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High prevalence of *Plasmodium falciparum pfcrt* K76T mutation in children with sickle cell disease at a tertiary hospital in north-western Tanzania

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Abstract: The high prevalence of sickle cell disease (SCD) and trait in Sub-Saharan Africa coincides with the distribution of *Plasmodium falciparum* malaria. Due to prolonged heavy use of chloroquine (CQ) as an antimalarial, drug resistance has developed. Many countries including Tanzania abandoned the use of CQ for uncomplicated malaria, except its use as prophylaxis in patients with sickle cell disease. This study investigated the prevalence of malaria in SCD patients and mutations associated with CQ resistance. Children diagnosed with sickle cell disease attending both outpatient clinic and those admitted at Bugando Medical Centre in north-western Tanzania were screened for malaria using thick blood smear. A dried blood spot on Whatman filter paper was also taken for polymerase chain reaction (PCR) and restriction fragment length polymorphism. Among 123 known patients with sickle cell disease, the prevalence of malaria by blood smear microscopy was 3.2% and by PCR was 13.8%. The prevalence of K76T mutation among the patients was 81.3%. The majority of the patients (72.4%) were using chloroquine prophylaxis. In conclusion, the prevalence of malaria parasitaemia among children with sickle cell disease attending BMC is low (3.2%) by microscopy but several children maintain sub patent infection detectable by PCR. The prevalence of chloroquine resistant *P. falciparum* in these children was higher than that previously seen in normal population in Tanzania. We recommend special attention to be paid to patients with sickle cell disease while studying the dynamics of drug resistant parasites.

Keywords: malaria, sickle cell disease, children, pcrt mutation, chemoprophylaxis, Tanzania

Introduction

The high prevalence of the sickle cell disease (SSD) and its trait in Sub-Saharan Africa coincides with the distribution of *Plasmodium falciparum* malaria (Serjeant *et al*., 2003). The unusually high frequency of sickle cell trait (HbAS) is maintained because carriers of that trait have lower rates of mortality from malaria infection. HbAS confers protection against severe malaria by limiting disease progression. Infections less often progress to the point at which either symptoms are evident (mild disease) or complications ensue (severe disease) (Williams *et al*., 2005). The occurrence of symptomatic malaria in individuals with homozygous sickle cell (HbSS) is however a major determinant of morbidity and mortality (Serjeant *et al*., 2003).

Previously, the World Health Organization (WHO) and many local national treatment guidelines advocated regular malaria chemoprophylaxis with chloroquine (CQ) among patients with sickle cell disease (MoHSW, 2006; WHO, 2006). The tremendous success of chloroquine and its heavy use through the decades eventually led to chloroquine resistance in *Plasmodium falciparum* (Wellems *et al*., 2001). Following the increase in drug resistance to CQ many countries including Tanzania changed treatment policy from CQ to sulphadoxine-pyrimethamine (SP) and later to artemisinin based combination therapies (ACTs). The changed treatment guidelines continued to advocate use of CQ for prophylaxis in SCD patients for some time (MoHSW, 2006). The recent national malaria treatment guideline clearly states that CQ has no benefits but does not give an alternative drug for chemoprophylaxis in these patients (MoHSW, 2013). A study done in Uganda showed that children with sickle cell anaemia receiving weekly chloroquine were two times more likely to get malaria compared to those on monthly SP. This showed that SP was

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more protective, as it reduced the prevalence of malaria by 50% compared to chloroquine (Nakiibuka *et al*., 2009).

Resistance against chloroquine in *P. falciparum* is associated with an amino acid change from lysine (K) to threonine (T) in codon 76 of the *P. falciparum* chloroquine resistance transporter gene (*pfcrt*), which codes for a transporter protein in the parasite's digestive vacuole membrane (Wellems *et al*., 2001; Ginsburg *et al*., 2006). After discontinuation of CQ use, several studies in African countries have reported different prevalence of *pfcrt* K76T mutation. In some countries there is clear re-emergence of wild type strains coupled with CQ sensitivity (Kublin *et al*., 2003; Lauffer *et al*., 2006; Ginsburg *et al.,* 2006; Frosch *et al*., 2011; Kamugisha *et al*., 2012; Mohamed *et al.,* 2013; Malmberg *et al.,* 2013). In other countries the level of CQ resistance is going down very slowly or is fixed (Frosch *et al*., 2011). Reasons for the difference in these countries are not clear but continued use of CQ for other purposes or combined treatment regimens for malaria have been proposed (Frosch *et al*., 2011).

