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Original article

Cerebral malaria is frequently associated with latent parasitemia among the semi-immune population of eastern Sudan

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Abstract

The accurate diagnosis of malaria starts with clinical suspicion, confirmed by reliable laboratory results. A hospital-based study, described here, was carried out in a malaria mesoendemic area in eastern Sudan, where the inhabitants are semi-immune to malaria, and the fever threshold of parasitemia is not above the detection level of microscopy. Thus, we hypothesized that patients with symptoms highly suggestive of cerebral malaria (CM), but aparasitemic by microscopy, may have submicroscopic parasitemia. Patients in our malaria clinic were screened by microscopy, and 120 individuals were selected for the study, including febrile patients with and without microscopically detectable parasitemia, and apparently healthy individuals. In the two former groups there were patients with severe anemia and deep coma. Polymerase chain reaction (PCR) for parasite detection and ELISA tests for measuring serum antibody levels were carried out on all blood samples. A majority of the febrile patients who were parasite negative by microscopy showed the presence of a *Plasmodium falciparum* infection by PCR. The occurrence of *P. falciparum* infection with parasitemia below the detection level of microscopy was recognized more often in patients with CM symptoms than in those with severe malarial anemia (SMA), and in older rather than younger patients. Patients clinically suspected (CS) of having CM (^{CS}CM) mostly were infected with a single clone, and a large proportion of them acquired antibodies (Abs) against merozoite surface protein (MSP) antigens (Ags). The therapeutic response to quinine treatment was comparable between patients with ^{CS}CM and CM. In conclusion, uniquely in this setting, CM can be associated with sub-patent parasitemia; thus, a diagnostic tool more sensitive than microscopy is needed.
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Keywords: *Plasmodium falciparum*; Cerebral malaria; Sub-patent; PCR

1. Introduction

Plasmodium falciparum malaria is a threat not only in the tropics and subtropics but also all over the world, especially during this era of globalization. Irrespective of the accuracy of morbidity and mortality estimates reported by the World Health Organization [1,2], the burden of malaria is rising [3]. Among the factors that have hampered malaria control efforts are epidemiological diversity and instability of malaria transmission, both factors having a direct influence on host immunity [4] and possibly on host and parasite evolution in the long term. Accordingly, in each epidemiological setting, malaria presentation, diagnosis and management are a local issue and cannot be universally standardized.

In equatorial sub-Saharan Africa, where malaria transmission is intense and stable, severe malaria (SM) in the form of anemia occurs early in life, after trans-placental maternal immunity has faded [5]. Cerebral malaria (CM) is a major

Abbreviations: Abs, antibodies; Ags, antigens; CM, cerebral malaria; CS, clinically suspected; ELISA, enzyme linked immunosorbent assay; MF, malaria-free; MSP, merozoite surface protein; PCR, polymerase chain reaction; RBC, red blood cells; SM, severe malaria; SMA, severe malarial anemia.

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fatal complication, peaking at the age of 2–3 years, and by the age of 5 years, malaria mortality declines sharply [6–8]. In these areas, the diagnosis of malaria is based on parasitemia above a cut-off level, since not all febrile conditions are attributed to malarial parasitemia [9]. In the other extreme, non-immune individuals and travelers from malaria-non-endemic areas are prone to develop the full clinical spectrum of SM at any age, although the risk of mortality increases with each decade of age [10]. Diagnosis is based on detection of parasitemia at any level. Mortality associated with *P. falciparum* infection is in these cases, commonly due to organ failure and other acute complications rather than chronic anemia [11]. In the dry savannah of sub-Saharan Africa, like in the Sudan, malaria is mesoendemic, and transmission is confined to the months that follow the rainy season [12]. Parasitemia of any count could be associated with malaria morbidity and mortality, although it is occasionally higher in fatal malaria. CM is more common during adolescence and early adulthood, and it is the commonest cause of malaria mortality in this area [13].

