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Dynamics of *Plasmodium falciparum* alleles in children with normal haemoglobin and with sickle cell trait in western Uganda

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Summary We describe the diversity of *Plasmodium falciparum* populations in western Uganda and assess the role that asymptomatic malaria carriers with sickle cell trait (HbAS) may be playing on the *Plasmodium* population structure. We genotyped *P. falciparum* in 291 samples using merozoite surface protein (MSP) 1 and 2 loci. Extensive genetic diversity was detected among symptomatic children in Mbarara (20 MSP1 alleles; 31 MSP2 alleles) and Kagando, Kasese (19 MSP1 alleles; 30 MSP2 alleles). Multiplicity of infection (MOI) was significantly higher in Kagando, Kasese than in Mbarara, with 2.7 and 2.1 genotypes/PCR positive sample with MSP2 marker, respectively. Similar strains were circulating in the two sites; however, a few strains specific to individual sites were observed. Prevalence of HbAS was 36% (12/33) among asymptomatic children in Kisinga sub-county, Kasese. In asymptomatic children, MOI was age-dependent and higher in HbAS carriers than HbAA, suggesting that HbAS carriers harbour a wider range of *P. falciparum* genotypes. Sickle cell trait may influence rapid acquisition of premunition by creating a reservoir of variant parasite strains in the host. The high level of genetic diversity demonstrated here shows that even in areas with low or seasonal transmission, high levels of parasite polymorphism can occur.

1. Introduction

Malaria is the leading cause of morbidity and mortality in children under 5 years of age in Uganda. *Plasmodium falciparum* populations are highly diverse,[1,2] and this genetic diversity varies geographically. Implementation of malaria

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control measures, such as use of effective drugs or vaccines, begins with obtaining information on the genetic polymorphism of the parasites in the human host in the community to be investigated. Because of their extensive polymorphisms, merozoite surface protein (MSP) 1 and MSP2 genes have been used to characterize *P. falciparum* infections[3,4] and specifically to determine the multiplicity of *P. falciparum* infections in residents from endemic areas.[2,5,6]

In Kasese district, Uganda, where most out-patient cases among the under fives are due to malaria, sickle cell trait (HbAS) frequency is assumed to be high. While it has been suggested that the protective effect of HbAS is linked to some modulation of the immune response against malaria,[7,8] recent observations show that it also involves accelerated acquired immunity to the parasite;[9,10] however, the mechanisms of protection are still unclear.[9,11] There is scanty information on the composition of *P. falciparum* populations in regions with relatively low malaria transmission, such as western Uganda. Many HbAS carriers have asymptomatic infections and so may serve as 'good' reservoirs of the parasite; however, their influence on the turnover and/or genetic diversity of *P. falciparum* is still unclear. Description of the local *Plasmodium* population might lead to greater understanding of the molecular basis of vector—human—parasite interactions. Presence of various genotypes for a specific parasite locus in a host may increase the possibility of cross-fertilization and meiotic recombination in the mosquito vector.[12] This would necessitate use of molecular markers for monitoring and evaluation of efficacy of antimalarials. Our objectives were to describe the diversity of *P. falciparum* populations in children aged three months to 15 years and assess the role that asymptomatic malaria carriers with HbAS play on this population genetic structure. This paper aims at improving our understanding on how carriage of HbAS may be influencing the natural history of the disease.

2. Materials and methods

2.1. Study sites

We targeted children reporting to Mbarara University Teaching Hospital (MUTH) and Kagando Hospital (KH). MUTH is the regional referral hospital in Mbarara district. Kagando Hospital is in Kisinga sub-county, Kasese district. The districts are 153 km apart. Mbarara district (average altitude 1420 m) has low malaria transmission. Kasese (average altitude 940 m) has strongly seasonal transmission.[13] Raper[14] reported 40—45% HbAS gene frequencies in Bundibugyo district, which is near Kasese. The Sickle Cell Association of Uganda estimates 40% HbAS frequency in Bundibugyo/Kasese and 1—4% in Mbarara; the present situation is not known.

