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# Cold Agglutinin Disease Prevalence and Immunoglobulin M levels in Patients with Splenomegaly from Endemic Areas for Malaria in Rwanda

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## Abstract

**Background:** Inhabitants from malaria endemic zones often present with enlarged spleen, mainly due to hyper reactive malarial splenomegaly (HMS), and it is seen more commonly associated with elevated levels of Immunoglobulin M (IgM). Cold agglutinin disease is an acquired autoimmune hemolytic anemia (AIHA) that is usually due to cold-reacting IgM autoantibodies directed against red cell antigens. The study was conducted in response to the observed high frequency of transfusion dependent anemia in patients with splenomegaly from malaria endemic zones in southern province of Rwanda. The objectives of this study were to determine the prevalence of cold agglutinin disease and to assess the distribution of IgM antibodies among these patients. **Methodology:** This was a descriptive, cross-sectional study conducted over a period of six months from June 2016 to December 2016. The study enrolled adult population from malaria endemic areas of the southern province of Rwanda with unexplained splenomegaly. Blood samples for testing IgM levels, cold agglutinin, FBC, and markers of hemolysis were collected from peripheral health settings and analyzed at the laboratory of Butare University Teaching Hospital. **Results:** During the study period, we enrolled 188 participants with enlarged spleen. One hundred twenty-five (66%) were females and 34% were males. The mean (±SD) age of the study participants was 35.6 ± 15.2 years. Out of 188 participants, only 4.8% were found with significantly positive Cold Agglutinin Titer (CAT) (≥1:64) and 84% of participants were found to have elevated serum IgM level. Hemoglobin level, white blood count and platelets count decrease with severity of splenomegaly, while IgM

level increases with spleen size. **Conclusion:** Among patients with splenomegaly from malaria endemic areas, IgM levels correlate with the stages of splenomegaly while cold agglutinin disease plays a small role in the etiology of anemia.

## Keywords

Cold Agglutinin, Hyperreactive Malarial Splenomegaly, Malarial Endemic Areas, Rwanda

## 1. Introduction

Malaria is a major public health problem in sub-Saharan countries. Rwanda is among the endemic countries for malaria and its entire population is at risk of getting it [1] [2]. In 2016, malaria cases in Rwanda were estimated at 1.8 million adults and 443,000 children under 5 years of age and pregnant women, respectively, according to the report by Rudasingwa Guillaume and Sung-II Cho [1]. Hyper-reactive Malaria Splenomegaly (HMS) is globally seen in malaria-endemic zones, generally in tropical Africa as a result of recurrent malaria infection [3]. HMS has a wide prevalence in different countries, ranging from 1% - 2% in Nigeria up to 80% in Papua New Guinea [4]. One study revealed that 6.2% of 210 healthy students from eastern province of Rwanda had HMS [5].

When the spleen becomes enlarged beyond 12 cm, it is termed *splenomegaly* [3] [6] [7]. The Hackett's classification of splenomegaly is the most used classification in cross-sectional studies [8]. Diagnostic criteria for HMS were proposed by Fakunle in 1981. These include major criteria: persistent gross splenomegaly extending more than 10 cm below the costal margin, elevated anti-malarial antibodies, IgM titer >2SD (≥2.5 g/L) above the local mean value, and favorable clinical and immunological response to long-term malarial treatment; and minor criteria including hypersplenism, lymphocyte proliferation, and occurrence within the family [6] [8] [9].

One of the complications of HMS is hemolytic anemia [3]. In malaria, cold agglutinins are sometimes implicated in the pathogenesis of hemolysis [3] [10] [11]. Cold agglutinins are antibodies capable of agglutinating erythrocytes at a temperature below 37˚C, and they were first described by Landsteiner in 1903. The association of cold agglutination with hemolysis was described by Rosenthal and Corten in 1937 [10] [12] [13]. Cold agglutinin disease is an acquired autoimmune hemolytic anemia (AIHA) that is usually due to cold-reacting IgM autoantibodies directed against red cell antigens [12]. Cold agglutinin (CA) disease is diagnosed when the following criteria are met: chronic hemolysis, CA titer of ≥1:64 at 4˚C and direct antiglobulin test (DAT) characterized by a positive polyspecific test confirmed with monospecific test positive for complement protein C3d and negative for IgG [14] [15] [16].

