ORSTOM
Institut Français de Recherche Scientifique pour le développement en Coopération

DOSSIER D'HABILITATION A DIRIGER DES RECHERCHES

présenté par

Simone Frédérique Brenière

Juillet 1995

SOMMAIRE

CURRICULUM VITAE

PUBLICATIONS

CARRIERE SCIENTIFIQUE

RESUME DES TRAVAUX

CURRICULUM VITAE

1 - ETAT CIVIL

Nom : Brenière épouse Campana
Prénoms : Simone Marie Viviane, prénom usuel : Frédérique
Née le : 24 mars 1955 au Plessis Trévise, Val-de-Marne
Nationalité : Française
Mariée : 3 enfants.

Chargée de recherche de 1ère classe à l'Institut Français de Recherche Scientifique pour le Développement en Coopération, ORSTOM : Unité Mixte de Recherche CNRS/ORSTOM n° 9926 "Génétique Moléculaire des Parasites et des Vecteurs", B.P. 5045, 34032 Montpellier Cédex, France.

Adresse actuelle : ORSTOM CP 9214, La Paz-Bolivie, fax : 39 18 54, tel. 37 52 80.

2 - ETUDES SUPERIEURES ET DIPLOMES

1972-1973 - Préparation à l'école Vétérinaire de Maison Alfort.

1973-1974 - **D.U.E.S. de Chimie-Biologie** - Université de Paris VI - Mention AB.

1975-1976 - **Certificat de Pathologie et Immunologie Parasitaire** - C.H.U. La Pitié Salpétrière - Service des Maladies Tropicales Parasitaires - Professeur M. Gentilini.

1974-1976 - **Maîtrise de Biologie Animale** - Université de Paris VI - Mention AB.

1976-1978 - **Maître Es Sciences** - Université de Montréal, Département de Microbiologie et Immunologie - Laboratoire du Professeur Pierre Viens - Montréal - Canada - (Boursière "France-Québec").

1978-1982 - **Thèse de Doctorat en Sciences de Biologie de la Reproduction et du Développement (3ème cycle)**, "Infection humaine par *Trypanosoma cruzi* (maladie de Chagas) en Bolivie à différentes altitudes : réponse immune humorale", Laboratoire du Professeur André Capron - Université des Sciences et Techniques de Lille - Mention Très bien.

3 - FONCTIONS ET STRUCTURES D'ACCUEIL

3.1. Avancements

- Assistant-chercheur de la Coopération technique, affecté à l'Institut Bolivien de Biologie d'Altitude (1979-1982)
- Elève chercheur de l'Institut Français de Recherche Scientifique pour le Développement en Coopération, ORSTOM , Novembre 1982-1984.
- Chargée de Recherche 2ème classe, Janvier 1985.
- Chargée de Recherche 1ère classe, Janvier 1991.

3.2. Affectations

- De Novembre 1979 à Décembre 1987 : Affectation au titre de la Coopération puis de l'ORSTOM à l'Institut Bolivien de Biologie d'Altitude de La Paz en Bolivie.
- De Janvier 1988 à Novembre 1991 : Affectation au centre ORSTOM de Montpellier, laboratoire de Génétique des Parasites et des Vecteurs (devenu en janvier 1992 l' UMR CNRS/ORSTOM 9926 : "Génétique Moléculaire des Parasites et des Vecteurs").
- A partir de Novembre 1991 : Affectation à l'Institut Bolivien de Biologie d'Altitude de La Paz, en Bolivie.

4 - ENSEIGNEMENT

4.1. Académique

Université de Montréal

Date : 1977-1978. **Durée** : 20 heures. **Fonction** : Moniteur de travaux pratiques de Protozoologie (cursus Sciences Biologiques), Professeur responsable P. Viens.

Date : 1978. **Durée** : 30 heures. **Fonction** : Moniteur de travaux pratiques d'histologie (cursus médecine), Professeur responsable P. Messier.

Institut Bolivien de Biologie d'Altitude (IBBA) - La Paz -

Cours International d'Immunologie Parasitaire et Maladie de Chagas

Date : 1980. **Durée** : 6 heures. **Thèmes présentés** :

- Réponse immune humorale au cours de la maladie de Chagas
- Réponse autoimmune au cours de la maladie de Chagas

Cours International d'Entomologie Médicale, nouveaux progrès

Date : 1994. **Durée** : 3 heures. **Thèmes présentés** :

- Génétique moléculaire : généralités
- Techniques directes d'analyse du génome
- Isolement, culture et caractérisation génétique des souches de *Trypanosoma cruzi*.

Société Bolivienne de Biochimie et Clinique

Date : 1982. **Durée** : 2 heures. **Thème présenté** :

- Relation hôte/parasite au cours de la maladie de Chagas.

Date : 1992. **Durée** : 4 heures. **Thèmes présentés** :

- Electrophorèse d'isoenzymes
- La théorie clonale chez les Protozoaires
- La technique de PCR
- Caractérisation par PCR des clones majeurs de *Trypanosoma cruzi*

Université "Mayor de San Andres" - La Paz -

Date : 1982. **Durée** : 2 heures. **Thème présenté** :

- Réponse humorale au cours de la Maladie de Chagas dans le cadre d'un séminaire sur les Maladies Tropicales.

Date : 1992. **Durée :** 1 heure. **Thème présenté :**

- La technique de PCR - Cursus de la Faculté des Sciences de Pharmacie et Biochimie, cours d'Immunoparasitologie.

Date : 1994. **Durée :** 2 heures. **Thème présenté :**

- Application de la technique de PCR au diagnostic de la maladie de Chagas et en taxonomie de *Trypanosoma cruzi.* Cours international de "post-grado" : techniques de PCR, et applications au diagnostic biomédical et contrôle du milieu ambiant.

Date : Juin 95 - Novembre 95. **Durée** : 2 heures / semaine. **Fonction** : Direction de travaux dirigés dans le cadre de la "Maestria de biologie médicale" mention biologie moléculaire. Cette mention concerne 5 étudiants.

Programme : bases théoriques de la biologie moléculaire, entraînements à l'expression orale et écrite.

Centre National des Maladies Tropicales (CENETROP)-Santa-Cruz

Cours de recyclage en immunologie parasitaire.

Date : 1984. **Durée :** 3 heures. **Thème présenté :**

- Le diagnostic immunologique.

Cours basique de Médecine tropicale.

Date : 1994. **Durée :** 4 heures. **Thèmes présentés :**

- Diagnostics de la maladie de Chagas, des Leishmanioses et du Paludisme
- La technique de PCR.

Université des Sciences du Languedoc - Montpellier -

Cursus DEA de Parasitologie

Date : 1990. **Durée :** 2 heures. **Thème présenté :**

- La maladie de Chagas

Université de Pharmacie de Valence - Espagne -

Cursus Maîtrise Européenne des Maladies Parasitaires Tropicales

Date : 1990. **Durée** : conférence 4 heures, travaux pratiques 4 heures. **Thème présenté :**

- La maladie de Chagas

4.2. Formation à la recherche

4.2.1. Formation d'étudiants

Etudiants en thèse

Susana Revollo : Thèse de licence en Biochimie-Pharmacie, Université "Mayor de San Andrès de La Paz, Bolivie, 1987. "Analyse antigénique des différentes souches isoenzymatiques de *Trypanosoma cruzi* : reconnaissance des épitopes du composant antigénique 5 à l'aide d'anticorps monoclonaux." Encadrement sous mon entière responsabilité. Ce travail a également fait l'objet de la publication A32 et d'une communication à un congrès (C11). J'ai par ailleurs assuré l'encadrement de Susana Revollo en ce qui concerne la génétique des populations et le typage des souches de parasites. Dans ce cadre, Susana Revollo est co-auteur des publications suivantes : A6, A16, C8, C12, C13. Par ailleurs, en 1991 j'ai rédigé une demande de bourse auprès de l'OMS pour que Susana Revollo puisse effectuer son doctorat d'Université à Montpellier sous la responsabilité du Michel Tibayrenc, dans la mesure où ma nouvelle affectation me ramenait en Bolivie.

Françoise Mathieu-Daudé : Thèse de doctorat, Université de Montpellier II, 1991. Encadrement de la partie biologie moléculaire de son travail. Les résultats ont été exploités dans sa thèse : "Mode de reproduction de *Trypanosoma brucei* dans ses populations naturelles : implications taxonomiques et épidémiologiques", dans l'article A4 et ~~au cours~~ de congrès (C3 et C4).

Adrian Ovando : Thèse de licence en Biologie, Université "Mayor de San Simon" de Cochabamba, Bolivie, 1993. " Analyse génétique par isoenzymes de *Triatoma infestans* (Hemiptera-Reduviidae) vecteur principal de la maladie de Chagas en Bolivie." Cette thèse, déposée pour corrections auprès du directeur, n'a pas été soutenue pour raisons personnelles au candidat. Encadrement sous mon entière responsabilité. A l'occasion des "Journées Internationales de Biophatologie Andine" organisées en 1992 à l'IBBA, l'étudiant a présenté oralement une partie de ses travaux (C5).

Jenny Telleria : Thèse de licence en Biochimie-Pharmacie, Université "Mayor de San Andrès" de La Paz, Bolivie, 1995 " Détection de deux clones majeurs de *Trypanosoma cruzi* dans des vecteurs domestiques et sylvestres : implications épidémiologiques." Encadrement sous mon entière responsabilité. Cette étudiante est co-auteur de deux publications de rang A (A1 et A2), d'un article publié dans une revue argentine (A27) et de communications lors de congrès (C1 et C5).

Jose Luis AlcazarDalenz : Thèse de licence en Biologie, Université Mayor de "San Simon" de Cochabamba, Bolivie. " Etude de la microdistribution des populations de*Triatoma infestans* et des clones majeurs de *Trypanosoma cruzi* dans une zone sub-urbaine de la ville de Cochabamba." Cette thèse est sous co-responsabilité avec le Dr. F. Noireau. En cours.

Willson Morochi : Thèse de licence en Biochimie-Pharmacie, Université "Mayor de San Andrès de La Paz, Bolivie. "Caractérisation génétique des clones de *Trypanosoma cruzi* circulant dans les insectes du complexe *Triatoma sordida* par isoenzymes et PCR. En cours. Encadrement sous mon entière responsabilité.

Vient de s'ouvrir à l'Université de La Paz une nouvelle formation post-doctoral (maestria) en biologie médicale dont 3 étudiants réaliseront leur travail dans le laboratoire.

Jenny Telleria : Thèse de "maestria" mention biologie moléculaire. Son sujet concerne le développement de sondes de leishmanies circulant en Bolivie : outils épidémiologiques. Encadrement sous mon entière responsabilité. Débute actuellement.

Giovanni Rodrigo Garcia Rada : Thèse de "maestria" mention biologie moléculaire. Son sujet concerne la recherche de nouveaux marqueurs du complexe *Triatoma sordida* par la tecnique du RAPD. Encadrement sous mon entière responsabilité. Débute actuellement.

Jorge Antonio Nogales Vera : Thèse de "maestria" mention biologie moléculaire. Son sujet concerne l'étude de l'éfficacité de traitements de la maladie de Chagas en fonction de la variabilité génétique de *Trypanosoma cruzi*. Encadrement en collaboration avec S. Revollo . Débute actuellement.

Etudiants en DEA

Katarzyna Lewicka : DEA de Parasitologie, , 1991) : Université de Montpellier II, 1991. Encadrement complet de la rédaction du DEA : Etude de la variabilité génétique de *Trypanosoma cruzi* en Guayane française. Ces résultats sont également présents dans l'article A 3.

Katiaj Neubauer : DEA de l'Université de Constance (Allemagne) 1992. Encadrement de la partie biologie moléculaire de son travail. Ces résultats sont exploités dans son mémoire : Microevolution of *Trypanosoma cruzi* natural clones in Chile and Paraguay.

Volontaires du Service National (VSN)

Eric Valette, VSN à l'IBBA de 1984 à 1985. Formation de base aux isoenzymes et étude du réservoir sylvestre de *Trypanosoma cruzi* sous mon entière responsabilité. Résultats dans l'article A31.

Eric Prina, VSN à l'IBBA de 1984 à 1985. Etude des antigènes de surface de leishmanies. Encadrement partagé.

Ivan Araniki, VSN à l'IBBA 1987-1988. Etude des antigènes de surface de différents clones de *T. cruzi* sous mon entière responsabilité. Exploitation des résultats dans l'article A8.

4.2.2. Formation et encadrement de personnel technique

Clara Camacho et *Hortencia Miguez* **Statut :** techniciennes IBBA, **Années :** 1981 à 1983, **Durée :** 2 ans, **Thème :** Formation aux techniques sérologiques et immunologiques appliquées à la maladie de Chagas (voir les publications A2, A36 et C5).

Nina Yaksic, **Statut :** technicienne IBBA, **Années :** 1992-1993, **Durée :** 2 ans, **Thème :** formation aux techniques d'isoenzymes et de biologie moléculaire. Cette technicienne travaille sous mon entière responsabilité depuis 3 ans. (voir les publications A1, A2, A5, et C5).

Marie France Bosseno, **Statut :** technicienne puis assistant ingénieur depuis 2 ans ORSTOM, **Année :** 1991, **Durée :** 1 an, **Thème :** Formation aux techniques de base de la biologie moléculaire. *Marie-France Bosseno*, travaille sous mon entière responsabilité depuis 1990. Sa collaboration à Montpellier puis actuellement en Bolivie, est d'une aide précieuse dans mes fonctions d'animation et d'encadrement du laboratoire. D'autre part, nous la soutenons dans son effort de rédaction de résultats. (voir les publications A1, A2, A4, A5, A6, A27, A28, A29, A30, C1 à C6, C8 et C10

Fernando Vargas, **Statut :** Etudiant travailleur Bolivien, **Année :** 1992, **Durée :** 1 an, **Thème :** Formation aux techniques d'isoenzymes appliquées aux vecteurs et aux parasites de la maladie de Chagas.Travaille sous ma co-responsabilité avec le Dr F. Noireau depuis 3 ans (voir les publications A1, A2, A28 et C5)

Lieve Carlier, technicienne temporaire, a travaillé sous ma responsabilité pendant 1 an à des essais de clonage des sondes utilisées dans le laboratoire. Le travail développé par cette technicienne a été poursuivi par Jenny Telleria et les résultats exploités dans sa thèse de licence.

4.2.3. Encadrement de stagiaires

Philippe Lena, **Statut :** post doctorat, boursier du MRT, **Année :** 1990, **Durée :** 6 mois, **Thème :** Application des marquages froids aux sondes de *T. cruzi* spécifiques des clones majeurs.

Brigitte Bastrenta , **Statut :** post doctorat, **Année :** 1991, **Durée :** 1 an, **Thème :** Etude du polymorphisme de longueur des fragments générés par les enzymes de restriction (RFLP) de l'ADN satellite de *Trypanosoma cruzi.*

Dr. *François Noireau*, **Statut :** Chercheur ORSTOM, **Année :** 1992, **Durée :** 1 an, **Thème :** Formation théorique et pratique à l'étude de la génétique des populations de vecteurs par isoenzymes.

Jean-Charles Soria, **Statut :** Etudiant en médecine (Paris 4ème année) **Année :** 1992, **Durée :** 3 mois, **Thème :** Application de la technique de polymerisation en chaîne (PCR) à la détection de *Trypanosoma cruzi* dans les fèces de vecteurs. Rapport de stage.

Dr. *Graciela Russomando*, **Statut :** Chercheur de l'IICS d'Asunción, Paraguay **Année :** 1992, **Durée :** 15 jours, **Thème :** Caractérisation des clones de *Trypanosoma cruzi* par les techniques de polymerisation en chaîne (PCR) et d'hybridation.

Dr. *Patricia Dorn*, accompagné d'un étudiant, **Statut :** Chercheur du " Hope College, Michigan, USA **Année :** 1992, **Durée :** 2 mois, **Thème :** Techniques de terrain appliquées en épidémiologie de la maladie de Chagas ; techniques de caractérisation des clones de *Trypanosoma cruzi.*

Jenny Lopez, **Statut :** Chargée de cours à l'Université Catholique d'Aréquipa, Pérou **Année :** 1992 et 1993, **Durée :** 3 semaines, **Thème :** Etude de la variabilité

isoenzymatique de populations de *Triatoma infestans* et *Trypanosoma cruzi* de la région d'Aréquipa.

Dr. *Mariana Ceriotto*, **Statut :** Chercheur de la Faculté de Médecine de l'Université de Buenos- Aires, Argentine **Année :** 1994, **Durée :** 1 semaine, **Thème :** Application de la technique de PCR au xénodiagnostic.

Marleny Montilla, **Statut :** Etudiante de l'Université "de los Andes" de Bogota, Colombie **Année :** 1994, **Durée :** 3 semaines, **Thème :** Caractérisation isoenzymatique d'isolats Colombiens de *T. cruzi* .

Nicolas Schweigmann, **Statut :** Chercheur de la Faculté des Sciences de l'Université de Buenos- Aires, Argentine **Année :** 1994, **Durée :** 1 semaine, **Thème :** Détection de *T. cruzi* dans des vecteurs sylvestres par PCR.

PUBLICATIONS

1 - MEMOIRES

Titre : L'infection à *Trypanosoma musculi* chez la souris nouveau-née et le souriceau.
Lieu : Université de Montréal, Canada
Date : 25 Septembre 1978
Directeur : Dr. Pierre Viens
Grade obtenu : Maître Es Sciences

Titre : Génétique et taxonomie biochimique de *Trypanosoma cruzi* et quelques implications en immunologie et pathologie.
Lieu : Recherches effectuées à l'IBBA de La Paz, Bolivie
Date : Septembre 1984
Directeur : Dr. Michel Tibayrenc
Grade obtenu : Titularisation ORSTOM

2 - THESE

Titre : Infection humaine par *Trypanosoma cruzi* (maladie de Chagas) en Bolivie à différentes altitudes : réponse immune humorale.
Lieu : Université de Sciences et Technologies de Lille - Recherches effectuées à l'IBBA de La Paz -
Date : Janvier 1982
Jury : Président , Pr Vivier; rapporteur, Pr Carlier, Membres, Pr Capron, Pr Dhainaut, Dr Afchain
Grade obtenu : Doctorat de 3ème cycle en Biologie de la Reproduction et du Développement, option : Biologie Cellulaire, Protistologie et Parasitologie.

3 - REVUES SCIENTIFIQUES A COMITE DE LECTURE

(La mention "sous presse' signifie : "définitivement accepté par le journal")

3.1. Articles de rang A

1995

A1 - **Brenière** S.F., Bosseno M.F., Telleria J., Carrasco R., Vargas F., Yaksic N. & Noireau F. - Field application of PCR diagnosis and strain typing of *Trypanosoma cruzi* in Bolivian triatomines. *Am. J. Trop. Med. Hyg.*, sous presse.

A2 - Noireau F., Bosseno M.F., Carrasco R., Telleria J., Vargas F., Camacho C., Yaksic N. & **Brenière** S.F. - Sylvatic triatomines in Bolivia : trends toward domesticity and infection with *Trypanosoma cruzi* clones known from the domestic cycle. *J. Med. Entomol.*, sous presse.

A3 - Lewika, K., **Brenière**, S.F., Barnabé, C., Dédet, J.P. & Tibayrenc, M. An isoenzyme survey of *Trypanosoma cruzi* genetic variability in sylvatic cycles from French Guiana. *Exp. Parasitol.*, sous presse.

1994

A4 - Mathieu-Daudé F., Bicard-See A., Bosseno M.F., **Brenière** S.F. & Tibayrenc M. 1994. - Identification of *Trypanosoma brucei gambiense* group I by a specific kinetoplast DNA probe. *Am. J. Trop. Med. Hyg.*, **50**, (1), 13-19.

A5 - Wincker P., Bosseno M.F., Britto C., Yaksic N., Cardoso M.C., Morel C.M. & **Brenière** S.F. 1994 - High correlation between Chagas'disease serology and PCR-based detection of *Trypanosoma cruzi* kinetoplast DNA in Bolivian children living in an endemic area. *FEMS Microbiology letters*, **124** : 419-424.

1992

A6 - **Brenière** S.F., Bosseno M.F., Revollo S., Rivera M.T., Carlier Y. & Tibayrenc M. 1992. - Direct identification of *Typanosoma cruzi* natural clones in vectors and mammalian hosts by polymerase chain reaction amplification. *Am. J. Trop. Med. Hyg*, **46** (3), 335-341.

A7 - Solari A., Muñoz S., Venegas J., Wallace A., Aguilera X., Apt W., **Brenière** S.F. & Tibayrenc M. 1992. - Characterization of Chilean, Bolivian and Argentinian *Trypanosoma cruzi* populations by restriction endonuclease and isoenzyme analysis. *Exp. Parasitol.*, **75** , 187-195.

1991

A8 - **Brenière** S.F., Araniki I., Le Ray D.& Tibayrenc M. 1991. - L'analyse SDS des protéines et antigènes de surface révèle une forte hétérogénéité chez les clones naturels de *Trypanosoma cruzi*, corrélée à la variabilité isoenzymatique. *C.R.Acad. Sci. Paris*, **t. 312, Série III**, 449-454.

A9 - **Brenière** S.F., Braquemond P., Solari A., Agnèse J.F. & Tibayrenc M. 1991. - An isoenzyme study of *Trypanosoma cruzi* natural clones isolated from the two sides of the West Andes Highland. *Trans. R. Soc. Trop. Med. Hyg.*, **85**, 62-66.

A10 - Kutner S., Pellerin P., **Brenière** S.F., Desjeux P.& Dedet J.P. 1991. - Antigenic specificity of the 72-kilodalton major surface glycoprotein of *Leishmania braziliensis braziliensis. J. Clin. Microbiol.* , **23** , 26-59.

A11 - Tibayrenc M., Kjellberg F., Arnaud J., Oury B., **Brenière** S.F., Dardé M.L. & Ayala F.J. 1991. - Are eucaryotic microorganisms clonal or sexual ? A population genetics vantage. *Proc. Natl. Acad. Sci. USA* , **88** , 5129-5133.

A12 - Veas F., **Brenière** S.F., Cuny G., Brengues C., Solari A. & Tibayrenc M. 1991. - General procedure to construct highly specific kDNA probes for clones of *Trypanosoma cruzi* for sensitive detection by Polymerase Chain Reaction. *Cel. Molec. Biol.* , **37 (1)**, 73-84.

1990

A13 - Kutner S., Pellerin P. & **Brenière** S. F. 1990. - Identification of cytoplasmic soluble antigens related to the major surface antigens of *Leishmania braziliensis braziliensis* and of *Leishmania donovani chagasi. Parasitol. Research* ,**76**, 185-191.

A14 - Veas F., Cuny G., **Brenière** S.F. & Tibayrenc M. 1990. - Subspecific kDNA probes for major clones of *Trypanosoma cruzi. Acta Tropica* **48 (1)**, 79-82.

1989

A15 - **Brenière** S.F., Carrasco R., Antezana G., Desjeux P. & Tibayrenc M. 1989. - Association between *Trypanosoma cruzi* zymodemes and specific humoral depression in chronic chagasic patients. *Trans. R. Soc. Trop. Med. Hyg,* **83**, 517.

A16 - **Brenière** S.F., Carrasco R., Revollo S., Aparicio G., Desjeux P. & Tibayrenc M. 1989. - Chagas'disease in Bolivia : clinical and epidemiological features and zymodeme variability of *Trypanosoma cruzi* strains isolated from patients. *Am. J. Trop. Med. Hyg,* **41 (5)**, 521-529.

1988

A17 - Le Pont F., **Brenière** S.F., Mouchet J. & Desjeux P. 1988. - Leishmaniose en Bolivie. III, *Psychodopygus carrerai carrerai* (Barretto, 1946) nouveau vecteur de *Leishmania braziliensis* en milieu sylvatique de régions subandine basse. *C. R. Acad. Sci. Paris,* **307**, 279-282.

1987

A18 - **Brenière** S.F., Carrasco R., Molinedo S., Lemesre J.L., Desjeux P., Afchain D. & Carlier Y. 1987. - Specific immunodiagnosis of Chagas'disease : Immunodiffusion test using a specific serum anti - *Trypanosoma cruzi* component 5. *Trop. Geograph. Med.*, **39**, 281-286.

A19 - Legrand D., Desjeux P., Le Pont F., **Brenière** S.F., Lemesre J.L., Santoro F. & Capron A. 1987 - Identification of a major 72 kilodalton surface antigen in twelve

isolates of *Leishmania braziliensis braziliensis*. *Mol. Biochem. Parasitol.*, **24**, 117-124.

1986

A20 - Legrand D., Desjeux P. Prina E., Le Pont F. & **Brenière** S.F. 1986. - Résultats préliminaires en faveur de l'existence d'un antigène majeur de surface spécifique de *Leismania braziliensis braziliensis*. *C. R. Acad. Sci. Paris*, **303**, 607-612.

A21 - Lemesre J.L., Afchain D., Orozco O., Loyens M., **Brenière** S.F., Desjeux P., Carlier Y., Martin U., Nogueira-queiror A., Le Ray D. & Capron A. 1986. - Specific serodiagnosis of Chagas'disease by competitive inhibition enzyme immunoassay using a *Trypanosoma cruzi* species specific monoclonal antibody. *Am. J. Trop. Med. Hyg.*, **35 (1)**, 86-93.

A22 - Ribaute E., Lemesre J.L., Rodriguez C., Carrasco R., **Brenière** S.F., Antezana G., Raynaud J. & Carlier Y. 1986. - Bioenergetic and cardiovascular response to exercice in resident at 2850 m, with asymptomatic Chagas'disease. *Trop. Geograph. Med.*, **38**, 150-157.

1985

A23 - **Brenière** S.F., Carrasco R., Miguez H., Lemesre J.L. & Carlier Y. 1985. - Comparison of immunological tests for serodiagnosis of Chagas'disease in Bolivian patients. *Trop. Geograph. Med.*, **37**, 231-238.

A24 - **Brenière** S.F., Tibayrenc M., Antezana G., Pabon J., Carrasco R., Selaès H. & Desjeux P. 1985. - Résultats préliminaires en faveur d'une relation faible ou inexistante entre les formes cliniques de la maladie de Chagas et les souches isoenzymatiques de *Trypanosoma cruzi*. *C. R. Acad. Sci. Paris.*, **300 (15)**, 555-558.

1983

A25 - Tibayrenc M., Echalar L., **Brenière** F., Lemesre J.L., Barnabé C. & Desjeux P. 1983. - Sur le statut taxonomique et médical des souches isoenzymatiques de *Trypanosoma cruzi*. Considérations sur la valeur taxonomique et immunogénique des différentes isoenzymes. *C. R. Acad. Sci. Paris*, **296**, 721-726.

1980

A26 - **Brenière** S.F. & Viens P. 1980. - *Trypanosoma musculi* : transfer of immunity from mother to litter. *Can. J. Microbiol.*, **26**, 1090-1095.

3.2. Autres

1995

A27 - Bosseno M.F., Torrico F., Telleria J., Noireau F.& **Brenière** S.F. - Reacción de polimerización en cadena : detección y caracterización de cepas de *Trypanosoma cruzi*, en niños chagásicos. *Medicina.*, sous presse.

A28 - Noireau F., Bosseno M.F., Vargas F. & **Brenière** S.F. - Apparent trend to domesticity observed in *Panstrongylus rufotuberculatus* (Hemiptera : Reduviidae) in Bolivia. *Research and Rewiews in Parasitology,* sous presse.

1993

A29 - **Brenière** S.F., Bosseno M.F., Barnabé C., Urdaneta Morales S. & Tibayrenc M. 1993. - Copy number differences in the 195 bp repeated satellite DNA from *Trypanosoma cruzi* and *Trypanosoma rangeli* : potential use for epidemiologic survey. *Mem. Inst. Oswaldo Cruz,* **88 (1)**, 163-165.

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1987

A32 - **Brenière** S.F., Revollo S., Caillard T., Valette E., Legrand D., Afchain D. & Desjeux P. 1987. - *Trypanosoma cruzi* : expression of antigenic component 5 among 35 laboratory clones obtained from 18 isozymic variants. *Rev. Inst. Med. Trop. Sao Paulo,* **29**, 80-85.

1985

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A34 - Carlier Y., **Brenière** S.F., Lemesre J.L., Carrasco R., Desjeux P. & Afchain D. 1985. - The interest of immunoprecipitation tests in the immunological diagnosis of Chagas'disease. *Ann. Soc. belge Méd. trop.*, **65 (suppl. 1)**, 85-94.

A35 - Carlier Y., Ribaute E., Lemesre J.L., Rodriguez C., Carrasco R., **Brenière** S.F. & Raynaud J. 1985. - Bioenergetic studies in residents of high altitude (2850 m) with asymptomatic chagas'disease. *Ann. Soc. belge Méd. trop.*, **65 (suppl. 1)**, 51-57.

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A37 - Tibayrenc M., **Brenière** S.F., Barnabé C., Lemesre J.L., Echalar L. & Desjeux P. 1985. - Isozymic variability of *Trypanosoma cruzi* : biological and epidemiological significance. *Ann. Soc. belge Méd. trop.*, **65 (suppl. 1)**, 59-61.

1984

A38 - **Brenière** F., Poch O., Selaès H., Tibayrenc M., Lemesre J.L., Antezana G. & Desjeux P. 1984. - Specific humoral depression in chronic patients infected with *Trypanosoma cruzi*. *Rev. Med. Trop. Sao Paulo*, **26 (5)**, 254-258.

1983

A39 - **Brenière** S.F., Bailly M., Carrasco R. & Carlier Y. 1983. - Transmission transplacentaire des anticorps anti *Trypanosoma cruzi*. *Cah. ORSTOM, sér. Ent. méd. et Parasitol.* ,**21 (3)**, 139-140.

A40 - **Brenière** S. F. & Tibayrenc M. 1983. - Activité Glutamate déshydrogénase Nadp+ d'une fraction antigénique immunogénique de *Trypanosoma cruzi*. *Cah. ORSTOM sér. Ent. méd. parasitol.*, **21 (1)**, 53-55.

1981

A41 - Tibayrenc M., **Brenière** S.F., Echalar L. & Carlier Y. 1981. - Données isoenzymatiques pour onze souches boliviennes de *Trypanosoma cruzi*. Interprétation génétique et calcul de distances. *Cah. ORSTOM sér. Ent. méd. Parasitol.*, **19 (2)**, 129-134.

4 - REVUES SCIENTIFIQUES SANS COMITE DE LECTURE

Publications en espagnol internes à l'Institut Bolivien de Biologie d'Atitude

Traduction en espagnol : Brenière S.F., Tibayrenc M., Antezana G., Pabon J., Carrasco R., Selaès H. & Desjeux P. 1985.- Résultats préliminaires en faveur d'une relation faible ou inexistante entre les formes cliniques de la maladie de Chagas et les souches isoenzymatiques de *Trypanosoma cruzi*. *C. R. Acad. Sci. Paris.*, **300 (15)**: 555-558. **Dans :** *Anuario de l'IBBA*, 1986-1987.

Traduction en espagnol : Brenière S.F., LLanos B., Tibayrenc M. & Desjeux P. 1985. - Isoenzymic studies and epidemiological data of *Trypanosoma cruzi* from Arequipa (Peru), Pacific side. *Ann. Soc. belge méd. trop.* **65 (suppl.1)**: 63-66. **Dans :** *Anuario del IBBA*, 1986-1987.

Traduction en espagnol : Carlier Y., **Brenière** S.F., Lemesre J.L., Carrasco R., Desjeux P. & Afchain D. 1985. - The interest of immunoprecipitation tests in the immunological diagnosis of Chagas'disease. *Ann. Soc. belge Méd. trop.*, **65 (suppl. 1)**, 85-94. **Dans :** *Anuario de l'IBBA*,, 1986-1987.

Traduction en espagnol : Carrasco R., **Brenière** S.F., Poch O., Miguez H. Selaes H., Antezana G., Desjeux P. & Carlier Y. 1985. Chagas serology and its problems. *Ann. Soc. belge Méd. trop.*, **65 (suppl. 1)**, 79-84. **Dans :** *Anuario de l'IBBA*, 1986-1987.

Traduction en espagnol : Brenière S.F., Revollo S., Caillard T., Valette E., Legrand D., Afchain D. & Desjeux, P. 1987. - *Trypanosoma cruzi* : expression of antigenic component 5 among 35 laboratory clones obtained from 18 isozymic variants. *Rev. Inst. Med. Trop. Sao Paulo*, **29**, 80-85. **Dans :** *Anuario de l'IBBA*,, 1986-1987.

Brenière S.F., Carrasco R., Kutner S. 1988. - Inmunoparasitología en la enfermedad de Chagas. *IBBA Bodas de plata*, 1963-1988.

Brenière S.F., Carrasco R., Kutner S. 1988. - Leishmaniasis en Bolivia - Estudios epidemiológicos y bioquímicos. *IBBA Bodas de plata*, 1963-1988.

5 - COMMUNICATIONS A DES CONGRES ET RENCONTRES SCIENTIFIQUES

C1 - V Congrès Latino-Americain de Médicine Tropicale
Lieu : Guayaquil, Ecuador Date : 16-20 Mai 1993. Titre et auteurs de la communication : Detección de una infección infra-microscópica por *Trypanosoma cruzi* en triatominos silvestres con la téchnica PCR - Bosseno M.F., Noireau F., Telleria J., Carrasco R. & **Brenière** S. F.
Statut : co-auteur de poster

C2 - CYTED. Réunion sur la Biologie Moléculaire des Leishmanies et de *Trypanosoma cruzi.*
Lieu : Caracas, Vénézuela. Date : 1993. Titre et auteurs de la communication : Population genetics of *Trypanosoma cruzi* and *Trypanosoma rangeli* : taxonomical and epidemiological purpose - **Brenière** S.F., Bosseno M.F., Barnabé C., Urdaneta Morales S. & Tibayrenc M.
Statut : orateur invité et chairman de section.

C3 - Réunion annuelle de recherche fondamentale sur la maladie de Chagas.
Lieu : Caxambu, Brésil. Date : 1993. Titre et auteurs de la communication : PCR field aplication : geographic repartition of major *Trypanosoma cruzi* clones in vectors, advantage of isoenzyme typing - **Brenière** S.F. , Bosseno M.F., Mathieu Daudé F. & Tibayrenc M. 1993. Résumé : *Mem. Inst. Oswaldo Cruz, Rio de Janeiro*, V. 88, suppl., p.175.
Statut : ~~co-auteur~~ de Poster.

C4 - IV congrès régional de Protozoologie. Table ronde, développement technologique appliqué au diagnostic des parasitoses.
Lieu : Catamarca, Argentine Date : Novembre 1993. Titre et auteurs de la communication : Caracterisación de cepas de *Trypanosoma cruzi* con PCR y hybridización con sondas de DNA - **Brenière** S.F. , Bosseno M.F., Mathieu Daudé F. & Tibayrenc M.
Statut : co-auteur de communication orale.

C5 - III Journées internationales de Biopathologie Andine.
Lieu : La Paz, Bolivia. Date : 15-18 Septembre 1992. Titre et auteurs des communications :
*Estudio comparativo de la epidemiología de la enfermedad de chagas en dos áreas cercanas de la región de los Yungas Altas (Dpt. La Paz) - Ovando A., Vargas F., Camacho C., Carrasco R., Soria J.C., Noireau F., Bosseno M.F.& **Brenière** S.F.
*Repartición en mosáico de la infección por *Trypanosoma cruzi* en zona Sub-Andina alta (Dept. La Paz) - Vargas F., Ovando A., Urquieta R., Camacho C., Yaksic N., Tellerin J., Carrasco R., Noireau F., Bosseno M.F.& **Brenière** S.F.
*Técnica de PCR y de hibridación de ADN para la caracterización genética de los clones de *Trypanosoma cruzi* - Bosseno M.F., Soria J.C., Torrico F.,Telleria J., Camacho C., Tibayrenc M.& **Brenière** S.F.
*Estudios isoenzimáticos y de ADN de poblaciones de *Trypanosoma rangeli* y *Trypanosoma cruzi* permiten diferenciar los dos taxones - **Brenière** S. F., Bosseno M.F., Barnabé C., Yaksic N., Le Ray D.& Tibayrenc M.
*Presencia de varios vectores de la enfermedad de Chagas en zona sub-andina baja (departamento de La Paz) : implicaciones epidemiológicas - Noireau F., Carrasco R.,

Vargas F., Ovando A., Bosseno M.F. & **Brenière** S.F.
*Prevalencia de la infección humana por *T. cruzi* en Bolivia - Carrasco R., Miguez H., Camacho C.& **Brenière** S.F.
Résumés : *Acta Andina*, V2, 1993
Statut : orateur, co-auteur et organisateur de la section parasitologie.

C6 - Réunion annuelle de recherche fondamentale sur la maladie de Chagas.
Lieu : Caxambu, Brésil. Date : 7-9 Septembre 1992. Titre et auteurs de la communication : Population genetic analysis determine the taxonomic units for further biological and medical studies. Brenière S.F. , Bosseno M.F., Mathieu Daudé F. & Tibayrenc M.
Résumé : *Mem. Inst. Oswaldo Cruz, Rio de Janeiro,* 87 suppl 2, 104.
Statut : Présentation de poster.

C7 - Symposium de génétique moléculaire des parasites.
Lieu : Montpellier, organisé par le Laboratoire de Génétique des Parasites et des Vecteurs de l'ORSTOM. Date : 2-5 Février 1991. Titre et auteurs de la communication : Sondes d'ADN spécifiques des clones majeurs de *Trypanosoma cruzi* - **Brenière** S.F.
Statut : orateur, membre du comité organisateur.

C8 - ICASEP I
Lieu : Valencia, Espagne. Date : 1-5 Juillet 1991. Titre et auteurs de la communication : Characterization of *Trypanosoma cruzi* majors clones by PCR DNA probes : a new epidemiologic approach in Chagas'disease - **Brenière** S.F., Bosseno M.F., Revollo S., Tibayrenc M.
Statut : orateur

C9 - FLAP, X congrès Latinoamericain de Parasitologie.
Lieu : Caracas, Vénézuéla. Date : Juillet 1991. Titre et auteurs des communications :
* Estudio comparativo en medio de cultivo acelular de un stock de *Trypanosoma cruzi* con dos clones geneticamente homólogos - Penin P., Tibayrenc M, **Brenière** S.F.& de Diego J.A.
* Caracterización patológica y genética de un stock Boliviano de *Trypanosoma cruzi* - Penin P., Tibayrenc M, **Brenière** S.F., Gamallo C. & de Diego J.A.
Statut : co-auteur de communications orales

C10 - ICOPA
Lieu : Paris, France. Date : Août, 1990. Titres et auteurs des communications :
*Caractérisation des clones majeurs de *Trypanosoma cruzi* grâce à la "PCR" : quand et comment ? - **Brenière** S.F., Veas F., Cuny G., Bosseno M.F., Rivera M.T.& Tibayrenc M.
*Sondes d'ADN spécifiques des clones majeurs de *Trypanosoma cruzi* : instrument de recherche et de diagnostic pour la maladie de Chagas - Veas, F. **Brenière**, S.F., Cuny, G., Brengues, C.& Tibayrenc, M.
*Paralel evolution between kDNA and nuclear markers in *Leishmania* genus - Veas F., **Brenière** S.F., Bonhomme F., Cuny G. & Tibayrenc M.
*Specific recognition by sera from mucocutaneous leishmaniasis patients of 72 kDa antigens of *Leishmania braziliensis braziliensis* - Kutner S., Pellerin P., **Brenière** S.F., Desjeux P.& Dedet J.P

Résumés: *Bull. Soc. Fr. Parasitol.* 8 suppl. 1 : 708, 259, 258, 489.
Statut : orateur et co-auteur de posters

C11 - Réunion annuelle de recherche fondamentale sur la maladie de Chagas.
Lieu : Caxambu, Brésil. Date : Septembre 1988. Titres et auteurs des communications :
*Specific recognition of the surface major 72 kDa antigen from *Leishmania braziliensis braziliensis*. by sera from patients suffering of cutaneous and mucocutaneous Leishmaniasis - Kutner, S. Pellerin P., **Brenière** S.F. & Dedet J.P.
*Identification and purification of a 72 kDa cytoplasmique glycoprotein analogous to the major surface antigen from *Leishmania braziliensis braziliensis* - Kutner S., Pellerin P., Desjeux P. & **Brenière** S.F.
*Analisis antigénico de differentes cepas isoenzymáticas de *Trypanosoma cruzi* : reconocimiento de epitopes del componente antigénico 5 por medio de anticuerpos monoclonales - Revollo S., **Brenière** S.F., Caillard T. & Desjeux P.
Résumés: *Mem. Inst. Oswaldo Cruz, Rio de Janeiro*, 83 suppl 1: p75, p125
Statut : co-auteur de posters.
Trypanosoma cruzi* : major clones rather than principal zymodemes - Tibayrenc M. & **Brenière S.F.
*Identification and purification of a 72 kDa antigen of *Leishmania braziliensis braziliensis* present on the surface and in the cytoplasme of the promastigotes and its specific recognition by sera from mucocutaneous Leishmaniasis patients - Kutner S. , Pellerin P., **Brenière** S.F., Desjeux P. & Dedet J.P.
Résumés : *Mem. Inst. Oswaldo Cruz, Rio de Janeiro,* 83 suppl. 1, 249-255 et 431-433.
Statut : co-auteur de conférences.

C12 - II congrès régional de Protozoologie
Lieu : Cordoba, Argentine. Date : Novembre 1987. Titres et auteurs des communications :
*Isozymic variability of *Trypanosoma cruzi* strains in Bolivia : relationship between zymodemes and pathology of Chagas'disease - **Brenière** S.F., Carrasco R., Revollo S., Antezana G. & Desjeux P.
*Biochimical and immunological characterization of main antigens of *Leishmania braziliensis braziliensis* and *Leishmania donovani chagasi* - Kutner S., **Brenière** S.F., Kahane B., Legrand D. & Desjeux P.
Résumés : *Protozool.*, 36 (2), 34A. et p.200, 1989.
Statut : orateur et co-auteur de poster.

C13 - Réunion annuelle de recherche fondamentale sur la maladie de Chagas.
Lieu : Caxambu, Brésil. Date : Septembre 1986. Titres et auteurs des communications :
*Isoenzymic patterns of Bolivian human stocks : relation with symptomatic forms of Chagas'disease - **Brenière** S.F., Carrasco R., Antezana G., Pabon J., Revollo S. & Desjeux.
*Evidence for a 72 kd specific major surface antigen in *Leishmania braziliensis braziliensis* - Legrand D., Desjeux P., Le Pont F., Kahane B. & **Brenière** S.F.
Résumés: *Mem. Inst. Oswaldo Cruz, Rio de Janeiro,* 81 suppl : p97, p90
Statut : co-auteur d'une présentation et de poster

C14 - Colloque international sur la maladie de Chagas
Lieu : Santa-Cruz de la Sierra, Bolivia. Date : Mars 1984. Titre et auteurs de la

communication : Estudios isoenzimáticos y datos epidemiológicos de *Trypanosoma cruzi* del lado pacífico, Arequipa Perú. **Brenière** S.F., LLanos B., Tibayrenc M. & Desjeux P.
Statut : orateur

C15 - ICOPA V
Lieu : Toronto. Date : Juillet 1982. Titre et auteurs de la communications : Characterization of specific 5 antigen of *Trypanosoma cruzi* in culture medium. Lemesre J.L. & **Brenière** S.F.
Résumés : *Mol. Biochem. Parasitol*
Statut : co-auteur de posters.

C16 - XIII Congrès Latino Americain de Pathologie, VII Congrès Bolivien de pathologie
Lieu : La Paz Bolivia. Date : Octobre 1981. Titre et auteurs de la communication : "Enzimo-inmuno-analisis" - S.F. **Brenière**
Statut : orateur.

CARRIERE SCIENTIFIQUE

1 - HISTORIQUE

1.1.Etapes chronologiques

1976-1978 : Université de Montréal, Canada, étudiante
1978-1979 : Institut Pasteur de Lille (CIBP), étudiante
1980-1982 : Ministère de la Coopération (affectation IBBA en Bolivie), assistant de recherche
1983-1984 : Elève ORSTOM (affectation IBBA en Bolivie)
1984-1987 : Chargée de Recherche 2ème classe ORSTOM (affectation IBBA en Bolivie)
1988-1991 : Chargée de Recherche 2ème classe puis 1ère classe (affectation ORSTOM Montpellier, France)
1992-1994 : Chargée de Recherche 1ère classe (affectation IBBA en Bolivie)

1.2. Période pré-Orstomienne

La période précédant mon recrutement à l'ORSTOM (1976-1982) comprend deux étapes :

La première a été celle de ma formation à la recherche, effectuée pour partie au Canada de 1976 à 1978 et m'a permis d'obtenir un "Master" de microbiologie. Elle a été suivie par un stage d'un an en 1979 à l'Institut Pasteur de Lille au laboratoire du Professeur André Capron.

La deuxième qui est l'étape de mise en oeuvre en situation réelle d'un programme de recherche, s'est déroulée en Bolivie à l'Institut Bolivien de Biologie d'Altitude situé à La Paz, en qualité d'assistant de recherche dépendant du Ministère Français des Affaires Etrangères.

Ma première recherche qui était développée au sein du laboratoire du Professeur Pierre Viens à l'Université de Montréal dans le cadre d'une bourse France-Québec, consistait en l'étude du transfert de l'immunité entre mère et souriceaux dans le modèle expérimental de l'infection par *Trypanosoma musculi* de souris CBA/J.

De retour en France, j'ai été accueillie dans le laboratoire du professeur André Capron afin d'y préparer une thèse de troisième cycle. Mais quelques mois plus tard, le professeur André Capron me proposait un poste d'assitant de recherche pour travailler en

coopération à l'IBBA, Institut Bolivien de Biologie d'Altitude à La Paz. Le travail s'inscrivait dans un vaste programme concernant la maladie de Chagas engagé par le co-directeur français de cet institut, le Dr. Yves Carlier, détaché de l'Institut Pasteur de Lille.

En acceptant cette proposition, j'ai dû abandonner mon premier sujet de thèse (Antigènes circulants dans l'onchocercose) afin de me préparer à ces nouvelles fonctions. Il s'agissait en effet de monter un nouveau laboratoire, de transférer les techniques de bases applicables à l'étude de la maladie de Chagas (culture des parasites, préparation d'antigènes, développement des techniques de diagnostic sérologique), et de former à ces techniques des techniciens de l'IBBA.

Par la suite, le laboratoire étant devenu fonctionnel, j'ai étudié la réponse immune humorale de patients chagasiques en phase chronique de l'infection choisis dans des populations boliviennes vivant à différentes altitudes. Ce travail s'est exécuté en collaboration avec le département de Cardiologie de l'IBBA tandis que se constituait le département de Parasitologie de l'Institut.

Ce département, actuellement dirigé par le Dr. Céleste Rodriguez, comporte deux points forts, l'étude de la maladie de Chagas et celle des Leishmanioses.

A l'issu de ces deux premières années de Coopération en Bolivie, j'ai présenté ma thèse de 3ème cycle à l'Université des Sciences et Techniques de Lille "Infection humaine par *Trypanosoma cruzi* (Maladie de Chagas) en Bolivie à différentes altitudes : réponse immune humorale".

1.3. Carrière ORSTOM

1.3.1. Période d'élève et première affectation en Bolivie, 1983-1987

Dès 1980, deux chercheurs ORSTOM joignent le département de Parasitologie de l'IBBA pour travailler sur la Maladie de Chagas et la Leishmaniose (Dr. Michel Tibayrenc et François Le Pont). Ils y resteront tous deux plusieurs années, et le Dr. Michel Tibayrenc sera le responsable de ma formation durant les deux années d'élève ORSTOM passées à La Paz (1983-1984).

J'ai alors entrepris des recherches dans le domaine de la génétique des populations appliquée à l'agent de la maladie de Chagas, *Trypanosoma cruzi*. Mes travaux ont porté sur :

- L'étude de la variabilié génétique de *T. cruzi* dans le sud du Pérou, au Chili et en cycles sylvestres boliviens.

- L'étude comparée d'antigènes composants des souches génétiquement différentes de *T. cruzi.*

- L'étude des relations entre la pathologie de la Maladie de Chagas et les différentes souches de *T. cruzi* .

Les résultats ont conditionné la poursuite de mes recherches à Montpellier.

Pendant cette période, j'ai aussi entrepris différentes recherches sur la Leishmaniose en collaboration avec les chercheurs de l'Institut. En 1987 j'ai demandé mon affectation à Montpellier dans le laboratoire du Dr. Michel Tibayrenc qui ne comptait à l'époque qu'un seul étudiant.

1.3.2. Affectation à Montpellier, 1988-1991

Le centre de Montpellier ne fonctionnait que depuis un an. Ma première tâche a donc été de collaborer à la mise en place du laboratoire de Génétique des Parasites et des Vecteurs dirigé par le Dr. M. Tibayrenc. J'ai également participé à l'encadrement d'étudiants et à la rédaction de projets de recherche afin d'obtenir des fonds externes indispensables à l'installation.