Historically, a remarkably unstable rainfall characterizes the eastern horn of Africa, including the northern two thirds of the Sudan. This results in unstable malaria transmission in the region, and the inhabitants of these areas are described as semi-immune. The diagnosis of malaria in this area is sometimes problematic, partly due to clustering of annual morbidity into a few busy months and partly due to the occurrence of symptomatic and even SM with low parasitemia. This is in addition to the inherent limitations in the use of microscopy [14,15] and inexperienced microscopists. These limitations, especially in naïve and semi-immune communities, could lead to delays in the diagnosis or misdiagnosis of malaria due to false negative results. In contrast, the overestimation of malaria (false positive) or diagnosis made basically on clinical judgment can have serious implications, as many diseases could mimic malaria symptoms. Thus, the physician's awareness of the possibility of malaria infection and the unbiased consideration of other febrile diseases are complementary for improving malaria diagnosis in these situations.

This study investigated whether submicroscopic parasitemia exists in patients who present with clinical manifestations highly suggestive of CM. The rationale was based on three observations that are unique to this setting: (a) there is sometimes no correlation between severity of malaria and parasite count; (b) a typical clinical presentation of malaria is not uncommonly associated with a negative blood smear; (c) asymptomatic parasitemia is rare. To test for the above association, we deployed the more sensitive and specific technique of polymerase chain reaction (PCR) for detection of malaria parasites [16], with emphasis on clinically suspected (CS) CM (^{CS}CM) patients. PCR is a molecular tool, which revolutionized the epidemiology of malaria, but for case diagnosis, it is neither practical nor diagnostic in areas where malaria is hyper-endemic, where parasite detection in symptomatic cases is not a problem. Parasite detection in early symptomatic malaria may be a problem in malaria non-endemic countries or areas of low malaria transmission, where low parasitemia is associated with disease manifestations. On the other hand, the serological diagnosis of current malaria infection by detection of generally long-lasting antibodies (Abs) against malaria antigens (Ags) is unreliable [17], but it has been tried as a crude marker for recent exposure [18].

2. Material and methods

2.1. Study area

This study was carried out in New-Halfa Hospital, eastern Sudan, 600 km from Khartoum. New-Halfa is a relatively large town center surrounded by more than 100 small villages on the outskirts of the town; it has a population of around 250,000, with approximately 2000–3000 individuals in each village. Several ethnic groups, including Afroarab tribes, were settled in the area in 1964. A large sugar cane industry and associated agricultural schemes represent the backbone of the economy in the area. The agriculture depends on rainfall and irrigation from Atabra Nile, and many canals are found around and between the villages.

The malaria infections in the region are predominantly (95%) caused by *P. falciparum,* and transmission is seasonal and unstable. Although malaria is mesoendemic in this area, severe epidemics intersperse the continuous flow of the seasonal malaria. The health facilities of the area are constituted of one referral hospital, where severely ill patients are brought (the study site), one single-doctor hospital and twenty dispensaries.

2.2. Study design

This study was designed to be part of a large study on the pathogenesis of SM in eastern Sudan. The data were obtained in the period from November 2000 to February 2001. The study received ethical clearance and national endorsement by the Sudan Ministry of Health. A medical doctor examined patients presenting with malaria-like symptoms in New-Halfa Hospital, in a malaria outpatient clinic. The diagnosis of malaria was by examination of thick and thin blood smears on light microscopy. Patients with confirmed malaria were treated with chloroquine (10 mg/kg body weight on each of the first 2 days and 5 mg/kg body weight on the third day) and/or sulfadoxine/pyrimethamine. A sulfadoxine/pyrimethamine single dose of 1500 mg sulfadoxine and 75 mg pyrimethamine (three tablets) was given to adults; children with body weight 5–10 kg were given 0.5 tablet; 11–20 kg one tablet; 21–30 kg 1.5 tablets and 31–45 kg two tablets. Patients with SM or showing infections resistant to the former drugs were treated with quinine (10 mg base/kg body weight 8-hourly) for 7 days.

