Microsatellite characterization of *Plasmodium falciparum* from symptomatic and non-symptomatic infections from the Western Amazon reveals the existence of non-symptomatic infection-associated genotypes

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In Western Amazon areas with perennial malaria transmission, long term residents frequently develop partial immunity to malarial infection caused either by Plasmodium falciparum *or* P. vivax, *resulting in a considerable number of non-symptomatically infected individuals. For yet unknown reasons, these individuals sporadically develop symptomatic malaria. In order to identify if determined parasite genotypes, defined by a combination of eleven microsatellite markers, were associated to different outcomes – symptomatic or asymptomatic malaria – we analyzed infecting* P. falciparum *parasites in a suburban riverine population. Despite of detecting a high degree of diversity in the analyzed samples, several microsatellite marker alleles appeared accumulated in parasites from non-symptomatic infections. This result may be interpreted that a number of microsatellites, which are not directly related to antigenic features, could be associated to the outcome of malarial infection. The result may also point to a low frequency of recombinatorial events which otherwise would dissociate genes under strong immune pressure from the relatively neutral microsatellite loci.*

Key words: non-symptomatic malaria - *Plasmodium falciparum* - microsatellites - genotyping

Malaria is one of the main infectious diseases of the world, more specifically in developing or underdeveloped tropical regions of the planet. Worldwide, 2-3 million individuals, mostly children under 5 years, succumb to *Plasmodium falciparum* caused disease. More than 2 billion people are at risk to acquire malarial infection and 500 million or more cases are estimated to occur annually (Snow et al. 2005). In the Western Amazon, acute malarial infection with deaths are encountered mainly in gold digger or wood logging camps with a high influx of malaria-naïve individuals (Sawyer 1988), whereas stable settlements along rivers rarely report fatal cases but show a high prevalence of non-symptomatic infections (Alves et al. 2002) which function as a silent source of transmission (Alves et al. 2005). Recently, during a major malaria control effort in the Candelária suburb of Porto Velho, capital of Rondônia, many non-symptomatic individuals infected with either *P. falciparum* or *P. vivax* were detected. Interestingly, between the cross-sectional surveys involving most of the citizens in the area, symptomatic malaria attacks were also reported in the same population. We wondered if different parasite populations could be responsible for the different outcome of malaria infections in this area. In a previous report from parasite samples from French-Guyana (Ariey et al. 2001), different genotypes of *P. falciparum* and certain alleles of polymorphic genes were associated to severe disease. In another study in Vietnam (Ferreira et al. 2002), where the circulating *P. falciparum* were genotyped using neutral microsatellites, the clinical outcome of the individual malaria cases could not be associated to specific *P. falciparum* genotypes.

In the Western Amazon, circulating *P. falciparum* genotypes seem to share a high number of variant antigens suggesting a low frequency of truly recombinatory events and favoring inbreeding of circulating strains (Albrecht et al. 2006). Taking into account the relatively low transmission (Gil et al. 2003) in the study area, we asked if distinct *P. falciparum* strains could be identified in the observed symptomatic or non-symptomatic infections in the same population. For this, multilocus microsatellite genotyping was conducted on the circulating strains of 43 cases of uncomplicated symptomatic and non-symptomatic *P. falciparum* infections, occurring during a 1 year-interval in a population exposed to the same risk of infecting mosquito bites.

MATERIALS AND METHODS

Study area, patients and origin of parasite samples - The study area Vila Candelária (8º 47' 08" S, 63º 55' 04" W) is situated at 2 km of the centre of Porto Velho, capital of the state of Rondônia, Brazil. The climate is

