Human and Animal Trypanosomes in Côte d’Ivoire Form a Single Breeding Population

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Abstract

Background: Trypanosoma brucei is the causative agent of African Sleeping Sickness in humans and contributes to the related veterinary disease, Nagana. T. brucei is segregated into three subspecies based on host specificity, geography and pathology. T. b. brucei is limited to domestic animals (excluding some primates) throughout sub-Saharan Africa and is non-infective to humans due to trypanolytic factors found in human serum. T. b. gambiense and T. b. rhodesiense are human infective subspecies. T. b. gambiense is the more prevalent human pathogen, causing over 97% of human cases. Study of T. b. gambiense is complicated in that there are two distinct groups delineated by genetics and phenotype. The relationships between the two groups and local T. b. brucei are unclear and may have a bearing on the evolution of the human infectivity traits.

Methodology/Principal Findings: A collection of sympatric T. brucei isolates from Côte d’Ivoire, consisting of T. b. brucei and both groups of T. b. gambiense have previously been categorized by isoenzymes, RFLPs and Blood Incubation Infectivity Tests. These samples were further characterized using the group 1 specific marker, TgSGP, and seven microsatellites. The relationships between the T. b. brucei and T. b. gambiense isolates were determined using principal components analysis, neighbor-joining phylogenetics, STRUCTURE, FST, Hardy-Weinberg equilibrium and linkage disequilibrium.

Conclusions/Significance: Group 1 T. b. gambiense form a clonal genetic group, distinct from group 2 and T. b. brucei, whereas group 2 T. b. gambiense are genetically indistinguishable from local T. b. brucei. There is strong evidence for mating within and between group 2 T. b. gambiense and T. b. brucei. We found no evidence to support the hypothesis that group 2 T. b. gambiense are hybrids of group 1 and T. b. brucei, suggesting that human infectivity has evolved independently in groups 1 and 2 T. b. gambiense.

Introduction

The parasite Trypanosoma brucei is the causative agent of African Sleeping Sickness in humans and one of several pathogens that cause the veterinary disease Nagana. These diseases have a wide distribution across sub-Saharan Africa and affect some of the poorest areas of the world. T. brucei is traditionally segregated into three morphologically identical sub-species based on host, geography and pathology. T. b. brucei is limited to domestic and wild animals throughout sub-Saharan Africa and is non-infective to humans (and some primates) due to sensitivity to trypanosome lytic factors found in their serum [1]. T. b. gambiense and T. b. rhodesiense are human infective sub-species, named due to their relative geographic locations. While T. b. rhodesiense can infect humans due to possession of a serum resistance associated (SRA) gene [2], this gene is not present in the more prevalent West and central African trypanosome subspecies, T. b. gambiense [3–5], causative agent of over 95% of reported cases of sleeping sickness [6,7]. How this sub-species is able to resist lysis by human trypanolytic factors is still unknown, although there is a decrease in uptake of lytic factor due to modification of the HpHbR cell surface receptor that contributes to resistance [8–12]. The study of T. b. gambiense is complicated in that there are two distinct groups within the sub-species that can be delineated by genetics and several phenotypic characteristics [13]. Group 1 T. b. gambiense causes a chronic infection, is invariably resistant to human serum and is by far the more prevalent group of the two. It appears to be a largely a disease limited to humans, although some animal reservoirs have been described [14–19]. Conversely, group 2 T. b. gambiense are reported to be more virulent and exhibit a variable human serum resistance mechanism in a manner similar to T. b. rhodesiense [16,19]. Unlike group 1 T. b. gambiense, group 2 T. b. gambiense does not possess the modification to the cell surface receptor HpHbR to avoid lysis by human serum and appears to utilize a different method to counter lytic factors that is distinct
Table 1. Details of the isolates used in the study, including the isolate name, the host each strain was isolated from, the serum resistance profile resulting from a 5 rodent BIT (R = highly resistant, I = intermediate or sub-resistant and S = sensitive) and the geographic location of the isolate when collected within Côte d’Ivoire.