During the study period, all patients admitted to the hospital in a coma and suspected of having malaria, but with negative blood smear for malaria parasites (^{CS}CM), were

included in the study after the consent of their guardians was obtained. The enrolled patients were treated according to the health policy and management protocol adopted by the local health authorities. They were initially treated by quinine infusion and, after suitable clinical improvement, by quinine tablets (10 mg/kg body weight, 8-hourly, for a total duration of 7 days). Two patients were further treated with benzyl penicillin (10^6 IU/6-hourly, for 5 days), and one of them was given gentamicin (80 mg/8-hourly for 5 days) in addition; all were given in parallel with quinine from day 1. Only a small fraction of individuals from other groups with and without microscopically confirmed *P. falciparum* malaria were included in the study, as controls, in addition to apparently healthy donors.

2.3. Malaria definition and diagnosis

A patient was defined as suffering from malaria if he/she complained of fever or had body temperature measured with an oral probe of > 37.5 °C, plus microscopically detected asexual parasitemia. Patients classified as having CM were in unarousable coma persisting for at least 30 min, while patients with severe anemia had normocytic anemia with hematocrit < 15% or hemoglobin < 5 g/dl, in the presence of microscopically detectable parasitemia [1]. Patients CS to have SM (^{CS}SM) had the above symptoms, but parasitemia was not detectable by microscopy, and the other possible diagnoses were excluded; the latter was done by meticulous clinical examination, consultancy of clinical experts and by performing basic routine and feasible investigations such as urine/stool general, complete hemogram, chest X-ray, lumbar puncture and etc.

For the diagnosis of malaria, thick and thin blood smears were prepared, stained with Giemsa and examined by microscopy for detection of malaria parasites under a magnification of × 1000, by the clinic's technician. Blood smears were considered negative after examination of 200 fields without detection of parasites. Another two expert malaria microscopists using quality control-evaluated microscopes at the malaria reference laboratory rechecked all slides. Ideally, more blood smears should be taken 12–24 h later from microscopy-negative patients to exclude cases where the infections are synchronous and the parasites are sequestered. However, that is not practical, as in comatose patients a decision needs to be taken immediately (emergency situation). Furthermore, during the peak of malaria transmission in remote rural areas with limited resources and personnel and a large number of febrile patients, redoing microscopy for all smear negative patients is not feasible. All patients with SM or ^{CS}SM were treated with quinine. Patients with SMA were further treated with cross-matched, infection-screened blood transfusion.

2.4. Blood sampling

Blood samples (5–10 ml) were drawn into heparinized vacutainers from patients with malaria or suspected to have malaria at the time of diagnosis and before treatment, and from malaria symptom-free donors. From each of those donors, a drop of blood was blotted on filter paper, dried and sealed in a separate plastic bag.

2.5. Detection and genotyping of malaria parasites by PCR

The malaria parasite DNA was extracted from blood in filter papers using the protocol described by Plowe et al. [19]. In brief, sterile 0.5% saponin in 1 × PBS was used overnight (at 4 °C) for lysis of red blood cells (RBCs). Then, hemolyzed RBCs were washed with PBS (autoclaved), and 50 µl of stock 20% chelex-100 solution (100 °C) was used for DNA extraction. After repeated vortex and heating and finally spinning, a DNA solution was obtained, transferred to a fresh tube and stored at –20 °C until use.

PCR was used for detection of *P. falciparum* parasite DNA by genotyping, essentially as described by Ranford-Cartwright et al. [20] and Paul et al. [21], using primer pairs for allele families IC1 and FC27 of the *P. falciparum msp-2* gene.

