SCREENING RED CELL ALLOANTIBODIES IN SCA PATIENTS SEEN AT MNH SCD CLINIC-DAR ES SALAAM, TANZANIA

By

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A dissertation submitted in partial fulfilment of the requirements for the Degree of Master of Medicine (Haematology and Blood Transfusion) of Muhimbili University of Health and Allied Sciences

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CERTIFICATION

The undersigned that they have read and hereby recommended for acceptance by Muhimbili University of Health and Allied Sciences a dissertation entitled: ***"SCREENING RED BLOOD CELLS ALLOANTIBODIES IN SCA PATIENTS SEEN AT MNH SCD CLINIC"*** in partial fulfillment of the requirements for the degree of Master of Medicine (Haematology and Blood Transfusion) of Muhimbili University of Health and Allied Sciences

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DECLARATION AND COPYRIGHT

I, Dr Elineema Meda declare that to the best of my knowledge this dissertation is my own original work, and has not been presented and will not be presented to any other University for a similar or any other degree award.

Signature .....

Date 29.11.2010

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DEDICATION

"To my Wife Rhoda, my son Nathan Collin Jr. and my parents for their support and encouragement"

ABSTRACT

Background: Red blood cell transfusion in sickle cell patients is a major form of supportive care and long term transfusion is recommended for patients with a risk of stroke. Many patients with SCA attend at MNH but no data are available on the frequency and pattern of alloantibodies among those frequently transfused.

Objective: This study aimed at determining the frequency and pattern of red blood cell alloantibodies in sickle cell patients attending at MNH.

Materials and Methods

This was a descriptive cross -sectional study that was conducted at Muhimbili National Hospital between August and November 2009. Informed consent was obtained from the patients, their parents or guardians for those less than 18 years. Information on social demographic and clinical characteristics was collected from the medical files and interview from the parents or guardians. After physical examination, laboratory tests on blood were done for each study subject. ABO and Rhesus blood group and alloantibody screening were performed on every patient's sample and alloantibody identification on those found with positive screening test. The overall prevalence of RBC alloantibodies was determined and expressed as a percentage of all recruited patients during the time of data collection. All information was recorded using questionnaires and analysis was done using SPSS version 15.

Results

The study involved a total of 471 SCA patients aged 6 months and above attending paediatric and general haematology SCD clinic. Of these, 365 (77.5%) had of received a blood transfusion with 1184 total life time episodes transfusion (median, 2; range, 1-40).No records of transfusion documented in 106 (25.4%).The alloimmunization rate was 3.2% (15/471) among the SCA patients and 4.1% (15/365) of those who had been transfused. Anti-Kell was the most prevalent 20.7% and Rhesus blood group constituted 13.8% of total alloantibodies. The risk of alloimmunization was found to increase with episodes of RBC transfusion. Rather unusual that sickle and pregnancy did not elicit antibody development. Life threatening anaemia and splenomegaly were encountered in alloimmunized individuals.

Conclusion

The alloimmunization was evident among the transfused SCA patients. The presence of clinically significant alloantibody in transfused SCA patients shows the relevant role of RBC transfusion in the risk of alloimmunization

Recommendations

It is recommended that blood transfusion guideline be observed so that transfusion is appropriately used in management of SCA patients. This should involve an adequate pre-transfusion antibody screen and IAT cross-match. Policy of perfection would incorporate limited/partial phenotype matching of donor RBC for Kell, D, E and C prior to commencing chronic transfusion in order to minimize the risk of alloimmunization.

Finally, further prospective studies are required to track the formation, clearance and features associated with alloimmunization in SCA patients.

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ABBREVIATIONS

ABO: ABO blood grouping system

CBC: Complete blood count

DAT: Direct Antiglobulin Test

DHTRs: Delayed Haemolytic Transfusion Reactions

MOH: Ministry of Health

MNH: Muhimbili National Hospital

NBTs: National Blood Transfusion Service

OPD: Out-Patient Department

RBC: Red blood cells

SCA: Sickle cell disease

IgM: Immunoglobulin M

NHSBT: National Health Service Blood & Tissues (formerly National Blood Service)

NISS: Normal Ionic Strength Saline

AHG: Anti-Human Globulin

IAT: Indirect Anti-Globulin Test

DAT: Direct Anti-Globulin Test

ACW: Automatic Cell Washer

Definition of Terms

Anaemia was defined as hemoglobin level of less than 11g/dl. It was graded as follows; (basing on WHO classification)

Grade 1 anaemia (mild); Haemoglobin level (g/dl) of 9.5-10.9

Grade 2 anaemia (moderate); Haemoglobin level (g/dl) of 8.0-9.4

Grade 3 anaemia (severe**);** Haemoglobin level (g/dl) of 6.5-7.9

Grade 4 anaemia (life-threatening); Haemoglobin level (g/dl) of less than 6.5g/dl

INTRODUCTION

Sickle cell disease (SCA) is a genetic disorder that is characterized by a chronic haemolytic anemia. The clinical manifestations arise from the tendency of hemoglobin S to polymerize and deform red cells into the characteristic sickle shape. Homozygous state (HbSS or sickle cell anemia) is the commonest form of sickle cell disease [5].

Red blood cell transfusion in patients with sickle cell anaemia (SCA) is given as a definitive treatment when they present with vaso-occlusive crisis, severe anaemia and long term transfusion is recommended for those patients with risk of stroke. Unfortunately, this is limited by the development of alloantibodies to red blood cells (RBC) [1, 2, 30].

A blood group system consists of group of antigens encoded by alleles at a single gene locus or at gene loci so closely linked that crossing over does not occur or is very rare. Red blood cells (RBC) bear numerous cell surface structures that can be recognized as antigens by the immune system of individuals who lack that particular structure. Recipients of a blood transfusion may produce antibodies to an entire structure, or a single or limited number of epitopes [5, 7].

The ABO is the most clinically important blood group system is the ABO and consists of blood groups A, B, AB and O with further subdivisions of A and B [5, 7].

Rhesus system (Rh) forms the second most important blood group in transfusion. RBC with positive Rh antigens is capable of immunizing antigen negative individuals through transfusion and pregnancy. Rh antigens are determined by a complex of two closely linked genes: one encodes the protein carrying D antigen (RhD); the other encodes the protein carrying C or c and E or e antigens (RhCE). In the Rh system, eight common antigen combinations or haplotypes are possible: Dce (R_0, Rh_0), DCe (R_1, Rh_1), DcE (R_2, Rh_2), DCE (R_Z, Rh_z), ce (r, rh), Ce (r', hr'), cE (r″, hr″), and CE (ry, rh″). The letter "d" is used to designate the lack of D, but there is no d antigen or anti-d [5, 7].

