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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*, [1]). 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 [2, 3]. 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. [4] 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. [5]. 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 [6-8, 127] 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, [9]). In the original Blood Incubation Infectivity Test (BIIT) developed by Rickman et al. [10], 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 [11, 12], 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; [13]). *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 [14]. 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 [15-18]. The repetitive DNA probes specific for *T. brucei* detect all 3 subspecies, however [19], 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 [20-39]. The main conclusions from this data are discussed in the later 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 [40]. 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 [41, 42]. 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 [31, 43, 44]. 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 [45].

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 [46, 47]. 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 [47]. 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*, [48]).

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 [49-51], and variant surface glycoprotein (VSG) genes [52-55]. 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 later 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 [56, 57].

Two PCR tests for Group 1 *T. b. gambiense* have been devised, based on a conserved VSG gene and kDNA minicircles, respectively [42, 58]. 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* [54]. 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 [58]. 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 [42].

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 [59, 60]. 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 Figure 1.

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 [59-62]. 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 [63]. 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, [64]). 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 [65-67]. 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 [66, 68, 69].

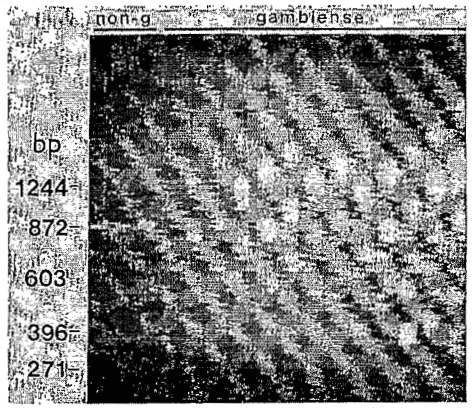

Fig. 1. Comparison of RAPD patterns obtained with a single primer from genomic DNAs of various *T. brucei* ssp. stocks. From left to right, lanes 1-3 non-gambiense stocks: *T. b. brucei*, Zaire; *T. b. brucei*, Côte d'Ivoire; *T. b. rhodesiense*, Zambia. Lanes 4-16 Group 1 *T. b. gambiense*: 3 stocks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

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 [70]. 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 data 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 [71, 72], 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 [73].

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 [28, 67, 126]. 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 [23] used classical Hardy-Weinberg statistics to reveal genetic exchange in African trypanosomes from the field, although a later study by Cibulskis [74] underlined some of the pitfalls associated with single-locus Hardy-Weinberg analysis when sample sizes are small. The randomization approach developed by Cibulskis [74] was extended by Stevens & Welburn [75] to study genetic exchange in epidemic populations, together with a multilocus approach. Multilocus analyses offer a robust extension to single locus methods [76] 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 [70, 73].

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, [74]). 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. [77-79]), association indices [71, 80] or a combination of the two [81]. 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 [60, 77, 78], while an extended Mantel test [82] has been used to study a range of *T. brucei* [61, 62, 79]. Association indices have also been employed for defining population structure in a range of *T. brucei* populations, e.g. Maynard Smith et al. [71], the Lambwe Valley, Kenya; Hide et al. [51], Uganda; Stevens & Tibayrenc [72], Côte d'Ivoire, Uganda, Zambia, and for investigating associations between parasite genotype and host/location [83].

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 [76] 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 (β) 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 [74, 84]. Once β is known, steps can be taken to reduce it to less than 5%, usually by increasing the sample size. If β 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 [79] 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 [85] is often difficult or impossible to use [73].

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 [86], and a range of computer based methods are now available to elucidate relationships by means of phylogenetic trees [87, 88]. A measure of confidence can be attached to a given tree by additional bootstrap analysis [89]; 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 [90, 91]. 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 [92], simple matching coefficient [93]. 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 [73]. 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* [22], a host of other studies using phenetic classification systems have followed, e.g. Hide et al. [49, 50], Cibulskis [83], Stevens & Godfrey [41], Truc & Tibayrenc [94], Mathieu-Daudé & Tibayrenc [38], Enyaru et al. [95]. 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 [96], Nei's method [97], Neighbour joining [98] and the Wagner method [99] (see Nei [100] 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 [51, 74] and Nei's distance being employed for broader phylogenetic studies of *Trypanozoon* (e.g. Tait [27]; Stevens [35]; Hide [51]; Mathieu-Daudé [38]).

