

Direct Antiglobulin Reactions in *Plasmodium falciparum* Parasitized Patients in Sokoto, North Western Nigeria

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Abstract

Background: Malaria is a global public health problem affecting people particularly in tropical and sub-tropical regions of the world. Immune mediated haemolysis is thought to occur in malaria infection. The aim of this study was to investigate the incidence of direct antiglobulin positivity among 100 patients with *P. falciparum* malaria in Sokoto, North Western Nigeria.

Method: Evidence of immune mediated haemolysis with characteristic positive direct Coombs test was investigated among a cohort of 100 *Plasmodium falciparum* parasitized subjects aged 6 to 45 years and mean age 26.9 ± 8.25 years, made of 56 males (56%) and 44 females (44%) resident in Sokoto, North Western, Nigeria.

Result: Amongst the 100 subjects with uncomplicated malaria infection, 3 (3%) had a positive Direct Antiglobulin Test (DAT). The incidence of positive DAT was concentrated among subjects in the 6-15 years age groups ($p=0.001$). There was no gender-related differences in the incidence of positive DAT among the subjects.

Conclusion: These findings indicate that a positive DAT is common in *Plasmodium falciparum* parasitized Nigerians. Malaria-related positive DAT may be responsible for the anaemia seen in patients with malaria. There is the need for the routine monitoring of malaria parasitized subjects, particularly those with anaemia in the area.

Keywords: Malaria; *Plasmodium falciparum*; Haemolysis; Parasitemia

Introduction

Malaria is one of the world's deadliest diseases affecting people, particularly in tropical and sub-tropical regions of the world. Malaria remains the most complex and overwhelming health problem facing humanity [1]. About 300 to 500 million cases and 2 to 3 million deaths occur per year. The disease imposes serious effect on the blood resulting in the haemolysis of red blood cells. Immune mediated haemolysis is thought to occur in some diseases [2,3]. Adherence of complement component C3 and C4 and sensitization with IgG is often associated with a positive direct Coombs test in malaria infection [4]. Association between a high incidence of DAT positivity and *P. falciparum* parasitaemia associated with raised serum antibody titres to falciparum schizonts has been suggested [5]. A previous report of DAT testing performed on 243 malaria parasitized children in Kenya indicated that 70% had their RBCs coated with IgG, C3 and C4 either separately or together [6]. Severe anaemia and intravascular haemolysis with characteristic positive direct Coombs test are associated with severe forms of *Plasmodium falciparum* malaria [7]. Complement factors have been implicated in the mechanism leading to excess anaemia in acute *P. falciparum* infection [8]. Cases of three patients with malaria who developed post malaria immune mediated haemolysis has been reported [9].

The DAT test was introduced by Coombs [10] in 1945. It can detect the presence of immunoglobulins or complement, in particular IgG and C3d, attached to red cell membranes [11]. Sokoto State in North Western, Nigeria is an area hyperendemic for *P. falciparum* malaria. There is paucity of data on DAT in *P. falciparum* parasitized individuals in Nigeria. The aim of this study was to investigate the incidence of positive DAT results among 100 patients with *P. falciparum* malaria in Sokoto, North Western Nigeria.

Subjects

The subjects for this case-control study included one hundred consecutively-recruited plasmodium parasitized participants aged 6 to 45 years with mean age 26.9 ± 8.25 made of 56 males (56%) and

44 females (44%) resident in Sokoto, North Western Nigeria. The 100 patients used in this work are dispersed in the whole state. Only participants who met the inclusion criteria of age (≥ 6 years), confirmed *Plasmodium falciparum* parasitemia and willingness to give written informed consent after counselling were enrolled into the study. One hundred age and gender matched healthy non-parasitized individuals were monitored as controls.

Study Area

This present research work was carried out at the Haematology Department in the Faculty of Medical Laboratory Science of Usmanu Danfodiyo University, in collaboration with the Haematology Department of Usmanu Danfodiyo University Teaching Hospital in Sokoto, in the North West geo-political zone of Nigeria. Sokoto State is located in the extreme North Western part of Nigeria near to the confluence of the Sokoto River and the Rima River. With an annual average temperature of 28.3°C (82.9°F), Sokoto, is on the whole, a very hot area. However, maximum daytime temperatures are for most of the year generally under 40°C (104.0°F). The warmest months are February to April when daytime temperatures can exceed 45°C (113.0°F). The rainy season is from June to October during which showers are a daily occurrence. There are two major seasons, wet and dry which are distinct, and are characterized by high and low malarial transmission, respectively. Sokoto state had a population of 4.2 million as at the 2006

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Participant groups	Number (%) antiglobulin positive	Number (%) antiglobulin negative	Total tested
Subjects	3 (3%)	97 (97%)	100
Controls	0 (0%)	100 (100%)	100

Table 1: Direct antiglobulin Test reactivity in malaria infected and non-infected subjects.

