1 **Molecular Epidemiology of African Sleeping Sickness**

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3 **GEOFF HIDE[a] AND ANDREW TAIT[b]**

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5 [a]*Centre for Parasitology and Disease, Biomedical Sciences Research Institute, School of*

6 *Environment and Life Sciences, University of Salford, Salford, UK, M5 4WT*

7

8 [b]*Faculty of Veterinary Medicine, Division of Infection and Immunity, University of Glasgow,*

9 *Bearsden Road, Glasgow, G61 1QH, Scotland, U.K.*

10

11 Corresponding Author:

12 Geoff Hide

13 Centre for Parasitology and Disease, Biomedical Sciences Research Institute, School of

14 Environment and Life Sciences, University of Salford, Salford, UK, M5 4WT

15 Telephone No:          0161 295 3371

16 Fascimile No:          0161 295 5015

17 E-mail No:          g.hide@salford.ac.uk

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1    SUMMARY

2    Human sleeping sickness in Africa, caused by *Trypanosoma brucei* spp. raises a number of

3    questions. Despite the widespread distribution of the tsetse vectors and animal

4    trypanosomiasis, human disease is only found in discrete foci which periodically give rise to

5    epidemics followed by periods of endemicity   A key to unravelling this puzzle is a detailed

6    knowledge of the etiological agents responsible for different patterns of disease – knowledge

7    that is difficult to achieve using traditional microscopy. The science of molecular

8    epidemiology has developed a range of tools which have enabled us to accurately identify

9    taxonomic groups at all levels (species, subspecies, populations, strains and isolates). Using

10   these tools, we can now investigate the genetic interactions within and between populations

11   of *Trypanosoma brucei* and gain an understanding of the distinction between human- and

12   nonhuman-infective sub species. In this review, we discuss the development of these tools,

13   their advantages and disadvantages and describe how they have been used to understand

14   parasite genetic diversity, the origin of epidemics, the role of reservoir hosts and the

15   population structure. Using the specific case of *T.b.rhodesiense* in Uganda, we illustrate how

16   molecular epidemiology has enabled us to construct a more detailed understanding of the

17   origins, generation and dynamics of sleeping sickness epidemics.

## INTRODUCTION

Human sleeping sickness in Africa is a puzzling disease. It is caused by the parasite *Trypanosoma brucei* (see table 1). Despite the widespread distribution of the tsetse vectors and animal trypanosomiasis, human disease is found in very specific foci and appears as epidemics followed by periods of endemicity (reviewed in Hide, 1999). The locations of these foci are very stable over time but occasionally new epidemics emerge in new localities. For example, the Busoga focus of Uganda has had epidemics recorded from 1900 to the present day while a new epidemic has emerged in the nearby Soroti region – an area free of sleeping sickness prior to 1998 (Welburn *et al.,* 2001). An understanding of the factors which cause these epidemics could clearly lead to an understanding of how to eradicate the disease. Sleeping sickness was one of the earliest examples of the application of molecular epidemiology to a parasitic disease and, as will be seen, some important insights have been made into the origins and dynamics of these epidemics. In this review, we will trace the development of molecular epidemiological approaches with a particular emphasis on African sleeping sickness. As the journal "Parasitology" has played a significant role in publishing these developments it is an appropriate topic for the centenary issue.

## MOLECULAR EPIDEMIOLOGY

The molecular epidemiology of parasites describes the application of DNA based techniques to the understanding of parasite epidemiology and diversity (Hide & Tait, 1991). The approach was prompted by an emerging understanding that parasites could not be easily packaged into groups of organisms that produced defined diseases in defined hosts. Instead, there were complex epidemiological cycles involving multiple hosts, sometimes multiple

