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GENERAL PROCEDURE TO CONSTRUCT HIGHLY SPECIFIC kDNA PROBES FOR CLONES OF TRYPANOSOMA CRUZI FOR SENSITIVE DETECTION BY POLYMERASE CHAIN REACTION

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Abstract - Our strategy of probes designing for major clones of Trypanosoma cruzi was performed taking into account the : (i) clear identification of the major clones under multilocus study ; (ii) hypothesis of a parallel evolution between the extranuclear and nuclear markers ; (iii) structure of kDNA which allowed to amplify high variable regions of the minicircle (HVRm) by PCR. The large production of HVRm was very useful to test their ability to be used as probes for detection of DNA from a diversified genetic panel of T. cruzi. Our success in designing such probes has important implications on : the enhancement of 2 evolutive hypothesis about the clonal structure of T. cruzi and the parallel evolution of their extranuclear and nuclear genetics markers; direct diagnosis in patients and vectors. Studies on bio-clinical significance of major clones are discused. This procedure could be used as a general strategy to generate DNA probes for Kinetoplastida.

Key words : Chagas'disease, kinetoplast, minicircle, clonal structure, PCR, kDNA.

PROCEDURE GENERALE POUR LA CONSTRUCTION DE SONDES D'ADNk SPECIFIQUES DE CLONES DE TRYPANOSOMA CRUZI POUR UNE DETECTION SENSIBLE PAR LA REACTION DE POLYMERISATION EN CHAINE

Résumé - Notre stratégie pour construire des sondes de clones majeurs de Trypanosoma cruzi a été dévelopée en tenant compte de : (i) une claire identification de clones majeurs selon une analyse multilocus ; (ii) l'hypothèse d'une évolution parallèle entre l'ADN nucléaire et extra-nucléaire ; (iii) la structure particulière de l'ADNk qui a permis d'amplifier des régions variables du minicercle (HVRm) par PCR. Les quantités importantes d'HVRm ont été très utiles pour tester leur capacité à être utilisées en tant que sondes pour la détection d'ADN sur un groupe de stocks génétiquement diversifiés. Le succès de cette stratégie a des implications théoriques et pratiques très importantes sur : le renforcement de deux hypothèses évolutives, à savoir : la structure clonale de populations de T. cruzi et l'évolution parallèle des marqueurs génétiques nucléaires et extranucléaires ; le diagnostic direct chez les patients et les vecteurs. Ainsi, des études sur la signification bio-clinique des clones majeurs sont discutées. Cette approche pourra être utilisée comme stratégie générale pour générer des sondes d'ADN pour d'autres Kinétoplastidés.

Mots clés - Maladie de Chagas, kinétoplaste, minicercle, structure clonale, PCR, ADNk.

INTRODUCTION

The protozoan parasite : Trypanosoma cruzi, is the etiological agent of Chagas'disease which threc several million people in the American continent. A great geographic diversity is observed in its severity

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prevalence. In most of the cases, the initial phase is benign with a level of parasitemia sufficient to allow direct detection in blood and subsequent humoral specific response settled 2 to 3 months later, conversely, during the last phases, parasites circulate in blood at a very low level and the direct examination failing to parasite detection introducing dramatical problems in diagnosis particularly within transfusion centers.

These last years, population studies based on genetic markers such as isoenzymes and kDNA RLFPs have evidenced a high genetic variability in T. cruzi populations (Miles et al., 1977; Morel et al.,1980; Tibayrenc et al., 1986)

Extensive population genetic analysis have evidenced a typical clonal structure in T. cruzi (Tibayrenc et al., 1986, Tibayrenc and Ayala, 1988, Tibayrenc et al.,1990). The formerly described zymodemes can be equated to natural clones of the parasite. Some of these clones appear as ubiquist and frequently sampled in endemic areas, and were referred to "major clones" for this reason (Tibayrenc and Brenière,1988) .

The complex kDNA network composition and functions of T. cruzi are reviewed by Simpson (1987). Briefly, one of the substructures of kDNA are minicircles families, each minicircle presents 4 (120 bp) high conserved similar regions (HCRm) localized at 90° intervals (Mattei et al., 1977) and four dissimilar high variable regions (HVRm). Populations of T. cruzi have been grouped in subpopulations having similar kDNA minicircle RFLPs (schizodemes) and the evidenced high variability of these schizodemes were hypothetized as having possible concordance with zymodeme patterns (Carreno et al.,1987, Morel et al., 1980;. Tibayrenc and Ayala (1988) found a high correlation between schizodemes variability and clones of T. cruzi defined by genetic isoenzyme analyses.

Having regard to the high genetic variability of major clones, the pleomorphisme of the disease (WHO, 1974) and the biological diversity of T. cruzi (Dvorak, 1984) high biological and medical differences can be expected between radically genetic dissimilar clones. Then a correlation between biomedical characteristics should be searched because this important question stay still unanswered.

Up to date, usual techniques employed for subspecific characterization of T. cruzi populations lack of sensitivity and need an obligatory amplification step of the parasite by xenodiagnosis and/or culture. During this processing a clone-selection can occur, and interpretation of the obtained data could suffer of bias, particularly concerning their medical importance.

