UNIVERSITE MONTPELLIER II
Université des Sciences et Techniques du Languedoc

Dossier de Travaux et Publications

HABILITATION A DIRIGER DES RECHERCHES

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par

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Sommaire

1- Travaux de recherche effectués

1-1 Initiation à la recherche

- Stage de DEA

Il avait pour but de comparer la morphologie de divers trypanosomes et de sauroleishmanies (spécifiques de reptiles) avec leurs caractéristiques génétiques. Ce travail m'a permis de m'initier d'une part, à la culture *in vitro* des trypanosomes de mammifères, de reptiles et de batraciens, et, d'autre part, à l'électrophorèse d'isoenzymes. Cette dernière était la technique de référence pour l'étude taxonomique des leishmanies, mais aussi pour l'étude de trypanosomes humains et animaux (*Trypanosoma brucei, T. congolense, T. evansi, T. cruzi*).

La recherche sur les leishmanioses étant l'activité principale du laboratoire d'accueil, j'ai pu aussi apprendre le diagnostic sérologique et parasitologique de la leishmaniose viscérale, et effectuer deux missions en Espagne et dans les Cévennes françaises pour la recherche des parasites chez les phlébotomes vecteurs.

Mes travaux ont permis de trouver des corrélations entre formes de culture et caractéristiques génétiques, une nette individualisation taxonomique des différentes espèces, et de décrire une nouvelle espèce de trypanosomes du blaireau (autre que *T. pestanai*) dans le sud de la France, sans pour autant la nommer.

Publication : 91-2

- Thèse de doctorat

Mon travail de DEA et mon souhait de travailler sur le terrain ont été des éléments déterminants pour le choix de mon sujet de thèse. Les études menées par l'IRD/ORSTOM à cette époque concernaient l'étude génétique par isoenzymes de *T. cruzi*, agent de la maladie de Chagas. Il m'a été proposé par Jean Louis Frézil et Michel Tibayrenc (IRD/ORSTOM) de vérifier l'apport d'une étude des trypanosomes par marqueurs génétiques pour la taxonomie de *T. brucei* et à l'épidémiologie de la Trypanosomiase Humaine Africaine (THA).

Problématique

Depuis le début du siècle, il est admis que la THA ou maladie du sommeil sévit sous deux formes : le type "gambien" ou forme chronique en Afrique de l'Ouest et Centrale, et la forme "rhodésienne" ou forme aiguë, dont le cycle inclut un réservoir animal sauvage en Afrique de l'Est. Ces caractéristiques extrinsèques du parasite ont servi à définir la classification sub-spécifique de l'espèce *Trypanosoma brucei : T. b. gambiense* correspond à la forme chronique, *T. b. rhodesiense* à la forme aiguë, *T. b. brucei* à la forme non pathogène chez l'homme, mais souvent pathogène chez l'animal. Les trois sous-espèces sont indifférenciables au microscope par la morphologie des formes circulantes dans le sang, que ce soit chez l'homme ou l'animal. Elles sont transmises par des glossines ou mouches tsé-tsé. La classification de *T. brucei* en 3 sous-espèces reste encore incertaine, car elle n'est pas corroborée par l'analyse génétique.

Chez l'animal circulent aussi des souches de trypanosomes que les isoenzymes et autres marqueurs génétiques ont supposés être identiques aux souches isolées de l'homme. L'animal domestique devient alors un réservoir potentiel de THA. Ce résultat pose le problème du rôle de ce réservoir animal dans la dynamique de transmission de la maladie du sommeil.

Une étude génétique permet aussi d'approfondir nos connaissances sur le mode de reproduction des trypanosomes. S'il est admis que le mode de reproduction est classiquement clonal, de récents travaux font état d'échanges génétiques qui plaideraient en faveur d'une reproduction sexuée. La connaissance du mode de reproduction est importante par exemple pour le suivi épidémiologique, les problèmes de pathogénicité ou de résistance aux médicaments.

En Afrique Centrale, la THA sévit toujours dans de nombreux foyers historiques. La transmission péridomestique et l'asymptomatisme de la maladie chez l'homme seraient des paramètres importants dans la dispersion et le maintien de la maladie en particulier au Congo.

Objectifs

- Vérifier la validité de la taxonomie sub-spécifique de *T. brucei* en Afrique Centrale.
- Préciser le mode de reproduction des trypanosomes dans l'aire Congo-ex Zaïre-Cameroun.
- Étudier la persistance et la propagation de la maladie par la circulation des réservoirs humains et animaux.

Méthodologie

Isolement et culture des trypanosomes

Les différentes techniques, en particulier le KIVI (publication 92-2), sont évoquées dans le paragraphe (1-2-1 A).

Électrophorèse d'isoenzymes sur acétate de cellulose

En 1987, si le protocole technique était opérationnel pour l'étude de *Leishmania et T. cruzi*, il était inadapté à l'étude des trypanosomes africains. J'ai donc standardisé les protocoles techniques de migration et révélation pour plus d'une trentaine d'enzymes métaboliques, cibles de choix car contenues en grande quantité dans le cytoplasme des parasites. Une vingtaine d'enzymes ont ensuite été retenues (publications 90-1, 91-1, 93-1), représentant 24 loci génétiques, permettant d'obtenir un niveau de résolution suffisant pour mes études.

Analyse du polymorphisme des fragments de restriction de l'ADN kinétoplastique (RFLP)

Après avoir extrait l'ADN total, puis séparé l'ADN nucléaire de l'ADN kinétoplastique, j'ai testé plusieurs enzymes de restriction.

Résultats et corollaires épidémiologiques

Par l'étude isoenzymatique

On distingue deux sous-ensembles de zymodèmes (zymodème = ensemble de souches présentant le même profil enzymatique) :

- Un groupe rassemblant la majorité des zymodèmes isolés de l'homme, un zymodème isolé de l'homme et de l'animal ainsi qu'un zymodème isolé uniquement de l'animal.
- Un groupe rassemblant la majorité des zymodèmes isolés de l'animal et un zymodème isolé de l'homme et de l'animal.

D'une manière générale, on constate qu'il y a stabilité des lignés parasitaires dans l'espace et dans le temps. L'hypothèse d'une reproduction sexuée n'est pas corroborée par les résultats, donc le mode de reproduction clonal semble confirmé.

Par le RFLP

L'identification des souches par cette technique corrobore les résultats obtenus par isoenzymes.

Discussion

Taxonomique

Les résultats reflètent une variabilité génétique qui reste relativement faible en comparaison à celle observée pour d'autres trypanosomes, par exemple l'espèce *T. cruzi* .

Les deux groupes de zymodèmes identifiés correspondent aux deux sous-espèces classiques (*T. b. gambiense* et *T. b. brucei*).

Les tests statistiques menés sur les résultats isoenzymatiques, en majorité très significatifs, permettent de rejeter fermement l'hypothèse d'une recombinaison sexuée libre, dans l'aire Congo, mais aussi dans l'aire Congo-ex Zaïre-Cameroun.

Le fait que les résultats isoenzymatiques soient corroborés d'une manière générale par les résultats du RFLP est un exemple particulier de déséquilibre de liaison, très illustratif, car impliquant génomes nucléaire (isoenzymes) et extranucléaire (ADNk). Ce sont autant d'arguments qui plaident en faveur d'un mode de reproduction clonal.

Épidémiologique

La répartition géographique des zymodèmes n'est pas aléatoire. Elle se fait le long des axes de communication : les fleuves (le Congo et le Kasaï), les axes routiers et l'axe ferroviaire. Connaissant le mode de transmission péridomestique de la maladie, il est logique de constater que la répartition des zymodèmes est superposable à la répartition des principales agglomérations. Par ailleurs, il n'est pas surprenant que de nombreux zymodèmes se localisent le long des cours d'eau, qui sont les gîtes préférentiels des glossines du groupe *palpalis*.

Il ne semble pas exister de spécificité vectorielle des stocks de *T. brucei* au Congo, puisque des stocks appartenant à un même zymodème peuvent être transmis par des vecteurs différents (glossines de l'espèce *fuscipes* ou de l'espèce *palpalis*).

Si un même zymodème peut se retrouver en des points géographiquement distants, la majorité des zymodèmes apparaît inféodée à des aires géographiques précises. La distribution des zymodèmes s'explique par l'intense circulation humaine le long des axes principaux de communication. Les trypanosomés asymptomatiques, inconscients du danger qu'ils représentent en tant que réservoir potentiel de trypanosomes, propagent la maladie à travers tout le pays, à l'occasion d'un déplacement professionnel ou personnel.

Un même zymodème regroupe des stocks humains et des stocks animaux. Ceci est compatible avec l'existence d'un réservoir animal au Congo et en ex-Zaïre.

Conclusion

L'étude isoenzymatique a permis de confirmer la taxonomie connue (publications 90-2, 93-4, 94-3). L'analyse statistique des résultats isoenzymatiques montre que la reproduction des trypanosomes est essentiellement clonale dans notre échantillonnage. Les zymodèmes ou clones naturels sont les unités taxonomiques à considérer, en particulier dans les recherches médicales.

L'importance d'un réservoir humain asymptomatique, principal responsable passif de la persistance de la maladie à l'état endémique, et le caractère endémo-épidémique de la THA en Afrique Centrale (localisation géographique limitée de certains clones et ubiquité d'autres clones), nécessitent impérativement le dépistage de toute la population vivant dans les foyers endémiques, *a fortiori* pendant les périodes épidémiques (publications 93-2, 93-4).

Même si l'importance du réservoir animal semble plus faible au Congo qu'en ex-Zaïre, il ne doit pas être sous-estimé dans les deux cas mais au contraire considéré comme un paramètre non négligeable, pouvant être responsable du maintien à bas bruit de la maladie entre les périodes épidémiques. En ce sens, il est primordial de continuer l'examen systématique des élevages domestiques.

Si l'histoire indique que l'apparition des premières épidémies décrites de THA au Congo et en ex-Zaïre s'est faite en des lieux géographiquement très proches les uns des autres (le long du fleuve Congo), au même moment (vers 1885), l'origine de la THA se situe certainement bien avant. Il est probable que la THA a la même origine ancestrale au Congo qu'en ex-Zaïre, et que les voies de communications naturelles (les fleuves), puis que l'apparition des routes et voies ferrées, aient favorisé l'expansion de la maladie grâce essentiellement au déplacement des malades trypanosomés

asymptomatiques. Enfin, l'isolement géographique de la plupart des agglomérations entre elles aurait favorisé la persistance de la maladie à l'état endémique.

Publications : 90-1, 90-2, 91-1, 92-2, 93-1, 93-2, 93-4, 94-3
Communications : 91-1, 92-1

1-2 Recherches opérationnelles

Problématique générale

Depuis une trentaine d'années, la THA connaît une très nette recrudescence, en particulier en Afrique Centrale. Environ 60 millions de personnes exposées, plus de 300.000 personnes infectées, 100 décès officiels par jour, prés de 300.000 nouveaux cas par an, selon les données de l'OMS (1998). L'absence presque totale de dépistage actif, les problèmes sociaux et économiques des pays les plus touchés, la pauvreté de l'arsenal thérapeutique et ses redoutables effets secondaires font de la THA la troisième maladie parasitaire majeure en Afrique.

Il est impératif de mener des recherches opérationnelles dans le but d'améliorer rapidement la lutte contre cette maladie. Ainsi, pour les recherches épidémiologiques, il faut isoler le parasite sur le terrain quel que soit l'hôte, et l'identifier par marqueurs génétiques. Pour optimiser les techniques de lutte, il faut évaluer et améliorer les techniques de dépistage, de diagnostic, de contrôle de l'efficacité des traitements thérapeutiques. Enfin, il faut aussi perfectionner les techniques d'étude sur les glossines vectrices afin de faciliter les recherches sur la transmission de la maladie.

J'ai donc orienté mes travaux selon les axes suivants :

- Chez l'homme :

- Peut-on simplifier les méthodes d'isolement de souches de trypanosomes sur le terrain ?
- Peut-on améliorer les techniques de dépistage, de diagnostic et de suivi post-thérapeutique ?
- Existe-t-il uniquement une forme chronique de THA due *à T. b. gambiense* en Afrique occidentale et centrale ?
- Sinon, quelles sont les autres formes cliniques et quels types de trypanosomes en sont responsables ?
- Peut-on mettre au point des techniques de détection spécifiques de chaque type de trypanosomes, chez l'homme, le vecteur, l'animal ?
- Quelle est la cause des phénomènes d'échec du traitement par mélarsoprol ?

- Chez l'animal :

- L'animal domestique ou sauvage est-il un réservoir de THA à *T. b. gambiense* ?
- Quelle est l'importance réelle de ce phénomène dans les foyers ?

- Chez la glossine :

- Peut-on améliorer la détection des trypanosomes ?
- Peut-on améliorer les techniques d'analyse des repas de sang ?

Les produits de ces recherches devraient faciliter le travail :

- Des Programmes Nationaux de lutte contre la THA ;
- Des équipes vétérinaires nationales ou internationales ;
- Des chercheurs travaillant sur la THA, en particulier en entomologie et en épidémiologie.

1-2-1 Chez l'homme

A/ Simplification de l'isolement des trypanosomes: la mise au point et l'évaluation du KIVI (Kit for *In Vitro* Isolation of trypanosomes)

Problématique

Sur le terrain, la technique d'inoculation de sang parasité à des rongeurs était utilisée depuis le début du siècle. Les conditions de transport difficiles, le faible taux de survie des animaux inoculés et le faible pourcentage de succès à l'isolement (dû en particulier à la faible virulence des souches humaines), rendaient cette technique trop aléatoire. De plus, des phénomènes de sélection de souches étaient suspectés, en particulier dans le cas des infections mixtes de trypanosomes d'espèces.

Enfin, le risque lié à la manipulation était important. Immobiliser un rat dans une main et manipuler une seringue remplie de sang humain contaminé avec l'autre main constituaient pour le manipulateur un risque permanent d'inoculation accidentelle de divers pathogènes.

Objectif

Mettre au point une méthode simple et efficace d'isolement de terrain des trypanosomes, peu dangereuse pour le manipulateur, et facilement transportable.

Méthodologie

Nous avons adapté, avec le Pr Le Ray et le Dr Aerts de l'IMT d'Anvers, un milieu de culture à l'isolement des trypanosomes africains. Le sang du malade était ensemencé directement dans des flacons en verre contenant ce milieu de culture. Un kit a été élaboré pour le travail de terrain, le KIVI (Kit for *In Vitro* Isolation of trypanosomes). Il a été testé au Congo pour la première fois, à partir de sang de malades trypanosomés (publication 92-2).

J'ai simplifié par la suite le protocole puis évalué cette technique à grande échelle (publications 92-1, 94-1). J'ai enfin mis au point et testé une version miniature du KIVI (publication 96-1).

Résultats

Le KIVI est un outil fiable, permettant d'obtenir entre 70 et 90 % de succès à l'isolement. Par contre, il ne permet la culture que des formes dites « procycliques », semblables aux formes de multiplication dans le tube digestif de la glossine vectrice. Ces formes ne présentent plus leurs manteaux antigéniques, mais ne sont plus infectantes. Le patrimoine génétique étant identique aux autres formes du cycle parasitaire, elles constituent un matériel adapté aux études génétiques.

Conclusion

Le KIVI est maintenant considéré comme la technique de référence pour l'isolement de terrain et comme outil de diagnostic parasitologique complémentaire de la THA (OMS, Rapport du Comité d'Experts, 1998).

Publications : 92-1, 92-2, 93-2, 94-1
Communications : 93-1

B/ Conception et évaluation préliminaire de la version miniature du KIVI : miniKIVI

Objectif

Chercher à simplifier le protocole technique du KIVI afin de réduire son coût tout en lui conservant un maximum d'efficacité. La simplification consistait en une réduction des volumes de sang prélevé et du milieu de culture, le tout conditionné dans un tube en plastique stérile à bouchon à vis de 10 ml. Le sang était prélevé par ponction digitale. Le choix de ce type de prélèvement et de tube avait pour but d'éviter l'usage de seringue et d'aiguille, réduisant ainsi les risques d'accident pour le manipulateur. Le nouveau prototype devait alors être testé sur plusieurs prélèvements de sang de malades.

Méthodologie

Un KIVI et un miniKIVI étaient testés en parallèle pour chaque malade.

Résultats et discussion

Le pourcentage de succès à l'isolement du miniKIVI est de 75 %. 93 % de succès sont obtenus avec un lot de KIVI classique.

Conclusion

Un plus grand confort pour le malade (plus de "piqûre"), une diminution du risque d'accident pour le manipulateur et une réduction importante du coût du KIVI pour un pourcentage de réussite acceptable.

Publication : 96-1
Communication : 95-2

C/ Evaluation en parallèle du CATT pour le dépistage sérologique

Problématique

Dans le cadre du dépistage de masse de la THA, la technique utilisée est un test d'agglutination directe Antigènes-Anticorps : le test CATT sur sang total. En mélangeant quelques gouttes de sang à du réactif, une agglutination bleue est visualisable en cas de positivité. Il faut 10 minutes pour réaliser le test et sa réponse est immédiate. Cependant, un suspect positif au CATT n'est pas obligatoirement malade. Il faut impérativement détecter le trypanosome dans le sang ou les ganglions lymphatiques pour affirmer que la personne est réellement atteinte par la THA (OMS, 1995).

J'ai pu évaluer le CATT au Congo et en Côte d'Ivoire en parallèle au KIVI et à d'autres méthodes de diagnostic.

Objectif

Vérifier l'efficacité du CATT pour le dépistage de la maladie.

Méthodologie

Au total, 8974 personnes ont été examinées en Côte d'Ivoire.

Résultats

Des KIVI positifs obtenus de sujets ayant des CATT et des examens parasitologiques négatifs montrent que le KIVI a un rôle pour le diagnostic parasitologique différé. Les résultats obtenus chez l'homme confirment que le KIVI est une excellente méthode d'isolement de *T. b. gambiense*.

Conclusion

Des infections précoces chez des sujets négatifs au CATT et aux examens parasitologiques classiques sont détectées par le KIVI. Le CATT n'est pas un outil parfait, mais il est simple à utiliser sur le terrain et donne une réponse immédiate contrairement à d'autres méthodes (IFI ou ELISA).

Publication : 94-1

D/ Evaluation du CATT sur latex

Problématique

Un nouveau test sérologique, le CATT sur latex, est évalué de façon préliminaire en Côte d'Ivoire, en comparaison avec le CATT classique et un examen parasitologique systématique. Cette comparaison est effectuée dans une zone de forte endémie et dans une zone de faible endémie afin d'une part, de comparer la sensibilité des deux tests et, d'autre part, de comparer les spécificités.

Objectif

Tester une nouvelle méthode de dépistage sérologique CATT sur latex afin d'éliminer les faux positifs détectés par le CATT classique.

Méthodologie

Nous avons comparé les tests CATT sur latex et CATT classique dans une zone de forte endémie (Sinfra), et dans une zone de faible endémie (Bonoufla) en Côte d'Ivoire.

Un examen parasitologique QBC est aussi effectué systématiquement.

Résultats et discussion

Comparaison des deux protocoles techniques : CATT et CATT latex

D'un point de vue technique, il s'est avéré que les deux protocoles sont facilement réalisables sur le terrain.

Comparaison des résultats CATT et CATT latex

La valeur prédictive positive (VPP) du CATT est plus faible (12,4 %) que celle du CATT latex (35,3 %). En d'autres termes, la probabilité qu'un individu positif au latex soit trypanosomé est de 0,35, alors que la probabilité qu'un individu positif au CATT soit trypanosomé n'est que de 0,12. Dans le cadre de cet échantillonnage, le CATT latex est plus performant pour conduire à un diagnostic parasitologique de THA.

Conclusion

Ces résultats confirment que le CATT latex pourrait permettre de réduire le coût d'une prospection médicale, en éliminant de la chaîne de diagnostic les CATT classiques faiblement positifs non confirmés parasitologiquement (Jamonneau *et al.*, soumis pour publication). L'utilisation des antigènes bruts fixés sur des particules de latex était à l'étude déjà depuis plusieurs années dans mon laboratoire (publications 96-3, 99-4). En réduisant le nombre de sujets à examiner, on diminue la durée de la prospection médicale, donc on réduit le coût total. Ces paramètres sont très importants à l'heure où le nombre de trypanosomés augmente de façon alarmante dans toute l'Afrique et où les moyens financiers des Programmes Nationaux de lutte sont extrêmement réduits.

Publications : 96-3, 99-4, (Jamonneau *et al.*, soumis pour publication)

E/ Etude du mode de prélèvement de sang pour le diagnostic parasitologique et comparaison des techniques mAECT et QBC

Problématique

Durant certaines prospections médicales, il m'a paru troublant que l'examen parasitologique soit négatif malgré la très forte sérologie positive de patients associée parfois à des signes cliniques typiques de THA.

Avant d'incriminer la technique, j'ai voulu vérifier si le mode de prélèvement sanguin n'était pas responsable de ces résultats douteux suspectant que la parasitémie pouvait être différente entre la circulation sanguine superficielle et interne. J'ai donc comparé la ponction veineuse digitale (en accord avec le protocole classique), avec la ponction veineuse au pli du coude, les deux ponctions étant réalisées au même moment. Les méthodes mAECT et QBC ont été comparées aussi sur chaque type de ponction.

Objectif

Optimiser le diagnostic parasitologique par mAECT ou QBC.

Méthodologie

Les sujets présentant des CATT positifs sur sang et plasma ont été prélevés au pli du coude (par seringue héparinée : sang veineux), et au bout du doigt (ponction digitale avec un microtube hépariné : sang périphérique). Chaque prélèvement a été testé par mAECT et QBC en parallèle par deux manipulateurs.

Résultats

Grâce au sang veineux, 27 des 33 sujets positifs en mAECT étaient aussi positifs par QBC. 8 patients étaient positifs par mAECT et QBC, sur sang veineux ou sang périphérique. 19 patients étaient négatifs par mAECT et/ou QBC sur sang périphérique, alors qu'ils étaient positifs pour l'une et/ou les deux techniques sur sang veineux.

Discussion

La ponction veineuse permet de diagnostiquer plus de patients que la ponction digitale. Le QBC donne des résultats similaires à la mAECT. Cependant, le QBC nécessite en théorie un prélèvement digital , l'un des avantages majeurs de cette technique. Le test QBC est réalisé en 15 minutes contre 45 minutes pour la mAECT.

Conclusion

La ponction digitale est à proscrire, quel que soit le test utilisé (mAECT ou QBC), et la ponction veineuse est recommandée. Le choix de la technique mAECT ou QBC dépend des moyens financiers des équipes de terrain et du nombre de personnes à examiner. Dans le cas d'un nombre important de sujets à tester, l'usage du QBC peut permettre un gain de temps considérable, pour un coût similaire à la mAECT (2$ chaque test).

Publication : 98-3

F/ Simplification de la mAECT, méthode de diagnostic parasitologique

Problématique

La mAECT est la technique classique la plus sensible pour le diagnostic parasitologique de la THA. Cependant, son coût est élevé (2$), compte tenu des moyens financiers des programmes nationaux de lutte. J'ai donc cherché à simplifier cette technique tout en lui conservant son efficacité. Le principe technique reste inchangé, c'est-à-dire faire traverser du sang à travers de la cellulose contenue dans une colonne. Les éléments figurés du sang sont retenus par la cellulose, et seuls les trypanosomes sont collectés à la sortie de la colonne dans un éluat de tampon glucosé. Cet éluat est ensuite centrifugé, puis examiné au microscope.

Objectif

Réduire le coût de la méthode mAECT pour le diagnostic parasitologique.

Méthodologie

La colonne classique est constituée d'un corps de seringue de 10 ml. En remplaçant celui-ci par une pipette Pasteur, on peut supprimer le réservoir à tampon placé en dessus de la colonne, la pipette jouant alors le double rôle de colonne et de réservoir. Le volume de cellulose reste inchangé,

ainsi que la méthode de stérilisation et le volume de sang testé. Les deux types de mAECT (classique et « Pasteur ») ont été évalués sur des sujets séropositifs.

Résultats

27 sujets étaient positifs en mAECT classique, dont 26 en mAECT « Pasteur ».

Conclusion

L'usage de la mAECT « Pasteur » est recommandé, car son prix est la moitié de celui de la mAECT classique, pour une efficacité similaire. Nous avons initié la production de ce test à l'Institut Pierre Richet de Bouaké. Il est disponible pour les PNLT et les équipes de recherche.

Publication : 98-1

G/ Mise au point et évaluation préliminaire de la détermination de la période et le suivi post-thérapeutique par la technique PCR

Problématique

La THA évolue chez l'homme selon deux périodes cliniques : la première ou période lymphaticosanguine (pratiquement asymptomatique), la seconde ou période d'atteinte du système méningoencéphalique (troubles nerveux et psychiques). Cette dernière conduit au coma, puis à la mort en absence de traitement.

La détermination de la période conditionne le traitement. Elle se base sur trois examens biologiques du LCR : dosage des protéines totales, numération des lymphocytes et recherche du trypanosome par microscopie après simple ou double centrifugation du LCR. Un taux de protéines supérieur à 37 mg/100 ml, un comptage supérieur à 5 cellules/mm3, avec ou sans détection de parasite, détermine une seconde période et donc le traitement approprié (mélarsoprol ou éflornithine).

À la fin du traitement, les mêmes examens sont répétés pour vérifier l'efficacité du traitement. Cependant, le nombre de cellules et le taux de protéines restent anormaux pendant plusieurs mois. L'absence de parasite par double centrifugation dans le LCR est alors le seul test biologique permettant aux cliniciens de déclarer une totale guérison.

La PCR s'est avérée efficace pour détecter les trypanosomes dans le sang. J'ai ainsi adapté cette technique pour la détection des trypanosomes dans le LCR, afin de confirmer la détermination de la période de la maladie et pour vérifier l'efficacité du traitement.

Objectif

Adapter une technique de détection de l'ADN parasitaire au diagnostic de phase et au suivi post-thérapeutique des malades.

Méthodologie

Les LCR ont été prélevés de patients confirmés parasitologiquement dans le sang :
- avant le traitement ;
- immédiatement après le traitement ;
- à la visite de contrôle, soit 1 mois après le traitement.

A chaque prélèvement, des aliquots sont effectués. Un aliquot est traité immédiatement (numération de cellules, dosage des protéines, recherche des trypanosomes par double centrifugation). Les autres aliquots sont immédiatement congelés dans l'azote liquide. Ces tubes sont ensuite décongelés, puis la PCR est conduite selon le protocole décrit (99-1), en utilisant les amorces spécifiques de *T. brucei* ssp..

Résultats

Parmi 20 patients trypanosomés, 15 étaient en seconde période d'après le nombre de cellules et la taux de protéines. Il étaient tous PCR positifs, alors que 11 seulement avaient une double centrifugation positive.

Les 5 autres patients étaient en première période (cellules<5, et protéines< 37), excepté un qui avait 24 cellules. Ces 5 patients étaient PCR négatifs, et doubles centrifugations négatives.

Pour les LCR prélevés après traitement et au contrôle de 1 mois, tous étaient PCR négatifs et double centrifugation négatives, sauf pour deux malades en seconde période qui étaient PCR positifs mais double centrifugation négatives. Pour ces deux derniers, les contrôles à 1 mois étaient aussi PCR positifs mais doubles centrifugations négatives.

Discussion

La PCR confirme le stade de la maladie déterminé par les dosages classiques. Dans le cas particulier d'un malade à 24 cellules, donc théoriquement en seconde période, la PCR est négative, ainsi que la double centrifugation. Cet argument soulève à nouveau les discussions autour de ces valeurs de 5 cellules et de 37 mg de protéines. En effet, certaines études attestent que de tels patients (jusqu'à une vingtaine de cellules) peuvent être traités à la pentamidine au lieu du mélarsoprol. La toxicité des deux médicaments n'étant pas la même, la question est importante à vérifier.

Pour les deux patients toujours PCR positifs après traitement, la double centrifugation est négative, mais les nombres de cellules et le taux de protéines sont toujours anormaux. La sensibilité de cette double centrifugation n'est peut être pas satisfaisante, et la PCR plus sensible. Il est probable que le traitement n'a pas été totalement efficace pour ces patients, et qu'une rechute soit prévisible.

Conclusion

Cette technique par PCR permet de conforter la détermination de la période de la maladie. Elle peut aussi permettre de distinguer les cas douteux pour lesquels un traitement à la pentamidine peut être préférable au mélarsoprol. Enfin, elle peut être utile à la détection des cas d'échec du traitement (fréquents en Angola, Ouganda et Soudan). De nouvelles évaluations sont nécessaires, avec un suivi longitudinal des patients.

Publication : 99-1

H/ Pathogénicité des trypanosomes africains

Problématique

En 1960, l'équipe médicale du Dr Pierre Richet suspectait, en Côte d'Ivoire, différents types « d'agressivité » des trypanosomes, selon l'ancienneté du foyer de THA. Des souches très virulentes, provoquant un tableau clinique aigu type « rhodésien », s'opposaient à ces trypanosomes d'Afrique occidentale classiquement dénommé *T. b. gambiense*, responsable de la forme chronique de THA.

Inversement, une équipe de Liverpool décrivait en Afrique de l'Est des tableaux cliniques caractéristiques de la forme chronique, alors que c'est une zone à *T. b. rhodesiense*, classiquement responsable de la forme aiguë de THA.

Enfin, des trypanosomes étaient isolés en Afrique occidentale de l'homme et de l'animal domestique, mais leur identification génétique ne correspondait pas au groupe « gambiense 1 », seule entité génétiquement homogène. Certains les ont dénommés « gambiense 2 », d'autres « rhodesiense-like », mais les analyses génétiques montraient qu'il s'agissait plus probablement de *T. b. brucei*, normalement parasite exclusif du bétail.

Dans tous les cas, si les souches de trypanosomes étaient nombreuses et disponibles, aucune donnée clinique des patients porteurs de ces trypanosomes n'avait été enregistrée.

Je me suis intéressé à ce problème, en recherchant les dossiers cliniques des patients pour lesquels nous avions déjà identifié les trypanosomes. Ces malades ivoiriens se regroupaient en deux ensembles :

- Ceux qui présentaient une forme de THA chronique (80 %) ;
- Ceux qui présentaient des atteintes sévères de système nerveux central et un état général très altéré.

Le premier groupe de malades était porteur de *T. b. gambiense* (groupe 1) ;

Le second de *T. b. brucei* groupe Bouaflé, parasite que l'on ne trouve normalement que chez l'animal.

Cette corrélation troublante n'est pas sans doute le seul fait du hasard. Si l'on compare avec la forme rhodésienne, il est admis que *T. b. rhodesiense* serait à l'origine un trypanosome circulant chez l'animal, qui aurait été ensuite transmis accidentellement chez l'homme, d'où sa forte virulence.

Pour la THA classique chronique, la « chronicité » de la maladie est difficile à vérifier pour plusieurs raisons :

- Au questionnaire d'entrée en clinique, le malade est le plus souvent incapable d'évaluer depuis combien de temps se manifestent les signes cliniques de THA ; les nombreuses autres pathologies comme le paludisme et les infections intestinales, ou des symptômes non spécifiques comme la fatigue ou les céphalées, rendent incertaines les réponses données;

- La majorité des cas de THA est diagnostiquée au Centre de Santé, par présentation spontanée des malades. Ils sont pour la presque totalité déjà en seconde période.

En conséquence:

- La notion de durée de séjour en permanence dans le foyer peut être un indicateur de temps d'incubation ;
- Des dosages biologiques dans le sang ou le LCR semblent beaucoup plus fiables (IgM, NO, anticorps particuliers).

Objectifs

Une meilleure définition clinique de la THA en Côte d'Ivoire et une identification des agents pathogènes (importance des infections non dues à *T. b. gambiense*).

Méthodologie

Le protocole était le suivant :
- Intégrer une centaine de malades dépistés activement, mais aussi passivement ;
- Collecter avant et après le traitement, du sérum, du sang sur confettis, du LCR, effectuer deux KIVI à 3 jours d'intervalle avant traitement ;
- Effectuer un questionnaire épidémiologique et un examen clinique poussés, en insistant sur toute indication de durée d'incubation et sur les manifestations cliniques d'atteinte nerveuse ;
- Effectuer des examens biologiques complets, visant en particulier à détecter d'autres infections ou parasitoses concomitantes ;
- Effectuer un marquage isoenzymatique et par amorces aléatoires RAPD/PCR sur les souches isolées, parfois en double pour chaque malade ;
- Effectuer des dosages spécifiques dans le sérum et le LCR (indicateurs d'atteinte du système nerveux) : NO, IgM, latex/IgM, anticorps anti-NO-tryptophane ou anti-NO-cystéine, anti-galactocérébroside, anti-neurofilaments ; (indicateurs de spécificité *gambiense*) : trypanolyse, ELISA, Latex/gambiense Litat 1.3, 1.5, 1.6.
- Collecter au contrôle postcure (1, 3, 6 et 12 mois), du sérum, du sang sur confettis, du LCR, pour le même type d'analyses et dosages qu'évoqué précédemment.

Résultats

Les analyses finales sont en cours. Cependant, nous pouvons dire :

- Que la majorité des souches isolées correspond à *T. b. gambiense* groupe 1 classique (publication 98-3, 99-2) ;
- Que des souches de *T. b. brucei* et *T. congolense* ont été isolées de patients présentant des tableaux cliniques graves ou de patients asymptomatiques (publications 97-1, 98-1, 98-2) ;
- Que la technique isoenzymatique est moins résolutive que les RAPD ou les microsatellites (publication 99-5) ;
- Que des infections multiples impliquant des clones différents par RAPD ont été identifiés chez plusieurs malades (Oury *et al.*, en préparation).

Produits attendus

- Meilleure définition clinique de la (des) forme (s) occidentale (s) de THA ;
- Identification des agents pathogènes associés à des types cliniques particuliers, et détermination des séquences nucléotidiques spécifiques pour élaborer des amorces PCR permettant d'amplifier, quel que soit l'hôte, l'ADN de ce type de trypanosomes ;
- Détection des infections multiples à *T b. gambiense* ou à d'autres trypanosomes (PCR et PCR/RAPD) et recherche de leurs impacts sur l'évolution clinique ;
- Standardisation de la séquence des tests biologiques indicateurs d'atteinte du système nerveux central (donc de seconde période de la maladie).

Publications : 97-1, 98-2, 98-3, 99-2, 99-5, (Oury *et al.*, en préparation)
Communications : 95-1, 95-3, 96-1, 97-3, 98-1, 98-3, 98-4, 99-1, 99-2

1-2-2 Chez l'animal domestique et sauvage

Son rôle de réservoir à THA

Problématique

L'animal domestique est suspecté d'être un réservoir potentiel de la forme chronique de THA. L'animal sauvage est connu comme étant porteur de trypanosomes pathogènes pour l'homme ; il est une composante importante du cycle épidémiologique de la forme aiguë d'Afrique de l'Est à *T. b. rhodesiense*. Il a été aussi suspecté dans le cas de la forme chronique gambienne, en particulier le cobe de Buffon et le lion mais sur la seule base de résultats sérologiques.

Objectif général

Évaluer la prévalence des infections à trypanosomes grâce à une méthode performante de détection parasitologique et d'isolement de souches (KIVI) tout en tenant compte de la morphologie des trypanosomes, de l'identité génétique des souches détectées ou isolées chez l'animal domestique et sauvage. Comparer les résultats isoenzymatiques obtenus avec ceux d'un ensemble de souches de référence de Côte d'Ivoire.

Méthodologie

Les souches ont été isolées par KIVI, les frottis de sang effectués par prélèvement sanguin à l'oreille.

Le test de détection des antigènes circulants par ELISA sur sérum a été réalisé grâce aux anticorps monoclonaux (*T. brucei, T. congolense* et *T. vivax*), le CATT classique effectué sur 5 µl de plasma.

L'électrophorèse d'isoenzymes a été effectuée sur 16 loci.

L'interprétation taxonomique sera faite en comparaison avec des souches de référence identifiées comme *Trypanosoma brucei gambiense* groupe 1, *T. brucei* groupe "Bouaflé" et *T. congolense* groupes forêt, savane et kilifi.

A/ Animaux domestiques au Congo

L'isolement et l'identification des souches animales et humaines, en particulier le mouton et le porc, montrent que des souches isolées de ces animaux sont identiques ou très proches de

souches isolées de l'homme. Il existerait un réservoir animal domestique au Congo, dont l'importance reste à définir.

B/ Animaux domestiques et sauvages de Côte d'Ivoire

Animaux domestiques

Résultats du KIVI et des frottis

Le KIVI a été effectué sur 292 animaux (164 moutons, 60 chèvres, 24 porcs, 44 chiens) dans un foyer de THA. Chez l'animal, le KIVI a permis de détecter et d'isoler des trypanosomes chez 20 animaux négatifs par microscopie (5 moutons, 2 chèvres et 13 porcs), et d'isoler les souches de 4 animaux trypanosomés : 1 chèvre, 2 porcs et 1 chien.

Animaux sauvages

Résultats du KIVI et des frottis

Les prélèvements ont été effectués dans la réserve de la Comoé et à Daloa. Les animaux ont été anesthésiés par dartage. 98 prélèvements et ensemencements KIVI ont été effectués à partir de :
- 9 hippotragues ;
- 10 buffles ;
- 18 cobes Defassa ;
- 54 cobes de Buffon ;
- 3 phacochères ;
- 3 éléphants ;
- 1 guib harnaché.

Résultats et discussion

Isolement des souches, résultats sérologiques et identification génétique des souches

Sur les 98 animaux sauvages prélevés, 88 étaient positifs au CATT. Cet examen sérologique révèle la présence de *T. b. gambiense*, bien que des réactions croisées avec *T. b. brucei* ou *T. congolense* aient été décrites.

Pour le test ELISA, il apparaît que la concordance est satisfaisante pour *T. congolense* entre ELISA et isoenzymes, ainsi qu'entre ELISA et CATT. Par contre, les résultats sont largement discordants pour *T. brucei* (en particulier les cobes de Buffon). Dans ce cas particulier de la détection de *T. brucei* chez l'animal sauvage, les résultats indiquent que le test ELISA n'est pas satisfaisant alors que le CATT apparaît beaucoup plus sensible.

Sur les 98 animaux prélevés, 16 présentaient des frottis positifs, 79 ont donné un KIVI positif et 19 des KIVI négatifs. Les 19 KIVI négatifs correspondent à 3 hippotragues, 1 buffle, 1 cobe Defassa, 9 cobes de Buffon, 2 phacochères et 3 éléphants.

L'examen microscopique des frottis effectués sur le terrain (16 animaux trypanosomés) révèlent la présence de trypanosomes du type *brucei* et *congolense*. Des formes plus grandes correspondraient à *T. vivax*.

Nous avons individualisé 37 zymodèmes.

Trois groupes s'individualisent :

- Un groupe *T. brucei* Bouaflé ;
- Un groupe *T. b. gambiense* ;
- Un groupe *T. congolense* .

Par ailleurs, 5 zymodèmes qui sont isolés de l'animal sauvage (cobe de Buffon, buffle et phacochère) ne se rattachent à aucun des groupes précités.

La seule lecture des distances génétiques permet de distinguer des lignées parasitaires très hétérogènes. Deux ensembles principaux de souches se distinguent aisément :
- Le premier regroupant les souches appartenant à l'espèce *T. brucei ;*
- Le second, génétiquement très distinct du précédent, rassemble les souches correspondant à l'espèce *T. congolense.* Cette hypothèse est corroborée par les résultats sérologiques ELISA, par la morphologie des trypanosomes, c'est-à-dire des formes courtes sans flagelle libre.

Conclusion

Le KIVI permet aussi l'isolement de *T. brucei spp.* et *T. congolense* chez l'animal domestique ou sauvage et la détection de ces parasites chez des animaux négatifs à l'examen parasitologique classique (utilité au diagnostic parasitologique complémentaire : publications 92-1, 97-1, 97-2).

Le CATT est recommandé pour le dépistage de *T. brucei* et l'ELISA pour *T. congolense* chez les animaux sauvages, le CATT pour *T. brucei* chez les animaux domestiques (publications 92-1, 94-2).

En Afrique de l'Ouest et Centrale, l'existence d'un réservoir animal domestique à THA est confirmée, celle d'un réservoir animal sauvage est démontrée (90-1, 91-1, 93-4, 97-1, 99-2).

Un groupe de zymodèmes s'individualise au sein de cet ensemble *brucei*. Il s'agit de trypanosomes isolés d'animaux sauvages uniquement, ne se rattachant pas de façon significative aux autres groupes. J'ai proposé de le nommer le groupe « Comoé » en regard de la provenance géographique des animaux concernés. Un « nouveau » groupe de *T. congolense* semble aussi avoir été identifié chez l'animal sauvage (publication 97-2).

Publications : 90-1, 91-1, 92-1, 93-4, 94-2, 97-1, 97-2, 99-2
Communications : 91-1, 92-1, 95-3, 97-2, 97-3

1-2-3 Chez la glossine

J'ai effectué la détection de trypanosomes par PCR et amorces spécifiques de *T. brucei* et *T. congolense* sur des glossines sauvages capturées dans un foyer de forte transmission en Côte d'Ivoire (publication 96-2). Une proportion importante d'infections mixtes a été détectée. Les conséquences de ce phénomène sont à étudier.

L'analyse des repas de sang pris par les glossines a fait l'objet de certains de mes travaux conduisant à la mise au point de deux nouvelles techniques (isoenzymes et PCR/HeteroDuplex Assay HDA, publications 97-3, 99-3). La technique de PCR/HDA a été modifiée puis évaluée avec l'Université d'Alabama (USA).

A/ Analyse des repas de sang de glossines par isoenzymes et PCR/HDA

Problématique

En matière d'épidémiologie de la THA, plusieurs paramètres peuvent être appréciés grâce à l'analyse des repas de sang des glossines. Dans un premier temps, pour calculer l'indice de risque de contamination en un point donné, il faut connaître le nombre de repas « humains » ou « non-humains » pris par des glossines à cet endroit. Une technique simple permettant de distinguer le sang humain de tous les autres types de sang de mammifères est nécessaire et suffisante.

Ensuite, pour les études sur le réservoir animal de THA, pour détecter tout changement de préférence trophique chez les glossines, ou pour préciser la transmission des trypanosomiases animales, une technique plus précise est nécessaire, permettant d'identifier l'espèce de l'hôte mammifère sur laquelle la glossine a pris son repas de sang. La technique PCR/HDA a été mise au point et testée avec succès sur des simulies, vectrices de l'onchocercose, en permettant avec une très grande précision et sensibilité d'identifier l'espèce de l'hôte mammifère. Cette dernière technique permet bien sûr de distinguer aussi les repas humains des non-humains.

Objectif

Mettre au point deux nouvelles techniques d'analyse des repas de sang de glossines adaptées au type de recherche effectué :
- Distinguer les repas pris sur l'homme des autres types de repas : mise au point et évaluation de la technique par isoenzyme.

- Identifier l'espèce de l'hôte sur laquelle la glossine s'est gorgée : mise et point et évaluation de la technique par PCR/HDA.

Méthodologie

1/ Isoenzymes : Révélation de la SOD

L'analyse qualitative d'une enzyme contenue en grande quantité dans les cellules sanguines, la SuperOxydeDismutase (SOD), a été adaptée par électrophorèse sur acétate de cellulose.

Les glossines sont disséquées. Le tube digestif est prélevé et déposé sur du papier Whatman. L'ensemble est stocké dans un bocal en verre renfermant du silicagel et conservé à 4°c jusqu'à utilisation. Sur chaque plaque, 22 repas sanguins sont analysés, plus deux témoins (sang humain et tube digestif de glossine non gorgée).

Les protocoles électrophorètiques et de coloration sont décrits par Diallo *et al.* (1997 : publication 97-3). La lecture doit se faire immédiatement après coloration.

2/ PCR/HDA

Les glossines sont placées dans un tube eppendorf sur le terrain et conservées à 4°c jusqu'au laboratoire. Ensuite, elles sont directement broyées sans être disséquées, puis le protocole classique d'extraction de l'ADN total est effectué, suivi de l'amplification par PCR et de la migration/révélation. Cette technique est basée sur l'amplification spécifique d'un fragment de 350 paires de bases du cytochrome B contenu dans le sang de l'ADN ingéré. Les produits d'amplification sont ensuite classés par la technique HDA. L'ADN sonde utilisé est soit celui du buffle, soit du chimpanzé. Après deux amplifications successives, des homoduplexes et des hétéroduplexes sont formés. Les hétéroduplexes migrant plus lentement que les homoduplexes sur un gel partiellement dénaturé de 5 % d'acrylamide enrichi en urée, il est possible de distinguer des changements d'une seule paire de base sur une séquence totale d'environ 550 paires de bases (publication 99-3).

Résultats et Discussion

Isoenzymes

Dans un premier temps, une étude sur la variabilité de la SOD au sein des populations humaines et glossiniennes a été menée. Cette variabilité est pratiquement nulle, ce qui permet une localisation spécifique des patterns SOD du sang humain et des tissus de la glossine, nettement différenciables entre eux.

Par contre, une grande variabilité existe entre les différentes espèces de mammifères. La SOD ainsi révélée (exemples : bétail, porc, mouton, cheval et rongeurs) migre dans tous les cas moins rapidement que la SOD humaine et que la SOD glossinienne. Il est donc aisé de distinguer l'origine humaine des repas de sang par la seule révélation de la SOD. En Côte d'Ivoire, sur 2800 analyses effectuées, 30 % sont d'origine humaine, 60 % d'origine animale et 10 % n'ont pas permis d'identification. Dans ce dernier cas, il est probable que la dégradation de l'enzyme durant la digestion du sang par l'insecte en soit probablement responsable. A ce jour, plus de 4000 analyses ont été effectuées, et les résultats sont concordants avec les valeurs de l'indice de risque de contamination calculé en partie grâce à ces données.

PCR/HDA

Les primers utilisés pour chaque amplification ont été choisis de façon à n'amplifier que l'ADN de sang de mammifères (exemples : porc, bétail, bubale, antilope, homme, rongeur sauvage et rongeur domestique) et pas d'insectes. Chaque hétéroduplexe observé est spécifique d'une espèce mammifère.

De la même manière que pour la SOD, une étude préliminaire de la variabilité par HDA au sein d'une population humaine a été conduite afin de valider la stabilité du marqueur génétique. Une légère variabilité a été décelée, et deux types humains A et B ont été retenus comme témoins.

Ensuite, il était nécessaire de vérifier après combien de temps suivant le repas de sang la technique HDA permettait encore une identification de l'hôte. Jusqu'à 72 heures suivant le repas de sang, il est possible de l' identifier. Ces résultats sont identiques à ceux qui sont obtenus pour les simulies, vectrices de l'onchocercose.

Enfin, sur un échantillonnage de glossines capturées dans la région de Sinfra en Côte d'Ivoire, cette technique a permis de distinguer des repas de sang pris sur le porc et l'homme. Dans un but de vérification, l'un des produits d'amplification identifié comme provenant de porc a été séquencé. Sa séquence correspond à 100 % avec la séquence connue et publiée de cette partie du gène pour le porc.

Conclusion

Bien que la technique HDA devrait être testée encore sur d'autres glossines de foyers différents, les deux techniques SOD et HDA sont complémentaires. La SOD permet à moindre coût et rapidement de distinguer les repas de sang humain de non-humain (publication 97-3), en particulier afin de calculer l'indice de risque de contamination (publication 97-4). La PCR/HDA permet de distinguer très spécifiquement l'espèce hôte en évitant la dissection de la glossine (publication 99-3), et devrait faciliter les études sur le réservoir animal à THA et sur la biologie comportementale des glossines (changement de régime alimentaire).

Publications : 96-2, 97-3, 97-4, 99-3
Communications : 97-1, 97-3, 98-2

2- Perspectives de recherche

Les problèmes techniques sur le plan du dépistage, du diagnostic et du traitement des malades, et évidemment la gravité de la situation actuelle de la THA en Afrique, nous obligent à mener en priorité des recherches opérationnelles afin d'élucider le maximum de problèmes précédemment évoqués et de fournir rapidement des outils fiables de détection et de suivi post-thérapeutique des malades aux Programmes nationaux de lutte (PNLT). Les produits de ces recherches, menées en étroite collaboration avec des partenaires Sud et Nord, seront transférés sous forme de formation des chercheurs africains et des responsables techniques des PNLT.

En ce sens, trois projets de recherche pour lesquels je suis responsable ou collaborateur sont en cours :

- L'évaluation et l'amélioration des méthodes de dépistage et de diagnostic en Afrique Centrale et Occidentale (financement FAC/OMS) ;
- La détermination des zones à risque de transmission de THA selon les densités de campements en zone forestière de Côte d'Ivoire (financement FAC/OMS).
- Une étude sur les facteurs biologiques impliqués dans les cas d'échec du traitement par mélarsoprol en Angola (financement FAC/OMS/STI, sous la responsabilité du Swiss Tropical Institute).

Ensuite, il est nécessaire d'effectuer des études sur la réelle pathogénicité des trypanosomes africains, sur le schéma suivi en Côte d'Ivoire, afin d'apprécier l'importance de l'asymptomatisme et des infections transitoires non pathogènes, et enfin l'éventualité d'une forme aiguë de la maladie en Afrique Centrale, région la plus touchée par la THA.

3- Application et valorisation des résultats de la recherche

- Le KIVI est considéré comme la technique de référence pour l'isolement de terrain et comme outil de diagnostic parasitologique complémentaire de la THA (OMS, Rapport du Comité d'Experts, 1995).

- Le mode de prélèvement sanguin au pli du coude permet d'optimiser le diagnostic parasitologique.

- La minicolonne « Pasteur », par son coût réduit, devrait permettre aux PNLT et aux équipes de recherche de diminuer le coût global des prospections médicales sur le terrain.

- L'utilisation de la PCR pour déterminer la période de la maladie et l'efficacité du traitement des malades en seconde période, devrait être évaluée à grande échelle. S'il se confirme que cet outil est utile, en particulier pour la détection précoce des échecs au traitement, il faudra vulgariser la technique comme test de laboratoire classique, et le transférer dans les cliniques concernées en assurant la formation du personnel.

- Le Compact Disk sur la THA conçu par l'OMS et pour lequel j'ai contribué à la conception, sera largement distribué pour aider les étudiants, les élèves, les chercheurs et toutes les personnes impliquées dans les Programmes de lutte contre la THA.

- Les techniques d'analyses des repas de sang devraient permettre de faciliter les recherches sur la transmission et les réservoirs animaux de la THA.

4- Diffusion de l'information

1- Liste des publications

99-1 **Truc P.**, Jamonneau V., Cuny G. & Frézil J.L.. Polymerase Chain Reaction for Human African Trypanosomiasis stage determination and follow-up. *Bulletin of the World Health Organization,* sous presse.

99-2 Gibson W. C., Stevens J. & **Truc P.** (1999). Identification of trypanosomes: from morphology to molecular biology.: "*Progress in Human African Trypanosomiasis sleeping sickness*", Springer Publ., Paris, 7-29.

99-3 Boakye D., Tang J., **Truc P.**, Merriweather A. & Unnasch T. (1999). Identification of blood meals in hematophagous Diptera by polymerase chain reaction and heteroduplex analysis. *Medical and Veterinary Entomology*, sous presse.

99-4 Diallo P. B., **Truc P.,** Méda H. A. & Kamenan A. (1999). Diagnostic sérologique de la Trypanosomiase Humaine Africaine à *Trypanosoma brucei gambiense*. 2- Obtention et utilisation des glycoprotéines de surface des trypanosomes pour améliorer la spécificité et la sensibilité des techniques ELISA et THP. *Bulletin de la Société de Pathologie Exotique*, sous presse.

99-5 Biteau N., Bringaud F., Gibson W.C., **Truc P.** & Baltz T. (1999). Characterization of *Trypanozoon* isolates using genetic micro- and minisatellite markers. *Molecular and Biochemical Parasitology*, sous presse.

98-1 **Truc P.**, Jamonneau V., N'Guessan P., Diallo P.B. & Bustigier X. (1998). Simplification of the mini-anion exchange technique for the parasitological diagnosis of Human African Trypanosomiasis. *Transactions of the Royal Society of Tropical Medicine and Hygiene*, 92, 5, 512.

