Molecular and Biochemical Parasitology, 42 (1990) 175–188
Elsevier

MOLBIO 01383

Schizodeme analysis of *Trypanosoma cruzi* stocks from South and Central America by analysis of PCR-amplified minicircle variable region sequences

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(Received 5 February 1990; accepted 18 April 1990)

Kinetoplast DNA (kDNA) was isolated from 56 stocks of *Trypanosoma cruzi* isolated from human patients, animals and insects from Brazil, Venezuela, Colombia and Costa Rica. Comparison of the patterns of digested kDNA on acrylamide gels led to the grouping of several stocks into two schizodemes. Schizodeme analysis was also performed using a set of 330-bp fragments representing all the variable regions of the minicircle DNA molecules, which were obtained by PCR amplification of the kDNA using conserved region primers. The results of this analysis were consistent with the analysis using total kDNA, but the more informative restriction profiles allowed the construction of additional schizodemes. In addition, two oligomers were generated from variable region sequences of cloned minicircles from a Y and a Cl strain, and these were used as schizodeme-specific probes to detect homologous sequences in the amplified minicircle DNAs. The results indicate that a combination of restriction enzyme fingerprinting and hybridization of amplified variable region minicircle DNA with schizodeme-specific probes can be used for both sensitive detection and classification of *T. cruzi*.

Key words: *Trypanosoma cruzi*; Polymerase chain reaction; Schizodeme; Minicircle DNA

Introduction

The kinetoplastid protozoan *Trypanosoma cruzi* is the causal agent of Chagas' disease, which is characterized by an acute phase after the initial infection and then a chronic phase in which there is a progressive degenerative disorder of the cardiac and/or gastrointestinal systems. Different isolates or stocks of *T. cruzi* exhibit a great deal of heterogeneity in terms of isoenzyme patterns, surface antigens, nuclear DNA content, morphology and kinetoplast minicircle DNA sequences. Differences in isoenzyme patterns and kinetoplast minicircle DNA sequences were used to distinguish related stocks or strains of *T. cruzi* which were operationally labeled 'zymodemes' [1] or 'schizodemes' [2], and theoretically defined [3,4] as non-sexually interacting clonal lines which have been evolutionarily separated for a long time. Schizodeme analysis using total kinetoplast DNA (kDNA) has been shown to represent the method of choice for distinguishing different stocks and strains of this parasite [2]. However, this analysis requires at least 10^8 cells, and it has been shown that the required outgrowth of parasites in insects, culture or animals may result in artificial selection of parasites from the original population present in the infected host [5,6]. We have shown previously that the polymerase chain reaction (PCR) can be used to amplify species-specific and possibly strain-specific fragments of minicircle DNA [7] from as few as 10 molecules, thereby allowing direct detection and classification of small numbers of parasites from infected patients or animals. In this paper we have established the generality of this method by analyzing 56 stocks of *T. cruzi* from different geographical areas of South and Central America, and have shown that the amplified variable region of the minicircle DNA is an appropriate species- and strain-specific marker for

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Abbreviations: kDNA, kinetoplast DNA; PCR, polymerase chain reaction.

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detection and classification of this parasite.

Materials and Methods

Trypanosoma cruzi stocks. Table I summarizes the host and geographical origin of the samples analyzed.

Isolation of total kDNA. Total kDNA was isolated as described previously [2,8].

Schizodeme analysis of total kDNA. Total kDNA was digested with a restriction enzyme and the fragments separated by electrophoresis in 5.5–10% acrylamide gradient gels. The patterns were visualized by silver staining [9,10].

Polymerase chain reaction amplification of minicircle sequences. Two pg of each kDNA sample were amplified in a 100 μl reaction using Taq DNA Polymerase (Perkin-Elmer Cetus). The reaction conditions were: 10 mM Tris-HCl pH 8.4/50 mM KCl/6 mM $MgCl_2$/0.01% gelatin/1 mM of each deoxytrinucleotide/300 pmol of each primer/2 units of Taq polymerase. 100 μl of light mineral oil was layered on top of each reaction to prevent evaporation. The reactions were amplified for 35 cycles using a DNA Thermal Cycler (Perkin-Elmer Cetus). The cycling profile was as follows: 20 s denaturation at 94°C, 20 s annealing at 55°C, and a 1 min elongation at 72°C. An initial denaturation step of 4 min at 94°C and final elongation step of 10 min at 72°C were also included. A 5 μl aliquot of each reaction was run on a 2% agarose gel to check the efficiency and specificity of the amplification reaction.

Schizodeme analysis of amplified minicircle kDNA. The amplified DNA was extracted once with chloroform to remove the mineral oil. The samples were then ethanol precipitated in the presence of 0.3 M sodium acetate. The pellets were washed once with ethanol, vaccuum dried, and resuspended in 10 mM Tris-HCl pH 7.5/1 mM EDTA. One third of each amplification reaction was used for a single restriction endonuclease digestion. Restriction enzymes were used according to the instructions of the manufacturer (Bethesda Research Laboratories). Digested samples were run in 5–15% gradient acrylamide gels in 1× Tris-borate-EDTA electrophoresis buffer, at 100 V for 16 h. The gels were stained in 0.5 $\mu g\ ml^{-1}$ ethidium bromide for 30 min and photographed with 300 nm UV trans-illumination [11].

Oligonucleotides. Oligonucleotides were synthesized by standard phosphoramidite methods in an ABS DNA Synthesizer 381A. The PCR primers:
P35: 5′-AAATAATGTACGGG(T/G)GAGATG-CATGA-3′ and
P36: 5′-GGGTTCGATTGGGGTTGGTGT-3′
hybridize to the conserved region within the minirepeats of *T. cruzi* kDNA minicircles. The resulting amplification product is approximately 330 bp long and covers the adjacent variable region sequences in addition to a portion of the minirepeat [7]. The hybridization oligonucleotide probe:
P67: 5′-TGGTTTTGGGAGGGG(C/G)(G/C)-(T/G)TCAA(A/C)TTT
hybridizes to the conserved region of the minirepeats. The oligonucleotide probes:
P173: 5′-TTACTTAATGAAAGTGTATCTG-AAG-3′ and
P174: 5′-AGACGATACTCAGATTGTAGTA-GAG-3′
were obtained from the variable regions of two cloned kDNA minicircles from a Y and a Cl strain, respectively [12].

