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The Application of Molecular Biological Tools to Epidemiology of African Trypanosomosis

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Difficulties have often been encountered in the field surveys due to a lack of definitive morphological characters, particularly where mixed infections are expected. To address this problem, some molecular biological techniques such as DNA probe hybridization, restriction fragment length polymorphism (RFLP) analysis, the polymerase chain reaction (PCR), analyses of ribosomal DNA, and pulsed-field gel electrophoresis (PFGE), have been applied to the analysis of field samples collected during epidemiological surveys of African trypanosomosis. Concurrent natural infection of different individual tsetse flies and mammalian hosts with different species of the trypanosomes have been demonstrated, through the use of a combination of specific DNA probe hybridization and the PCR. Molecular karyotypes of *Trypanosoma brucei* species were analyzed by PFGE in 45 - 2,000 kb range. There are distinctive differences in intermediate and mini-chromosomes among the strains. We have compared the nucleotide sequences of ribosomal DNAs of the parasites by PCR techniques. From this data new phylogenetic tree can be inferred. It is apparent that these technologies can provide powerful tools for identification and diagnosis of trypanosomes in their hosts and vectors, and for their more accurate phylogenetic classification.

Keywords : African trypanosomosis, Epidemiology, Molecular biological tools, Pulsed-field gel electrophoresis, Ribosomal DNA

INTRODUCTION

African trypanosomes cause severe diseases in man and livestock in Africa and other parts of the world [26]. Depending on the host, the trypanosomosis can vary in clinical severity and magnitude of epidemic in the affected areas in Africa. The trypanosomes naturally undergo profound physiological and morphological differentiation in mammalian hosts and tsetse vectors, making it difficult to identify any of them unequivocally based upon these characteristics. One of the remarkable advances in research on African trypanosomosis has been the application of tools and reagents derived from molecular biology to the study of parasite species or types, their spatial distribution and their population genetic structure, in both the hosts and the vectors. These studies have resulted in new observations concerning many aspects of the biology of African trypanosomes. In this paper, we show some data on pulsed-field gel electrophoresis (PFGE) and review some related technologies and approaches available for the epidemiology.

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MATERIALS AND METHODS

(1) Pulsed-field gel electrophoresis of *Trypanosoma brucei* species.

Molecular karyotypes on African trypanosomes especially among strains of *T. brucei* species were analyzed by PFGE. Six strains of cloned *T. brucei* subspecies, *T. b. brucei* GuTat 3.1, *T. b. brucei* tc221 KO, *T. b. brucei* tc221 IL, *T. b. brucei* 427W, *T. b. rhodesiense* IL1501, *T. b. gambiense* Wellcome #9.2, *T. b. gambiense* IL1852 and *T. brucei* IL2343 were tested.

Procyclic trypomastigotes of *T. b. brucei* 427W and *T. b. rhodesiense* IL1501 were cultivated in flask at 27℃. Bloodstream forms of *T. b. brucei* tc221 KO were cultivated in flask at 37℃. They were isolated from media by DE-52 column chromatography. On the other hand, these 7 different parasites except *T. b. brucei* 427W were injected and propagated in BALB/c mice. Bloodstream trypomastigotes were isolated from mouse blood cells by DE-52 column chromatography [1]. The purified live parasites were embedded in 0.5% low melting agarose blocks and lysed *in situ* at the final concentration of 3.3×10^8 ml^{-1}. The parasites in the blocks were digested with proteinase K (Boehringer Manheim Co.Ltd.) at 65℃ for 2 days. After the blocks were rinsed in TE buffer, they were kept at 4℃ until use.

Pulsed-field gel electrophoresis was carried out using a pulsed-field gel apparatus (Gene Navigator System, Pharmacia Co.Ltd.). Agarose gels (1%) were run in 0.5×TBE at 195 volts at 8℃. Switch time was 35 seconds for 7.5 hours, followed by 60 seconds for 6 hours, and then 100 seconds for 10.5 hours, resulting in a clear separation of mini and intermediate-chromosome-sized DNA in 45 - 2,000 kb range. *Lambda* ladder DNA and yeast chromosomes, *Saccharomyces cerevisiae* (Bio-Rad Co. Ltd.) were used as size markers for the range 48.5 - 145.5 and 225 - 2,200 kb, respectively. Gels were stained with ethidium bromide and photographed by ultraviolet transillumination.