Several authors have developed nuclear or kinetoplast DNA probes specific to the whole taxon T. cruzi used for parasite detection in host blood or in Triatominae feces (Gonzalez et al., 1984, Greig and Ashall, 1987, Sturm et al., 1989). Macina et al. (1987) have developed probes specific to "stocks", genetically and pathologically undefined. Recently, Moser et al. (1989) have proposed the polymerase chain reaction (PCR) for diagnosis purpose of the Chagas'disease. In all the cases we have merely stating that up to date there are any clear strategy to get good defined probes for a determined pathology or for a determined population of T. cruzi contrarely to the bacterias or other Protozoan parasites Tannich et al. (1989).

Taking into account the facts treated above we have defined three working hypothesis necessary to construct our probes : (i) clonal structure of natural population of T. cruzi (ii) parallel evolution between nuclear (isozymes) and extranuclear DNA (kDNA) and (iii) conservation of the peculiar structural characteristics of kDNA. If the three hypothesis are verified then we could generate good candidate sequences for probes by applying PCR DNA amplification technology created by Mullis and Faloona (1987).

MATERIAL AND METHODS

Parasites

In order to check the specificity of probes, a set of 38 T. cruzi stocks representing various natural clones (characterized by 15 isozymes loci or kDNA RFLPs) and some other related parasites (T. cruzi marenkellei , T.

kDNA probes for T. cruzi clones

STOCKS	Species	Host/Vector	Locality	Clone*
X10 cl1 "Z1"	*T. cruzi*	human	Belém, Brazil	17
CUTIA	*T. cruzi*	*Dasyprota agutii*	Esperito Santo, Brazil	19
OPS21	*T. cruzi*	human	Cojedes, Venezuela	19
13379 cl 7	*T. cruzi*	human (acute case)	Santa Cruz, Bolivia	19
SO30	*T. cruzi*	*Triatoma infestans*	Calcha, Bolivia	rel 19
LGN	*T. cruzi*	human (chronic case)	IV region, Chile	rel 19
Florida	*T. cruzi*	*Triatoma sanguisuga*	Ganesville, USA	rel 19
SO34	*T. cruzi*	*Triatoma infestans*	Toropalca, Bolivia	20
P/11	*T. cruzi*	human (chronic case)	Cochabamba, Bolivia	20
ESQUILO	*T. cruzi*	*Sciurus aestruans ingrami*	Sao Paulo, Brazil	20
CUICA	*T. cruzi*	*Opossum cuica philander*	Sao Paulo, Brazil	20
OPS4	*T. cruzi*	*Didelphis marsupialis*	Carabobo, Venezuela	21
OPS22	*T. cruzi*	*Panstrongylus geneculatus*	Cojedes, Venezuela	21
Can III cl1"Z3"	*T. cruzi*	human	Belém, Brazil	27
Esmeraldo cl3"Z2"	*T. cruzi*	human	Bahia, Brazil	30
TU18 cl2	*T. cruzi*	*Triatoma infestans*	Tupiza, Bolivia	32
MXCH88	*T. cruzi*	human	Cuncumen, Chile	32
CBB	*T. cruzi*	human	IV region, Chile	33
MCH3	*T. cruzi*	*Triatoma infestans*	Arrayan, Chile	33
MXCH53	*T. cruzi*	human	Chile	33
MXCH89	*T. cruzi*	human	Illapel, Chile	**
MXCH46	*T. cruzi*	human	Salamanca, Chile	**
MXCH80	*T. cruzi*	human	Chanaral alto, Chile	**
Bug 1738	*T. cruzi*	*Triatoma infestans*	Las Ramadas, Brazil	**
M6241 cl6	*T. cruzi*	human (acute case)	Belém, Brazil	35
M5631 cl5	*T. cruzi*	*Didelphis novemcinctus*	Belém, Brazil	36
SC43 cl1	*T. cruzi*	*Triatoma infestans*	Santa Cruz, Bolivia	39
SO3	*T. cruzi*	*Triatoma infestans*	Otavi, Bolivia	39
TPK1	*T. cruzi*	*Triatoma infestans*	Khala Khala, Bolivia	39
NR	*T. cruzi*	human	IV region, Chile	39
MN	*T. cruzi*	human	IV region, Chile	39
Bug 2145	*T. cruzi*	*Triatoma infestans*	Rio Grande do Sul, Brazil	39
Bug 2148	*T. cruzi*	*Triatoma infestans*	Rio Grande do Sul, Brazil	39
GR	*T. cruzi*	human (chronic case)	IV region, Chile	rel 39
Tulahuen FKIIA cl2	*T. cruzi*	*Triatoma infestans*	Chile	43
MCV	*T. cruzi*	human	Chile	**
MVB	*T. cruzi*	human	Chile	**
Unnamed	*T. cruzi*		Chile	§
Rangeli (Basel)	*T. rangeli*	*Aotus*	Brazil	
TMB7	*T. marenkellei*	*Phyllostomum discolor*	Sao Felipe, Brazil	
LEM 1139	*L. infantum*	man	Spain	MON-28
LEM 536	*L. donovani*	man	Saudi Arabia	MON-31
LEM 250	*L. infantum*	*Canis familiaris*	Cevennes, France	MON-01
LEM 765	*L. infantum*	*man*	France	MON-01
MTRG/UG/66/EATRO/125	*T. brucei s.p.*	*Tragelaphus scriptus*	Mavubwe South,Uganda	
MHOM/SD/82 Biyamina	*T.b.gambiense.*	human	Yambio, Soudan	
MSUS/CI/82/TW53	*T. b. brucei*	pig	Kouassi-Perita, Ivory Coast	

Table 1 : Geographic origine, host origin and zymodeme pattern identification of studied stocks.