tropical humid with a rainy season from October to April (around 2,500 mm rain precipitation) and a relatively dry season from May to September (500 mm or less of rain precipitation). The temperature ranges from 18º to 38ºC and the humidity, with exception of short periods in the dry season, is always higher than 80%. Vila Candelária extends from the right Madeira bank to more elevated areas, around 400 m distant from the riverbank. The population of Candelária (~300 individuals) is relatively stable and of mixed indigenous, negro and Caucasian origin. Bate Estaca is located immediately upstream of Candelária and has similar populational characteristics, but only 80-90 inhabitants. The herein employed blood samples were collected during two cross-sectional surveys and continuous surveillance of the population during April and September 2001 in Candelária from people between 3 and 49 years of age and several years of residence in malaria-endemic areas. Individuals presenting symptomatic infections between the cross-sectional surveys in Candelária or Bate Estaca actively sought treatment in the Candelária health post. In total, 24 samples from asymptomatic infections and 10 samples from symptomatic samples were collected in the Candelária area. Another part of the blood samples from symptomatic patients (n = 9) was collected in the Malaria and Dengue Outpatient Clinic of the Centre of Tropical Medicine in Porto Velho. These patients acquired their infections at different locations throughout the Porto Velho county in a diameter of approx. 80 km. All participating patients gave their written consent before contributing blood samples. Participants were considered non-symptomatic if they (i) did not report any febrile episodes two weeks before blood retrieval, (ii) did not present any symptoms on the day of blood retrieval and throughout the following 60 days. Parasitemias in these samples were sub-microscopical (approx. 1-100 IRBC per µl erythrocytes). Non-symptomatic individuals were not treated for malaria since they chose to do so.

Symptomatic, non-severe participants with parasitemias between 0.1 and 2% presenting typical malaria symptoms such as fever, myalgia, nausea, sweating, and headache were immediately treated after malaria diagnosis and blood retrieval. Ethical clearance was obtained beforehand from the Federal Ethics commission.

Preparation of parasite genomic DNA - Five ml of blood were collected in heparinized vacutainer tubes and centrifuged for 6 min at 2000 g at room temperature (RT). Fractions of plasma, buffy coat and infected red blood cells (IRBC) were separated and frozen at –20°C until use. Genomic DNA of parasites was purified following the previously published protocol (Schlichtherle et al. 2000) and frozen at –20°C until use.

Parasite genotyping and microsatellite PCR - Initially, each genomic DNA was tested for the presence or not of *P. falciparum* genomic DNA (gDNA). For this purpose, the oligonucleotides and PCR conditions introduced by Snounou (1996) were applied. Briefly, each gDNA was amplified with primers specific for the ribosomal RNA genes and afterwards with a second set of primers, detecting either *P. falciparum P. vivax,* or *P. malariae.*

For the evaluation of the circulating *P. falciparum* haplotypes, defined by a combination of marker sizes of eleven microsatellite loci, gDNAs were amplified in the nested PCR protocol published by Anderson et al. (1999) using the oligonucleotide primers used therein. In the second PCR reaction, one Cy5-labeled primer was used to permit semi-automatic analysis of the marker sizes in an ALF2 (Amersham Pharmacia) automated sequencer. For the size analysis of fragments, a molecular weight marker containing an internal standard of 60 bp added to every sample, and length markers of 60 bp, 97 bp, 175bp, and 219 bp were used.

Data analysis - Fragments detected by the ALF2 sequencer were size-determined using the AlleleLinks 1.0 software (Amersham Pharmacia). When multiple peaks were detected per microsatellite marker and the additionally observed peak was at least 33% the size of the largest peak, the infecting parasite population was classified as polyclonal. For further analysis, only the main peaks were considered. The microsatellite markers of different gDNAs samples were analyzed using the Arlequin 2000 software (Schneider et al. 2000). The molecular diversity index for each sample was calculated and the population differentiation was expressed as Reynolds' distance (R_{ST}) for the three groups of parasite DNAs as listed in Table I.

Significance of association tests with single microsatellite markers testing for uneven distribution in gDNAs stemming from symptomatic versus asymptomatic infections including or not the outside-Candelária sample SCP were performed using Pearson's Chi-square tests (Yates corrected) in SPSS (version 11). Associations were considered significant when *p* values were below 0.05.