RBC antibodies are immunoglobulin (Ig) products of B lymphocytes that may be naturally occurring or that follow stimulation by an antigen. They bind at a specific site of the antigen. These antibodies are either alloantibodies or auto antibodies. Whilst the immunogenetic

hypothesis argues that while the ability to manufacture antibodies is inherited, isoantibodies become demonstrable only if the antibody-producing machinery of the host is exposed to exogenous cross-reactive or homologous antigens [41]. The ABO antibodies are not present at birth but start to appear in third month of age probably in response to micro organisms and other inhaled or ingested substances [5, 7].

The IgM RBC antibodies, cause acute haemolytic transfusion reactions; due to antibodies binding to the red cell or compliment system activation, whereas IgG antibodies (Rhesus, Kidd, Kell, Duffy or Ss) are associated with extra vascular haemolysis, [7].

Delayed haemolytic transfusion reactions (DHTRs) occur between 5 to 10 days (frequently 2 weeks) after transfusion in previously immunized patients by transfusion or pregnancy. Infrequently brisk primary immune response can result in DHTRs after an initial transfusion and in about 50% of the immunized patients, anti-RBC antibody titers drop below detectable levels, allowing incompatible units to be transfused which re-stimulate memory cells thus increasing the antibody titer [7].

An acute transfusion haemolytic reaction is characterized by premature destruction of transfused red cell reacting with antibodies in the recipient and is usually due to ABO- Rh incompatibility. The delayed transfusion reaction occurs 5 to 10 days after transfusion and is due to antibodies not detected at the time of compatibility testing. It has been found that 30 percent or more of the antibodies to red blood cell antigens may disappear with time, but the probability of recipients to mount severe haemolytic transfusion reaction following further stimulation by transfusion is great [5]. The haemolytic transfusion reaction worsens the anaemia, may result into onset of painful crisis, and sometimes death.

There is increasing risk of alloantibodies with repeated exposure to incompletely matched donor red cells. At MNH, the practice is to issue red cells for transfusion after an immediate spin cross match has been done. No IAT cross match, antibody screen or donor red cell phenotyping is done.

Studies on the magnitude of this problem among children with sickle cell disease (SCA) in Tanzania are not available. Most of such studies have been done outside Tanzania with few African studies and far less than in the West or in Asia. Therefore this study was conducted to

provide the baseline data describing the magnitude of the alloantibody problem in the SCA patients and use this to improve transfusion services with our limited resources.

LITERATURE REVIEW

Epidemiological aspect

Incidence of alloantibodies in transfused SCA patients

The use of RBC transfusion in SCA patients is complicated with increased incidence of alloimmunization [3, 14, 18-22, 30]. This condition causes difficulties in obtaining compatible blood and results in a high incidence of haemolytic transfusion reactions.

The red blood cell antigens in transfused patients with SCA vary and the documented incidence show a range of 8% to 50% in studies from USA and UK [3, 4]. In some studies, in the United States of America and Europe on SCA patients who had a transfusion history, an incidence of alloimmunization ranging between 5% to 50% been reported [3,4,9-11].

However, an incidence of as low as 2% has been reported in some studies done in transfused SCA patients [8].

A study done at the King Fahd hospital, Saudi Arabia demonstrated the prevalence of alloantibodies in SCA to be 13.7% with the highest mode in group O patients. [13]. A similar study carried out in one of the provinces in Saudi Arabia reported alloimmunization rate of 34.2% with development of clinically significant alloantibodies [14].

Risk factors for alloimmunization

The probability that a transfused individual will form alloantibodies is related to several factors such as age, frequency of transfusion and phenotypic difference between the donor and the recipient [23-32]. Some studies have estimated the risk of alloimmunization ranging from 0.9% to 3.1% from a single unit that has not been phenotypically matched and the risk was higher below the age of fourteen years [30]. A study conducted in Brazil, comparing phenotypic compatibility between transfused SCA patients and donors, reported increased frequency of C antigen in donors (66.7% compared with 46.0% in patients; $P < 0.01$),although there was no difference noted in the rest of red blood cell antigens [22]. Similar findings were reported in recent study done in Uganda that demonstrated that the risk of alloimmunization was associated with number of units transfused [38].

A comparative study between transfused Jamaican and UK SCA patients found higher occurrence of multiple alloantibodies in UK patients than in the Jamaican cohort. The heterogeneity of UK donor population was thought to be the cause of this difference [31] .Although one study reported female preponderance with respect to alloimmunization, another study conducted in Brazil reported no gender difference with respect to alloimmunization [27, 30, 37].

Common alloantibodies

The clinically significant red blood cell antibodies has been demonstrated by several studies to occur the following descending order anti-E 16- 40%; Kell 5-40%; D 8-33%; c 4-15%; Jk^a 2-13%; FY 4-12%; C 2-10%; e 2-3%; and Jk^b 2% [2,11,33]. The relevant role of these alloantibodies in patients is the ability to cause transfusion reactions [5, 7].

Rare clinico-laboratory findings in alloimmunized individuals

In about 50% of the immunized patients, anti-RBC antibody titers drop below detectable levels, allowing incompatible units to be transfused which re-stimulate memory cells thus increasing the antibody titer, thus resulting into delayed haemolytic transfusion reaction (DHTR) [7].

DHTR is the commonest manifestation of alloantibodies following transfusion and has an incidence of about 11% in alloimmunized individuals and usually may go undetected as it mimics the clinical features of sickle cell anaemia [20, 25]. A rare finding but well documented cause of DHTR is the ant-Cob that was associated with worsening haemoglobin levels in primigravida [40]. Worsening clinical condition of transfusion dependent patient following transfusion should raise suspicion of DHTR especially when the patient presents with haemolysis and vaso-occlusive like symptoms [10, 20, 25]. Hemoglobin falls rapidly at approximately one week after transfusion, in many cases the DAT is negative and in many occasions no antibodies are detected in serum [21, 24, 35]. Hyper-hemolysis may be associated with spleen enlargement and attempts to correct anaemia through vigorous transfusion have been associated with clinically palpable or

radiological splenomegaly 46].