*	: clone number are referred to Tibayrenc and Ayala (1988)
**	: kDNA RFLP characterized strains are referred to Carreno et al. (1987)
§	: genetically non identified
rel n°	: genetically related to the associated clone number

rangeli, T. brucei and Leishmania) were studied. The geographic, host origin, isozyme and kDNA RFLP characterization (Carreno et al.,1987) of the stocks studied in the present work are summarized in Table 1 in which numbering of T. cruzi clones is according to Tibayrenc and Ayala (1988).

Parasite cultivation

Stocks of T. cruzi T. rangeli and T. cruzi marenkellei were cultured in LIT medium, Leishmania stocks were cultured in RPMI 1640 medium supplemented with 10% heat inactivated fetal calf serum. Parasites were harvested by centrifugation (300 g during 8 min. at 4°C). African trypanosome stocks were amplified by passage through rats and recovered according to Lanham and Godfrey (1970).

DNA purification

(i) Total DNAs were purified from parasite pellets of 100 mg (wet weight) and incubated in 5 ml of the following lysis buffer : TE (Tris-HCl pH 8.0 10 mM ; EDTA 1 mM), SDS 1% and proteinase K (200 µg/ml, Sigma) at 37°C overnight, followed by 3 phenol/chloroforme (24/1) extractions and subsequently precipitated with 0.3 M sodium acetate in 3 V/V of pure ethanol. DNAs were pelleted by centrifugation at 15,000 rpm for 15 min. and vacuum dried under centrifugation with a Speed Vac apparatus (Savant, USA). The dried pellets were finally resuspended in 2 ml of Tris-HCl 5 mM pH 8.0. (ii) Kinetoplast DNAs were purified according to Morel et al. (1980). All DNA extractions were treated with 2 µg/ml of RNase (Sigma, USA) and DNA concentrations were assessed by either spectrophotometry, with a DU 70 apparatus (Beckman, USA) or colorimetry, with DNA Dipstick (Invitrogene, USA).

Parasite lysates

The way used here, to avoid DNA purification step, was to prepare parasite lysates (dilutions from 1 to 10^4 parasite cells in 100 µl) by two freezing-thawing cycles, followed by a 5 min. boiling process, Milli-Q water was added (V/V), and finally a centrifugation was done at 15,000 tpm for 10 min. An aliquot of 20 µl of supernatant contains enough DNA to carry out the PCR amplification.

Oligoprimers

Figure 1 shows the location of the two oligoprimers, CV 1 and CV 2 used as templates in all PCR experiments carried out in this work. Their sequences are slightly modified sequences (synthetized by CRBGC, Toulouse, France) from previously high conserved regions of minicircle (HCRm) described by others authors (Degrave et al.,1988; Gonzalez, 1986; Macina et al.,1986). These sequences were established in order to anneals flanking sites of the high variable regions of the minicircle (HVRm) of the kDNA. CV 1 : $^{5'}$GATTGGGGTTGGAGTACTACTAT$^{3'}$ and CV 2 : $^{5'}$TTGAACGGCCCTCCGAAAAC$^{3'}$.

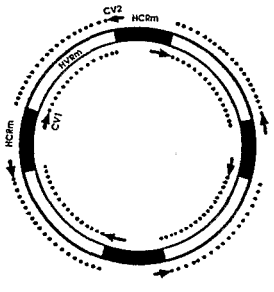

Fig. 1 : Schematic representation of an individualizec kDNA minicircle of T. cruzi and location of CV 1 and CV 2 oligoprimers. Black areas represent the HCRm sequences and white areas represent HVRm sequences. Arrows indicate the sense of DNA elongation during the PCR.

PCR amplification

The HVRm sequences were amplified using CV 1 and CV 2 with 2.5 U/sample of Thermus aquaticus polymerase, (Biolabs, USA) per sample. PCR were carried out within 30 temperature cycles in PHC-2 apparatus (Techne, UK) starting from purified templates : 1 µg of total DNAs or 100 ng of kDNAs. The polymerase buffer was composed following the manufacturer's conditions : Tris-HCl (pH 8.8) 67 mM, $(NH_4)_2SO_4$ 19 mM, $MgCl_2$ 6.7 mM, β-mercaptoethanol 11.4 mM ; 5 µl of BSA 100 µg/ml and 6.0 µl of dNTP 200 mM in a total volume reaction of 50 µl completed with Milli-Q water (Millipore, USA). Thirty cycles were carried out as follow : (i) the first cycle is composed of a first step at 95°C during 5 min. for denaturation, a second step at 48°C during 2 min. for the accomplishment of annealing of oligoprimers and a third step 2 min. at 70°C for the elongation of CV 1 and CV 2 sequences ; (ii) the following cycles consist of 5 sec.at 95°C, 30 sec. at 48°C and 2 min. at 70°C ; (iii) a final cycle of 5 sec. at 95°C, 30 sec. at 48°C and 15 min. at 70°C. At the end of PCR, 5 µg of each samples were electrophoresed (80 V, 30 min.) in 0.8% agarose minigel and visualized by ultraviolet light after ethidium bromide staining.

