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Multiple-strain infections of *Trypanosoma brucei* across Africa

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Abstract

It is becoming increasingly clear that parasitic infections frequently contain multiple strains of the same parasite species. This may have important consequences for the parasite dynamics in the host and thus alter disease and transmission dynamics. In *Trypanosoma brucei*, the causal agent of human African trypanosomiasis (sleeping sickness), multiple-strain infections have previously been demonstrated to occur. Here, we analyzed field isolates of *T. b. gambiense*, *T. b. rhodesiense*, and *T. b. brucei*, isolated throughout Africa to assess the commonness of multiple-strain infections across the natural range of this parasite. Using eight highly variable microsatellite loci, we found multiple strains in 8.8% of our isolates. Due to the technical challenges of detecting multiple infections this number represents a minimum estimate and the true frequency of multiple-strain infections is likely to be higher. Multiple-strain infections occurred across the entire East-West range of the parasite. Together with previous results, these findings strongly suggest that multiple-strain infections are common for this parasite and that their consequences for epidemiology and parasite evolution should be investigated in detail.

Keywords

Trypanosoma brucei; microsatellites; co-infection; multiple infection; strains; isolates; sleeping sickness; nagana

1. Introduction

Parasitic infections commonly consist of heterogeneous mixes of genetically distinct parasites. Concomitant infections with multiple parasite species are thought to be the norm rather than the exception, at least in the developing world (Utzinger and de Savigny, 2006). However, it is becoming increasingly clear that infections also often contain multiple strains of the same parasite species, a phenomenon that may have important epidemiological and evolutionary implications. For the purpose of this paper, we define the term ‘strain’ as meaning all parasite individuals that are indistinguishable by genetic markers. This is in contrast to the term

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'isolate', which we use to refer to a sample taken from an infected host or vector, cultured or not, that may contain parasites from several strains.

We investigated multiple-strain infections in *Trypanosoma brucei*, the causative agent of African sleeping sickness (human African trypanosomiasis), a vector-borne disease that is fatal if untreated (Barrett et al., 2003) and ranks second among parasitic diseases in sub-Saharan Africa only to malaria in terms of mortality (WHO, 2002). Previous work has shown that multiple-strain infections occur in *T. brucei* in hosts and vectors (Scott, 1981; Letch, 1984; Stevens et al., 1994; MacLeod et al., 1999; MacLeod et al., 2000; Truc et al., 2002; Koffi et al., 2007). However, these studies were restricted to three countries (Ivory Coast, Uganda, Kenya) and some had very low sample sizes, making inferences about the frequency and distribution of multiple-strain infections problematic. Only one study (Godfrey et al., 1990) investigated multiple-strain infections across Africa. But this study employed isoenzymes, which are known to have lower resolution than modern markers, and reported surprisingly low levels of multiple-strain infections well below those reported in the other, geographically restricted, studies. It is therefore still unclear how common multiple-strain infections are in general in this parasite and if they are restricted to certain locations or host species. We therefore analyzed available cryo-preserved *T. brucei* isolates from throughout Africa to reevaluate the frequency of multiple infections among infected human and non-human vertebrate hosts and the tsetse fly (*Glossina sp.*) vector. We employed modern microsatellite markers to increase resolution and sampled previously unsampled areas to cover almost the entire range of the parasite.

