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Full Length Research Paper

Haptoglobin gene polymorphism influences the effect of malaria infection on host haptoglobin plasma level but not susceptibility to the disease

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A cohort of 344 children aged less than five years were followed for one year to establish the relationship between haptoglobin genotype, haptoglobin plasma level and malaria infection and susceptibility. Haptoglobin genotyping was undertaken using PCR, with plasma haptoglobin levels measured using indirect ELISA. The number of children carrying the genotypes Hp^{1-1}, Hp^{1-2} and Hp^{2-2} were 85, 171 and 88, respectively. The state of malaria infection was assessed using standard microscopy method to detect parasitemia. Higher plasma haptoglobin level tended to be associated with negative parasitemia (P=0.01). During malaria infection, haptoglobin level decreased in all individuals and, the decrease significantly varied by genotype, whereby the decrease was greater in Hp^{2-2} individuals than the other genotypes. A longitudinal examination of blood smears revealed a slightly higher proportion of children carrying the Hp^{2-2} genotype (12.4%) to be positive for malaria, as compared to children with the genotype Hp^{1-1} (11.7%) and Hp^{1-2} (11.6%). The present study has demonstrated that malaria infection in children affects the haptoglobin plasma level and the effect is influenced by haptoglobin genotype. The study has also revealed a limited influence of haptoglobin genotype to malaria infection and disease development.

Key words: Haptoglobin, malaria, parasitemia.

INTRODUCTION

Malaria is a vector-borne infectious disease caused by a protozoan parasite of the genus Plasmodium. Four species of the plasmodium parasite can infect humans; the most serious forms of the disease are caused by *Plasmodium falciparum* and *P. vivax.* Other related species (*P. ovale*, *P. malariae*) can also infect humans, and a fifth species *P. knowlesi* has recently been observed to naturally infect humans as a zoonosis in Southeast Asia (Daneshvar, 2009).

It is known that some human genetic factors play a key role in disease susceptibility, progression and outcome. These include red blood cell polymorphisms like ABO blood group, sickle-cell trait (Hill et al., 1986), glucose-6-phosphate dehydrogenase deficiency (G6PD) deficiency as well as point mutations in the mannose binding protein (MBP) and in the promoter regions of both the *TNFa* and *NOS2* genes. Other genetic factors shown to influence susceptibility to malaria are haptoglobin (Hp) and Heme Oxygenase-1 (HO-1) polymorphisms. The two proteins (Hp and HO-1) have a key role in malaria pathogenesis, being involved in free hemoglobin and heme breakdown, respectively.

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Abbreviations: Hp, Haptoglobin; **PCR,** polymerase chain reaction; **ELISA,** enzyme linked immunosorbent assay.

Haptoglobin is an acute phase protein that binds haemoglobin released during the intravascular lysis of erythrocytes. Cell free plasma haemoglobin is a potent pro-oxidant and Haptoglobin is thought to be important in removing it from circulation. In humans, Hp is polymorphic with two co-dominant alleles, Hp^1 and Hp^2 encoded by a single gene on chromosome 16, resulting in three phenotypes Hp^{1-1}, Hp^{1-2} and Hp^{2-2}. Hp polymorphisms have been associated with a variety of different functional capacities and outcomes (Langlois and Delanghe, 1996) including haemoglobin binding affinity (Okazaki and Nagai, 1997; Okazaki et al., 1997), markers of oxidant stress (Langlois and Delanghe, 2006; Asleh et al., 2005), iron delocalisation within monocytes (Langlois et al., 2004) and immune regulation (Arredouani et al., 2003). Moreover, Hp polymorphisms have been associated with a range of disease outcomes, including contradictory evidence for a protective effect of the Hp^2 allele against clinical malaria (Elagib et al., 1998; Quaye et al., 2000; Aucan et al., 2002; Minang et al., 2004; Bienzle et al., 2005; Atkinson et al., 2007).