| Isolate Name       | Host         | Serum Resistance | Location          |
|--------------------|--------------|------------------|-------------------|
| MHOM/CI/78/TH112   | Human        | S                | Bouafle           |
| MHOM/CI/78/TH114   | Human        | R                | Bouafle           |
| MHOM/CI/78/TH126   | Human        | I                | Bouafle           |
| MSUS/CI/78/TSW065  | Pig          | S                | Bouafle           |
| MSUS/CI/78/TSW100  | Pig          | I                | Bouafle           |
| MSUS/CI/78/TSW113  | Pig          | I                | Bouafle           |
| MSUS/CI/78/TSW115A | Pig          | R                | Bouafle           |
| MSUS/CI/78/TSW168  | Pig          | R                | Bouafle           |
| MSUS/CI/78/TSW175  | Pig          | S                | Bouafle           |
| MSUS/CI/78/TSW18   | Pig          | R                | Bouafle           |
| MSUS/CI/78/TSW182  | Pig          | I                | Bouafle           |
| MSUS/CI/78/TSW19   | Pig          | I                | Bouafle           |
| MSUS/CI/78/TSW190  | Pig          | I                | Bouafle           |
| MSUS/CI/78/TSW196  | Pig          | S                | Bouafle           |
| MSUS/CI/78/TSW209  | Pig          | I                | Bouafle           |
| MSUS/CI/78/TSW251  | Pig          | S                | Bouafle           |
| MSUS/CI/78/TSW308  | Pig          | I                | Bouafle           |
| MSUS/CI/78/TSW332  | Pig          | S                | Bouafle           |
| MSUS/CI/78/TSW38   | Pig          | S                | Bouafle           |
| MSUS/CI/78/TSW390  | Pig          | S                | Bouafle           |
| MSUS/CI/78/TSW65   | Pig          | S                | Bouafle           |
| MHOM/83/DAL387     | Human        | R                | Bouafle           |
| MHOM/83/DAL598     | Human        | R                | Daloa             |
| MHOM/83/DAL642     | Human        | R                | Daloa             |
| MHOM/83/DAL645     | Human        | R                | Daloa             |
| MHOM/83/DAL403     | Human        | R                | Daloa             |
| MHOM/83/DAL633     | Human        | R                | Daloa             |
| MHOM/83/DAL672     | Human        | R                | Daloa             |
| MHOM/83/DAL654     | Human        | R                | Daloa             |
| MHOM/83/DAL596     | Human        | R                | Daloa             |
| MHOM/83/DAL607     | Human        | R                | Daloa             |
| MHOM/83/DAL609     | Human        | R                | Daloa             |
| MHOM/78/DAL069     | Human        | R                | Daloa             |
| MHOM/83/DAL595     | Human        | R                | Gagnoa            |
| MHOM/83/DAL543     | Human        | R                | Vavoua            |
| MHOM/78/TH1        | Human        | R                | Vavoua            |
| MSUS/CI/78/TSW155  | Pig          | S                | Vavoua            |
| MSUS/CI/78/TSW158  | Pig          | I                | Vavoua            |
| MSUS/CI/78/TSW187  | Pig          | I                | Vavoua            |
| MHOM/83/DAL542     | Human        | R                | Vavoua            |
| MHOM/84/DAL740     | Human        | R                | Vavoua            |
| MHOM/CI/82/DAL494  | Human        | R                | Vavoua            |

Table 1. Cont.

| Isolate Name       | Host     | Serum Resistance | Location          |
|--------------------|----------|------------------|-------------------|
| MHOM/Ci/52/ELIANE  | Human    | R                | Côte d’Ivoire     |

from group 1 T. b. gambiense or T. b. rhodesiense [20]. This group of T. b. gambiense has only been described in Côte d’Ivoire, Cameroon and Burkina Faso to date, and always in geographical areas where group 1 T. b. gambiense is also found [13,21]. This geographical overlap of the two groups of T. b. gambiense raises interesting questions as to whether they share a close genetic relationship and possibly the ability to undergo sexual recombination.

Investigation of the population genetics of T. b. gambiense would reveal if such a relationship exists, however most research on T. b. gambiense field populations has focused on the more prevalent group 1. Studies with isoenzymes and RFLPs have indicated that group 1 T. b. gambiense populations exhibit low genetic variation and appear distinct from T. b. brucei populations [16,19,21–26] and recent comprehensive microsatellite genotyping techniques appear to have confirmed this for some populations [27,28]. This is in contrast to high genetic variation found in T. b. brucei populations [16,19,22–26,29]. It has also been shown that while group 1 T. b. gambiense are clonal within a disease focus, there are genetic differences between isolates from different geographic locations [27]. During the decades of investigation into the population structure of T. brucei, a second group of human infective T. b. gambiense from Côte d’Ivoire and Burkina Faso (formally Upper Volta) were identified that possess different isoenzyme and RFLP profiles from the classical group 1 T. b. gambiense isolates. These isolates also showed greater genetic variation and appeared more similar to local T. b. brucei populations [16,19,22–26,29,30]. The greater variation in genetic markers and similarity to the T. b. brucei population suggested that this second group of T. b. gambiense might be genetically competent, in contrast to group 1. This has subsequently been confirmed by laboratory crosses between T. b. gambiense group 2 and both T. b. brucei and T. b. rhodesiense strains [31–33]. The possibility that different mating structures may exist in the two groups of T. b. gambiense has implications in evaluating the relationships between them and the evolution of several traits in the population, including human infectivity. It is possible that this trait has evolved twice in separate populations of T. b. gambiense or conversely evolved once but due to mating events has become invariant in group 1 and variable in group 2 T. b. gambiense.

A recent study of microsatellite multi-locus genotypes from many T. brucei isolates from across Africa has found that group 1 T. b. gambiense form a clade separate from T. b. brucei, T. b. rhodesiense and group 2 T. b. gambiense [29]. Group 2 T. b. gambiense individuals formed a cluster positioned between T. b. brucei and group 1 T. b. gambiense, suggesting that group 2 T. b. gambiense may be a hybrid of group 1 and T. b. brucei and would perhaps share a mechanism for human infectivity. However, the T. b. brucei used in the study were mostly from East Africa and are not representative of West African T. b. brucei that are sympatric with the two T. b. gambiense groups. In order to resolve the relationships between the two groups of T. b. gambiense and local T. b. brucei, we used a microsatellite genotyping approach to examine a collection of T. brucei isolates from Côte d’Ivoire [15,16,34]. The isolates have been characterized by the Blood Incubation Infectivity Test (BIT) [35,36] and defined as
Figure 1. Genetic analysis of the *Trypanosoma brucei* population at the Côte d'Ivoire focus. a. Dendrogram of multi-locus genotypes (MLG) for the *T. brucei* isolates collected from several townships in Côte d'Ivoire, over the period of time 1978–1983 in addition to DAL972 and ELIANE. Bootstrap values from 100 iterations are indicated for branch nodes with a bootstrap value above 10. The presence of *TgSGP* using primers spanning the 5' and 3' ends is indicated by *. Isolates that displayed human serum resistance, the classical *T. b. gambiense* isoenzyme profile and possess *TgSGP* can be inferred to be group 1 *T. b. gambiense* (Red). Isolates that display a degree of resistance or were isolated from humans but did not possess the classical isoenzyme profile or *TgSGP* were determined to be group 2 *T. b gambiense* (Blue). Strains that exhibited no human serum resistance and were isolated from animals are most likely *T. b. brucei* (Black). b. Predicted structure of the Côte d'Ivoire *T. brucei* focus for the most likely population number (K=2). The proportion of each population that an isolate is a member of is indicated by red and blue in the histogram. c. Principal component analysis (PCA) of the Côte d'Ivoire *T. brucei* isolates using a pair-wise genetic distance comparison between each isolates MLG. The x-axis
highly resistant (all of 5 rodents infected), intermediate or sub-resistant (1–4 rodents were infected) or sensitive (none of 5 rodents infected) (Table S1 | Table S2). They have also been typed as classical gambiense (group 1) or non-gambiense by isoenzyme or RFLP profiles [16,21]. All of these samples were collected from a similar time period (1978–1983) and from a geographic area that contains both groups of *T. b. gambiense*. This collection of isolates allows the analysis of the sub-species genetics at this time point and the elucidation of the relationships between them, especially those between group 1 and 2 *T. b. gambiense*, which has implications for the evolution of human infectivity traits.