98-2 **Truc P.**, Jamonneau V., N'Guessan P., N'Dri L., Diallo P. B. & Cuny G. (1998). *Trypanosoma brucei* spp. and *Trypanosoma congolense* : mixed human infection in Côte d'Ivoire. *Transactions of the Royal Society of Tropical Medicine and Hygiene*, 92, 5, 537-538.

98-3 **Truc P.**, Jamonneau V., N'Guessan P., Diallo P.B. & Garcia A. (1998). Parasitological diagnosis of human African trypanosomiasis : a comparison of the QBC® and the miniature anion-exchange centrifugation technique. *Transactions of the Royal Society of Tropical Medicine and Hygiene*, 92, 288-289.

97-1 **Truc P.**, Formenty P., Diallo P. B., Komoin-Oka C.& Lauginie F. (1997) Confirmation of two distinct classes of zymodemes of *Trypanosoma brucei* infecting patients and wild mammals in Côte d'Ivoire : suspected difference in pathogenicity. *Annals of Tropical Medicine and Parasitology*, 91, 8, 951-956.

97-2 **Truc P.**, Formenty P., Duvallet G., Komoin-Oka C., Diallo P. B. & Lauginie F. (1997). Identification of trypanosomes isolated by KIVI from wild mammals in Côte d'Ivoire : diagnostic, taxonomic and epidemiological considerations. *Acta Tropica*, 67, 187-196.

97-3 Diallo P. B., **Truc P.** & Laveissière C. (1997). A new method for identifying blood meals of human origin in tsetse flies. *Acta Tropica*, 63, 61-64.

97-4 Laveissière C., Sané B., Diallo P. B. & **Truc P.** (1997). Le risque épidémiologique dans un foyer de maladie du sommeil en Côte d'Ivoire.*Tropical Medicine and International Health*, 2, 8, 729-732.

96-1 **Truc P.**. A miniature kit for the *in vitro* isolation of *Trypanosoma brucei gambiense* : a preliminary field assessment on sleeping sickness patients in Côte d'Ivoire (1996). *Transactions of the Royal Society of Tropical Medicine and Hygiene*, 90, 246-247.

96-2 Masiga D. K., McNamara J., Laveissière C., **Truc P.** & Gibson W.C. (1996). A high prevalence of mixed trypanosome infections in tsetse flies in Sinfra, Côte d'Ivoire, detected by DNA amplification. *Parasitology*, 112, 75-80.

96-3 Diallo P. B., **Truc P.**, Méda H. A. & Kamenan A. (1996). Diagnostic sérologique de la Trypanosomiase Humaine Africaine à *Trypanosoma brucei gambiense*. 1- Obtention et utilisation d'antigènes bruts dans les tests ELISA et d'agglutination au latex. *Bulletin de la Société de Pathologie Exotique*, 89, 262-268.

94-1 **Truc P.**, Bailey W., Doua F., Laveissière C. & Godfrey D. G. (1994). A comparison of parasitological methods for the diagnosis of gambian trypanosomiasis in an area of

low endemicity in Côte d'Ivoire. *Transactions of the Royal Society of Tropical Medicine and Hygiene*, 88, 419-421.

94-2 Komoin-Oka, C., **Truc P.**, Bengaly, Z., Formenty, P., Duvallet, G., Lauginié, F., Raath, J. P., N'Depo, A. E. & Leforban, Y. (1994). Etude de la prévalence des infections à trypanosomes chez différentes espèces d'animaux sauvages du parc national de la Comoé en Côte d'Ivoire : résultats préliminaires sur la comparaison de trois méthodes de diagnostic. *Revue Elevage et Médecine vétérinaire des Pays tropicaux*, 47, 2, 189-194.

94-3 Gashumba J., Komba E. K., **Truc P.**, Allingham R., Ferris V. & Godfrey D. G. (1994). The persistence of genetic homogeneity among *Trypanosoma brucei rhodesiense* isolates from patients in north-west Tanzania. *Acta Tropica*, 56, 341-348.

93-1 Ben Abderrazak, S., Guerrini, F., Mathieu Daudé, **Truc P.**, Neubauer, K., Lewicka, K., Barnabé, C. & Tibayrenc, M., (1993). Isoenzyme electrophoresis for Parasite characterization. In : *Protocols in Molecular Parasitology, The Humana Press,* 21, 27, 361- 382.

93-2 Authie, E., Cuisance, D., Force-Barge, P., Frézil, J. L., Gouteux, J. P., Jannin, J., Lancien, J., Lavoissière, C., Lemesre, J. L., Mathieu-Daudé, F., Nitcheman, S., Noireau, F., Penchenier, L., Tibayrenc, M. & **Truc P**. (1993). Some new prospects in epidemiology and fight against human African trypanosomiasis. *Research and Reviews in Parasitology*, 51 (1-4): 29-46.

93-4 **Truc P**. & Tibayrenc, M. (1993) Population genetics of *Trypanosoma brucei* in Central Africa : taxonomic and epidemiological significance. *Parasitology*, 106, 137-149.

92-1 **Truc P.,** Aerts D., McNamara J. J., Claes Y., Allingham R., Le Ray D. & Godfrey D.(1992). The direct *in vitro* isolation of *Trypanosoma brucei* from man and animals, and its potential value for the diagnosis of Gambian trypanosomiasis. *Transactions of the Royal Society of Tropical Medicine and Hygiene*, 86, 627-629.

92-2 Aerts, D., **Truc P.**, Penchenier, L., Claes Y. & Le Ray, D., (1992). A kit for *in vitro* isolation of trypanosomes in the field : First trial with sleeping sickness patients in the Congo. *Transactions of the Royal Society of Tropical Medicine and Hygiene*, 86, 394-395.

91-1 **Truc P.**, Mathieu Daudé, F. & Tibayrenc, M., (1991). Multilocus isoenzyme identification of *Trypanosoma brucei* stocks isolated in Central Africa: evidence for an animal reservoir of sleeping sickness in Congo. *Acta Tropica*, 49, 127-135.

91-2 **Truc P.**, Rioux, J. A. & Pratlong, F., (1991). A propos du trypanosome du Blaireau : *Trypanosoma pestanai* Bettencourt et Franca, 1905 est-il seul en cause? *Annales de Parasitologie Humaine et Comparée,* 66, 1, 45-46.

90-1 **Truc P.**, Mathieu Daudé, F. & Tibayrenc, M, (1990). Etude isoenzymatique de *Trypanosoma brucei* en Afrique Centrale: corollaires épidémiologiques. *Bulletin de la Société Française de Parasitologie*, 8, 257.

90-2 Mathieu Daudé, F., **Truc P.** & Tibayrenc, M., (1990). Diversité génétique comparée des taxons *Trypanosoma brucei* s. l. et *Trypanosoma cruzi. Bulletin de la Société Française de Parasitologie* , 8, 247.

2- Publications soumises

Jammoneau V., **Truc P.**, Büscher P., Magnus E. & Van Meirvenne N. Evaluation of the Latex/*T. b. gambiense* test and others alternatives of the TestrypCATT/*T. b. gambiense* for diagnosis of Human African Trypanosomiasis in Côte d'Ivoire.

3- Publications en préparation

Oury B., Jamonneau V., Tibayrenc M. & **Truc P.** Detection *of Trypanosoma brucei gambiense* multiple infections by mutiprimers RAPD fingerprinting in patients in Côte d'Ivoire.

Diplôme d'Etudes Approfondies

(1987). Morphométrie et électrophorèse de quelques *Trypanosoma* et *Sauroleishmania.*

Thèse de Doctorat d'Université

(1991). Apport de la Génétique des Populations à la taxonomie de *Trypanosoma brucei* et à l'épidémiologie de la Trypanosomiase Humaine en Afrique Centrale. Thèse de Doctorat, Université de Montpellier II, France.

3- Communications à des Séminaires ou Congrès Internationaux

99-1 **Truc P.,** Jamonneau V., Tibayrenc M. & Oury B. Molecular epidemiology of Human African Trypanosomiasis. 4th International workshop on Molecular Epidemiology and Evolutionary Genetics of Pathogenic Microorganisms. CDC/CNRS/ORSTOM, Dakar, Sénégal (juin 1999).

99-2 Biteau N., Bringaud F., Gibson W.C., **Truc P**. & Baltz T. Caractérisation des isolats de trypanosomes à l'aide de marqueurs de polymorphisme. IIème Réunion Biennale de Parasitologie CNRS/DGA/DCSSA/MENESR, Cibles thérapeutiques et vaccinales en parasitologie: paludisme, leishmanioses, trypanosomes, schistosomiase, Montpellier, France (février 1999).

98-1 **Truc P.**, Jamonneau V. & Oury B. Genetic and Pathogenesis of African trypanosomes in man. International Colloquium « Sleeping sickness rediscovered », Anvers, Belgique (décembre 1998).

98-2 **Truc P.**, Merriweather A., Diallo B.P., Jamonneau V. & Unnasch T. R.. Molecular biology: a new tool for identifying blood meals in tsetse flies, vector of sleeping sickness in Africa. IX International Congress of Parasitology (ICOPA), Makuhari Chiba, Japon (août 1998).

98-3 **Truc P.**, Jamonneau V., N'Guessan P., N'Dri L. & Diallo P.B.. Pathogenicity of African trypanosomes: a new approach of the epidemiology of gambian sleeping sickness. IX International Congress of Parasitology (ICOPA), Makuhari Chiba, Japon (août 1998).

98-4 **Truc P.,** Jamonneau V., N'Guessan P., N'Dri L., Diallo P.B & Garcia A.. *Trypanosoma brucei gambiense* is not the only agent of the Human African Trypanosomiasis in West Africa. *Third International workshop on Molecular Epidemiology and Evolutionary Genetics of Pathogenic Microorganisms*. CDC/CNRS/ORSTOM, Rio de Janeiro, Brézil (juin 1998).

97-1 **Truc P.,** Merriweather A., Diallo P.B. & Unnasch T.R.. Isoenzyme and PCR for identifying blood meals in tsetse flies. *Proceedings of the 24rd ISCTRC Meeting (International Scientific Council for Trypanosomiasis Research and Control)*, Maputo, Mozambique, septembre 1997), 119, 166-169.

97-2 **Truc P.**, Diallo P.B. & Jamonneau V.. Identification of different classes of zymodemes of *Trypanosoma brucei* s. p. circulating in patients and wild mammals in West Africa. *International workshop on Molecular Epidemiology and Evolutionary Genetics of Pathogenic Microorganisms*. CDC/CNRS/ORSTOM, ORSTOM Montpellier, France (mai 1997).

97-3 **Truc P.**, Diallo P.B. & Jamonneau V.. Circulation chez l'homme, la glossine et le porc d'un même zymodème non gambiense groupe 1 en Côte d'Ivoire: conséquences en matière de contrôle de la THA. XVIIIème Conférence Technique de l'OCEAC, Yaoundé, Cameroun (Mai 1997).

96-1 Baker J.R. & **Truc P.**. Human African Trypanosomiasis: towards an epidemic pattern in Africa. *International workshop on Molecular Epidemiology and Evolutionary Genetics of Pathogenic Microorganisms*. Center for Disease Control and prevention, Atlanta, USA (juin 1996).

95-1 **Truc P.** , Diallo P. B. & Godfrey D.G.. Genetic identification and pathogenicity of *Trypanosoma brucei* s. l. in Man: an acute form of HAT is suspected in Côte d'Ivoire. *Proceedings of the 23rd ISCTRC Meeting (International Scientific Council for Trypanosomiasis Research and Control)*, Banjul, Gambia, 118, 128 (1995).

95-2 **Truc P.**, Diallo P. B., N'Guessan P. & Le Ray D..The kit for *in vitro* isolation of african trypanosomes (KIVI): a new simple procedure for a field use. *Proceedings of the 23rd ISCTRC Meeting (International Scientific Council for Trypanosomiasis Research and Control)*, Banjul, Gambia, 118, 143 (1995).

95-3 **Truc P.**. Genetic and Trypanosomiasis: epidemiological consequences. Malaria Division, OMS/WHO Center, Faculty of Sciences, Chulalongkorn University, Bangkok, Thailande (août 1995).

93-1 **Truc P.**, Formenty P., Godfrey D.G, Le Ray D.. The kit for *in vitro* isolation of African trypanosomes: diagnosis and new prevalence of human trypanosomiasis *Proceedings of the 22rd ISCTRC Meeting (International Scientific Council for Trypanosomiasis Research and Control)*, Kampala, Uganda, 117, 42 (1993).

92-1 **Truc P.**. Populations Genetics of *Trypanosoma brucei* and epidemiology of sleeping sickness in the Congo Republic, Université de Californie, Irvine, USA (avril 1992).

91-1 **Truc P**.. Isoenzyme characterization of *Trypanosoma brucei* stocks isolated from Congo and Zaire: epidemiological significance. *Proceedings of the 21rd ISCTRC Meeting (International Scientific Council for Trypanosomiasis Research and Control)*, Yammoussoukro, Côte d'Ivoire, 116, 248 (1991).

5- Formation

A- Cours d'enseignement supérieur dispensés

- Advanced Methods in Medical and Veterinary Vector Control, Université de Bristol, Royaume-Uni (1991/92).

- Cours d'Isoenzymologie appliquée : applications à l'étude des maladies parasitaires humaines, Institut Pierre Richet, Bouaké, Côte d'Ivoire (1994).

- Cours de Protozoologie Médicale : Systématique et biologie moléculaire appliquées à l'étude de parasitoses humaines à transmission vectorielle. Diplôme d'Etudes Approfondies en Entomologie Médicale, Centre Universitaire de Formation en Entomologie Médicale et Vétérinaire, Bouaké, Côte d'Ivoire (1994).

B- Formation et encadrement d'étudiants et chercheurs

1. Entre 1993 et 1998, j'ai participé à la formation de deux chercheurs de l'IPR en thèse de doctorat, et de plusieurs étudiants qui ont été accueillis au sein du laboratoire. Ces derniers ont intégré l'équipe « THA » et ils ont participé ainsi à un travail multidisciplinaire sur le diagnostic et l'épidémiologie, ainsi que sur l'identification génétique des trypanosomes.

1-A Thèses de doctorat

- Un chercheur sénégalais: Papa Boubacar Diallo, dans le cadre de la préparation d'une thèse de troisième cycle (Université des Sciences d'Abidjan, Coccody, Côte d'Ivoire, Directeur de thèse: Pr Diopoh Kore Jacques), qu'il a passé avec succès et félicitations du jury.

J'ai initié ce chercheur à l'utilisation et l'évaluation des nouveaux outils de diagnostic, à l'isolement de terrain et la culture *in vitro* des trypanosomes, à l'électrophorèse d'isoenzymes grâce à laquelle nous avons mis au point une nouvelle technique d'analyse de repas de sang de glossines, et identifier de nouveaux génotypes virulents chez l'homme. La formation de ce chercheur ne s'est donc pas limitée à son travail de thèse, mais à de nombreux aspects de l'épidémiologie de la THA car il devait prendre ma succession à la direction du laboratoire après mon départ en 1998.

Titre de la thèse :

Utilisation des fractions antigéniques des trypanosomes pour le diagnostic de la Trypanosomiase Humaine Africaine (THA), 1993, Université d'Abidjan (Côte d'Ivoire).

Publications :

Diallo P. B., Truc P., Méda H. A. & Kamenan A. (1999). Diagnostic sérologique de la Trypanosomiase Humaine Africaine à *Trypanosoma brucei gambiense*. 2- Obtention et utilisation des glycoprotéines de surface des trypanosomes pour améliorer la spécificité et la sensibilité des techniques ELISA et THP. *Bulletin de la Société de Pathologie Exotique*, sous presse.

Diallo P. B., Truc P. & Laveissière C. (1997). A new method for identifying blood meals of human origin in tsetse flies. *Acta Tropica*, 63, 61-64.

Diallo P. B., Truc P., Méda H. A. & Kamenan A. (1996). Diagnostic sérologique de la Trypanosomiase Humaine Africaine à *Trypanosoma brucei gambiense*. 1- Obtention et utilisation d'antigènes bruts dans les tests ELISA et d'agglutination au latex. *Bulletin de la Société de Pathologie Exotique*, 89, 262-268.

Truc P., Jamonneau V., N'Guessan P., **Diallo P.B.** & Garcia A. (1998). Parasitological diagnosis of human African trypanosomiasis : a comparison of the QBC® and the miniature anion-exchange centrifugation technique. *Transactions of the Royal Society of Tropical Medicine and Hygiene*, 92, 288-289.

Truc P., Jamonneau V., N'Guessan P., **Diallo P.B.** & Bustigier X. (1998). Simplification of the mini-anion exchange technique for the parasitological diagnosis of Human African Trypanosomiasis. *Transactions of the Royal Society of Tropical Medicine and Hygiene*, 92, 5, 512.

Truc P., Jamonneau V., N'Guessan P., N'Dri L., **Diallo P. B.** & Cuny G. (1998). A mixed infection in man with *Trypanosoma brucei* spp. and *Trypanosoma congolense* in Côte d'Ivoire : a case report. *Transactions of the Royal Society of Tropical Medicine and Hygiene*, 92, 5, 537-538.

Laveissière C., Sané B., **Diallo P. B.** & Truc P. (1997). Le risque épidémiologique dans un foyer de maladie du sommeil en Côte d'Ivoire. *Tropical Medicine and International Health*, 2, 8, 729-732.

Truc P., Formenty P., **Diallo P. B.**, Komoin-Oka C.& Lauginie F. (1997) Confirmation of two distinct classes of zymodemes of *Trypanosoma brucei* infecting patients and wild mammals in Côte d'Ivoire : suspected difference in pathogenicity. *Annals of Tropical Medicine and Parasitology*, 91, 8, 951-956.

Truc P., Formenty P., Duvallet G., Komoin-Oka C., **Diallo P. B**. & Lauginie F. (1997). Identification of trypanosomes isolated by KIVI from wild mammals in Côte d'Ivoire : diagnostic, taxonomic and epidemiological considerations. *Acta Tropica*, 67, 187-196.

• Un second chercheur sénégalais : Bocar Sané, dans le cadre de la préparation d'une thèse de troisième cycle (Faculté des Sciences d'Abidjan, Coccody, Côte d'Ivoire, Directeur de thèse : Pr Foua Bi Kouaho), qu'il a passé avec succès et félicitations du jury.

Avec cet étudiant, nous avons insisté sur l'aspect entomologique de la transmission de la THA. En l'initiant à la nouvelle technique d'analyse de repas de sang de glossines mise au point avec P.B. Diallo, et en évaluant ensemble cette technique sur le terrain, il a pu ensuite différencier les repas de sang « humains » des « non humains », permettant ainsi de calculer l'index de risque de contamination par la THA mis au point par Claude Laveissière (cf. Laveisssière et al., 1997).

Titre de la thèse :

Contribution à l'étude du rôle épidémiologique et au contrôle de *Glossina palpalis palpalis* (Robineau-Desvoidy, 1830) dans la région de Zoukougbeu, 1997 (Faculté des Sciences d'Abidjan, Côte d'Ivoire).

Publication :

Laveissière C., **Sané B.,** Diallo P. B. & Truc P. (1997). Le risque épidémiologique dans un foyer de maladie du sommeil en Côte d'Ivoire. *Tropical Medicine and International Health*, 2, 8, 729-732.

• Un étudiant français ORSTOM : Vincent Jamonneau. Ce stagiaire a été formé et encadré pour sa thèse de doctorat (Université de Montpellier II, Directeur de thèse Dr J.L. Frézil, Directeur de recherche IRD Montpellier, France) au sein du laboratoire. Vincent Jamonneau est actuellement en troisième année de thèse.

J'ai initié Vincent Jamonneau à plusieurs techniques d'isolement et de culture *in vitro* des trypanosomes, à l'épidémiologie de la THA (dépistage, diagnostic, pathogénicité, réservoir animal). Son travail comporte une composante terrain importante, en particulier dans le cadre de sa thèse, par le suivi longitudinal avant et après traitement des malades. Nous avons ensemble étudié de nombreux cas atypiques de THA (forme aiguë, guérison spontanée), en collaboration avec les Universités de Limoges (Pr Dumas), de Bordeaux (Pr Vincendeau) et l'Institut de Médecine tropicale d'Anvers (Belgique : Pr Büscher). Ce travail, corrélé à l'identification génétique des souches, constitue la partie principale de son travail de thèse.

Titre de la thèse :

Apport des outils biomoléculaires au diagnostic de la THA et à l'étude de la pathogénicité des trypanosomes africains chez l'homme.

Publications :

Truc P., **Jamonneau V.**, Cuny G. & Frézil J.L. (1999). Polymerase Chain Reaction for Human African Trypanosomiasis stage determination and follow-up. *Bulletin of the World Health Organization*, in press.

Truc P., **Jamonneau V.**, N'Guessan P., Diallo P.B. & Garcia A. (1998). Parasitological diagnosis of human African trypanosomiasis: a comparison of the QBC® and the miniature anion-exchange centrifugation technique. *Transactions of the Royal Society of Tropical Medicine and Hygiene*, 92, 288-289.

Truc P., **Jamonneau V.**, N'Guessan P., Diallo P.B. & Bustigier X. (1998). Simplification of the mini-anion exchange technique for the parasitological diagnosis of Human African Trypanosomiasis. *Transactions of the Royal Society of Tropical Medicine and Hygiene*, 92, 5, 512.

Truc P., **Jamonneau V.**, N'Guessan P., N'Dri L., Diallo P. B. & Cuny G. (1998). *Trypanosoma brucei* ssp. and *Trypanosoma congolense* : mixed infection in Côte d'Ivoire. *Transactions of the Royal Society of Tropical Medicine and Hygiene*, 92, 5, 537-538.

• Une étudiante ivoirienne : Murielle Koffi, dans le cadre de la préparation d'une thèse de troisième cycle (Université des Sciences d'Abidjan, Coccody, Côte d'Ivoire, Directeur de thèse Pr Yao N'Guessan Thomas). Pour des raisons familiales, cette étudiante a abandonné sa préparation de thèse après 18 mois passés au laboratoire.

Nous avons effectué des extractions aqueuses et alcooliques à partir de feuilles, de tiges et de racines de plantes connues pour leurs effets trypanocides (selon les tradipraticiens comme *Detarium microcarpum* ou *Fagara xantixyloides*), puis effectué des tests de sensibilité *in vitro* sur des souches de *T. b. gambiense* en dilutions décroissantes. Malgré des résultats encourageants, ces travaux n'ont pu être poursuivis pour la raison évoquée dans le précédent paragraphe.

Titre de la thèse :

Rôle des extraits végétaux issus de la Médecine traditionnelle Africaine dans le traitement de la Trypanosomiase Humaine Africaine.

• Deux étudiants ivoiriens (Mrs Aguido Apende Abraham et Konan Yao Jacques) de la Faculté de Pharmacie d'Abidjan (responsables de thèse : Dr Jean Frédéric Brunel et Pr Djénéba Koné) ont effectué des stages de formation à la culture *in vitro* des trypanosomes et à l'action trypanocide *in vitro* d'extraits végétaux issus de la Pharmacopée traditionnelle africaine. Ils ont ensuite soutenu des thèses d'exercice, essentiellement bibliographiques sur la Pharmacopée traditionnelle africaine dont les titres et exemplaires ne m'ont jamais été communiqués.

1- B Diplôme d'Etudes Approfondies (DEA)

• Un étudiant français ORSTOM : Vincent Jamonneau. Ce stagiaire a été formé et encadré pour son stage de DEA de Parasitologie (Université de Montpellier II, responsable Dr J.L. Frézil, Directeur de recherche IRD Montpellier, France) au sein du laboratoire.

J'ai initié Vincent Jamonneau à toutes les techniques de dépistage et de diagnostic, de la THA. Son travail était d'améliorer le diagnostic sérologique de la THA sur le terrain.

Titre du DEA de Parasitologie :

Évaluation préliminaire d'une nouvelle technique de dépistage de la Trypanosomiase Humaine Africaine en Côte d'Ivoire : le TestrypCATT sur Latex, 1996 (Université Montpellier II).

Publication :

Jamonneau V., Truc P., Büscher P. & Magnus E.. Preliminary evaluation of Latex/*T. b. gambiense* alternative versions for mass screening of Human African Trypanosomiasis in Côte d'Ivoire. *Acta Tropica*, soumis pour publication.

1-C. Maîtrise

Entre 1994 et 1998, j'ai participé à la formation de trois étudiants soit à Bouaké, soit en stage au centre ORSTOM de Montpellier. Ces trois étudiants se sont familiarisés à la culture *in vitro* des trypanosomes, et à leurs identifications génétiques par isoenzymes et PCR/RAPD.

Maîtrise de Biologie des populations et des Ecosystèmes, option Parasitologie (Université d'Orléans, Vincent Jamonneau, 1994, responsable de stage Pr Kuentin) : Culture *in vitro* et variabilité génétique de *Trypanosoma brucei gambiense* .

Maîtrise de Biologie des populations et des Ecosystèmes, option Parasitologie (Université de Montpellier II, David Courtin, 1997/98, responsable de stage Dr Bruno Oury) : Diversité génétique de *Trypanosoma brucei gambiense*, agent responsable de la maladie du sommeil chez l'homme en Côte d'Ivoire.

Maîtrise de Biologie cellulaire et physiologie, mention Génétique moléculaire (Université de Rennes I : Edgar Brice Ngoungou, 1997/98, responsable de stage Dr Bruno Oury): Caractérisation génétique de *Trypanosoma brucei gambiense*, agent responsable de la maladie du sommeil chez l'homme en Côte d'Ivoire.

2- Formation et encadrement de professionnels pour la lutte contre la THA

Le laboratoire de l'IPR est toujours le laboratoire de référence pour l'Afrique de l'Ouest dans le cadre du Programme International de Surveillance et de lutte contre la THA, sous le contrôle de l'OMS. Il a été complètement rénové et équipé. Il est opérationnel pour :

- La culture *in vitro* ;
- La fabrication des milieux de culture ;
- L'identification biomoléculaire des trypanosomes (isoenzymes et PCR) ;
- La fabrication des tests mAECT pour le diagnostic de la THA ;
- Le diagnostic de laboratoire et de terrain de la THA ;
- L'analyse des repas de sang de glossines ;
- La saisie et gestions des données par informatique ;
- La cryobanque de référence de stocks de trypanosomes humains et animaux.

J'ai contribué à la formation du personnel du laboratoire de l'IPR que j'ai dirigé pendant 6 ans. Les principaux résultats sont :

- Mon successeur, chercheur africain senior, Papa Boubacar Diallo, a obtenu une source de financement pour sa recherche personnelle, a publié plusieurs articles scientifiques et a participé à de nombreuses présentations dans des séminaires ou congrès internationaux ;

- L'ingénieur de recherche africain, Paul N'Guessan, maitrise parfaitement toutes les techniques nécessaires au fonctionnement du laboratoire. Cet ingénieur a pu suivre en particulier une formation de deux mois à l'Institut de Médecine Tropicale d'Anvers en Belgique, qui lui a permis de se perfectionner, lui donnant ainsi de nouveaux atouts pour l'évolution de sa carrière scientifique ;

- Le technicien de laboratoire, Louis N'Dri, a atteint un bon niveau scientifique et technique et peut aider l'ingénieur précité dans le fontionnement du laboratoire ;

- L'auxiliaire de laboratoire, Alain N'Goran, formé aux différentes techniques de base, est chargé en particulier de la production des tests mAECT et de la supervision de l'animalerie.

J'ai participé à la formation des responsables des programmes nationaux de lutte contre la THA d'Afrique de l'Ouest aux techniques de surveillance et de dépistage de la THA. J'ai participé aussi à la formation des responsables des PNLT « Afrique Centrale » à l'OCEAC de Yaoundé (Cameroun).

6- Administration et Direction de la recherche

Implications dans des programmes de Recherche

- Étude de la technique du microCATT dans 4 foyers de Trypanosomiase Humaine en Afrique de l'Ouest et en Afrique Centrale. Financement "Fonds d'Aide à la Coopération", Ministère des Affaires Etrangères, Secrétariat d'Etat à la Coopération et à la Francophonie, France, 1998/99 (responsable de programme).

- Investigations on unsual Sleeping Sickness Cases Refractory to treatment with Melarsoprol. Pharmacological, immunological and biological investigations. Financement FAC/OMS/STI, sous la responsabilité du Swiss Tropical Institut, Angola, 1998/99 (collaborateur).

- Délimitation des zones à risques de Maladie du Sommeil d'après les densités de campements. Région forestière de Côte d'Ivoire. "Fonds d'Aide à la Coopération", Ministère des Affaires Etrangères, Secrétariat d'Etat à la Coopération et à la Francophonie, 1998/1999 (collaborateur).

- Pathogénicité et variants génétiques de *Trypanosoma brucei* chez l'Homme en Côte d'Ivoire : conséquences épidémiologiques. Financement "Fonds d'Aide à la Coopération", Ministère des Affaires Etrangères, Secrétariat d'Etat à la Coopération et à la Francophonie, France, 1996/1998 (responsable de programme).

- Étude longitudinale de sujets séropositifs au CATT sans confirmation parasitologique : signification, stabilité de la séropositivité dans le temps et recherche de concentration familiale de sujets séropositifs. "Fonds d'Aide à la Coopération", Ministère des Affaires Etrangères, Secrétariat d'Etat à la Coopération et à la Francophonie, France, 1996/1998 (collaborateur).

- Détermination et prédiction de l'émergence des zones à risques de maladie du sommeil. De la télédétection à l'action. Financement "Organisation Mondiale de la Santé, Recherches sur les Maladies Tropicales", Genève, Suisse, 1996/97 (collaborateur).

- Dispersion de *Glossina palpalis palpalis* et dissémination du trypanosome dans le foyer de maladie du sommeil de Zoukougbeu (Côte d'Ivoire). Financement "ORSTOM", Paris, 1995/96 (collaborateur).

- Étude de la circulation des trypanosomes, agents de la maladie du sommeil, par marqueurs génétiques, entre les différents foyers de Côte d'Ivoire : conséquences en matière de contrôle et de lutte. Financement "ORSTOM", Paris, 1995/96 (collaborateur).

- Installation d'un Laboratoire d'Isoenzymologie et de biologie moléculaire à l'Institut Pierre Richet : appui aux programmes de Recherche et de Lutte contre la Maladie du Sommeil en Côte d'Ivoire. Financement "Fonds d'Aide à la Coopération", Ministère des Affaires Etrangères, Secrétariat d'Etat à la Coopération et à la Francophonie, France, 1993/1994 (responsable de programme).

- Mise au point, perfectionnement et évaluation d'une nouvelle technique d'isolement *in vitro* des trypanosomes africains au Congo et en Côte d'Ivoire : Financements "Medical Research Council", Royaume Uni (collaborateur), "Organisation Mondiale de la Santé, Recherches sur les Maladies Tropicales", Genève, Suisse, 1992/1994 (responsable de programme).

- Génétique et Epidémiologie de la Trypanosomiase Humaine ou Maladie du Sommeil en Afrique de l'Est et de l'Ouest : financements "Medical Research Council", Royaume Uni (collaborateur), "Organisation Mondiale de la Santé, Recherches sur les Maladies Tropicales", Genève, Suisse, 1992/1993 (collaborateur).

- Génétique de la Trypanosomiase Humaine ou Maladie du Sommeil en Afrique Centrale : financement "Fondation pour la Recherche Médicale", "Région Languedoc-Roussillon", France, 1989/1991 (collaborateur).

7- Autres activités scientifiques

Actions de Conseil, Coordination Scientifique et Technique

- Membre du Comité de Conseil Technique de la Division de Lutte contre les Maladies Tropicales CTD/OMS (Technical Advisory Group).

- Conseiller temporaire du Comité Directeur du Program Against African Trypanosomiasis (PAAT) OMS/FAO/IAEA/OUA IBAR.

- Responsable du Laboratoire de référence pour le diagnostic et la sérologie de la THA en Afrique de l'Ouest. Financement "Fonds d'Aide à la Coopération", Ministère Français de la Coopération et du Développement, France, 1997/1998.

- Secrétaire Local (Côte d'Ivoire) de la Société Royale de Médecine Tropicale et d'Hygiène, Londres, Royaume-Uni.

- Participation pour l'IPR à l'organisation de la Réunion de coordination des activités de lutte contre la THA, programme FAC, Maputo (Mozambique) les 25 et 26 septembre 1997.

- Organisation de la Réunion de coordination des activités de lutte contre la THA, programme FAC, IPR, Abidjan du 3 au 5 février 1997.

- Organisation du séminaire de formation des Responsables nationaux de lutte contre la THA en Afrique de l'Ouest, Coordination de la lutte contre la THA, programme FAC, IPR, Bouaké du 27 au 31 janvier 1997.

8- Glossaire

CATT :	Card Agglutination Test for Trypanosomiasis
CATT latex :	Card Agglutination Test for Trypanosomiasis sur billes de latex
FAC :	Fonds d'Aide à la Coopération
KIVI :	Kit for *In Vitro* Isolation of trypanosomes
LCR :	Liquide Céphalo-Rachidien
MAECT :	mini-Anion Exchange Centrifugation Technique
OCCGE :	Organisation de Coopération et de Coordination pour la lutte contre les Grandes Endémies
OCEAC :	Organisation de Coordination pour la lutte contre les Endémies en Afrique Centrale
PAAT :	Program Against African Trypanosomiasis
PCR :	Polymerase Chain Reaction
PNLT :	Programme National de Lutte contre la Trypanosomiase
QBC :	Quantitative Buffy Coat
RAPD :	Random Amplified Polymorphic DNA
TAG :	Technical Advisory Group
TDR :	Tropical Diseases Research
THA :	Trypanosomiase Humaine Africaine

9- Quelques publications

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Multilocus isozyme identification of *Trypanosoma brucei* stocks isolated in Central Africa: evidence for an animal reservoir of sleeping sickness in Congo

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Six Congolese and 3 Zairian *Trypanosoma brucei* stocks were studied by isozyme cellulose acetate electrophoresis. Twenty isozyme systems were used, of which only 5 showed variability. These 5 polymorphic systems made it possible to identify 5 different zymodemes. Zymodemes isolated from man were recorded both from pig and sheep too, which confirms the results of previous authors. This favors the existence of an animal reservoir of human African trypanosomiasis in the Congo, which could play a role in the transmission of the disease, at least by the maintenance of residual foci.

Key words: *Trypanosoma brucei*; Epidemiology; Strain characterization; Genetic interpretation

Introduction

The existence of an animal reservoir of human African trypanosomiasis has been established in Western Africa (Mehlitz, 1977; Mehlitz et al., 1982; Gibson et al., 1978, 1980; Godfrey et al., 1990). A similar situation was suspected for Central Africa, particularly in the Congo (Scott et al., 1983; Paindavoine et al., 1986; Noireau et al., 1989) by use of genetic markers (isozymes and DNA probes). But these data remain scanty due to the small number of stocks studied (only one stock was isolated from animals in the work by Scott et al., 1983). In this study, we present isozyme data on 9 stocks from Central Africa (3 from Zaire, 6 from the Congo). 21 loci are analysed by cellulose acetate electrophoresis. The results are compared with those obtained by previous authors (Tait et al., 1984; Paindavoine et al., 1986; Scott et al., 1983).

Material and Methods

Preparation of samples for electrophoresis:

The origin of the stocks is listed in Table 1. Primary isolates were intraperitonally inoculated into Wistar rats, which made it possible to obtain 5×10^8 parasites/ml.

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TABLE 1

Historical and geographical data about the stocks under study

Stock	Host	Country	Area	Year
TB26	pig	Congo	Kinzaba (Niari)	1983
LIICL2	pig	Congo	Brazzaville (Pool)	1985
D12K	sheep	Congo	Kounzoulou (Couloir)	1980
OK	man	Congo	Makoua (Cuvette)	1974
PA	man	Congo	Comba (Niari)	1975
FY	man	Congo	Mbinda (Lekoumou)[a]	1985
1811	man	Zaire	Kinshasa	1974
1821	man	Zaire	Gemena	1974
C126	man	Zaire	Kinshasa	1971

[a]Imported case from Bouenza area.

Parasites were separated from rat blood by passage through DEAE-cellulose columns (Lanham and Godfrey, 1970), and pellets of trypanosomes were obtained by centrifugation at $1440 \times g$. Cells were mixed with an equal volume of enzyme stabilizer (Miles and Ward, 1978) and kept on ice for 20 min. Cells were then pelleted by centrifugation at $8000 \times g$ and discarded. The supernatant was stored at $-70°C$.

Cellulose acetate electrophoresis:

Electrophoresis and staining procedures (see Tables 2 and 3), were adapted to African trypanosomes from the recipes of Cariou (1977), Kreutzer et al. (1980, 1981, 1983), Lanham et al. (1981), Richardson et al. (1986), Shaw and Prasad (1970), and Tibayrenc et al. (1985). The 20 enzyme systems studied are: AK (EC 2.7.4.3), ALAT (EC 2.6.1.2), ALP (EC 3.1.3.1), EST (EC 3.1.1.1), FK (EC 2.7.1.1), G6PDH (EC 1.1.1.49), GPI (EC 5.3.1.9), GAPD (EC 1.2.1.12), GOT (EC 2.6.1.1), GSR (EC 1.6.4.2), ICD (EC 1.1.1.42), LAP (EC 3.4.11 or 13.*.), MDH (EC 1.1.1.37), ME (EC 1.1.1.40), NH (EC 3.2.2.1), PEP2 (L-Leucyl-L-alanine EC 3.4.11 or 13.*.), 6PGD (EC 1.1.1.44), PGM (EC 2.7.5.1), TDH (EC 1.1.1.103), and UGPP (EC 2.7.7.9). The Helena system was used for all experiments.

Results

The 20 enzyme systems made it possible to identify 21 putative genetic loci (NH exhibits the activity of two separate loci, NH1 and NH2). Of these 21 loci, 16 were monomorphic for the stocks under study: AK, ALP, EST, FK, G6PD, GAPD, GPI, GSR, LAP, MDH, ME, NH1, NH2, 6PGD, TDH and UGPP. The 5 variable loci were ALAT, GOT, IDH, PEP 2 and PGM. The variability of these 5 loci produced 5 different zymodemes, which are listed in Table 4.

In order to initiate population genetic studies on *Trypanosoma brucei* (see Cibulskis, 1988), we tried to interpret our data in terms of Mendelian genetics, an approach initiated by Tait (1980) in the case of *T. brucei* and successfully used by us for *Trypanosoma cruzi*, the agent of Chagas' disease (Tibayrenc et al., 1981, 1986). When possible, electromorphs were equated to alleles, following the hypothesis of

TABLE 2

Buffers used in the present study

Code	Cell buffers	Code	Reaction buffers
A	0.5 M Tris versene borate pH 8.0	1	1 M Tris HCl pH 7
B	Tris barbital sodium barbital (Helena HR) pH 9.0	2	1 M Tris HCl pH 8
C	0.1 M Tris 0.1 M maleic acid 0.01 M EDTA 0.01 M $MgCl_2$ pH 7.4	3	0.076 M Tris 0.005 M citric acid pH 7.9
D	0.1 M Tris 0.0044 M maleic acid 0.01 M EDTA 0.01 M $MgCl_2$ pH 8.6	4	0.2 M Tris 0.2 M maleic acid pH 6.0
E	0.015 M Tris 0.005 M EDTA 0.01 M $MgCl_2$ 0.01 M boric acid pH 7.8	5	0.1 M Tris adjust to pH 6.5 with 1 M maleic acid
F	0.66 M Tris 0.083 M citric acid pH 8.6	6	0.025 M NaH_2PO_4 0.08 M Na_2HPO_4 pH 7.4
G	0.2 M Tris 0.025 M tricine 0.01 M KCl pH 8.0		
H	0.15 M citric acid 0.24 M NaH_2PO_4 pH 6.3		
I	Add 0.2 M NaH_2PO_4 to 0.2 M Na_2HPO_4 reach pH 7.0		

TABLE 3

Electrophoresis and staining procedures. The cell buffers F, C and D must be diluted for electrophoresis, respectively at 3/4, 1/2 and 1/4. The quotient in brackets indicated after each cell buffer (CB) is the dilution used for cellulose acetate plate soaking; RB = reaction buffer (10 ml in every recipe) with the appropriate dilution in brackets. ALAT and EST both visualized under UV light. All chemicals were obtained from Sigma

Enzyme	CB	Voltage	Time	RB	Staining solution
AK[a]	C (75/100)	160	20	2 (1/10)	60 mg α-D-glucose, 6 mg ADP, 20 units hexokinase (from bakers' yeast), G6PDH (from Torula yeast), 50 mg $MgCl_2$ ($6H_2O$), 5 mg NADP, 5 mg NBT, 1.5 mg PMS
ALAT[a]	F (1/15)	200	45	6	5 mg α-ketoglutaric acid, 10 mg L-alanine, 15 units LDH (from bovine heart), 2 mg NADH
ALP[a]	H (5/10)	80	35	3	50 mg β-naphthyl acid phosphate, 20 mg $MgCl_2$, 2 mg $MnCl_2$ (4 H_2O), 15 mg Fast Blue RR in 200 µl acetone
EST[a]	E (25/100)	150	40	5	4 methylumbelliferylacetate 10 mg in 0.6 ml acetone 50%
FK[a]	C (5/10)	200	30	2 (6/100)	30 mg β-D-fructose, 17 units PGI (from rabbit muscle), 10 mg ATP, (Na_2 salt) (from equine muscle), 15 units G6PDH (from Torula yeast), 50 mg $MgCl_2$ ($6H_2O$), 5 mg NADP, 0.6 mg MTT, 1.5 mg PMS
G6PD[a]	B	200	25	1 (5/10)	5 mg glucose-6 phosphate, 50 mg $MgCl_2$, 5 mg NADP, 2.5 mg NBT, 3 mg PMS
GAPD	A (25/100)	200	25	1 (25/100)	12.5 mg frustose-1,6-diphosphate Na_4 salt, 0.7 unit aldolase (from rabbit muscle): incubate at 25°C for 60 min, then add 12.5 mg acid arsenic $Na_{\geq 2}$ salt, 12.5 mg pyruvic acid (Na), 5 mg NAD, 5 mg NBT, 1.5 mg PMS
GOT	A (5/10)	150	45	2 (1/10)	25 mg L-aspartic acid, 20 mg α-ketoglutaric acid, 3 mg pyridoxal-5 phosphate: readjust pH to 8.00 with 40% NaOH, then add 20 mg Fast Blue BB
GPI	A (25/100)	200	45	1 (25/100)	10 mg fructose-6 phosphate, 5 units G6PDH (from Torula yeast), 50 mg $MgCl_2$, 5 mg NADP, 5 mg NBT, 1.5 mg PMS

GSR[a]	I (1/10)	200	20	2 (25/100)	10 mg glutathione (oxidized form), 5 mg NADPH (Na_4 salt), 5 mg NBT
ICD	A (25/100)	160	25	2 (1/10)	10 mg DL-isocitric acid, 50 mg $MgCl_2$, 5 mg $MnCl_2$, 5 mg NADP, 2.5 mg NBT, 1.5 mg PMS
MDH[a]	B	200	20	1 (2/10)	0.9 ml 1 M malic acid pH 7 (DL-malic acid), 5 mg NAD, 5 mg NBT, 1.5 mg PMS
ME[a]	A (5/10)	150	30	1 (25/100)	0.6 ml 1 M malic acid pH 7, 50 mg $MgCl_2$, 5 mg NADP, 5 mg NBT, 1.5 mg PMS
NH	A	120	30	2 (1/10)	10 mg inosine, 0.6 units xanthine oxydase (from buttermilk), 0.6 mg MTT, 1.5 mg PMS
6PGD	D (25/100)	200	25	2 (25/100)	10 mg 6-phosphogluconic acid, 50 mg $MgCl_2$, 5 mg NADP, 5 mg NBT, 3 mg PMS
PEP 2	A	200	25	2 (1/10)	5 mg L-leucyl L-alanine, 1 mg L amino acid oxidase, 1 mg peroxidase, 30 mg $MgCl_2$, 3 mg $MnCl_2$, 5 mg 3-aminoethylcarbazole in 400 µl ethanol
LAP	A (25/100)	200	45	4 (15/100)	10 mg L-leucine β-naphthylamide, 5 mg fast black K salt
PGM	A (25/100)	200	45	2 (25/100)	20 mg glucose-1 phosphate, 8 units G6PDH, 50 mg $MgCl_2$, 5 mg NADP, 2.5 mg NBT, 3 mg PMS
TDH[a]	G	200	15	2 (1/10)	100 mg L-threonine, 150 mg KCl, 10 mg NAD, 1.8 mg MTT, 1.5 mg PMS
UGPP	B	200	20	2 (4/10)	50 mg uridine diphosphoglucose, 70 mg tetrasodium pyrophosphate, 10 mg NADP, 10 units G6PDH, 90 units phosphoglucomutase (from rabbit muscle), 1 mg glucose-1,6 diphosphate, 60 mg EDTA, 50 mg $MgCl_2$, 1.2 mg MTT, 3 mg PMS

[a]Cooled by using the cooling device provided by Helena.

TABLE 4

Zymodemes recorded in the present study. Alleles at any given locus are numbered starting from 1 for the fastest allele. The variability recorded for PEP 2 was interpreted phenotypically (see text)

ZYM	ALAT	GOT	IDH	PEP2	PGM	Stocks
1	4/4	1/1	1/1	I	2/2	1811
2	4/4	1/1	1/1	IV	2/2	1821
3	1/3	1/1	1/1	III	2/2	C126
4	2/2	2/2	1/2	II	1/1	FY,LIICL2
5	2/4	1/1	1/1	IV	2/2	D12K,TB26,PA,OK

diploidy established by Tait (1980), Gibson et al. (1980), Jenni et al. (1986) and Sternberg et al. (1989) for *T. brucei*. The different putative alleles at a given locus were numbered starting from 1 for the fastest allele. Table 4 gives the inferred genotypes for the 5 zymodemes recorded. The locus PEP2 was not suitable for allelic interpretation, and its variability was interpreted phenotypically.

Some of our results disagree slightly with the ones communicated by other workers (Tait et al., 1984), who studied the same isolates. This might be due to a different technical approach. These divergent results involve, for example, the D12K stock. These authors have recorded complex, multi-banded patterns for this stock, for the enzyme system PEP2, while our results show a one-banded pattern at this locus (see Fig. 1). Conversely, for the locus ALAT, we recorded a three-banded pattern (see Fig. 2), as Scott et al. did, while Tait et al. recorded a one-banded pattern.

Discussion

The present study is based on the use of 21 isozyme loci, which provide a sound picture of the actual genetic variability of the stocks. Indeed such a number of loci is considered as statistically significant in population and evolutionary studies: Avise and Aquadro (1982) chose a lower limit of 14 loci for accepting the phylogenetic value of genetic distances in vertebrates. Nevertheless, we do not want to give the zymodemes recorded here any definitive labelling or numbering. Indeed, delineation of zymodemes is highly dependent upon the methods used, and upon the range of

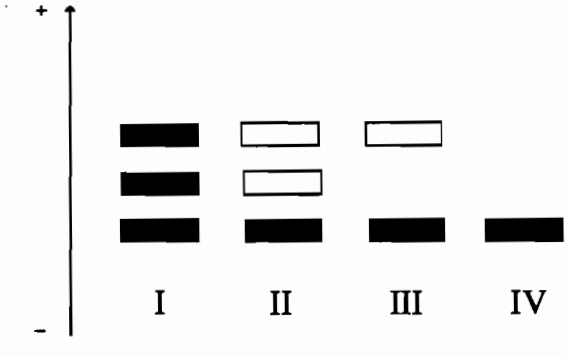

Fig. 1. Diagrams for the observed PEP2 patterns (see Table 4).

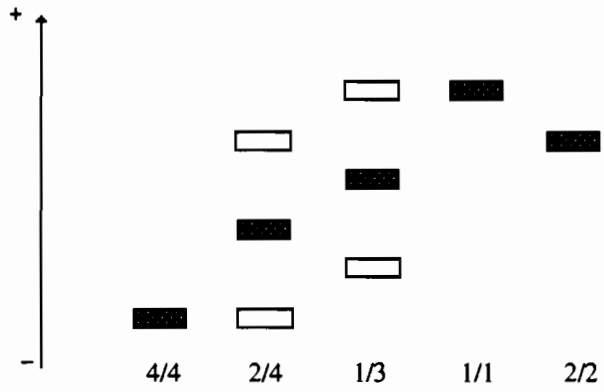

Fig. 2. Diagrams for the observed ALAT patterns, with putative allelic interpretations: 1/1 = homozygote for the allele 1, etc. (see Table 4).

isozyme markers under study (Tibayrenc and Ayala, 1988). The number of different zymodemes recorded is expected to increase with the range of markers and the number of stocks examined.

Although the present stock sample is still limited, our results confirm the presence of identical zymodemes both in animal and human hosts in the Congo, as already recorded by Scott et al. (1983). In particular zymodeme 5 in the present study was isolated four times, independently from a sheep, from a pig and from two patients. Zymodeme No. 4 was recorded twice, respectively from a pig and from a patient. So our results extend the data presented by Scott et al., suggesting the role of pigs as animal reservoirs in the Congo, as has been shown to be the case in West Africa (Mehlitz, 1977; Mehlitz et al., 1982; Gibson et al., 1978, 1980). These data are still too limited to allow the significance of animal reservoir hosts in the Congo to be ascertained precisely. Indeed, isolation of the same zymodeme both from man and from animal is not definitive evidence for communication between human and animal reservoirs. Nevertheless, the animal hosts are likely to be important in the maintenance of residual foci in this country (see Noireau et al., 1986, 1989). It is worth noting that the stock D12K, which was isolated from a sheep, was identified by Paindavoine et al. (1986) as *T. brucei gambiense*, which again favors the existence of an animal reservoir of human African trypanosomiasis in the Congo.

Work is in hand to survey a larger sample of stocks from the same region, in order to give a more accurate picture of the epidemiology of sleeping sickness in this area, and especially, to provide better estimates of the role of the animal reservoir in Central Africa.

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Short Report

A kit for *in vitro* isolation of trypanosomes in the field: first trial with sleeping sickness patients in the Congo Republic

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Low parasitaemias in patients infected with *Trypanosoma brucei gambiense*, with its limited infectivity to rodents, result in poor diagnosis and isolation (DUKES *et al.*, 1991). Procyclic forms grow readily in culture medium (e.g., BRUTSAERT & HENRARD, 1936; CUNNINGHAM, 1977), but the transformation of low numbers of bloodstream forms from rodent blood to the procyclic phase is difficult (BIENEN *et al.*, 1980; TRUC, 1991). DUKES *et al.* (1989) achieved such transformation using the natural cycle in the vector by feeding infected blood to susceptible laboratory-reared tsetse flies. However, in the field the usual approaches require either inoculation of rodents, which is unreliable, or deep-freezing of patients' blood.

To solve these problems, we have developed a kit for *in vitro* isolation of trypanosomes (KIVI). It allowed direct introduction of patients' blood into culture medium, with the subsequent transformation to, and multiplication of, procyclic trypanosomes. The medium (GLSH-DCA) comprised glucose, lactalbumin, serum and haemoglobin (LE RAY, 1975) diluted with an equal volume of Hanks's solution (HANKS & WALLACE, 1949; LE RAY, *et al.*, 1970) and complemented with 3 mM *cis*-aconitate (final concentration) according to BRUN & SCHÖNENBERGER (1981). Ten ml of blood were drawn into either a syringe containing 0.5 ml of 5% Liquoïde® Roche (sodium polyanetholesulphonate) anticomplementary anticoagulant (LE RAY *et al.*, 1970), or a Monovette® (Sarstedt) syringe containing heparin (lithium salt). The blood mixture was then dispensed equally into 2 vials (R1), each containing 10 ml of GLSH-DCA; one also contained a supplement of antibiotics (penicillin, 5000 iu/ml; gentamycin, 200 µg/ml; 5-fluorocytosine, 50 µg/ml). The vials were mixed by gentle manual agitation and kept at room temperature. In the initial laboratory tests, a minimum concentration of $2{\cdot}5 \times 10^4$ trypanosomes per ml could be transformed and cultured.

