Analysis of circulating populations of *Plasmodium falciparum* in mild and severe malaria in two different epidemiological patterns in Madagascar

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Summary

**Objective** To investigate whether the severity of *Plasmodium falciparum* attack in endemic areas was associated with the multiplicity of infection (MOI) and/or with a particular genotype(s).

**Method** In two areas of different malaria transmission pattern in Madagascar (Sainte-Marie – mesoendemic and Tsiraoanomandidy – hypoendemic) the number and the proportions of *msp-2* genotypes within isolates were determined for each patient using a capillary electrophoresis genotyping method. DNA sequencing was performed to identify the *msp-2* allelic family of dominant clones.

**Results** Eighty six uncomplicated and 33 severe cases were included in Sainte-Marie and 48 uncomplicated and 69 severe cases were included in Tsiraoanomandidy. We found no association between the MOI and severity of malaria as the same mean number of *msp-2* genotypes was found in isolates from uncomplicated and from severe malaria cases (3.72 and 3.73, respectively, P>0.05). The study of the association of dominant clones with clinical status showed no particular genotype or allelic family associated with malaria severity.

**Conclusions** Severity of malaria was not associated with higher MOI in our study. Severity did not appear restricted to some particular genotypes either. On the contrary, severe malaria appeared to be caused by very common genotypes in the studied areas. More comprehensive explorations including immunity and genetic factors of the host are needed to acquire new information about this complex condition.

**Keywords** *Plasmodium falciparum*, severe malaria, mild malaria, multiplicity of infection

Introduction

*Plasmodium falciparum* malaria remains one of the major causes of morbidity and mortality in sub-Saharan Africa, leading each year to the death of an estimated number of more than one million individuals, mostly children (Korenromp 2004). Individuals often harbour a mixture of genetically distinct parasites, which results in a major challenge to immune protection, drug efficacy and malaria control (Contamin et al. 1996). It is apparent that whether an individual develops mild or severe malaria must depend on a complex combination of host and parasite factors (Miller et al. 2002) but the development of immunity remains probably the key factor as older children and adults in endemic areas are less frequently victims of severe malaria. Variations in parasite virulence may also contribute to the wide spectrum of disease severity observed in *P. falciparum* malaria infection. The main factors of virulence are capacity of parasite multiplication (Chotivanich et al. 2000) and ability to induce binding of infected RBCs to the vascular endothelium (cytoadherence) and to non-infected erythrocytes (rosetting) or to other infected...
erythrocytes (autoagglutination), all phenomena which lead to local occlusions of post-capillary microvasculature. It has been reported that the ability to bind to multiple host receptors as well as the ability to form rosettes was found more frequently among isolates from children with severe vs. mild malaria (Heddini et al. 2001). There is speculation that a parasite that binds to multiple erythrocytes and endothelial receptors may cause more obstruction than one that only adhere with single-receptor specificity in the microvasculature. It remains unknown whether multi-adhesion of the pRBCs found in the patients with severe malaria is due to the coexpression of multiple binding events in a single clonal population of parasites, or if the observation reflects multiclonal infection, where each clone infecting the patient adheres with a distinct receptor specificity (without excluding the possibility that individual clones are multiple adhesive). These binding events lead infected erythrocytes to sequester and avoid clearance by the spleen in addition to cause microvascular inflammation and obstruction (Fairhurst & Wellems 2006). In addition to cytoadherence, multiple infections may present diverse abilities to induce rosetting or autoagglutination. Multiple infections may also induce the production and/or release of various pro-inflammatory cytokines and may be more difficult to control by immunity. The association of multiplicity of P. falciparum infection (MOI) with severity of clinical episodes is poorly explored and data are controversial (Robert et al. 1996; Mockenhaupt et al. 2003; Ranjit et al. 2005; A-Elbasit et al. 2007; Mayengue et al. 2007, Amodu et al. 2008). Independent of MOI, some authors have reported the association of particular genotypes with severe malaria in French Guyana and India (Ariey et al. 2001; Legrand et al. 2005; Ranjit et al. 2005). In areas of higher malaria transmission, others failed to see such an association (Robert et al. 1996; Shigidi et al. 2004).

We examined the genetic complexity of the parasites obtained in subjects having severe malaria or uncomplicated cases from two areas in Madagascar. The aim of this study was to investigate whether the severity of malaria attack was associated with the multiplicity of infection and/or with a particular genotype. For that purpose, we have used a capillary electrophoresis genotyping method that enumerates the clones and quantify their proportions within P. falciparum isolates (Jafari et al. 2004).

