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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).

Table 1 Baseline characteristics of study children aged 3 months to 15 years recruited in Mbarara and Kasese districts in western Uganda

	MUTH[a] *n* (%)	KH[b] *n* (%)	CK[c] *n* (%)
No. subjects recruited	61	68	33
Type of haemoglobin			
HbAA (*n*)	59 (97)	66 (97)	21 (64)
HbAS (*n*)	2 (3)	2 (3)	12 (36)
No. aged ≤2 years	30 (49)	23 (34)	7 (21)
MOI (parasitaemia[d])	1.9	2.6	1.6 (231/190)
No. aged >2−≤5 years	19 (31)	25 (76)	3[e] (9)
MOI (parasitaemia[d])	2.3	2.8	3.0 (1624/−)
No. aged >5 years	12 (20)	20 (29)	23 (70)
MOI (parasitaemia[d])	2.2	2.6	3.1 (626/84)
Parasitaemia[d,f]	8866	10 753	314 (593/103)

MOI: multiplicity of infection, estimated as indicated in Materials and methods; −: no value, because the age group had no HbAS carrier.
[a] Symptomatic malaria carriers recruited at Mbarara University Teaching Hospital, Mbarara, Uganda.
[b] Symptomatic malaria carriers recruited at Kagando Hospital, Kasese, Uganda.
[c] Asymptomatic malaria carriers recruited in Kisinga sub-county, Kasese, Uganda.
[d] Geometric mean parasite density (parasites/μl) of microscopically positive samples according to type of haemoglobin. This was determined only for the CK cohort. Parasitaemia for HbAA carriers is given first followed by the corresponding value for HbAS.
[e] Children in the age group were all HbAA carriers.
[f] Geometric mean parasite density (parasites/μl) of microscopically positive samples of all individuals in the cohort.

PCR conditions were as follows: for MSP1, initial denaturation of 5 min at 95 °C, 38 cycles of 30 s at 95 °C, 30 s at 58 °C, 45 s at 72 °C, and a final extension of 3 min at 72 °C; for MSP2, initial denaturation of 5 min at 96 °C, 38 cycles of 30 s at 96 °C, 30 s at 55 °C, 45 s at 90 °C, and extension of 3 min at 72 °C in iCycler (Bio-Rad Laboratories Ltd). Nested PCR products were separated on 1.4% agarose gel, stained with ethidium bromide and visualized under ultraviolet light (UV-Transilluminator; Herolab GmbH, Wiesloch, Germany). A software package called E.A.S.Y. Win32 (Herolab GmbH) was used to analyse the separated DNA fragments. Representative DNA fragments were sequenced using PE Applied Biosystems automated sequencer (Perkin-Elmer, Warrington, UK).

2.5. Data analysis

The diversity of the *Plasmodium* population was determined as the number of distinct alleles detected in day 0 samples. Alleles were considered the same if molecular weights were within 20 bp, except RO33-type alleles. Strains were categorized into 20 bp ranges and average values were taken as the size of the alleles. For RO33 alleles, a 30 bp size range was used because of a 30 bp variation in alleles when electrophoresis was repeated several times. This categorization of alleles was confirmed by DNA sequencing of selected fragments. Multiplicity of infection (MOI) was calculated by dividing the total number of DNA bands by the number of samples positive in PCR reaction in the group under consideration. The duration of carriage of infection in HbAA and HbAS carriers was estimated using the Kaplan–Meier analysis with censoring of retained and/or newly acquired alleles and the difference in survival of alleles was compared using the log-rank test. Statistical procedures were done using SPSS, version 12 (SPSS Inc., Chicago, IL, USA); statistical significance was $P < 0.05$.

3. Results

3.1. Frequency of type of haemoglobin

HbAS was low among symptomatic children but high [36% (12/33)] among asymptomatic children (Table 1). We analysed the association of haemoglobin type with outcome variables of parasite populations only for asymptomatic children (Table 1), because the number of HbAS among symptomatic children could not allow statistical analysis. The difference between day 0 parasitaemia of asymptomatic children with HbAS was significantly higher than that with HbAA (ANOVA F; $P = 0.012$).