Dot blot hybridizations. An aliquot of each amplification reaction containing the equivalent amount of DNA was denatured in 200 μl of 0.4 M NaOH, 25 mM EDTA and applied to a Nytran (Schleicher and Schuell) filter. The DNA was fixed onto the membrane by cross-linking with short wavelength ultraviolet light. Hybridization conditions were: 6 × SSC, 1 × Denhardt's, 0.05% sodium pyrophosphate, 1% SDS and 20 $\mu g\ ml^{-1}$ yeast tRNA. The oligonucleotide probes were 5′-end-labeled with [^{32}P]ATP to a specific activity of 10^9 cpm μg^{-1}. The filters were hybridized at 45°C and then washed at 55°C (low stringency) or 65°C (high stringency) in 3 M tetramethylammonium chloride solution [13] with 1% SDS.

TABLE I

Origin of T.cruzi kDNA samples

#	Sample	Host	Geographical Origin	Group
043[a,1]	clone	Human	Bambui,MG,Brasil	α, S1
853[a]	stock	Human	Bambui,MG,Brasil	α, S1
240[a]	clone	Human	Bambui,MG,Brasil	α, S1
020[a]	clone	Human	Bambui,MG,Brasil	α, S1
272[a]	stock	Human	Bambui,MG,Brasil	α, S1
852[a]	stock	Human	Bambui,MG,Brasil	α, S1
822[a]	stock	Human	Bambui,MG,Brasil	α, S1
269[a]	stock	Human	Bambui,MG,Brasil	α, S1
270[a]	stock	Human	Bambui,MG,Brasil	α, S1
017[a]	clone	Human	Bambui,MG,Brasil	β, S4
121[a]	stock	Human	Bambui,MG,Brasil	β, S4
118[a]	stock	Human	Bambui,MG,Brasil	β, S4
123[a]	stock	Human	Bambui,MG,Brasil	β, S4
119[a]	stock	Human	Bambui,MG,Brasil	β, S4
600[a]	stock	Triatomine	Itacarambi,MG,Brasil	
036[a]	clone	Human	Bambui,MG,Brasil	S2
037[a]	clone	Human	Bambui,MG,Brasil	S2
512[a]	stock	D.marsupialis	Bambui,MG,Brasil	S2
730[b,2]	Cl strain		laboratory strain	S2
1006[c,3]	Daza strain	Human	Monterrey, Colombia	
605[a]	stock	Triatomine	Belo Horizonte,MG,Brasil	
264[a]	stock	Human	Bambui,MG,Brasil	
997[d,2]	stock	D.marsupialis	Manaus,Brasil	
677[d]	stock	D.marsupialis	Rio de Janeiro,RJ,Brasil	
709[d]	stock	D. marsupialis	Rio de Janeiro,RJ,Brasil	
590[d]	F strain		laboratory strain	
631[b]	Y+F strain		mixture isolated from mice	
598[a]	stock	Triatomine	Patos de Minas, MG, Brasil	
599[e,2]	stock	Triatomine	Virgen de Lapa, MG, Brasil	
586[f,4]	strain 28	D.marsupialis	Venezuela	S5
161[f]	Bert.strain	Human	Venezuela	
162[f]	stock	Rat	Venezuela	
184[f]	strain 17	D.marsupialis	Venezuela	S5
656[g,5]	clone	Human	Brasil	
160[f]	stock	Human	Brasil	
618[h,2]	stock	no information	Costa Rica	S3
619[h]	stock	no information	Costa Rica	S3
547[i,6]	stock	Calomys callosus	Goias,Brasil	
548[i]	stock	Triatomine	Goias,Brasil	
579[b]	Y strain		laboratory strain	
0001[d]	MPB strain	Bat	Venezuela	S6
655[j,7]	W strain	Guinea Pig	Recife,Brasil	
215[k,8]	stock	Human	Bahia,Brasil	
273[a]	stock	Human	Bambui,MG,Brasil	
0122[d]	stock	T.hastatus	Brasil	
733[f]	stock	Human	Venezuela	S5
642[k]	stock	no information	Colombia	
124[a]	stock	Human	Bambui,MG,Brasil	S4
221[k]	stock	Human	Montalvania,MG,Brasil	
2010[l,2]	Y strain		laboratory strain	S6
2011[l]	Y strain		laboratory strain	S6
3010[m,2]	Y strain		laboratory strain	S6
2020[l]	Cl strain		laboratory strain	S2
3020[m]	Cl strain		laboratory strain	S2
4020[n,9]	Cl strain		laboratory strain	
5030[o,9]	Costa Rica strain		laboratory strain	

Samples were obtained from the following laboratories:

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[c]Nancy Saravia
[d]Maria Deane
[e]Jose Borges Pereira
[f]Victor Contreras
[g]James Dvorak
[h]Gabriel Grimaldi
[i]Judith Kloetzel
[j]Andre Furtado
[k]Bernardo Galvao
[l]Wim Degrave
[m]Carlos Morel
[n]Jerry Manning
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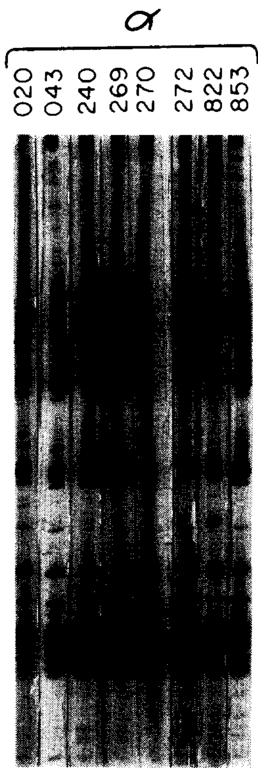
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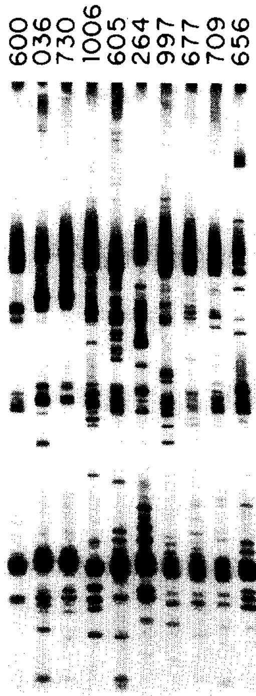
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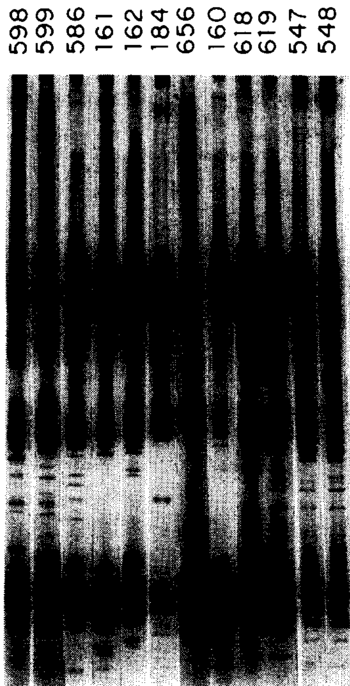
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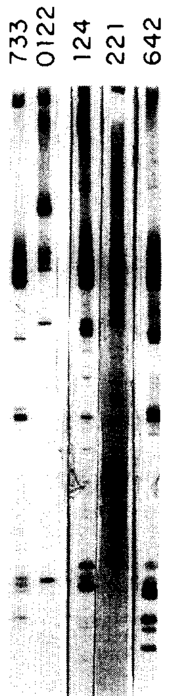
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273