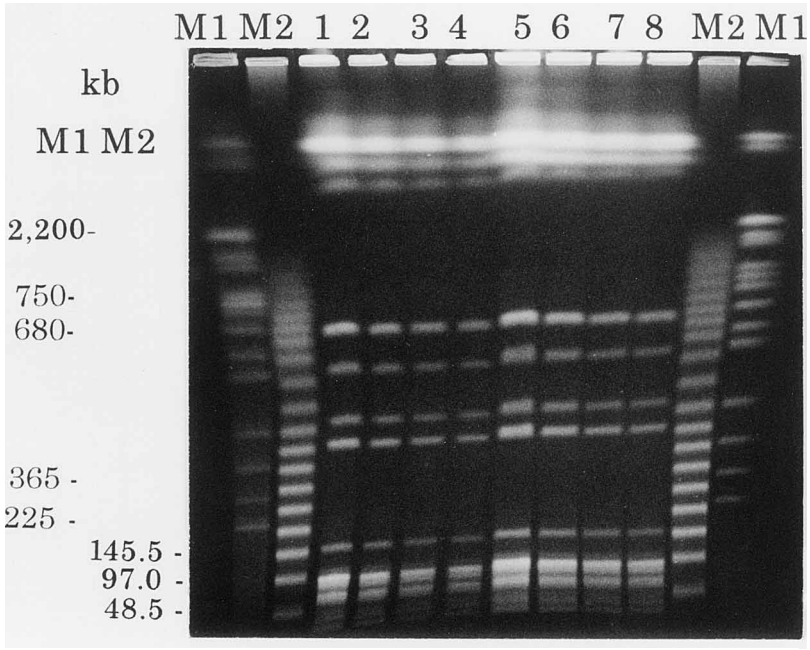

Fig. 1 Molecular karyotype patterns of *Trypanosoma brucei gambiense* Wellcome #9.2.
DNA size markers: M1 (*Saccharomyces cerevisiae*, 225 - 2,200kb, Bio-Rad), M2 (*Lambda* ladder, 48.5 - 1,000kb, Bio-Rad). Lanes 1: *T. b. gambiense* Wellcome #9.2, 1.25×10^8/ml, 2: 0.83×10^8/ml, 3: 0.62×10^8/ml, 4: 0.5×10^8/ml (1/40 of 2×10^9/ml), 5: 4.99×10^8/ml, 6: 3.33×10^8/ml, 7: 2.49×10^8/ml, 8: 1.99×10^8/ml (Ca. 1/10 of 2×10^9/ml).

Relative sizes of all DNA bands that appeared in PFGE agaroses were measured as distance from the origin of agarose. Totally 35 of different sizes of bands in intermediate- and mini-chromosome areas were listed. Present band was marked as 1, and absent band was marked as 0. Bands in each PFGE agarose lane were scored using MacClade 3.0 software for Macintosh. *Trypanosoma brucei* IL2343 for example, 1, 0, 0, 0, 1, 1, 1, ・・・, 0, 1 as score, in 1, 2, 3, 4, 5, 6, 7, ・・・, 34, 35 as number from origin agarose, respectively. Cluster analyses of molecular karyotype patterns for the bloodstream and procyclic trypomastigotes were analyzed with data of MacClade 3.0 by PAUP 3.1.1 software for Macintosh.

RESULTS

(1) Size-fractionation of the chromosome-sized DNA by PFGE among strains of *T. brucei* species.

Molecular karyotypes of the parasites especially some strains of *T. brucei* species were analyzed by PFGE, resulting in a clear separation of chromosome-sized DNA in about 45 - 2,000 kb range (Fig. 1). Figure 1 shows comparisons between low and high number of *T. b. gambiense* Wellcome #9.2 parasites in agarose block per 1 ml (lanes 1 - 8 in Fig.1). 3.33×10^8/ml was selected as the number of parasites for further experiments (lane 6 in Fig.1). There are distinctive differences in intermediate and mini-chromosomes among the strains of *T. brucei* species. *T. brucei* IL2343 and *T. b. brucei* tc221 KO have different number and size of bands in intermediate-chromosomes (lanes 1-4 and 5-6 in Fig. 2). And even between two strains, *T. b. brucei* tc221 KO and *T. b. brucei* tc221 IL which were derived from a clone, different size DNA patterns in intermediate-chromosomes in the about 145.5 - 450 kb area, were seen (lanes 4 and 5 in Fig. 3).

