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Anemia in Patients Receiving Sulphonamide Therapy for Malaria: Role of Glucose-6-Phosphate Dehydrogenase Deficiency and Antibodies to Sulphonamide

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Abstract
Sulphonamide can cause anemia by antibody production as well as in individuals who are glucose–6–phosphate dehydrogenase (G6PD) deficient. Against the background of no data on the relationship between G6PD deficiency, sulphonamide antibody production and anemia among patients with malaria, this study was conducted. The study was conducted between April and November 2016. Blood specimens were collected from 500 patients with symptoms of malaria who had taken sulphonamide-containing anti-malaria. Screening for G6PD deficiency, detection of antibody to sulphonamide, in-vivo and in-vitro sensitization, and hemoglobin concentration were determined using standard techniques. A total of 110 (22%) and 79 (15.80%) out of the 500 patients had antibodies to sulphonamide and were G6PD deficient respectively. There was no significant association between sulphonamide antibody production and G6PD deficiency (OR=1.055, 95%CI=0.594, 1.874; P=0.9717). Presence of sulphonamide antibodies, G6PD deficiency, in-vivo and in-vitro sensitization were significantly associated with anemia (P<0.001). In-vivo and in-vitro sensitizations were significantly associated with sulphonamide antibody production and G6PD deficiency. The study observed no relationship between sulphonamide antibody production and G6PD deficiency in causing anemia. Also, sulphonamide antibody was associated with sensitization (in vivo and in vitro) of red blood cells.
Keywords: Anemia, Malaria, Sulphonamide antibody, Glucose–6–phosphate dehydrogenase, Sensitazation

Introduction
Anemia is a known complication of malaria (1). It has a profound effect on the quality of life of people by inducing such symptoms as loss of stamina, rapid heart rate and shortness of breath (2). It has also been reported that over half of malaria related deaths are attributable to severe anemia (3). There are three main causes of anemia, that due to blood loss, decreased red blood cell production and due to increase in red blood cell breakdown. Causes of blood loss include trauma and gastrointestinal bleeding, among others, while decrease production can result from iron deficiency, lack of vitamin B12, thalassemia and a number of neoplasms of the bone marrow. Cause of increase in red blood cell breakdown includes a number of genetic conditions such as sickle cell anemia, infections like malaria and certain auto-immune hemolytic anemia. Anemia occurs in malaria due to invasion and destruction of red blood cells by malaria parasite, splenic sequestration and ineffective erythropoiesis (4).
Glucose-6-phosphate dehydrogenase (G6PD) catalyzes the reduction of nicotinamide adenine dinucleotide phosphate (NADP) to NADPH, which protects cells from oxidative damage (5). Erythrocytes do not generate NADPH in any other way (6) as such G6PD deficient erythrocytes are destroyed during oxidative stress. Hereditary condition, drug-induced hemolytic anemia, most often accompanies a glucose-6-phosphate dehydrogenase (G6PD) enzyme deficiency. G6PD deficiency is a disorder of the hexose monophosphate shunt, which is responsible for producing NADPH in red blood cells, which in turn keeps glutathione peroxidase, an enzyme that removes peroxidase from red blood cells, thus protecting them from oxidative stress (5). Without reduced glutathione, oxidative drugs can oxidize the sulfhydryl groups of hemoglobin, removing them prematurely from the circulation that will cause hemolysis. Hemolysis may be precipitated in G6PD deficient individuals by infection, fava bean and drugs (7). Malaria is an infection that can result in anemia. *Plasmodium falciparum* can cause direct lyses of red blood cells and drugs used in course of treatment can also destroy red cells. Sulfonamide-containing drugs cause anemia by production of antibodies and in G6PD deficient individuals (8). It has also been reported that patients with malaria infection have positive direct Coomb's test (9). In our locality, there is paucity of report on the relationship between G6PD deficiency, sulphonamide antibodies production, presence of *in vivo* and *in vitro* antibody sensitization, and anemia among patients with malaria. Hence, the study aimed to determine the relationship between G6PD deficiency and antibodies to sulphonamide.