2.6. Measurement of Abs against MSP Ags by ELISA

Anti-MSP1 Abs were measured by indirect enzyme linked immunosorbent assay (ELISA), using a protocol described by Cavanagh et al. [22]. In brief, 96-well microtiter plates were coated at 4 °C for 3 days with $MSP1_{19}$ (fused with Glutathione-S Transferase, GST) or with GST (50 ng, pH 9.4). Plates were washed by washing buffer (PBS-Tween) and were blocked by skimmed milk. One hundred microliter of diluted plasma (1:500) was added in duplicates to coated plates and the latter incubated overnight at 4 °C. After washings, plates were incubated sequentially with 100 µl horseradish peroxidase-conjugated rabbit antihuman IgG Abs (1:5000 dilution) and 100 µl substrate buffer, then the reaction was stopped with 2 M H_2SO_4. The optical density (OD) was measured using an ELISA reader at 492 nm, and the mean value of each pair of wells (after subtraction of that of GST) was calculated. A pool of adult African hyperimmune sera and sera obtained from 17 Danish blood donors known not to have been exposed to malaria, were used as positive and negative controls, respectively, in each plate. The same steps were followed for measurement of Abs against MSP2 Ags, GF and T9 [23]. The cut-off level for positive plasma was considered as the mean plus 3 × S.D. of OD readings of the Danish blood donors. The OD readings of serial dilution of the hyperimmune sera were used as a reference for grading of Ab levels into high, intermediate and low levels.

2.7. Statistical analysis

SigmaStat software was used for statistical analysis. For comparison of the clone number between all clinical subgroups, we used Kruskal–Wallis one-way analysis of variance on rank, and when comparing between any two sub-

groups, we used Mann–Whitney rank sum test. The Fisher exact test was used for comparison of proportions of antibody responders and prevalence of merozoite surface protein (MSP) alleles between the study groups.

3. Results

3.1. Clinical and parasitological categorization of patients

From among the patients seen in the malaria clinic, 120 individuals were recruited to the study. Three study groups were identified based on clinical and parasitological data: 52 febrile patients CS of having malaria but having negative blood smears, 40 febrile patients with microscopically confirmed malaria, and 28 apparently malaria-free (MF) donors (Table 1). In the former two groups of patients, there were three clinically diverse sub-groups of patients: patients with or suspected of having uncomplicated malaria (UM), severe malarial anemia (SMA) or CM.

The CM patients.

Among the CS group of malaria patients, 24 patients were brought to the hospital in a coma, suspected of having CM (^{CS}CM), the microscopic examinations failed to confirm the clinical diagnosis. The age of ^{CS}CM patients (age data are available for 21 out of 24 patients) falls between 1 and 70 years of age (including one child and three adolescents), with an overall mean (S.D.) age of 31.2 ± 21.8 years. Half of the patients were males. All the comatose patients were treated initially with quinine infusion: in addition, injectable antibiotics were given to two patients. Nineteen patients (79.2%) fully recovered, and five (20.8%) patients died (mean age was 46.2 ± 22.4 years).

The other clinically diagnosed malaria patients with negative blood smear included 14 patients suspected of having SMA (^{CS}SMA; hemoglobin—mean ± S.D. of 24.3 [± 4.5]%). Their mean age was 18.6 ± 19.3 (range 1–58) years (age data are available for nine out of 14 patients). Fourteen patients with a mean age of 4.1 ± 3.4 (range 1–12) years, had symptoms suggestive of uncomplicated malaria (^{CS}UM).

The control groups were patients with microscopically confirmed malaria and MF donors; the former group included 40 patients, 17 of which had uncomplicated malaria (UM) and 23 SM (10 patients had CM and 13 had SMA). The mean age ± S.D. (range) of the patients with UM, CM and SMA were 8.2 ± 9.4 (1–30), 8.4 ± 9.1 (2–33) and 1.6 ± 1.1 (0.3–4). In addition, a group of 28 apparently healthy donors (MF) was included in the study, with mean age ± S.D. (range) 6.8 ± 4.8 (1–21) years.

The ^{CS}SM groups (^{CS}CM and ^{CS}SMA) had a mean age much greater than that of the corresponding representative group of the confirmed SM; CM and SMA. However, the mean age for all selected groups represented the overall age pattern of malaria prevalence in the area ([24], unpublished data).