Cladistics (often referred to as the parsimony method [101]) and the closely allied technique of maximum likelihood, use discrete character data and work on changes in character states [102]; while cladistics evolved largely from studies of morphological data, maximum likelihood was developed specifically for molecular data [103]. The suitability 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 [104, 105]. 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 [30]. Since then Mathieu-Daudé et al. [61] have extended this work with a RAPD-based parsimony analysis of *T. brucei*, while Fernandes et al. [106] and Maslov et al. [107] have used the technique with sequence data for broad studies of evolution in the Trypanosomatidae.

Maslov et al. [107] 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 [70, 73]) 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 [73].

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; [21, 22, 24, 26, 29, 35], and by a set of RFLPs for VSG genes, notably AnTat 1.8 and 11.17 [52-55] and ribosomal genes [49]. Besides these molecular markers, Group 1 *T. b. gambiense* stocks have a restricted antigenic repertoire [108, 109], a small genome and fewer small chromosomes compared to other *T. brucei* ssp. [66, 68, 69]. 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 [29, 36]. 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* [110].

Group 2 *T. b. gambiense* is defined as a virulent form of *T. b. gambiense* from foci of gambian sleeping sickness [24, 66]. 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 [110]; whether it also differs in clinical features is presently under examination [128]. By molecular characterization this trypanosome falls outside homogeneous Group 1 and does not share its characteristic isoenzyme and RFLP markers (Fig. 2, [19, 24, 31, 49, 111]). Although often referred to as "*T. b. rhodesiense*-like", Group 2 *T. b. gambiense* is more akin to West African *T. b. brucei* stocks [49, 111] and indeed may represent a zoonotic form of sleeping sickness in West Africa [24, 128].

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 [19, 72, 77]. While genetic exchange has been demonstrated for Group 2 *T. b. gambiense* in the laboratory [112, 113], for Group 1 *T. b. gambiense* such experiments are rendered difficult by its poor tsetse transmissibility.