Methods

Study areas

Two areas of different malaria transmission pattern (Sainte-Marie – mesoendemic and Tsiroanomandidy – hypoendemic) were chosen for the study. Sainte-Marie is a 60-km long island along the East coast of Madagascar at an altitude of 2 m, at 17°05’ S, 49°49’ E. The mean annual rainfall is 2500 mm, with no dry season. The climate is tropical with mean temperatures greater than 20 °C all year. Malaria transmission is stable and perennial at an estimated more than 30 infective bites per person per year (Mouchet et al. 2004). The population (estimated 19 000 inhabitants) is poor and relies on fishing and subsistence agriculture. This island attracts a lot of tourists. Tsiroanomandidy is located on the north-western foothill area at an altitude of 700 m, at 18°47’ S, 46°03’ E. The mean annual rainfall is 1496 mm, with four months without precipitation (May to August). The mean annual temperature is 22.5 °C, with maximal temperatures in November (near 30 °C) and the mean temperature of the coldest months, July and August, ranging between 16 °C and 19 °C. Malaria transmission is moderate; the annual entomological inoculation rate (number of bites of infected anophelines per adult person) was estimated as less than 3 (Mouchet et al. 2004). P. falciparum is the dominant species (79%) but P. vivax, P. malariae and P. ovale are also present. Tsiroanomandidy is considered as representative of an intermediate stratum between coastal high transmission areas and unstable malaria transmission characteristic of the highlands, due to its ecological characteristics and on the basis of parasitologic evidence. The population (approximately 25 000 inhabitants) is mostly rural; cultivating rice is the main occupation.

Selection of patients

As part of the routine malaria survey within the National Malaria Control Program, this study was conducted over three consecutive malaria seasons (1998–2000). Severe malaria patients and mild patients were included at the same period at each site. Mild malaria patients were recruited from subjects attending the outpatient clinic at basic health centres in town (not far from the District Hospital) or attending the outpatient clinic of the District Hospitals. Uncomplicated malaria was defined as fever (axillary temperature greater than 37.5 °C) with P. falciparum parasitemia (sexual parasites >1000/μl) and no sign of gravity, following the 1990 WHO definition (Warrell et al. 1990). Severe malaria patients were recruited at the District Hospitals of Tsiroanomandidy and Sainte Marie. Hospitalized children suffered respectively from severe anaemia (haemoglobin concentration of <5 g/dl), cerebral malaria (defined as a Blantyre coma score of less than 2 or as being comatose with simultaneous inability to localize a painful stimulus), or other severe symptoms if they were prostrated or had repeated
generalized convulsions (more than two episodes in the preceding 24 h) but did not meet the criteria of severe anaemia or cerebral malaria (Warrell et al. 1990). Children with other detectable infections or cause for the clinical presentation were not included in the study. Malaria was diagnosed by microscopy. Thick and thin blood smears were prepared following the standard procedures and 100 microscopic fields (approximately 20 leukocytes/field at 1000x = approximately 0.25 μl of blood) of the thick blood smear were examined. The *Plasmodium* species was identified on the thin blood smear. Malaria patients were treated according to the national recommendation. Clinical *P. falciparum* isolates (2 ml of venous blood) were collected by venipuncture into EDTA-coated tubes from consenting patients before treatment has started (the procedure did not delay the treatment). The isolates were transported to the MoH Malaria Department laboratory in Antananarivo and kept at −20 °C until use. The study protocol was reviewed and approved by the Direction d’Etude et de Planification within the Ministry of Health and informed consent was obtained from the children’s guardians.

**Parasite DNA preparation and clonal analysis**

Parasite DNA was extracted from 200 μl of red blood cell pellets by phenol–chloroform purification (Ariey et al. 1999). The number and the proportions of genotypes within isolates were determined for each patient using a previously published fragment analysis method (Jafari et al. 2004). Briefly, the method was based on the polymorphism of the gene encoding merozoite surface protein 2. The amplification of a part of merozoite surface protein-2 (*msp*-2) gene by polymerase chain reaction (PCR) with a primer pair including a sense primer 5’ labelled with fluorescein, followed by capillary gel electrophoresis, was used to discriminate alleles of different base pair sizes. Each genotype was characterized by the size and the area under the curve of the peak corresponding to its *msp*-2 PCR products measured during the capillary electrophoresis. The description of each isolate included the number of *msp*-2 genotypes, the size of the corresponding PCR products and the proportion of each genotype (given in percentage) within the isolate. Block 3 of the *msp*-2 domain was amplified on 2 μl of DNA solution in the following conditions: in a 50 μl reaction mixture containing 15 pmol of each primer 5’-GAAGGTATTTA-AACATTGTC-3’ (sense) and 5’-GACACCTCGTCGTTG-TAGGGAG-3’ (antisense) (Genset SA Europe), buffer (15 mmol/l Tris–HCl [pH 8.0], 50 mmol/l KCl, and 6 mmol/l MgCl2) 200 μmol/l dNTP, and 1.25 U of AmpliTaq Gold DNA (Applied Biosystems). The thermal cycling conditions were as follows: 7 min at 95 °C, followed by 40 cycles for 30 s at 95 °C, 30 s at 42 °C, and 30 s at 72 °C. Amplifications products were then processed in an ABI PRISM 3100 Genetic Analyser (Applied Biosystems) to enumerate and quantify fluorescent fragments as previously described (Jafari et al. 2004).