Extent of alloantibody screening

Studies have shown the importance of issuing phenotypically cross-matched blood for transfusion-dependent patients such as SCA, in order to reduce the rate of alloimmunization and related complications [3, 37,38].

Many protocols for screening alloantibodies exist and controversy is to what extent of antigen screening should be performed so as to minimize incidence of alloantibodies in SCA patients. The use of extensive matching protocol in which 9 or more red cell antigens are screened is expensive and may not be feasible in under resourced countries, so the use of a limited approach where by only 3-5 red cell antigens are screened is recommended [39].

A comparative study exploring the clinical efficacy and cost effectiveness between the two protocols found that the use of a limited approach that is, screening for Kell, E and C only had relatively clinical efficacy and was cost effective [36].

Management of SCA patients with alloantibodies

The contributing factors that seem to increase risks of alloimmunization include phenotypic difference between the patient and the donor, number of units of blood transfused, and whether the recipient was previously alloimmunized. To minimize and prevent alloimmunization haemolytic transfusion reactions, routine screening for alloantibodies in patients, screening of the blood supply and proper documentation is mandatory. Clinicians and those involved in patients' management need to know the alloimmunization status of their patients as this may have a role during management. Limiting the number of units transfused is also important. The antigenic mismatching can be reduced by using donors of the same ethnic background as the patient. Patients should have a phenotype determined for common haemolytic minor antigens before regular transfusion to minimize the risk of alloimmunization and to assist in selecting units for transfusion if multiple antibodies develop. Since some observations have documented the occurrences of alloimmunization and clinical features suggesting DHTRs in patients with sickle cell diseases, it is recommended that pre-

transfusion testing for at least Rh and Kell red blood cell antigens is done in patients with alloantibody already or who are being enrolled in a transfusion program [33]. Therefore it is important that chronically transfused patients be transfused with phenotypically matched red cells, as clinical benefits outweigh the higher cost of the process [40].

Laboratory investigations

Antibody Screening

Antibody screening undertaken in advance of the requirement to provide blood for transfusion alerts the clinician to possible delay in the supply of compatible blood if the antibody screen is positive. It also provides the laboratory with time to identify irregular antibodies and select suitable units. Antibody screening may be more reliable and sensitive than cross matching against donor RBC, therefore it is recommended that antibody screening should be performed in all pre-transfusion testing [16].

Choice of techniques

Several techniques are available for alloantibody screening. These include;

- Indirect antiglobulin test
- Column agglutination method
- Liquid phase tube methods have
- Solid-phase methods
- First-stage enzyme
- Two-stage enzyme and polybrene methods

All methods may be used; however, IAT is superior to the others due to its proficiency in the performance [16].

Indirect Ant globulin Test.

The IAT using red cells suspended in normal-ionic strength solution (NISS) was used in this study. Although, it takes longer time to incubate and lower sensitivity and specificity than

lower-ionic strength solution (LISS), NISS has minimal false positive thus does not need experience in using it.

Reagent red cells for use in antibody screening

Red cells for antibody screening should be preserved in a temperature-controlled environment i.e. between 2-8^{o}c in a medium shown to minimize loss of blood-group antigens during the recommended storage period. For screening purposes, the cells expressing the following minimum antigens are used: C; c; D; E; e; K; k; Fya; Fyb; Jka; Jkb; S; s; M; N and Lea. These are the antigens associated with significant clinical complications

Antibody Identification

When an alloantibody is detected in the screening procedure, its specificity is determined and its likely clinical significance is correlated with a clinical history. Once the antibodies have been identified a phenotype should be performed on the patient's red cells.

Principles of antibody identification

The patient's serum is tested by an appropriate technique (according to manufacturer's specification) against an identification panel of reagent red cells. As a starting point, the technique by which the antibody is detected during screening is used.

The specificity of the antibody is only assigned when it is reactive with at least two examples of reagent red cells carrying the antigen and non reactive with at least two examples of reagent red cells lacking the antigen. The presence of anti-Jk (a), anti-Jk(b), anti-S, anti-s, anti-Fy(a) and anti-Fy(b) are excluded by using red cells having homozygous expressions of the relevant antigen. An identification panel consists of red cells from eight or more group O donors [16].

STATEMENT OF THE PROBLEM

Red blood cell transfusion is an important an integral part of management of patients with sickle cell anaemia. Due to increased survival of SCA patients, the number of patients receiving red cell transfusion continues to increase. Repeated blood transfusions increase risks of development of alloantibodies this lead to DHTR, which is difficult to treat. This increases burden on transfusion and hospital services, as further transfusion is required to correct worsening anaemia, therefore increasing patient morbidity and mortality.

Studies on the magnitude of this problem among children with SCA in Tanzania are not available. Most of such studies have been done outside Tanzania, only few in Africa [38] and far less than in the West or in Asia.

It is important to know the magnitude of prevalence of alloantibodies in SCA in order to decide about the level of investment in alloantibody screening and phenotyping in our setting where resources are limited.

STUDY RATIONALE

The reason for doing this study was to establish baseline data on the prevalence, and pattern of alloantibodies in SCA patients at MNH because such baseline data were not available in this population and there were very few studies from Africa as a whole.

Evidence from these studies indicated that pre-transfusion workout of patients and donors for potential clinical significant antibodies reduced morbidity and mortality especially those associated with multiple blood transfusions.

This study's results would be used to improve transfusion service through provision of evidence based clinical laboratory findings which would be reflected through patients' outcome and efficiency of blood use.

RESEARCH QUESTION

1. What was the frequency and pattern of alloantibodies among the transfused SCA patients at seen at Muhimbili National Hospital SCD Clinic?

STUDY OBJECTIVES

Broad Objective

To determine the prevalence and pattern of alloantibodies in SCA patients, seen at Muhimbili National Hospital SCD Clinic

Specific objectives

i) To determine the prevalence of alloantibodies in SCA patients seen at Muhimbili National Hospital SCD Clinic

ii) To describe the pattern of alloantibodies in SCA patients seen at Muhimbili National Hospital SCD Clinic

iii) To describe the clinical features of patients with alloantibodies in SCA patients seen at Muhimbili National Hospital SCD Clinic

iv) To describe the laboratory features of patients with alloantibodies in SCA patients seen at Muhimbili National Hospital SCD Clinic

METHODOLOGY

Study Design

This was a descriptive, cross-sectional study nested within a pre-existing Cohort study titled: 'Defining the spectrum of sickle cell disease in East Africa: A clinico-epidemiological study'. The Cohort study was started in 2004, where I worked as a research assistant and Dr Julie Makani as the principal investigator.