Despite the high prevalence of SCD patients in malaria endemic areas, very little research has been done to assess the actual prevalence of *P. falciparum* and chloroquine resistance markers among this special population after deployment of CQ. This study was therefore, conducted to determine the prevalence of malarial parasitaemia and chloroquine resistant *P. falciparum* among children with sickle cell disease attending Bugando Medical Centre in north-western Tanzania.

Materials and Methods

Study site

This was a hospital-based prospective cross-sectional study. The study was conducted from January to April 2012 at Bugando Medical Centre (BMC), a referral, research and teaching hospital, located in the North-western part of Tanzania. BMC serves a population of around 13 million people. There is a sickle cell disease outpatient clinic conducted once weekly whereby each patient is seen once a month. At the time of this study, there were about 225 children registered in this clinic.

Study subjects

All children with confirmed sickle cell disease under 12 years of age attending BMC inpatient and outpatient departments and the parent/guardian signed informed consent were eligible. Those with sickle cell disease and had history of antimalarial use other than chloroquine within two weeks prior to outpatient visit or admission, were excluded. Children with confirmed sickle cell disease by haemoglobin electrophoresis were screened as they reported into the paediatric outpatient clinic and inpatient wards. Data and clinical findings were collected using a standardized questionnaire after interviewing the parents/guardians and doing a thorough clinical examination to children.

Laboratory analysis

Malaria parasitaemia was confirmed with the use of Giemsa-stained thick blood films following standard methods. *P. falciparum* densities were assessed by counting the number of asexual-stage parasites per 200 white blood cells and expressed as parasites per microliter of whole blood. DNA extraction from dried blood spots was done using Tris-EDTA as previously described (Bereczky *et al*., 2005). PCR and restriction fragment length polymorphism (RFLP) of the *pfcrt* gene was done using primers and conditions as previously established (Djimde *et al*., 2001). Full blood counts were performed with the use of haematology analyser CELL DYN 3700 (Abbott Laboratories, USA).

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Ethical considerations

The ethical clearance for conducting the study was granted from the Joint BMC-Catholic University of Health and Allied Sciences Publication and Ethical Committee. The signed informed consent was obtained from the respective parent or guardian before enrolment of the child. Treatment was done according to the hospital's paediatric department protocol for management of sickle cell disease and the Tanzania National Malaria Treatment Guidelines (MoHSW, 2006).

Results

A total of 123 children with SCD were enrolled of which 79 (64.2%) were males. A majority of these children, 108 (87.8%) were outpatients. The average age of diagnosis with SCD was 2 years and 4 months. The most common symptom on presentation was pain, which was reported in 14.6% of the children. The majority (68.3%) of enrolled children had a palpable liver and only 29.3% had a palpable spleen. Hypoxemia was found in 21 (17.1%) with a transcutaneous arterial oxygen saturation (SaO_2) of less than 90%. The mean haemoglobin level was 7.2g/dl and 11.2% of those with severe anaemia had haemoglobin levels less than 5g/dl (Table 1).

Table 1: Clinical Presentation of Children with Sickle Cell Disease attending Bugando Medical Centre between January and April 2012

Clinical Presentation	Number (%) or Median (IQR)
Splenomegaly	36 (29.3%)
Hepatomegaly	84 (68.3%)
Hypoxemia (SaO_2 <90% in Room Air)	21 (17.1%)
Haemoglobin level (g/dL)	7.2 (1.2 – 13.7)
Mild anaemia (7 – 10g/dL)	64 (55.2%)
Moderate anaemia (5 – 6.9g/dL)	39 (33.6%)
Severe anaemia (<5g/dL)	13 (11.2%)
Mean corpuscular volume (fL)	82.4 (31.9 – 114)
Reticulocyte count (%)	3.6 (0.7 – 14.5)
White blood cell count ($x10^9$/L)	13.0 (2.78 – 38.2)

Of the children enrolled, 89 (72.4%) were regularly taking chloroquine as antimalarial chemoprophylaxis, and 98.9 % of them purchased the drug from local pharmacies and the remaining obtained them from dispensaries and health centres.