### Materials and Methods

#### Ethical Statement

Ethical approval for the human derived isolates in this study have been previously published [16,17].

#### Isolate Library

The collection of 43 *T. brucei* isolates used for population analysis was collected from a disease focus in Côte d’Ivoire that encompasses the townships of Gagnoa, Vavoua, Daloa and Bouaflé [16,21] from a range of hosts, including humans, between 1978 and 1983 (Table 1). The townships are approximately 50km apart. Samples were obtained as purified DNA or blood spots from stabilates on FTA cards that were washed and prepared as per the manufacturer’s instructions before analysis (Whatman).

The collection comprises both classical group 1 *T. b. gambiense* and group 2 *T. b. gamiense* as identified by isoenzymes and RFLPs [35,36], and also non-human infective *T. b. brucei* determined by biit. A 5 rodent Biit [37] was used to assess whether each isolate possessed the potential for human infectivity [11]. In addition to the main sample collection, the following reference isolates were also included: the group 1 *T. b. gambiense* genome strain MHOM/Ci/86/DAL972 [27,30], the 2 *T. b. gambiense* strain STIB386 (MHOM/Ci/78/TH114/78E) [also included in the main collection [39]] used in the *T. b. gambiense* genetic map [27,30] and the comprehensively studied group 1 *T. b. gambiense* strain MHOM/Ci/52/ELIANE were also included.

#### Genotyping

Samples were genotyped for population analysis using previously published nested PCR primers for eight microsatellite markers; Ch1/18, Ch1/D2/7, Ch2/PLC, Ch2/5, Ch5/JS2, Ch11/110, Ch3/I/J15/1 and Ch4/M12C12 [40]. The Ch4/ M12C12 marker proved to be monomorphic for these samples and was excluded. Markers on the same chromosome are at opposite ends of the chromosome to each other and are unlikely to be linked [27]. All of the primers used to amplify the microsatellites have been previously identified and verified for both *T. b. brucei* and *T. b. gambiense* populations [41]. The PCR conditions for each nested reaction were 95°C 50 secs, 55°C 50 secs and 65°C 60 secs, for 30 cycles. PCR was performed in a total volume of 30 µl with the primers at a final concentration of 10 μM each and Taq polymerase final concentration of 0.25 units/µl (Thermo Scientific). If a sample was homozygous at a marker, it was repeated, otherwise all reactions were performed once. To determine allele size, one of the second round primers was tagged using either AFAM or HEX fluorescent dye to allow accurate sizing on a capillary ABI sequencer against ROX labeled size standards (Dundee Sequencing Unit). The size of the tagged PCR product was measured to within 2bp using the Peak Scanning® software package (Applied Biosystems). Each distinct allele size was given a number and the two alleles for each locus were catalogued for each isolate. This allowed the creation of a multi-locus genotype (MLG) for each isolate. In addition to the microsatellite MLG, the samples were further characterized using the previously published diagnostic PCR for the presence/absence of the TgsGP gene [42,43] and an improved set of nested TgsGP primers that span the ‘‘s’’ and ‘‘g’’ regions of the gene [44]. The PCR conditions used for these primers were 95°C 50 secs, 55°C 50 secs and 65°C 120 secs, for 35 cycles. TgsGP PCR was performed in a total volume of 10 µl.

#### Population Analysis

The multi-locus genotype (MLG) of each isolate studied was used to create a dendrogram to infer relationships within the Côte d’Ivoire *T. brucei* population. Clustering calculator (http://www. biology.uabberta.ca/jbrzusto/cluster.php) was used to create a Phypill Drowtree string (analysed using neighbor-joining clustering and Canberra Distances). A bootstrap with 100 iterations was also generated. This Drowtree string was then converted into a dendrogram using Figtree software (http://tree.bio.ed.ac.uk/). Heterozygosity, Nei’s genetic distance and FST were calculated using the GenePop v4.0.10 [45–47] and LIAN v3.5 [47] software packages. The likelihood of replicated genotypes occurring due to sexual recombination was examined using MLGsim 2.0 [48], using Psex values calculated for each replicated MLG compared to 10,000 simulations for a population of the same size. Additionally, a principal component analysis (PCA) of the genetic distance was performed on the three populations using the GenAlEx software package [41]. k-means clustering was calculated from the eigenvalues from the PCA using the kmeans function of the R software package [49]. Finally, a sub-structuring modeling analysis was performed on the MLG using the STRUCTURE software package [50]. The models were formulated assuming an admixture model and correlated allele frequencies and a burn-in...
value of 50,000 and replication value of 250,000 [27,28]. Several estimates of population size (K) were investigated for K = 2 to 12. The most likely estimate for K was inferred using both the posterior probability of 5 repeat runs for each value of K [40,51,52] and an estimate of delta K [40].