Molecular karyotypes of *T. brucei* IL2343, *T. b. gambiense* IL1852 and *T. b. rhodesiense* IL1501 were recognized as different DNA size patterns (lanes 1 - 3 in Fig. 3). About 8

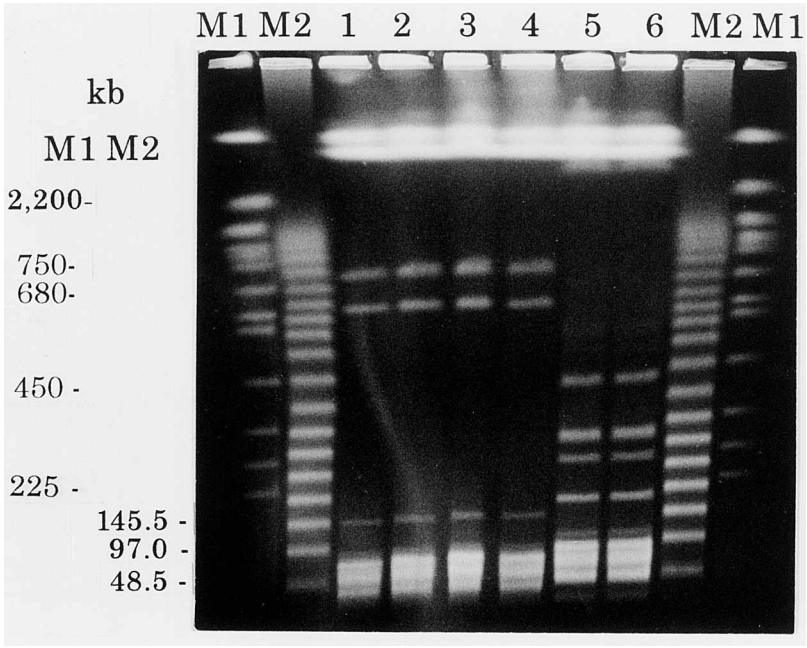

Fig. 2 Molecular karyotype patterns of *Trypanosoma brucei* IL2343 and *T. b. brucei* tc221 KO.
DNA size markers: M1 (*S. cerevisiae*, 245 - 2,200kb), M2 (*Lambda* ladder, 48.5 - 1,000kb). Lanes 1: *T. brucei* IL2343, 3.2×10^8/ml, 2: IL2343, 3.33×10^8/ml, 3: IL2343, 3.74×10^8/ml, 4: IL2343, 1.86×10^8/ml (Ca. 1/10 of 2×10^9/ml), 5: *T. b. brucei* tc221 KO, 3.33×10^8/ml, 6: tc221 KO, 3.33×10^8/ml (Ca. 1/6 of 2×10^9/ml).

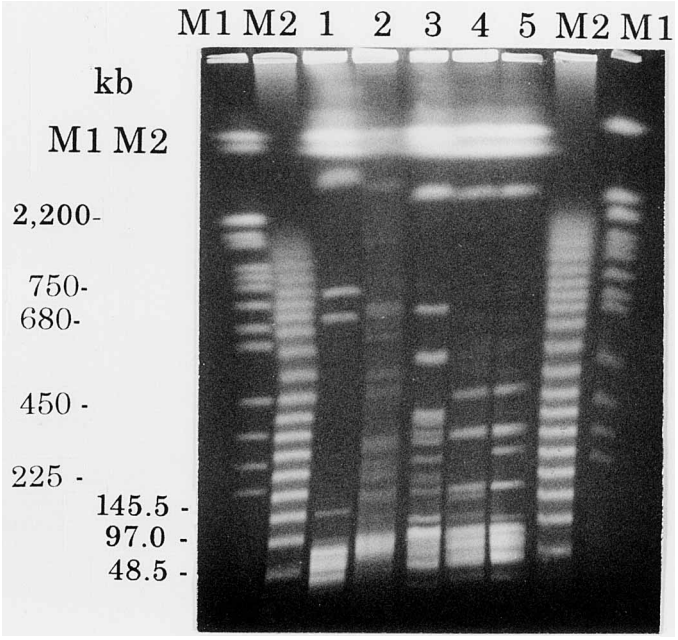

Fig. 3 Molecular karyotype patterns of *Trypanosoma brucei* IL2343, *T. b. gambiense* IL1852, *T. b. rhodesiense* IL1501, *T. b. brucei* tc221 IL and *T. b. brucei* tc221 KO.
DNA size markers: M1: *S. cerevisiae* (225 - 2,200kb), M2: *Lambda* ladder (48.5 - 1,000kb). Lanes 1: *T. brucei* IL2343, 1.86×10^8/ml (Ca. 1/10 of 2×10^9/ml), 2: *T. b. gambiense* IL1852, 3.33×10^8/ml (Ca. 1/6 of 2×10^9/ml), 3: *T. b. rhodesiense* IL1501, 3.33×10^8/ml, 4: *T. b. brucei* tc221 IL, 3.33×10^8/ml, 5: *T. b. brucei* tc221 KO, 3.33×10^8/ml.