diseases, reservoir hosts, specific strains, emerging drug resistance, genetic exchange between strains and even the apparent emergence of new parasite species. Thus tools were needed which could be used to gain a higher level of resolution than morphology in defining the organisms involved. In fact, the discipline of molecular epidemiology strictly emerged from several earlier strands of research. Principally this was the use of phenotypic approaches such as isoenzyme (or allozyme) analysis as a tool to study the epidemiology of parasitic infections. One of the key epidemiological questions in parasitology is determining the correct identity (diagnosis) and taxomomic level of a given parasite (eg. species, subspecies, strains, variants). This has often proved challenging especially in the case of microscopic parasites (e.g. parasitic protozoa) where morphological distinctions are not easily identified. Two linked problems exist – defining the scope of a taxonomic unit and establishing unambiguous markers for each unit. The scope of a taxonomic unit could be relatively easily established in the absence of any form of gene exchange between units such as occurs in clearly distinct species or clonally reproducing parasites. However in earlier studies the precise identity of parasitic species or the role of gene exchange was often not known. An inability to clearly define taxonomic units therefore makes it difficult to identify markers that can be used diagnostically to distinguish that taxonomic unit. There is unfortunately a degree of circularity in the problem. There are two levels of identification needed: (1) diagnosis – generally referring to diagnostic tools capable of discriminating to the species level and defining a particular association between causative agent and disease and (2) epidemiological tools – generally referring to markers that can be used at the sub-species or strain level to investigate detailed interactions within parasite transmission cycles. This problem of circularity in developing appropriate markers can be overcome by using independent markers that are not linked to the, often very subjective, medical criteria by

1    which parasites were traditionally classified. DNA based molecular methods offered a

2    potential solution to these problems.

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4    FROM BIOCHEMICAL TO MOLECULAR EPIDEMIOLOGY

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6    The advent of protein based technologies such as gel electrophoretic separation of

7    isoenzymes (allozymes) enabled distinction of morphologically identical organisms and could

8    be used as a tool for genetic studies. These approaches were initially used with the free-

9    living protozoa both as markers for studying genetic exchange and for defining species

10   (previously defined as syngens) as illustrated by work with *Paramecium aurelia* (Tait, 1970a;

11   Tait, 1970b). Key centres in Edinburgh, London and subsequently Bristol then applied these

12   approaches to investigating the genetics and taxonomy of the medically important protozoa,

13   *Plasmodium* and *Trypanosoma*. For example these approaches were used for identifying

14   different levels of taxonomic units (eg species, subspecies and strains) in *Plasmodium*

15   (Carter & Walliker, 1975) and *Trypanosoma* spp (Kilgour and Godfrey, 1973; Miles *et al.,*

16   1977; Godfrey ,1978; Gibson *et al.,* 1978; Gibson *et al.,* 1980; Tait *et al.,* 1984, 1985).

17   Furthermore, using these techniques, genetic exchange was shown to occur in *Plasmodium*

18   spp (Walliker *et al.,* 1971, 1973) and *Trypanosoma spp* (Tait,1980; Gibson *et al.,* 1980; Jenni

19   *et al.,* 1986; Gibson *et al.,* 1989).

20   When used to analyse the African trypanosomes causing human sleeping sickness, these

21   approaches revealed a complex taxonomic situation. The classical taxonomy described three

22   subspecies, *Trypanosoma brucei brucei*, (non-human infective) *Trypanosoma brucei*

23   *rhodesiense* (East African, human infective) and *Trypanosoma brucei gambiense* (West

24   African, human infective). Multilocus enzyme electrophoresis (MLEE) studies revealed that

1   there were clearly subgroups or strains within both *T.b.gambiense* and *T.b.rhodesiense* as

2   well as a high degree of variation between isolates of *T.b.brucei* (Gibson *et al.,* 1980; Tait *et*

3   *al.,* 1984, 1985). Furthermore, these studies showed that different levels of taxonomy

4   appeared to be operating – the genetic relationships showed that *T.b.gambiense* appeared to

5   behave like a separate subspecies while *T.b.rhodesiense* appeared to be a host range

6   variant of *T.b.brucei.*

7   It was becoming apparent that the question of what defines a species in parasitic protozoa

8   (and other micro-pathogens) was a complex one to address. Traditionally, the species

9   concept is based on presence or absence of genetic exchange between taxonomic units. In

10  the case of *T.brucei* sspp, the extent of the occurrence of genetic exchange in natural

11  populations was unclear. Tibayrenc *et al.,* 1990 conducted a reanalysis of MLEE data from a

12  wide range of pathogens including *T.brucei* and *Plasmodium* and proposed a clonal theory,

13  based on a range of measures such as high levels of heterozygosity, linkage disequilibrium

14  and over representation of particular genotypes. In the case of *T.b.rhodesiense* there was

15  evidence for clonality but the results with *T.b.brucei* were less convincing. Further analysis of

16  a large collection of isolates from across Africa (Mathieu-Daude & Tibayrenc, 1994) was

17  interpreted as showing limited genetic exchange although geographical sub-structuring could