Study site

Muhimbili:

This consists of the Muhimbili University of Health and Allied Sciences (MUHAS) and the Muhimbili National Hospital (MNH). MUHAS is the only public higher learning institution in health sciences in Tanzania and is responsible for training all cadres of health care workers including doctors, nurses, laboratory technologists and pharmacists. It is closely affiliated to MNH. It serves approximately 1,000 outpatients every day (Monday to Friday) and 1,000 admissions a week for inpatient care.

Study subjects

The study was carried out both at the paediatric sickle cell and general haematology clinics. The hospital runs two SCA clinics; one for children every Thursday with an average attendance of 30-40 patients, and the other one for adults every Friday with an average attendance of about 45 patients

Inclusion criteria:

All consecutive patients attending at MNH were included if;

- They had HbSS with history of transfusion and/or pregnancy
- They were six months old or older
- They or their parent or guardian had freely consented to participate.

Controls

- They had HbSS without history of transfusion or pregnancy; this was set out as a comparative group as experience showed less than 1% of those without record of transfusion or pregnancy would test positive for antibodies.

Exclusion criteria:

Patients with the following were excluded initially from this study if

- They were non-sicklers (HbSS).
- They were less than six months old.
- All SCA-inpatients.
- They refused to participate.

Sample size estimation

A minimum sample size for the study was obtained using the sample size estimation formula of $N=Z^2 P (1-P)/d^2$ whereby

N=Minimum sample size

Z=Standard normal deviate corresponding to two sided specified significant level. This is 1.96 (at 95%c confidence interval)

P= Prevalence of alloantibodies per literature review is taken to be 26%

d=Margin of error (taken as 5% in this study)

Putting P=26%, d=5%, N=300

Note: To get a more precise estimate of the prevalence of alloantibodies, 471 patients were recruited in this study.

Sampling Procedure

All SCA patients were involved in this study. Subjects were consecutively recruited from SCASCD CLINIC clinics after they had signed an informed written consent. For those less than 18 years of age, the parents or guardian had to sign on their behalf.

Clinical procedures

Clinical information was collected at SCD CLINIC visit using preformed questionnaire (see Appendix I) Information that was collected included demographic data and clinical history. The clinical history detailed all previous transfusions and clinical episodes occurring immediately after or in the two weeks following blood transfusion.

Sample collection and processing

4 ml of blood was collected. 2 ml in EDTA vacutainer tubes for Full Blood Picture (FBP) and 2ml in serum separator for ABO & Rh blood grouping, alloantibody screening and identification.

ETHICAL CONSIDERATIONS

Before recruiting study participants, they asked for their informed consent. Parents or guardians were also subjected to this process where participants are less than 18yrs old. The purpose of the study was described; that was, ***to determine the frequency and pattern of alloantibodies in SCA patients, seen at MNH SCD CLINIC***. Study procedures, risks, benefits and alternatives to participation (see Appendix IV). Information was provided on how participants or their parents or guardians with concern related to the study could access members of the research team and the chair of the MUHAS Research and Publications Committee. Willing participants or their parents or guardians were required to provide signed consent witnessed by researcher/ research assistants (see Appendix IV). The voluntary nature of study participation was explained to each potential participant, their parents or guardian and that refusal to participate would in no way affect their rights to standard treatment offered at MNH. The alternative to participate provided as not to participate.

For those study participants with alloantibodies, arrangements were made between physicians and blood bank to make sure patients would get appropriate blood when they need transfusion.

Measures to insure confidentiality of the information provided was addressed, this included use of name only for identification, ID numbers were used , and safe handling and storage of data collected by the researcher alone till the end of analysis when all records would destroyed The data would utilized only for the purpose stated.

No data collection began before ethical clearance to conduct the study was attained. This was received from the Director, University Research and Publications Committee of MUHAS, and permission from MNH management.

LABORATORY TESTS

Blood grouping

ABO and Rh blood grouping were performed as per SOP of the haematology departments at MNH (see Appendix II).

Interpretation of results

Agglutination was assessed macroscopically. Reactions of agglutination were graded as 0, 1, 2, 3, and 4 depending on the strength of the reactions. The characteristics of reaction strengths are given below. Cell grouping had to agree with serum group where applicable (see Appendix II).

Antibody Screening and Identification Procedure

Antibody screening was used to detect the presence of RBC antibodies to the antigens of the main blood group systems. Their clinical significance as regards transfusion or Haemolytic Disease of the Newborn was assessed.

- .

First the patient's serum was tested against the 2 UK NHSBT cell panel by NISS IAT at 37°C. **(See Appendix IV).**

For screening purposes, the cells expressing the following minimum antigens were used: C; c; D; E; e; K; k; Fya; Fyb; Jka; Jkb; S; s; M; N and Lea. These are associated with significant clinical complications.

Then all positive screening tests were subjected to a wider panel of 10 for identification **(See Appendix V).**

- To confirm the presence of a specific antibody, a minimum of 2, and preferably 3, positive reactions against the appropriate antigen were required.
- To exclude the presence of a specific antibody, a minimum of 2, and preferably 3, negative reactions against the appropriate homozygous antigen were required.

Care was taken to look for weak antibodies that only reacted with homozygous expression of the antigen by confirming reaction under microscope

Materials

- Automatic Cell Washer (ACW) – it was important to ensure that this device was functioning properly by checking that there was adequate saline in the reservoir and that it was set to 4 wash cycles.
- 37°C Water Bath
- RT30 plastic test tubes
- Disposable Pasteur pipettes

Sources of reagents used

Reagents	Manufacturer	Catalogue Number
2-Cell Screen	NHSBT	PR121
NHSBT Panel	NHSBT	PR141
Alba Panel	Alpha labs	960050
Weak anti-Rh positive control	NHSBT	PN046
AB serum negative control	NHSBT	PN061
AHG	Lorne Laboratory	435010
Sensitized cells	NHSBT	PR092
PBS Tablets	Sigma Aldrich	P 4417

Interpretation and Validation of Results

Reaction Grades (all macroscopically visible) according to manufacturer's instructions

Grade	Description
5+	Cell button remains in one clump.
4+	Cell button dislodges in to several clumps.
3+	Cell button dislodges in too many small clumps.
2+	Cell button dislodges in to finely granular small clumps.
+	Cell button dislodges in to fine granules or microscopic result
-	Negative
±	Inconclusive

- All results were recorded on Antibody Identification Panel sheets

Negative Investigation

One drop of sensitized cells was added to each test tube to validate all negative reactions.

