Original Article **OPEN ACCESS**

Submicroscopic and multiple *plasmodium falciparum* infections in pregnant Sudanese women

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Citation: Omer S, Khalil E, Ali H, Sharief A. Submicroscopic and multiple *plasmodium falciparum* infections in pregnant Sudanese women. **North Am J Med Sci** 2011; 3: 137-141.
Doi: 10.4297/najms.2011.3137
Availability: www.najms.org
ISSN: 1947 – 2714

Abstract

Background: Control of malaria during pregnancy remains a major public health challenge in developing countries. Microscopic parasite detection represents a pivotal step in malaria control, while modern molecular techniques are deemed to improve detection rates markedly. **Aims**: This study aimed to investigate the frequency of submicroscopic and multiple *Plasmodium falciparum* (*P. falciparum)* infections during pregnancy, using the *P. falciparum* merozoite surface protein1 (MSP-1) gene as a polymorphic marker. **Materials and Methods**: The study was a cross-sectional, analytical study that was conducted at Omdurman Maternity Hospital, Sudan, between July 2003 and December 2004. Following informed consent, 836 pregnant women between the ages of 16-47 years with different gestational ages were enrolled in the study. Thin and thick blood films were stained with Giemsa and examined by experienced microscopists. Parasite DNA was extracted using Chelex method. Nested polymerase chain reaction **(**PCR) assays specific for *P. falciparum* were carried out to detect infections below the threshold of microscopy and to genotype different strains in the samples using merozoite surface protein-1. **Results:** More than a quarter of the study participants (219/836; 26.2%) were smear-positive for malaria infection. The results of the PCR-based assays showed that 41.8 % (257/617) of the smear-negative women were PCR positive and therefore had submicroscopic infections. The mean number of genetically different *P. falciparum* parasites detected was 2.7 (range 1–9). The multiplicity of infection identified by at least two alleles of *MSP-1* was significantly higher among paucigravidae (45.6%) compared to multigravidae (28.9%), with mean number of alleles of 2.4 and 1.9, respectively (*p=0.009*). This likely indicates the gradual acquisition of immunity. **Conclusion:** Conventional microscopy underestimates the actual extent of malaria infections during pregnancy in endemic regions. Multiplicity of infection may be an important factor in the gradual acquisition of strain-specific immunity.

Keywords: Pregnancy-associated malaria, submicroscopic infection, multiplicity of infection, P. falciparum merozoite surface protein1.

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Introduction

It has been well documented that pregnant women are more susceptible to *Plasmodium falciparum* (*P. falciparum*) infection as opposed to their non-pregnant counterparts [1-4]. In malaria endemic areas, the prevalence of clinical and asymptomatic malaria is highest in young women and those in their first and second pregnancies. The susceptibility decreases with the number of pregnancies, suggesting that women acquire a gravidity form of immunity, resulting in decreased prevalence and severity of the disease [5-8]. High proportions of submicroscopic *P. falciparum* infections have been demonstrated by polymerase chain reaction (PCR) assays in children and non-pregnant adults in Sub-Saharan Africa [9, 10]; however, there is little information with respect to pregnant women. DNA methods for the detection of parasite showed that at

least twice as many pregnant women are infected with malaria as indicated by microscopy [4, 8, 11, 12].

It has been shown that approximately one in every three of the pregnant Sudanese women who had been found to be smear-negative for malaria had PCR-based evidence of submicroscopic *P. falciparum* infection [13]. PCR is even more sensitive in the detection of malaria during pregnancy than the microscopy of placenta impression smears; 65% of women negative for malaria by microscopy parasites were positive by PCR [11, 14]. The increased susceptibility of pregnant women, especially primigravidae to *P. falciparum* infection and clinical malaria, has been attributed to specific strains of *P. falciparum* that are able to adhere to placental tissue [15]. Studies carried out in pregnant women for parasite polymorphism showed that multiplicity of infection (MOI), the number of genetically-different parasites, is common in pregnant women [16]. It is also notable that the occurrence of different parasite alleles at different time points has been found in asymptomatic pregnant women. The number and type of parasite clones of alleles found in genotyping were used for calculating MOI for *P. falciparum* and can vary between 69% to 98% in some endemic areas [8,17-19].