Results from 10 patients sampled on 2 separate occasions (1989, 1990) in the Bouenza focus, Republic of the

Table. Trypanosome isolation by KIVI and by rat inoculation from ten sleeping sickness patients in the Congo Republic

Patient	CATT[a]	LN[b]	MHC[c]	Rat[d]	Anticoagulant[e]	KIVI R1[f]	KIVI R2[g]	Stock no.
November 1989								
Minja	+	NS	+	+41	Li	27–36	20→4	ITMAP 2202
Balpa	+	NS	+	NEG	He+Li	24–36	20→4	ITMAP 2203
Silou	+	T+	++	+38	He+Li	31–36	20→16	ITMAP 2204
Bissi	+	T+	+	NEG	Li	27–36	20→4	ITMAP 2205
Pave	+	NS	+	NEG	Li	NEG	20→NEG	–
April 1990								
Dicar	+	T+	+	NEG	Li	18–25	15→5	ITMAP 2208
					He	NEG	ND	
Koa	NEG	T+	++	+26	Li	10–54	10→3	ITMAP 2209
					He	NEG	ND	
Bousa	+	T+	++	NEG	Li	15–54	15→3	ITMAP 2210
					He	NEG	ND	
Houm	+	NEG	+++	ND	Li	NEG	13→NEG	–
					He	NEG	13→NEG	
Babi	+	T+	+	NEG	Li	NEG	14→NEG	–
					He	NEG	ND	

[a]Card agglutination test for trypanosomes (MAGNUS *et al.*, 1978).
[b]Lymph nodes: NS, not swollen; T+, trypanosomes seen in lymph fluid.
[c]Microhaematocrit centrifugation (WOO, 1970): +, 1–5; ++, 6–15; +++, >15 trypanosomes.
[d]Rat inoculation: time (days) to positive by thin blood film examination; NEG: negative by wet smear over 2 months; ND, not done.
[e]Li: Liquoïde (see text); He: heparin
[f]R1: first and last days of patency in initial culture following inoculation. NEG: negative.
[g]R2: age in days of R1 when subinoculation into R2 was performed; arrow indicates day R2 became positive. NEG: negative.

Congo, are presented in the Table; at the same time, 1 ml of blood from each patient was inoculated into a rat. The inoculated KIVIs were sent or brought back to Europe, where 2 to 4 weeks after the initial inoculation, they were examined. Subinoculation (R2) was performed into blood-agar (TOBIE, 1949) and Cunningham's medium (CUNNINGHAM, 1977). R1 and R2 vials were kept under observation for one month.

Of the 10 sleeping sickness patients with low-grade parasitaemias, 7 provided a positive culture in KIVI whereas only 3 were infective to rats. Isoenzyme characterization for 24 loci (TRUC, 1991) showed that all the isolates belonged to classical *T. b. gambiense*. Our results also confirm the low infectivity of *T. b. gambiense* in Central Africa to rodents.

In this preliminary study, KIVI was more effective than rat inoculation in isolating human parasites. Liquoïde (Roche) was confirmed to be the best anticoagulant (WEINMAN, 1960). The operational value of KIVI in field work was demonstrated by the long period during which it sustained the growth and viability of procyclic trypanosomes (25–54 d; average 40 d). Work is now in progress to improve the KIVI and test it in other areas of Africa, and to evaluate its diagnostic value for hosts with subpatent infections.

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Population genetics of *Trypanosoma brucei* in Central Africa: taxonomic and epidemiological significance

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SUMMARY

In order to estimate the value of population genetics for both the taxonomy of trypanosomes belonging to the species *Trypanosoma brucei* and a better understanding of Human African Trypanosomiasis (HAT), we undertook a cellulose acetate electrophoresis isoenzyme study involving 55 stocks isolated from man and animals in Congo, Zaire and Cameroun. Out of the 24 loci surveyed, 15 exhibited variability, which made it possible to delimit 23 zymodemes, divided into 2 groups. The first group equated to the classical subspecies *Trypanosoma brucei gambiense*, while the second corresponded to the classical subspecies *Trypanosoma brucei brucei*. These results broadly agree with the current taxonomy, and are corroborated by RFLP analysis of kDNA. Statistical analysis indicates a basically clonal reproduction system of the trypanosomes in the area studied; the zymodemes are equivalent to natural clones (or a family of closely related clones), stable in space and time. Epidemiological hypotheses are proposed according to the geographic distribution of the clones in this area.

Key words: population genetics, *Trypanosoma brucei*, human African trypanosomiasis, isoenzymes, linkage disequilibrium, clonal structure, epidemiology.

INTRODUCTION

Sleeping sickness, or human African trypanosomiasis (HAT), has been described since the beginning of the century and is still present in many African countries. The Gambian type or chronic form is found in West and Central Africa while the Rhodesian type or acute form, with a cycle including wild and domestic animal reservoirs, is located in East Africa. Based upon these features, a subspecific taxonomy of *Trypanosoma brucei* has been generally accepted. *Trypanosoma brucei gambiense* refers to the chronic form, *T. b. rhodesiense* to the acute form, and *T. b. brucei* to a form that is not infective to man, but only to animals.

Unfortunately none of these behavioural characteristics presents satisfactory objective criteria for a sound taxonomy, and indeed evidence exists of variability in each feature (Apted, 1970; Hoare, 1972). Biochemical and molecular markers, such as isoenzymes (Godfrey & Kilgour, 1976; Gibson, Marshall & Godfrey, 1980), and kinetoplast DNA RFLPs (Borst *et al.* 1980*a*, *b*; Borst, Fase Fowler & Gibson, 1981; Gibson, Borst & Fase Fowler, 1985*a*), have been extensively used in an attempt to establish a reliable classification. These new methods of identification were able to individualize, within the trypanosomes that are pathogenic for man in West Africa, a group of parasites that is genetically relatively homogeneous. This group appears to represent about 80% of all West African human isolates, and was named *Trypanosoma brucei gambiense* group I (Gibson, 1986); the status of the other subspecies remained doubtful. These new methods showed that some trypanosome stocks circulating in animals are similar to others found in man (Gibson *et al.* 1978, 1980; Mehlitz *et al.* 1982; Gibson & Gashumba, 1983; Scott *et al.* 1983; Zillman, Mehlitz & Sachs, 1984; Gibson & Wellde, 1985; Mehlitz, 1986; Noireau *et al.* 1989; Mihok, Otieno & Darji, 1990; Truc, 1991; Truc, Mathieu-Daudé & Tibayrenc, 1991). Thus domestic animals are suspected to be a potential reservoir of HAT.

Genetic analysis also makes it possible to understand better the mode of reproduction of trypanosomes. This aspect is closely linked to taxonomic problems; the mode of reproduction of the parasite in field conditions is the main feature influencing genetic isolation of the populations from one another.

The notion generally accepted until recently was that trypanosomes reproduce only mitotically. Nevertheless, Tait (1980) and Tait, Babiker & Le Ray (1984), on the basis of isoenzyme analysis of natural populations of the parasite, proposed that the agent of HAT was a sexual organism, and even that its populations had a potentially panmictic structure. That is to say, genetic exchange occurs at random, the only possible obstacles to gene flow being

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geographical ones. Later, successful mating experiments in the laboratory (Jenni *et al.* 1986; Steinberg *et al.* 1988) seemed to confirm this hypothesis. Such experiments, however, show only that the potentiality for genetic exchange is not lost, and say nothing about the actual impact of it in natural populations (Tibayrenc, Kjellberg & Ayala, 1990).

Based upon population genetic results, Cibulskis (1988) did not consider the earlier evidence (Tait, 1980, 1983) for a panmictic structure valid and proposed the hypothesis of separate evolution of distinct parasitic lines. In a recent review, Tait & Turner (1990) in the light of Cibulskis' assumptions, questioned the panmictic model earlier proposed. Tibayrenc *et al.* (1990, 1991), within the frame of a general model for parasitic protozoa, postulated that *T. brucei* populations had a basically clonal population structure. Such a hypothesis has important consequences on the subspecific taxonomy of the parasite. The possibility of identifying, within a given species, discrete lines that might be studied separately is inversely proportional to the extent of sexual reproduction. In the case of *T. brucei*, it is apparent that discrete genetic entities develop within the species that are stable in space and time. In the quasi-panmictic model, such discrete units do not exist, and the entities defined by genetic markers amount to mere individual variants.

In Central Africa, HAT still persists in the main historical foci. Peridomestic transmission of sleeping sickness and its asymptomatic character (Frezil, 1983) would be two important factors accounting for the spread and persistence of this disease, especially in the Congo (Truc, 1991). On the other hand, the existence of an animal reservoir has been also suspected in this area (Scott *et al.* 1983; Noireau *et al.* 1986, 1989; Truc *et al.* 1991). But some trypanosomes which are considered as non-infective for man also circulate among domestic animals, especially in adjoining Zaire (Kageruka, Colaert & Ngimbi, 1977; Makumyaviri *et al.* 1989), and could be equated to the *T. b. brucei* subspecies.

In order to evaluate the validity of *T. brucei* subspecific taxonomy, and to clarify the mode of reproduction of trypanosomes in the Congo, an isoenzyme study involving 55 stocks isolated from man and animals was carried out. Twenty-two of these stocks were analysed for kDNA restriction fragment length polymorphism (RFLP) too. The results, analysed in terms of population genetics, show the dominant reproduction system in this area, and make it possible to propose some hypotheses on the persistence and spread of the disease.

MATERIALS AND METHODS

Isolation and cultivation of trypanosome stocks

The stocks surveyed are detailed in Table 1. Included are 19 reference stocks formerly identified as either *T. b. gambiense* or *T. b. brucei* by isoenzyme or DNA probe analyses (Gibson, Marshall & Godfrey, 1980; Scott *et al.* 1983; Tait *et al.* 1984, 1989; Paindavoine *et al.* 1986, 1989; Noireau *et al.* 1989; Godfrey *et al.* 1990; Hide *et al.* 1990).

Thirty-six other stocks were isolated from man and animals between 1986 and 1989 in the Congo and Zaire. Positive patients were diagnosed using the Testryp CATT test (Magnus, Vervoort & Van Meirvenne, 1978), and parasitologically confirmed by the microhaematocrit test (Woo, 1970). Animals were submitted to a parasitological test only. A blood sample was taken from each positive patient or animal. An intraperitoneal inoculation of 1 ml of blood to a Wistar rat allowed successful isolation of most stocks. Then, because of the low virulence of bloodstream forms of *T. b. gambiense* from Central Africa in rodents, we performed a transformation into procyclic trypomastigote forms either by feeding stabilates to laboratory-reared *Glossina morsitans morsitans* (Dukes *et al.* 1989) or by a direct transfer of rat blood into a semi-defined medium (Bienen, Hammadi & Hill, 1980; Truc, 1991). Some isolations were achieved by transfer of fresh human blood into a semi-defined medium (Aerts *et al.* 1992). The procyclic forms so obtained can easily be cultivated in semi-defined medium (Cunningham, 1977) for isoenzyme electrophoresis and RFLP analysis.

After centrifugation from the medium, the pellets of procyclic forms were finally mixed with an equal quantity of enzyme stabilizer (Miles & Ward, 1978), and left on ice for 20 min to lyse the cells. After centrifugation in an Eppendorf microfuge at 11000 *g* for 10 min, aliquots of the supernatant fractions were kept at −70 °C until used for isoenzyme electrophoresis, while pellets of the broken cells were stored at −70 °C for DNA analyses.

Isoenzyme electrophoresis

The following 19 enzyme systems were studied by cellulose acetate electrophoresis: alanine aminotransferase (Alat, EC 2.6.1.2), alkaline phosphatase (Akp, EC 3.1.3.1), diaphorase (Dia, EC 1.6.*.*), glyceraldehyde-3-phosphate dehydrogenase (Gapd, EC 1.2.1.12), aspartate aminotransferase (Asat = Got, EC 2.6.1.1), glucose phosphate dehydrogenase (Gpi, EC 5.3.1.9), glucose 6-phosphate dehydrogenase (G6pd, EC 1.1.1.49), glutathione reductase (Gsr, EC 1.6.4.2), isocitrate dehydrogenase (Icd, EC 1.1.1.42), leucine amino peptidase (Lap, EC 3.4.11 or 13.*), malate dehydrogenase (Mdh, EC 1.1.1.37), malic enzyme (Me, EC 1.1.1.40), nucleoside hydrolase (Nh, EC 3.2.2.1), peptidase (substrate L-leucyl-L-alanine: Pep2, EC 3.4.11 or 13.*), 6-phosphogluconate dehydrogenase (6Pgd, EC 1.1.1.44), phosphoglucomutase (Pgm, EC 2.7.5.1), superoxide dismutase (Sod, EC 1.15.1.1), threonine dehydrogenase (Tdh,

Table 1. Stocks under study, with zymodeme (Z) and schizodeme (S) identification

(See also Tables 3 and 8.)

Stock	Host	Country	Focus/Area	City/Village	Year	Z	S	Reference
MBA	Man	Zaire	?	Kinshasa	1974	1	C	3, 4, 7
SH86	Sheep	Zaire	Bandundu	Bandundu	1986	2	A	—
EATRO 1125	Bushbuck	Uganda	South Busoga	Mavubwe	1966	3	B	4, 5, 7, 9
SW3/87	Pig	Zaire	Bandundu	Djumuna	1987	4	E	—
SW1/87	Pig	Zaire	Bandundu	Djumuna	1987	5	E	—
SW2/87	Pig	Zaire	Bandundu	Djumuna	1987	5	E	—
SW4/87	Pig	Zaire	Bandundu	Djumuna	1987	5	E	—
SW161/87	Sheep	Zaire	Boma	Boma	1987	5	E	—
SW94/87	Pig	Zaire	Boma	Boma	1987	5	E	—
SW165/87	Pig	Zaire	Boma	Boma	1987	5	N.D.	—
SH109/87	Sheep	Zaire	Boma	Boma	1987	6	N.D.	—
DIMONA	Man	Congo	Bouenza	Kinsaka	1989	7	N.D.	—
BALPA	Man	Congo	Bouenza	Kinsaka	1989	7	N.D.	—
BISSI	Man	Congo	Bouenza	Tanga	1989	7	F	—
SILOU	Man	Congo	Bouenza	Mayanama	1989	7	N.D.	—
MINJA	Man	Congo	Bouenza	Mayanama	1989	7	F	—
KOA	Man	Congo	Bouenza	Minga	1989	7	N.D.	—
DICAR	Man	Congo	Bouenza	Minga	1989	7	N.D.	—
SANNA	Man	Congo	Bouenza	Kinsaksa	1989	7	N.D.	—
DIANTETE	Man	Congo	Bouenza	Madiadia	1989	7	N.D.	—
NSOUNGUI	Man	Congo	Bouenza	Mayanama	1989	7	F	—
NITOUL	Man	Congo	Bouenza	Mayanama	1989	7	F	—
MABOU	Man	Congo	Bouenza	Kinsaka	1989	7	N.D.	—
BIH	Man	Congo	Bouenza	Tanga	1989	7	F	—
NSIMBA	Man	Congo	Bouenza	Mayanama	1989	7	N.D.	—
BAKOU	Man	Congo	Bouenza	Kinsaka	1989	7	N.D.	—
MABIA	Man	Congo	Bouenza	Kimbanda	1989	7	N.D.	—
DEMBA	Man	Congo	Bouenza	Mayanama	1989	7	N.D.	—
MAZOUM	Man	Congo	Pool	Kingoli*	1989	7	N.D.	—
BALEM	Man	Congo	Bouenza	Madiadia	1989	7	N.D.	—
DI2K	Sheep	Congo	Couloir	Kounzoulou	1980	8	N.D.	2, 3, 4, 7, 8
PA	Man	Congo	Bouenza	Comba	1975	8	G	2, 3, 4, 7, 8, 9
TB26	Pig	Congo	Bouenza	Kinzaba	1983	8	N.D.	—
OK	Man	Congo	Cuvette	Makoua	1974	8	N.D.	2, 3, 4, 7, 8
LIICL2	Pig	Congo	Pool	Brazzaville	1985	9	N.D.	6
FY	Man	Congo	Bouenza	Mbinba†	1985	9	N.D.	6
BB	Man	Congo	Bouenza	Kinzaba	1973	10	N.D.	2
KIKOU	Man	Congo	Bouenza	Loudima	1989	11	H	—
PEYA	Man	Congo	Couloir	N'gabe	1980	12	N.D.	—
NGABELA	Man	Congo	Couloir	N'gabe	1989	12	F	—
MABAYA	Man	Congo	Couloir	N'gabe	1989	12	F	—
MBOULANG	Man	Congo	Cuvette	Mossaka	1989	13	F	—
MALOUNDA	Man	Congo	Bouenza	Loudima	1989	13	F	—
MA	Man	Congo	Cuvette	Mossaka	1974	14	N.D.	3, 4, 7, 9
BOULA	Man	Congo	Bouenza	Minga	1989	15	N.D.	—
MATSOUMA	Man	Congo	Bouenza	Mayanama	1989	16	N.D.	—
MBOUSSA	Man	Congo	Couloir	N'gabe	1989	17	N.D.	—
SANE	Man	Cameroon	Bafia	Guefigue	1976	18	N.D.	3, 4, 7
MOS	Man	Cameroon	Bafia	Ombessa	1974	19	N.D.	3, 4, 7, 9
BIM	Man	Cameroon	Campo	Campo	1974	19	N.D.	3, 4, 7, 9
JUA	Man	Cameroon	Fontem	Fontem	1974	20	N.D.	3, 4, 7
KEMLO	Man	Zaire	Haut-Zaïre	Gemena	1974	21	N.D.	3, 4, 7
C126	Man	Zaire	?	Kinshasa	1971	22	D	1, 8
ALJO	Man	Zaire	Bandundu	Mushie	1970	23	N.D.	—
BOSENDJA	Man	Zaire	?	Kinshasa	1972	23	A	3, 4, 7

* Imported case.
† Near Mindouli. N.D., not done.
(1) Gibson, Marshall & Godfrey (1980).
(2) Scott *et al.* (1983).
(3) Tait, Babiker & Le Ray (1984).
(4) Paindavoine *et al.* (1986).
(5) Tait *et al.* (1989).
(6) Noireau *et al.* (1989).
(7) Paindavoine *et al.* (1989).
(8) Godfrey *et al.* (1990).
(9) Hide *et al.* (1990).

EC 1.1.1.103) and uridine diphosphoglucose pyrophosphorylase (Ugpp, EC 2.7.7.9). Staining and electrophoresis procedures were performed according to the method of Truc, Mathieu-Daudé & Tibayrenc (1991), except for Sod, which was performed according to Stevens *et al.* (1989). All chemicals were obtained from Sigma, and the Helena® system was used for the electrophoresis.

Statistical analysis of the isoenzyme results

Specific population genetic tests (Tibayrenc *et al.* 1990) were performed in order to check the clonal hypothesis. These tests consider the two main consequences of sexuality in nature, that is to say segregation of alleles at given loci, and recombination of genotypes among loci.

For segregation tests, precise identification of the alleles is required; interpretation of isoenzyme electrophoreses is based on the assumption that *T. brucei* is a diploid organism (Borst *et al.* 1980*a*, *b*; Tait, 1980; Shapiro *et al.* 1984; Tait *et al.* 1984; Gibson *et al.* 1985*b*; Jenni *et al.* 1986; Kooy *et al.* 1989; Tait *et al.* 1989). For recombination tests, no allelic reading is required; each distinguishable and reproducible pattern at a given locus is considered a distinct genotype (Tibayrenc & Ayala, 1991; Tibayrenc *et al.* 1991).

The tests take as a null hypothesis a panmictic model in which gene exchange occurs at random. Under this hypothesis, circumstantial evidence for clonality is provided by the departure of different genotypes from the expected numbers; for segregation tests, the expected frequency of a given unilocus genotype is given by Hardy–Weinberg statistics. In the case of recombination tests, the expected frequency of a given multilocus genotype is the product of the observed frequencies of the component unilocus genotypes (Tibayrenc *et al.* 1990, 1991; Tibayrenc & Ayala, 1991). Expectations can be simply checked by a χ^2 test (Tibayrenc *et al.* 1991). When numerous loci are surveyed, the expected frequencies and numbers of individual genotypes quickly become very low, which make these tests very powerful, but prevent the use of χ^2 tests, because the expected numbers are far lower than 5. To solve the problem, tests based upon either combination methods or Monte Carlo simulations, run with specific Turbo Pascal softwares, were designed (Tibayrenc *et al.* 1990).

The various tests are presented in Table 2. Tests a and b have been fully described by Tibayrenc *et al.* (1990). Test c, the classic Hardy–Weinberg statistic, can also be used with the χ^2 test when expected numbers are equal to or above 5. When expected numbers are not sufficient, either Monte Carlo simulations or combinatorial methods can be used, as for recombination tests. Tests d2, e and f are based on Monte Carlo simulations, while test d1 is based on a combinatorial method (Tibayrenc *et al.* 1990). This test gives the probability of observing a given genotype with its actual frequency, under the null hypothesis of random mating. Test d2 gives the probability of observing any genotype with as many or more individuals actually observed for the most abundant genotype. Test e, which is analogous to the test performed by Cibulskis (1988) in a population genetic survey of Lambwe valley isolates, gives the probability of observing as few or less different genotypes actually observed in the sample; in the case of inhibition of interbreeding, a lower number of different genotypes is generally expected than in the case of free recombination. Test f refers to a 'classical' linkage disequilibrium test, checking non-random association among loci. Based on the observed genotype frequencies at the various loci under survey, the result gives the probability of observing, during free recombination, a linkage disequilibrium as high as actually observed. Combination of isoenzyme results and kDNA RFLP analysis (see below) make it possible to perform the g test of correlation between independent sets of genetic markers, which provides especially strong evidence for clonality.

Table 2. Criteria and tests of clonality

(See Tibayrenc *et al.* 1990 and Materials and Methods section.)

Criterion	Description
Segregation (within locus)	
a	Fixed heterozygosity*
b	Absence of segregation genotypes*
c	Deviation from Hardy–Weinberg expectation†
Recombination (between loci)	
d	Overrepresented, identical genotypes widespread (statistical tests d1 and d2)*†
e	Absence of recombinant genotypes†
f	Linkage disequilibrium†
g	Correlation between independent sets of genetic markers†

* Used qualitatively, without statistical calculations.
† Used with statistical calculations.

It has been claimed (Dye, 1991) that linkage disequilibrium was not in itself evidence of clonality. It must be underlined that in our approach, evidence for clonality does not come from the rough results of departures from Hardy–Weinberg expectations, or of a positive linkage disequilibrium, considered in isolation. Rather we use the levels of significance of the tests, and even more, the qualitative analysis of the different components of the results, namely, distribution of genotypes in excess or in deficit, and their geographical and temporal distribution. These points are specifically discussed later (see Discussion section). It should be underlined that the approach

used here is a quite classical one in population genetics of uniparental organisms (bacteria, parthenogenetic insects or plants, etc.). In such studies, both linkage disequilibria and strong departures from Hardy–Weinberg expectations are considered as strong, although circumstantial, evidence for clonality (see for example Suomalainen *et al.* 1976; Selander & Levin, 1980). Deviation from panmixia expectations that are generated by geographical separation as an example indeed exhibit different patterns (see Discussion section).

Restriction fragment length polymorphism (RFLP)

Techniques for kinetoplast DNA extraction and electrophoresis procedures were performed according to Gibson, Borst & Fase Fowler (1985*a*). *Hind* III is the restriction enzyme used in this study.

RESULTS

Isoenzyme electrophoresis

From the assumption that *T. brucei* is diploid (Borst *et al.* 1980*a*, *b*; Tait, 1980; Tait *et al.* 1984, 1989; Shapiro *et al.* 1984; Gibson *et al.* 1985*b*; Jenni *et al.* 1986; Kooy *et al.* 1989) we performed an allelic interpretation of most of the enzyme systems under study. However, allelic reading was not possible for the genotypes recorded at the *Got*, *Pep* 2, *Sod a* and *Sod b* loci (Fig. 1). Eight out of the 24 loci studied were monomorphic (*Dia*, *Gapd*, *Gpi*, *Gsr*, *Mdh*, *Mel*, *Nh a* and *Nh b*). The level of polymorphism was hence 0·666 (number of variable loci/total number of loci). The variability recorded at the 16 other loci (average number of alleles for all variable loci = 2·83) produced 23 different zymodemes (Table 3). Two types of distances were calculated, namely Nei's standard genetic distance (Nei, 1972), and Jacquard's distance (Jacquard, 1973). The first has an actual genetic meaning (average number of codon differences per gene between any two populations – here stocks). The second one is a mere estimation of the percentage of isoenzyme band mismatches between any two stocks. Since the two measures are based on similar data (isoenzyme bands), they are usually highly correlated. Both (Tables 4 and 5) were used to build distance dendrograms. Nei's distance, although it has a more precise genetic definition, presents the drawback of requiring an allelic interpretation of the zymograms. Since this was possible here only for 20 loci, some zymodemes appeared identical to each other, while they differed if 24 loci were used. The 4 remaining loci, which were phenotypically interpreted, were very discriminant since they identified 5 additional zymodemes. The main distance values were as follows: average Nei's distance (Dn) = 0·246, with

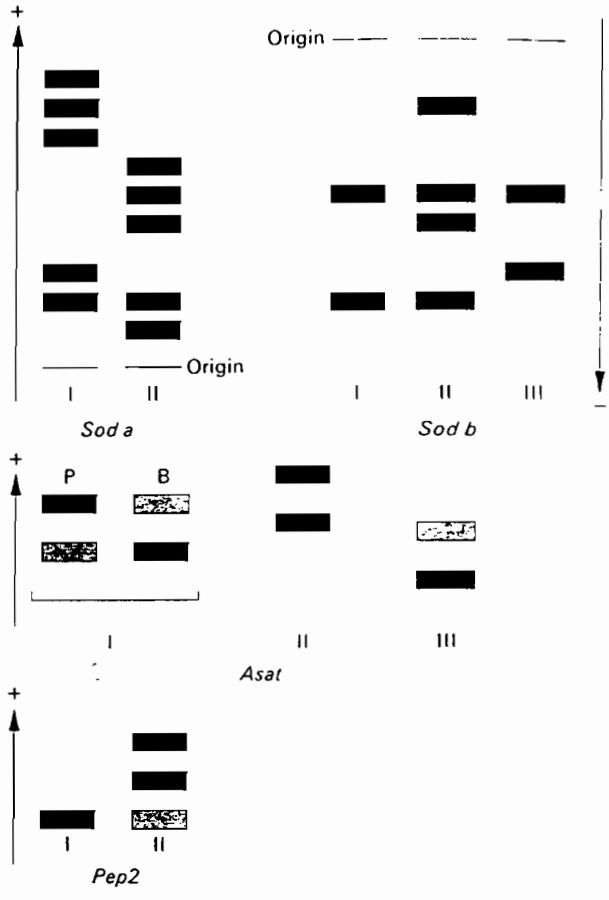

Fig. 1. Non-allelic genotypes recorded at the loci *Sod a*, *Sod b*, *Asat* (*Got*) and *Pep 2*. P = procyclic form; B = bloodstream form.

standard deviation = 0·149, maximum Dn = 0·72, minimum Dn = 0·02; average Jacquard's distance (Dj) = 0·381, standard deviation = 0·161, maximum Dj = 0·71, minimum Dj = 0·06.

Dendrogram visualization of distance matrices

Jacquard's distance, which takes into account all the loci examined, was used to build a dendrogram that clearly distinguished 2 groups of zymodemes (Fig. 2). Group A included most of the zymodemes isolated from man, a zymodeme both from man and animals (Z8), and a zymodeme only from an animal (Z2). Group B included most of the zymodemes isolated from animals and a zymodeme from man and animal (Z9). *Sod a* and *Sod b* appeared discriminant, since they made it possible to distinguish group A (*Sod a* II with *Sod b* III) from group B (*Sod a* I with *Sod b* I or II). Nei's distance yielded a similar dendrogram which is not shown.

Population genetic tests

In order to minimize possible biases due to geographical structure (Tibayrenc *et al.* 1991) and/or

Table 3. Zymodemes recorded in the present study, and corresponding isoenzyme patterns

Z	*Alat*	*Alp*	*Got**	*G6pd*	*Idh*	*Lap*	*Mc2*	*Nhc*	*Pep2**	*Pgm*	*Pgd*	*Soda**	*Sodb**	*Tdh*	*Ugppi*	*Ugpp2*	*Nb*†
1	*4/4*	*2/2*	I	*1/1*	*1/1*	*1/1*	*1/2*	*2/2*	II	*1/2*	*1/1*	II	III	*2/2*	*1/2*	*1/1*	1
2	*2/2*	*2/2*	I	*1/1*	*1/1*	*1/1*	*1/2*	*2/2*	II	*1/2*	*1/1*	II	III	*1/1*	*1/2*	*1/1*	1
3	*2/2*	*2/2*	I	*1/1*	*1/2*	*1/1*	*1/1*	*1/2*	II	*1/1*	*1/1*	I	II	*2/2*	*2/2*	*1/1*	1
4	*2/2*	*2/2*	I	*1/1*	*1/1*	*2/2*	*1/1*	*2/2*	II	*1/2*	*1/1*	I	II	*2/2*	*2/2*	*1/1*	1
5	*2/2*	*2/2*	I	*1/1*	*1/1*	*2/2*	*1/1*	*2/2*	II	*1/2*	*1/1*	I	I	*2/2*	*2/2*	*1/1*	6
6	*1/3*	*2/2*	I	*1/1*	*1/1*	*1/1*	*1/1*	*2/2*	I	*1/2*	*0/0*	I	I	*2/2*	*2/2*	*1/1*	1
7	*2/4*	*2/2*	I	*1/1*	*1/1*	*2/2*	*1/2*	*2/2*	II	*2/2*	*1/1*	II	III	*2/2*	*1/2*	*1/1*	19
8	*2/4*	*2/2*	I	*1/1*	*1/1*	*2/2*	*0/0*	*2/2*	I	*2/2*	*1/1*	II	III	*2/2*	*1/2*	*1/1*	4
9	*2/2*	*2/2*	III	*1/1*	*1/2*	*2/2*	*1/1*	*2/2*	I	*1/1*	*1/1*	I	II	*2/2*	*2/2*	*1/1*	2
10	*2/4*	*2/2*	I	*1/1*	*1/1*	*2/2*	*1/2*	*2/2*	I	*2/2*	*1/1*	II	III	*2/2*	*1/2*	*1/1*	1
11	*4/4*	*2/2*	I	*1/1*	*1/1*	*2/2*	*1/2*	*2/2*	II	*2/2*	*1/1*	II	III	*2/2*	*1/2*	*1/1*	1
12	*2/2*	*2/2*	I	*1/1*	*1/1*	*2/2*	*1/2*	*2/2*	II	*2/2*	*1/1*	II	III	*2/2*	*1/2*	*1/1*	3
13	*4/4*	*2/2*	I	*1/1*	*1/1*	*2/2*	*1/2*	*2/2*	II	*2/2*	*1/1*	II	III	*1/1*	*1/2*	*1/1*	2
14	*4/4*	*2/2*	I	*1/1*	*1/1*	*2/2*	*1/2*	*2/2*	I	*2/2*	*1/1*	II	III	*2/2*	*1/2*	*1/1*	1
15	*2/4*	*2/2*	I	*1/1*	*1/1*	*2/2*	*1/2*	*2/2*	II	*2/2*	*1/1*	II	III	*1/2*	*2/2*	*1/1*	1
16	*2/2*	*2/2*	I	*1/1*	*1/1*	*2/2*	*1/2*	*2/2*	II	*2/2*	*1/1*	II	III	*2/2*	*2/2*	*1/1*	1
17	*2/4*	*2/2*	I	*1/1*	*1/1*	*2/2*	*1/2*	*2/2*	II	*2/2*	*1/1*	II	III	*1/2*	*1/2*	*1/1*	1
18	*2/4*	*1/1*	I	*1/1*	*1/1*	*2/2*	*0/0*	*2/2*	I	*2/2*	*1/1*	II	III	*2/2*	*2/2*	*1/2*	1
19	*2/4*	*1/1*	I	*1/1*	*1/1*	*2/2*	*0/0*	*2/2*	I	*2/2*	*1/1*	II	III	*2/2*	*2/2*	*1/1*	2
20	*2/4*	*1/1*	I	*2/2*	*1/1*	*2/2*	*0/0*	*2/2*	I	*2/2*	*1/1*	II	III	*2/2*	*2/2*	*1/1*	1
21	*4/4*	*2/2*	II	*1/1*	*1/1*	*1/1*	*0/0*	*2/2*	I	*2/2*	*1/1*	II	III	*2/2*	*2/2*	*1/1*	1
22	*2/4*	*2/2*	II	*1/1*	*1/1*	*2/2*	*0/0*	*2/2*	I	*2/2*	*1/1*	II	III	*2/2*	*2/2*	*1/1*	1
23	*2/4*	*2/2*	I	*1/1*	*1/1*	*2/2*	*0/0*	*2/2*	I	*1/2*	*1/1*	II	III	*2/2*	*2/2*	*1/1*	2

* Indicates that these genotypes did not allow an allelic reading (see Fig. 1).
† Nb, number of stocks for the corresponding zymodemes.

Table 4. Matrix of values for Nei's standard genetic distance

Z/Z	1	2	3	4*	6	7**	8	9	11***	12	16	17	18	19	20	21	22
1	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·
2	0·21	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·
3	0·23	0·23	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·
4*	0·26	0·26	0·17	·	·	·	·	·	·	·	·	·	·	·	·	·	·
6	0·23	0·36	0·26	0·28	·	·	·	·	·	·	·	·	·	·	·	·	·
7**	0·15	0·27	0·36	0·09	0·36	·	·	·	·	·	·	·	·	·	·	·	·
8	0·23	0·36	0·45	0·17	0·45	0·07	·	·	·	·	·	·	·	·	·	·	·
9	0·32	0·32	0·12	0·04	0·34	0·2	0·28	·	·	·	·	·	·	·	·	·	·
11***	0·12	0·36	0·45	0·17	0·38	0·02	0·1	0·28	·	·	·	·	·	·	·	·	·
12	0·23	0·23	0·32	0·07	0·38	0·02	0·1	0·17	0·1	·	·	·	·	·	·	·	·
16	0·26	0·26	0·28	0·04	0·34	0·05	0·12	0·14	0·12	0·02	·	·	·	·	·	·	·
17	0·19	0·19	0·4	0·12	0·4	0·02	0·1	0·23	0·05	0·05	0·07	·	·	·	·	·	·
18	0·43	0·58	0·61	0·28	0·61	0·23	0·15	0·41	0·26	0·26	0·22	0·27	·	·	·	·	·
19	0·38	0·53	0·55	0·25	0·55	0·2	0·12	0·36	0·22	0·22	0·19	0·23	0·02	·	·	·	·
20	0·53	0·69	0·72	0·36	0·72	0·32	0·22	0·5	0·34	0·34	0·3	0·36	0·12	0·09	·	·	·
21	0·12	0·34	0·36	0·32	0·3	0·22	0·14	0·45	0·19	0·3	0·27	0·25	0·24	0·21	0·32	·	·
22	0·26	0·38	0·41	0·14	0·41	0·09	0·02	0·25	0·12	0·12	0·09	0·12	0·12	0·09	0·19	0·11	·
23	0·23	0·36	0·32	0·12	0·38	0·12	0·05	0·17	0·15	0·15	0·12	0·15	0·15	0·12	0·22	0·14	0·02

* Z4 = Z5; ** Z7 = Z10 = Z15; *** Z11 = Z13 = Z14 (see text).

too small sample sizes, these tests were performed on the Congolese stocks only.

(*a*) *Segregation tests at given loci.* Hardy–Weinberg equilibrium was checked separately for the isolated Bouenza region of the Congo (Table 6), and for all the Congolese stocks (Table 7). Sympatry is really satisfactory only in the first area, which is geographically limited, and where all the samples were collected during the same year. In the total Congolese sample, it is important to keep in mind possible biases caused by geographical separation and genetic drift.

(*b*) *Recombination tests.* The d1 test shows that, with random mating, the probability of observing zymodeme 7 with as many or more individuals as actually observed is $1{\cdot}34 \times 10^{-3}$; the expected number

Table 5. Matrix of values for Jacquard's distance

Z/Z	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22
1	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·
2	0·22	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·
3	0·49	0·49	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·
4	0·45	0·45	0·27	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·
5	0·44	0·44	0·32	0·06	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·
6	0·49	0·56	0·45	0·41	0·37	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·
7	0·22	0·32	0·56	0·37	0·36	0·59	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·
8	0·37	0·45	0·63	0·47	0·46	0·58	0·19	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·
9	0·63	0·63	0·33	0·29	0·33	0·51	0·57	0·56	·	·	·	·	·	·	·	·	·	·	·	·	·	·
10	0·29	0·37	0·6	0·43	0·42	0·55	0·08	0·12	0·52	·	·	·	·	·	·	·	·	·	·	·	·	·
11	0·17	0·36	0·59	0·41	0·4	0·59	0·06	0·24	0·6	0·14	·	·	·	·	·	·	·	·	·	·	·	·
12	0·27	0·27	0·52	0·33	0·32	0·59	0·06	0·24	0·54	0·14	0·12	·	·	·	·	·	·	·	·	·	·	·
13	0·36	0·17	0·59	0·41	0·4	0·65	0·17	0·33	0·6	0·24	0·22	0·12	·	·	·	·	·	·	·	·	·	·
14	0·24	0·41	0·63	0·47	0·46	0·55	0·14	0·17	0·56	0·06	0·08	0·19	0·29	·	·	·	·	·	·	·	·	·
15	0·27	0·36	0·59	0·41	0·4	0·59	0·17	0·33	0·6	0·24	0·12	0·22	0·22	0·19	·	·	·	·	·	·	·	·
16	0·27	0·36	0·52	0·33	0·32	0·56	0·06	0·24	0·54	0·14	0·12	0·12	0·22	0·19	0·12	·	·	·	·	·	·	·
17	0·32	0·22	0·56	0·37	0·36	0·62	0·12	0·29	0·57	0·19	0·17	0·06	0·06	0·24	0·17	0·17	·	·	·	·	·	·
18	0·52	0·59	0·69	0·54	0·53	0·64	0·37	0·22	0·62	0·32	0·41	0·41	0·49	0·36	0·41	0·33	0·45	·	·	·	·	·
19	0·49	0·56	0·66	0·5	0·49	0·61	0·33	0·17	0·59	0·27	0·37	0·37	0·45	0·32	0·37	0·29	0·41	0·06	·	·	·	·
20	0·56	0·62	0·71	0·57	0·56	0·67	0·41	0·27	0·65	0·36	0·45	0·45	0·52	0·4	0·45	0·37	0·49	0·17	0·12	·	·	·
21	0·37	0·52	0·63	0·6	0·59	0·55	0·45	0·32	0·62	0·4	0·41	0·49	0·56	0·36	0·41	0·41	0·52	0·4	0·36	0·44	·	·
22	0·49	0·56	0·66	0·5	0·49	0·61	0·33	0·17	0·52	0·27	0·37	0·37	0·45	0·32	0·37	0·29	0·41	0·27	0·22	0·32	0·17	·
23	0·37	0·45	0·57	0·39	0·38	0·52	0·29	0·12	0·49	0·22	0·33	0·33	0·41	0·27	0·33	0·24	0·37	0·22	0·17	0·27	0·32	0·17

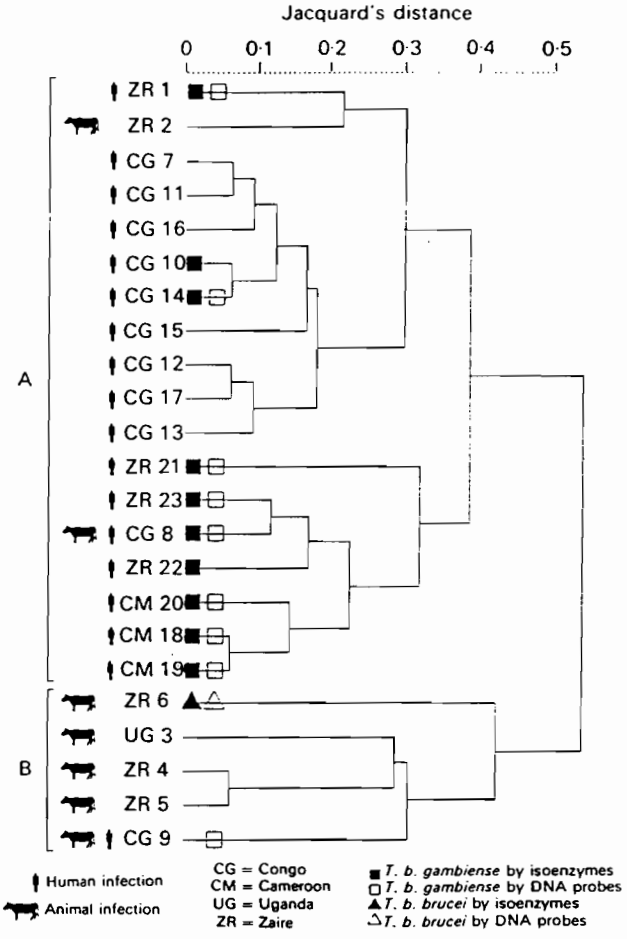

Fig. 2. UPGMA dendrogram built from Jacquard's distance matrix (see Table 5). Square and triangular symbols indicate identification of reference stocks by previous workers (see Table 1).

Table 6. Hardy–Weinberg equilibrium test concerning the Bouenza area. (P = level of significance (at the 0·05 level).)

Genotype	Observed	Expected	χ^2	P*
Alat 2/2	1	5		
Alat 2/4	19	11	11·67	$< 10^{-3}$
Alat 4/4	2	6		
Me2 1/1	0	5·5		
Me2 1/2	22	11	22	$< 10^{-3}$
Me2 2/2	0	5·5		
Ugppl 1/1	0	4·5		
Ugppl 1/2	20	11	22	$< 10^{-3}$
Ugppl 2/2	2	6·5		

* P = level of significance at the 0·05 level.

is 10 while 19 were found: $\chi^2 = 11{\cdot}21$; $P < 10^{-3}$. The same probability for zymodeme 8 is $5{\cdot}5 \times 10^{-4}$. Out of 10^4 Monte Carlo simulations, repetition of any genotype with 19 individuals or more, as for zymodeme 7, was observed only 41 times (test d2; probability = $4{\cdot}1 \times 10^{-3}$). Cases with only 11 different genotypes, as in the whole sample, or less

Table 7. Hardy–Weinberg equilibrium tests concerning the whole Congolese sample

(The d1 test (see Table 2) is used instead of χ^2 when expected size is lower than 5. P = level of significance (at the 0·05 level).)

Genotype	Observed	Expected	Test used	P
Alat 2/2	6	10·08		
Alat 4/4	4	7·92	χ^2	$10^{-2} \ll 10^{-3}$
Alat 2/4	26	18		
Idh 1/1	34	34·2	d1	0·73
Idh 1/2	2	2·16	d1	0·64
Idh 2/2	0	0·032	d1	1
Me2 1/2	30	14·04	d1	6×10^{-5}
Me2 1/1	2	7·92	d1	0·9
Me2 0/0	4	0·43	d1	$8{\cdot}9 \times 10^{-4}$
Me2 2/0	0	3·31	d1	1
Me2 1/0	0	3·7	d1	1
Me2 2/2	0	6·12	d1	1
Pgm 1/1	2	0·108	d1	$5{\cdot}3 \times 10^{-3}$
Pgm 2/2	34	31·68	d1	0·176
Pgm 1/2	0	4·06	d1	0·1
Tdh 1/1	2	0·216		
Tdh 2/2	32	30·24	χ^2	0·4
Tdh 1/2	2	5·4		
Uggp1 1/1	0	7·2		
Ugpp1 1/2	32	17·64	χ^2	$< 10^{-3}$
Ugpp1 2/2	4	10·8		

(test e) were recorded only 24 times (probability = $2{\cdot}4 \times 10^{-3}$). The probability of observing a linkage disequilibrium as high as actually observed (test f) is 0 (no case observed out of 10^4 randomization runs). Apart from these examples of approximate statistical results, the data are worth analysing qualitatively. Zymodeme 7 was isolated in geographically distant places (Kingoli and Minga; Fig. 3) and revealed the presence of heterozygotes at 3 different loci. This fixed heterozygosity is an extreme case of the excess of heterozygotes mentioned above. Zymodeme 8 is represented in the Congo in even more geographically separated places. In addition the stocks in Z12 were isolated in the same place at a nine-year interval. Thus, Z8 exhibits a genetic stability in space and time while Z12 shows a permanency in time.

We have not performed statistical analyses separately for the two clusters evidenced in the present study (see Fig. 2). Indeed one could consider that if these two clusters represent two distinct biological species, this could bias our tests and provide unreasonably strong evidence for clonality. Nevertheless, it should be emphasized that the tests were performed on the Congolese stocks only, which represent almost the totality of cluster 1 (only 2 Congolese stocks in cluster 2). So it is extremely improbable that these tests become non-significant only by discarding 2 stocks out of 36 from the calculations. Apart from the statistical results themselves, the assumptions made on qualitative analy-

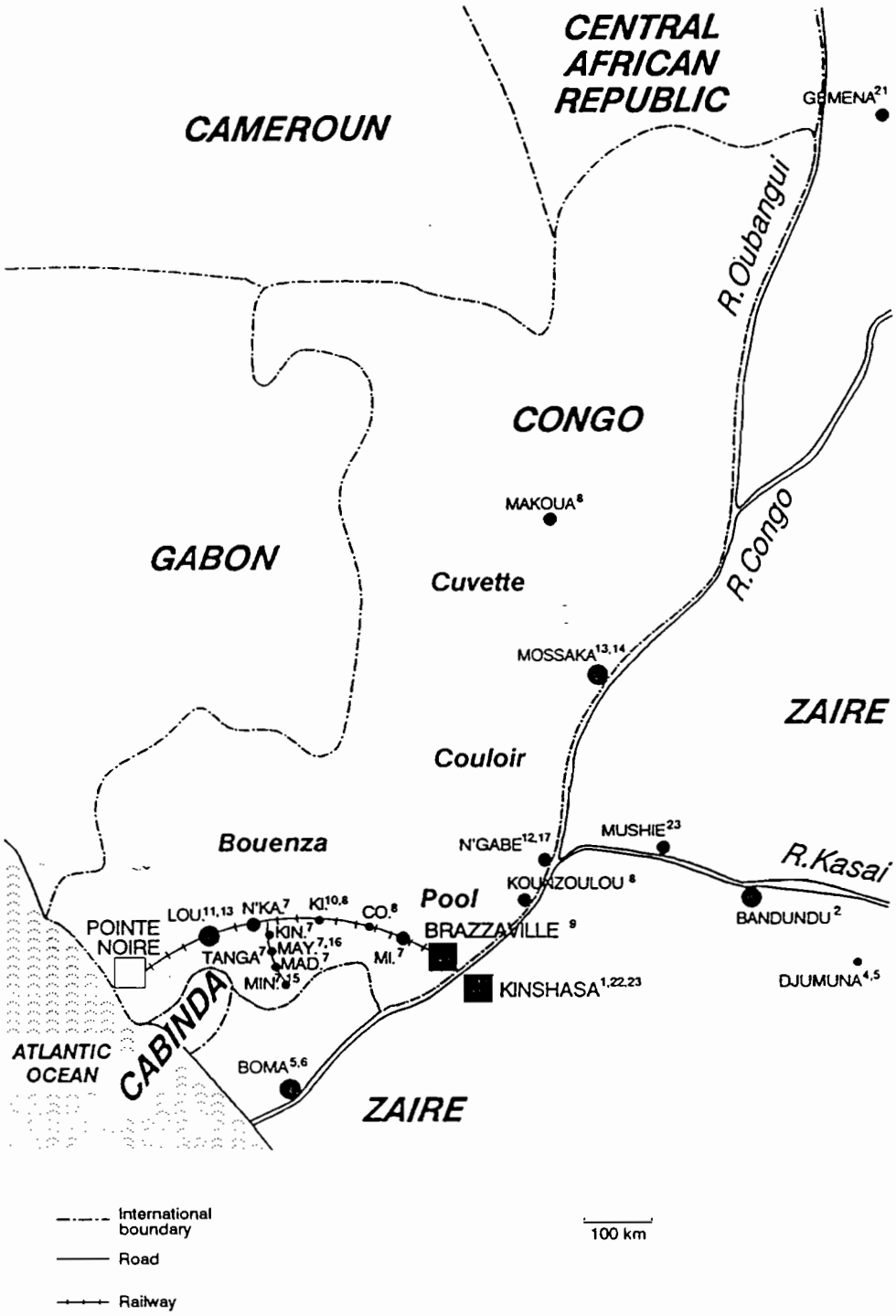

Fig. 3. Geographical distribution of the zymodemes recorded in the Congo and Zaire (see Tables 1 and 2). Numbers refer to zymodemes found in an area (see text). CO = Comba, KI = Kinzaba, KIN = Kinsaka, LOU = Loudima, MAD = Madiadia, MAY = Mayanama, MI = Mindouli, MIN = Minga, N'KA = N'Kayi.

sis of the data (fixed heterozygosity, permanency of the zymodemes in space and time: see above) would remain true even by discarding these two stocks.

RFLP

The interpretation is based on the linearized physical maps of the maxicircles set by Borst & Fase Fowler (1979). By estimating the size of the linear DNA fragment including the variable region, we can distinguish 8 different types or schizodemes. The results are shown in Table 8. Although the two sets of genetic markers, isoenzymes and RFLP, generally are in agreement, RFLP appears as less discriminant than isoenzymes since stocks in the same schizodeme can belong to different zymodemes; for example, SH86 and BOSENDJA belong to schizodeme A although their respective zymodemes are Z2 and

Table 8. List of the 8 schizodemes identified in the present study

(SFVR = Size of the fragment including the Variable Region (see also Table 1) in kilobases.)

Schizodemes	SFVR
A	7·8
B	8·2
C	8·8
D	9·0
E	10·0
F	8·0
G	9·2
H	8·6

Z23. This finding corroborates the results of Gibson *et al.* (1985*a*). The same zymodeme was never found in several schizodemes.

DISCUSSION

General genetic variability

The differences in genetic distances recorded are not negligible (average Nei's distance (Dn) = 0·72, with standard deviation = 0·149, maximum Dn = 0·72, minimum Dn = 0·02), but they are less than that obtained with another parasite, with a comparable level of isoenzyme resolution. In *Trypanosoma cruzi*, Tibayrenc & Ayala (1988) found Nei's standard genetic distances ranging from 0·017 to 2·015, with an average of 0·757 and a standard deviation of 0·478. Although the values observed in *T. brucei* are increased by considering a wider geographical range (Mathieu-Daudé, 1991), the maximum values recorded for *T. brucei* remain clearly less than the ones recorded in the Chagas' disease agent.

Reproduction system in field conditions

Results of the statistical tests favour a predominantly clonal population structure in the Congolese sample, which is in agreement with the same general hypothesis proposed for the taxon *T. brucei* s.l. as a whole (Tibayrenc *et al.* 1990, 1991). Segregation tests not only exhibit strong departures from Hardy–Weinberg expectations, but also show that these results are mainly due to an excess of heterozygotes. Two facts are worth emphasizing: (*a*) on a much wider geographical scale, for comparable sample scales and with similar electrophoretic data, human populations show weak or null deviations from panmixia (Tibayrenc *et al.* 1991); (*b*) deviations from Hardy–Weinberg expectations that are observed here are difficult to explain by geographical structure. Moreover, it is worth underlining that deviations from Hardy–Weinberg expectations are frequently due to excess of heterozygotes. Now, if genetic drift leading to allelic frequency differences among localities (geographical structure) was the explanation of the observations, a deficit of heterozygotes (Whalund effect) would be expected (Tibayrenc & Ayala, 1991; Tibayrenc *et al.* 1991). Similarly, when recombination tests are considered, the multilocus genotypes in excess (zymodemes 7 and 8) have no tendency to be localized but, on the contrary, are widespread over large geographical areas. We have also shown (Tibayrenc *et al.* 1991) that when linkage disequilibria are due to allelic frequency differences among localities, the excess multilocus genotypes that are generated tend to be restricted to particular localities.