- Tubes were return to the Automatic Cell Washer and select 'mix-cent' the sample. This mixes and then be followed by a spin cycle.
- The tests were read macroscopically - all tests read positive.
- A negative test at this stage invalidated the antibody investigation and necessitated a repeat of the test.

Quality Assurance

Patients' serum samples collected were store frozen at -80°C for alloantibody screening and identification were done under supervision by Teresa Marlow, a senior manager of the Haematology Laboratory at the Whittington Hospital, London UK. She provided the technical support in sample analysis.

A weak anti-c as positive control and AB serum as negative control were set up with each batch of antibody screens and identifications to validate antibody screening.

Sensitized cells were added to all negative IAT results to give a positive reaction and thus validated that they were not falsely negative owing to a failure in the washing stage.

Full blood picture (FBP)/Complete blood count (CBC)

Full blood picture was done using CBC Coulter Counter (Coulter Corp, Miami, FL), haematological analyzer. The samples were analyzed according to a standard protocol, which involves running control reagents to ensure that the machine is performing well before running the blood samples.

STATISTICAL METHODS

Data handling:

Data were entered into a computer and cleaned using the SPSS package software Version 15.0.The prevalence alloantibodies was determined as a percentage among all antibody screened SCA patients during the study period. To compare the distribution of RBC alloantibodies by gender, a pair wise comparison was conducted using Chi squared test or Fisher's exact test .Univariate logistic regression analysis was performed to identify possible risk factors associated with risk of alloimmunization in SCA patients. All analyses were conducted with SPSS v 15.0. Test of significance was 2-sided with a probability cut-off value of 0.05.

RESULTS

The study was done at MNH between August and November 2009. Among the 992 excluded individuals, 146 had not been screened for SCA, 20 patients had incomplete questionnaires and remaining 826 patients were those who had been seen in previous visits. However, the minimum sample size for this study (i.e.300 patients) was achieved.

Figure 1 below: A flow chart showing how patients were recruited and analyzed

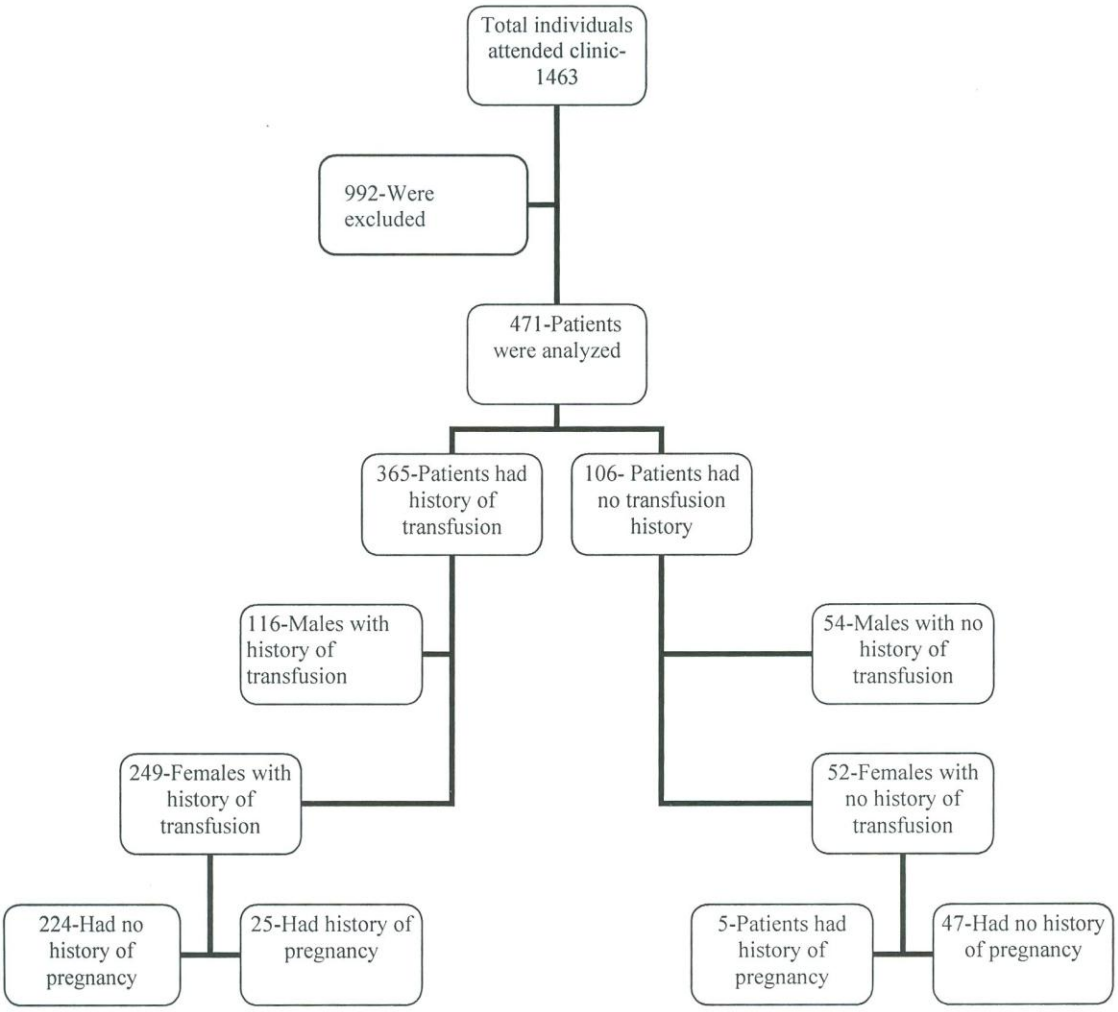

Table 1 below summarizes the characteristics of SCA patients by sex. In this study, a total of 471 patients were recruited. The male to female ratio was 1:1.17, and the overall median age was 16 years (range 6 months to 49 years). The proportion of transfused among study population by gender was 75.1% and 79.5% for males and females respectively. The difference was not statistically significant (P=0.15). In this study patients had received a median of two RBC transfusions (range 1-40).