Les recherches entreprises pendant cette affectation, ont eu pour but de disposer d'outils capables d'identifier sur le terrain les clones de *T. cruzi*.. Ces outils devaient permettre : (i) de pallier aux défauts des techniques connues, (ii) de développer une étude plus fine des relations entres les clones et l'épidémiologie de la maladie.

Ces recherches m'ont demandé une formation de base en biologie moléculaire. Après avoir obtenu des sondes d'ADN spécifiques des principux clones circulant en Bolivie, j'ai présenté à l'OMS un projet de collaboration avec l'IBBA (Bolivie) pour évaluer l'apport de ce nouvel outil à l'épidémiologie de la maladie de Chagas. Ce projet ayant été accepté, l'ORSTOM m'a affectée en Bolivie.

1.3.3. Deuxième affectation en Bolivie, 1992-1995

Ma nouvelle affectation en Bolivie s'accompagnait de celles du Dr. François Noireau (Chercheur ORSTOM) et de Marie-France Bosseno (ITA ORSTOM). Cette décision prise par l'ORSTOM d'affecter une équipe à un programme est à notre avis une

excellente formule car elle nous a permis de développer un travail de groupe sur une aire d'étude très large et géographiquement très diversifiée. Nos activités au laboratoire et sur le terrain se sont donc effectuées en équipe. Afin de compléter et de prolonger le financement du projet initial, plusieurs nouveaux projets ont été présentés à des instances internationales par les deux chercheurs du groupe. Par ailleurs, le financement de l'OMS initialement prévu pour trois ans, avait été sérieusement réduit, sans doute à cause de la restructuration de cet organisme et en raison de la conjoncture économique. Heureusement, nous avons récemment obtenu des moyens financiers (TDR Chagas et CEE) pour des recherches concernant les vecteurs secondaires de la maladie de Chagas et l'étude de l'importance épidémiologique des cycles sylvestres en Bolivie.

L'objectif de notre groupe de coopération ne se limite pas à l'exécution de programmes scientifiques. Il doit conduire à la formation d'un partenariat bolivien en matière de génétique des populations de parasites et de vecteurs. Dans ce contexte, notre ancienne étudiante bolivienne, Susana Revollo, est actuellement en France dans le laboratoire du Dr. M. Tibayrenc pour y préparer son Doctorat d'Université. A son retour, ce jeune chercheur pourra intégrer l' IBBA pour former le premier noyau du corps des chercheurs nationaux spécialisés. Le reste du groupe national est actuellement composé de 3 étudiants (facultés de médecine et de biochimie-pharmacie) et d'une technicienne de l'IBBA.

Le programme actuel se développe autour de deux grands thèmes :

- les cycles sylvestres de la maladie de Chagas en Bolivie,
- les conséquences médicales de la variabilité génétique de *Trypanosoma cruzi* l'agent de la maladie de Chagas.

La génétique des populations appliquée aux vecteurs comme aux parasites reste notre principal instrument de recherche.

2 - ANIMATION DE LA RECHERCHE

2.1. Formation académique

Tout au long de ma carrière j'ai assuré quelques cours de spécialité, à la demande de différentes instances d'enseignement. En Bolivie j'ai souvent été sollicitée par l'université de La Paz et par la Société Bolivienne de Biochimie et de Pharmacie. En France, lors de mon séjour à Montpellier, j'ai pris part au DEA de Parasitologie et en Espagne à la maîtrise de Parasitologie Tropicale de Valence.

En octobre dernier, un cours d'Entomologie Médicale a été organisé à l'IBBA en collaboration avec l'Institut Pasteur de Paris, et j'y ai participé en donnant quelques heures d'enseignement sur les aspects de la biologie moléculaire.

Enfin, j'ai présenté sur demande, un dossier de candidature auprès de l'OPS-ONUDI dont j'attends la réponse, pour organiser un cours de "Techniques avancées de diagnostic de la maladie de Chagas et des leishmanioses" à l'IBBA durant l'année 1995. Ce cours, d'envergure régionale comprendra une importante partie pratique et aura pour objectif de rassembler des professionnels afin d'établir de futures collaborations.

La liste des cours que j'ai donnés est présentée dans la partie "enseignement" du *Curriculum Vitae.*

2.2. Formation à la recherche et encadrement scientifique

J'ai été chargée d'encadrer en permanence des travaux de recherche. Le personnel que j'ai formé est très divers : des étudiants boliviens ou français, le plus souvent jeunes et non expérimentés, des techniciens boliviens de formation incomplète, des stagiaires français et étrangers de niveaux très différents. Dernièrement, nous avons intensifié notre activité de formation en recevant des chercheurs pour des stages de courte durée dans le laboratoire de l'IBBA.

Dès mon premier séjour en Bolivie, j'ai formé un petit groupe de techniciennes et chercheurs de l'IBBA aux techniques de parasitologie et d'immunologie appliquées aux recherches sur la maladie de Chagas. Par la suite, j'ai dirigé le travail de fin d'étude de Susana Revollo étudiante en Biochimie Pharmacie de l'Université "Mayor de san Andres" (La Paz) ainsi que le travail de plusieurs VSN (volontaires du service national) affectés à l'IBBA. Il s'agissait le plus souvent de jeunes gens n'ayant aucune formation de recherche ou de laboratoire et dont l'encadrement demandait beaucoup de temps et de soin. J'ai

essayé le plus vite possible de les faire participer à un programme de recherches avec un sujet propre. Le plus souvent, leur travail a pu être valorisé par une publication.

Dès sa création en 1988, j'ai rejoins le laboratoire de Génétique des Parasites et des Vecteurs de l'ORSTOM dirigé par le Dr. M. Tibayrenc à Montpellier. Mon rôle a consisté à le seconder dans l'encadrement des étudiants arrivés rapidement en assez grand nombre (DEA et étudiant en thèse). En 1990, Marie-France Bosseno, ITA de l'ORSTOM, venait se joindre à moi, motivée par la nouveauté des recherches en cours (productions de sondes d' ADN) et des techniques employées. Je l'ai épaulée dans ses efforts de reconversion et par la suite elle m'a suivi en Bolivie pour développer le programme sur la maladie de Chagas, financé par l'OMS.

Depuis mon retour en Bolivie, j'ai pris de nouveau en charge avec mes collègues orstomiens, la formation d'étudiants et de techniciens boliviens afin de disposer d'un petit groupe d'excellence dans le domaine de l'étude de la variabilité génétique des Parasites et des Vecteurs.

A plusieurs occasion j'ai déposé des demandes de bourse pour des jeunes chercheurs boliviens ou français. Dans le cadre de la collaboration avec l'IBBA, avant mon départ en Bolivie, j'avais soumis à l'OMS, en accord avec le Dr. Tibayrenc, un dossier de bourse pour Susana Revollo (chercheur bolivien) afin qu'elle réalise une thèse d'Université au sein de son laboratoire. Son séjour en France s'achève actuellement, et son intégration au sein de l'IBBA permettra d'assurer la direction bolivienne de l'équipe

2.3.Collaborations externes

la première partie de mes recherches sur le diagnostic et l'immunobiologie de la maladie de Chagas se sont développées en collaborations avec l'Institut Pasteur de Lille (laboratoire du Pr. A. Capron). Cette collaboration bilatérale a permis de bénéficier de "produits biologiques" comme des sérums hyper-immuns, des anticorps monoclonaux, et en retour nous avons envoyé à l'Institut Pasteur des sérums bien documentés.

A partir de mon entrée à l'ORSTOM, les thèmes de recherche ayant changés, la collaboration avec l'Institut Pasteur de Lille n'a plus eu cours. Deux collaborations informelles se sont alors établies, une avec le Chili et l'autre avec le sud du Pérou. Ces collaborations ont consisté en échanges de souches de *T. cruzi* et mission de terrain avec exploitation conjointe des données pour publications. Actuellement la collaboration avec le Chili se manifeste par des invitations à participer à des évènements scientifiques organisés par ce pays, celle avec le Pérou est plus technique puisque qu'un travaille est en cours sur des vecteurs et des stocks de *T. cruzi* en provenance de cette région.

Actuellement j'ai deux types de collaboration :

une demande de chercheurs sud et nord américains motivés par l'approche de biologie moléculaire développée dernièrement ; reçus dans le laboratoire de l'IBBA nous avons expérimenté avec eux leurs échantillons ; les laboratoires impliqués sont deux d'Argentine, un de Colombie, un du Paraguay et un d'Amérique du Nord (voir la liste des stagiaires dans le *Curriculum Vitae*).

une recherche de collaborations pour étendre le champs d'étude et améliorer les techniques ; la colloboration avec une équipe brésilienne travaillant sur le diagnostic de la maladie de Chagas par PCR a été exellente comme j'en ai largement parlé dans l'exposé des travaux. L'étude sur la relation entre souches et immunobiologie et pathologie de la maladie de Chagas se fait en collaboration avec plusieurs équipes. L'Institut Pasteur de Paris se charge d'une partie de l'étude de la réponse immune des patients, deux groupes d'Argentine nous communiquent des protéines recombinantes marqueurs des infections précoces et de la pathologie cardiaque. J'ai également une collaboration avec un groupe nord américain qui nous a envoyé une protéine recombinante également marqueur de la pathologie.

Je ne manquerais pas de signaler ma collaboration permanante, interne à l'ORSTOM, avec le groupe de Montpellier dirigé par le Dr Michel Tibayrenc. Cette collaboration consiste en des échanges scientifiques constants, et des co-participations dans plusieurs programmes de recherche.

2.4. Propositions de projets à des instances financières externes à l'ORSTOM

Les approches développées en France et en Bolivie, requièrent une solide structure de laboratoire, ainsi que d'importants moyens financiers de fonctionnement. La recherche de financements complémentaires au budget alloué par l'ORSTOM figure en bonne place parmis mes activités.

Instance financière : OMS, "Re-entry Grant", **1995**

Titre : Evaluation de l'efficacité des traitements de la maladie de Chagas chez des jeunes enfants et des nouveaux-nés.
Statut : Chercheur associé.
Décision : Accepté

Instance financière : OMS, "Applied Field Research committee", **1995**
Titre : Tegumentary leishmaniasis : Risk factors and self protection.
Statut : Chercheur associé.
Décision : Accepté

Instance financière : CEE, STD3, **1994**
Titre : Sylvatic Triatomines in Bolivia : trend toward domesticity, population dynamics, potential vector role and feasibility of control.
Statut : Chercheur associé.
Décision : Accepté comme addendum à un projet plus vaste

Instance financière : OMS, TDR Chagas, **1994**
Titre : Epidemiological significance of *Triatoma sordida* complexe in Bolivia
Statut : Chercheur associé.
Décision : Accepté

Instance financière : CEE, Coopération Scientique internationale **1993**
Titre : Human Chagas'disease : multicentric study of medical implications of infections by major natural clones of *Trypanosoma cruzi*, in Bolivia, Chile, Colombia and Paraguay.
Statut : Chercheur principal.
Décision : Refusé

Instance financière : Ministère de l'environnement, **1992**
Titre : Génétiques des populations de vecteurs et parasites impliquées dans la maladie de Chagas : interactions cycles sylvestres cycles domestiques.
Statut : Chercheur principal.
Décision : Refusé

Instance financière : CEE, STD 3, **1992**
Titre : Etude des cycles de la maladie de Chagas en Uruguay à l'aide de marqueurs génétiques, en particulier étude des dangers de réinfestation des structures domestiques par les vecteurs sylvestres.
Statut : Chercheur associé.
Décision : Accepté

Instance financière : TDR Chagas, juin **1991**, OMS
Titre : Study of the epidemiological relevance of *Trypanosoma cruzi* major clones in Bolivian sylvatic and domestic cycles by the use of specific PCR kDNA probes.
Statut : Chercheur principal
Décision : Accepté.

Instance financière : TDR Chagas, juin **1991**, OMS
Titre : Experimental study of the impact of population clonal structure on relevant biological and medical properties of *Trypanosoma cruzi*.
Statut : Chercheur associé, rédaction du projet.
Décision : Accepté - Projet réalisé à Montpellier

Instance financière : MRT. **1991**
Titre : Identification par sondes d'ADN des souches de *Trypanosoma cruzi* en Bolivie : implications épidémiologiques en cycles sylvestre et domestique.
Statut : Chercheur principal.
Décision : Accepté.

Instance financière : INSERM **1990,** Collaboration avec l'unité mixte INSERM 167 - CNRS 624 de Lille
Titre : Comparaison des caractéristiques biologiques et médicales des clones majeurs de *Trypanosoma cruzi*
Statut : Chercheur associé, rédaction du projet.

Décision : Refusé.

2.5. Demandes de bourses

J'ai également rédigé des demandes de bourse d'étude auprès de l'OMS et du MRT pour :

Nom : Philippe Lena
Instance financière : MRT
Type de bourse : post doctorale
Laboratoire : Génétique des Parasites et des Vecteurs, ORSTOM Montpellier.
Durée : un an (1990)
Décision : Acceptée

Nom : Susana Revollo
Instance financière : OMS
Type de bourse : Thèse Française
Laboratoire : Génétique des Parasites et des Vecteurs, ORSTOM Montpellier.
Durée : trois ans (1992-1994)
Décision : Acceptée

Nom : Katarzyna Lewicka
Instance financière : MRT
Type de bourse : Thèse
Laboratoire : Génétique des Parasites et des Vecteurs, ORSTOM Montpellier et IBBA.
Durée : 2 ans (1990)
Décision : Refusé

Nom : Jenny Telleria
Instance financière : OMS
Type de bourse : post doctoral
laboratoire : IBBA
Durée : 1 an (1995)
Décision : en cours

4 - OBJECTIFS

4.1. Objectif scientifique général

La connaissance des maladies parasitaires à transmission vectorielle repose sur de nombreux aspects dont certains ont pris récemment une importance toute nouvelle. C'est en effet seulement depuis quelques années qu'on a découvert le rôle prépondérant de la variabilité génétique des organismes concernés : les parasites, leurs vecteurs et les hôtes. Les espèces, définies le plus souvent sur les seuls critères morphologiques, étaient prises comme des unités homogènes et les études tenaient rarement compte du rôle de la variabilité génétique dans le jeu de la transmission et de l'évolution de la maladie.

De nombreux exemples peuvent être présentés pour illustrer l'intérêt de la génétique des populations en matière de maladies parasitaires. Pour *Trypanosoma cruzi,* l'agent de la maladie de Chagas, les études de génétique des populations dirigées par le Dr. Michel Tibayrenc ont montré que ces populations sont composées de clones ; c'est-à-dire que les évènements de recombinaison sont si rares (s'ils existent) que le modèle clonal est le plus parcimonieux. Entre certains clones les distances génétiques sont comparables à celles mesurées entre des souches de leishmanies (agents des leishmanioses) connues appartenant à des "espèces" différentes. Autrement dit, la variabilité génétique du taxon *T. cruzi* est énorme. Des études menées sur un seul clone de ce parasite (vaccination, résistance aux drogues, mécanisme de pénétration etc...) risquent de n'apporter que des informations partielles, c'est-à-dire non applicables à l'ensemble du taxon. Nous devons noter que ce modèle clonal, quoique généralement bien accepté pour *T. cruzi* , fait encore l'objet de débats (Maynard Smith, 1993, *Proc. Nat. Acad. Sci.* **90** : 4384-4388) et peu d'équipes s'interessent au conséquences biologiques de ce modèle.

De la même façon, la morphologie ne suffit pas à l'identification des espèces d'insectes vecteurs comme c'est le cas des complexes *Triatoma sordida* et *Rhodnius.* Encore dernièrement, l'expertise morphologique de spécimens du complexe *T. sordida* capturés en Bolivie, effectuée par deux équipes spécialisées a donné des résultats contradictoires. Dans un tel cas, la génétique des populations utilisant les isoenzymes comme marqueurs peut résoudre ces problèmes de spéciation.

Plusieurs questions fondamentales justifient l'étude de la variabilité génétique des populations des parasites et des vecteurs :

Quelles sont les unités taxonomiques biologiquement importantes ?

Dans le cas des insectes vecteurs (reproduction sexuée) il est fondamental de reconnaître tout d'abord les espèces ; c'est la première unité taxonomique à considérer puisqu'une espèce est composée de membres qui partagent un même "pool" génique différent de celui d'une autre espèce. Le biologiste s'attend à ce que chaque espèce présente des propriétés particulières tant au niveau écologie que capacité vectorielle.

Chez les Protozoaires parasites, l'étude du mode de reproduction a montré qu'une reproduction asexuée est très souvent privilégiée dans la nature. Les unités à étudier sont donc des clones dont l'identification se fait au moyen de marqueurs génétiques choisis par l'expérimentateur. Cette recherche doit se faire en étroite liaison avec des études fondamentales de génétique des populations telles que nous les réalisons au sein de l'Unité mixte de Recherche CNRS/ORSTOM qui a sa base à Montpellier.

Quelles sont les relations évolutives entre les taxons ?

L'analyse phylogénique des espèces permet d'appréhender les similitudes biologiques auquelles on peut s'attendre et de mieux comprende leur évolution. Dans le cas des clones elle permet de mesurer leurs parentés génétiques et d'individualiser des groupes génétiquement homogènes qui peuvent alors être considérés comme unité taxonomique.

Quelle est l'étendue de la variabilité génétique à l'intérieur de chaque taxon ?

Toute espèce présente une variabilité génétique. Il est important de la connaître et de l'évaluer entre populations géographiques ou biologiques ; existe -t-il des races biologiques et géographiques identifiables ? Dans le cas de la clonalité, chaque clone est défini par un certain nombre de marqueurs partagés par l'ensemble des membres du clone mais il peut exister une variabilité résiduelle. Cette variabilité peut être analysée par des marqueurs supplémentaires. Grâce à des sondes d'ADN s'observent des mêmes clones en cycles domestique et sylvestre en Bolivie ; ce résultat est en faveur d'un contact entre ces deux cycles. Ce marquage correspond à des stocks qui partagent environ 15 loci enzymatiques. Une analyse supplémentaire de la variabilité génétique des clones peut

permettre de distinguer de nouveaux clones et d'évaluer ainsi si les contacts entre les cycles sont actuels (clones similaires) ou plus anciens (clones génétiquement apparentés).

Comment se structurent les populations dans la nature ?

L'analyse génétique des populations géographiques permet de définir le mode d'expansion d'une espèce. Ces études présentent des retombées directes sur les mesures de contrôle. Les populations de *Triatoma infestans* des Yungas (région sub Andine) sont particulièrement séparées les unes des autres et des mesures de contrôle sont alors envisageables dans une communauté sans risque de réinfection par une autre, même voisine.

Actuellement peu de données sont disponibles sur la répartition géographique des clones. Leur étude peut permettre, (i) de comprendre quels sont les facteurs de dispersion des clones (circulation des vecteurs, circulation des hôtes mammifères), (ii) d'évaluer leur capacité géographique de dispersion, (iii) de mieux comprendre quels sont les interactions entre cycles sylvestre et domestique. Dans une des communautés de la région des Yungas a été observé un taux important de clones différents de ceux des autres villages chez *T. infestans* ; ce résultat permet de poser l'hypothèse d'un apport externe de clones qui pourraient avoir une origine sylvestre.

Mon objectif est d'approfondir peu à peu l'ensemble de ces connaissances pour certains modèles parasitaires puis d'analyser les conséquences épidémiologiques et médicales des variabilités observées.

La recherche des unités biologiquement importantes repose sur l'étude du mode de reproduction des organismes ce qui permet d'identifier les espèces (cas des vecteurs) et de préciser le concept d'"espèce" et de souches chez les Protozoaires parasites. D'autre part, pour connaître l'étendue de la variabilité génétique de chaque taxon il est important d'étudier les populations naturelles sur toute l'aire d'endémie. Dans ce but, il est essentiel d'obtenir la contribution des équipes nationales des pays où se situent les zones d'endémie et notre équipe de chercheurs expatriés affectée à ces zones aura pour tâche de coordonner et de faciliter la mise en oeuvre d'un tel programme.

D'ailleurs, les infrastructures de recherche des pays concernés permettent dès à présent d'assurer la formation théorique et technique nécessaire.

L'étude de la variabilité génétique des populations naturelles d'une espèce comprend celle de la dynamique de sa population, et présente des implications

épidémiologiques directes. Je propose de mener en parallèle ces études sur les vecteurs et sur leurs parasites afin de mieux comprendre les modes de transmission et d'expansion des maladies.

Les propriétés biologiques des sous-populations de vecteurs et des clones de parasites constituent un vaste sujet qui implique la contribution de plusieurs diciplines, mais il est nécessaire d'en assurer la coordination pour parvenir à déterminer les conséquences de la variabilité génétique des divers organismes en l'épidémiologie.

Mon expérience pré-orstomienne, de par la formation de biologie et d'immunologie quelle m'a apportée, me permet de mieux appréhender l'étude de telles études.

4.2. Coopération

Les travaux sur la variabilité des parasites sont nombreux, mais leurs approches manquent totalement de standardisation et ne sont pas comparatives. L'équipe de Montpellier a proposé en juin 1994 de créer un nouveau laboratoire de référence et de formation, le Centre d'Etudes sur le polymorphisme des microorganismes (C.E.P.M.). Le projet a été accepté par les autorités de l'ORSTOM et celles du CNRS et devrait être mis en application en 1996. Dans cette perspective, étant donné que les liaisons entre l'Europe et les divers pays concernés de l'Amérique du sud risquent de rester souvent difficiles, (notamment en raison de difficultés de financement), il paraît souhaitable de créer une équipe de relais sud américaine. Elle présentera une relation privilégiée avec le futur C.E.P.M. de Montpellier et sera capable d'assurer le transfert des techniques, de compléter la formation théorique et pratique des personnels locaux et de coordonner l'ensemble des collaborations. Ces dernières années, j'ai reçu différents stagiaires régionaux (Paraguay, Argentine, Pérou) au sein de l'IBBA, et nous comptons sur la formation de l'équipe de l'IBBA pour dans le futur assurer cette irradiation dans la région sud Amérique.

L'étude de la génétique des populations de vecteurs est une approche qui présente plusieurs aspects. Le premier, est de clarifier le statut taxonomique de certaines espèces difficile à distinguer morphologiquement. Le second est l'étude de la variabilité génétique des populations d'une espèce. Ces approches ne sont pratiquement pas encore développées et les efforts de mon collègue J.P. Dujardin, auquel je m'associe, consistent à former un réseau de laboratoires sud américains focalisant leur effort sur ce type de recherches.

Mon objectif immédiat est de fortifier le partenaire Bolivien afin de structurer une bonne équipe multidiciplinaire capable de développer des recherches de haut niveau.

Au niveau du continent sud américain, le but est de tisser des contacts avec différentes équipes afin de faire école en ce qui concerne l'importance des approches de génétique des populations de parasites et de vecteurs.

4.3. Objectifs particuliers à la maladie de Chagas

La maladie de chagas est en voie de disparition dans certains pays de l'aire d'endémie comme au Brésil et en Argentine, mais elle sévit encore dans beaucoup d'autres où des programmes nationaux et internationaux se mettent en place. Ces programmes proposent la lutte contre les vecteurs, ceci impose une prise en charge nationale complétée par des financements internationaux et un labeur continu de plusieurs années. Cette action dépend aussi de l'état de l'infrastructure sanitaire des pays.

En Bolivie malheureusement, cette infrastructure ne fait pas de grand progrès, les voies de communication manquent et le contrôle de toute l'aire d'endémie semble difficile à réaliser. Les recherche doivent s'orienter vers l'aide au développement de ces actions de contrôle.

Dans ce cadre de mon expérience les actions que je propose sont les suivantes :

- la recherche des vecteurs secondaires dont la répartition et l'importance épidémiologique est actuellement inconnue en Bolivie. L'approche de génétique des populations permet de préciser les espèces vectorielles impliquées, de comparer des populations domiciliées de vecteurs avec des populations sylvestres et d'évaluer les échanges entre ces populations, de détecter et d'identifier les clones de *T. cruzi* circulants et de les comparer avec les clones du cycle domestique,

- les études parallèles de la génétique des populations des vecteurs et des parasites permet l'analyse de la dynamique des populations et des modalités d'expansion de la maladie. Elle conduit à des applications directes sur les mesures de contrôle,

- la connaissance des propriétés biologiques et médicales des principaux clones de *T. cruzi* en Bolivie présente des applications importantes ; l'efficacité du traitement de patients infectés par différents clones de *T. cruzi* est totalement inconnue ; de même, les

propriétés pathologiques des clones sont peu connues. En Bolivie, existent deux groupes de clones génétiquement très différents et tous deux très fréquents en cycle domestique ; ce modèle naturel est idéal pour ces études.

4.4. Objectifs particuliers à l'étude des Leishmanioses

Les chercheurs de l'Institut Bolivien d'Altitude ont permis de mieux connaître les principaux foyers de Leishmaniose en Bolivie : plusieurs vecteurs ont été identifiés, les populations à risque sont connues pour certains foyers, des études isoenzymatiques ont précisé quels étaient les agents pathogènes. A l'initiative de chercheurs de l'institut, un projet Andin est actuellement proposé à la communauté européenne pour identifier les facteurs qui jouent sur le risque de contact homme/vecteur afin d'obtenir des méthodes de prévention.

Dans le cadre de ce programme, j'ai choisi de développer des techniques moléculaires permettant de détecter et d'identifier génétiquement les agents pathogènes directement dans les vecteurs, les mammifères qui seraient des réservoirs potentiels et chez l'homme. L'approche de génétique des populations développée au sein de l'UMR et les méthodes utilisées sont applicables sur la pluspart des organismes. Les dernières connaissances de la structure des populations de leishmanies mettent en évidence un mode de reproduction essentiellement clonal dans la nature. Ce modèle se rapproche étrangement de celui de *T. cruzi* , ce qui nous permet de supposer que les travaux de biologie moléculaire appliqués à *T. cruzi* peuvent s'adapter au modèle Leishmanie. L'association d'une détection des parasites hautement sensible, par la technique de PCR, avec celle de la caractérisation fine des agents pathogènes (identification des clones) permettra de décrire avec précision quels sont les cycles des parasites. D'autre-part, cette approche d'épidémiologie moléculaire dotera l'institut d'un outil utile au développement d'autres thèmes de recherche comme l'étude du diagnostic précoce de la maladie et l'étude après traitement des critères de cure.

RESUME DES TRAVAUX

1 - INTRODUCTION

La majeure partie de mon activité scientifique a porté sur l'étude de la Maladie de Chagas et plus particulièrement sur une approche ciblée des conséquences médicales et épidémiologiques de la variabilité génétique du parasite.

Les différentes étapes de ma carrière m'ont cependant amenée à diversifier mes recherches. Tout d'abord, au Canada (1976-1982) j'ai abordé le domaine de l'immunologie à travers le modèle expérimental de l'infection murine par *Trypanosoma musculi* et acquis une bonne expérience du modèle murin. Ensuite, dans le laboratoire du professeur André Capron à l' Institut Pasteur de Lille, j'ai reçu un complément de formation en immunochimie et acquis notamment une connaissance des techniques appliquées alors à l'étude de l'immunologie de la maladie de Chagas (1979).

A l'issue de cette même année, le ministère de la Coopération Française m'a affectée en Bolivie (fin 1979), pour participer à un programme sur l'immunologie de la Maladie de Chagas. Pendant les premières années de ce séjour, j'ai dû mettre en place un nouveau laboratoire et assurer la formation de son personnel technique. J'ai aussi acquis pendant cette période, une certaine expérience de terrain.

A partir de mon recrutement à l'ORSTOM (fin 1982) j'ai surtout développé des recherches sur la génétique des populations de *Trypanosoma cruzi* sous la direction du Dr. M. Tibayrenc (1983-1987).

En France, je me suis formée aux techniques de biologie moléculaire et par la suite développé des recherches plus fondamentales avec pour objectif leur application sur le terrain (1988-1991).

Depuis 1992, de retour en Bolivie, mes recherches sont principalement orientées sur les propriétés épidémiologiques et médicales des principales souches de *T. cruzi* en Bolivie. Ce programme que j'ai défini en coordination avec l'équipe de Montpellier, se développe sous ma responsabilité de façon indépendante.

Ma deuxième tâche est de fortifier notre organisme partenaire Bolivien afin de structurer une bonne équipe multidiciplinaire capable de développer des recherches de haut niveau.

Mon troisième objectif est de tisser des contacts avec différentes équipes sud américaines afin de faire école en ce qui concerne l'étude de la variabilité génétique des protozoaires parasites de l'homme et de développer des projets coordonnés entre plusieurs pays.

Mes recherches sont présentées par thèmes en se référant le plus souvent aux publications. Les codes des publications indiqués entre parenthèses et précédés de la lettre "A" se rapportent à mes travaux dont la liste figure dans le chapitre *Curriculum* Vitae.

2 - MODELE EXPERIMENTAL DE L'INFECTION PAR *TRYPANOSOMA MUSCULI*

Trypanosoma musculi est un parasite naturel de la souris qui provoque chez l'adulte une forte parasitémie spontanément contrôlée vers le 25ème jour. Les animaux sont alors immuns et les parasites qui ont disparu de la circulation sanguine se trouvent confinés dans les vasa recta du rein. Ce type d'infection procure un modèle d'équilibre hôte-parasite puisque les animaux ne sont par tués par l'agent pathogène. La nature des paramètres qui assurent cet équilibre, l'intervention des mécanismes immunologiques humoraux et cellulaires, ont été partiellement étudiés chez l'adulte (Viens *et al.* 1974, *Clin. Exp. Immunol.* **16 :** 279-294).

J'ai abordé l'étude de l'évolution parasitaire et immunologique de cette infection chez des nouveau-nés et chez de jeunes souriceaux ainsi que les relations mère-progéniture. Aucune donnée n'étaient alors disponible. L'ensemble des résultats apporta la confirmation de la nature humorale du mécanisme de contrôle des parasites dans le sang.

- l'infection chez la souris nouveau-née et le souriceau se distingue de celle des souris adultes par une amplitude plus forte de la parasitémie (3 fois plus élevée) et par une plus longue durée de l'infection, 60 jours au lieu de 25. Malgré la très forte parasitémie, les animaux survivent et finissent par la contrôler. Des anticorps spécifiques anti-*T. musculi* se développent à partir de la deuxième semaine d'infection, la production d'IgM (immunoglobulines de classe M) précède celle des IgG (immunoglobulines de classe G) de seulement 2 jours,

- l'étude de portées de souris nées de mère immune ou normale et allaitées par une mère immune ou non a permis de mettre en évidence le transfert mère-progéniture d'une certaine protection. La protection acquise par transfert placentaire est partielle puisque des nouveau-nés, issus de mère immune et nourris dès la naissance par une mère normale, s'infectent mais présentent une parasitémie plus basse que les témoins. D'autre-part, les souris nées de mère normale et allaitées par une mère immune sont entièrement protégées ; cette protection est totale mais temporaire puisque après deux mois ces souris peuvent être infectées et présentent alors des parasitémies similaires aux souris témoins. Dans tous les cas de protection, des anticorps spécifiques des classes IgG 1 et IgG 2 et parfois IgM ont été mis en évidence. On observe également une décroissance de ces anticorps au cours du

temps, mais le degré de résistance immunitaire des souriceaux n'est pas corrélé à la quantité d'anticorps maternels transférés.

Ce travail a fait l'objet d'un mémoire pour l'obtention du titre de Maître Es Sciences (M.Sc.) Canadien et d'une publication (A25).

3 - LA MALADIE DE CHAGAS

Mes recherches sont à rattâcher à trois grands volets de la maladie de Chagas :

- le diagnostic,
- l'immunobiologie,
- la variabilité génétique des populations de *Trypanosoma cruzi* et son impact épidémiologique et médical.

Depuis 2 ans j'ai également entrepris l'étude de la variabilité génétique des vecteurs afin de mieux appréhender les relations hôte/parasite en fonction de cette double variabilité.

3.1 - Diagnostic

L' infection humaine à *Trypanosoma cruzi* se caractérise par trois phases successives :

- une forte parasitémie s'observe peu de jours après l'infection. Le diagnostic au cours de cette première phase est parasitologique, avec la mise en évidence des formes trypomastigotes circulantes dans le sang (observation microscopique),
- après environ deux semaines, les formes sanguines sont contrôlées et deviennent très rares. Ainsi débute la deuxième phase nommée "indéterminée" ; le diagnostic est essentiellement sérologique et aucune pathologie n'est encore développée. La "phase indéterminée" peut durer plusieurs années ou même toute la vie,
- enfin, la troisième phase généralement tardive (plusieurs années après l'infection initiale) fait apparaître une pathologie principalement cardiaque ou digestive, les parasites circulants étant toujours très rares et le diagnostic restant sérologique.

Finalement le diagnostic sérologique de la maladie de Chagas est d'autant plus important que les signes cliniques sont inconstants et peu évocateurs. L'infection provoque une forte réponse humorale spécifique dont les anticorps, dirigés contre le parasite, se maintiennent toute la vie si aucun traitement précoce n'a pu être mené. Ces anticorps permettent le diagnostic immunologique. De nombreuses techniques ont été et sont utilisées, depuis la réaction de fixation du complément, technique

pionnière pour la maladie de Chagas, jusqu'à la technique ELISA (Enzyme Linked Immunosorbent Assays).

Dernièrement, une nouvelle technique, basée sur les progrès de la biologie moléculaire est à l'étude : la PCR ("Polymerase Chain Reaction") permet la détection d'infimes quantités d'ADN du parasite dans le sang de patients chagasiques. Cette technique de diagnostic parasitologique pourrait atteindre la sensibilité des techniques sérologiques.

Comme pour toute maladie, le diagnostic doit être spécifique et le plus sensible possible. Au cours de mes recherches sur les diagnostics immunologiques et parasitologiques, ces deux aspects ont retenu mon attention.

3.1.1. Spécificité : étude de l'antigène 5 de *Trypanosoma cruzi*

Dans beaucoup d'aires géographiques les populations présentent des infections multiples avec des agents pathogènes succeptibles de provoquer des fausses positivités des tests sérologiques. Les principales maladies succeptibles de provoquer des anticorps pouvant reconnaître une partie des antigènes composant *T. cruzi* sont les Leishmanioses. Elles sévissent souvent dans les mêmes zones géographiques que la maladie de Chagas comme c'est le cas dans la région des Yungas en Bolivie. D'autre part, *Trypanosoma rangeli*, parasite considéré comme non pathogène pour l'homme, circule chez les mêmes vecteurs et hôtes que *T. cruzi* : en Colombie et au Vénézuela il est très souvent rencontré chez l'homme, ce qui n'est pas le cas en Bolivie où il a été seulement rencontré chez des mammifères sauvages (A30 et D'Alessandro *et al.* 1986, *Am. J. Trop. Med. Hyg.*, **35** : 285-289) ; sa constitution antigénique est voisine de celle de *T. cruzi*, et des individus infectés par ce parasite présentent des anticorps capables de réagir avec certains antigènes de *T. cruzi*. (Guhl *et al.* 1987, *Parasitol.*, **94** : 475-484). Afin d'améliorer la spécificité des tests, au lieu d'utiliser des antigènes totaux du parasite, les chercheurs ont étudié des antigènes spécifiques du parasite.

J'ai approfondi l'étude de l'antigène 5 spécifique de *T. cruzi*, décrit antérieurement (Afchain *et al.* 1979, *J. Parasitol.* **65** : 507-514). L' objectif du travail développé en Bolivie était d'évaluer sa valeur diagnostique (A17, A22, A33 et A35). Les tests d'immunoélectrophorèse et de double diffusion en gels d'agarose ont montré que :

- la grande majorité des patients chagasiques en phase chronique de l'infection présentent des anticorps anti-antigène 5 (70 % et 80 % respectivement) en quantité suffisante pour être détectés par ces techniques assez peu sensibles,

- ces tests sont spécifiques puisque des sérums de patients atteints de leishmaniose cutanéo-muqueuse ne donnent aucune positivité.

Par la suite, un de nos collègues de l'IBBA (J.L. Lemesre), de retour en France au sein du laboratoire du CIBP de Lille (Directeur Pr. André Capron), a développé un test d'immunocompétition (A20) à l'aide d'un anticorps monoclonal produit à partir de la purification biochimique partielle de l'antigène 5. La spécificité et la sensibilité de ce test ont été controlées en utilisant les sérums Boliviens de mes études précédentes.

Les souches de *T. cruzi* (isolats naturels) présentent une grande variabilité génétique mise en évidence par des études isoenzymatiques et d'ADN (Toyé, 1974, *Trans. R. Soc. Med. Hyg.* **68** : 147; Miles *et al.*, 1980, *Trans. R. Soc. Med. Hyg.* **74** : 221-237; Tibayrenc and Ayala, 1988, *Evolution* **42** : 277-292). Elles présentent également une grande diversité biologique (infectivité, virulence, pathogénicité, Dvorak, 1984 *J. cell. Biochem.*, **24** : 357-371.) et une variabilité de leur composition antigénique. Etant donné cette diversité, j'ai étudié l'expression de l'antigène 5 dans 18 variants isoenzymatiques de référence génétiquement diversifiés. Afin de travailler sur des populations génétiquement homogènes, les variants isoenzymatiques ont été clonés au laboratoire de l'IBBA, selon la technique de micromanipulation (micro-goutte) apprise à l' Institut Prince Leopold d'Anvers en Belgique. L'expression de l'antigène 5 a été identifiée par plusieurs anticorps monoclonaux fournis par le laboratoire du CIBP de Lille. Les résultats ont montré l'universalité de l'antigène 5 dans le taxon *T. cruzi* et ainsi renforcé la valeur diagnostique de cet antigène. Ce travail a fait l'objet d'une thèse de licence bolivienne de Biochimie Pharmacie de l'Université "Mayor de San Andrès" de La Paz, présentée par Susana Revollo et d'une publication (A 31).

Les études de cet antigène 5 ont bien démontré que cette molécule pouvait donner cours a un meilleur diagnostic de la maladie de Chagas, cependant à l'époque aucune commercialisation n'a été faite. Actuellement les techniques ont changé, plusieurs autres molécules sont proposées en vue du diagnostic immunologique parmi lesquelles des protéines recombinantes et des peptides. Par exemple le clone recombinant JL7, obtenu d'une librairie génomique, est reconnu par 96 % des patients chagasiques en phase chronique de l' infection (Levin *et al.* 1991, *FEMS Microbiol. Immunol.* **89** : 11-20). La commercialisation en kit de diagnostic de ce type de protéines est en cours au Brésil et en Argentine mais les seuls kits de diagnostic commercialisés actuellement en Bolivie utilisent l'ensemble des antigènes du parasite ainsi que les réactions d'agglutination et d'hémagglutination.

3.1.2. Sensibilité : comparaison de différentes techniques sérologiques

Les techniques sérologiques utilisées sont nombreuses et afin de standardiser le diagnostic sérologique de la maladie de Chagas à l'IBBA, j'ai évalué à partir de 405 sérums de patients boliviens en phases indéterminée et chronique de la maladie, les sensibilités relatives des tests d'ELISA, d'immunofluorescence (IF), de fixation du complément (CFT), et d'immunoélectrophorèse (IEP). Pour chaque test, l'antigène utilisé est produit au laboratoire à partir d'une même souche de *T. cruzi*. Les résultats permettent de classer ces techniques selon leur pourcentage de sensibilité.

ELISA > IF > CFT > IEP

Toutefois, aucune différence significative de sensibilité n'est observée entre les différentes techniques. L'ensemble de ces résultats est consigné dans les publications A22 et A35.

La plus grande source d'erreur de diagnostic provient, à notre avis, des erreurs de laboratoire (inversion de sérums, erreurs de dilutions ...) aussi il est fortement recommandé de n'établir un diagnostic qu'après son contrôle par au moins deux techniques différentes. Néanmoins pour des enquêtes sérologiques d'envergure une seule technique devrait suffire.

3.1.3. Immunodépression naturelle spécifique

Pendant les phases indeterminée et chronique de la maladie de Chagas, le xénodiagnostic est le diagnostic parasitologique classique. En cas de positivité, le traitement spécifique de la maladie de Chagas est souvent appliqué bien que les dernières recommandations de l'OMS soient de le supprimer durant ces phases. Au cours de mes travaux, j'ai identifié plusieurs patients présentant une sérologie négative et un xénodiagnostic positif. Ce statut particulier n'avait pas été rapporté dans la littérature c'est pourquoi la sérologie de ces cas a été contrôlée indépendamment par 2 autres laboratoires. Pour certains de ces patients, j'ai confirmé l'absence d'anticorps anti-*T.cruzi* établie par plusieurs techniques de sérologie classique, par la technique d'immunoprécipitation des antigènes de surface marqués à l'iode 125 (données non publiées). De plus, cet état de dépression humorale spécifique ne semble pas lié à la pathologie. Ces résultats permettent de supposer l'absence de réponse immune humorale spécifique chez certains patients (A37). Des cas similaires m'ont été signalés oralement par des chercheurs brésiliens et chiliens.

Afin d'approfondir cette étude, j'ai alors examiné le taux des différentes types cellulaires de lymphocytes grâce à leurs marqueurs de surface par la technique d'immunofluorescence (résultats non publiés) : les lymphocytes T de type OkT3, OkT4 et OkT8 ainsi que les lymphocytes B OkLa chez 7 chagasiques présentant une dépression humorale spécifique, 20 chagasiques présentant une réponse humorale normale et 22 non chagasiques. L'analyse statistique des résultats n'a montré qu'une légère augmentation significative des lymphocytes B dans le groupe des patients présentant une dépression humorale spécifique. La poursuite de ces travaux nécessiterait l'étude de l'état fonctionnel des cellules et non pas seulement l'étude quantitative des sous populations de lymphocytes.

Etant donné que le diagnostic de la maladie de Chagas est chez l'adulte essentiellement sérologique, ces patients chagasiques (xénodiagnostic positif et sérologie négative) échappent au diagnostic classique. La proportion de patients en zone endémique pouvant présenter ce statut reste pour le moment indéterminée mais la technique de Polymérisation en Chaîne (voir ci-dessous) pourrait nous permettre d' évaluer la fréquence de ce phénomène.

3.1.4. Nouveau diagnostic parasitologique par PCR ("Polymerase Chain Reaction")

La technique de PCR découverte par Mullis-Saiki en 1985 a été appliquée à l'ADN de *Escherichia coli.* Elle consiste à synthétiser *in vitro* de manière exponentielle des séquences choisies d'ADN. Elle est spécifique de ces séquences et ne nécessite pas la purification initiale de l'ADN cible. C'est une technique rapide qui présente des applications multiples dont le diagnostic d'agents pathogènes pour l'homme.

J'ai utilisé cette technique pour améliorer le diagnostic parasitologique de la maladie de Chagas, toujours difficile et peu sensible, spécialement pendant la phase chronique de l'infection. La phase aiguë de la maladie se définit de façon arbitraire par une parasitémie détectable à l'examen microscopique direct et correspond à la toute première phase de l'infection. Elle est accompagnée de la production d'anticorps spécifiques de classe IgM mais ces anticorps ne sont malheureusement pas toujours faciles à détecter. Chez les enfants, elle passe le plus souvent inaperçue. Au cours de cette phase, le traitement est efficace et l'utilisation de bons outils pour sa détection est d'une grande importance épidémiologique. Actuellement, son diagnostic se fait par l'observation directe au microscope des parasites présents dans le sang après leur

concentration par centrifugation. Cette technique simple mais laborieuse demande des observations microscopiques prolongées et manque de sensibilité.

Au cours de la phase chronique, les parasites sont très peu abondants dans le sang et le test parasitologique employé est le xénodiagnostic. Il consiste à faire gorger sur les patients une quarantaine de larves de triatomes d'élevage (stades 3 ou 4) non infectées afin de concentrer les parasites. Ce test, dont le résultat ne peut être donné qu'un mois après l'examen, est peu sensible. C'est donc le diagnostic sérologique qui doit être employé préférentiellement au cours de cette phase

L'ADN cible choisi, est composé des parties hyper variables des minicercles de l' ADN kinétoplastique (ADN extranucléaire formant le kinétoplaste) car ces séquences présentent les caractéristiques suivantes :

- elles sont très répétées (plusieurs milliers de copies),
- elles sont encadrées par des parties constantes concensus de toutes les souches de *T. cruzi* ,
- elles permettent l'identification génétique des clones (voir paragraphe 3.3)

L'amplification est positive pour tous les variants génétiques de *T. cruzi*, donnant une bande majeure de 270 paires de bases. Les souches de leishmanies ne donnent aucune amplification tandis que les souches de *T. rangeli* donnent une amplification positive mais la bande majeure est de taille supérieure à celle des souches de *T. cruzi*. Cette dernière donnée a été observée pour un nombre réduit de souches de *T. rangeli* et demande des études complémentaires.

Une approche similaire a été développée par une équipe de chercheurs brésiliens. Ils ont également choisi comme ADN cible les parties hyper variables des minicercles. Leurs "primers" légèrement différents, génèrent des fragments d'environ 320 bp qui comprennent toute la partie hyper variable et une partie de la séquence constante des minicercles.

La sensibilité théorique de la technique PCR est remarquable mais il faut cependant relever que cette grande sensibilité entraîne de sérieux inconvénients car elle fait de cet outil une technique délicate qui risque de faire apparaître de nombreux faux positifs quand elle n'est pas développée dans des règles strictes.

Les résultats préliminaires évaluant la sensibilité des tests PCR en phases aiguë et chronique de la maladie, chez des patients chagasiques d'âge différents, montrent que la technique brésilienne est nettement plus sensible que la notre (A4 et A26). Les différentes procédures utilisées pour préparer les échantillons doivent être à l'origine de cette différence de sensibilité.

En appliquant la technique brésilienne à 280 échantillons de sang prélevés chez des enfants boliviens entre 6 et 10 ans habitant une zone endémique, nous confirmons la haute concordance entre ce test PCR et la sérologie (article en préparation).

Un test PCR de haute sensibilité, appliqué au diagnostic de la maladie de Chagas présente divers avantages :

- il est utile quand les tests sérologiques ne sont pas applicables ; c'est le cas de la phase initiale de l'infection, quand la réponse immune de l'hôte n'est pas encore développée ; sa contribution au diagnostic du Chagas congénital risque d'être importante puisque ce diagnostic n'est réalisé que par l'examen direct des parasites dans le sang. C'est aussi le cas des patients en phase chronique de l'infection qui présentent une réponse humorale spécifique déprimée (voir paragraphe 3.1.3.),
- sa haute spécificité permet d'appliquer le test dans les cas où la sérologie reste douteuse (réactions croisées avec d'autres maladies),
- enfin ce test parasitologique, va apporter de nouvelles données sur l'histoire naturelle de l'infection (suivi de la parasitémie) et sera important pour le contrôle des traitements. C'est un outil de recherche de grande portée.

Le projet proposé par S. Revollo (chercheur IBBA) : "Evaluation de l'efficacité des traitements de la maladie de Chagas chez des jeunes enfants et nouveaux-nés" s'incrit dans la suite logique de ce développement technologique.

Dernièrement, une équipe du Paraguay a montré (Russomando *et al.* 1992, *J. Clin.. Microb.* **30**: 271-280) qu'en utilisant comme cible d'ADN, l'ADN satellite de *T. cruzi* (ADN nucléaire hautement répété), une réaction de PCR positive peut être obtenue à partir de sérum de patients chagasiques en phase chronique de l'infection. Une collaboration établie entre nos deux groupes va permettre de comparer nos techniques et d'approfondir les recherches à partir des sérums de patients.

3.2 - Immunobiologie de la maladie de Chagas en phase chronique de l'infection

Les résultats principaux sont consignés dans ma thèse : "Infection humaine par *Trypanosoma cruzi* (maladie de Chagas) en Bolivie à différentes altitudes : réponse immune humorale".

En Bolivie l'aire d'endémie est très vaste, elle occupe les deux tiers du territoire et présente une diversité géographique importante due en partie aux différences d'altitude. L'étude porte sur la comparaison des caractéristiques de

l'infection de plusieurs populations boliviennes vivant à différentes altitudes et évalue l'importance du facteur "altitude" sur la réponse immune de patients chagasiques en phase chronique de l'infection.

3.2.1. Réponse immune humorale non spécifique

Le modèle expérimental de souris montre clairement qu'une activation polyclonale non spécifique des lymphocytes B est provoquée dès le début de l'infection (Hontebeyrie-Joscowicz, 1992, *Mem. Inst. Oswaldo Cruz, Rio de Janeiro,* **87**: 101-103). Une des conséquences de cette activation serait la rupture de la tolérance chez l'hôte aux antigènes du soi et en conséquence une production importante d'anticorps autoimmuns. Peu de données existent chez l' homme en phase aiguë de l'infection et les résultats sont contradictoires. Pour la phase chronique, la littérature rapporte des résultats différents sur l'augmentation des classes d'immunoglobulines. Ma propre étude a permis de conclure à une faible augmentation des immunoglobulines IgG et IgM , durant la phase chronique de la maladie, indépendante de l'altitude mais variable selon les populations. Il est possible que l'étude des sous classes d'immunoglobulines révèle une augmentation sélective.

J'ai également recherché les immuns complexes circulants et observé comme dans d'autres travaux une augmentation significative des immuns complexes chez les patients chagasiques adultes sans qu'il y ait de relation avec la cardiopathie. Ces immuns complexes se déposant dans les tissus joueraient un rôle au niveau de la pathologie ; toutefois, leur origine (réponse autoimmune ou spécifique) n'a pas été déterminée.