Results

All studies of the population structure of group 1 T. b. gambiense to date have revealed foci to be largely clonal and relatively homogeneous [40]. Isoenzymes and RFLP analyses suggest that group 1 T. b. gambiense is genetically distinct from sympatric T. b. brucei and from group 2 T. b. gambiense. Here we have examined parasites isolated from both human patients and animals in four neighboring foci of infection in the Côte d'Ivoire in the late 1978 and 1983. The samples can be segregated into three different classes based on previous isoenzyme and RFLP studies and by the ability to resist lysis by human serum measured using the BIIT. Isolates that are invariably resistant to lysis and possess the classical isoenzymes of T. b. gambiense are classified as group 1 T. b. gambiense. A second group of parasites that also show resistance (either by BIIT or due to being isolated from a human host) but lack the isoenzyme markers characteristic of group 1 T. b. gambiense, are defined as non-gambiense or group 2 T. b. gambiense. Isoenzyme profiles of these isolates were highly variable [11]. The third group of isolates were sensitive to human serum in the BIIT and were not isolated from human patients; they were therefore classified as T. b. brucei and the isoenzyme profiles of these isolates were also highly variable [11].

TgSGP Genotyping

The cell surface receptor TgSGP has been proposed as a diagnostic marker of group 1 T. b. gambiense [52] and PCR targeting the 3' end of the gene was recommended to test for the presence of the gene as a diagnostic for T. b. gambiense group 1 [52]. Unfortunately, several group 2 T. b. gambiense are also positive for the published PCR diagnostic test, limiting its use in foci with both groups of T. b. gambiense [40]. Further work has now shown that the 3' end of the TgSGP gene is closely related to the VSG Tb10.v4.0178 and this VSG is likely the progenitor of this section of the TgSGP gene [47]. Tb10.v4.0178 is present in several strains, including T. b. brucei, T. b. rhodesiense and group 2 T. b. gambiense isolates [27,28], so there is a possibility of false positives when using the originally proposed primers. In our analysis using the original primers targeted to the 3' end of the TgSGP gene [27,28], 21 isolates were positive for the TgSGP gene diagnostic, of which, 18 were previously characterized as group 1 T. b. gambiense on the basis of isoenzymes and RFLPs. However, two isolates (MSUS/C1/78/TSW209 and MSUS/C1/78/TSW158) that would be putatively identified as group 2 T. b. gambiense on the basis of human serum resistance phenotype in the BIIT but non-classical isoenzyme profiles, also showed positive for the 3' end of the TgSGP gene. Additionally, a serum sensitive isolate (MSUS/C1/78/TSW308) was also positive. However, when using nested PCR primers that span both the 5' and 3' regions of the gene, the TgSGP gene was only shown to be present in the 18 isolates previously identified as group 1 T. b. gambiense (Figure 1a). This would suggest that the three non-group 1 T. b. gambiense isolates are false positives and possess either VSG Tb10.v4.0178 or a closely related VSG. To investigate the relationships between the isolates from this disease focus, the isolates were further characterized using microsatellite genotyping.

Microsatellite Genotyping

To investigate the relationships between the group 1 and 2 T. b. gambiense and T. b. brucei populations, the genotype of each of the 45 samples was determined using seven microsatellite markers. All seven markers are polymorphic, featuring several alleles at each locus (Table S1). For the entire sampled population, the majority of markers display a level of heterozygosity that is in agreement with Hardy-Weinberg distribution (Table 2). Using these multilocus genotypes (MLG), a dendrogram of relatedness was created to visualize the relationship between isolates in the study (figure 1a). The dendrogram revealed a discrete population containing only the 18 isolates characterized as group 1 T. b. gambiense by isoenzymes, RFLPs, BIIT and the presence of TgSGP. This clade also included the genome reference strain MHOM/CI/86/DAL972 and the well characterized group 1 strain MHOM/CI/52/ELIANE. The bootstrap value (100) indicates a high degree of support for the node that separates this group.

Table 3. Nei's genetic distance between the populations of group 1 and 2 T. b. gambiense and T. b. brucei at the Côte d'Ivoire focus.

|                  | T. b. brucei (N = 9) | Gp 2 T. b. gambiense (N = 16) | Gp 1 T. b. gambiense (N = 20) |
|------------------|----------------------|------------------------------|-----------------------------|
| T. b. brucei     | 0.000                | –                            | –                           |
| Gp 2 T. b. gambiense | 0.026                | 0.000                        | –                           |
| Gp 1 T. b. gambiense | 0.357                | 0.321                        | 0.000                       |

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Table 4. Fst proportion indicating genetic distance between the populations of group 1 and 2 T. b. gambiense and T. b. brucei at the Côte d'Ivoire focus.

|                  | T. b. brucei (N = 9) | Gp 2 T. b. gambiense (N = 16) | Gp 1 T. b. gambiense (N = 20) |
|------------------|----------------------|------------------------------|-----------------------------|
| T. b. brucei     | 0.000                | –                            | –                           |
| Gp 2 T. b. gambiense | 0.138                | 0.000                        | –                           |
| Gp 1 T. b. gambiense | 0.277                | 0.264                        | 0.000                       |

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remaining 27 isolates formed a separate cluster distinct from the
group 1 T. b. gambiense, containing both serum sensitive T. b. brucei
(n = 9) and serum resistant group 2 T. b. gambiense isolates (n = 18).
There is little bootstrap support for any of the branching nodes
within this clade, suggesting this cluster represents a single
population and that T. b. brucei and group 2 T. b. gambiense are
largely indistinguishable from each other.