2.2. Parasite samples

2.2.1. Patients

The study was conducted at the end of each of two dry seasons into the rainy seasons that followed: January—March 2004 at MUTH; October through November 2004 at KH. One hundred and twenty-nine children were recruited (Table 1) after obtaining informed consent from their parents/guardians. Criteria for inclusion: children aged 3 months to 15 years, uncomplicated clinical malaria confirmed microscopically to be *P. falciparum*-positive. Exclusion criteria: complicated malaria, sickle-cell disease (HbSS), severe anaemia (packed cell volume <21%). Thin and thick blood films of every subject were made and stained using 10% Giemsa stain. Fingerprick blood samples for PCR assay were collected on Whatman paper #3. A little blood from the same fingerprick was collected in heparinized tubes for haemoglobin electrophoresis. Follow-up blood samples were collected on days 4, 18 and 31.

2.2.2. Asymptomatic malaria carriers

To study natural host—parasite interactions, 33 asymptomatic children (CK cohort) in Kisinga sub-county, Kasese were recruited (Table 1) and followed-up during the dry season, from late December 2004 through January 2005. The children had not had symptomatic malaria 2 weeks before and 1 week after day 0, and had axillary temperature $\leq$37.3 °C and a positive microscopic blood slide. Blood samples were collected at Kajwenge primary school and from the homes of the children, within a radius of 3 km from KH, on days 0, 4, 8 and 14.

2.3. Typing of haemoglobin

Haemoglobin in samples was separated by electrophoresis on cellulose acetate membrane in alkaline buffer (pH 8.4—8.6) at 350 V (TITUN PLUS, power supply) for 20 min and stained with Ponceau S stain (Helena Laboratories; Beaumont, TX, USA) following the manufacturer's protocol.

2.4. DNA extraction and PCR genotyping

Genomic DNA of *P. falciparum* was extracted using the Chelex-100 method[15] with modifications. Extracted DNA was stored at −20 °C until air-freighted to Germany for PCR assays.

Nested PCR typing strategy was used based on a primary duplex PCR using highly conserved primers flanking a polymorphic domain of the gene block 2 of MSP1 and block 3 of MSP2, and a second reaction driven by an internal set of primers.[12] All primary PCR reactions took place in a total volume of 25 μl, containing 1.25 units of SuperHot polymerase (Bioron GmbH, Ludwigshafen, Germany), 200 μmol/l of each of the four deoxyribonucleotides (dNTPs), 100 pmol of each of the primers, 2.5 μl 10× complete buffer (Bioron GmbH) containing 670 mmol/l Tris-HCl pH 8.8, 160 mmol/l $(NH_4)_2SO_4$, 25 mmol/l $MgCl_2$, 0.1% Tween-20 and 2.0 μl DNA. PCR conditions were 5 min at 96 °C followed by 38 cycles of 30 s at 96 °C, 30 s at 55 °C, 90 s at 72 °C and a final step of 3 min at 72 °C in iCycler (Bio-Rad Laboratories Ltd, Hertfordshire, UK). Nested PCR took place in a total volume of 25 μl, containing 1 μl and 2 μl primary PCR product (diluted 1:100 with PCR-quality water) used as template for MSP1 and MSP2, respectively. PCR reaction mix contained 1.25 units of Fire Pol polymerase (Bioron GmbH), 200 μmol/l of each of the four dNTPs, 100 pmol of each specific primer to amplify MSP1 alleles, MAD20, K1, RO33, and MSP2 alleles, FC27 and IC, 2.5 mmol/l $MgCl_2$ for MSP1 (3.0 mmol/l $MgCl_2$ for MSP2) and 2.5 μl 10× incomplete buffer (800 mmol/l Tris-HCl pH 9.4, 200 mmol/l $(NH_4)_2SO_4$, 0.2% w/v Tween-20).