De nombreux anticorps autoimmuns ont été décrits dont les anticorps EVI hétérophiles (endocardium vascular interstitium) qui selon les auteurs peuvent être corrélés à la cardiopathie. Ces anticorps pourraient aussi intervenir directement dans la destruction cellulaire. Ces anticorps reconnaîtraient des structures antigéniques communes au parasite et à l'hôte mammifère. En effet les sérums de patients présentent des réactions positives sur des coupes de coeurs normaux de souris. L'origine de ces anticorps est cependant mal connue, elle peut être la conséquence d'une activation polyclonale (voir ci-dessus) mais également en rapport avec l'existence d'antigènes communs entre les cellules d'hôte et *T. cruzi*. Parmi deux populations boliviennes, j'ai observé des taux d'anticorps EVI significativement plus élevés chez les patients chagasiques sans corrélation avec la pathologie cardiaque. Malgré de nouvelles recherches, l'origine et le rôle des anticorps autoimmuns dans la maladie de Chagas restent en partie inconnus.

3.2.2. Réponse immune humorale spécifique

Comme nous l'avons vu à propos du diagnostic sérologique de la maladie de Chagas, de nombreuses techniques sont utilisées pour doser les anticorps spécifiques. La plupart des techniques sont quantitatives et utilisent un antigène total du parasite.

Aspects quantitatifs de la réponse

Plusieurs points sont à souligner : tout d'abord, selon les populations étudiées, les taux des anticorps sont différents. Les taux élevés pourraient dépendre de l'endémicité et ainsi être en rapport avec des réinfections. Ce dernier aspect est pour le moment strictement spéculatif puisque la littérature scientifique ne rapporte pas d'études sur la dynamique de réinfection des populations humaines. Sur ce point, l'identification directe des différents clones de *T. cruzi* dans le sang de patients chagasiques grâce à la PCR pourrait donner un élément de réponse : en effet, les patients se trouvent souvent infectés par plusieurs clones (A15 et A26) et l'identification de ces mélanges de clones en fonction de l'âge des patients permettrait d'évaluer le taux des réinfections.

Une cohorte de patients a été soumise à l'examen parasitologique classique : le xénodiagnostic. Les résultats montrent qu'au cours des deux phases indéterminée et chronique 40% à 60 % des patients présentent un test positif. De plus, le taux des anticorps spécifiques est significativement plus élevé chez les patients chagasiques présentant un xénodiagnostic positif. Il est possible que les formes trypomastigotes provenant du cycle de multiplication intracellulaire provoquent une stimulation d'anticorps impliqués dans la régulation de la parasitémie. Ce type d'anticorps nommés dans la littérature "anticorps de lyse", sont spécifiques du parasite et semblent jouer un rôle essentiel pour l'élimination des parasites circulants.

Enfin, le taux des anticorps pourrait être en rapport avec l'endémicité et la parasitémie, mais ne possède aucun lien avec les pathologies cardiaques et gastriques de la maladie ni avec les différences d'altitude.

Aspects qualitatifs de la réponse

La technique d'immunoélectrophorèse appliquée à 201 sérums de patients met en évidence l'importante production des précipitines en phases indéterminée et chronique. La réponse est hétérogène et selon les sérums le nombre de précipitines varie, mais l'arc 5 correspondant à l'antigène 5 spécifique de *T. cruzi* est majeur (A33). En phases indéterminée et chronique de l'infection, la fraction 5 serait la plus

immunogène. Cependant, la présence d'anticorps anti-antigène 5 n'est ni en relation avec la positivité du xénodiagnostic ni avec le taux des anticorps totaux. L'approche qualitative de la réponse immune par l'électrophorèse est limitée par la faible sensibilité de la technique mais permet d'orienter les recherches vers certaines fractions antigéniques. Actuellement cette approche est dépassée. La biologie moléculaire permet d'affiner les études des antigènes candidats à la vaccination mais jusqu'à présent aucune n'est satisfaisante. Si la pathologie de la maladie de Chagas dépend de phénomènes autoimmuns, il n'est pas exclu qu'un vaccin protégeant contre le parasite (développement de la lyse parasitaire dès l'entrée du parasite dans l'hôte) induise les mécanismes autoimmuns responsables de la pathologie.

3.3 - Variabilité génétique de *Trypanosoma cruzi*

3.3.1. Structure clonale de la population

L'étude de la variabilité génétique des souches de *T. cruzi* a été abordée depuis plusieurs années par l'analyse isoenzymatique (Toyé, 1974, *Trans. R. Soc. Med. Hyg.* **68** : 147; Miles *et al.*, 1980, *Trans. R. Soc. Med. Hyg.* **74** : 221-237; Tibayrenc and Ayala, 1988, *Evolution* **42** : 277-292). Les isoenzymes sont des protéines présentant une même spécificité enzymatique mais un déplacement électrophorétique différent. Le déplacement électrophorétique dépend principalement des structures primaire et tertiaire des enzymes qui sont elles mêmes dépendantes de la séquence d'ADN codante. Les variations de séquences de l'ADN dépendent essentiellement des mutations ponctuelles dont le taux est constant. Cet ensemble de caractéristiques permet d'analyser, au sein d'une population, l'étendue de la variabilité génétique ainsi que la structure de la population (mode de reproduction des individus).

L'application de cette technique à des souches de *T. cruzi* a mis en évidence leur grande variabilité génétique. De plus, l'interprétation des profils enzymatiques en terme de génétique des populations (étude des flux géniques entre individus) faite dès 1980 par le Dr. Michel Tibayrenc et ses collaborateurs a permis de comprendre quel est le mode de reproduction de ce parasite dans la nature. En bref, l'étude électroenzymatique à plusieurs locus (MLEE) appliquée à de nombreux isolats de *T.cruzi* provenant d'hôtes et d'origines géographiques différents fait clairement apparaître l'absence de reproduction sexuelle. Il est possible que certains échanges génétiques interviennent entre les individus mais ils sont rares. Dernièrement, l'approche a été standardisée grâce au développement de "critères de clonalité". Ce sont des critères qualitatifs et quantitatifs qui permettent, sans erreur possible, de rejeter l'hypothèse de l'existence d'une reproduction sexuée au sein d'un taxon. Le

modèle clonal est actuellement bien accepté pour *T. cruzi* quoique très peu d'équipes cherchent à en évaluer les conséquences. Ma contribution à l'ensemble de ces travaux est traduite par différents articles : A8, A10, A15, A24, A32, A36, A40.

T. cruzi se caractérise par la présence d'un kinétoplaste, organite cytoplasmique composé d'ADN organisé en séquences circulaires, les maxicercles et les minicercles. Lorsque cet ADN est digéré par des enzymes de restriction, il génère en électrophorèse une série de bandes reproductibles, le schizodème, dont le profil est variable selon les souches. Plusieurs travaux ont montré que ces profils étaient corrélés à ceux obtenus par la technique des isoenzymes (Morel *et al.*, 1980, *P. N. Acad. Sc. USA*, **77**: 6810-6814; Tibayrenc and Ayala, 1987, *C. R. Acad. Sc. Paris* **304**: 89-92, A6), c'est-à-dire que la variabilité isoenzymatique qui dépend de gènes situés dans l'ADN du noyau, est liée à celle de l'ADN kinétoplastique. La liaison entre ces deux marqueurs (isoenzymes et kinétoplaste) fait donc rejeter l'hypothèse qui assimilait le kinétoplaste à un plasmide interchangeable entre souches et renforce celle de la propagation clonale des souches de *T. cruzi* dans la nature. En effet, l'ensemble de l'ADN nucléaire et extranucléaire (kinétoplaste) serait transmis en bloc inchangé à chaque génération.

L'étude de la variabilité génétique des populations naturelles de *T. cruzi* fait ressortir deux résultats importants :

- les populations de *T. cruzi* sont composées de clones qui se propagent entre hôtes mammifères et vecteurs et qui restent génétiquement semblables à eux-même (aux mutations près) dans le temps,
- ces clones sont nombreux et peuvent présenter des différences génétiques importantes (fortes distances génétiques) ; les conséquences biologiques de cette variabilité sont pour le moment fort mal connues mais elles pourraient expliquer la diversité observée entre les souches de *T. cruzi.*

En conclusion, toutes les études, quelles soient fondamentales ou appliquées, devront tôt ou tard tenir compte de la variabilité génétique.

3.3.2. Limites du taxon *Trypanosoma cruzi*

Dans le cas de populations présentant une structure clonale, il devient impossible de définir l'espèce car celle ci présente une définition biologique basée sur la reproduction sexuée. En quelque sorte, chaque clone séparé génétiquement du voisin (absence d'échanges génétiques) pourrait être considéré comme une espèce

particulière. Ainsi se pose le problème de la définition de l' espèce "*T. cruzi* " et de ses limites.

J'ai abordé l'étude comparée des taxons *T. cruzi* et *T. rangeli* dont la différenciation n'est pas toujours facile. Ces parasites se distinguent actuellement par des critères morphologiques (appliquée aux formes sanguicoles) et biologiques (comportement chez l'animal, localisation salivaire de *T. rangeli* chez les vecteurs). L'étude de marqueurs génétiques est nécessaire car ces critères ne sont pas suffisants pour séparer nettement les deux taxons. J'ai donc développé une étude isoenzymatique de souches considérées comme appartenant à l'un et l'autre des deux taxons. Cette analyse met en évidence une grande variabilité génétique des souches de *T. rangeli,* analogue à celle observée dans le taxon *T. cruzi.* Ceci confirme les résultats de quelques études isoenzymatiques antérieures sur des souches de *T. rangeli* (Kreutzer et Sousa, 1981, *Am. J. Trop. Med. Hyg.* **30**: 308-317).

Etant donné l'étendue de la variabilité génétique de chacun des deux taxons, l'approche isoenzymatique n'est pas suffisante pour décider de la position taxonomique de certains isolats : ainsi, certains stocks isolés de singes du Pérou, présentaient des parentés génétiques fortes avec des stocks de référence d'un des deux taxons mais d'autres n'étaient génétiquement apparentés à aucune référence (A29).

Par ailleurs cette étude préliminaire nous a permis de poser l'hypothèse d'une structure clonale des souches de *T. rangeli.* En effet, trois souches de référence de *T. rangeli*, isolées au Venézuéla (deux souches) et en Bolivie (une souche) présentent le même profil isoenzymatique multilocus. L'existence dans une population de membres présentant un même génotype multilocus et d'origines géographiques différentes fait partie d'un des critères de clonalité développés par le Dr. Michel Tibayrenc.

T. rangeli et *T. cruzi* seraient deux taxons voisins composés chacun de clones. Pour faire de cet ensemble de clones deux taxons différents, nous devons supposer que les membres de chaque taxon partagent un certain nombre de marqueurs génétiques mais qu'ils sont différents entre les taxons. De récentes analyses m'ont permis d'identifier deux marqueurs qui pourraient différencier ces taxons, le premier isoenzymatique et le second est l'ADN satellite de *T. cruzi* (A28 et A29).

D'autres auteurs proposent des marqueurs différents comme :

- les profils de restriction du l'ADN kinétoplastique générés par l'enzyme *Ras* I (Gonçalves *et al.* 1991, *Mem. Oswaldo Cruz*, **86**, 477-478),

- les séquences d'ADN correspondantes aux parties variables des gènes des mini exons du RNA (Murthy *et al.* 1992, *Mol. Cell. Probes*, **6**, 237-243).

Ces techniques appliquées à un nombre réduit de souches appartenant à notre étude (données non publiées), ont permis de classer les souches entre les deux taxons de la même manière que le classement effectué par nos marqueurs. En conclusion, ces marqueurs apparaîssent tous associés les uns aux autres et pourraient donc identifier les taxons. L'étude doit être toutefois étendue à un plus grand nombre de stocks.

3.3.3. Données supplémentaires sur l'ADN satellite de *T. cruzi*

L'ADN satellite de *T. cruzi* est composé de nombreuses copies répétées d'une séquence de 195 pb, et représente environ 10 % de l'ADN nucléaire (Sloof *et al.*, 1983, *J. Mol. Biol.*, **167**, 1-21). Notre étude a montré que cette séquence est universelle au sein du taxon *T. cruzi* (A28).

J'ai aussi analysé la variabilité intra spécifique de cet ADN par la technique du polymorphisme de fragments de restriction (RFLP). Les profils observés par trois enzymes de restriction (*Eco* RI, *HAE* III et *Hinf* I) sont les suivants :

- une seule bande de 195 pb, le site de restriction existe dans toutes les copies,
- quelques grands fragments peu résolus, le site existe seulement dans quelques copies,
- un continuum de fragments (échelle) formé par des polymères de l'unité de base.

Ces profils sont en accord avec l'existence de séquences d'ADN composées d'unités de répétition répétées en tandem et dispersées en gros blocs.

La variabilité des profils générés par enzyme de restriction, a été analysée pour 15 stocks clonés au laboratoire appartenant à 2 groupes de clones génétiquement très distincts. A l'intérieur de chaque groupe, les profils sont très apparentés mais très différents entre les deux groupes. L'évolution des ADN répétés est très mal connue. Des processus d'hybridization croisée et de délétion massive ont été évoqués pour expliquer les phénomènes d'homogénisation de séquences de ces ADN (apparition ou disparition d'un site de restriction dans toutes les unités de répétition). Cette évolution au sein d'organismes à structure clonale reste cependant à approfondir avec un plus grand nombre d'enzymes de restrictions sur un échantillonage de stocks largement diversifié. Comme ce marqueur ne semble pas être extrêmement variable, il pourrait déterminer les grandes lignées évolutives au sein du taxon *T. cruzi* (cette étude préliminaire n'a pas été publiée).

3.3.4. L'unité taxonomique opérationnelle : le clone

Les travaux menés sur le terrain consistent à identifier des clones de *T. cruzi* dans les hôtes naturels et à en analyser les conséquences épidémiologiques. Il est nécessaire de tenir une réflexion à propos de la définition du clone et des outils qui nous permettent de l'identifier. Cet intermède dans l'exposé des résultats permettra de mieux comprendre le développement de la suite de mes recherches.

Définition du clone

La définition biologique d'un clone est celle d'un organisme dont les descendants sont génétiquement semblables à lui même aux mutations près. L'analyse génétique de plusieurs locus permet d'élucider le mode de reproduction des organismes et a mis clairement en évidence la structure clonale des populations naturelles de *T. cruzi* (voir le paragraphe 3.3.1.). Les clones naturels peuvent être définis comme des unités biologiques génétiquement isolées. Ils sont identifiés expérimentalement par une série de marqueurs génétiques. Ce marquage est forcément imparfait, et en augmentant la résolution des techniques, une variabilité additionnelle peut être mise en évidence au sein de chaque "clone naturel". Pour prendre en compte cette difficulté, le terme de "clonet" a été proposé pour désigner chez un organisme à structure clonale, l'ensemble des membres identiques pour une série de marqueurs génétiques (Tibayrenc *et al.*, 1991, *Bioscience*,**41** : 767-774).

La clonalité implique que les propriétés spécifiques de chaque clone ne soient guère modifiées aux cours du temps, puisqu'il n'intervient pas d'échange génétique entre les clones. Le clone est donc l'unité biologique a considérer lors des études d'agents pathogènes dont la reproduction est clonale.

Dans le cas des populations de *T. cruzi*, la diversité clonale est très grande (existence de nombreux clones), de plus, une grande différence génétique (grande distance génétique) peut les séparer. Ces données nous permettent de proposer l'existence de propriétés spécifiques à chaque clone naturel, d'autant plus nombreuses que les clones présentent entre eux une plus grande distance génétique.

Choix des marqueurs

Quels sont les marqueurs génétiques à utiliser pour définir les clones ? Ceux qui sont soumis à une forte sélection doivent être rejetés car dans ce cas des phénomènes de convergence peuvent apparaître. Les marqueurs stochastiques, dont la variation dépend essentiellement de mutations ponctuelles, sont les meilleurs pour

mesurer les distances évolutives entre les organismes. Les isoenzymes sont largement employées à cet effet.

L'ensemble des profils isoenzymatiques observés définissent un zymodème. Un clone naturel se définit par l'ensemble des individus présentant le même zymodème. Toutefois, l'analyse d'un nombre supplémentaire de systèmes enzymatiques peut distinguer des sous groupes génétiques. Dans ce cas, on est en présence d'un mélange de clones. L'utilisation d'un nombre réduit de marqueurs risque de regrouper des clones qui présentent encore de grandes distances génétiques. Il est important de tester la validité des unités taxonomiques choisies par l'utilisation d'un plus grand nombre de marqueurs.

D'autres marqueurs que les isoenzymes ont été proposés pour étudier la variabilité du taxon *T. cruzi*.

L'ADN kinétoplastique incubé en présence d'enzymes de restriction génère des profils de bandes très variables d'un stock à l'autre : les schizodèmes. L'analyse de cette variabilité sur de nombreux stocks de *T. cruzi* a montré qu'elle est corrélée à la variabilité isoenzymatique (voir le paragraphe 3.3.1.). Les profils de bandes sont cependant très complexes et nécessitent une analyse par ordinateur pour une exploitation optimale.

De même, la variabilité peut être recherchée par la technique de RAPD (Random Amplification Polymorphism DNA). Cette technique consiste a générer un polymorphisme de produits d'amplification de l'ADN en utilisant des "primers" choisis au hasard. Le polymorphisme obtenu pour un large échantillon de stocks de *T. cruzi* est aussi corrélé à celui des isoenzymes (Tibayrenc *et al.*, 1993, *Proc. Natl. Acad. Sci. USA*, **90** : 1335-1339).

L'intérêt de multiplier les analyses à l'aide de marqueurs différents est de comparer les groupes phylogéniques obtenus et de sélectionner ceux qui sont identifiés par l'ensemble des analyses.

L'étude de la liaison entre la variabilité génétique et celle des caractéristiques biologiques de populations clonales doit s'effectuer sur un modèle représentatif de l'ensemble du taxon. Il est nécessaire de tester plusieurs individus génétiquement apparentés ou dissemblables en choisissant des stocks d'origines géographiques variées ainsi que d'hôtes différents. A partir d'un tel modèle, il est possible de tester l'impact de la variabilité génétique du parasite sur ces propriétés biologiques.

- Les membres d'un même clone ou de clones génétiquement très apparentés partagent-ils les mêmes propriétés ?

- Les membres de clones génétiquement différents presentent-ils des propriétés radicalement différentes ?

- Comment les propriétés biologiques se distribuent-elles dans l'ensemble du taxon ?

Le choix des clones à étudier est laissé au jugement de l'expérimentateur. L'identification des clones est dépendante de marqueurs et une étude plus approfondie de la variabilité génétique de son échantillon peut déboucher sur une nouvelle interprétation de ses résultats.

3.3.5. Développement de marqueurs spécifiques de clones

Sondes d'ADN kinétoplastique de *T. cruzi*

La caractérisation des souches de *T. cruzi* nécessite habituellement un isolement (fèces d'insectes vecteurs ou sang de mammifères) et une production massive pour obtenir le matériel nécessaire à l'analyse isoenzymatique multilocus ou à la purification d'ADN. Cette étape de culture provoque la sélection de certains clones quand l'échantillon initial en contient plusieurs. Pour mener des études sur la répartition naturelle des clones de *T. cruzi* j'ai entrepris le développement de techniques permettant la caractérisation génétique directe des clones dans les fèces et le sang des différents hôtes. La première partie du travail, développée en 89-90, a consisté à trouver des sondes d'ADN spécifiques de clones ou groupes de clones génétiquement apparentés (A11 et A13).

L' ADN kinétoplastique (kDNA) est constitué de deux types de chaînes circulaires, les maxicercles et les minicercles. Ces nombreuses chaînes concaténées (environ 50 maxicercles et 10^4 minicercles) forment un réseau complexe. Dans le cas de *T. cruzi*, les séquences des minicercles présentent 4 zones de même taille et de séquence analogue séparées par des séquences hyper variable (HVRm).

La Réaction de Polymérisation en Chaîne (PCR), est basée sur l'amplification enzymatique d'une séquence choisie d'ADN à l'aide de deux amorces d'oligonucleotides complémentaires des extrémités de la séquence cible. De cette manière il est possible de produire en grande quantité les séquences correspondantes aux HVRm du kDNA en utilisant des amorces situées dans les parties constantes des minicercles, juste en bordure des parties variables. Les résultats ont montré que les produits d'amplification correspondants aux HVRm, étaient spécifiques de clones (définis par 15 loci enzymatiques). Il était donc possible de développer des sondes d'ADN permettant l'identification génétique de clones de *T. cruzi*.

Par la suite, deux sondes de kDNA spécifiques de deux groupes de clones génétiquement très différents et fréquemment identifiés en Bolivie (A1, A5 et A13) ont été utilisés dans mes travaux.

Modèle généralisable aux Kinetoplastidae

Cette approche a été appliquée avec succès pour distinguer des sous-groupes génétiques au sein du taxon *T. brucei* sp.(A3). Une sonde kDNA est capable de reconnaître les stocks de *Trypanosoma brucei gambiense* groupe I responsables de la pathologie humaine en Afrique centrale et de l'ouest. De la même manière, au sein du taxon *T. rangeli*, des sondes de kDNA reconnaissent seulement des groupes de stocks génétiquement apparentés, ces données non publiées doivent cependant être confirmées par une étude d'un plus grand nombre de stocks. La généralisation de la spécificité clonale des HVRm du kDNA peut être envisagée pour les leishmanies car ces parasites présentent également une structure clonale. De plus, les études des profils générés par digestion à l'aide d'enzymes de restriction (RFLP) sur l'ADN kinétoplastique montrent une variabilité importante corrélée avec les profils isoenzymatiques (voir le résumé des présentations d'ICOPA 1990, volume 1, p.22)

3.3.6 - Caractéristiques épidémiologiques et médicales des clones de *T. cruzi*

Hypothèse

Les populations de *T. cruzi* se composent de clones naturels stables dans l'espace et dans le temps. Parmi ces nombreux clones, certains sont très fréquents et sont nommés clones majeurs (voir le résumé de la présentation de la réunion annuelle de recherche fondamentale sur la maladie de Chagas, 1988, volume 1, p.21). En Bolivie, les études isoenzymatiques ont mis en évidence la circulation en cycle domestique de deux clones majeurs qui sont génétiquement très différents (grandes distances évolutives). Selon notre hypothèse, ils pourraient présenter des caractéristiques biologiques différentes (transmission, pathogénicité, réponse immune de l'hôte mammifère, spécificité d'hôte et de vecteur, résistance au traitement etc...). Ce sujet rapproche l'immunologie et la biologie de la génétique des populations et ma formation est bien adaptée à cette situation.

Techniques d'identification des clones

Caractérisation isoenzymatique

La caractérisation isoenzymatique reste une technique de choix qui apporte une très bonne information génétique de base indispensable aux approches utilisant des sondes d'ADN. Cependant elle présente quelques limitations :

- elle nécessite l'isolement des souches. Cette étape est de faible rendement chez l'homme où le taux des parasites circulants est bas,
- les quantités de parasite requises pour une étude sur de nombreux locus demande une étape de culture massive ; comme nous l'avons déjà évoqué au chapitre précédent, l'étape de culture sélectionne certains clones quand la population initiale en est composée de plusieurs.

J'ai en général utilisé 11 systèmes enzymatiques qui correspondent à 13 loci. Ces études m'ont permis de faire des analyses du mode de reproduction du parasite et de mieux connaître les relations phylogéniques entre clones isolés de différents hôtes et zones géographiques.

Détections directe des clones par PCR et sondes d' ADN dans les liquides biologiques

Il s'agit de combiner deux techniques. La première consiste à amplifier par PCR, directement dans les liquides biologiques, les séquences d'ADN correspondantes aux HVRm Ce test a valeur de diagnostic et permet en cas de positivité (obtention de produits d'amplification de 270 pb) de démontrer l'infection par *T. cruzi*.

La deuxième est la caractérisation génétique des souches infectantes. Elle repose sur la propriété des séquences des HVRm qui sont spécifiques des différents clones. Ainsi les produits d'amplification obtenus à partir des liquides biologiques infectés par des souches de *T. cruzi* présentent une variabilité selon les souches infectantes. Ces produits ne peuvent être hybridés que par des séquences d'ADN homologues, donc des sondes kDNA (HVRm) élaborées à partir de stocks génétiquement apparentés.

Notre technique de PCR à partir d'échantillons de sang de patients en phase chronique de l'infection n'est pas très sensible comparées à celle de l'équipe brésilienne (voir le chapitre 3.1.4.). Leurs produits d'amplification de 320 pb comprennent aussi les parties HVRm et une plus grande séquence de partie constante. Des essais d'hybridization de ces produits obtenus de stocks de référence (caractérisation isoenzymatique) avec nos sondes montrent les mêmes profils

d'hybridation que sur les produits obtenus dans nos conditions d'amplification (données non encore publiées). Une combinaison des deux approches permet d'allier la haute sensibilité de la première technique avec l'identification spécifique des clones.

Résultats

Comparaison des clones circulants des deux côtés des Andes (Chili, Bolivie et Pérou).

Une étude isoenzymatique sur de nombreux stocks isolées de part et d'autre des Andes en Bolivie, au Chili et au Pérou (A8 et A32) a été menée. Trois mêmes groupes de clones ont été trouvés en Bolivie et au chili. Il convient de remarquer que l'un des trois groupes est présent uniquement au Chili ainsi qu'au sud de la Bolivie. Cette région présente avec le Chili des contacts commerciaux intenses qui peuvent être responsables du transport des triatomes.

Les souches isolées au sud du Pérou dans la région d'Aréquipa (1985) ont montré une diversité clonale moyenne de 27,7 % mais la majorité de ces clones (94 %) sont génétiquement apparentés. Une étude similaire effectuée dernièrement dans une des communautés précédemment étudiées fait apparaître un indice de diversité clonale plus élevé (38 %) mais tous ces clones sont comparables à ceux de la première étude. Ces nouvelles données sont en faveur de la stabilité des populations de parasites dans cette région.

Cette deuxième étude menée au Pérou apporte des résultats interressants sur la micro-diversité des clones de *T. cruzi* à l'échelle d'une même communauté. Les critères de clonalité ont été appliqués à cet échantillon qui répond à des conditions strictes de sympatrie (une même maison), alors que peu d'échantillon sont dans ce cas. Ces résultats sont en cours d'exploitation.

Etude de la variabilité génétique de souches de Colombie.

Grâce à une récente collaboration avec l'Université de "Los Andes" de Bogota nous avons examiné 37 stocks colombiens isolés de différents vecteurs et hôtes impliqués en cycle domestique et sylvestre. Plusieurs stocks des deux cycles pourraient appartenir au taxon *T. rangeli* qui est un parasite très abondant en Colombie. L'ensemble des autres stocks appartiendrait au taxon *T. cruzi* (29 souches). La diversité clonale de ces isolats est élevée (82,7 %) et le reste remarquablement quelque soit l'origine des stocks (cycle domestique ou sylvestre). La diversité géographique des isolats pourrait expliquer le nombre important de

génotypes obtenus ; toutefois, dans le même département de Cundinamarca, les 10 souches isolées (3 de cycle domestique et 7 de cycle sylvestre) présentent 10 génotypes différents. Ainsi, la diversité génotypique en Colombie semble très importante et supérieure en cycle domestique à celle observée en Bolivie.

Des stocks appartenant à chacun des deux cycles sont génétiquement très apparentés ; ceci est en faveur d'un contact étroit entre les deux cycles.

L'exploitation de ces données est en cours en collaboration avec mes collègues colombiens.

Etude de la variabilité génétique de souches isolées en Guyane française

La Guyane française n'est pas une région endémique pour la maladie de Chagas bien que quelques cas sporadiques de malades y ont été décrits. Il existe cependant un cycle sylvestre dont plusieurs vecteurs et hôtes mammifères sont porteurs de *T. cruzi.*

L'ensemble des stocks étudiés a pour origine le cycle sylvestre. La diversité clonale des 27 stocks est très élèvée (81,4 %) et la majorité forme un groupe génétiquement apparenté à un des clones majeurs circulant en Bolivie. Toutefois, ce groupe présente encore une grande variabilité génétique interne. Malgré la petite taille de l'échantillonnage, quelques considérations epidémiologiques peuvent être avancées : nous n'observons pas de spécificité d'hôtes ni de spécificité géographique en ce qui concerne la distribution des clones. Ces observations sont semblables à celles de Colombie.

Les tests de critères de clonalité appliqués à cet échantillonnage sont aussi hautement significatifs. Ces résultats font l'objet d'une publication qui est actuellement soumise.

Circulation des deux groupes de clones majeurs dans 4 espèces de vecteurs sylvestres et domestiques en Bolivie : identification par PCR et hybridation.

Les esssais de PCR réalisés à Montpellier en 1991 sur un nombre réduit de fèces de *Triatoma infestans* étaient très prometteurs mais une étude plus importante avec différentes espèces de triatomes d'origines géographiques différentes était nécessaire pour mieux évaluer l'interêt de cette approche épidémiologique.

Nous avons utilisé la technique de PCR suivie de la méthode d'hybridation pour identifier les clones infectants dans 345 fèces de triatomes. Pour l'espèce *T. infestans,* les techniques de PCR et l'examen microscopique sont en accord dans 85%

des cas. Certains échantillons présentent des parasites à l'examen microscopique bien qu'ayant une PCR négative. Ce profil est observé dans 18 % des triatomes positifs à l' observation microscopique ; cependant nous avons noté que ces faux négatifs en PCR se limitent à certaines localitées et sont pratiquement inexistants dans d'autres. La préparation de l'échantillon ne consiste pas en une extraction d'ADN et donc l'extrait peut encore contenir des facteurs inhibiteurs de l'amplification.

D'autre part, certains échantillons présentant un examen microscopique négatif sont positifs en PCR. Cette discordance est observé dans 10 % des fèces microscopiquement négatifs. Dans le cas des espèces sylvestre étudiées (*Erathyrus mucronatus*, *Triatoma sordida* et *Rhodnius pictipes*), seule la PCR a pu détecter le parasite dans respectivement 19,1 %, 12,5 % et 52,8% des échantillons. Le pourcentage de détection par la PCR d'infections infra-microscopiques dans l'espèce *R. pictipes* est particulièrement élevé et doit être confirmé. Cette première partie met en évidence l'interêt de la technique de PCR pour évaluer en particulier dans des espèces sylvestres la présence de *T. cruzi* (A1 ; A2 ; A5 et A27).

L'identification par hybridation, des deux clones majeurs circulants en Bolivie chez les triatomes présente divers résultats qui démontrent l'intérêt de cette approche épidémiologique :

- Identification des clones majoritaires, pour *T. infestans*, les résultats des hybridations confirment la présence majoritaire des deux clones correspondant aux sondes utilisées. Ces clones sont détectés dans 74 % et 64 % des insectes et seulement 7,6 % des insectes ne seraient infectés par aucun des deux. Jusqu'à présent, les travaux de caractérisation isoenzymatique ne rendaient pas compte d'une telle fréquence de ces deux clones.

- Origine sylvestre et domestique des clones, ces deux clones majeurs sont aussi observés avec une moins grande fréquence chez des espèces considérées comme sylvestres : *E. mucronatus* et *R. pictipes*. Les spécimens de *E. mucronatus* ont été capturés à l'intérieur de maisons et en milieu péridomestique dans le village d'Apolo situé dans le département de La Paz. Cette espèce est associée avec *Triatoma sordida* dans plusieurs habitations mais *T. infestans* y est absent. *E. mucronatus* et *T. sordida* sont des espèces habituellement rencontrées en cycle sylvestre qui peuvent prendre domicile dans certains contextes. Une enquête sérologique (390 personnes) conduite dans la population d'Apolo n'a mis en évidence aucun cas d'infection chagasique. L'ensemble de ces données laissent supposer que les clones majeurs pourraient avoir une origine sylvestre. Dans le cas de *Rhodnius pictipes*, les spécimens trouvés exclusivement en péridomestique, proviennent de localités où la coexistance d'autres espèces est mal connue. La nature sylvestre de l'un des deux

clones est ici mise en évidence pour la première fois et ceci par la technique de PCR. De plus, grâce à la collaboration d'une équipe américaine de mammalogistes (Université de Nebraska-Lincoln, NE, USA) nous confirmons la présence de ce clone dans des mammifères sylvestres en appliquant la technique de PCR des tissus d'organes.

- Infections multiples, la détection directe chez les vecteurs des clones majeurs de *T. cruzi* met en évidence pour la première fois des taux très importants (26% en moyenne) d'infections doubles. L'analyse isoenzymatique menée sur des stocks boliviens n'a mis en évidence que 10 % d'infections doubles chez les vecteurs (Tibayrenc *et al.*, 1985, *Genetica*, **67** : 223-230). Chez *T. infestans* les taux d'infections doubles sont variables d'une communauté à l'autre et non corrélés aux taux des infections des vecteurs. Chez les espèces sylvestres des infections doubles sont aussi observées mais en moins grande abondance. L' analyse à l'aide du test du chi 2 (hypothèse nulle) est en faveur d'une absence d'interaction et de sélection entre les deux clones majeurs chez le vecteur. Ces résultas sont en cours d'exploitation et font l'objet d'un article soumis à publication.

- Distribution géographique des clones, la détection directe des clones dans les fèces permet d'étudier la distribution spaciale des clones. Cette méthode permet de traiter un grand nombre d'échantillons. Les deux clones majeurs ont été détectés dans 8 populations de *T. infestans* issues de 8 localités distantes de 29 à 400 km et situées dans deux département de Bolivie. Les résultats sont en cours d'exploitation.

Propriétés immunobiologiques et pathologiques des clones

Dès 1985, j'ai entrepris l'étude des relations des clones de *T. cruzi* avec la pathologie de la maladie de Chagas. De nombreux stocks de patients chagasiques de pathologie connue ont été caractérisés par analyse isoenzymatique ; les résultats (A15 ; A23) ne montrent pas de relation entre la pathologie et les clones. Dès cette étude, j'ai souligné les limites de la méthode employée. En effet, l'étude isoenzymatique ne permet pas d'identifier l'ensemble des patients présentant des infections multiples car la mise en culture du parasite est une étape de sélection des clones. Cette méthode introduit donc un biais. J'ai repris ce sujet en applicant les sondes kDNA développées à Montpellier et en ciblant l'étude sur de jeunes patients chagasiques dont le nombre d'infections multiples devrait être réduit. Cette étude qui est en cours, est menée en parallèle avec l'étude de la réponse immune spécifique et non spécifique des patients ainsi qu'avec l'étude des anomalies cardiaques détectées par électrocardiogramme. L'étude préliminaire que nous avons menée montre bien que les infections multiples sont nombreuses et probablement plus abondantes chez des sujets infectés depuis de nombreuses années (A26).

Comme je l'ai signalé au chapitre du diagnostic de la maladie de Chagas, certains patients présentent un statut particulier, avec une sérologie négative et un xénodiagnostic positif. La caractérisation isoenzymatique des stocks de parasite montre que ces patients présentent de façon significative un seul type de clone (A14). Toutefois, cette étude demande à être poursuivie sur un plus grand nombre de cas.

3.4. - Variabilité génétique des vecteurs de la maladie de Chagas

Comme signalé en tête du chapitre sur la maladie de Chagas, j'ai intégré à mes recherches depuis seulement 2 ans, l'étude de la variabilité génétique des vecteurs. Des études parallèles de génétique des populations des parasites et des vecteurs menées dans différentes zones endémiques ne peuvent qu'apporter des informations précieuses sur la dynamique de dispersion de la maladie. De plus, l'application de cette approche à l'étude des cycles sylvestre et domestique permet d'évaluer les interactions entre les deux cycles et de mieux adapter les mesures de contrôle.

3.4.1. - Méthode d'étude

Les premiers travaux sur la variabilité génétique des populations de *Triatoma infestans*, vecteurs principal de la maladie de Chagas en Bolivie, ont été développés à partir de 1981 (Tibayrenc *et al.*, 1981, *Cah. ORSTOM Sér. Ent. méd. Parasitol.*, **19** : 125-127 ; Dujardin et Tibayrenc, 1985, *Ann. Soc. belge Méd. trop.*, **65** : 165-169). Ces études ont été menées par une analyse isoenzymatique de 19 loci. La même analyse a été appliquée d'une part à des populations provenant de deux Départements de Bolivie, qui présentent des contextes épidémiologiques différents et d'autre part à des populations péruviennes étudiées 10 ans plus tôt par le Dr. J.P. Dujardin. L'analyse de la distribution des clones principaux de *T. cruzi* dans ces même zones est en cours.

Les premiers travaux et les nôtres mettent en évidence la faible variabilité génétique de *T. infestans* puisque seulement 3 loci sur 19 sont variables. Cette faible variabilité limite les interprétations génétiques aussi je me suis associée au Dr. J.P. Dujardin pour la recherche de nouveaux marqueurs. La technique de RAPD (Random Amplified Polymorphic DNA) est actuellement développée à l'IBBA sur *T. infestans*. Cette technique de PCR génère un profil reproductible complexe de produits d'amplification pour un individu et variable d'un individu à l'autre. L'analyse populationnelle de cette variabilité permet de faire des études de

phylogénie. Il est aussi possible, à partir des produits d'amplification, de faire une recherche de variabilité allélique et je prendrais ce thème en charge.

3.4.2. - Résultats

Les résultats sont en cours d'exploitation. Dans la région des Yungas, zone sub Andine, la stucturation des populations de *T. infestans* est particulièrement forte. D'autre part, les distances génétiques ne sont pas proportionnelles aux distances kilométriques. La population péruvienne provenant d'une vallée endémique proche de la ville d'Aréquipa, montre des fréquences alléliques comparables à celles observées 10 ans plus tôt. Quelques spécimens de la ville d'Aréquipa sont radicalement différents de ceux de cette proche vallée. Une étude de plus grande envergure peut déterminer l'origine de ces nouvelles populations qui envahissent actuellement les quartiers périphériques de la ville d'Arequipa.

4 - LES LEISHMANIOSES

L'arrivée du Dr. Philippe Desjeux au poste de co-directeur de l'IBBA en 1982, a permis de développer un programme sur l'épidémiologie des leishmanioses en Bolivie.

Les études cliniques et parasitologiques ont montré que la leishmaniose viscérale et la leishmaniose cutanéo-muqueuse peuvent co-exister dans certains foyers Boliviens. Cependant la leishmaniose cutanéo-muqueuse est la plus répandue. Ces études ont fait l'objet d'une synthèse de notre collègue F. Le Pont comprenant : l'identification des espèces vectrices, l'incidence de la maladie et la détermination des populations à risque dans plusieurs foyers, la description des modes de transmission ainsi que la caractérisation génétique des parasites. Nous avons collaboré à ce travail par l'identification d'"espèces" de leishmanie à l'aide d'anticorps monoclonaux (A17).

Dès lors, a été menée une étude fondamentale sur les protéines de surface des agents parasitaires dans un but d'amélioration du diagnostic. Cette approche était à l'époque très développée. Plusieurs jeunes chercheurs ont successivement mené ces recherches, toutes effectuées à l'IBBA, et j'y ai participé par un suivi journalier des expériences et des discussions fréquentes sur l'évolution à donner à ces recherches. Les chercheurs qui ont pris en charge ce travail avaient tous une formation de biochimie, notre collaboration a été complémentaire et en retour j'ai bénéficié de leurs connaissances.

4.1. Méthode d'étude

Ces travaux ont fait appel à des techniques d'analyse de protéines de surface des parasites par marquage radioactif, puis à des techniques biochimiques de purification et enfin à des électrophorèses en gels de polyacrylamide.

4.2. Résultats

La première étude a montré que les souches caractérisées comme appartenant à l'espèce *Leishmania braziliensis* présentaient un antigène spécifique majeur de surface de 72 kDa (A18 et A19). Par la suite cet antigène a été partiellement purifié par une méthode biochimique simple. Une protéine cytoplasmique de même poids moléculaire, présentant des parentés antigéniques fortes avec l'antigène de surface, a été identifiée (A12). La spécificité antigénique de la protéine 72 Kda de surface a été démontré à l'aide d'une trentaine de sérums boliviens de patients infectés par *Leishmania braziliensis* (A9). Le diagnostic sérologique classique, effectué par la

technique d'immunofluorescence indirecte, est souvent mis en échec par le faible taux des anticorps en phase initiale de l'infection (lésion cutanée primaire) et par le manque de spécificité à tous les stades de la maladie. L'ensemble de ces travaux montre que cet antigène est un bon candidat pour le diagnostic sérologique des infections humaines par *Leishmania braziliensis* : il est spécifique de l'espèce *Leishmania braziliensis* et les sérums de faibles taux d'anticorps présentent une bonne reconaissance de cet antigène.

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5 - PERSPECTIVES

J'ai développé dans le chapitre de ma carrière scientifique mes objectifs scientifiques généraux et spécifiques et je précise ici, quelques points.

Les programmes engagés en Bolivie sont encore en développement : l'apport à la connaissance de l'approche conjointe de la génétique des vecteurs et de celle des parasites est en cours d'évaluation et pourra dès lors servir de modèle d'étude de l'épidémiologie de la maladie de Chagas dans différentes aires d'endémie. L'effort est aussi porté sur l'exploitation de nouveaux outils afin d'étudier plus facilement la génétique des populations naturelles.

Les quelques collaborations que nous avons développées avec d'autres pays que la Bolivie, nous montrent que la diversité de l'épidémiologie de la maladie de Chagas est grande et des études standardisées sont nécéssaires pour mieux caractériser les profils épidémiologiques permettant ainsi d'adapter les méthodes de contrôle.

En conclusion, les études en cours en Bolivie apportent non seulement une meilleure connaissance de la maladie de Chagas dans ce pays mais testent une approche afin d'évaluer ses retombées scientifiques sur la connaissance.

Ce vaste projet demande à mon sens un effort soutenu de notre équipe en Bolivie durant encore quelques années mais doit se développer par la suite dans de nouvelles zones d'endémie. Pour la génétique des populations de vecteurs, seulement quelques équipes en Uruguay, au Chili et au Brésil l'étudient. Le manque de standardization dans la caractérisation génétique des souches de *T. cruzi* ne permet pas de regrouper l'ensemble des données et pour le moment l'approche d'"épidémiologie moléculaire" n'est pas développée. Le déplacement de notre équipe dans de nouvelles affectations, accompagné de son renforcement en effectif permettrait réellement de faire école et de renforcer la cohérence de notre UMR : recherche fondamentale au sein du futur centre (C.E.P.M.) et application sur le terrain par une partie de ses membres.

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PRINCIPALES PUBLICATIONS

présentées par

Simone Frédérique Brenière

Juillet 1995

Sommaire

1 - Diagnostic de la maladie de Chagas

Wincker P., Bosseno M.F., Britto C., Yaksic N., Cardoso M.C., Morel C.M. & **Brenière** S.F.(**1994**) - High correlation between Chagas'disease serology and PCR-based detection of *Trypanosoma cruzi* kinetoplast DNA in Bolivian children living in an endemic area. *FEMS Microbiology letters*, **124** : 419-424

Brenière S.F., Carlier Y., Carrasco R., Molinedo S., Lemesre J.L., Desjeux P., Afchain D. & Carlier Y. (**1987**) - Specific immunodiagnosis of Chagas'disease : Immunodiffusion test using a specific serum anti - *Trypanosoma cruzi* component 5. *Trop. Geograph. Med.*, **39**, 281-286.

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2 - Immunobiogie de la maladie de Chagas

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3 - Variabilité génétique de *T. cruzi* et conséquences épidémiologiques

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1 - DIAGNOSTIC DE LA MALADIE DE CHAGAS

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High correlation between Chagas' disease serology and PCR-based detection of *Trypanosoma cruzi* kinetoplast DNA in Bolivian children living in an endemic area

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Abstract: The detection of *Trypanosoma cruzi* kinetoplast DNA by polymerase chain reaction (PCR) amplification is a potentially powerful tool for the parasitological diagnosis of Chagas' disease. We have applied this technique in a field situation in Bolivia, where 45 children from a primary school were subjected to serological testing, buffy coat analysis and PCR diagnosis. 26 of the 28 serology-positive individuals were also positive by PCR. In addition, two serology-negative children gave a positive result by PCR, including one who was positive in the buffy coat test. These results suggest that PCR detection of *T. cruzi* DNA in blood can be a very useful complement to serology in Chagas' disease diagnosis in Bolivia.

Key words: Trypanosoma cruzi; Chagas' disease; Kinetoplast DNA; PCR diagnosis

Introduction

Chagas' disease, caused by the parasitic protozoan *Trypanosoma cruzi*, is an important public health problem in most countries of Latin America. Its direct diagnosis is difficult, due to the low concentration of parasites in the blood of infected persons. Because of this limitation, Chagas' disease diagnosis relies mainly on serological techniques. The sensitivity of these serological methods is generally high, but their use presents two main problems. First, the existence of cross-reactive epitopes between *T. cruzi* and other parasites circulating in the same geographical area may lead to false-positive results [1]. Second, the clinical status of a patient can be unlinked to his humoral response, as for example during the first weeks of an infection (when no serological reac-

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tion is yet observed) or after specific treatment (when an immune response can persist for years even if the treatment has been successful [2]). These considerations have justified the quest for a more efficient method of parasite detection in chagasic patients. The principal technique that has been tested for *T. cruzi* detection in blood samples is the polymerase chain reaction (PCR). Two main systems, leading respectively to the amplification of kinetoplast minicircle DNA [3–5] or of nuclear satellite sequences [6,7] have been described. Reconstitution experiments have suggested that these techniques are potentially able to detect a single parasite cell in 20 ml of blood [4,8]. However promising, these methods have yet to be validated in clinical samples originating from different geographical regions, due to the great variations in Chagas' disease incidence and clinical manifestations in the Americas. One large-scale study has been conducted in an endemic region of Brazil, showing that PCR can attain a high level of sensitivity in this situation [9]. The present work is aimed at testing the efficacy of PCR diagnosis of Chagas' disease in a radically different situation in Bolivia.

Materials and Methods

Patients and clinical samples

The individuals examined in this study belonged to two different groups. The first was composed of 45 children from the Mizque locality, Campero province, Cochabamba department, Bolivia. They were 5 to 8 years old, and were previously selected by a serological and parasitological (buffy coat) diagnosis for Chagas' disease when they were at school. The second was composed of 8 control individuals from Rio de Janeiro and La Paz who had never lived in endemic areas.

Ten ml of blood were collected from each individual, 5 ml of which were mixed immediately with an equal volume of 6 M guanidine HCl/0.2M EDTA for further processing for the PCR test [4]. The remaining 5 ml were used for serum preparation and buffy coat testing.

Serology and buffy coat tests

Serology was performed by indirect hemagglutination using the HEMAVE kit (Polychaco, Argentina), indirect immunofluorescence (IIF) and enzyme-linked immunosorbent assay (ELISA). A patient was considered serology-positive when his serum reacted in at least two of the three tests. Buffy coat testing was performed in quadruplicate and observed by 2 independent people.

DNA preparation

The guanidine-EDTA-blood lysates were heated for 15 min in boiling water in order to shear the minicircle molecules that constitute most of the kDNA into moderately-sized pieces, and equalise their overall concentration [8]. One hundred μl was used for DNA preparation. After phenol-chloroform and chloroform extraction, the material was precipitated with ethanol. The pellet was resuspended in 50 μl of distilled water and stored at −20°C. All these and subsequent steps were performed in a laminar flow hood in a separate room not used for manipulation of amplified products, with dedicated micropipettes and filter-protected tips.

PCR conditions

The amplification reactions were performed in a volume of 75 μl using the 'hot-start' protocol with a solid paraffin barrier separating the Taq DNA polymerase from the oligonucleotides [10]. The lower phase consisted of 4 μl of the 10 × Taq polymerase buffer (100 mM Tris-HCl, pH 8.3; 500 mM KCl), 1.8 μl of a 10 mM dNTPs solution, 13.5 μl of a 25 mM $MgCl_2$ solution, 200 ng of *T. cruzi*-minicircle specific primers (5′-AAATAATGTACGGG(T/G)GAGATGCATGA 3′ and 5′ GGTTCGATTGGGGTTGGTG-TAATATA 3′ [11]), and water up to 40 μl, in a thin-walled reaction tube. An Ampliwax PCR Gem Bead (Perkin-Elmer) was added, melted by placing the tube at 80°C for 5 min, and solidified at room temperature. The upper phase consisted of 7.5 μl of the DNA sample, 3.5 μl of 10 × Taq DNA polymerase reaction buffer, 2.5 U of Taq DNA polymerase and water up to 35 μl. The PCR reaction was performed using 2 cycles at 98°C for 1 min and 64°C for 2 min, 33 cycles at

94°C for 1 min and 64°C for 1 min, and one extension step at 72°C for 10 min. The amplified products were electrophoresed in a 2% agarose gel and visualised by ethidium bromide-staining.