PCR controls

Internal negative controls of PCR were brought by Milli-Q water (instead DNA targets) were added at the end and at the start of sampling preparation. Positive controls were brought by samples giving known positive results. Special cares were observed all over the PCR manipulation to avoid false positives or negatives (Saiki et al., 1989). A final control was brought by DNA hybridization.

Probes purification

Six T. cruzi stocks representing 5 majors clones (see Table 1) were selected in order to generate clone-specific probes. Starting from a positive PCR amplification of DNA target, around 80% of the HVRm (50 µg) were recovered as follows : amplified DNAs were electrophoresed in preparative low melting point 0.8% ultrapure agarose gel (BRL, UK) and DNA was subsequently eluted using beads according to the manufacturer's instructions of Genclean (Bio 101, USA). DNAs were double digested by *Sau* 96 I and *Sca* I (BRL, UK) restriction enzymes. Finally after ethanol precipitation, the DNAs were resuspended in 100 µl of Milli-Q (Millipore, USA) sterilized water and stored at -20°C until use.

Southern blotting

RFLPs were generated by Hae III (purchased at Boehringer, FRG) restriction digestion of total DNA electrophoresed into of 1.5 % agarose gels. Transfers were carried out after alkaline denaturation treatment (NaOH 1.5 M and NaCl 0.5 M) onto charged nylon membranes Hybond N+ (Amersham, UK) by pocket-blotting procedure according to Cuny et al.(1990).

Slot blotting

Using the same alkaline denaturation conditions (above mentioned), two different DNA concentrations : 300 ng, 30 ng for total DNA and 30 ng, 3 ng for kDNA were transferred onto nylon membranes Hybond-N^+ using a slot blot apparatus (Millipore, USA) under constant vacuum conditions (-25 kPa).

Probe labeling and hybridization

Twenty nanograms of HVRm purified fragments were labelled with α^{32}PdCTP (Amersham, UK) according to the manufacturer's instructions of Rapid Hybridization System-Multiprime (Amersham, UK) while 1 µg of HVRm were used in non-radiolabeling procedure according to manufacturer's instructions (Boehringer, FRG).

RESULTS

DNA products of PCR amplification

For all T. cruzi stocks from which total- or K-DNA were PCR-amplified, CV 1 and CV 2 oligoprimers generate a major DNA product correspondong to HVRm (Fig. 2a). The used templates were total DNAs or purified kDNA, from 38 stocks (Table 1). Two other products of 600 bp and 900 bp were generally observed (Fig. 2a). On the upper part of the gel a more or less intense DNA smear was observed, probably due to the presence of relatively high quantities of kDNA (100 ng), used as target template at the starting PCR. Hybridization of these products with the radiolabelled HVRm from SC43 cl1 stock evidence high homology between the HVRm, 600 and 900 PCR products on one hand, and with smear products on the other hand (Fig. 2b). These facts sugget ends binding between the fragments newly generated. T. rangeli and T. cruzi marenkellei (close related species of T. cruzi) were also successfully PCR-amplified with CV 1 and CV 2 oligoprimers. T. cruzi marenkellei exhibit two major products having higher molecular weights than those of T. cruzi. In the same conditions, with our African trypanosomes and Leishmania stocks used as template targets, PCR failed to generate amplified products.

PCR detection sensitivity

The sensitivity of PCR was tested using purified DNA and pure parasite pellets as targets. Sensitivity was determined by titration experiment and the minimal template target amount of total DNA required to visualize the 250 bp product (after gel electrophoresis, identified by ethidium bromide staining) was 10^{-4} fg. After limit dilution, the DNA from lysed parasites (see Material and Methods) was used as template and gave a positive amplification from as few as 1 to 5 parasites (epimastigote or trypomastigote culture forms).