Association between MOI in pregnant women and malaria morbidity and mortality is not a straightforward issue and emphasis has been given to the possible role of MOI and infection outcome [20, 21]. Repeated infections with a broad range of antigenetically diverse strains are thought to result in cross-reactive immunity that may prevent super-infection by additional strains and clinical disease [22]. MOI in pregnant women may be an important factor for the acquisition and maintenance of immunity against malaria [16]. In Sudan, no studies elucidating the distribution and possible role of multiple *P. falciparum* infections as a determinant of malarial disease in pregnant women have been published. In this study, we assessed the rates of submicroscopic and the multiplicity of *P. falciparum* infections among pregnant women of different gravidity.

Materials and Methods

Study Design

Ethical and scientific approvals were obtained from the scientific committees of the Institute of Endemic Diseases, University of Khartoum and the Directorate of Research, Federal Ministry of Health, Khartoum, Sudan. Following informed consent by the women and their spouses, a total of 836 pregnant women at the routine antenatal clinics at Omdurman Maternity Hospital were enrolled in the study during the period from July 2003 to December 2004. A standard questionnaire containing demographic and clinical data was completed for all volunteers. Finger-prick blood samples were used to make thick and thin blood films and for hemoglobin concentration measurement. About 50µl of blood were collected on filter paper which was dried, sealed individually in plastic bags and stored for DNA extraction. The pregnant women at the antenatal clinic were given one month's supply of iron (II) sulfate tablets and folic acid tablets.

Laboratory Methods

Duplicate thick and thin blood films were stained with Giemsa and read by experienced microscopists to determine parasite density and species. Asexual stages were counted per 200 leukocytes. Hemoglobin concentration was measured using the cyanomethaemoglobin method. Anemia was defined as Hb level <11g/dL. DNA was extracted from the dried spots on the filter paper using the Chelex extraction method as described by Plowe et al[23].

Genotyping of P. falciparum Isolates

Following DNA extraction, nested *P. falciparum*-specific PCR assays were performed. The PCR reaction was performed in a mix with a final volume of 22 µl, containing 100nM of each primer in a 22 µl pre-mix containing 100nM of each primer, 75µM each of dNTP, and 1 Unit Taq DNA polymerase in buffer (10mM Tris-Cl pH8.8, 50mM KCl, 1.5mM $MgCl_2$). The cycling parameters used were: (94^0 C for 25 sec; 50^0 C for 35 sec; 68^0 C for 1 min and 30 sec) for 30 cycles and 68^0 C for 10 minutes. In the nested reaction, primers specific for the *merozoite surface protein-1* (*MSP-1*) family sequences MAD20, 3D7 and RO33 were used. The cycling parameters were: (94^0 C for 30 sec; 50^0 C for 1 min and 30 sec; 70^0 C for 1m) for 30 cycles for each of three sequence-specific reactions.

Nested PCR products were analyzed on 3% Metaphor agarose gels, with each band considered an individual strain. Strains were considered to be the same if their bands were of the same size on the agarose gel. For each sample, the MOI was determined using the genetic marker with the largest number of fragments found at the three allelic families. Allele-specific positive controls and DNA-free negative controls were included in each set of reactions.