Results and Discussion

In the PCR assay used in this study, each blood sample to be tested was subjected to two independent DNA purifications, in order to avoid any problems arising from inhibition in any particular DNA preparation. Each PCR test included 5 samples in duplicate, one DNA preparation from an individual from a non-endemic area (also in duplicate), an amplification reaction without DNA (as a negative control) and an amplification reaction with DNA from a previously characterised chagasic patient (as a positive control). Typical results are shown in Fig. 1. In this way, we analysed 28 blood samples from serology-positive children living in the endemic area. Twenty-six of them (93%) were detected in our PCR assay, while only four of them had parasites detectable in buffy coat testing (Table 1). A previous study has indicated the high sensitivity of PCR when compared to xenodiagnosis in the detection of *T. cruzi* in blood samples collected in Brazil [9]. Until the advent of PCR, xenodiagnosis was considered to be the most sensitive parasitological technique available for Chagas'

Table 1

Comparison of the PCR results of all individuals with serology and buffy coat test results

Patient status	Patient no.	PCR	
		Positive	Negative
Bolivian children			
Serology-positive with positive buffy coat test	4	4	0
with negative buffy coat test	24	22	2
Serology-negative with positive buffy coat test	1	1	0
with negative buffy coat test	16	1	15
Non-chagasic individuals	8	0	8

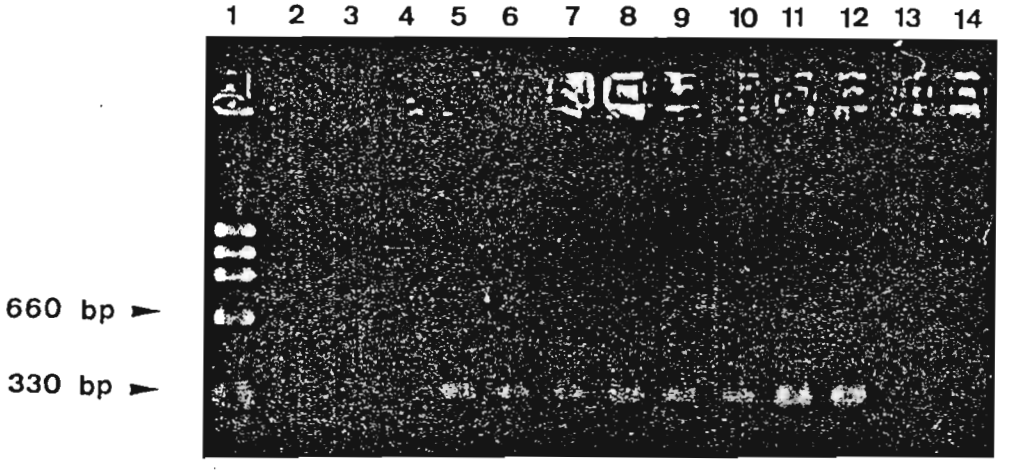

Fig. 1. Ethidium bromide-stained agarose gel from a PCR diagnosis experiment performed on 5 Bolivian children and one individual from a non-endemic area. All DNA preparations from blood samples and PCR amplifications were performed in duplicate for each patient. Lane 1, ΦX174 DNA digested with *Hae*III; Lane 2, amplification reaction without added DNA; lanes 3 and 4, amplifications from a control individual; Lanes 5 and 6 to 11 and 12, amplification products from four Bolivian children with positive serology; lanes 13 and 14, amplifications from a serology-negative Bolivian child. Fifteen μl of the total reaction volume (75 μl) was loaded on this gel. The arrows indicate the expected products of the minicircle DNA amplification: a 330 bp band corresponding to a single variable region and a 660 bp corresponding to a dimer.

disease diagnosis [12]. The results of the present study extend these findings to a different geographical area and suggest that PCR may find extensive applications in situations where other methods for parasitological diagnosis of Chagas' disease are currently being used.

Seventeen blood samples from serology-negative children were also submitted to our PCR assay, and 2 of them were positive (Table 1). Three arguments make the possibility of this result having occurred due to DNA contamination improbable. Firstly, we did not detect any contamination in 8 DNA preparations from control individuals processed in duplicate at the same time as the blood samples from the Bolivian children, while the two serology-negative, PCR positive samples gave an amplification in both of the duplicate DNA preparations. Secondly, the endemic region where these children live is considered to be an area of active transmission for Chagas' disease [13]. This renders the existence of young individuals who are serology-negative, but present a detectable parasitemia, explicable in cases of recently acquired infections, as the time necessary to mount a detectable humoral response is estimated to be several weeks [14]. Finally, one of the two serology-negative, PCR-positive children had *T. cruzi* cells in his blood as observed in buffy coat testing (Table 1). It should be noted that this result is consistent with the observation that parasitemia levels are generally high in the initial acute phase of Chagas' disease, when no antibody responses are yet detectable [15].

The high level of sensitivity and specificity of PCR detection of *T. cruzi* DNA in blood, observed in this and previous studies in an endemic region with very different characteristics, suggests that this technique will be a very valuable tool to study the evolution of Chagas' disease in patients in different epidemiological situations.

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(17)

SPECIFIC IMMUNODIAGNOSIS OF CHAGAS DISEASE: IMMUNODIFFUSION TEST USING A SPECIFIC SERUM ANTI-TRYPANOSOMA CRUZI COMPONENT 5

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Abstract A micro double diffusion test (MD), allowing the identification of precipitation band 5 by identity reaction, using a rabbit specific anti-component 5 serum, was evaluated for the immunological diagnosis of Chagas' disease. The previous studies on the *Trypanosoma cruzi* specificity of component 5 [g] were completed, showing it to be absent in *Leishmania brazilienis*, but present in different strains of *T. cruzi*. 200 sera from Bolivian patients were studied. (88 with a positive xenodiagnosis, 45 with mucocutaneous leishmaniasis but without Chagas' disease, and 67 controls). Band 5 was found in 74 (84.1%) of the sera with positive xenodiagnosis but was never found either in the leishmaniasis or in the control groups. MD, allowing an easy detection of *T. cruzi* specific band 5, cheap and simple to perform, can be recommended in association with other serological tests, when highly specific immunodiagnosis of Chagas' disease is required

Key words: specific immunodiagnosis, Chagas' disease, Trypanosoma cruzi component 5, immunodiffusion; Bolivia

Introduction

The importance of the immunodiagnosis of chronic Chagas' disease has already been widely emphasized. However, many human sera with positive serological reactions for *Trypanosoma cruzi* also have positive reactions with antigens of other flagellates such as *T. rangeli, Leishmania donovani* or *L. braziliensis* [1-8]. The existence of shared epitopes between flagellate antigens [9-11] can explain such observations. Consequently various methods have been proposed to increase the specificity of the immunodiagnosis of Chagas' disease, using: 1. other parasite forms, like amastigotes or trypomastigotes, assumed to be more specific for *T. cruzi* than epimastigote forms [5, 12, 13]; 2. other antigenic extraction procedures [7]; 3. absorption of the positive human sera with the cross-reacting antigens, before determination of the anti-*T. cruzi* antibody level [3]; 4. specific purified antigen of *T. cruzi* [14, 15].

Such a *T. cruzi* antigen, the so-called component 5, has been demonstrated without shared epitopes with *L. donovani, L. mexicana, T. brucei* or *T. rangeli* [9, 16]. Moreover, using immunoelectrophoresis (IEP), anti-component 5 precipitating antibodies could be demonstrated in 72.6% of sera from patients chronically infected by *T. cruzi* [17]. The aims of this work was to complete the specificity study of component 5 and to evaluate a micro double diffusion test (MD), allowing the identification of precipitation band 5 by identity reaction using a specific rabbit anti-component 5 serum, for specific immunodiagnosis of Chagas' disease. MD, previously used for

specific diagnosis of other parasitic disease [18], was expected to be simple to perform, avoid, ig preparation of a large quantity of purified component 5, and higher sensitive than IEP.

Materials and Methods

Human sera.
Sera were obtained from 200 Bolivian patients, divided into three groups according to their geographical origin. The first group (1) contained 88 patients (mean age 39 ± 13 years) with positive xenodiagnosis. They came from southern lowlands, areas known to be highly endemic for Chagas' disease, but where leishmaniasis had never been found. They were asymptomatic or with cardiac or digestive symptoms compatible with the chronic phase of Chagas' disease. The second group (2) contained 45 patients (mean age 29 ± 9 years) with clinical evidence of mucocutaneous leishmaniasis (35 with typical primary cutaneous ulcerations with surrounding indurations and 10 with typical mutilations of the face). They lived in northern lowlands ('Beni' and 'Alto Beni'), areas known to be endemic for leishmaniasis but free of Chagas' disease. The third group (3) was a control group of 67 asymptomatic patients (mean age 25 ± 5 years), from highland areas ('Altiplano') exempt of both infections and who had never travelled in the endemic areas.

T. cruzi and L. braziliensis antigens.
T. cruzi epimastigotes (Tehuantepec strain) were cultivated in cell free GLSH monophasic medium at 28°C [19]. After 7 culture days, the parasites were harvested by centrifugation at 800 g for 15 min at 4°C and washed three times with Hank's balanced salt solution. Six grams (wet weight) of epimastigotes were suspended in 36 ml of 1% NaCl, frozen and desintegrated five times in a hydraulic press (LKB X press) at 18000 psi and then centrifuged at 26.000 g for 1h at 4° C. The supernatant was dialyzed against distilled water for 24h at 4°C, lyophilized and used as a soluble extract of *T. cruzi*. Soluble antigenic extracts of other six different isoenzymic strains of *T. cruzi*, classified as 1, 1b, 1c. 2, 2a and 2b according to Tibayrenc *et al.* [20] were prepared as the *T. cruzi* Tehuantepec strain for the complementary study of specificity. *L. braziliensis* promastigotes (LV65 strain) were obtained from cell-free culture on NNN medium, modified according to Deker-Jackson & Honigberg [21]. A. *L. braziliensis* soluble extract was prepared as for *T. cruzi*.

T. cruzi component 5. 20 mg of *T. cruzi* (Tehuantepec strain) soluble antigenic extract were resuspended in 4 ml of distilled water and added with an equal volume of chloroform/methanol solution (2/1). The mixture was shaken for 30 min at room temperature and centrifuged at 1.000 g for 30 min at 4°C. The aqueous phase was collected and extracted twice in the same way. Organic solvents were evaporated and glycoproteins were precipitated by the addition of 3 volumes of ethanol for 4 hours at -20°C. After centrifugation (1.000 g - 30 min), the precipitate was washed with ethanol, dried, and resuspended in 2 ml of distilled water. The solution was centrifuged, dialyzed against distilled water for 24 hours at 4°C and lyophilized to obtain 2.5 mg of a component 5-rich fraction.

Rabbit immune sera.
One rabbit was immunized with the total soluble extract of *T. cruzi*, for the specificity study of component 5. Another rabbit was injected with fraction 5 and used in the diagnosis evaluation of MD. The immunization procedure used simultaneous multiple intradermic injections, according to Vaitukaitis *et al.* [22] with 2 mg of antigen. The rabbits were boosted by weekly subscapular injections of 1 mg of antigen over six weeks. The presence of precipitating anti-component 5 antibodies in the rabbit sera was controlled in IEP and in immunodiffusion by identity reaction with a reference monospecific anti-component 5 serum, prepared according to Afchain *et al.* [9].

Micro double diffusion test (MD).
MD was performed on microscope slides (25 × 80 mm) covered with 4 ml of 1% agarose (IBF-France) in 0.1 M veronal buffer, pH 8.2. Three patterns of wells for sera and antigenic extract were punched for one slide, allowing simultaneous study of 12 different human sera (*figure 1*). 60 µl of human sera, concentrated to 12 µl by lyophilization, was placed in peripheral wells whereas 12 µl of 1/4 diluted rabbit anti-5 immune serum was put in the central wells. The two micro-wells were filled with 2 µ (24 µg) of *T. cruzi* antigenic extract.

Then, the slides were incubated in a moist chamber, at room temperature for 24h. They were washed by immersion in 0.1 M veronal buffer pH 8.2 for 48h, demineralized in distilled water for 1h at room temperature, dried and stained by Coomassie blue. The test was positive when one of the precipitation bands produced by the human sera showed an identity reaction with the band 5 obtained with the anti-component 5 immune serum.

Results

Complementary study on the T. cruzi specificity of component 5. As shown in *figure 2*, precipitation band 5 was demonstrated to be present and identical in the 6 studied different isoenzymic strains of *T. cruzi*, but it was not found in the *L. braziliensis* antigenic extract.

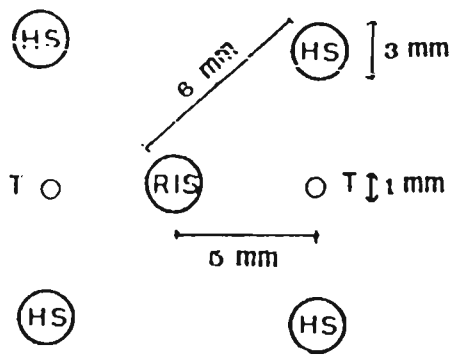

Figure 1. MD pattern (RIS = anti-component 5 rabbit immune serum; HS = human serum; T = soluble antigenic extract of *T. cruzi*).

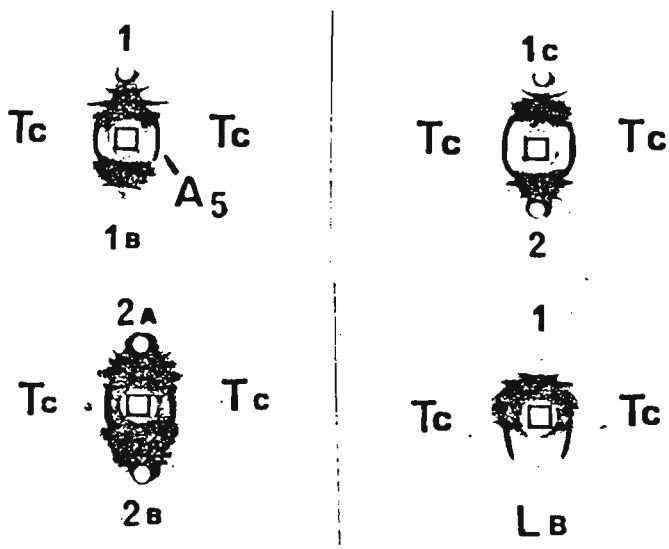

Figure 2. Immunodiffusion test studying the specificity of component 5 (1, 1B, 2, 2A, 2B = soluble antigenic extract of different isoenzymic strains of *T. cruzi* containing component 5 (A5); Tc = component 5 fraction (*T. cruzi* Tehuantepec strain; LB = soluble antigenic extract of *L. braziliensis*□ = anti *T. cruzi* rabbit serum).

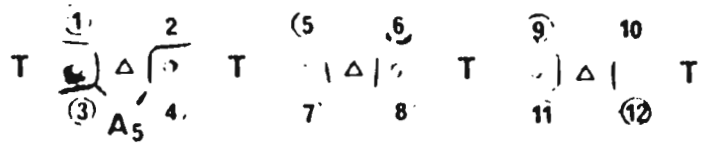

Figure 3. MD with human sera (1, 2, 3, 9, 10, 11: human sera showing precipitation band 5 (A5); 4, 5, 6, 7, 8, 12 = band 5 negative sera; T = *T. cruzi* antigenic extract Δ = anti-component 5 rabbit immune serum).

Evaluation of the micro double diffusion test to identify the precipitation band 5. 74 out of 88 (84,1%) sera from the group 1 presented a well-defined precipitation band, identical to the band obtained with the specific anti-component 5 sera in MD, as shown in *figure 3*. No sera of leishmaniasis group 2 and of control group 3 presented a precipitation band.

Discussion

The *T. cruzi* specificity of component 5 in relation to *T. brucei*, *T. rangeli*, *L. donovani*, and *L. mexicana* was previously demonstrated by Afchain *et al.* [9, 16]. The present results completed this specificity study, showing its presence in different *T. cruzi* strains but the absence of component 5 in *L. braziliensis*. Moreover band 5 was not found in sera from patients with mucocutaneous leishmaniasis or Bolivian controls. Consequently it can be affirmed that the presence of anti-component 5 antibodies in a serum ensures the diagnosis of Chagas' disease.

The MD sensitivity of band 5 detection was 84.1%, which is higher than the 72.6% previously obtained using IEP [17]. Indeed MD avoids the electrophoresis step, necessary to identify component 5 in IEP, increasing the antigen concentration available for the diffusion step. Moreover, in MD, the identification of band 5 in human serum is easy using the identity reaction with a rabbit specific anti-component 5 immune serum.

MD requires little human or rabbit serum or *T. cruzi* antigenic extract and allows simultaneous study of many sera using microscope slides. The specific anti-component 5 serum is easy to produce. 40 ml of a high quality serum, from one immunized rabbit, allow 40.000 patient sera to be tested. Moreover human sera with a predominant precipitation band 5 could be progressively selected by IEP or immunodiffusion to be used instead of rabbit serum.

Since other serological tests, such as immunofluorescence, complement fixation test or ELISA, also with Bolivian sera, though less specific, obtained a better relative sensitivity (99-100%) [17], the routine use of MD for the immunodiagnosis of Chagas' disease, instead of more sensitive tests, is unsuitable. However, when the classical serological tests are positive for different flagellate antigens, this can be due to shared epitopes between flagellates or mixed infections with various flagellates. Indeed, most South american areas are known to be endemic for both leishmaniasis and Chagas' disease and it is essential to obtain a confirmation of the diagnosis of Chagas' disease, since clinical and therapeutic management of leishmaniasis and Chagas' disease are very different. In such cases, in association with other serological tests, MD, with identity reaction, cheap and simple to perform (not requiring enzyme or fluorochrome-labelled reagents), can be recommended as a highly specific tool for immunodiagnosis of Chagas' disease.

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COMPARISONS OF IMMUNOLOGICAL TESTS FOR SERODIAGNOSIS OF CHAGAS DISEASE IN BOLIVIAN PATIENTS

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Abstract. Enzyme linked immunosorbent assay (ELISA) and immunoelectrophoresis (IEP) were evaluated and compared to the classical immunofluorescence (IF) and complement fixation test (CFT) in the immunological diagnosis of Chagas' disease, using 407 sera from Bolivian patients. 72.7 to 79.5% of randomised sera, coming from patients living in endemic areas for Chagas' disease were considered as positive, according to the test limits, previously determined. The techniques could be classified according to their percentage detection as ELISA > IF > CFT > IEP.

The quantitative correlations between the tests were excellent ($p < 0.001$). 92.8% of the sera were positive or negative for the four tests, 6.1% for three tests and 1.1% for only two tests. The agreement between the tests ranged from 94.6 to 99.2%, co-positivity from 95.5 to 100% and co-negativity from 88.5 to 100%. IF gave the best results, and could be considered as the reference test since it was easy and rapid to perform. However to avoid errors or discrepancies between laboratories, two tests, such as IF and CFT, might be associated. ELISA can be used if higher sensitivity is required. IEP showed 1 to 14 precipitation bands in 96% of the sera from infected patients. The precipitation band 5, previously demonstrated as *Trypanosoma cruzi* specific, was present in 73% of these sera, indicating the interest to use immunoprecipitation test, if more specificity is required for the immunodiagnosis of Chagas' disease.

Key words: Chagas' disease; *Trypanosoma cruzi*; serodiagnosis; ELISA; immunoelectrophoresis; immunofluorescence; complement fixation test; comparisons of immunological tests; Bolivia.

Introduction

In the acute phase of Chagas' disease, blood trypomastigotes of *Trypanosoma cruzi* are easy to detect by direct microscopy. By contrast, in the chronic stage of the infection, parasitological investigations such as xenodiagnosis or blood culture only lead to 31 to 50% of diagnosis [1-4]. In chronic cases without blood forms, the diagnosis of *T. cruzi* infection must be based only on the presence of anti-*T. cruzi* circulating antibodies. This demonstrates the importance of the immunological diagnosis of Chagas' disease.

Various techniques have been applied: the complement fixation test (CFT), pioneered by Guerreiro & Machado [5] direct agglutination [6], hemagglutination (IHA) [7-10], immunoprecipitation [11, 12], immunofluorescence (IF) [13-17] and more

recently enzyme-linked immunosorbent assay (ELISA) [18-23] and thin layer immunoassay [24]. However few comparative studies between more than two tests have been performed. Camargo [25] evaluated CFT, IF, IHA and direct agglutination together recommending the association of two tests. Fuchs [26] compared CFT, IHA, IF and ELISA and showed IF and ELISA more related to clinical pictures of Chagas' disease.

In the present study, ELISA, which is largely used for sensitive diagnosis of other parasitosis [27, 28] and immunoelectrophoresis (IEP), recommended for specific diagnosis of helminthiasis [29], were evaluated and compared to the classical IF and CFT in the immunological diagnosis of Chagas' disease in Bolivian patients. Moreover, advantage was taken of the immunoelectrophoretic analysis of human antibodies to study the frequency of precipitating antibodies anti-antigen 5, previously demonstrated as specific of *T. cruzi* and without cross-reactions with other flagella [30, 31].

Material and methods

Antigenic extract of T. cruzi. Epimastigotes of *T. cruzi* (Tehuantepec strain) were obtained from culture in cell-free GLSH monophasic medium at 28°C [32]. The parasites were collected by centrifugation at 2000 g, washed three times in Hank's balanced solution and divided into two samples. The first one was directly used in smears for immunofluorescence. The second was suspended in NaCl 1% o, frozen three times for cell disintegration using an hydraulic press at 18000 PSI (× press I.KB). It was then centrifuged at 26.000 g for 1 h. at 4°C. The supernatant was dialysed and lyophylized. Such crude *T. cruzi* antigenic extract was used for IEP, CFT and ELISA.

Human sera. For preliminary determination of the limits of each test, well referenced sera (group 1) were used: 76 came from *T. cruzi* infected Bolivian patients with positive xenodiagnosis; 68 were from control Bolivian patients living in highlands (altiplano) without Chagas' disease, never having travelled in known Bolivian endemic areas and for which parasitological and clinical investigations were negative; 10 were from European control patients living outside the Bolivian chagasic endemic areas and without parasitological or clinical Chagas' disease.

For the comparative study between the four serological tests, 263 randomised sera (group 2) came from patients living in various endemic areas of Bolivia.

Serological tests. IF was performed using *T. cruzi* epimastigotes fixed by 1% glutaraldehyde [16]. The FITC labelled anti-human immunoglobulins conjugate (Institut Pasteur Production-Paris) was used diluted 1/200.

CFT was carried out according to Kent and Fife [33].

ELISA was performed according to Carlier [27]. Preliminary assays showed the optimal *T. cruzi* antigenic extract coating concentration to be 10 μg/ml. The sera were diluted 1/100. The peroxydase-labelled anti-human immunoglobulins (Institut Pasteur Production, Paris) were used at 500 ng/ml with H_2O_2 and orthodianizine as substrate. Extinction values were measured at 405 nm.

IEP was carried out according to Biguet [29] in 1% agarose using sera three times concentrated by lyophylisation. The precipitation band 5 was identified by its particular intensity and position on immunoelectrophoresis [31].

The same *T. cruzi* antigenic extract was used for these three last techniques though each test was performed independently and the results compared *a posteriori.*

Results

Determination of the specific limit of the tests. The results of the group 1 sera are expressed in *table 1.* The detection limits of 1/40 for IF and 1/2 for CFT were selected. The limit extinction value of ELISA was determined as $m + 2\sigma$ of the control Bolivian group: $0.07 + 2 \times 0.05 = 0.17$.

The IEP was considered positive with the presence of, at least, one well-defined precipitation band.

Table 1. Frequency of the different results obtained for group 1 of selected sera.

Tests	*Results*	*Control Europeans*	*Control Bolivians*	T-cruzi *infected Bolivians (positive xenodiagnosis)*
	⩽10	10	64	0
IF	20	0	4	0
titre	40	0	0	7
	80	0	0	10
	⩾160	0	0	59
	<2	10	67	0
CFT	2	0	1	5
titre	4	0	0	16
	8	0	0	24
	⩾16	0	0	31
	0	10	68	3
IEP	1-2	0	0	2
(precipi-	3-4	0	0	22
tation	5-6	0	0	21
bands)	>6	0	0	28
ELISA	Ext.values	0.07 ± 0.03	0.07 ± 0.5	0.39 ± 0.07

Results obtained in the randomised sera group. The frequency of the different titres, number of precipitation bands and extinction values obtained for the group 2 sera are expressed in *table 2*. IEP showed 1 to 14 precipitation bands with a mean of 6 ± 3. 72.7 to 79.5% of these sera were considered as positive and the techniques can be classified according to their percentage detection as ELISA > IF > CFT > IEP.

Correlation studies between IF, CFT, and ELISA. The quantitative correlation values between the logarithmic transformations of the titres of IF and CFT and the arithmetic extinction values of ELISA, of the group 2 sera were obtained by analysis of the regression curves and are expressed in *table 3*.

Agreement between the tests. Among the 263 randomised sera, 244 (92.8%) were positive or negative for the four tests, 16 (6.1%) for three tests and 3 (1.1%) for only two tests (doubtful results).

Consequently, only 260 sera could be classified as positive or negative according to any three of the four tests. The agreement, co-positivity and co-negativity of each test, alone or associated, with such a classification are expressed in *table 4*. The general agreement ranged from 94.6 to 99.2% as IF > ELISA, CFT > IEP, co-positivity from 95.5 to 100% as IF, ELISA > CFT > IEP, and co-negativity from 88.5 to 100% as IEP > IF. CFT > ELISA. However, no significant differences could be noted between these results.

Frequencies of the precipitation band 5 in IEP. The frequencies of the band 5, and of the total number of precipitation bands observed in IEP are expressed in *table 5*. There is no difference between the results of the group 1 of sera with positive xenodiagnosis (72.6%) and the group 2 (73.3%).

Table 2. Frequency of different results obtained for group 2 of randomised sera.

IF			*CFT*			*IEP*			*ELISA*		
titre	*n*	%	*titre*	*n*	%	*bands*	*nb*	%	*O.D.*	*n*	%
<40	60	22.8	<2	62	23.5	0	72	27.3	<0.17	54	20.5
40	29		2	28		1-2	12		0.17-0.27	17	
80	59		4	38		3-4	51		0.28-0.37	54	
160	65	77.2	8	62	76.5	5-6	62	72.7	0.38-0.47	111	79.5
320	34		16	59		7-8	40		0.48-0.57	27	
≥640	16		≥32	14		≥9	26		0.58-0.67	0	

Table 3. Results of the quantitative correlation studies between IF, CFT and ELISA (r = correlation coefficient; t = value of student's t; p = probability).

Correlations	*r*	*t*	*P*
CFT/ELISA	0.32	4.76	0.001
IF/ELISA	0.43	9.36	0.001
IF/CFT	0.23	3.41	0.001

Table 4. Agreement, co-positivity and co-negativity between the tests alone or associated with the 260 sera classified as positive or negative according to any 3 of the 4 tests.

Tests	*agreement*		*co-positivity*		*co-negativity*	
	n	%	*n*	%	*n*	%
IF	258	99.2	199	100.0	59	96.7
ELISA	257	98.8	199	100.0	58	95.1
CFT	257	98.8	198	99.5	59	96.7
IEP	252	96.9	191	96.0	61	100.0
IF/ELISA	255	98.0	199	100.0	56	91.8
IF/CFT	255	98.0	198	99.5	57	93.4
CFT/ELISA	234	97.6	198	99.5	56	91.8
IF/IEP	250	96.1	191	96.0	59	96.7
IEP/ELISA	249	95.7	191	96.0	58	95.1
IEP/CFT	249	95.7	190	95.5	59	96.7
ELISA/IF/	252	96.9	198	99.5	54	88.5
ELISA/IF/IEP	247	95.0	191	96.0	56	91.8
CFT/IEP/IF	247	95.0	190	95.5	57	93.4
ELISA/CFT/IEP	246	94.6	190	95.5	56	91.8

Table 5. Frequencies of the precipitation band 5 in IEP in group 1 (positive xenodiagnosis) and group 2 (randomised) sera.

Total	*positive xenodiagnosis sera*			*group 2 sera*		
bands	*n*	*band 5*	%	*n*	*band 5*	%
0	3	0	0.0	72	0	0.0
1-2	2	1	50	12	6	50.0
3-4	22	12	54.5	51	33	64.7
5-6	21	17	80.9	62	44	70.9
>6	28	23	82.1	66	57	86.3
	73	53	72.6	191	140	73.3

Discussion

The high frequency of positive results with the studied sera coming from patients living in endemic areas underlines the frequency of *T. cruzi* infection in Bolivia, where 35% of the total population is considered as infected [34].

The comparative study shows a good agreement between CFT, IF, ELISA and IEP, since no significant differences could be observed between the results. The co-positivity can be considered as a parameter of the relative sensitivity of the tests and the co-negativity as a relative specificity parameter. The results obtained are in accordance with those of Camargo [25] who obtained relative sensitivity of 99.9 for IF and 99.2 for CFT and Voller [18] who obtained 98% agreement for IF and ELISA. Fuchs [26] obtained sensitivity of 98.5% with ELISA, 95.1% with IF and 73.1% with CFT. Spencer [21] noted 87.4% agreement between ELISA, IF and CFT. Our slightly higher results than in these two last studies could be explained by the use of the same batch of *T. cruzi* antigenic extract, prepared from fresh *T. cruzi* epimastigotes and lyophilized to avoid conservation problems.

IF gave the best results, and can be considered as the reference test, since it is easier and more rapid to perform than CFT, which confirms many previous works [13, 16, 17, 25, 26].

IEP was able to detect 96% of the sera with positive xenodiagnosis (group 1) or serology (group 2), with the highest relative specificity. The *T. cruzi* specific band 5 could be identified in 73% of the two groups of sera having precipitating antibodies, bringing the certitude of the *T. cruzi* infection. Such results are in accordance with a preliminary work of Afchain [35] performed with few sera. They indicate the high immunogenecity in man of the antigen 5 and the interest to use such immunoprecipitation test, cheap and simple to perform, for the immunodiagnosis of Chagas' disease.

ELISA appears with a high sensitivity but a lower specificity than in the other tests. This could be due to the use of a crude *T. cruzi* antigenic extract [28].

The use of purified specific antigens as the 90 Kd molecular weight glycoprotein [23] or anti-antigen 5 monoclonal antibody in competition EIA [36] allows considerable improvement in specificity. However, at the present time, such reagents are not available for routine study and only crude antigenic extract can be used.

Such comparisons also clearly show that the use of two or three associated tests do not improve the relative sensitivity or specificity of the serodiagnosis. One single, well-chosen, test can be sufficient. However to avoid discrepancies or errors between laboratories [37] it is preferable to associate two techniques.

In terms of equipments and cost, IF and ELISA are more expensive than CFT and IEP. Indeed IF needs a fluorescent microscope and fluorescein conjugate, while ELISA needs a spectrophotometer and enzyme conjugate (it is also possible to use ELISA as a semiquantitative test, using serum dilution and visual determination of a titer, avoiding the use of a spectrophotometer). CFT and IEP need only disposable material (plates, slides). The antigen consumption is higher in IEP and CFT than in IF, using smears of epimastigote forms and ELISA using only very low amount of antigenic extract. The required technical skill is quite the same for all the tests. IEP, CFT and ELISA are more time consuming than IF. Such considerations on easy handiness, rapidity, slow antigen consumption, sensitivity and specificity lead to the conclusion that IF is the best test, beside the need of fluorescence microscope which can be used in many other applications in a routine laboratory. According to the possibilities of the laboratory, IF and CFT could be recommended or IF and ELISA, if more sensitivity is necessary or IF and IEP, if more specificity is required.

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SPECIFIC HUMORAL DEPRESSION IN CHRONIC PATIENTS INFECTED BY TRYPANOSOMA CRUZI

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SUMMARY

We performed a comparative study between xenodiagnosis and serological tests for Chagas'disease. 150 Patients from several endemic areas were studied. Four of them appeared to have a peculiar status with positive xenodiagnosis and negative serology carried out with four classical techniques (Immunofluorescence test, ELISA: Enzyme Linked Immunosorbent Assay, Complement fixation test and Immunoelectrophoresis). One serum out of the four patients presenting humoral depression showed a high quantity of circulating antigen proved by immunoelectrophoresis. The Authors suggest the use of one serological test for detecting circulating antigens of **Trypanosoma cruzi** in addition to the classical serology. It would allow the diagnosis of Chagas' disease in patients with low production of specific antibodies.

INTRODUCTION

Chronic stage of Chagas' disease is characterized by a high production of specific antibodies which allows an easy diagnosis (CAMARGO & TAKEDA[5]). During this stage, several serological tests are available for detecting circulating antibodies, i.e.: complement fixation test, immunofluorescence test, Enzyme Linked Immunosorbent Assay (GUERREIRO & MACHADO[9], LELCHUK et al.[12], VOLLER et al.[21]). Antibodies are present during all the infection and even after treatment (BARCLAY et al.[3], COURA et al.[7]). Xenodiagnosis test allows a parasitological confirmation of the infection, but it is of low sensitivity compared with the serological diagnosis.

We studied 150 chagasic patients proceeding from endemic areas; they were systematically investigated by parasitological and serological tests, looking for a possible correlation between both tests. Among them, four patients presented a peculiar status, with a positive xenodiagnosis and a negative serology.

The Authors discuss the origin of this humoral immunossuppression for total specific antibodies to **Trypanosoma cruzi** in these four cases.

MATERIAL AND METHODS

Patients — 150 patients from endemic areas, having lived for a few years in La Paz City (non endemic area), were investigated by serological diagnosis, and then tested by xenodiagnosis according to the methods described underneath.

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Xenodiagnosis — Patients were exposed for 30 minutes to 30 **Triatoma infestans** specimens of third larval stage. Faeces control was carried out one, two and three months after the insect's bite. This observation was performed on microscope slides pooling faeces from three triatomes.

Serological diagnosis was performed with four techniques:

(1) Immunofluorescence test (IFT) according to WELLER & COONS [22];

(2) Enzyme Linked Immunosorbent Assay (ELISA) according to BOUT et al.[4];

(3) Complement fixation test (CFT) according to GUERREIRO & MACHADO[9] method, modified by KENT & FIFE[10]. A soluble epimastigote antigen was used at a dilution of 0.2 mg/ml;

(4) Immunoelectrophoresis (IEP) was carried out for each serum as described in details by AFCHAIN et al.[1]. The electrophoregrams' interpretation was established with 100 Bolivian sera from non endemic areas and 15 European sera as controls: the test was considered as positive when more than three bands were observed, or only one or two bands if they were strong.

Serological diagnosis was considered as positive when at least three out of the four techniques proved positive, and vice-versa for negativity. Criteria for positivity were respectively: titers $\geq$ 1/40 (IFT), optical density $>$ 0.17 (ELISA), titers $\geq$ 1/2 (CF).

Detection of T. cruzi circulating antigens — 10 European sera and four sera from chagasic Bolivian patients were tested in IEP against an immune rabbit serum (IRS) obtained by immunization (Vaitukaitis method) with **T. cruzi** antigenic fraction; this fraction was obtained by precipitation of **T. cruzi** total extract with a major immune rabbit serum anti-antigen 5 (LEMESRE [13]).

Isolation and isoenzyme typification of T. cruzi stocks — a simple method for obtaining stocks of T. **cruzi** from guts of triatome bug vectors was used (TIBAYRENC et al.[18]). The isoenzyme typification was performed with five enzymatic systems: phosphoglucomutase (E.C.2.7.1., PGM), malate deshydrogenase Nadp+ or malic enzyme (E.C.1.1.1.40.,ME), glucose phosphate isomerase (E.C.5.3.1.9., PGI), 6-phosphogluconate deshydrogenase (E.C.1.1.1.44., 6PGD) and isocitrate deshydrogenase (E.C.A.A.A. 42., ICD). The procedures and determination of the zymostrains were according to TIBAYRENC et al.[19].

RESULTS

Results of parasitological and serological examinations for 150 chagasic sera are summarized in Table I. Among the patients presenting a positive serology (97.3%), 61.3% showed a positive xenodiagnosis, and 36% a negative one. These results express the low sensitivity of this parasitological test, in contrast with the serological diagnosis established with four techniques. Four patients out of 96 with positive diagnosis (4.2%) presented a peculiar status, with a positive xenodiagnosis together with a negative serology.

TABLE I
Distribution of patients tested by xenodiagnosis and Chagas'serology

Patients number	Serology	Xenodiagnosis	Percentage of patients
92	P	P	61.3%
54	P	N	36.0%
4	N	P	2.7%

P : Positive
N : Negative

Patient No. 1 had a negative serology, more than one year after a previous positive test with a negative xenodiagnosis which became then positive. Patient No. 2 maintained a negative serology two years after the first test, while the xenodiagnosis, initially positive, had turned negative. Patient No. 3 presented a negative serology one month after a first negative test, and had also a positive xenodiagnosis. Patient No. 4 showed also a negative serology and a positive xenodiagnosis (Table II).

Stocks from patients No. 1 and 3 were typified by isoenzyme technique, confirming their belonging to **T. cruzi** complex (zymostrain 1, TIBAYRENC et al.[19]).

All four sera were investigated looking for circulating antigens of **T. cruzi**. As shown in Fig. 1A and B, serum from patient No. 3 reacts

TABLE II

Serology and xenodiagnosis of four patients with depression of specific humoral immunity to Trypanosoma cruzi

	Age	Dates tests	IFT titers	ELISA (D.O.)	IEP (nb. of bands)	Xenodiagnosis
P/140		Nov. 1981	1/40	0.31	2	N
Patient 1	18	Nov. 1982	< 1/40	0.08	0	P
		May 1981	< 1/40	0.02	0	P
Patient 2	48	Jan. 1983	< 1/40	0.09	0	N
P/184		Nov. 1982	< 1/40	0.05	0	P
Patient 3	30	Dic. 1982	< 1/40	0.01	0	—
Patient 4	31	Apr. 1980	< 1/40	0.07	0	P

P : Positive
N : Negative

positively against the IRS, and gives e pattern with 2 precipitation bands. On the other hand, we tested sera from 15 Europeans by the IEP technique; all sera presented with the IRS only one precipitation band, which can be observed also in Patient No. 3. This constitutes probably a non specific reaction between the normal sera and the IRS. The second band, of cathodic localization, was only observed in serum of Patient No. 3, and never in Europeans sera. This band seems specific of **T. cruzi**, and proves the presence of an antigenic component of **T. cruzi** in huge quantity in this patient's serum.

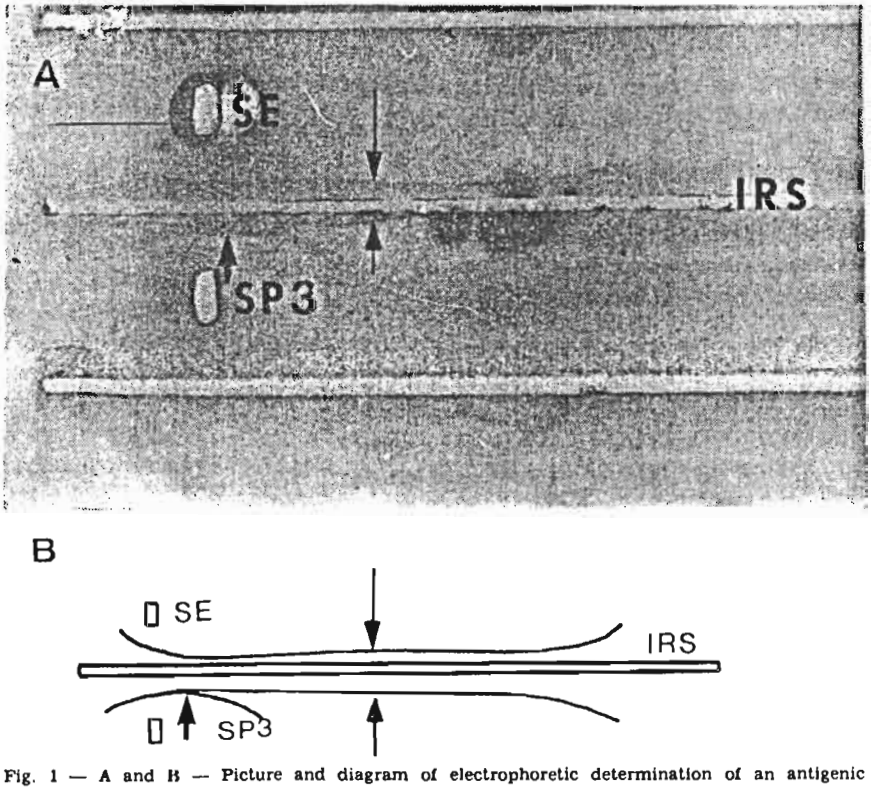

Fig. 1 — A and B — Picture and diagram of electrophoretic determination of an antigenic component of T. cruzi in the serum of patient presenting positive xenodiagnosis and negative serology. Serum of patient No. 3 (SP3), serum of Europeans (SE), Immune Rabbit Serum to an antigenic fraction of T. cruzi (IRS), non specific band (⟶), specific band of T. cruzi (⟶). All sera were concentrated three times.

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DISCUSSION

Our results confirm the supremacy of serology for establishing a diagnosis of Chagas' disease in the chronic stage. In fact, in the studied population, only 61.3% of the patients with a positive serology presented a positive xenodiagnosis. Nevertheless, the four cases we report here were not detected as Chagas' disease by the classical serological tests; on the contrary, in spite of its low sensitivity, xenodiagnosis permitted the diagnosis.

In the present study, a low percentage of patients (2.6%) presented an important depression of specific humoral antibodies' production to T. cruzi, but the selection of the patients generally done by the serology does not allow a rigorous evaluation of these cases. Only an epidemiological study, with parasitological and serological diagnosis, could reveal the real frequency of this humoral suppression.

The immunossuppression phenomenons during the evolution of the parasite infections are very frequent and have been demonstrated in various protozoan infections (TERRY [17]). Up to now, non specific immunossuppression in experimental Chagas' disease has been described only during the acute phase of the infection (KIERSZENBAUM [11]). Some Authors assessed that in human infection, a non specific immunossuppression occurs in some acute cases (TEIXEIRA et al.[16]), but probably not in chronic cases (TSCHUDI et al.[20]). However, our results shown in few cases a specific immunossuppression during the chronic period of the infection. This phenomenon could be related to mixed infections (viral, bacterial or parasitological infections associated with Chagas' disease: COX [8], SALAMAN [14], SCHWAB [15]), but in our 4 cases a general clinical examination did not show any intercurrent affection. In addition, the immunossuppression seems to be a lasting phenomenon, one of the patients still presenting a negative serology two years after the first examination. A further immunossuppression study by lymphoblast transformation test would allow to define with more accuracy the origin of this suppression: cellular suppression, or a possible non specific or specific humoral factor such as circulating antigen (CAPRON et al.[6]).

Moreover, the IEP reveals the presence of a specific band to a rabbit immune serum with Patient No. 3 serum; this points to the presence of a rather great quantity of antigen in this serum. The IEP can only detect high quantity of antigenic proteins because of its low sensitivity, which could explain the absence of any band in the sera of patients No. 1, 2 and 4. ARAUJO et al.[2] showed the presence of T. cruzi circulating antigens in some sera of chronically infected patients, using ELISA test with Fab'2 coating; this test could be improved using a purified antigen fraction, which could get a more sensitive diagnosis with higher specificity. In these cases with immunossuppression, the systematic investigation of circulating antigens would be useful for Chagas' diagnosis.

RESUMO

Depressão humoral específica em pacientes crônicos infectados pelo Trypanosoma cruzi

Realizamos um estudo comparativo entre o xenodiagnóstico e os testes sorológicos para a doença de Chagas. Cento e cincoenta pacientes de algumas áreas endêmicas foram estudados. Quatro deles pareceram revelar um estado particular com um xenodiagnóstico positivo e uma sorologia negativa, esta realizada com quatro diferentes técnicas clássicas (teste de immunofluorescência, ELISA: Enzyme Linked ImmunoSorbent Assay, teste de fixação do complemento e teste de immuno-eletroforese).

O soro de um dos pacientes que apresentou depressão humoral específica mostra elevada quantidade de antígenos circulantes comprovada pela técnica da immuno-eletroforese. Os Autores sugerem o uso de um teste sorológico para detectar a presença de antígenos circulantes de T. cruzi, além da utilização de testes sorológicos clássicos. Isto permitiria o diagnóstico da doença de Chagas em pacientes com uma baixa (ou mesmo inexistente) produção de anticorpos específicos.

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TRYPANOSOMA CRUZI: EXPRESSION OF ANTIGENIC COMPONENT 5 AMONG 35 LABORATORY CLONES OBTAINED FROM 18 DIFFERENT ISOZYMIC VARIANTS

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SUMMARY

Two monoclonal antibodies anti-component 5 of **Trypanosoma cruzi** (I-35/115 and II-190/30) were tested in IFA and ELISA respectively against 35 **T. cruzi** laboratory clones. Among the 35 clones tested, 18 different isozyme patterns were detected. All clones were recognized by both monoclonal antibodies except one clone which did not react with II-190/30. These results support the universal expression of specific component 5 within the taxon **T. cruzi**.

KEY WORDS: **Trypanosoma cruzi**: Antigenic components; Clones.

INTRODUCTION

Trypanosoma cruzi, the causative agent of Chagas' disease, infects 24 million people in America [27]. It displays a large heterogeneity in both morphology and biology [6]: for example, differences are observed in virulence, tissue tropism, pathogenicity, drug resistance and antigen composition.

T. cruzi stocks have been characterized taxonomically by means of isoenzyme studies. Analysis of zymograms enabled MILES et al.[13,14] and READY & MILES [16] to distinguish three major groups of isozymic strains which were called "zymodemes". An extensive isozyme study on 121 stocks of **T. cruzi** [25], isolated from various regions of South and Central America, described 43 isozyme variants which exhibited large genetic variability and did not fall into the 3 main clusters that had been proposed initially [13,14,16].

Diagnosis of Chagas' disease during indeterminate and chronic phases is only possible by serologic techniques since circulating levels of parasites are too low. However, techniques used in standard serology are not specific enough to discriminate **Leishmania** and **T. cruzi** infections [2,10]. Furthermore, several mixed infection areas have been found in Central and South America [2,22]. Thus, for diagnostic purposes, for an effective immunological approach to Chagas' disease, it would be important to demonstrate the presence of universal antigens among the taxon **T. cruzi**.

Comparative studies of epimastigote cultures of **T. cruzi** and other **Trypanosomatidae** have demonstrated the existence of a **T. cruzi** specific antigenic component: component 5 [1]. This antigen exhibits several characteristic features. It possesses a great level of immunogenicity in natural [4] or experimental infections as in immunization experiments [1] and has been found at the surface of epimastigotes and bloodstream trypomastigotes [9]. Three murine monoclonal antibodies against component 5 of

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T. cruzi have been purified and characterized [15]. Monoclonal antibody I-35/115 has been shown to bind the epimastigote cell surface (immunofluorescence study) and monoclonal antibodies II-190/30 and II-160/18 to bind internal organelles. Immunoprecipitation of T. cruzi iodinated soluble antigen with these monoclonal antibodies, followed by polyacrylamide gel analysis. has led to the identification of four molecules whose respective molecular weights are: 72 kD, 51 kD, 43 kD and 24 kD [15]. These proteins were not recognized with the same intensity by the three monoclonal antibodies. Finally, 96.6% of chronic chagasic patient sera have been detected by competitive enzyme immunoassay using anti-component 5 monoclonal antibody (II-190/30). This test is still in evaluation in several laboratories in South America, with W.H.O. grant support.

Here, we evaluate T. cruzi component 5 with 2 monoclonal antibodies (I-35/115 and II-190/30) in the taxon T. cruzi. Among 35 clones tested, 18 isoenzyme patterns were detected (TIBAYRENC et al. classification, 25), and represent a large proportion of the genotypes classified up to now.

MATERIALS AND METHODS

1 — Parasites:

Parasites were grown in LIT medium. Twenty T. cruzi stocks representing 20 different isozymic strains classified according to TIBAYRENC et al. [25] have been cloned by micromanipulation; 35 laboratory clones were so obtained. The original stocks were isolated from mammals or bug vectors from various geographic origins (see Table 1). Control stocks were **Leismania mexicana amazonensis** (WHO IFLA/BR/67/PH-8), **Leishmania brasiliensis braziliensis** (WHO MHOM/BR/75/M-2904), 6 **Leishmania braziliensis braziliensis** Bolivian stocks [7] and **Trypanosoma rangeli** RBG strain.

2 — Isoenzyme analysis of T. cruzi clones:

Isoenzyme analysis of the clones was performed using 9 enzyme systems: glucose 6 phosphate dehydrogenase (E.C.1.1.1.49), glucose 6 phosphate isomerase (E.C. 5.1.3.9), glutamate dehydrogenase NADP+ (E.C.1.4.1.4), isocitrate dehydrogenase (E.C.1.1.1.42), malate dehydrogenase (E.C.1.1.1.37), malate dehydrogenase (oxalo-acetate decarboxylating) NADP+ or malic enzyme (E.C.1.1.1.40), phosphoglucomutase (E.C.5.4.2.2, formerly E.C.2.7.5.1), phosphogluconate dehydrogenase (E.C.1.1.1.44) and mannose phosphate isomerase (E.C.5.3.1.8). Methods were described previously [24].