2. Materials and methods

2.1. Isolates

We investigated 137 cryo-preserved isolates and laboratory strains of *T. brucei* isolated throughout Africa between 1959 and 2003 (Tab. 1). These included 63 *T. b. gambiense*, which cause chronic sleeping sickness in West and Central Africa, 22 *T. b. rhodesiense*, which cause acute sleeping sickness in East Africa, and 41 non-human infective *T. b. brucei* from vertebrate hosts; and 10 *T. b. brucei* and 1 *T. b. rhodesiense* from tsetse flies. The isolates were initially isolated and (in parts) subsequently cultivated by a range of methods. We were unable to establish the exact history for a large portion of them and so cannot take isolation and cultivation history into account in our analyses. However, no isolates were included in this study that have been cloned in the past because they cannot contain multiple strains anymore. The isolates were kindly provided by four different laboratories (Serap Aksoy, Yale University; Reto Brun, Swiss Tropical Institute; Wendy Gibson, University of Bristol; and Pascal Grébaut and Anne-Clarisse Lékané Likeufack, CIRAD-IRD/LRCT, Montpellier) either as extracted DNA or as cryo-preserved blood samples from the original host or from a rodent used to culture the original isolate.

2.2. Molecular analysis

Parasite DNA of isolates not received as extracted DNA already was extracted using the DNeasy Blood & Tissue Kit (Qiagen) following the manufacturer's protocol and resuspended in 50 microliters of distilled water. Eight microsatellite markers (TB1/8, TB2/19, TB5/2, TB6/7, TB8/11, TB9/6, TB10/5, and TB11/13), each amplifying a single polymorphic locus on a different chromosome (Balmer et al., 2006), were amplified for every isolate using the following PCR profile: 1 cycle of : 4 min 94°, 35 cycles of: 45 sec 94°, 30 sec 53°, 45 sec 72°, 1 cycle of: 7 min 72°. Reactions contained 1× PCR buffer II with MgCl₂ (Applied Biosystems), 0.8 mM of each dNTP (Promega), 0.2 μM of each primer, 0.25 U AmpliTaq polymerase (Applied Biosystems). Allele sizes were determined using an ABI3100 Genetic Analyzer (Applied Biosystems) and GeneMapper 3.5 software (Applied Biosystems, 2003).

The microsatellite loci amplified are diploid. Therefore, the presence of more than two alleles at any locus in an isolate was used as indication for a multiple-strain infection. The proportion of detected multiple-strain infections was used as minimum estimate of the true multiple-strain infections rate. In isolates where more than two alleles were found at any locus, PCR and allele size determination were repeated for that locus at least once to confirm the presence of multiple strains. For *T. b. gambiense* and *T. b. rhodesiense*, humans and other vertebrate hosts were treated together as 'vertebrate hosts'.

3. Results

Multiple strains were detected in 12 (8.8%) of the 137 analyzed isolates. The majority (126) of the isolates were from vertebrate hosts (including humans). Of those, 12 (9.5%) contained multiple-strain infections: 5 of 63 (7.9%) *T. b. gambiense*, 6 of 41 (14.6%) *T. b. brucei*, and 1 of 22 (4.5%) *T. b. rhodesiense* isolates (Tab. 2).

The frequency of multiple-strain infections in *T. b. brucei* and *T. b. rhodesiense* did not differ significantly (Fisher's exact test, $p=0.41$), so our results provide no evidence that these two taxa, which differ only by the presence of the SRA gene conferring human infectivity to *T. b. rhodesiense* (Xong et al., 1998; Gibson, 2005), differ in terms of multiplicity of infection. Multiple-strain infections were found in isolates from vertebrate hosts from the entire East-West range sampled (Tab. 2). Among the 11 isolates from tsetse flies, no multiple-strain infections were detected.