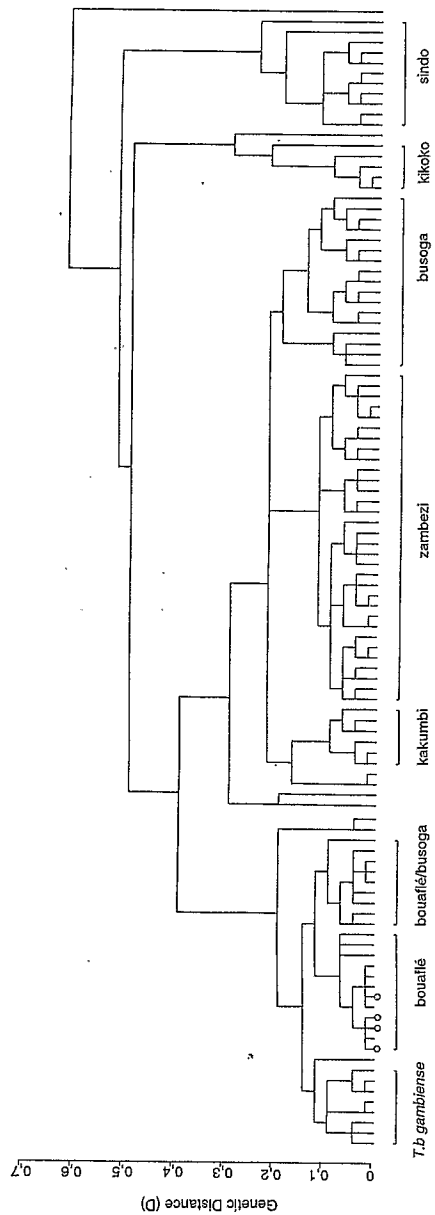

Fig. 2. Dendrogram summarizing the intraspecific taxonomy of *T. brucei*. Branch end points correspond to zymodemes defined by isoenzyme characterization studies. The major groups defined are: *T. b. gambiense*, corresponding to Group 1 *T. b. gambiense; bouaflé*, corresponding to *T. b. brucei* in West 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. [22, 47], Godfrey et al. [30], Stevens & Godfrey [41] 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 [97] by allelic interpretation of ten enzyme systems (Stevens et al. [35]).

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 [22, 25, 28, 30, 35, 49-51, 111]. 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) [22], and this result was confirmed by RFLP analysis [28, 49, 111]. The human pathogens are more closely related to their 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 [115]; however, *T. b. brucei/T. b. rhodesiense* by no means constitutes a panmictic group in East Africa [72].

T. b. rhodesiense stocks in general are highly variable and each epidemic focus appears to have its own set of associated trypanosomes [22, 25, 27, 28, 30, 32, 33, 35, 37, 39, 51]. Ormerod [116] 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 [22, 30, 35, 37, 50, 51]. 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 [28, 130].

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; [22, 30, 35, 47]). 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 [117].

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 infections 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 [24, 26, 34, 53, 118-121, 128]. 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. Figure 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 [24, 128, 129].

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 [122, 123]. 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 [28, 51]. 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 [28]. Reservoir hosts that remain contagious in the longterm 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 [29, 35, 53, 55, 62, 94]. For example, stocks from Cameroon had a divergent gene for VSG LiTat 1.3, which is the antigen targeted in the CATT [124], while stocks from the Moyo focus in north-west Uganda lacked another *gambiense-*

specific VSG gene, AnTat 11.17 [36]. Thus, Group 1 *T. b. gambiense* appears to originate from a single strain, which spread widely and then diverged locally by mutations [19].

For rhodesian sleeping sickness, the story is more complex. Three areas have been intensively studied: Busoga in south-east Uganda [25, 27, 37, 50, 51, 75], the Lambwe valley in south-west Kenya [28, 32, 33], and the Luangwa valley in north-eastern Zambia [22, 35, 50]. Limited data is also available from foci in Tanzania and Ethiopia [22, 39, 130]. 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 [25, 28].

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; [25, 30, 37, 51]). This is an example of epidemic clonal propagation from an underlying sexual population [71, 72, 77].

On the other hand, new as well as old strains may be associated with an outbreak [25, 28, 37, 95]. 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 [33, 83]. 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 [25, 28, 32, 33, 37, 95]. Since genetic exchange in *T. brucei* occurs in the fly [112], this was a possible 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 [61, 72, 77, 83, 114]. Part of this controversy has arisen from attempting to combine data from unrelated epidemics, instead of considering each focus as a separate population [72].

Conclusion

From the panmictic view of *T. brucei* originally proposed by Tait [23], the consensus at present is that *T. b. brucei/T. b. rhodesiense* are basically sexual, but undergo periods of clonal expansion [71, 72, 125], while the majority of evidence suggests a lower degree of genetic exchange in classical *T. b. gambiense*.

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PROGRESS
IN HUMAN AFRICAN TRYPANOSOMIASIS,
SLEEPING SICKNESS

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Human African trypanosomiasis (HAT) or sleeping sickness is an old disease now considered to be as reemergent. HAT is endemic in 36 sub-Saharan African countries, in areas where tsetse flies are found. The public health importance of HAT is underestimated, but the disease causes severe social disruption in many rural areas.

The present publication covers new findings about HAT, from biology to clinical features, diagnosis and treatment.

Written by specialists who are very experienced in their respective fields, the contributions provide an indispensable tool for all practitioners and scientists as well as those working in this field, as others interested by host-parasite relationship or chemotherapy.

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