Infection, Genetics and Evolution 30 (2015) 128–133

Contents lists available at ScienceDirect

Infection, Genetics and Evolution

journal homepage: www.elsevier.com/locate/meegid

Population genetics of *Trypanosoma brucei gambiense* in sleeping sickness patients with treatment failures in the focus of Mbuji-Mayi, Democratic Republic of the Congo

Patient Pati Pyana [a,1], Modou Sere [b,1], Jacques Kaboré [b,c], Thierry De Meeûs [b,d,*], Annette MacLeod [e], Bruno Bucheton [f,d], Nick Van Reet [g], Philippe Büscher [g], Adrien Marie Gaston Belem [c], Vincent Jamonneau [b,d]

[a] Département de Parasitologie, Institut National de Recherche Biomédicale, Av. de la Démocratie, BP 1197, Kinshasa Gombe, Congo
[b] Centre International de Recherche-Développement sur l'Élevage en zones Subhumides (CIRDES), Unité de recherches sur les bases biologiques de la lutte intégrée, Institut de Recherche pour le Développement, Unité Mixte de Recherche IRD-CIRAD 177 INTERTRYP, 01 BP 454 Bobo-Dioulasso 01, Burkina Faso
[c] Université Polytechnique de Bobo-Dioulasso, 01 BP 1091 Bobo-Dioulasso 01, Burkina Faso
[d] Institut de Recherche pour le Développement, Unité Mixte de Recherche IRD-CIRAD 177 INTERTRYP, TA A-17/G, Campus International de Baillarguet, F-34398 Montpellier, France
[e] Wellcome Centre for Molecular Parasitology, University of Glasgow, Biomedical Research Centre, 120 University Place, Glasgow G12 8TA, United Kingdom
[f] Programme National de Lutte contre la Trypanosomiase Humaine Africaine, Conakry BP 851, Guinea
[g] Department of Biomedical Sciences, Institute of Tropical Medicine, Nationalestraat 155, B-2000 Antwerp, Belgium

ARTICLE INFO

Article history:
Received 8 October 2014
Received in revised form 11 December 2014
Accepted 12 December 2014
Available online 20 December 2014

Keywords:
Trypanosoma brucei gambiense
Sleeping sickness
Population genetics
Treatment failure
Democratic Republic of the Congo

ABSTRACT

Human African trypanosomiasis (HAT) in the Democratic Republic of the Congo (DRC) is caused by the protozoan *Trypanosoma brucei gambiense*. Until recently, all patients in the second or neurological stage of the disease were treated with melarsoprol. At the end of the past and the beginning of the present century, alarmingly high relapse rates in patients treated with melarsoprol were reported in isolated HAT foci. In the Mbuji-Mayi focus of DRC, a particular mutation that confers cross resistance for pentamidine and melarsoprol was recently found for all strains studied. Nevertheless, treatment successfully cured a significant proportion of patients. To check for the existence of other possible genetic factors of the parasites, we genotyped trypanosomes isolated from patients before and after treatment (relapsing patients) with eight microsatellite markers. We found no evidence of any genetic correlation between parasite genotype and treatment outcome and we concluded that relapse or cure probably depend more on patients' factors such as disease progression, nutritional or immunological status or co-infections with other pathogens. The existence of a melarsoprol and pentamidine resistance associated mutation at such high rates highlights an increasing problem, even for other drugs, especially those using the same transporters as melarsoprol and pentamidine.

1. Introduction

Sleeping sickness or human African trypanosomiasis (HAT) in the Democratic Republic of the Congo (DRC) is caused by the protozoan *Trypanosoma brucei gambiense*. Until recently, in DRC as well as in other *gambiense* HAT endemic countries, patients in the second or neurological stage of the disease were treated with melarsoprol, a drug that has been in use for more than 60 years now. For several decades, little concern was given to treatment failure and relapse rates in patients with some exceptions (Ginoux et al., 1984). Only at the end of the past century and the beginning of the present century, alarmingly high relapse rates (50–80%) in patients treated with melarsoprol were reported in isolated foci in different Central African countries such as DRC, Uganda, Angola and Sudan (now South Sudan) (Legros et al., 1999b; Brun et al., 2001; Burri and Keiser, 2001a; Moore and Richer, 2001; Ollivier and Legros, 2001; Stanghellini and Josenando, 2001; Maina et al., 2007; Robays et al., 2008).