Results

Origins of T. cruzi kDNA samples. The origins of the 56 kDNA samples are shown in Table I. Several of these samples are from cloned cell lines, but most are from uncloned stocks, and therefore may possibly consist of more than one strain. The parasites were isolated and cultured as epimastigotes from human patients, animals and insects from several regions of Brazil, Venezuela, Colombia and Costa Rica. The largest group of stocks came from Bambui, Brazil. Four laboratory stocks of *T. cruzi* which were labeled as Cl strain and four which were labeled as Y strain were also analyzed. Sequences of several cloned kDNA minicircles from one of the Cl strains and one of the Y strains have been published [12].

Schizodeme analysis using digestion of total kDNA. Total kDNA was isolated from approximately 10^9 epimastigote cells of each stock and digested with restriction enzymes. The *Eco*RI acrylamide gel profiles are shown in Fig. 1. Comparison of these patterns allowed the classification of the Bambui stocks into two schizodemes, the α- and β-groups. The remaining 34 samples are tentatively unclassified into schizodemes. However, it is clear by qualitative examination of the restriction profiles of the 34 unclassified stocks in Fig. 1 that these fall into three subgroups as shown. Further analysis is required to determine if these subgroups are valid. Samples 852, 037, 512 and the laboratory strains (except samples 730 and 590) were not analyzed in terms of total kDNA restriction fingerprints.

Polymerase chain reaction amplification of the kDNA minicircle variable region from 56 T. cruzi stocks. The P35/P36 primer set amplifies a 330 bp variable region fragment extending from one minirepeat region to the next in each kDNA minicircle molecule. This DNA amplification has been shown previously to be *T. cruzi* species-specific and, apparently, *T. cruzi* strain-independent [7]. The latter conclusion, however, was based only on the results from amplification of minicircle DNA from three *T. cruzi* laboratory stocks, which were labeled as the Cl, Y, and Peru strains.

In this paper we have analyzed an additional 54 *T. cruzi* stocks and strains, plus the Y and Cl stocks previously tested. 2 pg of total kDNA, isolated from each of the 56 *T. cruzi* stocks, was amplified using the P35/P36 primer set. The expected 330 bp band was obtained for all 56 samples tested except one (0122), in which a slighter faster migrating product was obtained (Fig. 2). This indicates that the amplification of the variable region of the kDNA minicircle is probably general for all strains of *T. cruzi*.

Schizodeme analysis of the amplified variable region minicircle DNA. The 330-bp PCR products were digested with several restriction enzymes and the fragments separated in 5–15% acrylamide gradient gels. The *Rsa*I, *Taq*I and *Hin*fI restriction profiles are presented in Figs. 3–5. It is apparent that the two schizodemes obtained from analysis of total kDNA digests are also valid for the digestion patterns obtained with the 330-bp fragments. It is also apparent that the amount of information contained in the digestion patterns of the amplified 330-bp fragments is much greater than that contained in the total kDNA digestion patterns, where much of the information is obscured by the 1/4, 2/4, 3/4 fragment pattern due to cleavage at one or more of the four conserved regions in the minicircle molecule. In order to differentiate the schizodeme classifications obtained using the amplified variable region minicircle DNA from those obtained using total kDNA, different designations are used. As shown in Table I, the kDNA schizodemes are indicated as α and β, whereas the amplified variable region schizodemes are indicated as S1-S6.

Fig. 1. Schizodeme analysis using total kDNA. Total kDNA from 46 of the samples listed in Table I was digested with *Eco*RI and the fragments separated in 5.5–10% acrylamide gradient gels. The gels were silver stained. The samples are grouped according to the similarities of the gel patterns. Two schizodeme groups, α and β (labeled 1 in Goncalves et al., 1990), are apparent. Samples 590 through 548 (grouped in the figure from left to right sequentially) were electrophoresed in separate gels from the other unclassified samples.

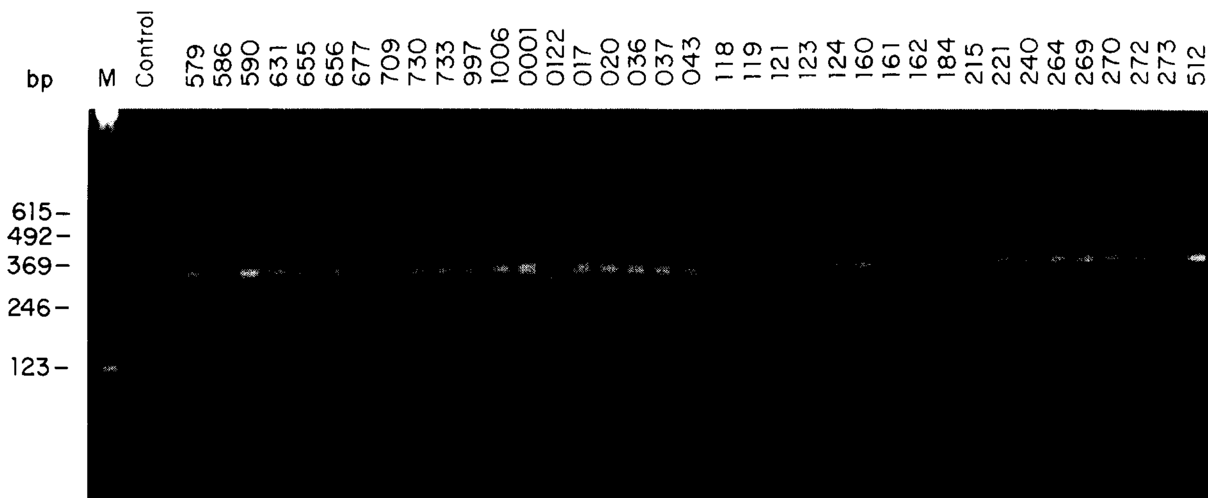


Fig. 2. Amplification of the 330-bp minicircle DNA variable region from 37 *T. cruzi* stocks. All samples yielded the expected 330-bp PCR fragment except No. 122. Lane M is a 123-bp ladder size marker. 2% agarose.