Zymodeme and schizodeme analyses (see Table 1) show that isoenzyme variability and kDNA RFLP generally are in agreement. This correlation between two independent sets of genetic markers is classical evidence of clonality (test f; Tibayrenc & Ayala, 1988; Tibayrenc *et al.* 1990). It is a particular case of linkage disequilibrium, revealed when different genomes are considered; isoenzyme loci are considered as nuclear genes, while the kinetoplast is an extranuclear genome.

It has been proposed (Cibulskis, 1992) that, although *T. brucei* zymodemes behave as clones in the short run, recombination events make these clones unstable, with no permanency in space and time. The present data do not seem to support this hypothesis. Several multilocus genotypes or zymodemes were recorded over wide geographical areas and long periods of time (see Tables 1 and 3): Z7 was sampled over the whole Bouenza region (5 different localities) and in the Pool region; Z8 was observed from 1974 to 1983, in three different regions of the Congo, and three different mammalian hosts; Z12 was isolated at dates ranging from 1980 to 1989; Z5 and Z23 were sampled from Zairian localities distant from one another. The clonal hypothesis proposed (Tibayrenc *et al.* 1990) states that the clones observed in many parasitic protozoa are stable in space and time; it does not imply that they must be worldwide distributed. The present data show that several *T. brucei* multilocus genotypes were recorded throughout a whole country. The same distribution was also observed even at much larger geographical scales (Godfrey *et al.* 1990; Mathieu-Daudé, 1991).

We hence take as a working hypothesis that the zymodemes observed in the present work can be equated to natural clones, or families of closely related clones, stable in space and time, that deserve to be considered as useful taxonomic units in epidemiological studies. As previously emphasized (Tibayrenc & Ayala, 1988; Tibayrenc *et al.* 1990), the hypothesis does not rule out the possibility of occasional genetic exchanges but states that these exchanges are not frequent enough to break the prevalent pattern of a basically clonal population structure.

Taxonomic clustering

Group A (see Fig. 2) includes the majority of the stocks isolated from man in the present study; included are some reference stocks identified by other authors as *T. b. gambiense*. This group could represent either the classical *T. b. gambiense* subspecies, or, more restrictively, *T. b. gambiense* group 1 (Gibson, 1986). The present set of data does not allow us to choose between these two possibilities. Group B would be equated to the classical subspecies *T. b. brucei*, if Z9 did not include a stock isolated from man; however, on examining the records of isolation we are uncertain of the true provenance of FY and thus group B could be *T. b. brucei*. Alternatively, the presence of a human stock in cluster B would be consistent with the hypothesis that cluster A is equivalent to *T. brucei gambiense* group 1.

Linkage disequilibrium is total between the genotypes recorded at the *Sod a* locus on the one hand, and *Sod b* on the other hand; *Sod a* II is systematically associated with *Sod b* III, and likewise *Sod a* I with either *Sod b* I or II. The first association is specific for group A, while group B can be characterized by the second association. These patterns correspond to those previously described (Truc, 1991; Stevens *et al.* 1992).

Although both groups show a rough correspondence with the two commonly accepted subspecies, it is important to underline that each of them represents a set of stocks that are genetically heterogeneous. The genetic variability recorded in the whole sample (0·1219) is not notably higher than the ones recorded in each group (0·0721 for group A, 0·1023 for group B). It is hence not certain that these two clusters are the only meaningful subdivisions to be distinguished for epidemiological purposes or clinical surveys, within the present set of stocks. Until further information is obtained, we propose that the natural clones that subdivide the species *T. brucei* should be considered as the relevant taxonomic units in all applied studies.

Epidemiological considerations

The present sample is still limited and not random in the geographical area under survey and firm epidemiological conclusions cannot be reached. However, it may be that the geographical distribution of the zymodemes is not random, but follows communication lines, such as rivers (Congo-river, Kasai river), roads (Brazzaville–Pointe Noire road) and the railway line.

In the case of Z7, the dominant zymodeme in Bouenza, the distribution appears to reflect an epidemic pattern. This is difficult to explain by tsetse fly migration alone, because the distances separating villages are large, in excess of 200 km although tsetse are known to be transported by vehicles. On the other hand, it seems unlikely that the same multilocus genotype could be generated in different places, far from one another, by convergence from a common ancestor. Human movements would be the most parsimonious hypothesis to account for clone spread; asymptomatic forms of the disease could have a notable impact (Truc, 1991).

Z8 includes both human stocks (PA and OK) and animal stocks (TB26 and D12K). It is interesting to note that D12K was identified as *T. b. gambiense* by isoenzymes, DNA probes and high resistance to human plasma (Scott *et al.* 1983; Paindavoine *et al.* 1986). Although isolation of the same zymodeme both from man and from animals is not definitive evidence for communication between human and animal reservoirs, this result is in agreement with the hypothesis of an animal reservoir in the Congo. The epidemiological role of this animal reservoir is considered as negligible during epidemic periods, considering the high prevalence in man (Noireau *et al.* 1986). It could nevertheless be partly responsible for the inter-epidemic persistence of the sickness (Noireau *et al.* 1989). It is necessary to check parasitologically all the domestic animals in an endemic area in order to assess the effectiveness of animal reservoirs.

The prevalence of *T. brucei* infections in animals is notably higher in Zaire (16% of pigs and 6·2% of sheep, Makumyaviri *et al.* 1989), than in the Congo (0·5% in all animals, Noireau *et al.* 1986). Even if most of the stocks isolated from animals in Zaire belong to group B (*T. b. brucei*), and so are probably not pathogenic to man, this supposed non-pathogenicity must be confirmed by further extensive population genetic studies in this area.

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Short communication

A new method for identifying blood meals of human origin in tsetse flies

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Abstract

A new sensitive technique using the electrophoresis of superoxide dismutase to distinguish between tsetse blood meals of human and non-human origin is described. In Côte d'Ivoire, 602 blood meals were collected; 170 were from man (28.3%), 377 from animals (62.6%) and 55 were unidentified (9.1%) because no pattern was observed. When calculating the index of epidemiological risk, it is strongly correlated with the incidence of sleeping sickness cases.

Keywords: Blood meal; Human trypanosomiasis; Isoenzyme; Tsetse fly

1. Introduction

The calculation for a given area of the index of epidemiological risk should lead to major improvements in human African trypanosomiasis control (Laveissière et al., 1994). An important component in such an estimate is the number of blood meals taken from man by the tsetse fly vector, which may, for instance, increase at the beginning of a period of high transmission. There may be no need to identify

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the source of every blood meal, as is normally done and the widely used complement fixation test may fail to identify more than 20% of blood meals collected (Staak et al., 1986). Instead the proportion of human to animal feeds would suffice for many purposes, particularly if changes in feeding patterns are suspected. If valid results are to be obtained, samples must be protected against deterioration, which may be rapid in tropical conditions.

This paper presents a sensitive technique using the electrophoresis of superoxide dismutase to distinguish between tsetse blood meals of human and non-human origin.

2. Materials and methods

2.1. Collection of midguts

After dissecting a trapped tsetse fly in saline on a microscope slide, the whole midgut was removed and placed at a marked position on a Whatman No. 1 filter paper. The samples were stored in a glass vial, containing silica gel, and kept refrigerated until required.

2.2. Sample preparation and isoenzyme electrophoresis

The piece of filter paper carrying the squashed midgut from each fly was cut out and place into 30 μl of enzyme stabilizer (Miles and Ward, 1978), in a well of a microtitre plate kept on ice. After 20 min, the extraction product was re-suspended thoroughly by repeated gentle pipetting. Five applications in one position totalling 2 μl from each extract were then placed at the origin of a cellulose acetate plate, ready for electrophoresis. Ten samples were placed on each plate, together with control extracts from human blood and from the midgut of a starved fly. Electrophoresis, by the Helena system, and staining for superoxide dismutase (SOD; EC 1.15.1.1) are described by Truc and Tibayrenc (1993).

3. Results and discussion

In man, two autosomal loci SODa and SODb determine respectively the soluble and mitochondrial forms of the enzyme (Creagan et al., 1973); at pH 7.0 the SODa isoenzymes migrate anodally and the SODb cathodally. SODa is found in all tissues except polymorphonuclear leucocytes (Beckman et al., 1973). A preliminary study showed that SOD from human red blood cells had a different electrophoretic mobility than SOD from tsetse midgut. Furthermore, no SODa variability was observed among different human blood samples nor among several midguts from wild flies. When comparing human blood with blood of other mammals (cattle, pig, sheep, horse and mouse), the patterns observed for human blood was clearly different from other bloods and also from the midguts of starved tsetse flies.

Although tsetse can feed on others species of animals, previous studies show that 90% of meals are taken either from man, pig or dog by *Glossina palpalis* in Côte d'Ivoire (Staak et al., 1986). Thus, our technique could distinguish human from non-human blood in a tsetse midgut.

After staining, the patterns appeared as white zones on a orange background. Interpretation must be done immediately because sometimes an indistinct pattern, presumably as a result of a small amount of enzyme, disappeared when fixed with acetic acid. The patterns observed clearly showed those from man had a significantly faster mobility of SOD than any non-human blood meal.

In Côte d'Ivoire, 602 blood meals were collected from *G. p. palpalis* in the Sinfra area; 170 were found to be from man (28.3%), 377 from animals (62.6%) and 55 were unidentified (9.1%) because no pattern was observed. These unidentified cases are probably due to digestion of a blood meal, so that SOD had been wholly degraded.

When calculating the index of epidemiological risk (Laveissière et al., 1994) from those results, it is strongly correlated with the incidence of sleeping sickness cases (Laveissière, unpublished results). A large number of human blood meals in an area indicates a high risk of disease transmission.

Although this technique does not allow species identification but only distinguishing human from non-human feeds, the number of unidentified blood meals seems lower than using the technique of Staak et al. (1981); 9.1% instead of 23.3%. Work is in progress in order to reduce the cost of the new electrophoretic approach. Further comparative trials should be carried out, espacially with the ELISA technique used for rapid detection of human blood meals in mosquitoes (Savage et al., 1991).

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Identification of trypanosomes isolated by KIVI from wild mammals in Côte d'Ivoire: diagnostic, taxonomic and epidemiological considerations

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Abstract

In Côte d'Ivoire, a comparative study was carried out on 122 wild mammals by parasitological and serological examination and by in vitro isolation of trypanosomes from fresh blood (KIVI). Thirteen isolated stocks were studied by isoenzymes and compared with *Trypanosoma congolense* and *T. brucei* bouaflé group reference stocks. Of the 122 animals, only 22 were positive on blood smears while 88 were KIVI positive and 92 were CATT/*T. b. gambiense* positive. For six stocks identified by isoenzymes as *T. congolense*, the agreement between ELISA and CATT was good (75%). As compared with CATT, antigen detection ELISA was not satisfactory for *T. brucei* (20%). Out of 18, 16 stocks represented a separate zymodeme (seven *T. congolense* and nine *T. brucei*) and a high genetic heterogeneity was observed. For *T. congolense*, savanna, kilifi and forest groups were represented by one zymodeme each. The four remaining zymodemes while put into this *T. congolense* group, were strongly independent of each other. Morphology indicated that those new zymodemes correspond to *T. congolense*. In the other hand, five new zymodemes fit into *T. brucei* classification. © 1997 Elsevier Science B.V.

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Keywords: *Trypanosoma brucei*; *Trypanosoma congolense*; Wild animals; Diagnostic; Isoenzymes; Taxonomy

1. Introduction

In East Africa, wild animals are a component of the epidemiological cycle of the acute form of sleeping sickness caused by *Trypanosoma brucei rhodesiense* (e.g. Ashcroft, 1959). For the gambian chronic form of this disease, a wild animal reservoir has also been suspected by serological investigations, especially the kob (*Kobus kob*) and lion (*Panthera leo*, Guedegbe et al., 1992). In Burkina-Faso, several wild animals are infected by trypanosomes belonging to the subgenera *Trypanozoon*, *Duttonella*, *Nannomonas* and *Megatrypanum* (see Mehlitz, 1986; Mattioli et al., 1990). Isolation of the various trypanosomes, except for *Megatrypanum*, was always achieved by inoculating rodents (*Mastomys natalensis*, see Mehlitz, 1986) with fresh blood from kob or waterbuck (*Kobus defassa*). A few years ago, the Kit for in vitro Isolation of trypanosomes (KIVI; Aerts et al., 1992; Truc et al., 1992) was developed for in vitro isolation of trypanosomes in the field. Following its use for isolation of human and domestic animal trypanosomes (Truc et al., 1994), a first trial with wild animals was carried out in the Côte d'Ivoire (Komoin-Oka et al., 1994). However, no accurate identification of the trypanosomes detected was achieved. The main objective of the present study was the precise identification by isoenzyme electrophoresis of trypanosomes from wild animals.

2. Materials and methods

2.1. Study area

All new stocks were isolated in the Comoé National Park, in northern Côte d'Ivoire. The tsetse flies in this area are *Glossina longipalpis*, *G. tachinoides*, *G. medicorum*, *G. fusca fusca* and *G. palpalis palpalis* (Laveissière and Challier, 1981).

2.2. Collection of samples and parasitological and serological examinations

In total, 122 animals were bled in the field. From each, blood was inoculated into KIVI and a blood smear and plasma collected. The microscopic examination of the stained blood smears, the antigen detection ELISA (Nantulya and Lindqvist, 1989), the card agglutination test CATT/*T. b. gambiense* (Magnus et al., 1978) and a follow-up of the cultures over 60 days were carried out in the laboratory. KIVI inoculation and subcultures were done according to Truc et al. (1992, 1994).

The isolated stocks were compared with reference stocks KK39 (kob) and AB14 (hartebeest) previously identified as *T. brucei* bouaflé group (Godfrey et al., 1990;

Young and Godfrey, 1983), TSW103 (pig) identified as *T. congolense* forest group, WG84 (sheep) identified as *T. congolense* kilifi group and 1/148 (cattle) identified as *T. congolense* savanna group (Gashumba et al., 1988).

For the antigen ELISA test, monoclonal antibodies (*T. brucei*, *T. congolense* and *T. vivax*) were provided by courtesy of the International Laboratory for Research on Animal Diseases (ILRAD), Nairobi. Serum dilution for *T. brucei* detection was 1/120 and 1/10 for *T. congolense* and *T. vivax*. Optical density (OD) was determined between test and control sera provided by courtesy of the ILRAD with a Labsystems Multiskan ND MCC/340 at 414 nm wave length. The test was regarded as positive if the OD difference was 0.05 for *T. brucei* and *T. congolense* and 0.02 for *T. vivax*.

CATT/*T. b. gambiense* test was performed with 5 μl of plasma (Magnus et al., 1978). This test was originally designed for *T. b. gambiense* detection in human blood, but also allows other *T. brucei* subspecies antibodies to be detected in animal blood (e.g. Noireau et al., 1986).

2.3. Isoenzyme electrophoresis

The following 14 enzyme systems (17 loci) were studied by cellulose acetate electrophoresis: alanine aminotransferase (ALAT, EC 2.6.1.2.), glyceraldehyde-3-phosphate dehydrogenase (GAPD, EC 1.2.1.12.), aspartate aminotransferase (ASAT = GOT, EC 2.6.1.1.), glucose phosphate dehydrogenase (GPI, EC 5.3.1.9.), isocitrate dehydrogenase (IDH, EC 1.1.1.42.), malate dehydrogenase (MDH, EC 1.1.1.37.), malic enzyme (ME, EC 1.1.1.40.), nucleoside hydrolase (NH, EC 3.2.2.1.) using two different substrates; inosine (NHi) and deoxyinosine (NHd), peptidase (substrate L-leucyl-L-alanine: PEP2, EC 3.4.11 or 13.*.), 6-phosphogluconate dehydrogenase (PGD, EC 1.1.1.44.), phosphoglucomutase (PGM, EC 2.7.5.1.), superoxide dismutase (SOD, EC 1.15.1.1.) and threonine dehydrogenase (TDH, EC 1.1.1.103.) Sample preparation, staining and electrophoresis procedures were performed according to Truc et al. (1991) and Truc and Tibayrenc (1993), except for SOD, which was performed according to Stevens et al. (1989). All chemicals were obtained from Sigma, and the Helena® system was used for the electrophoresis.

Zymodemes were defined by phenotype interpretation of patterns. A dendrogram was constructed by the unweighted pair group method using arithmetic averages (UPGMA) from the Jacquard's distance (JD) matrix. This UPGMA showed the taxonomical relationships between zymodemes (Serres and Roux, 1986).

3. Results

3.1. In vitro isolation and serological tests

Of 122 animals, 22 were positive on blood smears and 88 were KIVI positive (Table 1). Only five KIVI were contaminated with other microorganisms. The KIVI became positive in an average of 10.2 days (minimum, 6 days; maximum, 23 days).

Because of the poor growth of most stocks isolated in vitro, only 13 were compared by isoenzymes with reference stocks.

Microscopical examination of the 22 blood smears suggested the presence of *T. brucei*, *T. congolense* and *T. vivax*. However, the bad quality of the smears and the very low parasitaemia did not allow an accurate morphological identification. These results are not shown or discussed. In any case, the parasitological examination gave fewer positives (18%) than the KIVI (72%). On the other hand, nine animals with positive blood films gave negative KIVI (Table 1).

Of the 122 animals, 92 were CATT positive. Those results are not shown in this study, except for those stocks analysed by isoenzymes which are compared with the ELISA results and isoenzyme identification (Table 2). The results are detailed in Table 2. For those stocks identified by isoenzymes as *T. congolense*, the agreement between ELISA and CATT is good (4/6). On the other hand, as compared with CATT, ELISA was not satisfactory for *T. brucei* detection because only 2/7 animals were ELISA positive while 5/7 were CATT positive.

The 34 negative KIVI corresponded to 12 kob, one waterbuck, eight bushbuck (*Tragelaphus scriptus*), three roan antelope, five red duiker (*Cephalophus*), two warthog (*Phacochoerus aethiopicus*) and three elephant (*Loxodonta africana*).

3.2. Isoenzyme characterization

Five stocks were isolated from roan antelope (*Hippotragus equinus*), four stocks from buffalo (*Syncerus caffer*), one from waterbuck (*Alcelaphus buselaphus*) and four stocks from kob (Table 3). In the present study, one stock was previously

Table 1
Blood smear and KIVI results obtained from 122 wild animals

No. of animals	Blood smear	KIVI
61 kob	4+	1+
	57−	48+
18 waterbuck	5+	5+
	13−	12+
10 buffalo	1+	1+
	9−	9+
10 bushbuck	6+	2+
	4−	4−
9 roan antelope	5+	3+
	4−	3+
7 red duiker	7−	2+
3 warthog	1+	1+
	2−	2−
3 elephant	3−	3−
1 hartebeest	1−	1+
Total: 122 animals	22+	13+
	100−	75+
Positive (%)	18.03	72.13

Table 2
Results of ELISA, CATT and isoenzyme identification in individual wild animals

Animal	*n*	ELISA			CATT	Isoenzyme identification
		T. brucei	*T. congolense*	*T. vivax*		
Roan antelope	2	–	–	–	+	*T. congolense*
	8	+	+	+	+	*T. congolense*
	42	–	+	–	+/–	*T. congolense*
	43	+	+	–	+/–	*T. congolense*
Buffalo	20	–	+	–	–	*T. brucei*
	21	+	+	–	–	*T. congolense*
	23	–	+	–	+	*T. brucei*
	24	+	+	+	–	*T. brucei*
	26	+	+	–	+/–	*T. congolense*
Kob	110	–	+	–	+	*T. brucei*
	123	–	–	–	+	*T. brucei*
	132	–	–	–	+	*T. brucei*
Waterbuck	51	+	+	–	+	*T. brucei*

identified by isoenzymes as *T. b. gambiense*, with the absence of the subspecific SODa and SODb phenotypes, 3-6-7-11-13 and 3-5, respectively (Truc et al., 1997), equivalent of the phenotypes 3 and 5 for the same loci described by Stevens et al. (1992).

Isoenzyme electrophoresis revealed 16 zymodemes for 18 stocks (Table 4). Almost each stock corresponded to a separate zymodeme, except Z20 (stocks 42 and 43) and Z22 (stocks 21 and 26). Generally however, a high genetic heterogeneity was observed. The dendrogram is shown in Fig. 1. The two main groups are: (i) a *T. brucei* group of nine zymodemes (JD < 0.5). A subgroup (Z31, 37, 33 and 30) appeared to be genetically distinct from other zymodemes (JD < 0.3). Compared to the reference zymodemes Z33 and 37, previously identified as the *T. brucei* bouaflé group (Stevens et al., 1992), this subgroup could be equated to the same group; (ii) a *T. congolense* group of seven zymodemes (three reference zymodemes and Z19, 20, 22 and 26). Within this group, high genetic heterogeneity occurred. Z19, Z20, Z22 and Z26 seemed to be closely related to the known *T. congolense*.

4. Discussion

The high number of positive KIVI confirmed its potential value as a diagnostic test for trypanosome infection. The fact that nine animals with positive blood films remained KIVI negative is probably due to species of trypanosomes involved (e.g. *T. vivax*) which cannot grow in the KIVI culture medium.

The present version of CATT aims at *T. b. gambiense* antibodies detection for diagnosis of the Human African Trypanosomiasis. However, cross reactions with

antibodies in animal blood infected by other *T. brucei* subspecies or by *T. congolense* have been described (e.g. Noireau et al., 1986). Thus, because of the simple use of the CATT in the field and the immediate result, an evaluation of this serological test system, or alternative versions thereof, on a large scale might be interesting for animal trypanosomiasis surveys.

A limited number of studies based on a small range of stocks has been carried out by isoenzymes comparing *T. congolense* and *T. brucei* (e.g. McNamara et al., 1994). The distinction between the two species is clear with only a few isoenzyme bands identical in both. The bouaflé group is distinct within *T. brucei* sp. by its specific patterns observed for the isoenzyme superoxide dismutase (SOD; SODa phenotype 1-2-5-12-14, SODb phenotype 3-7), confirming the observations of Stevens et al. (1992).

For *T. congolense*, an important variation is observed in this study. Savanna, kilifi and forest groups are represented by one zymodeme each. They are clearly independent from each other, confirming previous results (Young and Godfrey, 1983; Gashumba et al., 1988); ICD isoenzyme is still specific for the savanna group. On the other hand, ME1, NHd, PGD and PGM distinguish these three groups of *T. congolense* (see Table 4). The others zymodemes (Z19, 20, 22 and 26) while put into this *T. congolense* group, are strongly independent of each other, except for Z20 and Z26 which are genetically close ($JD < 0.15$). This heterogeneity may indicate that the zymodemes from wild animals do not necessarily fit into the previous classification of *T. congolense*. No DNA amplification was obtained when

Table 3
Stocks under study, with zymodeme (Z) and taxonomic identification

Stock	Host	Year	Area	Country	Z	Identification	Ref.
2	Roan antelope	1993	Comoé	CI	19	*T. congolense*	—
42	Roan antelope	1993	Comoé	CI	20	*T. congolense*	—
43	Roan antelope	1993	Comoé	CI	20	*T. congolense*	—
23	Buffalo	1993	Comoé	CI	21	New brucei	—
21	Buffalo	1993	Comoé	CI	22	*T. congolense*	—
26	Buffalo	1993	Comoé	CI	22	*T. congolense*	—
1/148	Cattle	1960	River Donga	NGR	24	Congolense savanna	1
WG84	Sheep	1981	Matuga	KEN	25	Congolense kilifi	1
8	Roan antelope	1993	Comoé	CI	26	*T. congolense*	—
TSW103	Pig	1977	Sanniquelle	LIB	27	Congolense forest	1
24	Buffalo	1993	Comoé	CI	28	New brucei	—
110	Kob	1993	Comoé	CI	29	New brucei	—
132	Kob	1993	Comoé	CI	30	Brucei bouaflé	—
123	Kob	1993	Comoé	CI	31	Brucei bouaflé	—
KK39	Kob	1980	Comoé	BRK	33	Brucei bouaflé	2
20	Buffalo	1993	Comoé	CI	34	New brucei	—
51	Waterbuck	1993	Comoé	CI	35	New brucei	—
AB14	Hartebeest	1980	Comoé	BRK	37	Brucei bouaflé	3—

1, Gashumba et al., 1988; 2, Young and Godfrey, 1983; 3, Stevens et al., 1992. CI, Côte d'Ivoire; NGR, Nigeria; KEN, Kenya; LIB, Liberia; BRK, Burkina-Faso.

Table 4

Z	ALAT	GAPD	GOT	GPI	IDH	MDH	ME1	ME2	NHi1	NHi2	NHd	PEP2	PGD	PGM	SODa	SODb	TDH
19	1	1-2	4-6-9	8	3	2-3-10-11	1-6	99	1	2	6	3-7-10	5-6	5-7	11-18-19	3-7	99
20	3	1	9	9	3	2-11	1	99	3	1	5	6	6	8-10	15-16-17	1-2-4	99
21	4-5-6	4	5-7-8	7	5	5-6-8	2	3	3	3	9-11	1-2	7	11–12	8-9-12-14	2-4	3
22	3	2	3	4	1-6	3-10	6	99	2	2	3	6	6	1-2	99	2-4	4-5
24	3	3	4	2-3	4	4-7	7	99	2	1	7	7-9	2	4	4-12	1	1
25	9	3	4	3	3	4-9	6	99	4	1	10	5-8	1	3	4-12	1-2	2
26	3	1	9	9	3	2-11	1	99	3	1	5-6	7	6	8-10	15-16-17	1-2-4	99
27	3	3	4	1	5	4-7	3-4-5	99	3	1	2-3-4	7-9	4	6-9	4-12	1–4	2
28	3	4-5	2-8	5-7	2-5	5-6-8-12	2	3	3	3	9-11	1-4	3	11-12	1-2-5-12-14	3-7	3
29	2	4	1-7	6	5-7	1-5-6-8	2	3	3	3	9-11	1-4	7	11-12	1-2-3-13-14-19	3-6-7	3
30	6	4	7	7	5	5-6-8	2	3	3	3	1-12	1–4	7	11-12	1-2-5-1-12-14	3-7	3
31	6	4	5-7-8	7	5	5-6-8	2	3	3	3	9-11	1-2-4	7	11-12	1-2-5-12-14	3-7	3
33	5-6	4	8	7	5	5-6	2	3	3	3	9-11	1–4	7	11-12	1-2-5-12-14	3-7	3
34	4	4	5-7-8	7	5	5-6-8	2	3	3	3	9-11	1-2	7	11 -12	8-9-12-14	2-4	3
35	6	4	5-7	7	5	5-6	2	3	3	3	9-11	1-2-4	7	11-12	8-9-12-14	2-4	3
37	5-6	4	5-7-8	7	5	5-6	2	3	3	3	9-11	1-2-4	7	11-12	1-2-5-12-14	3-7	3

Zymodemes recorded and corresponding isoenzymes patterns. Numbers indicate presence or absence of patterns at a given locus, 99 = absence of pattern.

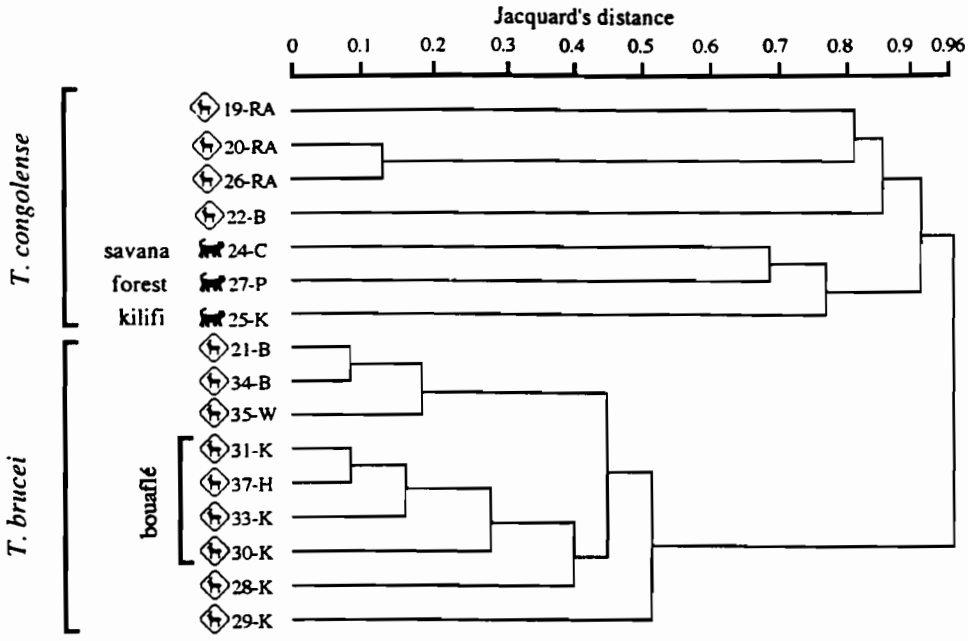

Fig. 1. A UPGMA dendrogram built from Jacquard's distance matrix (not shown). Deer symbol, wild animal infection; cat symbol, domestic animal infection. Numbers refer to zymodemes and initials of host: RA, roan antelope; B, buffalo; C, cattle; P, pig; K, kob; W, waterbuck and H, hartebeest (see Tables 3 and 4). Savanna, forest and kilifi indicate *T. congolense* reference zymodemes (see Table 3).

testing Z19, 20, 22 and 26 by PCR with *T. godfreyi* and *T. simiae* specific primers, according to Masiga et al. (1992) and McNamara et al. (1994). In particular, case Z19, taking into account its isoenzymes and the antigen ELISA negativity for *T. congolense*, it may belong to a stercorarian trypanosome, perhaps *T. (Megatrypanum) theileri*. However, despite its poor quality, we observed in the Z19 blood smear small trypanosomes (20–30 μm), without a free flagellum and the kinetoplast is in a posterior location. These observations do not fit with *Megatrypanum* description which is large in size and has a free flagellum. Despite the isoenzymes and PCR results, morphology indicates that this new zymodeme could be equated to a new group of *T. congolense* never described before. It is not surprising that new strains of parasites are found, because of the use of the new technic for isolation (KIVI) and because of the rare opportunity to sample from wild animals. Further investigations are required in order to precise the taxonomic status of these new zymodemes.

For *T. brucei*, the bouafle group was represented by three zymodemes isolated from kob (Z30, Z31 and Z33) and one from hartebeest (Z37). It has been suggested that human infectivity of bouafle group was not entirely excluded (Godfrey et al., 1990), but recently this human infectivity has been confirmed (Truc et al., 1997). This gives rise to the suspicion of the existence of a wild animal reservoir of HAT in West Africa.

In the present study, five other zymodemes belonged to *T. brucei* (Z21, 34, 35, 28 and 29). They were all isolated from wild animals and are not closely related to other zymodemes within *T. brucei*. Although they may be a new type of *T. brucei* (see Table 1), it is impossible at present to clearly define their accurate taxonomic status. Further morphological and genetic investigations are required.

Finally, this study shows the necessity to use other genetic markers in order to improve the accuracy of identification. For the bouaflé group, its taxonomic status is clear and studies are in progress on the infectivity to man. The hypothesis of a potential wild animal reservoir of HAT in West Africa must also be investigated.

Using the KIVI technique for isolation in the field, it is not surprising to detect some new genetic types of parasites in wild animals. However, again further investigations are required.

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Short Report

Simplification of the miniature anion-exchange centrifugation technique for the parasitological diagnosis of human African trypanosomiasis

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Keywords: trypanosomiasis, human African trypanosomiasis, *Trypanosoma brucei* sspp., miniature anion exchange centrifugation technique, diagnosis

The miniature anion exchange centrifugation technique (m-AECT) to demonstrate trypanosomes in blood has been developed and used in the field for the detection of low parasitaemias (LUMSDEN *et al.*, 1979). In 1982, a sterile m-AECT kit was adapted successfully for field use (P. Cattand, personal communication) and is available from Projet de Recherche Clinique sur la Trypanosomiase (PRCT), Daloa, Côte d'Ivoire. A modified version was later adapted for laboratory use (ZILLMAN *et al.*, 1996). For the sterile m-AECT kit in the field, it was suggested that blood collection be done by finger-prick using a heparinized Caraway tube (CARAWAY & FANGER, 1955). More recently, a study has shown that blood collected by venepuncture using a heparinized syringe substantially increased test sensitivity (TRUC *et al.*, 1998).

The current sleeping sickness epidemic in Côte d'Ivoire has reinforced the need to find highly sensitive, yet cost-effective, techniques to diagnose the disease in the field. With this in mind, we have further improved the sterile m-AECT kit by reducing the amount of material required for its manufacture, thus reducing its cost while retaining its high sensitivity. The syringe barrel containing the anion-exchange diethylaminoethyl (DEAE) cellulose has been replaced by a Pasteur pipette which also serves as a filling reservoir (see Figure). The tip of the Pasteur pipette is cut 10 mm from the shoulder of the pipette. The same amounts of DEAE cellulose and blood are used. The need for a filling reservoir is eliminated since only about half of the pipette is filled with the cellulose. The column is then heat sterilized and stored in a heat-resistant plastic tube with a rubber stopper. The manufacturing process and reduction in material required have halved the production cost, from US$ 2 to US$ 1. The blood is discharged from the syringe (TRUC *et al.*, 1998) on to the DEAE cellulose bed and allowed to permeate it (LUMSDEN *et al.*, 1979). A centrifuge tube is provided to receive the eluate, and phosphate-buffered saline is added to the top of the column until the centrifuge tube is almost filled.

During 3 surveys in Guinea and Côte d'Ivoire, 71 individuals gave positive results in the card agglutination trypanosomiasis test (CATT; MAGNUS *et al.*, 1978) using whole blood and plasma. Each individual was subsequently bled by venepuncture and 2 mL of blood were collected in a heparinized syringe (Sarsdtedt®). Both the original sterile m-AECT kit and the Pasteur pipette version were used to test this blood and the results were compared. Microscopical examination of the eluate was done by 2 experienced technicians. The time required

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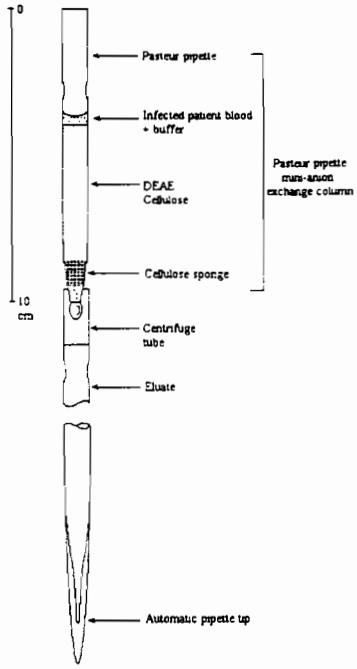

Figure. Pasteur pipette miniature anion-exchange centrifugation kit.

to perform either version of the test was similar, approximately 30–45 min.

Twenty-seven of the 71 individuals had trypanosomes demonstrated in their blood by the original m-AECT kit, and 26 did so with the Pasteur pipette version. Considering the similarity in test implementation and performance, we suggest the use of the Pasteur pipette version which would substantially reduce the cost of the diagnostic procedure. The new version of the m-AECT is manufactured at Institut Pierre Richet (01 B.P. 1500, Bouaké, Côte d'Ivoire) and is available to order.

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Confirmation of two distinct classes of zymodemes of *Trypanosoma brucei* infecting man and wild mammals in Côte d'Ivoire: suspected difference in pathogenicity

Although, since the first studies on the isoenzymes of *Trypanosoma brucei* s. l. (Bagster and Parr, 1973; Kilgour and Godfrey, 1973), use of genetic markers has substantially increased our knowledge of human African trypanosomiasis (HAT), numerous taxonomic and epidemiological questions remain. For example, the exact infra-specific classification of *T. brucei* s.l., the full spectrum of pathogenicity to man and many details of the animal reservoirs have yet to be elucidated (Baker, 1995).

The sole genetically clear group is the one defined and termed *T. b. gambiense* Group 1 (Gibson, 1986); other trypanosomes, including some parasites of man and other animals in West Africa (Mehlitz *et al.*, 1982; Truc *et al.*, 1997) and the causative organism of acute human trypanosomiasis in other parts of Africa (Godfrey *et al.*, 1990; Tibayrenc *et al.*, 1993), cannot be recognized so distinctly as homogeneous genetic entities. Despite analysis of other genetic markers such as RFLP (Paindavoine *et al.*, 1989; Hide *et al.*, 1990), isoenzyme electrophoresis remains the most useful method for identification of *T. brucei* subgroups.

In Côte d'Ivoire, the organisms apparently causing classical, chronic, sleeping sickness are thought to be sympatric with a genetically different variant. The latter is of uncertain pathogenicity but is known to have an animal reservoir (Mehlitz, 1986; Truc *et al.*, 1997). In the present study, the improved sampling resulting from use of the KIVI method (based on a commercial kit for in-vitro isolation of trypanosomes; Aerts *et al.*, 1992; Truc *et al.*, 1992, 1994) was combined with precise genetic identification of the isolates (by isoenzyme analysis) to investigate the types of trypanosome present in humans and other animals in Côte d'Ivoire.

The six reference stocks used for comparison (DAL072, DAL967, TH2, TSW53, KK39 and AB14; see Table) had been isolated in the past by rodent inoculation and identified as *T. brucei gambiense* or belonging to the bouaflé strain-group of *T. brucei brucei* (Stevens and Godfrey, 1992). The other 37 stocks of parasites investigated had been detected during surveys based on active case detection. Most (29) of them, whether from humans (see below) or other animals (Komoin-Oka *et al.*, 1994; P. B. Diallo, unpubl. obs.), had been isolated using the KIVI method in or after 1991, the other eight having been isolated by rodent inoculation (see Table). Each infected human subject detected had been examined clinically according to standard (WHO) guidelines: samples of blood, lymph-node aspirate and cerebrospinal fluid (CSF) had been checked for trypanosomes and concentrations of protein and the host's cells in the CSF had been estimated. Each subject had been interviewed using a questionnaire and asked how long he or she had felt unwell. The subjects had not been asked whether they had had any of the typical symptoms of HAT.

Each of the 43 trypanosome stocks was bulked up by cultivation in Cunningham's medium (Cunningham, 1977) before protein extraction for isoenzyme analysis (Truc and Tibayrenc, 1993). Thirteen enzyme systems [alanine aminotransferase (ALAT; EC 2.6.1.2), aspartate aminotransferase (ASAT; EC 1.2.1.12), glucose phosphate dehydrogenase (GPI; EC 5.3.1.9), isocitrate dehydrogenase (IDH; EC 1.1.1.42), malate dehydrogenase (MDH; EC 1.1.1.37), malic enzyme (ME; EC 1.1.1.40), nucleoside hydrolase (EC 3.2.2.1) using inosine (NHI) or deoxyinosine substrate (NHD), peptidase (EC 3.4.11 or EC 3.4.13) using L-leucyl-L-alanine substrate (PEP2), 6-phosphogluconate dehydrogenase (PGD; EC 1.1.1.44), phosphoglucomutase (PGM; EC 2.7.5.1), superoxide

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TABLE

Details of the 43 stocks investigated, including the zymodeme (Z) to which each stock was assigned

Stock	*Host*	*Year*	*Village*	*Area and country**	*Z*	*Identification†*	*Reference*
DAL072	Man	1978	Vavoua	Vavoua, CI	1	T.b.g. 1	Stevens *et al.* (1992); Mathieu-Daude and Tibayrenc (1994)
TRAZIE	Man	1991	Sinfra	Sinfra, CI	2	T.b.g. 1	–
SIQUE	Man	1991	Ouragahio	Sinfra, CI	3	T.b.g. 1	–
DAL967	Man	1986	Daloa	Daloa, CI	3	T.b.g. 1	Stevens *et al.* (1992); Mathieu-Daude and Tibayrenc (1994)
SH002	Man	1988	Gbieguhe	Daloa, CI	4	T.b.g. 1	–
SH028	Man	1992	Zahirougbeu2	Daloa, CI	5	T.b.g. 1	–
SH109	Man	1990	Dediafla	Vavoua, CI	4	T.b.g. 1	–
SH133	Man	1990	Gregbeu	Daloa, CI	8	T.b.g. 1	–
SINF19	Man	1992	Senikro	Sinfra, CI	3	T.b.g. 1	–
SINF22	Man	1992	Senikro	Sinfra, CI	3	T.b.g. 1	–
SINF16	Man	1992	Senikro	Sinfra, CI	3	T.b.g. 1	–
SINF1	Man	1992	Koffi Yaokro	Sinfra, CI	11	T.b.g. 1	–
SINF5	Man	1992	Gouenefla	Sinfra, CI	12	T.b.g. 1	–
PT312	Man	1992	Gbieguhe	Daloa, CI	3	T.b.g. 1	–
PT228	Man	1992	Ziguedia	Daloa, CI	3	T.b.g. 1	–
PT198	Man	1992	Zoboua	Daloa, CI	3	T.b.g. 1	–
PT272	Man	1992	Gbieguhe	Daloa, CI	3	T.b.g. 1	–
PT16	Man	1992	Gboguhe	Daloa, CI	3	T.b.g. 1	–
PT230	Man	1992	Ziguedia	Daloa, CI	3	T.b.g. 1	–
PT270	Man	1992	Guedekiprea	Daloa, CI	3	T.b.g. 1	–
1972	Man	1994	Sinfra	Sinfra, CI	38	T.b.g. 1	–
D25/41	Man	1994	Sinfra	Sinfra, CI	39	T.b.g. 1	–
Bub6	Hartebeest	1994	Game reserve	Marahoué, CI	40	T.b.g. 1	–

SI1196	Man	1990	Gueguigbeu	Daloa, CI	7	T.b. bouaflé	–
SI1276	Man	1992	Zoukougbeu	Daloa, CI	10	T.b. bouaflé	–
SI1017	Man	1989	Aboisso	Aboisso, CI	6	T.b. bouaflé	–
SI1136	Man	1988	Ketro-Bassam	Vavoua, CI	7	T.b. bouaflé	–
TI12	Man	1978	Koudougou	Daloa, CI	14	T.b. bouaflé	Mehlitz *et al.* (1986)
TSW53	Pig	1982	Kouassi-Perita	Bouaflé, CI	15	T.b. bouaflé	Stevens *et al.* (1992)
104	Kob	1993	Game reserve	Comoé, CI	16	T.b. bouaflé	–
PORC3	Pig	1988	Balea	Daloa, CI	17	T.b. bouaflé	–
PORC9	Pig	1988	Balea	Daloa, CI	18	T.b. bouaflé	–
KP465	Pig	1991	Kouassi-Perita	Bouaflé, CI	23	T.b. bouaflé	–
24	Kob	1993	Game reserve	Comoé, CI	28	T.b. bouaflé	Komoin-Oka *et al.* (1994)
132	Kob	1993	Game reserve	Comoé, CI	30	T.b. bouaflé	Komoin-Oka *et al.* (1994)
123	Kob	1993	Game reserve	Comoé, CI	31	T.b. bouaflé	Komoin-Oka *et al.* (1994)
150	Kob	1993	Game reserve	Comoé, CI	32	T.b. bouaflé	–
KK39	Kob	1980	Game reserve	Comoé, BRK	33	T.b. bouaflé	Stevens *et al.* (1992)
125	Kob	1993	Game reserve	Comoé, CI	36	T.b. bouaflé	–
AB14	Hartebeest	1980	Game reserve	Comoé, BRK	37	T.b. bouaflé	Stevens *et al.* (1992)
23	Buffalo	1993	Game reserve	Comoé, CI	21	T.b. comoé	–
20	Buffalo	1993	Game reserve	Comoé, CI	34	T.b. comoé	–
51	Waterbuck	1993	Game reserve	Comoé, CI	35	T.b. comoé	–

* CI, Côte d'Ivoire; BRK, Burkina Faso.
† T.b.g. 1, *Trypanosoma brucei gambiense* Group 1; T.b., *Trypanosoma brucei*.

dismutase (SOD; EC 1.15.1.1), and threonine dehydrogenase (TDH, EC 1.1.1.103)], representing 15 loci, were subjected to electrophoresis on cellulose acetate (in a Helena® system; Stevens *et al.* 1989; Truc *et al.*, 1991; Truc and Tibayrenc, 1993). Thirty different zymodemes were seen among the 43 isolates studied (see Table); 12 of the human isolates were identical for all 15 loci and thus belonged to a single zymodeme (Z3). The Jacquard (1973) method and the computer programme described by Serres and Roux (1986) were used to construct a dendrogram which arranged the zymodemes into three major groups (Fig.). Of the 11 zymodemes in one of these groups, which was clearly equivalent to *T. b. gambiense* Group 1 as described by Gibson (1986), 10 were from man and one (Bub6) from a hartebeest. This group was characterized by a particular pattern for SODb (3.5) plus one of three variants in SODa (3.6.7.11.13, 3.6.7.10.11.13 or 3.6.7.10.11), confirming the observations of Stevens *et al.* (1992). Most human isolates belong in this group (Mehlitz *et al.*, 1982; Gibson, 1986; Mehlitz, 1986; Truc and Tibayrenc, 1993; Mathieu-Daude and Tibayrenc, 1994) but, as demonstrated in the present study, it does occur in other animals.

Another of the major groups in the dendrogram comprised just three zymodemes (21, 34 and 35, each represented by a single stock from waterbuck or buffalo) with enzyme patterns which do not seem to have been described previously; SODb 2.4 with SODa 8.9.12.14 appeared to be especially characteristic. As this group appeared so distinct from the other two and all the stocks assigned to it came from Comoé it was named the comoé strain-group.

The third major group, containing 16 zymodemes, was the most heterogeneous and corresponded to the previously described bouaflé strain-group (Godfrey *et al.*, 1990). Gibson (1986) classed all such stocks isolated from human subjects as *T. b. gambiense* Group 2. As Mehlitz *et al.* (1982) and Truc *et al.* (1997) also observed, most of the stocks assigned to this group are found in wild and domestic animals but some may be found in man.

The present results therefore confirm the existence of two classes of zymodeme in Côte d'Ivoire each of which occurs in man and other animals: *T. b gambiense* Group 1 and the bouaflé strain-group (So far, the comoé strain-group has not been found in man.) Interestingly, there was an indication that the course of disease following human infection with Group 1 differs from that following infection with a bouaflé isolate. Twelve of the 20 subjects found infected with Group 1 presented with early-stage disease and had no signs of trypanosomiasis other than swollen lymph nodes. The subjects with Group 1 infections reported feeling unwell for a mean of 8 months. In contrast, three of the four subjects infected with bouaflé strains were in the second stage of the disease, had trypanosomes and 460–672 other cells/ml in their CSF and were in a critical general condition. The subjects with bouaflé infections reported feeling unwell for a mean of just 2 months.

Whether the bouaflé isolates from man are genetically distinct from those in other animals remains to be seen. If all are infective to man then the epidemiology of the bouaflé group will have to be seriously re-appraised. Similarly, the possibility that Group 1 and bouaflé cause different patterns of human disease must be explored further as this would, if confirmed, affect diagnosis, treatment and control and possibly explain some of the variation in response to chemotherapy.

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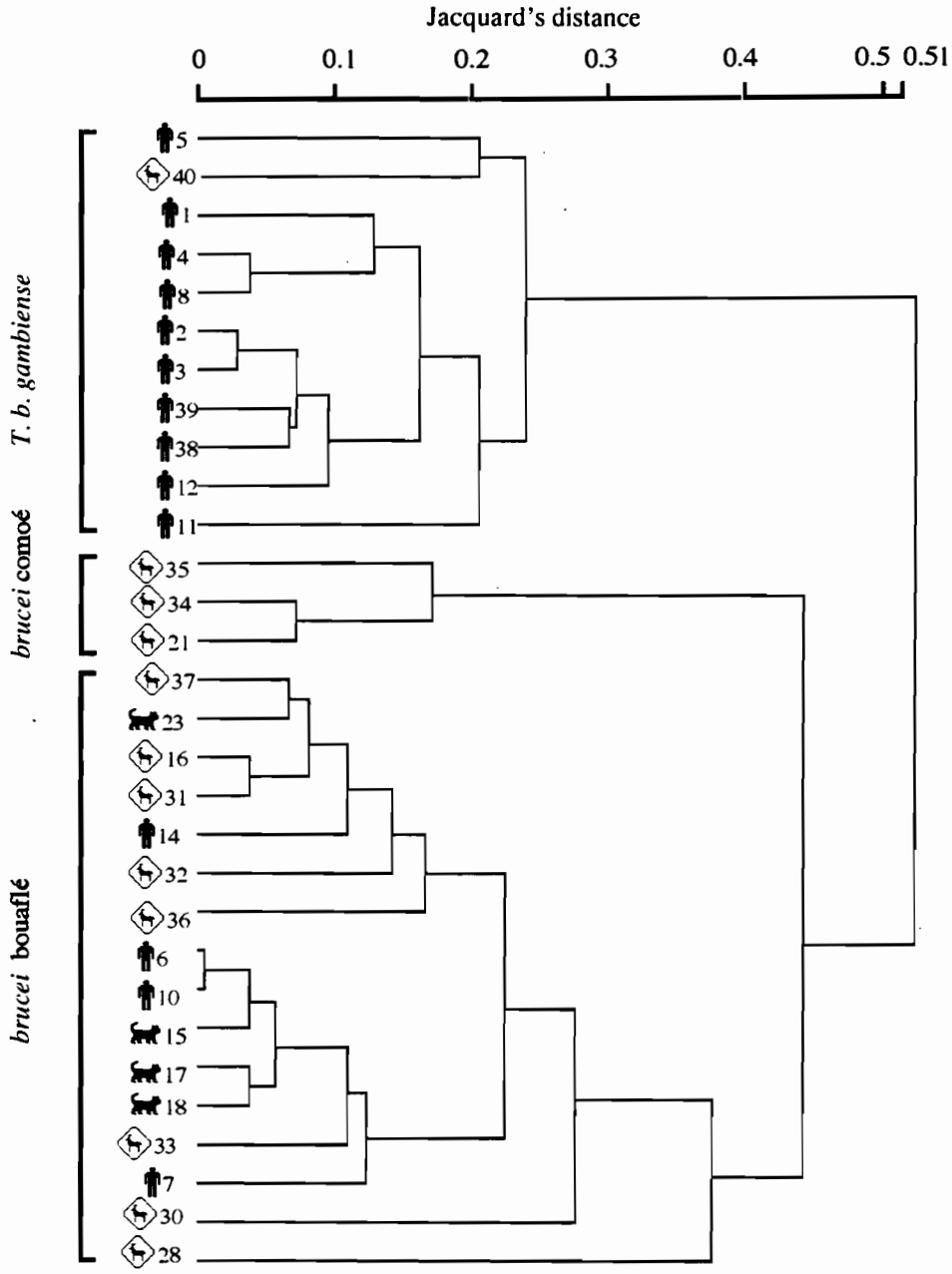

Fig. A UGPMA dendrogram built from a matrix of Jacquard's distances (not shown). The numbers following the pictograms indicate zymodemes, which came from human subjects ( ), domestic animals ( ) and wild animals ( ).

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Short Report

Trypanosoma brucei ssp. and *T. congolense*: mixed human infection in Côte d'Ivoire

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It is generally agreed that human African trypanosomiasis (HAT) in West and Central Africa is caused by *Trypanosoma brucei gambiense*. However, recent studies have found patients harbouring other *T. brucei* subspecies identified mainly by isoenzyme analysis, such as the bouaflé strain group (STEVENS & GODFREY, 1992). Furthermore, TRUC *et al.* (1997) have shown that the disease in these patients followed a more rapid and severe course; characterization of the parasite isolates was confirmed by isoenzyme electrophoresis using cellulose acetate (TRUC *et al.*, 1991).

Due to the toxicity of the available drugs to treat HAT, the presence of parasites must be confirmed before therapy is started. In the field, active case detection commonly involves establishing a suspicion of infection by serology, followed by the microscopical examination of blood or lymph of seropositive cases. The card agglutination trypanosomiasis test (CATT) is commonly used, on serum or plasma (MAGNUS *et al.*, 1978), followed by one or more parasitological tests, such as the capillary centrifugation test, the mini-anion exchange centrifugation technique (m-AECT) (LUMSDEN *et al.*, 1979) or the quantitative buffy coat (QBC®) technique.

