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Experimental *Trypanosoma cruzi* biclonal infection in *Triatoma infestans*: detection of distinct clonal genotypes using kinetoplast DNA probes

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Abstract

Monitored biclonal densities of parasites were offered to third-stage larvae of *Triatoma infestans* via an artificial feeding device and 30 days later, the gut contents of the insects were processed for microscopic examination and polymerase chain reaction (PCR) detection of *Trypanosoma cruzi* kinetoplast DNA [kDNA]). A total of 15 mixtures involving nine different stocks attributed to the 19/20, 32 and 39 major clonal genotypes of *Trypanosoma cruzi* were used. The presence of each *T. cruzi* clonal genotype after completion of the cycle through the insects was investigated by hybridising the PCR amplification products with genotype-specific minicircle kDNA probes. Sixty-five out of 90 examined insects (72.2%) were positive for parasites by microscopic examination and 85 (94.4%) were positive by PCR. The results show that almost half of the biclonal infections are not detectable after completion of the cycle, and that there are important differences in detection of such biclonal infections according to the clonal genotypes considered. Moreover, elimination of a clonal genotype by another is a frequent, but not constant, pattern in biclonal infections of *T. infestans*. The use of PCR and kDNA probes makes it possible to avoid the culture phase, which makes detection of mixed infections much easier in epidemiological surveys. Moreover, the fact that *T. infestans*does not transmit different *T. cruzi* clonal genotypes with the same efficiency has strong implications for the reliability of xenogiagnosis.

Keywords: Vector; Chagas disease; Transmissibility; Clonal genotype; Clonal competition

1. Introduction

Chagas disease remains a major public health problem in Latin America where it has been estimated that 16–18 million people are chronically infected by *Trypanosoma cruzi* [1]. The parasite is transmitted by triatomine bugs and the widely distributed species *Triatoma infestans* is considered to be responsible for the contamination of more than one half of infected people (Dujardin J.P., The use of genetic markers in *Triatoma infestans* and its relevance for vector control, 2nd CDC/ORSTOM/CNRS International Workshop on Molecular Epidemiology and Evolutionary Genetics of Pathogenic Microorganisms, Montpellier, France, 1997). Population genetic analyses have shown that *T. cruzi* undergoes predominant clonal evolution [2]. Long-term experimental studies by our group have shown that the biological behaviour of *T. cruzi* natural clones is linked to their phylogenetic diversity. Those clones that are phylogenetically distant tend to show very distinct properties for various biological parameters such as doubling time in axenic cultures, in vitro sensitivity to antichagasic drugs, infectivity to cell cultures, pathogenicity in mice and transmissibility through *T. infestans* [3–6]. In the field, it is very frequent to sample isolates from the same host (either man or triatomine bug) that contain two or more distinct clonal genotypes of *T. cruzi* [7–11]. The present work aimed at exploring the dynamics of such mixed infections in laboratory experiments based on artificially-fed *T. infestans* [4,5], and the characterization of

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Table 1
Laboratory code, host and geographic origin of the *Trypanosoma cruzi* stocks used in the present study

Clonal Genotype	Stock	Host	Country	Region
19/20	Esquilo cl 1	Man, acute phase	Venezuela	Cojedes
	SO34 cl 4	*Triatoma infestans*	Bolivia	Potosi
	OPS21 cl 11	*Sciurus aestuans ingramini*	Brazil	Sao Paulo
32	CBB cl 3	Man, chronic phase	Chile	Tulahuen
	MVB cl 8	*Triatoma infestans*	Bolivia	Tupiza
	TU18 cl 2	Man, chronic phase	Chile	Santiago
39	SC43 cl 1	*Triatoma infestans*	Bolivia	Sante Cruz
	Bug2148 cl 1	*Triatoma infestans*	Brazil	Rio Grand do Sul
	MN cl 2	Man, chronic phase	Chile	Santiago

stocks composed of two distinct clonal genotypes by PCR amplification and use of specific kinetoplast DNA probes [7,11,12].