different faint bands in *T. b. gambiense* IL1852 and 9 bands in *T. b. rhodesiense* IL1501 were observed in the about 145.5 - 750 kb area (lanes 2 and 3 in Fig. 3), however *T. brucei* IL2343 showed only 3 bands in the area (lanes 1 in Fig. 3).

Differences between bloodstream and procyclic trypomastigotes were compared in different culture conditions (Fig. 4). DNA size band patterns of *in vitro* bloodstream trypomastigotes of *T. b. brucei* tc221 KO that were cultivated at 37℃ showed the same patterns as bloodstream trypomastigotes of *T. b. brucei* tc221 KO (lane 5 in Fig. 3, lane 2 in Fig. 4) as well as procyclic trypomastigotes of *T. b. brucei* 427W (lane 3 in Fig. 4). Same DNA size patterns were observed between bloodstream and procyclic trypomastigotes of *T. b. rhodesiense* IL1501 (lane 3 in Fig. 3, lane 4 in Fig. 4).

Cluster analyses of molecular karyotype patterns for *T. brucei* subspecies including bloodstream and procyclic trypomastigotes was performed (Fig. 5). Five strains of *T. b. brucei* were in one cluster. *T. b. rhodesiense* IL1501 relatively close to *T. b. brucei* cluster. *T. b. gambiense* Wellcome #9.2 and *T. b. gambiense* IL1852 were relatively far from *T. b. brucei* cluster than *T. b. rhodesiense* IL1501. However *T. brucei* IL2343 was in between the two *T. b. gambiense* strains.

DISCUSSION

(1) PFGE of *T. brucei* species.

We have compared the molecular karyotypes of African trypanosomes from different subspecies within species *T. brucei* by PFGE. Observation of molecular karyotypes of the parasites resulted in a clear separation of chromosome-sized DNA in 45 - 2,000 kb range. However mini-chromosomes, about 50 kb were changed at shorter interval than 35 seconds depending on PFGE conditions.

There were distinctive differences in inter-

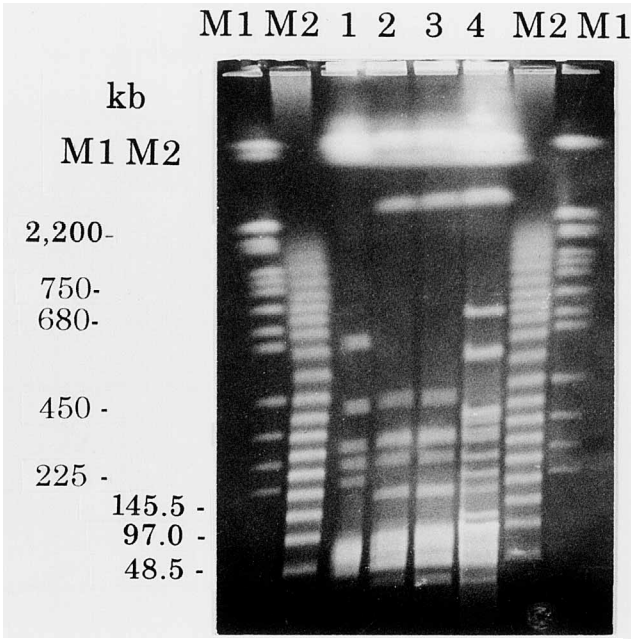

Fig. 4 Molecular karyotype patterns of *Trypanosoma brucei brucei* GuTat 3.1, *T. b. brucei* tc221 KO, *T. b. brucei* 427W and *T. b. rhodesiense* IL1501.
DNA size markers: M1: *S. cerevisiae* (225 - 2,200kb), M2: *Lambda* ladder (48.5 - 1,000kb). Lanes 1: *T. b. brucei* GuTat 3.1 (BSF in a mouse), 6.63×10^8/ml, 2: *T. b. brucei* tc221 KO (BSF cultivated at 37℃), 3.33×10^8/ml, 3: *T. b. brucei* 427W (procyclic form cultivated at 27℃), 3.33×10^8/ml, 4: *T. b. rhodesiense* IL1501 (procyclic form cultivated at 27℃), 3.33×10^8/ml (Ca. 1/6 of 2×10^9/ml).