18  be an explanation given the diversity of the origins of the strains. These conclusions

19  conflicted with published data showing the existence of genetic exchange (eg Tait and

20  Turner, 1990). Analysis of a large collection of isolates from the Lambwe Valley focus, in

21  kenya, provided a partial resolution of the debate by showing that the population had an

22  epidemic population structure (Maynard-Smith *et al.,* 1993) i.e. that is there was underlying

23  genetic exchange masked by the local expansion of a small number of genotypes. Further

24  analysis of populations from Cote d'Ivoire, Zambia and Busoga, Uganda, (Stevens and

1    Tibayrenc, 1995) showed a diversity of population structures with evidence for clonality in

2    some populations but an epidemic structure in others (Uganda). This diversity was also

3    illustrated by MLEE analysis of sympatric stocks collected during a sleeping sickness

4    epidemic in Tororo, Uganda (Figure 1). In this study, it was demonstrated that the population

5    structure of the *T.b.brucei* stocks appeared to conform to a random mating population

6    structure while the population structure of *T.b.rhodesiense* was recognized as epidemic (Hide

7    *et al.,* 1994). The debate over the role of genetic exchange in *T.brucei* was not fully resolved

8    using MLEE.

9    MLEE is a highly informative technique and enabled epidemiological analyses to be based on

10    a genetic interpretation, with the essential conclusions for African trypanosomes remaining

11    unchallenged today. However, these methods require relatively large quantities of pure

12    parasite material and so are cumbersome for large scale epidemiological analysis. Initially,

13    blood from patients and animals had to be isolated, followed by several passages through

14    mice (or in culture) and further mouse amplification to produce enough material for analysis.

15    In some cases, the Kit for In Vitro Isolation (KIVI) of trypanosomes has been used to isolate

16    trypanosomes (Truc *et al.,* 1992). During these processes, cloning from individual

17    trypanosomes was also often necessary with several years work required to analyse an

18    appropriately sized sample set.  An additional problem associated with these methods is the

19    potential of the methods to select for particular types of trypanosomes, such as those better

20    adapted to growth in mice, and thereby introduce bias into the distribution of parasites

21    recovered (Koffi *et al.*, 2007).

22    Initially, DNA technology was focussed on developing diagnostic tools to identify

23    *Trypanosoma* species. As it is the subject of another review in this issue (Gibson, 2009), it

24    will not be considered further here and we will consider the development of DNA based tools

1    for measuring intraspecific variation (Table 2). Initial DNA studies on intraspecific variation in

2    *T.brucei* were based on analysis of restriction enzyme site variation in the kinetoplast DNA

3    (Borst *et al.,* 1981). However with limited stocks available and a relatively small number of

4    restriction enzyme sites covered, it was not possible to discriminate between the three

5    subspecies. A larger scale analysis (Gibson *et al.,* 1985) however demonstrated that a

6    difference could be detected in stocks originating from East and West Africa. Restriction

7    Fragment Length Polymorphism (RFLP) of VSG antigen genes and numerical taxonomy,

8    based on those RFLPs, revealed that *T.b.gambiense* was quite distinct from *T.b.rhodesiense*

9    or *T.b.brucei* but that the latter two subspecies could not be distinguished (Paindavoine *et al.,*

10   1986). However, it was discovered that two forms of *T.b.gambiense* existed (Paindavoine *et*

11   *al.,* 1986; 1989) one of which was probably the classical *T.b.gambiense* (Type 1) and one

12   which has been called either non-gambiense or Type 2 or "Bouafle" (Gibson, 1986;

13   Paindavoine *et al.,* 1989; Gibson, 2007). This latter group is relatively rare and may constitute

14   a variant of *T.b.brucei* that has acquired human infectivity (Gibson, 2007).  RFLP methods

15   were developed using repetitive DNA sequences including the ribosomal RNA genes to

16   investigate RFLP variation using multiple loci (Hide *et al.,* 1990). Although these data could

17   not be interpreted genetically and could not detect mixtures of trypanosomes, they offered a

18   highly discriminatory approach to "fingerprinting" trypanosome isolates. The RFLP data

19   combined with numerical taxonomy clearly distinguished the two types of *T.b.gambiense* and

20   showed that the Type 2 appeared to be a subset of West African *T.b.brucei* strains (Hide *et*

21   *al.,* 1990). Furthermore, comparison of two *T.b.rhodesiense* foci in Zambia and Uganda