TABLE I

Genetic distance of genotypes from different samples. The genetic distances (R_{ST} values) between all samples were calculated with Arlequin

	AVC	SVC/SBE
AVC	0	
SVC/SBE	0,41 ($p < 0,001$)	0
SCP	0,16 ($p = 0,036$)	0,17 ($p = 0,009$)
SCP+SVC/SBE	0,24 ($p < 0,001$)	

RESULTS

Forty-three field DNA samples were analyzed and all infections were solely caused by *P. falciparum* and no mixed infection with *P. vivax* were detected by species-specific PCR (data not shown). A total of 28 showed more than one haplotype, identified by two alleles for at least one microsatellite marker. From the 43 dominant haplotypes, 37 were different (Table III).

In the following, the genetic heterogeneity of the different samples was computed on the basis of micro-satellite data. All parasite samples showed a high molecu-

TABLE II

Significant associations of microsatellite markers from parasites causing symptomatic (S) or non-symptomatic (NS) infections

A - Comparison of all genotypes, classified for "symptomatic/non-symptomatic"

TA1 Allele size	171	174	177	180	183		Total
S	4	4	1		10		19
NS		11	10	1	2		24
Sum	4	16	11		11		43

POLY-α Allele size	152	155	164	182	185	191	Total
S	2		2	9	4	1	18*
NS	13	2	1	8			24
Sum	15	2	3	17	4	1	42

TAA60 Allele size	75	78	84	87	93	Total
S	13	3	1	1	1	19
NS	8	16				24
Sum	21	19	1	1	1	43

TAA42 Allele size	188	191	194	197	200	203	Total
S	4	1	1		3	10	19
NS	10			10		4	24
Sum	14	1	1	10	3	14	43

B - Comparison of genotypes from the Candelária area, classified for "symptomatic/non-symptomatic"

TA1 Allele size	174	177	180	183	Total
S	3	1		6	10
NS	11	10	1	2	24
Sum	14	11	1	8	34

POLY-α Allele size	152	155	164	182	185	Total
S		1	5	3		9
NS	13	2	1	8		24
sum	13	2	2	13	3	33

TAA42 Allele size	188	191	194	197	200	203	Total
S	2	1	1		1	5	10
NS	10			10		4	24
sum	12	1	1	10	1	9	34

A: all genotypes and locations were tested. B: results from genotypes of parasites stemming exclusively from the Candelária area (including Bate Estaca). The significance (p < 0.05) of allele distribution differences was calculated using Pearson´s chi square test. * means that of one gDNA sample the indicated microsatellite marker could not be amplified.

lar diversity index (MDI), with the highest value found in parasites from different origins outside the Candelária area. The symptomatic infection-associated genotypes SVC/SBE were less divergent than the both other samples (SVC/SBE group, MDI: 0.43 ± 0.27 versus AVC group MDI: 0.49 ± 0.27 and SCP group, MDI: 0.53 ± 0.32). One genotype was found in all groups (corresponding to AVC4, Table III).

When the different parasite genotypes were compared between the three groups considering the genetic distance expressed as R_{ST} values, a significant differentiation of genotype samples was observed and all samples were different from each other. Genotypes from the asymptomatic sample AVC showed the most significant difference to the sample from symptomatic infections of the same area (SVC/SBE, Table I). When the genotypes were classified in parasite genotypes from symptomatic or non-symptomatic infections, a significant difference between the two groups could be observed (Table I).

Given the apparent population differentiation, we asked which microsatellite loci were associated with symptomatic or non-symptomatic forms of infection. After analysis of the frequencies of allele distribution per microsatellite marker, comparing the group of *P. falciparum* parasites isolated from symptomatic (SVC/SBE/SCP) with non-symptomatic infections (AVC), in 4 of 11 loci significantly different allele distributions were detected. When only parasite genotypes from the Candelária district were considered, still 3 of 11 markers showed significant differences in their allele distribution upon comparison of symptomatic versus non-symptomatic associated genotypes (Table II). Notably, of the locus Ta1, the 174bp and 177bp alleles, in the locus Taa42 the alleles 188bp and 197bp and the allele of 152bp of the Polya locus were associated with non-symptomatic parasite genotypes considering or not the SCP sample. When the SCP sample was considered, the loci TAA60 showed also strong and significant accumulation of the 78 bp allele in the asymptomatic sample.