mediate and mini-chromosomes among the strains. The gels illustrated the large differences in the complement of intermediate and mini-chromosomes between the subspecies. Even in *T. b. brucei* tc221 IL and *T. b. brucei* tc221 KO strains, at least one DNA band in intermediate-chromosomes in the 145.5-450 kb area, were different. *Trypanosoma b. brucei* tc221 KO was divided and distributed from *T. b. brucei* tc221 IL in 1987. Since the size differences in chromosomes from the same origin are due to incidental gene rearrangement, further analyses for biological difference are needed.

Trypanosoma brucei IL2343, a clone derivative of STIB386AA [9] that was derived from TH144/78E(020), isolated originally from a patient in Ivory Coast in 1978 [4] and has been previously classified as a 'non-*gambiense*' stock by isoenzyme [18] and antigen gene probe criteria [23] and more recently classified as *T. b. rhodesiense* base on the repetitive DNA sequences [6]. Since *T. brucei* IL2343, originally considered to be *T. b. gambiense*, has been classified as a *T. b. rhodesiense* stock [6], it is needed to examine whether a serum-free medium (HMI-224) can also support the continuous growth of 'true-*gambiense*' bloodstream trypomastigotes [7].

(2) Comparative analyses of ribosomal DNAs (rDNAs) of African trypanosomes.

In order to investigate questions surrounding the phylogenic relationships among African trypanosomes, the nucleotide sequences of rDNAs of the parasite strains of 5 different trypanosomes: *T. b. brucei* (2), *T. congolense* [savanna-type (5), West African-riverine-forest type (1), Kilifi-type (1), Tsavo-type (1)], *T. vivax* (1), *T. simiae* (1) and *T. thaileri* (2) have been compared by PCR techniques. The ribosomal RNA genes, including 18S rDNA, 5'ITS, 5.8S rDNA, 3'ITS and

28s-LS1, were obtained either as genomic DNA clones in *Lambda* phage or as PCR products from trypanosomes genomic DNA, and were either sequenced directly or subcloned into plasmid vectors prior to the sequencing [28].

The rDNAs of four different types of *T. congolense* had significant heterogeneity among the sequences, making each of the trypanosomes clearly distinguishable on this basis. The sequence differences were observed in the spacer regions, the 18S and the 28S-LS1 rDNA segments. Among 5 isolates of savanna-type from different parts of Africa, an insignificant number of nucleotide substitutions were observed in the 18S rDNA region. Of the different types of *T. congolense*, the West African-riverine-forest type was found to be closest to the savanna-type, with 97% matches, followed by the Kilifi-type with 93% matches [28]. Curiously, the Tsavo-type *T. congolense* was phylogenetically closer to *T. simiae* than it was to the savanna-type.

These data strongly suggest that the four trypanosomes now grouped together as *T. congolense* could be classified into individual species. This situation contrasts with what we observed in the analysis of *T. brucei* and related trypanosomes. The subgenus *Trypanozoon* comprises three species: *T. brucei*, *T. evansi* and *T. equiperdum*. In addition to host range, the major phenotypic difference among trypanosomes in this subgenus is the absence of kDNA maxicircle, which is correlated with the capacity for cyclical development in the tsetse fly. A few nucleotide base substitutions in both the 18S rDNA and the spacer regions were found when the rDNA sequences of these trypanosomes were compared; there was no data for *T. equiperdum*. It is thought that *T. evansi* evolved from *T. brucei*, and then spread from Africa to other parts of the world in the recent past. Although the two trypanosomes have different lifestyles, no evidence was found from the analyses of rDNA genes to support their classification into different species. For simplicity from evolutionary point of view they may be

