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Short communication

Multiple-strain infections of *Trypanosoma brucei* across Africa

Oliver Balmer[a,b,c,*], Adalgisa Caccone[a,d]

[a] *Department of Ecology and Evolutionary Biology, Yale University, 165 Prospect Street, New Haven, CT 06511, USA*
[b] *Swiss Tropical Institute, Socinstrasse 53, 4051 Basel, Switzerland*
[c] *Institute of Zoology, University of Basel, Vesalgasse 1, 4051 Basel, Switzerland*
[d] *Yale Institute for Biospheric Studies, Molecular Systematics and Conservation Genetics Laboratory, Yale University, PO Box 208106, New Haven, CT 06520-8106, USA*

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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.

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 '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 trypanosomosis), 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, 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 re-evaluate 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

* Corresponding author. Current address: Swiss Tropical Institute, Socinstrasse 53, 4051 Basel, Switzerland. Tel.: +41 61 284 82 32; fax: +41 61 284 81 01.
E-mail address: oliver.balmer@aya.yale.edu (O. Balmer).

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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 (Table 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) subsequentially 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 μl 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 °C; 35 cycles of 45 s 94 °C, 30 s 53 °C and 45 s 72 °C; 1 cycle of 7 min 72 °C. Reactions contained 1× PCR buffer II with $MgCl_2$ (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 (Table 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

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]
T. b. brucei	Burkina Faso	GAOUA89
	Kenya	KETRI1738, KETRI1814[c], KETRI1902[c], KETRI2090, KETRI2108, LF1, LUMP266(MRC241flyK4), LUMP1342(LUMP450), LVBG118N, M249, RB67, RUMP503
	Somalia	STIB794A
	Tanzania	RUMP501, STIB056, STIB201, STIB202, STIB204, STIB205, STIB206[c], STIB207, STIB209, STIB210, STIB211[c], STIB213, STIB214[c], STIB215[c], STIB216, STIB217, STIB218, STIB219, STIB221, STIB247, STIB286, STIB316, STIB337
	Uganda	EATRO1296, Katerema41, STIB340, STIB390, STIB776, STIB783, STIB795
	Zambia	H3, J10, TRPZ239, TRPZ260, TRPZ286(pop1), TRPZ320(pop1), TRPZ323
T. b. rhodesiense	Botswana	**STIB338**
	Ethiopia	STIB707, **STIB809**
	Kenya	**EATRO0237**, **STIB365**, **STIB706**
	Mozambique	**KETRI2538**
	Tanzania	STIB236, STIB241-A, STIB243, STIB250, STIB262, STIB263, STIB324, **STIB389**[c], **STIB704**
	Uganda	**EATRO0240**, STIB391, **STIB799**, **STIB848**, **STIB849**, **STIB851**, **STIB854**
T. b. gambiense	Angola	**001K1Angola**, **003K1**[c]
	Cameroon	**BIP04**, **BIP08**, **BIP09**, **BIP40**, **BIP42**, **C3359**, **DOUME1**, **JUA**[c], P7F, P8F, P16F, **P26F**, **SEMI**, **SOMABc**, **TSEMESO**
	CAR	**BAT10**, **BAT31**, **BAT37**, **BAT39**, **BAT40**, **BAT42**, **BAT45**, **BAT51**, **BAT58**, **BAT60**, **BIBIANA**, **MBADI**
	Chad	**NATONDJI**
	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**[c], **STIB754**, **STIB755**
	Liberia	**STIB756**
	Sudan	**K00014JD**, **K0303028**[c], **K0303030**, **K0303043**, **K0303045**, **K0303048**
	Uganda	**F43UG**, **R56UG**, **R60UG**, **STIB368**, **STIB887**[c]

[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. *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.
[c] Isolates containing multiple strains.

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

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

[a] Isolate name with country, location, host and year of isolation underneath.
[b] Fragment lengths in basepairs. '–' denotes loci that did not amplify.

range sampled (Table 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.*

Table 3
Reports of multiple-strain infections in *T. brucei*

Host	Multiple infections	Method	Countries	Reference
Pig	–	Isoenzymes	Ivory Coast	Scott (1981)
Glossina p. palpalis	–	Isoenzymes	Ivory Coast	Letch (1984)
Glossina p. palpalis	–	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)
Glossina sp.	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 ca. 900)[b]	Isoenzymes	All Africa	Godfrey et al. (1990)
All species	8.8% (12 of 137)	Microsatellites	All Africa	This study

Frequency estimates of multiple-strain infections are given only where the sample size was judged sufficient.

[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 (ca. 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%.

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 (Table 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 (Table 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, 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 (ca. 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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