3.2. Genetic diversity

In total, 291 samples were genotyped: 129 day 0 and 102 follow-up samples from the MUTH and KH cohorts; 33 day 0 and 27 follow-up samples from the CK cohort. All the major allelic families described for MSP1 and MSP2 reviewed in Miller et al.[16] and Fenton et al.,[17] respectively, were detected (Table 2). In all samples, the most prevalent MSP2 allelic family was FC27 and K1 for MSP1 (Table 2).

Size polymorphism was highest in the MSP2 allelic families. Unlike in KH, all RO33-type infections in the MUTH cohort had one band (Supplementary Table 1). The distribution of some individual MSP1 and MSP2 alleles differed in cohorts (Figures 1–3), particularly K1, FC27 and IC alleles (Figures 1B, 2A and 2B), respectively.

3.3. Multiplicity of infection

Most of the samples had multiple infections with at least two parasite clones (Table 2). The MOI was higher in

Table 2 Characteristics of *Plasmodium falciparum* infections genotyped for the merozoite surface protein (MSP) 1 and MSP2 locus in study children aged 3 months to 15 years recruited in Mbarara and Kasese districts in western Uganda

Cohort		Allelic family				
		MSP1			MSP2	
		MAD20	K1	RO33	FC27	IC/3D7
MUTH[a]	Positive samples, *n* (%)	21 (34)	42 (69)	24 (39)	48 (79)	37 (61)
	DNA bands[b] (*n*)	24	48	24	74	45
	Range of band sizes (bp)	137—272	150—379	124—180	186—504	385—705
	PMAF[c] (%)	25	50	25	62	38
	Alleles (*n*)	7	10	3	17	14
	MOI[c]	MSP1 = 1.7; 45%			MSP2 = 2.1; 63%	
KH[d]	Positive samples *n* (%)	32 (54)	49 (83)	42 (71)	43 (73)	48 (81)
	DNA bands[b] (*n*)	37	66	45	84	62
	Range of band sizes (bp)	134—256	154—325	126—234	202—510	429—723
	PMAF[c] (%)	25	45	30	58	42
	Alleles (*n*)	7	8	4	15	15
	MOI[c]	MSP1 = 2.5; 78%			MSP2 = 2.7; 75%	
CK[e]	Positive samples *n* (%)	11 (33)	17 (52)	9 (27)	17 (52)	21 (64)
	DNA bands[b] (*n*)	13	26	9	41	30
	Range of bands sizes (bp)	156—271	158—268	134—162	213—480	319—713
	PMAF[c] (%)	27	54	19	58	42
	Alleles (*n*)	6	6	2	13	14
	MOI	MSP1 = 2.0; 58%			MSP2 = 2.7; 62%	

MOI: multiplicity of infection, estimated as indicated in Materials and methods followed by percentage of samples with multiple infections.
[a] Symptomatic malaria carriers recruited at Mbarara University Teaching Hospital, Mbarara, Uganda.
[b] 'DNA bands', the total DNA fragments identified for the locus analysed. Individual alleles were detected as indicated in Materials and methods.
[c] Prevalence of MSP1 and MSP2 allelic families.
[d] Symptomatic malaria carriers recruited at Kagando Hospital, Kasese, Uganda.
[e] Asymptomatic malaria carriers recruited in Kisinga sub-county, Kasese, Uganda.

the KH cohort than in the MUTH cohort, and the difference was statistically significant ($P < 0.001$ for MSP1 and $P = 0.036$ for MSP2). In the asymptomatic group, the MOI was higher than in the MUTH cohort, but the difference was not statistically significant ($P = 0.48$). Different combinations of alleles were detected in multiple infections; none was associated with the clinical disease. Allelic combinations of FC27-305+405 bp, FC27-325+405 bp, FC27-345+IC-435 bp and FC27-325+IC-515 were detected in both MUTH and KH cohorts (Supplementary Table 2).

3.4. Multiplicity of infection and age

We used the most polymorphic marker, MSP2, to analyse the influence of MOI on parasitaemia in different age groups (Table 1). Among symptomatic children, most multiple infections were seen in children below 3 years of age. There was no correlation between MOI and actual age of asymptomatic children (Spearman's ρ, $P = 0.262$). However, using an age of 3 years as a cut-off value showed a significant correlation between age and MOI (Spearman's ρ, $P = 0.028$).