3 — IFA — indirect fluorescent antibody test:

Following the technique described by OROZCO et al. [15], this test was carried out on epimastigote forms in their initial stationary phase. The monoclonal antibody (Mc Ab I-35/115) was used. The conjugate was fluorescein conjugated anti-mice IgG (H+L) (Institut Pasteur Production, Paris, France). All assays were performed in duplicate.

4 — ELISA — Enzyme Linked Immunosorbent Assay:

Epimastigote parasites from the initial stationary phase were washed 3 times in Hanks-Wallace solution, resuspended in NaCl 9% ($100x10^6$ p/100 ul), sonicated and centrifuged at 26,000 g for one hour at 4°C. Subsequent steps have been described previously [3]. Purified Mc Ab II-190/30 was labelled with alkaline phosphatase [12]. All assays were performed in duplicate. Extinction values were measured at 405 nm.

RESULTS

1 — Isozymic variability of T. cruzi cloned strains:

Laboratory clones, compared to their original stocks on the basis of isozyme patterns, appeared to be similar in all cases, except one strain with isoenzyme pattern number 19. The original stocks differed for glucose 6 phosphate isomerase (2 bands instead of one observed in 4 clones obtained from this stock). GPI is a dimeric enzyme and the 2 bands obtained for the original stock were probably the result of a mixed population.

2 — Expression of component 5 in clones of T. cruzi:

All results are summarized in Table 1. Tested clones were isolated from different mam-

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TABLE I

Expression of antigenic component 5 in T. cruzi isozymic clones

Number of T. cruzi	Country of origin	Source of isolates	No. of isozyme strains[a] of original stocks	Mc Ab recognition of Component 5 IFA[b] Mc Ab I-35/115	ELISA Mc Ab II-190/30 Extinction values[c]
1	Chile	Human	43	+	0.43
4	French Guiana	Wild mammal	3	+	1.00
			5 (2 clones)	+	0.22, 0.70
			11	+	0.35
4	French Guiana	Triatoma[d]	1 (4 clones)	+	0.73, 0.94, 0.95, 1.02
11	Bolivia	Human	10 (3 clones)	+	0.95, 1.12, 1.60
			16	+	1.47
			19 (5 clones)	+	0.36, 0.43, 0.82, 1.16, 1.48
			39	+	1.01
			39	+	0.72
1	Bolivia	Wild mammal	28	+	0.26
3	Bolivia	Triatoma infestans	32	+	0.21
			9	+	0.31
			32	+	0.08
3	Bolivia	Triatoma[d]	25 (3 clones)	+	0.75, 1.06, 1.28
7	Brazil	Human	not classified (2 clones)	+	0.26, 1.18
			17	+	1.57
			30 (2 clones)	+	0.9, 1.04
			34	+	1.23
			35	+	1.10
1	Brazil	Wild mammal	36	+	1.25
Control strains					
L. m. a.	Panama		—	—	0.03
L. b. b.	6 Bolivia	4 Human	—	—	0.01, 0.02, 0.03, 0.06
		2 Sandfly	—	—	0.02, 0.07
	1 Brazil	Human	—	—	0.03
T. rangeli	Venezuela	Dog	—	—	0.03

a — The isozymic strains were classified according to TIBAYRENC et al.[36]. Among 35 T. cruzi clones tested, 18 different isozyme patterns were observed.

b — Mc Ab I-35/115 was diluted at 1/10 and 1/25.

c — The extinction values of ELISA were from duplicate assays; the limit extinction value was 0.096 determined as m + 3 × (standard deviation) of the 9 control strains.

d — Triatoma other than T. infestans.

mals and vectors. Only one Bolivian clone isolated from Triatoma infestans (isozymic strain No. 32) produced a negative result in ELISA with Mc Ab II-190/30. All other clones were recognized by both Mc Ab I-35/115 and Mc Ab II-190/30 tested, respectively, in IFA and ELISA. Extinction values obtained in ELISA, which ranged from 0.08 to 1.57, demonstrate a high level of heterogeneity. IFA controls consisting of a L. m. amazonensis strain, 7 L. b. braziliensis strains and a T. rangeli strain were negative. The limit extinction value of ELISA was determined to be m + 3 times the standard deviation of the 9 control strains: 0.0338 + (3 × 0.027) = 0.096.

DISCUSSION

1 — Clonal diversity in a single isolate of T. cruzi:

Our data support the presence of a mixed population in one T. cruzi human isolate. This confirms genetic heterogeneity of single T. cru-

zi isolates. Similar findings based on isoenzyme patterns have been reported previously for natural populations of T. cruzi isolated from both triatomine bug vectors [23] and humans [5]. All these results emphasize the usefulness of cloning before experimental studies of T. cruzi are conducted [1], as recommended by DVORAK et al. [8].

2 — Specific antigens of T. cruzi and their diagnostic application:

WHO (1975) [26] recommends the use of specific antigens in the diagnosis of Chagas' disease. Candidate proteins must be expressed in all isoenzyme variants and must be absent in Leishmania and T. rangeli strains.

Several antigens have been proposed and warrant further research.

a) The 90 kD glycoprotein antigen semi-purified by lectin affinity chromatography was tested in ELISA system [18]. This test is quite sensitive but reacts with sera of patients infected with Leishmania. The authors suggest that purification of this antigen is necessary.

b) The 25 kD glycoprotein has been proposed as specific to T. cruzi, but was only tested on 8 T. cruzi strains [17]. This protein was not detected on either T. rangeli or Leishmania [17]. 96.5% of chagasic patients sera were positive in immunoprecipitation against this purified protein, and all 23 Leishmania human sera were negative in this test [17]. However, no information was given concerning the possible crossed-reactions of these control sera in standard serology for Chagas' disease.

c) A 72 kD surface glycoprotein isolated from both epimastigote and metacyclic trypomastigotes was purified by monoclonal affinity chromatography [20]. Monoclonal antibodies directed against various epitopes of 72 kD glycoprotein have been obtained. These epitopes were shown to be strain- or species specific [6, 11,19]. Some of these monoclonal antibodies are good candidates for use in Chagas' diagnosis.

d) Lastly, our results support the universal expression of T. cruzi component 5 among varied set of T. cruzi genotypes. Indeed, 18 isozyme variants from different regions and isolate origins were recognized by Mc Ab I-35/115. However, it is worth noting that quantitative differences were observed in the recognition of the clones using the second Mc Ab II-190/30 in ELISA. This could be due to different levels of expression of the epitope recognized by Mc Ab II-190/30. These quantitative differences seem independent of genotype: for genotype 19 we tested 5 clones which range from 0.36 to 1.48, and as great a quantitative difference is observed for genotype 1 (4 clones tested) and genotype 5 (2 clones tested). Both Mc Ab used in this work are directed against a 72 kD glycoprotein (component 5), and the identity of this antigen with the 72 kD glycoprotein of SNARY et al. [20] has been suggested [15]. Nevertheless, further studies are required to show the identity of monoclonal antibodies against the 72 kD protein, and others against component 5. Finally, a specific test using the Mc Ab II-190/30 has been proposed [12]. Our results confirm the utility of this test in specific diagnosis, but appropriate controls of Leishmania sera must be tested to ascertain its application in areas with mixed leishmanial and chagasic infections.

RESUMO

Trypanosoma cruzi: Expressão do componente antigênico 5 entre 35 clones de laboratório obtidos de 18 variantes isoenzímicas.

Dois anticorpos monoclonais anticomponente 5 de Trypanosoma cruzi (I-35/115 e II-190/30) foram testados respectivamente em IFA e ELISA sobre 35 clones de T. cruzi isolados no laboratório. Entre estes 35 clones testados, 18 perfis isoenzímicos diferentes puderam ser detectados. Todos os clones foram reconhecidos exceto um clone que não reagiu com o anticor po monoclonal II-190/30. Estes resultados são a favor da expressão constante do componente 5 no seio do taxón T. cruzi.

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3 - VARIABILITE GENETIQUE DE *T. CRUZI* ET CONSEQUENCES EPIDEMIOLOGIQUES

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FIELD APPLICATION OF POLYMERASE CHAIN REACTION DIAGNOSIS AND STRAIN TYPING OF *TRYPANOSOMA CRUZI* IN BOLIVIAN TRIATOMINES

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Abstract. A new approach for direct identification and characterization of *Trypanosoma cruzi* stocks in biological samples was tested for field applicability on an extensive sample of feces collected from triatomine vectors from four different species found in Bolivia. The first step of the technique is polymerase chain reaction (PCR) amplification of the hypervariable region of kinetoplast DNA minicircles of *T. cruzi* parasites. In this report, 345 fecal samples were analyzed and the PCR results were compared with microscopic examination. For *Triatoma infestans*, the principal Bolivian vector, both techniques were in concordance 85.3% of the time. For the three other species, *Rhodnius pictipes*, *Eratyrus mucronatus*, and *Triatoma sordida*, the fecal samples were all negative by microscopic examination whereas PCR results showed several *T. cruzi*–infected insects in each species. The second step of the procedure is the characterization of the *T. cruzi* clones by means of hybridization of the PCR products with clone-specific probes generated by the PCR. We used two probes corresponding to major clones circulating in high frequency in Bolivia (as shown by previous population genetic studies using isoenzyme characterization). We obtained four primary results: 1) we confirm the importance of two major clones in Bolivia in two distinct regions; 2) we report high rates of mixed infections (multiple clones in a single vector) in *Triatoma infestans*, up to 22% and 35% in Cochabamba and La Paz departments, respectively; 3) the results favor the absence of interaction between different clones; and 4) we find, for the first time, evidence of the major clones circulating in three species of triatomines that are known as mainly sylvatic species. The origin of these clones, sylvatic or domestic, is also discussed.

Trypanosoma cruzi, the causative agent of Chagas' disease, is present as numerous natural clones as evidenced by population genetic studies; these natural clones appear to be evolving with time and dispersing over geographic locations without genetic exchange between organisms, thus maintaining genetically similar populations.[1] Some natural clones are ubiquitous and are the ones most frequently isolated from domestic vectors and from humans; these have been designated as major clones.[2] Until recently, the characterization of the different clones of *T. cruzi* has been performed by multilocus enzyme electrophoresis (MLEE) after isolation and massive culture of the parasite to obtain sufficient quantities for this analysis. The process of culturing clearly selects for particular clones and may reduce an initial isolate that is composed of several clones to a single clone.[3] Even by MLEE analysis, infection of an individual vector with multiple clones is found in approximately 10% of the cases, as has been previously demonstrated by the visualization of double isoenzyme patterns.[4] This percentage is probably an underestimate because of the necessity of first culturing the parasites.

We and other investigators have recently described a new technique for direct identification of *T. cruzi* stocks in the feces of triatomine vectors and in mammalian blood based on polymerase chain reaction (PCR) amplification of a portion of the minicircle of kinetoplast DNA (kDNA).[5–7] Moreover, we have demonstrated the clone specificity of the hypervariable region of the kDNA minicircle (HVRm) based on a population genetics approach.[6] This result makes possible the development of clone-specific DNA probes that may be used for direct genetic characterization of the natural clones found in the various hosts.[7] In this paper, we report the construction of two probes by the PCR that correspond to two major clones of *T. cruzi* circulating in Bolivia.[6] We hybridized these probes to PCR-amplified kDNA from field samples of triatomine feces to determine the clone infecting each vector, and we assessed the field applicability of this technique by testing a large number of samples from different Bolivian triatomines species, both domestic and sylvatic.

MATERIALS AND METHODS

Vectors. The triatomines tested include four different species found in Bolivia: *Triatoma infestans*, *Rhodnius pictipes*, *Eratyrus mucronatus*, and *Triatoma sordida*. *Triatoma infestans* is a domestic vector whereas the remaining are usually considered sylvatic vectors. The different specimens were captured in the field at different locations (see Table 1 for numbers and collection sites). Moreover, we used as negative controls 28 laboratory-reared, noninfected insects (*Triatoma infestans*).

Microscopic observation. The microscopic observation was considered positive if flagellated parasites were observed in the feces of a triatome specimen during a 5-min examination of a drop of feces mixed with phosphate-buffered saline at a 400 × magnification.

Processing of triatomine feces for the PCR. Feces samples were prepared by the addition of distilled water, followed by boiling and centrifugation. Briefly, 10–20 μl of triatomine feces were individually collected in sterile Eppendorf (Hamburg, Germany) tubes using forceps and gloves rinsed in bleach between handling of each sample, and the samples were stored −20°C. The samples were collected in the entomologic room that was separate from the other room where the PCR procedure was undertaken. They

TABLE 1
Geographic origin and collection site of the vectors

Department	Province	Triatomine species*	No. of communities sampled	Collection sites	No. of triatomines analyzed
Cochabamba	Campero	*T. infestans*	1	Houses/chicken coop	6
	Capinota	*T. infestans*	6	Houses/chicken coop	34
La Paz	Nor Yungas	*T. infestans*	12	Houses/chicken coop	124
	Caranavi	*T. infestans*	1	Houses/chicken coop	53
	Sud Yungas	*R. pictipes*	1	Chicken coop	36
	Franz Tamayo	*E. mucronatus*	1	Houses/chicken coop	68
		T. sordida	1	Houses/chicken coop	24

* *T. = triatoma; R. = Rhodnius; E. = Eratyrus.*

were diluted by the addition of 200 µl of distilled water. The parasites were lysed by boiling for 10 min followed by two 10-min centrifugation at 8,000 × *g*. As a control, one tube with water was subjected to the same treatment as the fecal samples (boiled and centrifuged). We believe that the water template is a good negative control because it is free of the possible presence of PCR inhibitors. Ten microliters of the supernatant was used as a template in each of the PCR assays. One-tenth of the PCR product was analyzed by electrophoresis on a 0.8% agarose gel and visualized by staining with ethidium bromide.

Polymerase chain reaction. The PCR was performed according to a previously described method.[7] Briefly, the sequences of the oligonucleotide primers used were CV1: 5′-GATTGGGGTTGGAGTACTACTAT-3′ and CV2: 5′-TTGAACGGCCCTCCGAAAAC-3′ (chosen to amplify all *T. cruzi* isolates). They were obtained from the Genset Laboratory (Paris, France).[6] Two restriction sites (*Sca* I and *Sau* 96 I) were artificially introduced at the 3′ end of each oligonucleotide and used for purification of the HVRm-amplified sequence away from the oligonucleotide primers that contain part of the conserved region of the minicircle. Samples were amplified in 67 mM Tris-HCl (pH 8.8), 16.6 mM $(NH_4)_2SO_4$, 6.7 mM $MgCl_2$, 10 mM mercaptoethanol, 0.01 mg/ml of bovine serum albumin, 75 pM of each deoxynucleotide triphosphate, and 75 pM of each oligonucleotide in a total reaction volume of 50 µl. A systematic aliquoting of this amplification buffer was done in 1.5-ml microtubes. Each aliquot was covered with 50 µl of paraffin to prevent evaporation and was stored frozen at −20°C. For the amplification, which was performed using a Trio thermoblock PCR device (Biometra, Gottingen, Germany), 2.5 U of *Thermus aquaticus* DNA polymerase (Promega, Madison, WI) was used. The amplification involved three distinct steps: 1) an initiation step with DNA denaturation (95°C for 5 min), oligonucleotide primer annealing (48°C for 2 min), and elongation (72°C for 2min); 2) an amplification step with 30 cycles (95°C for 5 sec, 48°C for 30 sec, and 72°C for 1 min), and 3) a cooling step (4°C for variable times). Each run include 1) one positive control of total DNA template and 2) two negative controls with water instead of DNA template, with one of them subjected to the same treatment as each feces sample as described above. A systematic aliquoting of the PCR components (buffer, primers, oligonucleotides, and water) was done in 30–40 sterile microtubes. Each aliquot was covered with 50 µl of paraffin to prevent evaporation and was stored at −20°C until use. The absence of contamination in the aliquots was previously checked by a test using a water template.

Sensitivity of detection. The sensitivity was evaluated by the addition of 10 fg to 20 pg of *T. cruzi* total DNA (reference strain) in the feces samples from different laboratory-reared *Triatoma infestans.*

Southern blot. One-tenth of each PCR-positive sample was subjected to electrophoresis on a 0.8% agarose gel and transferred after alkali denaturation (0.5 N NaOH, 1.5 M NaCl, twice for 15 min) onto charged nylon membranes (Hybond N+; Amersham, Buckinghamshire, UK) by vacuum blotting. For the vacuum transfer, the gel was placed on a prewetted membrane, which consisted of filter paper backed with a piece of diaper. This sandwich was covered with plastic wrap and placed on a gel dryer (Bio-Rad, Richmond, CA) and vacuum was applied for 10 min without heat.

Probes. The two clone-specific probes (20 and 39) were purified from their respective HVRm DNA fragments produced by the PCR from TPk1 (clone 39) and So34 c14 (clone 20) *T. cruzi* stocks.[1,4,8] Briefly, the PCR-amplified 270-basepair HVRm fragments from 10 runs were purified by electrophoresis on 0.8% preparative agarose gel (Sigma, St Louis, MO). The fragments were eluted by electroelution using a 422 electro-eluter devise (Bio-Rad) according to the manufacturer's instructions, precipitated with 0.2 M NaCl in 2.5 volume/volume (v/v) of pure ethanol, and resuspended in 100 µl of Tris-EDTA buffer. The DNA was digested with the restriction endonucleases *Sau* 96 I and *Sca* I (Promega) to eliminate part of the oligonucleotide primers selected in the conserved region of the minicircle. After digestion, the DNA was precipitated with ethanol and resuspended in a volume of 100 µl, as described above. The amount of DNA was quantitated by electrophoresis of sequenced dilutions. These probes have previously been shown to be highly specific for *T. cruzi* clones 20 and 39 and genetically closely related clones.[6,7]

Labeling and hybridization conditions. The probes were labeled and the filters were hybridized using the enhanced chemiluminescence gene detection system according to the manufacturer's recommendations (Amersham). Briefly, the membranes were incubated at 42°C in hybridization buffer (0.12 ml/cm²) for 15 min. At the same time, each of the purified probes was labeled for 10 min at 37°C. A total of 10 ng of labeled probe was added to the membranes per milliliter of hybridization buffer. Hybridization was performed at 42°C overnight in a rotary oven (Appligen, Illk-

TABLE 2

Detection of parasites in feces: comparison of microscopic observation and the polymerase chain reaction (PCR) technique

Triatomine species*	No.	Microscopic results + / PCR results +	Microscopic results + / PCR results −	Microscopic results − / PCR results +	Microscopic results − / PCR results −	Comparison of both techniques: % sensitivity† MOD	% sensitivity† PCR	% concordance‡	% discrepancy§ A	% discrepancy§ B
T. infestans	217	95	22	10	90	92.1	82.7	85.3	10.1	4.6
E. mucronatus	68	0	0	13	55	—	—	80.9	—	19.1
T. sordida	24	0	0	3	21	—	—	87.5	—	12.5
R. pictipes	36	0	0	19	17	—	—	47.2	—	52.8

* *T.* = *Triatoma*; *E.* = *Eratyrus*; *R.* = *Rhodnius*.
† The sensitivity of each technique was calculated by the following formula: ni/N with ni = positive by a particular technique and N = positive by either technique. MOD = microscopic observation.
‡ % concordance = % of samples positive or negative by both techniques.
§ % discrepancy A = % of samples positive by microscopy and negative by PCR; B = % of samples positive by PCR and negative by microscopy.

irch, France). To remove nonspecific hybridization products, the membranes were washed twice under highly stringent conditions (6 M urea, 0.1× SSC [1× SSC = 0.15 M NaCl, 0.015 M sodium citrate] at 42°C for 10 min), and then twice in 2× SSC at room temperature. Two exposures were performed (1 min and 30 min) on autoradiography film (Hyperfilm®-MP; Amersham).

RESULTS

To test the field applicability and sensitivity of PCR-based diagnosis of *T. cruzi*, we analyzed 345 fecal samples from four triatomine species obtained from domestic and peridomestic habitats (Table 1) for the presence of parasites by direct microscopic observation and by PCR. The results are summarized in Table 2.

Among the 217 fecal samples recovered from *Triatoma infestans*, 117 showed flagellated parasites by microscopic observation, whereas only 105 showed an amplified band by PCR. The two techniques were in agreement 85.3% of the time. Discrepancy between the techniques occurred with two different patterns. The first showed a negative PCR result when the microscopic observation was positive; this pattern occurred in 10% of the insects examined. This rate of false-negative results was higher than expected. The second showed a positive PCR result when the microscopic observation was negative; this pattern occurred in 5% of the samples examined. Considering the sum of positive samples (positive by microscopic observation and/or positive by PCR), we compared the sensitivity of both techniques. Microscopic observation appears to be significantly more sensitive than the PCR technique (Yates' $\chi^2 = 5.18$, $P < 0.05$). We did not find significant differences in the sensitivity of the PCR technique between locales (La Paz and Cochabamba Departments). The 28 fecal samples from laboratory-reared *Triatoma infestans* were all PCR-negative as expected. Likewise, the sensitivity evaluated by the addition of *T. cruzi* total DNA to fecal samples of laboratory-reared *Triatoma infestans* insects always gave a positive amplification with 0.2 pg of DNA. On the other hand, the PCR was positive in only 30% of fecal samples artifically infected with either 10 or 100 fg of DNA. If one considers that a cell contains approximately 150–200 fg of DNA, the PCR method permitted the detection of one parasite in a feces sample. A discrepancy in the sensitivity of the PCR-based diagnosis was observed when comparing the results of studies of the 217 fecal samples recovered from the field with the fecal samples from artificially infected, laboratory-reared insects.

The relative sensitivities of the PCR and microscopic observations were reversed (higher) for the three sylvatic species compared with the results obtained with *Triatoma infestans*. All of the fecal samples collected from *R. pictipes*, *E. mucronatus*, and *Triatoma sordida* species were negative by microscopic observation. However, we obtained 52.8%, 19.1%, and 12.5% positive results, respectively, by the PCR.

We then proceeded to identify the particular clones present in the infected triatomines (Figure 1). A total of 136 PCR-positive samples were hybridized with probes specific for the two *T. cruzi* clones previously identified by isoenzyme analysis as major clones circulating in the domestic cycle in Bolivia (clones 20 and 39, see Materials and Methods). A large majority of the samples tested (77.9%) were recognized by one, or in the case of mixed infections, both probes (Table 3). Clones 20 and 39 were present in both domestic and sylvatic vectors. However, 30 samples (22.1%) were not recognized by either probe and the percentage of unrecognized samples was higher among sylvatic species of vectors (51.4%) than in domestic ones (11.9%) (Table 3).

We detected a much higher percentage of mixed infections in *Triatoma infestans*, up to 22.7% and 35.4% in Cochabamba and La Paz samples, respectively, (Table 4) than had been previously reported; 10% of the mixed infections were identified by isoenzyme analysis.[4,9] Mixed infections were also observed in *E. mucronatus* and *Triatoma sordida*. To determine whether or not infection with one clone affected the likelihood of infection with the other (inhibition or facilitation), we compared the observed rates of mixed infections with theoretical rates based on observed rates of infection of each clone in the population. The calculated numbers (N × (% 20 × % 39, with N = total population number, % 20 = observed % of triatomines infected by clone 20 and % 39 = observed % of triatomines infected by clone 39) did not differ significantly from the observed rates when they were compared by Yates' modified chi-square test (Table 4).

DISCUSSION

In this study, we have demonstrated the applicability of PCR-based diagnosis and strain typing for epidemiologic studies of *T. cruzi*. The first effort was to evaluate the sensitivity of the PCR technique. The level of sensitivity of the

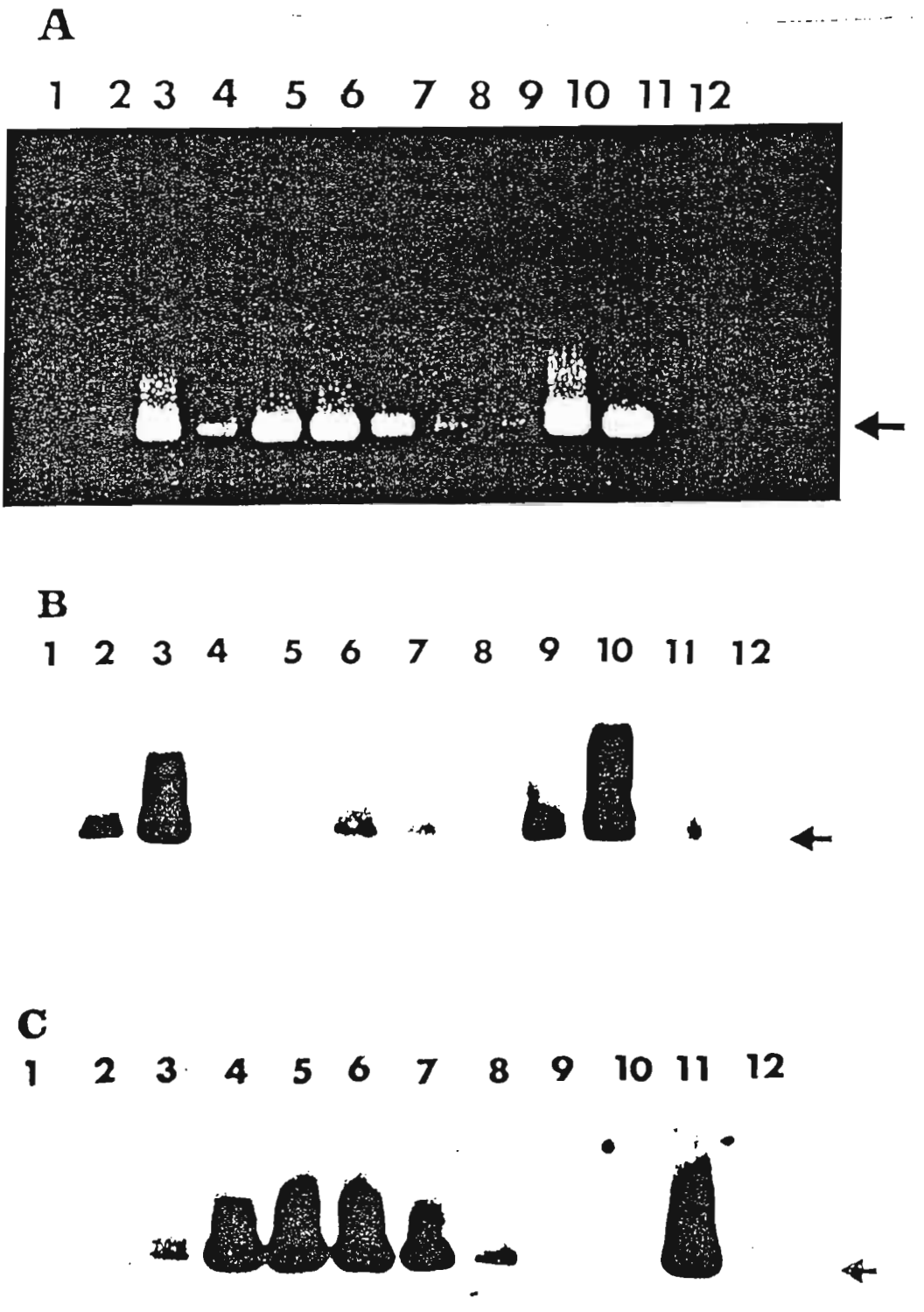

FIGURE 1. A, ethidium bromide–stained 0.8% agarose gel containing polymerase chain reaction products from *Trypanosoma cruzi*–infected *Triatoma infestans* feces. B and C, hybridization patterns of these products with clone-specific probes corresponding to natural clones 20 and 39, respectively. Lanes 1 and 12, control samples (using distilled water as the template); lanes 2–10, positive *Triatoma infestans* feces from Cochabamba Department; lane 11, positive control (using 10 ng of total DNA from the reference *T. cruzi* stock pertaining to natural clone 39 as the template). The arrows indicate the major amplified bands (270 basepairs).

TABLE 3

Identification of major clones 20 and 39 of *Trypanosoma cruzi* by specific DNA probes in polymerase chain reaction–amplified feces of vectors

Triatomine species*	Department	No.	Clone identification, no. (%)†			
			20 only	39 only	20 + 39	neither 20 nor 39
T. infestans	Cochabamba	22	15 (68.2)	0 (0)	5 (22.7)	2 (9.1)
	La Paz	79	24 (30.4)	17 (21.5)	28 (35.4)	10 (12.7)
Total		101	39 (38.6)	17 (16.8)	33 (32.7)	12 (11.9)
E. mucronatus	La Paz	13	1 (7.7)	2 (15.4)	2 (15.4)	8 (61.5)
T. sordida	La Paz	3	1 (–)	0 (–)	1 (–)	1 (–)
R. pictipes	La Paz	19	8 (42.1)	2 (10.5)	0 (0)	9 (47.4)
Total		35	10 (28.6)	4 (11.4)	3 (8.6)	18 (51.4)

* *T.* = *Triatoma*; *E.* = *Eratyrus*; *R.* = *Rhodnius*.
† – = not calculated (sample too small).

PCR reached 82.6% with samples from *Triatoma infestans*, a rate lower than expected considering the high theoretical sensitivity of the PCR. This may be explained by the lack of DNA purification. In fact, we have chosen a very simple method of template preparation, involving just boiling the feces in water, to facilitate processing large numbers of samples and to avoid cross-contamination. This crude lysate may contain various factors that are inhibitory in the PCR. Some of these inhibitors include blood breakdown products, such as heme,[10] which may vary in quantity and quality, depending on the digestive status of blood present in the gut of individual bugs. It is worth noting that the relationship between the percentage positive by microscopic observation and the percentage negative by PCR-based diagnosis varies between localities and in some, the false-negative PCR responses are absent. However, we have observed 35.7% and 58.3% false-negative PCR samples in two localities. The estimated sensitivity of the PCR technique is approximately one parasite per sample. The presence of PCR inhibitors in bug feces may explain the failure to detect a single parasite in some samples. The PCR technique was able to identify *T. cruzi* in some samples that could not be identified by microscopic examination (4.6% for *Triatoma infestans*, 19.1% for *E. mucronatus*, 12.5% fpr *Triatoma sordida*, and 52.8% for *R. pictipes*). Most notably, the PCR detected *T. cruzi* in three sylvatic species that were not detected by microscopic examination. We believe that the discrepancy in the techniques is due to the low parasite burden in these sylvatic species, which is missed by microscopic examination but detected by the PCR. These results bring to light a potential vector role for these sylvatic species in Bolivia.

Contamination with previous amplification products is the main problem with the PCR technique, but we do not believe it is an explanation for the discrepancy between negative microscopic examination results and positive PCR results in this study because of the following arguments. Among positive samples that showed a negative result by microscope observation, some give weak PCR amplification but others give strong amplification (generally contamination gives a weak band), and the negative and positive controls of each protocol always gave appropriate results. The hybridization patterns (see below) indicate the presence of several clones of *T. cruzi* in a group of triatomine samples tested in each experiment, and false-positive PCR results (contamination) occurred mostly due to the same source (the same clone of *T. cruzi*). It is worth noting that some samples were not recognized by either of the two probes that were used.

The PCR has the advantage of providing a more specific diagnosis than one that relies only on the detection of flagellated parasites in areas where other parasites, such as *T. rangeli* or *T. cruzi marenkellei*, could be present as well.

The second part of the investigation was to characterize the clones circulating in the vectors. We used two probes made with the PCR that encompass the HVRm of kDNA and hybridized these to PCR products from each sample of feces. We found high levels of two major clones that were previously reported from the same area using nuclear DNA markers (isoenzymes). Clones 20 and 39 (closely related

TABLE 4

Prevalence of mixed *Trypanosoma cruzi* clone infections: comparison of the observed and theoretical rates

Triatomine species*	Department	No.	No. (%) recognized†		Mixed infections‡		
			By probe 20	By probe 39	Observed	Expected	
					No. (%)		P§
T. infestans	Cochabamba	22	20 (90.9)	5 (22.7)	5 (22.7)	5 (22.7)	>0.05
	La Paz	79	52 (65.8)	45 (57.0)	28 (35.4)	30 (38.0)	>0.05
E. mucronatus	La Paz	13	3 (23.1)	4 (30.8)	2 (15.4)	1 (7.7)	>0.05
T. sordida	La Paz	3	1 (–)	0 (–)	1 (–)	–	
R. pictipes	La Paz	19	8 (42.1)	2 (10.5)	0 (0)	1 (5.3)	>0.05

* *T.* = *Triatoma*; *E.* = *Eratyrus*; *R.* = *Rhodnius*.
† – = not calculated (sample too small).
‡ Mixed infections observed = number and % of fecal samples hybridizing with both probes 20 and 39. Mixed infections expected = number of fecal samples calculated by the formula: N × (%20 × %39) with N = number of studied samples and %20 and %39 = observed % of fecal samples hybridized by probes 20 and 39, respectively. The expected % are deducted dividing the expected numbers by the total number of samples.
§ By Yates' chi-square test, ddl = 1.

clones) were identified from the domestic vector (*Triatoma infestans*). The PCR-based analysis is therefore a powerful tool for determining the distribution of clones because of the facility of the method in dealing with large numbers of samples without the time, expense, and loss of clones during culturing, which is required for isoenzyme analysis.

The results of the PCR with *Triatoma infestans* confirm earlier work with isoenzymes indicating the importance of major clones 20 and 39 in the domestic cycle in Bolivia.[1] Although the sample sizes are as yet too small to allow epidemiologic conclusions, this technique is clearly able to identify the microdistribution of particular clones: e.g., in our sample, the Department of Cochabamba showed a significantly higher percentage of clone 20 (90.9%) than the Department of La Paz (65.8%, Table 3, Yates' $\chi^2 = 5.65$, $P < 0.05$).

Of particular interest is finding clones 20 and 39 in three nondomestic species. This is the first report of the presence of these major clones, known from the domestic cycle, in sylvatic species in Bolivia. Since these normally sylvatic triatomines were collected in domestic and peridomestic areas, it is not clear whether these vectors are tending towards domesticity and acquiring domestic clones or whether clones 20 and 39 exist in the sylvatic situation as well. The origin of these major clones, sylvatic or domestic, remains to be determined. Application of this PCR-based technique to sylvatic mammals and triatomines should resolve this issue. Contrary to what was observed in *Triatoma infestans*, the majority of the clones present in *E. macronatus* and *R. pictipes* are neither clone 20 nor clone 39 and thus likely represent infections by additional clones whose taxonomic status remains to be determined.

Amplification with the PCR followed by hybridization was also a much more powerful technique than MLEE for detecting mixed infections. The probes used here do not cross-hybridize, as previously verified on a large range of reference strains (characterized by isoenzymes) pertaining to natural clones 20, 39, and others.[6] This result strongly suggests the detection of mixed infections (clone 20 and clone 39).[6] As more specific *T. cruzi* clone probes are developed and more strains are identified, we expect to find even higher rates of mixed infections. Since the expected rates of mixed infections with clones 20 and 39 (Table 4) did not differ significantly from the observed rates, we have no evidence for interaction between the clones in which the presence of one would affect the presence or development of the other.

The approach developed here should allow characterization of many clones from different regions. When the PCR products from an infected host (vector or mammal) do not hybridize with the probes that are used, it will be possible to develop a new probe from these PCR products. This new probe will be hybridized first with strains that have been genetically characterized (by MLEE) to determine its specificity and then used to test biological samples for epidemiologic studies if the specificity results are satisfactory. If we consider the clonal nature of *T. cruzi* populations as well as the linkage between HVRm DNA and nuclear DNA, it should be possible to develop specific probes from HVRm kDNA. Moreover, this approach, when applied to human infection, should allow investigation of the medical consequences of infection with a particular clone or multiple clones.

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Population genetics of *Trypanosoma cruzi* and *Trypanosoma rangeli:* taxonomical and epidemiological purpose

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A genetic analysis of a set of Trypanosoma cruzi and Trypanosoma rangeli stocks was performed by two combined approaches, namely multilocus enzyme electrophoresis (MLEE) and labeling by DNA probes. A considerable genetic variability was evidenced within each of the two species. Since the upper level of resolution of the isoenzyme method was reached, it was impossible to draw any definite discrimination between the two species by usual clustering methods. Nevertheless, two markers appeared as species-specific, namely the malic enzyme, and a probe that hybridizes highly-repeated sequences of T. cruzi.

INTRODUCTION

Trypanosoma cruzi, the agent of Chagas' disease, and *Trypanosoma rangeli*, usually considered as non pathogenic, can be both found in human and in the same mammalian hosts. Morphologic discrimination between the two species is sometimes difficult. Hence, the development of reliable molecular markers able to distinguish between the two species is needed. Toward reaching this goal, we report the results of a multilocus enzyme electrophoresis (MLEE) study of *T. cruzi* and *T. rangeli* populations. Moreover, we propose two candidate markers able to reliably identify the two species.

METHODS

Parasites. The sample of parasites involves: (i) 7 stocks communicated from different laboratories, and formerly identified as *T. rangeli* (this group is referred to as *T. rangeli* reference stocks in the present work); (ii) 4 stocks previously characterized as *T. cruzi* by MLEE (Tibayrenc and Ayala, 1988); they were selected because of the considerable genetic distances recorded among them, which reflect the total genetic variability of the species; and (iii) 11 unidentified trypanosomatid stocks isolated from 7 Peruvian *Saimiri* monkeys. Hosts and geographic origins of the stocks are summarized in Table I.

Multilocus Enzyme Electrophoresis (MLEE). Parasites were cultured in LIT medium, harvested by centrifugation and the extracts were perfomed according to Tibayrenc *et al.* (1985). Eleven enzyme systems were assayed, namely: diaphorase (E.C. 1.8.1.4, DIA); glutamate dehydrogenase NAD^+ (E.C. 1.4.1.2., GDH1 NAD^+); glutamate dehydrogenase $NADP^+$ (E.C. 1.4.1.4., GDH2 $NADP^+$); glutamate oxaloacetate transaminase (E.C.2.6.1.1, GOT); glucose phosphate isomerase (E.C. 5.3.1.9, GPI); isocitrate dehydrogenase (E.C. 1.1.1.42, IDH); malate dehydrogenase (E.C.

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TABLE I

Hosts and geographic origins, zymodemes patterns of 7 *T. rangeli*, 4 T. *cruzi* reference stocks and 11 stocks isolated from 7 Peruvian monkeys

Stocks Nb.	Name	Country	Host	Genotypes 13 loci
	T. rangeli			
1	Riera	Venezuela	Man	A
1	Palma	Venezuela	*Rhodnius robustus*	A
1	R2008	Bolivia	*Coendou prehensilis*	A
1	R2010	Bolivia	*Coendou prehensilis*	B
1	ITMAP 1140	Unknown	*Aotus trivirgatus*	C
1	Basel	Venezuela	dog	D
1	RGB	Venezuela	dog	E
	T. cruzi			
1	Tehuentepec cl1 (Cl12)*	Mexico	*Triatominae*	F
1	Sc43 cl1 (Cl 39)*	Bolivia	*Triatoma infestans*	G
1	Tula FKIIA (Cl 43)*	Chile	Human	H
1	Can III cl1 Z3 (Cl 27)*	Brazil	Human	I
	Peruvian stocks Monkey : stock n°.			
2	S243 : 7, 8	Peru	*Saimiri Sciureus*	J
1	S300 : 1	Peru	*Saimiri Sciureus*	K
1	S317 : 1	Peru	*Saimiri Sciureus*	L
1	S329 : 5	Peru	*Saimiri Sciureus*	M
2	S329 : 9, 11	Peru	*Saimiri Sciureus*	N
1	S364 : 4	Peru	*Saimiri Sciureus*	O
1	S377 : 5	Peru	*Saimiri boliviensis*	P
1	S415 : 11	Peru	*Saimiri boliviensis*	Q
1	S415 : 18	Peru	*Saimiri boliviensis*	R

* These stocks have been previously characterized: Cl (= natural clone) 12, 27, 39 and 43 referred to numbering classification of Tibayrenc and Ayala, 1988.

1.1.1.37., MDH); malic enzyme (E.C. 1.1:1.40., ME); peptidase 1 (ficin) (E.C. 3.4.22.3. [formerly E.C. 3.4.4.12.], substrate: leucyl-leucyl-leucine, PEP 1); peptidase 2. (bromelain) (E.C. 3.4.22.4. [formerly EC 3.4.4.24.], substrate: leucyl-L-alanine, PEP 2); and phosphoglucomutase (E.C. 5.4.2.2. [formerly E.C. 2.7.5.1.], PGM). Electrophoreses were performed on cellulose acetate plates as described by Tibayrenc *et al.* (1985) with slight modifications. The DIA and GOT were new enzyme systems and the electrophoretic conditions were as follows: the migrations were run respectively for 20 min at 200 volts and 30 min at 160 volts in the buffer III of Shaw and Prasad (1970). Before migration, the plates were soaked in the same buffer, diluted V/V.

Dot-blotting hybridization. Crude parasite extractions were performed by harvesting, centrifugating and freezing the parasites. 3 µl of pellet were subsequently resuspended in

100 µl of distilled water, boiled for 10 min and centrifuged for 10 min at 8,000 g. The supernatants were subsequently 10-fold diluted in NaOH 1.5 M, NaCl 0.5 M (final concentration) and 50 µl of each dilution were dot-blotted (6 dilutions were done) onto charged nylon membranes (Hybond H+; Amersham, Buckinghamshire, UK). Then, the membranes were rinsed out in 2 x SSC buffer and stored in "Saran" film paper (Poly-Labo, Strasbourg, France) until hybridization assay.

DNA probe preparation. The probe was produced by PCR technique. The primers, respectively referred to as TCZ1 and TCZ2 (ordered from Genset, Paris, France) were designed according to Moser *et al.* (1989) in order to anneal sites at the extremities of the 195-bp repeat of the nuclear DNA of *T. cruzi* (satellite DNA; Sloff *et al.*, 1983). The sequence of TCZ1is: 5'CGAGCTCTTGCCCACACG-GGTGCT3'; the sequence of TCZ2 is: 5'CCTCCAAGCAGCGGATAGTTCAGG3'. Buffer and cycle conditions were according to Brenière *et al.* (1992) using 1 IU of *Thermus aquaticus* DNA polymerase (Promega, Madison, USA). The major amplified fragment of 195 bp was purified from a preparative gel of 1% lowmelting point agarose, by using the enzymatic preparation GELase™, according to the manufacturer's instructions (Epicentre Technologies Corporation, Madison, USA).

Probe labeling and hybridization. Labeling and hybridization conditions were according to the ECL gene detection system based on enhanced chemiluminescence (Amersham, Buckinghamshire, UK). Simultaneously, the membranes were incubated at 42° C in the hybridization buffer (0.25 ml cm^{-2}) for 15 min, and the purified probe (20 ng ml^{-1} DNA) was simultaneously labeled for 10 min at 37° C. The hybridization was performed at 42° C overnight in a rotary oven (Appligene, France). Before the detection step, the membranes were washed, first twice in a highly stringent solution (6 M urea / 0.1% SDS / 0.1 SSC at 42° C), and then twice in a 2 SSC solution at room temperature. Serial expositions were performed (1 min, 5 min and 20 min) on autoradiography films (Hyperfilm™-MP, Amersham, UK).

Data Analysis. Phylogenetic relationships among the zymodemes were evaluated by using a cladistic analysis (Felsenstein, 1978). The CLIQUE program, based on the compatibility principle, was used to construct the network (Le Quesne, 1974; Eck and Dayhoff, 1966). This algorithm uses a matrix of presence/absence (1/0) of the enzymic characters.

RESULTS

Isoenzyme studies. Among the 22 studied stocks, 18 different zymodemes (which can be equated to multilocus genotypes) could be discriminated (Table II). A striking pattern was noticeable for the malic enzyme: several stocks, including all *T. rangeli* reference stocks, exhibited one-banded zymograms only. This profile probably corresponds to the activity of one locus only. In contrast, all *T. cruzi* reference stocks exhibited two-banded patterns attributable to the activity of two different loci, as previously recorded (Tibayrenc and Ayala, 1988).

The standard genetic distances of Nei (1972), calculated between any two pairs of multilocus genotypes, are presented in Table III. They can be summarized as follows: (i) all *T. rangeli* reference stocks present high genetic distances from any *T. cruzi* reference stock, ranging from 1.32 to infinite (stocks which share no common allele); (ii) nevertheless some *T. rangeli* reference stocks (namely, genotypes A and E) present higher genetic distances between them (1.44) than with some *T. cruzi* stocks (genotypes F and A, 1.32) (see discussion); (iii) among the Peruvian *Saimiri* stocks, genotypes J and P are more closely related to *T. cruzi* reference stocks (genotypes I and F, respectively) than to any *T. rangeli* reference stock; on the contrary, all L, N, Q, R, and M genotypes are more closely related to any *T. rangeli* reference stocks than to any *T. cruzi* stock; (iv) genotypes O and K (from *Saimiri* monkeys) are equally distant from both *T. rangeli* and *T. cruzi* reference stocks.

The evolutionary relationships among the different zymodemes are visualized in Figure 1 by the phylogenic network obtained by use of the CLIQUE program from a matrice of presence/absence of the isoenzyme characters. The *T. rangeli* reference stocks are clustered into two distinct groups, while the different *T. cruzi* stocks are distantly related from one

another, and do not appear as a clustered group, which corroborates previous results (Tibayrenc and Ayala, 1988). The relative position of the *Saimiri* stocks confirms analysis of the data from Nei's distance (see above).

Hybridization with satellite DNA probe from T. cruzi. The satellite DNA probe produced by PCR was hybridized with crude extracts of the different stocks under study. Positive hybridization signal was observed only: (i) to the *T. cruzi* reference stocks; and (ii) among the *Saimiri* monkey set, to those stocks (genotypes J and P) that appear to be more closely related to two *T. cruzi* reference stocks (Figures 1 and 2). All other stocks were negative, including two control stocks identified respectively as *Trypanosoma brucei s.l.* and *Leishmania sp.*

DISCUSSION

Distinction between T. rangeli and T. cruzi. Since the discrimination between the two species is sometimes difficult, the population genetic approach brings hope to build reliable tools towards reaching this goal. Nevertheless, the clustering study perfomed here failed in drawing a clear distinction between these two species. The reason is that the upper level of resolution of the isoenzyme method is reached

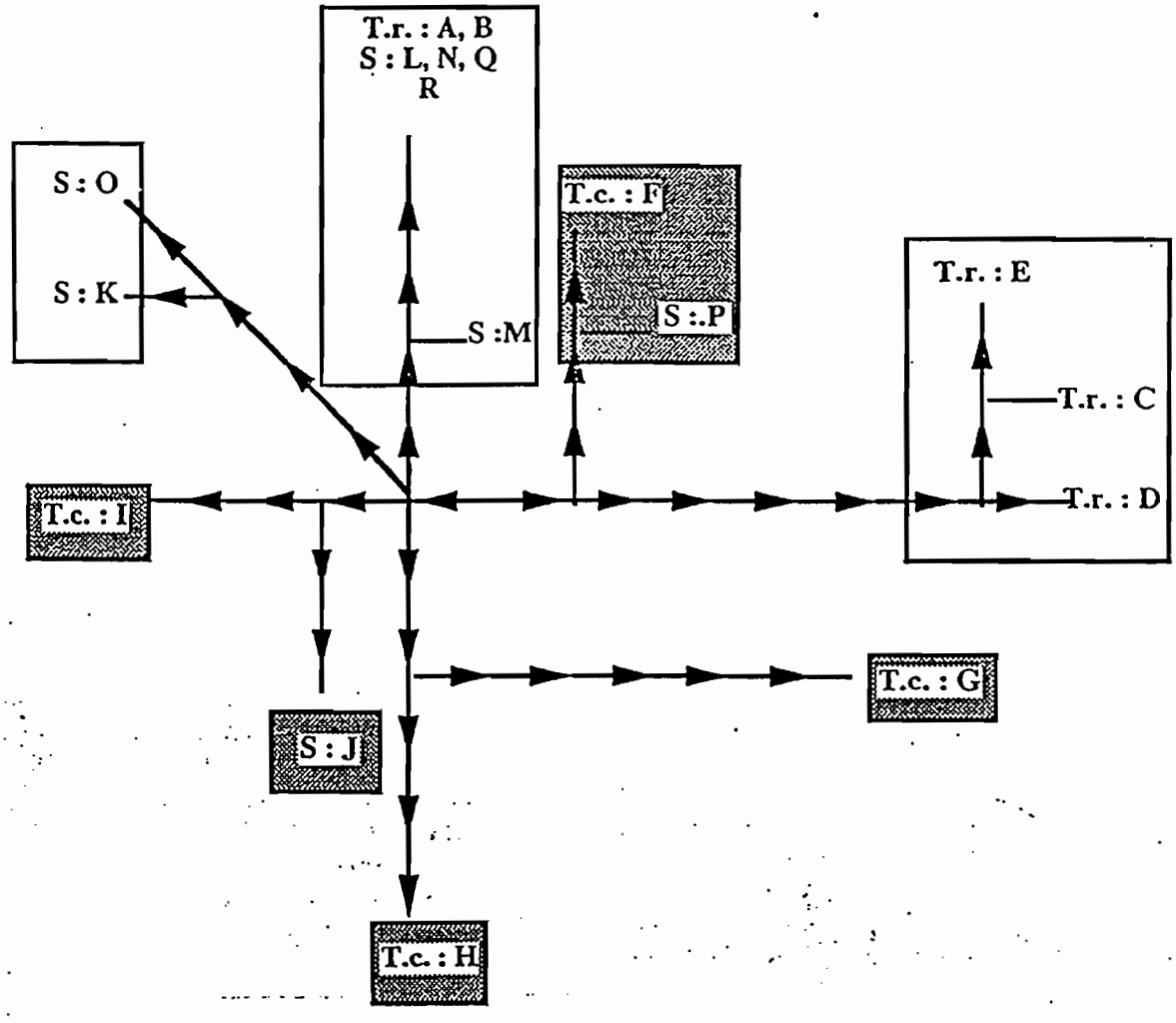

Fig. 1: Phylogenetic network showing evolutionary relationships among stocks under study, obtained by "CLIQUE" program from a matrix of presence/absence of enzymic characters. T. r = *T. rangeli* reference stocks; T. c. = *T. cruzi* reference stocks; S = Peruvian stocks isolated from *Saimiri* monkeys. Letters A to R correspond to different zymodemes (or multilocus genotypes) identified. Grey areas include stocks presenting two-banded patterns (two loci) for Malic enzyme and a positive hybridization with satellite DNA probe from *T. cruzi*. White areas include stocks presenting one-banded patterns (one locus) for Malic enzyme and a negative hybridization with satellite DNA probe from *T. cruzi*.