4. Discussion

The key finding of this study is that multiple-strain infections are quite common in *T. brucei* and that they occur over the entire range of the parasite. A considerable percentage of *T. brucei* infections are made up of multiple *T. brucei* strains. The reported 8.8% frequency of detected multiple-strain infections is likely an underestimate for several reasons. Probably the biggest problem is that co-infecting strains that are at a much lower density than the dominant strain are hard to detect by PCR. Koffi *et al.* (2007) demonstrated that *T. brucei* strains become undetectable when their density falls below one tenth that of a co-infecting strain, a finding that matches our own results (unpublished) using the same approach. Densities of co-infecting *T. brucei* strains can easily vary by more than two orders of magnitude (unpublished), especially in human infections with *T. b. rhodesiense* or if isolates are passaged through laboratory rodents, where very high parasitaemias are reached. It is therefore possible that in some isolates a co-infecting strain was missed because it was at too low density. Also, some of the isolates used were cultured or passaged through non-natural hosts before analysis. This introduces the risk that strains originally present in the isolates were lost (Jamonneau et al., 2003), again leading to an underestimation of multiple-strain infections. On the other hand, contamination with other strains during prolonged passages in the laboratory is possible and would lead to an overestimation. However, we regard this as a minor factor as similar levels of multiple-strain infections were observed by several independent groups (Tab. 3) and also in isolates that were analyzed without or after minimal passaging.

The reports of multiple-strain infections in *T. brucei* available to date (Tab. 3) can be divided into three groups. Three early reports (Scott, 1981; Letch, 1984; Stevens et al., 1994) used isoenzyme analysis to demonstrate that multiple-strain infections occur but did not quantify how common they are. A second group, employing microsatellite or minisatellite markers, quantified the frequency of multiple-strain infections but were geographically restricted to Ivory Coast (Truc et al., 2002; Koffi et al., 2007) or Kenya and Uganda (MacLeod et al., 1999; MacLeod et al., 2000). Truc *et al.* (2002) using two microsatellite loci found a frequency of multiple-strain infections of 8.7% in 23 human sleeping sickness patients in the Ivory Coast,

which is similar to our numbers. A recent study based on the same samples but employing randomly amplified polymorphic DNA (RAPD) reported a higher frequency of multiple infections than in the previous one (Oury et al., 2004). However, we trust the first results more because microsatellite results are generally more reliable than RAPD results (Palumbi, 1996). The study by MacLeod *et al.* (1999) has a limited sample size as well (28 flies from 2 countries), but they report frequencies of 36-47%. The frequency estimates they provide are therefore still reliable because frequencies close to 50% can be better estimated with small sample sizes than the lower frequencies reported in the other studies. Finally, the study by Godfrey *et al.* (1990) and this study have considerably higher sample sizes and cover the entire geographic range of *T. brucei*. Our study provides a minimum estimate of the frequency of multiple-strain infections in line with most other studies but notably higher than Godfrey *et al.* (1990), which found high levels of multiple-strain infections in Zambia (c. 10%) but virtually none (<0.5%) across the rest of Africa. We attribute the much higher frequency of multiple-strain infections reported in our study to two methodological factors. First, our microsatellite markers are more variable than the isoenzymes used by Godfrey *et al.* (1990) allowing us to distinguish strains that may be indistinguishable by isoenzymes. Second, in contrast to microsatellite analysis, isoenzyme analysis requires the cultivation of the parasites to increase the amount of enzyme, which can lead to a selective amplification of only one strain (Jamonneau et al., 2003), so that multiple-strain infections are not detected anymore. The collection of isolates analyzed in this study contains some long cultured isolates as well, but also a large number with no or very limited culturing. The much higher frequency of multiple-strain infections reported from Zambia by Godfrey *et al.* (1990) would suggest the circulation in that country of strains that are either more genetically diverse and/or generally easier to culture.

The results of MacLeod *et al.* (1999) stand out because of the high levels of multiplicity of infection in tsetse flies they report. The frequency of multiple-strain infections in tsetse flies reported here is significantly lower (Fisher's exact test, $p=0.009$). Our data thus do not support the high levels reported by MacLeod *et al.* (1999), as such high frequencies would be apparent even with our limited sample size. This difference may at least in part reflect differences in the sensitivity of the molecular methods applied in these studies (minisatellites generally being even more variable than microsatellites) or in the treatment of the isolates before and during analysis. Caution must be used in general when comparing studies with different methodology (Jamonneau et al., 2003).