3.5. Genetic diversity and type of haemoglobin

The effect of haemoglobin type on genetic diversity could not be analysed among symptomatic children, as mentioned earlier. In asymptomatic children aged above 5 years, multiple infections were detected more in HbAS carriers than in HbAA carriers (Fisher's exact test, $P = 0.04$).

Both FC27 and IC type alleles were frequent (78%) in HbAS carriers, but there was no significant influence on haemoglobin type on the distribution of these allelic families. There was no association between the distribution of day 0 MSP1 and MSP2 allelic families and haemoglobin type (Mann-Whitney *U* test, $P = 0.45$, $P = 0.12$, $P = 0.069$, $P = 0.195$ and $P = 0.45$ for MAD 20, K1, RO33, FC27 and IC, respectively).

3.6. Follow-up visits of symptomatic children

Following treatment, alleles transiently disappeared, or additional alleles appeared (and eventually disappeared), or some day 0 alleles reappeared during follow-up but parasitaemia, reflected by geometric mean parasite density, was lower than at day 0. Changes in parasitaemia were not

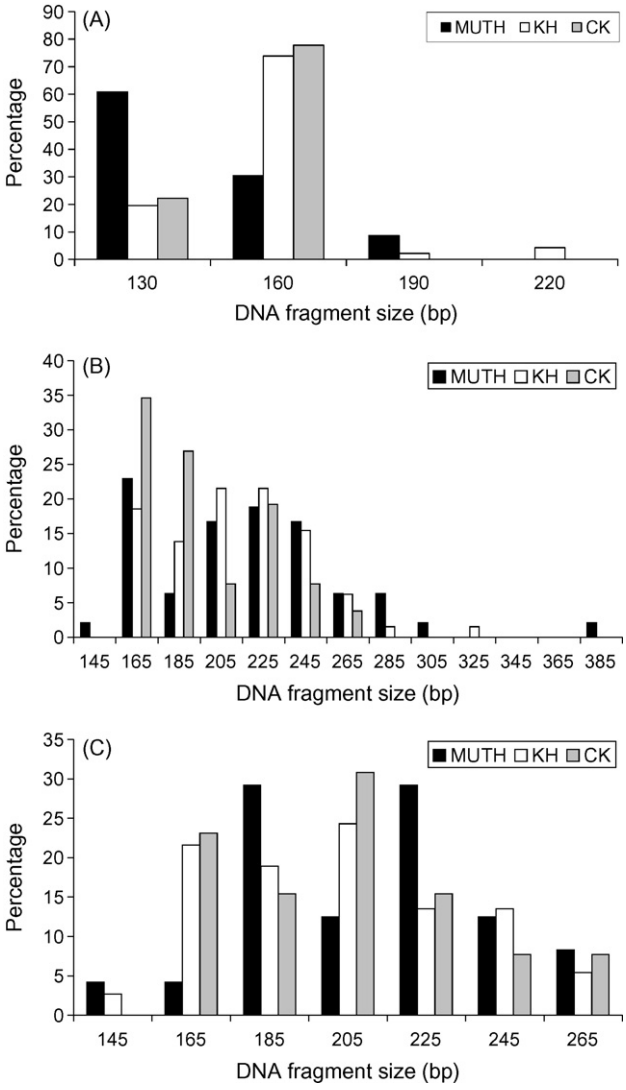

Figure 1 Comparison of the distribution of day 0 merozoite surface protein (MSP) 1 alleles in isolates from children aged 3 months to 15 years recruited in Mbarara and Kasese districts in western Uganda. (A) RO33-type, (B) K1-type and (C) MAD20-type MSP1. MUTH: symptomatic cohort recruited at Mbarara University Teaching Hospital; KH: symptomatic cohort recruited at Kagando Hospital, Kasese; CK: asymptomatic cohort recruited in Kisinga community, Kasese.

related to the fluctuation of alleles (Spearman test for MOI and parasitaemia, $P = 0.7$). No specific genotype was associated with the clinical disease.