3.2. Parasite prevalence by PCR

P. falciparum parasites were detected by PCR in 17 out of 24 (70.8%) blood samples obtained from the ^{CS}CM patients (Table 2). All the five comatose patients who died had parasitemia revealed by PCR (Fig. 2A). For the 14 ^{CS}SMA, the parasite rate as detected by PCR was 42.8% (6/14). The parasite detection rate by PCR was 100% for the 14 patients who had negative blood smears and clinical features suggestive of uncomplicated malaria (^{CS}UM). Parasitemia was also detected by PCR in patients with microscopically confirmed SMA, CM and UM, except for one patient with UM (PH08), for who repeated PCR did not reveal any visible band. On the other hand, parasitemia was detected by PCR in only five of the 28 asymptomatic (MF) donors (17.8%), and two of them had parasitemia also detected by microscopy (7%). As shown

Table 1
Number of individuals, number of patients with coma and mortality rate of comatose patients in the three study groups

Study groups	Confirmed Malaria (+ve blood smear)	^{CS}Malaria (–ve blood smear)	Apparently healthy (MF donors)
Number of individuals (male/female ratio)	40 (27/13)	52 (24/28)	28 (17/11)
Number of comatose patients	10	24	0
Mortality rate of comatose patients	20% (2/10)	20.8% (5/24)	0% (0/28)

Grouping of study individuals was based on presence of malaria symptoms and microscopic detection of parasites. CS = clinically suspected, MF = malaria-free.

Table 2
Parasitemia detected by microscopy and by PCR and the mean number of clones, in different study groups or subgroups

Parasitemia	Patients with +ve BF			Patients with –ve BF			Apparently healthy, MF donors (n = 28)
	UM (n = 17)	CM (n = 10)	SMA (n = 13)	^{CS}UM (n = 14)	^{CS}CM (n = 24)	^{CS}SMA (n = 14)	
Parasite rate by microscopy	100%	100%	100%	0%	0%	0%	7.1%
Parasite rate by PCR	94.1%	100%	100%	100%	70.8%	42.8%	€17.8%
Mean clone number	1.69 ± 0.70	1.70 ± 0.82	1.62 ± 0.77	1.86 ± 0.66*	1.23 ± 0.56*	1.17 ± 0.41	1.20 ± 0.45

BF = blood film for malaria parasite. UM = uncomplicated malaria, SMA = severe malarial anemia, CM = cerebral malaria, MF = malaria-free donors and CS = clinically suspected. *Comparison of the number of parasite clones between ^{Cs}CM and ^{Cs}UM, $P = 0.014$. € Comparison of parasite rates (PCR) between MF donors and the other patient groups, $P < 0.05$ for all comparisons.

in Table 2, the differences were statistically significant between the prevalence of parasitemia as detected by PCR between malaria symptom-free donors (MF) and the other malaria patient categories, UM, ^{CS}UM, CM, ^{CS}CM and SMA, ^{CS}SMA (17.8% for MF versus 94.1, 100, 100, 100, 70.8 or 42.8, respectively), $P < 0.05$ for all comparisons.

3.3. Molecular characterization of parasite isolates

The predominant sub-patent malaria infections of the comatose patients (^{CS}CM) were single-clone infections (82.3%, 14/17), with a mean clonal number (MCN) of 1.23 ± 0.56, while only 50% of the CM infections with patent parasitemia, were single-clone infections, with an MCN of 1.70 ± 82 (Table 2 and Fig. 2). The uncomplicated malaria infections were relatively more commonly multiclonal, both patent UM (56%, 9/16; with an MCN of 1.69 ± 0.70), and sub-patent ^{CS}UM (71.4%, 10/14; with a MCN of 1.86 ± 0.66). Although there were great variations in clone number between the groups, the mean clonal number was not statistically significantly different between all the groups taken together (P = 0.060, Kruskal–Wallis one-way analysis of variance on rank). However, when comparing ^{CS}CM with ^{CS}UM the difference was statistically significant (1.23 ± 0.56 versus 1.86 ± 0.66, P = 0.014, Mann–Whitney rank sum test). The genotype IC1 was detected in all PCR-detectable infections except in seven patients (7/80). The genotype FC27, was recognized in only three infections in ^{CS}CM (17.6%, 3/17), while it was recognized in 7/10, 9/14, and 8/17 parasite isolates obtained from CM, ^{CS}UM and UM patients, respectively. The differences were statistically significant when comparing ^{CS}CM to CM or ^{CS}UM, (P = 0.013 and 0.012, respectively, Fisher exact test).