This study was conducted in order to provide insights on the influence of haptoglobin gene polymorphism on malaria infection in children, and to evaluate the effects of malaria infection of the level of plasma haptoglobin level.

MATERIALS AND METHODS

Study site

The study was conducted in Morogoro, an eastern region of Tanzania. Samples were collected from two sites; Morogoro Regional hospital and Melela, a rural health centre located 50 km away from Morogoro Regional hospital.

Sampling

The study was conducted on a cohort design and was under an ongoing Mother Offspring Malaria Studies (MOMS) project studying malaria in pregnant mothers and children up to five years of age. Participants were recruited from mothers who attended the two health centres for maternity services. Mothers, who consent to participate after adequate sensitisation on the project objectives and possible benefits, were enrolled to the study for themselves (if pregnant) or for their babies. The mothers were scheduled to attend (with babies) after every four weeks for clinical check up and sampling at the project clinics. However, if a baby shows any sign of being sick, the mother was advised to take him/her to the clinic at any time. During clinic visits, any child diagnosed for any clinical condition is treated accordingly. For the purpose of this study, a cohort of 344 children was purposively selected. Children with not more than five years of age, with at least six months of follow up, and at least one positive malaria parasitemia record were selected.

Sample analysis and data collection

Parasite detection

Thick blood smears were stained with Giemsa for parasitological examination by microscopy. The parasites detected were scored against 200 white blood cells and the parasite densities calculated.

Haptoglobin genotyping

Genomic DNA was extracted from peripheral blood (dry blood spots) using the QIAamp DNA blood mini kit as suggested by the supplier (Qiagen, USA). Genotyping was undertaken by amplifying the Haptoglobin gene by polymerase chain reaction (PCR) then separating the PCR products in 0.7% agarose gels. The PCR protocol was that used by Koch et al. (2002).

Quantification of plasma haptoglobin

Plasma haptoglobin (Hp) levels were determined by an enzyme-linked immunosorbent assay (ELISA) using 96-well plates coated with chicken anti-human Hp (GenWay, USA) as capture antibody and monoclonal anti-human Hp (GenWay, USA) as detection antibody. Immunoplates (Immulon, USA) were coated overnight at 4°C with the capture antibody diluted in coating buffer (0.05 M Carbonate-Bicarbonate, pH 9.6) to a final concentration of 5 µg/mL (100 µL/well). Wells were washed three times with 200 µL/well of wash buffer (0.05% Tween in PBS pH 7.4). Non fat dairy milk (1%) in PBS was used to block the wells (200 µL/well for 1 h at room temperature), followed by three washes as described above. Samples were diluted 1:1,000 in diluent (50 mM Tris, 0.14 M Nacl, 1% BSA, 0.05% Tween 20) and Hp standards (500 ng/mL, 250 ng/mL, 125 ng/mL, 62.5 ng/mL, 31.2 ng/mL, 15.6 ng/mL and 7.8 ng/mL) prepared using pooled Hp (GenWay) in diluent (50 mM Tris, 0.14 M Nacl, 1% BSA, 0.05% Tween 20). Aliquots of 100 µL of diluted samples and Hp standards were added to wells in duplicate, incubated for 1 h at room temperature and washed five times in washing buffer. Detection was undertaken by adding 100 µL of detection antibody in each well for 1 h diluted in the same diluent to a concentration of 0.025 µg/mL. This was followed by addition of chromogen enzyme substrate and incubation for 20 min. Optical densities were read at 405 nm using SOFTmax PRO spectrophotometer. A standard curve was plotted from which sample levels were extrapolated and multiplied by dilution factor, giving sample Hp concentrations.

Statistical analysis

Using StatView® package, chi-square tests were undertaken to assess the frequency distribution analysis. The association between malaria infection, haptoglobin genotype and plasma level were assessed using Kruskal-Wallis tests. Exclusions included subjects whose Hp levels could not be determined (n = 12) missing values (n = 8) and outliers (n = 5).