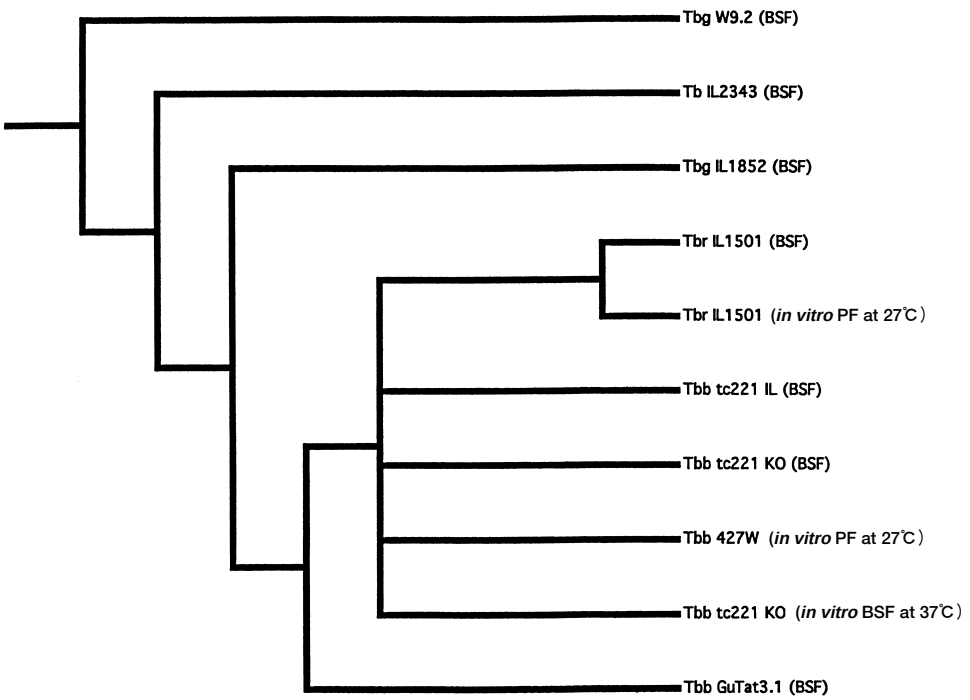

Fig. 5 Cluster analyses of molecular karyotype patterns for *Trypanosoma brucei*, *T. b. brucei*, *T. b. rhodesiense* and *T. b. gambiense* strains.
DNA band patterns of each strains which were appeared in Figure 1 to 4 were analyzed with MacClade (version 3.0) and PAUP (version 3.1.1) analysis software.

regarded as subspecies or variant of *T. brucei*. From our data new phylogenetic tree may be inferred [28].

(3) Other molecular biological tools.

It is important to identify the parasitic infectious agent. And knowledge of disease incidence and transmission dynamics are crucial to such studies, as are accurate diagnosis and treatment of cases as they arise due to infection by the agent. Some molecular biological techniques such as DNA probe hybridization, restriction fragment length polymorphism (RFLP) analysis, rDNA analysis, the polymerase chain reaction (PCR), and PFGE have been applied to the analysis of field samples collected during epidemiological surveys of African trypanosomosis. The above-mentioned strategies may provide a basis for a rapid identification in future molecular epidemiological studies of parasites in Africa.

(3.1) Deoxyribonucleic acid (DNA) probe-based methods for the detection of trypanosomes.

Accurate identification of an infectious organism facilitates the study of epidemiology of the disease it causes and makes possible rational evaluation of the relative success of different control strategies. Protocols have been adapted for use with these DNA probes in the detection of trypanosomes in the blood or buffy coat samples from mammalian hosts and the saliva of live tsetse flies.

Deoxyribonucleic acid probe hybridization is gaining popularity [30]. However, methods which utilize the PCR [24] offer an attractive alternative because they are sensitive, rapid, and require very little amount of the test sample. Majiwa *et al.* [16] have cloned highly repetitive, tandemly arranged DNA sequences unique to each trypanosome species, type or sub-type. These probes have been used in combination with the PCR to detect trypanosomes in buffy-coat samples from antigenaemic but aparasitaemic cattle, and in the saliva of live infected tsetse flies. These assays will make it feasible to trace patterns of trypanosome infection in populations of mammalian hosts and in the tsetse fly vectors, to identify individual trypanosome species or types that persist after treatment of the infected individuals, and to detect cases of co-infection or reinfection.

(3.2) Recombinant plasmids for hybridization assays.

There exists a need for (a) development of improved methods for accurate identification and characterization of different species of the African trypanosomes in the tsetse vector and the mammalian host blood, and (b) simplification of such methods, as far as possible, specifically to exclude the use of radioactive reporter molecules in the detection of the parasites.

The criteria adopted in selection of the prospective DNA probes included the requirements that the probe be specific for the trypanosome type and, secondly, that the probe be sensitive enough to detect a small number of the parasites. Details of the identification and cloning of the trypanosome repetitive DNA sequences with these qualities have been described [10,14,15] and their uses reviewed [5,13,22].