**DNA sequencing**

DNA sequencing was performed for monoclonal isolates and multiclone isolates having a dominant clone corresponding to more than 80% of total parasitemia. The block 3 of *msp*-2 was amplified by nested PCR using the primers and the PCR conditions described previously (Snounou et al. 1999), allowing the detection of the FC27 and 3D7 alleles. PCR products were purified by using a QIAquick PCR Purification Kit (Qiagen) and sequenced by using an ABI PRISM Big Dye Terminator Cycle sequencing Kit (Applied Biosystems) in an ABI PRISM 310 Genetic Analyser following the manufacturer’s protocol.

**Statistical analysis**

Means and proportions were compared between groups using Student *t*- and chi-square tests, respectively. Since the MOI variable was not normally distributed, a non-parametric Mann–Whitney *U*-test was used to compare the MOI between severe and uncomplicated malaria cases in each site. *P* values less than 0.05 were considered significant. All analyses were performed using stata statistical software, version 9.0 (StataCorp LP, College Station, TX, USA).

**Results**

**Characteristics of malaria cases**

The study population included 236 patients, of whom 119 were recruited in Sainte-Marie (33 severe and 86 mild malaria cases) and 117 in Tsirioanomandidy (69 severe and 48 mild malaria cases). Main clinical manifestations and characteristics of the patients are summarized in Table 1. In Tsirioanomandidy, the mean age of patients was lower in severe malaria compared to uncomplicated malaria (9.8 and 17.5 years respectively, *P* = 0.008), whereas the opposite was seen in Sainte-Marie (mean age 18.0 and 12.2 years, respectively, *P* = 0.02). Two people died in Sainte-Marie (one severe and one uncomplicated case) and nine people died in Tsirioanomandidy (all severe cases). Mean parasite density was higher in patients with severe than in those with uncomplicated malaria, although not significantly (40 421/μl vs. 23 401/μl, *P* = 0.08).
We found no association between the MOI and severity of malaria as the same mean number of msp-2 genotypes was found in isolates from uncomplicated and severe malaria cases (3.72 and 3.73, respectively, \(P > 0.05\)). In Tsiroanomandidy, the mean MOI tended to be higher among severe cases when compared to non severe (3.52 vs. 3.10, \(P = 0.06\)) but without reaching statistical significance, and in Sainte Marie, there was no difference in mean MOI between the two groups (4.15 vs. 4.07, \(P = 0.83\)).

There was a slight variation of MOI according to age with higher MOI occurring in 7–20 years old subjects in Sainte-Marie (mean MOI = 4.4) and in 3–20 years old subjects in Tsiroanomandidy (mean MOI = 3.42). However, according to age groups, there was no association between MOI and severity of malaria in each site of transmission and in both merged sites.

On an average, patients in Sainte-Marie harboured more clones than patients in Tsiroanomandidy: 4.09 vs. 3.35, \(P = 0.002\). The diversity of genotypes was comparable in both sites with 39 different msp-2 alleles observed in Sainte-Marie vs. 40 in Tsiroanomandidy (Figure 1). DNA sequencing showed that the ratio of genotypes belonging to the FC27 family/genotypes belonging to the 3D7 family was 0.86 in Sainte-Marie and 1.11 in Tsiroanomandidy. As most alleles were present in both areas, the cumulative number of identified alleles was 44. Some genotypes were over represented in isolates from both sites but others were found on fewer than 10 occasions (Table S1). The study of the association of dominant clones within isolates with clinical status showed that no particular genotype or msp-2 allelic family was found associated with malaria severity.