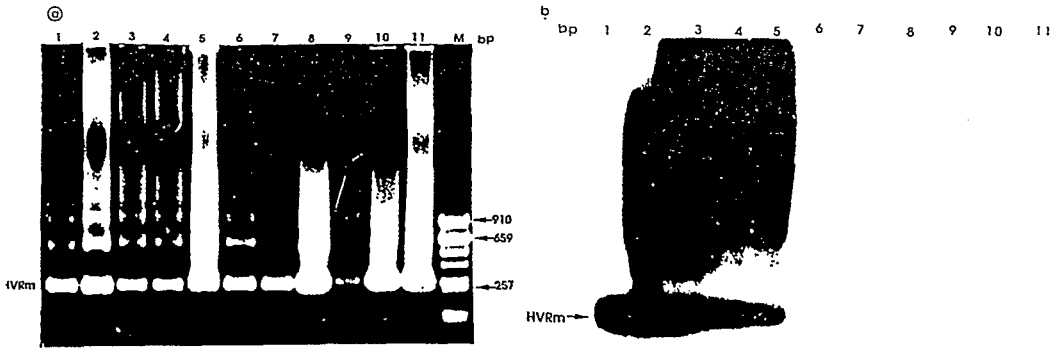

Fig. 2 : (a) Electrophoresed and ethidium bromide stained 0.8% agarose gel exhibiting PCR products from 1 µg of total DNA or 100 ng of kDNA of T. cruzi stocks (indicated here into the brackets). Lanes 1 to 3 : clone 39 (SC43 cl1, NR, Bug 2148), lane 4 to 11 : related to clone 39 (GR), clone 36 (M5631 cl5), clone 43 (Tulahuen FKIIA cl2), clone 33 (CBB), clone 20 (SO34), clone 19 (Cutia), clone 30 (Esmeraldo cl3 "Z2"), clone 27 (CANIII cl1 "Z3") ; lane M : molecular weight marker pBR-Alu I ; (b) Hybridization of the blotted gel with SC43 cl1 HVRm 39 radiolabelled probe, high washing stringency was monitored at 0.1 x SSPE and 0.1% SDS at 68°C and then autoradiographed on MP films with intensifying screens (Amersham, UK) for 20 hours at -70°C.

Subspecies specificity of HVRm probes isolated from T. cruzi stocks

HVRm PCR-amplified and purified products obtained from 6 different T. cruzi major clones were radiolabelled and hybridized with all stocks listed in Table 1. Low stringency washing conditions (2 x SSPE, 0.1% SDS at 68°C) allowed us to observe that the set of six HVRm fragments hybridize with different subsets of tested stocks of T. cruzi, the T. rangeli and T. cruzi marenkellei, but constantly failled to hybridize with Leishmania or African trypanosomes stocks (data not shown).

In the present work we have obtained 4 HVRm probes, which clone-specifity for the different majors clones, previously identified, is illustrated on Fig. 3 : showing clearly the genetical recognition of clone-targets present in the Wagner network previously established (Tibayrenc and Ayala, 1988) and their distribution in South America. These results have been evidenced by hybridization of these radiolabelled probes with transferred (i) total DNA or purified kDNAs slot-blotted onto nylon membranes (Fig. 5), and (ii) RFLPs from total DNA digested with Hae III restriction enzyme (Fig. 4) and (iii) PCR products (Figs. 2, 6 and 7).

- SC43 cl1 HVRm 39 probe is specific of all stocks pertaining to major clone n° 39 (Figs. 2, 4 and 5a). A positive hybridization was also noted too for the GR stock which genotype is closely related to clone 39 (Nei's standard genetic distance = 0.10).

- SO34 HVRm 20 probe is specific of all stocks pertaining to major clone n° 20 in conditions of the hybridization on PCR products in our usual stringency washing conditions. In slot blots conditions (Fig. 5a), we have noted a very light hybridization signal with some stocks pertaining to clone 19 or related to clone 19 (which is closely related to clone 20, as appear in the Wagner network of the Fig. 3).

- TU18 cl2 HVRm 32 probe is highly specific of all stocks pertaining to major clone n° 32 in conditions of the hybridization on PCR products in the usual stringency washing conditions. A slight hybridization signal was recorded with some stocks identified as clone 33 (Fig. 5a) but the hybridization is highly specific on HVRm from PCR as shown in Fig. 6b. We have observed an hybridization with MCV stock identified by RFLP for 5 restriction enzymes (Carreno et al., 1987) having different RFLP from the stocks identified as belonging to clone 33.

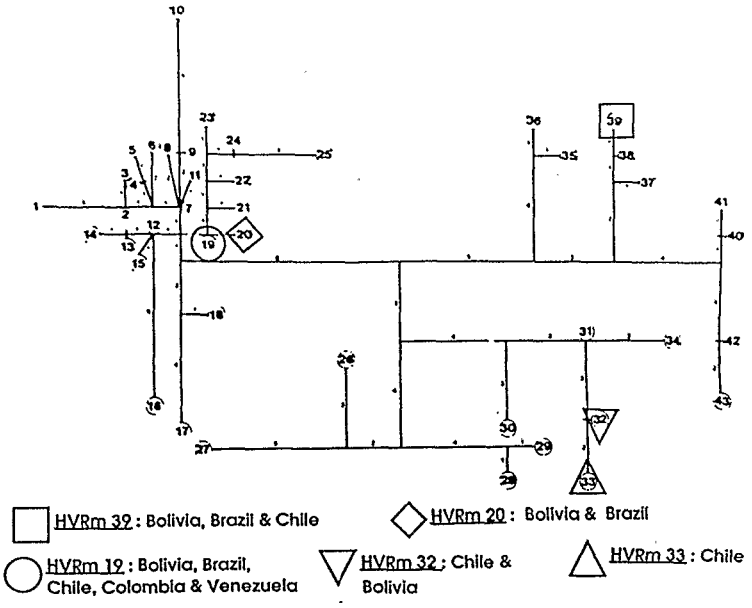

Fig. 3 : Wagner network established from T. cruzi natural population sampling from Tibayrenc and Ayala (1988) exhibiting the genetic targets and geographic distribution of the HVRm probes.