DISCUSSION

In the rural areas of the Western Amazon, cases of non-symptomatic malarial infections caused either by *P. falciparum* or by *P. vivax* (Alves et al. 2002) are frequently encountered and individuals of these areas usually have generally strong responses against malarial antigens (Ferreira et al. 1994). Among these populations, symptomatic infections are also observed. In order to find parameters which are responsible for the malarial infections in an otherwise semi-immune population, the genotypes of the infecting *P. falciparum* parasites were analyzed by multilocus microsatellite typing.

We first looked at the polyclonality of infections. According to previous results from Druilhe and colleagues (Druilhe et al. 1998), a high overall proportion of mixed genotypes per infection was identified, which does not necessarily mean the occurrence of profoundly different parasites in terms of major antigens (Hoffmann et al. 2006). The observed frequency of mixed infections was significantly lower in the groups SCP and SVC (symptomatic infections) when compared to AVC (asymptomatic), however, we believe that this result does not exclude that indeed more than one parasite genotype is circulating in the actual infections. We rather believe that the applied method may be prone to underestimate the presence of additional genotypes when one is clearly dominant, as is frequently the case in natural symptomatic infections (Jafari et al. 2004).

Two of the three parasite samples were from a rather limited area spanning no more than 4 km in length and 500 m wide along the Madeira River. We expected in

TABLE III

Microsatellite markers from the isolates. AVC isolates from non-symptomatic *Plasmodium falciparum* infections were identified in two cross-sections, and all other isolates were from symptomatic infections from the same area (SBE and SVC) or from the medical attendance post (SCP)