3.7. Duration of carriage of genotypes: follow-up of asymptomatic children

We investigated duration of carriage of specific genotypes among asymptomatic malaria carriers using IC-type alleles, because no HbAS sample carried any FC27-type infection by day 4. The duration of carriage of IC-type alleles over the 14 day follow-up was studied in 22 children: 9 HbAS and 13 HbAA. HbAA carriers were infected longer (mean of 12 d; 95% CI 10—13) than HbAS carriers (mean of 7 d; 95% CI 5—10).

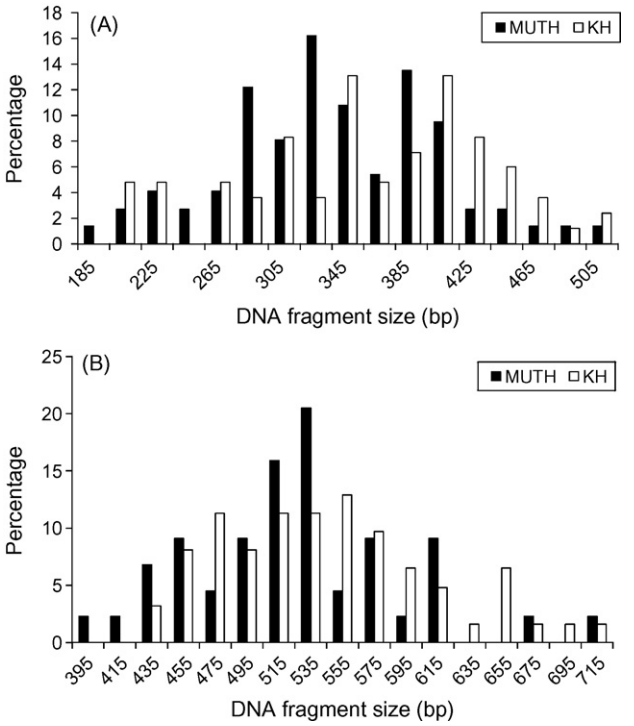

Figure 2 Comparison of the distribution of day 0 merozoite surface protein (MSP) 2 alleles in isolates from children aged 3 months to 15 years recruited in Mbarara and Kasese districts in western Uganda. (A) FC27-type and (B) IC-type MSP2. MUTH: symptomatic cohort recruited at Mbarara University Teaching Hospital; KH: symptomatic cohort recruited at Kagando Hospital, Kasese.

The difference in duration of infection was statistically significant; log rank, $P = 0.011$.

There was a high turnover of IC-type genotypes in individual children, characterized by acquisition of new genotypes and reappearance of clones that had disappeared previously: no HbAS carrier carried the same genotype on two follow-up visits. Among HbAA individuals, only IC-type alleles were detected in the same individuals after day 0. Children with HbAA carried specific genotypes longer [mean of 9 d (95% CI 8—10)] than HbAS carriers [mean of 7 d (95% CI 5—10)], but the difference was not statistically significant (log rank, $P = 0.15$).

We compared duration of infection of FC27 and IC alleles in asymptomatic children with HbAA to determine the MSP2 allelic family most responsible for long *P. falciparum* infection in these children. Interestingly, FC27-type infections, which were not detected by day 4 in HbAS carriers, showed a higher probability of carriage beyond day 14 than IC-type infections in HbAA carriers. Duration of carriage of infection was a median of 14 d for IC-type infection and more than 14 d for FC27-type infections, but the difference was not statistically significant (log rank, $P = 0.057$).

4. Discussion

We characterized the *P. falciparum* population using MSP1 and MSP2 loci as genetic markers and provided evidence that HbAS increases MOI.