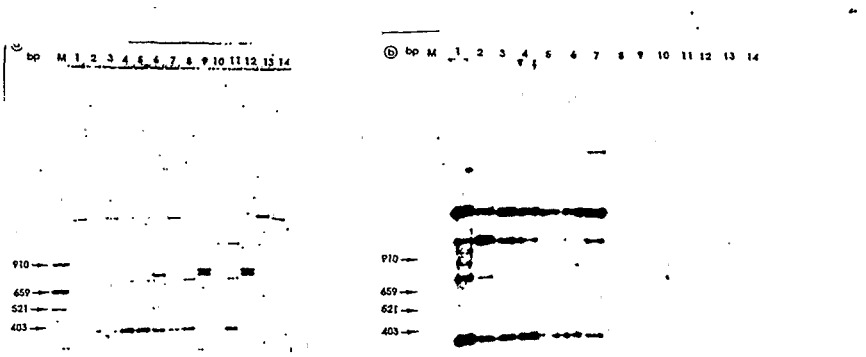

Fig. 4 : (a) Agarose electrophoresed total DNA RFLPs generated by Hae III : lane M : molecular weight marker λ–Pst1, lanes 1 to 7 : clone 39 (SC43 cl1, MN, NR, TPK1, Bug 2145, Bug 2148, SO3), lanes 8 : clone 33 (CBB), lane 9 : clone 32 (TU18 cl2), lane 10 : clone 30 (Esmeraldo cl3 "z2"), lane 11 : clone 43 (Tulahuen FKII 2), lane 12 : clone 27 (Can III cl1 "z3"), lane 13 : T. rangeli (Rangeli, Basel), lane 14 : T. cruzi marinkelei (TMB7) ; (b) Specific hybridization of SC43 cl 1 HVRm 39 probe with the lanes (1 to 8) containing only strains pertaining to clone 39 and slight differences between the patterns. Post treatment hybridizations were carried out under high stringency conditions (0.1 x SSPE and 0.1% SDS at 68°C) and then autoradiographed on MP films with intensifying screens (Amersham, UK) for 20 hours at -70°C.