In the Aboisso HAT focus in south-eastern Côte d'Ivoire, a 50 years old woman gave a weakly positive CATT result using whole blood but a negative result with plasma; she also gave a negative result in the latex agglutination test (BUSCHER *et al.*, 1991), using whole blood. Parasites were detected by the m-AECT using heparinized whole blood collected by venepuncture, as recently recommended by TRUC *et al.* (1998), over 100 trypanosomes being seen in the eluate. The morphology of these trypanosomes was different from that of *T. brucei* spp.; they were shorter, without a free flagellum, and were only weakly motile. No *T. brucei* slender form was observed and the trypanosomes died in the m-AECT collector tube in less than 20 min, unlike *T. brucei* ssp., which survive for several hours.

The extremely bad physical condition of this patient indicated that she was in the advanced stage of the disease. However, no trypanosome was found in her cerebrospinal fluid by double centrifugation, and the cell count was only 1/mm^3. No malaria parasite was found in a blood film, and no intestinal parasite was detected by faecal examination.

The blood collected for the m-AECT was divided into aliquots and deep frozen in liquid nitrogen, inoculated into a kit for the *in vitro* isolation of trypanosomes (KIVI) (AERTS *et al.*, 1992), and inoculated intraperitoneally to a white mouse. Both attempts to isolate trypanosomes *in vitro* and *in vivo* failed after 60 d follow-up.

Deoxyribonucleic acid (DNA) was isolated from the whole trypanosomes (PENCHENIER *et al.*, 1996) and amplified by the polymerase chain reaction (PCR) (MASIGA *et al.*, 1992) using specific primers for *T. brucei* spp. and *T. congolense* (Kilifi, forest and savannah groups). DNA amplification, done in 2 different laboratories, gave clearly positive results when using primers for the *T. congolense* savannah group (MAJIWA & OTIENO, 1990) and for *T. brucei* ssp. (MOSER *et al.*, 1989). Positive and negative controls confirmed the specificity of the PCR and the absence of contamination by parasite DNA.

The initial microscopical observation of the trypanosomes suggested that they might be *T. congolense* and the failure to isolate parasites *in vitro* or *in vivo* confirmed our suspicion that they were not *T. b. gambiense*. Furthermore, the weak CATT reaction and the negative results in the other serological tests corroborated this. The patient was seronegative for human immunodeficiency virus infection in the Murex® 200A Ice Pack enzyme-linked immunosorbent assay and the Innogenetics® 1036 line immunoassay.

Resistance of *T. congolense* to human serum has been suggested previously (JOSHUA, 1989). In a previous study, 3 stocks of *T. congolense* isolated from goats and sheep in Nigeria were resistant to human serum in the blood incubation infectivity test (RICKMAN & ROBSON, 1970).

The fact that this patient was treated successfully with pentamidine (7 intramuscular injections over 14 d), with no parasite being seen on follow-up examination immediately after treatment and one month later and her general health improving within 2 weeks after starting the treatment, suggests that she might have had a mixed infection. However, the presence of *T. brucei* was indicated only by the weak CATT reaction and the PCR result.

Mixed infections in humans involving allegedly non-infective trypanosomes, confirmed by DNA identification, have never, to our knowledge, been described.

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Whether our patient's disease was due to *T. brucei* and not to *T. congolense* is irrelevant; this case report simply shows that *T. congolense* can infect humans. The importance of this in endemic countries, as well as the possible pathogenicity of such parasites, remains to be determined.

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CHAPTER 2

Identification of trypanosomes: from morphology to molecular biology

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Introduction

One of the enduring problems in the epidemiology of sleeping sickness is that there are 3 morphologically indistinguishable subspecies of *Trypanosoma brucei* involved in a complex transmission cycle between humans, tsetse and reservoir hosts. Two subspecies, *T. b. gambiense* and *T. b. rhodesiense*, are infective to man and cause gambian and rhodesian sleeping sickness respectively. The third subspecies *T. b. brucei* cannot by definition infect humans, but coexists with the other trypanosomes in reservoir hosts and vectors.

The advent of molecular methods for taxonomy brought the hope that unequivocal biochemical markers for the 3 subspecies would quickly be found. In particular, markers for the human infective trypanosomes would have enabled identification of reservoir hosts without recourse to experiments with human volunteers or serum resistance tests. However, the results of molecular characterization revealed a much more complex picture, with several subdivisions within *T. brucei*, rather than the 3 expected. Most isolates of *T. b. gambiense* fitted into one clearly demarcated group, but the other subdivisions did not correspond to the recognised subspecies. More significantly, analysis of the data using population genetics methods led to the discovery of genetic exchange in *T. brucei*. The importance of genetic exchange in generating diversity among *T. brucei* stocks in the field is still controversial, but the principle of gene flow destroys any remaining hope that stable markers for *T. brucei* subspecies exist.

In this chapter, we will first describe the methods for isolation and characterization of trypanosomes from the field and the mathematical methods of data analysis, before considering the implications of this work for epidemiology.

Isolation of trypanosomes stocks from the field

Before trypanosome characterization, stocks must be isolated from the field. The sampling process inevitably introduces bias: some regions may be geographically remote, some hosts may be easier to sample than others, some hosts may contain higher numbers of organisms. Most of the characterization methods described below require outgrowth from the original isolate and thus more slowly dividing trypanosomes may be lost from mixed isolates. Recent developments in isolation methods have tried to address these issues.

Rodent subinoculation

Since Bruce's original discovery, the standard method of isolation of bloodstream form *T. brucei* has been by inoculation of host blood, CSF or lymph into experimental rodents. The same method can be applied to infected saliva or macerated salivary glands from tsetse flies. Subsequent rodent subpassages will increase the virulence of the initial isolate until the rodent blood literally swarms with trypanosomes. This method works well for *T. b. rhodesiense* and *T. b. brucei*, even in subpatent parasitaemias, since in principle one organism is sufficient for infection. However, classical *T. b. gambiense* is of much lower virulence and therefore isolation by rodent subinoculation may fail unless susceptibility is increased by, for example, the use of neonatal rodents, immunosuppression or particular rodent species (e.g. *Mastomys natalensis*; Mehlitz, 1978). These methods are not very practical for field use.

In vitro methods

Procyclic trypanosomes, as found in the tsetse midgut, grow readily in *in vitro* culture and a number of media have been described, of which the semi-defined liquid media, SDM-79 and Cunningham's medium, are probably most widely used (Cunningham, 1977; Brun, 1979). Bloodstream form trypanosomes (bsf's) are more difficult to establish *in vitro* and do not grow to such high densities as procyclics (typically 10^5 ml^{-1} for bsf's compared to 10^7 ml^{-1} for procyclics). However, bsf's readily transform to procyclics on transfer from 37°C to a lower temperature (~25°C), and this feature has been exploited to develop *in vitro* isolation methods. Dukes et al. (1989) successfully isolated Group 1 *T. b. gambiense* from Cameroon by feeding cryopreserved blood from patients to experimental tsetse flies and then culturing the resultant midgut procyclics.

A more convenient and generally applicable method is the KIVI (Kit for in vitro isolation) developed by Aerts et al (1992). The KIVI consists of a pre-prepared small bottle containing sterile growth medium plus antibiotics, into which 5-10 ml of sterile blood is introduced via an airtight rubber seal. After inoculation the bottles can be kept at ambient temperature, before transfer to the laboratory, where they can be opened and examined for trypanosome growth under sterile conditions. Since the trypanosomes take a few days to transform into procyclics and the volume of medium is large, 3 to 4 weeks may elapse between inoculation and necessity to subpassage. Thus the KIVI is ideal for field isolation and has proved its worth in areas of gambian sleeping sickness (Truc, 1992; McNamara, 1995; Truc, 1996) and may also be used for isolation of *T. b. brucei* and *T. b. rhodesiense*.

Cryopreservation

Following collection and initial passage, trypanosome stocks can be cryopreserved, seemingly indefinately, in liquid nitrogen. Either glycerol (10% final volume) or DMSO (7.5% final volume) are used routinely as cryoprotectants. With care, there is little loss of viability; however, less virulent organisms may be lost from mixed isolates after freeze-thawing. Major collections of cryopreserved trypanosome isolates have been built up since the introduction of cryopreservation in the 1960's and these now form an important resource for longitudinal studies.

Determination of human infectivity

Unless a *T. brucei* stock has been isolated from a human patient, its status with regard to human infectivity is uncertain. This question was resolved in the past by the inoculation of human volunteers, and latterly by *in vitro* tests involving incubation of trypanosomes with human blood or serum. These tests rely on the differential killing of *T. b. brucei* by the trypanolytic factor (TLF) in human blood (reviewed by Hajduk, 1992). In the original Blood Incubation Infectivity Test (BIIT) developed by Rickman et al (1970), the viability of trypanosomes following incubation with human blood was tested by inoculation of rodents. However, TLF resistance was found to change with antigenic variation (Van Meirvenne, 1976; Rickman, 1977), and a more reliable way of carrying out the test was developed using metacyclic trypanosomes direct from the fly, which have a smaller and more stable antigenic repertoire (Human Serum Resistance Test, HSRT; Brun, 1987). *In vitro* tests in their various guises have been

used extensively to characterise field isolates from flies and non-human hosts for the trait of human infectivity (Rickman, 1992). These results will be linked with those from molecular characterization methods in the following section "epidemiological implications".

Molecular characterization

Bloodstream form trypanosomes of the 3 principle tsetse-transmitted subgenera (*Trypanozoon*, *Nannomonas*, *Duttonella*) can be distinguished by characteristic morphological features visible by microscopy. Within the vector, however, these morphological differences disappear. To address this problem, DNA probes based on repetitive DNA elements (e.g. satellite DNA) were developed and have proved very useful for the identification of trypanosomes in wild caught tsetse (Kukla, 1987; Gibson, 1988; Majiwa, 1994; Masiga, 1995). The repetitive DNA probes specific for *T. brucei* detect all 3 subspecies, however (Gibson, 1986), indicating that they constitute a closely related group of organisms. Therefore subspecific identification of *T. brucei* stocks relies on molecular "fingerprinting" techniques and comparison with reference isolates. The various methods that have been widely used are described below.

Isoenzyme electrophoresis

Isoenzyme analysis was the first technique to be widely applied to the characterization of isolates within the *T. brucei* species and well over 1000 stocks from all over the continent have now been characterized in this way (Bagster, 1973; Godfrey, 1976; Gibson, 1980; Tait, 1980; Mehlitz, 1982; Gibson, 1983; Tait, 1984, 1985; Gibson, 1985; Godfrey, 1987, 1990; Richner, 1989: Otieno, 1990; Mihok, 1990; Truc, 1991; Stevens, 1992; Enyaru, 1993a,b; Mathieu-Daudé, 1994; Gashumba, 1994). The main conclusions from this data are discussed in the following section "epidemiological implications". Developed in the 1950's, isoenzyme electrophoresis is comparatively cheap and robust, and, given a good choice of enzymes, its usefulness for characterization purposes has not been significantly superseded by DNA-based techniques.

Isoenzyme analysis is performed on highly concentrated extracts of cytoplasmic proteins and thus requires large numbers of trypanosomes (minimum 100 million). The proteins are separated by gel electrophoresis and particular enzymes are visualised by specific staining reactions based on the catalytic properties of the enzyme. Multiple molecular forms of an

enzyme (isoenzymes) give rise to multiple bands on the gel if the molecules differ in electrophoretic mobility. In practice, only about a quarter of amino acid substitutions give rise to changes in electrophoretic charge (Harris, 1976). Therefore, a range of different enzymes need to be screened and the use of 10-20 enzymes, which give clear and reproducible results, is recommended (Stevens, 1992; Mathieu-Daudé, 1994). Note that the natural tendency to use enzymes which show up differences between isolates, rather than those which are conserved, accentuates the observed level of dissimilarity.

Since the metabolism of insect and bloodstream forms of *T. brucei* is different, the isoenzyme bands seen may differ in number, mobility or intensity depending on the lifecycle stage used; this is not a problem for DNA-based characterization. Both bloodstream forms or culture grown procyclics give satisfactory isoenzyme results (Kilgour, 1980; Richner, 1989; Kaukus, 1990). Various media have been used for electrophoretic separation of *T. brucei* isoenzymes, with starch (both thick and thin layer) and cellulose acetate plates the most widely used for reasons of efficacy and economy (Lanham, 1989).

Restriction fragment length polymorphisms

Like isoenzyme electrophoresis, RFLP analysis requires large numbers of trypanosomes, but is more costly in terms of materials and reagents. The starting material is DNA, which first has to be purified from protein and other cellular debris. Both nuclear and kinetoplast (= mitochondrial) DNA have been used. The purified DNA is digested with various restriction enzymes, each of which recognises a specific short sequence of bases and cuts the DNA at this point. The resulting DNA fragments are separated by gel electrophoresis, either in agarose or acrylamide, and are visualised by staining with ethidium bromide, which binds to the DNA. If 2 samples differ by a single base change in the recognition sequence of a particular restriction enzyme, the enzyme will no longer cut at this position and a change in fragment length will be observed.

Such RFLPs can be detected in purified kDNA by simple gel electrophoresis. The ~20 kb kDNA maxicircles, which correspond to the mitochondrial DNA of other eukaryotes, are homogeneous in sequence and therefore appear as one or more discrete fragments after electrophoresis depending on the restriction enzyme used (Borst, 1981; Gibson, 1985a). Analysis of maxicircles from 32 *T. brucei* ssp. stocks, including *T. b. gambiense* and *T. b. rhodesiense*, showed little variation, except for 2 subgroups

of *T. b. brucei* (*kiboko* and *sindo*) with distinctive RFLPs (Gibson, 1985a). The minicircles of *T. brucei* are heterogeneous in sequence. Linearisation by a single cut anywhere in the circle produces a 1 kb fragment, while enzymes which cut more than once yield a complex pattern of fragments smaller than 1 kb. The extreme heterogeneity of *T. brucei* minicircles makes them less useful for identification purposes than those of other trypanosomes (e.g. *T. cruzi*, (Morel, 1980).

RFLPs are detected in nuclear DNA by Southern analysis. After electrophoresis the DNA is single-stranded *in situ* by alkali treatment of the gel and then transferred to a solid support (e.g. nitrocellulose or nylon membrane) by Southern blotting. The blot is incubated in a solution containing a labelled DNA probe, allowing the single-stranded DNA probe to hybridise with its complementary sequence on the blot. Unbound probe is then washed away and the position of hybridisation on the blot is visualised by autoradiography or other means, depending on the method used to label the probe. Various DNA probes have been used, but the largest data sets come from analysis of ribosomal DNAs (Hide, 1990, 1991, 1994), and variant surface glycoprotein (VSG) genes (Pays, 1983; Paindavoine, 1986; Thi, 1991; Kanmogne, 1996a). The repetitive nature of ribosomal and VSG genes means that multiple bands are produced for subsequent mathematical analysis. These results are discussed together with the isoenzyme data in the following section "epidemiological implications".

Polymerase chain reaction-based methods

The chief advantage of polymerase chain reaction (PCR)-based methods is that far fewer trypanosomes are required than for isoenzyme or RFLP analysis. Two approaches have been tried: firstly the development of PCR identification methods for *T. b. gambiense* based on specific sequences, and secondly the random amplification of polymorphic DNA (RAPD) technique (Welsh, 1990; Williams, 1990).

Two PCR tests for Group 1 *T. b. gambiense* have been devised, based on a conserved VSG gene and kDNA minicircles respectively (Bromidge, 1993; Mathieu-Daudé, 1994). In this approach, the sequence of the target DNA must be known before suitable PCR primers can be chosen; these primers are then used to amplify the specific DNA fragment, which is visualised by gel electrophoresis and may be further characterized by hybridisation. For the first test, VSG gene AnTat 11.17 was the target, since this gene was found to be unique to Group 1 *T. b. gambiense* (Thi, 1991). This PCR test was capable of distinguishing Group 1 *T. b. gambiense* from

T. b. brucei in most foci of Gambian sleeping sickness, except north-west Uganda, and gave positive results only with 24 Group 1 stocks in a total sample of 39 *T. brucei* ssp. isolates of diverse origins (Bromidge, 1993). In the second PCR test, the minicircle variable region was targeted for amplification by virtue of a conserved 122 bp sequence and the PCR product from 12 Group 1 *T. b. gambiense* stocks of 26 *T. brucei* ssp. stocks examined, was shown to be unique by hybridisation (Mathieu-Daudé, 1994).

RAPD is a PCR-based technique, which uses arbitrary 10-mer primers to amplify random fragments from a genomic DNA template. A 10-mer primer has a theoretical chance of finding its complementary sequence roughly every million bases in random DNA. Thus no sequence information about the target DNA is necessary and trial and error will show which 10-mer primers produce suitable amplification. Individual primers can yield a fingerprint consisting of 10 or so bands, and thus the use of several primers will rapidly generate large volumes of characterization data for strain comparison (Waitumbi, 1993; Tibayrenc, 1993). The PCR reaction is carried out on purified template DNA in solution. Each reaction requires only about 10 - 20 ng of DNA, equivalent to tens of thousands rather than the billions of trypanosomes required for isoenzyme or RFLP analysis. Amplification products in the size range ~200 - 1000 bp are visualized by gel electrophoresis and staining with ethidium bromide. A typical result is shown in Fig 1.

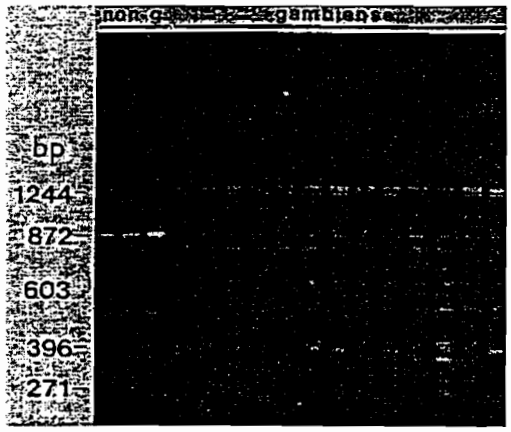

Fig. 1. Comparison of RAPD patterns obtained with a single primer from genomic DNAs of various *T. brucei* ssp. stocks. From left to right, lanes 1-3 non-gambiense stocks: *T. b. brucei*, Zaire; *T. b. brucei*, Côte d'Ivoire; *T. b. rhodesiense*, Zambia. Lanes 4-16 Group 1 *T. b. gambiense*: 3 stocks from Côte d'Ivoire, 2 stocks from Uganda, 6 stocks from Congo and Zaire, 2 stocks from Cameroon.

RAPD is clearly the present method of choice for quick and easy characterization of trypanosome isolates. The results are reproducible and agree with those derived from isoenzyme or RFLP studies (Waitumbi, 1993; Tibayrenc, 1993; Mathieu-Daudé, 1995; Kanmogne, 1996b). The main disadvantage is that the target sequences amplified are unknown and hence the data is not open to interpretation in terms of individual loci and alleles; RAPD data is therefore analysed by particular mathematical methods – see section "mathematical analysis of characterization data". A further problem with *T. brucei*, is that an unknown proportion of bands will derive from VSG genes, which comprise roughly 5% of the genome, have a non-diploid organisation and evolve rapidly (Van der Ploeg, 1982). In addition, contamination of trypanosome DNA with DNA from other sources must be avoided, since RAPD primers are not specific.

Molecular karyotype

Molecular karyotypes are produced by size fractionation of chromosomal DNAs by PFGE (pulsed field gel electrophoresis; Schwartz, 1984). Trypanosomes of the *T. brucei* species have over 100 chromosomes of sizes ranging from 50 kb to several Mb, which are subject to relatively frequent rearrangements and length alterations, thus giving rise to unique karyotypes (Van der Ploeg, 1984; Gibson, 1986; Gottesdiener, 1990). In practice, karyotypes are highly variable and, as with kDNA minicircles, the results are more useful for identification of individual isolates by fingerprinting than characterization of populations. However, some karyotypic features have been found to be characteristic of Group 1 *T. b. gambiense* e.g. size and number of minichromosomes (Gibson, 1986; Dero, 1987; Kanmogne, 1997).

Mathematical analysis of characterization data

Numerical methods for analysing molecular data derived from characterization studies of microorganisms fall into two basic categories: population genetics methods and phylogenetic methods. The former, as their name suggests, are used at the population level, while the latter are used across a broad evolutionary range from the population level upwards. In addition, a wide range of more general epidemiological and/or ecological methods (which lie outside the scope of this chapter) exist for mapping disease movements and population changes, certain of which have

proved useful in the study of trypanosomiasis. The choice of method is generally related to the type of molecular data obtained.

The availability and use of numerical methods for studying trypanosomiasis has developed in relation to a range of factors, including the introduction of new characterization methods, the increased availability and processing power of computers, and the development of new theories in the field of trypanosome population genetics. In this section, we review those techniques commonly used in trypanosomiasis research, together with a number of newer, somewhat more sophisticated techniques which will undoubtedly be of use in the near future.

Population genetics methods

At the base of population genetics methods lies the concept of gene flow within and between populations. In particular, population genetics methods test for the presence of subdivisions within a given population, between which gene flow is either restricted or absent (Tibayrenc, 1995). The null hypothesis (H_0) assumes that the population is randomly mixing i.e. panmictic; a significant variation from H_0 implies a non-panmictic population structure. Statistics used to evidence departures from panmixia consider either the lack of segregation or the lack of recombination of markers (see below), and thus equate to indirect measures of gene flow. The use of such measures to analyse field dat, can provide valuable information concerning the frequency and impact of sexual reproduction in natural populations of *T. brucei*. However, the possibility of population sub-structuring (Maynard Smith, 1993; Stevens, 1996), which can also affect gene flow, should also be considered. Furthermore, although all tests rely on the same basic principle (departure from panmixia), levels of resolution will differ between tests, possibly leading to divergent conclusions (Tibayrenc, 1996).

Segregation tests

Segregation tests are based on the concept of Hardy-Weinberg equilibrium, where there is random reassortment of different alleles at a given locus. Such tests require that alleles are identifiable and that the ploidy level of the organism being studied is known and greater than one. Although the ploidy of the smaller chromosomes of *T. brucei* is problematic, the larger chromosomes which contain housekeeping genes are diploid (Gibson, 1985; Gibson, 1986; Gottesdiener, 1990). Therefore, diploidy can be used as a working hypothesis for population studies based on isoenzyme

markers and segregation tests have been used in a number of important studies of *Trypanozoon* trypanosomes.

The seminal work of Tait (1980) used classical Hardy-Weinberg statistics to reveal genetic exchange in African trypanosomes from the field, although a later study by Cibulskis (1988) underlined some of the pitfalls associated with single-locus Hardy-Weinberg analysis when sample sizes are small. The randomization approach developed by Cibulskis (1988) was extended by Stevens & Welburn (1993) to study genetic exchange in epidemic populations, together with a multilocus approach. Multilocus analyses offer a robust extension to single locus methods (Workman, 1969) and form the basis of the next section covering recombination and linkage disequilibrium tests.

Recombination/linkage methods

Recombination tests offer a powerful alternative to segregation methods, with the advantage that mandatory requirements for segregation tests (e.g. knowledge of ploidy and allelic loci) can be avoided. Importantly, such tests can be performed not just on individual loci, but groups of loci, even when the alleles within such groups are not precisely defined. The only requirement is that the loci or groups of loci are independent from one another (Tibayrenc, 1995, 1996).

Practically, the tests rely on evidencing departures from random assortment, where the expected frequency of a given genotype is simply the product of the observed frequencies of the individual genotypes which make it up. Data which are randomly assorted conform to a random distribution; this is the only state for which statistical criteria can be readily defined and is taken as the null hypothesis (H_0). Studies of organisms known to be undergoing regular genetic exchange also indicate that disequilibrium between loci is rare and that departures from equilibrium are not generally observed (reviewed by Cibulskis, 1988). Thus, a significant variation from H_0 implies a non-panmictic population structure. Such variation can be measured by any one of a number of statistics based on randomization methods (e.g. Tibayrenc, 1990, 1991; Stevens, 1995), association indices (Brown, 1981; Maynard Smith, 1993) or a combination of the two (Souza, 1992). All explore different aspects of the same variation: departures from panmixia or linkage disequilibrium (non-random association among loci, where the predictions of expected probabilities for multilocus genotypes are no longer satisfied).

Of course, while such tests permit departures from panmixia to be demonstrated, the statistics say nothing about the underlying cause.

Obstacles to gene flow can be classified under two main headings: physical (genetic isolation in either space or time) and biological (natural selection, physical linkage of different genes on the same chromosome, cryptic speciation, clonality). The relative importance of one or other will obviously vary, depending on the population being considered.

Many of these methods have been employed in population studies of African trypanosomes. Linkage analyses have been used for broad studies of the population structure of parasitic microorganisms including trypanosomes (Tibayrenc, 1990, 1991, 1993), while an extended Mantel test (Mantel, 1967) has been used to study a range of *T. brucei* (Stevens, 1995; Mathieu-Daudé, 1995; Kanmogne, 1996b). Association indices have also been employed for defining population structure in a range of *T. brucei* populations, e.g. Maynard Smith et al (1993), the Lambwe Valley, Kenya; Hide et al (1994), Uganda; Stevens & Tibayrenc (1996), Côte d'Ivoire, Uganda, Zambia, and for investigating associations between parasite genotype and host/location (Cibulskis, 1992).

Finally, it is important to realize the somewhat unconventional nature of tests for departures from equilibrium, as they are effectively tests of the null hypothesis (Workman, 1969) and are heavily dependent upon the richness of the data under study. Accordingly, as the richness of the data declines, H_0 is sometimes accepted when, in reality, all that has been shown is that there is not sufficient evidence for accepting the alternative hypothesis, H_1. This is not statistically valid and is known as a Type II error. For segregation methods, the probability (b) of making a Type II error can, in certain cases, be calculated from the number of arrangements of genotype frequencies which conform to H-W equilibrium by chance (Fairburn, 1980; Cibulskis, 1988). Once b is known, steps can be taken to reduce it to less than 5%, usually by increasing the sample size. If b cannot be reduced, then at least a probability of having avoided a Type II error can be attached to results to provide some measure of confidence in the conclusions.

The very nature of randomization methods does not permit the calculation of such formal statistics and their use remains dependent on the richness of the data being analysed and on the discriminative power of the technique employed. For example, using RAPD analysis Stevens & Tibayrenc (1995) identified thirteen genetically distinct populations, originating (cloned) from two primary isolates of *T. brucei* from tsetse; isoenzyme characterization of the same stocks revealed only eight zymodemes. Correspondingly, all linkage analyses of the RAPD data evidenced significant association, while only 70% of analyses of the

isoenzyme data showed significant linkage; levels of significance obtained from analysis of the isoenzyme data were also much reduced, being at least one order of magnitude lower.

Phylogenetic methods

Phylogenetic techniques are complementary to population genetics methods and address higher levels of divergence between taxa, i.e. generally above the species level. Indeed, phylogenetic criteria are especially informative for defining taxa in microorganisms in general, for which the biological species concept (Dobzhansky, 1937) is often difficult or impossible to use (Tibayrenc, 1996).

At their most basic, phylogenetic methods can be thought of as trees describing evolutionary relationships between taxa. Due to the use of more general classes of marker for phylogenetic analysis, it has been possible to employ standard techniques for studies of African trypanosomes. Based on the level of phylogenetic divergence to be explored, different classes of genetic marker (e.g. gene sequences, restriction sites or fragments), with different levels of resolution, have been used. It is, however, rare that one marker provides a level of resolution satisfactory across all levels of phylogenetic divergence and many studies now combine data from a range of markers, with various 'clock' speeds. Details are available in standard texts, e.g. Avise (1994), and a range of computer based methods are now available to elucidate relationships by means of phylogenetic trees (Felsenstein, 1993; Swofford, 1993). A measure of confidence can be attached to a given tree by additional bootstrap analysis (Felsenstein, 1985); the merits of such techniques for analysing molecular data remain much in debate.

Phylogenetic methods fall into two main categories: numerical methods and "true" phylogenetic methods.

Numerical methods

Numerical, or more appropriately phenetic methods, were developed primarily to explore bacterial taxonomy (Sneath, 1973; Dunn, 1982). Phenetic methods cluster taxa on the basis of overall similarity (or dissimilarity), e.g. the unweighted pair-group method using arithmetic averages (UPGMA). The measure of similarity is calculated according to the presence or absence of the characters chosen and an equal weight is given to all available characters, e.g. Jaccard's coefficient (Jaccard, 1908), simple matching coefficient (Sokal, 1958). Significantly, such

methods assume an equal rate of evolution in all lineages. Relationships between the individuals in a population can be visualized by a phenogram (dendrogram). However, such dendrograms cannot be regarded as true phylogenetic trees unless all the individuals sampled represent discrete phylogenetic lineages between which gene flow is absent or severely reduced, e.g. natural clones or distinct biological species (Tibayrenc, 1996). For *T. brucei* ssp., this is probably most often not the case, and such dendrograms provide only a pictorial representation of individual variability in the population being studied.

Nevertheless, due in part to their relatively early appearance in the field of biosystematics and to their ease of use, a range of phenetic measures continue to be widely used in trypanosome systematics. Since the first numerical taxonomic study of *Trypanozoon* (Gibson, 1980), a host of other studies using phenetic classification systems have followed, e.g. Hide et al (1990, 1991), Cibulskis (1992), Stevens & Godfrey (1992), Truc & Tibayrenc (1993), Mathieu-Daudé & Tibayrenc (1994), Enyaru et al (1997). Surprisingly, despite improvements in both molecular and mathematical characterization methods, the broader relationships described by these phenetic based studies have remained largely unchanged.

True phylogenetic methods

Unlike phenetic methods, true phylogenetic methods do not assume a uniform evolutionary rate along all phylogenetic lines. Accordingly, many also offer the option to weight calculations according to the importance of different character state changes. There are two major classes of phylogenetic inference methods which can be applied to molecular data: distance methods and cladistic methods.

Phylogenetic distance methods, as for phenetic methods, use procedures which cluster intertaxon genetic distances derived from paired comparisons of the molecular data. Indeed, the two categories of method have much in common and differ primarily in the calculation of the underlying distance measure. Whereas phenetic distances rely simply on scoring the presence or absence of reaction products, e.g. all bands on an isoenzyme electrophoretic plate, phylogenetic distances are derived from a genetic (in the case of isoenzymes, allelic) interpretation of the patterns/fragments observed.

As noted, a range of methods exist including, the Fitch-Margoliash method (Fitch, 1967), Nei's method (Nei, 1972), Neighbour joining

(Saitou, 1987) and the Wagner method (Farris, 1970) [see Nei (1991) for a review of the relative efficiencies of different tree making methods]. Of these, Nei's method and the Wagner method have been widely used for the study of African trypanosomes, with the Wagner method being used for studies of phylogenetic relationships in Kenyan and Ugandan trypanosomes (Cibulskis, 1988; Hide, 1994) and Nei's distance being employed for broader phylogenetic studies of *Trypanozoon* (e.g. Tait, 1985; Stevens, 1992; Hide, 1994; Mathieu-Daudé, 1994).

Cladistics (often referred to as the parsimony method – (Hennig, 1966) and the closely allied technique of maximum likelihood, use discrete character data and work on changes in character states (Scotland, 1992); while cladistics evolved largely from studies of morphological data, maximum likelihood was developed specifically for molecular data (Cavalli-Sforza, 1967). The suitabilty of cladistics for analysing biochemical and molecular data is, therefore, much in debate as problems concerning the relative importance of varying levels of homoplasy, sequence gaps and alignments, variation in molecular clock speed between markers, deletions and insertions all remain to be resolved (Siebert, 1992; Williams, 1992). Nevertheless, a number of apparently highly informative cladistic, parsimony based, studies of African trypanosomes have been undertaken. Parsimony analysis which, as the name suggests, seeks to define the phylogenetic tree requiring the least number of evolutionary changes (the most parsimonious tree) was first used to study evolutionary relationships within subgenus *Trypanozoon* using isoenzyme data (Godfrey, 1990). Since then Mathieu-Daudé et al (1995) have extended this work with a RAPD-based parsimony analysis of *T. brucei*, while Fernandes et al (1993) and Maslov et al (1996) have used the technique with sequence data for broad studies of evolution in the Trypanosomatidae.

Maslov et al (1996) also used the maximum likelihood as an alternative to parsimony when reconstructing a phylogeny for a number of kinetoplastid species based on small and large subunit rDNA sequence data. To date, this method and cladistic methods in general, seem to have been little used in trypanosome taxonomy. In the case of maximum likelihood this is probably due to computing power requirements, while in the case of parsimony methods, the suitability of a procedure originally developed for morphological data remains unknown. Indeed, the phylogenetic value of electrophoretic data in general is still a subject of much debate (see Tibayrenc, 1995) and the high degree of homoplasy present in nearly all molecular data (e.g. DNA sequences, RAPD, RFLP, isoenzymes) must be considered.

Finally, it should be remembered that all mathematical methods for analysing biochemical and molecular data are totally reliant on the quality of the original data. Consequently, the inability of a particular mathematical approach to produce a meaningful result is probably due as much to the resolution of the chosen molecular marker as to the numerical method used - irrespective of the genetic variation being studied, a computer is always able to generate a dendrogram, even if the data have no phylogenetic value (Tibayrenc, 1996).

Epidemiological implications

The impact of molecular characterization techniques on our understanding of the epidemiology of trypanosomiasis has been considerable. In this section we will highlight the major points relevant to the clinical disease and control strategies: firstly, the question of the identity of *T. b. gambiense* and *T. b. rhodesiense*; secondly, the identification of animal reservoir hosts; and thirdly, the evolution of epidemics.

The identity of the trypanosomes

T. b. gambiense

A conclusion reiterated in turn by isoenzyme, RFLP and RAPD analyses is that the majority of *T. b. gambiense* isolates form a homogeneous group. This group (Group 1) conforms to the classical concept of *T. b. gambiense*, which runs a chronic course in the human patient and is of low virulence to experimental animals. Group 1 *T. b. gambiense* is characterized by particular isoenzyme patterns (Fig 2; Godfrey, 1976; Gibson, 1980; Mehlitz, 1982; Tait, 1984; Godfrey, 1987; Stevens, 1992), and by a set of RFLPs for VSG genes, notably AnTat 1.8 and 11.17 (Pays, 1983; Paindavoine, 1986; Thi, 1991; Kanmogne, 1996a) and ribosomal genes (Hide, 1990). Besides these molecular markers, Group 1 *T. b. gambiense* stocks have a restricted antigenic repertoire (Gray, 1972; Jones, 1981), a small genome and fewer small chromosomes compared to other *T. brucei* ssp. (Gibson, 1986; Dero, 1987; Kanmogne, 1997). This group of *T. b. gambiense* is widespread through tropical Africa, eastwards from Senegal to Zaire, and including adjoining foci in south-west Sudan and north-west Uganda (Godfrey, 1987; Enyaru, 1993a). Considering biological characteristics, in the past *T. b. gambiense* was distinguished from

T. b. rhodesiense by its susceptibility to tryparsamide; currently DFMO is effective for treatment of gambian but not rhodesian sleeping sickness, suggesting that Group 1 T. b. gambiense is also characterized by susceptibility to DFMO. *T. b. gambiense* is typically transmitted by tsetse flies of the *palpalis* rather than *morsitans* group and this association appears to be a further characteristic of Group 1 *T. b. gambiense* (Richner, 1988).

Group 2 *T. b. gambiense* is defined as a virulent form of *T. b. gambiense* from foci of gambian sleeping sickness (Mehlitz, 1982; Gibson, 1986). In contrast to Group 1, Group 2 *T. b. gambiense* grows well in experimental rodents and is easy to tsetse-transmit via *morsitans*-group flies in the laboratory (Richner, 1988); whether it also differs in clinical features is presently under examination (Truc, unpublished). By molecular characterization this trypanosome falls outside homogeneous Group 1 and does not share its characteristic isoenzyme and RFLP markers (Fig 2; Mehlitz, 1982; Gibson, 1986; Paindavoine, 1989; Richner, 1989; Hide, 1990). Although often referred to as "*T. b. rhodesiense*-like", Group 2 *T. b. gambiense* is more akin to West African *T. b. brucei* stocks (Paindavoine, 1989; Hide, 1990) and indeed may represent a zoonotic form of sleeping sickness in West Africa (Mehlitz, 1982).

The homogeneity of Group 1 *T. b. gambiense* may reflect lack of sexuality and the wide distribution of this stable genotype has been interpreted as evidence of clonal expansion (Gibson, 1986; Stevens, 1996). While genetic exchange has been demonstrated for Group 2 *T. b. gambiense* in the laboratory (Jenni, 1986; Gibson, 1997), for Group 1 *T. b. gambiense* such experiments are rendered difficult by its poor tsetse transmissibility.

T. b. rhodesiense and T. b. brucei

Biochemical markers for *T. b. rhodesiense* and *T. b. brucei* have proved far more elusive than for Group 1 *T. b. gambiense*. By isoenzyme and RFLP analyses, *T. b. rhodesiense* and *T. b. brucei* stocks fall into several groups (Fig 2), largely according to geographical origin (Gibson, 1980; Gibson, 1983; Gibson, 1985; Paindavoine, 1989; Godfrey, 1990; Stevens, 1992; Hide, 1990, 1991, 1994). Early on, isoenzyme analysis revealed that *T. b. brucei* stocks could be broadly divided into East and West African groups based on homozygosity at loci for phosphoglucomutase (PGM) and isocitrate dehydrogenase (ICD) (Gibson, 1980), and this result was confirmed by RFLP analysis (Gibson, 1985; Paindavoine, 1989; Hide, 1990). The human pathogens are more closely related to their

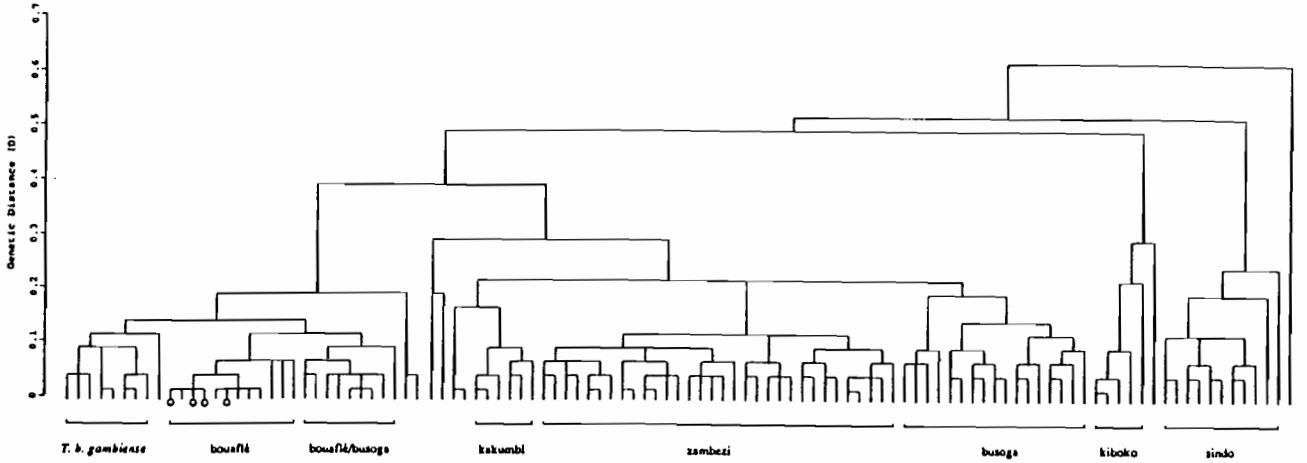

Fig. 2. Dendrogram summarizing the intraspecific taxonomy of *T. brucei*. Branch end points correspond to zymodemes defined by isoenzyme characterization studies. The major groups defined are: *T. b. gambiense*, corresponding to Group 1 *T. b. gambiense*; *bouaflé*, corresponding to *T. b. brucei* in West Africa, but including a number of stocks isolated from humans (o) which belong to Group 2 *T. b. gambiense*; five strain groups containing predominantly East African isolates, *busoga*, *kakumbi*, *kiboko*, *sindo* and *zambezi*, reflecting the complex relationships between *T. b. rhodesiense* and East African *T. b. brucei*. Of these, *busoga* and *zambezi* correspond to 'classical' *T. b. rhodesiense*, *busoga* being associated with northern sleeping sickness foci and *zambezi* with southern foci; the remaining three strain groups show differing degrees of man-infectivity and are strongly associated with wild animal reservoirs. See Gibson et al (1980; 1985a), Godfrey et al (1990), Stevens & Godfrey (1992) for further information on strain groups and subspecific taxonomy. The dendrogram was constructed by UPGMA from a matrix of genetic distances (D). D values between zymodemes were calculated according to Nei's method (1972) by allelic interpretation of ten enzyme systems (Stevens et al, 1992).

sympatric *T. b. brucei* than each other. Thus, in East Africa *T. b. rhodesiense* remains difficult to distinguish from *T. b. brucei*, except by its ability to infect humans. In one way this is not surprising considering that representatives of these 2 subspecies can mate (Gibson, 1989); however, *T. b. brucei*/*T. b. rhodesiense* by no means constitutes a panmictic group in East Africa (Stevens, 1996).

T. b. rhodesiense stocks in general are highly variable and each epidemic focus appears to have its own set of associated trypanosomes (Gibson, 1980; Gibson, 1983; Tait, 1985; Gibson, 1985; Godfrey, 1990; Otieno, 1990; Mihok, 1990; Stevens, 1992; Enyaru, 1993b; Hide, 1994; Gashumba, 1994). Ormerod (1967) identified a trend in decreasing virulence of rhodesian sleeping sickness from north to south and associated this with northern and southern strains of *T. b. rhodesiense*. Characterization data supports this idea to some extent, since clear isoenzyme and RFLP differences have been shown between *T. b. rhodesiense* from the extremes of the range, Zambia and Uganda (Gibson, 1980; Godfrey, 1990; Stevens, 1992; Hide, 1991, 1994; Enyaru, 1993b). However, results from endemic areas in between (Lambwe Valley, Kenya and Kigoma/Tabora, Tanzania) suggest that the observed dichotomy between northern and southern strains may be part of a larger mosaic of different genotypes in East Africa rather than a simple cline (Gibson, 1985; Komba, in preparation).

Although the majority of East African *T. b. brucei* and *T. b. rhodesiense* isolates fall into one broadly similar group, there are in addition 2 or 3 highly distinctive groups of *T. b. brucei*, which have emerged from isoenzyme and kDNA characterization (*kiboko*, *sindo* and *kakumbi*, see Fig 2; Gibson, 1980, 1985a; Godfrey, 1990; Stevens, 1992). These stocks appear to circulate in transmission cycles involving wild animals and tsetse, but not humans. Again, mating experiments indicate that these groups are probably not reproductively isolated (Gibson, 1991).

Reservoir hosts

Isoenzyme and RFLP analyses have made possible the identification of animal reservoir hosts of both T. b. gambiense and *T. b. rhodesiense* without recourse to human infectivity experiments or serum resistance tests. The observation that sporadic infections with *T. b. rhodesiense* could be contracted by visitors to areas populated only by wild animals quickly established that rhodesian sleeping sickness was a zoonosis. However, the epidemiology of gambian sleeping sickness suggested that human infec-

tions alone maintained transmission. As described above, Group 1 *T. b. gambiense* has characteristic isoenzyme and RFLP markers and can thus be readily identified in hosts other than humans. In this way pigs, dogs, sheep, goats, cattle and also wild antelope have been incriminated as reservoir hosts of sleeping sickness in West Africa (Gibson, 1978; Mehlitz, 1982; Scott, 1983; Zillmann, 1984; Tait, 1984; Paindavoine, 1986; Noireau, 1989; Truc, 1991). There is now no doubt that these animals harbour *T. b. gambiense*, but their actual importance in maintaining transmission of sleeping sickness remains to be established. Fig 2 shows the similarity between Group 2 *T. b. gambiense* and *T. b. brucei* isolates of the *bouaflé* group, illustrating the truely zoonotic nature of this infection (Mehlitz, 1982; Truc, unpublished).

In East Africa, the existence of wild and domestic animal reservoir hosts of rhodesian sleeping sickness was proved by the experimental infection of human volunteers (Heisch, 1958; Onyango, 1966). The role of molecular fingerprinting has been to quantify the risk; thus cattle have been shown to constitute a significant reservoir of *T. b. rhodesiense* in epidemics in Kenya and Uganda (Gibson, 1985; Hide, 1994). The presence of large numbers of infected animals in the peridomestic environment assumes particular importance if flies are also resident.

In the Kenyan study, trypanosomes tended to persist in the lymph glands and CNS of the cattle rather than the bloodstream, and may therefore not have been transmissible by tsetse after a few months; in addition, the cattle suffered heavy mortality from trypanosomiasis during this outbreak (Gibson, 1985). Reservoir hosts that remain contagious in the long-term and do not become sick themselves pose the greatest disease risk. Thus secretive wild animal hosts, such as bushbuck or bushpig, may be as important as domestic reservoir hosts in sustaining endemic foci, especially considering that these animals are favoured hosts of tsetse.

Evolution of epidemics

Large numbers of trypanosome isolates from several sleeping sickness foci have now been characterized by isoenzyme or RFLP analysis. Some studies have included stocks isolated over extended time periods, up to 30 years in some cases. For gambian sleeping sickness, the picture is constant, with the Group 1 trypanosome predominant, but showing a degree of microheterogeneity within and between different foci (Godfrey, 1987; Stevens, 1992; Paindavoine, 1986; Truc, 1993; Kanmogne, 1996a,b). For example, stocks from Cameroon had a divergent gene for VSG LiTat 1.3,

which is the antigen targeted in the CATT (Dukes, 1992), while stocks from the Moyo focus in north-west Uganda lacked another *gambiense*-specific VSG gene, AnTat 11.17 (Enyaru, 1993a). Thus, Group 1 *T. b. gambiense* appears to originate from a single strain, which spread widely and then diverged locally by mutations (Gibson, 1986).

For rhodesian sleeping sickness, the story is more complex. Three areas have been intensively studied: Busoga in south-east Uganda (Gibson, 1983; Tait, 1985; Hide, 1991, 1994; Enyaru, 1993b; Stevens, 1993), the Lambwe valley in south-west Kenya (Gibson, 1985; Otieno, 1990; Mihok, 1990), and the Luangwa valley in north-eastern Zambia (Gibson, 1980; Hide, 1991; Stevens, 1992). Limited data is also available from foci in Tanzania and Ethiopia (Gibson, 1980; Gashumba, 1994). The general points that emerge are as follows. Firstly, each focus has its own associated *T. b. rhodesiense* strains. For example, although epidemics occurred simultaneously in Lambwe valley and Busoga in the 1980's, *T. b. rhodesiense* strains from these 2 geographically close foci were unrelated, indicating that each epidemic had a separate origin (Gibson, 1983; Gibson, 1985).

Secondly, some strains of *T. b. rhodesiense* remain stable over many years in a given focus, while new strains also emerge. The stable strains are apparently well adapted to humans, but are nevertheless also found in reservoir hosts. An example is the *busoga* strain from the large focus spanning south-east Uganda and the neighbouring area of Kenya, which has been isolated repeatedly over the past 30 years or so and is characterized by both isoenzyme and RFLP data (Fig 2; Gibson, 1983; Godfrey, 1990; Enyaru, 1993b; Hide, 1994). This is an example of epidemic clonal propagation from an underlying sexual population (Tibayrenc, 1990; Maynard Smith, 1993; Stevens, 1996).

On the other hand, new as well as old strains may be associated with an outbreak (Gibson, 1983; Gibson, 1985; Enyaru, 1993b, 1997). The emergence of new strains may be interpreted as the adaptation to humans of strains previously circulating in reservoir hosts, or as the direct products of genetic exchange (Mihok, 1990; Cibulskis, 1992). These authors produced intriguing evidence of host selection for certain trypanosome genotypes, since cattle did not harbour all strains found in humans and had their own associated trypanosome genotypes. A further finding from epidemic foci in both Uganda and Kenya was that trypanosome isolates from tsetse were extremely variable (Gibson, 1983; Gibson, 1985; Otieno, 1990; Mihok, 1990; Enyaru, 1993b, 1997). Since genetic exchange in *T. brucei* occurs in the fly (Jenni, 1986), this was a possible

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explanation for the generation of new genotypes. Paradoxically, however, these new genotypes were rarely found among the isolates collected from vertebrate hosts, usually humans and domestic stock. This may reflect undersampling from wild animal hosts or alternatively may indicate a time lag between the generation of new genotypes in the fly and their appearance in an outbreak. The extent to which genetic exchange orchestrates strain variability in the field is still controversial (Tibayrenc, 1990; Gibson, 1990; Cibulskis, 1992; Mathieu-Daudé, 1995; Stevens, 1996). Part of this controversy has arisen from attempting to combine data from unrelated epidemics, instead of considering each focus as a separate population (Stevens, 1996).

Conclusion

From the panmictic view of T. brucei originally proposed by Tait (1980), the consensus at present is that T. b. brucei/*T. b. rhodesiense* are basically sexual, but undergo periods of clonal expansion (Maynard Smith, 1993; Stevens, 1996; Hide, 1996), while the majority of evidence suggests a lower degree of genetic exchange in classical *T. b. gambiense*.

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Characterization of *Trypanozoon* isolates using a repeated coding sequence and microsatellite markers

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Abstract

Genetic variation of microsatellite loci is a widely used method for linkage analysis, individual identification or inter-population studies. Here we analyse a repeated DNA coding sequence and eleven new microsatellites identified within the *Trypanosoma* (*Trypanozoon*) *brucei* genome. Ninety-seven isolates belonging to the five species and subspecies *Trypanosoma evansi*, *T. equiperdum*, *T. brucei brucei*, *T. b. rhodesiense* and *T. b. gambiense* were compared regarding the genetic patterns of these markers. The results reveal a great heterogeneity of the genotypes related to the repeated coding sequence and five microsatellites, some of which show a high degree of polymorphism. This allows us to define group-specific genotypes or alleles; in particular, we show that one specific pattern clearly segregates the human pathogen *T. b. gambiense* group 1.

Keywords: *Trypanosoma brucei*; Microsatellite repeats; Group specific markers; Human African trypanosomiasis; Classification; Polymorphism (Genetics)

1. Introduction

The classification and identification of the African trypanosomes, in particular within the *Trypanozoon* subgenus, remains a major problem. The five species and subspecies belonging to this subgenus (*Trypanosoma brucei brucei*, *T. b. rhodesiense*, *T. b. gambiense*, *T. evansi* and *T. equiperdum*) are morphologically identical and are classified according to host, type of disease and geographical distribution. In many cases, exceptions to these criteria were observed [1], necessi-

Abbreviations: RAPD, random amplification of polymorphic DNA; RFLP, restriction fragment length polymorphism; SSR, simple sequence repeat; VSG, variant surface glycoprotein.

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tating the development of biochemical and genotypic criteria such as isoenzyme patterns [2–8], restriction fragment length polymorphisms (RFLPs) [9] in variant surface glycoproteins (VSGs) [10,11] or ribosomal RNA genes [1], conserved antigenic repertoires [12] and specific kinetoplast DNA probes [13]. Among African trypanosomes, *T. b. gambiense* and *T. b. rhodesiense* are the causative agents of a human disease, African sleeping sickness. *T. b. gambiense* is associated with a chronic form of the disease, mainly in West and Central Africa, while *T. b. rhodesiense* is responsible for an acute disease in East Africa. *T. b. gambiense* has been subdivided into two groups depending on virulence to laboratory rodents (group 2 strains are more virulent than group 1 strains), biochemical and genetic markers, and the heterogeneity of antigenic repertoires (*T. b. gambiense* group 2 is more heterogeneous than group 1) [14].

The requirement for great quantities of parasite material constitutes a considerable disadvantage for the various techniques listed above, especially for epidemiological surveys. This problem was solved by the development of PCR based methods such as specific PCR amplification [10,15–17], random amplification of polymorphic DNA (RAPD) [18,19] or minisatellite analysis [20].

