peru
M/HOM/BR/00/12 SF
M/HOM/CO/00/Colombia
M/HOM/BR/00/Y strain
M/HOM/BR/00/CL strain
M/HOM/CH/00/Tulahuen
M/HOM/AR/74/CA-I
M/HOM/AR/74/CA-I/72[a]
M/HOM/AR/00/CA-I/78[a]
M/HOM/AR/00/Miranda 83[a]
M/HOM/AR/00/Miranda 88[a]
M/HOM/BR/82/Dm 28c[a]
M/HOM/BR/78?/Sylvio-X10-CL1[a]
M/HOM/BR/Sylvio/X-10-CL4[a]
M/HOM/BR/77/Esmeraldo CL3[a]
M/HOM/BR/68/CAN III CL1[a]
M/HOM/BR/68/CAN III CL2[a]
M/HOM/BO/80/CNT/92: 80 CL1[a]
I/INF/B0/80/SC43 CL1[a]
I/INF/PY/81/P63 CL[a]

a: derived from clonal populations.

Although there is circumstantial evidence, there is still no formal proof that infection with a particular *T. cruzi* strain determines a poor clinical prognosis. The high prevalence of *T. cruzi* Z2 in human infections in central and eastern Brazil, where megasyndromes are common and the contrasting high prevalence of *T. cruzi* Z1 in human infections in the north of South America, where megaoesophagus and megacolon are said to be rare, suggested that Z2 was more likely to cause chronic Chagas disease. Luquetti et al. (1986) isolated only Z2 from symptomatic chronic Chagas disease, but there was no proof that Z1 was not present earlier in the infections. Z1 and Z2 have each been isolated from symptomatic acute phase human infections and each could relapse after unsuccessful treatment. There are no conclusive studies of the relationship between human genotype and disease prognosis (Miles 1998).

It was soon demonstrated that biological clones of Z1, Z2 and Z3 differed radically in other characters, such as in kinetoplast DNA minicircle fragment patterns (schizodemes), in DNA content, in virulence to experimental animals, in extracellular and intracellular growth rates *in vitro*, in response to experimental chemotherapy, in antigenic profiles, in oxidative metabolism and in elemental composition of iron, zinc and potassium (Nozaki & Dvorak 1993, Miles 1998). Zymodeme groupings and schizodeme groupings of *T. cruzi* strains and the surface abundance of a particular antigen broadly correlated.

In further extensive studies of isoenzyme phenotypes of *T. cruzi*, Tibayrenc and colleagues subdivided the Z1, Z2 and Z3 groups into many individual zymodemes. They also demonstrated a strong correlation between the strain clusters established by isoenzyme phenotypes and by genotyping using random amplified polymorphic DNA (RAPD) profiles (Tibayrenc et al. 1993). Two further genotyping methods have been introduced for *T. cruzi* isolates, one based on analysis of polymorphisms in polymerase chain reaction (PCR) amplified ribosomal RNA genes and a second based on polymorphisms in mini-exon genes. These two methods have indicated the presence of at least two major *T. cruzi* genetic lineages, named genetic lineage 1 – which appears to correspond with Z2, and genetic lineage 2 – which appears to correspond with Z1 (Stothard et al. 1998, Fernandes et al. 1999). Microsatellite analysis and functional analysis of gene promoters have also partitioned *T. cruzi* into two major groups (Nunes et al. 1997, Oliveira et al. 1998).

Recent studies of sylvatic *T. cruzi* transmission cycles in Rio de Janeiro have confirmed the hitherto proposed link between genetic lineage 2 (presumed Z1) and *Didelphis*. It has also revealed a previously unsuspected association in Brazil between genetic lineage 1 (presumed Z2) and a primate host, the golden-lion tamarin, *Leontopithecus rosalia* (Fernandes et al. 1999). Genetic lineage 1 (again presumed Z2) has also been reported from lion-tailed macaques (*Macaca silenus*), from a ring-tailed lemur (*Lemur catta*) and from racoons (*Procyon lotor*) in the USA (Pung et al. 1998). In both these recent studies the principal zymodemes are

presumed from correlations established with reference *T. cruzi* strains and it appears that isoenzyme phenotypes were not determined for all the new *T. cruzi* isolates involved. The finding of *T. cruzi* genetic lineage 1 (presumed Z2) in primates is of particular interest as pre-adaption to primates may well have facilitated the establishment of *T. cruzi* Z2 in the human host and domestic transmission cycles. Furthermore, marmosets, especially *Callothrix* species, are common pets and this could have introduced *T. cruzi* Z2 into dwellings from sylvatic cycles. Based on a single isolate, the suspected triatomine vector of genetic lineage 1 (presumed Z2) in Rio de Janeiro is *Triatoma vitticeps*.

Potential for antigenic variation within biological clones of *T. cruzi* is not the primary focus of this brief article. Nevertheless, attention is drawn here to the presence of complex gene families in *T. cruzi*, which may have a role in evasion of the host immune response. The large family of putative mucin genes consists of hundreds of copies per haploid genome. The copies share a signal peptide on the N-terminus and a presumed glycosyl-phosphatidylinositol anchoring sequence on the C-terminus, with hypervariable central regions (Di Noia et al. 1998).

GENETIC EXCHANGE

As yet, genetic exchange in trypanosomes has only been proven for the African trypanosome, *Trypanosoma brucei*. Extensive experimental crosses have been performed, with virtually all combinations of *T. brucei* subspecies, by copassage of parental isolates through tsetse files. The inheritance of drug resistant markers has greatly facilitated the selection of hybrid progeny from these experiments (see Gibson & Stevens 1999 for review).