Definitions and Statistical Analysis

P. falciparum infections were classified as follows: i) microscopically confirmed parasitaemia; ii) PCR confirmed parasitaemia; iii) submicroscopic (negative thick film but positive for parasite DNA proven by PCR); iv) no infection (absence of *P. falciparum* parasite in thick blood smear by microscopy and PCR). MOI was defined as the number of distinct *MSP-1* genotypes detected in a patient. Double data entry, validation, and cleaning were done using EpiInfo version 3.3.2 software. For comparisons of proportions, the Chi-square test was used. Non-parametric tests were performed to compare quantitative variables (e.g. number of different *MSP-1* genotypes) between two or more groups (Mann-Whitney U tests and Kruskal-Wallis tests). The ANOVA test was used to compare normally distributed data and the Wilcoxon test for non-parametric data which were obtained by non-parametric statistics. Furthermore, multivariate linear regression was used to test for confounding and independent associations between age, gravidity parasite density and MOI. *P* values of <0.05 were considered to indicate statistical significance.

Results

Detection of Malaria Infections

All infections were due to *P. falciparum.* More than a quarter (219/836; 26.2%) of the enrolled pregnant women demonstrated smear-positive malaria infection. On the other hand, the overall frequency of *P. falciparum* infection detected by PCR was 56.3% (471/836). Forty one percent (257/617; 41.8%) of the smear-negative women showed PCR positivity (submicroscopic infections). Submicroscopic *P. falciparum* infections were not statistically different across maternal age (p=0.68). Both smear and PCR negativity decreased with gravidity—28.2% in bucigravidae and 79.3% in multigravidae (Table 1).

Table1 Malaria microscopic and submicroscopic infections according to gravidity

	Pucigraviade	*Multigravidae*	*P-value*
Age range	16-41	19-46	
Malaria infection (n)			
Microscopic (219)	30.2%	16.4%	<.001
Submicroscopic(257)	32.%	27.8%	0.01
Negative in both (360)	28.2%	79.3%	<0.001
Parasite density[a]	17625±1223.6	2882.4±163.7	<0.001
Parasite density[b]	1220 ±105.4	1059 ±53.7	0.01
Number of samples	593	243	

a:P. falciparum density (parasites/µl) ± SE on microscopically positive samples. *b:P. falciparum* density ± SE on submicroscopic infection

Prevalence and Distribution of MSP-1 Alleles

MSP-1 alleles were classified according to their family sequences 3D7, MAD20 and RO33. Nine distinguishable alleles of the *MSP-1* gene were identified (5 K1-types, 3 MAD 20-types and 1 RO33-type). The distribution of *MSP-1* allelic families showed that K1 allele was the most frequent representing 52.9%; the MAD20 allele represented 44.7%, while RO33 occurred at 35.2% (Table 2).

Table 2 The frequencies of *MSP1* alleles in addition to the different combinations of alleles in all gravidity

		Gravidity		
MSP-1 Alleles	All groups	G1	G2	G≥3
K1	120(25.5%)	50(41.7%)	40 (33.3%)	30(25.0%)
MAD20	79 (16.8%)	31(39.5%)	27 (34.2%)	21(26.6%)
RO33	38 (8.1%)	15(39.5%)	13 (34.2%)	10(26.3%)
K1/ MAD20	96 (20.4%)	38(39.6%)	34 (35.4%)	24(25.0%)
K1/ RO33	51 (10.8%)	25(49.0%)	15 (29.4%)	11(21.6%)
MAD20/RO33	48 (10.2%)	20(41.6%)	18 (37.5%)	10(20.8%)
K1/MAD20/RO33	39 (8.3%)	17 (43.6%)	13 (33.3%)	9 (23.1%)
Total	471	196 (41.6)	160(34.0%)	115(24.4%)

Association Between Multiplicity of P. falciparum Infection and Gravidity

The mean number of multiple infections (standard deviation [SD]) was 1.9 (0.9). Forty percent (207/471) of the PCR positive subjects carried more than one strain. The multiplicity of infection (MOI), identified by at least two alleles of *MSP-1*, was significantly higher among primigravidae compared to secungravidae and multigraviade (p<0.001) with mean number of alleles of 2.4, 1.9 and 1.7, respectively. Maternal age was not associated with the probability of multiple *P. falciparum* infections and was not significantly different between women ≤20 years and those >20 years (p=0.5) (Table 3**).** MOI was positively related to parasite density (p=0.004). However, no significant association was observed between MOI and anemia (OR = 1.12, 95% CI = 0.67–2.32, p = 0.6).