Principal components analysis (PCA) was performed using the

generic distance of the isolates to create a multi-dimensional

comparison (figure 1c). PCA identifies the independent factors

from a data set that explain the maximum amount of correlation.

Plotting these factors allows the structure of a population to be

visualized. Further structure could be inferred by utilizing k-mean

clustering and the eigenvalues from the PCA. This identified two
distinct clusters; one containing T. b. brucei and group 2 T. b.
gambiense populations and a second containing only isolates

previously identified as group 1 T. b. gambiense. When analyzed
by village, there is no evidence for any sub-structuring in the group
2 and T. b. brucei populations based on geography (data not shown)
suggesting that the parasites within the townships are part of the
same transmission cycle. Also, while not included in the analysis
due to low sample numbers, similar genotypes are present in group
2 T. b. gambiense and T. b. brucei isolates from neighboring Burkina
Faso, suggesting that there is one meta-population spanning the
two countries (Table S1).

In order to further examine the population structure of the Côte
d’Ivoire focus, STRUCTURE modeling was performed using the
MLG data set. The most likely population number (K) was found
to be 2 when estimated using both posterior probabilities and delta
K (figure 1b) [47]. Isolates are either predominantly a member of a
population containing only group 1 T. b. gambiense or a population
containing group 2 T. b. gambiense and T. b. brucei. The removal of
the group 1 isolates and then re-modeling with STRUCTURE did
not indicate any further structure in the T. b. brucei/group 2 T. b.
gambiense isolates (up to K = 12, data not shown).

Population Analysis

In order to determine the population structure within each
clearly distinct cluster as defined by the dendrogram, PCA and
STRUCTURE, each cluster was examined separately. Cluster 1
contained the 18 isolates that had previously been identified as
group 1 T. b. gambiense isolates and have subsequently been shown
to all possess TgSGP. Cluster 2 contained all other isolates (n = 27),
i.e. serum sensitive T. b. brucei and the non-classical gambiense
isolates that showed any degree of serum resistance and could be
termed group 2 T. b. gambiense. To further examine the differences
between the sub-species and groups, cluster 2 was sub-divided into
those isolates that previously showed any degree of resistance in a
BIT (putative group 2 T. b. gambiense, n = 18) and those that were
highly sensitive and could be described as T. b. brucei isolates
(n = 9).

The relationship between these populations was analyzed using
two statistical tests, Nei’s genetic distance and FST. Both Nei’s

generic distance and the FST statistic indicate that both popula-
tions within cluster 2 are distinct from cluster 1 and that within
cluster 2, there is very little genetic difference between T. b. brucei
and group 2 T. b. gambiense populations (Tables 3 & 4). As there is
so little difference between populations, subsequent analysis
considered the cluster 2 as a single population.

If random mating is occurring within the Côte d’Ivoire
population then the number of heterozygous genotypes within
the population should be a predictable proportion based on the
number and frequency of alleles present in the population, in
agreement with Hardy-Weinberg expectations. Analysis of the

genotype frequencies in cluster 2 (the combined T. b. brucei and
group 2 T. b. gambiense isolates) revealed that none of the seven
markers significantly deviated from Hardy-Weinberg equilibrium
(Table 5). These data are consistent with the hypothesis that some
degree of mating is occurring within this cluster i.e. within and
between the group 2 T. b. gambiense and T. b. brucei. In contrast,
analysis of markers for cluster 1 shows that the group 1 T. b.
gambiense population display a strong deviation from Hardy-
Weinberg, with markers being either monomorphic or possessing
heterozygote excess strongly disagreeing with the Hardy-Weinberg
hypothesis (Table 6).

A second method to assess the degree of mating within cluster 2
is to estimate the amount of linkage disequilibrium in allele and
genotype frequencies. The presence of mating in a population
will cause alleles at unlinked loci to be inherited in a randomly
assorted manner, leading to linkage equilibrium, while a population
with little mating will exhibit linkage disequilibrium. Analysis of
linkage equilibrium revealed that the overwhelming majority of allele
combinations do not show significant linkage disequilibrium in
cluster 2 (Table 2). Additionally, both the T. b. brucei and group 2
T. b. gambiense populations exhibit a standardized Index of
Association (I) that tends towards zero. I is a function of the
rate of recombination and is zero for complete linkage equilibri-

um, so our data are consistent with linkage equilibrium existing in
these populations (Table 8). Linkage equilibrium is also exhibited
when the T. b. brucei and group 2 populations are analyzed
together, suggesting linkage equilibrium between the populations.

These data, combined with the Hardy-Weinberg analysis suggest
that there is a degree of mating and re-assortment both between
and within the T. b. brucei and group 2 T. b. gambiense populations.