2. Materials and Methods

2.1. Parasites

The nine *T. cruzi* stocks used are listed in Table 1. They were selected from a standardized set of 21 stocks used in our laboratory for all biology studies dealing with *T. cruzi* [3–6]. All stocks have been fully characterized by both multilocus enzyme electrophoresis with 22 different genetic loci [13] and RAPD [14,15]. They have been cloned in the laboratory, with verification under the microscope. Information on the laboratory code, host, and geographic origin of the stocks under survey is given in Table 1. Three main categories of clonal genotypes are represented in the stocks under survey: 19/20, 32 and 39 (after the numbering by [2]). They illustrate different phylogenetic relationships. Indeed, 32 and 39 are more closely related to each other, whereas 19/20 is more distantly related to both 32 and 39. The nine stocks were previously analyzed for their transmissibility in *T. infestans* [4]. According to their transmission behaviour *in T. infestans*, they entered the composition of 15 different mixtures designed to represent various behavioral situations. For each genotype category, either 19/20 or 32 or 39, we selected three stocks, a 'fast' one (easily transmitted through *T. infestans*), a 'slow' one and a 'medium' one, according to the results obtained by Lana et al. [4]. For each case of biclonal infection (19/20 + 32, 19/20 + 39, 32 + 39), five different classes were designed, namely medium/medium, fast/fast, slow/slow, slow/fast and fast/slow, resulting in a total of 15 mixtures (Table 2). This protocol aims at eliminating a 'stock effect', that is to say: the differences observed are not due to the fact that the stocks under study pertain to a given clonal genotype, but rather, to the individual biological characteristics of these stocks. With this approach, meaningful results are obtained for mixtures of clonal genotypes rather than for mixtures of particular stocks.

2.2. Experimental conditions

Experiments were undertaken with *T. infestans* L3 reared in laboratory conditions, i.e. at 26–27°C, 65–70% of relative humidity and allowed to feed on chickens every 3 weeks. The insects originated from an outbred colony with individuals coming from Chile, Uruguay and Brazil. They were exposed to infection by using middle-log phase cultures forms from LIT medium, at 28°C in an artificial xenodiagnosis device through latex membrane. Eight milliliters of citrated mouse blood, containing exactly one half of each *T. cruzi* clonal genotype assayed at the final concentration of 5.0×10^5 parasite cells/ml, was used. The system was maintained at 37°C and continuously mixed with a magnetic stirrer. The insects were allowed to feed for 30 min and only the engorged individuals were retained for further analysis. Parasite-free experimental protocols were used as negative controls. Thirty days later, the whole digestive tube of each insect was removed and gently ground in 0.5 ml

Table 2
Mixtures of stocks representative of the 19/20, 32 and 39 *Trypanosoma cruzi* clonal genotypes used to infect third stage larvae of *Triatoma infestans*. Medium, fast, slow = behaviour in uniclonal infections

Genotype mixture	Stock mixture				
	Medium + medium	Fast + fast	Slow + slow	Slow + fast	Fast + slow
19/20 + 32	Esquilo cl1 + CBB cl3	SO34 cl1 + MVB cl8	OPS21 cl11 + TU18 cl2	OPS21 cl11 + MVB cl8	SO34 cl4 + TU18 cl2
19/20 + 39	Esquilo cl 1 + MN cl2	SO34 cl1 + SC43 cl1	OPS21 cl11 + Bug2148 cl1	OPS21 cl11 + SC43 cl1	SO34 cl4 + Bug2148 cl1
32 + 39	CBB cl3 + MN cl2	MVB cl8 + SC43 cl1	TU18 cl2 + Bug2148 cl1	TU18 cl2 + SC43 cl1	MVB cl8 + Bug2148 cl1

Eppendorf tubes containing 10 μl of phosphate buffered saline (PBS), pH 7.2. The suspension was then homogenized and 5 μl was used to prepare fresh slide smears covered with 22 × 22 mm lids. Two hundred microscope fields of each preparation were examined for the presence of parasites, with magnification of 200×.