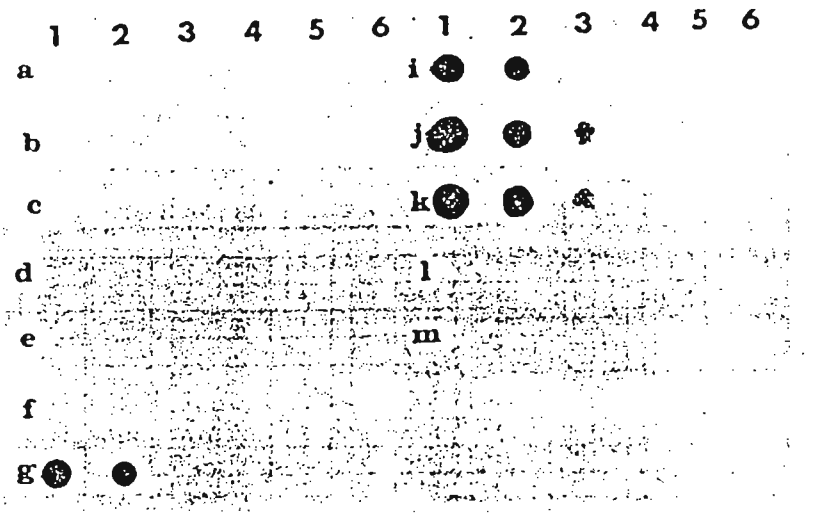

Fig. 2: Set of dot-blotted parasite stocks (crude extracts surpernatants are subsequently diluted 10 fold, columns 1 to 6) hybridized with satellite DNA probe from *T. cruzi*. Stocks are as follows: lane a: *Leishmania* sp.; lane b: *T. brucei s.l.*; lanes c and d: *T. rangeli* reference stocks (Riera and ITMAP 1140); lanes e, f, g, l and m: Peruvian *Saimiri* stocks, S415-18, S329-9, S377-5, S329-11 and S-364-4, respectively; lanes i, j and k: *T. cruzi* reference stocks.

here: the considerable genetic distances already recorded within each of the two species shows that some stocks of the same species share very few common allozymes. In other words, the theoretical maximum distances are close to be reached within each species. Phylogenetically speaking, the difference between Nei distances of 2, 3 and infinite is not relevant. Similar results were recorded in an attempt to classify various stocks pertaining to the genus *Phytomonas* (Guerrini *et al.*, 1992). Nevertheless, in the present case, qualitative analysis of the data reveals a potential diagnostic marker between the two species, that is to say, the Malic enzyme, where stocks identified as *T. cruzi* exhibit the actitivy of two different loci, while stocks classified as *T. rangeli* exhibit the activity of one locus only.

By using a satellite DNA probe from a *T. cruzi* stock, a positive hybridization signal was obtained with the *T. cruzi* reference stocks and not with the *T. rangeli* reference stocks. This molecular character is hence totally linked to the genotypes recorded for the Malic enzyme marker. Both are in agreement with the current taxonomic classification in the case of these reference stocks. The results on the *Saimiri* stocks confirm this linkage between the two markers: those stocks that appear as closely related to the *T. rangeli* reference stocks show the activity of only one locus for the Malic enzyme, and are not hybridized by the *T. cruzi* satellite DNA probe. On the contrary those stocks that are related to the *T. cruzi* reference stocks present the activity of two different locus for the Malic enzyme and a positive hybridization with the probe. On the whole sample of the stocks under study, a total linkage was observed between these two markers, which leads us to propose them for diagnostic characters between the two species.

Preliminary arguments for clonality in T. rangeli. Three *T. rangeli* reference stocks, respectively isolated in Venezuela (two stocks) and Bolivia (one stock), share the same multilocus genotypes. The occurrence of such identical multilocus genotypes recorded on wide geographical areas, which suggests that recombination is rare or absent, is a classical argument for clonal propagation (Tibayrenc *et al.*, 1990, 1991). Nevertheless, this result has to be confirmed on a larger sample of stocks.

In regard to these preliminary results, the population structure and genetic variability of *T. rangeli* seem to present some striking similarities with *T. cruzi* (considerable intraspecific genetic variability and, perhaps, clonal population structure). This is consistent with

TABLE II

Genotypes identified by 13 loci within 7 T. rangeli, 4T. cruzi reference stocks and 11 stocks isolated from Peruvian monkeys

Genotypes 13 loci Code	Nb.of stocks	DIA 1	DIA 2	GDH 1	GDH 2	GOT	GPI	IDH	MDH	ME 1	ME 2	PEP 1	PEP 2	PGM
A	3	1/1	1/4	3/3	7/7	1/4	3/4	2/2	2/2	absent	2/2	5/5	6/6	3/3
N	2	1/1	4/4	3/3	7/7	1/1	3/4	2/2	2/2	absent	2/2	5/5	6/6	3/3
B	1	1/1	1/4	abs	7/7	1/1	3/4	2/2	2/2	absent	2/2	5/5	6/6	3/3
R	1	1/1	1/3	3/3	7/7	1/1	3/4	2/2	2/2	absent	2/2	5/5	6/6	3/3
L	1	1/1	1/3	3/3	7/7	1/1	3/4	3/3	2/2	absent	2/2	5/5	6/6	3/3
M	1	1/1	4/4	abs	4/4	1/1	4/4	2/2	2/2	absent	2/2	5/5	6/6	3/3
Q	1	1/3	3/7	3/3	7/7	1/1	3/4	2/2	2/2	absent	2/2	4/4	3/3	3/3
D	1	2/2	1/4	4/4	2/2	5/5	5/5	2/2	2/2	absent	4/4	5/5	5/5	1/4
C	1	2/2	2/4	4/4	1/1	5/5	5/5	2/2	2/2	absent	4/4	5/5	5/5	1/4
E	1	2/2	1/4	4/4	1/1	5/5	5/5	1/1	2/2	absent	4/4	5/5	5/5	1/4
K	1	2/2	2/3	abs	5/5	5/5	4/6	1/1	1/1	absent	2/2	2/2	4/4	2/10
O	1	2/2	1/3	abs	5/5	7/7	4/6	1/1	1/1	absent	2/2	2/2	6/6	2/10
P	1	2/3	4/4	3/3	4/4	4/4	5/5	1/1	1/1	2/2	2/2	3/3	1/1	5/5
F (Cl 12)*	1	4/4	4/4	3/3	4/4	4/4	5/5	1/1	1/1	2/2	2/2	1/1	1/1	5/5
G (Cl 39)*	1	3/3	4/4	1/1	3/3	2/2	1/4	3/3	1/1	1/1	3/3	3/3	2/2	7/9
H (Cl 43)*	1	3/3	5/5	2/2	5/5	1/2	2/4	3/3	1/1	1/1	3/3	2/2	2/2	6/11
I (Cl 27)*	1	4/4	7/7	2/2	6/6	2/2	4/4	3/3	1/1	2/2	1/1	4/4	3/3	8/8
J	2	2/2	6/6	2/2	5/5	3/3	4/4	2/2	1/1	1/1	1/1	5/5	3/3	6/6
18 genotypes	22 stocks													

* These stocks have been previously characterized: Cl (= natural clone) 12, 27, 39 and 43 referred to numbering classification of Tibayrenc and Ayala, 1988.

TABLE III

Matrix of Nei (1972) genetic distances between the different multiloci genotypes observed in our sample, identified by assaying 13 enzymatic loci

		Genotype code																
		T.rangeli reference stocks					*T. cruzi* reference stocks				Peruvian *Saimiri* stocks							
	Genotype code	A	B	C	D	E	F	G	H	I	J	P	L	N	Q	R	M	O
T. rangeli reference stocks	A																	
	B	0.12																
	C	1.20	1.22															
	D	1.12	1.15	0.12														
	E	1.44	1.46	0.15	0.23													
T. cruzi reference stocks	F	1.32	2.04	2.04	2.04	1.50												
	G	2.26	2.69	3.09	3.09	3.07	1.75											
	H	3.04	2.66	∞	∞	∞	2.42	0.63										
	I	3.11	3.13	∞	∞	∞	1.79	1.19	1.03									
Peruvian *Saimiri* stocks	J	1.50	1.53	1.34	1.34	1.72	2.48	2.04	1.03	0.88								
	P	1.30	2.01	1.73	1.73	1.30	0.16	1.17	1.99	2.46	2.60							
	L	0.15	0.23	1.70	1.59	1.56	1.75	2.17	1.82	2.04	2.04	1.73						
	N	0.05	0.12	1.17	1.17	1.48	1.37	2.20	2.68	3.16	1.55	1.34	0.17					
	Q	0.34	0.43	1.68	1.68	2.35	1.72	2.66	2.13	1.32	1.50	1.59	0.39	0.32				
	R	0.05	0.12	1.30	1.22	1.56	1.75	3.78	2.66	3.13	1.53	1.73	0.10	0.07	0.26			
	M	0.28	0.14	1.19	1.19	1.50	1.39	2.04	2.42	2.48	1.39	1.37	0.43	0.21	0.63	0.30		
	O	1.44	1.12	2.37	2.15	1.54	1.32	2.15	1.17	2.01	1.17	1.14	1.36	1.59	1.95	1.36	1.17	
	K	2.13	1.56	1.56	1.68	1.44	1.32	2.15	1.17	2.01	1.17	1.14	1.97	2.17	1.95	1.97	1.50	0.24

the known similar biology of these two species. Although two potential diagnostic markers could be proposed from the present results, the actual phylogenetic relationships which exist between the two species will have to be assessed by the use of "conservative", slowly-evolving genetic characters, such as rRNA sequences.

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Research Note

COPY NUMBER DIFFERENCES IN THE 195 BP REPEATED SATELLITE DNA FROM *TRYPANOSOMA CRUZI* AND *TRYPANOSOMA RANGELI:* POTENTIAL USE FOR EPIDEMIOLOGIC SURVEYS

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Trypanosoma cruzi, the agent of Chagas' disease, and the apparently non-pathogenic species *Trypanosoma rangeli* can be both encountered in human and in the same mammiferous hosts. On fresh isolates, the discrimination between both species can be sometimes difficult. Hence the development of reliable molecular markers able to distinguish between the two species is sorely needed (F. Guhl et al., 1987, *Parasitol., 94:* 475-484). Toward reaching this goal, we report here further characterization of the 195 bp DNA repeat previously described in *T. cruzi* (P. Sloof et al., 1983, *J. Mol. Biol., 167:* 1-21). A closely related satellite DNA sequence was evidenced in *T. rangeli,* but apparently with a much lower number of repeats, hence providing a potential epidemiological tool for discrimination between the two taxa.

Fifteen different *T. cruzi* stocks as well 7 *T. rangeli* stocks were studied. These stocks represent for each taxon a high genetic variability, as verified by isoenzyme analysis at 11 polymorphic loci, according to the methods described by M. Tibayrenc & F. J. Ayala, (1988, *Evolution, 42:* 277-292). Oligonuclotide primers TCZ1 and TCZ2 previously described by D. R. Moser et al. (1989, *J. Clin. Microbiol., 27:* 1477-1482), were used for DNA amplification of the 195 bp DNA units from purified total DNA (F. Veas et al., 1991, *Cell. Mol. Biol., 37:* 73-84), according to the PCR amplification procedure described by S. F. Brenière et al. (1992, *Am. J. Trop. Med. Hyg., 46:* 335-341).

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As expected, all *T. cruzi* stocks gave a major amplified band of 195 bp on ethidium bromide-stained 2% agarose gels. For the 7 *T. rangeli* stocks studied, PCR products exhibiting a comparable molecular weight were obtained (Fig. 1). These 195 bp length PCR fragments were not observed with other Kinetoplastida species used as control (*Leishmania, T. brucei*), a result already noted by D. R. Moser et al. (1989, *loc. cit.*). The minimal amount of template DNA required to obtain a positive PCR amplification was 10 fg for one *T. cruzi* stock and 10 pg for one *T. rangeli* stock.

The sequence homology of *T. cruzi* and *T. rangeli* was evaluated by cross-hybridization of *T. cruzi* and *T. rangeli* probes with sequential diluted PCR products, electrophoresed and transfered onto nylon membranes (S. F. Brenière et al., 1992, *loc. cit*). Both probes were prepared as follows: the PCR fragments of one *T. cruzi* stock and one *T. rangeli* stock were purified by electrophoresis on 0.8% preparative low melting ultra pure agarose gels (Bethesda Research Laboratories, Uxbridge UK). The fragments were eluted from the agarose using Glass beads (Gene Clean kit) according to instructions of the manufacturer (Bio 101, La Jolla, CA USA). Non radioactive labeling of purified products was performed by using ECL gene detection system based on enhanced chemiluminescence (Amersham, Buckinghamshire, UK). Briefly membranes were incubated at 42 °C in hybridization buffer (0.1 ml/cm^2) for 15 mn and during the same time, each purified probe was labeled for 10 mn at 37 °C. The hybridization was performed at 42 °C overnight in a rotative oven. A similar sensitivity was obtained with either homologous or heterologous hybridizations (Fig. 2a, b, c). This result favours the hypothesis of strong sequence homology between 195 bp PCR products from *T. cruzi* and *T. rangeli.*

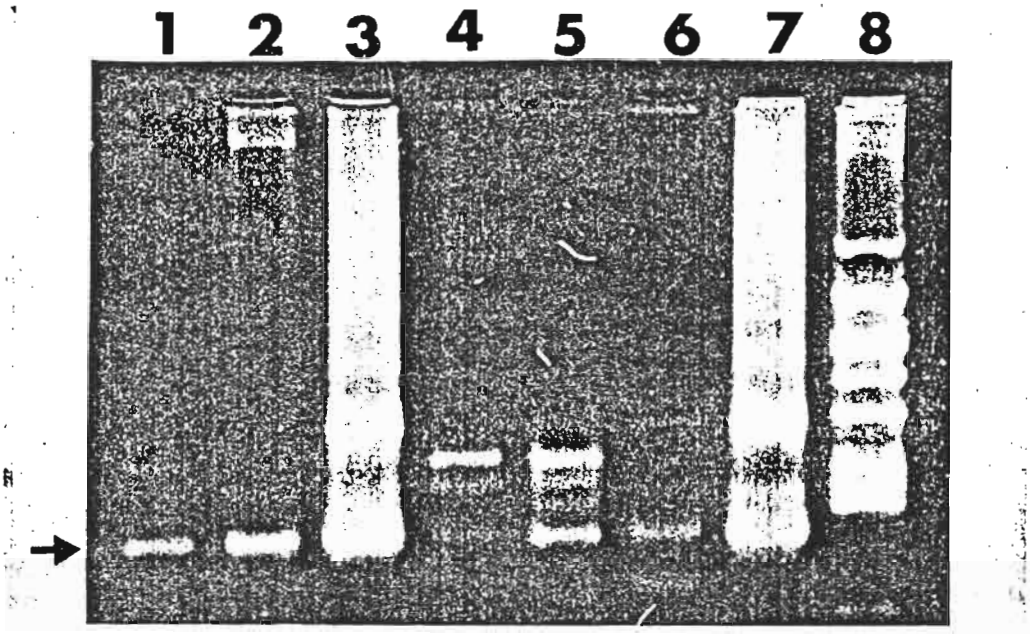

Fig. 1: electrophoresed and ethidium bromide stained 2% agarose gel exhibiting PCR products from: lanes 1, 2, 4 and 5, 1 µg of total DNA of reference *Trypanosoma rangeli* stocks (R2008, Basel, Riera and R2010 respectively); lanes 3 and 7, 1 µg of total DNA of *T. cruzi* stock SC43 cl 1; lane 6, 100 pg of total DNA of *T. cruzi* stock OPS21; lane 8, molecular weight marker PBR 322- *Alu* I. The arrow indicate the major amplified band of 195 bp.

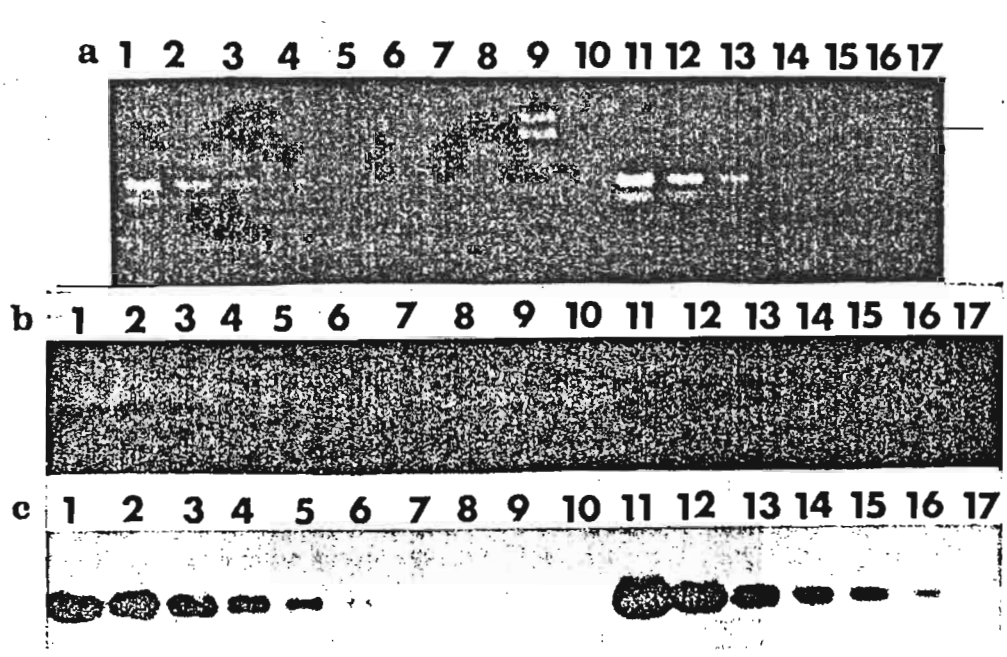

Fig. 2: a – Electrophoresed and ethidium bromide stained 0.8% agarose gel exhibiting – lane 1 to 8: serial dilutions (1/1, 1/2 to 1/128) of the PCR products from *Trypanosoma rangeli* Basel stock total DNA (1 mg); lanes 11 to 17 and 10: serial dilutions (1/1, 1/2 to 1/128) of the PCR products from *T. cruzi* NR stock total DNA (100 fg) respectively; lane 9: molecular weight marker PBR 322- *Alu* I. b and c – hybridizations of the blotted gel with NR *T. cruzi* and Basel *T. rangeli* non radioactive probes (5 ng/ml and 20 ng/ml respectively), high washing stringency was monitored twice in 6 M urea, 0.5 x SSC buffer for 20 mn at 42 °C and then twice in 2 x SSC buffer for 10 mn. The expositions were performed 10 mn on autoradiography films (Hyperfilm TM-MP Amersham, Buckinghamshire, UK).

We have dot-blotted serially-diluted total DNA extracts from the total range of 15 *T. cruzi* stocks and 7 *T. rangeli* stocks as well as one *T. cruzi marinkellei* stock, 2 *Leishmania* stocks and 3 *T. brucei* stocks, according to F. Veas et al. (1990, *loc. cit.*). All *T. cruzi* stocks gave a positive hybridization at a minimal concentration ranging from 0.01 to 10 ng, either with *T. cruzi* or *T. rangeli* probes (see above). Some *T. rangeli* stocks gave a positive hybridization but with a minimal concentration of 500 ng total DNA, while others remained negative, even with a concentration as high as 1 µg total DNA. With 500 ng total DNA, non hybridization was observed for the others species used as controls.

These results confirm the presence in *T. cruzi* of a satellite DNA composed of 195 bp repeat, and show that this DNA is constantly observed over a range of stocks of this parasite that are genetically highly diversified. Moreover, the presence of repeats that are either identical or similar was shown also on a set of *T. rangeli* stocks whose genetic heterogeneity is also very high. Nevertheless, the number os repeats appeared as far lower in the case of *T. rangeli* than for *T. cruzi;* we propose the hypothesis that the *T. rangeli* sequence is an ancestral one that has been duplicated and amplified in the species *T. cruzi.* Whatever be the explanation of it, this result (far higher number of repeats in *T. cruzi* than in *T. rangeli*) seems to be valid for any *T. cruzi* or *T. rangeli* stock, since it has been verified for two set of stocks belonging to the two taxa, that are genetically extremely diversified: this potentially provides a convenient tool to discriminate between the two species in the course of epidemiological surveys, by simple dot-blot hybridizations. Discrimination of both taxa by restriction endonuclease patterns of kDNA (A. C. C. Frasch, 1981, *Mol. Biochem. Parasitol., 4:* 163-170; A. M. Gonçalves et al., 1991, *Mem. Inst. Oswaldo Cruz, 86:* 477-478) and PCR amplification of mini-exon genes (V. K. Murthy, 1992, *Mol. Cell. Probes, 6:* 237-243) have been proposed. These authors do not contemplate the intraspecific genetic variability of both taxa which is particulary high (R. Kreutzer & O. E. Sousa, 1981, *Am. J. Trop. Med. Hyg., 30:* 308-317; M. Tibayrenc & F. J. Ayala, 1988, *loc. cit.*), nevertheless, the markers used in these experiments should be valid for discrimination of the taxa. Studies, in the light of population genetics, of the different proposed markers with extensive samples of stocks pertaining to both taxa, are highly recommended to investigate their possible correlation.

In our dot-blot experiments, the only *T. cruzi marinkellei* stock, which is considered a subspecies of *T. cruzi,* and hence, closely related to *T. cruzi sensu stricto* showed no hybridization signal. The close genetic relationship between *T. cruzi* and *T. cruzi mareikellei* is questioned by this unexpected result. This opens the more general and difficult problem of a reliable identification of the so-called *T. cruzi*-like organisms, whose epidemiological relevance and actual taxonomic relationship with *T. cruzi* remain unclear. Work is in hand to explore this problem by complete population genetics analysis.

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DIRECT IDENTIFICATION OF *TRYPANOSOMA CRUZI* NATURAL CLONES IN VECTORS AND MAMMALIAN HOSTS BY POLYMERASE CHAIN REACTION AMPLIFICATION

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Abstract. The polymerase chain reaction was used to amplify the highly variable region of the kinetoplast minicircle of *Trypanosoma cruzi* directly in biological samples (feces of infected Triatomine bugs, blood samples of experimentally infected mice, and artificially infected human blood samples). Hybridization of the amplified DNAs with reference stocks representing different genotypes (natural clones) enabled us to characterize the stocks infecting the biological samples under study. The main interest of this new approach is the diagnosis of *T. cruzi* infection and simultaneous direct identification of the different natural clones circulating in vectors and mammalian blood without isolation of the stocks. The suitability of this technique for epidemiologic studies is also discussed.

Natural populations of *Trypanosoma cruzi*, the agent of Chagas' disease, have a basic clonal structure, which has been analyzed by extensive population genetic studies.[1–3] The previously described zymodemes and schizodemes can be equated to natural clones, as shown by isozyme or restriction fragment length polymorphism analyses, respectively.[4,5] These natural clones should be considered as useful taxonomic units in any applied study.[6] Among the large number of different natural clones isolated, some appear to be ubiquitous and are frequently encountered in endemic areas. These dominant genotypes, which are called major clones, deserve special attention.[7] Nevertheless, the biological and medical peculiarities of these various clones remain poorly defined. At present, identification of these natural clones requires 1) isolation of the parasites from vectors or mammalian hosts, 2) culture amplification, and 3) multilocus characterization by isoenzyme techniques. Isolation of *T. cruzi* stocks from vectors is relatively simple, but it lacks sensitivity when attempted with chronically infected patients and mammalian hosts because of their low level of parasitemia.[8,9] Selection pressure may occur with natural clones through isolation from culture or mice.[10,11] Moreover, multilocus isozyme characterization is both time-consuming and expensive. For these reasons, fast, specific, and sensitive labeling of *T. cruzi* major clones is needed for field or experimental studies directed toward defining clinical characteristics.

Previous work has shown that the hypervariable region of the *T. cruzi* kinetoplast DNA (kDNA) minicircle (HVRm) exhibits sequences that are specific for the natural clones identified by our multilocus isozyme characterization. This has led to the development of DNA probes for some *T. cruzi* major clones.[12] These results are based upon and favor the hypothesis of linked evolution between nuclear DNA (labeled by isozyme analysis) and kinetoplast DNA.[13] Thus, the HVRm of kDNA can be considered a clone marker. In this report, we propose direct characterization of *T. cruzi* natural clones isolated from infected feces and blood using clone-specific HVRm DNA sequences as new markers that are easily obtained by the PCR technique.

MATERIALS AND METHODS

Samples

Four different types of samples were investigated: 1) triatomine feces, 2) blood from adult patients, 3) artificially-infected human blood, and 4) blood samples from experimentally infected mice. All triatomines *(Triatoma infestans)* were isolated in Bolivia. Some of them were collected

in endemic areas, while others originated from xenodiagnosis procedures performed on patients. Microscopic examinations for positive infection were performed (by checking at least 60 microscopic fields at 40× magnification) before recovering the fecal samples. All patient blood samples were collected in Bolivia. Some of the patients exhibited positive serology by both immunofluorescence analysis and enzyme-linked immunosorbent assay, while other patients had negative results for these tests.[14]

BALB/c mice were infected with 100–1,000 trypomastigote blood forms of a laboratory cloned stock of *T. cruzi* that was previously used in an experimental mouse model at the Laboratory of Parasitology at the University of Brussels. This clone is genetically related to clone 43, as shown by isozyme analysis. (In the present work, all clones are numbered according to the recommendations of a previous report.[1] The term clone denotes a natural clone of *T. cruzi* identified by population genetic analysis rather than a laboratory clone.) Parasitemia levels in infected mice were evaluated by examination of blood samples.[15]

Processing of fecal and blood samples for PCR analysis

Samples of triatomine feces (10–20 μl) were individually collected in microtubes, diluted with 200 μl of distilled water, boiled for 10 min, and centrifuged at 8,000 *g* for 10 min. Mouse blood samples (100 μl) were collected by intracardiac puncture, diluted with 100 μl of distilled water, boiled for 10 min, and centrifuged at 8,000 *g* for 10 min. Samples (100 μl) of artificially infected human blood were obtained by the addio of 10, 100, or 1,000 parasites (epimastigote culture forms) of various *T. cruzi* clones. These samples were diluted with 1 ml of distilled water, boiled for 10 min, and centrifuged at 8,000 *g* for 10 min. The supernatants were boiled and centrifuged again at 8,000 *g* for 10 min. Blood samples from naturally infected patients were also processed in an identical manner. Ten microliter samples of the different extracts were used as templates in all PCR assays. After PCR, samples were electrophoresed on either 0.8% or 2% agarose gels, the gels were stained with ethidium bromide, and PCR subsamples (1/10 of the original sample) were analyzed.

PCR procedure

The oligonucleotides were obtained from P. Zalta (Centre de Recherche en Biologie et Genetique Cellulaire, Toulouse, France). Sequences were selected to anneal sites flanking the HVRm, as previously described[12] (5′-GATTGGGGTTGGAGTACTACTAT-3′ and 5′-TTGAACGGCCCTCCGAAAAC-3′). Two restriction endonuclease sites (*Sca* I and *Sau* 96 I) were artificially introduced at the 3′ end of each oligonucleotide to allow purification of the HVRm-amplified sequence from the oligonucleotide primers complementary to the conserved part of minicircle. Samples were amplified in 67 mM Tris HCl (pH 8.8), 16.6 mM $(NH_4)_2SO_4$, 6.7 mM $MgCl_2$, 10 mM mercaptoethanol, 0.1 mg/ml of bovine serum albumin, 75 pM of each deoxynucleotide triphosphate, and 75 pM of each oligonucleotide in a total reaction volume of 50 μl. A systematic aliquoting of this amplification buffer was done in 1.5-ml microtubes. Each aliquot was covered with 50 μl of paraffin to prevent evaporation, and was stored frozen at −20°C until use. *Thermus aquaticus* DNA polymerase (2.5 units) (Genofit, Geneva, Switzerland) was used for the amplification, which was performed with a Techne (Cambridge, UK) PH2 PCR device. The amplification procedure consisted of DNA denaturation (95°C for 5 min), oligoprimer annealing (48°C for 2 min), elongation (72°C for 2 min), 30 amplification cycles (95°C for 5 sec, 48°C for 30 sec, 70°C for 1 min), and cooling (20°C for 10 min).

Characterization of PCR-positive samples

The PCR-amplified 270 base-pair HVRm fragment was purified by electrophoresis on 0.8% preparative low melting point ultrapure agarose gels (Bethesda Research Laboratories, Uxbridge, UK). The fragment was eluted from the agarose using glass beads (Geneclean kit) according to the instructions of the manufacturer (Bio 101, La Jolla, CA USA), and then digested with the restriction endonucleases *Sau* 96 I and *Sca* I (Bethesda Research Laboratories) to eliminate part of the oligonucleotide primers selected in the conserved region of the minicircle. After digestion, the DNA was precipitated with ethanol, and resuspended in 100 μl of sterilized, distilled water.

TABLE I

Direct characterization of Trypanosoma cruzi *clones in triatomine bug feces**

Origin of triatomine bugs	Number	Microscopic control	Positive by PCR	Natural clone no.
Endemic area	10	+	10	19, 20 (3 cases)
	12	−	2	
Xenodiagnosis	1	+	1	39 (1 case)
	35	−	1	43 (1 case)

* Natural clones are numbered according to ref. 1. PCR = polymerase chain reaction.

Non-radioactive labeling of purified HVRm (100 ng) was performed using digoxigenin (Boehringer, Mannheim, Germany) according to the instructions of the manufacturer. Amounts of DNA were quantitated using a Dipstick kit (Invitrogen, San Diego, CA). Samples (50 ng) of purified HVRm fragments were hybridized with DNA from various *T. cruzi* strains that were genetically characterized by isozyme analysis.[1] Briefly, total DNA from 1×10^4 parasites was amplified by PCR, electrophoresed on 0.8% agarose gels, alkali denaturated (two 15-min denaturations in 0.5 M NaOH, 1.5 M NaCl), and transferred onto charged nylon membranes (Hybond N+; Amersham, Buckinghamshire, UK) by the pocket-blotting procedure.[16]

RESULTS

Identification of natural clones from vectors

PCR sensitivity. Agarose gel electrophoretic analysis of PCR products from 58 triatomine fecal samples detected 14 positive specimens, as determined by visualization of a major intense band (270 bp) after staining the gels with ethidium bromide (Table I and Figure 1). All 11 fecal samples shown by microscopic examination to be infected with parasites were also positive by PCR analysis. Three triatomine specimens that were microscopically negative for parasites were PCR-positive. It is worth noting that one of these triatomes originated from a specimen that gave a negative result on a xenodiagnostic procedure. Of three other infected triatomes that had been collected in endemic areas, frozen, and shipped to our laboratory without cryoconservation, two were also PCR-positive (unpublished data).

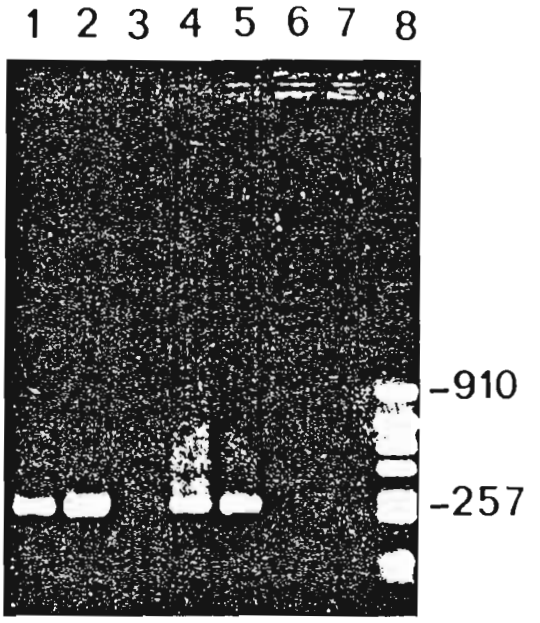

FIGURE 1. Ethidium bromide–stained 0.8% agarose gel containing polymerase chain reaction products of DNA isolated from a Bolivian triatomine bug fecal sample. Lanes 1, 2, 4, and 5, parasite-positive samples (positive by microscopic examination); lanes 3 and 6, parasite-negative samples (negative by microscopic examination); lane 7, control sample without template; lane 8, *Alu* I digest of pBR322. The amplified fragments have a length of approximately 270 base pairs. Values at right are size standards (basepairs).

Characterization of natural clones. Five PCR-positive samples from five triatomine fecal samples were tested. This procedure identified clones 39 (Figure 2A) and 43 (Figure 2B), and three isolates of either clones 19 or 20 (Figure 2C) (which are genetically related to each other). Isolates identified as clones 39 and 43 originated from xenodiagnosis of triatomine bugs. These clones had already been observed in Bolivian patients.[1, 17] The three other isolates stocks identified as clones 19 or 20 originated from triatomine bugs collected in an endemic area (the Yungas valley) where clone 20 had been already observed.[1, 18]

Detection of clones in experimentally-infected mouse blood

The results of PCR analysis of blood samples (100 μl) from a group of parasite-positive mice

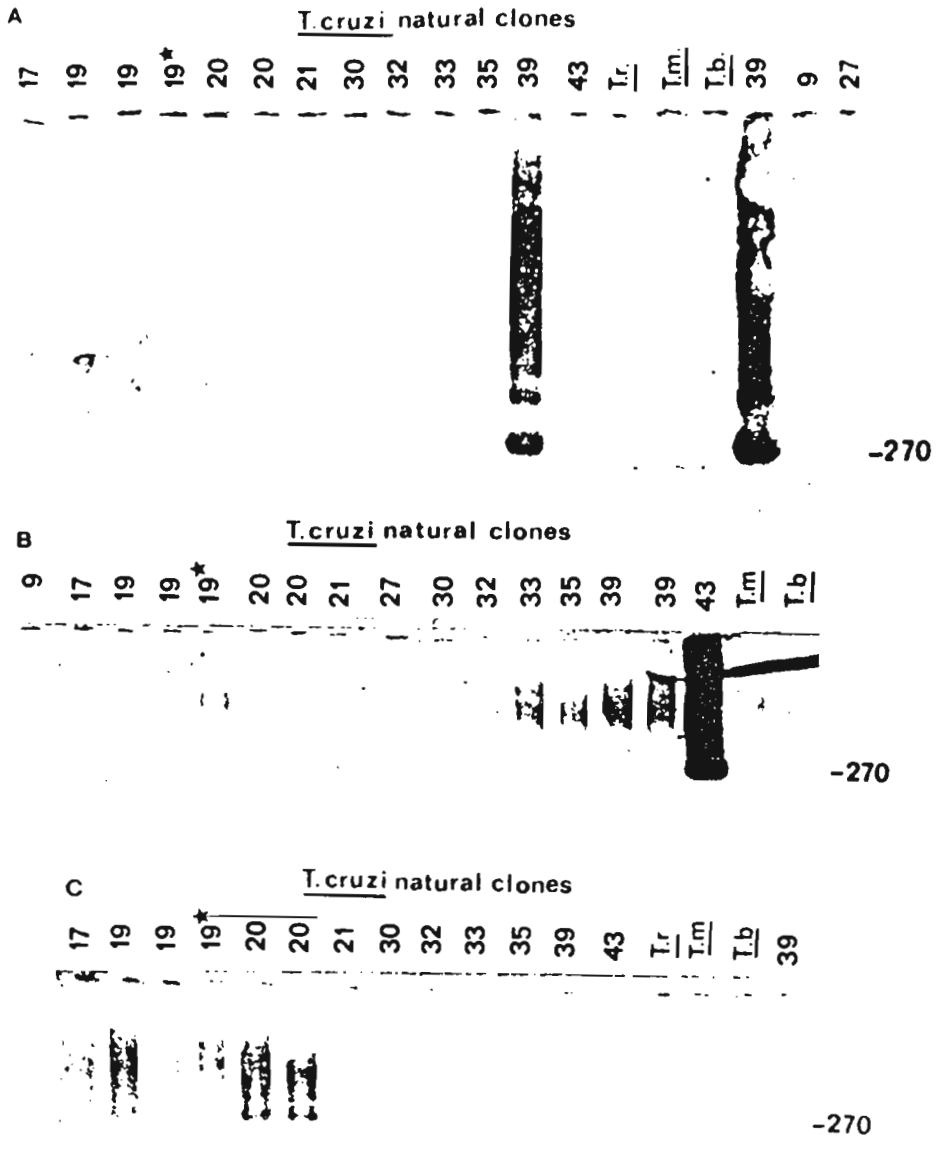

FIGURE 2. Hybridization patterns of three non-radioactively labeled highly variable region fragments of kinetoplast minicircle DNA from three different triatomine bug fecal samples obtained by polymerase (PCR) chain reaction amplification. These samples were hybridized with electrophoresed PCR products of total DNA of different *Trypanosoma cruzi* stocks that were transferred onto nylon membranes. The hybridization patterns were limited for the three different probes: 1) clone 39, **panel A**, lanes 12 and 17, TPK1 and bug 2146 stocks, respectively; 2) clone 43, **panel B**, lane 16, Tulahuen FK1IA c12 stock; 3) clones 19 or 20, **panel C**, lanes 2 and 3, clone 19 (Cutia, 13379 c17 stocks), lane 4, a clone closely related to clone 19 (19★) (LGN stock); lanes 5 and 6, clone 20 (Cuica, So34 c14 stocks). The other stocks of *T. cruzi* screened pertain to clones 9, 17, 21, 27, 30, 32, 33, and 35. *Trypanosoma rangeli* (T.r.), *T. marenkellei* (T.m.), and *T. brucei* (T.b.) control samples were also tested. Values at right are size standards (basepairs).

exhibiting either acute or chronic infections are shown in Table 2 and Figure 3A. All infected mice tested were PCR positive, except for animals in which parasitemia was less than 74 parasites/ml (no parasites were observed in 300 microscopic fields). This level of parasitemia appears to be the limit of detection under our experimental conditions. The hybridization of the

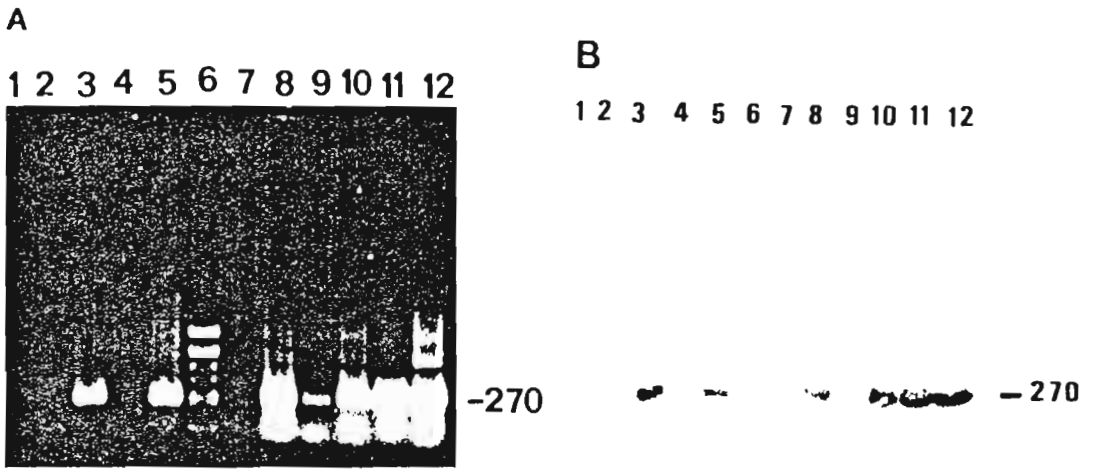

FIGURE 3. A, ethidium–bromide-stained 0.8% agarose gel containing polymerase chain reaction (PCR) products from DNA of mouse blood samples. B, hybridization pattern of these products with purified DNA from the highly variable region of the kinetoplast DNA minicircle of the Tulahuen c12 stock (clone 43) after transfer onto a nylon membrane. Lane 1, control sample (using distilled water as the template); lanes 2 and 7, uninfected mouse blood; lanes 3, 4, 5, 8, 9, and 12, blood from mice with a chronic infection at 190, 120, 120, 92, 140, 83 days, respectively, after infection; lanes 10 and 11, blood from mice in the acute phase of infection at 16 and 13 days, respectively, after infection; lane 6, *Alu* I digest of pBR322. Values at right are size standards (basepairs).

HVRm sequence with PCR products from mouse blood gave a pattern restricted to the Tulahuen c12 stock, which pertains to clone 43 (unpublished data). Moreover, HVRm purified sequences from the Tulahuen c12 stock and from triatomine bug fecal samples, both of which hybridize only with clone 43, showed strong hybridization patterns with PCR products from the different mouse blood samples (Figure 3B). Under the same conditions, no hybridization was observed with HVRm purified sequence from the Sc43 c11 stock pertaining to clone 39 (the most genetically related clone to clone 43 in our collection), for which the standard genetic distance to clone 43 is only 0.43.[1,19]

Detection of clones in human blood

The modification used in PCR sample preparation of human blood (greater sample dilution; 100 μl of blood diluted in 1 ml of distilled water) was required to obtain a PCR-positive result (at least 10 parasites/100 μl of blood) (Figure 4A). The assay of artificially-infected blood samples were performed with different stocks (So34 c14 and Sc43 c11 pertaining to clones 20 and 39, respectively). Hybridization with the corresponding specific probes confirmed the sensitivity of this assay (Figure 4B). This sensitivity appears to be comparable with results obtained with mice blood. Under these conditions, samples of both chronically-infected (six cases) and non-infected (eight cases) Bolivian patients were tested, but none of these samples gave a positive result by PCR analysis.

DISCUSSION

These results clearly demonstrate the successful use of the HVRm sequences to identify different *T. cruzi* clones circulating in vectors and

TABLE 2

Direct detection of Trypanosoma cruzi *infection in acute and chronic cases of experimentally infected mice**

Days postinfection	Parasitemia (parasites/ml)	PCR result
13	2.74×10^6	+
16	3.40×10^6	+
83	7.40×10^2	+
92	2.95×10^2	+
120	$<0.74 \times 10^2$	−
120	1.48×10^2	+
140	$<1.40 \times 10^2$	+
190	5.18×10^2	+

* PCR = polymerase chain reaction.

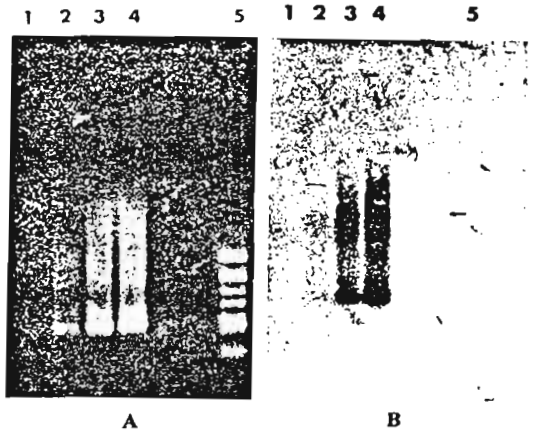

FIGURE 4. **A**, ethidium bromide–stained 0.8% agarose gel containing polymerase chain reaction (PCR) products from DNA of human blood samples (100 μl) artificially infected with the SC43 c1 11 stock (clone 39) of *Trypanosoma cruzi*. **B**, hybridization pattern of these products with purified DNA from the highly variable region of the kinetoplast DNA minicircle of the SC43 c1 1 stock after transfer to a nylon membrane. Lane 1, uninfected blood; lanes 2, 3, and 4, 100 μl of blood artificially infected with 10, 100, and 1,000 epimastigote culture forms, respectively; lane 5, *Alu* I digest of pBR322.

mammalian hosts. The preliminary results indicate that PCR sensitivity for vectors is quite satisfactory. Our results were consistent with microscopic evidence of parasite positivity, and in some instances, the PCR appears more sensitive than microscopic examination, which is expected. Therefore, the three samples that were parasite negative upon microscopic examination but parasite positive by PCR analysis were most likely infected triatomine bugs that presented with a very low level of parasitemia. The first consequence of these findings is the possibility of higher xenodiagnosis sensitivity. Our results indicate that xenodiagnosis, which is the most efficient method for parasitologic diagnosis of Chagas' disease, could be improved considerably by being used in conjunction with PCR analysis.

In addition, we were able to identify the parasitic clone circulating in five triatomine bugs by hybridization of amplified HVRm sequences with a set of *T. cruzi* clones that were fully characterized by isozyme analysis. These results are consistent with the available epidemiologic data concerning the various clones circulating in Bolivian patients and vectors. The clone specificity of the HVRm sequence has been clearly demonstrated for clones 39 and 20.[12] Some of our samples hybridized with both clones 19 and 20. Both of these clones are present in Bolivia.[1, 17, 18] One possible explanation for these non-specific hybridization patterns is the occurrence of mixed infections in vector studies already reported in Bolivia.[17, 20] There is also the possibility of cross-reacting hybridization of HVRm sequences from clone 19 with clone 20. Indeed, these clones are closely related, with a standard genetic distance of 0.02 (only one allelic difference recorded in 15 loci).[1]

The cross hybridization between the Tulahuen c12 stock and clone 43, which is the cloned stock used in the experimental infections as well as the stock circulating in a Bolivian patient, are consistent with isoenzyme multilocus characterization, and confirm the linkage between nuclear DNA and HVRm kinetoplast DNA evolution.[13]

The sensitivities for the detection of infection in mouse or human blood were comparable. Nevertheless, a weakly positive result must be interpreted cautiously, based on control samples (without DNA template) and hybridization results. This sensitivity level should be adequate enough in detecting acute human infections, where parasitemia levels are high, and this would make it possible to directly identify a specific clone in such cases. The assays results involving blood samples from chronic human infections were negative. In these cases, the small sample volume might be the main limiting factor for detection of positive PCR amplification. It should be emphasized that the classical xenodiagnosis test, which uses 30 L3 triatomine stages, requires approximately 2 ml of blood, while the present PCR experiments requires only 100 μl samples of blood.

The successful use of specific PCR probes under natural conditions (samples of blood and vector feces) will make it possible to establish a new standard for the comparison of the relevant medical properties (geographic distribution, clinical specificity, drug resistance, and virulence) of *T. cruzi* clones. This combination of a population genetics approach and a new technical tool provides an efficient means of investigating the long-standing, as yet unanswered, question regarding the medical consequences of *T. cruzi* genetic variability.

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An isoenzyme study of naturally occurring clones of *Trypanosoma cruzi* isolated from both sides of the West Andes highland

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Abstract

Seventy-two stocks of *Trypanosoma cruzi* isolated from both sides of the West Andes highland (Bolivia, Chile and Peru) were analysed by isoenzyme electrophoresis at 12 loci. The data, which were interpreted in terms of population and evolutionary genetics, corroborated the hypothesis of *T. cruzi* clonal population structure previously proposed, and indicated extensive genetic variability within the taxon *T. cruzi*. Fifteen different clones (or zymodemes) were identified, which could be grouped into 3 different clusters. Several clones from 2 of these clusters were isolated both in Chile and Bolivia, suggesting a significant circulation of invertebrate and/or vertebrate hosts of *T. cruzi* between these 2 countries. Low clonal variability in Peru suggested the occurrence of a 'founder effect' in this country. The potential usefulness of a cladistic approach in epidemiology is discussed.

Introduction

Isoenzyme electrophoresis has been widely used for the characterization of *Trypanosoma cruzi* populations (TOYÉ, 1974; MILES *et al.*, 1977, 1980) and extensive population genetical analysis has demonstrated a basically clonal structure in natural populations (TIBAYRENC *et al.*, 1986). The zymodemes described can be equated to natural clones of the parasite, as they can be identified by isozyme patterns. To date, 43 different natural clones have been described from an extensive sample of stocks isolated from different geographical areas and from various hosts. The distribution of these clones is noteworthy in two respects: (i) radically dissimilar clones are frequently sampled in close geographical proximity, in the same house or from the same individual host, either triatomine bug or human (BRENIÈRE *et al.*, 1985b; TIBAYRENC *et al.*, 1985); and (ii), while most of the clones are rarely recorded, a limited number appear to be widely distributed: for example, clone 19 was recorded in Brazil, Bolivia, Columbia and Venezuela (TIBAYRENC & AYALA, 1988). These predominant clones, the medical importance of which might be considerable, were termed 'major clones' (TIBAYRENC & BRENIÈRE, 1988).