Also it was noted that, there were no difference by gender in distribution of other features such as, pallor, jaundice, splenomegaly, and mean haemoglobin level.

Table 1: Characteristics of patients studied

Variables	Male n %	Female n %	Total N (%)	*P-value*
All	217(46.1)	254(53.9)	471(100)	
Age category (years)$^{\Theta}$				0.001
0-4.9	26(11.9)	19(7.5)	45(9.6)	
5-9.9	61(28.1)	43(16.9)	104(22.1)	
≥10	130(59.9)	192(75.6)	239(50.7)	
Median age(range) in years**	13(1-43)	16(0.5-49)	15(0.5-49)	
Pregnancy history		30(11)		
History of blood transfusion				
Yes	163(75.1)	202(79.5)	365(77.5)	0.15
No	54(24.9)	52(20.5)	106(22.5)	
Median (range) episodes of BT**	2(1-40)	2(1-20)	2(1-40)	
Physical examination				
% Pallor	195(89.9)	235(92.5)	430 (91.3)	0.19
% Splenomegaly	13(5.9)	12(4.7)	25(5.31)	0.34
% Jaundice	86(39.6)	108(42.5)	194(41.2)	0.29
Laboratory findings				
Mean haemoglobin ±(SD) *	7.4±1.39	7.4±1.28	7.4±1.33	

Note: *indicate numbers and not %. SD: standard deviation. ** Number 1-indicates median and numbers in brackets range respectively. BT: Blood Transfusion

$^{\Theta}$ Age stratification based on findings from other studies [30, 37]

In this study it was found that the overall rate of alloimmunization among SCA patients was 3.2%. Male sex had higher proportion of alloantibody (60.0%) as compared to female (2.4%) but the difference was not statistically significant (P=0.20). The age category ≥10 years had highest proportion of alloantibodies (80%) compared to other age groups, however, these could be reasoned out of number rather than age, and difference was not statistically significant (P=0.26).The rate of alloimmunization among the transfused patient was 4.1%.

Table 2: Proportion of SCA patients with alloantibodies by gender, age groups and episodes of life-time BT

Variables	Alloantibody Status		Total	*P-value*
	Positive n (%)	Negative n (%)	N (%)	
All	15 (3.2)	456 (96.8)	471 (100.)	
Sex				
Male	9 (4.1)	208 (95.9)	217 (46.1)	0.20
Female	6 (2.4)	248 (97.6)	254 (53.9)	
Age category (years)				
0-4.9	2 (13.3)	43 (9.4)	45 (9.6)	
5-9.9	1 (6.6)	103 (22.6)	104(22.1)	0.26
≥10	12 (80.0)	310 (67.9)	322 (68.4)	
History of BT and pregnancy	0 (0.0)	25(100)	25(100)	
Episodes of life time BT				
No BT	0 (0.0)	106 (100)	106 (22.5)	
Overall with BT	15 (4.1)	350 (95.9)	365 (77.5)	
1-10	12 (80.0)	341 (97.4)	353 (74.9)	
11-20	1 (6.6)	8 (2.3)	9 (1.9)	<0.001
21-30	1 (6.6)	1 (0.3)	2 (0.4)	
>30	1 (6.6)	0 (0.0)	1 (0.2)	

Note: BT means Blood Transfusion.

The table below summarizes the specificities of the identified alloantibodies as well as patients' characteristics. Some patients had 2 alloantibodies, while others had multiple antibodies. One patient had inconclusive result as the test was pan reactive. Alloimmunized patients had a total of 110 episodes of RBC transfusion and developed a total of 58 alloantibodies.

Table3: Profile of SCA patients who developed alloantibodies

Patients' characteristics					**Alloantibody specificity**
Serial no.	Sex	Age (years)	BT***	Blood group	Most likely or could not be excluded[§]
55	M	10	3	O+ve	Kell, Le^b
86	M	20	40	B+ve	D, E, S, P,Le^a, Kell, Kp^a,Fy^b, Jk^b and Cob
122	M	18	5	B+ve	Weak Le^a and weak Kell
208	F	22	2	B+ve	C, Cw, Lu^a, Kell, Le^b and Fy^b
212	F	17	5	O+ve	Cw, M, s, Lu^a, Kell, Kp^a, Le^a, Fy^b, and Jk^a
213	M	17	2	B+ve	Weak Le^a and weak Kell
243	M	15	1	B+ve	Weak Le^a and weak Kell
218	F	19	2	AB+ve	Le^a, Cob, Kell, and Lu^a
249	F	22	1	A+ve	Weak Kell, Cob +, unidentified antibody.
256	M	5	1	O+ve	N, s, weak Le^a and Kp^a
369	M	16	1	A+ve	D, C, Cob, weak Kell.
371	M	3	1	O+ve	Weak Le^a weak Kell
434	F	18	1	B+ve	Pan agglutinin
478	F	10	15	A+ve	E, Cw
504	M	17	30	A+ve	D, E, S, P, Le^a, Kell, Kp^a, Fy^b, Jk^b and Cob

Key: * Indicates life-time episodes of RBC transfused, BT =Blood Transfusion**

[§]All specificities add to 58

The proportion of specific alloantibodies encountered in 15 patients are given below .It was found that the most encountered alloantibody was anti-Kell (20.7%) followed by anti-Le^b. The antibodies (anti-C, anti-D and anti-E) against Rhesus group constituted about 13.8% of all alloantibodies.

Table 4: Proportion of specific alloantibodies encountered in 15 patients

RBC antibody specificity	Number of occurrence	Prevalence (%)
Anti-C	2	3.4
Anti-D	3	5.2
Anti-E	3	5.2
Anti-Kell	12	20.7
Anti-Le^a	10	17.2
Anti-Le^b	4	6.9
Anti-Jk^b	2	3.4
Anti-M	1	1.7
Anti-S	2	3.4
Anti-P	2	3.4
Anti-Co	9	15.5
Anti-Fy^b	4	6.9
Anti-Kp^a	4	6.9
Total	58	100.0

Table 5 summarizes the clinical features of SCA patients by the prevalence of alloantibodies. It was found that males had about twice the rate of alloimmunization (60%) than females. However, the difference was not statistically significant (P=0.20). The age group ≥10 years had the highest rate of alloimmunization, (80.0%) as compared to other age groups but the difference was not statistically significant (P=0.26).