(3.3) Features of the results in restriction enzyme digests of parasite genomic DNA.

Parasite genomic DNA resolved by electrophoresis in an agarose gel, the repetitive DNA sequences can be seen as bands of intense fluorescence above a background smear of numerous fragments of DNA. Such bands can be purified and then cloned in plasmids. Alternatively, the highly repetitive DNA sequences can be identified by low $Cot_{½}$ hybridization using as probe total genomic DNA from homologous and heterologous species of trypanosomes. Once identified, the DNA can be recovered by cloning from total genomic DNA digested with an appropriate restriction enzyme. The cloning is normally done in plasmids which can be grown in bacteria.

(3.4) Detection of parasites in vectors.

All the DNA probes so identified are repetitive, being arranged either in tandem arrays or as dispersed repeats in the genome. They hybridize specifically with DNA from the respective trypanosome species. The DNA probes have been used after labeling with [a^{32}P]-dCTP a radioactive isotope. Examples of their use to detect trypanosomes present in naturally infected tsetse flies have been described [12,19,21]. The probes, when labeled to high specific activities, can detect

the parasites present in both the mouth parts and the guts.

Furthermore, it is possible to detect mixed infections in a number of the tsetse flies, both in the mouth parts and in the guts of tsetse flies. One of the observations made in a number of such studies [12,21], was that some trypanosomes present in some of the tsetse flies could not be detected by hybridization with any of the panel of probes used. This observation implied the existence of trypanosome types genetically distinct from those for which DNA probes were available.

It was possible to demonstrate the existence of such a trypanosome after *in vitro* technologies were developed to grow in culture trypanosomes isolated directly from tsetse fly proboscis, salivary glands or the guts [19].

In vitro technologies have been exploited to grow a number of trypanosomes including at least two new *Nannomonas* which had failed to react with the panel of probes used in the earlier studies. For one of these trypanosomes, a specific DNA probe has been identified and cloned [14].

(3.5) Polymerase chain reaction-based methods for the detection of trypanosomes.

Combined with PCR, the method detects trypanosomes in buffy-coat samples from antigenaemic but aparasitaemic cattle, and in the saliva of live infected tsetse flies. The majority of these recombinant DNA probes are presently available from various sources.

(3.5.1) Oligonucleotide primers for PCR assays.

The nucleotide sequence composition has been determined either in part or in total for the majority of the repetitive DNA probes. It has therefore been possible to design oligonucleotides for PCR amplification of DNA to detect low parasite numbers. The sensitivity of the detection can be increased by hybridization of the products with a specific probe. One additional advantage of this approach is that primers specific for different trypanosome species can be placed in a single reaction tube, and the products obtained hybridized with the specific probes.

(3.5.2) Detection of the parasites secreted from the proboscis of tsetse fly.

For the anticipated practical field application, it is essential that methods are appropriately adapted. One of the adaptations [14] investigated involved obtaining saliva from live tsetse flies. To reveal trypanosomes in the saliva of the tsetse flies, each infected (or mock-infected) fly is induced to probe into 10 μl of PSG at 37℃ in a lymphocyte migration plate; 2 μl of this is used in a PCR amplification without further manipulation.

Alternatively, the proboscis of each fly is used in a PCR amplification. Gut contents of infected tsetse are used in diagnostic PCR assays. This approach has been used in the amplification of samples from naturally as well as experimentally infected tsetse flies caught in different areas of Africa [14,17]. In this way it was possible to demonstrate that a new *Nannomonas* designated the Tsavo-type *T. congolense* is present in other places than the Tsavo area in which it was initially identified. A similar approach was used in the identification of trypanosomes in buffy coat samples from animals which had been shown to be antigenaemic by antigen ELISA but aparasitaemic by classical parasitological procedures [16].

(3.6) Non-radioactive DNA probes.

One of the problems in nucleic acid hybridization for the detection of parasites in vectors has been the almost mandatory use of radioisotopes, in particular ^{32}P in order to achieve maximum sensitivity. However, the use of radioisotopes brings another layer of complexity to laboratory procedures, including short half-life of 14.3 days, and hazardous usages of radioisotope. The development of non-radioactive detection of hybridization has made it possible to detect the parasites with a sensitivity approaching that of equal to procedures using ^{32}P.