The Bolivian domestic cycle is now well known: it is characterized by a large number of different clones (13) with a heterogeneous distribution throughout the whole endemic area, and by the presence of 3 predominant clones (19, 20 and 39), the last being radically dissimilar from the other two (large genetic distances). These 3 clones are found in sympatric circulation in most of the areas studied (TIBAYRENC *et al.*, 1986). In Chile, isoenzyme studies of *T. cruzi* stocks suggest the existence of 2 separate transmission cycles (domestic and sylvatic) in northern Chile with possible overlap between the 2 cycles (APT *et al.*, 1987). In southern Peru, BRENIÈRE *et al.* (1985a) reported the existence of a prevalence of stocks related to zymodeme 1 of READY & MILES (1980) and exhibiting a low variability. The purpose of the present study was to elucidate the distribution of natural clones of *T. cruzi* on either side of the Andean highlands, to check whether the Andes represent an efficient geographical barrier for *T. cruzi*, and to elucidate the phylogenetic relationships between clones of *T. cruzi*.

Material and Methods

Parasites. Table 1 gives all data about the origin of the 72 stocks studied.

Isoenzyme analysis. Eleven enzyme systems, as previously listed, corresponding to 12 genetic loci, were assayed (BRENIÈRE *et al.*, 1989). Electrophoresis was performed on cellulose acetate plates as described by TIBAYRENC *et al.* (1985), with slight modifications. Genetic interpretation and clone (or 'zymodeme') numbering was according to TIBAYRENC & AYALA (1988). The following stocks, already fully characterized (TIBAYRENC & AYALA, 1988), were used as references for allelic identification: SC43, cl.2, C8 cl.1, Mil3, Tulahuen, Tehuentepec, A107, Can III cl.1 (Z3f), Esmeraldo cl.3 (Z2) and 27R27. Isoenzymic analysis of the Bolivian and Peruvian stocks was done soon after isolation (one month), while the Chilean stocks were analysed after a longer period in culture.

Taxonomic clustering. Phylogenetic relationships among the clones were evaluated by using two different methods. (i) Based on the standard genetic distance matrix of NEI (1972), a dendogram was built by using the KITSCH program of hierarchized agglomeration (LANCE & WILLIAMS, 1966; LEGENDRE & LEGENDRE, 1979). (ii) After transforming the allelic frequency matrix into an allelic presence/absence (1/0) matrix, a cladistic network was built by using both the MIX program of Wagner algorithm (FARRIS, 1970), based on the parsimony principle (FELSENSTEIN, 1978, 1982) and the CLIQUE program, based on the compatibility principle (LE QUESNE, 1974; ECK & DAYHOFF, 1966). Several runs of the MIX program

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were carried out, by shuffling clone presentation order, to obtain the most parsimonious tree and check clustering stability. All programs pertain to the PHYLIP package communicated by J. Felsenstein.

Results

Further genetic variability in T. cruzi

Fifteen different clones, with the genotypes detailed in Table 2, were identified (see Table 1). Among them, 5 have already been reported, namely clones 2, 19, 20, 33 and 39 (TIBAYRENC & AYALA, 1988). The others were closely related to them, since they presented at most 6 allelic differences out of 24 possible ones, by comparison with the previously identified clones. We did not number any of the new clones, since more complete genotype characterization is required for fuller understanding of their phylogenetic relationships with the previously described clones.

For the locus *Pep 1*, a putative three-banded heterozygous aspect of a dimeric enzyme (the central band being more intensely stained than the two extreme ones) was observed for the first time in *T. cruzi*.

Stocks presenting isoenzyme patterns typical of two mixed clones

Some stocks from Bolivia (Potosi, 5 stocks) and Peru (Majes valley, 1 stock) exhibited patterns typical (TIBAYRENC *et al.*, 1985) of a mixture of two different clones (Table 1). We did not observe such patterns in the other localities under study. The patterns have been extensively described previously (BRENIÈRE *et al.*, 1985b; TIBAYRENC *et al.*, 1985). The fact that they are due to the presence of mixtures of various genotypes has been ascertained either by cloning experiments (TIBAYRENC & MILES, 1982) or by preparing artificial mixtures of cloned stocks (our unpublished data).

Phylogenetic relationships and geographical distribution of the clones

Standard genetic distances (NEI, 1972) are shown in

Table 1. Geographical and hostal origin and genotypes of the 72 *Trypanosoma cruzi* stocks studied[a]

Number of stocks	Designation	Country	Place	Host	Genotype
4	TPA 1–4	Bolivia	Trinidad Pampa Yungas (1600 m)	*Triatoma infestans*	cl.20
2	TPC 1,2	Bolivia	Coripata Yungas (1800 m)	*Triatoma infestans*	cl.20
3	TPL 3,4,7	Bolivia	Chillamani Yungas (1800 m)	*Triatoma infestans*	cl.20
2	TPN 12,13	Bolivia	Parrarani Yungas (1600 m)	*Triatoma infestans*	cl.20
1	SO 34	Bolivia	Toropalca Potosio (2700 m)	*Triatoma infestans*	cl.20
2	SO 44, 51	Bolivia	Vitichi Potosio (3000 m)	*Triatoma infestans*	cl.20
1	SO 18	Bolivia	Miculpaya Potosio (3000 m)	*Triatoma infestans*	cl.19
1	SP 104	Chile	IVa Region	*Triatoma spinolai*	cl.19
2	SO 16,28	Bolivia	Miculpava Potosi (3000 m)	*Triatoma infestans*	NP3
1	SO 30	Bolivia	Calcha Potosio (2700 m)	*Triatoma infestans*	NP3
1	SO 40	Bolivia	Vitichi Potosi (3000 m)	*Triatoma infestans*	NP3
1	LGN	Chile	IVa Region	Man (chronic case)	NP4
1	SPAII	Chile	IVa Region	*Triatoma spinolai*	NP5
1	SP 31	Chile	IVa Region	*Triatoma spinolai*	NP6
1	LQ	Chile	IVa Region	Man (chronic case)	NP7
1	A 97	Peru	Sihuas valley (1500 m)	*Triatoma infestans*	cl.2
1	A 105	Peru	Sihuas valley (1500 m)	*Triatoma infestans*	NP9
1	A 82	Peru	Sihuas valley (1500 m)	*Triatoma infestans*	NP10
4	A 1,4,6,7	Peru	Victor valley (2000 m)	*Triatoma infestans*	NP11
5	A 34, 38, 70, 76, 87	Peru	Sihuas valley (1500 m)	*Triatoma infestans*	NP11
5	A 136,138,141,144,146	Peru	Majes valley (600 m)	*Triatoma infestans*	NP11
2	SO19, 23	Bolivia	Miculpaya Potosi (3000 m)	*Triatoma infestans*	cl.39
1	SO 31	Bolivia	Calcha Potosi (2700 m)	*Triatoma infestans*	cl.39
1	SO 33	Bolivia	Toropalca Potosi (2700 m)	*Triatoma infestans*	cl.39
2	SO 35, 38	Bolivia	Huatina Potosi	*Triatoma infestans*	cl.39
2	SO 45, 48	Bolivia	Vitichi Potosi (3000 m)	*Triatoma infestans*	cl.39
3	SO 3–5	Bolivia	Otavi Potosi (3400 m)	*Triatoma infestans*	cl.39
1	TPK 1	Bolivia	Khala-khala Yungas (1700 m)	*Triatoma infestans*	cl.39
3	TPN 1,21,22	Bolivia	Parrarani Yungas (1600 m)	*Triatoma infestans*	cl.39
2	MN, RMS	Chile	IVa Region	Man (chronic case)	cl.39
1	NR	Chile	IIIa Region	Man (chronic case)	cl.39
1	V2X	Chile	IVa Region	*Triatoma infestans*	cl.39
2	PCM, MCC	Chile	IVa Region	Man (chronic case)	NP13
1	GR	Chile	IIIa Region	Man (chronic case)	NP13
1	CBB	Chile	IVa Region	Man (chronic case)	cl.33
1	SO 21	Bolivia	Miculpaya Potosi (3000 m)	*Triatoma infestans*	NP15
1	SO 50	Bolivia	Vitichi Potosi (3000 m)	*Triatoma infestans*	NP15
2	SO 22, 25	Bolivia	Miculpaya Potosi (3000 m)	*Triatoma infestans*	NP3+NP15
2	SO 36,37	Bolivia	Huatina Potosi	*Triatoma infestans*	cl.39+NP3
1	SO 15	Bolivia	Miculpaya Potosi (3000 m)	*Triatoma infestans*	cl.39+NP15
1	A 141	Peru	Majes valley (600 m)	*Triatoma infestans*	NP11+cl.39
Total=72					

[a]Fifteen different genotyes were identified; clone numbers (cl.) indicate genotypes described by TIBAYRENC & AYALA (1988), NP indicates provisional numbers (see footnote to Table 2). For discussion of relationship with zymodemes defined by READY & MILES (1980), see TIBAYRENC *et al.* (1986) and text.

Table 2. Genotypes of *Trypanosoma cruzi* clones identified in Bolivia, Chile and Peru[a]

	Genotypes[b]										
	G6pd	*Gpi*	*Gd1*	*Gd2*	*Idh*	*Me-1*	*Me-2*	*Pep-1*	*Pep-2*	*Pgm*	*6Pgd*
cl. 20	5/5	5/5	3/3	2/2	1/1	2/2	4/4	1/1	1*/1*	3/3	2/4
cl. 19	5/5	5/5	3/3	2/2	1/1	2/2	4/4	1/1	1*/1*	3/3	4/4
NP3	5/5	5/5	3/3	2/2	1/1	2/2	4/4	3/3	1*/1*	3/3	4/4
NP4	5/5	5/5	3/3	2/2	1/1	2/2	4/4	1/4	1*/1*	3/3	4/4
NP5	6/6	5/5	3/3	2/2	1/1	2/2	4/4	1/1	1*/1	3/3	4/4
NP6	6/6	5/5	3/3	2/2	1/1	2/2	4/4	1/1	1*/1*	3/3	4/4
NP7	6/6	5/5	3/3	2/2	1/1	2/2	4/4	1/1*	1*/1*	3/3	4/4
cl. 2	6/6	5/5	3/3	2/2	1/1	2/2	4/4	3/3	1 /1	3/3	4/4
NP9	6/6	5/5	3/3	2/2	1/1	2/2	4/4	1/1	3 /3	8/10	4/4
NP10	6/6	5/5	3/3	2/2	1/1	2/2	4/4	4/4	1*/1*	3/3	2/4
NP11	6/6	5/5	3/3	2/2	1/1	2/2	4/4	1/1	1*/1*	3/3	2/4
cl. 39	4/4	2/4	1/1	1/1	2/2	1/1	5/5	5/5	1 /1	6/10	1/4
NP13	4/4	2/4	1/1	1/1	2/2	1/1	6/6	5/5	1 /1	6/10	1/4
cl. 33	2/2	3/3	2/2	5/5	2/2	2/2	6/6	4/4	1 /1	10/12	1/1
NP15	2/2	3/3	2/2	5/5	2/2	2/2	5/5	5/5	1 /1	10/12	1/1

[a]For each locus, allele 1 codes for the fastest electromorph. The malate dehydrogenase enzyme system is not listed as it was monomorphic in this sample (*Mdh*=2/2). Each electromorph was determined according to TIBAYRENC & AYALA (1988) using appropriate reference clones. Clone numbering (cl.) refers to TIBAYRENC & AYALA (1988), the other genotypes (NP) are newly recorded. For better understanding, provisional numbering is given to the new genotypes recorded here; nevertheless, they need additional characterization to confirm their phylogenetic position (see text).

[b]The asterisks (*) indicate new allelic positions, slightly faster than the fastest position previously recorded (=1).

Genotypes	cl 20	cl 19	NP3	NP4	NP5	NP6	NP7	cl 2	NP9	NP10	NP11	cl 39	NP13	cl 33	NP15
cl 20	-	1	3	2	5	3	5	7	8	6	5	26	26	24	24
cl 19	0 02	-	2	1	4	2	4	6	7	5	4	25	25	23	23
NP3	0 11	0 09	-	3	6	4	6	8	9	7	6	27	27	25	25
NP4	0 40	0 02	0 09	-	5	3	5	7	8	6	5	26	26	24	24
NP5	0 21	0 18	0 29	0 21	-	2	4	2	5	5	4	21	21	19	19
NP6	0 11	0 09	0 18	0 11	0 09	-	2	4	5	5	2	23	23	21	21
NP7	0 21	0 18	0 18	0 16	0 18	0 09	-	6	7	3	2	25	25	23	23
cl 2	0 32	0 29	0 16	0 27	0 09	0 18	0 18	-	7	7	6	21	21	19	19
NP9	0 03	0 27	0 58	0 30	0 16	0 16	0 27	0 27	-	8	7	26	26	24	24
NP10	0 19	0 21	0 21	0 14	0 21	0 16	0 11	0 21	0 30	-	1	26	26	24	24
NP11	0 09	0 11	0 21	0 14	0 11	0 02	0 11	0 21	0 19	0 09	-	25	25	23	25
cl 39	2 17	2 01	2 01	1 20	1 50	2 01	2 01	1 50	1 84	2 17	2 17	-	2	10	10
NP13	2 17	2 01	2 01	1 20	1 50	2 01	2 01	1 50	1 84	2 17	2 17	0 10	-	10	10
cl 33	1 75	1 77	1 77	1 57	1 36	1 77	1 77	1 36	1 63	1 34	1 75	1 07	0 42	-	4
NP15	1 75	1 77	1 77	1 75	1 36	1 77	1 77	1 36	1 63	1 75	1 75	0 65	0 04	0 19	-

Fig. 1. Matrix of patristic or evolutionary distances (above the diagonal: see Fig. 3) and Nei's standard genetic distances (below the diagonal) between the 15 genotypes identified by 12 isoenzyme loci in *Trypanosoma cruzi* stocks isolated from Bolivia, Chile and Peru. Clone numbering (cl.) refers to TIBAYRENC & AYALA (1988); the other genotypes (NP) are newly recorded (see Table 2).

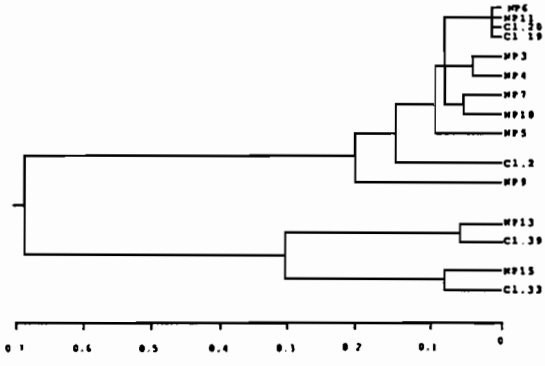

Fig. 2. Dendogram obtained from the genetic distance matrix of NEI (1972) for the different clones of *Trypanosoma cruzi*, using the KITSCH program (package PHYLIP). The P parameter was fixed by (=0) in order to obtain a treatment similar to UPGMA.

Fig. 1. As previously noted (TIBAYRENC *et al.*, 1986), some pair-wise comparisons exhibited very high values (up to 2·17) while others showed values as low as 0·02.

Three clusters can be discerned (Figs 2, 3). (i) A cluster including 10 different multilocus genotypes (clones) which share 14 alleles out of 24 possible allelic positions, with allelic differences among the clones ranging from 1 to 6. Within this group, 3 clones have been recorded previously, namely clones 2, 19 and 20 (TIBAYRENC & AYALA, 1988). Stocks pertaining to this cluster have been isolated from all three countries studied. The Peruvian stocks were all included in this cluster, except one stock which exhibited a weak pattern for glucose phosphate isomerase, suggesting a mixture with clone 39, which is radically different (see below). (ii) A second cluster included 2 clones (one corresponding to the formerly described clone 39, and the other closely related to it). These clones differed from any clone of the first cluster by 19 to 21 allelic differences. In the present sample, clone 39 (identified as a 'major clone': TIBAYRENC & BRENIÈRE, 1988) was frequently found in Bolivia as well as in Chile, and was possibly recorded in Peru (see Table 1). (iii) The third cluster included two different clones, the previously recorded clone 33 and another closely related to it. These two clones differed from any clone of the two other clusters in at least 17 allelic positions. Relationships between these various genotypes and the previously described zymodemes I, II and III (READY & MILES, 1980) have been extensively discussed by TIBAYRENC *et al.* (1986) and TIBAYRENC & AYALA (1988). Briefly, zymodemes II and III are clearly distinct from any of the stocks

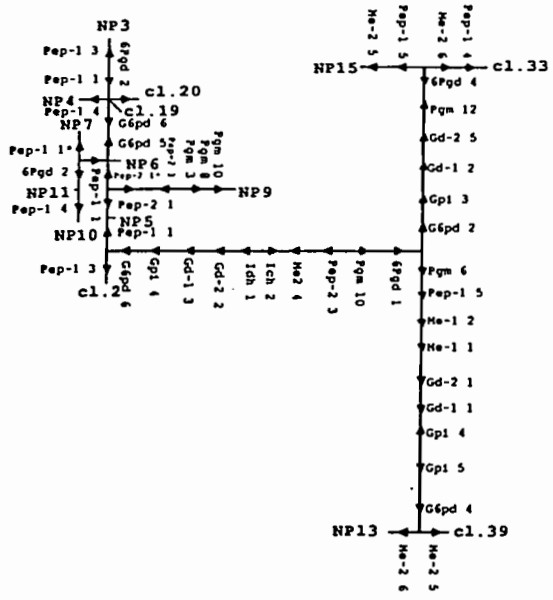

Fig. 3. Phylogenetic network showing the evolutionary relationships between the *Trypanosoma cruzi* clones using the MIX program; CLIQUE program yielded a quite similar pattern (see text). The matrix was built from the presence/absence criterion for 38 alleles. The cl. and NP numbers refer to the genotypes identified in this study (see Table 2). The arrows represent the different evolutionary steps between the clones, either by gain or loss of an allele.

surveyed here (minimum genetic distance 0·87; maximum genetic distance 1·79). Zymodeme I appears to be related to the stocks from the first cluster (see above) only, although the minimum genetic distance (between clone 2 and zymodeme I) was not negligible (0·28).

Discussion

Several stocks of *T. cruzi* from Bolivia, Chile and Peru presented new electrophoretic patterns which differed at one to three loci from clones previously described by TIBAYRENC & AYALA (1988). These results are consistent with the clonal structure of *T. cruzi* populations proposed by TIBAYRENC *et al.* (1986): when stock sampling increases and genetic labelling improves, the number of clones identified is expected to become higher. Nevertheless, the main results indicating clonal structure are fully confirmed by the present study, that is to say fixed heterozygosity of some genotypes (see clone 39) and strong linkage disequilibrium; if the alleles recorded here combined randomly, the total number of possible genotypes would be considerable, so that frequent isolation of the same multilocus genotype, as in this work, would be extremely improbable.

Some clones were identified on both sides of the Andes, in Chile and Bolivia (clones 19 and 39), which suggests the existence of communication between the two countries for the invertebrate and/or vertebrate hosts of *T. cruzi*: this could be explained by the important commercial contacts between the two countries. Nevertheless, although stock sampling is still limited, some clones appear to be specific for each of the two countries (see Table 1). This suggests that communication between the two countries is not sufficient to allow complete mixing of the clone populations between them. In Peru, the low genetic variability existing among the clones (maximum genetic distance=0·30) and the presence of a predominant clone (defined by genotype NP11) circulating in the three Peruvian valleys studied suggest the occurrence of founder effects in this area (see below).

The rate of mixed stocks recorded is similar to those in previous results—about 10% (BRENIÈRE *et al.*, 1985a; TIBAYRENC *et al.*, 1985). Due to technical limitations, this is probably an underestimation of the real rate. This is particularly true for the present sample, where Chilean stocks were maintained in culture for a long time, which probably led to the elimination of some clones in mixtures.

Three clusters of clones were identified. This clustering pattern, and the relative configuration of the clones, seem to be 'robust', since they remained unchanged when studied by the different methods used, namely the hierarchized agglomeration technique, the MIX program (with several runs) based on the parsimony principle, and the CLIQUE program based on the compatibility principle: this program rejects incompatible characters (9 out of 38 in the present case) implicating reversion, convergence or an error of interpretation.

The first cluster is rather heterogeneous, and exhibits a complex topology. Within this cluster, Peruvian clones (see Table 1) cannot be equated with a monophyletic group which could be distinguished from the other clones belonging to this first cluster. Although founder effects can be inferred to explain the present genetical variability of *T. cruzi* in Peru, it is probable that the Peruvian stocks do not derive from a unique common ancestor which had differentiated locally: the divergence among NP11/NP10, NP9 and clone 2 seems to have occurred before their introduction in Peru. To date we have no indication about the possible geographical origin of these *T. cruzi* populations from Peru. A larger sample of stocks isolated from *Triatoma spinolai* and human hosts in Chile is needed to understand better the overlap between wild and domestic cycles in this country.

Stock characterization based upon the use of a sufficient range of loci, and on the working hypothesis of clonality, provides an efficient means of studying spatial and temporal distribution of *T. cruzi* populations. Moreover, cladistic analysis makes it possible to establish firmly the phylogenetic relationships among the clones, and to infer working hypotheses concerning their patterns of geographical divergence. Work is in hand to complete the present study by increasing the number of stocks and of isoenzyme loci studied, and by comparing the results with those obtained using other genetic markers, such as kinetoplast deoxyribonucleic acid fragment polymorphism (VEAS *et al.*, 1990).

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Note added in proof. Since admission of this paper, an additional patient has relapsed, with 262 WBC/μl and trypanosomes in the CSF 15 months after nifurtimox treatment.

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Short Report

Association between *Trypanosoma cruzi* zymodemes and specific humoral depression in chronic chagasic patients

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We previously reported four autochthonous cases of Chagas disease in Bolivia, presenting a particular pattern of negative serology with positive xenodiagnosis (BRENIÈRE *et al.*, 1984). To date, we have observed 13 similar cases (8 women and 5 men), 12 from Bolivia and one from Argentina. The age of these patients ranged from 18 to 58 years (mean 39±13). The clinical features exhibited were diversified: 5 patients presented a cardiac pathology (electrocardiogram) or a digestive pathology (megacolon), or both, while the 8 other patients were asymptomatic. In the present preliminary study, we explored a possible association between the humoral depression and *Trypanosoma cruzi* zymodemes. Eight stocks isolated from patients with negative serology and positive xenodiagnosis and 52 stocks from patients with positive serology and positive xenodiagnosis were characterized by 11 enzyme systems (12 genetic loci), using the genetic interpretation and zymodeme numbering used by TIBAYRENC *et al.* (1986); definitions of positive and negative serology were according to BRENIÈRE *et al.* (1984). Eight different zymodemes were recorded, of which 3 represent more than 90% of our sample, namely zymodemes 19, 20 and 39. Zymodemes 19 and 20 are closely related (only one allelic difference) and were plotted together in the statistical analyses. On the contrary, zymodeme 39 is radically different from 19 and 20. Twenty-nine patients (48%) were infected with either zymodeme 19 or 20, while 18 patients (30%) had zymodeme 39, and 7 patients (11·6%) exhibited a mixture of zymodeme 39 with either 19 or 20. A statistically significant association was observed between specific humoral depression and zymodeme: all serologically negative patients had either zymodeme 19 or 20, and none had zymodeme 39. Yate's corrected χ^2 was 4·19, one degree of freedom and $P<0·05$.

These results confirm the existence of specific humoral depression in some chronic chagasic patients, and shows that this phenomenon can be associated with typical chagasic symptomatology. The association between negative serology and *T. cruzi* zymodeme, although statistically significant in the present set of patients, must be confirmed on a more extensive sample.

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CHAGAS' DISEASE IN BOLIVIA: CLINICAL AND EPIDEMIOLOGICAL FEATURES AND ZYMODEME VARIABILITY OF *TRYPANOSOMA CRUZI* STRAINS ISOLATED FROM PATIENTS

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Abstract. We performed serological and pathological studies on 495 patients with Chagas' disease from different areas of Bolivia. Eighty-nine *Trypanosoma cruzi* strains, isolated by xenodiagnosis, were characterized by 12 isoenzyme loci and were related to the presence of cardiac changes and enteric disease with megacolon. There was a high heterogeneity of human zymodemes, presenting evidence of 2 predominant zymodemes genetically dissimilar from each other and ubiquitous in Bolivia. The frequencies of these predominant zymodemes among strains from patients were compared to strains from triatomine bugs previously studied. We observed mixtures of different zymodemes within the same patient, a phenomenon seen previously in Bolivian patients. There was no apparent difference of pathogenicity between the 2 more frequent zymodemes isolated from humans.

Trypanosoma cruzi, the agent of Chagas' disease, is associated with 2 main clinical forms, cardiac and intestinal. These clinical forms are not equally distributed within the geographical range of the disease, and it has been proposed that *T. cruzi* zymodeme distribution could explain this fact.[1] We present here pathological, serological, and parasitological studies, involving 495 patients from Bolivia, showing the diversity of the clinical forms and the distributions of these clinical forms and *T. cruzi* zymodemes isolated from human. The relationships are presented and discussed.

MATERIALS AND METHODS

Patients

Two groups were examined at different times and under different conditions. Group I, consisting of 364 patients, was from the villages of Chiwisivi (Department La Paz) at 2,800 meters above sea level (mas), Salinas (Department Santa-Cruz) at 800 mas, and Camiri (Department Santa-Cruz) at 800 mas (Table 1). After clinical examination, electrocardiography was performed. Serodiagnostic procedures included indirect immunofluorescence IgG (IMF), and ELISA.[2] These patients were then classified as follows, according to the results of these tests:

Positive serology and positive cardiopathy—IMF and ELISA tests positive, ECG with abnormalities as in Groups II and III of the WHO classification.[3]

Positive serology and negative cardiopathy—IMF and ELISA tests positive, normal ECG.

Negative serology and positive cardiopathy—IMF and ELISA tests negative, ECG with abnormalities as in Groups II and III of the WHO classification.[3]

Negative serology and negative cardiopathy—IMF and ELISA tests negative, normal ECG.

The second group was composed of 131 patients, all exhibiting a positive serology (serodiagnosis as noted above). They originated from different endemic regions of Bolivia, although 108 had been living in La Paz (3,600 mas) for at least 5 years (average time spent in La Paz: 10 years). The endemic areas where the patients had spent their childhoods or the first endemic area where they had spent > 1 year were considered the geographical origins of infection (Table 2). The following tests were conducted: ECG, x-ray of the right anterior side of the esophagus, and x-ray of colon after barium washing (4 x-ray films. Group II patients were then classified as follows:

Asymptomatic—positive serology, normal ECG, normal esophagus and colon.

Cardiopathy—positive serology, ECG with

TABLE 1

Serology, cardiac pathology, and zymodemes of Group I patients by locality

Localities	Sex	n	Positive serology		Negative serology		Zymodemes*
			C+†	C-†	C+	C−	
Chiwisivi	M	113	27	37	11	38	9
Salinas	M	24	7	16	0	1	
	F	22	6	15	0	1	—
Camiri	M	88	13	63	0	12	2, 37, 38, 39, 42
	F	117	17	71	0	29	
Total		364	272 (74.7%)		92 (25.3%)		

* After Tibayrenc and others[8-12]; zymodemes isolated from *Triatoma infestans*
† C+ = ECG presenting abnormalities Groups II and III of the WHO classification.[3]
‡ C− = Normal ECG.

abnormalities as in Group II and III of the WHO classification,[3] normal esophagus and colon.

Megacolon—positive serology, colon with abnormal length (DI, DII, or DIII, according to the degree of hypertrophy) and/or enlargement (MI, MII, or MIII, according to the degree of hypertrophy), normal ECG, normal esophagus. The abnormal length of the colon (DI) observed in the patients having spent >5 years in highland was considered insignificant because this is frequently seen in individuals living in highlands (La Paz, 3,800 mas; Cochabamba and Sucre, 2,600 mas).

Megaoesophagus—esophagus with abnormal dilatation,[4] normal ECG and colon.

Associated—positive serology, ECG with abnormalities, and megaoesophagus and/or megacolon as mentioned above.

Zymodeme sampling

By drawn xenodiagnosis, 35 strains were isolated from patients of the Group II; 10 others were isolated from patients who had ECG exam only, and 44 others were isolated from patients who had not been examined otherwise. All patients originated from various Bolivian endemic areas (Table 3) and all presented a positive serology.

Zymodeme identification

T. cruzi strains were isolated by xenodiagnosis using a method previously described.[5] The xenodiagnosis was performed with 30 laboratory-reared *Triatoma infestans* (third larval stage). Strains of *T. cruzi* from these patients were grown in LIT medium. Eleven enzyme systems corresponding to 12 genetic loci were assayed: glucose-6-phosphate dehydrogenase (E.C.1.1.1. 49, G6PD), glucose-6-phosphate isomerase (E.C.5.3.1.9., GPI), glutamate dehydrogenase NAD+ (E.C.1 4.1.2., GDH-NAD+), glutamate dehydrogenase NADP+ (E.C.1.4.1.4., GDH-NADP+), isocitrate dehydrogenase (E.C.1.1.1.42, IDH), malate dehydrogenase (E.C.

TABLE 2

Pathologies of 131 chronic Bolivian chagasic patients from various endemic areas

Geographical origin of patients	n	Clinical groups*				
		A	C	MC	MO	AS
Camargo	1	1	0	0	0	0
Camiri	4	3	0	1	0	0
Cochabamba	34	15 (44.1%)	7 (20.5%)	10 (29.4%)	0 (0%)	2 (5.8%)
Potosi	5	3	1	1	0	0
Santa-Cruz	1	2	3	5	0	0
Sucre	38	20 (52.6%)	8 (21%)	6 (15.8%)	0 (0%)	6 (15.8%)
Tarija	7	2	0	4	0	1
Tupiza	9	2	4	3	0	0
Valle Grande	5	3	1	0	1	0
Yungas	7	4	0	3	0	0
Undetermined	9	4	2	2	0	1
Total	131	59 (45%)	26 (19.8%)	35 (26.7%)	1 (0.07%)	10 (7.6%)

All of these patients were examined in La Paz and presented a positive serology.
* A = asymptomatic, C = cardiopathy, MC = megacolon, MO = megaoesophagus, and AS = associated.

TABLE 3

Numbers of patients infected by given zymodemes, or mixtures of zymodemes, with reference to their geographical origin

Geographical origin of patients	n	Only one zymodeme								Two zymodemes within the same patient			
		NP1	7	19	20	32	39	40	43	NP2	20 + 39	20 + 40	7 + 20
Cochabamba	33	0	1	2	6	0	22	0	0	0	1	1	0
Potosi	3	1	0	0	1	0	1	0	0	0	0	0	0
Santa-Cruz	10	0	0	2	0	0	6	1	0	0	1	0	0
Sucre	16	0	1	4	2	1	6	0	0	0	2	0	0
Tarija	1	0	0	0	1	0	0	0	0	0	0	0	0
Tupiza	2	0	0	0	1	0	1	0	0	0	0	0	0
Valle Grande	1	0	0	0	1	0	0	0	0	0	0	0	0
Yungas	2	0	0	0	0	0	0	0	0	0	1	0	1
Unknown	21	0	0	3	6	0	5	0	2	1	4	0	0
Total	89	1	1	11	18	1	41	1	2	1	9	1	1

NP = new profile.
Zymodeme numbering is according to Tibayrenc and others.[6]

1.1.1.37, MDH), malate dehydrogenase (oxaloacetate decarboxylating, NADP+), or malic enzyme (E.C.1.1.1 40, ME), peptidase 1 (Ficin) (E.C.3.4.22.3., formerly E.C.3.4.4.12, PEP-1, substrate: Leucyl-leucyl-leucine), peptidase 2 (Bromelain) (E.C.3.4.22.4., formerly E.C.3.4.4.24, PEP-2, substrate: Leucyl-L-alanine), phosphoglucomutase (E.C.5.4.2.2., formerly E.C.2.7. 5.1., PGM), and 6-phosphogluconate dehydrogenase (E.C.1.1.1.44, 6PGD). Electrophoreses were performed on cellulose acetate plates using the method of Tibayrenc and others[6] with slight modifications. Genetic interpretation of the zymograms and zymodeme numbering were performed as previously described.[7,8]

Statistical methods

The chi-square (χ^2) test or the modified χ^2 test (small samples) were used for the different comparisons. The phylogenetic relationships among the zymodemes evidenced have been evaluated by calculation of Nei's standard genetic distance[9] (measure of the number of codon differences per gene between 2 populations) and by the minimum-length Wagner network clustering method.[8,10,11]

RESULTS

Serological and electrographic study of Group I patients

The study of 364 patients from 3 endemic areas revealed high rates of positive serology, ranging from 56.6% in Chiwisivi to 95.6% in Salinas. Women from Camiri presented a significantly higher number of positive serologies than men ($\chi^2 = 3.89$, $df = 1$, $P < 0.05$); this result was not observed in Salinas. Men from Chiwisivi exhibited a significantly lower rate of positive serology than men from Camiri and Salinas ($\chi^2 = 20.66$, $P < 10^{-3}$ and $\chi^2 = 13.12$, $P < 10^{-3}$, $df = 1$, respectively). The difference observed between Camiri and Salinas was not statistically significant (0.85, $P > 0.05$). High rates of ECG abnormalities in the whole populations were observed (Table 1): 14.6% in Camiri, 28.2% in Salinas, and 33.6% in Chiwisivi. In Camiri and Salinas, ECG abnormalities were constantly associated with positive serology. In contrast, in Chiwisivi, 11 patients with negative serology presented ECG abnormalities, although there was a significant association between positive serology and cardiopathy ($\chi^2 = 5.22$, $df = 1$, $P < 0.05$). Among the patients exhibiting a positive serology, the index of cardiopathy was higher in Chiwisivi than in Salinas, and higher in Salinas than in Camiri (statistically significant for Chiwisivi and Camiri only [$\chi^2 = 10.71$, $df = 1$, $P < 10^{-2}$]).

Clinical forms observed in Group II patients

Table 2 summarizes the differences within Group II. Only 1 case of megaoesophagus without cardiac change was identified (0.07%). Considering the different clinical forms (asymptomatic, cardiac, digestive, and associated), no significant difference was observed between pa-

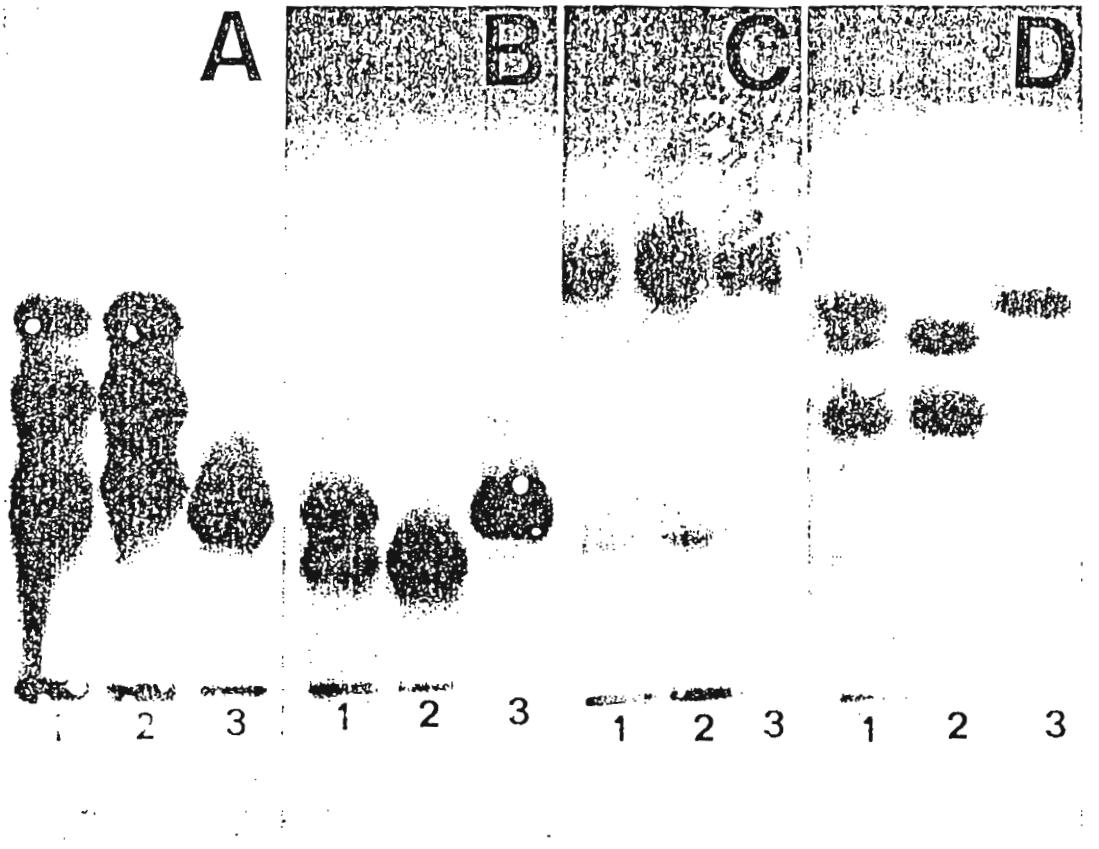

FIGURE 1. Electrophoretic profiles of 3 strains of *T. cruzi* isolated from patients by xenodiagnosis. Enzyme systems shown are (A) GPI, (B) IDH, (C) ME, and (D) PGM. Lanes 2 and 3 show strains isolated from 2 xenodiagnosis triatomine bugs from a single patient (typical patterns of zymodemes 39 and 20, respectively). Lanes 1 show stock isolated from a single xenodiagnosis triatomine bug from a single patient (typical pattern of a mixture of zymodemes 39 and 20).

tients infected either in Cochabamba or Sucre. Samples from others cities were too small for comparisons. Among the patients who presented with gastrointestinal changes, 42.2% had a severe megacolon (Grade III), 50.3% had a moderate megacolon (Grade II), and 7.5% had a megacolon with minor changes (Grade I). The different frequencies of cardiac abnormalities were as follows: Bradycardia, 63.8%; right branch block, 50%; left anterior hemiblock, 38.8%; ventricular premature beat, 8.3%; and incomplete right bundle branch block, ischemia, and atrial fibrillation, 2.7% each. Of these patients, 36% presented with 2 associated cardiac abnormalities, while 16.6% presented with 3 associated abnormalities. Two patients had pacemaker devices related to Chagas cardiopathy.

Zymodeme variability of T. cruzi *strains isolated from humans*

T. cruzi zymodemes identified among strains isolated from 89 Bolivian patients are presented in Table 3. Eleven patients from this sample (12.3%) presented with mixed infections of 2 different zymodemes. These existed within 2 different sets of circumstances: a typical mixed isoenzyme pattern (which could be checked for 4 enzyme systems) of 1 stock isolated from 1 xenodiagnosis triatomine bug from a single patient, and 2 different isoenzymic patterns in 2 strains from 2 xenodiagnosis triatomine bugs from a single patient (Fig. 1). In the present sample, 9 different zymodemes (the genotypes of which are presented in Table 4) were identified. Seven zym-

TABLE 4

Genotypes of Bolivian zymodemes isolated from humans identified by assaying 12 isozyme loci

Zymodeme*	Locus and genotype										
	G6pd	*Gpi*	*Gdh-1*	*Gdh-2*	*Idh*	*Me-1*	*Me-2*	*Pep-1*	*Pep-2*	*Pgm*	*6pgd*
NP1†	5/5	5/5	3/3	2/2	1/1	2/2	4/4	3/3	1/1	3/3	4/4
7	6/6	5/5	3/3	2/2	1/1	2/2	4/4	1/1	1/1	3/3	4/4
19	5/5	5/5	3/3	2/2	1/1	2/2	4/4	1/1	1/1	3/3	4/4
20	5/5	5/5	3/3	2/2	1/1	2/2	4/4	1/1	1/1	3/3	2/4
32	2/2	3/3	2/2	5/5	2/2	2/2	6/6	5/5	1/1	10/12	1/1
39	4/4	2/4	1/1	1/1	2/2	1/1	5/5	5/5	1/1	6/10	1/4
40	4/4	3/4	1/1	1/1	2/2	1/1	5/5	4/4	1/1	4/11	1/4
43	3/3	3/4	2/2	4/4	2/2	1/1	5/5	4/4	1/1	4/11	1/4
NP2	6/6	5/5	3/3	4/4	1/1	3/3	4/4	—‡	1/1	4/4	2/4

* Zymodeme numbering according to Tibayrenc and others.[8]
† This zymodeme differed from zymodeme 2 by 1 locus: genotype *5/5* for *G6pd* instead of *6/6*.
‡ New electrophoretic position located between bands corresponding to genotypes *1/1* and *2/2*.
NP = new profile.
For each locus, allele 1 codes for the fastest electromorph. Mdh is not listed because it is monomorphic within this sample (genotype *2/2*).

odemes had been previously described,[8] and 2 had never been observed before; 1 of the latter appeared to be closely related to zymodeme 2,[8] with 2 allelic differences out of 24 alleles distinguishable using 12 enzyme loci. The other zymodeme appeared to be related to zymodeme 6[8] (6 allelic differences out of 24 alleles) (Table 4). We do not give any numbers to these new zymodemes, since their phylogenic relationship to those formerly described have to be assayed for a broader range of isozyme loci. In this sample, the most frequent zymodemes were 19, 20, and 39,[8] which were already recorded in triatomine bugs sampled from various regions of Bolivia (Cochabamba, Sucre, Santa-Cruz),[12] and which have been observed in wide geographical ranges in South America.[7,8]

The genetic and patristic[8,10,11] (or evolution-

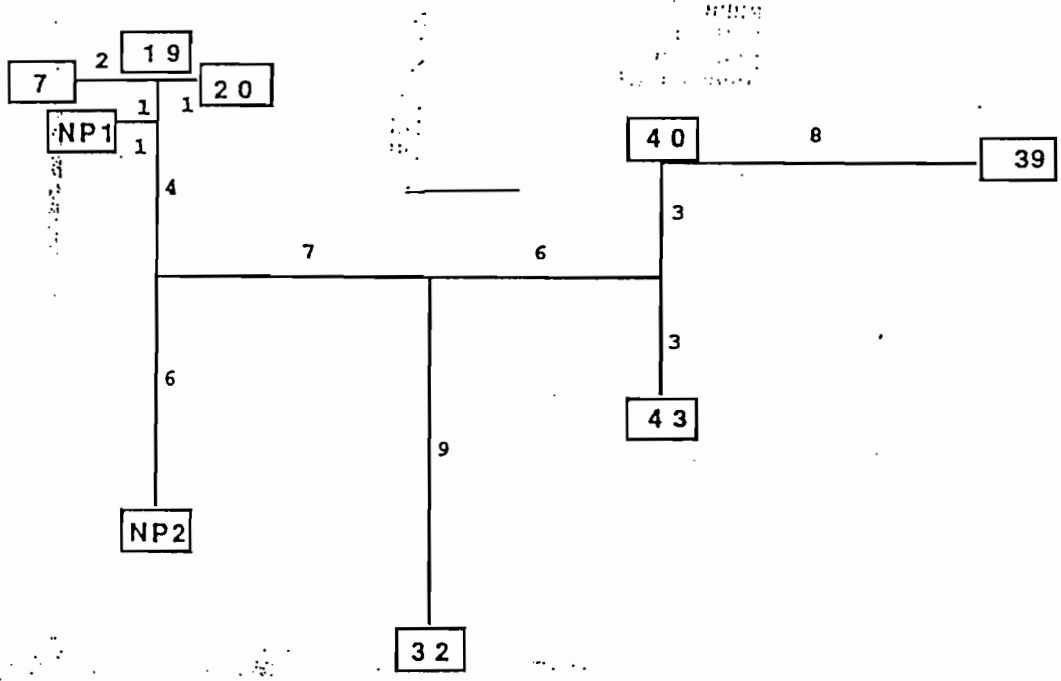

FIGURE 2. A minimum-length unrooted Wagner network of 51 steps linking the 9 zymodemes of *T. cruzi* identified in the present study. The boxed numbers correspond to the zymodemes, according to the numbering of Tibayrenc and others.[8] The numbers along the branches are the patristic (or evolutionary) distances among the zymodemes.

TABLE 5

Matrix of patristic (above the diagonal) and genetic (below the diagonal) distances among 9 T. cruzi zymodemes isolated from 89 Bolivian patients using 12 isoenzyme loci

	Zymodeme								
Zymodeme	NP1	7	19	20	32	39	40	43	NP2
NP1	—	4	2	3	21	29	21	21	11
7	0.18	—	2	3	23	31	23	23	13
19	0.08	0.08	—	1	21	29	21	21	11
20	0.13	0.13	0.02	—	24	30	22	22	12
32	1.36	1.36	1.36	1.34	—	26	18	18	22
39	1.60	1.60	1.60	1.58	0.89	—	8	14	30
40	1.60	1.60	1.60	1.58	1.14	0.18	—	6	22
43	1.60	1.60	1.60	1.58	1.14	0.60	0.37	—	22
NP2	0.54	0.39	0.54	0.52	1.65	1.49	1.29	0.98	—

ary) distances observed among zymodemes are given in Table 5. The Wagner network[8,10,11] depicting their phylogenic relationships is shown in Figure 2.

Zymodemes and pathologies

Among patients presenting with a given anatomic picture and infected by only 1 zymodeme, a statistical analysis was carried out for checking the possible association between anatomic changes and the more frequent *T. cruzi* zymodemes found in our study: we plotted into 1 group those patients infected with either zymodemes 7, 19, or 20, since these zymodemes are closely related to each other.[8] The second group was composed of patients infected with zymodeme 39, which is radically dissimilar from zymodemes 7, 19, and 20.[8] The other zymodemes were scarcely sampled, which made their use for statistical analyses impossible. Table 6 summarizes the pathologies observed in the 2 groups of patients defined above.

Several patients from these 2 different sets pre-

TABLE 6

Numbers of patients infected by given zymodemes by clinical form

			Zymodemes‡	
Clinical groups	ECG abnormalities*	Megacolon†	7, 19, or 20	39
Asymptomatic (n = 11)	N	N	6	5
Cardiac (n = 10)	B	N	2	1
	LAH	N	1	1
	VPB	N	0	1
	RBBB	N	1	0
	LAH + B	N	0	1
	RBBB + B	N	1	0
	RBBB + LAH	N	1	0
Megacolon (n = 6)	N	DI	1	0
	N	DII	3	1
	N	DIII	1	0
Associated (n = 3)	B	DII	1	0
	RBBB + AF + VPB	MI, DIII	1	0
	RBBB + LAH	DII	1	3
Not classified (n = 7)	N	not done	3	2
	RBBB	not done	1	0
	AVB + B	not done	0	1
Total (n = 37)			24	16

* N = normal, B = bradycardia, LAH = left anterior hemiblock, VPB = ventricular premature beat, RBBB = right bundle branch block, AF = atrial fibrillation, and AVB = auriculo ventricular block.
† DI, DII, DIII, MI, MII, and MIII = abnormal length or enlargement of the colon according to the degree of hypertrophy.
‡ Zymodeme numbering according Tibayrenc and others.[8]

sented a cardiac and/or digestive pathology. Both groups of zymodemes were found significantly pathogenic (χ^2 test with the null hypothesis being that the zymodemes are non-pathogenic. For patients infected by zymodemes 7, 19, or 20, $\chi^2 = 23.33$, $P < 10^{-3}$, and df = 1. For patients infected by zymodeme 39, $\chi^2 = 9.5$, $P < 10^{-2}$, df = 1. We did not find any statistical differences between the 2 groups regarding the alternative presence/absence of pathogenicity ($\chi^2 = 0.19$, $P > 0.30$, df = 1). Numbers of patients from each clinical group were too small to allow a statistical study of a possible specific association between a given zymodeme and the existence of either cardiopathy or a megacolon.

DISCUSSION

Clinical and serological features of Chagas' disease in Bolivia

The high rates of positive serology observed in the 3 endemic areas studied clearly shows the extensive distribution of Chagas' disease in Bolivia. We observed important differences in the index of serology between Chiwisivi and Salinas, and between the rates of cardiopathy in the 3 areas. The different anatomic changes observed in Bolivian patients have already been reported in the framework of Chagas' disease; however, we observed a particularly low frequency of megaoesophagus in this country compared to the frequencies observed in endemic areas of Bahia, Goias,[13,14] and Minas Gerais.[15] These results confirm the heterogenity of the different anatomic changes seen in Chagas' disease endemic areas.[1]

Zymodeme variability: human hosts from Bolivia

Extensive isoenzyme studies of *T. cruzi* isolated from *T. infestans*, the main domestic vector in Bolivia, have been done.[12] We report zymodeme variability among strains isolated from humans in the same country. Our results show that the zymodeme variability is comparable among strains isolated from triatomine insects[12] and from humans. If we compare strains isolated from triatomine insects with strains isolated from humans in the areas of Cochabamba, Sucre, and Santa-Cruz, the numbers of strains pertaining to zymodemes 19 and 20 and to zymodeme 39 are not significantly different ($\chi^2 = 2.22$, df = 1, $P > 0.05$). This strongly suggests a lack of selection, either by humans or by triatomine bugs, for any of the 3 zymodemes.