Table 5: Clinical features of SCA patients by prevalence of alloantibodies

Variables	Alloantibody Status		Total	*P value*
	Positive n %	Negative n %	N %	
Sex (All)	15(3.20)	456(96.80)	471(100)	
Male	9(60)	208(45.6)	217(46.1)	0.20
Female	6(40)	248(54.4)	254(53.9)	
Age group (years)#				
0 – 4.9	2(13.3)	43(9.4)	45(9.5)	
5 – 9.9	1(6.7)[μ]	103(22.6)	104(22.1)	NA
≥10	12(80.0)	310(67.9)	322(68.4)	
Median age(range) years*	13(1-43)	17(0.5-49)	16(0.5-49)	0.90
History of pregnancy	0(0.0)	30(100)	30(11.81)	0.36
Episodes of life time BT				
No BT	0(0.0)	106(100)	106(22.5)	
1-10	12(80.0)	341(97.4)	353(74.9)	
11-20	1(6.7)	8(2.3)	9(1.9)	<0.005
21-30	1(6.7)	1(0.3)	2(0.4)	
>30	1(6.7)	0(0.0)	1(0.2)	
Pallor				
Yes	14(93.3)	416(91.2)	430(90.2)	0.61
No	1(6.7)	40(8.2)	41(9.8)	
Jaundice				
Yes	8(53.3)	269 (58.9)	277(58.8)	0.42
No	7 (46.7)	187 (41.0)	194 (41.2)	
Splenomegaly				
Yes	3(20.0)	22(4.8)	25(5.3)	0.03
No	12 (80.0)	434(95.2)	446 (94.7)	

NB: NA=Not applicable; [μ] cell had expected count less than 5; *indicate numbers not %;

[#]Age stratification based on findings from other studies [30, 37]

In this study it was also noted that none of the female individuals with a history of pregnancy had positive alloimmunization status and was not statistically significant (0.36). The positive alloimmunization status was evident among transfused patients while there was none among un-transfused patients and the difference was statistically significant (P=0.001). The proportion of patients with splenomegaly was higher in alloimmunized patients (20.0%) as compared to 4.8 % in non-alloimmunized patients; the difference was statistically significant (P=0.03).

Table 5 shows the distribution of laboratory features of SCA patients by the prevalence of alloantibodies. The mean WBC distribution among alloimmunized patients was 13.46±3.54, and this did not differ significantly from the non-alloimmunized patients (14.86± 7.02) (*P*=0.78).It was found that the mean haemoglobin level (6.18±2.10) was lower in alloimmunized patients as compared to non-immunized patients (7.47±1.28) and the difference was statistically significant (P<0.001).The highest proportion of alloimmunized patients (53.3%) was found to have life threatening anaemia as compared to 18.6% of non immunized patients and the difference was statistically significant(*P*=0.01).

Table6: Laboratory features of SCA patients by prevalence of alloantibodies

Variables	**Alloantibody Status**		**Total**	**P-*value***
	Positive n %	**Negative** n %	N %	
All	15(3.2)	456(96.8)	471(100)	
Mean WBC ± SD*	13.46±3.54	14.86±7.02	14.81±6.94	0.77
Mean Hb± SD*	6.18±2.10	7.47±1.28	7.43±1.33	< 0.0001
No anaemia (Hb) ≥ 11.0	0(0.0)	4(0.9)	4(0.8)	0.01
Mild anaemia (Hb) 9.5-10.9	0(0.0)	25(5.5)	25(5.3)	
Moderate anaemia (Hb) 8.0-9.4	3(20.0)	122(26.8)	125(26.5)	
Severe anaemia (Hb) 6.5-7.9	4(26.7)	220(48.2)	224(47.6)	
Life threatening anaemia (Hb) <6.5	8(53.3)	85(18.6)	93(19.7)	
Mean MCH ±SD*	26.8±2.70	26.76±3.64	26.76±3.61	0.48
Mean MCHC±SD*	31.03±1.05	31.04±1.28	31.04±1.27	0.51
Mean total bilirubin ±SD*	80.13±39.72	58.27± 45.19	58.97±45.15	0.74
Mean direct bilirubin ±SD*	15.39±11.94	13.69±8.03	13.75±8.17	0.44
Mean reticulocytes ± SD*	14.41± 4.41	12.11±6.23	12.17±6.19	0.16
Mean LDH±SD*	825±367.40	775.8±527.97	777.4 ±523.33	0.36
ABO blood groups				0.18
A	4(26.7)	96(20.7)	100(21.2)	
B	5(33.3)	82(18.1)	87(18.5)	
AB	1(6.7)	21(4.6)	22(4.7)	
O	5(33.3)	257(56.6)	262(55.6)	
Rhesus D status				0.87
Positive	12(80.0)	455(99.)	467(99.2)	
Negative	3(20.0)	1(0.8)	4(0.8)	

Note: *indicates numbers not % Note: *indicates numbers not %

There was no difference in red cell indices (MCH and MCHC) between the two groups. Although the mean total bilirubin was higher in alloimmunized patients (80.13±39.72) than in non-alloimmunized patients(58.27± 45.19), the difference just fell short of statistical significance (P=0.74).It was also noted that the mean reticulocyte count was higher in alloimmunized patients (14.41± 4.41) as compared to non-alloimmunized patients (12.11±6.23); however, this was statistically not significant (P=0.16).The mean lactate dehydrogenase (LDH) was higher in the alloimmunized patients (825±367.40) than in non-alloimmunized patients (775.8±527.97) although the difference was not statistically significant (P= 0.36).The blood group AB participants comprised the lowest proportion (6.7%) among the alloimmunized individuals than the rest of the blood groups; however, the difference observed was not statistically significant (P=0.18).It was also noted that none of the alloimmunized patients had a negative Rhesus blood group.

DISCUSSION

This was a descriptive cross -sectional study that was conducted at Muhimbili National Hospital between August and November 2009. The study aimed at determining the magnitude and pattern of red blood cell alloantibodies in sickle cell anaemia patients seen at MNH SCD Clinic.

The study involved a total 471 SCA patients, out of these 254 (53.9%) were female. The overall median age for the study subjects was 15 years with range of 6months to 49 years. The proportion of patients with history of transfusion was 77.5% and the total life time episodes of transfusion was 1184 (median, 2; range, 1-40).The transfusion frequency appears to lower about half that was reported in Uganda, in which the total episodes of transfusion was 2463 (median, 3; range, 2-80) [38]. In this study, it was difficult to establish the units if RBC transfused due to poor record keeping and relying on the figures given by patients alone would inflate or deflate the true value of the estimate (recall bias).