There have been no studies demonstrating the prevalence of IgM positivity or

cold agglutinin disease among patients with splenomegaly in Rwanda. The objective of this study was to determine the prevalence of cold agglutinin disease and the distribution of IgM antibodies in patients with unexplained splenomegaly from the malaria-endemic zones of the southern province of Rwanda.

## 2. Methods and Materials

**Study design:** This was a descriptive cross sectional study

**Study sites:** The study has enrolled adult patients with splenomegaly who were followed at Kibilizi District Hospital and its affiliated health centers, and at Centre Hospitalier Universitaire de Butare (CHUB).

**Study time:** It was conducted over a period of 6 months, from June 2016 to December 2016.

**Sample size:** Patients aged 16 years and above who reside in Malaria endemic areas in Southern Province of Rwanda and who was found with splenomegaly.

**Study procedure:** The study was approved by UR (University of Rwanda) and CHUB (Centre Hospitalier Universitaire de Butare) ethics committees. Included in patients were those hospitalized for decompensated anemia on background of tropical splenomegaly. Outpatients living with enlarged spleens (commonly called *"igisyo"* in Kinyarwanda, the local language*)* were recruited by Community Health workers and then interviewed and examined. Prior to participation in the study, the patients were briefly introduced to the study design, objectives and the possible impact of results. Patients who were included in the study gave written informed consent.

Data collection was in the form of a questionnaire. This included the name initials, sex, age, occupation and address, Clinical data including history of abdominal swelling, history of recurrent malaria infection, history of documented hematological malignancy and chronic liver disease. Spleen size was assessed by abdominal examination by a Medical Doctor, using a tape measure from the left coastal margin. The participant without palpable spleen was excluded at this step. Hematological, biochemical, and immuno-serological analysis was conducted at CHUB. Blood samples were conserved and transported in an appropriate laboratory box conserving at room temperature.

**Studies variables:** The variables in this study include: demographic (age, sex,), clinical (splenomegaly stage) and biological (cold agglutinin titer, this was considered significant or pathological if ≥1:64, Hemoglobin, platelet and white blood count, coombs test and Ig M level).

**Inclusion criteria**

- Participants aged 16 years old and above, residing from malaria endemic areas
- Participants from malaria endemic areas with enlarged spleen on physical examination

**Exclusion criteria**

- Participants less than 16 years old

- Patients not willing to participate
- Patients with a documented alternative cause of splenomegaly such as chronic liver disease, hematological malignancy (leukemia, lymphoma), etc.

**Ethical consideration:** The validity of the study was assessed by School of Medicine and Pharmacy staff members who provided relevant advice to be observed throughout the study. Permission to carry out this study was obtained from the research committee of the College of Medicine and Health sciences IRB (Institutional Review Board). Prior to being enrolled in the study, researchers obtained a signed consent form from the study participant or his surrogate after brief introduction of the study. The participants' data was kept under conditions of strict confidentiality.

**Data collection, entry and analysis plan**

For each enrolled participant in this study, the demographic information, clinical information and laboratory data were collected on a data collection form. The entry and descriptive statistical analysis of collected data were performed using EPIDATA and SPSS version 16.

## 3. Results

During the period of the study (between June and December, 2016), we enrolled 188 subjects from malaria endemic areas, with enlarged spleen. One hundred twenty-five (66%) were females and 34% were males. The mean (±SD) age of the subjects in the study group was 35.6 ± 15.2 years. The mean age group of patients with significant positive CAT was 34.9 ± 15.6 years old. The demographics, laboratory and clinical parameters of the study subjects according to the presence or absence of significant cold agglutinin titers, the mean IgM level high at was 13.5 g/L and Direct comb test are shown in Table 1.