In conclusion, our results show two things. First, multiple-strain infections with *T. brucei* occur over the entire range of the parasite and are not restricted to certain areas. Second, multiple-strain infections are common enough that they cannot be neglected and that their consequences should be investigated further. A frequency of multiple-strain infections of above 10% appears to be the norm in *T. brucei* across its distribution.

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Table 1

Names and origin of *Trypanosoma brucei* isolates from humans, other vertebrates or tsetse flies screened for multiple-strain infections

Taxon	Origin ^a	Isolates ^b
<i>T. b. brucei</i>	Burkina Faso	GAOUA89
	Kenya	KETRI1738, KETRI1814 *, KETRI1902 *, KETRI2090, KETRI2108, <u>LF1</u> , LUMP266(MRC241flyK4), <u>LUMP1342(LUMP450)</u> , LVBG118N, M249, RB67, RUMP503
	Somalia	STIB794A
	Tanzania	RUMP501, STIB056, STIB201, STIB202, STIB204, STIB205, STIB206 *, STIB207, STIB209, STIB210, STIB211 *, STIB213, STIB214 *, STIB215 *, STIB216, STIB217, STIB218, STIB219, STIB221, STIB247, STIB286, STIB316, STIB337
	Uganda	<u>EATRO1296</u> , Katerema41, STIB340, STIB390, <u>STIB776</u> , <u>STIB783</u> , <u>STIB795</u>
<i>T. b. rhodesiense</i>	Zambia	H3, J10, TRPZ239, TRPZ260, <u>TRPZ286(pop1)</u> , <u>TRPZ320(pop1)</u> , <u>TRPZ323</u>
	Botswana	STIB338
	Ethiopia	STIB707, STIB809
	Kenya	EATRO0237 , STIB365 , STIB706
	Mozambique	KETRI2538
<i>T. b. gambiense</i>	Tanzania	STIB236, STIB241-A, STIB243, STIB250, STIB262, STIB263, STIB324, STIB389 * , STIB704
	Uganda	EATRO0240 , <u>STIB391</u> , STIB799 , STIB848 , STIB849 , STIB851 , STIB854
	Angola	001K1Angola , 003K1 *
	Cameroon	BIP04 , BIP08 , BIP09 , BIP40 , BIP42 , C3359 , DOUME1 , JUA * , P7F , P8F , P16F , P26F , SEMI , SOMABc , TSEMESO
	CAR	BAT10 , BAT31 , BAT37 , BAT39 , BAT40 , BAT42 , BAT45 , BAT51 , BAT58 , BAT60 , BIBIANA , MBADI
<i>T. b. gambiense</i>	Chad	NATONDI
	Congo	DEMBA , MALOUNDA
	DRC	ITMAP020578 , ITMAP141267 , ITMAP160986 , ITMAP1780 , ITMAP210879 , ITMAP211290 , ITMAS060401
	Eq. Guinea	13.97D , 14.97D , 15.97D
	Ivory Coast	DAL069 , DAL1086 , DAL1086R , DAL1402 , STIB386 , STIB733 , STIB739 , STIB754 , STIB755
<i>T. b. gambiense</i>	Liberia	STIB756
	Sudan	K00014JD , K0303028 * , K0303030 , K0303043 , K0303045 , K0303048
	Uganda	F43UG , R56UG , R60UG , STIB368 , STIB887 *

^a CAR, Central African Republic; DRC, Democratic Republic of Congo.

^b Isolate names are coded by host species: **bold, human**; plain, other vertebrate; underlined, tsetse fly; *italics*, host unknown. Sources: Serap Aksoy, Yale University; Reto Brun, Swiss Tropical Institute; Wendy Gibson, University of Bristol; Pascal Grébaut and Anne-Clarisse Lékané Likeufack, CIRAD-IRD/LRCT, Montpellier.

* denotes isolates containing multiple strains.