To characterise isolates, we developed a very convenient PCR approach based on the analysis of a repeated DNA coding sequence and microsatellite length polymorphism. One remarkable property of microsatellites is the hypervariable copy number of the basal motif within one microsatellite locus. Simple sequence repeat (SSR) microsatellites are specific DNA sequences of tandemly repeated short basic motifs, generally less than 5 nucleotides in length. These microsatellites are ubiquitous among eukaryotes, from mammals [21] to parasites [22–25] and most of them occur interspersed in the genome, depending on the repeated motif and the species considered. Generally they arise from slippage mechanisms which occur during replication or DNA repair according to the Stepwise Mutation Model [26–28]. The mutation rate of SSR microsatellites, and thus the variability, is higher than that observed for isoenzyme or RFLP markers [29,30], where the variability is probably mainly due to recombination mechanisms [31]. Thus, SSR microsatellite analysis is highly suitable for studying the relationships between closely related species or within populations of the same species [32].

In this report, we analysed the length polymorphism of one repeated DNA coding sequence (3′ extremity of *ORF2*) and 11 SSR microsatellite loci in 97 well-characterised stocks of the *Trypanozoon* subgenus. We show that this method provides a rapid and simple method for the distinction of *T. b. gambiense* group 1 from the other subspecies and we demonstrate that the data obtained can be used for epidemiological studies. Microsatellite loci provide a useful and complementary addition to other genetic markers, since the microsatellite polymorphisms are based on a different mechanism.

2. Materials and methods

2.1. Parasite and nucleic acids isolation

The trypanosome stocks used were either procyclic forms grown in axenic liquid culture medium or bloodstream forms purified by anion exchange chromatography [33]. The parasites (10^9–10^{10}) were collected and standard genomic DNA preparation was performed [34].

2.2. Polymerase chain reaction and detection

All PCR amplifications were carried out with the GeneAmp® PCR System 2400 (Perkin Elmer) in 50 μl reaction mixtures containing 200 μM of each dATP, dTTP, dCTP, dGTP, 0.2 μM of each primer (primers designed for each microsatellite and for the ORF2 locus are listed in Table 1) and between 10 to 50 ng of purified genomic DNA.

For the ORF2 analysis, the reaction was hot started at 95°C for 12 min in the presence of 0.5 U AmpliTaq Gold polymerase (Perkin Elmer), 1 × reaction buffer containing 3 mM $MgCl_2$, to activate the enzyme. Then, the samples were processed through 30 cycles consisting of 30 s at 95°C, 30 s 60°C and 3 min at 72°C followed by

Table 1
Repeated coding sequence and microsatellite markers

Marker	Ref.[a]	Accession Number	Strand[b]	PCR Primers 5′→3′	5′-labelling for detection	Repeat sequence	Size range alleles, bp	Repeats	Number of different alleles found
MORF2-REP	A	AF031926	P	TGCATG-GCAATAGC-GATGGGC	–	102 bp	478–≈5000	3–40	16
		AF032098	M	ATCGTCAC-CTGGTG-TACTTCTC	–				
MORF2-CA	A	AF031926	P	TTTATCTCA-CATTACTCG-GCG	–	(CA)n	115–253	6–75	40
		AF032098	M	GCGTCGAT-CATGTCTAC-CGTAC	6-FAM[c]				
M18D10-CA	D	GATAR46TPB	P	CGACATTC-CATGTGT-GAAC	–	(CA)n	–	–	–
			M	CTTTGTAT-ACAGGCTTGC-TAC	–		–	–	–
M6C8-CA	D	GATBV16TJ	P	CTTTCAAC-CGCCT-TATCAGC	6-FAM[c]	(CA)n	84–>360	13–>150	56
			M	GGCTAGTTA-CACTG-TAGTTCTC	–		–		
M10B2-TA	D, E	GATAJ14TVB	P	GCAAAT-ACAGACGGGGT GAG	–	(TA)n	–	–	–
			M	AGTGGATTT-GAGGTGC-GAAG	–		–	–	–
M25D5-TA	D, E	GATGC15TV	P	CGAGAG-CAATGCTA-CACGAG	–	(TA)n	–	–	–
			M	TCGGTCGTG-GTTTCAAT-TAAG	–		–	–	–
MT3033-AC/TC	C, E	W84128	P	GAGTGA-CAATGGT-GAAGATCG	6-FAM[c]	(AC/TC)n	119–201	13–54	26
			M	TTTTTCTTTG-GTGCTTGT-GAG	–				

Table 1

Marker	Ref.[a]	Accession Number	Strand[b]	PCR Primers 5′→3′	5′-labelling for detection	Repeat sequence	Size range alleles, bp	Repeats	Number of different alleles found
MT3033-AT			P	CTCACAAGCAC-CAAAG	–	(AT)n	164–178	8–15	7
			M	ATGGAACTTCG-CAAGTGTG	HEX[c]				
MT3328-CA	C, E	AA063702	P	TGAGTCATTG-GATTGTTCAC	–	(CA)n	–	–	–
			M	AACAGCGCT-GCCACGAAAG	–		–	–	–
MEST19-AT/GT	-	-	P	TACA-CAAAACGTTCT-CAAC	6-FAM[c]	(AT/GT)n	257–340	18–60	28
			M	GACAGAGTAT-ACGAGAAGTG	–				
MAOX5′-TA	B, E	U52964	P	TGAATGCATG-CATTTGTG	–	(TA)n	–	–	–
			M	CCTTACATCC-GATGAGTTC	–		–	–	–
MT1145-TA	C, E	W00257	P	GACTTTGAGGT-GCCTGGTTTCG	–	(TA)n	–	–	–
			M	CTGACAGTCT-CACACGTTGC	–		–	–	–

[a] Ref.: References are listed in the reference list and correspond to: A: [35]; B: [36]; C: [37]; D: *Trypanosoma brucei* genome project at TIGR (http://www.tigr.org/tdb/mdb/tbdb/index.html); E: African trypanosome DNA database at the EBI (http://mercury.ebi.ac.uk/parasites/kineto_bs.html).

[b] P and M stand for Plus and Minus DNA strand.

[c] 6-FAM and HEX are fluorescent dye molecules used for the 5′ end labelling of the primers.

one elongation step of 10 min at 72°C. PCR products were separated and analysed on a 1.5% agarose gel and visualised by ultra violet transillumination.

For the microsatellite PCR reactions, the reaction mixture also contained 0.5 U of DyNAzyme™ II DNA Polymerase (Finnzymes Oy, Espoo, Finland) and 1 × reaction buffer supplied with the enzyme. The amplification conditions were as follows: 95°C for 3 min (denaturation step), then 2 cycles of 45 s at 95°C, 45 s at 60°C and 45 s at 72°C, followed by 28 cycles of 45 s at 95°C, 45 s at 55°C and 45 s at 72°C, and 5 min at 72°C. The resulting microsatellite amplified products were first analysed on a 2% agarose gel and 1 μl of a 1/20 to 1/50 dilution were mixed with 3 μl of loading buffer containing 2 μl deionised formamide, 0.5 μl PRISM™ Genescan-350 ROX labeled standard DNAs (Perkin Elmer, Applied Biosystems) and 0.5 μl Blue Dextran (MW 2 000 000 Da)/EDTA (25 mM). Samples were denatured for 2 min at 90°C, loaded onto a 36 cm 4.25% denaturing polyacrylamide gel and electrophoresed at 3000 V, 60 mA, 200 W and 51°C for 2.5 h on a ABI 377 DNA sequencer (PE, Applied Biosystems). The PCR product length was determined automatically by the Genescan software (PE, Applied Biosystems) using the local Southern method.

In order to verify the correlation between the calculated size of the PCR products and the microsatellite repeat number at each locus, we undertook sequence analysis of some homozygous and heterozygous PCR products (bold figures in Table 2). Electrophoresis of PCR products labelled with 6-FAM or HEX fluorescent molecule on an ABI sequencer using internal size standards showed that the calculated and the real size are well correlated with a maximum 1 bp difference probably due to the labelling itself.

2.3. Southern analysis

Digestion of 3 μg of parasite genomic DNA by the restriction enzyme α-*Taq*I and separation on 0.8% agarose gel were performed as described by Sambrook et al. [38]. After electrophoresis, DNA was transferred onto neutral nylon membranes (Appligene®, Oncor®) and hybridised with a [α-^{32}P]dCTP-labelled (Rediprime™ II kit -Amersham Pharmacia Biotech) DNA purified fragment containing 6 ORF2 repeats.

2.4. Sequencing

After purification using the GFX™PCR purification kit (Amersham Pharmacia Biotech), PCR products were sequenced on an automated ABI 377 DNA sequencer (PE, Applied Biosystems) by using the PRISM™ Dye Terminator Cycle Sequencing Ready Reaction kit (Applied Biosystems) as described by the manufacturer. The sequences were determined from both directions using the same unlabelled primers designed for the PCR analysis.

3. Results

We analysed 97 trypanosome stocks representing the five species and subspecies of the subgenus *Trypanozoon* namely *T. evansi*, *T. equiperdum*, *T. b. brucei*, *T. b. rhodesiense and T. b. gambiense*. The origin (Central, West and East Africa) and the subspecific identification references of these stocks are listed in Table 2.

3.1. Repeated coding sequence

The 3' end of *ORF2*, previously identified within the downstream region of the glucose transporter gene cluster (Fig. 1) located on chromosome X in *T. brucei* ssp. [54], contains a variable number of 102 bp (34 aa) repeats, which constitute up to 2/3 of the total gene length (40 repeats) [35].

To study the DNA polymorphism, our initial approach was to use, as a genetic marker, the tandemly repeated coding sequence (*MORF2-REP*). Two specific primers (Fig. 1B) flanking the repeats were designed for PCR analysis. DNA fragments ranging from 274 bp to 1.6 kb, corresponding to 1 to 14 repeats respectively, could be distinguished easily by agarose gel separation (Fig. 2). The limit of this approach is reached above 14 repeats, due to the weakness of the

Table 2
Summary of information on the origin of the trypanosome isolates and characteristic repeated coding sequence and microsatellite patterns.

Group and stock designation	(focus) Origin	Year	Host	Ref.[a]	MORF2-REP		MORF2-CA		M6C8-CA		MT3033-AC/TC		MT3033-TA		MEST19-AT/GT	
					All.[b] 1	All. 2	All. 1	All. 2	All. 1	All. 2	All. 1	All. 2	All. 1	All. 2	All. 1	All. 2
Trypanosoma evansi																
E9/CO	(Carimagua) Columbia	1973	Horse	[5]	4	6	51	54	17	32	16	20	8	10	22	23
2187	Brazil	1989	Canine	[39]	4	6	51	51	17	30	16	20	8	10	22	23
2191	Brazil	1989	Canine	[39]	4	6	51	52	17	31	16	20	8	10	22	23
SH	(Shangaï) China	1988	Bovine	[39]	5	6	42	56	18	39	16	20	-	-	23	23
ZJ	(Zhejiang) China	1985	Buffalo	[39]	5	6	42	56	18	39	16	20	8	11	23	23
NJ	(Nanjing) China	1988	Bovine	[39]	5	6	42	56	18	39	16	20	8	11	23	23
GX	(Guangxi) China	1988	Mule	[39]	5	6	42	54	-	-	16	20	8	10	-	-
JX	(Jiangxi) China	1988	Buffalo	[39]	5	6	42	54	19	38	16	20	8	10	23	24
TC	Chad	1988	Camel	[39]	3	4	47	56	58	70	22	23	8	10	31	38
ET	Ethiopia	1988	Camel	[39]	6	7	37	37	18	46	16	20	8	10	22	22
KETRI 2480	(Ngurunit) Kenya	1980	Camel	[40,41,8]	14	-	22	36	17	47	13	13	8	8	19	22
Trypanosoma equiperdum																
SA	South Africa		Horse	[39]	11	-	17	18	28	67	-	-	-	-	20	20
BJ	(Beijing) China	1979	Horse	[39,52]	6	7	43	49	20	52	16	22	8	10	23	24
BoTat-1		1961	Horse	[42]	13	20	22	36	15	27	13	13	8	8	19	22
Trypanosoma brucei brucei																
AnTat-5/1	Gambia	1975	Bovine	[C]	5	6	36	45	18	65	16	22	-	-	30	37
LM 55	Ivory Coast	1992	Pig	[11]	6	13	12	48	32	34	18	22	-	-	24	25
LM 118	Ivory Coast	1992	Pig	[11]	6	-	46	46	38	54	14	24	-	-	18	22
LM 184	Ivory Coast	1992	Pig	[11,19]	5, 6	7	13, 20	48, 56	23, 27	37, 71	14, 15	22	-	-	22	24, 25
LM 225	Ivory Coast	1992	Pig	[11]	5	5	20	56	37	71	15	15	-	-	22	24
KP10	Ivory Coast	1997	Pig	[A]	6	7	12	20	37	48	16	22	8	10	31	58
PTAG 130(IPR-01130)	(Dalou) Ivory Coast	1985	Pig	[8,10,51]	6	6	50	50	29	35	23	23	-	-	21	26
KP2	(Kouassi-Perita) Ivory Coast	1982	Tsetse fly	[8,49]	6	13	23	50	30	32	14	14	-	-	30	30
DiTat-1	Burkina Faso			[43][D]	3	7	13	13	30	43	26	26	9	9	23	37
B8/18	(Nsukka) Nigeria	1962	Pig	[5,8,10]	3	3	45	46	24	47	26	26	-	-	21	29
SW3/87	(Bandundu) D.R. Congo	1987	Pig	[11,19,45]	6	-	38	38	42	80	21	22	-	-	32	35
SW4/87	(Bandundu) D.R. Congo	1987	Pig	[11,45]	7	7	38	38	42	80	22	22	-	-	32	35
SW 161/87	(Boma) D.R. Congo	1987	Sheep	[11,45]	7	7	35	75	20	96	21	23	-	-	29	29
STIB 345	(Kiboko) Kenya	1969	Tsetse fly	[B]	10	10	43	43	33	33	16	16	-	-	29	31
STIB-777.AE	Uganda	1971	Tsetse fly	[43]	9	11	8	47	18	33	20	22	-	-	31	34
AnTat-1/1	Uganda	1966	Antelope	[39,42][C]	40	40	57	57	67	129	17	23	8	10	21	35
EATRO-427	Uganda	1960	Sheep	[5]	12	35	35	57	28	32	17	22	11	12	21	43
STIB-247.LFB	(Serengeti) Tanzania	1971	Hartebeest	[9]	20	20	44	51	49	50	16	16	-	-	31	31
STIB 348	(Serengeti) Tanzania	1971	Hartebeest	[5][B]	10	20	44	51	32	50	16	17	-	-	29	31
Trypanosoma brucei rhodesiense																
IL-2025	(Nyanza) Kenya	1961	Human	[44]	10	35	16	57	65	83	17	18	-	-	31	43
ETat-1/1	Uganda	1960	Tsetse fly	[42][C]	10	35	16	57	65	83	17	18	-	-	31	43
UTat 1/8	Uganda	1977	Human	[42][C]	10	35	16	57	68	144	18	18	8	8	43	44
STIB 859	(UTRO Hp) Uganda	1991	Human	[B]	10	35	16	57	73	>150	18	18	8	10	22	43
STIB 863	(UTRO Hp) Uganda	1990	Human	[B]	10	30	16	57	65	>150	18	18	-	-	31	43
STIB 704	(Ifakara) Tanzania	1982	Human	[9][B]	9	9	41	41	35	79	18	18	8	8	43	60
STIB 362	(Serengeti) Tanzania	1971	Human	[B]	8	20	17	51	16	49	18	18	8	8	41	42
TMRS001	(Taboru) Tanzania	1991	Human	[50]	9	11	40	44	15	90	18	19	-	-	31	45

Table 2 (Continued)

Group and stock designation	(focus) Origin	Year	Host	Réf.[a]	MORF2-REP		MORF2-CA		M6C8-CA		MT3033-AC/TC		MT3033-TA		MEST19-AT/GT	
					All.[b] 1	All. 2	All. 1	All. 2	All. 1	All. 2	All. 1	All. 2	All. 1	All. 2	All. 1	All. 2
TMRS002	(Kasulu) Tanzania	1991	Human	[50]	10	11	37	43	17	19	13	20	–	–	22	54
TMRS003	(Kasulu) Tanzania	1991	Human	[50]	10	11	37	43	17	19	13	20	–	–	22	54
TMRS005	(Kasulu) Tanzania	1991	Human	[50]	10	11	37	43	17	19	13	20	–	–	22	54
TMRS006	(Kasulu) Tanzania	1991	Human	[50]	10	11	37	43	17	19	13	20	–	–	22	54
TMRS007	(Kasulu) Tanzania	1991	Human	[50]	10	11	37	43	17	19	13	20	–	–	22	54
TMRS123	(Kasulu) Tanzania	1994	Human	[50]	10	11	37	43	17	19	13	20	–	–	22	54
TMRS008	(Kasulu) Tanzania	1991	Human	[50]	6,10	11	17, 31	37, 43	17,19	21,42	13,17	19,20	–	–	22, 31	32, 54
TMRS106	(Kibondo) Tanzania	1994	Human	[50]	6	11	17	31	21	42	17	19	–	–	31	32
TMRS009	(Kasulu) Tanzania	1991	Human	[50]	14	–	26	27	16	21	13	17	–	–	29	31
TMRS010	(Kasulu) Tanzania	1991	Human	[50]	7	9	33	44	60	100	17	20	–	–	31	33
TMRS109	(Kibondo) Tanzania	1994	Human	[50]	7	–	33	44	16	53	16	17	10	10	32	33
TMRS119	(Kibondo) Tanzania	1994	Human	[50]	7	–	34	35	15	17	16	17	–	–	32	33
TMRS124	(Kasulu) Tanzania	1994	Human	[50]	8	–	38	47	16	66	17	18	–	–	22	32
TMRS117	(Kibondo) Tanzania	1994	Human	[50]	8	10	15	69	17	31	16	18	–	–	31	32
TMRS108	(Kibondo) Tanzania	1994	Human	[50]	8	9,10	15,37	43,68	17	31,109	13	16,17	–	–	29.31	32
TMRS120	(Kibondo) Tanzania	1994	Human	[50]	9	10	16	43	16	16	17	18	–		31	32
TMRS122	(Kibondo) Tanzania	1994	Human	[50]	9	10	45	54	16	55	13	17	–	–	32	37
TMRS127	(Mpanda) Tanzania	1994	Human	[50]	9	10	45	54	16	55	13	17	–	–	31	32
AnTat 12/1	Rwanda	1971	Human	[42] [C]	8	9	34	56	16	17	17	17	9	11	22	40
Zambia	Zambia		Human	[C]	9	9	21	42	43	48	16	17	8	10	31	34
Trypanosoma brucei gambiense group 1																
Yao	(Sinfra)Ivory Coast	1997	Human	[A]	7	10	16	16	13	53	30	48	8	10	22	22
1972	(Sinfra)Ivory Coast	1993	Human	[A]	7	10	16	16	13	53	30	48	8	10	22	22
2147	(Sinfra)Ivory Coast	1995	Human	[A]	7	10	16	16	13	53	30	48	8	10	22	22
C4D/1	(Sinfra)Ivory Coast	1996	Human	[A]	7	10	16	16	13	53	30	48	8	10	22	22
27/7	(Sinfra)Ivory Coast	1995	Human	[A]	7	10	16	16	13	53	30	48	8	10	22	22
C55/1	(Sinfra)Ivory Coast	1995	Human	[A]	7	10	16	16	13	53	30	48	8	10	22	22
2390	(Daloa)Ivory Coast	1996	Human	[A]	7	10	16	16	13	53	30	48	8	10	22	22
Zakaria	(Sinfra)Ivory Coast	1995	Human	[A]	7	10	16	16	13	53	30	49	8	10	22	22
ITMAP-1842	(Bouenza) Congo	1973	Human	[11,19,46]	7	11	16	16	13	37	30	30	8	8	22	22
ITMAP-1843	(Bouenza) Congo	1975	Human	[11,19,46]	7	11	16	16	13	37	30	30	8	8	22	22
TB26	(Bouenza) Congo	1983	Pig	[11,19,45]	7	11	16	16	13	37	30	30	8	8	22	22
MOS	(Mbam) Cameroon	1974	Human	[6,8–11,19]	7	11	16	16	13	38	30	30	8	8	22	22
5 guinée équat.	Equat. Guinea	1996	Human	[A]	7	11	16	16	13	38	30	30	8	10	22	22
4 guinée équat.	Equat. Guinea	1996	Human	[A]	7	11	16	16	13	38	30	30	8	10	22	22
AnTat-13/1	D.R. Congo	1974	Human	[C]	7	11	16	16	13	36	30	46	8	10	22	22
1135	(Daloa) Ivory Coast		Human	[A]	7	11	16	16	13	43	30	44	–	–	22	22
LiTat-1/1	Ivory Coast	1952	Human	[42][C]	7	11	16	16	13	44	30	43	8	10	22	22
A005	(Fontem) Cameroon	1988	Human	[8,11,19,47]	7	11	16	16	13	49	30	38	8	10	22	22
Bida 3	(Bida) Nigeria	1968	Human	[5,8,11,10]	7	11	16	16	13	49	30	33	–	–	22	22
NW7	Uganda	1992	Human	[11,19,48]	7	11	16	16	13	49	32	46	8	10	22	22
NW6	Uganda	1992	Human	[11,19,48]	7	11	16	16	13	50	32	46	8	10	22	22
Mamaissata	(Conakry) Guinea	1996	Human	[A]	7	11	16	16	13	59	30	42	8	10	22	22
46/5	(Sinfra) Ivory Coast	1995	Human	[A]	7	11	16	17	13	41	30	54	8	10	22	22
Suzena	(Yambio) Sudan	1982	Human	[4]	7	11	16	6	13	52	30	45	–	–	22	22
1829 (Aljo)	(Bandundu) D.R. Congo	1970	Human	[11,19,45]	7	11	16	6	13	55	30	45	8	10	22	22
SH 86	(Bandundu) D.R. Congo	1986	Sheep	[11,19,45]	7	13	16	16	13	48	30	46	–	–	22	22
Salif	(Conakry) Guinea	1996	Human	[A]	7	13	16	16	13	53	30	42	8	10	22	22

Table 2 (Continued)

Group and stock designation	(focus) Origin	Year	Host	Réf.[a]	MORF2-REP		MORF2-CA		M6C8-CA		MT3033-AC/TC		MT3033-TA		MEST19-AT/GT	
					All.[b] 1	All. 2	All. 1	All. 2	All. 1	All. 2	All. 1	All. 2	All. 1	All. 2	All. 1	All. 2
1898	D.R. Congo	1974	Human	[6,11,19]	7	9	16	16	13	39	30	46	8	10	22	22
Tsuaa	(Tsuaa) Nigeria	1968	Human	[5,8,10]	7	8	16	15	13	56	30	30	-	-	22	22
Trypanosoma brucei gambiense group 2 (Bouaflé)																
HTAG/107-1	(Daloa) Ivory Coast	1986	Human	[8,10]	6	7	13	48	23	37	14	14	14	15	24	25
2171	(Zoukougbeu) Ivory Coast	1997	Pig	[A]	3	6	48	49	29	46	15	15	11	12	25	25
TH-126	(Koudougou) Ivory Coast	1978	Human	[8,3]	6	7	13	49	38	48	19	22	10	10	31	40
TH-112	(Koudougou) Ivory Coast	1978	Human	[8,3]	6	7	13	49	38	48	19	22	10	10	31	40
TJ12 (78E)	(Koudougou) Ivory Coast	1978	Human	[10,3]	6	7	13	49	38	48	19	22	-	-	31	40
HTAG 15/5	(Daloa) Ivory Coast	1985	Human	[8,10,51]	8	14	12	43	16	34	17	22	-	-	25	28
2178	(Zoukougbeu) Ivory Coast	1997	Pig	[A]	8	14	12	43	31	35	14	28	11	12	24	31

[a] For space saving, publication references have been listed in the reference list. Other references correspond to: A: Isolates from I.P.R. (Institut Pierre Richet, Bouaké, Côte d'Ivoire); B: Isolates from Swiss Tropical Institute, Basel; C: Isolates from Institute for Tropical Medecine, Antwerpen; D: Isolate from G. Duvalet CIRAD, Montpellier.

[b] Indicates the number of repeats perallele, as examplified in Fig. 4. Bold figures correspond to sequenced PCR products.:

[c] Indicates alleles that have not been determined. All the isolates designated as *T. b. brucei* and studied in previous papers have been tested for human serum sensitivity, details in cited references.

signal and the high background (see lane 3 in Fig. 2). Therefore, Southern blot analysis was performed for stocks not presenting the expected two bands by PCR analysis (Fig. 3). On the basis of the PCR and Southern blot analysis, a total of 16 distinct alleles were identified (Table 1) ranging from 3 to 40 repeats of the 102 bp unit. Most of the isolates could be characterised by PCR alone, since more than 93% were in the 3 to 14 repeat range. The analysis of the genotypes shows a high degree of heterozygosity at this locus. From the 89 strains totally analysed, only 10, which belong to the *T. b. brucei* and *T. b. rhodesiense* groups, are homozygous. From this analysis it appears that 2 genotypes (7/10 and 7/11) are associated only with the *T. b. gambiense* group 1 isolates and two others (10/35 and 10/11) with the *T. b. rhodesiense* subspecies. However, only 87% (*T. b. gambiense* group 1) and 40% (*T. b. rhodesiense*) of the stocks analysed had the respective subspecies-specific genotype. Therefore, to improve the genetic characterization of the *T. brucei* group, we looked for SSR microsatellite markers, which are known to present a high degree of polymorphism.

3.2. Microsatellites

We analysed two putative microsatellites: MORF2-CA previously identified between the *RAB1* gene and *ORF2* [35] (Fig. 1A) and MEST19-AT/GT (Fig. 4) randomly isolated from a cDNA data bank previously constructed in our laboratory (data not shown). In addition, 9 microsatellite sequences were characterised from the

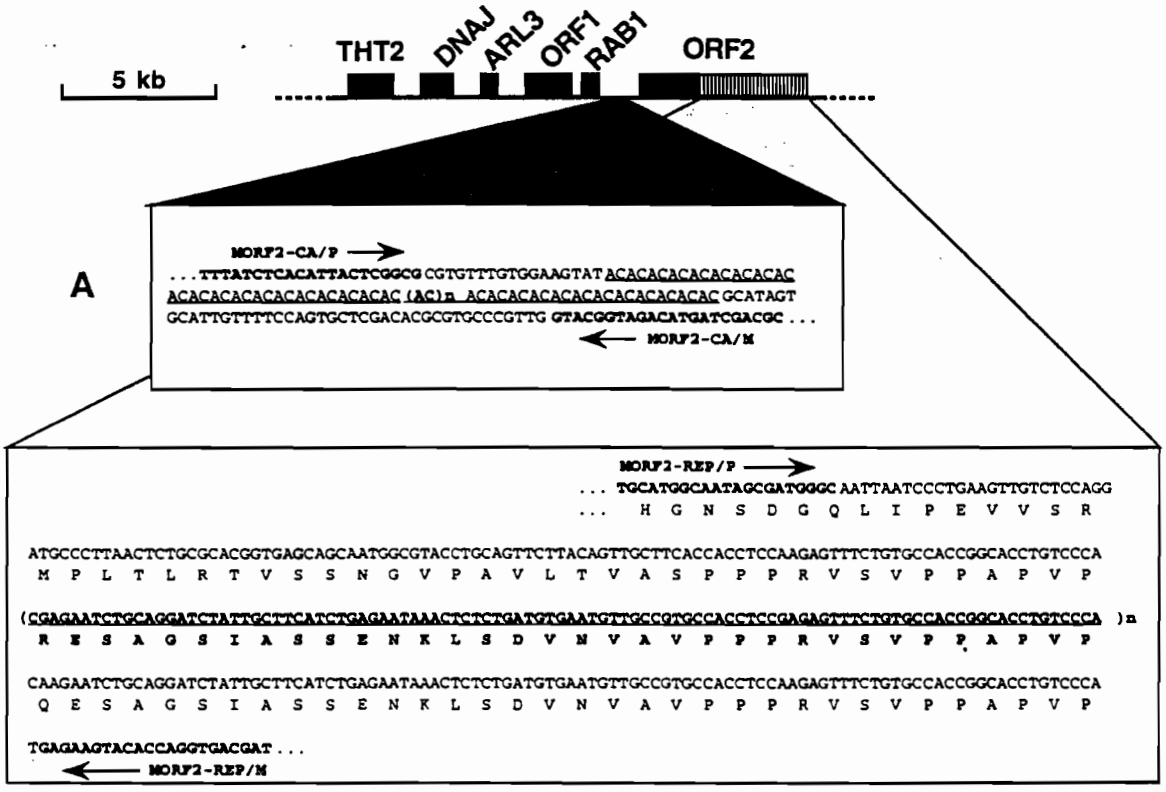

Fig. 1. Genomic organization of the downstream region of the glucose transporter gene cluster in *T. brucei ssp.* Black boxes indicate the genes and the shaded region of the ORF2 gene indicates the 3' end location of the repeat. A: Location and DNA sequence of the MORF2-CA microsatellite. B: Nucleotide and corresponding amino acid sequences of the repeated coding sequence *MORF2-REP*. Arrows indicate positions of primers.

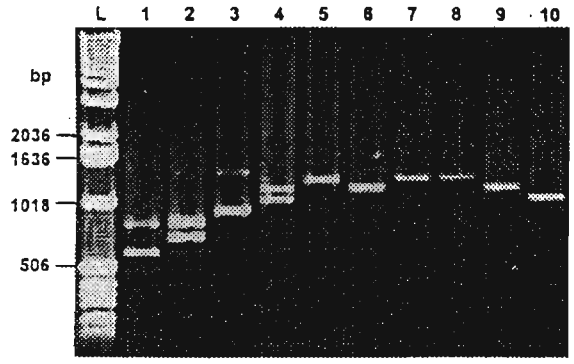

Fig. 2. PCR amplification and allele agarose gel characterization at the *MORF2-REP* locus from a range of different *T. brucei* isolates. Lanes and identified alleles are, respectively; L: 1 Kb marker ladder – BRL; 1–2: *T. evansi* CO (4/6) and SH (5/6); 3: *T. b. gambiense* group 1 LiTat-1 (7/11); 4–10: *T. b. rhodesiense* AnTat 12 (8/9), UTat 1/8 (10/35), Zambia (9/9), STIB 859 (10/35), STIB 863 (10/30), STIB 704 (9/9), STIB 362 (8/20). In brackets, alleles identified directly from the agarose gel are underlined while homozygous or new alleles characterized after Southern blot analysis are not.

African trypanosome DNA database at the EBI (European Bioinformatics Institute-EBI, Wellcome Trust Genome Campus Hinxton Cambridge CB10 1SD UK.) and the *T. brucei* BAC and P1 End Sequence database at the TIGR (The Institute for Genomic Research, Rockville, MD, Washington, DC). Primers for the SSR microsatellites were defined in order to obtain amplified PCR products of lengths compatible with the Genescan 350 ROX marker (Fig. 1A and Fig. 4). To determine the variability of the selected microsatellite loci, a few strains belonging to each subspecies were analysed by PCR and agarose gel separation to estimate the potential size variability (results not shown). Among the 11 new microsatellites characterised, 6 were not analysed further due to the lack of size polymorphism (Table 1).

The 5 selected microsatellite loci (MORF2-CA, M6C8-CA, MT3033-AC/TC, MT3033-AT and MEST19-AT/GT) are highly polymorphic with 7–56 different alleles ranging from 6 to more than 150 repeats (see Table 1). All the DNA samples analysed, except LM 184, TMRS008 and TMRS108 stocks, presented one or two alleles for the 5 SSR microsatellite loci studied, indicating that these markers are present at a single locus in the *T. brucei* genome. The *T. b. brucei* LM 184 and the two *T. b. rhodesiense* TMRS008 and TMRS108 stocks had 3 or 4 different alleles for all the analysed markers, suggesting that these 3 isolates contain at least 2 different strains. To test the mitotic stability of the markers, we analysed each satellite on three different *T. equiperdum* (BoTat) antigenic variants obtained after several passages through rabbits and mice. No difference was observed between these variants, indicating that the SSR microsatellites and the ORF2 repeats are stable through several generations (data not shown). From these results it seems that microsatellite variations, which arise from slippage mutations, are sufficiently frequent to maintain a high degree of polymorphism within populations, but not frequent enough to occur in successive generations.

Since these new genetic markers were highly polymorphic, present only once in the *T. brucei* genome and sufficiently stable, we undertook a systematic analysis of many isolates belonging to the *Trypanozoon* subgenus.

3.3. *T. evansi and T. equiperdum*

The few *T. equiperdum* stocks analysed are very different and are not characterised by specific alleles, while, despite the small number of stocks studied, 3 geographically-determined clusters of *T. evansi* appear: South America (CO, 2187, 2191), China (SH, ZJ, NJ, GX, JX), and Africa (TC, ET, KE). In the South American cluster, only small differences at the MORF2-CA and the M6C8 loci were observed between the 3 stocks, although the different strains were isolated over a 16 year period. In the Chinese cluster, 3 isolates (SH, ZJ and NJ) originating from the middle east of China are identical for the analysed markers, while slight differences are observed with the GX (south-west) and JX (south-east) stocks. Within the African cluster, no relationship could be observed, and only the ET stock shares the MT3033-AC/TC (16/20) and MT3033-TA (8/10) genotypes and the *MORF2-REP* (6), the M6C8-CA (18) and MEST19-AT/GT (22) alleles with the other *T. evansi* clusters.

3.4. T. b. brucei and T. b. rhodesiense

All the analysed genetic markers showed remarkable genetic diversity within the *T. b. brucei* group and there were no obvious relationships between the analysed isolates. Indeed, none of the West African *T. b. brucei* stocks, even if isolated the same year, from the same host type and in the same geographical area, displayed identical genotypes and no consistent pattern of allele distribution could be observed. Nevertheless, two East African stocks and stocks isolated from the D.R. of Congo (SW3/87/SW4/87) or Tanzania (STIB 247/STIB 348) share common genotypes and alleles.

From the results (Table 2), it can be seen that three cohesive clusters arise within the *T. b. rhodesiense* stocks, corresponding to different geographical areas. The first group comprises 4 Ugandan and Kenyan stocks characterised by the

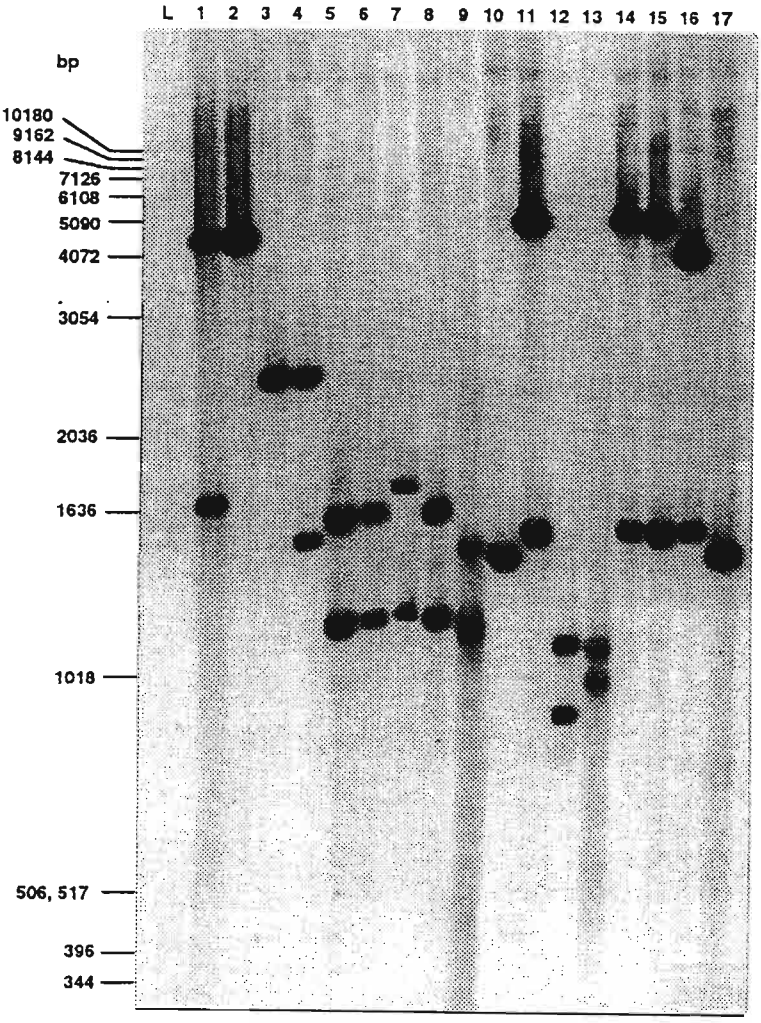

Fig. 3. Southern hybridization of selected trypanosome DNA stocks digested to completion with α*TaqI* and probed with a PCR amplified fragment containing 6 ORF2 repeats. Lanes are, respectively; L: 1 Kb marker ladder (BRL); 1–4: *T. b. brucei* EATRO-427, AnTat 1P15, STIB 247 and STIB 348; 5–9: *T. b. gambiense* group 1 5GE, NW6, Salif, LiTat-1 and 27/7; 10 and 11: *T. b. rhodesiense* Zambia and UTat 1/8; 12 and 13: *T. evansi* 2187 and SH; 14–17: *T. b. rhodesiense* ETat 1.1, STIB 859, STIB 863 and STIB 704.

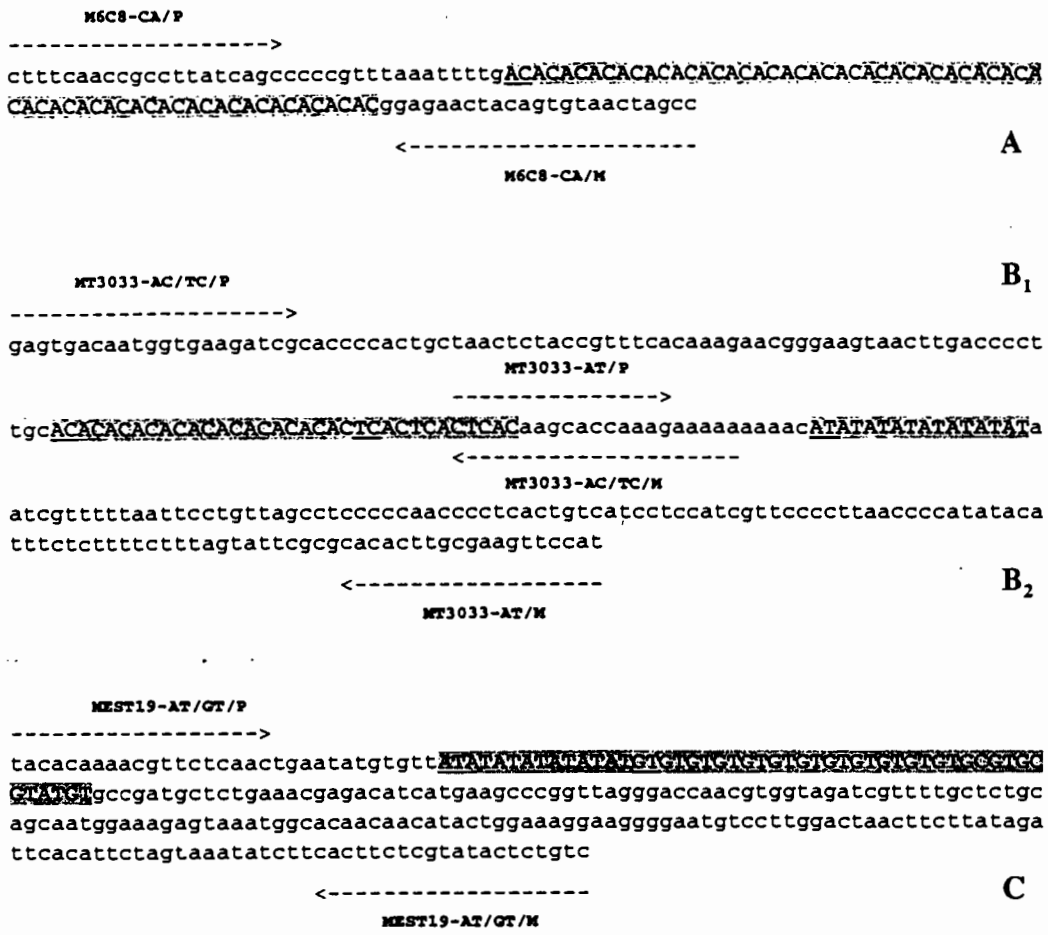

Fig. 4. PCR amplification and sequenced alleles of the A: M6C8-CA (Strain *T. b. brucei* STIB 345; allele 33); B_1: MT3033-AC/TC, B_2: MT3033-AC/TC (Strain *T. b. gambiense* group 2 1D3; MT3033-AC/TC allele 17 and MT3033-AT allele 8); and C: MEST19-AT/GT (Strain *T. b. gambiense* group 2 2171; allele 25) loci. The corresponding primers of each system are indicated by arrows. The microsatellite repetitions of each locus are shaded and the repeat unit underlined.

MORF2-CA (16/57) genotype and also sharing common alleles such as MEST19-AT/GT (43) and MT3033-AC/TC (18). In addition, all of them have the *MORF2-REP* (10/35) genotype, except the STIB 863 isolate (10/30). The second group is formed by seven Kasulu Tanzanian stocks, six of which (TMRS-002, -003, -005, -006, 007, -008) were isolated in 1991 and one (TMRS123) in 1994. The third group comprises two Tanzanian stocks (TMRS122 and −127) that differ only for one allele of the MEST19-AT/GT genotype.

It is noteworthy that some *T. b. brucei* and *T. b. rhodesiense* isolates from the same region share common alleles specific to these stocks. This is the case for the MORF2-CA (57) allele, which is found in all the Ugandan *T. b. brucei* and *T. b. rhodesiense* stocks and in one Kenyan *T. b. rhodesiense* stock. Similarly, the *MORF2-REP* (35) and MORF2-CA (44) alleles are specific to Ugandan and Tanzanian isolates, respectively.

3.5. *T. b. gambiense* groups 1 and 2

For the 6 analysed markers, identical genotypes or common alleles were observed for geographically unrelated *T. b. gambiense* group 1 stocks

isolated over a 40 year period. All the group 1 strains were characterised by two very specific markers: (i) the M6C8-CA (13) allele; and (ii) the MT3033-AC/TC (>30) alleles, since only group 1 stocks have more than 30 repeats in both alleles for this marker. Moreover, all the *T. b. gambiense* group 1 stocks are characterised by the systematic presence of the *MORF2-REP* allele (7), the MORF2-CA (16) repeats and the non-specific homozygous MEST19-AT/GT (22/22) genotype which was also found in the *T. evansi* ET strain. As the *MORF2-REP* and MORF2-CA markers are linked, we deduce that the corresponding haplotype (7–16) is specific for the 25 *T. b. gambiense* group 1 isolates, which are homozygous at the MORF2-CA locus, and could also occur in the four remaining isolates. None of the *MORF2-REP* or MORF2-CA genotypes found within *T. b. gambiense* group 1 are found in other groups. Despite the overall homogeneity of the group, two clusters can be observed. The first is composed of 8 of the 11 Ivory Coast stocks where all, but one, patterns were identical. The second cluster contains three Congo stocks from the same focus isolated from humans or pig over a 10 year period, the MOS stock from Cameroon and the two stocks from Equatorial Guinea. For these stocks, only small differences were observed in the M6C8-CA and MT3033-TA alleles. All these stocks, except the two not previously analysed Guinean stocks, were clustered together by RFLP and RAPD analysis [11,19]. Two other stocks (NW7 and SH 86) that differed slightly in their RFLP pattern [11] and were also included in this cluster, seem to have a more distantly related microsatellite pattern, as 3 or 4 alleles differ.

All the stocks belonging to group 2 *T. b. gambiense* were isolated from the Ivory Coast. These stocks were highly heterogeneous, except for TH-112 and TH2, which were isolated in the same year in the same area and had identical genotypes. Despite the similar origin of the stocks, none of the analysed markers identified common alleles for this group, in contrast to group 1 *T. b. gambiense*.

4. Discussion

Specific PCR at five microsatellite and one repeated coding sequence loci revealed a high degree of size polymorphism among trypanosomes of subgenus *Trypanozoon*. Depending on the number of stocks analysed, the number of alleles ranged from 7 to 56. In general, the level of polymorphism of microsatellites increases with the length of the perfect repeat [21]. Thus it is not surprising that the M6C8-CA and MORF2-CA loci are more polymorphic than the MT3033-AC/TC, MEST19-AT/GT and MT3033-AT loci. These markers reveal far greater levels of genetic variation than previous isoenzyme and RFLP analyses and require only small amounts of DNA, making them highly suitable for genotyping isolates on a large scale. Conclusions from a preliminary analysis of 97 subgenus *Trypanozoon* isolates of various origins are described below.

The South American and the Chinese *T. evansi* clusters are highly homogeneous and share the specific genotype MT3033-AC/TC (16/20) and the non-specific alleles *MORF2-REP* (6) and MEST19-AT/GT (23), suggesting that all these stocks could have arisen from a single lineage. In contrast, the African cluster is far more heterogeneous; the alleles diverge totally from all the other analysed *T. evansi* stocks, except for the ET stock, which shares some alleles with the other two clusters. Similarly, the three *T. equiperdum* stocks are largely heterogeneous, although one stock (BoTat-1) has identical genotypes at 4 loci to the Kenyan stock (KETRI 2480) and the Chinese stock (BJ) shares common alleles with the Chinese *T. evansi* cluster. These results are broadly in agreement with those obtained by RFLP analysis including most of these *T. evansi* and *T. equiperdum* stocks; all the Chinese *T. evansi* stocks were identical and *T. equiperdum* BoTat-1 was found to be closely related to KETRI 2480 [39]. Isoenzyme and molecular RFLP studies demonstrated a very close relationship between *T. evansi* and *T. equiperdum* [5,39,52,55,56]. However, our results suggest that this interpretation may be simplistic, now that we have been able to characterise these stocks more finely.

Concerning the *T. b. rhodesiense* group, our results confirm previous findings that isolates from various foci (Tanzania, Uganda/Kenya, Zambia) are quite distinct [57,58]. Nevertheless, several lines of evidence indicate that isolates from the same region are similar. The relative homogeneity of the Ugandan/Kenyan group suggests that these isolates may have arisen from a single parasite by clonal expansion over a 31 year period. Indeed, two stocks (Ugandan ETat-1.1 and north-west Kenyan IL-2025), isolated at one year distance from a tsetse fly and a human respectively, have identical patterns. This group probably corresponds to 'busoga' [5], the *T. b. rhodesiense* epidemic strain identified by isoenzymes and RFLPs found in the regions of Uganda and Kenya bordering the north-west shore of Lake Victoria, which was shown to have an epidemic population structure (defined as a population which exhibits explosive clonal outbursts from an underlying sexual base) [59–61]. Similarly, six Tanzanian isolates have a unique and stable genotype suggesting that one strain was responsible for this epidemic over a 3 year period. Despite their different origins, two other Tanzanian stocks (TMRS122, Kibondo and TMRS127, Mpanda) have closely related genotypes. Nevertheless the Mpanda patient was described to be a recent immigrant from Burundi, suggesting that he could have been infected before or during his transfer through the Kibondo area [50].

In contrast, the remaining 12 Tanzanian *T. b. rhodesiense* stocks, including all the Kibondo stocks isolated in 1994, were heterogeneous. The only Zambian stock analysed shares few alleles with isolates from further north. Analysis of more stocks from southern Africa might resolve the issue of whether distinct northern and southern genotypes of *T. b. rhodesiense* exist [50,62] and the proposed clonal structure of Zambian *T. b. rhodesiense* isolates [63].

All the analysed stocks of *T. b. gambiense* group 1 form a homogeneous group, characterised by the [(7/x)-(16/x)-(13/x)-(> 30/ > 30)-(22/22)] genetic pattern of the corresponding *MORF2-REP*, MORF2-CA, M6C8-CA, MT3033-AC/TC and MEST19-AT/GT markers. Importantly, the M6C8-CA (13) and MT3033-AC/TC (> 30) alleles, which were strictly specific to this group here, appear to constitute a reliable and specific diagnostic tool for the characterization of *T. b. gambiense* group 1. Some of the markers studied present a relatively high degree of polymorphism (M6C8-CA, MT3033-AC/TC), while others are weakly polymorphic (*MORF2-REP*, MORF2-CA) or monomorphic (MEST19-AT/GT). These results, which show variable levels of heterogeneity depending on the marker analysed, are consistent with previously published work on cluster analysis; *T. b. gambiense* group 1 was shown to be homogeneous using isoenzyme or RFLP data [1,64], but showed a higher degree of heterogeneity using RAPD or other RFLP data [9,11,12,19]. The weak variability of these stocks over a 40 year period and their characterization in man and in animal reservoirs correlates with the clonal structure of this population [63] and clearly indicates the persistence of these genotypes in the field.

Our results with a first selection of SSR microsatellite markers confirm the observations of earlier studies showing that *T. evansi* and *T. equiperdum* are closely related, that for *T. b. rhodesiense* isolates, there is restricted diversity within a focus, but a high degree of diversity between foci and that *T. b. gambiense* group 1 forms a genetically homogeneous group. Furthermore, we show that polymorphic microsatellite markers allow the characterization of clusters within the *T. brucei* group that reflect their geographical origin with remarkable accuracy. Development of other microsatellite markers will certainly extend the possibilities of such characterization even further.

From the 97 subgenus *Trypanozoon* isolates, three (LM 184, TMRS008, TMRS108) were found to be characterised by more than two bands for all the analysed markers, suggesting that they contain a mixed population. Indeed, the LM 184 stock, which contains all the alleles of the LM 225 stock, is probably a blend of the LM 225 stock and another unidentified stock similar to the *T. b. gambiense* group 2 HTAG-107 stock. In accord with this hypothesis, both LM 184 and LM 225 isolates are pig stocks isolated the same

year in the Ivory Coast and the HTAG-107 stock also originates from Ivory Coast. Similarly, the double genotype of the TMRS008 stock probably indicates a mixture of one strain belonging to the Tanzanian Kasulu cluster (TMRS-002 to -007 and TMRS123) and the TMRS106 strain (Fig. 5), while the TMRS108 stock contains all but two alleles of the TMRS117 stock. Characterization of mixed populations of the same or different trypanosome species within a naturally infected host or in tsetse has been reported [65–67]. Nevertheless, it is also possible that these results reflect laboratory contamination. Furthermore, genetic exchange has been shown to occur between trypanosome populations when they infect the tsetse fly [68]. Thus, it is clear that microsatellite marker analysis provides a very useful method to identify multiple infection.

In conclusion, in addition to increasing our fundamental knowledge, polymorphic marker analysis can be valuable in the epidemiological field. Indeed, these markers can be used for the genetic characterization of metacyclic and bloodstream forms and can thus improve taxonomic knowledge, characterise the hybrid products of genetic exchange, take part in the study of the dynamics of the parasitic populations or contribute to the identification of possible animal reservoirs of human trypanosomiasis. This PCR based method is sufficiently sensitive to identify trypanosomes directly in the vector, which constitutes a prerequisite for more efficient vector control in the field. It may also allow the characterization of drug resistant populations and can constitute a rapid method to distinguish between relapse and reinfection, or mixed infections.