Genetic and patristic distances calculated between the zymodemes studied here, which represent a geographically limited sample, reveal high genetic variability (Table 5). Clustering the zymodemes into a few, strictly separated subgroups was impossible (Fig. 2). This confirms previous data.[8]

Close sympatrical circulation of Bolivian zymodemes

The existence of mixtures of ≥2 different zymodemes has been reported from Bolivia in triatomine vectors as well as in humans.[6,12,16] We report here new cases of mixed human infections and describe several cases of double infections with zymodemes genetically unrelated[8] (20 and 39, 40 and 20) or, in contrast, closely related to each other[8] (20 and 7). Thus, mixed infections appear to be common in Bolivia. This phenomenon has not been noted neither in Brazilian nor in Chilean endemic areas.[17,18] This may be because other authors have used different sampling, culture, and electrophoresis techniques. Tibayrenc and others, using techniques comparable to the present ones, have described cases of mixed strains in Chilean samples.[7,8]

Zymodemes and pathology

Serological and clinical data of the present study have shown important differences between the Chiwisivi area and the Camiri and Salinas areas, which are only 50 km apart. It is worth noting that previous studies have identified very different zymodeme variability in the Chiwisivi and Camiri regions. Of 141 strains of *T. cruzi* isolated from *T. infestans* in Chiwisivi, 99% presented a similar isozyme profile corresponding to zymodeme 9. In Camiri, a much higher range of genetic variability has been recorded: 5 different zymodemes from only 15 strains isolated from *T. infestans* (Table 1).[12] This observation is consistent with the working hypothesis of specific pathogenic properties of the zymodemes, but has of course to be examined on a broader scale.

In view of the clinical diversity of Chagas' disease and the presence in Bolivia of 2 radically dissimilar, widespread zymodemes, we decided to check for a possible statistical association be-

tween pathologies and zymodemes. Such an association was impossible to evidence, which confirms our previous results.[16] Moreover, the 3 more frequent zymodemes (19, 20, and 39) did not show significant differences in their pathogenicity, and have all been found in patients presenting cardiac or gastrointestinal (megacolon) or associated alterations. Although the present sample is too small for statistical analysis of a possible association between zymodeme variability and specific pathological changes, these results suggest an absence of strict association. Nevertheless, they do not rule out the hypothesis of zymodeme pathogenic specificity. Indeed, several methodological difficulties could explain our negative results. For instance, xenodiagnosis, culture, and host immune response could act as "filters," which could select given parasite subpopulations from a mixed infection, in which case the parasite population studied by isozymes would not be a true picture of the 1 from the patient. Furthermore, mixed infections of at least 2 different zymodemes are very frequent in Bolivia (Table 3) and are probably heavily underestimated, which means that human Chagas' disease in this country is definitely not a pure model for studying the problem of zymodeme pathogenic specificity.

The question of the clinical consequences of *T. cruzi* genetic variability is still an unanswered one. In natural cycles, valuable circumstantial evidence could come from extensive comparisons of ecologically similar regions, each harboring a different type of zymodeme. To avoid biases due to culture and xenodiagnosis, zymodeme specific DNA probes would be extremely valuable. Such studies should be compared to experimental works on animals involving statistically representative samples of cloned strains pertaining to various zymodemes.

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Antigenic Specificity of the 72-Kilodalton Major Surface Glycoprotein of *Leishmania braziliensis braziliensis*

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We examined the expression and the antigenicity of the major surface polypeptides of *Leishmania braziliensis braziliensis* and *Leishmania donovani chagasi*, parasites which commonly coexist in the same endemic areas of Bolivia. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis protein profiles from surface-iodinated promastigotes showed the presence of a unique iodinatable polypeptide of 72 kDa on the *L. b. braziliensis* surface and of two major components of 65 and 50 kDa exposed at the surface of *L. d. chagasi*. Comparison of the peptide digestion profiles of the major iodinated polypeptides of both strains showed no similarity between the maps of the 72- and the 65-kDa polypeptides of *L. b. braziliensis* and *L. d. chagasi*, respectively. Immunoprecipitation of surface-labeled *L. b. braziliensis* Nonidet P-40 extracts with 35 serum specimens obtained from Bolivian patients with cutaneous and mucocutaneous leishmaniasis showed that all serum specimens recognized predominantly the 72-kDa antigen and high-molecular-mass proteins in some cases. The recognition patterns were independent of the geographical origin of the patient, the type of lesion, and the serum antibody titer. Serum specimens from children with visceral leishmaniasis did not precipitate the *L. b. braziliensis* 72-kDa antigen. Hamster hyperimmune serum against *L. b. braziliensis* also recognized the 72-kDa surface antigen. However, this recognition was inhibited in the presence of the homologous nonlabeled antigen but not in the presence of heterologous (*L. d. chagasi* and *Trypanosoma cruzi*) antigens. The specific recognition of the 72-kDa surface antigen in both natural and experimental *L. b. braziliensis* infections suggests that this antigen could be a good candidate for use in the differential immunodiagnosis and prognosis of the disease.

Human leishmaniasis includes a group of diseases with different clinical manifestations: cutaneous, mucocutaneous, and visceral. These diseases occur throughout the world and, in endemic tropical areas, represent a great public health problem (6, 20). The etiological agents of leishmaniasis include many complex and epidemiologically diverse species from the genus *Leishmania* (16).

In South America, particularly in Bolivia, the species of *Leishmania* present are mainly *Leishmania braziliensis braziliensis* and *Leishmania donovani chagasi*; they are the causative agents of human mucocutaneous and visceral leishmaniasis, respectively (19). Both types of leishmaniasis are frequently endemic in the same areas of the country (i.e., the Yungas valleys, Department of La Paz), rendering epidemiological studies difficult (7, 8).

During the past few years, particular emphasis has been given to characterization of antigenic components on the promastigote surface as a tool in obtaining specific *Leishmania* antigens for parasite identification as well as for specific diagnosis and immunoprophylaxis. Several promastigote cell surface antigens have been well characterized. Among them, the promastigote surface protease, a predominant surface glycoprotein (gp63) with an apparent molecular mass of 63 to 65 kDa, was identified as a highly conserved and cross-reactive antigen shared by most of the *Leishmania* species tested so far (1–3, 5, 10, 11, 18). However, Legrand and coworkers (17) recently demonstrated the presence of a polypeptide of 72 kDa as the predominant surface antigen in 12 different isolates of *L. b. braziliensis* (10 local Bolivian strains isolated from patients or sandflies and 2 Brazilian reference strains) instead of the gp63-gp65 major surface antigens present in other Old and New World *Leishmania* species, including *L. d. chagasi*.

Based on the findings described above, we performed a comparative study of the major surface polypeptides of *L. b. braziliensis* and *L. d. chagasi* and analyzed their antigenic properties in experimental and natural infections.

MATERIALS AND METHODS

Parasites and culture conditions. Promastigotes of an *L. b. braziliensis* reference strain (MHOM/BR/75/M-2903) and an *L. d. chagasi* reference strain (MHOM/BR/74/M-2682) were grown at 28°C in Schneider *Drosophila* medium (GIBCO Bio-Cult, Paisley, United Kingdom) supplemented with 15% (vol/vol) heat-inactivated fetal bovine serum (Serva, Heidelberg, Federal Republic of Germany).

Human and hamster sera. Serum specimens from 35 Bolivian patients with cutaneous and mucocutaneous leishmaniasis were collected in our laboratory. All the patients were clinically diagnosed and exhibited a positive skin test reaction, and their sera showed positive serology for *L. b. braziliensis*, as detected by immunofluorescence (in most cases, the antibody titers were very low, in the range of 1/20 to 1/40, and in some cases they were negative) (9). In some cases the parasite was isolated from the lesions and characterized as *L. b. braziliensis* by isoenzyme electrophoresis (7). In addition, serum specimens from two Bolivian patients

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with visceral leishmaniasis infected with *L. d. chagasi* were used (8).

Hamster hyperimmune antiserum against *L. b. braziliensis* was collected 1 year after footpad inoculation of hamsters with viable promastigotes of the strain MHOM/BO/84/LPZ-688.

Surface iodination and detergent solubilization of proteins. Late-log-phase promastigotes (2×10^8) were surface iodinated with 200 μCi of carrier-free $Na^{125}I$ (ORIS, Gif-sur-Yvette, France) and Iodo-Gen (1,3,4,6-tetrachloro-3α,6α-diphenylglycouril; Sigma) as described by Legrand et al. (17). After iodination and washing of the unbound ^{125}I, the cell pellets were extracted for 2 h at 4°C with 200 μl of 0.5% Nonidet P-40 in 20 mM Tris (pH 7.4)–300 mM NaCl containing aprotinin (100 U/ml). The detergent-insoluble material was removed by centrifugation, and lysates were stored at −70°C until use.

Gel electrophoresis and autoradiography. Proteins were separated by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) on slab gels containing 7.5% polyacrylamide (15). After staining of the proteins with Coomassie blue, the gels were dried and autoradiographed at −70°C with X-OPMAT AR film (Eastman Kodak, Rochester, N.Y.) in conjunction with Cronex intensifying screens (E. I. du Pont de Nemours & Co., Inc., Wilmington, Del.). Bands in the autoradiograms were quantified by densitometry (Hoefer Scientific Instruments, San Francisco, Calif.).

Peptide mapping. Partial proteolytic maps of the major surface-iodinated proteins were performed as described by Cleveland et al. (4). Proteins were separated by SDS-PAGE on 7.5% polyacrylamide gels. After electrophoresis, gel pieces containing the major iodinated proteins were excised and run on a second 12.5% polyacrylamide gel with sample buffer containing *Staphylococcus aureus* V8 protease (1 to 50 μg/ml; Boehringer, Mannheim, Federal Republic of Germany). The products of partial proteolysis were revealed by autoradiography.

Immunoprecipitation and immunocompetition. Immunoprecipitation was carried out by the method of Kessler (14). A detergent extract of labeled promastigotes (10 μl; 10^7 cells) was diluted in 450 μl of 10 mM Tris (pH 8)–150 mM NaCl–2 mM EDTA–0.5% (vol/vol) Nonidet P-40–100 U of aprotinin per ml and incubated with 50 μl of serum for 1 h at room temperature with constant agitation. Immune complexes were absorbed for 1 h with 5 mg of protein A–Sepharose 4B-CL (Pharmacia, Uppsala, Sweden) and suspended in the buffer described above. Absorbed antigens were solubilized in 20 μl of sample buffer and stored at −20°C for SDS-PAGE.

For immunocompetition experiments, 10 μl of the labeled extract was incubated with the serum in the presence of 50 μl of homologous or heterologous nonlabeled antigen extract and processed for absorption with protein A–Sepharose as described above.

RESULTS

Identification and peptide mapping analysis of surface-labeled *L. b. braziliensis* and *L. d. chagasi* polypeptides. *L. b. braziliensis* and *L. d. chagasi* promastigotes were surface iodinated and extracted with Nonidet P-40, and the proteins were resolved by SDS-PAGE. The autoradiographic profiles revealed a unique surface-labeled polypeptide of 72 kDa in the *L. b. braziliensis* extract (Fig. 1, lane 1) and two major labeled proteins of 65 and 50 kDa in the *L. d. chagasi* extract (Fig. 1, lane 2).

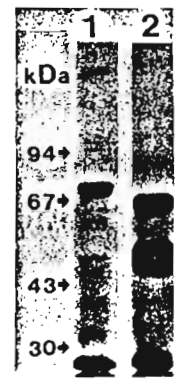

FIG. 1. Autoradiographic patterns of iodinated surface proteins of *L. b. braziliensis* and *L. d. chagasi* promastigotes. Samples of radioiodinated *L. b. braziliensis* and *L. d. chagasi* were analyzed by SDS-PAGE followed by autoradiography. A total of 2×10^7 promastigotes was loaded into each slot. Lane 1, *L. b. braziliensis*; lane 2, *L. d. chagasi*. The arrows indicate the migration of the protein standards.

In order to determine a structural relationship between the 72- and 65-kDa proteins of *L. b. braziliensis* and *L. d. chagasi*, peptide mapping analysis of both polypeptides was carried out (Fig. 2). Peptide digestion profiles of the 72- and 65-kDa proteins with the V8 protease of *S. aureus* showed that both proteins were sensitive to digestion by the enzyme; however, the overall patterns obtained were significantly different. No similarity was found in the apparent molecular weights of any of the partial proteolysis products. Distinct peptide patterns were also obtained upon cleavage of the proteins at tryptophan by using *N*-chlorosuccinimide (to be published elsewhere). These results might indicate that the 72- and 65-kDa polypeptides of *L. b. braziliensis* and *L. d. chagasi* are not structurally related.

Antigenic recognition of major surface polypeptides in experimental *L. b. braziliensis* infection. Hamster hyperimmune serum to *L. b. braziliensis* was used to precipitate labeled *L. b. braziliensis* and *L. d. chagasi* surface antigens from parasite extracts. SDS-PAGE immunoprecipitation patterns showed that the hamster serum recognized strongly and specifically the 72-kDa protein expressed on the surface

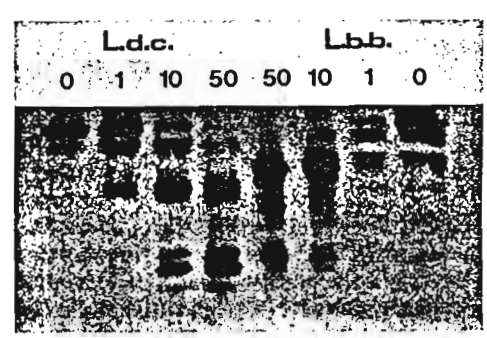

FIG. 2. Peptide mapping of the major *L. b. braziliensis* (L.b.b.) and *L. d. chagasi* (L.d.c.) surface polypeptides. Labeled 72- and 65-kDa polypeptides were excised from the gel shown in Fig. 1 and submitted to proteolytic digestion with different concentrations of *S. aureus* V8 protease (numbers above the lanes indicate the protease concentration, in micrograms per milliliter). The products of partial proteolysis were separated by SDS-PAGE and were autoradiographed.

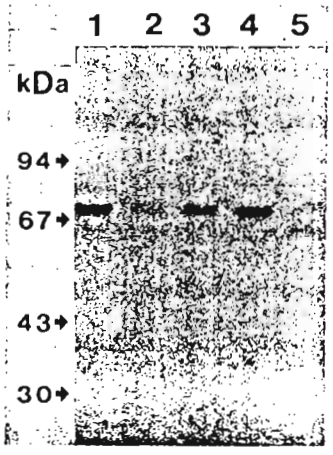

FIG. 3. Immunoprecipitation of *L. b. braziliensis* and *L. d. chagasi* surface proteins with hamster hyperimmune serum and immunocompetition. Nonidet P-40 extracts from surface-iodinated promastigotes were reacted with serum from an *L. b. braziliensis*-infected hamster, and the precipitated proteins were resolved by SDS-PAGE and autoradiographed. Lanes 1 and 5, precipitation patterns of radioiodinated *L. b. braziliensis* and *L. d. chagasi* extracts, respectively; lanes 2 to 4, immunocompetition-precipitation of radioiodinated *L. b. braziliensis* extract with the anti-*L. b. braziliensis* serum in the presence of either homologous *L. b. braziliensis* (lane 2) or heterologous *L. d. chagasi* (lane 3) and *T. cruzi* (lane 4) nonlabeled extracts.

of *L. b. braziliensis* promastigotes (Fig. 3, lane 1). However, hamster serum recognized very poorly (less than 20%) the 65-kDa surface polypeptide of *L. d. chagasi* (Fig. 3, lane 5).

In order to assess the degree of cross-reactivity between the major surface antigens of these two *Leishmania* subspecies, immunocompetition experiments were carried out. Immunoprecipitation of labeled *L. b. braziliensis* Nonidet P-40 extracts with the hamster serum was performed in the presence of nonlabeled *L. b. braziliensis* or *L. d. chagasi* antigen extracts. A *Trypanosoma cruzi* antigen extract was also tested in the competition experiments because Chagas'

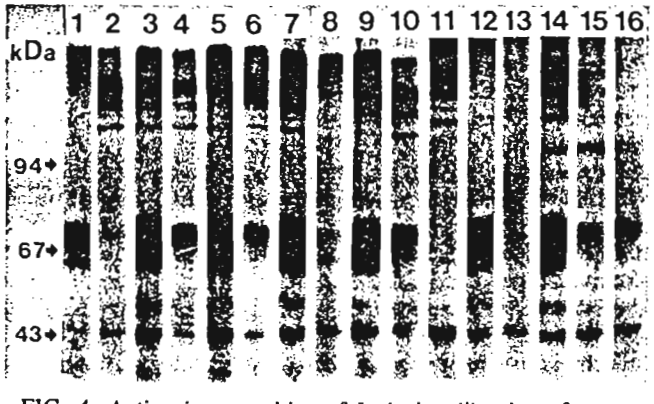

FIG. 4. Antigenic recognition of *L. b. braziliensis* surface proteins by sera from Bolivian patients with cutaneous and mucocutaneous leishmaniasis. Immunoprecipitation patterns of iodinated *L. b. braziliensis* surface proteins precipitated with different sera after SDS-PAGE and autoradiography. Lanes 1 to 12 and 14 to 16, *L. b. braziliensis* extracts; lane 13, *L. d. chagasi* extract. The descriptions of the different serum specimens are given in Table 1 (serum specimen numbers in Table 1 correspond to lane numbers).

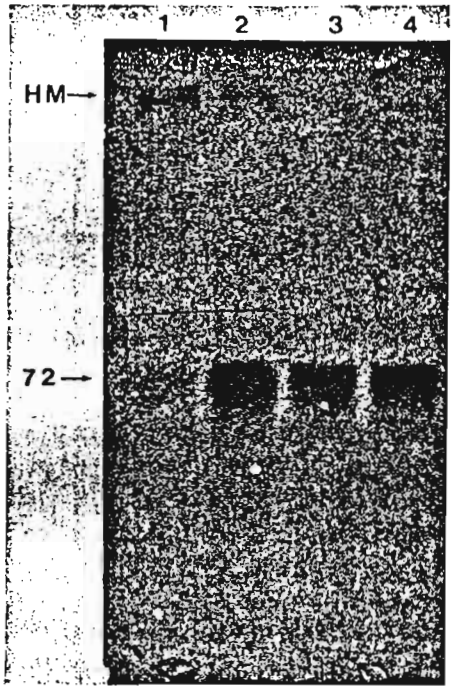

FIG. 5. Antigenic recognition of *L. b. braziliensis* surface proteins during the course of infection of a single patient. Extracts from surface-iodinated *L. b. braziliensis* were immunoprecipitated with sera from a patient collected at different times during the course of an *L. b. braziliensis* infection. Lane 1, Immunoprecipitation with serum collected at the beginning of the infection (primary lesion in November 1984); lane 2, serum collected after Glucantime treatment (March 1985); lane 3, serum collected 1 year after treatment; lane 4, serum collected at the beginning of a mucous lesion (February 1988). HM, High-molecular-mass antigens; 72, the 72-kDa major surface antigen.

disease is frequently coendemic with mucocutaneous and visceral leishmaniasis in Bolivia. Up to 92% inhibition was obtained when the precipitation was carried out in the presence of the homologous *L. b. braziliensis* antigen (Fig. 3, lane 2). However, only a slight inhibition was observed when heterologous *L. d. chagasi* and *T. cruzi* antigens were used (24 and 13%, respectively) (Fig. 3, lanes 3 and 4). The inability of the heterologous antigens to fully inhibit the precipitation reaction suggested that there is a low degree of cross-reactivity between them and the 72-kDa surface antigen of *L. b. braziliensis*.

Antigenic recognition of the surface antigens in natural *L. b. braziliensis* infections. Serum specimens from 35 *L. b. braziliensis*-infected patients presenting cutaneous or mucous lesions and originating from different endemic areas of Bolivia were chosen arbitrarily. These serum specimens were used to immunoprecipitate iodinated *L. b. braziliensis* extracts, and the results for 16 representative samples are shown in Fig. 4. Up to 95% of the serum specimens recognized the 72-kDa antigen of *L. b. braziliensis*, and some of them revealed additional high-molecular-mass components (lanes 1 to 12 and 14 to 16). Serum from a patient with visceral leishmaniasis did not recognize any of the labeled components on the *L. b. braziliensis* surface (Fig. 4, lane 13).

No apparent correlation could be found between the different patterns of recognition obtained, on the one hand, and the geographical origin of the patient, the clinical man-

TABLE 1. Recognition of *L. b. braziliensis* antigens by sera from Bolivian patients with cutaneous and mucocutaneous leishmaniasis

Serum specimen no.	Geographical area	Type of leishmaniasis	Antibody titer	Antigen recognition	
				72-kDa antigen	High-molecular-mass antigen
1	San Borja (Beni)	Cutaneous	1/40	++	–
2	San Borja (Beni)	Cutaneous	1/40	+	+++
3	Alto Beni	Cutaneous	1/20	+++	+++
4	San Borja (Beni)	Cutaneous	1/40	++	++
5	Alto Beni	Cutaneous	1/40	+++	+++
6	Chapare	Cutaneous	1/20	+	+++
7	Ixiamas (Alto Beni)	Cutaneous	Negative	+++	+++
8	Chulumani (Yungas)	Mucocutaneous	1/20	+	–
9	Chapare	Cutaneous[a]	1/80	+++	+++
10	Chapare	Cutaneous[a]	1/40	++	++
11	Alto Beni	Cutaneous	1/40	–	+++
12	Caranavi (Yungas)	Mucocutaneous	1/40	+++	–
13	Yungas	Visceral	1/80	–	–
14	Coroico (Yungas)	Mucocutaneous	1/80	+++	+++
15	Quillabamba (Peru)	Mucocutaneous	1/40	+	+
16	Yungas	Mucocutaneous	1/80	+	–

[a] Patients with associated Chagas' disease.

ifestation of the disease, and the antibody titer of the sera, on the other (Table 1). Nevertheless, a correlation between the immune recognition of the 72-kDa antigen and the progress of the infection could be evaluated. This was carried out by using sera from a patient infected with *L. b. braziliensis* who was monitored from the beginning of the infection until the appearance of the mucous lesion (Glucantime [meglumine antimoniate] was used to treat the patient, but it did not cure the patient; there was a low antibody titer [1/20]). SDS-PAGE immunoprecipitation profiles of *L. b. braziliensis* extract reacted with the patient sera, which were collected at various stages of the disease (Fig. 5), showed that at the beginning of the infection, at the time of the appearance of the primary lesion, the serum recognized mostly the high-molecular-mass components and, to some extent, the 72-kDa antigen (Fig. 5, lane 1). With the progression of the infection (Fig. 5, lanes 2 and 3), recognition of the 72-kDa antigen increased, while, inversely, that of the high-molecular-mass antigen decreased until the mucous lesion became manifested (Fig. 5, lane 4).

DISCUSSION

The expression of a 72-kDa antigen on the surface of *L. b. braziliensis* promastigotes was previously reported by Legrand and coworkers (17) in a comparative study with 12 different *L. b. braziliensis* isolates. Other New World *Leishmania* species belonging to the same *L. b. braziliensis* complex (*L. braziliensis panamensis* and *L. braziliensis guyanensis*) or to other complexes (*L. m. amazonensis* and *L. d. chagasi*) were shown to express a 63- to 65-kDa polypeptide on their surfaces, as demonstrated by surface radioiodination and immunoprecipitation protein profiles (2).

In the present study we extended the studies described above and structurally compared, by peptide mapping analysis, the major 72- and 65-kDa polypeptides expressed on the surface of *L. b. braziliensis* and *L. d. chagasi*, respectively. Our results demonstrated that these polypeptides not only differ in their molecular masses but are structurally different, inasmuch as no homology could be observed in their peptide digestion profiles. This result is in disagreement with those previously reported by other investigators (5, 10) regarding the structural homology of the major surface antigens from various *Leishmania* strains, including *L. b. braziliensis*. Further sequencing of these proteins is required in order to fully prove structural differences.

In the same work, Legrand and coworkers (17) showed that the *L. b. braziliensis* 72-kDa major surface antigen was not recognized by sera from patients with visceral leishmaniasis or Chagas' disease, although recognition of this antigen was achieved by serum from a patient with mucocutaneous leishmaniasis and by serum from a hamster infected with *L. b. braziliensis*.

In this study we demonstrated that specific recognition of the *L. b. braziliensis* 72-kDa antigen is a property of the majority of the serum specimens from 35 Bolivian patients naturally infected with *L. b. braziliensis*. Moreover, the structural differences we observed in the present study correlated with the antigenic recognition obtained both by sera from hamsters with experimental infections and by patient sera. In either case, the 72-kDa antigen was demonstrated to be highly immunogenic and specifically recognized by the serum specimens from hamsters experimentally infected and from patients with *L. b. braziliensis* but not by the serum specimens from patients with visceral leishmaniasis. However, under the same experimental conditions the hamster sera failed to recognize the 65-kDa antigen of *L. d. chagasi*. Those results, together with the inability of the *L. d. chagasi* and *T. cruzi* antigens to fully inhibit the 72-kDa antigen-antibody recognition, demonstrate a low level of cross-reactivity and might suggest an antigenic specificity of the 72-kDa polypeptide expressed on the surface of *L. b. braziliensis*.

The heterogeneity in *Leishmania* cell surface antigens has been postulated by several groups of investigators (2, 12, 13). The need to study those antigens in more depth has been stressed, since their expression may differ among the various *Leishmania* species. This is particularly true if we take into consideration the diversity of *Leishmania* strains, the variety of vectors, the geographical habitats, the reservoir hosts, the different tissue tropisms, and the various clinical manifestations of the disease. Even though the existence of

common antigens among *Leishmania* species could help in designing immunoprophylactic protocols against the disease, the characterization of species- and subspecies-specific antigens such as the 72-kDa antigen of *L. b. braziliensis* may be important for both taxonomic studies and practical identification techniques. Such a specific antigen can also represent a good candidate for the differential diagnosis and prognosis of mucocutaneous leishmaniasis in endemic areas where various types of *Leishmania* and other protozoan parasites coexist.

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5 - VARIABILITE GENETIQUE DES SOUCHES DE LEISHMANIES ET DE *TRYPANOSOMA BRUCEI* SP.

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Parallel evolution between kDNA and nuclear markers in *Leishmania* genus

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Key words : kDNA, taxonomy, DNA polymorphism, *Leishmania*.

Quantified kDNA RFLP analysis of diversified sample of Old World *Leishmania* previously characterized using 15 isoenzyme loci show highly significant correlation between these two sets of genetical markers. Moreover, the data involving maxicircle as well as minicircle variabilities are correlated together too. These results clearly show that kinetoplastic and nuclear genomes do not evolve independently in Old world *Leishmania*, and that, if some recombination occurs in the kinetoplast, it is not sufficient to to break the general pattern of linked evolution between the two genomes on the evolutionary scale. This fact has important consequences for stock typing and strain specific kDNA probe designing.

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IDENTIFICATION OF *TRYPANOSOMA BRUCEI GAMBIENSE* GROUP I BY A SPECIFIC KINETOPLAST DNA PROBE

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Abstract. A set of 26 *Trypanosoma brucei* stocks from various African countries, previously characterized by multilocus enzyme electrophoresis (MLEE) for 18 polymorphic loci, have been selected to be representative of the three *T. brucei* classic subspecies. The kinetoplast DNA minicircle variable regions from these stocks have been amplified using the polymerase chain reaction (PCR) technique, and hybridized with the amplified variable regions of three *T. brucei* reference stocks, previously identified as *T. brucei brucei*, *T. brucei gambiense*, and *T. brucei rhodesiense*, respectively. Both *T. b. brucei* and *T. b. rhodesiense* probes hybridized only with their own stocks, but the *T. b. gambiense* probe specifically hybridized with a group of 12 stocks that represented most of the human stocks from West and Central Africa in our sample. These stocks, which appeared as a clearly separable cluster based on previous MLEE analysis, probably correspond to *T. brucei gambiense* group I. No other stock hybridized with this amplified fragment. Since the *T. b. gambiense* probe obtained is specific for many isolates that are pathogenic for humans in Central and West Africa, it appears to be a promising tool for epidemiologic and medical surveys.

The kinetoplast DNA of *Trypanosoma brucei s.l.*, the agent of human sleeping sickness and of a cattle disease in Africa, is composed of a network of maxicircles and minicircles. The one-kilobase minicircles (approximately 10,000 copies in a single cell), contain a 122-basepair (bp) sequence that appears to be conserved within the whole taxon *T. brucei*.[1] These minicircles also contain a nonconserved sequence (hypervariable region or HVR), which seems to be very heterogeneous within a single organism. Indeed, 200–300 different sequence classes of variable frequencies can be found within the same cell.[2] The HVR of *T. cruzi* was successfully used as a target sequence to design probes[3,4] that are specific for the natural clones of this parasite previously demonstrated by a population genetics approach.[5] The purpose of the present study was to use a similar approach to design specific markers for the *T. brucei s.l.* genotypes and clusters of genotypes characterized by a previous population genetics study.[6]

MATERIALS AND METHODS

Parasite sampling

Twenty-six *T. brucei* stocks isolated from various hosts (humans, wild and domestic mammals, tsetse flies) in Central, West, and East Africa were analyzed. The origin of these stocks is summarized in Table 1. They were selected to represent the three classic subspecies of *T. brucei*, namely *T. b. brucei*, *T. b. gambiense*, and *T. b. rhodesiense*. This subspecific identification was performed by other investigators (see references ~~in Table 1~~).

Parasite culturing

Trypanosomes were grown by culturing procyclic forms in Cunningham's medium containing 20% fetal calf serum and 20 μg/ml of gentamicin.[7]

Polymerase chain reaction

The primers, obtained from Centre de Recherche en Biologie et Genetique Cellulaire (Toulouse, France), were selected to anneal DNA sequence at both ends of the 122-bp conserved region of the kinetoplast DNA (kDNA) minicircle described by Chen and Donelson.[1] A restriction site (*Msp* I) was artificially introduced at the 3'-end of each oligonucleotide to facilitate the subsequent isolation of the amplified variable sequence. The sequences of the primers were as

TABLE 1

Trypanosoma brucei *stocks used in the study*

Stock (World Health organization identification)	Host	Country	Year	References*	Subspecies identification and references†
MKOK/BF/80/KK 39	Waterbuck	Burkina Faso	1980	20, 21	
MHOM/CM/74/MOS	Human	Cameroon	1974	21–25	Tbg; 21–24
MHOM/CM/75/BIM	Human	Cameroon	1975	22–25	Tbg; 22–24
MHOM/CM/88/A 004	Human	Cameroon	1988	21	Tbg; 21
MHOM/CM/88/B 014	Human	Cameroon	1988	21	Tbg; 21
MHOM/CM/88/M 001	Human	Cameroon	1988	21	Tbg; 21
MHOM/CG/80/PEYA	Human	Congo	1980	21, 25	Tbg; 21
MHOM/ET/67/GAMBELA I	Human	Ethiopia	1967	26	
MHOM/CI/52/ELIANE	Human	Cote d'Ivoire	1952	22–24	Tbg; 22–24
MHOM/CI/78/DAL 069	Human	Cote d'Ivoire	1978	20–22, 27	Tbg; 21, 22, 27
MHOM/CI/78/TH 112	Human	Cote d'Ivoire	1978	21	
MHOM/CI/78/TH 149	Human	Cote d'Ivoire	1978	20, 21, 27	Tbg; 21, 27
MHOM/CI/78/TH 2	Human	Cote d'Ivoire	1978	20, 27	
MHOM/CI/86/DAL 967	Human	Cote d'Ivoire	1986	21	Tbg; 21
MHOM/CI/??/NANA	Human	Cote d'Ivoire	19??	21, 28	Tbg; 21, 28
GPAL/KE/69/EATRO 1535	Fly	Kenya	1969		
GPAL/KE/69/S 29	Fly	Kenya	1969		
GMOS/NG/70/NITR 40.12	Fly	Nigeria	1970	22–24	Non-g; 22, 23 Tbb; 24
MBOT/NG/77/IBADAN 22 cl.A	Cattle	Nigeria	1977	21, 26	Tbb; 26
MHOM/RW/70/0404	Human	Rwanda	1970		
MHOM/SD/82/BIYAMINA cl.B	Human	Sudan	1982	28	Tbg; 28
MTRG/UG/66/EATRO 1125	Bushbuck	Uganda	1966	22, 24, 25	Non-g; 22 Tbb; 24
MHOM/ZR/71/C 126	Human	Zaire	1971	21, 25, 26	Tbg; 21, 26
MBOI/ZM/82/TRPZ 166	Zebu	Zambia	1982		
MHOM/ZM/74/058 cl.A3	Human	Zambia	1974	26	Tbr; 26
MHOM/ZM/82/TRPZ 227	Human	Zambia	1982	21	

* References of previous studies on the stock.

† Tbg = *Trypanosoma brucei gambiense*; non-g = *non-gambiense*; Tbb = *T. brucei brucei*; Tbr = *T. brucei rhodesiense*.

follows: 5'-CGCCCATAAGATTTCCGGTT-3' and 5'-GGTGTAATACTCACCCGGTT-3'. Buffer and incubation conditions were performed according to Breniere and others.[4] Amplification was performed using 1 IU of *Thermus aquaticus* DNA polymerase (Genofit, Geneva, Switzerland) and a PHC-2 apparatus (Techne, Cambridge, UK). Parasite cells (10^4) were harvested by centrifugation, boiled in 100 μl of distilled water for 5 min, and centrifuged in an Eppendorf (Madison, WI) microfuge at 5,000 × *g* for approximately 30 sec. Ten microliters of the supernatant obtained by this procedure was used as template.

Probe purification

The PCR-amplified fragments were purified from a 1% preparative gel (low-melting point ultrapure agarose in Tris-acetate-EDTA [TAE] buffer) using the enzymatic preparation GELase® (Epicentre Technologies Corporation, Madison, WI) according to the manufacturer's instructions. The extracts were then digested with *Msp* I restriction endonuclease to eliminate part of the oligonucleotide primers selected in the conserved region of the minicircle. The DNA was precipitated with ethanol and resuspended in 100 μl of distilled water. The DNA concentration was assessed with a DU 70 spectrophotometer (Beckman, Palo Alto, CA).

Southern blotting

The PCR products were electrophoresed in a 0.8% grade agarose gel (Bethesda Research Laboratories, Uxbridge, UK) in TAE buffer for 40 min at 100 volts. The gels were stained with ethidium bromide and were then transferred onto charged nylon membranes (Hybond-N⁺; Amersham, Buckinghamshire, UK) after alkaline denaturation (15 min twice in 0.5 M NaOH, 1.5 M NaCl) by the pocket-blotting procedure.[8] Subsequently, the membranes were washed in 2× SSPE (1 × SSPE = 0.15 M NaCl, 0.01 M sodium diphosphate, 0.001 M EDTA), dried, and stored in a protective film until the hybridization stage.

Labeling and hybridization conditions

The ECL® (Amersham) gene detection system based on enhanced chemiluminescence was used according to the manufacturer's instructions. Simultaneously, the membranes were incubated at 42°C in hybridization buffer supplied by the manufacturer (0.25 ml/cm² of blot) for 15 min, and the purified probe (20 ng/ml of hybridization buffer) was labeled for 10 min at 37°C. The hybridization was performed at 42°C overnight in a rotary oven (Appligene, Illkirch, France). Before the detection step, the membranes were washed twice in a highly stringent solution (6 M urea, 0.1% sodium dodecyl sulfate, 0.1× SSC [SSC = 0.15 M NaCl, 0.015 M sodium citrate]) at 42°C, and twice in a 2× SSC at room temperature. Serial exposures were performed (1, 5, and 20 min) on autoradiography films (Hyperfilm®; Amersham).

RESULTS

All 26 *T. brucei* stocks showed positive amplification. The amplified band, when analyzed by electrophoresis, had a length of approximately 930 bp, which corresponds to the expected molecular weight of the minicircle HVR. Figure 1A shows the amplification results for some of these stocks. As a control, amplification was tested on stocks belonging to the following other species: *T. evansi*, *T. cruzi*, and *Leishmania mexicana* (one stock each). *Trypanosoma cruzi* and *L. mexicana* both showed no amplification, while *T. evansi* exhibited a slight signal at the same molecular weight as the *T. brucei* band. These results suggest that the sequence homology observed by Chen and Donelson actually involves a conserved sequence that is specific for the whole taxon *T. brucei*.[1] This sequence would be different from the minicircle conserved regions characterized in *T. cruzi* and *Leishmania*,[9,10] while it would be very similar to the *T. evansi* sequence, as indicated by Bajyana Songa and others.[11]

Three *T. brucei* PCR products were selected to be representative of the three classic subspecies and were used as probes. The stocks from which these amplified products were obtained are DAL 967 (*T. b. gambiense*), NITR 40.12 (*T. b. brucei*), and 058 cl.A3 (*T. b. rhodesiense*) (Table 1). After purification, the amplified variable regions of these stocks were hybridized with the membrane-blotted PCR products of the whole set of 26 stocks. Two radically different kinds of hybridization patterns were observed (Table 2). The two probes obtained from the NITR 40.12 and 058 cl.A3 stocks hybridized only to their own stock. The DAL 967 probe showed a pos-

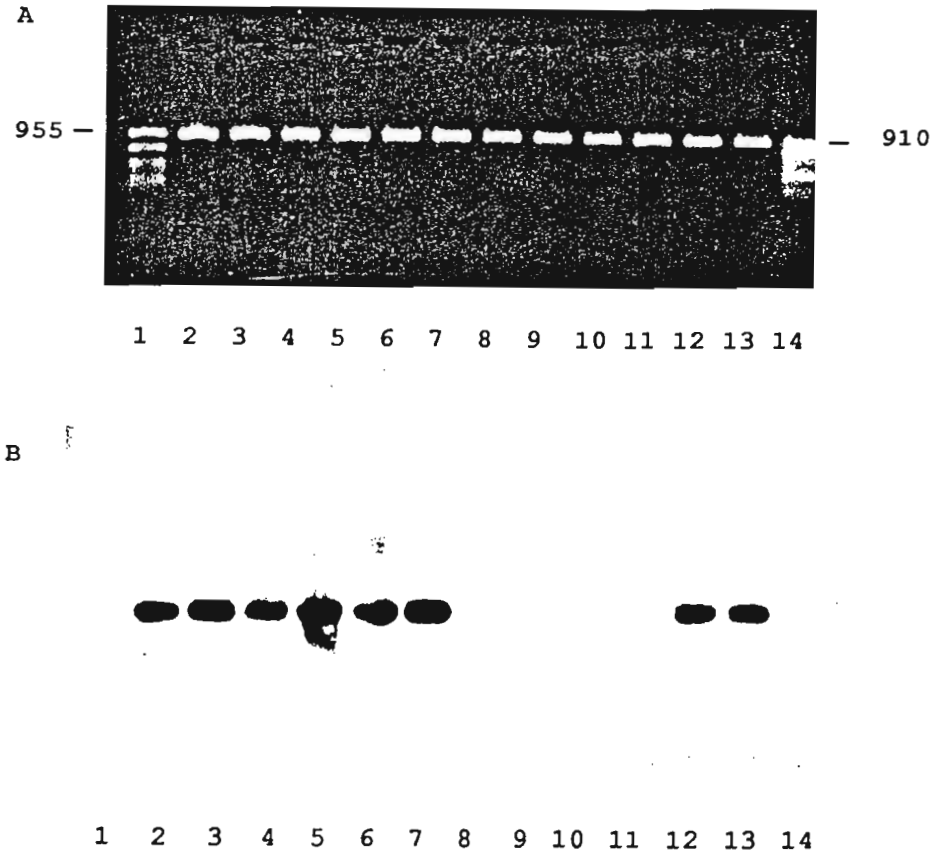

FIGURE 1. **A,** polymerase chain reaction (PCR) amplification of the kinetoplast DNA minicircle variable region of *Trypanosoma brucei*. The PCR products were analyzed by electrophoresis on a 0.8% agarose gel run in Tris-acetate-EDTA buffer and stained with ethidium bromide. The amplified fragment had a length of approximately 930 basepairs. Lanes 1 and 14, DNA size markers (*Sau* 3A I digest of pUC18 and *Alu* I digest of pBR322, respectively); lanes 2–13, PCR products of *T. brucei* stocks DAL 069, DAL 967, ELIANE, TH 149, PEYA, MOS, TH 112, BIYAMINA, EATRO 1125, 058 cl.A3, A 004, and M 001, respectively. Molecular weight standards (in basepairs) are shown on both sides of the gel. **B,** Southern blot of the agarose gel in **A,** showing hybridization with the DAL 967 kinetoplast DNA probe and chemiluminescent detection. No bands were detectable in lanes 8–11, while strong signals were exhibited in lanes 2–7 and 12 and 13.

itive hybridization, not only with its own stock, but also with 11 other stocks. Figure 1B shows the hybridization of the DAL 967 probe with seven of these stocks.

When previous MLEE phylogenetic results based on 18 polymorphic loci are considered, it is interesting to note that those 12 stocks that hybridize with the DAL 967 probe can be assigned to a clearly separate cluster.[6] Moreover, it is worth emphazing that this cluster involves most of the human stocks from Central and West Africa studied here. Figure 2 shows a dendrogram constructed using the unweighted pair group method with arithmetic mean (UPGMA)[12] from the Jaccard distance matrix obtained from the MLEE data among the 26 stocks studied, which corresponds to 22 zymodemes. The group of zymodemes indicated by the brace in Figure 2 corresponds to this separate cluster of human stocks. This specific clustering pattern was confirmed for 78 *T. brucei s.l.* stocks,[6] not only by UPGMA dendrograms as shown here, but also by both Wagner networking and principal component analysis.[13, 14]

DISCUSSION

It is quite probable that this cluster of human stocks corresponds to *T. brucei gambiense* group 1.[15] Since this group of trypanosomes involves

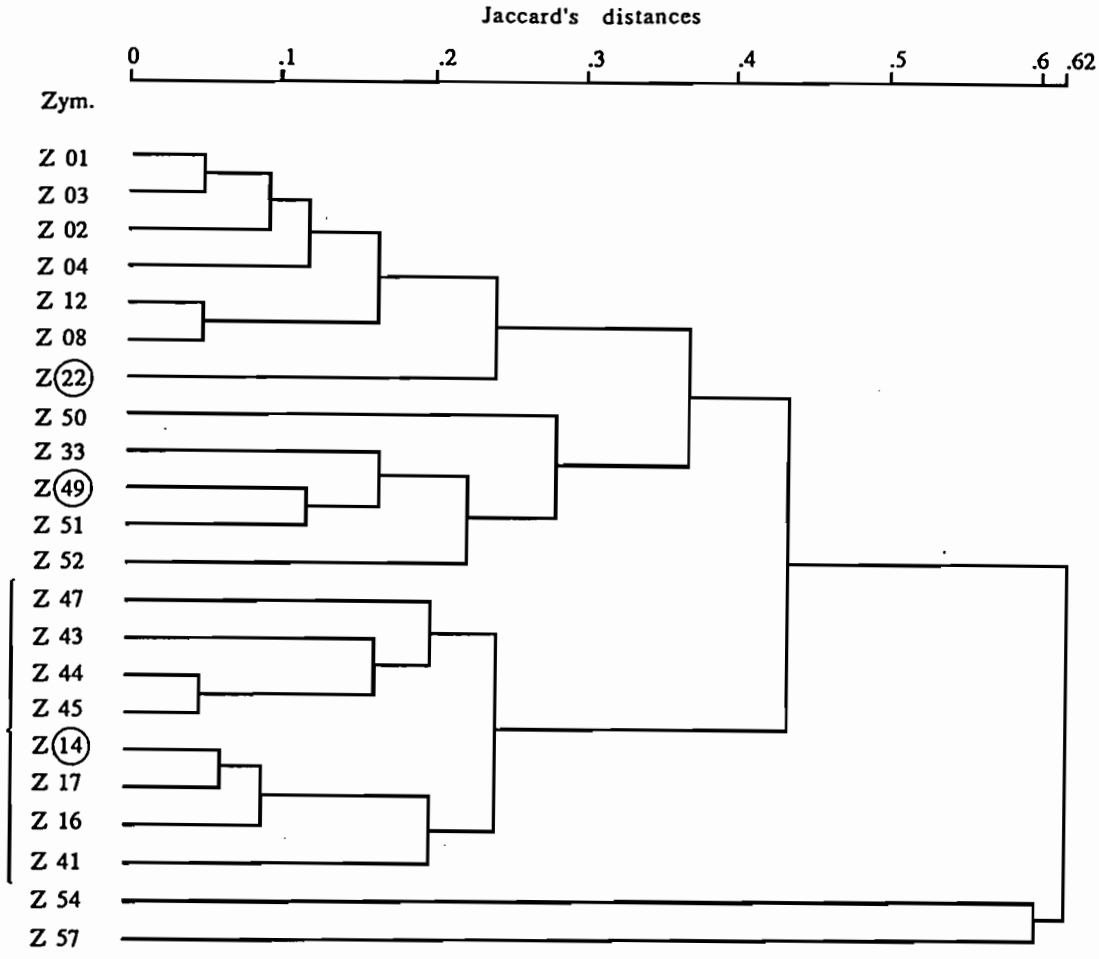

FIGURE 2. Dendrogram constructed using the unweighted pair group method with arithmetic mean from a Jaccard distance matrix, based on a multilocus enzyme electrophoresis study on 18 polymorphic loci.[6] Zymodemes (Zym.) Z22, Z49, and Z14 (circled) indicate the three stocks used as probes (NITR 40.12, 058 cl.A3, and DAL 967, respectively). The brace delimits the zymodemes of the 12 stocks that hybridize with the DAL 967 probe. This cluster corresponds to *Trypanosoma brucei gambiense* group I.[15]

most of the human isolates from Central and West Africa, its medical and epidemiologic relevance is considerable. Thus, the kDNA probe designated in the present study appears to be a promising tool that can be used to specifically identify the stocks belonging to this group of trypanosomes in tsetse flies, as well as in patients and potential mammalian reservoirs.

From a population genetics point of view, successful designing of this probe is consistent with two hypotheses that are not incompatible with each other. First, it can be considered that the cluster identified by the probe is genetically isolated from any other stock studied here. If not, there would be no reason to observe this strong linkage disequilibrium between the kDNA sequence of the probe and nuclear genes that cause the isoenzyme variability, i.e., the probe should randomly hybridize with any stock of the present sample. Second, this result is consistent (although not definitive evidence by itself) with the more general hypothesis that natural populations of *T. brucei s.l.* have a basically clonal evolutionary pattern.[6, 16, 17] Such a hypothesis does not mean that genetic recombination is absent in *T. brucei*, but only that it does not occur frequently enough to alter a prevalent pattern of clonal population structure.

These results also have implications regarding the question of kDNA evolution. It has been

TABLE 2

Hybridization results for the Trypanosoma brucei *probes tested**

Stock	Zymodeme†	Probe: DAL 967	Probe: NITR 4012	Probe: 058 cl.A3
BIYAMINA	1	−	−	−
IBADAN 22	2	−	−	−
TH 112	3	−	−	−
TH 2	4	−	−	−
KK 39	8	−	−	−
GAMBELA 1	12	−	−	−
DAL 069	14	+	−	−
DAL 967	14	+	−	−
NANA	14	+	−	−
TH 149	16	+	−	−
ELIANE	17	+	−	−
NITR 40.12	22	−	+	−
EATRO 1125	33	−	−	−
MOS 84	41	+	−	−
C 126	43	+	−	−
A 004	44	+	−	−
B 014	44	+	−	−
M 001	44	+	−	−
PEYA	45	+	−	−
BIM	47	+	−	−
058 cl.A3	49	−	−	+
TRPZ 227	50	−	−	−
TRPZ 166	51	−	−	−
0404	52	−	−	−
S 29	54	−	−	−
EATRO 1535	57	−	−	−

* − = negative; + = positive.
† Identified using 18 polymorphic loci.*

postulated ~~that *T. brucei*~~ minicircle HVR sequences had a very high rate of evolution due to both nucleotide substitutions and segmental rearrangements.[2] This rapid sequence evolution of the minicircle has up to now precluded its use for taxonomic purposes.[18,19] The results obtained with both the NITR 40.12 and 058 cl.A3 probes are consistent with this hypothesis, but the result for the Dal 967 probe at least shows that this hypothesis is not constantly verified, since it indicates limited evolution of an HVR sequence within a whole group of parasites that exhibit non-negligible genetic variability (Figure 2). On the other hand, since several classes of minicircle sequences coexist within a single cell,[2] the present result suggests that within the group of trypanosomes specifically labeled by the DAL 967 probe, a dominant class of minicircles exists that shares an identical or closely related HVR sequence.

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