2.3. Polymerase chain reaction (PCR)

DNA extraction was performed as described in [16,17]. A 7 μl aliquot of the resuspended DNA was amplified by PCR using *T. cruzi*-specific minicircle primers S35 (5′-AAATAATGTACGG(T/G)GAGATGCATGA-3′) and S36 (5′-GGGTTCGATTGGGGTT GGTGT-3′) [18]. The primers were annealed to the four conserved regions of each *T. cruzi* minicircle molecule [19,20]. PCR conditions have been described previously [16,19]. Denaturation, annealing, and elongation steps were performed for a total of 35 cycles, for 1 min each at 94, 60, and 72°C, respectively. A 330 bp PCR amplification product which encompassed all of the adjacent variable regions and a small portion of the conserved region (approximately 70 bp) was generated. The amplified products were electrophoresed in a 0.8% agarose gel and visualized by ethidium bromide staining. Samples from uninfected insects and DNA-free (negative template control) were also included in each PCR run.

2.4. Probes, labeling and hybridisation

Three probes specific for *T. cruzi* clonal genotypes [7,12] were used. The PCR-amplified 270 bp HVRm fragment was purified by electrophoresis on 0.8% preparative low melting point ultrapure agarose gels (BRL). The fragments were eluted from the agarose using glass beads according to the instructions of the manufacturer (Bio 101), and then digested with the restriction endonucleases *Sau*96 I and *Sca*I (BRL). After digestion, the DNA was precipitated with ethanol and resuspended in 100 μl of distilled water. Oligonucleotide probes were labeled by ^{32}P. The PCR electrophoresed samples were transferred onto charged Hybond N+ nylon membranes (Amersham) by vacuum blotting. Each membrane included a negative control (uninfected insects) and three PCR positive controls corresponding to the three categories of clonal genotypes 19/20, 32 and 39.

Membranes were hybridized at 27°C in 6 × SSC (1 × SSC is 0.15 M NaCl plus 0.015 M sodium citrate)-0.1 SDS1 × Denhart's solution-0.05 Na Ppi, washed in 6 × SSC-0.1% SDS at 37°C for 20 min, and then washed under high stringency conditions in 3 M tetramethyl ammonium chloride at

Table 3
Detection of *Trypanosoma cruzi* 19/20, 32 and 39 clonal genotypes in experimentally-infected *Triatoma infestans*

Genotype and stock mixture	Number of examined insects	Positive microscopic examination	Positive HVRm-PCR amplification	Specific probe recognition			
				First genotype only	Second genotype only	Neither first nor second genotype	Both genotypes
19/20 + 32							
Medium + medium	6	5	6	1	0	0	5
Fast + fast	6	6	6	6	0	0	0
Slow + slow	6	3	5	0	5	1	0
Slow + fast	6	5	6	0	6	0	0
Fast + slow	6	6	6	6	0	0	0
Total	30	25 (0.83)	29 (0.96)	13 (0.43)	11 (0.36)	1 (0.03)	5 (0.16)
19/20 + 39							
Medium + medium	6	5	6	0	0	0	6
Fast + fast	6	2	6	4	0	0	2
Slow + slow	6	5	5	0	5	1	0
Slow + fast	6	4	4	1	0	2	3
Fast + slow	6	6	6	1	0	0	5
Total	30	22 (0.73)	28 (0.93)	6 (0.20)	6 (0.20)	3 (0.10)	16 (0.53)
32 + 39							
Medium + medium	6	4	6	0	3	0	3
Fast + fast	6	2	5	0	0	1	5
Slow + slow	6	3	5	0	0	1	5
Slow + fast	6	3	6	0	0	0	6
Fast + slow	6	6	6	0	0	0	6
Total	30	18 (0.60)	28 (0.93)	0 (0.0)	3 (0.10)	2 (0.06)	25 (0.83)
Total general	90	65 (0.72)	85 (0.94)	19 (0.21)	20 (0.22)	6 (0.06)	46 (0.51)

65°C for 30 min [16]. Detection of the ^{32}P-labeled probe was performed by exposure of the blot to X-ray film at −80°C.