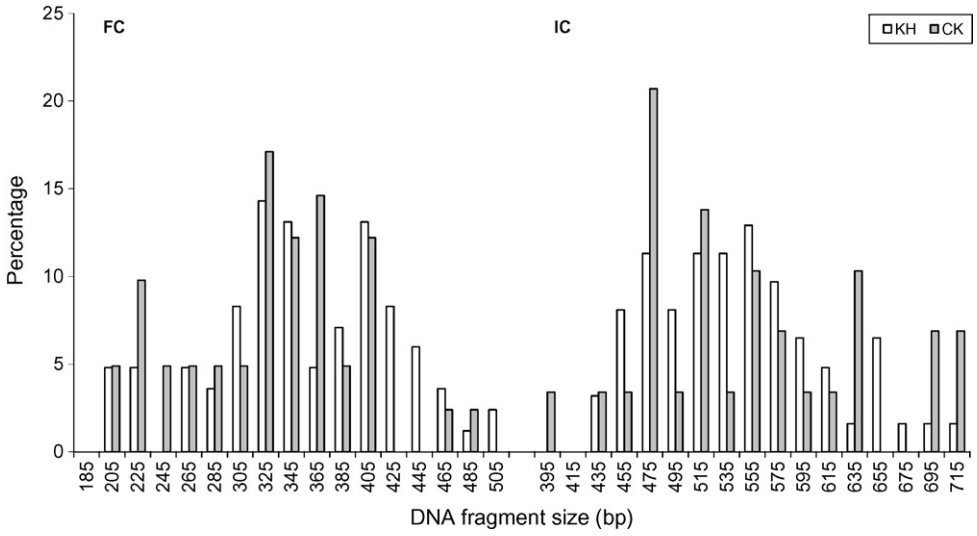

Figure 3 Comparison of the distribution of day 0 merozoite surface protein 2 alleles in isolates from children aged 3 months to 15 years recruited in Kasese district in western Uganda. KH: symptomatic cohort recruited at Kagando Hospital; CK: asymptomatic cohort recruited in Kisinga community.

We encountered a small number of HbAS carriers at the two hospitals. This may be because of its protective effect against high parasitaemia and malaria morbidity. HbAS frequency was 36% among asymptomatic children in Kisinga, Kasese. The small number of asymptomatic children could have biased the figure. This figure was lower 16% (13/79) among all asymptomatic children examined for malaria before recruitment (data not shown). The 40% HbAS prevalence observed in the region[14] needs to be reassessed in a larger study.

Asymptomatic children with HbAS had lower parasitaemia than HbAA carriers between age groups, confirming earlier findings[18] that the HbS gene reduces parasite density. In HbAS carriers, parasitaemia declined with increasing age, although the trend was not definite.

All the five allelic families were detected in the study areas, with FC27 being the most frequent MSP2 allelic family, contrasting with other data[19] in which the IC allelic family was predominant. This can be explained as geographical variation in *P. falciparum* diversity.[20] In this study, although the allelic families in the samples analysed were highly diverse, a similar distribution pattern for MSP1 and MSP2 allelic families was detected in Mbarara and Kasese, suggesting that at the time of the study, the same strains were circulating in the areas. Lack of significant difference in allelic family distribution is very unlikely to be caused by travel between the study sites, as noted by Peyerl-Hoffmann et al.;[19] the two study areas are 153 km apart, and there is limited travelling by residents of the study area in Kasese. One possible explanation may be migration of mosquitoes.

In all cohorts, K1 was the most abundant MSP1 allelic family, contrasting with the findings of Apio et al.,[21] who reported that the most predominant MSP1 allelic type in Ugandan isolates was MAD20. Our findings are similar to other data.[19,22] An explanation for this could be an increase in selection pressure of *P. falciparum* in families that are more polymorphic. In areas with unstable malaria, even distribution of the MSP1 allelic families has been reported.[23] We detected three RO33-type alleles in the MUTH and KH cohorts, and four in the CK cohort, contrasting with other studies[22,24] but similar to those of Jordan et al.[25] and Ntoumi et al.[26] In an earlier study in Uganda, Peyerl-Hoffmann et al.[19] observed four different fragments but could not confirm whether they were unspecific bands or new RO33 variants. Our study confirms variation in the RO33 allelic family in western Uganda, thereby extending the Peyerl-Hoffmann et al.[19] findings. DNA sequencing revealed a deletion in the RO33-160 bp allele that yielded the RO33-130 bp allele.

RO33 alleles 190 and 220 bp were detected in 7% (5/69) of RO33-positive samples from symptomatic children. Although this is a small contribution, the existence of these uncommon alleles reveals a greater degree of diversity at this locus than previously reported and highlights increasing polymorphism in *Plasmodium*. Two samples in the KH cohort showed multiple RO33-alleles, similarly to the findings of Jordan et al.[25] These could have been new inoculations of other RO33 alleles circulating in the area. In all cohorts, RO33 alleles were short-lived. It is assumed that many are cleared by effective treatment.