5. Uncited Reference

[53]

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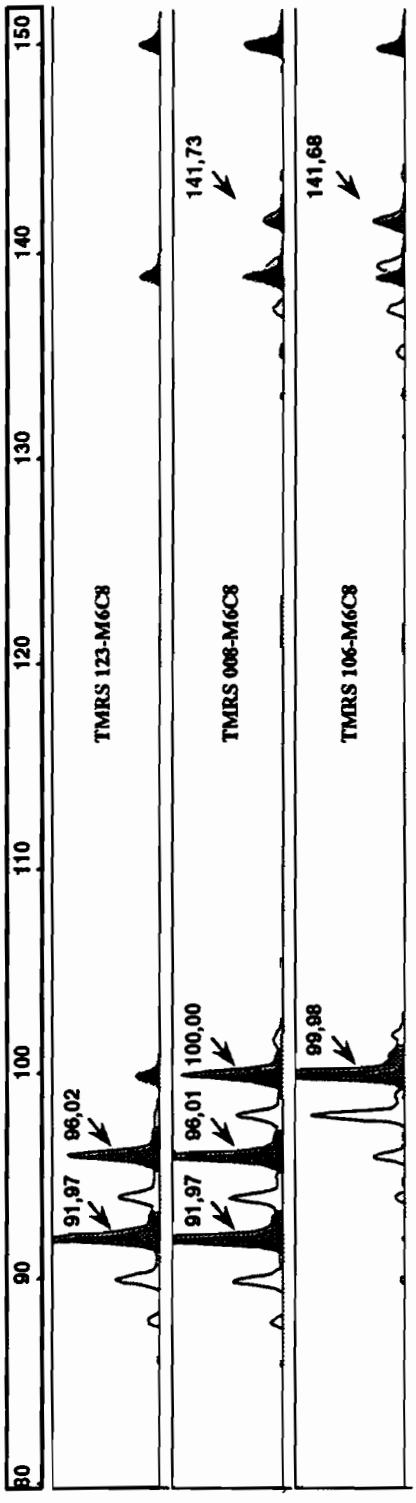

Fig. 5. Typical microsatellite electrophoresis profiles obtained from the M6C8-CA locus. Fluorescent PCR products from the TMRS-123, -008 and -106 isolates were compared. The red peaks correspond to the internal ROX-350 marker and the blue peaks, indicated by arrows, correspond to the alleles of each isolate. The numbers (calculated and ladder) refer to the sizes (bp) of the PCR products.

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Identification of bloodmeals in haematophagous Diptera by cytochrome B heteroduplex analysis

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Abstract. We developed a DNA assay for bloodmeal identification in haematophagous insects. Specific host cytochrome B gene sequences were amplified by PCR and classified on the basis of their mobility in a heteroduplex assay. In the blackfly *Simulium damnosum s.l.* (Diptera: Simuliidae), human cytochrome B DNA sequences were identifiable up to 3 days following ingestion of the bloodmeal. In the tsetse *Glossina palpalis* (Diptera: Glossinidae) collected from tsetse traps in Ivory Coast, bloodmeals were identified as taken from domestic pigs on the basis of their heteroduplex pattern and DNA sequence. Evidently the cytochrome B sequence shows sufficient interspecific variation to distinguish between mammalian host samples, while exhibiting minimal intraspecific variation. The stability of DNA in bloodmeals, for several days post-ingestion by haematophagous insects, allows PCR-HDA assays to be used reliably for host identification.

Key words. *Glossina*, *Simulium*, cytochrome B, heteroduplex analysis, polymerase chain reaction, species identification, Ivory Coast.

Introduction

Identification of the bloodmeal taken by a haematophagous insect provides information on host preferences under natural conditions. The anthropophilic index (percentage feeding on humans) is a vital component of vectorial capacity, while knowledge of other hosts reveals the relative importance of reservoirs of vector-borne zoonotic or enzootic infections.

Contemporary procedures for bloodmeal identification are generally based upon the detection of host antigens by the complement fixation test (Staak *et al.*, 1981) or by enzyme-linked immunoabsorbant assays (ELISA), using polyclonal antibodies raised against blood components from potential host vertebrates (Chow *et al.*, 1993). This method, however, requires the preparation of immune sera against the blood of each potential host species, a difficult and laborious process. Pre-adsorption steps are also needed to eliminate cross reactions when using this technique (Hunter & Bayly, 1991).

Analysis of DNA sequences of the host species (Gokool *et al.*, 1993) can provide a more specific approach than the serological methods. For example, Kirstein & Gray (1996) developed a method to identify bloodmeals in the tick *Ixodes ricinus* (L.) (Acari: Ixodidae) based upon polymerase chain reaction (PCR) amplification of a 638-bp fragment of the cytochrome B (cytB) gene encoded in the host vertebrate mitochondrion. Based upon a combination of restriction fragment length polymorphism (RFLP) and hybridization analysis, they used cytB PCR products to classify the tick bloodmeal to the genus level. RFLP and hybridization analyses, however, are cumbersome when analysing large numbers of samples.

Heteroduplex analysis (HDA) is a simple and powerful method of detecting small differences between closely related DNA sequences (Tang & Unnasch, 1995). The PCR amplified DNA to be classified (the sample) is mixed with a closely related probe sequence (the heteroduplex driver), the DNA products denatured and allowed to re-anneal. This results in the formation of four products, consisting of: two homoduplexes resulting from the re-annealing of the completely complementary DNA strands to one another; and two heteroduplex molecules formed from annealing a DNA strand derived from the heteroduplex driver with the complementary strand derived from the sample. Heteroduplex molecules can be separated from homoduplex molecules by electrophoresis on a partially denaturing polyacrylamide gel (Tang & Unnasch, 1997). Mobility of the heteroduplex products is always retarded

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relative to that of the homoduplexes in this system. Moreover, each heteroduplex molecule exhibits a unique mobility, which is dependent on the number, type and position of the mismatches present in the heteroduplex molecule (Zimmerman *et al.*, 1995). HDA has been applied in many situations requiring a method to rapidly classify large numbers of samples. These include distinguishing between, for example, closely related species of blackflies (Tang *et al.*, 1995; Zimmerman *et al.*, 1995), HIV 1 subtypes (Delwart *et al.*, 1995) and different histocompatibility complex alleles in humans (Zimmerman *et al.*, 1993).

We found it possible to amplify host-specific cytB sequences present in the bloodmeals of medically-important Dipteran insects. Here we present a method to determine the host bloodmeal origin, based on the mobility of cytB heteroduplex products in an HDA. The utility of this method is demonstrated by the identification of bloodmeals from Afrotropical blackflies of the *Simulium (Ewardsellum) damnosum* Theobald complex (Diptera: Simuliidae) and wild-caught tsetse, *Glossina palpalis* Robineau-Desvoidy (Diptera: Glossinidae).

Materials and Methods

Specimens

Tissue and blood samples for use in the production of HDA standards were obtained from Abidjan Zoo, Ivory Coast, and from commercial meat vendors and animal facilities of the University of Alabama at Birmingham. The vertebrate species tested included domestic cattle (*Bos taurus* L.), domestic pig (*Sus scrofa* L.), human (*Homo sapiens* L.), domestic chicken (*Gallus gallus* L.), water buffalo (*Bubalus bubalis* L.), antelope (*Gazella spekei* Blythe), cotton rat (*Rattus fuscipes* Waterhouse) and mouse (*Mus musculus* L.). The mosquito *Culex pipiens* L. (Diptera: Culicidae) from our North American strain was used as the negative standard of comparison for insect cytB.

Glossina palpalis tsetse were obtained from tsetse traps set near the village of Sinfara (6°37′N, 5°54′E) in the Ivory Coast. *Simulium damnosum s.l.* larvae were obtained from the Nzi river, near Fétékro (6°38′N, 4°42′E), Ivory Coast, and reared to adulthood by the system of Raybould *et al.* (1982). Young adult nulliparous female blackflies were collected from the rearing system, allowed to take a bloodmeal from a human volunteer and kept separately at ambient temperature (25–38°C) for up to 5 days.

DNA extraction

DNA extraction from whole blood-fed flies and from vertebrate samples followed the procedure of Steiner *et al.* (1995). Blood and tissue samples were disrupted by mechanical homogenization in a buffer containing 10 mM Tris-HCl (pH 8.0) 312.5 mM EDTA, 1% (w/v) sodium lauryl sarcosine and 1% polyvinylpyrollidone. Approximately 15 µl of buffer was used per 1 mg of tissue. The homogenates were heated to 90°C for 20 min and chilled on ice for 5 min. Samples were subjected to centrifugation at 13 000 *g* for 5 min at room temperature. The supernatant was removed and diluted 20-fold in 10 mM Tris-HCl (pH 8.0) 1 mM EDTA (TE).

PCR amplifications were undertaken in 25 µl of a solution containing 10 mM Tris-HCl (pH 8.3 at 25°C) 50 mM KCl, 1.5 mM MgCl2, 0.001% gelatin 200 µM dATP, dCTP, dGTP and TTP, 0.5 µM each primer, 1 unit of AmpliTaq DNA polymerase (Perkin Elmer, Branchburg, NJ, U.S.A.) and 2.5 µl of the DNA template solution, prepared as described above. The sequence of primers used in the PCR was as follows: 5′ CCCCTCAGAATGATATTTGTCCTCA 3′ and 5′ CCATCCAACATCTCAGCATGATGAAA 3′. Reactions began by incubation at 95°C for 3.5 min, followed by 36 cycles comprising 30 s at 95°C, 50 s at 60°C and 40 s at 72°C. The reaction was completed by incubation at 72°C for 5 min

Heteroduplex analysis (HDA) of PCR products used equal volumes (4 µl) of the sample and driver (water buffalo) PCR products mixed with 8 µl TE and overlaid with 10 µl of mineral oil. The mixture was denatured for 2 min at 98°C and heteroduplex products were allowed to form by slow cooling to room temperature over a period of 30 min. An aliquot (14 µl) of each heteroduplex solution was mixed with 6 µl loading buffer (0.25% bromophenol blue, 0.25% xylene cyanol, 30% glycerol). A total of 10 µl of this mixture was loaded onto a 5% polyacrylamide/urea gel (19 : 1 acrylamide : bis acrylamide, 2.7 M urea) prepared in 108 mM Tris-boric acid (pH 8.3), 2.4 mM EDTA. Electrophoresis was performed on 20 cm × 20 cm Protean II Xii system (Bio-Rad, Hercules, CA, U.S.A.) at 12 mA per gel for 18 h in 90 mM Tris-boric acid (pH 8.3), 2 mM EDTA. Gels were stained in 2 µg/ml ethidium bromide and the homoduplex and heteroduplex patterns were visualized under UV light.

Results

In order to develop a PCR-based assay to identify bloodmeals in haematophagous Diptera, it was first necessary to identify PCR conditions capable of amplifying DNA from the bloodmeal in the presence of relatively large amounts of insect DNA. Furthermore, it was necessary to identify a sequence sufficiently divergent to distinguish between different host species, but conserved enough for unambiguous identification of each potential host species. Previous studies had revealed that the cytB gene might meet these criteria (Kocher *et al.*, 1989; Bartlett & Davidson, 1992). Thus we sought PCR conditions for amplification of vertebrate cytB sequences, but not cytB of insects. As described in the Materials and Methods section, we established PCR conditions to amplify cytB sequences from both mammals and birds, without amplifying the corresponding gene from insect genera representing three important families of haematophagous Diptera, i.e. *Culex*, *Glossina* or *Simulium* (Fig. 1).

When DNA was prepared from extracts of human blood-fed *Simulium damnosum s.l.* and used as a template in the PCR amplification reaction, human DNA was identified in the

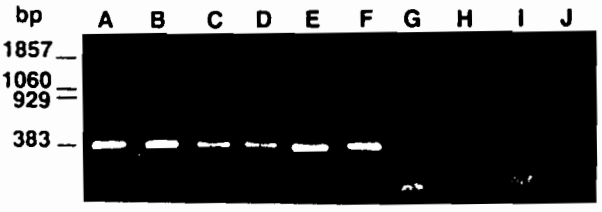

Fig. 1. PCR amplifications of vertebrate and insect cytB sequences. Template DNAs for each lane were as follows: A = domestic pig (*Sus scrofa*); B = water buffalo (*Bubalus bubalis*); C = domestic cow (*Bos taurus*); D = human (*Homo sapiens*); E = chicken (*Gallus gallus*); F = chimpanzee (*Pan troglodytes*); G = *Simulium damnosum s.l.*; H = *Culex pipiens*; I = *Glossina palpalis*; J = PCR negative control (no template).

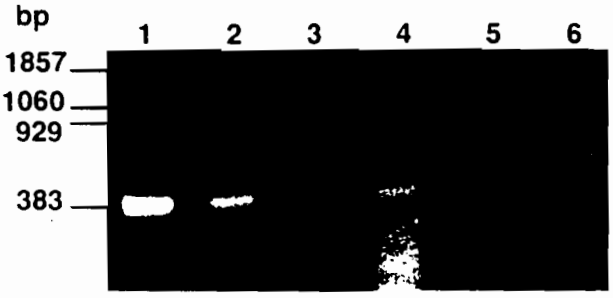

Fig. 2. Time course of detection of bloodmeals in blackflies (*Simulium damnosum s.l.*) fed upon a volunteer and held at ambient temperature for varying periods of time. DNA was extracted from the flies, and cytB sequences amplified from the bloodmeal as described in Materials and Methods. Lane 1 = positive control; Lane 2 = fly held for 24 h following ingestion of the bloodmeal; Lane 3 = fly held for 48 h; Lane 4 = fly held for 72 h; Lane 5 = DNA extracted from an unfed fly; Lane 6 = PCR negative control.

specimens processed up to 72 h postingestion of the bloodmeal (Fig. 2), but no signal was detected in flies kept for longer periods under local conditions of 35°C and 80% r.h.

When potential drivers were tested against cytB PCR products derived from different vertebrate species, the cytB PCR product from water buffalo was more versatile than those derived from chicken, chimpanzee, human and pig cytB PCR products. When tested against itself, as expected, the water buffalo PCR product produced only a homoduplex band (lane 4, Fig. 3). All vertebrate species tested produced HDA patterns readily distinguishable from each other. The PCR product derived from domestic cattle gave a heteroduplex product with the fastest mobility (lane 3, Fig. 3) while those from chicken gave products with the slowest mobility relative to the homoduplex (lane 1, Fig. 3). This is consistent with the expectation that electrophoretic retardation of any heteroduplex product is proportional to the genetic distance between the driver and sample species (Delwart *et al.*, 1994; Tang & Unnasch, 1997).

HDA is an extremely sensitive method for detecting minor sequence differences (Tang & Unnasch, 1995) and it is well known that intraspecific sequence variation exists in the mitochondrial genome of many organisms, including humans (Cann *et al.*, 1984). Such intraspecific variation, if sufficiently large, might interfere with the interpretation of results obtained in the HDA assay described above. To determine if this was the case, DNA from two or more individuals of three species of potential host animals was examined for intraspecific variation in the HDA. No intraspecific variation (data not shown) was seen in the cytB HDA from cattle ($n = 2$, from Africa and North America), water buffalo ($n = 2$) and pigs ($n = 7$).

In contrast, some intraspecific variation was noted for human samples tested in the cytB HDA. Of 368 individuals from five ethnic groups (Africans, African Americans, Asians, European and North American Caucasians) tested in the cytB HDA, 96% (354) gave cytB products that were indistinguishable in the HDA. In the remaining 14 individuals, minor variations in mobility of the cytB products were detectable (Fig. 4). This variation was insufficient to interfere with the correct identification of the sample as human derived (Fig. 4).

To confirm the utility of the HDA for identification of bloodmeals, DNA was extracted from two female *S. damnosum s.l.* that had blood fed on a human volunteer, and five *G. palpalis* tsetse flies collected in the village of Sinfara, Ivory Coast. In the cytB PCR, both of the blackflies and three of the tsetse produced detectable PCR products that were then classified using the HDA assay. As expected, both blackfly specimens gave heteroduplex patterns corresponding to human DNA, whereas the three tsetse bloodmeals gave PCR products identical in mobility to those obtained from domestic pig DNA (Fig. 5). To confirm the identity of these tsetse hosts, the remaining PCR products from the three tsetse bloodmeals were subjected to DNA sequence analysis. The DNA sequence obtained from the bloodmeal PCR products was found to match that of the pig cytB gene sequence deposited in the Genbank sequence database (Accession number X56295), confirming the HDA identification.

Discussion

The results presented above demonstrate that it is possible to use vertebrate-specific PCR primers to amplify DNA present in a bloodmeal from haematophagous Diptera. Furthermore, the identity of the bloodmeal can be established by subsequent analysis of the PCR product using HDA. The PCR-HDA assay is somewhat more complicated to apply than is the case for ELISA-based assays (e.g. Chow *et al.* 1993) to identify bloodmeals in field-collected insects. PCR HDA, however, has some advantages over ELISA. First, in order to unambiguously identify a given bloodmeal with ELISA it is necessary to produce polyclonal antisera against the blood of the host in question. Second, it is also often necessary to carry out pre-absorption steps against the blood antigens of other species before the specificity of the antibody reagent can be ensured. Third, it is necessary to test the specimen with antisera produced against all potential hosts in order to unambiguously identify the bloodmeal. In some cases, this is simply impossible, given that the supply of antigen obtained from a given insect specimen may be limited. Finally, it may not be

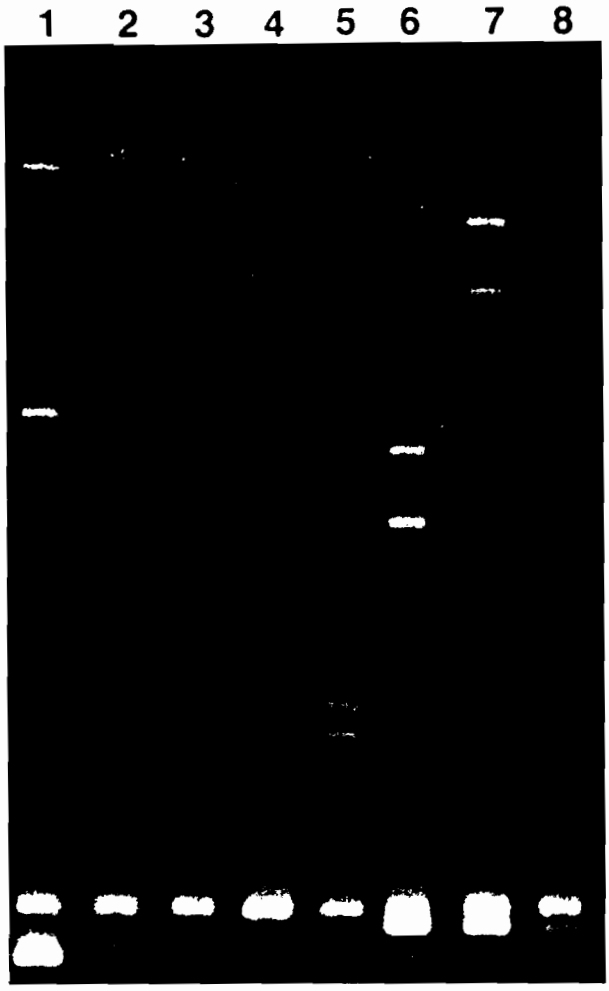

Fig. 3. HDA analysis of cytB PCR products from different species. HDA was carried out using a PCR product derived from water buffalo as a heteroduplex driver. Lane 1 = chicken; Lane 2 = domestic pig; Lane 3 = domestic cow; Lane 4 = water buffalo; Lane 5 = gazelle; Lane 6 = human; Lane 7 = cotton rat; Lane 8 = mouse.

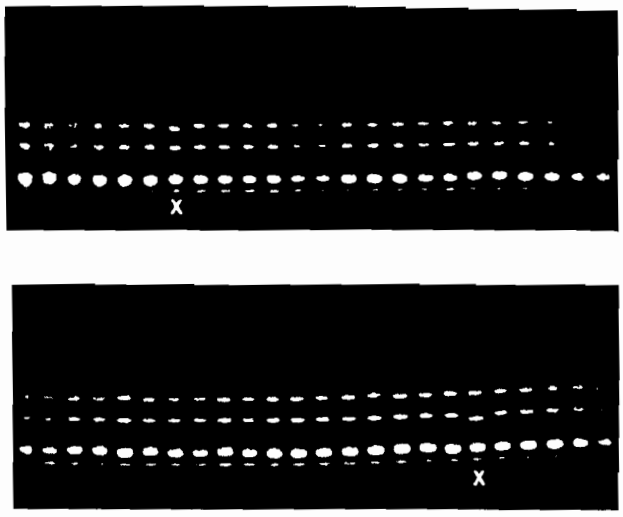

Fig. 4. Intraspecific variation of human cytB PCR products as detected by HDA carried out as described in Materials and Methods. Lanes marked with an X indicate individuals with HDA pattern that differed from the most common allele (samples in unmarked lanes).

possible to identify a particular bloodmeal if a suitable antibody is not available.

In contrast to the ELISA-based method, the HDA-PCR-based method relies upon the mobility of the heteroduplex molecule formed by hybridizing sample and driver PCR products to give the identification. Thus, with an optimized heteroduplex driver and a collection of reference DNA samples, it is possible to identify the bloodmeal using a single assay. Furthermore, bloodmeals for which standards are not available will be detected in the HDA, because they will produce heteroduplex products with novel mobilities. The identity of products producing novel HDA patterns can be further analysed by DNA sequence analysis. In many cases, a comparison of the resulting DNA sequence to others deposited in the DNA sequence databases should allow one to identify the source of the bloodmeal unambiguously. This approach has proved useful in the identification of meats in agricultural studies (Bartlett & Davidson, 1992; Forrest & Carnegie, 1994). If a complete match to the unknown DNA sequence is not present in the databases, the sequence data may be subjected to phylogenetic analyses. This will identify the species most closely related to the unknown sample, providing important clues to its true identity. The identity of the bloodmeal can then be confirmed by obtaining a small DNA sample from the most likely hosts, as inferred from the phylogenetic analysis. Once a given bloodmeal is identified, the PCR product can be used to generate a new reference sample for the PCR HDA.

HDA is a very sensitive method of detecting minor sequence differences (Tang & Unnasch, 1995), so the assay could be compromised by intraspecific polymorphisms. To minimize the likelihood of this occurring, the cytB gene was targeted in the PCR-HDA, because it represents one of the more conserved portions of the mitochondrial genome (Cann *et al.*, 1984). We also examined the degree of intraspecific polymorphism within the cytB fragment in four species, as detected by the PCR HDA assay. We found no intraspecific polymorphisms detectable by HDA in the small number of pigs, domestic cattle and water buffalo samples tested. Furthermore, among > 350 humans, 96% were found to contain a single cytB allele as determined by HDA. In the remaining 4%, some intraspecific variation was detected, but these resulted in very minor shifts in the mobility of the heteroduplex products, insufficient to preclude the correct identification of the product as human derived. Thus, the HDA detected a limited level of intraspecific heterogeneity that does not compromise the assay itself.

Some segments of the mitochondrial genome of humans do contain significant levels of intraspecific variation. This is particularly true of the control or D loop domain, which is sufficiently hypervariable to be useful for the identification of

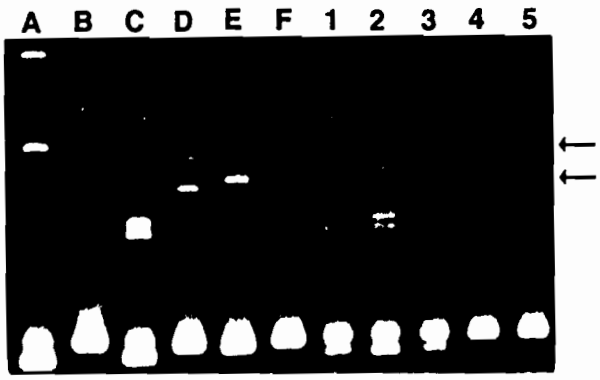

Fig. 5. Identification of bloodmeals by cytB PCR-HDA in wild-caught tsetse and experimentally fed blackflies – PCR amplification and HDA were carried out as described in Materials and Methods, using the water buffalo PCR product as a heteroduplex driver. Lanes A–E represent standards derived from known species. Lane A = chicken; lane B = water buffalo; lane C = domestic pig; lane D = human; lane E = chimpanzee. Lanes 1–5 represent PCR products derived from bloodmeals. Lanes 1–3 represent bloodmeals from three wild-caught tsetse flies, and lanes 4 and 5 represent bloodmeals from *S. damnosum s.l.* experimentally fed on a human volunteer. Arrows highlight the position of the bands seen in the *Simulium* bloodmeals.

individual humans in forensic studies (Stoneking *et al.*, 1991; Alonso *et al.*, 1996; Butler & Levin, 1998). The demonstration that it is possible to amplify a portion of the cytB gene from human-derived bloodmeals suggests that it may be possible to amplify more variable segments of the human mitochondrial genome from the bloodmeal as well. If this is the case, it may be possible to assign the source of a given bloodmeal to a particular individual, or family group. This may be useful in microepidemiological studies addressing the dispersion of haematophagous insects following bloodmeal uptake under natural conditions.

As a first test of the PCR-HDA to identify bloodmeals from field-collected insects, we amplified and characterized the DNA present in the bloodmeals of three wild-caught tsetse flies. Each contained blood identified by HDA as derived from domestic pig, confirmed by direct sequence analysis. This finding was in accordance with previous studies employing an ELISA-based assay to identify bloodmeals of *G. palpalis* (Staak *et al.*, 1986). In this study, the majority of bloodmeals from *G. palpalis* collected in the Ivory Coast were classified as derived from pigs. Our results support the conclusion from this previous study that pigs are a common host of *G. palpalis* under natural conditions.

Results presented above indicate that a bloodmeal is detectable using the cytB PCR assay for a period of up to 72 h following ingestion by *S. damnosum s.l.*, sufficient to allow the PCR-based assay to be used to study the feeding preferences of wild-caught *Simulium* females. Previous studies have shown that a large proportion of *S. damnosum s.l.* females oviposit and begin searching for the next bloodmeal within 72 h after the previous bloodmeal (Thompson, 1976). The period during which we were able to identify the bloodmeal in African *S. damnosum s.l.* was approximately 40 h less than for the ELISA method applied to North American blackflies (Hunter & Bayly, 1991). This may be attributed to the warmer temperature (25–38°C) for Afrotropical blackflies than for those in North America (9–22°C). Whereas the ELISA detects antigens that make up the bulk of the bloodmeal, the PCR relies on detection of a component (host genomic DNA) present in only a small proportion of cells in the bloodmeal. Further comparative tests are needed, at a range of controlled temperatures, to ascertain whether the antigen target of the ELISA does persist for longer than the DNA target of the PCR HDA in bloodmeals of haematophagous arthropods.

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Use of polymerase chain reaction in human African trypanosomiasis stage determination and follow-up

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Stage determination of human African trypanosomiasis is based on the detection of parasites and measurements of biological changes in the cerebrospinal fluid (CSF) (concentration of white blood cells > 5 cells per mm^3 and increased total protein levels). The patient is treated accordingly. Demonstration of the absence or presence of trypanosomes by the double centrifugation technique is still the only test available to clinicians for assessing treatment success. In this study, however, we evaluate the polymerase chain reaction (PCR) as a tool for assessing the disease stage of trypanosomiasis and for determining whether treatment has been successful. All 15 study patients considered to be in the advanced stage of the disease were PCR positive; however, trypanosomes were demonstrated by double centrifugation in only 11 patients. Of the five remaining patients, who were considered to be in the early stage, PCR and double centrifugation were negative. Following treatment, 13 of the 15 second-stage patients were found to be negative for the disease in at least two samples by PCR and double centrifugation. Two others were still positive by PCR immediately and one month after the treatment. Trypanosome DNA detection using PCR suggested that the two positive patients were not cured but that their possible relapse could not be identified by a search for parasites using the double centrifugation technique. Further evaluation of the PCR method is required, in particular to determine whether PCR assays could be used in studies on patients who fail to respond to melarsoprol, as observed in several foci.

Voir page 747 le résumé en français. En la página 747 figura un resumen en español.

Human African trypanosomiasis evolves from the haematolymphatic phase (first or early stage) to the meningoencephalitic stage (second or advanced stage). Although neurological signs and symptoms do occur during the first stage, they become more pronounced and more frequent during the second stage (*1*). The stage determination of the disease is currently based on measurements of the biological changes occurring in the cerebrospinal fluid (CSF) and the presence of parasites. The generally accepted criteria for CSF biological alteration are the same as those currently used in neurology: concentration of white blood cells >5 cells per mm^3 and increased total protein levels (>37 mg per 100 ml in a dye-binding protein assay). In human African trypanosomiasis, an increased cell count and/or protein level, with or without the presence of trypanosomes, consequently leads to the conclusion that the disease is at an advanced stage, and the patient will be treated accordingly. The presence of trypanosomes alone without any CSF alteration is considered by some workers to be insufficient indication for central nervous system involvement and second-stage diagnosis (*2*). Consequently, they have suggested various techniques for identifying changes in CSF that would allow the diagnosis of second stage of the disease, such as the presence of antitrypanosomal-specific antibodies determined by immunofluorescence (*3*), autoantibodies directed against neurofilament proteins (*4*), trypanosome-specific antibody (*5*), and elevated trypanosome-specific and nonspecific IgM levels (*6*). It should be noted that IgM levels can now be determined through a recently developed latex agglutination test (Latex/IgM) which combines stability, sensitivity and simplicity (*7*), and which has demonstrated its many advantages for application in the field.

It has been shown that, following treatment, it is usually several months before the number of cells and total protein level in CSF return to normal. Demonstration of the absence or presence of trypanosomes by the double centrifugation technique (*8*) is still the only means available to clinicians for assessing treatment success. However, despite its good sensitivity, double centrifugation is hampered by the fluctuation of parasite numbers in CSF. Given the efficiency of the polymerase chain reaction (PCR) in detecting the presence of trypanosomes in whole blood (*9*), we report in the present article on the use of this technique as a tool for diagnosing disease stage and for determining treatment success or failure.

CSF samples were obtained from 20 patients from Côte d'Ivoire. For each patient, samples had been taken prior to treatment, at the end of the

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treatment, and at 1, 3, 6 and 12 months after treatment. Samples were aliquoted. One aliquot was used immediately to test for the presence of parasites by double centrifugation, cell count and determination of the protein level. Another aliquot of each sample was frozen in Nunc (Nalge Nunc International, Rochester, NY, USA) and stored in liquid nitrogen for further studies.

Aliquots were not available from all samples for this study. CSF double centrifugation, cell counts, determinations of protein levels and PCR could only be performed for each patient on samples collected before treatment and on at least two samples collected after treatment.

Each frozen 1.5-ml aliquot of CSF was thawed at room temperature and 500 µl was transferred to a 0.6-ml Eppendorf conical tube and centrifuged at 13 000 *g* for 20 min at 4 °C. By means of a pipette, 450 µl of the supernatant was gently removed and discarded. The pellet was resuspended in the remaining liquid and vortexed for 5 min. Subsequently, 100 µl of 5% Chelex solution in sterile purified water (Chelex® 100 Resin, Bio-Rad Laboratories, CA, USA) was added to each tube. The tubes were then vortexed for 1 min, centrifuged at 13 000 *g* for 1 min and incubated at 56 °C for 1 hour. This was followed by a second incubation at 95 °C for 30 min. After incubation, the tubes were again centrifuged for 5 min at 13 000 *g*. The supernatant, now containing single strands of DNA, was used directly or after storage at 4 °C for no longer than 2 days.

PCR was performed according to the method described by Penchenier et al. (*9*) using *Trypanosoma brucei*-specific primers (*10*). The amplification conditions were as follows: 40 cycles with the denaturation step at 94 °C for 1 min, the annealing step at 56 °C for 1 min, and the polymerization step at 72 °C for 1 min. The final elongation was at 72 °C for 5 min. Samples of 10 µl of each reaction product were run in 2% agarose gel with 1x 40mM Tris-acetate, 1mM EDTA (TAE) buffer at 100 V for 30 min. The gel was then stained for 20 min with a 0.5 µg/ml solution of ethidium bromide before being visualized under ultraviolet light.

Table 1 shows the PCR, double centrifugation, protein level and cell count results for the pre-treatment samples taken from each patient. Of the 15 patients considered to have second-stage trypanosomiasis according to the number of cells and/or protein content, all were PCR positive; however, trypanosomes were demonstrated by double centrifugation in only 11 of these patients. For the five remaining patients, who were considered to be first stage, the PCR and double centrifugation results were negative. Among these, one (patient 2522) had a cell count of 24, and might be in an early-late stage of the disease, a result which supports the controversial value of 5 cells for the cut-off between the first and second stages (*11*). Furthermore, patients with up to 20 cells in their CSF have been successfully treated with pentamidine rather than melarsoprol (*12*).

For the post-treatment samples, 13 of the 15 patients considered to be in the second stage were found to be negative in at least two samples by PCR and double centrifugation. Two others (patients 2585 and 2552) were still PCR positive both immediately and 1 month after receiving treatment. For these two patients, the double centrifugation did not disclose the presence of parasites but their cell counts and protein levels were abnormal.

The detection of parasites either by double centrifugation or by parasite DNA detection using PCR seems to be the only reliable technique for assessing the efficacy of trypanosomiasis treatment, since cell number and protein level are slow to return to normal and remain high even 3 months after treatment has ended. Detection of trypanosome DNA using PCR in the two positive patients indicated that they had not been cured but that their potential relapse could not be identified using the double centrifugation technique since it is not sufficiently sensitive.

Occasionally, CSF samples with a normal cell count and protein level have been found to harbour trypanosomes (*13*). In such cases, the patients are considered to be in the first stage of the disease. Other occasional observations indicate a difference in CSF analysis depending on the site of the spinal puncture (suboccipital or lumbar) (*14*). PCR may be an appropriate method for studying such unusual cases.

Our study indicates that the PCR technique described could be useful for determining the stage of the disease in human African trypanosomiasis. It might also be useful for identifying early treatment

Table 1. **Results of PCR, double centrifugation, protein level determination and cell count for patients with human African trypanosomiasis prior to treatment**

Patient	PCR	Double centrifugation	Protein level (mg/100 ml)	Cell count	Stage
2501	+	+	70	872	2
2555	+	+	70	274	2
2565	+	+	71	1044	2
2585	+	+	53	122	2
2591	+	+	38	930	2
2511	+	+	60	728	2
2552	+	+	127	132	2
2589	+	+	60	278	2
2592	+	+	71	228	2
637	+	+	NP[a]	36	2
649	+	+	NP	30	2
2564	+	–	87	450	2
2567	+	–	45	260	2
2571	+	–	71	1152	2
2494	+	–	68	252	2
2520	–	–	19	0	1
2521	–	–	26	2	1
2522	–	–	29	24	1
2586	–	–	24	4	1
2543	–	–	34	0	1

[a] NP = not performed.

failures. Further evaluation of the method is required, in particular to determine whether PCR assays could be used in studies on patients who fail to respond to melarsoprol, as observed in several foci (e.g. Angola, Sudan, and Uganda). ■

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Résumé

Amplification génique (PCR) appliquée à la détection du stade et au suivi de la trypanosomiase africaine humaine

La détermination du stade de la trypanosomiase africaine humaine est actuellement fondée sur la recherche du parasite et la mesure des modifications biologiques du liquide céphalorachidien (LCR) : une concentration des leucocytes >5 par µl et une augmentation de la protéinorachie (>37 mg/100 ml avec la méthode colorimétrique). Une augmentation de la cellularité et/ou de la concentration en protéines, avec ou sans présence de trypanosomes, débouche sur un diagnostic de stade avancé (deuxième stade), et le patient sera traité comme il se doit. La mise en évidence de l'absence ou de la présence de trypanosomes par double centrifugation demeure le seul moyen dont dispose le clinicien pour juger de la réussite du traitement. Compte tenu de l'efficacité de la PCR pour détecter la présence de trypanosomes dans le sang total, nous avons étudié cette technique en tant qu'instrument permettant d'évaluer le stade de la maladie et de déterminer le succès ou l'échec du traitement. On a obtenu des prélèvements de LCR pour 20 patients de Côte d'Ivoire. Sur 15 patients considérés comme ayant une trypanosomiase de deuxième stade en fonction de leurs cellulorachie et protéinorachie, tous étaient PCR positifs; toutefois, la double centrifugation n'a montré la présence de trypanosomes que chez 11 de ces patients. Les résultats de la PCR et de la double centrifugation pour les 5 patients restants, considérés comme en étant au premier stade, étaient négatifs. Un de ces patients avait 24 cellules par µl et pouvait se trouver à un stade soit précoce soit tardif de la maladie, ce qui vient à l'appui du chiffre contesté de 5 cellules par µl comme étant le seuil entre le premier et le deuxième stade. Des prélèvements postérieurs au traitement effectués chez 13 des 15 malades présumés au deuxième stade ont été trouvés négatifs au moins à deux occasions, à la fois par la PCR et par la double centrifugation. Les deux autres sont demeurés positifs pour la PCR, immédiatement après le traitement et un mois suivant la fin de ce dernier. La recherche du parasite soit par double centrifugation soit par la PCR pour mettre en évidence l'ADN du parasite semble être la seule technique fiable, car le nombre des cellules comme la concentration en protéines ne reviennent à la normale que lentement et demeurent élevés même trois mois après le traitement. Les résultats de la PCR donnent à penser que les deux patients positifs n'étaient pas guéris, mais que leur rechute éventuelle n'a pu être identifiée par une recherche du parasite à l'aide de la double centrifugation, cette technique n'étant pas suffisamment sensible. Il faut évaluer plus avant la PCR, notamment pour déterminer si elle peut être utilisée dans les études sur les patients qui ne répondent pas au mélarsoprol ainsi qu'on a pu l'observer dans plusieurs foyers.

Resumen

Empleo de la reacción en cadena de la polimerasa para la determinación de la fase de la tripanosomiasis africana humana y su seguimiento

La determinación de la fase de la tripanosomiasis africana humana se basa actualmente en la detección de los parásitos y en la medición de los cambios biológicos del líquido cefalorraquídeo (LCR): concentración de leucocitos superior a 5 células por µl y aumento de las proteínas totales (>37 mg/100 ml usando un método colorimétrico). Así pues, un aumento del número de células y/o de la concentración de proteínas, con o sin presencia de tripanosomas, permitirá diagnosticar una fase avanzada (segunda fase) de la enfermedad y tratar al paciente en consecuencia. La demostración de la ausencia o presencia de tripanosomas mediante la técnica de doble centrifugación es todavía el único medio de que dispone el personal clínico para evaluar el éxito del tratamiento. En vista de la eficiencia de la reacción en cadena de la polimerasa (RCP) como medio de detección de la presencia de tripanosomas en sangre entera, hemos evaluado el uso de esta técnica para determinar la fase de la enfermedad y el éxito o el fracaso del tratamiento. Se obtuvieron muestras de líquido cefalorraquídeo de 20 pacientes de Côte d'Ivoire. Los 15 pacientes a quienes se consideraba en la segunda fase de tripanosomiasis, según el recuento leucocitario y el nivel de proteínas en su LCR, eran todos RCP-positivos. En cambio, la doble centrifugación sólo mostró tripanosomas en 11 de esos pacientes. Los resultados

de la RCP y de la doble centrifugación para los cinco pacientes restantes, a quienes se consideraba en la primera fase, fueron negativos. Uno de esos pacientes tenía 24 células por µl y podía hallarse tanto en una fase temprana como en una fase avanzada de la enfermedad, lo que respaldaría la consideración del controvertido valor de 5 células por µl como el límite entre las fases primera y segunda. Las muestras posteriores al tratamiento de 13 de los 15 pacientes a quienes se consideraba en la segunda fase fueron negativas por lo menos en dos ocasiones tanto en la RCP como en la doble centrifugación. Los otros dos seguían siendo RCP-positivos inmediatamente después del tratamiento y un mes más tarde. La detección de los parásitos, ya sea por doble centrifugación o empleando la RCP para identificar el ADN del parásito, parece la única técnica fiable, ya que tanto el recuento leucocitario como los niveles de proteínas tardan en volver a la normalidad y siguen siendo altos incluso tres meses después del tratamiento. Los resultados de la RCP sugieren que los dos pacientes positivos no estaban curados, pero no se hubiese podido prever su eventual recaída buscando los parásitos mediante la técnica de doble centrifugación, ya que ésta no es lo bastante sensible. Es necesario evaluar mejor el método de la RCP, en particular para determinar si puede emplearse en los estudios de pacientes que no responden al melarsoprol, como se ha observado en varios focos.

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UNIVERSITE MONTPELLIER II
Université des Sciences et Techniques du Languedoc

Dossier de synthèse de Recherches

HABILITATION A DIRIGER DES RECHERCHES

présentée et soutenue le 9 décembre 1999

par

Philippe Truc

Membres du Jury :

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Professeur Philippe Desjeux
Professeur Philippe Vincendeau
Docteur Jean Louis Frézil

Sommaire

Préambule sur la Trypanosomiase Humaine Africaine

La Trypanosomiase Humaine Africaine ou THA est une maladie orpheline. Plus connue sous le nom mythique de « Maladie du sommeil », elle est pourtant d'une cruelle réalité. Elle ne frappe que des populations rurales africaines, souvent très éloignées des structures de santé. Sournoise, elle se maintient à bas bruit, attendant le moment propice à une explosion épidémique, notamment à la faveur de graves problèmes politiques et sociaux qui sévissent régulièrement dans des pays d'Afrique centrale.

La THA n'a malheureusement pas le soutien médiatique que connaissent d'autres parasitoses tropicales comme le paludisme ou l'onchocercose. Pourtant, un rapport de l'Organisation Mondiale de la Santé en 1998 estime entre 300 et 500.000 le nombre de patients non traités, 300.000 nouveaux malades chaque année, et enfin 100 décès journaliers « officiellement » répertoriés dans toute l'Afrique. Cette parasitose mortelle en absence de traitement, dont l'expansion exponentielle dépasse les capacités de réaction des quelques structures locales de santé publique, est et sera à nouveau à l'origine de l'anéantissement de nombreux villages.

La recherche sur la THA a débuté à la fin du XIXe siècle, en particulier par les travaux de David Bruce. Malgré les moyens techniques de l'époque, pas plus de 20 années ont été nécessaires pour connaître les grandes lignes de l'épidémiologie de cette maladie : hôtes, vecteurs, distribution et pathologies. Puis, à la faveur de la présence coloniale, les efforts se sont concentrés efficacement sur la lutte. Des résultats spectaculaires ont été obtenus, grâce en particulier aux équipes médicales mobiles, œuvres d'Eugène Jammot. La prévalence de la maladie redevenait alors « tolérable » pour les populations africaines.

À partir de 1965, soit peu de temps après l'indépendance de la majorité des pays africains francophones, la THA connaît une nette recrudescence due principalement à l'arrêt des campagnes des équipes mobiles. Depuis lors, et malgré des efforts notables en matière de recherche permettant d'approfondir nos connaissances sur la THA, cette maladie ne cesse de se propager.

1- Résumé de l'itinéraire scientifique et des activités de recherche

La THA sévit sous deux formes basées sur l'aire de répartition de la maladie, l'espèce du vecteur (*glossina*) ou mouche tsé-tsé, et la pathogénicité chez l'homme. Ainsi, *Trypanosoma brucei gambiense*, transmis par *Glossina palpalis*, est responsable de la forme chronique en Afrique de l'Ouest et Centrale, *Trypanosoma brucei rhodesiense*, transmis par *G. morsitans*, provoque la forme aiguë en Afrique de l'Est. Dans ce dernier cas, un réservoir animal sylvatique est connu depuis le début du siècle. Enfin, une troisième sous-espèce de *T. brucei*, *T. b. brucei*, est responsable de la nagana chez les animaux d'élevage, essentiellement bovidés. La nagana est la première cause de mortalité dans ces cheptels. Par définition, *T. b. brucei* n'infecte pas l'homme.

L'épidémiologie de la THA est complexe. Malgré des travaux précédents et compte tenu de nombreuses observations de terrain, plusieurs questions restaient posées en 1987 sur la validité de la taxonomie classique, la pathogénicité chez l'homme, la suspicion d'un réservoir animal pour la forme chronique, le dépistage et le diagnostic de la maladie. Ainsi, j'ai décidé d'organiser mes recherches en plusieurs étapes, basées sur des problématiques de terrain et m'intéressant non seulement aux différents aspects de l'épidémiologie mais aussi à la lutte contre la THA. Les méthodes d'isolement des parasites, l'identification des parasites chez l'homme ou l'animal, la pathogénicité des trypanosomes chez l'homme, puis le diagnostic sérologique et parasitologique et l'identification des repas de sang chez les glossines ont été mes principaux sujets de recherche.

Le but de ces travaux était une meilleure compréhension de l'épidémiologie de la THA, dans l'espoir de contribuer à améliorer le combat à mener contre cette redoutable parasitose. Ce travail a été réalisé au sein d'équipes multidisciplinaires, comprenant des entomologistes, des biologistes, des médecins, professionnels universitaires, d'EPST ou d'Organisations Internationales, tout en participant à l'initiation et à la formation de jeunes chercheurs français ou étrangers, que ce soit dans le cadre de stages de licence, maîtrise, DEA de parasitologie ou d'entomologie médicale, ou en préparation de thèse de doctorat.

Je me suis familiarisé à la recherche en biologie appliquée aux maladies tropicales durant mon stage de DEA au sein du laboratoire d'Ecologie Médicale de la faculté de Médecine de Montpellier. Bien que ce laboratoire effectuât des recherches principalement sur les leishmanioses, j'ai pu travailler sur des trypanosomes de mammifères et de reptiles, et trouver des corrélations entre les mesures morphométriques des trypanosomes et leurs caractéristiques génétiques définies par électrophorèse d'isoenzymes. Nous avons décrit chez le blaireau ce qui pourrait correspondre à une nouvelle espèce autre que *T. pestanai*, sans pour autant la nommer (publication 91-2).

Je souhaitais ensuite continuer l'étude des agents pathogènes parasitaires par marquage génétique. L'IRD/ORSTOM m'a permis de préparer mon doctorat. Le sujet était « Apport de la

génétique des Populations à la taxonomie de *Trypanosoma brucei* et à l'épidémiologie de la Trypanosomiase Humaine ou Maladie du sommeil en Afrique Centrale ». La connaissance du terrain et de l'épidémiologie de la THA conditionnait la collecte de matériel parasitaire et l'étude génétique.

Au Congo, j'ai effectué mes premières prospections médicales, me familiarisant aux techniques de diagnostic de la THA tout en collectant les souches de parasites nécessaires à mon travail.

De retour à Montpellier, j'ai terminé la mise au point technique des protocoles électrophorétiques sur acétate de cellulose (publications 93-1, 93-3), puis effectué mes analyses et rédigé ma thèse.

Les conclusions de mon travail de thèse étaient essentiellement la confirmation du mode de reproduction clonal des trypanosomes (publications 90-2, 93-4), l'existence d'un réservoir animal domestique au Congo (publication 91-1), la description d'une épidémisation de la THA au Congo en 1989 due à un type particulier de trypanosomes (zymodème), la comparaison des foyers du Congo, ex-Zaïre et Cameroun, en remarquant que l'endémicité de chaque foyer était caractérisée par un type particulier et unique de zymodème (publications 90-1, 93-4). Enfin, l'homme se confirmait être le principal responsable de la dispersion de la maladie, l'isolement géographique des foyers renforçant ce caractère endémique.

Mon premier poste de chargé de recherche : Bristol (U.K.)

En 1990, 12 mois avant ma soutenance de thèse, le Dr David Godfrey, responsable du Tsetse Research Laboratory à Bristol, m'a proposé un poste. J'ai pris mes fonctions de chargé de recherche à Bristol en Janvier 1991.

Ce contrat de 4 ans présentait plusieurs avantages :

- L'immersion dans un cadre universitaire anglophone, permettant des contacts nouveaux, par exemple avec nos collègues de l'Afrique de l'Est (ougandais et kenyans), et la pratique de l'enseignement supérieur ;
- L'intégration dans une équipe pluridisciplinaire qui maîtrisait les différents aspects de la recherche sur la THA (entomologie, parasitologie, isoenzymologie et biologie moléculaire) ;
- La poursuite de mes activités de recherche, car les travaux du laboratoire étaient alors très similaires à ceux que je souhaitais poursuivre, en particulier en matière de perfectionnement des

outils de terrain (isolement des parasites et diagnostic), et d'identification génétique des trypanosomes appliquée à l'épidémiologie de la THA.

Dès 1991, j'ai poursuivi mes recherches, en commençant par la mise au point d'une nouvelle technique d'isolement *in vitro* des trypanosomes : le KIVI (kit for isolation *in vitro* of trypanosomes: publications 92-1, 92-2). Puis mes travaux m'ont conduits à vérifier l'existence d'un réservoir animal en Côte d'Ivoire, à effectuer des infections expérimentales de porcs domestiques par des glossines infectées, à étudier les variants génétiques de trypanosomes isolés de l'homme en Afrique de l'Est, en particulier en Tanzanie (publications 93-2, 94-3).

Durant mon contrat en Angleterre, j'ai pris des contacts internationaux qui me sont toujours utiles à l'heure actuelle. J'ai donné des cours de Parasitologie à l'Université de Bristol, et pu ainsi diversifier mes activités par l'encadrement d'étudiants vétérinaires en stage de Parasitologie tropicale au sein de notre laboratoire.

En 1992, j'ai été contacté par le Dr Christian Marchal du Ministère français de la Coopération. Ce dernier m'a proposé un poste contractuel d'assistant technique de la Coopération afin de redynamiser le laboratoire d'isoenzymes de l'Institut Pierre Richet (IPR) de Bouaké. J'ai accepté et pris ma nouvelle affectation à Bouaké à la fin du mois de décembre 1992.

Mon second poste de chargé de recherche : Bouaké (Côte d'Ivoire)

Les grandes lignes de ma lettre de mission étaient les suivantes : *« Le chef du laboratoire d'isoenzymes, en accord avec la lettre de mission signée par M. le Ministre de la Santé de Côte d'Ivoire et M. le Chef de la Mission Française de Coopération et d'Action Culturelle d'Abidjan, a pour attributions essentielles de cordonner les activités du laboratoire, de rédiger des programmes de recherche et de trouver des financements, de rédiger la synthèse des résultats de la recherche et de les faire publier dans des revues de qualité, et de former un homologue devant lui succéder ».*

À mon arrivée à Bouaké, aucun programme ni financement n'était en cours. Le laboratoire, situé à l'Institut Pierre Richet de l'Organisation de Coopération et de Coordination pour la lutte Contre les Grandes Endémies (OCCGE), nécessitait une rénovation et un équipement complet. Ceci a pu être réalisé grâce au soutien financier du Fonds d'Aide à la Coopération (FAC). Afin de ne pas perdre de temps, les premiers programmes de recherche (FAC et OMS/Tropical Diseases Research ou TDR), ont été menés en grande partie en accueil dans le laboratoire de Pathologie Animale de Bingerville (Côte d'Ivoire), durant les travaux de rénovation du laboratoire de Bouaké.

Quand le laboratoire fut opérationnel à Bouaké, j'ai pu accueillir des stagiaires et intensifier mes travaux de recherche et de formation. Dans le même temps, j'ai développé des contacts scientifiques nationaux et internationaux. Par exemple, les collaborations avec les Services vétérinaires ivoiriens et la World Wild Life Fundation, m'ont permis de travailler sur le réservoir animal de THA en Côte d'Ivoire. Ces travaux ont prouvé par identification génétique l'existence d'un réservoir animal sauvage dans le cycle épidémiologique de la maladie (publications 94-2, 97-1, 97-2).

La mise au point de kits d'isolement *in vitro* des parasites a été déterminante. Ce travail a été initié à Anvers puis poursuivi à Bristol, et enfin finalisé à Bouaké (publications 94-1, 96-1), permettant la production et la distribution du KIVI. Ce kit est reconnu par l'OMS et l'ensemble des chercheurs comme l'outil de référence pour l'isolement des trypanosomes de l'espèce *T. brucei* sur le terrain (OMS, 1998).

J'ai suspecté la présence d'une forme aiguë de THA en Côte d'Ivoire, s'opposant à la forme chronique gambienne, à la vue des données cliniques disponibles de patients dont nous avions déjà les souches de trypanosomes (publication 97-1). Ces résultats m'ont incité à proposer un projet de recherche sur la pathogénicité des trypanosomes africains chez l'homme, projet qui est en cours de finalisation. Nous avons ainsi découvert le premier cas d'infection à *Trypanosoma congolense* chez l'homme (publication 98-2), alors que ce parasite est exclusivement animal. D'autres cas d'infections à *T. b. brucei* (trypanosomes d'intérêt vétérinaire responsable de la nagana) chez l'homme ont été aussi confirmés par mon laboratoire (publication 97-1). Selon l'importance de ces phénomènes dans les populations naturelles, les conséquences en matière d'épidémiologie de la THA et des stratégies de lutte pourraient être importantes. De plus, nous avons affiné grâce à la Polymerase Chain Reaction (PCR) l'identification des trypanosomes effectuée par isoenzymes (publication 99-5), et nous avons détecté chez un même malade plusieurs clones de trypanosomes de la sous-espèce *T. b. gambiense* (Oury *et al.*, en préparation). Les regroupements des parasites, par forme clinique, devraient nous permettre de mettre au point des techniques de détection spécifiques de chaque groupe défini sur ses caractéristiques pathologiques. Cette recherche constitue mon principal sujet de travail à l'heure actuelle.