Parasite- and vector- specific DNA probes are labeled with non-radioisotope agents such as biotin using nick translation (Bethesda Research Laboratory Inc., BRESA Inc., Tropics Inc., etc.), horseradish peroxidase (Amersham Inc.), and digoxigenin (Boehringer Mannheim Inc.). Simple non-radioactive protocols have been adapted for the detection of African trypanosomes in the blood of mammalian hosts and the saliva of live tsetse flies. The detection of parasite

DNA relies upon hybridization with parasite type-specific DNA probe labeled with digoxigenin, followed by revealing the hybridized probe using anti-digoxigenin antibodies conjugated to alkaline phosphatase and the addition of enzyme substrates that result either in visually discernible color or chemiluminescence detectable by autoradiography. The method has been used in combination with the PCR to detect trypanosomes in buffy-coat samples from antigenaemic but aparasitaemic cattle, and in the saliva of live infected tsetse flies. It has also been used to demonstrate concurrent natural infections of single tsetse flies with different species of African trypanosomes.

Concurrent natural infection of different individual tsetse flies and mammalian hosts with different species of the trypanosomes have been demonstrated, through the use of a combination of specific DNA probe hybridization and the PCR.

(3.6.1) Non-radioactive labeling kits.

Several commercial companies are developing reagents for non-radioactive labeling of DNA probes used in the diagnosis of various infectious organisms and genetic disorders [8,27]. Some of these are available as kits. Among the commercial kits whose ease of use we investigated, the one from Boehringer-Mannheim was the most robust. The kit is based on digoxigenin coupled to dUTP, a nucleotide which can be incorporated into the DNA probe by the random priming reaction. It is subsequently revealed by color reaction or light emission.

(3.6.2) Availability of the probes.

For purposes of research, the majority of the DNA probes are available free of charge from the scientists who have developed them. It is possible to receive the probes as recombinant plasmids which can be propagated by growth in bacteria. By similar arrangements, it should also be possible to obtain the probes pre-labeled with a stable, non-radioactive reporter molecule, ready for use in hybridization assays. Where one has access to the parasites, it would be just as easy to amplify the specific DNA sequences directly from DNA of the respective parasites. This is a feasible particularly because the nucleotide sequences of the primers specific for a majority of the probes have been published.

(4) Cautionary notes.

Some of the novel technologies and reagents mentioned above and others under development will soon become part of routine methods for medical diagnosis and forensic analyses. Because of their exquisite sensitivities, one needs to be aware of the dangers that may result from lack of care in the design and execution, particularly if these lead to contamination. Of these procedures, the PCR will be likely to be the most widespread in use. Indeed it has been observed by many investigators that, because of its extreme sensitivity, diagnostic PCR can be fraught with contamination. Contamination is the most pernicious problem plaguing the PCR technology.

In diagnostic assays, it is essential that the only DNA present in the sample is that either added by the investigator or naturally present in the test sample. Thus, the user of this PCR technology should ensure that the work is performed in DNA-free and clean environment.

(4.1) Control of contamination.

Control of contamination must involve prevention of transfer of DNA between amplified samples, and between positive and negative test samples (among the test samples). Contamination of test samples from many of these sources has been documented [11,25,29] and suggestions made [3] to minimize or exclude altogether the chances of contamination.

These have included the physical separation of the entire diagnostic procedure into three parts: the location for sample preparation, pre-PCR location and the post-PCR location. The test samples should be prepared using the simplest method that involves least number of manipulations, with utmost care being taken to avoid sample-to-sample contamination.

To test the success of any number of precautions in the exclusion of contamination, each set of assays must have appropriate controls, including at least positive and negative controls. The negative control must contain all the reagents used in each reaction except test DNA. This sample is also known as "no DNA". The positive control should have all components of the PCR reaction

including the template DNA from which the primers are known to produce fragment of a particular size upon amplification. In assembling reaction components, DNA polymerase should be added last.

(5) Future format.

The application of recombinant DNA technology and the reagents derived from it to the diagnosis of both genetic and infectious diseases is rapidly becoming popular [2,5]. It is anticipated that the majority of these diagnostic assays presently involving a combination of the PCR, agarose gel electrophoresis and blot hybridization will be converted such that they can be performed in an enzyme-linked-immunosorbent (ELISA) format. They will then be relatively easier to carry out in many laboratories which presently use the ELISA as a routine assay in the diagnosis of various infectious diseases. The ELISA-based assays have several advantages and, when combined with the PCR and hybridization, have the sensitivity far greater than blot hybridization alone in the detection of DNA from an infectious organism [20].

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