While 158 of the 188 (84%) of the study subjects with splenomegaly had elevated Ig M level, only 9 (4.8%) had significant cold agglutinin titers of 1:64 or higher (shown in Table 3). The majority of these patients had moderate to severe splenomegaly categorized as stage III and IV with 76 (40%) and 49 (26%) respectively (shown in Table 1). The severity of the anemia, pancytopenia and IgM hypergammaglobulinemia correlated with the spleen size (shown in Table 4).

The mean hemoglobin level of the study subjects was 11.8 (± 2.6) g/dL. Patients with negative cold agglutinin test results had a mean hemoglobin level of 12.0 (± 2.5) g/dL while those with positive test had a mean hemoglobin level of 11.1 (± 2.9) g/dL. About 57% of the patients (men and women) had normal level of hemoglobin and 42% had less than normal hemoglobin level (mild to severe anemia) as shown in Table 2.

The pathological Cold Agglutinin Titer (CAT) > 1:64, Table 3 shows that only 9 (4.8%) subjects of the study population had significant or pathologic Cold Agglutinin Titer. As the total number of subjects who had anemia exceeds widely the total cases of Pathological CAT, this is suggestive the minor role of cold agglutinin in anemia among patients with splenomegaly in malaria endemic areas.

**Table 1.** Cold agglutinin test results compared to different study population characteristics.

| | Total or Mean value | CAT− (<1/64) | CAT+ (≥1/64) |
|---|---|---|---|
| **Demographics** | | | |
| Gender | **Total** | | |
| *Male* | 63 | 61 | 2 |
| *Female* | 125 | 118 | 7 |
| | **Mean** | | |
| Age | 35.6 +/− 15.2 | 35.6 +/− 15.2 | 34.9 +/− 15.6 |
| **Hematological parameters** | **Mean** | | |
| Hemoglobin (g/dL) | | | |
| *Male* [13-17.5 *g/dL*] | 12.3 | 12.3 | 11.6 |
| *Female* [12 - 15.5 *g/dL*] | 11.6 | 11.8 | 7.9 |
| Reticulocyte count [0.5 - 2.1] | 2.9 | 2.9 | 4.3 |
| MCV [80 - 94 fl] | 88.9 | 88.6 | 94.4 |
| WBC [4 -11,000/ml] | 4.1 | 4.1 | 3.3 |
| Platelets [150 - 400 * 1000/l] | 121.2 | 120.2 | 140.7 |
| **Immunology/biochemistry** | **Mean** | | |
| IgM [0.4 - 2.3 g/L] | 13.5 | 13.6 | 11.8 |
| LDH [114 - 237 IU/L] | 306.1 | 301.6 | 393.9 |
| Direct Coombs | negative | negative | negative |
| **Splenomegaly stage** | **Total** | | |
| Stage II | 43 | 42 | 1 |
| Stage III | 76 | 72 | 4 |
| Stage IV | 49 | 45 | 4 |
| Stage V | 20 | 20 | 0 |

WBC: White Blood count, MCV: Mean Corpuscular Volume, LDH: Lactate Dehydrogenase, IgM: Immunoglobulin M.

**Table 2.** Distribution of Malarial patients according to the level of hemoglobin and results of CAT.

| Hemoglobin (g/dL) | Cold agglutinin test | | Total n (%) |
|---|---|---|---|
| | Negative n (%) | Positive n (%) | |
| Below normal (men & women) | 75 (41.9) | 9 (100.0) | 84 (44.7) |
| Normal (men & women) | 103 (57.8) | 0 (0.0) | 103 (54.8) |
| Above normal | 1 (0.6) | 0 (0.0) | 1 (0.5) |
| Total | 179 (100.0) | 9 (100.0) | 188 (100.0) |
| Mean Std dev | 12 ± 2.5 | 11.1 ± 2.9 | 11.8 ± 2.6 |