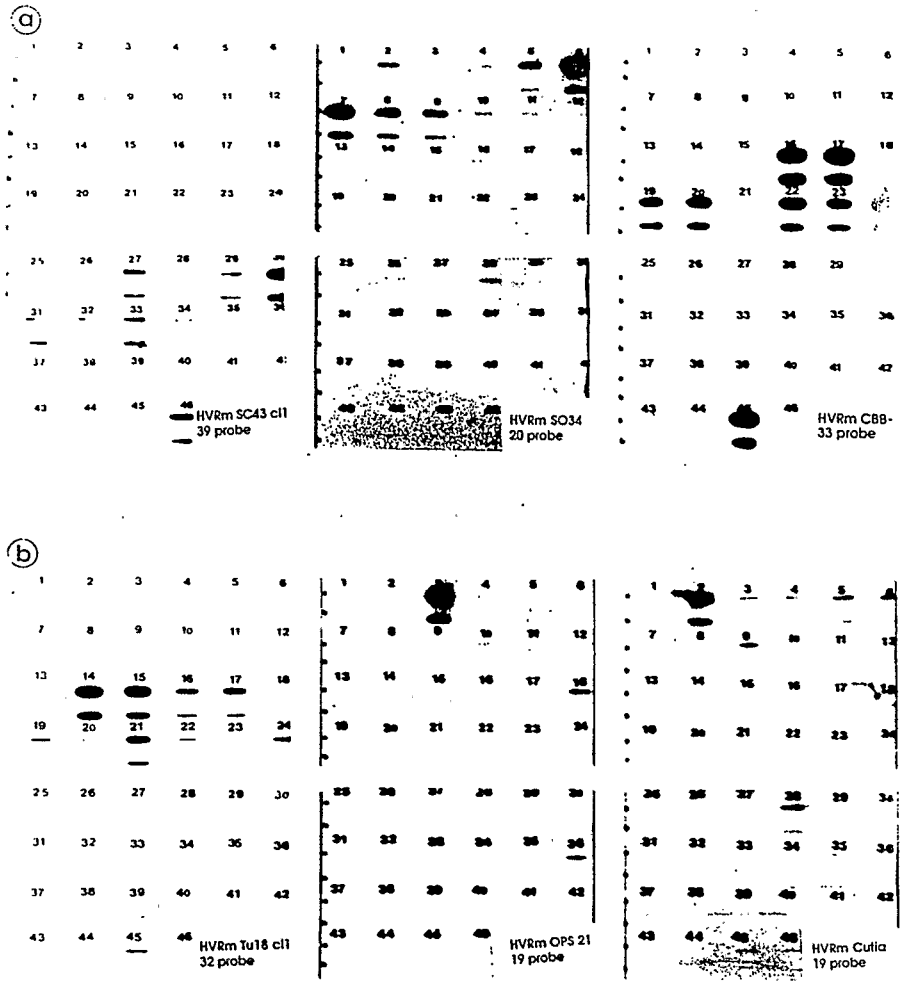

Fig. 5 : Six replicated sets of 46 slot blotted stocks (of total DNAs at 2 concentrations : (a) : 300 ng, (b) : 30 ng or purified kDNAs at 2 concentrations (a) : 30 ng, (b) : 3 ng were challenged for hybridization with six differents HVRm probes : HVRm SC43 cl1-39 probe, HVRm SO 34-20 probe ; HVRm CBB-33 probe ; HVRm TU 18 cl 2-32 probe ; HVRm OPS 21-19 probe and HVRm Cutia-19 probe. The T. cruzi stocks were disposed as follows : Slots 1 : clone 17 (X10 cl1 "Z1"), slots 2-4 : clone 19 (Cutia, OPS21, 13379 cl7), slots 5 : related to clone 19 (LGN), slots 6-9 : clone 20 (SO34, P/11, Esquilo, Culca), slots 10,11 : clone 21 (OPS4, OPS22), slots 12 : clone 27 (CANIII cl1 "Z3"), slots 13 : clone 30 (Esmeraldo cl3 "Z2"), slots 14,15 : clone 32 (TU18 cl2, MXCH88), slots 16,17 : clone 33 (CBB, MCH3), slots 18 : stock without isoenzyme characterisation (unnamed), slots 19,20,21,22-24) related to clone 33 (MCV, MXCH89, Bug 1738, MXCH46, MXCH80), slots 25 : clone 35 (M6241 cl6), slots 26 : clone 36 (M5631 cl5), slots 27 : clone 39 (SC43 cl1), slots 28 : related to clone 19 (SO30), slots 29-33 : clone 39 (TPK1, NR, MN, Bug 2145, Bug 2148), slots 34 : related to 39 (GR), slots 35 : clone 43 (Tulahuen FKIIA cl2), slots 36 : related to clone 19 (Florida), slots 37 : T. rangeli (Rangeli, Basel), slots 38 : T. cruzi marenkellei (TMB7), slots 39-41 : African trypanosomes (Byamina, TW53, Eatro), slots 42-44 : Leishmania (Lem1139, Lem 536, Lem 250), slots 45 : clone 33 (CBB), slots 46 : clone 39 (MN). Slots DNAs of n° 15, n° 17 to 24, n° 34 and n° 42 to 46 were purified kDNAs the others were total DNAs. The different radiolabelled probes were hybridized with stocks pertaining to different clones of T. cruzi ; the membranes were washed under high stringency conditions (0.1 x SSPE and 0.1% SDS at 68°C) and then autoradiographed on MP films with intensifying screens (Amersham, UK) for 20 hours at -70°C.

- CBB HVRm 33 probe is highly specific of all stocks pertaining to major clone n° 33 identified by isoenzymes and with MXCH 88, MXCH 46, MXCH 89 and Bug 1738 stocks presenting identical kDNA RFLP profiles (Carreno et al., 1987) to those stocks as clone 33 as shown on slot blots (Fig. 5a) and on HVRm from PCR as shown in Figure 6a.

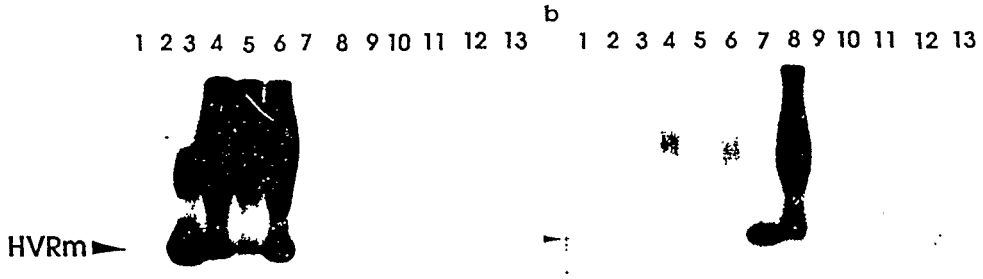

Fig. 6 : Specificity of radiolabelled (a) CBB HVRm 33 and (b) TU 18 cl 2 HVRm 32 probes on challenge of 13 different electrophoresed HVRm targets, lane 1 : clone 39 (SC43 cl1), lane 2 : clone 43 (Tulahuen FKIIA cl2), lanes 3and 4 : clone 33 (CBB, MCH3), lanes 5 and 6 : isoenzymatically undeterminated clones (Bug 1738, MCV), lane 10: clone 19 (Cutia), lane 11: clones 30 (Esmeraldo cl3 "Z2"), lane 12 : clone 27 (CAN III cl1 "Z3"), lane 13 : clone 17 (X10 cl1"Z1"). Hybridization and washing condition were the same as above.

Probes exhibiting self-specificity

HVRm purified fragments from stocks OPS 21 and Cutia (both stocks pertaining to clone 19) shows a high hybridation signal restricted only to themselves. These results were evidenced by hybridation with slot blotted DNAs (Fig. 5b) and with pocket-blotted RLFP of total DNAs and PCR products from different T. cruzi stocks (Fig. 7). However, the Cutia HVRm 19 probe exhibited a weak hybridization with stocks pertaining to either clone 19 or to clone closely related to itself. The OPS 21 HVRm 19 probe unlike Cutia HVRm 19 probe exhibit a weak hybridization with Florida stock (clone closely related to clone 19).