Age groups (Years)	Number(%) Antiglobulin Positive	Number(%) Antiglobulin Negative	Total number (%)	Z-value	P-value
6-15	3 (3%)	22 (22%)	25 (25%)	1.8	0.001
16-25	0 (0%)	12 (12%)	12 (12%)		
26-35	0 (0%)	37 (37%)	37 (37%)		
36-45	0 (0%)	26 (26%)	26 (26%)		

Table 2: Direct Antiglobulin Test in malaria infected subjects based on age groups.

census. The metropolis is estimated to have a population of 427,760 people [12].

Methods

Malaria infection was confirmed using thin films made by push wedge technique prepared from the EDTA-anticoagulated blood and stained with Giemsa stain. Conventional manual tube method was used for the determination of direct antiglobulin test. Polyspecific Antihuman Globulin reagents (Lorne Laboratories, UK) was used for DAT testing. Lorne anti-human globulin reagents detect non-agglutinating antibody molecules, as well as molecules of complement attached to red cells, following *in vivo* or *in vitro* antigen-antibody reactions. When used by the recommended techniques, the reagents will react with immunoglobulins and/or complement attached to the red cell surface, resulting in agglutination (clumping) of adjacent sensitized cells. Cells not sensitized will not be agglutinated. In summary, we added 1-2 drops of well mixed EDTA anticoagulated patient's red cells to a tube. Washed the red cells three times with isotonic saline. After the third wash, we decanted completely the supernatant saline and re-suspended the red cell button to prepare a 3% suspension from the washed cells. We added one drop of the washed 3% suspension to a well labelled tube. We washed these tubes one more time and decanted completely the supernatant saline and blotted dry the tube with an absorbent wipe. Immediately, we added one drop Polyspecific Antihuman Globulin and mixed gently to allow for adequate mix between the red cell button and the AHG reagent. We incubated at room temperature 5 minutes and centrifuged the tube lightly for 1 minute at 1,000 rpm. We immediately re-suspend gently and examined macroscopically for agglutination using the lighted agglutination viewer. When the result was negative, we examined for agglutination under the microscope. If result was still negative microscopically, we added one drop of IgG-coated Coombs Control Cells to the tube and centrifuged lightly for 1 minute at 1,000 rpm on a centrifuge. We immediately re-suspended gently and examined macroscopically for agglutination. A positive reaction at this state confirmed a negative test. If the result was negative after the addition of the IgG-coated Coombs Control Cells, the test result was reported as invalid and test repeated. The DAT detects immunoglobulin, and/or complement on the surface of red blood cells, which agglutinate with the addition of antihuman globulin. The agglutination is detected visually as agglutination of red blood cells. The most common causes for false negative results are improper or under-washing or under-centrifugation of the sample resulting in residual unbound antibodies remaining in the tube and adsorb the AHG reagent [13]. The failure to add AHG reagents or the addition of inactive AHG reagents (outdated or sub- optimally stored AHG reagent) may lead to false negative results. A delay in the addition of AHG after washing and over agitation

at the time of result interpretation can potentially cause false negatives. In the conventional tube method, major causes of false positive include; poor housekeeping associated with the reuse of improperly cleaned glass tubes and bacterial-infected saline [14]. False positive results tend to arise when specimens degrade sufficiently to cause non-specific binding of the DAT reagents. Causes of false positive results include over-centrifugation, which causes the RBC to be packed too tightly, under agitation at the time of result interpretation, a prolonged delay in testing, a clotted specimen, reagent issues, and patient factors, such as spontaneous agglutination [13,14].

Statistical Analysis

Statistical analyses were conducted using SPSS (version 11) software. Comparisons were assessed using mean and chi-square test. A p-value of ≤ 0.05 was considered statistically significant in all statistical comparison. Correlation was compared using SPSS linear regression analysis.

Results

Amongst the 100 subjects with uncomplicated malaria infection, 3 (3%) had a positive direct antiglobulin test (DAT). Table 1 show the antiglobulin test reactivity in malaria infected subjects and non-infected controls. The incidence of positive DAT was concentrated among subjects in the 6-15 years age groups ($p=0.001$). Table 2 shows the direct Antiglobulin Test in malaria infected subjects based on age groups.