22   showed that there were two genetically distinct groups in the two foci (Hide *et al.,* 1991). A

23   detailed analysis of the 1988 sleeping sickness epidemic in Tororo, Uganda, showed that the

24   human isolates (ie *T.b.rhodesiense*) were genetically homogeneous and could be clearly

distinguished from non-human isolates (ie *T.b.brucei*) by numerical taxonomy (Hide *et al.,* 1994) using RFLP data. Furthermore, the *T.b.rhodesiense* stocks were found to be frequent in cattle and were closely related to isolates from previous epidemics since the 1960s in that region. Combining molecular and traditional epidemiological data it was shown that humans were five times more likely to acquire sleeping sickness via cattle than from other humans (Hide *et al.,* 1996), thus demonstrating the importance of cattle as a reservoir for sleeping sickness during an epidemic (Hide, 1999). Comparison of epidemic and endemic areas of sleeping sickness (Hide *et al.,* 1998) and areas free of sleeping sickness (Hide *et al.,* 2000) revealed the presence of the same human infective strains in patients, cattle and tsetse suggesting that the presence of a human infective strain was not the only factor determining whether an epidemic developed.

It was observed that *T.b.rhodesiense* isolates circulating in the Tororo region of Uganda were genetically homogeneous whilst the sympatric *T.b.brucei* isolates were much more diverse (Hide *et al.,* 1994). This raised the question as to the contribution of genetic exchange in "field" populations of trypanosomes. High levels of genetic exchange would facilitate the "spread" of phenotypes, such as human infectivity and drug resistance, potentially into new populations and could possibly explain the emergence of new epidemics. This collection of stocks offered an opportunity to investigate this question since they were isolated at the same place and time. However, as suitable DNA based techniques were not available, it was necessary to resort to MLEE analysis to show that genetic exchange was frequent in *T.b.brucei* but epidemic in *T.b.rhodesiense* (Hide *et al.,* 1994).

This suggested that genetic exchange could contribute to the genetic variation in trypanosome isolates in the field and importantly could contribute to the epidemiology of the disease. Two problems beset further analysis of these questions. Firstly, the inability to

1     genetically interpret multilocus RFLP markers and, secondly, despite DNA based methods

2     providing higher levels of resolution, the requirement for sufficient pure parasite material for

3     analysis.

4

5     PCR BASED MOLECULAR EPIDEMIOLOGICAL TOOLS

6     The advent of PCR to amplify and identify DNA at source or from small amounts of parasite

7     material – such as a single trypanosome (Macleod *et al.,* 1997; Cox *et al.,* 2005) - offered

8     tremendous advantages particularly in the area of diagnosis. In terms of epidemiological

9     analysis, where multilocus analysis was clearly important, this was more difficult. Some

10     single locus typing such as PCR-RFLP were found to be of limited use (Tilley & Hide, 2001).

11     Random Amplification of Polymorphic DNA (RAPD) analysis was one of the initial solutions to

12     PCR amplification of multiple loci (Mathieu-Daude *et al.,* 1995; Stevens *et al.,* 1995). This

13     technique relied on the use of a short single PCR primer which randomly annealed to the

14     DNA sample, a banding pattern was produced for each stock and numerical taxonomy used

15     to assess the relatedness of stocks with each other. This was a good multilocus approach,

16     useful with small amounts of DNA, which probably sampled the full extent of the genome

17     rather than being restricted to certain genes or parts of the genome. However, this method is

18     highly susceptible to contaminating host or other DNA – bands will be generated from any

19     DNA template, it is not possible to interpret genetically and is unable to detect mixed

20     infections of trypanosomes. A similar approach, Amplified Fragment Length Polymorphism

21     (AFLP), uses PCR primers based on restriction enzyme sites to look for variation between

22     trypanosome stocks (Agbo *et al.,* 2002) and has the same advantages and disadvantages of

23     RAPD analysis. To overcome the problem of contaminating host DNA, a technique termed

24     MGE-PCR (Mobile Genetic Element PCR) was developed utilising the positional variation of

1    mobile genetic elements to detect genetic variation between parasite isolates (Hide & Tilley,

2    2001; Terry *et al.,* 2001; Tilley *et al.,* 2003). Using a single primer designed to be specific for

3    the repeat regions of the RIME mobile genetic element of *T.brucei*, amplification of bands

4    between adjacent RIME elements generates a banding pattern which varies from stock to

5    stock based on differences in the positions of RIME elements and so can be used to identify

6    groups of similar isolates by numerical taxonomy. This approach has the advantage that it is

7    PCR-based, specific to *T.brucei* DNA, multilocus, representative of a significant portion of the

8    genome and generates a single "fingerprint" for each isolate or stock. The disadvantages are

9    that it cannot detect mixed infections and it cannot be interpreted genetically.