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Materials and Methods

Study Population: This study (cross-sectional) was carried out in Central Hospital, Benin City, Nigeria, among out-patients with signs and symptoms of malaria infection between April and November 2016. A total of 500 patients consisting of 223 males and 277 females were recruited for this study. All patients used for this study had consumed sulphonamide-containing drugs at least once within one month prior to specimen collection. Patients who had not consumed sulphonamide-containing drugs and those confirmed not to have malaria infection were excluded from the study. Informed consent was obtained from each patient or their parents or guardians in case of children prior to specimen collection. This study was approved by Ethical Committee of Central Hospital, Benin City.

Sample Collection and Processing: From each patient, 10 ml of blood was collected and dispensed in 5 ml amount into plain and ethylene diamine tetra-acetic acid (EDTA) containers. The samples in plain containers were allowed to clot and the sera obtained were used to detect antibody to sulphonamide – sulfadoxine, and for indirect Coomb's test. The blood sample in EDTA containers were used to determine hemoglobin concentration, glucose-6-phosphate dehydrogenase (G6PD) deficiency and to perform direct Coomb's test.

Determination of Hemoglobin Concentration: Hemoglobin concentration was determined using a hematology auto-analyzer – Sysmex K21N (Sysmex Co-operation, Kobe, Japan). Anemia was defined as hemoglobin concentration less than 12g/dl for female and less than 13g/dl for male (10).

Determination of glucose–6–phosphate dehydrogenase (G6PD) deficiency: G6PD deficiency was detected using the method described by Cheesbrough (11). Briefly, three glass test tubes were labeled test, normal and deficient. Into the test tubes labeled test and deficient, 0.1ml of freshly prepared sodium nitrite glucose reagent was added. Into the test tube labeled test, 0.1ml methylene blue reagent was added. To all test tubes (Test, Normal and Deficient), 2 ml of patient's blood was added. The tubes were stopper mixed and incubated in a water-bath at 37^0C for 3 hours. From each of the test, normal and deficient test tubes, 0.1ml of well mixed content were transferred to 3 respective test tubes labeled test, normal and deficient containing 10 ml of distilled water. This was mixed and the color of each tube was examined. If the color of the test tube labeled test is the same as the tube labeled normal, the patient is deemed to have normal G6PD activity. But if the color matches the tube labeled deficient, the patient is deemed to be G6PD deficient. If the color of the tube labeled test is mid-way between the tube labeled normal and the tube labeled deficient, a reduced G6PD activity (heterozygous) was inferred and was also taken as G6PD deficient.

Direct Coomb's test: The direct Coomb's test was performed according to the method describe by Zantek *et al* (12). Briefly, patient's anticoagulated red blood cells were washed with normal saline. This was done by adding 0.5ml of anticoagulated blood into a test-tube followed by 5ml of normal saline into the same test tube. This was centrifuge at 1000 rpm for 5 minutes. Supernatant was discarded and another 5ml normal saline added. The process was repeated two times. After the last wash, supernatant was completely discarded and anti-human globulin was added. This was centrifuged at 1000rpm for a minute and was observed for agglutination or hemolysis. The presence of agglutination or hemolysis was taken as a positive or direct Coomb's test.

Indirect Coomb's test: A modification of the method described by Mahapatra *et al.,* (13) was used. Briefly, blood from 12 groups O donors were washed in separate containers four times with normal saline. The respective washed blood sample was then pooled together. Into a clean test tube 2 drops of pooled group 0 red cell was placed and follow by 4 drops of patient's serum. The mixture was incubated at 37^0C for 15 minutes. After incubation the mixture was washed four times. Two drops of antihuman globulin (AHG) reagent was added to the red cell in the test tube after washing. This was centrifuged briefly and agglutination or hemolysis was watched out for. The presence of agglutination or hemolysis was considered positive for the indirect Coomb's test.

Detection of Sulfadoxine antibodies: Sulfadoxine antibodies were detected using two methods-drug absorption and immune complex methods, as previously described (14). Briefly, 0.5 grams of sulfadoxine was dissolved into 10ml of sterile normal saline and used for the detection of sulfadoxine antibodies using the two methods mentioned earlier. For *drug-absorption method*, equal volume of group 0 washed red blood cell and sulfadoxine solutions were placed in test tube and incubated at 37^0C for 30 minutes. This was washing four times with normal saline to remove excess drug. Equal volume of patient's serum and drug-red cell suspension was placed inside test tube and incubated for 30 minutes at 37^0C. After incubation, the content of the test tube was centrifuged briefly and was observed for the presence of agglutination or hemolysis. When negative the mixture was washed four times with normal saline and antihuman globulin (AHG) was added. This was centrifuged briefly and observed for agglutination or hemolysis. Controls were carried out as above but consist of:

1. Patient serum and group O red cell without drug.
2. Drug-red cell suspension without patient serum.

Both controls were negative (No agglutination or hemolysis were observed).