Microsatellite marker → Isolate	TA1	Polya	TAA60	ARA2	Pfg377	PfPK 2	TAA87	TAA109	TAA81	TAA42	2490
AVC1	174/*180*	155	78	66	96	162/*171*	*96*/114	166	121	197	93
AVC2	174/*183*	182	78	66	96	171	114	166	121	203/*188*	87
AVC3	177	155	75	66	96	177	114	163	124	188	84
AVC4[#]	183	182	75	66	96	171	114	166	121	203	87
AVC5	*174*/180	182	78	66	96	171	99	166	121	188	93
AVC6	174	182	75/78	66	96	177	111/*114*	166	121	197	93
AVC7	174	182	78	66	99	162/171	114	166	*115*/121	197	93
AVC8	174	164	78	66	96	*171*/177	114	166	*121*/124	197	93
AVC9	174	152	78	66	96	177	114	166	121/*127*	197	87
AVC10	177/*183*	182	78	66	96	177	99/*114*	163	*121*/139	203	87
AVC11	174	152	75	66	96/99	*171*/177	114	163	121	197	87
AVC12	177	152	75	66	96	177	99	166	121	188	93
AVC13	177	*149*/152	75	66	96	177	99	166	121	188	84
AVC14	174	182	78/*84*	66	99	177	111	163	118	188	90
AVC15[#]	183	182	75/78	66	96	171	114	166	121	203	87
AVC16[$]	177	152	78	66	96	177	99	166	121	188	84
AVC17	177	152	78	66	96	171/*177*	99	166	121	188/*197*	84
AVC18	177	152	75/*78*	66	96	177	99	175	121	188	84
AVC19[$]	177	152	78	66	96	177	99	166	121	188	84
AVC20	177	152	78	66	96/99	171/*177*	114	175	121	*203*/197	93
AVC21	174	152	78	66	96	171	114	166	121	197/*203*	93
AVC22[$]	177	152	78	66	96	*171*/177	99	166	121	188/*197*	84
AVC23	174	152	78	66	96	*171*/177	114	175	121	197/*203*	93
AVC24	174	152/*170*	78	66	96	171	*93*/114	178	121/*124*	197	93
H_{obs}	0,59	0,66	0,43	0,00	0,16	0,60	0,67	0,53	0,24	0,65	0,71
H_{exp}	0,70	0,83	0,52	0,00	0,52	0,70	0,83	0,78	0,78	0,70	0,78
SVC1	183	182	78	66	96	171	114	166	121	203	93/*87*
SVC2[#]	183	182	75	66	96	171	114	166	121	203	87
SVC3	183	185	75	66	96	171	114	166	121	203	93
SVC4	*168*/174	*176*/185	78	66	96/*99*	171	114	166/*175*	121	194	93
SVC5	*177*/180	-	93	66	-	171	114	175	-	200	-
SVC6	174	182	84	66	99	177	111	175	118	191	90
SBE1	*174*/183	182	75/78	66	96/*99*	171/*177*	114	166	124	203	87
SBE2	174/*177*	164	78	66	99	177	114	166	124	188	84
SBE3	183	185	75/*84*	66	96	171/*177*	114	166	118	188/*203*	90
SBE4[#]	*174*/183	182	75/*78*	66	96	171	114	166	121	197/*203*	87
H_{obs}	0,71	0,77	0,71	0,00	0,52	0,53	0,20	0,36	0,74	0,84	0,83
H_{exp}	0,83	0,83	0,83	0,00	0,56	0,56	0,56	0,56	0,74	0,93	0,83
SCP1	183	191	75	66	96	171	99	166	124	203	87
SCP2	171	*152*/185	75	66	96	177	99	166	124	203	84
SCP3	183	152	87	72	96	174	99	175	121	203	84
SCP4	183	152	87	72	96	174	99	175	121	203	84
SCP5[#]	183	182	75	66	96	171	114	166	121	203	87
SCP6	174	164	75	66	99	177	114	166	124	188	84
SCP7	171	182	75	66	99	177	99	166	124	200	84
SCP8	171	152	75	66	99	174	99	163	121	188	84
SCP9	171	182	75	66	99	*168*/177	114	166	*118*/121	203	84/*87*
H_{obs}	0,67	0,75	0,22	0,22	0,56	0,72	0,50	0,56	0,56	0,67	0,39
H_{exp}	0,75	0,84	0,56	0,56	0,56	0,75	0,56	0,75	0,56	0,75	0,56
H_{obs} sym	0,72	0,76	0,62	0,11	0,56	0,65	0,53	0,44	0,65	0,74	0,74
H_{exp} sym	0,84	0,94	0,90	0,56	0,56	0,75	0,75	0,75	0,75	0,94	0,84
H_{obs} all	0,74	0,73	0,57	0,05	0,38	0,62	0,60	0,49	0,46	0,75	0,73
H_{exp} all	0,82	0,90	0,82	0,51	0,51	0,77	0,82	0,77	0,77	0,85	0,77

"-" means that no readable signal was obtained after two repetitions. Numbers in *italic* indicate non-dominant additional peaks in mixed isolates. Symbols "#" and "$" highlight identical haplotypes encountered in different isolates. The observed heterozygosity (H_{obs}) values for each marker (dominant values) in subgroups were calculated as follows: $H_{obs}= n/(n-1) * (1-S^{k}(p_i)^2)$, where n is the number of gDNAs per subgroup, k the number of different alleles and p the observed relative frequency of each allele. The expected heterozygosity (H_{exp}) was $H_{exp}= n/(n-1)*(1-k*((n/k)/n))$. "$H_{exp}$ sym", "H_{obs} sym", "H_{exp} all" and "H_{obs} all" show the heterozygosity values for gDNAs from all symptomatic (SCP/SBE/SVC) and all infections, respectively.