### Discussion

The main finding of the study was the mean number of circulating genotypes in severe malaria cases appeared similar to that of the uncomplicated ones. Our results were consistent with those of a study in Gabon (Mayengue et al. 2007) and of another study in Sudan (A-Elbasit et al. 2007) but were in contrast with those of smaller series performed in India in which mild cases had fewer clones compared to severe ones (Ranjit et al. 2004, 2005). Our results did not confirm either the reports from Senegal (Robert et al. 1996) or Nigeria (Amodu et al. 2008), where severe malaria had lower MOI than mild cases. Those previous studies had limitations. Robert et al. included patients regardless of their ethnic origin: French military personnel were considered in the same group as locals (Robert et al. 1996). Mockenhaupt et al. (2003) studied MOI in severe anaemia vs. uncomplicated malaria but used ill-defined criteria of inclusion (7 g/dl haemoglobin concentration threshold instead of 5 g/dl in the WHO severe malaria...
Figure 1 Frequency distribution of merozoite surface protein-2 dominant alleles in patients with mild or severe malaria (only one dominant clone per isolate is shown). (a) Isolates from Sainte-Marie; (b) isolates from Tsiraoanomandidy.
criteria). Ranjit et al. (2004, 2005) included severe cases in a tertiary care centre when severe complications developed after treatment at rural health care centres, which could have reduced isolates complexity. Differences in transmission levels according to study sites may result in hazardous comparisons regarding MOI of isolates (Amodu et al. 2008). Differences in genotyping methods and/or interpretation of data may also explain in part the variability of results between studies.

A particular 3D7 msp-2 allele, having a PCR product of near 550 bp (540–559 bp, according to the studies), was over represented in severe malaria cases in India (Ranjit et al. 2005). In another study, the same allele (albeit with a different name) and another one (300–319 bp FC27 msp-2) were found as possibly (but ‘still within the range of statistical chance’) associated with severe malaria in Ghana (Mockenhaupt et al. 2003). Other authors reported that severe malaria associated an association of a specific msp-1 allele with a specific var gene in French Guiana (Ariey et al. 2001; Legrand et al. 2005). These studies were performed in limited areas and their results cannot be extrapolated to other regions. Moreover, they did not consider the proportion of the genotypes within the isolates because the used methodology was unsuited to doing so. The capillary electrophoresis genotyping method used enabled us to enumerate the clones and to quantify their proportions; thus we found that the 536 bp 3D7 msp-2 allele was globally over represented but also that this allele was rarely dominant within isolates. When majority genotypes were considered, no particular allele was associated with severity of malaria attacks. We did not notice either over-representation of an allelic family, 3D7 or FC27, in severe malaria. Our results are consistent with those of A-Elbasit et al. (2007) and with those of Färnert et al. (2002) on this point as these authors did not find an association between severity of disease and allelic msp-1 or msp-2 types in Swedish travelers.

The MOI paralleled the levels of malaria endemicity in the study areas as the mean number of clones was significantly higher in isolates from Sainte-Marie than from Tsiranoamandidy. These results are consistent with previous findings in other areas (Zwetyenga et al. 1998; Hoffmann et al. 2001). The difference of transmission between the study areas was also perceptible as the mean MOI in Tsiranoamandidy tended to be higher among severe cases than among uncomplicated cases, which was not seen in Sainte-Marie. However, this trend was not statistically significant and thus did not prevent the merger of data of both sites for a larger analysis.

Hypoendemic areas represent a favourable epidemiological context in the search for parasite-derived elements contributing to virulence, as acquired immunity rapidly reduces the clinical severity of malaria infections. One factor that might potentially compromise a comparison of genotypic characters of isolates collected from patients experiencing a clinical episode is uncontrolled drug intake before presentation to the hospital. Another limitation of the present work was that we studied only circulating parasites and not those which sequestered in the tissues. A recent work by Dembo et al. (2006) suggested that among children with severe malaria the dominant clones sequestered in deep organs are usually the same as those in peripheral circulation. Another study of circulating and sequestered population of P. falciparum in fatal paediatric malaria did not show a significant difference between the average number of genotypes detected in brain and other profound tissues in comparison with peripheral blood (Montgomery et al. 2006).

The capillary electrophoresis genotyping method using the polymorphism of the msp-2 gene proved to be a powerful tool for genotyping. Previous works have demonstrated that considering msp-2 gene alone may be suitable for P. falciparum genotyping studies (Jafari et al. 2004; A-Elbasit et al. 2007). The global allelic diversity in both sites of this study was high and very similar to that reported by Mockenhaupt et al. (2003) who detected 40 msp-2 different alleles in a series of 366 children in northern Ghana.

In conclusion, severity of malaria was not associated with higher MOI in our study and did not appear either restricted to some particular genotypes or even to some allelic msp-2 family. On the contrary, severe malaria appeared to be caused most often by very common genotypes in the studied areas. More comprehensive explorations including immunity and genetic factors and also the nutritional and sociologic status of the host should probably be performed to acquire new information about this complex condition.

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References


Supporting Information

Additional Supporting Information may be found in the online version of this article:

Table S1. Number and proportion of clones (in percentages) within isolates. Each isolate is named by a letter (i stands for Tsiranoamandidy; H for Sainte-Marie)
followed by an inclusion number in the study. Each clone is characterized by its proportion in isolate, from first (C11 column) to ninth (C91 column) and by the size of its msp-2 PCR products in capillary electrophoresis, from C12 to C92 columns, respectively.

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