In 2006, we started an investigation into the origin of high treatment failure rates encountered in second stage *gambiense* patients that were treated with melarsoprol in the HAT focus of Mbuji-Mayi, East Kasai. To this end, we isolated the parasite strains from patients before treatment and from relapsing patients after treatment. Thus

* Corresponding author at: Centre International de Recherche-Développement sur l'Élevage en zones Subhumides (CIRDES), Unité de recherches sur les bases biologiques de la lutte intégrée, Institut de Recherche pour le Développement, Unité Mixte de Recherche IRD-CIRAD 177 INTERTRYP, 01 BP 454 Bobo-Dioulasso 01, Burkina Faso.
E-mail address: thierry.demeeus@ird.fr (T. De Meeûs).
[1] These authors contributed equally to this work.

http://dx.doi.org/10.1016/j.meegid.2014.12.017
1567-1348/

we established a collection of *T. b. gambiense* strains, that allowed us to confirm reduced *in vivo* sensitivity to melarsoprol of the *T. b. gambiense* strains from Mbuji-Mayi (Pyana et al., 2011, 2014). We further could link this phenotype to a particular mutation that confers cross resistance for pentamidine and melarsoprol (Graf et al., 2013). In all strains from Mbuji-Mayi the genes coding for aquaglyceroporin 2 and 3 (TbAQP2, TbAQP3) are replaced by chimeric TbAQP2/3 genes. Analysis of the gene coding for the adenosine 2 transporter did not reveal any particular mutation that is known to confer resistance to melarsoprol (Pyana et al., 2014). Since the chimeric TbAQP2/3 genes are present in strains from patients who relapsed as well as who were cured after melarsoprol treatment in Mbuji-Mayi, other factors must be involved that define the treatment outcome in a particular patient. Such factors can be related to genetic or cultural background of patients as well as to the immunological status of the host, to the progression of the disease or to parasite-specific factors.

In this paper we genotyped trypanosomes isolated from patients before melarsoprol treatment and from relapsing patients, with eight microsatellite loci. We tested whether these individual genotypes (those which survived, those that did not survive and those that appeared as new genotype after treatment) can be considered as randomly sampled from the same pool of parasites representative of the investigated HAT focus. We found a total absence of genetic differentiation between trypanosomes isolated before and after melarsoprol treatment and that no specific trypanosome genotype can be associated to treatment failure. We discuss what other parameters could explain treatment failures.

2. Materials and methods

2.1. Trypanosome strains

A list of the studied *T. b. gambiense* strains is given in Table 1. Forty-one stains were isolated between 2005 and 2008 in Mbuji-Mayi, a HAT focus in East Kasai Province, DRC, with high melarsoprol treatment failure rates. The alias name of each strain indicates whether it was isolated before treatment (BT) or after treatment (AT). Eleven strains were isolated from patients that became cured after melarsoprol treatment. For first stage patients, cure was defined as <6 white blood cells/μl of cerebrospinal fluid and no parasitological evidence of relapse during 24 months of follow-up, while for second stage patients, cures was defined as <21 white blood cells/μl of cerebrospinal fluid and no parasitological evidence of relapse during 24 months of follow-up (WHO, 2007). Thirty strains were isolated from patients that relapsed after treatment and among those, twenty are "couples" since they were isolated from the same patient before treatment and after relapse (Pyana et al., 2011). Seven patients provided a genotyped strain only after treatment and three only before treatment (see Table 1). Four other strains were isolated in 2011 in Masi-Manimba, a HAT focus in Bandundu Province in DRC (Pyana et al., 2014). These strains were used as an outgroup as compared to the other strains studied here. All the strains were kept as 250 μl cryostabilates in liquid nitrogen. All strains were confirmed by TgsGP specific PCR as *T. b. gambiense* type I (Radwanska et al., 2002; Pyana et al., 2014).