RESULTS

Malaria status in relation to haptoglobin genotype

Children were randomly recruited into the study at approximately one year of age (Table 1). Age comparison in the three genotypes was done by Kruskal-Wallis test and was found to be non-significant (*P=0.94*). 85 (24.5%) of the children carried the Hp^{1-1} genotype, whereas 88 children (25.5%) carried the Hp^{2-2} genotype and 171 children (50%) had the Hp^{1-2} genotype.

Malaria status was studied in relation to haptoglobin genotypes. Malaria status was defined by level of parasitemia or clinical characteristics. Individuals identified to be positive for malaria as detected by

Table 1. General characteristics of the study children. Age comparison in the three genotypes was done by Kruskal-Wallis test (*P=0.94*).

Parameter	Genotype			*P*-value
	Hp^{1-1}	Hp^{1-2}	Hp^{2-2}	
Number of children	85	171	88	
Female count	37	71	39	
Male count	48	100	49	
Mean age at recruitment (years)	1.03	1.07	1.05	0.94

Table 2. Comparison of Malaria status observations in relation to Haptoglobin genotypes.

Parameter	Genotype			Kruskal-Wallis test *P*- value
	Hp^{1-1}	Hp^{1-2}	Hp^{2-2}	
Total slides	1,949	4,344	2,284	
Negative observations	1,721	3,839	2,000	
Positive observations	228	505	284	*0.52*
Asymptomatic	187	409	242	*0.43*
Symptomatic	41	96	42	*0.74*

microscopy were further classified either as symptomatic or asymptomatic based on their clinical status at the time of sample collection. Symptoms considered included one or more among the following; fever (> 37°C), depression, vomiting, convulsions, pale mucous membranes, diarrhoea, signs of dehydration and any reported body aches. The distribution of positive, non-positive, symptomatic and asymptomatic observations across the three genotypes are presented in Table 2.

Parasitological characteristics of the patients

A total of 8577 blood samples were collected during all clinic visits in this study. From each sample, a blood slide was prepared making an average of 24.9 slides per child. The number of blood slides examined per genotype were 1949 (Hp^{1-1}), 4344 (Hp^{1-2}) and 2284 (Hp^{2-2}). Of all the slides examined microscopically, 1017 (11.9%) were positive giving an estimated average of 1 positive malaria parasitemia in every eight clinic visits. Comparison of the three haptoglobin genotypes showed no significant difference in the distribution of the positive slides (*P = 0.52*). The data showed that 228 (11.7%) slides from Hp^{1-1} children were positive for malaria parasitemia while 505 (11.6%) and 284 (12.4%) were positive from Hp^{1-2} and Hp^{2-2} children, respectively.

Frequency of malaria positive observations

The frequency of positive malaria parasitemia observations per child varied from 0 to 14. Out of the 344 study children, only three did not show any case of positive parasitemia for the whole study period. These children of which two of them were males and one female, were under inspection for 6, 16 and 17 months, respectively.

Clinical characteristics of the patients

Out of the 1017 positive parasitemia cases, 179 (17.6%) were accompanied with malaria symptoms. The symptomatic cases were unevenly distributed among the three genotypes with 41 cases in Hp^{1-1}, 96 cases in Hp^{1-2} and 42 cases in Hp^{2-2}. When these cases were expressed over the total number of positive cases within each genotype, it was found that symptomatic cases were 18, 19, and 14.8% of all positive cases for genotypes $Hp1^{-1}$, Hp^{1-2} and Hp^{2-2}, respectively (Figure 1).