Discussion

In this present study, we had observed a prevalence of direct antiglobulin positivity of 3% in our cohort of 100 *Plasmodium falciparum* parasitized subjects. Our observed prevalence although lower is consistent with previous reports among Gambian children, which observed DAT positivity of 38.8% among their cohort of 134 children with *P. falciparum* malaria. In a significant number of the children, positive DAT was due to the adherence of C3 while the others had sensitization with IgG, as well as C3 [4]. Similarly, Abdalla et al. [6] performed DAT on 243 children in Kenya. They observed a high incidence of positive DAT (70%) among in children with malaria. A significant number of the children had their RBCs coated with IgG, C3 and C4 either separately or together compared to only 12% in paediatric patients with conditions other than malaria. Helegbe et al. [8] investigated 484 *Plasmodium* parasitized children for direct antiglobulin test and observed a positive DAT reactivity in 27% of subjects. Out of which, 87.8% were positive for C3d alone, while 12.2% were positive for either IgG alone or both IgG and C3d. Also, Merry et al. [3] in Eastern Thailand investigated evidence of immune mediated haemolysis in their cohort of 83 patients with *P. falciparum* malaria and obtained a prevalence of DAT positivity of 16.4%. The possible reason for our observed lower prevalence is that we used a manual tube method using a polyspecific AHG reagent that detects IgG, and or complement coating on red cells, unlike other reports that used more sensitive gel microcolumn, affinity microcolumn and flow cytometric methods. The manual tube method using a polyspecific AHG reagent is a readily available and affordable alternative to the more expensive gel microcolumn, affinity microcolumn and flow cytometric methods in resource-limited settings. There has been a mixed opinion on the potential cause/s of Direct Coombs Test (DCT) positivity in malaria parasitized subjects. A previous report [5] that investigated the erythrocytes taken from malaria parasitized children with a high IgG DAT titre indicated that the eluted IgG had specific antibody activity against *P. falciparum* schizont antigen, as demonstrated by

means of the indirect fluorescent antibody technique. The finding and report by Abdulla [15] seems to provide convincing evidence, which tend to implicate *falciparum* malaria in the aetiology of positive DAT result in malaria parasitized individuals. It is probable that erythrocyte sensitization results from passive attachment of circulating complement-fixing malaria antigen-antibody complexes.

Plasmodium falciparum malaria associated with positive DAT has been implicated to contribute to the immunohaemolytic process that contributes to the anaemia seen in patients with *Plasmodium falciparum* malaria. Salloum and Lundberg [2] also observed incidence of haemolytic anaemia with positive direct antiglobulin test in patients with spontaneous cytomegalovirus (CMV) infection. There is growing advocacy to include malaria serology and serology of other diseases that are associated with positive DAT routinely, as a part of haemolysis work-up investigation of patients with haemolytic anaemia [7,9].

In this study, our observed prevalence was lower than prevalence's observed from other previous reports [3,5,6,8]. The reason for the lower prevalence obtained in this study may not be far-fetched. In this present study, detection of immunoglobulin or complement bound to RBCs was carried out using the traditionally manual method performed by tube agglutination using polyspecific (anti-IgG or anti-C3d). Previous reports [2] that compared the tube agglutination DAT to gel microcolumn, affinity microcolumn and flow cytometric DATs on RBCs coated *in vitro*, and on patient RBC samples indicates that detection of IgG bound to RBCs was not consistent with the methods described. Gel microcolumn DATs was more sensitive than tube agglutination and affinity microcolumn DATs [16]. Given the varied results of these assays, it may be worthwhile for reference laboratories not to rely on a single method for DATs. More comprehensive testing should be performed when the tube agglutination DAT is negative in a patient with suspected immune-mediated hemolytic anemia. Further comparisons may be required to determine the proficiency of flow cytometric assays [17,18].

We observed that DAT reactivity was concentrated among children in the age group 6-15 years. The reason for this observation is unknown. Our finding is, however, consistent with previous report, which indicated that children with positive IgG DAT, were of a significantly older age group and had significantly higher parasitaemias at presentation than children with complement components only or with a negative DAT [4]. Similarly Helegbe et al. [8] investigated *Plasmodium falciparum* infected children in Ghana and observed that DCT positivity was more common in the age group relative to children who were 5 years or older. Our finding is, however, at variance with a previous report [6] that investigated DAT among Children in Kenya, which indicated that most positive DATs occurred in association with malaria parasitaemia in children between 18 months and five years of age. The finding from this study indicates that the direct antiglobulin test (DAT) remain an important test that detects immunoglobulin, and/or complement on the surface of red blood cells. The utility of the DAT is vital to differentiate between haemolysis of an immune or non-immune etiology [19]. The DAT is, therefore used in investigations of immune haemolytic anaemia. Testing typically starts with polyspecific AHG containing both anti-IgG and anti-complement with positive reactions repeated with monospecific AHG to individually detect the presence of IgG or complement [20].

Conclusion

These findings indicate that a positive IgG DAT is common in *Plasmodium falciparum* parasitized individuals. This finding may be responsible for the anaemia seen in patients with malaria in Nigeria.

There is the need for the routine monitoring of malaria parasitized subjects, particularly those with anaemia in the area for DAT.

Competing Interest

The author confirm that there is no conflict of interest.

Authors Contribution

Uko EK, Erhabor O, Isah IZ and Yakubu Y designed the study; Kabiru M, Bello Y, Okwesili AN, Buhari HA and Onuigwe FU recruited the subjects and carried out the laboratory testing, while Erhabor O, Mainasara Y and Uko EK were involved in the statistical analysis and writing up the manuscript.

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