3. Results

Table 3 shows the numerical results obtained in the present study. Some typical results are presented on Fig.1. As expected, PCR amplification was more efficient for parasite detection in triatomine bugs than the direct microscopic examination (94.4 and 72.2 %, respectively). This rate of successful amplification was roughly the same whatever the pattern of mixture considered. Despite the heavy exposure to infection permitted by the present protocol, we observed some insects in which the presence of parasites was not detectable by PCR (see Tables 2 and 3). The overall rate of successful detection of the two genotypes after completion of the cycle was 51.1%. This rate varied greatly according to the mixture considered: 16.6% for 19/20 + 32, 53.3% for 19/20 + 39, 83.3% for 32 + 39. These differences were statistically significant, as verified by χ^2 analysis (19/20 + 32 vs. 19/20 + 39: $10^{-2} > P > 10^{-3}$; 19/20 + 32 vs. 32 + 39: $P < 10^{-3}$;19/20 + 39 vs. 32 + 39: $2.5 \times 10^{-2} > P > 10^{-2}$.

4. Discussion

PCR amplification is significantly more sensitive than microscopic examination for the detection of parasites after completion of the cycle, as verified by χ^2 analysis ($P > 0.001$). In our results, the overall rate of successful detection of biclonal infection after completion of the cycle was only 51.1 %. In a previous study [5], the combined use of