For *immune complex method***,** equal volume of sulfadoxine solution, patient's serum and 5% washed group O red blood cell were placed inside a test tube and incubated at 37^0C for 1 hour. After the incubation period it was centrifuged briefly and observed for agglutination or hemolysis. When negative the mixture was washed 4 times

with normal saline and AHG added. This was followed by brief centrifugation and observed for agglutination or hemolysis. Control was carried out as above but consists of.

1. Patient's serum, red blood cell and normal saline without sulfadoxine solution.
2. Red blood cell, sulfadoxine solution and normal saline, without patient serum. Both controls were negative (No agglutination or hemolysis).

Statistical Analysis: The data obtained were analyzed with Chi square (X^2) test using the statistical software INSTAT ® (Graph Pad Software Inc. La Jolla, CA, USA).

Results

Of the 500 patients used in this study 22% had antibody to sulphonamide and 15.80% were G6PD deficient. There was no significant association (OR = 1.055, 95%. CI = 0.594, 1.874; P=0.9717) between G6PD deficiency and presence of sulphonamide antibody (Table 1).

Presence of Sulphonamide antibodies, G6PD deficiency, direct Coomb's test and indirect Coomb's test were all significantly (P<0.001) associated with anemia (Table 2).

In-vivo sensitization (direct Coomb's test) and *in-vitro* sensitization (indirect Coomb's test) were significantly (P<0.0001) associated with presence of sulphonamide antibodies (Table 3) and G6PD deficiency (Table 4)

Table 1: Relationship between sulphonamide antibodies and glucose-6-phosphate dehydrogenase deficiency

Characteristics	**Glucose-6-phosphae dehydrogenase deficiency positive (%)**	**Glucose-6-phosphates dehydrogenase deficiency negative (%)**	**Total**
Sulphonamide antibody positive	18(22.78)	92 (21.85)	110(22)
Sulphonamide antibody negative	61(77.22)	329 (78.15)	390(78)
Total	79(15.8)	412(84.2)	500

Figures in parenthesis are percentages; OR = 1.055 95% CI = 0.5941, 1.874; P = 0.9717

Table 2: Factors Associated with Anemia

Characteristics	**No. Tested**	**No. with Anemia (%)**	**OR**	**95% CI**	**P Value**
Presence of Sulphonamide antibodies	110	88 (80.00)	4.677	2.808,7.756	<0.0001
Absence of Sulphonamide antibodies	390	180(46.15)			
Presence of G6PD deficiency	79	58(73.42)	2.775	1.626,4.736	0.0002
Absence of G6PD deficiency	421	210(49.88)			
Direct Coomb's Test					
Positive	153	108(70.59)	2.805	1.867,4.214	<0.0001
Negative	347	160(46.11)			
Indirect Coomb's Test					
Positive	98	69(70.41)	2.427	1.508,3.906	0.0003
Negative	402	199(49.51)			

G6PD = Glucose–6-Phosphate Dehydrogenase

Table 3: Association of sulphonamide antibodies with *in vivo* and *in vitro* sensitization

Characteristics	**No. Tested**	**No. Positive for Sulphonamide Antibodies (%)**	**OR**	**95% CI**	**P-Value**
Direct Coomb's test					
Positive	153	98(64.05)	49.742	25.608,96.624	<0.0001
Negative	347	12(3.46)			
Indirect Coomb's test					
Positive	98	65(66.33)	15.626	9.278,26.318	<0.0001
Negative	402	45(11.19)			

Table 4: Association of G6PD deficiency with *in vivo* and *in vitro* sensitization

Characteristics	No. Tested	No. Positive for G6PD Deficiency (%)	OR	95% CI	P Value
Direct Coomb's test					
Positive	153	58(37.91)	9.478	5.473,16.417	<0.0001
Negative	347	21(6.05)			
Indirect Coomb's test					
Positive	98	40(40.82)	6.419	3.812,10.808	<0.0001
Negative	402	39(9.70)			

G6PD = Glucose-6–phosphate dehydrogenase

Discussion

Anemia is a known complication of malaria and over half malaria related death is attributable to severe malaria (1, 3). The malaria parasite, G6PD deficiency, presence of antibody against anti-malaria agent and presence of immune antibodies has been reported to cause anemia (4, 7). No study has looked at the relationship between G6PD deficiency, sulphonamide antibodies production and presence of *in-vivo* and *in-vitro* sensitization, and anemia among patients with malaria in our locality. Hence this study was conducted.