Typical triple-banded heterozygous isenzyme patterns for dimeric enzymes, such as glucose phosphate isomerase, were an early sign that led to the suggestion that genetic exchange might occur in *T. cruzi*. Nevertheless, most population genetics analyses of allozyme frequencies among *T. cruzi* populations have found no evidence of randomly mating (panmictic) populations. Departures from Hardy-Weinberg equilibrium, and linkage disequilibrium, have consistently been found using either isoenzyme or RAPD characters (Tibayrenc 1998). It should be noted, however, that such studies have largely involved *T. cruzi* strains from widely dispersed geographical regions, rather than multiple samples from sympatric *T. cruzi* populations in a single locality and they might therefore have missed heterogeneity through lack of sampling. Thus, although clonal propagation may predominate, genetic exchange in *T. cruzi* cannot be entirely excluded. Two studies of multiple *T. cruzi* isolates from single localities have found some evidence of genetic exchange. In both studies putative homozygotes and heterozygotes were found circulating sympatrically (Bogliolo et al. 1996, Carrasco et al. 1996). Among 36 isolates of *T. cruzi* Z1 from the Amazon basin Carrasco et al. (1996) observed parental and hybrid RAPD profiles, and although Hardy-Weinberg equilibrium analysis was extremely limited, allozyme frequencies for phosphoglucomutase did accord with expectations for panmixia. Taken together these observations are highly indicative that *T. cruzi* has the capacity for genetic exchange, and that genetic exchange may well be a current phenomenon in undisturbed sylvatic transmission cycles.

CROSSING EXPERIMENTS WITH EPISOMAL TRANSFORMANTS

Development of genetic transformation methods has provided a means of introducing drug-resistant markers to trypanosome populations. Transfection of neomycin phosphotransferase confers resistance to neomycin (G418) and of hygromycin phosphotransferase confers resistance to hygromycin. Putative parental *T. cruzi* populations that have been genetically transformed to carry different single drug-resistant markers enable selection of dual drug-resistant progeny after crossing experiments.

Ideally, parents for such experimental crosses should be *T. cruzi* isolates from a single locality (sympatric isolates) and also from what is considered to be a single type of transmission cycle. Accordingly, we have undertaken some experimental crosses with the putative parental *T. cruzi* isolates described by Carrasco et al. (1996), both assigned to Z1, and occurring sympatrically in the Amazon basin. A detailed account of these experiments will be published elsewhere (Frame, Stothard and Miles, unpublished data). Briefly, we have used a plasmid carrying neomycin resistance and a second plasmid carrying hygromycin resistance to confer drug resistance, stable for sustained passage, as described by Kelly (1997). Following copassage of putative parental *T. cruzi* populations carrying single drug-resistant markers, dual drug-resistant populations were selected by growth in medium containing predetermined optimal combinations of both neomycin and hygromycin. Appropriate controls included single drug-resistant transformants and mock transfected *T. cruzi* populations. Biological clones of *T. cruzi* were prepared from dual drug-resistant populations.

A multiplex PCR was designed to examine the basis of dual drug resistance in the biological clones that were the product of crossing experiments. Two

oligonucleotide primer pairs were made, one which gave a specific amplification product size to detect neomycin resistance and a second which gave a PCR product of a different size to detect hygromycin resistance. As the primer pairs do not cross react both can be included in a single (multiplex) PCR reaction. The size difference of the products enables rapid, simple determination of the presence of neomycin resistance, hygromycin resistance, or both forms of resistance in very small numbers of organisms. A particular advantage of the multiplex PCR is thus the rapid screening of large numbers of progeny populations without growing them in bulk. This multiplex PCR has confirmed the carriage of both episomal plasmids in dual drug-resistant *T. cruzi* populations obtained from crossing experiments. Acquisition of both episomal constructs in dual drug-resistant biological clones is also confirmed by Southern hybridisation. Furthermore, preliminary karyotype analysis suggests that recombination of parental genotypes is not confined to the extranuclear genome (Frame, Stothard & Miles, unpublished data).

CONCLUSION

Both phenotypic and genotypic characterisation have radically changed our understanding of the diversity and epidemiology of *T. cruzi*. There is evidence that *T. cruzi* can undergo genetic recombination in natural populations, and now some preliminary indication that genetic exchange can also be obtained experimentally. Although clonal propagation may predominate in transmission cycles involving humans, genetic exchange in natural populations might yield *T. cruzi* strains with new combinations of biological properties, such as virulence and drug resistance, with potential for spread to human populations.

However, significant unanswered questions remain that are worthy of further research. What is the relationship between the two main genetic lineages of *T. cruzi* and what are their origins? Are primates indeed an early host of Z2 and what is the full range of its geographical distribution? Will further studies confirm the ancient link between *T. cruzi* and trypanosomes of Australasia, proposed by Stevens et al. (1999)? Were the antecedents of *T. cruzi* monoxenous parasites of primitive mammals such as *Didelphis*, which can have infective forms in anal glands, or parasites of insects? What are the vectors, if any, of *T. cruzi* clade trypanosomes in Australasia? What mechanisms are involved in genetic recombination in *T. cruzi*? Does infection with particular *T. cruzi* strains predict a poor prognosis for Chagas disease? To what extent does human genotype predispose to chronic Chagas disease, or protect against it?

Although human Chagas disease can eventually virtually be eliminated by control of domestic triatomine vector populations and prevention of blood transfusion transmission these research questions are nevertheless of interest and some of the answers may affect intervention strategies. It is certain that, with time and coordinated effort, all the necessary technologies are now available to answer these questions.

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