Plasma haptoglobin level in relation to haptoglobin genotype and malaria

The levels of plasma haptoglobin varied between individuals regardless of their malaria status or genotype. As shown in Table 3, mean plasma haptoglobin levels in malaria negative children were 180.7 µg/ml in Hp^{1-1} children, 203.9 µg/ml in Hp^{1-2} individuals and 245.7 µg/ml in children carrying the Hp^{2-2} genotype. The haptoglobin levels in malaria negative individuals significantly varied between the three genotypes (*P*=0.03). The overall mean haptoglobin level across all genotypes was higher in malaria negative children (208.3 µg/ml) than malaria

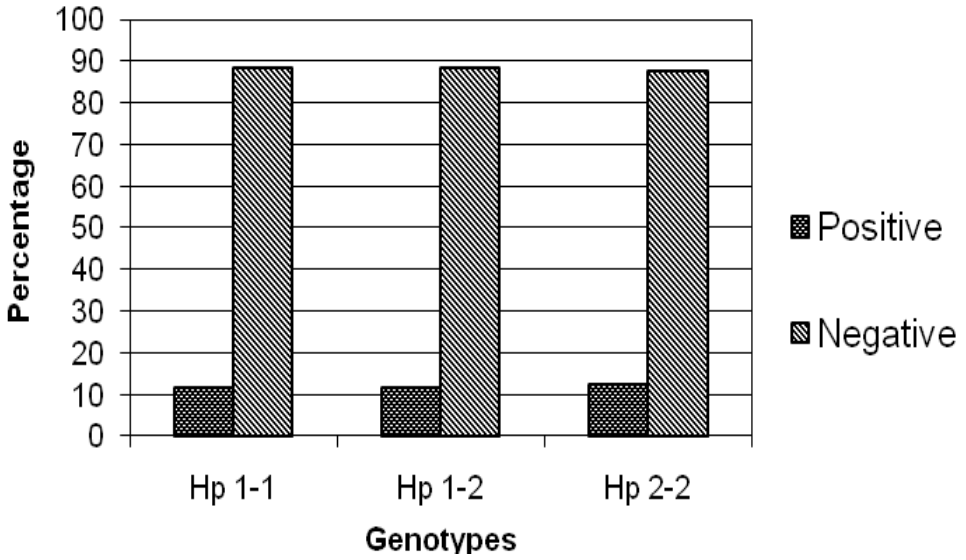

Figure 1. Percentage distribution of negative and positive blood slides in the three genotypes.

Table 3. Plasma haptoglobin levels and difference between positive and negative samples in the three Haptoglobin genotypes.

Parameter	Mean plasma Hp level (µg/ml) (range)	Unpaired t-test against Hp^{2-2} p-value	Kruskal-Wallis test p-value
Malaria negative			
Hp^{1-1}	180.7 (29.4 - 496.9)	0.01	
Hp^{1-2}	203.9 (9.5 - 458.6)	0.08	0.03
Hp^{2-2}	245.7 (35.6 - 491.2)		
Malaria positive			
Hp^{1-1}	170.8 (10.6 - 474.9)	0.15	
Hp^{1-2}	167.5 (14.4 - 330.8)	0.07	0.19
Hp^{2-2}	133 (8.9 - 325.3)		

positive children (159.3 µg/ml) as indicated in Figure 2.

DISCUSSION

The present study has documented for the first time in Tanzania the relationship between malaria prevalence and haptoglobin genotypes and its plasma level in children. All three haptoglobin genotypes were present in the Tanzanian population and their occurrence in the ratio 1:2:1 for Hp^{1-1}, Hp^{1-2} and Hp^{2-2}, respectively further supports the influence of genetic and environmental factors on the distribution of haptoglobin phenotypes within populations (Atkinson et al., 2007).