The low variability in genotypes of the group 1 T. b. gambiense
isolates was tested for clonality using MLGsim. This indicated that all
observed Psex values for each MLG expansion were highly significant at
the <0.01 threshold, suggesting that all of the samples are clonal. Conversely, the
same analysis on the T. b. brucei and group 2 T. b. gambiense populations
shows that most genotypes have likely arisen due to sexual
recombination and only two of the samples possess possible clonal
genotypes at the p<0.01 significance threshold, MSUS/CI/78/
TSW158 and MSUS/CI/78/TSW117.

It would appear from these data that the T. b. brucei and group 2
T. b. gambiense populations exhibit some degree of mating, both
within and between sub-species. The group 1 T. b. gambiense
population is clonal and distinct from the sympatric T. b. brucei
and group 2 T. b. gambiense populations.

Discussion

These data reinforce studies from other foci that populations of

group 1 T. b. gambiense are likely clonal [27,28]. The reasons for the
clonality of group 1 T. b. gambiense are unclear as the genes
necessary for meiosis are present [37] and are expressed during
tsetse infection (Peacock and Gibson, unpublished data). Another
possible reason for the low frequency of mating observed in group
1 T. b. gambiense is that, due to the limited number of genotypes
present, the chances of a mixed genotype infection occurring in the
tsetse vector is unlikely. This precludes any mating other than
selling, which has been observed in laboratory crosses for T. b.
brucei [53]. However a high frequency of selfing would result in a population with an excess of homozygotes rather than the observed excess of heterozygotes, and is therefore unlikely.

The observed homogeneous population of group 1 T. b. gambiense could also be due to a bottleneck effect that occurred in the past or if group 1 T. b. gambiense underwent clonal expansion, possibly after it gained the human infectivity trait. Despite questions as to how the clonal population structure has developed, these data show that the group 1 T. b. gambiense genome reference strain MHOM/CI/86/DAL972 is clearly a good representative of group 1 strains from this area. Previous studies have shown that the MLG in the populations of various group 1 T. b. gambiense loci are significantly different from each other [27] and examination of the microsatellite alleles in this study with previous studies indicates that there are few shared alleles at each marker. Indeed, the necessity to include new markers specific to this study reinforces the fact that the focus possesses a different clonal population than that found in other foci.

This study has also shown that the STIB386 strain (MHOM/CI/78/TH114) used in genetic mapping studies [54] is a good population of group 1 to reinforce the fact that the focus possesses a different clonal population with an excess of homozygotes rather than the observed excess of heterozygotes, and is therefore unlikely.

Table 5. Hardy-Weinberg analysis for the combined population of group 2 T. b. gambiense and T. b. brucei at the Côte d’Ivoire focus (N = 25).

| Locus     | DF | $\chi^2$ | Probability | Significance |
|-----------|----|----------|-------------|-------------|
| Ch5/J52   | 15 | 21.021   | 0.136       | Not Significant |
| Ch1/110   | 3  | 1.800    | 0.982       | Not Significant |
| Ch11/51   | 3  | 1.073    | 0.982       | Not Significant |
| Ch1/18    | 15 | 18.798   | 0.223       | Not Significant |
| Ch1/D2/7  | 6  | 6.762    | 0.343       | Not Significant |
| Ch2/PLC   | 10 | 15.693   | 0.109       | Not Significant |
| Ch3/J15/1 | 6  | 6.382    | 0.559       | Not Significant |

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Table 7. Linkage disequilibrium for each genetic marker (statistically significant disequilibrium highlighted in bold) between pair wise polymorphic loci in the combined T. b. brucei and group 2 T. b. gambiense population at the Côte d’Ivoire focus (N = 25).

| Locus 1 | Locus 2 | Probability | Standard Error |
|---------|---------|-------------|----------------|
| Ch5/J52 | Ch11/110| 0.345       | 0.014          |
| Ch5/J52 | Ch11/51 | 0.053       | 0.006          |
| Ch11/110| Ch11/51 | 1.000       | 0.000          |
| Ch5/J52 | Ch1/18  | 0.299       | 0.029          |
| Ch11/110| Ch1/18  | 0.136       | 0.008          |
| Ch11/51 | Ch1/18  | 0.410       | 0.012          |
| Ch5/J52 | Ch1/D2/7| 0.172       | 0.019          |
| Ch11/110| Ch1/D2/7| 0.351       | 0.009          |
| Ch11/51 | Ch1/D2/7| 1.000       | 0.000          |
| Ch1/18  | Ch1/D2/7| 0.040       | 0.008          |
| Ch5/J52 | Ch2/PLC | 0.081       | 0.012          |
| Ch11/110| Ch2/PLC | 0.648       | 0.003          |
| Ch11/51 | Ch2/PLC | 0.573       | 0.018          |
| Ch1/18  | Ch2/PLC | 0.007       | 0.032          |
| Ch1/D2/7| Ch2/PLC | 0.293       | 0.010          |
| Ch5/J52 | Ch3/J15/1| 0.446      | 0.015          |
| Ch11/110| Ch3/J15/1| 0.527      | 0.023          |
| Ch11/51 | Ch3/J15/1| 0.431      | 0.021          |
| Ch11/110| Ch3/J15/1| 0.431      | 0.021          |
| Ch1/18  | Ch3/J15/1| 0.721      | 0.023          |
| Ch1/D2/7| Ch3/J15/1| 0.721      | 0.023          |
| Ch2/PLC | Ch3/J15/1| 0.525      | 0.021          |

The data were analyzed using the Genepop 4.0 software package.

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breeding population. There is a suggestion that this breeding population extends to neighboring Burkina Faso, as similar microsatellite alleles are found in both countries. However, this may be due to the seasonal, economic migration of the Mossi people between the two nations [55] that transports parasites rather than a large contiguous population. Nevertheless, group 2 T. b. gambiense can be seen as an extended host range variant T. b. brucei.