Nous avons aussi évalué sur le terrain et perfectionné des tests de dépistage sérologique (publications 96-3, 99-4) et de diagnostic parasitologique : en particulier le CATT latex (Jamonneau *et al.*, soumis pour publication), le Quantitative Buffy Coat (publications 94-1, 98-3), la minicolonne ou mAECT (publication 98-1), la détermination du stade de la maladie par PCR dans le liquide céphalo-rachidien ou LCR et le suivi post-thérapeutique (publication 99-1). Ces résultats devraient permettre d'améliorer en partie la lutte contre la THA (publication 99-2).

Faire de la recherche sur la THA implique des études sur le trypanosome bien sûr, le réservoir animal, mais aussi sur le vecteur. Ainsi, j'ai aussi travaillé sur les glossines, par détection des trypanosomes par PCR (publication 96-2), et la mise au point de deux nouvelles techniques d'analyse des repas de sang (publications 97-3, 99-3). La connaissance des préférences trophiques des glossines, par des méthodes peu coûteuses et fiables, est importante pour une meilleure compréhension de la dynamique de transmission de la THA (publication 97-4).

J'ai initié un programme de tests *in vitro* d'extraits végétaux issus de la Pharmacopée traditionnelle, ayant un effet trypanocide, en collaboration avec la Faculté de Pharmacie d'Abidjan et celle de Montpellier (France). Malgré des résultats préliminaires encourageants, ces travaux n'ont pas été poursuivis car il fallait créer une nouvelle équipe travaillant uniquement sur ce sujet.

Actuellement, chargé de recherche contractuel (Montpellier, France)

Au sein du Département « Société et Santé » de l'IRD/ORSTOM, je suis responsable d'un projet de recherche sur l'évaluation et le perfectionnement des outils de surveillance et de dépistage de la THA, financé par le Ministère français des Affaires Etrangères, qui est réalisé en Afrique de l'Ouest et Centrale, en collaboration avec les Organisations Internationales africaines : l'OCCGE/IPR de Côte d'Ivoire, l'Organisation de Coordination pour la lutte contre les Endémies en Afrique Centrale (OCEAC) de Yaoundé, et les Programmes Nationaux de Côte d'Ivoire et de République Centrafricaine. Je suis aussi collaborateur pour un programme sur la détermination des zones à risque de transmission de THA selon les densités de campements en zone forestière de Côte d'Ivoire (financement Fonds d'Aide à la Coopération FAC/OMS).

Toujours pour améliorer les stratégies de lutte contre la THA, j'ai débuté un projet financé par l'OMS, le Ministère français des Affaires Etrangères et l'Université de Bâle (Suisse), sur la recherche des facteurs biologiques impliqués dans les cas d'échec au traitement par mélarsoprol en Angola. Ce programme est coordonné par l'Institut Suisse de Médecine tropicale à Bâle, et j'assure, comme collaborateur au projet, la caractérisation d'éventuelles souches résistantes en recherchant des marqueurs génétiques spécifiques de ces trypanosomes. Le mélarsoprol, un sel d'arsenic, ayant une très forte toxicité, est le médicament utilisé pour traiter les malades en phase d'atteinte nerveuse. Ces phénomènes de résistance sont inquiétants (environ 20 % des malades de la région M'Banza Congo dans le nord de l'Angola), et doivent, à mon avis, être étudiés en priorité.

2- La Coordination régionale d'un Programme International de Lutte

L'OMS a initié en 1995 un Programme International de Surveillance et de lutte contre la THA en Afrique. Si la coordination centrale est assurée par l'OMS/Division de lutte contre la THA à Genève, ce programme s'appuie sur deux organisations régionale africaines, l'OCCGE/IPR à Bouaké et l'OCEAC de Yaoundé.

Dans le cadre de ce programme, j'ai assumé de 1995 à 1998 les rôles de Coordonnateur Technique Régional et de responsable du Laboratoire de Référence pour le diagnostic et la sérologie de la THA en Afrique de l'Ouest.

J'étais alors impliqué dans des actions de lutte, sous forme d'appui technique, d'expertise ou de formation pour les 14 états concernés par ce programme.

3- Formation

A- Cours d'enseignement supérieur dispensés

- Advanced Methods in Medical and Veterinary Vector Control, Université de Bristol, Royaume-Uni (1991/92).

- Cours d'Isoenzymologie appliquée : applications à l'étude des maladies parasitaires humaines, Institut Pierre Richet, Bouaké, Côte d'Ivoire (1994).

- Cours de Protozoologie Médicale : Systématique et biologie moléculaire appliquées à l'étude de parasitoses humaines à transmission vectorielle. Diplôme d'Etudes Approfondies en Entomologie Médicale, Centre Universitaire de Formation en Entomologie Médicale et Vétérinaire, Bouaké, Côte d'Ivoire (1994).

B- Formation et encadrement d'étudiants et chercheurs

- Entre 1993 et 1998, j'ai participé à la formation de deux chercheurs de l'IPR en thèse de doctorat, et de plusieurs étudiants qui ont été accueillis au sein du laboratoire. Ces derniers ont intégré l'équipe « THA » et ils ont participé ainsi à un travail multidisciplinaire sur le diagnostic et l'épidémiologie, ainsi que sur l'identification génétique des trypanosomes.

1- Thèses de doctorat

- Un chercheur sénégalais : Papa Boubacar Diallo, dans le cadre de la préparation d'une thèse de troisième cycle (Université des Sciences d'Abidjan, Coccody, Côte d'Ivoire, Directeur de thèse : Pr Diopoh Kore Jacques), qu'il a passé avec succès et félicitations du jury.

J'ai initié ce chercheur à l'utilisation et l'évaluation des nouveaux outils de diagnostic, à l'isolement de terrain et la culture *in vitro* des trypanosomes, à l'électrophorèse d'isoenzymes grâce à laquelle nous avons mis au point une nouvelle technique d'analyse de repas de sang de glossines, et identifier de nouveaux génotypes virulents chez l'homme. La formation de ce chercheur ne s'est donc pas limitée à son travail de thèse, mais à de nombreux aspects de l'épidémiologie de la THA car il devait prendre ma succession à la direction du laboratoire après mon départ en 1998.

Titre de la thèse :

Utilisation des fractions antigéniques des trypanosomes pour le diagnostic de la Trypanosomiase Humaine Africaine (THA), 1993, Université d'Abidjan (Côte d'Ivoire).

- Un second chercheur sénégalais : Bocar Sané, dans le cadre de la préparation d'une thèse de troisième cycle (Faculté des Sciences d'Abidjan, Coccody, Côte d'Ivoire, Directeur de thèse : Pr Foua Bi Kouaho), qu'il a passé avec succès et félicitations du jury.

Avec cet étudiant, nous avons insisté sur l'aspect entomologique de la transmission de la THA. En l'initiant à la nouvelle technique d'analyse de repas de sang de glossines mise au point avec P.B. Diallo, et en évaluant ensemble cette technique sur le terrain, il a pu ensuite différencier les repas de sang « humains » des « non humains », permettant de calculer l'index de risque de contamination par la THA mis au point par Claude Laveissière (cf. Laveissière *et al.*, 1997).

Titre de la thèse :

Contribution à l'étude du rôle épidémiologique et au contrôle de *Glossina palpalis palpalis* (Robineau-Desvoidy, 1830) dans la région de Zoukougbeu, 1997 (Faculté des Sciences d'Abidjan, Côte d'Ivoire).

- Un étudiant français IRD/ORSTOM : Vincent Jamonneau. Ce stagiaire a été formé et encadré pour sa thèse de doctorat (Université de Montpellier II, Directeur de thèse Dr J.L. Frézil, Directeur de recherche IRD Montpellier, France) au sein du laboratoire. Vincent Jamonneau est actuellement en troisième année de thèse.

J'ai initié Vincent Jamonneau à plusieurs techniques d'isolement et de culture *in vitro* des trypanosomes, à l'épidémiologie de la THA (dépistage, diagnostic, pathogénicité, réservoir animal). Son travail comporte une composante de terrain importante, en particulier dans le cadre de sa thèse, par le suivi longitudinal avant et après traitement des malades. Nous avons ensemble étudié de nombreux cas atypiques de THA (forme aiguë, guérison spontanée), en collaboration avec les Universités de Limoges (Pr. Dumas), de Bordeaux (Pr. Vincendeau) et l'Institut de Médecine tropicale d'Anvers (Belgique : Pr. Büscher). Ce travail, corrélé à l'identification génétique des souches, constitue la partie principale de son travail de thèse.

Titre de la thèse :

Apport des outils biomoléculaires au diagnostic de la THA et à l'étude de la pathogénicité des trypanosomes africains chez l'homme.

- Une étudiante ivoirienne : Murielle Koffi, dans le cadre de la préparation d'une thèse de troisième cycle (Université des Sciences d'Abidjan, Coccody, Côte d'Ivoire, Directeur de thèse Pr. Yao N'Guessan Thomas). Pour des raisons familiales, cette étudiante a abandonné sa préparation de thèse après 18 mois passés au laboratoire.

Nous avons effectué des extractions aqueuses et alcooliques à partir de feuilles, de tiges et de racines de plantes connues pour leurs effets trypanocides (selon les tradipraticiens comme *Detarium microcarpum* ou *Fagara xantixyloides*), puis des tests de sensibilité *in vitro* sur des souches de *T. b. gambiense* en dilutions décroissantes. Malgré des résultats encourageants, ces travaux n'ont pu être poursuivis pour la raison évoquée dans le précédent paragraphe.

Titre de la thèse :

Rôle des extraits végétaux issus de la Médecine traditionnelle Africaine dans le traitement de la Trypanosomiase Humaine Africaine.

- Deux étudiants ivoiriens (Mrs Aguido Apende Abraham et Konan Yao Jacques) de la Faculté de Pharmacie d'Abidjan (responsables de thèse : Dr Jean Frédéric Brunel et Pr. Djénéba Koné) ont

effectué des stages de formation à la culture *in vitro* des trypanosomes et à l'action trypanocide *in vitro* d'extraits végétaux issus de la Pharmacopée traditionnelle africaine. Ils ont ensuite soutenu des thèses d'exercice, essentiellement bibliographiques sur la Pharmacopée traditionnelle africaine dont les titres et exemplaires ne m'ont jamais été communiqués.

2- Diplôme d'Etudes Approfondies (DEA)

- Un étudiant français IRD/ORSTOM: Vincent Jamonneau. Ce stagiaire a été formé et encadré pour son stage de DEA de Parasitologie (Université de Montpellier II, responsable Dr J.L. Frézil, Directeur de recherche IRD Montpellier, France) au sein du laboratoire.

J'ai initié Vincent Jamonneau à de nombreuses techniques de dépistage et de diagnostic de la THA. Son travail était d'améliorer le diagnostic sérologique de la THA sur le terrain.

Titre du DEA de Parasitologie :

Évaluation préliminaire d'une nouvelle technique de dépistage de la Trypanosomiase Humaine Africaine en Côte d'Ivoire : le TestrypCATT sur Latex, 1996 (Université Montpellier II).

3- Maîtrise

Entre 1994 et 1998, j'ai participé à la formation de trois étudiants soit à Bouaké, soit en stage au centre ORSTOM de Montpellier. Ces trois étudiants se sont familiarisés à la culture *in vitro* des trypanosomes, et à leurs identifications génétiques par isoenzymes et PCR/RAPD.

Maîtrise de Biologie des populations et des Ecosystèmes, option Parasitologie (Université d'Orléans, Vincent Jamonneau, 1994, responsable de stage Pr. Kuentin) : Culture *in vitro* et variabilité génétique de *Trypanosoma brucei gambiense*.

Maîtrise de Biologie des populations et des Ecosystèmes, option Parasitologie (Université de Montpellier II, David Courtin, 1997/98, responsable de stage Dr Bruno Oury): Diversité génétique de *Trypanosoma brucei gambiense*, agent responsable de la maladie du sommeil chez l'homme en Côte d'Ivoire.

Maîtrise de Biologie cellulaire et physiologie, mention Génétique moléculaire (Université de Rennes I : Edgar Brice Ngoungou, 1997/98, responsable de stage Dr Bruno Oury):

Caractérisation génétique de *Trypanosoma brucei gambiense*, agent responsable de la maladie du sommeil chez l'homme en Côte d'Ivoire.

C- Formation et encadrement de professionnels pour la lutte contre la THA

Le laboratoire de l'IPR est toujours le laboratoire de référence pour l'Afrique de l'Ouest dans le cadre du Programme International de Surveillance et de lutte contre la THA, sous le contrôle de l'OMS. Il a été complètement rénové et équipé. Il est opérationnel pour :

- La culture *in vitro* ;
- La fabrication des milieux de culture ;
- L'identification biomoléculaire des trypanosomes (isoenzymes et PCR) ;
- La fabrication des tests mAECT pour le diagnostic de la THA ;
- Le diagnostic de laboratoire et de terrain de la THA ;
- L'analyse des repas de sang de glossines ;
- La saisie et gestions des données par informatique ;
- La cryobanque de référence de stocks de trypanosomes humains et animaux.

J'ai contribué à la formation du personnel du laboratoire de l'IPR que j'ai dirigé pendant 6 ans. Les principaux résultats sont :

- Mon successeur, chercheur africain senior, Papa Boubacar Diallo, a obtenu une source de financement pour sa recherche personnelle, a publié plusieurs articles scientifiques et a participé à de nombreuses présentations dans des séminaires ou congrès internationaux ;

- L'ingénieur de recherche africain, Paul N'Guessan, maîtrise parfaitement toutes les techniques nécessaires au fonctionnement du laboratoire. Cet ingénieur a pu suivre en particulier une formation de deux mois à l'Institut de Médecine Tropicale d'Anvers en Belgique, qui lui a permis de se perfectionner en lui donnant de nouveaux atouts pour l'évolution de sa carrière scientifique ;

- Le technicien de laboratoire, Louis N'Dri, a atteint un bon niveau scientifique et technique et peut aider l'ingénieur précité dans le fonctionnement du laboratoire ;

- L'auxiliaire de laboratoire, Alain N'Goran, formé aux différentes techniques de base, est chargé en particulier de la production des tests mAECT et de la supervision de l'animalerie.

J'ai participé à la formation des responsables des programmes nationaux de lutte contre la THA d'Afrique de l'Ouest aux techniques de surveillance et de dépistage de la THA. J'ai participé aussi à la formation des responsables des PNLT « Afrique Centrale » à l'OCEAC de Yaoundé (Cameroun).

4- Participation à des Comités Internationaux et Expertises

Dans le cadre des actions de lutte contre la THA, l'OMS/Division de lutte contre les Maladies Tropicales m'a demandé d'être membre de leur comité de conseil technique (TAG), et conseiller temporaire du comité de coordination pour la lutte contre les trypanosomiases humaines et animales (PAAT). L'OMS m'a chargé aussi d'effectuer, en collaboration avec eux, l'évaluation des PNLT du Togo et de la Guinée.

5- Collaborations Scientifiques et Partenariat

La recherche ne peut s'effectuer que dans le cadre de collaborations scientifiques pluridisciplinaires, et en partenariat avec divers organismes, universités et instituts.

Étant étudiant, j'ai collaboré avec le PNLT du Congo, l'Institut de Médecine Tropicale d'Anvers, le Tsetse Research Laboratory de Bristol, différentes équipes de l'IRD/ORSTOM au Congo et en France.

Durant mon contrat en Angleterre, j'ai continué à collaborer avec l'IRD/ORSTOM, que ce soit à l'OCCGE, à Brazzaville ou à Montpellier. Les PNLT de Côte d'Ivoire et du Congo ont été d'excellents partenaires.

Pendant mon affectation en Côte d'Ivoire, j'ai pu diversifier les collaborations selon les programmes de recherche suivis. L'IRD/ORSTOM a été un partenaire fiable, les Services Vétérinaires ivoiriens et la WWF ont permis la réalisation des recherches sur le réservoir animal, le PNLT de Côte d'Ivoire a permis la réalisation du projet sur la pathogénicité des trypanosomes, l'Université de Bristol et celle d'Alabama (USA) ont facilité mes travaux sur les glossines, que ce soit pour la détection des trypanosomes ou l'analyse des repas de sang.

Les Universités de Limoges et de Bordeaux sont d'excellents collaborateurs, en particulier dans le cadre du projet sur le pathogénicité des trypanosomes.

L'OMS s'est avérée un excellent collaborateur et partenaire, pour ma formation sur la THA et le paludisme, ainsi que les actions de coordination du Programme International de lutte contre la THA. J'ai ainsi participé à l'élaboration d'un Compact Disk sur la THA, conçu par l'OMS à Genève.

Enfin, la Coopération française s'est avérée un employeur partenaire et un bailleur de fonds très méritant pour mes recherches sur la THA.

6- Conclusion et perspectives

Ma formation à l'IRD/ORSTOM a contribué à l'obtention de mon premier poste de chargé de recherche à Bristol. Les deux années passées en Angleterre ont été bénéfiques sur le plan de la recherche, de la formation et de la collaboration scientifique.

Durant les 6 années passées en Côte d'Ivoire, j'ai poursuivi des recherches opérationnelles, dans le but d'élucider certaines questions posées en matière d'épidémiologie de la Trypanosomiase Humaine Africaine afin d'optimiser les méthodes de lutte contre cette maladie. Les résultats obtenus m'incitent à poursuivre ce travail, en souhaitant pouvoir encadrer de jeunes chercheurs.

En ce sens, trois projets de recherche pour lesquels je suis responsable ou collaborateur sont en cours :

- L'évaluation et l'amélioration des méthodes de dépistage et de diagnostic en Afrique Centrale et Occidentale (financement Fonds d'Aide à la Coopération FAC/OMS) ;
- Une étude sur les facteurs biologiques impliqués dans les cas d'échec du traitement par mélarsoprol en Angola (financement FAC/OMS/STI, sous la responsabilité du Swiss Tropical Institut).;
- La détermination des zones à risques de transmission de THA selon les densités de campements : zone forestière de Côte d'Ivoire (financement Fonds d'Aide à la Coopération FAC/OMS).

Enfin, il semble utile d'effectuer des études sur la réelle pathogénicité des trypanosomes en Afrique Centrale, sur le schéma suivi en Côte d'Ivoire, afin d'apprécier l'importance des infections transitoires non pathogènes et l'éventualité d'une forme aiguë de la maladie dans cette autre zone géographique durement touchée par la THA.

7- Liste des publications

99-1 **Truc P.**, Jamonneau V., Cuny G. & Frézil J.L.. Polymerase Chain Reaction for Human African Trypanosomiasis stage determination and follow-up. *Bulletin of the World Health Organization,* sous presse.

99-2 Gibson W. C., Stevens J. & **Truc P.** (1999). Identification of trypanosomes: from morphology to molecular biology .: "*Progress in Human African Trypanosomiasis sleeping sickness*", Springer Publ., Paris, 7-29.

99-3 Boakye D., Tang J., **Truc P.**, Merriweather A. & Unnasch T. (1999). Identification of blood meals in hematophagous Diptera by polymerase chain reaction and heteroduplex analysis. *Medical and Veterinary Entomology*, sous presse.

99-4 Diallo P. B., **Truc P.,** Méda H. A. & Kamenan A. (1999). Diagnostic sérologique de la Trypanosomiase Humaine Africaine à *Trypanosoma brucei gambiense*. 2- Obtention et utilisation des glycoprotéines de surface des trypanosomes pour améliorer la spécificité et la sensibilité des techniques ELISA et THP. *Bulletin de la Société de Pathologie Exotique*, sous presse.

99-5 Biteau N., Bringaud F., Gibson W.C., **Truc P.** & Baltz T.. Characterization of *Trypanozoon* isolates using genetic micro- and minisatellite markers. *Molecular and Biochemical Parasitology*, sous presse.

98-1 **Truc P.**, Jamonneau V., N'Guessan P., Diallo P.B. & Bustigier X. (1998). Simplification of the mini-anion exchange technique for the parasitological diagnosis of Human African Trypanosomiasis. *Transactions of the Royal Society of Tropical Medicine and Hygiene*, 92, 5, 512.

98-2 **Truc P.**, Jamonneau V., N'Guessan P., N'Dri L., Diallo P. B. & Cuny G. (1998). *Trypanosoma brucei* spp. and *Trypanosoma congolense* : mixed human infection in Côte d'Ivoire. *Transactions of the Royal Society of Tropical Medicine and Hygiene*, 92, 5, 537-538.

98-3 **Truc P.**, Jamonneau V., N'Guessan P., Diallo P.B. & Garcia A. (1998). Parasitological diagnosis of human African trypanosomiasis: a comparison of the QBC® and the miniature anion-exchange centrifugation technique. *Transactions of the Royal Society of Tropical Medicine and Hygiene*, 92, 288-289.

97-1 **Truc P.**, Formenty P., Diallo P. B., Komoin-Oka C.& Lauginie F. (1997) Confirmation of two distinct classes of zymodemes of *Trypanosoma brucei* infecting patients and wild mammals in Côte d'Ivoire: suspected difference in pathogenicity. *Annals of Tropical Medicine and Parasitology*, 91, 8, 951-956.

97-2 **Truc P.**, Formenty P., Duvallet G., Komoin-Oka C., Diallo P. B. & Lauginie F. (1997). Identification of trypanosomes isolated by KIVI from wild mammals in Côte d'Ivoire : diagnostic, taxonomic and epidemiological considerations. *Acta Tropica*, 67, 187-196.

97-3 Diallo P. B., **Truc P.** & Laveissière C. (1997). A new method for identifying blood meals of human origin in tsetse flies. *Acta Tropica*, 63, 61-64.

97-4 Laveissière C., Sané B., Diallo P. B. & **Truc P.** (1997). Le risque épidémiologique dans un foyer de maladie du sommeil en Côte d'Ivoire.*Tropical Medicine and International Health*, 2, 8, 729-732.

96-1 **Truc P.**. A miniature kit for the *in vitro* isolation of *Trypanosoma brucei gambiense* : a preliminary field assessment on sleeping sickness patients in Côte d'Ivoire (1996). *Transactions of the Royal Society of Tropical Medicine and Hygiene*, 90, 246-247.

96-2 Masiga D. K., McNamara J., Laveissière C., **Truc P.** & Gibson W.C. (1996). A high prevalence of mixed trypanosome infections in tsetse flies in Sinfra, Côte d'Ivoire, detected by DNA amplification. *Parasitology*, 112, 75-80.

96-3 Diallo P. B., **Truc P.**, Méda H. A. & Kamenan A. (1996). Diagnostic sérologique de la Trypanosomiase Humaine Africaine à *Trypanosoma brucei gambiense*. 1- Obtention et utilisation d'antigènes bruts dans les tests ELISA et d'agglutination au latex. *Bulletin de la Société de Pathologie Exotique*, 89, 262-268.

94-1 **Truc P.**, Bailey W., Doua F., Laveissière C. & Godfrey D. G. (1994). A comparison of parasitological methods for the diagnosis of gambian trypanosomiasis in an area of low endemicity in Côte d'Ivoire. *Transactions of the Royal Society of Tropical Medicine and Hygiene*, 88, 419-421.

94-2 Komoin-Oka, C., **Truc P.**, Bengaly, Z., Formenty, P., Duvallet, G., Lauginié, F., Raath, J. P., N'Depo, A. E. & Leforban, Y. (1994). Etude de la prévalence des infections à trypanosomes chez différentes espèces d'animaux sauvages du parc national de la Comoé en Côte

d'Ivoire : résultats préliminaires sur la comparaison de trois méthodes de diagnostic. *Revue Elevage et Médecine vétérinaire des Pays tropicaux*, 47, 2, 189-194.

94-3 Gashumba J., Komba E. K., **Truc P.**, Allingham R., Ferris V. & Godfrey D. G. (1994). The persistence of genetic homogeneity among *Trypanosoma brucei rhodesiense* isolates from patients in north-west Tanzania. *Acta Tropica*, 56, 341-348.

93-1 Ben Abderrazak, S., Guerrini, F., Mathieu Daudé, **Truc P.**, Neubauer, K., Lewicka, K., Barnabé, C. & Tibayrenc, M., (1993). Isoenzyme electrophoresis for Parasite characterization. in: *Protocols in Molecular Parasitology, The Humana Press*, 21, 27, 361- 382.

93-2 Authie, E., Cuisance, D., Force-Barge, P., Frézil, J. L., Gouteux, J. P., Jannin, J., Lancien, J., Laveissière, C., Lemesre, J. L., Mathieu-Daudé, F., Nitcheman, S., Noireau, F., Penchenier, L., Tibayrenc, M. & **Truc P**. (1993). Some new prospects in epidemiology and fight against human African trypanosomiasis. *Research and Reviews in Parasitology*, 51 (1-4): 29-46.

93-4 **Truc P**. & Tibayrenc, M. (1993) Population genetics of *Trypanosoma brucei* in Central Africa: taxonomic and epidemiological significance. *Parasitology*, 106, 137-149.

92-1 **Truc P.,** Aerts D., McNamara J. J., Claes Y., Allingham R., Le Ray D. & Godfrey D.(1992). The direct *in vitro* isolation of *Trypanosoma brucei* from man and animals, and its potential value for the diagnosis of Gambian trypanosomiasis. *Transactions of the Royal Society of Tropical Medicine and Hygiene*, 86, 627-629.

92-2 Aerts, D., **Truc P.**, Penchenier, L., Claes Y. & Le Ray, D., (1992). A kit for *in vitro* isolation of trypanosomes in the field: First trial with sleeping sickness patients in the Congo. *Transactions of the Royal Society of Tropical Medicine and Hygiene*, 86, 394-395.

91-1 **Truc P.**, Mathieu Daudé, F. & Tibayrenc, M., (1991). Multilocus isoenzyme identification of *Trypanosoma brucei* stocks isolated in Central Africa : evidence for an animal reservoir of sleeping sickness in Congo. *Acta Tropica*, 49, 127-135.

91-2 **Truc P.**, Rioux, J. A. & Pratlong, F., (1991). A propos du trypanosome du Blaireau : *Trypanosoma pestanai* Bettencourt et Franca, 1905 est-il seul en cause. *Annales de Parasitologie Humaine et Comparée*, 66, n°1, 45-46.

90-1 **Truc P.**, Mathieu Daudé, F. & Tibayrenc, M, (1990). Etude isoenzymatique de *Trypanosoma brucei* en Afrique Centrale : corollaires épidémiologiques. *Bulletin de la Société Française de Parasitologie*, 8, p. 257.

90-2 Mathieu Daudé, F., **Truc P**. & Tibayrenc, M., (1990). Diversité génétique comparée des taxons *Trypanosoma brucei* s. l. et *Trypanosoma cruzi*. *Bulletin de la Société Française de Parasitologie* , 8, p. 247.

Publication soumise

Jamonneau V., **Truc P**., Büscher P. & Magnus E.. Serodiagnosis of Human African Trypanosomiasis : preliminary evaluation of Latex/*T. b. gambiense* and alternatives versions of CATT/*T. b. gambiense* for mass screening of the population at risk in Côte d'Ivoire. *Acta Tropica*, submitted for publication.

Publication en préparation

Oury B., Jamonneau V., Tibayrenc M. & **Truc P**. Detection *of Trypanosoma brucei gambiense* multiple infections by mutiprimers RAPD fingerprinting in patients in Côte d'Ivoire.

8- Communications à des Séminaires ou Congrès Internationaux

99-1 **Truc P.**, Jamonneau V., Tibayrenc M. & Oury B. Molecular epidemiology of Human African Trypanosomiasis. 4th International workshop on Molecular Epidemiology and Evolutionary Genetics of Pathogenic Microorganisms. CDC/CNRS/ORSTOM, Dakar, Sénégal (juin 1999).

99-2 Biteau N., Bringaud F., Gibson W.C., **Truc P**. & Baltz T. Caractérisation des isolats de trypanosomes à l'aide de marqueurs de polymorphisme. IIème Réunion Biennale de Parasitologie CNRS/DGA/DCSSA/MENESR, Cibles thérapeutiques et vaccinales en parasitologie: paludisme, leishmanioses, trypanosomes, schistosomiase, Montpellier, France (février 1999).

98-1 **Truc P.**, Jamonneau V. & Oury B. Genetic and Pathogenesis of African trypanosomes in man. International Colloquium « Sleeping sickness rediscovered », Anvers, Belgique (décembre 1998).

98-2 **Truc P.**, Merriweather A., Diallo B.P., Jamonneau V. & Unnasch T. R.. Molecular biology: a new tool for identifying blood meals in tsetse flies, vector of sleeping sickness in Africa. IX International Congress of Parasitology (ICOPA), Makuhari Chiba, Japon (août 1998).

98-3 **Truc P.**, Jamonneau V., N'Guessan P., N'Dri L. & Diallo P.B.. Pathogenicity of African trypanosomes: a new approach of the epidemiology of gambian sleeping sickness. IX International Congress of Parasitology (ICOPA), Makuhari Chiba, Japon (août 1998).

98-4 **Truc P.,** Jamonneau V., N'Guessan P., N'Dri L., Diallo P.B & Garcia A.. *Trypanosoma brucei gambiense* is not the only agent of the Human African Trypanosomiasis in West Africa. *Third International workshop on Molecular Epidemiology and Evolutionary Genetics of Pathogenic Microorganisms.* CDC/CNRS/ORSTOM, Rio de Janeiro, Brézil (juin 1998).

97-1 **Truc P.,** Merriweather A., Diallo P.B. & Unnasch T.R.. Isoenzyme and PCR for identifying blood meals in tsetse flies. *Proceedings of the 24rd ISCTRC Meeting (International Scientific Council for Trypanosomiasis Research and Control)*, Maputo, Mozambique, septembre 1997), 119, 166-169.

97-2 **Truc P.**, Diallo P.B. & Jamonneau V.. Identification of different classes of zymodemes of *Trypanosoma brucei* s. p. circulating in patients and wild mammals in West Africa. *International workshop on Molecular Epidemiology and Evolutionary Genetics of Pathogenic Microorganisms.* CDC/CNRS/ORSTOM, ORSTOM Montpellier, France (mai 1997).

97-3 **Truc P.**, Diallo P.B. & Jamonneau V.. Circulation chez l'homme, la glossine et le porc d'un même zymodème non gambiense groupe 1 en Côte d'Ivoire: conséquences en matière de contrôle de la THA. XVIIIème Conférence Technique de l'OCEAC, Yaoundé, Cameroun (Mai 1997).

96-1 Baker J.R. & **Truc P.**. Human African Trypanosomiasis: towards an epidemic pattern in Africa. *International workshop on Molecular Epidemiology and Evolutionary Genetics of Pathogenic Microorganisms.* Center for Disease Control and prevention, Atlanta, USA (juin 1996).

95-1 **Truc P.** , Diallo P. B. & Godfrey D.G.. Genetic identification and pathogenicity of *Trypanosoma brucei* s. l. in Man: an acute form of HAT is suspected in Côte d'Ivoire. *Proceedings of the 23rd ISCTRC Meeting (International Scientific Council for Trypanosomiasis Research and Control)*, Banjul, Gambia, 118, 128 (1995).

95-2 **Truc P.**, Diallo P. B., N'Guessan P. & Le Ray D..The kit for *in vitro* isolation of african trypanosomes (KIVI): a new simple procedure for a field use. *Proceedings of the 23rd ISCTRC Meeting (International Scientific Council for Trypanosomiasis Research and Control)*, Banjul, Gambia, 118, 143 (1995).

95-3 **Truc P.**. Genetic and Trypanosomiasis: epidemiological consequences. Malaria Division, OMS/WHO Center, Faculty of Sciences, Chulalongkorn University, Bangkok, Thailande (août 1995).

93-1 **Truc P.**, Formenty P., Godfrey D.G, Le Ray D.. The kit for *in vitro* isolation of African trypanosomes: diagnosis and new prevalence of human trypanosomiasis *Proceedings of the 22rd ISCTRC Meeting (International Scientific Council for Trypanosomiasis Research and Control)*, Kampala, Uganda, 117, 42 (1993).

92-1 **Truc P.**. Populations Genetics of *Trypanosoma brucei* and epidemiology of sleeping sickness in the Congo Republic, Université de Californie, Irvine, USA (avril 1992).

91-1 **Truc P.**. Isoenzyme characterization of *Trypanosoma brucei* stocks isolated from Congo and Zaire: epidemiological significance. *Proceedings of the 21rd ISCTRC Meeting (International Scientific Council for Trypanosomiasis Research and Control)*, Yammoussoukro, Côte d'Ivoire, 116, 248 (1991).

UNIVERSITE MONTPELLIER II
Université des Sciences et Techniques du Languedoc

Curriculum vitae

HABILITATION A DIRIGER DES RECHERCHES

présentée et soutenue le 9 décembre 1999

par

Philippe Truc

Membres du Jury :

Professeur André Raibaut
Professeur Michel Dumas
Professeur Philippe Desjeux
Professeur Philippe Vincendeau
Docteur Jean Louis Frézil

28/10/99

CURRICULUM VITAE

Truc
Philippe
né le 16 avril 1961, Béchar (Algérie)
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Nationalité: Française

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Situation militaire: Dégagé des obligations militaires

Etudes suivies

1980: Baccalauréat série D (Montpellier).
1981: Diplôme d'Etudes Universitaires Générales, 1ère année, Sciences de la Nature et de la Vie (Université de Perpignan).
1982: Diplôme d'Etudes Universitaires Générales, 2ème année, Sciences de la Nature et de la Vie (Université de Perpignan).
1984: Licence ès Sciences Naturelles (Université de Montpellier II).
1985: Maîtrise ès Sciences Naturelles (Université de Montpellier II).
1986: Certificat de Parasitologie et de Pathologie Parasitaire (Université de Montpellier II).
1987: Diplôme d'Etudes Approfondies en Parasitologie, Ecologie, Pathologie (Université de Montpellier II).
1991: Doctorat de l'Université de Montpellier II, spécialité "Physiologie, Biologie des Organismes et des Populations"; mention Parasitologie.

Langues:

Anglais: écrit et parlé couramment.
Espagnol: niveau élémentaire.

EXPERIENCE PROFESSIONNELLE

Octobre 98 à ce jour: Chargé de Recherche contractuel (CDD), Institut de Recherche pour le Développement (ex ORSTOM), Société et Santé, Montpellier, France.

Janvier 1993 au 31 Août 1998: Chargé de Recherche (assistant technique contractuel) au titre de la Coopération Française au sein de l'OCCGE (Organisation de Coordination et de Coopération pour la lutte contre les Grandes Endémies). Responsable du Laboratoire de Biologie des Parasites et Vecteurs à l'Institut Pierre Richet, Bouaké, Côte d'Ivoire.

Juillet 1995: stage de 3 semaines au Centre OMS/Malaria à Bangkok, Thaïlande (responsable: Pr S. Thaithong). Sujet: Isolement, culture et identification biomoléculaire de *Plasmodium falciparum*, agent responsable du Paludisme sévère. Tests de sensibilité *in vitro* aux molécules antipaludiques des agents pathogènes.

Janvier 1991 à décembre 1992: Chargé de Recherche au sein du Medical Research Council, Tsetse Research Laboratory, University of Bristol, Department of Veterinary Medicine, Langford House, Langford, Bristol, BS 18 7DU, U.K.

Janvier 1990 à Juin 1990: Allocataire de recherche ORSTOM: affectation Montpellier.

Avril 1990: Stage d'une semaine au Tsetse Research Laboratory (University of Bristol, Department of Veterinary Medicine, U.K.. Responsable: Dr D. G. Godrey). Sujet: Initiation à la culture des trypanosomes africains sur mouches vectrices de la maladie du sommeil (Dr P. Dukes), et aux techniques de biologie moléculaire pour l'étude génétique des trypanosomes (Dr W. C. Gibson).

Mars 1989 à Décembre 1989: Allocataire de recherche ORSTOM: affectation au centre ORSTOM de Brazzaville (République du Congo), dans le cadre de la préparation de la thèse de doctorat. Plusieurs missions effectuées sur le terrain.

Août 1988: Stage d'une semaine à l'Institut de Médecine Tropicale d'Anvers (Belgique: Laboratoire de Protozoologie, Pr D. Le Ray). Sujet: Initiation aux techniques de culture *in vitro* des trypanosomes africains.

Mai 1988 à Février 1989: Allocataire de recherche ORSTOM.

Février 1988 à Mai 1988: Stage à l'ORSTOM (Laboratoire de Génétique des Parasites et des Vecteurs) dans le cadre de la thèse de doctorat.

Janvier 1987 à Septembre 1987: Stage pratique de DEA dans le laboratoire d'Ecologie Médicale (Faculté de Médecine de Montpellier: Professeur J.A. Rioux). Titre du rapport de stage: "Morphométrie et électrophorèse de quelques *Trypanosoma* et *Sauroleishmania*".

Septembre 1981 à Mai 1988 : Surveillant d'externat à plein temps, Ministère de l'Education Nationale (Collège "Les Deux Pins" Frontignan, Lycée Clémenceau Montpellier, Collège "Les Garrigues" Montpellier, Lycée d'Enseignement Professionnel "Charles Blanc" Perpignan).

Implications dans des programmes de Recherche

- Etude de la technique du microCATT dans 4 foyers de Trypanosomiase Humaine en Afrique de l'Ouest et en Afrique Centrale. Financement "Fonds d'Aide à la Coopération", Ministère Français des Affaires Etrangères, Coopération et Développement, France, 1998/99 (responsable de programme).

- Détermination des zones à risques de transmission de la Trypanosomiase Humaine Africaine (THA) en Côte d'Ivoire. Financement "Fonds d'Aide à la Coopération", Ministère Français des Affaires Etrangères, Coopération et Développement, France, 1998/99 (responsable de programme).

- Investigations on unsual Sleeping Sickness Cases Refractory to treatment with Melarsoprol. Pharmacological, immunological and biological investigations. Financement FAC/OMS/STI, sous la responsabilité du Swiss Tropical Institut, Angola, 1998/99 (collaborateur).

- Pathogénicité et variants génétiques de *Trypanosoma brucei* chez l'Homme en Côte d'Ivoire: conséquences épidémiologiques. Financement "Fonds d'Aide à la Coopération", Ministère Français des Affaires Etrangères, Coopération et Développement, France, 1996/1998 (responsable de programme).

- Etude longitudinale de sujets séropositifs au CATT sans confirmation parasitologique: signification, stabilité de la séropositivité dans le temps et recherche de concentration familiale de sujets séropositifs. "Fonds d'Aide à la Coopération", Ministère Français des Affaires Etrangères, Coopération et Développement, France, 1996/1998 (collaborateur).

- Détermination et prédiction de l'émergence des zones à risques de maladie du sommeil. De la télédétection à l'action. Financement "Organisation Mondiale de la Santé, Recherches sur les Maladies Tropicales", Genève, Suisse, 1996/97 (collaborateur).

- Dispersion de *Glossina palpalis palpalis* et dissémination du trypanosome dans le foyer de maladie du sommeil de Zoukougbeu (Côte d'Ivoire). Financement "ORSTOM", Paris, 1995/96 (collaborateur).

- Etude de la circulation des trypanosomes, agents de la maladie du sommeil, par marqueurs génétiques, entre les différents foyers de Côte d'Ivoire: conséquences en matière de contrôle et de lutte. Financement "ORSTOM", Paris, 1995/96 (collaborateur).

- Installation d'un Laboratoire d'Isoenzymologie et de biologie moléculaire à l'Institut Pierre Richet: appui aux programmes de Recherche et de Lutte contre la Maladie du Sommeil en Côte d'Ivoire. Financement "Fonds d'Aide à la Coopération", Ministère Français de la Coopération et du Développement, France, 1993/1994 (responsable de programme).

- Mise au point, perfectionnement et évaluation d'une nouvelle technique d'isolement *in vitro* des trypanosomes africains au Congo et en Côte d'Ivoire: Financements "Medical Research Council", Royaume Uni (collaborateur), "Organisation Mondiale de la Santé, Recherches sur les Maladies Tropicales", Genève, Suisse, 1992/1994 (responsable de programme).

- Génétique et Epidémiologie de la Trypanosomiase Humaine ou Maladie du Sommeil en Afrique de l'Est et de l'Ouest: financements "Medical Research Council", Royaume Uni (collaborateur), "Organisation Mondiale de la Santé, Recherches sur les Maladies Tropicales", Genève, Suisse, 1992/1993 (collaborateur).

- Génétique de la Trypanosomiase Humaine ou Maladie du Sommeil en Afrique Centrale: financement "Fondation pour la Recherche Médicale", "Région Languedoc-Roussillon", France, 1989/1991 (collaborateur).

Chargé de Cours d'Enseignement Supérieur

- Advanced Methods in Medical and Veterinary Vector Control, Université de Bristol, Royaume Uni (1991/1992).

- Cours d'Isoenzymologie appliquée: applications à l'étude des maladies parasitaires humaines, Institut Pierre Richet, Bouaké, Côte d'Ivoire (1994).

- Cours de Protozoologie Médicale: Systématique et biologie moléculaire appliquées à l'étude de parasitoses humaines à transmission vectorielle. Diplôme d'Etudes Approfondies en Entomologie Médicale, Centre Universitaire de Formation en Entomologie Médicale et Vétérinaire, Bouaké, Côte d'Ivoire (1994).

Présentations à des séminaires ou Congrès Internationaux

- Some trypanosomes of the world: from morphology to molecular biology. Segundo Simposio sobre Trypanosomes del Nuevo Mundo y otros Hemoparasitos (SSNWTH'99). Universidad Romulo Gallegos, San Juan de Los Morros, Guarico, Venezuela, (octobre 1999).

- Isolation and diagnosis of trypanosomes in the field : lacks and needs. . Segungo Simposio sobre Trypanosomes del Nuevo Mundo y otros Hemoparasitos (SSNWTH'99). Universidad Romulo Gallegos, San Juan de Los Morros, Guarico, Venezuela, (octobre 1999).

- Simple and multiple infections with *Trypanosoma brucei gambiense* and pathogencity of Human African Trypanosomiasis in patients in Côte d'Ivoire. 25th International Scientific Council for Trypanosomiasis Research and Control, Mombasa, Kenya (octobre 1999).

- Further improvment of the Latex/IgM assay for cerebrospinal fluid of sleeping sickness patients. 25th International Scientific Council for Trypanosomiasis Research and Control, Mombasa, Kenya (octobre 1999).

- Molecular epidemiology of Human African Trypanosomiasis. 4th International workshop on Molecular Epidemiology and Evolutionary Genetics of Pathogenic Microorganisms. CDC/CNRS/ORSTOM, Dakar, Sénégal (juin 1999).

- Caractérisation des isolats de trypanosomes à l'aide de marqueurs de polymorphisme. IIème Réunion Biennale de Parasitologie CNRS/DGA/DCSSA/MENESR, Cibles thérapeutiques et vaccinales en parasitologie: paludisme, leishmanioses, trypanosomes, schistosomiase, Montpellier, France (février 1999).

- Genetic and Pathogenesis of African trypanosomes in man. International Colloquium « Sleeping sickness rediscovered », Anvers, Belgique (décembre 1998).

- Molecular biology: a new tool for identifying blood meals in tsetse flies, vector of sleeping sickness in Africa. IX Congrés International de Parasitologie (ICOPA), Makuhari Chiba, Japon (août 1998).

- Pathogenicity of African trypanosomes: a new approach of the epidemiology of gambian sleeping sickness. IX Congrés International de Parasitologie (ICOPA), Makuhari Chiba, Japon (août 1998).

- *Trypanosoma brucei gambiense* is not the only agent of the Human African Trypanosomiasis in West Africa. *Third International workshop on Molecular Epidemiology and Evolutionary Genetics of Pathogenic Microorganisms.* CDC/CNRS/ORSTOM, Rio de Janeiro, Brézil (juin 1998).

- Isoenzyme and PCR for identifying blood meals in tsetse flies. *24ème Réunion de l'ISCTRC (International Scientific Council for Trypanosomiasis Research and Control,* Maputo, Mozambique, septembre 1997).

- Identification of different classes of zymodemes of *Trypanosoma brucei* s. p. circulating in patients and wild mammals in West Africa. *International workshop on Molecular Epidemiology and Evolutionary Genetics of Pathogenic Microorganisms.* CDC/CNRS/ORSTOM, ORSTOM Montpellier, France (mai 1997).

- Circulation chez l'homme, la glossine et le porc d'un même zymodème non gambiense groupe 1 en Côte d'Ivoire: conséquences en matière de contrôle de la THA. XVIIIème Conférence Technique de l'OCEAC, Yaoundé, Cameroun (Mai 1997).

- Situation actuelle de la Trypanosomiase Humaine en Afrique: Emergence d'une situation épidémique. Présentation effectuée avec J. R. Baker (UK). *International workshop on Molecular Epidemiology and Evolutionary Genetics of Pathogenic Microorganisms.* Center for Disease Control and prevention, Atlanta, USA (juin 1996).

- The kit for *in vitro* isolation of African trypanosomes (KIVI): a new simple procedure for a field use. *23ème Réunion de l'ISCTRC (International Scientific Council for Trypanosomiasis Research and Contro*, Banjul, Gambie, septembre 1995).

- Genetic identification and pathogenicity of *Trypanosoma brucei* s. l. in Man: An acute form of HAT is suspected in Côte d'Ivoire. *23ème Réunion de l'ISCTRC (International Scientific Council for Trypanosomiasis Research and Control,* Banjul, Gambie, septembre 1995).

- Genetic and Trypanosomiasis: epidemiological consequences. Malaria Division, OMS/WHO Center, Faculty of Sciences, Chulalongkorn University, Bangkok, Thailande (août 1995).

- *In vitro* isolation of African trypanosomes and diagnostic of human trypanosomiasis. *22ème Réunion de l'ISCTRC (International Scientific Council for Trypanosomiasis Research and Control,* Kampala, Ouganda, octobre 1993).

- Populations Genetics of *Trypanosoma brucei* and epidemiology of sleeping sickness in the Congo Republic, Université de Californie, Irvine, USA (avril 1992).

- Isoenzyme characterization of *Trypanosoma brucei* stocks isolated from Congo and Zaire: epidemiological significance. *21ème Réunion de l'ISCTRC (International Scientific Council for Trypanosomiasis Research and Control,* Yammoussoukro, Côte d'Ivoire (octobre 1991).

- Etude isoenzymatique de *Trypanosoma brucei* en Afrique Centrale: corollaires épidémiologiques. VII Congrès International de Parasitologie (ICOPA), Paris, France (aout 1990).

Participation à des Réunions Scientifiques

- Advances in Gene Technology: Molecular Biology and Human Diseases. Miami Winter Symposia, Miami, Floride, USA (février 1994).

- Séminaire sur la Trypanosomiase, Institut de Médecine Tropicale d'Anvers, Belgique (décembre 1991).

- 20ème Réunion de l'ISCTRC, Mombassa, Kenya (avril 1989).

Actions de Conseil, Coordination Scientifique et Technique et formation

- Formateur (diagnostic sérologique et parasitologique des trypanosomes) : Primer Curso Internacional para graduados sobre : Diagnostico y Control de Hemoparasitos y sus vectores. Universidad Romulo Gallegos, San Juan de Los Morros, Guarico, Venezuela, Octobre 1999.

- Membre du Comité de Conseil Technique de la Division de Lutte contre les Maladies Tropicales CTD/OMS (Technical Advisory Group), 1997/98.

- Conseiller temporaire du Comité Directeur du Program Against African Trypanosomiasis (PAAT) OMS/FAO/IAEA/OUA IBAR, 1998.

- Responsable du Laboratoire de référence pour le diagnostic et la sérologie de la THA en Afrique de l'Ouest. Financement "Fonds d'Aide à la Coopération", Ministère Français de la Coopération et du Développement, France, 1997/1998.

- Secrétaire Local (Côte d'Ivoire) de la Société Royale de Médecine Tropicale et d'Hygiène, Londres, Royaume-Uni, 1997/1998.

- Participation pour l'IPR à l'organisation de la Réunion de coordination des activités de lutte contre la THA, programme FAC, Maputo (Mozambique) les 25 et 26 septembre 1997.

- Organisation (et participation) de la Réunion de coordination des activités de lutte contre la THA, programme FAC, IPR, Abidjan du 3 au 5 février 1997.

- Organisation (et formateur) du séminaire de formation des Responsables nationaux de lutte contre la THA en Afrique de l'ouest, Coordination de la lutte contre la THA, programme FAC, IPR, Bouaké du 27 au 31 janvier 1997.

- Formation dans le cadre du séminaire de formation des Responsables nationaux de lutte contre la THA en Afrique Centrale, Coordination de la lutte contre la THA, programme FAC, OCEAC, Yaoundé (Cameroun) en octobre 1997.

- Formation et encadrement technique d'un étudiant-stagiaire ORSTOM en DEA de Parasitologie, 1996, IPR, Bouaké, Côte d'Ivoire.

- Formation et encadrement technique d'un étudiant-stagiaire de l'Université des Sciences de Montpellier (France) en stage de licence de Biologie, 1997, IPR, Bouaké, Côte d'Ivoire.

- Formation et encadrement technique d'un étudiant allocataire de recherche ORSTOM en thèse de doctorat es-sciences, 1997/1999, IPR, Bouaké, Côte d'Ivoire.

- Formation et encadrement technique d'une étudiante ivoirienne financée par l' ORSTOM en thèse de doctorat es-sciences, 1997/1999, IPR, Bouaké, Côte d'Ivoire.

- Participation à la formation et encadrement technique de deux stagiaires ivoiriens en thèse d'exercice de Pharmacie, (Faculté de Pharmacie d'Abidjan), 1996/1997, IPR, Bouaké, Côte d'Ivoire.

- Participation à la formation et encadrement technique de deux chercheurs sénégalais en thèse de troisième cycle, (Faculté des Sciences d'Abidjan), 1994/1997, IPR, Bouaké, Côte d'Ivoire.

- Représentant du Responsable Santé de la Mission de Coopération Française d'Abidjan (Dr B. Floury) au Conseil de Gestion 1993 de l'Institut Pierre Richet de Bouaké (Côte d'Ivoire).

Contacts Scientifiques Nationaux et Internationaux

- Projet de Recherche Clinique sur la Trypanosomiase, Daloa, Côte d'Ivoire (Dr Doua).
- Laboratoire de Pathologie Animale, Bingerville, Côte d'Ivoire (Dr N'Depo).
- Direction des Parcs Nationaux et World Wildlife Fundation, Abidjan, Côte d'Ivoire (Dr Lauginié).
- Faculté de Pharmacie, Abidjan, Côte d'Ivoire (Pr Malland).
- OCEAC, Yaoundé, Cameroun.
- Université de Bordeaux II, Immunologie et Biologie Moléculaire des Protistes Parasites, France (Pr Baltz).
- UMR 9926 CNRS/ORSTOM, Génétique Moléculaire des Parasites et Vecteurs, Montpellier, France (Dr Tibayrenc).
- Université de Bordeaux II, Immunologie et Biologie parasitaires, France (Pr Vincendeau).
- Université de Limoges, Neurologie Tropicale, France (Pr Dumas et Dr Bouteille).
- Malaria Division, OMS/WHO Center, Faculty of Sciences, Chulalongkorn University, Bangkok, Thailande (Pr Thaithong).
- Institute of Health Research, Chulalongkorn University, Bangkok, Thaïlande (Pr Chantaraprateep).
- Wellcome-Tropical Medicine Research Programme, Faculty of Tropical Medicine, Mahidol University, Bangkok, Thaïlande (Dr White).
- Laboratoire de Pharmacognosie, Faculté de Pharmacie, Montpellier, France (Pr Bastide, Dr Pélissier).
- Department of Medicine, University of Alabama at Birmingham, USA (Dr Unnasch).
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Divers

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