In the present study, the haptoglobin genotypes were not shown to increase the risk of malaria parasitemia or development of the disease. The Hp^{2-2} genotype has been associated with 30% reduction in malaria episodes in the first two years of life but not protection as reported in several previous studies (Atkinson et al., 2007, Aucan et al., 2002; Bienzel et al., 2005). The protective effect of haptoglobin against malaria seems to be age dependent and this may suggest a role of the Hp^{2-2} genotype to accelerate acquisition of immunity against malaria as has been suggested with sickle cell trait (Williams et al., 2005; Atkinson et al., 2007). The influence of haptoglobin genotypes is not on malaria alone. It has been found that the Hp^{1-1} genotype is associated with significant protection against non-malarial febrile illnesses (Atkinson et al., 2007, McDermid and Prentice, 2006; Friis et al., 2003). Moreover, a child below two years of age may not benefit from the protective role of Hp^{2-2} genotype against malaria but the protective role of Hp^{1-1} against febrile diseases. Accordingly, the findings of this study have indicated that the haptoglobin genotypes may not influence malaria infection in children, but may determine

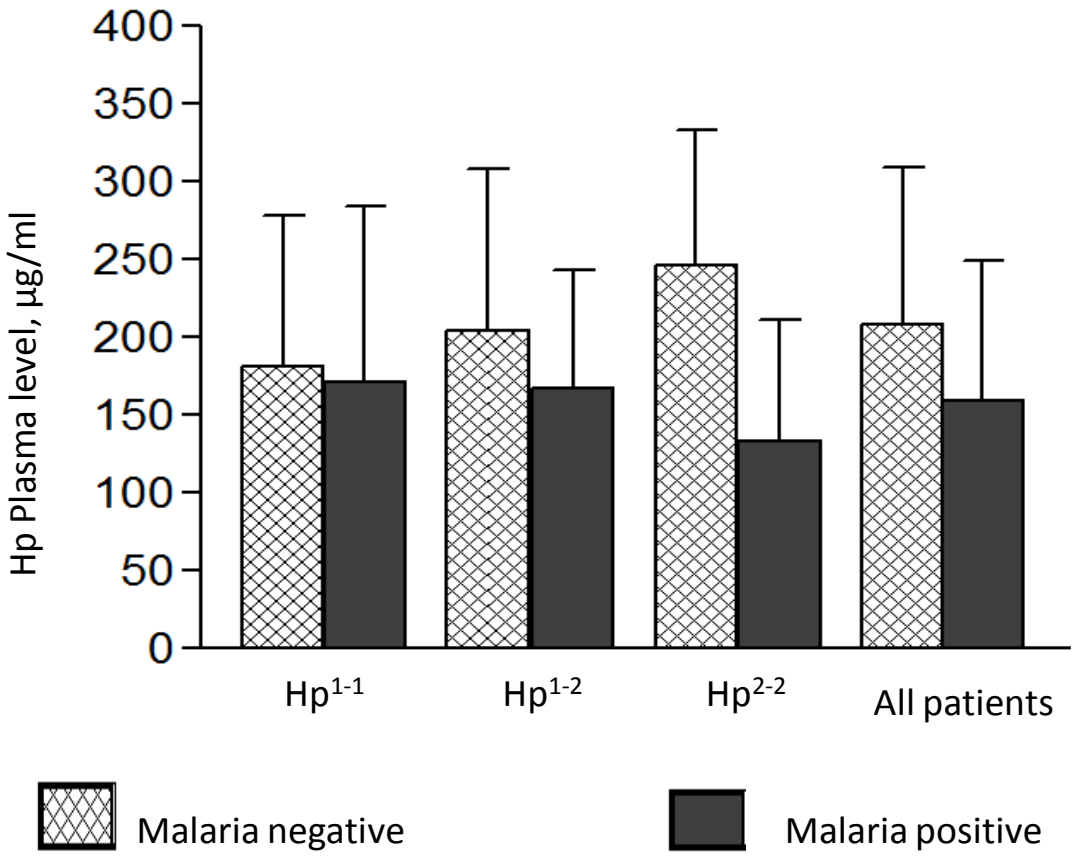

Figure 2. Relative difference in Plasma Hp level between malaria negative and malaria positive samples.

the fate of the infection in young children.