3.4. Prevalence of antibodies against MSP Ags

The prevalence of Abs against any or all the three MSP fragments ($MSP1_{19}$, $MSP2_{GF}$, $MSP2_{T9}$), in plasma collected at diagnosis and before treatment, was calculated for the three major groups of donors. Plasma from the ^{CS}SMA subgroup was not obtained.

As seen in Fig. 1, the positivity rate of anti-MSP Abs was 84% (16/19), 68% (26/38), 36% (8/22), and 14% (2/14) in the groups of patients, the ^{CS}CM, microscopically confirmed malaria, MF and ^{CS}UM groups, respectively. The difference in the proportion of responders between the ^{CS}CM and microscopically confirmed malaria group (UM & SM) was not significant (P = 0.339, Fisher exact test). But the difference between ^{CS}CM and MF or ^{CS}UM donors was statistically significant (P = 0.004 and 0.001, respectively), as was the difference between the confirmed malaria group and MF or ^{CS}UM (P = 0.029 and 0.001, respectively). When considering the clinical subgroups, the Ab prevalence rate in comatose patients, with negative blood smear and negative PCR, was 86% (6/7), while it was 100% (5/5) in comatose patients who died thereafter. While the prevalence of anti-MSP1 Abs was low in the MF donors, most of the responders (80%) had the highest level of Abs compared with the other groups as seen in the semi-quantitive estimation of antibody levels (Fig. 2A, B).

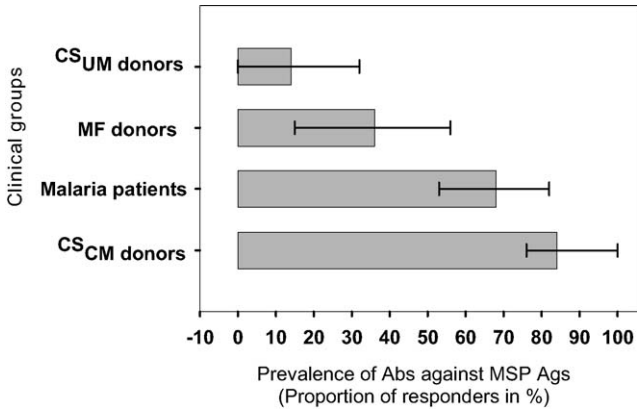

Fig. 1. The proportions (percentage and 95% confidence interval) of antibody responders to any of the MSP fragments ($MSP1_{19}$, $MSP2_{GF}$ and $MSP2_{T9}$), in the different study groups. Malaria patients were patients with microscopically confirmed *P. falciparum* malaria, including CM, SMA, and uncomplicated malaria patients (in declining order of frequency of responders, but the difference between ^{CS}CM and MF or ^{CS}UM donors was statistically significant (P = 0.004 and 0.001, respectively), as was the difference between the confirmed malaria group and MF or ^{CS}UM (P = 0.029 and 0.001, respectively). For the abbreviations ^{CS}UM, ^{CS}CM and MF see Table 2.

4. Discussion

SM, including CM, has been an important cause of human death since time immemorial and still poses a great challenge. Both the delay or failure of treatment and the delay and failure of diagnosis of malaria are important factors in malaria mortality [11]. The proper diagnosis of malaria begins with clinical suspicion [25], not only in non-immune travelers but also for the semi-immune inhabitants of malaria endemic regions. This study was not a random survey, since most of the ^{CS}SM patients were included in the study, while only small numbers of patients from the other clinical categories were enrolled. The discrepancy between PCR and microscopy in diagnosis of malaria in CS patients (^{CS}CM, ^{CS}UM) but not in others (CM, UM, or MF) was mainly due to preselection of the groups by microscopy before the deployment of PCR. Our data clearly suggests that a proportion of patients in this area can develop acute fulminant and often fatal CM symptoms with scanty, submicroscopic parasitemia. This phenomenon was predominantly recognized in adults, whose infections were revealed by PCR. Furthermore, the therapeutic response and fatality rates after quinine treatment were comparable with that of CM with patent parasitemia in the same region [13].