Schizodeme S1, which corresponds to the α schizodeme from total kDNA, is clearly very homogeneous, as shown in Figs. 3A–5A. The use of several enzymes allowed a finer discrimination to be performed. For example, with *Rsa*I digestion, all samples appeared very similar except for 822. With *Taq*I digestion all samples, including 822, appeared similar. With *Hin*fI digestion, samples 240, 269 and 270 formed a related subgroup, and samples 272 and 852 formed another related subgroup. We conclude that it is important to compare digestion patterns obtained with several enzymes to make any firm deductions regarding the relatedness of various stocks.

Schizodeme S2 consists of stocks 036, 037, 512 and 730. Since stock 730 was labeled a Cl laboratory strain, we also designate this the 'Cl schizodeme'. Three other putative Cl laboratory strains were also analyzed, but only two of them (2020 and 3020) were found to belong to the Cl schizodeme. Three of these stocks (036, 037 and 512) were obtained from Bambui, Brazil. A schizodeme group not apparent with total kDNA, S5, consists of stocks 184, 586 and possibly 733. The relatedness of these three Venezuelan stocks, however, could only be visualized from the *Taq*I digestion patterns (Fig. 4A and 4C). The *Rsa*I digestion patterns (Fig. 3C) suggested that two Costa Rican stocks 618 and 619 are related (= schizodeme S3). It should be noted that gel analysis of the stocks belonging to schizodeme S6 is not presented in Figs. 3–5, but is presented below in Fig. 6.

From total kDNA digestion patterns, the β schizodeme is a relatively homogeneous subgroup (Fig. 1). However, comparison of the digestion patterns of the amplified DNA led to the conclusion that only four stocks within this schizodeme – 118, 119, 121 and 123 – are very similar with all three enzymes (Figs. 3D–5D). In addition, it is possible that stock 124, which was unclassified in Fig. 1, is also a member of this β schizodeme. As is the case with all of the above well defined schizodemes, these stocks are derived from a single geographical area. In addition, a single geographical area can contain several schizodemes. For example, the stocks from Bambui, Brazil comprise three schizodemes – S1 (or α), S2, and S4 (or β).

We conclude that restriction enzyme digestion of amplified minicircle variable region DNA provides more complex and richer profiles than digestion of total kDNA, allowing a more precise determination of the relatedness of *T. cruzi* strains. Digestion of total kDNA always contains the major 1/4, 2/4 and 3/4 unit length bands which are derived from the conserved minirepeats, and this pattern often obscures the diagnostic complexities