10   The markers that offer the greatest prospects are the mini- and micro-satellite markers (eg

11   Macleod *et al.,* 1997; Biteau *et al.,* 2000). These short repeated sequences vary in length or

12   sequence between trypanosome strains and can be amplified and analysed by population

13   genetic methods (MacLeod *et al.,* 2001a). Such markers can be used to detect mixed

14   infections (MacLeod *et al.,* 1999; Koffi *et al.*, 2007), are highly specific and sensitive tools for

15   detection of *T.brucei ssp* and can be used without the need to amplify parasites in rodents

16   (Morrison *et al.,* 2007). The development of PCR based methods for detailed analysis of

17   trypanosome stocks has opened up many avenues of research. In the remainder of this

18   review we will focus on the contributions of these techniques to our understanding of the

19   diversity of trypanosome isolates in the field, the role of genetic exchange in the

20   epidemiology of sleeping sickness, the nature of human infective strains and the generation

21   of sleeping sickness epidemics.

22   Many studies have examined the diversity of *T.brucei* stocks obtained from field isolates.

23   What is clear is that the initial biochemical and molecular studies were correct in suggesting

24   substructuring within each of the subspecies *T.b.gambiense* and *T.b.rhodesiense*. Analyses

1    of stocks of *T.b.gambiense* Type 1 clearly show this group to be genetically highly

2    homogeneous within a focus and distinguishable from *T.b.brucei* or *T.b.rhodesiense* by

3    RAPD (Jamonneau *et al.,* 2002) microsatellite analysis (Biteau *et al.,* 2000; Jamonneau *et*

4    *al.,* 2002), minisatellite analysis (MacLeod *et al.,* 2001a; MacLeod 2001b; MacLeod 2001c),

5    MGE-PCR (Simo *et al.,* 2005) and AFLP (Simo *et al.,* 2008). In most cases, Type 1

6    *T.b.gambiense* has been shown to be genetically homogeneous despite differing symptoms

7    in humans being associated with identical parasite genotypes (Jamonneau *et al.,* 2002).

8    However, more detailed studies have revealed significant differences between isolates from

9    different foci within the Type 1 group (Truc *et al.,* 2002; Morrison *et al.,* 2008; Koffi *et al.,*

10    2009). Interestingly, a new group of *T.b.gambiense* was identified by micro- and mini-

11    satellites that was associated with asymptomatic disease in humans (Jamonneau *et al.,*

12    2004a). It is clear from the microsatellite studies that *T.b.gambiense* Type 1 is clonal and

13    very limited, if any, genetic exchange occurs in the field (Morrison *et al.,* 2008; Koffi *et al.,*

14    2009).

15    The clonal nature of *T.b.gambiense* has allowed it to be clearly identified and shown to be

16    zoonotic. Early isoenzyme studies demonstrated its presence in dogs, pigs, bovines and wild

17    game (Mehlitz *et al.,* 1982) suggesting a possible animal reservoir. Recent research using

18    PCR based tools have shown that pigs (Njiokou *et al.,* 2006; Simo *et al.,* 2006) and wild

19    fauna (Njiokou *et al.,* 2006) have the potential to be animal reservoirs. Although in another

20    study, using microsatellite analysis, pigs were not considered to be an active reservoir

21    (Jamoneau *et al.,* 2004b). It is also possible that untreated, parasitologically unconfirmed,

22    seropositive individuals could also act as a human reservoir (Garcia *et al.*, 2006; Checchi *et*

23    *al.*, 2008). In a clonal pathogen, such as *T.b.gambiense*, the lack of genetic exchange is

1  helpful since it ensures a reasonable level of stability and reproducibility in genetic markers

2  for use in epidemiological studies.