In febrile patients with microscopically unproved malaria, presentation with coma narrows the differential diagnosis by exclusion of febrile conditions like pneumonia, typhoid, and other infectious diseases that are not a common cause of coma. Still other clinical conditions like meningitis, encephalitis and

A

Patient ID	Age year	Genotype IC1	FC27	CN	Anti-MSP Ab level	Clinical category
CH01	60					^{cs}CM
CH02	NK					^{cs}CM
CH04	35				0	^{cs}CM
CH05	32					^{cs}CM
CH06	NK					^{cs}CM
CH07	NK					^{cs}CM
CH08	65					^{cs}CM
CH03	5			1		^{cs}CM
CH09	4			1		^{cs}CM
CH10	50			1		^{cs}CM
CH13	25			1	0	^{cs}CM
CH15	1			1	ND	^{cs}CM
CH16	33			1	0	^{cs}CM
CH17	40			1		^{cs}CM
CH20	17			1		^{cs}CM
CH19	13			2		^{cs}CM
CH18	13			2		^{cs}CM
SH13	5.5			1		^{cs}CM
SH19	26			1		^{cs}CM
CH12	30			1		^{cs}CM$_{died}$
CH14	70			1		^{cs}CM$_{died}$
CH22	55			1		^{cs}CM$_{died}$
SH01	16			1		^{cs}CM$_{died}$
CH11	60			3		^{cs}CM$_{died}$
PH08	2			0		UM
PH02	4			1		UM
PH03	30			1	0	UM
PH09	2			1		UM
PH10	5			1		UM
PH15	2			1	0	UM
PH18	7			1		UM
PH21	1			1	0	UM
PH13	28			2		UM
PH04	5.5			2		UM
PH05	2			2	0	UM
PH06	14			2		UM
PH29	1.5			2		UM
PH30	22			2		UM
PH31	5			2		UM
PH14	5			3		UM
PH07	4			3	0	UM

B

Patient ID	Age year	Anti-MSP Ab level
NH02	21	0
NH04	20	
NH17	11	0
*NH01	9	
NH09	9	0
NH13	9	0
NH14	9	
NH22	9	0
NH16	8	0
NH29	8	
NH10	7	0
NH11	7	
NH18	7	0
NH24	7	
*NH26	7	0
NH23	6	
NH28	6	0
+NH19	5	0
NH27	5	0
NH06	4	
NH21	3.5	0
NH25	2.5	0
NH03	2	0
NH05	2	0
NH20	2	0
*NH15	1.5	0
+NH08	1	0
NH12	1	0

+ Blood film & PCR positive
* Only PCR positive
CN = clone number
Ab = antibodies
ND = not done
CS = clinically suspected
CM= cerebral malaria
UM= uncomplicated malaria
NK= not known

Fig. 2. Representative data from the three most diverse study groups: CS CM, microscopically confirmed uncomplicated malaria and MF donors. The test parameters are (a) parasite genotype (number of clones indicated by shading of small boxes; light shading = one clone, dark shading = two clones); and (b) level of anti-MSP Abs (blank, light, medium or dark, shaded large boxes for no response, low, intermediate and high level of Abs, respectively). Panel (A), patients with malaria symptoms (^{CS}SM and UM); panel (B), apparently MF donors. Patients with ID in bold were given quinine and benzyl penicillin, and patients with ID in *italic* were given gentamicin in addition.

some viral infections could mimic CM. Although meningitis can occur sporadically, it occurs in epidemics mainly before the rainy season, and it is mostly (but not absolutely) clinically distinct. However, the other infections occur sporadically but are not clustered during the short window of malaria transmission. Malaria and viral infections can co-exist in patients if either of them can be recognized independently of the other in the same community at the same time, but that was not the case for conditions like meningitis and viral encephalopathy, since no single case of either condition was recorded during the study period. Other non-febrile conditions presented with coma, like cerebro-vascular accidents (CVA) can be ruled out, since those patients do not present with fever. Practically, clinicians are confronted on many occasions with such clinical cases, where the diagnosis is a problem, and the decision to treat the patient as a malaria case or not, is critical and vital for patient survival.