Table 2
Names, origin, and microsatellite alleles per locus of *Trypanosoma brucei* isolates containing multiple strains

Isolate ^a	TB1/8	TB2/19	TB5/2	TB6/7	TB8/11	TB9/6	TB10/5	TB11/13
<i>Alles^b</i>								
<i>T. b. gambiense</i> 003 K1 Angola, Mbanza Coago, human, 1998	97 99	- -	88 99	- -	- -	126 134	- -	137 147
K0303028 Sudan, Mundri, Nyau 1, human, 2003	107 97 97	90 92	83 99	104 120	101 103	136 140	- -	125 129
JUA Cameroon, Fontem focus, human, 1974	97 99	90 92	99 101	116 120	101 103	128 132	85 89	147 157
STIB 739 (TH-162/78E (021)) Ivory Coast, human, 1978	99 99	- -	99 99	- -	101 103	140 140	- -	129 131
STIB 887 (UTRO 210393B) Uganda, Arua, West Nile, human, 1995	97 97	- -	- -	- -	115 101 103	126 136	89 89	129 147
<i>T. b. brucei</i> KETRI 1814 Kenya, Kiboko, rhinoceros, 1970	109 113	96 96	95 95	104 106	99 113	134 136	95 115	127 129 143 155 143 147
KETRI 1902 Kenya, Lambwe Valley, waterbuck, 1971	105 105	78 88	87 87	106 106	97 97	128 132	89 89	127 133 145 155 125 145
STIB 206 Tanzania, Serengeti N.P., lion, 1971	99 105	92 88 90	88 91	112 120	99 103	126 132	81 83	127 133 145 155 125 145
STIB 211 Tanzania, Serengeti N.P., lion, 1971	99 109 111	92 94	91 93 99	108 108	99 99	134 138 140	83 83	125 127
STIB 214 Tanzania, Serengeti N.P., hyena, 1971	99 103	96 98	103 95	106 108	99 109	134 136	83 87	125 127
STIB 215 Tanzania, Serengeti N.P., lion, 1971	107 113 109 113	90 100	99 101 93 95	106 106	115 117 99 115	140 130 134 142	91 83 89	125 125
<i>T. b. rhodesiense</i> STIB 389 (Mbulu strain) Tanzania, Up. Luangwe Valley, human, 1977	99 99	84 84	93 93	- -	103 109	126 126	81 85	135 135
							89	

^a Isolate name with country, location, host and year of isolation underneath.

^b Fragment lengths in basepairs. '-' denotes loci that did not amplify.

Table 3

Reports of multiple-strain infections in *Trypanosoma brucei*. Frequency estimates of multiple-strain infections are given only where the sample size was judged sufficient

Host	Multiple infections	Method	Countries	Reference
Pig	-	Isoenzymes	Ivory Coast	(Scott, 1981)
<i>Glossina p. palpalis</i>	-	Isoenzymes	Ivory Coast	(Letch, 1984)
<i>Glossina p. palpalis</i>	-	Isoenzymes, karyotype	Ivory Coast	(Stevens et al., 1994)
Human	8.7% (2 of 23)	Microsatellites	Ivory Coast	(Truc et al., 2002)
Human	67% (6 of 9) ^a	Microsatellites	Ivory Coast	(Koffi et al., 2007)
<i>Glossina sp.</i>	42.9% (12 of 28)	Minisatellites	Kenya, Uganda	(MacLeod et al., 1999)
Cattle	18% (9 of 50)	Minisatellites	Uganda	(MacLeod et al., 2000)
All species	3.1% (28 of c. 900) ^b	Isoenzymes	All Africa	(Godfrey et al., 1990)
All species	8.8% (12 of 137)	Microsatellites	All Africa	This study

^a only those data points where multiple strains were detected at the same time are reported here.

^b based on 945 "populations" derived from an undeclared number (c. 900) of primary isolates. 26 of the multiple infections are from Zambian isolates. The detected multiple infection rate in non-Zambian isolates is 0.22%.