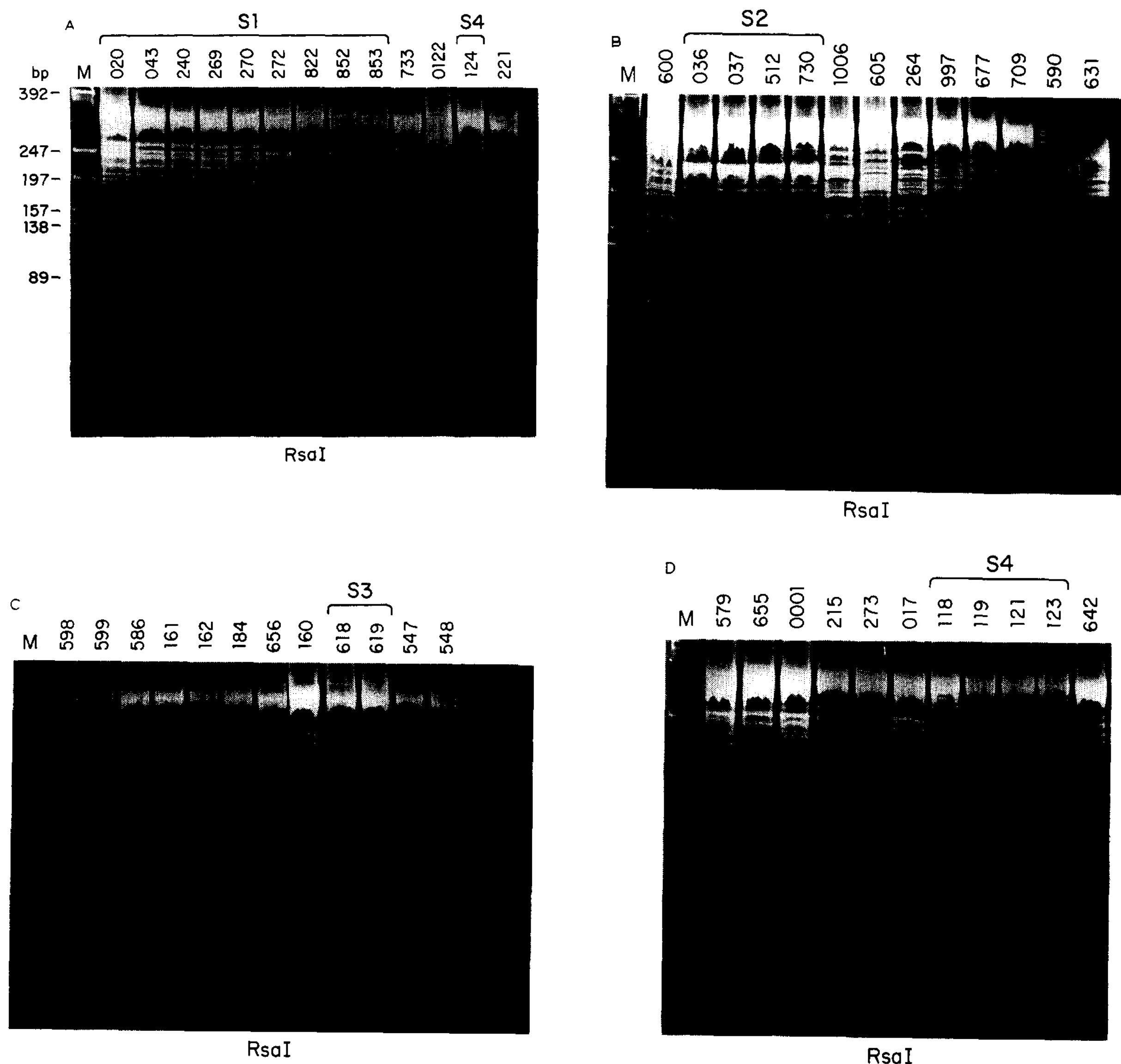


Fig. 3. Schizodeme analysis of *T. cruzi* stocks by restriction digestion of PCR-amplified minicircle variable region DNA. The 330-bp PCR products from the stocks listed in Table I were digested with *Rsa*I and the fragments separated on a 5–15% acrylamide gradient gel. Gels were stained with ethidium bromide. The samples are grouped as in Fig. 1 for ease of comparison. Samples belonging to a particular schizodeme group are denoted by the schizodeme S number. Lane M, ϕX174/*Rsa*I size marker.

of the restriction patterns.

Dot blot hybridization with variable region probes for Cl and Y strains. We have shown previously that differences in patterns of kDNA digestion products indicate differences in sequences of minicircle fragments [2]. However, since a single network contains thousands of minicircles composed of 10–20 different sequence classes as defined by the variable region sequences, the question must be addressed as to what is the actual variation in the frequency of different minicircle sequence classes between different schizodemes. If the variation is significant, then it might be possible to generate variable region hybridization probes

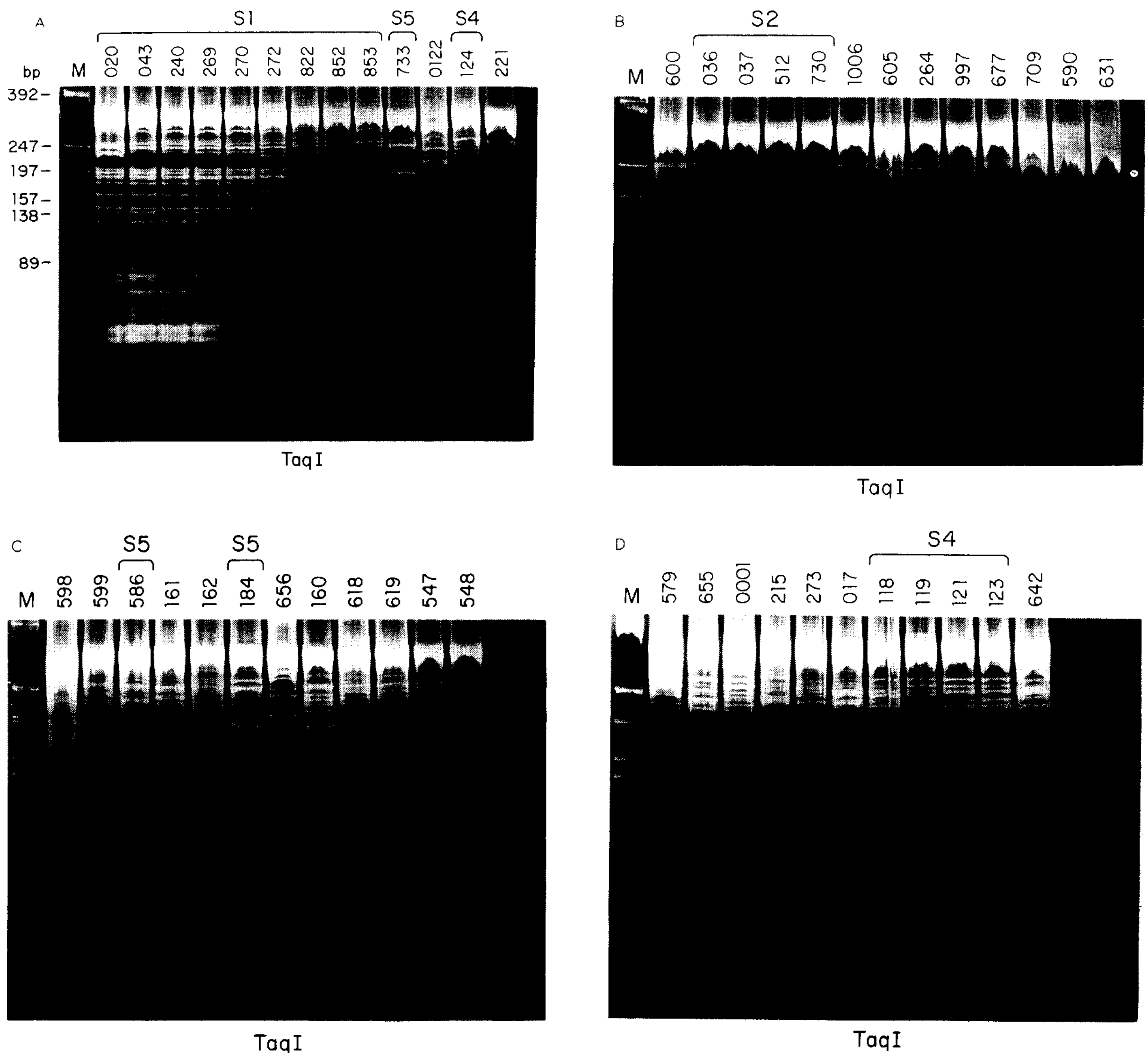


Fig. 4. Schizodeme analysis of *T. cruzi* stocks by restriction digestion of PCR-amplified minicircle variable region DNA. The 330-bp PCR products from the stocks listed in Table I were digested with *Taq*I. Conditions same as in Fig. 3. Lane M, ϕX174/*Rsa*I size marker.

which would be schizodeme-specific, and this might allow a rapid screening of amplified variable region minicircle DNA for the purpose of classification.