P. falciparum infection was detected in a majority of the CS cases of CM (^{CS}CM), as well as in all patients with CS uncomplicated malaria (^{CS}UM). Obviously, it was the periph-

eral parasitemia and not the absolute parasitic load, which was estimated by microscopy. In a recent post-mortem study carried out in Thailand and Vietnam, patients who died of CM showed a 26.6-fold higher parasitemia in the post-mortem brain vessels than in the pre-mortem peripheral blood [26]. In West Africa, Kurzhals et al. [27] reported that from 20% to 25% of the patients with anemia did not have microscopically detected parasitemia. In some non-immune travelers, malaria parasites were detected only when patients were terminally ill, or at post-mortem [11]. However, in general, peripheral hyperparasitemia is associated with complications of SM, and it is also considered an independent risk factor in malaria infection [1].

The occurrence of asymptomatic parasitemia is rare in the study region [24], in this study only a limited number of positive blood smears or PCR positives were observed among the malaria symptom-free (MF) donors. Although the MF donors were not followed to know whether their asymptomatic infections became overt malaria or not, there was a significant difference in the prevalence of PCR-proved submicroscopic parasitemia between MF donors and patients suspected of having CM. This appears different from the situation in Tanzania, where MF donors were shown to have the same prevalence of parasitemia as patients suspected of having CM [28]. In the latter setting, other neurological diseases were diagnosed and CM was excluded.

There was a significant difference between ^{CS}CM and uncomplicated malaria (UM and ^{CS}UM) infections in the mean clone number, which tends to be smaller in the former group. This is in agreement with other studies, where less complex infections were found to be more associated with CM [29]. However, in this study the mean clone number in CM with latent parasitemia was found to be comparable with that of UM.

Data obtained from large scale, longitudinal, village-based studies of infection and immunity in the region strongly support the description of the population as semi-immune to malaria [12,13,18,22,24]. Although prevalence and levels of Abs against MSP1 and MSP2, as serological markers for exposure, is a rather crude approach at the individual level, it was found to be informative in epidemiological surveillance in this setting [18,22]. Clinical, molecular and serological data were in favor of the diagnosis of malaria infection in most, if not all, of the comatose patients with microscopically undetectable parasitemia. Furthermore, this was supported by the occurrence of ^{CS}CM during the peak of the malaria transmission and the adequate therapeutic response of the ^{CS}CM patients to anti-malarial treatment. In undiagnosed patients with prolonged fever in Pakistan, the thick smears of bone marrow revealed *P. falciparum* malaria parasites in high proportions of patients with sub-patent parasitemia [30].

A possible explanation for the sub-patent parasitemia (microscopically negative) in patients with CM symptoms is sequestration of infected erythrocytes (IE) in the vascular bed of internal organs. The sequestration of the IE in deep tissues, with a preference for the brain, has been found to be associated with SM [31–33]. Thus, the sub-patent parasitemia was only time-related and coincided with the cytoadherence of the late stages in synchronized parasite growth. The single-clone infections (dominant in ^{CS}CM) logically should tend to synchronize easier than a multi-clonal infection. And by definition, parasites causing cerebral complications are more virulent and are characterized by the ability of cytoadhesion. However, the objective proof for sequestration would be the study of post-mortem brain specimens; the lack of such data are one of the limitations in this study.

In conclusion, CM may be associated with latent parasitemia in partially immune adults. One of the implications of this finding would be that the diagnosis of CM should not be ruled out if the blood film result is proved to be negative, and there is strong clinical suspicion of CM. That urges the development of robust and user-friendly molecular tools for diagnosis of scanty peripheral parasitemia, especially in naive and semi-immune populations. The development of such tools might also result in a reduction in malaria infection following use of microscopically screened blood for transfusions in areas of low malaria endemicity.

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