The extensive diversity of *P. falciparum* in a few asymptomatic children ($n = 33$) reflects the role that factors other than age might be playing in development of premunition in Kisinga sub-county. This diversity is an underestimate of the local parasite population, as shown by the appearance of new alleles during follow-up. No imbalanced distribution of alleles was detected between symptomatic and asymptomatic infections in Kasese. As enrolment of children in the two groups was done at different times, this suggests that both MSP1 and MSP2 alleles are maintained at similar balanced frequencies.[6,27]

High multiple infections have been reported among patients.[12] Biased detection of multiple infections using MSP1 and MSP2 markers in the MUTH cohort (Table 2) has been reported previously as due to a difference in sensitivity of the markers[3,28] and possible biological or immunological mechanisms underlying association/dissociation between

parasite lines.[12] This may have implications for the selection of vaccine candidate antigens. *Plasmodium falciparum* genotypes detected among symptomatic children were different, indicating that there was no 'pathogenic' type or allele combination but that the parasites causing the symptoms were those to which a child had not yet mounted an immune response.

Among symptomatic children, age did not influence MOI beyond statistical chance, as shown elsewhere.[22] The significantly higher MOI in KH than in MUTH cannot be explained by the difference in parasite load between the two groups, as the difference in mean parasite density of the groups was not statistically significant ($P = 0.58$). One reason may be variation in transmission levels.[1,22] Multiplicity in Kagando was similar to that reported in holoendemic areas[19] but lower than that in hyperendemic areas.[29]

The disappearance of alleles during follow-up could have been due to the efficacy of the drug, as reflected by decreasing parasitaemia and reappearance due to reinfection by a new anopheles bite or detection of genotype(s) present earlier at undetectable concentration. However, concomitant reinfection by several clones similar to those of day 0 would be unlikely because of the high polymorphism of *P. falciparum* in the study area.[5] As no clinical symptoms were reported, the acquired infections were assumed to be non-clinical episodes, suggesting that some children are able to restrict multiplication of some, but not all, newly inoculated parasite types.[30] Among asymptomatic children, HbAA carriers were infected with specific genotypes longer than HbAS carriers ($P = 0.011$). The high clonal fluctuation in HbAS could have reduced the power of the effect of HbAS on continuation of infection.

Although the effect of haemoglobin type on MOI is still unknown, the high clonal fluctuation in asymptomatic HbAS carriers suggests that several genotypes effectively evade the effects of the immune system, exposing the individual to a repertoire of strains, thereby promoting rapid development of premunition.

In follow-up of asymptomatic children, FC27-type alleles were more abundant in HbAA than in HbAS carriers. Some studies have shown the influence of haemoglobin status on the distribution of MSP1 and MSP2 allelic families.[20,26] Such an effect was not seen by Mockenhaupt et al.[29] More studies are needed to investigate this.

In conclusion, our findings show that even in areas with low or seasonal transmission, high levels of parasite polymorphism can occur. Some alleles detected in Mbarara were absent in Kagando, Kasese (153 km away) and vice versa, implying that the use of one malaria vaccine trial for the whole African continent, with endemic regions thousands of kilometres apart and different *P. falciparum* allelic composition, may not suffice.

Authors' contributions: HJ conceived the study; GNK and HJ designed the study protocol with the help of WKI; WKI supervised patient recruitment and microscopy; GNK carried out the PCR assays, haemoglobin electrophoresis and microscopy; KE and GNK analysed the results of the molecular studies and HJ, KE and GNK interpreted the data; GNK and WKI analysed the results from the haemoglobin electrophoresis and microscopy and HJ, WKI and GNK interpreted the data; GNK drafted the manuscript; KE, GNK and HJ revised the paper. All authors read and approved the final manuscript. GNK and WKI are guarantors of the paper.

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Conflicts of interest: None declared.

Ethical approval: The study was approved by the Faculty of Medicine Ethical Review Committee, Mbarara University, Mbarara, Uganda and its Institution Research Committee.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.trstmh.2008.07.023.

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