To answer this question, aliquots of the 330 bp minicircle variable region amplification products for each of the 56 *T. cruzi* stocks were spotted onto filters. One filter was hybridized with an oligonucleotide probe (P67) complementary to the minicircle conserved minirepeat region, in order to check for *T. cruzi*-specific minicircle DNA fragments: all samples showed positive hybridization (data not shown).

Replicate filters were hybridized with the oligonucleotide probes, P173 and P174, which represent sequences from the variable regions of cloned minicircles derived from a Y and a Cl strain [12]. As shown in Fig. 6A, the P173 probe

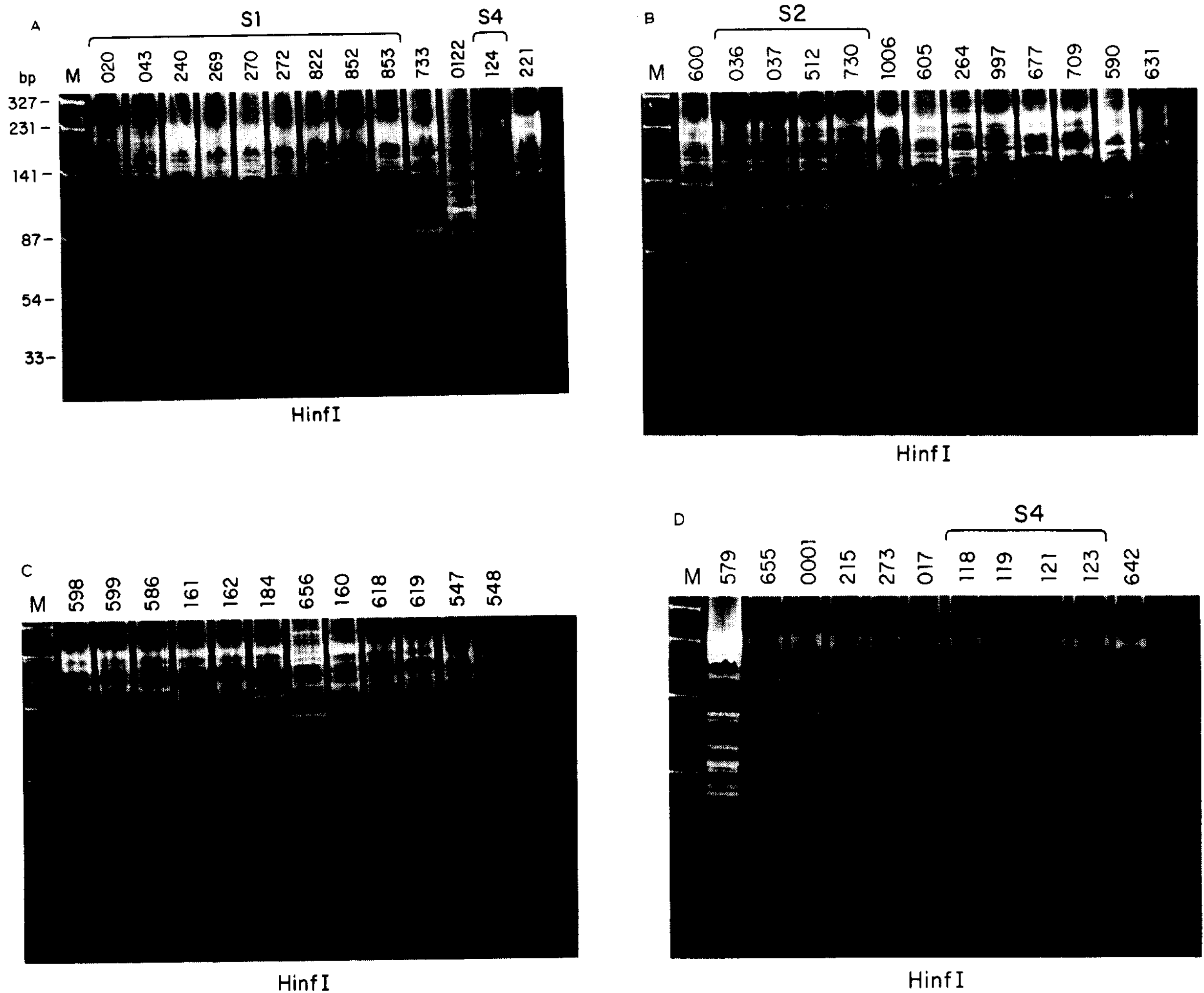
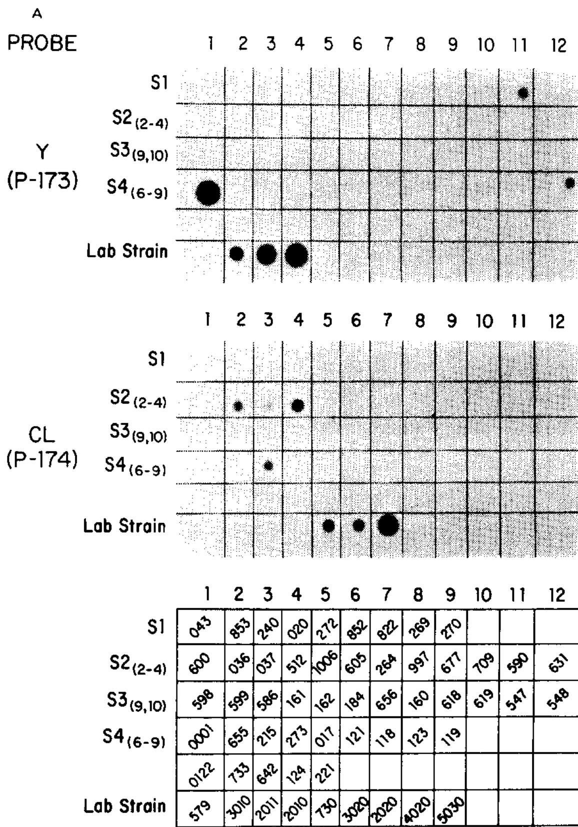


Fig. 5. Schizodeme analysis of *T. cruzi* stocks by restriction digestion of PCR-amplified minicircle variable region DNA. The 330-bp PCR products from the stocks listed in Table I were digested with *Hin*fI. Conditions same as in Fig. 3. Lane M, ϕX174/*Taq*I size marker.

hybridized with DNA from the actual cells from which the sequence was derived (2010) and also with DNA from two other samples which had been labeled as Y strains (3010, 2011) and a fourth sample obtained from a Venezuelan bat (0001). These four samples belong to the S6 schizodeme. The probe did not hybridize to sample 579, which had also been labeled as a 'Y strain' by the contributing laboratory. The P174 probe hybridized with seven other samples belonging to the S2 schizodeme (2020, 3020, 036, 037, 512, 215 and 730). No hybridization was detected with DNA from sample 4020, which had been labeled as a 'Cl strain' by the contributing laboratory. Both filters were rewashed at 65°C (high stringency, T_M-4°C). The four Y-strain specific hybrids with the P173 probe were stable even at this wash temperature (data not shown). The filter which was hybridized with the Cl-strain probe (P174) showed a loss of signal from sample 215 when washed at a high stringency (Fig. 6B), indicating substantial mismatch between the probe and that particular minicircle sequence.