**Table 3.** Study population according to cold agglutinin titer (CAT).

| | Cold agglutinin test | |
|---|---|---|
| | Frequency | Percent |
| Negative (No agglutination) | 157 | 83.5 |
| Positive at 4˚C at 1/8 dilution | 9 | 4.8 |
| Positive at 4˚C at 1/16 dilution | 7 | 3.7 |
| Positive at 4˚C at 1/32 dilution | 6 | 3.2 |
| Positive at 4˚C at 1/64 dilution | 4 | 2.1 |
| Positive at 4˚C at 1/128 dilution | 3 | 1.6 |
| Positive at 4˚C at 1/256 dilution | 2 | 1.1 |
| **Total** | **188** | **100.0** |

**Table 4.** Hematological parameters and IgM level stratified by splenomegaly stage.

| Splenomegaly Stage | Stage II | Stage III | Stage IV | Stage V |
|---|---|---|---|---|
| | Mean (std) | Mean (std) | Mean (std) | Mean (std) |
| Hemoglobin | | | | |
| *Male* [13 - 17.5 *g/dL*] | 13.4 (2.6) | 12.7 (2.6) | 12.5 (2.3) | 9.0 (2.0) |
| *Female* [12 - 15.5 *g/dL*] | 13.2 (1.4) | 11.5 (2.5) | 10.9 (2.4) | 9.5 (2.0) |
| MCV [80 - 94 fl] | 85.9 (7.0) | 88.0 (8.0) | 90.8 (7.4) | 94.1 (12.2) |
| WBC [4 - 11,000/ml] | 4.8 (1.6) | 4.2 (1.2) | 3.8 (1.1) | 2.9 (0.9) |
| Platelets [150 - 400 * 1000/l] | 147.7 (65.5) | 128.2 (55.2) | 110.2 (63.1) | 63.7 (36.1) |
| IgM [0.4 - 2.3 g/L] | 8.3 (10.1) | 12.5 (13.7) | 17.3 (14.2) | 19.3 (14.3) |

**Table 4** shows that hemoglobin level, white blood count and platelets account decrease with severity of splenomegaly, while IgM level increases with spleen size. The mean Corpuscular Volume (MCV) was normal in study subjects and normal across all stages of splenomegaly.

## 4. Discussion

Rwanda is one of sub-Saharan countries where malaria is endemic, the Eastern and Southern province are the most vulnerable provinces [1]. Residents from malaria endemic areas, usually present with enlarged spleen. Zeno Bisoffi et al. in their retrospective study have shown that splenomegaly is one of the findings among inhabitants of malaria endemic zones [7]. The splenomegaly that is usually massive, results from immune responses to malaria attacks [3] [7].

In the present study, of 188 study participants with female predominance (66%), we have demonstrated elevated IgM levels in 158 (84%) and cold aggluti-

nin titers of ≥1:64 in 9 (4.8%). Anemia was present in 104 of 188 patients (55%). It was observed that females (66%) are more affected than males, this correlates with the study that was conducted in Ghana by George Bedu-Addo *et al.* [17], however, other studies have shown males predominance [7] [18] [19].

The observed laboratory profile included mild macrocytosis and elevated LDH (Lactate Dehydrogenase) suggesting an element of hemolysis. These findings along with the negative direct Coombs test raise concern about other possible non-immune causes of hemolysis that may be playing a role in the etiology of the anemia, like G6PD (glucose-6-phosphate dehydrogenase deficiency) and megaloblastosis [20] [21].

These observations further support the concept of a multifactorial origin of the anemia, including a role for hypersplenism, given the positive correlation between spleen size and the degree of anemia, thrombocytopenia and leukopenia. These findings are similar with existing literature [19] [22].

The Level IgM is one of the major diagnostic criteria of Hyperactive malaria Splenomegaly (HMS) [6] [8] [9]. Previous observations of favorable response in patients on long-term antimalarial therapy included reduction in spleen size along with a decrease in serum IgM levels [9] [23] [24]. The IgM level measurement along with hematological and clinical parameters (measuring spleen size on Ultrasound and Physical examination spleen size) should be used to evaluate the response to anti-malaria therapy in patients from malaria endemic areas.

Cold agglutinin was found among patients with malaria, rarely but can contribute to anemia by causing hemolysis among the patients, the cause of anemia in patients with splenomegaly from malaria endemic areas is multifactorial and Cold agglutinin was reported to play a minor role [25]. In their study, Venkatesh V.N *et al.* found low prevalence of 2.6% in patients with malaria [11].