A total of 110 (22.00%) out of the 500 patients used in this study had antibodies to sulphonamide. The use of antimicrobial agents in Nigeria is unregulated and over the counters sales of antimicrobial agents are common (15-17). Sulphonamide-containing antimalarials are mainly preferred by those in our environment because of their single dose (2-3 tablets) compared with other antimalarials that may be taken more than once a day and for 2, 3 or more days (18).

The G6PD deficiency prevalence of 15.80% is in agreement with the 15.3% reported in children from South Western Nigeria (19), but lower than the geostatistically proposed prevalence of 31% for Nigeria (20). It is important to note that G6PD deficiency varies based on geographical location and ethnic population (21).

There was no significant association between sulphonamide antibodies production and G6PD deficiency (OR = 1.055 95%) CI = 0.5941 1.8) 4; p = 0.9717). G6PD deficiency is a genetic disorder of X-chromosome (22, 23), while sulphonamide antibodies production has not been associated with any genetic mutation. This may explain the finding in this study. That is, sulphonamide antibodies production is independent of G6PD deficiency. This means that both cause anemia independent of each other. However, G6PD deficiency prevalence of 15.80% and a sulphonamide antibody production of 22.00% may indicate that sulphonamide antibody production may be more likely to cause anemia than G6PD deficiency among the study population.

Anemia was significantly associated with sulphonamide antibody. The presence of sulphonamide antibodies (drug-absorption and immune complex mechanism) results in increased oxidative stress of red blood cell membrane resulting in extravascular and intravascular destruction of red cells (24, 25). In the case of G6PD deficiency the absence of the enzyme does not result in the generation of NADPH and the consequent exposure of such red blood cell to oxidative stress, which may be caused by infection, drugs etc, will result in intravascular destruction of red blood cells(5).

The antiglobulin test, also known as the Coomb's test is used to detect if red blood cells (RBC) have surface-bound IgG or complementor to detect erythrocyte directed IgG in plasma or serum (26). That is, they can be used to detect *in-vivo* sensitization (direct antihuman globulin test, DAT) and *in-vitro* sensitization (indirect antihuman globulin test, IAT) (13). In this study, *in-vivo* sensitization was associated with. *In-vivo* sensitization among malaria infected patients has been reported with low prevalence of 3% in Nigeria (9). It is important to note that the report of Uko *et al*. (9) did not state whether their patients were on any antimalarial drug, as the patients used in the study had taken sulphonamide-containing medications. *In-vivo* sensitized red blood cells are extravascularly destroyed in the spleen (24). Similarly, *in-vitro* sensitization result from antibodies which are capable of sensitizing red blood cells leading to intravascular destruction of the red blood cells (24). This was observed in this study. Thus, the anemia observed in the study appears to be mainly due to immune antibodies.

The presence of sulphonamide antibodies was significantly associated with *in-vivo* sensitization and *in-vitro* sensitization. Drugs have been known to cause drug-induced hemolytic anemia which may result in positive DCT and ICT (24, 26). This may explain the finding in the study.

The association between G6PD deficiency, *in-vivo* and *in-vitro* sensitization is surprising. This is because G6PD deficiency is associated with non-immune hemolytic anemia (7). Although, infants with G6PD have been

reported to have a negative DAT (27, 28). The observed finding may be as a result of immune antibodies produced during malaria infection (9) and the presence of sulphonamide antibody, which we represent in our study population. Further study with G6PD deficient individuals without malaria and who are not on any drugs that cause immune hemolytic anemia are needed to verify this.

In conclusion, this study observed no relationship between sulphonamide antibody production and G6PD deficiency in causing anemia in malaria infected patients. Sulphonamide antibody production was associated with immune antibodies (*in-vivo* and *in-vitro* sensitization) in causing anemia.

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