Women with microscopically detectable parasites had a statistically significant higher rate of multiple *P. falciparum* infections compared to those who were submicrosopic positive (OR = 2.70, 95% CI=1.44–6.11, p<0.001). Single clonal infection was detected in 45.2% (186/471) *P. falciparum* isolates, while 54.8% (258/471) had multi-clonal infections. More than two thirds (67.6%) of primigravidae had more than one clone and only 32.4 % harbored a single clone. There was a significant correlation between multi-clonal infections and microscopic positivity (p= 0.002). The mean number of genotypes in women with submicroscopic infections was 2.3 (range 1–7) compared to 3.1 (range 1–9) genotypes in slide-positive women (X^2 =3.92, p= 0.003).

Table 3 Mean (SD) number of *MSP-1* alleles (Multiplicity of infections; MOI) with age and gravidity

	n[a] total (%)	*n[b] (%)*	MOI±SD	P
Age (years)				
≤ 20	154(18.5%)	47 (30.5%)	2.1±0.7	0.500
> 20	682(84.5%)	184(27.0%)	2.2±0.5	
Gravidity				
1	355(42.5%)	109(29.3%)	2.4±0.4	
2	238(28.5%)	87 (23.5%)	1.9±0.4	0.009
≥3	243(29.1%)	35 (18.1%)	1.7±0.8	
Parasite density				
Microscopic	219(26.2%)	164(75.3%)	2.5±0.3	<0.001
Submicroscopic	257(41.8%)	147(57.2%)	1.9±0.3	
Anemia(HB<11g/dl)				
No	560(67%)	120(51.9%)	1.3±0.7	0.73
Yes	276 (33%)	111(48.1%)	1.7±0.4	

a: Total number of samples analyzed. b: Proportion of infected with more than one strain to total.

Discussion

This study showed that microscopically detectable *P. falciparum* parasitaemia in peripheral blood is a rather poor indicator of the actual prevalence of malarial infections in pregnancy. The underestimation of pregnancy-associated malaria could be harmful to the pregnant women and their fetuses, since even microscopically undetected infections are likely to be more clinically relevant during pregnancy. A number of studies suggested that malaria parasitaemia of any density may have a harmful effect on pregnant women and their developing fetuses [24, 25].

In many African countries, submicroscopic malaria infections have been found in up to 55% of the pregnant women [8,19, 21]. It is probable that a certain level of host immunity may be able to keep parasites at low and microscopically undetectable densities. Alternatively, these low-density infections may represent recently acquired infections, which would reach microscopical levels if infection is not treated. As in other African countries, Sudanese primigravidae are more susceptible to asymptomatic infections than multigravidae (p=0.009).

Reports regarding the acquisition of pregnancy-associated immunity and MOI are contradictory, since the MOI was reported to decrease with increasing gravidity in some studies but not in others [8, 17, 19, 21].The present study demonstrated absence of significant difference in the prevalence of MOI with increasing age of the pregnant women, but showed a definite increase of MOI in primigravidae and secundigravidae compared to those with multiple pregnancies (p=0.009). It is probable that women of higher gravidity have acquired efficient anti-parasite immunity against most of the local strains during successive pregnancies [17]. In successive pregnancies, women are repeatedly exposed to more and more strains, leading to the development of strain-specific and cross-reactive immunity [15]. Our study showed that MOI was not a significant risk factor for the development of anemia. This contradicts the report by Beck and colleagues that showed that the risk of anemia was significantly increased in highly complex infections among women with less than three pregnancies [16].

Conclusion

Microscopy underestimates malaria infection during pregnancy. MOI is inversely proportional to the gravidity and is not a risk factor for anemia in Sudanese pregnant women.

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