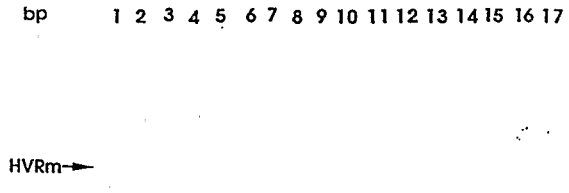

Fig. 7 : Different HVRm PCR-amplified and electrophoresed into a minigel, transferred onto nylon charged membrane and subsequently hybridized with the HVRm OPS 21 probe "cold-labelled" with digoxigenine from the non-radioactive kit of Boehringer. Lanes 1 to 4 : clone 20 (SO34, P/11, Cuica, Esquilo), lanes 5 to 7 : clone 19 (OPS21, Cutia, 13379 cl7), lane 8 : related to clone 19 (LGN), lanes 9, 10 : clone 21 (OPS22, OPS4), lane 11 : clone 17 (X10 cl1 "Z1"), lane 12 : clone 33 (CBB), lane 13 : clone 32 (TU18 cl2), lane 14 : clone 30 (Esmeraldo cl3 "Z2"), lane 15 : clone 27 (CANIII cl1 "Z3"), lane 16 : clone 43 (Tulahuén FKIIA cl2), lane 17 : clone 39 (SC43 cl 1). This Figure shows a single hybridization exhibiting an example of a self-specific probe of the OPS 21 stock (lane n° 5).

DISCUSSION

In a general way, DNA probes can be constructed to detect parasites following two starting approaches : (i) probes aiming to label stocks which share given relevant medical properties (virulence

(Tannich et al., 1989), resistance to drugs, etc), independently of any evolutionary or taxonomical consideration; (ii) conversely, probes based upon a firm taxonomical background, aiming to label actual taxa (subspecies, species, etc), independently, at least in a first time, of any medical consideration. In the present work, we have definitely followed the second approach: the precise goal was to get highly sensitive and specific probes to label discrete subdivisions of the taxon T. cruzi, namely the natural clones of this parasite. It should be emphasized that none of the probes obtained by other authors by use of various procedures (Macina et al., 1987 ; Moser et al., 1989 ; Sanchez et al., 1984; Sturm et al.,1989) presented such a precise subspecificity ascertained by a rigorous population genetic approach. Moreover, we have shown that PCR is an efficient method to generate large quantities of HVRm fragments (Veas et al., 1991) which can be used as clone-specific probes as well as a very sensitive method to detect parasites.

Our results gives strong elements to confirm our set of working hypothesis :

(i) lastly, our approach was based upon working hypothesis which in turn are corroborated by the successful obtention of the probes, namely the clonal structure of T. cruzi natural populations (Tibayrenc et al., 1981, 1986 ; Tibayrenc and Brenière, 1988)

(ii) the parallel evolution of nuclear and kDNA (Tibayrenc and Ayala, 1987 ; Veas et al., 1990) : if recombination occurred frequently in populations of the parasite, it would be impossible to elaborate probes specific of given parasitic lines: radically dissimilar T. cruzi genotypes (according to isozyme labeling) could hybridize with the same probe, while similar or identical genotypes could hybridize with different probes. The converse picture obtained here strongly suggests that the genes labelled by isozyme electrophoresis are strongly linked to those genes labelled by the probes, which is expected in the case of clonality. Moreover, this pattern shows that the strong linkage disequilibrium expected in clonality is not limited to the nuclear genome, but involves the kinetoplast genome too, as evidenced by the fair correspondence between kDNA-probe and isozyme labelings. The few observed exceptions could be explained by selection of some rare kDNA families of minicircles, or by some differences of evolution rates of minicircles from one natural clone to another. The rough fact remains that nuclear and kinetoplast evolutions are not independent in T. cruzi, in this aspect at least, minicircles cannot be equated to bacterial plasmids, as proposed elsewhere (Ray, 1987 ; Thomson and Lymberly, 1988). Indeed, in bacteria, chromosomal and plasmidic evolutions appear as largely independent (Miller and Hartl, 1986). As emphasized elsewhere (Tibayrenc and Ayala, 1988) the stocks characterized as identical on the basis of a few genetic markers are not necessarily a completely homogeneous set, but rather are families of related clones. A broader range of genetic markers would uncover additional variability within each set of "identical" clones. The PCR experiments developped here could be useful to resolve this residual variability, by using two approaches: (i) the probes could be used to resolve RFLPs by hybridization, in order to check genetical relationships among isolates, although this approach is difficult to interprete in a Mendelian fashion. (ii) PCR experiments could be used to study the different HVRm sequences which can be individualized in those natural clones where the probes are only self-specific (clone n°19).

(iii) It is interesting to note that the general structure of kDNA minicircle seems to be fairly conserved, as shown by the constant amplification of HVRm by PCR obtained in all T. cruzi stocks, as well as in the related taxa T. rangeli and T. cruzi marenkellei. In this latter taxon, HVRm exhibits a higher molecular weight, but this fact should be checked on a broad range of stocks. The conservation of this structure and PCR technology (with our oligoprimers CV1 and CV2) could allow specific (after hybridization) and non specific (after PCR amplification) detection to prevent transmission via blood transfusion.

Our probes, specific of circulating major clones in some endemic areas allow us to extend these work in running field studies in several South American countries with the aim to establish biogeographical

characteristics of these clones and to assess their prevalence levels and their consequences on mixed infections within the same host in transmission pathology. Furthermore, we can reasonably think that clinical accurate observations could help us to get a better understanding about the possible existant correlations between these genetic entities and well identified bioclinical factors at least as exist in Leishmania.

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