this population a rather limited repertoire of haplotypes similar to what was shown for other locations in the Amazon (Machado et al. 2004) which was not the case: The AVC group for example is as disperse as the SCP sample containing genotypes from infections from the entire county of Porto Velho. Upon R_{ST} analysis, genotypes from the Outpatient Clinic-group SCP showed significant genetic differentiation from the Candelária area, which was expected due to the different geographical origin of infections. Significant differences were also detected between genotypes infecting individuals in the Candelária area developing malaria symptoms or not. To our knowledge for the first time, it could be demonstrated that determined microsatellite marker sizes were associated with non-symptomatic outcomes. Two hypotheses may explain this finding. Firstly, the locally occurring asymptomatic-associated genotypes are less virulent than their symptomatic malaria-associated counterparts, in analogy to severe Malaria-associated genotypes in French Guyana (Ariey et al. 2001). This hypothesis is rather unlikely, since two parasite genotypes were found in asymptomatic and symptomatic samples. However, one must be aware that identical haplotypes may not necessarily mean a completely shared repertoire of virulence-relevant antigens.

Another hypothesis is that the population of Candelária may be immune against parasites bearing the genotypes (and their related antigens) encountered in the AVC sample, however, they are not immune against other genotypes which may be introduced to the area by 'outsiders' due to the significant tourist movement observed in Candelária, possibly explaining why mild symptomatic infections are still reported. It is not excluded that genetic host factors may also play a role.

Since strain-specific immunity to malarial infection is supposed to occur when the exposed individual developed competent immune responses to the majority of circulating variant antigens (Bull & Marsh 2002, Hviid & Staalsoe 2004), it may be hypothesized that the genotypes identified in the non-symptomatic infection-associated parasites are associated to variant antigens better recognized than the antigens encoded by variant genes associated to symptomatic infection-associated parasite genotypes. Again, as long as a differential immune response is not measured, any hypothesis in this direction is highly speculative.

Variant genes encoding erythrocyte surface exposed antigens such as the PfEMP1 antigen, encoded by the *var* gene family (Su et al. 1995) are mostly found in subtelomeric regions of all *P. falciparum* chromosomes (Gardner et al. 2002) and it is believed that these genes are rapidly redistributed during meiosis (Freitas-Junior et al. 2000). Accordingly, a fine-analysis of linkage disequilibrium of microsatellites along all *P. falciparum* chromosomes showed that the telomere ends are subject to far more recombination events than centromeric regions (Mu et al. 2005), where the herein used microsatellites are localized (Anderson et al. 1999). Therefore, it was not expected to see any association of the relatively selection-neutral (only the marker TA1 codes for antigen) markers with the observed form of infec-

tion caused by the analysed parasite genotype. We hypothesize that this association may only be found in situations with few recombination events due to low transmission, but never in the case of intense transmission and consequently high rates of recombination as in many African areas (Mu et al. 2005). Actually, it is unknown to what rate microsatellite markers of a given genotype change during a mosquito passage and subsequent reinfection.

A future task will be to identify if symptomatic infection-associated genotypes carry other variant gene repertoires than non-symptomatic associated genotypes. In a recent study, the global repertoire of variant genes, namely *var* genes, was estimated significantly smaller in Western Amazon parasites than from other areas (Albrecht et al. 2006). This turns the massive sequencing of symptomatic- and asymptomatic infection associated repertoires a feasible and not an never-ending task, as would be expected from African settings (see a study by Bull and colleagues (Bull et al. 2005).

What are the general implications for malaria epidemiology in riverine regions with populations such as those living in the Candelária suburb? On one hand, contact with new circulating parasite strains may cause any time novel infections in otherwise semi-immune individuals, and as occurred with the herein identified non-symptomatic associated strains, individuals may slowly mount resistance also against these novel strains. On the other hand, circulating parasites even at very low levels, which do not cause symptoms in the local population, are sufficient to contaminate present mosquito vectors in the area (Alves et al. 2005). These then may eventually put at risk malaria-naïve persons entering the area, very probably resulting to epidemics similarly to those observed in the past in this area (Cruz 1910, Sawyer 1988).

ACKNOWLEDGEMENTS

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