To correlate the above hybridization results with restriction enzyme patterns, the amplified variable region DNAs were digested with *Rsa*I and the



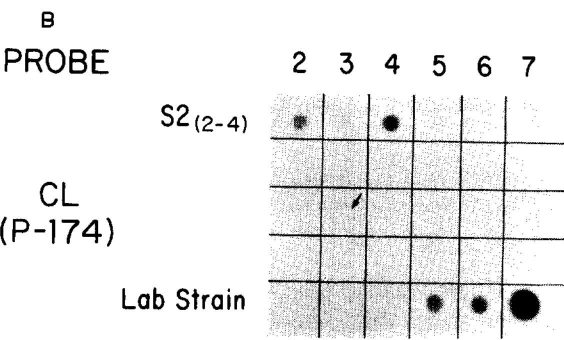


Fig. 6. Dot blot hybridizations of the amplified variable region DNAs with Y and Cl variable region oligonucleotide probes. (A) The 330-bp PCR-amplified DNAs from the 56 *T. cruzi* stocks were spotted onto Nytran filters which were hybridized with Y (P173) or Cl (P174) variable region oligonucleotide probes. Washed at low stringency (55°C, T_M-14°C). Subscripts by the schizodeme 'S' numbers denote the positions on the blot of the samples belonging to that particular schizodeme. Diagram below blots indicates the position of each of the 56 samples. (B) The same filter hybridized with the P174 oligonucleotide probe was rewashed at high stringency (65°C, T_M-4°C). Arrow indicates sample 215 which gives no signal when rewashed at high stringency.

acrylamide patterns compared, as shown in Fig. 7. This gel was blotted and the filter hybridized with the P173 probe, exposed, and then rehybridized with the P174 probe. The lanes on the right side of the blot in Fig. 7 were obtained from the initial hybridization with the Y probe, whereas the

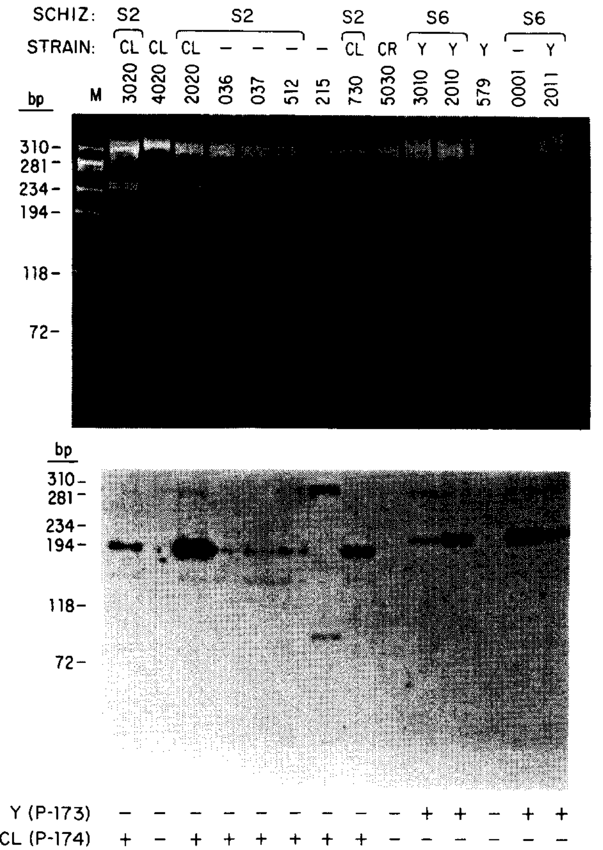


Fig. 7. Schizodeme analysis of the PCR-amplified variable region DNA from the putative Y and Cl strains. The amplified DNAs were digested with *Rsa*I and the restriction fragments separated in a 5–15% acrylamide gel. The gel was stained with ethidium bromide and then electroblotted onto Nytran filter. The blot was hybridized first with the P173 probe and then rehybridized with the P174 probe. The filter was washed at low stringency (55°C). Only the final exposure is shown, and the lanes which have bands hybridizing with either the Y or Cl probe are indicated by + or –. The autoradiograph lanes are aligned beneath the stained lanes. The stained sample lane 4020 was reproduced at a different exposure to better visualize the fragments. The putative strain classification of the samples is denoted above. Non-laboratory stocks are indicated by a – sign. Schizodeme analysis using the restriction fingerprints and the variable region oligomer probes agree, giving the same groupings. Schizodeme groups are denoted by the schizodeme 'S' number. Marker lane M is ϕX174/*Hae*III DNA.

lanes on the left side of the filter were obtained from the re-hybridization with the Cl probe. In the ethidium-stained gel, the relative homogeneity of the patterns from the 3020, 2020, 036, 037 and 512 stocks, which belonged to the Cl or S2 schizodeme, confirmed the close relatedness of these samples. The gel pattern of sample 215, which showed a faint hybridization with the Cl probe at low stringency (Fig. 6A) and none at high stringency (Fig. 6B), showed no similarity to the others, and the gel pattern of sample 4020, which showed no hybridization with the P174 probe even at low stringency, was also quite different from the others. The absence of hybridization of the P173 probe to sample 579 is shown, as is the absence of hybridization of the P174 probe to sample 4020. The cross-hybridizing *Rsa*I DNA band from sample 215 with probe P173 was larger than the major cross-hybridizing band in the other six samples, suggesting a restriction site polymorphism.