Interestingly, several previous studies have demonstrated that Hp^{2-2} genotype is associated with reduced susceptibility to malaria. In the study by Quaye et al. (2000), it was found that Hp^{2-2} phenotype was significantly less present in malaria patients as well as in complications of malaria disease. Elagib et al. (1998) conducted a similar study in Ghana with results suggesting that the Hp^{l-l} phenotype is associated with susceptibility to falciparum malaria and the development of severe complications. Atkinson et al. (2006) found in a study conducted in Kenya that Hp^{2-2} genotype was associated with reduced episodes of clinical malaria. However, the study by Bienzle et al. (2005) in northern Ghana found a limited influence of haptoglobin genotypes to malaria susceptibility. Summing from our own findings and those from other studies appear that the different haptoglobin genotypes may or may not influence reduced risk of malaria infection and development of the disease. The outcome of the relationship between haptoglobin genotypes and disease may be influenced by a multitude of disease determinants including age and environmental factors.

This study reveals a clear relationship between the level of plasma haptoglobin and malaria susceptibility, whereas malaria parasitemia was associated with reduced plasma haptoglobin level. In malaria-endemic areas, low levels of haptoglobin reflect recent parasitemia and malaria-induced haemolysis, as well as transmission intensity (Trape et al., 1988). Similar results were found when a survey study was done to patients of vivax malaria in South Korea (Bahk et al., 2010). In this study, haptoglobin levels were further associated with haptoglobin genotype in interaction with malaria parasitemia. Interestingly, with negative parasitemia, haptoglobin levels were higher in Hp^{2-2} when compared to Hp^{1-1} and Hp^{1-2}. In contrast, haptoglobin levels were lowest in Hp^{2-2}, followed by Hp^{1-2} and Hp^{1-1}, in the case of positive parasitemia. These findings are supported by the structural form of Hp^{1-2} and Hp^{2-2}, which are polymeric, whereas Hp^{1-1} is a dimer (Dobryszycka et al., 1997). This structural form and the ability to form disulfide bridges to other Hp^{2} molecules, leading to a wide range of oligomers explains the fact that at negative malaria parasitemia, haptoglobin level is higher in Hp^{2-2} when compared to Hp^{1-1} and Hp^{1-2}. As clearly shown in our study, haptoglobin level was reduced in malaria positive individuals, and the highest decrease was associated with Hp^{2-2} genotype. Level of haptoglobin is reduced by both chronic, low level parasitemia and possibly malaria-

associated immune complex destruction of infected erythrocytes, as well as clinical malaria (Trape et al., 1985; McGuire et al., 1996). Our findings are best explained by haemolysis, secondary to malaria as the only significant cause of hypohaptoglobinaemia as previously discussed in African populations by Trape et al. (1985). It can further be assumed that because of its larger size, the Hp^{2-2} haemoglobin complex is taken up more efficiently by macrophages as compared with Hp^{1-2} or Hp^{1-1}. A study by McGuire et al. (1996), found a generalized low levels of plasma haptoglobin in children that were not using bed nets. It has therefore been proposed that the distribution of haptogloblin levels may be useful in the evaluation of malaria control programs, where it can be used as a surrogate index of endemicity at the community level but not for individual malaria episodes (McGuire et al., 1996; Rougemont et al., 1988; Trape and Fribourg-Blanc, 1988; Sisay et al., 1992).

In summary, we have reported here that haptoglobin genotype may not influence susceptibility to malaria infection or development of the disease. However, haptoglobin genotype may influence the type of disease developed, whereby Hp^{2-2} individuals express more of complicated and severe types of malaria. Our findings on the relationship between haptoglobin genotype and frequency and susceptibility of malaria infection in this study unveiled the sensitivity of age factor in the relationship. Further, we have shown that haptoglobin genotype influences plasma haptoglobin level both in parasitized and unparasitized patients.

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