The differences observed in distribution on features such as, pallor, jaundice, splenomegaly, mean haemoglobin level and ABO blood groups by gender was not significant.

In this study, the overall rate of alloimmunization in the SCA cohort was found to be 3.2% and 4.1% in transfused subgroup. This rate (4.1%) is lower than that of a recent study conducted in Uganda which reported alloimmunization rate of 6.1% among transfused SCA patients [38].In this study, the lower rate could to some extent be explained by the less episodes of life time RBC transfusions observed (median, 2; range, 1-40) as compared to (median, 3; range, 2-80) that was reported in Uganda. Secondly, the proportion of female with history of pregnancy was 11.8% and none had alloantibodies. Another contributing factor could be phenotypic compatibility between the SCA patients and the blood donors but this needs to be established in further studies. Finally, as patients were not monitored for RBC antibodies after each transfusion, anti-RBC antibody titers could have dropped below detectable levels. This can occur in about 50% of the patients with alloimmunization allowing incompatible units to be transfused [7]. However, some studies have reported rates ranging from 2.1 to 47% [20, 22, 24-28, 31-35, 48].

The specificities of the antibodies found in the present study were similar to those found in other reports of alloimmunization in SCA. This was certainly attributed to the high immunogenicity of antigens C, D, E, and K [2, 11, 33, 38]. It might also be due to the fact that RBC matching was not routinely employed for all of these antigens, except for antigen D. About 40% of alloimmunized individuals had multiple antibodies and this is higher than (20.8%) that was recently reported in study done in Uganda [38]. Anti-Cob constituted about 15.52% of total alloantibodies, this is significant finding as it has been reported to cause delayed haemolytic transfusion reaction [40].

Of the 58 alloantibodies identified, 5.7% were anti-D, a figure lower than that reported in most studies [34]. A study done in Brazil reported 20% anti-D antibody among a total of 15 alloantibodies detected [30]. The comparatively low frequency of anti-D antibodies found in the present study may be explained by that, in this study, none of the patients with history of pregnancy was found to be alloimmunization hence absence of secondary alloimmunization related to pregnancy may have contributed. Also the low frequency of transfused patients with anti-D reflects the high frequency of Rhesus D positive in Tanzania (and elsewhere in sub-Saharan Africa).The presence of anti-D could reflect inadequate supervision with the transfusion service. The molecular study of these individuals would reveal their D –genotype.

In the present study, the rate of alloimmunization was found to be higher in males than in females; however, when univariate analysis was performed, the gender was not a risk factor for alloimmunization (P=0.20), similar to data reported by others [30, 37]. Some investigators have reported a higher risk for females [27, 28]. The alloimmunization status was evident in transfused groups and trend towards alloimmunization increased with episodes of RBC transfusion (P<0.001).The finding compares with study done in Uganda in which there was noted an increased trend towards alloimmunization status with episodes of transfusion although it just fell short of statistical significance, (P=0.08) [38].

Also it was observed that the proportion of patients with splenomegaly was higher in alloimmunized patients (20.0%) as compared to non-immunized individual (4.8%), the difference was statistically significant (P=0.039), this would partly explained by the fact that

those receiving transfusion were haemolysing more than those who did not need a transfusion. It is possible hyper-transfusion may have contributed to spleen regeneration observed in those individuals [39].

It was found that the mean haemoglobin level was lower in alloimmunized individuals, The highest proportion of alloimmunized SCA patients (53.3%) had a life threatening anaemia as compared to 18.6% of non-alloimmunized individuals and the difference was statistically significant(P=0.01). The low haemoglobin levels in alloimmunized individuals may simply reflect lower baseline haemoglobin levels. It could also represent a clinical phenomenon termed as hyperhaemolsis in which patient haemolyses own RBC and the transfused RBC and the resultant haemoglobin level is lower than the pre-transfusion haemoglobin [42]. The other proposed contributing factors (although out of scope of this study) to very low haemoglobin in the alloimmunized individuals is low serum erythropoietin levels due to renal damage and/or immune destruction of reticulocyte [33].The mean reticulocyte level reported in the present study need to be treated with caution as it was the steady state level reported during routine visit and not at the time of recruitment. Similar considerations apply for other haemolytic makers' i.e. bilirubin and LDH.

Study limitation

Due to an insufficient time and funds this study is limited:

- That being a hospital based is liable to selection bias.
- That being a cross sectional nature, it cannot track development and clearance of alloantibodies after transfusion and over time.
- The study also could have missed those with like-hood of having alloantibodies as they frequently unwell therefore they often miss their appointments.
- Phenotypic compatibility between donors and recipients was beyond the scope of this study. Further studies suggested.
- Failure to elicit antibodies in sickle patients with history of pregnancy.
- Capacity for full typing of alloantibodies is limited.

CONCLUSION

This was the first ever study documented in Tanzania to determine the magnitude and pattern of alloantibodies in SCA.

The rate of alloimmunization among transfused SCA patients was 4.1%. Anti-Kell was the most prevalent 20.7% and Rhesus group constituted 13.8% of total alloantibodies. All patients had two or multiple alloantibodies.

The alloimmunization status was evident among the transfused patients. No alloimmunization was documented among un-transfused patients and the difference was statistically significant (P=0.001).This confirmed the relevant role of RBC transfusion in the risk of alloimmunization. Again, no alloimmunization was documented in those with history of pregnancy.

It was also noted that the splenomegaly and anaemia were found to be associated with positive alloimmunization status.

RECOMMENDATIONS

Based on this study results, blood transfusion is associated with development of clinically significant alloantibodies and relevant based on practice. Therefore in the future, those involved with blood transfusion in these patients should consider the following:

- Improvement of transfusion service is required through adherence to guideline which includes thorough ABO and D grouping and IAT cross-match.
- Avoid unnecessary transfusion. When transfusion is warranted in SCA patients, then it should an adequate pre-transfusion antibody screen and IAT cross-match. A policy of perfection would incorporate limited/partial phenotype matching of donor RBC for Kell, D, E, and C to minimize the risk of alloimmunization.
- It is recommended that blood transfusion guideline be observed so that transfusion is appropriately used in management of SCA patients.
- Well designed prospective studies are required to track the formation, clinical and laboratory features of such alloantibodies in SCA patients.

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