Discussion

Current methods for detection and classification of *T. cruzi* (xenodiagnosis, isoenzyme analysis, schizodeme analysis of total kDNA) all require a cell amplification step in order to obtain enough material, thus possibly leading to selection of particular parasite strains in a mixed infection. The PCR schizodeme method overcomes this limitation by direct analysis of small quantities of parasite kinetoplast minicircle DNA obtained without cell outgrowth from a small sample of host blood or tissue [7].

Schizodeme analysis in general is dependent on the differential frequencies of kDNA minicircle sequence classes (defined by the variable region sequences) within a single network, and on the rapid rate of change of minicircle sequences in nature, leading to major differences between strains, especially in the case of *T. cruzi* [3]. The precise mechanism for the generation of minicircle sequence diversity is not well understood, and probably involves point mutations and recombination between different circles, as well as a possible selection of different minicircle sequence classes in the multiple clonal lines present in the *T. cruzi* parasite natural population [3,4]. 'Schizodeme' is an operational term for a group of parasites that are clearly genotypically related [2] and makes no assumptions about the cause of this relatedness. As such, schizodeme is a useful term for the operational classification of these parasites.

Whatever the mechanism for the generation of minicircle sequence diversity, this molecule has been shown to be a suitable genotypic marker for both species and strain of several kinetoplastids [14–16]. In addition to a variable region sequence that changes rapidly, the kinetoplastid minicircle contains another region, the sequence of which is fairly well conserved across the species. Several short subsequences within this conserved region are actually conserved across the kinetoplastid family, and are thought to be involved in the initiation of DNA replication [17,18]. In the case of *T. cruzi*, the conserved region is present as four conserved mini-repeats situated at 90° from each other. We have shown previously that these mini-repeats provide suitable species-specific primer sequences for amplification of the adjacent 330 bp variable region by the PCR method [7], and that the amplified variable region DNA represents the entire population of minicircle DNA. We have proposed to use this as a sensitive method for the diagnosis and classification of *T. cruzi*.

In this paper we have verified the generality of the PCR minicircle DNA amplification method for 56 strains and stocks of *T. cruzi* from widely separated geographical areas, and have also shown that schizodeme-specific variable region hybridization probes can be incorporated into this analysis method in addition to visualization of the acrylamide gel 'fingerprints'.

Due to the extreme heterogeneity of *T. cruzi* in nature, the schizodeme method is mainly useful for determination of closely related strains and for fingerprinting individual strains. We have limited the use of the term, schizodeme, to homogeneous subgroups of related strains, although more extended and more heterogeneous groupings can be observed with comparisons of total kDNA digestion profiles. It is possible that a more quantitative analysis of the digestion patterns, involving scoring the relative mobility and molar yield of each fragment and employing a statistical analysis of correlation coefficients may extend the range of this method.

A comparison of the schizodeme groupings

determined by acrylamide gel analysis of total kDNA or the amplified variable region minicircle DNA shows a good correspondence between both methods. However the digestion patterns obtained from the amplified variable region minicircle DNA appear to be richer in information than those obtained from total kDNA, allowing for the classification of strains into additional schizodemes.

It is interesting that the samples in each schizodeme group are derived from the same geographical areas in each case. The only apparent exception to this rule is the 0001 stock, obtained from a bat in Venezuela, which we included in the S6 schizodeme, the original member of which was isolated in Brazil. However, a laboratory strain mixup is always a possibility.

A single geographical area can contain more than one schizodeme group, as seen in the case of the strains from the Bambui area, which comprise at least three different schizodemes (S1, S2, and S4). A number of samples were not classified into schizodeme groups and may represent the sole representatives of multiple schizodemes from different geographical areas. Analysis of additional *T. cruzi* isolates from the same geographical areas should permit the formation of additional schizodemes and allow the determination of the geographical range of each individual schizodeme, which is an important epidemiological consideration. The sensitivity of detection combined with the high degree of distinction of different strains of *T. cruzi*, using PCR minicircle DNA amplification, should permit the study of patterns of transmission of this parasite within and between geographical localities.

Hybridization of the amplified variable region DNA with schizodeme-specific oligonucleotide probes is a faster and more practical way for classifying *T. cruzi* into different schizodeme groups than analysis of acrylamide gel profiles of DNA digests. Our results indicate that for at least two schizodemes (S2 and S6), there is a good correlation between the mobility of restriction enzyme fragments and the sequences of the fragments. The P173 probe hybridized with several samples which were labeled as Y strain, but did not hybridize with sample 579, which was also labeled as a Y strain. We attribute this to strain mixup, and point out that this demonstrates the power of this method. The relatively stronger hybridization to the 0001 sample derived from a Venezuelan bat is probably due to a higher abundance of that particular minicircle sequence class in that strain than in the homologous 2010 strain. Likewise, the P174 probe hybridizes with several samples that were labeled as Cl strains, but does not hybridize with the 4020 sample, which was also labeled as Cl strain. The likelihood of a laboratory strain mixup in this case is made plausible by the completely different restriction gel pattern for this sample. However, the hybridization of the P174 probe to the 215 sample, which has a different Rsa gel pattern than the Cl samples, might appear to indicate that this variable region probe is not schizodeme-specific. However, the fact that the hybridization vanished at high wash stringency indicates that there is substantial mismatch, and the different sizes of the hybridizing fragments indicates the presence of restriction site polymorphisms. Therefore, the two variable region probes used in this study appear to be schizodeme-specific based on the 56 samples analyzed. However, additional work is necessary to test the general hypothesis that specific variable region probes can be generated for every *T. cruzi* schizodeme.

The availability of schizodeme-specific probes would allow an analysis to be performed using the oligonucleotide bound to a solid state medium such as a microtiter well or a membrane filter [19,20]. A PCR-amplified unknown minicircle variable region sample could be tested against a library of such specific variable region probes by a single hybridization, and the results analyzed using non-radioactive technology. We suggest that this method should prove useful in the rapid and sensitive diagnosis of *T. cruzi* parasites in chronic Chagasic patients and in blood banks, as well as in epidemiological studies.

Acknowledgements

This work received financial support from the UNDP/World Bank/WHO Special Programme for Research and Training in Tropical Diseases and from a WHO/Rockefeller Foundation bilateral research grant. H. A. was supported by a Gradu-

ate Opportunity Fellowship and an NIH predoctoral Training Grant (AI 07323). We would like to thank Nancy Sturm and Wim Degrave for helpful discussions.

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