The degree to which mating occurs in a population is an important consideration due to the process acting as a major source of variation. While the human infectivity trait present in group 2 T. b. gambiense may have evolved once, it is now present in a wide range of genetic backgrounds. This is in contrast to group 1 T. b. gambiense which has limited genetic diversity at each discrete disease focus and hence a lower capacity to evolve. The genetic backgrounds of the group 2 T. b. gambiense infectivity trait may be in constant flux due to evidence suggesting the presence of mating within the population. This may go some way to explaining the emergence of a “new” group of T. b. gambiense in Côte d’Ivoire [56]. This new human infective group has been claimed as distinct from both group 1 T. b. gambiense and group 2 T. b. gambiense from the early 1980s. However, based on the heterogeneity and propensity for mating displayed by group 2 in our study, it is likely that this new group belongs to group 2, with the group 2 human serum resistance mechanism on a new genetic background. Unfortunately, the human serum resistance profile of the new group has not yet been described and it is unknown whether it also
Table 8. Standardised indices of association (Ia) for the populations of group 1 and 2 T. b. gambiense and T. b. brucei at the Côte d’Ivoire focus.

| Population analyzed | V0 | V2 | Ia |
|---------------------|----|----|----|
| Total Population (N = 45) | 2.739 | 1.476 | 0.143 |
| Combined T. b. brucei & gp 2 T. b. gambiense (N = 25) | 1.686 | 1.311 | 0.048 |
| Gp 2 T. b. gambiense (N = 16) | 1.716 | 1.227 | 0.067 |
| T. b. brucei (N = 9) | 1.441 | 1.360 | 0.099 |
| Gp 1 T. b. gambiense (N = 20) | – | – | ⋆ |

Data were analyzed using the UCLA software package to test the null hypothesis of linkage equilibrium. This null hypothesis is that the variance of loci that do not show linkage (V2) is equal to the expected variance of loci modelled under linkage equilibrium (V0).

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We have also shown that TgSGP is not present in the local group 2 T. b. gambiense or T. b. brucei populations by using nested primers that span the 5’ and 3’ ends of the gene. While this is counter to other published data [40], the primers described in the original diagnostic PCR are likely to show false positives due to similarity to the 3’ end of TgSGP and the VSG Th10:v.A0178 [52]. This observation adds further weight to the hypothesis that there is no mating between group 1 or group 2 T. b. gambiense, and that group 2 has not emerged as a result of hybridizing event.

The study presented here, in conjunction with other published data, suggests that human infectivity has arisen on at least three occasions (with some evidence of a fourth variant). In East Africa the evolution of SRA and the high levels of mating occurring there have allowed the human infectivity trait to spread through the T. brucei population, generating the T. b. rhodesiense sub-species [57,58]. However, there are also human infective trypanosomes in this area that do not possess SRA, suggesting a second novel mechanism [3,59]. In West and Central Africa, group 1 T. b. gambiense is the dominant cause of human African sleeping sickness. This group of parasites has evolved a constitutively expressed human serum resistance mechanism that does not depend on SRA. After evolution of this trait, the sub-species has expanded clonally in the human population. Just as in East Africa, there is also a human infective T. brucei population separate from the dominant form that appears to have a novel resistance mechanism. This group 2 T. b. gambiense population appears to be a host range variant of T. b. brucei and the human infectivity trait it possesses is freely transmitted among the local T. b. brucei population. The presence of mating amongst group 2 T. b. gambiense and T. b. brucei strains makes evolution of new traits more likely and may allow the human infectivity trait to be combined with new phenotypes that may increase its virulence. It also allows the human infectivity trait to persist in the local T. brucei animal reservoir through genetic transfer. Understanding whether the inter-sub-species mating and transfer of human infectivity traits exhibited in Côte d’Ivoire can also occur in other foci is becoming more pressing. In Uganda, the T. b. gambiense and T. b. rhodesiense foci are moving closer together and their ranges will begin to overlap [60,61]. Fortunately the possibility of mating between group 1 T. b. gambiense and T. b. rhodesiense appears unlikely due to the clonal nature of group 1 T. b. gambiense [27,28], and while T. b. rhodesiense mating with group 2 T. b. gambiense is certainly possible [33], the ranges of these two diseases are not converging at present. Taken together, all of these data indicate that T. brucei has a high zoonotic potential despite specific trypanolytic countermeasures that have been inherited by humans.

Supporting Information

Table S1 Overview of sample origin, host, human serum resistance phenotype and alleles present for each microsatellite marker. The presence absence of TgSGP is also indicated, whether it possesses the full gene (1) or the progenitor VSG (2). (XLSX)

Author Contributions

Conceived and designed the experiments: PC CD AM CMRT. Performed the experiments: PC CD AM CMRT. Analyzed the data: PC CMRT AM. Contributed reagents/materials/analysis tools: WG DM AT. Wrote the paper: PC WG DM AT.