3  As with *T.b.gambiense*, minisatellite analysis has been used to investigate whether

4  *T.b.rhodesiense* is clonal (MacLeod *et al.,* 2000). When the sympatric stocks from Tororo,

5  Uganda (Hide *et al.,* 1994), were examined *T.b.rhodesiense* was found to be clonal

6  suggesting that little or no genetic exchange is occurring in this population (MacLeod *et al.*,

7  2000). This is in contrast with earlier MLEE studies, using the same stocks, that suggested it

8  was epidemic (Hide *et al.,* 1994) – that is, genetic exchange masked by the amplification of a

9  small number of genotypes. This discrepancy might be explained by the more detailed and

10 sophisticated analyses available using minisatellites.

11 However, as predicted by the earlier MLEE and RFLP studies, genetic differences between

12 different geographical populations can be clearly detected by minisatellite DNA analysis such

13 as between Uganda and Zambia (MacLeod *et al.,* 2001b) and using polymorphism in the

14 SRA (Serum Resistant Associated) gene between populations in Uganda and Malawi or

15 Zambia (Gibson *et al.,* 2002; McLean *et al.,* 2004).

16 The SRA gene (De Greef *et al.,* 1989) has been shown to be capable of transforming a non

17 human-infective *T.brucei* to a human-infective phenotype (Xong *et al.,* 1998). This gene is

18 present in all human-infective field samples from East Africa and absent in animal isolates

19 and has consequently been used as a diagnostic tool for *T.b.rhodesiense* (Welburn *et al.,*

20 2001; Gibson *et al.,* 2002). Although, this gene looks promising as the candidate gene

21 determining human infectivity in *T.brucei*, it is clearly absent from both *T.b.gambiense* Type 1

22 and 2 (Turner *et al.,* 2004) showing that there is more than one mechanism that determines

23 human infectivity. There is growing evidence to suggest that in at least some epidemics, such

1    as the 1998 epidemic in Soroti in Uganda, that the spread of trypanosomes expressing the

2    SRA gene may be responsible for the generation of epidemics (Welburn *et al.,* 2001).

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4    MOLECULAR EPIDEMIOLOGY: THE ORIGINS AND DYNAMICS OF *TRYPANOSOMA*

5    *BRUCEI RHODESIENSE* SLEEPING SICKNESS EPIDEMICS

6    The 1988 Tororo sleeping sickness epidemic in Uganda resulted in a legacy of trypanosome

7    stocks isolated during an epidemic (Hide *et al.,* 1994). In addition to analysis by RFLP, they

8    have been analysed using minisatellite markers (MacLeod *et al.,* 2000), MGE-PCR (Tilley *et*

9    *al.,* 2003) and the SRA gene (Welburn *et al.,* 2001). A clear distinction has emerged between

10    the human infective stocks and non human-infective stocks in this region. The finding that

11    cattle are an important reservoir for human infective stocks (Hide *et al.,* 1996) has been

12    confirmed by more recent studies (Welburn *et al.*, 2001). Isolates collected from humans ten

13    years on from this epidemic have been shown by MGE-PCR to contain some identical

14    genotypes thus confirming the stability of genotypes within this focus (Tilley *et al.,* 2003). A

15    new epidemic started in December 1998 in Soroti, Uganda (Fevre *et al.,* 2001), and is

16    currently ongoing and spreading north and west (Fevre *et al.,* 2005). Based on the evidence

17    that cattle are an important reservoir for sleeping sickness, it has been postulated that cattle

18    infected with human-infective *T.brucei* were imported from the Tororo District into Soroti

19    (Fevre *et al.,* 2001). These cattle were then fed upon by local tsetse, passed onto the local

20    people and the epidemic started. Statistical analysis of the location of sleeping sickness

21    cases in relation to the Soroti cattle market (Fevre *et al.,* 2001) and the presence of SRA

22    positive *T.brucei* isolated from the cattle at this market has supported this view (Welburn *et*

23    *al.,* 2001). Other studies conducted on *T.brucei* isolates collected from Soroti suggest that

24    they have a different clinical disease profile (eg more chronic presentation) to isolates

1    collected in Tororo and also the corresponding microsatellite profiles were found to be

2    different (McLean *et al.,* 2007). This supports the view that, although some of the isolates

3    have probably originated in Tororo, there may be other origins of these *T.brucei* isolates.

4    These results clearly show that the importation of cattle from a high risk area may have been

5    responsible for the generation of this new epidemic. It also shows the importance of tracking

6    cattle movements particularly from high risk areas, such as Tororo, and the identification of

7    the geographical locations of high risk areas.