The prevalence of malaria by using blood smear microscopy was 3.3% (4/123) while when using PCR targeting a *pfcrt* gene segment, the prevalence of malaria was higher 13.8% (17/123) (Table 2). Restriction fragment length polymorphism was successful in 16 samples whereas one sample had not sufficient detectable digests by agarose gel electrophoresis. The prevalence of *pfcrt* mutations at position 76 was 81.3% (13/16) and the remaining showed the wild type allele. PCR picked more patients with malaria parasites that were not detected by smear microscopy giving it the sensitivity of 23.5% and specificity of 100% using blood smear microscopy as a gold standard test.

Table 2: Malarial Parasitaemia in children with Sickle Cell Disease

	PCR Positive	PCR Negative	Sensitivity	Specificity
BS Positive (n = 04)	4	0		
BS Negative (n = 119)	13	106	23.5%	100%
Total	17	106		

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Discussion

The prevalence of malaria by blood smear microscopy in children with sickle cell disease (SCD) in this study was low. This finding is similar to the low malaria prevalence found recently in Dar es Salaam among sickle cell anaemia patients (Makani *et al*., 2010). Low prevalence of malaria among patients with HbSS has also been reported in other studies in Africa (Freidman *et al*., 1978; Aluoch, 1997; Cholera *et al*., 2008; Komba *et al*., 2009; Sadarangani *et al*., 2009). This is lower than the prevalence among primary school children in Mwanza, which was found to be 20% (Kamugisha *et al*., 2011). These cited studies that reported low prevalence used only blood smear microscopy. In this study when PCR was used the prevalence of malaria increased four-fold. This may be explained by existence of sub patent infections undetectable by microscopy. PCR has been reported to be more sensitive than microscopy in the detection of malaria parasites (Kilonzo *et al*., 2014). The use of recently developed diagnostic methods for malaria including rapid diagnostic tests and PCR are likely to readily identify and provide more reliable information on sub patent malaria infections in endemic areas.

The majority of children with SCD in this study reported to be on regular chloroquine chemoprophylaxis. This shows that SCD patients are still adhering to the treatment guidelines and therefore the new changes in malaria treatment policy will have to be communicated properly to these patients. In our study the prevalence of *pfcrt* K76T was high. The continued presence of resistance markers in this study may be explained by the continuing use of chloroquine for malaria chemoprophylaxis thus maintaining a selective pressure on these parasites. Similar findings have been reported from a study in Nigeria where the prevalence of *pfcrt* K76T among patients with SCD using chloroquine prophylaxis was higher among those with sickle cell anaemia compared than those with sickle cell trait (SCT) (Tatfeng *et al*., 2008). This prevalence of *pfcrt* K76T mutations is higher than those reported previously in parasites from Mwanza in Tanzania (Kamugisha *et al*., 2012a,b). These two studies included parasites from all patients and not a special group of patients with sickle cell disease. Similarly, higher prevalence of these mutants at *pfcrt* have been reported recently from elsewhere Tanzania (Ngasala *et al*., 2011; Malmberg *et al.,* 2013; Mohamed *et al.,* 2013). However, in these other Tanzanian studies the prevalence of patients with SCD was not stated.

Finding more mutant and hence resistant parasites in special groups of people on malaria prophylaxis has been reported previously (Tatfeng *et al*., 2008; Bertin *et al*., 2005). However, the findings did not attract more attention. Resistant parasites are important when trying to understand the dynamics of drug resistant parasites. They are continuously experiencing a selective pressure of CQ at a sub-therapeutic level, which is evidently selecting mutant parasites and maintain them at a higher level than in the normal population. SCD patients seem to be a special group that maintains sub patent level of mutant parasites that are not static and probably continues to be transmitted to other individuals. This group should be considered while studying the re-emergence of *pfcrt* wild type parasites especially in countries showing slower progress as reported by Frosch *et al. (*2011). Unpublished data (A. Kheir, unpubl) have revealed that parasites continue to change in patients during the dry season in Sudan when there is no transmission and are capable of being transmitted to other patients at the beginning of next transmission season.

The prevalence of malaria parasitaemia among children with sickle cell disease attending a tertiary hospital in north-western Tanzania is low by microscopy but several children maintain sub patent infection detectable by PCR. The prevalence of chloroquine resistant *P. falciparum* in these children was higher than that previously seen in normal populations in Tanzania. We recommend special attention to be paid to patients with sickle cell disease while studying the dynamics of drug resistant parasites and the treatment guidelines to determine options for malaria chemoprophylaxis in this special group.

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