References

1. Pays E, Vanhollebeke B (2009) Human innate immunity against African trypanosomes. Current Opinion in Immunology 21: 493–498.
2. Xong HV, Vanhamme L, Chamekh M, Chiminfoswenge CE, Van Den Abbeele J, et al. (1998) A VSG expression site-associated gene confers resistance to human serum in Trypanosoma rhodesiense. Cell 95: 439–446.
3. De Greef C, Chimfwembe E, Kihang’a Wabacha J, Bajyana Songa E, Hamers R (1992) Only the serum-resistant bloodstream forms of Trypanosoma brucei rhodesiense express the serum resistance associated (SRA) protein. Ann Soc Belg Med Trop Suppl 1: 13–21.
4. Rautwańska M, Chamekh M, Vanhamme L, Claes F, Magee S, et al. (2002) The serum resistance-associated gene as a diagnostic tool for the detection of Trypanosoma brucei rhodesiense. Am J Trop Med Hyg 67: 684–690.
5. Turner C, McLellan S, Lindergard L, Bisoni L, Tait A, et al. (2004) Human infectivity trait in Trypanosoma brucei: stability, heritability and relationship to sra expression. Parasitology 129: 445–454.
6. World Health Organization (2006) Weekly epidemiological record: relevé épidémique hebdomadaire.
29. Balmer O, Beadell JS, Gibson W, Caccone A (2011) Phylogeography and
34. Mehlitz D, Brinkmann U, Haller L (1981) Epidemiological studies on the animal
32. Jenni L, Marti S, Schweizer J, Betschart B, Le Page RW, et al. (1986) Hybrid
12. Symula RE, Beadell JS, Sistrom M (2012)
21. Paindavoine P, Zampetti-Bosseler F, Coquelet H, Pays E, Steinert M (1989)
20. Capewell P, Veitch NJ, Turner CMR, Raper J, Berriman M, et al. (2011)
19. Zillmann U, Mehlitz D, Sachs R (1984) Identity of
25. Godfrey DG, Kilgour V (1976) Enzyme electrophoresis in characterizing the
27. Morrison LJ, Tait A, McCormack G, Sweeney L, Black A, et al. (2008)
23. Mathieu-Daude F, Bicart-See A (1994) Identification of
24. Tait A, Babiker EA, Le Ray D (1984) Enzyme variation in
31. Tait A, Babiker EA, Le Ray D (1984) Enzyme variation in Trypanosoma brucei sp.
32. Stevens JR, Tilyrene M (1996) Trypanosoma brucei ssp: evolution, linkage and the
33. Mathieu-Daude F, Bicart-See A (1994) Identification of Trypanosoma brucei gambiense group I by a specific kinetoplast DNA probe. Am J Trop Med Hyg 50: 13–19.
34. Raymond M, Rousset F (1995) GENEPOP (version 1.2): population genetics software for exact tests and ecumenicism. Journal of Heredity 86: 248–249.
31. Tait A, Babiker EA, Le Ray D (1984) Enzyme variation in Trypanosoma brucei sp.
36. Mehlitz D (1978) Untersuchungen zur Empfänglichkeit von Mastomys natalensis für Trypanosoma (Trypanozoon) brucei gambiense. Tropenmed Parasit 10: 101–107.
35. Jackson AP, Sanders M, Berry A, McQuillan J, Auld MA, et al. (2010) The genetic sequence of Trypanosoma brucei gambiense, causative agent of chronic human African trypanosomiasis. PLoS Negl Trop Dis 4: e658.
34. Mehlitz D, Beadell JS, Sistrom M (2012) Trypanosoma brucei gambiense Group I is distinguished by a Unique Amino Acid Substitution in the HpdH Receptor Implicated in Human Serum Resistance. PLoS Neglect... e:1728.
33. Mathieu-Daude F, Bicart-See A (1994) Identification of Trypanosoma brucei gambiense group I by a specific kinetoplast DNA probe. Am J Trop Med Hyg 50: 13–19.
32. Jenni L, Marti S, Schweizer J, Betschart B, Le Page RW, et al. (1986) Hybrid Trypanosomes of the Subgenus Trypanozoon stocks by isoenzymes and sensitivity to human serum. Tropenmedizin und Parasitologie 32: 134–140.
31. Tait A, Babiker EA, Le Ray D (1984) Enzyme variation in Trypanosoma brucei sp.
36. Mehlitz D (1978) Untersuchungen zur Empfänglichkeit von Mastomys natalensis für Trypanosoma (Trypanozoon) brucei gambiense. Tropenmed Parasit 10: 101–107.
35. Jackson AP, Sanders M, Berry A, McQuillan J, Auld MA, et al. (2010) The genetic sequence of Trypanosoma brucei gambiense, causative agent of chronic human African trypanosomiasis. PLoS Negl Trop Dis 4: e658.
34. Mehlitz D, Beadell JS, Sistrom M (2012) Trypanosoma brucei gambiense Group I is distinguished by a Unique Amino Acid Substitution in the HpdH Receptor Implicated in Human Serum Resistance. PLoS Neglect... e:1728.
33. Mathieu-Daude F, Bicart-See A (1994) Identification of Trypanosoma brucei gambiense group I by a specific kinetoplast DNA probe. Am J Trop Med Hyg 50: 13–19.
32. Jenni L, Marti S, Schweizer J, Betschart B, Le Page RW, et al. (1986) Hybrid Trypanosomes of the Subgenus Trypanozoon stocks by isoenzymes and sensitivity to human serum. Tropenmedizin und Parasitologie 32: 134–140.
31. Tait A, Babiker EA, Le Ray D (1984) Enzyme variation in Trypanosoma brucei sp.
36. Mehlitz D (1978) Untersuchungen zur Empfänglichkeit von Mastomys natalensis für Trypanosoma (Trypanozoon) brucei gambiense. Tropenmed Parasit 10: 101–107.
35. Jackson AP, Sanders M, Berry A, McQuillan J, Auld MA, et al. (2010) The genetic sequence of Trypanosoma brucei gambiense, causative agent of chronic human African trypanosomiasis. PLoS Negl Trop Dis 4: e658.