2.2. Expansion of parasite populations and preparation of pure trypanosome sediments

Each stabilate was thawed at 37 °C in a water bath and 250 μl of phosphate buffered saline glucose (PSG, 7.5 g/l Na_2 HPO_4 $2H_2O$, 0.34 g/l NaH_2 PO_4 H_2O, 2.12 g/l NaCl, 10 g/l D-glucose, pH 8) were added immediately. This suspension was inoculated intraperitoneally (IP) into two 1–2 months old female OF-1 mice (Charles River, Belgium) that were immunosuppressed by IP injection with 200 mg/kg body weight (BW) of cyclophosphamide, diluted in water (Endoxan, Baxter, Lessing, Belgium). Parasitaemia was monitored three times a week on 5 μl of tail blood according to the matching method of Herbert and Lumsden (1976). If needed, immunosuppression was repeated after 5 days, until the parasitaemia reached $\geqslant 10^{7.5}$/ml where after the mice were sacrificed and blood was collected on heparin by heart puncture. From this blood, the trypanosomes were separated by DEAE chromatography (1:6 blood:matrix ratio) (Lanham and Godfrey, 1970). The separated trypanosomes were washed three times with 5 ml of ice-cold PSG by centrifugation. After the last centrifugation, the supernatant was removed and the pure trypanosome sediment was frozen at −80 °C prior to DNA extraction.

2.3. DNA extraction

After thawing and addition of 200 μl of phosphate buffered saline, pH 8, genomic DNA was extracted with the Maxwell® 16 Tissue DNA Purification kit on the Maxwell® 16 robot (Promega Corporation, Madison, WI, USA) and DNA was stored at −20 °C. The DNA concentration was measured with the Nanodrop ND-1000 UV–Vis spectrophotometer (NanoDrop Technologies, Wilmington, USA) and adjusted to 10 ng/μl if appropriate.

2.4. Microsatellite markers

We used eight nested oligonucleotide primers for microsatellite markers Ch5/JS2, Ch1/18, Ch1/D2/7 (MacLeod et al., 2005), M6C8 (Biteau et al., 2000), Misatg4, Micbg5, Micbg6, Misatg9 (Koffi et al., 2007). One microliter of each DNA product was used as template for PCR, in a volume of 10 μl (Kaboré et al., 2013). Nested PCR amplification was carried out using the following conditions, PCR buffer (45 mM Tris–HCl pH 8.8, 11 mM $(NH_4)_2SO_4$, 4.5 mM $MgCl_2$, 6.7 mM 2-mercaptoethanol, 4.4 μM EDTA, 113 μg/ml BSA, 1 mM of each four deoxyribonucleotide triphosphates), 1 μM of each oligonucleotide primer, and 0.1 unit of Taq polymerase (ABgene) per 10 μl reaction. Nested PCR conditions for all markers for both rounds were 28 cycles of 50 s at 95 °C, 50 s at 55 °C and 1 min at 65 °C. 1 μl of a 1/280 dilution of the first round product was used as template in the second round PCR. PCR products were resolved by electrophoresis on a 3% Nusieve® GTG® agarose gel (Cambrex), and were stained with 0.2 μg/ml ethidium bromide in order to allow visualization under UV light. One primer out of every second round pair for the nested PCR amplification of microsatellite markers included a 5′-FAM or HEX modification, allowing size separation of products on a capillary-based sequencer (ABI 3100 Genetic Analyser; Applied Biosystems). The allele size was determined with the GeneMapper© software Version 4.0 (1999–2005 Applied Biosystems) based on the PCR amplicon. A set of standards size "GS400HD markers" (Applied Biosystems) was included, allowing determination of PCR amplicon size to the level of 1 bp.

2.5. Data analysis

With an automated sequencer, as was used here, homozygous individuals appear with a single peak at the expected size of the PCR product for the allele into study, while heterozygous individuals display two peaks. In clonal populations, like *T. b. gambiense* type 1 (Koffi et al., 2009; Simo et al., 2010), most, if not all, polymorphic microsatellite markers are expected to display two peaks (heterozygous). For the sake of maximum information conservation we have coded homozygous all single peaked individuals, though we know heterozygosity is almost totally fixed in *T. b. gambiense* type 1 (De Meeûs and Balloux, 2005; Koffi et al., 2009; Simo et al., 2010; Kaboré et al., 2011, 2013). This was particularly true for the