8    Based on the extensive molecular epidemiological data from Uganda, it is possible to

9    formulate a model of how sleeping sickness foci are generated and how epidemics develop.

10   It is clear from the data that foci, such as the Busoga focus, must have the presence of a

11   human infective *T.brucei* strain ("*T.b.rhodesiense*").  Molecular epidemiological studies

12   suggest that this may be a single predominant clonal strain which remains stable over time

13   (although multiple clonal strains could also be present). New epidemics can be generated by

14   the influx of a human strain into an area previously occupied by only animal strains. This

15   could be by the importation of a reservoir species, such as cattle, infected with a human

16   strain. Local socio-political customs or events may determine the amplitude and duration of

17   the epidemic. Despite having a wide antigenic repertoire, clonality will ensure that any given

18   *T.b.rhodesiense* strain will have reduced genetic variability. Consequently, with time, a local

19   human population will become immune/less susceptible or subjected to health interventions

20   causing the epidemic to decline. On entering a naïve population, such as Soroti, clonal

21   human strains would spread quickly. It is clear that different foci appear to have genetically

22   different human strains (*T.b.rhodesiense* or *T.b.gambiense*) which could be generated either

23   by the SRA gene being introduced into a new genetic background or by the introduction of

24   one of the other mechanisms of human infectivity emerging in a previously non human-

infective strain. This mechanism could involve genetic exchange between a non human- and a human-infective strain. With time and selection through humans, emergent human strains might become established and generate new foci. The data suggest that in the field genetic exchange occurs but is masked by the epidemic spread of a small number of strains. The relatively small numbers of human infective strains, the relatively small number of foci and the long term stability of foci might suggest that there is a minimal effect of genetic exchange in generating new human strains and foci. However, in practice, the generation of even a single new focus creates a significant health issue. Important questions arise from this model. Are there low frequencies of genetic exchange generating new human-infective strains or is the generation of new human-infective strains by genetic exchange frequent but fixation of those strains within the population (to produce a focus) infrequent? Also, given the relatively limited data from foci in Zambia, Tanzania, Malawi and Mozambique, are the conclusions from the Ugandan studies typical for *T.b.rhodesiense*? As can be seen from this review, the molecular tools are now available to address such questions.

As with any model, this one generates more questions than it answers. We have come along way to understanding the generation of sleeping sickness epidemics but further questions need to be addressed. The future of molecular epidemiology will be based on our increasing ability to be able to conduct large scale epidemiological studies which will involve carefully controlled sampling strategies, application of high throughput whole genome technologies for both host and parasite, development of Single Nucleotide Polymorphism (SNP) chips, development of bioinformatics tools and integration with mathematical models of disease transmission. These approaches and technologies provide opportunities for the future and offer the prospect of addressing some of the questions raised in this review.

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1    TABLE LEGENDS

2

3    Table 1. Characteristics of the subspecies of *Trypanosoma brucei*

4

5    Table 2  Molecular epidemiological methods for analysing African trypanosomes. The table

6    shows some of the characteristics of each method: 1. Subspecies discrimination, ability to

7    discriminate between the three subspecies of *T.brucei*. 2. Identification of populations or

8    groups, the ability to define populations or groups within the subspecies. 3. Population

9    genetics, the ability to be interpreted genetically to enable population genetics to be carried

10   out. 4. Detection of mixed infections, able to detect mixed infections or not to be confounded

11   by the presence of mixed infections. 5. PCR based amplification of DNA, can be used directly

12   on blood or tissue samples without the need to amplify parasites in experimental animals or

13   *in vitro*. 6. Requires pure parasites, parasites need to be amplified and purified. MLEE,

14   Multilocus Enzyme Electrophoresis; RFLP, Restriction Fragment Length Polymorphism;

15   PCR-RFLP, PCR amplified RFLP; RAPD, Randomly Amplified Polymorphic DNA; AFLP,

16   Amplified Fragment Length Polymorphism; MGE-PCR, Mobile Genetic Element – PCR;

17   Minisatellites and Microsatellites, analysis of length polymorphism in highly repeated

18   sequences; SRA, detection of the Serum Resistance Associated gene which is found in

19   human infective *T.b.rhodesiense*.

20

21

1    FIGURE LEGEND

2    Figure 1 A map of the Busoga focus of *T.b.rhodesiense* sleeping sickness in Uganda. Recent

3    epidemics occurred in Tororo (1988 to date) and Soroti (1998 to date).