The present study shows that splenomegaly is common in Residents of malaria endemic areas in Rwanda, paves a path for subsequent perspective studies and assesses patients' follow-up and response to anta-malarial therapy. We suggest it would be appropriate to include IgM levels in the clinical assessment of patients with splenomegaly, and consider using this as a surrogate for diagnosis of HMS. Further studies could be done to assess splenomegaly and IgM response to antibiotic prophylaxis.

### 4.1. Strength

The First detailed study to evaluate hematological and immunological characteristics of Inhabitants with Splenomegaly in Malaria endemic areas in Rwanda.

### 4.2. Limitation

In this study, it was not possible to assess response to anti-malarial therapy

The lack of determination of anti-plasmodium antibodies for fulfillment of the diagnostic criteria for hyperreactive malarial splenomegaly is a major limitation of this work.

## 5. Conclusion

The data from the present study, confirm like in other endemic areas, that splenomegaly is common among inhabitants in malaria endemic areas in Rwanda. IgM levels correlate with the stage of splenomegaly; thus this could potentially be used as diagnostic and follow-up tool for HMS in our population.

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## Ethical Approval and Consent to Participate

The approval was provided by University Teaching Hospital ($N^0$ 200/CMHS/IRB/2016) and patients provided informed consent after explanation about the study objectives

## Conflicts of Interest

The authors declare no conflicts of interest regarding the publication of this paper.

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## Appendix: Data Collection Form

| No | Question | Answer |
|---|---|---|
| | **A.DEMOGRAPHIC DATA** | |
| A1 | Hospital | 1= Kibirizi hospital<br>2 = CHUB |
| A2 | Patient ID | …… |
| A3 | Gender | 0 = Male 1 = Female |
| A4 | Age (years) | |
| A5 | Occupation | 1 = Farmer<br>2 = Student<br>3 = Office agent<br>4 = Other……..<br>5 = Unemployed |
| A6 | Address of participant | 1. Sector……<br>2. District…<br>3. Province:…… |
| A7 | Marital status | 1 = Single<br>2 = Married<br>3 = Widower<br>4 = Divorced |
| A8 | Ubudehe category<br>(Rwandan financial status category) | 1 = Category I (poorest)<br>2 =Category II<br>3 = Category III<br>4 = Category IV (Richest) |
| A9 | Health insurance | 1 = Community Based Health Insurance (CBHI)<br>2 = Rwanda Social Security Board (RSSB) insurance<br>3 = Others<br>4 = No insurance |
| | **B. CLINICAL DATA** | |
| B1 | Episodes of malaria in the last 5 years | 0 = never<br>1 = once<br>2 = two times<br>3 = three times<br>4 = four times<br>5 = five times<br>6 = above 5 times |
| | When was the last episode of malaria | |
| B2 | Stage of splenomegaly (on physical exam) | 1 = Stage II<br>2 = Stage III<br>3 = Stage IV<br>4 = Stage V |

**Continued**

| | C. LABORATORY RESULTS | |
|---|---|---|
| C1 | White blood cells count [4 - 12,000 /mL] | …… |
| C2 | Hemoglobin level (g/dL): Male [13 - 17.5 g/dL)<br>Female [12 - 15.5 g/dL] | …… |
| C3 | MCV [80 - 94 fl] | …… |
| C4 | Platelets count [150 - 450 × 1000/L] | …… |
| C5 | Reticulocytes index [0.5 - 2.1] | …… |
| C6 | LDH [114 - 237 IU/L] | …… |
| C7 | Direct Coombs test | 1 = Positive<br>0 =Negative |
| C8 | Cold Agglutinin Titer (CAT) | 1 = No agglutination<br>2 = Titer below 1:64<br>3 = Titer above 1:64 at 4˚C |
| C8 | IgM level (g/L) | 1 = normal 0.4 - 2.3 g/L<br>2 = 2.4 - 4.6 g/L<br>3 = 4.7 - 9.2g/L<br>4 = above 9.2 g/L |