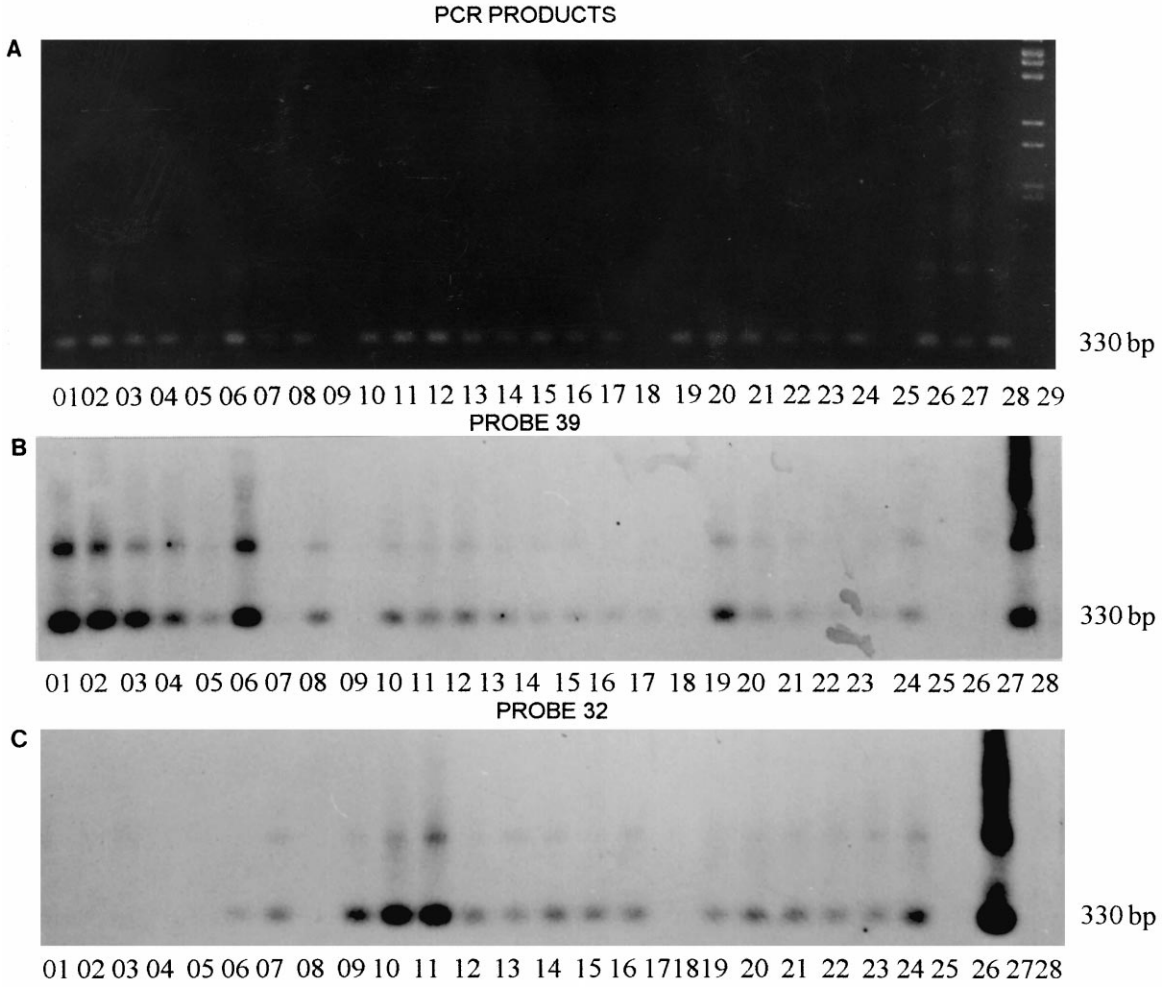

Fig. 1. PCR amplification and hybridisation with kDNA probes specific for given *Trypanosoma cruzi* clonal genotypes of various biclonal infections. Each set of six lanes corresponds to six different triatomine bugs infected with the same biclonal infection. Lanes 1 to 6: biclonal infection CBB cl3 (medium genotype 32) + MN cl2 (medium genotype 39); lanes 7 to 12: biclonal infection MVB cl8 (fast genotype 32) + SC43 cl1 (fast genotype 39): lanes 13 to 18: biclonal infection TU18 cl2 (slow genotype 32) + bug2148 cl1 (slow genotype 39); lanes 19 to 24: biclonal infection MVB cl8 (fast genotype 32) + bug 2148 cl1 (slow genotype 39); lane 25: negative control with unifected triatomine bug; lane 26: control probe 32; lane 27: control probe 39; lane 28: control probe 19/20. Medium, fast, slow refer to the stock behaviour previously estimated in uniclonal infections (Lana, et al., 1998). A = total amplification; B = hybridization with probe 39; C = hybridisation with probe 32.

MLEE and RAPD did much better, with a success rate of 87%. The difference is highly significant, as verified by χ^2 analysis ($P < 0.001$). This difference is probably due to the fact that both MLEE and RAPD rely on cultivation, and the amount of parasite cells available for detection is therefore much greater than that for the PCR amplification performed in the present study.

The present results clearly show that the composition of biclonal mixtures is not neutral for the successful identification of the two clonal genotypes after completion of the cycle. A genotype effect is apparent, as verified by the analysis above. The same tendency was raised in a previous study from our group [5] with the combined use of MLEE + RAPD, but the differences in that research were not statistically significant.

Moreover, for a given design of biclonal mixture (either 19/20 + 32 or 19/20 + 39 or 32 + 39), a stock effect may be suspected, but the small sample sizes make any statistical verification difficult. In the case of the 19/20 + 32 mixture, the only successfully identified mixture after completion of the cycle was with the medium/medium stocks. All other mixtures could not be detected. Yeates' corrected χ^2 is highly significant ($P > 0.001$). In the other mixtures, the stock effect is much less apparent.

It is worth noting that although a different efficiency of transmission through *T. infestans* was noted between *T. cruzi* clonal genotypes (by decreasing order: 19/20, 39, 32; [4]), in the present study, for any of the biclonal mixture patterns, there is no obvious tendency of one genotype to eliminate the other. This was already noted by looking for the persistence of mixtures with MLEE and RAPD [5]. This suggests that the behaviour of biclonal infections is different from a simple juxtaposition of the behaviour of the clonal genotypes they are composed of, and could explain the persistence in nature of many different clonal genotypes exhibiting a high diversity of biological parameters.

Moreover, when the problem of xenodiagnosis is considered, in confirmation with previous studies from our group [4,5], the present results show that the genotype composition of the stocks is definitely not neutral for the efficiency of parasite detection, since here, an obvious genotype effect could be detected.

Natural mixtures of *T. cruzi* clonal genotypes are very frequent [11]. We have proposed [20] that the biological relevance of these mixtures could be high (phenomena of clonal cooperation and clonal hitch-hiking). The methodological approach presented here, based on PCR detection of mixtures, although it is less sensitive than the combined use of MLEE and RAPD, is much more operational for extensive epidemiological studies aiming at evaluating the frequency of mixed infections in various ecological cycles and their impact on the transmission and pathogenicity of Chagas disease. As a matter of fact, the PCR approach avoids the tedious step of parasite culturing, which is required for MLEE and RAPD analysis.

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